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TESTING THE MICROSATELLITES-PCR MARKERS FOR GENETIC DIVERSITY RESEARCH OF ALIEN PONTO-CASPIAN AMPHIPOD *PONTOGAMMARUS ROBUSTOIDES* G. O. SARS, 1894

Aija Brakovska, Jana Paidere

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Microsatellites are successfully used for genetic studies for the monitoring of different Gammarus species. But microsatellites markers for the study of *Pontogammarus robustoides* population are not enough developed. Since the development of specific microsatellites primers requires time and material investments, therefore it may be used for research the primers designed to related species. We tested six developed microsatellites markers Dv-6, Dv-11, Gam-1, Gam-2, Gapu-9 and Gapu-17 for five related Gammarus species (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus roeselii*, *Gammarus orinos*, *Dikerogammarus villosus*), which showed the applicability of these markers for these species (Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014). Microsatellites-PCR based molecular markers are powerful tools for the analysis of genetic diversity of *Pontogammarus robustoides* specimens for which isolation of good quality genomic DNA and suitable microsatellites-PCR marker and protocol development is essential. This paper present a comparison of qualitative DNA isolation method and microsatellite-PCR markers for adults specimens of *Pontogammarus robustoides* population genetic study.

Key words: alien Ponto-Caspian amphipod, *Pontogammarus robustoides*, microsatellites-PCR.

Aija Brakovska, Jana Paidere. Institute of Life Sciences and Technology, Department of Ecology, Daugavpils University, Parādes str.1a, LV-5401, Daugavpils, Latvia, E-mail: aija.brakovska@inbox.lv

INTRODUCTION

Research on alien and invasive alien species is very relevant worldwide, as threats to biodiversity are linked not only to climate change and many other anthropogenic activities, but also to the progress, naturalization and impact of alien species. Ponto-Caspian Amphipoda species are in a constant focus of scientific research due to their successful biological invasion. Researches shows that introduced alien crustacean species (Amphipoda and other crustaceans) in the 1970s and 1980s, affect both the lower and higher stages of the food chain (phytoplankton, zooplankton, zoobenthos, juveniles of fish) with their aggressive (predatory) behavior at both structural and functional levels (Arbačiauskas et al. 2011, Arbačiauskas et al. 2013, Bacela & Konopacka 2005, Bacela-Spychalska & Van der Velde 2013, Bacela-Spychalska 2016,

Berezina 2007, Berezina 2016, Grabowski et al. 2007, Grudule et al. 2007, Gumuliauskaitė & Arbačiauskas 2008, Jazdzewski et al. 2004, Kobak et al. 2017, Šidagytė & Arbačiauskas 2016). One of the most aggressive group of peracaridan crustaceans in European freshwater is the amphipods (Grabowski et al. 2007). About twenty alien crustacean species from the Ponto-Caspian region are already known in European freshwaters (Berezina et al. 2011, Bij de Vaate 2002). One of the ways in which Amphipoda could enter European freshwaters was its introduction in the 1960s into the Kaunas Reservoir (Nemunas River) and into some Lithuanian lakes from the Black Sea (Arbačiauskas et al. 2011, Jażdżewska & Jażdżewski 2008), respectively, from here, they spread further (Arbačiauskas 2005). The introduction of Ponto-Caspian crustacean fauna into Latvia's inland waters was initially associated with their introduction as a valuable fish food base in the 1960s. It was realized based on such species as *Pontogammarus robustoides*, *Chaetogammarus warpachowskyi*, *Paramysis lacustris* and *Limnomysis benedeni*. *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 (Bodniece 1976, Grudule et al. 2007, Kachalova & Lagzdin 1968, Paidere et al. 2016, Paidere et al. 2019). The high invasiveness of species is due to its wide environmental tolerance, good adaptability, high fertility and behavior as an effective predator and omnivore (Arbačiauskas et al. 2013, Bacela & Konopacka 2005, Bacela-Spychalska & Van der Velde 2013, Bacela-Spychalska 2016, Berezina 2016, Früh et al. 2012, Grabowski et al. 2007, Hänfling et al. 2011, Havel et al. 2005, Johnson et al. 2008, Kobak et al. 2017, Ricciardi & MacIsaac 2011, Šidagytė & Arbačiauskas 2016). Alien species incl. the structures and reproductive parameters of the amphipoda population are one of the main preconditions for their successful invasion. Population stability depends on the age structure of the population, for example, a population with a high dominance of juveniles may indicate rapid population growth. It depends on many factors, such as the length of the reproductive period, the number of generations per year, the time it takes for an individual to reach sexual maturity, and other factors. Sometimes the age structure and reproductive performance of populations of even the same size but different alien amphipoda species or even different populations of the same species may differ and indicate a higher or lower potential of species (Hänfling et al. 2011, Havel et al. 2005, Johnson et al. 2008, Ricciardi & MacIsaac 2011). Various research approaches and methods are used to determine the success of the biological invasion of alien amphipoda in European freshwaters. However, there are still issues, especially in Latvia, about what processes regulate the spread of alien amphipoda species and the success of consolidation, what is the regional significance of these processes and the advantages in interaction with local communities, incl. local amphipoda at both the physiological and population structure and developmental levels. These studies are also paying increasing attention to genetic studies of alien species populations. 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 (Baltazar-Soares et al. 2017, Bock et al. 2015, Lawson et al. 2011, Lee 2002). Studies such as genetic diversity and their potential role in invasion success in the alien species *Pontogammarus robustoides* in Latvia have not been conducted so far. Accordingly, the aim of this study we made some modifications of other authors' methodologies available to us, in order to be able to find microsatellites-PCR based molecular 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. Consequently, to successfully carry out such studies are required the highest quality DNA material and suitable primers. Microsatellites are successfully used for genetic studies for the monitoring of different Gammarus species. But microsatellites markers

