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COMPARISON OF FOUR DNA EXTRACTION METHODS FROM INVASIVE FRESHWATER BIVALVE SPECIES (MOLLUSCA: BIVALVIA) IN ROMANIAN FAUNA

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Abstract. In this paper we propose an evaluation of four DNA extraction methods in terms of DNA quantity, quality and success of the subsequent PCR amplifications of nuclear and mitochondrial loci. Individuals from the following freshwater invasive mussels in Romania were used to assess the efficiency of the DNA extraction methods: *Dreissena polymorpha*, *Dreissena bugensis*, *Sinanodonta woodiana* and *Corbicula fluminea*. While the manual method provided the highest amount of DNA, the subsequent PCR manipulation showed variable success rate, possible because of the phenol contamination of the samples. The commercial kits assayed performed equally well with the subsequent PCR manipulations of the isolated DNA.

Résumé. On a comparé quatre méthodes pour l'extraction de l'ADN qui provenait des espèces suivantes, envahissantes en Roumanie: *Dreissena polymorpha*, *Dreissena bugensis*, *Sinanodonta woodiana* et *Corbicula fluminea*. Les méthodes commerciales essayées ont fourni de l'ADN en concentrations variables mais d'une qualité suffisante pour l'amplification par PCR. La méthode manuelle (protocole de Sokolov) a fourni une plus grande quantité d'ADN, mais celui-ci était contaminé par du phenol, ce qui a rendu l'amplification par PCR difficile à réaliser.

Key words: DNA, DNA extraction, PCR amplification.

INTRODUCTION

Studying invasive species is a hot topic in conservation biology because their impact on native species, communities, and ecosystems has been widely recognized for decades (Sakai et al., 2001, Simberloff & Von Holle, 1999). On the other hand, invasive species are now viewed as a significant component of global change (Sakai et al., op. cit., Vitousek et al., 1996).

Some of the aspects of the invasive process (number of colonization events, size of the founding population, pathway of the colonization, etc.) can be revealed by molecular biology studies, and as consequence, the use of molecular biology methods in studies of evolution and population genetics of invasive species has increased dramatically in the last years (Graputto et al., 2005, Timmermans et al., 2005).

Many genomic DNA extraction protocols have been described for prokaryotes and eukaryotes, from cell sample to specific tissues (Sambrook et al., 1989). However, the DNA isolation from molluscs proved to be a difficult task, because the polysaccharides present in the animals co-precipitate with the DNA. These polysaccharides can inhibit the activity of many enzymes used in molecular biology, such as polymerases, ligases and restriction endonucleases (Sokolov, 2000). Good quality DNA is essential to achieve good results in experiments, especially in the Polymerase Chain Reaction (PCR), in which excess of cell debris and proteins may inhibit the amplification process (Mullis et al., 1992).

In this paper we propose an evaluation of four DNA extraction methods in terms of DNA quantity, quality and success of the subsequent PCR amplifications of nuclear and mitochondrial loci.

MATERIAL AND METHODS

Samples

Individuals from the following freshwater invasive mussels in Romania were used to assess the efficiency of the DNA extraction methods: *Dreissena polymorpha*, *Dreissena bugensis*, *Sinanodonta woodiana* and *Corbicula fluminea*. The mussels were collected by hand and kept in 95% ethanol for one to two years. Depending on the size of the animals, various parts of the body were used for DNA extraction: the whole body (for juveniles of *D. polymorpha*), foot muscle tissue samples (for *S. woodiana*) and foot muscle and hepatopancreas tissue samples (for *D. bugensis* and *Corbicula fluminea*).

