



A Quick and Efficient Method for DNA Isolation from Freshwater Ostracods

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Abstract

Freshwater ostracods are ecologically and evolutionarily important small free-living crustaceans occurring in lakes, rivers and small water bodies. However, despite their importance, only a few genetic studies have been conducted so far in ostracoda due to isolation of insufficient amounts of DNA. We present a simple, efficient and cost-effective total DNA extraction protocol from freshwater ostracods for PCR amplification. Total DNA was extracted from four freshwater ostracods; *Heterocypris incongruens*, *Cypridopsis vidua*, *Eucypris virens* and *Herpetocypris chevreauxi*. We also compared the performance of our protocol with two manual DNA extraction protocols (HotSHOT, Chelex-100) and a commercial kit (Highpure PCR template preparation kit-Roche). The success of the isolation protocol was tested by PCR and sequencing of 18S rRNA and COI regions. Although our technique, Roche and HotSHOT methods resulted in acceptable DNA concentrations and showed an amplifiable band of expected size, both our protocol and Roche generally gave better results than HotSHOT, whereas the poor results were obtained from Chelex protocol. Therefore, we suggest that filter-based DNA isolation is more reproducible than other protocols. Our protocol requires no hazardous organic solvents and adaptable to 0.5 mL 8-strip PCR tubes format facilitating DNA extraction from a large number of samples.

Keywords: Total DNA, freshwater ostracods, PCR.

Introduction

Ostracods (“seed shrimp”) are small free-living aquatic crustaceans whose body size typically ranges from 0.2 to 32 mm. With approximately 8000 living species and wide geographic distribution, ostracods are considered as evolutionary and reproductively successful organisms (Horne & Martens, 2000; Horne, Cohen & Martens, 2002). According to recent studies, one quarter of all living ostracods (about 2000 species) are freshwater ostracods and they are found in lakes, springs, rivers, streams, small water bodies such as; pool and ponds (Martens *et al.* 2008). Species in ostracods have several unique morphological features in terms of interior body regions and well-calcified, bivalved shells that are taken into consideration in taxonomic studies of freshwater ostracods. These calcified valves (carapace) constitute an integral part of exoskeleton and provide an extraordinarily rich fossil record dating back to Ordovician (about 500 million years ago) (Schön *et al.* 2003). Ostracods have several attractive ecological and biological phenomena hidden within their systems probably governed by their unique genetic capacity. For instance, a research has suggested that

some ostracoda species (darwinulids) are fully parthenogenetic individuals and they have survived without sex for about 200 million years (Martens *et al.* 2003). Recent studies have also shown that the freshwater ostracod eggs have broader environmental tolerance and found to be extremely tolerant to digestive enzymes, high salinity, deep freezing, hydration, UV-B radiation, hypoxia and insecticide treatment (Vandekerkhove *et al.* 2013). Other intriguing feature of ostracods is the occurrence of filamentous giant sperm cells whose size can be reach up to ten times the animal’s body size (Yamada & Matzke-Karasch, 2012).

Richness in species and fossil records, wide geographic distribution and some intriguing phenomena, as explained above, make ostracods important invertebrate model systems for molecular phylogeny, population genetics, phylogeography, evolutionary research, reproductive biology, ecology and palaeo zoogeography. However, more recently, a relatively small number of genetic studies have been made to investigate reproductive mode, phylogeny and population genetics of freshwater ostracods (Yamaguchi & Endo 2003; Martens *et al.* 2005; Martins *et al.* 2009; Bode *et al.* 2010), since the

routine isolation of high quality DNA from ostracods (or other small crustaceans) is standing as a major drawback of studies on ostracod genetics. DNA extraction difficulties arise mainly from organisms' body structure due to the fact that adult ostracods have small body size with only eight pairs of appendages and their soft body tissues are extremely reduced and encapsulated with well-calcified carapace. Moreover, under some circumstances, classification of freshwater ostracods by traditional techniques can be insufficient, mainly because of their small size and few informative morphological characters. Thus, there exist a need for rigorous molecular phylogenetic studies, which is specifically required for extracted total DNA. So far DNA extractions from freshwater ostracods have been conducted mainly based on two approaches Chelex-100 (Schön *et al.* 1998; Schön *et al.* 2000; Schön *et al.* 2003) and commercial kits (Estronza *et al.* 2016; Nigro *et al.* 2016).