for the study of *Pontogammarus robustoides* population are not enough developed, to our knowledge, only few microsatellite loci have been developed for amphipods (Danancher et al. 2009, Wattier et al. 2006). Since the development of specific microsatellites primers requires time and material investments, therefore it may be used for research the primers designed to related species. Before microsatellite markers are used on a related species, they need to be optimized by testing them. We tested the developed markers for five related Gammarus species (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus roeselii*, *Gammarus orinos*, *Dikerogammarus villosus*) (Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014).

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 2020 (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. (Danancher et al. 2009, Ghareyazie & Mottaghi 2012, Harris et al. 2005).

Genomic DNA isolation

To screen for variability of these potential microsatellite loci, genomic DNA was isolated from muscle tissue of 94 *Pontogammarus robustoides* using *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany). DNA isolation are 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

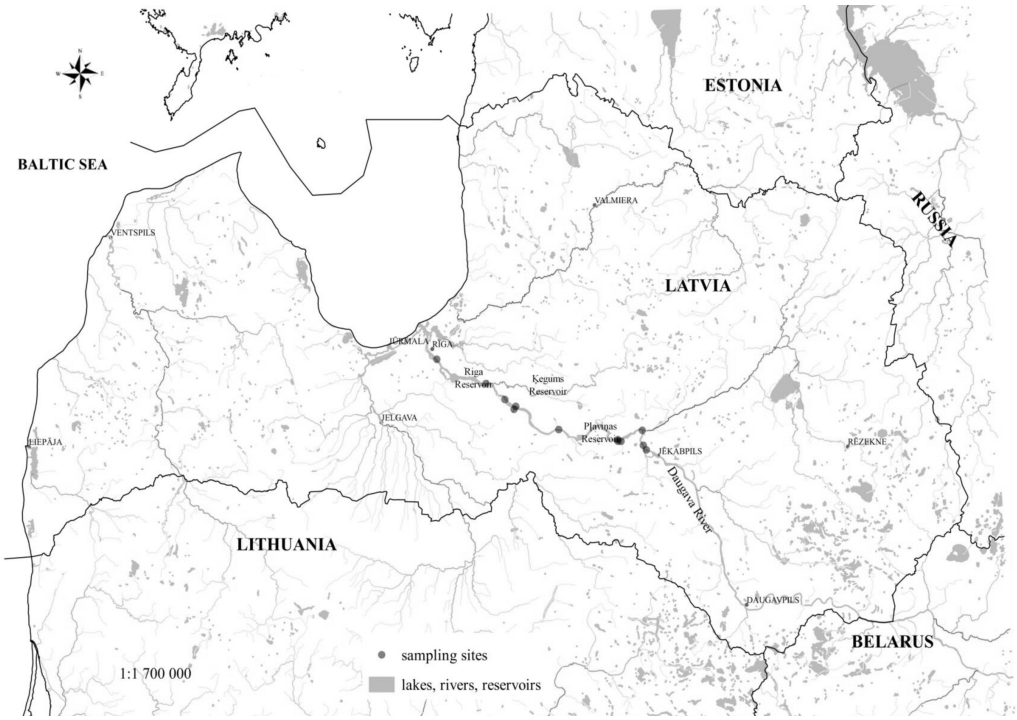

Fig.1. Localities of sampling sities in the Latvian reservoirs.

lysis is completed, centrifuge for 2 min at 11.000 g. Transfer supernatant into a new 1.5 ml tube, 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, Brakovska & Paidere 2019).