DNA extraction

Total genomic DNA was extracted from a small amount of wet tissue by means of the following four procedures:

1. *mi-Tissue Genomic DNA Isolation Kit* (Metabion GmbH, Martinsried, Germany)

Solutions:

- Proteinase K (20mg/ml), RNase A (10mg/ml);
- Cell Lysis Buffer, PPT Buffer, Column Binding Buffer, Column Wash Buffer
- RNase
- TE Buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0)
- PBS (150mM NaCl, 10mM KHPO₄ pH 7.2. The KHPO₄ is a mixture of KH₂PO₄ and K₂HPO₄ to make pH 7.2),

Equipment:

- Vortex
- Centrifuge
- Water bath (65°C)
- 1.5ml microcentrifuge tubes
- Spin columns
- Collection tubes

Protocol:

Extraction was performed according to the *mi-Tissue Genomic DNA Isolation Kit* protocol, with small modifications: approximately 30mg of animal tissue were cut in small pieces (about 1mm³) and the material was washed with PBS, centrifuged for 10 minutes at 12000g, then submitted to the lysis stage; for the lysis step the material was incubated at 55°C overnight, in order to achieve complete digestion; the elution step was performed with 100µl TE buffer pre-warmed at 70°C for 5 minutes.

2. *NucleoSpin Tissue* (Macherey-Nagel, Düren, Germany)

Solutions:

- PBS (fosfat buffer salin)
- T1 buffer, B3 buffer, BW buffer, B5 buffer, BE buffer
- Proteinase K solution
- Ethanol 96%

Equipment:

- Vortex
- Centrifuge
- Water bath (56°C)
- Thermoblock (70°C)
- Mini spin Column *NucleoSpin Tissue*
- 1.5ml microcentrifuge tubes
- 2ml collecting tube

Protocol:

The DNA extraction was performed according to the *NucleoSpin Tissue* protocol, with the following slight modification: approximately 25mg of animal tissue were cut in small pieces (about 1mm³) and the material was submitted to the lysis stage; the ellution step was performed for 10 minutes at 70°C with 100µl of BE buffer.

3. *DNeasy Tissue* (Qiagen Inc., Valencia, USA)

Solutions:

- ATL buffer, AL buffer, AW1 buffer, AW2 buffer, AE buffer
- Proteinase K solution

Equipment:

- Vortex
- Centrifuge
- Water bath (56°C)
- DNeasy Mini Spin Columns in 2ml Collectin Tubes
- 1.5ml microcentrifuge tubes
- Collection Tubes (2ml)

Protocol:

The DNA extraction was performed according to the *DNeasy Tissue* protocol, with the following slight modification: approximately 25mg of animal tissue were cut in small pieces (about 1mm³) and the material was submitted to the lysis stage; the ellution step was performed for 10 minutes at room temperature, with 100µl of AE buffer.

4. *Sokolov protocol*

Solutions:

- Lysis solution (TrisHCl 50mM, NaCl 100mM, EDTA 10mM, SDS 1%, Proteinase K 0.2 mg/ml)
- Precipitation solution : saturated solution of KCl
- Phenol/chloroforme/isoamyl alcohol (25:24:1).

- Isopropanol
- Ethanol 70%
- TE Buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0)
- RNase A solution (10 mg/ml)

Equipment:

- Centrifuge
- Water bath (65°C)
- 1.5ml microcentrifuge tubes

Protocol:

The homogenized tissue (50 mg) was added to a 2ml microcentrifuge tube containing 1ml of lysis buffer, briefly vortexed and incubated at 55°C until the tissue was completely lysed. 100µl saturated solution of KCl was added and mixed by inverting the tube. The solution was incubated on ice for 5 min. In this stage most of the polysaccharides and some proteins were precipitated along with the insoluble SDS.

The lysate was centrifuged at 14.000 rpm for 15 min, and the supernatant was collected in a clean tube and extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The clear supernatant was transferred to a new tube and the DNA was precipitated by the addition of an equal volume of isopropanol, followed by mixing by inversion and incubating the sample at room temperature for 5-10 min.

The DNA was recovered by centrifugation for 20 min at 15.000Xg, and the pellet was washed with 70% alcohol. The DNA was dried at room temperature then dissolved in 100µl of TE buffer.

After the DNA was completely dissolved, RNase A (10 mg/ml) was added to the final concentration of 10µg/ml and the tubes were incubated at 37°C for 30-60 min.

