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DNA extraction from crayfish exoskeleton

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Crayfish exoskeleton (CE) samples are generally less invasive and easy to be collected. However, it is difficult to extract DNA from them. This study was intended to investigate CE as a DNA source and design an easy and efficient DNA extraction protocol for polymerase chain reactions. Specific primer pair (PPO-F, PPO-R) was used to amplify extracted DNA from CE, and compared to crayfish tail muscle DNA sample. Moreover, seven microsatellites markers were used to amplify the CE DNA samples set. Since the extracted DNA from CE is suitable for gene amplification, the results present usefulness of CE as an easy and convenient DNA source for PCR-based population genetic research.

Keywords: Crayfish exoskeleton, DNA amplification, DNA extraction

Presently, crayfish tail muscles are primarily used for DNA extraction[1,2]. Collection of tail muscle samples is lethal and wasteful as the rest of the body parts are discarded. During sample collection, the crayfish exoskeleton (CE) are often wasted and the muscles left are too less to eat. Yet, if the samples are collected from some parts of crayfish body consisting principally of CE, such as chela, pleopod and pereiopod, the crayfishes are still alive and can be sold or eaten further. The CE samples are less invasive and can be easily obtained. A drawback of using CE as DNA source, however, is the difficulty of extracting genomic DNA from them, as their toughness makes them difficult to grind and lyse. Moreover, CE mostly contains copious astaxanthin which are not easy to precipitate and affect DNA quality[3]. Recently, Yue *et al*.[4] used the third pleopod as a DNA source for studying population genetic diversity of red swamp crayfish (RSC). They can obtain DNA samples successfully by a method[5] which was used to extract DNA from animal tissue since the third pleopod does not contain copious astaxanthin for DNA extraction. There are no reports on the DNA extraction method from the crayfish or other crustacean exoskeleton.

The present communication describes an efficient protocol for DNA extraction from RSC exoskeleton. Specific primers (PPO-F, PPO-R) are used to amplify extracted DNA from CE, and a comparison is made with tail muscle DNA sample to make sure the present method is reliable. Moreover, seven microsatellites markers are used to amplify the CE DNA samples to investigate the usefulness of CE as an easy and convenient DNA source for PCR-based population genetic research.

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Materials and Methods

Samples collection and CE samples processing—Red swamp crayfish (RSC; 50) individuals (body weight: 17.26 ± 0.38 g; body length: 6.34 ± 0.25 cm) were collected from a drain at Hengjin Village, Changzhou, Jiangsu Province, China. CE samples (50) were collected from each of RSC individuals' chela, and 30 tail muscle samples were also taken from these individuals. All samples were preserved separately in absolute alcohol and stored at -20°C for DNA extraction.

All the CE samples for DNA extraction were washed with distilled water, wiped with a paper towel, dried under natural conditions, cut into tiny pieces by a scissor and kept in 2 ml Eppendorf tubes. The tubes were labeled for subsequent use.

DNA extraction—DNA from 100 mg tail muscles was extracted according to Biase *et al*[6]. DNA from 100 mg CE was extracted using the modified protocol by extending the lysis time and adding an additional step for impurities precipitation based on the routine

salting-out protocol. In the modified protocol, 40 µl of 1 *M* DTT (dithiothreitol), 60 µl of 0.5 *M* EDTA and 10 µl proteinase K (20 mg/ml) along with 500 µl cell lysis buffer [100 m*M* Tris-HCl, *p*H 8.0; 50 m*M* EDTA, *p*H 8.0; 1% (w/v) sodium dodecyl sulphate] were respectively added into tubes containing pretreated CE samples before incubation at 65°C. The incubation step followed was extended from 3 to 4 h. After incubation, proteins and cellular debris were removed by adding 200 µl 7.5 *M* ammonium acetate and kept at 4°C for 10 min. Centrifugation was done at 12,000 rpm for 10 min. Additionally, 7.5 *M* ammonium acetate was added in the supernatant for the second time to completely remove impurities. The drying pellets were re-suspended in 50 µl sterile double-distilled water and DNA samples were stored at -20°C.