In our study, we present a simple, efficient and cost-effective total DNA extraction protocol from freshwater ostracods for PCR amplification. Our protocol requires no hazardous organic solvents and it is adaptable to "0.5 mL 8-strip PCR tubes" format facilitating DNA extraction from a large number of samples. Besides, we compared the effectiveness of our protocol with two commercial kits and two non-commercial protocols.

Material and Methods

Sample Collection

Specimens of four freshwater ostracods; *Heterocypris incongruens*, *Cypridopsis vidua*, *Eucypris virens* and *Herpetocypris chevreuxi* were collected in the northern shore of Lake Kucukcekmece (Table 1). Taxonomic status of the ostracods was defined morphologically according to Meisch (2000). After species identification, all mature samples were stored at -80°C deep freezer to prevent molecular deterioration until molecular analysis. For *C. vidua* species, these individuals were cultured in 50 ml tissue culture flasks containing only boiled tap water (after cooling) and wheat straw fragments at a temperature of 18-20°C with a standard photo period of 12 h light/12 h dark. *C. vidua* species were cultured in the flasks because these experiments were

conducted in 8-strip PCR tubes.

DNA Extraction Protocols for Individual Ostracods

All reagents, consumables and equipment used throughout the procedure must be sterilized before use. Commercial ultra-pure water or water with a similar purity grade is recommend to prepare all buffers. Prior to DNA extraction, considering that only a single organism was used in each experiment. Also, regardless of the extraction methods employed, carapace-valves of each ostracod were crushed against the side of the tube using a sterile pipette tip. After washing in PBS (1X) for 15 minutes, each species is placed in 0.5-ml microcentrifuge tube or 8-strip tubes containing 200 µl digestion buffer [600 mM NaCl, 5 mM CaCl₂, 100 mM Tris-HCl (pH 8.0) and 5 mM EDTA (pH 8.0)] and incubated for 2h at 70°C in a shaking incubator with gentle agitation (80 rpm). Following the incubation period, buffers were cooled down to 55°C and tissue digestion continued for a further 1 h by the addition of 16 µl proteinase K (20 mg/ml) and 20 µl SDS (10%) solution. As chaotropic agents, 150 µl of a pre-prepared binding solution (4M guanidine thiocyanate, 10 mM Tris-HCl, pH: 6.0-7.0) and 75 µl of 100% isopropanol were then added to this mixture. After transferring the whole homogenate from each tube into a 1.5-ml spin column using a pipette (multi-channel pipette for strip tubes), the homogenate was centrifuged at 10,000 g for 1 min. After centrifugation, 200 µl washing buffer (10 mM Tris, 0.5 mM EDTA, 50 mM NaCl and 50% absolute ethanol) was added and re-centrifuged at 8,000 g for 1 min. This washing step was repeated two times. Then, DNA from the spin column was eluted into a new 1.5-ml microcentrifuge tube (10,000 g for 2 min) using pre-heated TE elution buffer (10 mM Tris-HCl, 1 mM EDTA at 65°C). Isolated and PCR-ready DNA was stored at -20°C until further analysis (Figure 1).

As a commercial DNA extraction kit, High pure PCR template preparation kit (Roche Applied Science) was used according to the manufacturer's instructions. The DNA extraction kit contain four buffers; tissue lysis, binding, inhibitor removal and wash buffers. Briefly, each freshwater ostracoda was lysed using 200 µl tissue lysis buffer and 20 µl Proteinase K solution (20 mg/ml). After incubation at

Table 1. The summary of collection date and area of four freshwater ostracoda species

Species	Collection region	Sampling date	Number of sample
<i>Heterocypris incongruens</i>	Lake Kucukcekmece (40.030 N 28.442 E)	30-Apr-2013	36
<i>Cypridopsis vidua</i>	Lake Kucukcekmece (40.030 N 28.442 E)	30-Apr-2013	27
<i>Cypridopsis vidua</i>	Lake Kucukcekmece (41.004 N 28.454 E)	27-Jun-2013	44
<i>Eucypris virens</i>	Lake Kucukcekmece (40.592 N 28.440 E)	30-Jun-2013	31
<i>Herpetocypris chevreuxi</i>	Lake Kucukcekmece (40.592 N 28.440 E)	30-Jun-2013	26