DNA quality assessment

The DNA concentration and purity were assessed by spectrophotometry using the UV-VIS Nanodrop ND-1000 equipment (NanoDrop Technologies, Wilmington, USA). Complete spectra 220-350nm was recorded for every sample. DNA purity was determined by calculating the absorbance ratio A260/280. Pure DNA has a ratio of 1.8 ± 0.2 (Clark, 1997). Organic contamination was assessed by calculating the absorbance ratio A260/230 (Daneshwar et al., 2004).

PCR amplification

Two PCR amplification reaction were performed with the DNA samples extracted as described. The first PCR reaction amplified a fragment of nuclear ribosomal DNA while the second reaction amplified the COI gene from the mitochondrial DNA. The primers used are presented in table 1.

Table 1

Primer name	Nucleotide sequence 5'-3'	DNA target
ITS 1F	GGT GAA CCT GCG GAA GGA TCA	nuclear
ITS 1R	ACC CAC GAG CCG AGT GAT CC	nuclear
LCOI 1490	GGT CAA CAA ATC ATA AAG ATA TTG G	mitochondrial
HCOI 2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	mitochondrial

The first reaction (using primers for the ITS1 region) contained 20 to 30 ng of DNA, PCR buffer 1X (Fermentas UAB, Vilnius, Lithuania), 1.5mM MgCl₂, 100μM dNTPs, 0.2μM of each primer and 1 unit of Taq polymerase (Fermentas UAB, Vilnius, Lithuania) in a total reaction volume of 20μl. For the samples purified with the *mi-Tissue Genomic DNA Isolation Kit* the amount of DNA in the PCR reaction was 2μl of the DNA solution as presented in table 4, because of the low concentration level of these samples. The temperature profile of the PCR reaction is presented in table 2.

Table 2

No. of cycles	Denaturation	Annealing	Extension
1	94°C 2 min		
35	94°C 30 s	60°C 30 s	72°C 30 s
1			72°C 5 min

The second PCR reaction (the amplification of the COI gene) was performed in a total reaction volume of 20μL, containing 20-30ng of genomic DNA, PCR buffer 1X (Fermentas UAB, Vilnius, Lithuania), 2.5mM MgCl₂, 50μM dNTPs, 0.1μM of each primers and 0.5 units of Taq polymerase (Fermentas UAB, Vilnius, Lithuania). As in the previous PCR reaction, the DNA amount for the samples purified with the *mi-Tissue Genomic DNA Isolation Kit* was 2μl of the solutions presented in table 4. The temperature profile of the reaction is presented in table 3. In all cases the PCR amplifications were performed with a MJ Research PTC-100 equipment.

Table 3

No. of cycles	Denaturation	Annealing	Extension
1	94°C 1 min		
5	94°C 30 s	45°C 90 s	72°C 1 min
30	94°C 30 s	51°C 90 s	72°C 1 min
1			72°C 5 min

Electrophoresis conditions

The PCR products were analysed on 1.5% agarose gels in SB 1X (NaOH 100mM brought to pH 8.0 with H₃B₃O₄), containing 0.5μg/ml ethidium bromide. The same conductive media was used as electrophoresis buffer. The 100bp ladder mi-100bp+ DNA marker Go from Metabion was used to assign the size of the PCR fragments.

RESULTS

DNA extraction

The DNA concentration and the corresponding A230, A260 and A280 values, as well as the 260/280 and 260/230 ratios, for the four DNA isolation protocols are presented in table 4.