DNA quality and quantity were checked using the spectrophotometer method[7] on the following day. After about a week, DNA degeneration in duplicate samples was visualized by loading 10 µl DNA preparation (1 µl DNA sample, 1 µl loading buffer containing 2×GelRed™, and 8 µl sterile water) on 1.0% (w/v) agarose gel. A 1 Kb DNA ladder (TaKaRa) was used for size estimation by loading 5 µl DNA ladder mixture (4 µl DNA ladder and 1 µl loading buffer containing 2×GelRed™) on the gel.

Microsatellite genotyping—The concentration of DNA extracted from CE samples was uniformed to 100 ng/µl and arrayed to 96-well PCR plates for genotyping of microsatellites. Seven microsatellites were used to amplify the diluted DNA. Two CA- and GA-enriched microsatellites (*PCL02* and *PCL24*) were cloned from a partial genomic DNA library prepared by Zhu and Yue[8]. Additional five microsatellites (*PcLG-03, PcLG-07, PcLG-09, PcLG-29, PcLG-32*) were selected from Belfiore and May[9]. The PCR were performed on an Eppendorf Mastercycler gradient machine in 10 µl reaction volumes containing 50 ng DNA, 10× PCR buffer (TaKaRa) with 1.5 m*M* $MgCl_2$, 250 n*M* of each primer, 50 µ*M* of each dNTP and one unit of DNA polymerase (TaKaRa). Cycling conditions of reaction volume containing primers *PCL02* and *PCL24* were: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s with a final extension at 72°C for 5 min. For all other primers, the cycling conditions were: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s with a final extension at 72°C for 10 min.

Following amplification, each 6 µl PCR product mixture (4µl PCR product and 2 µl dye) was detected by DNA polyacrylamide gel electrophoresis (PAGE) and silver staining method. Sizes of alleles were determined according to a marker of puc18 DNA/*MspI* (TIANGEN).

DNA amplification—To see the quality of extracted DNA, the specific primer pair PPO-F (Forward primer: 5′-GCCAGGATAATACCCTACTC-3′) and PPO-R (Reverse primer: 5′-TGTCATGGCAGA ATGCCAGC-3′) was used to amplify the extracted DNA from CE and tail muscle. The PCR was performed in the 50 µl reaction system, optimized previously[10]. The PCR was performed as: 94°C for 5 min followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 45 s, and elongation at 72°C for 3 min, and 24 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and elongation at 72°C for 3 min, followed by a 10 min extension at 72°C and cooling to 4°C. The 5 µl PCR product mixture (4 µl PCR product and 1 µl dye) was run on 1% (w/v) agarose gels stained with GelRed™ (3×). DL2000 (TaKaRa) was used as DNA marker.

Statistical analysis—The number of alleles (*A*), the observed heterozygosity (H_O) and the expected heterozygosity (*He*) were determined by using the program POPGENE version 1.31[11]. Hardy-Weinberg departure value (*D*) was obtained using the equation, *D = (Ho–He)/He*. The polymorphism information content (PIC) was estimated according to the following formula[12]:

$$\mathrm{PIC}=1-\sum_{i=1}^{m} pi^2-\sum_{i=1}^{m-1}\sum_{j=i+1}^{m} 2pi^2pj^2 \qquad \ldots (1)$$

where p_i is the gene frequency of the *i* th allele, p_j is the gene frequency of the *j* th allele, and *m* is the allele number.

Results

Comparison of the quality of DNA extracted from tail muscles and CE—Spectrophotometric measurements of 30 different samples from tail muscles and CE indicated differences in DNA concentration and purity (Table 1). DNA extraction from tail muscle samples was successful in all 30 samples with an average of 673.1 ng/µl DNA concentration (range 148.1-1687.3 ng/µl) and an average of 336.55 ng/mg DNA yield (range 72.05-

843.65 ng/mg). DNA purity ranged from 1.78 to 2.07, with an average of 1.93. DNA was also successfully extracted from all CE, with DNA concentrations averaging 318.6 ng/µl (range 132.7-938.8 ng/µl) and DNA yields averaging 159.3 ng/mg (range 66.35-469.4 ng/mg). DNA purity ranged from 1.79 to 2.08, with an average of 1.95.

