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OPTIMISATION OF DNA EXTRACTION AND RAPD-PCR AMPLIFICATION FOR POPULATION GENETIC ANALYSIS OF *DAPHNIA CUCULLATA* SARS, 1862 (CRUSTACEA: CLADOCERA)

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Isolation of good quality DNA is a prerequisite for any PCR-based molecular tool therefore, the ability to prepare and isolate the genomic DNA from a variety of sources is an important step in many molecular techniques. In our research we present the optimization of DNA extraction protocols and PCR conditions for RAPD analysis of *Daphnia cucullata*. It should be noted that this modified DNA method can be applied by extracting the DNA from other small chitin containing zooplankton organisms. DNA extraction method modifications from *Daphnia cucullata* species provides high-quality and high-quantity DNA that can serve as a template in polymerase chain reaction (PCR). RAPD-PCR protocol was optimized based on 12 µl reaction volume and optimal amplification program with 46 cycles. Among three different RAPD-PCR mixes, we determined that more efficient was the one consisting of: 10 × Taq buffer with KCl; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). Nineteen 10-mer primers from Operon Kit A, B, C and F sets (Carl Roth, Germany) showed polymorphism of *Daphnia cucullata* among different populations. Thus, the results indicate that the optimized protocol for DNA extraction and RAPD-PCR is suitable for further analysis of genetic diversity of different zooplankton species and populations.

Key words: Cladocera, *Daphnia cucullata*, genetic diversity, DNA markers, RAPD

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INTRODUCTION

All organisms living in lakes are also in the interaction with each other and with their environment. They play particular roles in the circulation of substances and energies. These organisms interact with environmental factors in their own unique ways depending on their distinctive biological characteristics that help them to resist the changes in these environmental factors. Zooplankton plays an essential role in the transformation of substances and energy in bodies of water. Cladocera species are an important stage in the food chain. On the one hand, they regulate bacterial and detrital quantity; on the other hand they are an important component of the feed for juvenile fish, plankton-feeding fish and many other aquatic animals (Hebert 1982, Malone & McQueen 1983, Pinel-Alloul 1995, Wetzel 2001). Some Cladocera species such as

Daphnia magna, *Daphnia cucullata* are often used as bioindicators of water pollution. Thanks to the work conducted by Win-Fried Lampert and his colleagues worldwide, the common Cladocera species such as *Daphnia cucullata* have been used as a good model organism of international freshwater ecology (Adamowicz et al. 2004, Colbourne & Hebert 1996, Colbourne et al. 2006, Harris et al. 2012, Lampert 2006, Larsson &Weider 1995, Lubbock 1857, Schwenk et al. 2000, Weisse 2006). *Daphnia* has been considered to be a control organism in the freshwater as a kind of convergence model with the adaptive features in radically different habitats (Colbourne et al. 1997, Dodson & Hanazato 1995). For understanding this adaptive processes in Daphnia population under the changing environmental conditions the investigations of genetic structure are necessary. The first step in the population genetic investigation is extracting the pure DNA, which can be used for PCR reaction. Quite a lot of information on extracting the DNA from Daphniidae, Bosminiidae is available (Cousyn et al. 2001, Fitzsimmons & Innes 2005, Grosberg et al. 1996, Harris et al. 2005, Hellsten & Sundberg 2000, Martins et al. 2009, Schwenk et al. 1998). However, it should be noted that the methodologies proposed by other authors are usually applied to significantly bigger Daphniidae species (eg, *Daphnia pulex, Daphnia magna, Daphnia galeata*) and in our case they cannot be used to extract the DNA successfully, because of the significantly smaller size of Daphniidae species *Daphnia cucullata*.

Therefore we have made some modifications of other authors' methodologies available to us, in order to be able to extract the DNA from *Daphnia cucullata* specimens as quickly and as qualitatively as possible. The assessment of genetic variability in the population structure is the first step in evaluating the adaptative process while researching freshwater 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 microsatellites, AFLP. But RAPD-PCR markers are less expensive, than other approaches. Therefore they can be developed in each Hydrobiology laboratory.

MATERIAL AND METHODS

Material of *Daphnia cucullata*

Samples of *Daphnia cucullata* were collected from the deepest places of East Latvia lakes in May – September from 2007 to 2012. The collected material was preserved and stored in 70-98% ethanol. 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 *Daphnia cucullata* samples in 98% ethanol was cost-effective and appropriate for many molecular analyses. The samples had to be preserved immediately after harvesting to prevent individuals from biochemical and molecular degradation (Harris et al. 2005).