Table 4

<i>mi-Tissue Genomic DNA Isolation Kit</i>						
Sample ID	Coding	ng/ul	A260	A280	260/280	260/230
<i>Dreissena bugensis 1</i>	DB1M	2.13	0.043	0.009	4.94	-1.54
<i>Dreissena bugensis 2</i>	DB2M	2.58	0.052	0.055	0.93	-1.15
<i>Corbicula fluminea 3</i>	C3M	1.51	0.030	0.017	1.74	-0.44
<i>Corbicula fluminea 5</i>	C5M	1.44	0.029	0.022	1.33	0.07
<i>Sinanodonta woodiana 3</i>	W3M	5.76	0.115	0.069	1.68	0.19
<i>Sinanodonta woodiana 4</i>	W4M	6.54	0.131	0.095	1.38	3.49
<i>Dreissena polymorpha 1</i>	DD1M	1.30	0.026	0.018	1.41	-1.38
<i>Dreissena polymorpha 2</i>	DD2M	0.42	0.008	0.011	0.80	0.05
<i>NucleoSpin Tissue</i>						
Sample ID	Coding	ng/ul	A260	A280	260/280	260/230
<i>Dreissena bugensis 1</i>	DB1 or Db1	274.81	5.496	2.855	1.92	1.95
<i>Dreissena bugensis 2</i>	DB2or Db2	336.85	6.737	3.617	1.86	2.13
<i>Corbicula fluminea 1</i>	CF1 or Cf1	262.30	5.246	2.715	1.93	1.39
<i>Corbicula fluminea 2</i>	CF2 or Cf2	566.71	11.334	5.724	1.98	1.92
<i>Sinanodonta woodiana 1</i>	W1P,	419.44	8.389	4.058	2.07	2.06
<i>Sinanodonta woodiana 1</i>	W1M	93.51	1.870	0.886	2.11	2.00
<i>Sinanodonta woodiana 3</i>	W3M	459.57	9.191	4.477	2.05	2.03
<i>Dreissena polymorpha 1</i>	DP1 Dp1	513.83	10.277	5.152	1.99	1.85
<i>Dreissena polymorpha 2</i>	DP2 Dp2	953.18	19.064	9.270	2.06	2.05
<i>DNeasy Tissue</i>						
Sample ID	Coding	ng/ul	A260	A280	260/280	260/230
<i>Dreissena bugensis 1</i>	Db1	61.15	1.223	0.664	1.84	1.53
<i>Dreissena bugensis 2</i>	Db2	18.40	0.368	0.182	2.02	1.07
<i>Corbicula fluminea 1</i>	Cf1	53.81	1.076	0.564	1.91	0.77
<i>Corbicula fluminea 2</i>	Cf2	23.84	0.477	0.272	1.75	0.66
<i>Sinanodonta woodiana 1</i>	W1	80.83	1.617	0.736	2.20	2.24
<i>Sinanodonta woodiana 2</i>	W2	202.70	4.054	1.894	2.14	1.85
<i>Dreissena polymorpha 1</i>	Dp1	119.43	2.389	1.167	2.05	1.55
<i>Dreissena polymorpha 2</i>	Dp2	322.01	6.440	3.265	1.97	1.76
<i>Sokolov protocol</i>						
Sample ID	Coding	ng/ul	A260	A280	260/280	260/230
<i>Dreissena bugensis 1</i>	Db1	3125.71	62.514	33.522	1.86	1.89
<i>Dreissena bugensis 2</i>	Db2	3453.45	69.069	37.720	1.83	1.83
<i>Corbicula fluminea 1</i>	C1	851.07	17.021	9.191	1.85	1.54
<i>Corbicula fluminea 2</i>	C2	2874.14	57.483	30.466	1.89	1.70
<i>Sinanodonta woodiana 1</i>	W1	2402.08	48.042	24.768	1.94	1.51
<i>Sinanodonta woodiana 2</i>	W2	2245.59	44.912	22.683	1.98	1.81
<i>Dreissena polymorpha 1</i>	D1	38.40	0.768	0.584	1.31	0.43
<i>Dreissena polymorpha 2</i>	D2	3953.91	79.078	43.978	1.80	1.93

PCR amplification

In order to assess the utility of the extracted DNA in subsequent manipulation, we amplified a fragment of the nuclear ribosomal DNA ITS1 and one fragment of the CO1 gene from the mitochondrial DNA.

The results of the ITS1 amplification are shown in fig. 1 and fig. 2, while the results of the mitochondrial DNA amplification are presented in fig. 3 and fig. 4.