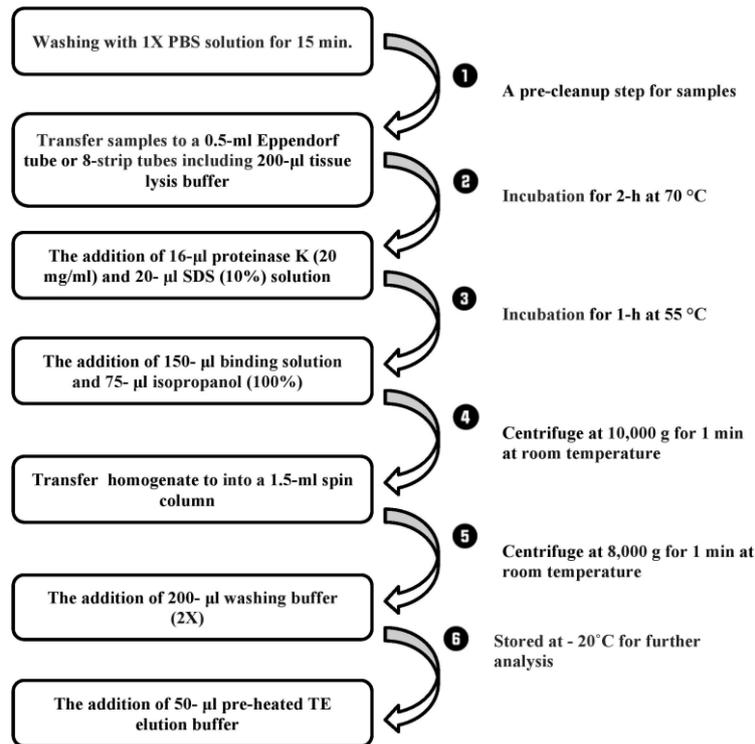


Figure 1. The schematic chart presents detailed step-by-step protocols for DNA isolation technique.

55°C for 1, 200 µl binding buffer and 100 µl isopropanol were added to lysate, following the manufacturer's spin-column protocol for animal tissues. The DNA bound on a spin-column membrane was cleaned and washed with inhibitor removal and washing buffer before elution. To maximize DNA yield, two successive elution steps (each 50 µl) were applied. Genomic DNA Purification Kit (GeneJET, Thermo scientific) was also used for DNA extraction from ostracoda according to the manufacturer's instructions. First, each sample were lysed in 400 µl of lysis solution and incubated at 65°C for 10 min. After the addition of 600 µl of chloroform, the mixture was centrifuged (10,000 rpm for 2 min) and upper aqueous phase containing DNA was transferred to a new tube including 800 µl precipitation solutions. Sample was re-centrifuged as above and supernatant was removed before resuspending the potential DNA pellet in 100 µl of NaCl. Then, 300 µl of cold ethanol was added to solution and finally, the DNA was washed with 70% ethanol, dried thoroughly and resuspended in 100 µl of sterile deionized water by gentle vortexing. Two manual DNA extraction techniques; Chelex-100 and HotSHOT extraction were also used for DNA isolation from freshwater ostracods. These DNA isolation procedures were conducted according to the method of Montero-Pau *et al.* (2008). Briefly, each ostracod was transferred individually to a 0.2 mL tubes containing 50 µl of a 5% Chelex solution, followed by incubation at 56°C for 30 min and boiling at 100°C for 8 min to lyse the cells and denature the proteins. The resulting

supernatant contains the DNA after centrifuge. As for HotSHOT extraction, individual ostracods were transferred into a 0.2 mL tube containing 50 µl of alkaline lysis buffer and carapace of ostracods were crushed against the side of the tube using a sterile pipette tip, allowing the lysis of inner tissues. After incubation at 95°C for 30 min, samples were stored on ice for 4 min and 50 µl neutralizing buffer was added to each tube. The samples were vortexed briefly and stored at -20°C until PCR reactions. Total DNA quantity and quality were assessed using a NanoDrop 2000c spectrophotometer.