Genomic DNA extraction

DNA extraction was performed using slightly modified methodology earlier described by Schwenk et al. (Schwenk et al. 1998), Fitzsimmons and Innes (Fitzsimmons & Innes 2005) and Harris et al. (Harris et al. 2005). The method Schwenk et al. (1998) consists of the following steps: Zooplankton samples were transferred to 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.

The method proposed by Fitzsimmons and Innes (2005) consists of the following steps: Zooplankton samples were transferred to 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.

The method proposed by Harris et al. (2005) consists of the following steps: Zooplankton 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). Lay the tubes on an agitator in an incubator at 37 ^{0}C. 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.

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 (Harris et al. 2005) with ethidium bromide.

RAPD analysis

55 random oligonucleotide primers from A, B, C and F sets (Carl Roth, Germany) were tested for possibility to use in population genetic studies of *Daphnia cucullata*. 9 primers from set A (OPA-02; OPA-03; OPA-04; OPA-05; OPA-07; OPA-09; OPA-10; OPA-12; OPA-13), 6 primers from set B (OPB-03; OPB-04; OPB-07; OPB-08; OPB-10; OPB-12), 2 primers from set C (OPC-11; OPC-20) and 1 primer from set F (OPF-10) were used (Table 1).

Three different PCR mixes were used for preparation of RAPD-PCR reactions. The polymerase chain reaction (PCR) was performed in 12 µl.

Table 1.Primers used in RAPD analyses

Primer	Primer sequence 5'⇨3'
Roth OPA-02	TGC CGA GCT G
Roth OPA-03	AGT CAG CCA C
Roth OPA-04	AAT CGG GCT G
Roth OPA-05	AGG GGT CTT G
Roth OPA-07	GAA ACG GGT G
Roth OPA-09	GGG TAA CGC C
Roth OPA-10	GTG ATC GCA G
Roth OPA-12	TCG GCG ATA G
Roth OPA-13	CAG CAC CCA C
Roth OPB-03	CAT CCC CCT G
Roth OPB-04	GGA CTG GAG T
Roth OPB-07	GGT GAC GCA G
Roth OPB-08	GTC CAC ACG G
Roth OPB-10	CTG CTG GGA C
Roth OPB-12	CCT TGA CGC A
Roth OPF-10	GGA AGC TTG G
Roth OPC-11	AAA GCT GCG G
Roth OPC-20	ACT TCG CCA C

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 (MBI Fermentas, Vilnius, Lithuania); 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 (Table 2).

The composition of second mix of PCR: 10 × Taq buffer with $(NH_4)_2SO_4$; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania); 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 (Table 2).

The composition of third mix of PCR: 10 × Taq buffer with KCl; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania); 4 µl of genomic DNA sample (20 ng/µl); 1.2 µl of Triton X-100 (Sigma-Aldrich); 0.6 µl of each RAPD primer (1pmol/µl) (Carl Roth, Germany); 3.46 µl dd H_2O (Table 2).

DNA amplification was performed in *GeneAmp® ABI 9700* (Applied Biosystems) PCR system following two amplification cycles. The first amplification cycle: 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: 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). After the PCR amplification the samples were stored in a refrigerator (4 ° C) until the analytical separation in agarose gel.

RAPD - PCR fragments are separated using horizontal electrophoresis equipment (Cleaven Scientific) in 1.4% agarose gel with TBE buffer (0.045M Tris, 0.001M EDTA, 0.045M H_3BO_3, pH 8.3-8.4) with regime 4.3 $V*cm^{-1}$ 15 min, 6.5 $V*cm^{-1}$ 2 hours.

Table 2. Comparison of three different RAPD-PCR mixes

RAPD-PCR mixes	**10 × Taq buffer with $(NH4)_2SO_4$**	**10 × Taq buffer with KCl**	**mix with Triton X-100**
10 × Taq buffer; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase; 4 µl of genomic DNA sample (20 ng/µl); 0.6 µl of each RAPD primer (1pmol/µl) 4.66 µl dd H_2O	+		-
10 × Taq buffer; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase; 4 µl of genomic DNA sample (20 ng/µl); 0.6 µl of each RAPD primer (1pmol/µl) 4.66 µl dd H_2O	-	+	-
10 × Taq buffer; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase; 4 µl of genomic DNA sample (20 ng/µl); 0.6 µl of each RAPD primer (1pmol/µl) 1.2 µl of 4.66 µl dd H_2O	-	-	+
Results of amplification	negative results of amplification	positive results of amplification	low amplification

Into the wells of the gel two-five µl of dye solution consisting of 50% glycerin, 0.1M EDTA, 0.002% bromophenol blue was added for the degradation of the samples and tracking of the electrophoresis time in gel (Atienzar *et al.* 1999; Atienzar & Jha 2004). RAPD-PCR products were used for analysis of all PCR reaction volume (12 µl). The DNA marker (GeneRuler™ 100bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) was used to identify the size of the RAPD-PCR fragments. The amplification products were separated electrophoretically in order to detect presence/absence of a band of a specific molecular weight. 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).