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 Elution Buffer (https://www.stratech.co.uk/wp-content/uploads/2016/10/Stratec-moolecular-DNA_tissue_cells.pdf; Brakovska & Paidere 2019). 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 (Ghareyazie & Mottaghi 2012; Harris et al. 2005) with ethidium bromide.

Microsatellite-PCR analysis and analytical seperation

Microsatellite markers developed for *Pontogammarus robustoides* were not found in the literature available to us, as well as in the gene bank database, therefore it may be used for this research the primers designed to related species (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus roeselii*, *Gammarus orinos*, *Dikerogammarus villosus*) (Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014). Before microsatellite markers can be used on a related species, for this purpose they must be optimized by testing. The sequences of the primers and nucleotides used in the study are summarized in Table 1.

We were testing microsatellite markers on *Pontogammarus robustoides* specimens using two different microsatellite-PCR mixes. 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); 2.10 µl of genomic DNA sample (20 ng/µl); 1.25 µl of each microsatellite marker (1 µM/µl) (Sigma-Aldrich Co.LLC) 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); 2.10 µl of genomic DNA sample (20 ng/µl); 1.25 µl of each microsatellite marker (1 µM/µl) (Sigma-Aldrich CoLLC.); dd H_2O.

DNA amplification was performed in *Eppendorf Mastercycler*® pro (Eppendorf) PCR system following two amplification cycles. - 98ºC 5 min; 40 cycles: 98°C 5 s (denaturation), X°C or 55°C (depending on primer melting temperature) 10 s (solicitation or primer annealing), 72°C 20 s (synthesis); final elongation step 72°C 1 min; 4°C (cooling). The second two step amplification cycle: initial denaturation - 95°C 3 min; 10 cycles: 95°C 45 s (denaturation), X°C or 55°C °C (depending on primer melting temperature) 40 s (solicitation or primer annealing), 72°C 40 s (synthesis) and 30 cycles: 95°C 30 s (denaturation), X°C or 55°C °C (depending on primer melting temperature) 40 s (solicitation or primer annealing), 72°C 40 s (synthesis); final elongation 72°C 2 min; 4°C (cooling). Both positive and negative controls (without DNA sample) were included in each series of amplification reactions. The products were

maintained at 4°C until loaded onto the gels.

Analytical separation of the amplification samples was performed with *Fragment Analyzer*™ Automated CE System (Advanced Analytical Technologies Inc.) with *PROSize* Data Analysis Software Revision 4.0.0.3. Accordingly, to check the quality of the microsatellite-PCR amplification, all manipulations were performed according to the manufacturer's recommendations.

RESULTS AND DISCUSSION

Genomic DNA isolation method for microsatellite-PCR analyse

Genomic DNA was isolated from muscle tissue of 94 *Pontogammarus robustoides* from different Daugava water reservoirs using *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany). Based on the received results from previous studies (Brakovska & Paidere 2019), we can conclude that DNA extraction method from *Pontogammarus robustoides* specimens 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 isolated DNA material and time consumption according to concentration of the DNA evaluated (DNA purity and quantity) in comparison to other methods who described by different autors (Danancher et al. 2009, Fitzsimmons & Innes 2005, Harris et al. 2005, Rewicz et al. 2014, Schwenk et al. 1998).

Optimization of microsatellite -PCR reaction

After the PCR reaction volume was optimized, instead of 10 µl (Danancher et al. 2009; Gergs et al. 2010; Rewicz et al. 2014), 12.5 µl (Westram et al. 2010) and 20 µl (Baltazar-Soares et al. 2017) 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, 33, or 35 (Baltazar-Soares et al. 2017, Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014, Westram et al. 2010). In our case, after several experiments had been carried out we concluded that the optimal microsatellite-PCR reaction volume for the studies of *Pontogammarus robustoides* population genetics was 12 µl with two step amplification type with 40 cycles (Fig. 2). Conversely, if we compare this data with other alternatives of microsatellite-PCR