PCR Amplification

To evaluate the success of DNA isolation, a fragment of the 18S rRNA and mitochondrial cytochrome c oxidase subunit I (COI) genes were amplified using specific primers. The forward and reverse primers for 18S rRNA gene are 5'-TGGCTCATTACATCAGTTATGGTT-3' and 5'-CGACGATCT AAGAATTTACCTCT-3', respectively (designed based on the sequence of *Heterocypris salina* 18S rRNA (GenBank Accession: KX228779.1) using Primer3 tool with default parameter)) whereas the forward and reverse primers for COI are 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' and 5'-GGTCAACAAATCATAAAGATATTGG-3' (Folmer *et al.* 1994), respectively. The PCR was performed using a TC-5000 gradient thermal cycler (Techne, UK) in 50 µl reaction volumes containing

27.6 µL sterilized deionized water, 0.4 µL Ex Taq polymerase (5 U/µL Takara), 5 µL Ex Taq buffer (Takara), 4 µL MgCl₂ (25 mM), 5 µL dNTP mix (2.5 mM each), 1.5 µL forward primer (10 pmol), 1.5 µL reverse primer (10 pmol) and 5 µL template DNA under the following conditions: one cycle of an initial denaturation at 94 °C for 5 min, 35 cycles of 30 s denaturation at 94 °C, 1 min annealing at 54°C (for COI gene), 56°C (for 18S rRNA) and 1.5 min extension at 72°C and followed by 10 min final extension at 72°C. The amplicons were separated and manually checked by electrophoresis in 1.5 % agarose gel with a negative control using 1X TBE buffer. The resulting PCR products were purified using a High Pure PCR product purification kit (Roche Applied Science) following the manufacturer's instructions. The completely purified products were directly sequenced on an automated sequencer at the GATC sequencing facility (Konstanz, Germany) using the above mentioned PCR primers. The forty-one nucleotide sequences of COI and 18S rRNA genes from each species have been submitted to GenBank and can be retrieved under accession numbers presented in Supplementary Table 1.

Results

In this study, we presented a practical and efficient DNA extraction technique, providing a sufficient amount of DNA from fresh water ostracods for approximately 40 PCR reactions (for 50 µL reaction volume). Our technique utilizes a joint lytic effect of high-salt buffer and proteinase K in a sequential manner. To evaluate the effectiveness of our technique, we applied four different techniques for extracting DNA from four different freshwater ostracod species. In our technique, the yield of total DNA was ranged from 174.6 to 48.3 ng/µL. The average DNA concentrations (\pm the standard error [SE]) were 100.6 ± 11.5 , 98.9 ± 10.5 , 111 ± 12.3 , 79.5 ± 8.1 ng/µL for *C. vidua*, *H. incongurens*, *E. virens* and *H. chevreuxi*, respectively. We also obtained a sufficient quantity of total DNA for a successful PCR reaction when applying Roche total DNA extraction method.

The nanodrop results for Roche extraction method ranged from 23.63 ± 1.1 to 113.50 ± 23.6 ng/µL (Table 2). By using GeneJET DNA purification kit, any DNA could not be recovered from this commercial kit even though it gave a good yield of DNA from a relatively big decapod crustacean (i.e. *Astacus leptodactylus* or *Parapenaeus longirostris*, data not shown). As a non-commercial method, the extraction with HotSHOT protocol yielded DNA in low concentrations ranged from 14.6 ± 1.71 to 28.1 ± 5.38 , nevertheless the protocol provide reliable amplification of the two investigated fragments (Table 2). The sizes of 18S rRNA and COI genes for each ostracod species were approximately 800 bp and 700 bp in length, respectively (Figure 2). As shown in

Figure 3a and 3b, both 18S rRNA and COI amplicons showed strong bands on agarose gels. Similarly, high pure PCR template preparation kit (Roche)-based DNA extraction method also gave strong bands on agarose (Supplementary Figure 1a and 1b). We had no DNA bands on agarose corresponding to the 18S and COI when applying GeneJET DNA purification kit.

As for other DNA extraction methods, HotSHOT protocol gave an acceptable performance in PCR amplification of these genes, but the protocol showed slightly weaker bands on agarose gels as compared to our protocol and Roche extraction method (Supplementary Figure 2a and 2b). Despite showing an amplifiable band of expected size, Chelex technique gave poor and inconsistent amplification results in terms of species and DNA fragments. For instance, 18S rRNA can be amplified by PCR for *C. vidua*, *E. virens* and *H. incongurens* but the expected band failed to amplify in the three independent PCR reactions for *H. chevreuxi*. An inconsistency has also occurred for amplifying *H. incongurens* using Chelex-100 method (Supplementary Figure 3a, lines 5-6). Unfortunately, using Chelex-100 technique, we could not obtain COI amplicons except for *C. vidua* although numerous temperature and Mg²⁺ concentration gradient-PCR experiments (Supplementary Figure 3a and 3b). We failed to amplify COI from *H. chevreuxi* DNA in all PCR reactions and it was omitted from further consideration probably due to the use of unsuitable primer pairs.