Degeneration of tail muscle DNA was slight to non-existent and there was even less apparent degeneration in the CE samples, while a clear band representing complete and good quality molecular DNA in the CE and tail muscle samples could be observed (Fig. 1).

PCR amplification—The genomic DNA fragment (about 1.7 kb) was obtained successfully by the specific primer pair (PPO-F, PPO-R) from CE and tail muscle sample DNA (Fig. 2).

Using CE as a DNA source for population diversity analysis—Total DNAs extracted from 50 individual CE samples were amplified using 7 microsatellites.

Table 1—CE and tail muscle DNA samples with their concentrations and purity

CE sample	Concentration (ng/µl)	Purity (OD260/280)	Tail muscle sample	Concentration (ng/µl)	Purity (OD260/280)
Ef1	197.8	2.05	Tf1	500.4	1.95
Ef2	754.3	1.99	Tf2	808.6	2.00
Ef3	336.0	2.00	Tf3	641	2.02
Ef4	925.7	1.95	Tf4	1214.1	2.04
Ef5	441.9	1.93	Tf5	703.3	2.03
Ef6	424.6	2.01	Tf6	1188.7	2.04
Ef7	352.6	2.08	Tf7	1221.2	2.02
Ef8	162.4	1.99	Tf8	1288.9	2.05
Ef9	282.1	2.03	Tf9	540.0	1.95
Ef10	277.1	2.04	Tf10	1010.5	2.02
Ef11	377.3	1.96	Tf11	1257.8	1.96
Ef12	319.7	2.04	Tf12	854.8	2.01
Ef13	208.7	1.96	Tf13	1687.3	2.06
Ef14	938.0	1.91	Tf14	849.9	2.02
Ef15	142.7	1.97	Tf15	1409.8	2.07
Em1	185.0	1.99	Tm1	203.5	1.83
Em2	177.9	1.99	Tm2	658.9	1.80
Em3	100.5	1.94	Tm3	192.6	1.84
Em4	361.0	1.88	Tm4	725.3	1.88
Em5	446.0	2.04	Tm5	281.7	1.84
Em6	369.9	1.96	Tm6	250.1	1.78
Em7	272.8	1.81	Tm7	202.1	1.87
Em8	212.0	2.01	Tm8	205.9	1.83
Em9	209.0	1.89	Tm9	173.9	1.80
Em10	132.7	1.80	Tm10	232.7	1.82
Em11	148.1	1.83	Tm11	148.1	1.91
Em12	186.3	1.79	Tm12	483.0	1.92
Em13	302.0	1.93	Tm13	220.6	1.80
Em14	169.1	1.87	Tm14	813.6	1.92
Em15	144.0	2.01	Tm15	224.7	1.79

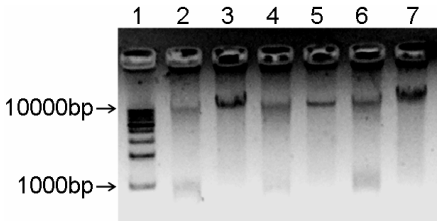

Fig. 1—Gel visualization of DNA degeneration in different crayfish exoskeleton and tail muscle samples. [Lane 1: 1Kb DNA ladder, Lane 2: DNA sample Tf2, Lane 3: DNA sample Ef2, Lane 4: DNA sample Tf5, Lane 5: DNA sample Ef5, Lane 6: DNA sample Tf14, Lane 7: DNA sample Ef14]

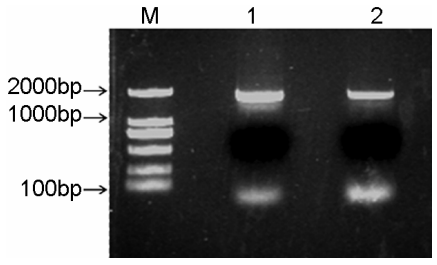

Fig. 2—PCR amplification comparison in DNA extracted from tail muscle and crayfish exoskeleton. [Lane M: DL2000 Marker; Lane 1: PCR products amplified from tail muscle sample; Lane 2: PCR products amplified from crayfish exoskeleton sample]

The results of amplification present clear polymorphic bands in the polyacrylamide gels as shown in Fig. 3. A total of 67 alleles were detected, ranging from a low of 3 alleles at locus *PCL24* to a high of 22 at *PcLG03*. The statistical results showed high-level genetic diversity in RSC (Tables 2 and 3).