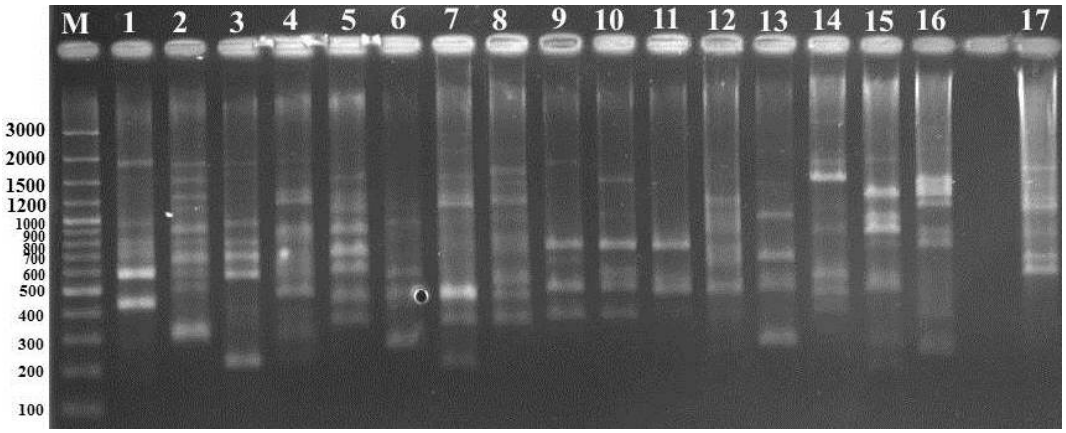

Fig.1. RAPD fingerprints results from different samples of *Daphnia cucullata* with primers OPA-03 and OPA-05 (M- marker, 1-16 runners- different samples of *Daphnia cucullata*; 17- control) using RAPD-PCR 10 × Taq buffer with KCl.

RESULTS AND DISCUSSION

Choice of genomic DNA extraction method

Genomic DNA was extracted from adult *Daphnia cucullata* individuals (20 – 30 specimens from each population), collected from four different lakes, which were preserved in 98% ethanol and stored at -20 C° (Fitzsimmons & Innes 2005, Harris et al. 2005, Hellsten & Sundberg 2000, Schwenk et al. 1998). Using DNA extraction methods following protocols developed by Schwenk et al. (Schwenk et al. 1998), Fitzsimmons and Innes (Fitzsimmons & Innes 2005) and Harris et al. (Harris et al. 2005), DNA was extracted with very low 0.2 ng / µl to 10 ng / µl concentrations or was not extracted at all. 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 body shell of *Daphnia cucullata*. First, organisms of *Daphnia cucullata* 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, cleaned glass sand was added to the sample. 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 40 ng / µl up to 318 ng / µl. In next step, the DNA extraction method proposed by Fitzsimmons and Innes (2005) was considered to be optimal. The method was used to *Daphnia pulex*. This method was the most optimal both in terms of reagent choice and time consumption. This DNA extraction method is the most appropriated also for DNA extraction from different small zooplankton species. DNA extracted by this method was used for randomly amplified DNA (RAPD) analysis.

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 (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, taking into account the fact that the PCR synthesis was performed using *GeneAmp® ABI 9700* (Applied Biosystems) PCR system, where the allowed reaction volume is 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

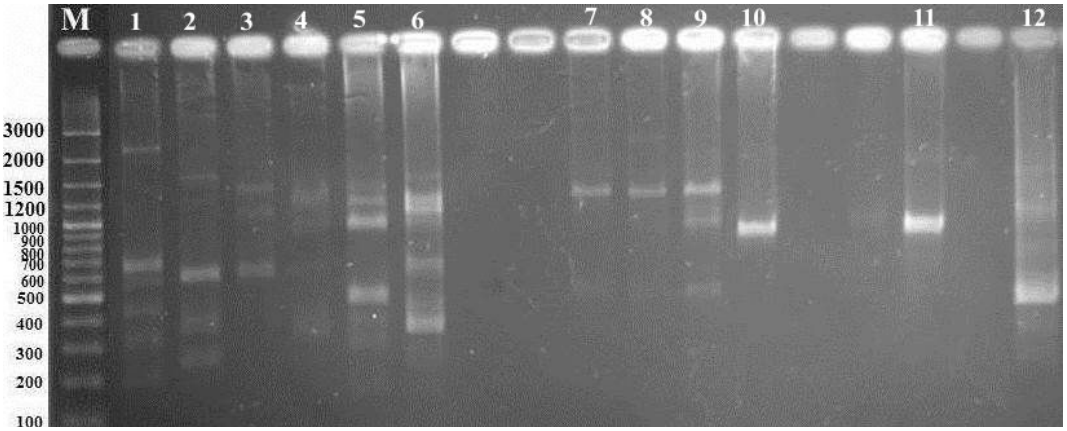

Fig. 2. RAPD fingerprints results from different samples of *Daphnia cucullata* with primers OPA-03 and OPA-05 (M- marker, 1-11 runners- different samples of *Daphnia cucullata*; 12- control) using RAPD-PCR 10 × Taq buffer with $(NH4)_2SO_4$.