Discussion

The success rate of a DNA extraction protocols from small animals (less than 0.3 cm) or zooplankton, diapausing eggs and cysts are usually measured indirectly by successful PCR amplification. As shown in Table 2 and Figure 2-3, the PCR-sufficient DNA concentration was obtained from each freshwater ostracods whose sizes ranged from 0.1 to 0.3 cm using our protocol. Although both our protocol and Roche-based isolation gave strong PCR bands (Figure 2 and Supplementary Figure 1a and 1b), DNA concentration obtained by our protocol were found to be slightly higher than Roche-based isolation (Table 2). This result can be attributed to the sequential disruptive effect of high-salt buffer, proteinase K and SDS solution. Because high temperature and saline buffer help to break ostracods into small pieces and the surface area of the tissues increases, proteinase K and SDS solution proteinase K and SDS solution can work more effectively to release DNA. It was suggested that HotSHOT (known as “hot sodium hydroxide and Tris”) and its modified versions (Ultra HotShot and HotSHOT with proteinase K) could be successfully applied to extract total DNA from diapausing eggs from aquatic invertebrates, zooplankton and copepod eggs (Montero-Pau et

Table 2. The summary of collection date and area of four fresh water ostracoda species

	<i>C. vidua</i>				<i>H. incongruens</i>				<i>E. virens</i>				<i>H. chevreuxy</i>			
	Ours	Roche	Chelex	HotShot	Ours	Roche	Chelex	HotShot	Ours	Roche	Chelex	HotShot	Ours	Roche	Chelex	HotShot
	174,6	30,8		7,2	149,3	48,3		12,7	122,3	79,5		9,4	107,6	19,2		6,2
	90,7	32,4		7,3	92,2	58,2		16,4	92,9	84,1		9,8	94,8	19,8		6,7
	84,1	33,9		28,6	86,7	66,0		17	81,4	86,9		9,9	87,5	21,2		16,7
	76,8	109,9		29,3	70,4	75,3		17,3	73,6	93,6		10,4	79,7	22,9		32,4
	56,7	112,8		6,1	58	76,8		17,6	66,0	96,7		17,1	50,5	23,7		33,9
	106,1	75,3		19,4	112,8	22,9		6,3	140,5	177,9		19,2	109,9	26,3		37,7
	107,4	76,8	NA	20,2	137,7	23,7	NA	26,9	146,5	259,3	NA	19,8	48,3	26,9	NA	43,5
	108,4	32,4		23,6	84,1	24,9		28,6	165	30,0		21,2	58,2	29,1		48,3
Mean	100,6	63,0		17,7	98,9	49,5		17,8	111,0	113,5		14,6	79,5	23,6		28,1
Standard Deviation																
	32,5	33,1		9,0	29,8	21,6		6,7	35,0	66,8		4,8	23,1	3,3		15,2
Standard Error																
	11,5	11,7		3,1	10,5	7,6		2,3	12,3	23,6		1,7	8,1	1,1		5,3

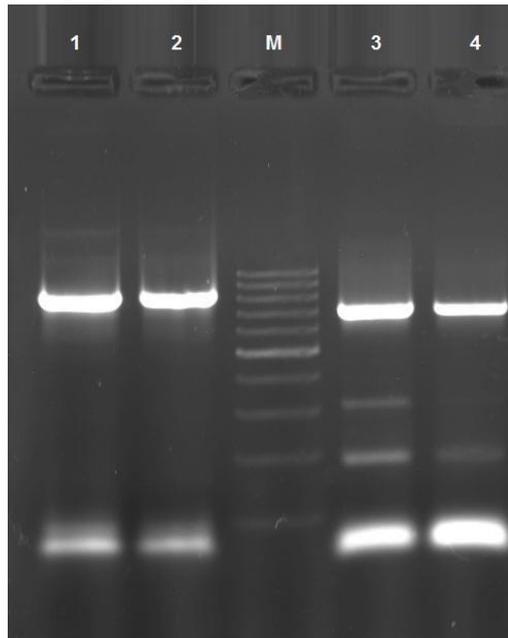


Figure 2. Amplicon sizes for 18S (Lane 1,2) and COI (Lane 3,4) were found to be ~800 and ~700 bp, respectively (DNA used PCR amplification extracted from *C. vidua* using our protocol).