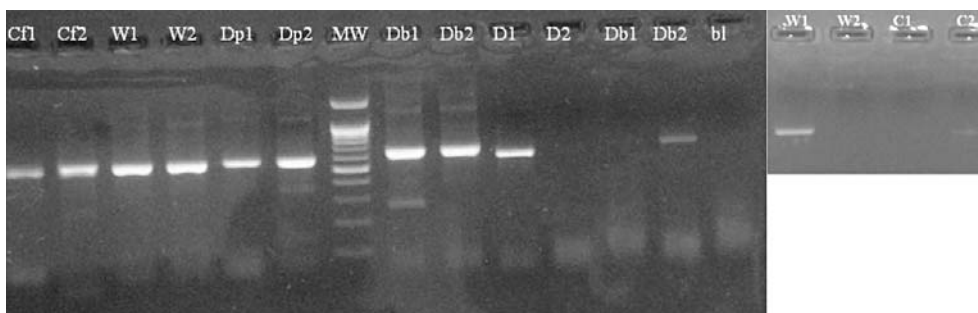


Fig. 1 – Amplification of ITS1 region from the nuclear genome. Agarose gel electrophoresis 1.5% with ethidium bromide 0.5µg/ml in gel; samples extracted with the Qiagen kit (Cf1, Cf2, W1, W2, Dp1, Dp2, Db1, Db2); samples extracted with the Sokolov protocol (D1, D2, Db1, Db2, W1, W2, C1, C2); MW –molecular weight marker mi-100bp+ DNA marker Go, Metabion; bl- PCR reaction negative control. The coding of the samples is the same as in table 4.

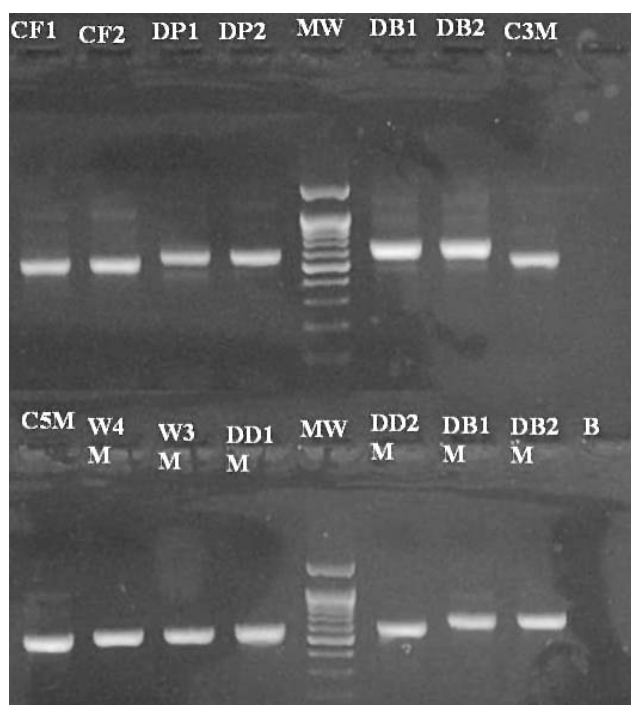


Fig. 2 – Amplification of ITS1 region from the nuclear genome. Agarose gel electrophoresis 1.5% with thidium bromide 0.5µg/ml in gel; samples extracted with the Macherey-Nagel kit (CF1, CF2, DP1, DP2, DB1, DB2); samples extracted with the Metabion kit (C3M, C5M, W4M, W3M, DD1M, DD2M, DB1M, DB2M); MW –molecular weight marker mi-100bp+ DNA marker Go, Metabion; B- PCR reaction negative control. The coding of the samples is the same as in table 4.

DISCUSSIONS

In this paper we compared four different methods of DNA extraction from freshwater mussels. The DNA isolation from molluscs is known to be challenging because of the high amount of mucopolysaccharides in the tissues of these animals, and the alcohol co-precipitation of these mucopolysaccharides with the DNA.

All three commercial kits, *mi-Tissue Genomic DNA Isolation Kit*, *NucleoSpin Tissue* and *DNeasy Tissue* are based on the principle of DNA binding to a silica matrix, followed by the elution of the DNA in an appropriate buffer. The Sokolov protocol is based on the KCl precipitation of polysaccharide content, followed by alcohol precipitation of the DNA.