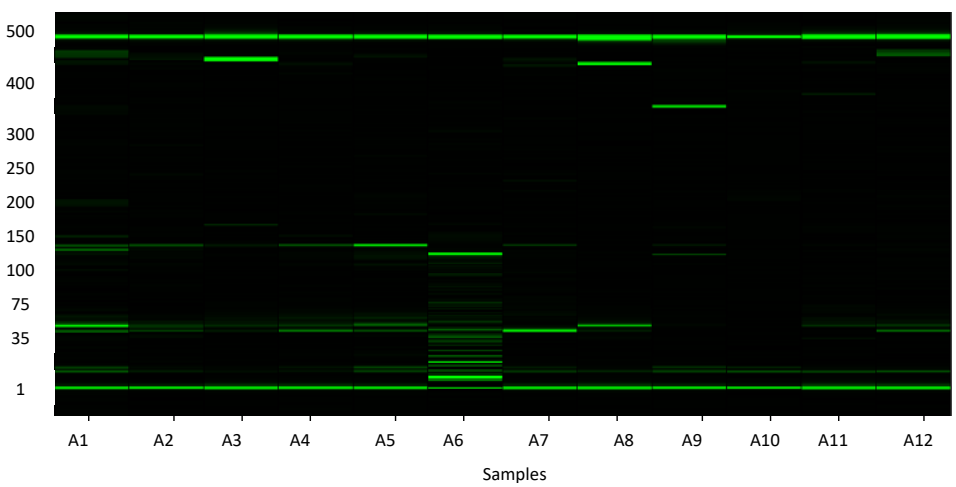

Fig. 2. Microsatellite fingerprints results from different samples of *Pontogammarus robustoides* from Pļaviņas Reservoir with primer Gapu-17 (A1-A12 runners- different samples of *Pontogammarus robustoides*) using microsatellite-PCR 10 × Taq buffer with KCl mix and two step amplification type with 40 cycles.

reactions, where the reaction volume is 12 µl, but the number of cycles is 40, but without two step amplification, the result will be different with poor amplification. It is known that one more factor determining the amplification of microsatellite-PCR is the choice of optimal microsatellite-PCR mix. Two different microsatellite-PCR mixes were tested to find a suitable optimal PCR mix. Based on the result of the research, the following microsatellite-PCR mix was found to be optimal allowing amplification with a well-defined contrast of the amplified fragments: 10 × Taq buffer with KCl; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (Thermo Fisher Scientific) (Fig. 2). Testing conducted with three repetitions.

Screening of microsatellite primers

Microsatellites are successfully used for genetic studies for the monitoring of different Gammarus species. But microsatellites markers for the study of *Pontogammarus robustoides* population are not enough developed. Since the development of specific microsatellites primers requires time and material investments, therefore it may be used for research the primers designed to related species. We tested six developed microsatellites markers Dv-6, Dv-11, Gam-1, Gam-2, Gapu-9 and Gapu-17 for five related Gammarus species (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus roeselii*, *Gammarus orinos*, *Dikerogammarus villosus*), which showed the applicability of these markers for these species (Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014).

All microsatellite markers who we used fom related Gammarus species (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus roeselii*, *Gammarus orinos*, *Dikerogammarus villosus*) (Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014) gave clear, but not reproducible banding patterns for *Pontogammarus robustoides* species specimens. The primers used and their nucleotide sequences were shown in the Table 1. The size of the scored polymorphic DNA fragments ranged from 67 bp to 489 bp. The number of alleles at each locus and the number of alleles on each microsatellite locus in investigated *Pontogammarus robustoides* population from some Daugava water reservoirs are differs (Table 1). These results do not reflect those recorded by other authors (Danancher et al. 2009, Gergs et al. 2010, Rewicz et al. 2014). The base pair lengths obtained in our study do not closely correspond to the Gammaridae base pair length range (Table 1, Fig. 3). This suggests that the obtained results are not interpretable and the selected markers six microsatellites loci Dv-6, Dv-11, Gam-1, Gam-2, Gapu-9 and Gapu-17 are unfortunately not available for future population genetic studies of *Pontogammarus robustoides*.