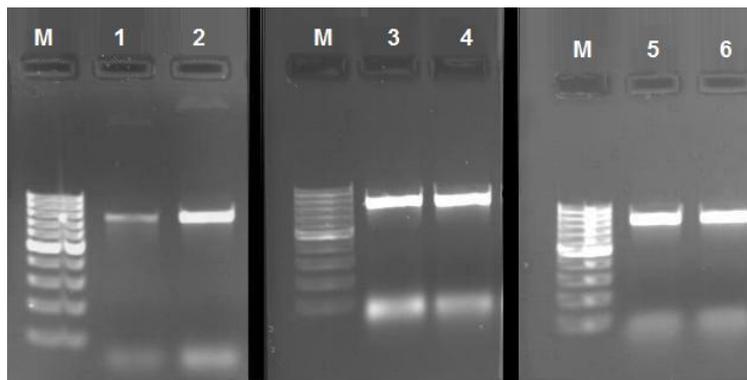


Figure 3a. Amplicons of 18S ribosomal RNA gene from the three freshwater ostracoda species using our technique. M: GeneRuler 100 bp DNA Ladder (Thermo); lanes 1-2: amplicons of *Herpetocypris chevreuxi*; amplicons of lanes 3-4: *Eucypris virens*; lanes 5-6: amplicons of *Heterocypris incongruens*. The reaction products were resolved on an 2% agarose gel at 90 volts for 1 hour. Please note that the amplicon for *C. vidua* shown in Figure 2.

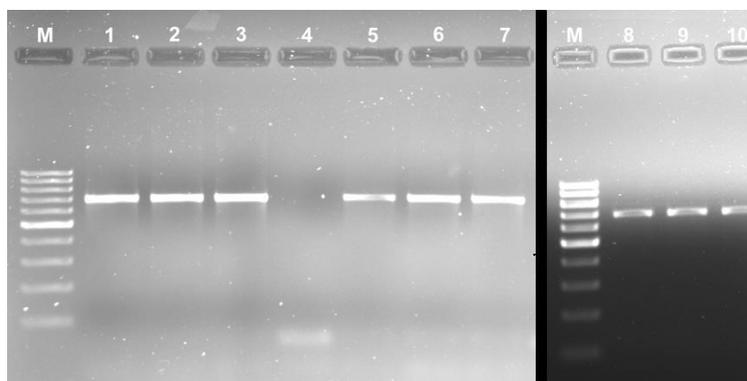


Figure 3b. PCR products for the COI (cytochrome c oxidase I) gene. M: GeneRuler 100 bp DNA Ladder (Thermo); lanes 1-3: amplicons of *H. incongruens*; lane 4: amplicon of *H. chevreuxi*; lanes 5-7: amplicons of *C. vidua*; lanes 8-9: amplicons of *Eucypris virens*.

al., 2008; Xu et al., 2011; Ishida et al., 2012). Although we were adhered to the HotSHOT procedure provided by Montero-Pau (2008) and it yielded sufficient DNA from ostracods for PCR reactions in a single tube, our protocol exhibited more superior performance in terms of amount of DNA and intensity of the positive bands. This result could be explained by the isolation protocols. Because, in filter-based DNA isolation techniques (our protocol and Roche), total DNA was recovered from a spin column which selectively retain DNA molecules and eluted into a buffer, whereas, in HotSHOT and Chelex-100 protocols, the cellular lysate and DNA molecules are not separated from each other. Both of them are rapid and cost-effective isolation protocols, but cellular debris contamination could be occurred especially during the PCR preparation though meticulous attention. Therefore, DNA extracted by filter-based DNA isolation techniques produced intense bands of appropriate size in all cases and they produced relatively high yields of total DNA compared to those obtained from nonfilter-based methods.

In conclusion, we provide consistent, cost-effective and PCR-amplifiable DNA isolation workflow for a freshwater ostracods. Our protocol can be used for routine use of PCR-based downstream applications such as; direct sequencing of amplicons, mini and microsatellite fingerprinting and small scale SNP identification as well as intersequence-specific PCR (ISSR).

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