experiments had been carried out we concluded that the optimal RAPD-PCR reaction volume for the studies of *Daphnia cucullata* population genetics was 12 µl with 46 cycles (Fig. 1).

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.2). It is known that one more factor determining the amplification of RAPD-PCR is the choice of optimal RAPD-PCR mix. Three different RAPD-PCR mixes were tested to find a suitable optimal PCR mix. RAPD-PCR mix of 10 × Taq buffer with $(NH4)_2SO_4$ didnot give any positive results and the mix with the addition of Triton X-100 gave poor results (Table 2). 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 (MBI Fermentas, Vilnius, Lithuania) (Fig. 1).

Screening of RAPD primers

Fifty-five 10-mer primers from Operon Kit A, B, C and F sets (Carl Roth, Germany) were used during the screening of the RAPD primers. The clear and reproducible banding patterns were selected. Out of these, only nineteen of the primers enabled amplification of DNA fragments showing polymorphisms (Table 1). Different primers produced different fragment pattern. Number of bands generated by each primer varied ranging from 0 to 13. Primers OPA-04, OPA-09 and OPC-20 formed the highest band number i.e. 13; primer OPB-08 formed the lowest band number i.e. 6. The sizes of bands ranged from 200 bp to 3000 bp. Different primers produced different fragments pattern (Table 3). These results reflect those recorded by other authors (Hasan et al. 2009; Parenrengi et al. 2000).

CONCLUSIONS

Despite the fact that literature describes a lot of DNA extraction methods from different zooplankton species, it is not always possible to apply these methods to our object without making some modifications. In our case, it was completely impossible to extract qualitatively the DNA from *Daphnia cucullata* using the methodology proposed by authors Schwenk et al. (Schwenk et al. 1998), Fitzsimmons and Innes (Fitzsimmons & Innes 2005) and Harris et al. (Harris et al. 2005) without modifications. In our case, the main problem was splitting the chitin shell of *Daphnia cucullata* specimens,

Table 3. RAPD analysis results of *Daphnia cucullata* using 19 primers

Primer	Ampified DNA fragment number	Lenght of the fragments of amplified DNA
Roth OPA-02	9	700 – 3000
Roth OPA-03	11	200 – 3000
Roth OPA-04	13	200 – 3000
Roth OPA-05	12	300 – 3000
Roth OPA-07	10	400 – 2000
Roth OPA-09	13	200 – 3000
Roth OPA-10	11	400 – 3000
Roth OPA-12	11	300 – 3000
Roth OPA-13	8	400 – 3000
Roth OPB-03	10	300 - 3000
Roth OPB-04	12	300 – 3000
Roth OPB-07	11	400 – 3000
Roth OPB-08	6	800 – 3000
Roth OPB-10	11	300 – 3000
Roth OPB-12	9	300 – 1500
Roth OPF-10	12	300 – 3000
Roth OPC-11	13	200 – 3000
Roth OPC-20	12	300 - 3000

and this problem was solved by homogenizing the samples at 100 ^{0}C, and the homogenization of samples by adding purified glass sand. Based on the received results, we can conclude that Fitzsimmons and Innes (Fitzsimmons & Innes 2005) proposed methodology with some modifications that allow to get the best extraction results for *Daphnia cucullata* according to concentration of the DNA evaluated by gel electropforesis and spectrophotometrically (DNA purity and quantity) in comparison to other methods. The screening of of RAPD 10-mer primers indicated that primers OPA-02, OPA-03, OPA-04, OPA-05, OPA-07, OPA-09, OPA-10, OPA-12, OPA-13, OPB-03, OPB-04, OPB-07, OPB-08, OPB-10, OPB-12, OPF-10, OPC-11, OPC-20 are suitable to be used in genetic studies of *Daphnia cucullata*. The DNA markers were polymorphic segments with band sizes from 200 bp to 3000 bp. The best amplification results were achieved with 46 cycles of amplification. Comparing three different mixes of PCR more efficient was mix consisting of: 10 × Taq buffer with KCl; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania).

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