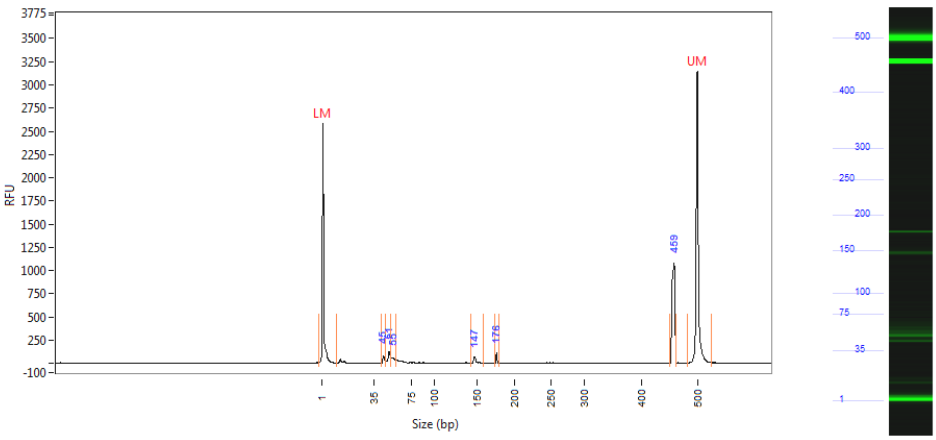

Fig. 3. Microsatellites amplification of *Pontogammarus robustoides* from Pļaviņas Reservoir used primer Gapu-17.

Table 1. Primers used in microsatellite analyses, sequence and sizes of general bands (* Data from literature sources: Danancher et al. 2009, Gergs et al. 2010; Rewicz et al. 2014 ; ** Our data)

Species	Locus	Primer sequence	Size (bp)
	Gam-1	**F**: TGCTACTCCTACCAACTACAAC **R**: GCGCAACTAACCAGTGAGC	
Gammarus fossarum *			337-346
Gammarus roeselii *			-?
Gammarus pulex *			334-346
Gammarus orinos *			340-343
Dikerogammarus villosus *			-?
Limnomysis benedeni *			-?
Pontogammarus robustoides **			134-185
	Gam-2	**F** : G C C A C A T A C A T A T A C G A ATACATACAC **R**: ATCGCAGTGGCTCTCTGAC	
Gammarus fossarum *			176-319
Gammarus roeselii *			-?
Gammarus pulex *			173-184
Gammarus orinos *			168-184
Dikerogammarus villosus *			-?
Limnomysis benedeni *			-?
Pontogammarus robustoides **			125-480
	Gapu-9	**F**: CTATGCCCAAGCTGGTTGTT **R**: TTCGCGTCATTCACTCGTAG	
Gammarus fossarum *			-
Gammarus roeselii *			149
Gammarus pulex *			-?
Gammarus orinos *			-?
Dikerogammarus villosus *			-
Limnomysis benedeni *			219
Pontogammarus robustoides **			67-112
	Gapu-17	**F**: ACTTGTACCGCCATCTGGAA **R**: GGTTGAGCATCGAATCTGGT	
Gammarus fossarum *			230
Gammarus roeselii *			234
*Dikerogammarus villosus**			228-240
Limnomysis benedeni *			234
Pontogammarus robustoides **			134-471
	Dv-6	**F**: ACACTGCCTATGTTTCCCCA **R**: AGGAAGCAAGGATTTAGGGC	
Gammarus fossarum *			-?
Gammarus roeselii *			-?
Dikerogammarus villosus *			150–190
Limnomysis benedeni *			-?
Pontogammarus robustoides **			134-489
	Dv-11	**F**: ATATGTCTGAGAGCATTTTGCC **R**: GTCGGTAAATCGACGCAT	
Gammarus fossarum *			-?
Gammarus roeselii *			-?
Dikerogammarus villosus *			190-194
Limnomysis benedeni *			-?
Pontogammarus robustoides **			94-110

CONCLUSIONS

Despite the fact that literature describes a lot of microsatellite-PCR analysis methods and primers designed to related Gammaridae species of *Pontogammarus robustoides*, it is not always possible to apply these methods and markers to our object without making some modifications. But not always making the necessary modifications can give the exact expected result. Based on the received genomic DNA isolation results, we can conclude that DNA isolation method of *Pontogammarus robustoides* specimens proposed by *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany) for microsatellite-PCR analysis was considered to be optimal. This method was the most optimal both in quality of isolated 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.

Optimization of microsatellite-PCR protocols has resulted in an optimal reaction protocol that can be used to perform genetic studies of *Pontogammarus robustoides* populations using species-specific microsatellite primers. Accordingly, in this case, our selected microsatellite-PCR loci (Dv-6, Dv-11, Gam-1, Gam-2, Gapu-9 and Gapu-17) from closely related Gammaridae species unfortunately cannot be used in genetic studies of *Pontogammarus robustoides* populations because they don't provide reliable and interpretable results for *Pontogammarus robustoides* species. There is a need for further studies to test the others microsatellite primers.

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