The concentration and purity of the extracted DNA was assessed by spectrophotometric reading of the absorbance at the 260nm, respectively the ratio between the corresponding absorbance at 260nm/280nm and 260nm/230nm. Minimal protein contamination of the DNA sample is considered at ratios 260/280 between 1.8 - 2.0 (Clark, 1997), while minimal organic contamination occurs at ratios 260/230 between 1.8 - 2.2 (Daneshwar et al., 2004).

Taking in consideration only the spectrophotometric assay, the *mi-Tissue Genomic DNA Isolation Kit* appears to perform not so well, regarding both the amount and the quality of the extracted DNA (Tab. 4), in all four species. However, the PCR amplification of the ITS1 region from the nuclear genome and the COI gene from the mitochondrial genome was successful in all samples.

The *NucleoSpin Tissue* yielded high concentration (the average DNA concentration was 431.13ng/ μ l) and pure DNA solutions (see table 4). The PCR amplifications performed well, with all the samples being amplified.

The *DNeasy Tissue* yielded an average DNA concentration of 110.27ng/ μ l, about four times smaller than the previous kit. The DNA quality appears also to be inferior. However, the PCR amplification of both nuclear and mitochondrial DNA was very effective in all samples.

The *Sokolov* protocol, yielded the highest average DNA concentration (2368.04ng/ μ l). While the protein purity of the samples appeared to be satisfactory (most samples had 260/280 values above 1.8), the organic contamination appeared to be higher than in the other protocols (most 260/230 ratios are below 1.8); the fact is not surprising, since the protein extraction was performed with phenol-chloroform in this method. The PCR amplification of the ITS1 region showed variable efficiency, with only four samples out of nine successfully amplified. For the COI gene amplification, only six samples out of eight were successfully amplified.

Among the four tested methods, the manual DNA extraction (Sokolov protocol) yielded the highest amount of DNA, but the phenol contamination of the samples is probably responsible for the variable efficiency of the subsequent PCR amplifications.

All three commercial kits performed equally well in the PCR amplifications, both with nuclear and mitochondrial oligonucleotide primers. The highest quality and average amount of DNA was provided by the *NucleoSpin Tissue* kit. The cost per DNA extraction reaction is variable among these kits, with the Qiagen product being the most expensive, followed by the Macherey-Nagel kit and the Metabion kit. However, the Metabion kit does not provide the proteinase K solution needed for the extraction and the quantity of the extracted DNA seems to be low.

In this paper we evaluated the success of three commercial kits and one manual protocol for the DNA extraction from four species of freshwater invasive mussels in the Romanian fauna. While the manual method provided the highest amount of DNA, the subsequent PCR manipulation showed variable success rate,

possible because of the phenol contamination of the samples. The commercial kits assayed performed equally well with the subsequent PCR manipulations of the isolated DNA. The choice among these kits is to be made according to the goals of the conducted research project. A long term available DNA collection needs a high DNA amount and quality. While an estimation of the average amount of the DNA isolated with these kits was obtained during the present study, the resilience of quality in time of the extracted DNA remains to be assessed by further studies.

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COMPARAREA A PATRU METODE DE EXTRAȚIE ADN DIN BIVALVE (MOLLUSCA: BIVALVIA) DULCICOLE INVAZIVE ÎN FAUNA ROMÂNIEI

REZUMAT

În acest studiu au fost evaluate patru metode de extracție ADN din patru specii de moluște bivalve invazive în fauna României: *Dreissena polymorpha*, *Dreissena bugensis*, *Sinanodonta woodiana* și *Corbicula fluminea*. Dintre cele patru metode testate, una (protocolul Sokolov) a furnizat o cantitate mare de ADN, dar calitatea acestuia a fost afectată de contaminarea cu fenol, ceea ce a condus probabil și la succesul variabil al amplificărilor ulterioare prin PCR a ADN obținut prin această metodă. Celelalte trei metode testate, sub forma unor kit-uri comerciale, au furnizat cantități variabile în limite destul de largi pentru ADN obținut, dar calitatea acestuia a permis amplificarea prin PCR cu succes în toate cazurile.

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