Discussion

Most of the studies have been done on astaxanthin extraction or characterization from crustacean waste including CE[13,14]. But few studies have been undertaken on the DNA extraction from the crayfish or other crustacean exoskeleton. In fact, the CE samples are less invasive and easy to be collected, and can be cleaned before storing or extraction to avoid the unexpected external pollution sources as well. Because dithiothreitol can be used to break-up disulfide bonds in protein[15], high salt can be used to precipitate impurities including astaxanthin[16,17]. Dithiothreitol and ammonium acetate were used in the present modified protocol, and results showed that the purity of DNA isolated from the CE was similar to crayfish tail muscle samples (Table 1). Additionally, CE samples showed less apparent DNA degeneration (Fig. 1), which is possibly due to the reason that DNA in CE was protected by tough tissues in the samples collecting and storing period.

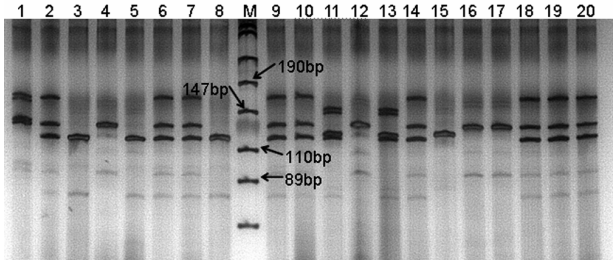

Fig. 3—Microsatellite amplification using the primer *PcLG*-07. [Lane M: puc18 DNA/*MspI* marker; Lane 1-20: the results of PCR amplification with microsatellite primer *PcLG*-07]

Moreover, as Table 1 showed, DNA yield of tail muscles averaged 336.55 ng/mg, which was similar to that obtained by Biase *et al.*[6] from swine solid tissue and Atmadja *et al.*[18] from human organs, and far less than that obtained by Aranishi[19] from single fish egg. DNA yield of CEs averaged 159.30 ng/mg, which was similar to that obtained by Yoshida-Yamamoto *et al.*[20] from nail clippings and Kumar *et al.*[21] from fish scales, and DNA yield was far less than that isolated by Eguchi *et al.*[22] from snake cast-off skin. The variability in DNA quantities of tail muscles may be due to the fact that different amounts of supernatant transferred to another new tube varied widely by different people. The variation in DNA yields of CE reported in the present study apart from the cause above, may be also due to the fact that different amounts of muscle tissue present on the CE or due to CE from different part of body. The DNA samples visualized by agarose gels seemed that the quantities of DNA were not consistent with the results of spectrophotometric measurement, which may be due to the fact that the DNA preparation contained the limited loading buffer which contained the scanty GelRed™ (Fig. 1).

In general, concentration of approximately 100 ng/μl DNA template prepared was used for polymerase chain reactions[4,23,24]. In the present study, the concentration of DNA extracted from CE (Table 1) demonstrates that all sample concentrations are adequate for PCR-based procedures.

Amplification success of using the primer pair PPO-F and PPO-R in crayfish tail muscle samples is

Table 2—Polymorphism information content (*PIC*), observed heterozygosity (H_o), expected heterozygosity (H_e) and Hardy-Weinberg departure value (*D*) of 7 microsatellite loci in the red swamp crayfish population

Loci	Number of alleles	Polymorphism information content (*PIC*)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)	Hardy-Weinberg departure value (*D*)
PCL-02	4	0.6232	0.6591	0.6878	-0.04173
PCL-24	3	0.3280	0.0612	0.3936	-0.8445
PcLG-03	22	0.9140	0.7143	0.9291	-0.2312
PcLG-07	7	0.6003	0.3542	0.6539	-0.4583
PcLG-09	6	0.7220	0.3061	0.7684	-0.6016
PcLG-29	14	0.8442	0.2449	0.8670	-0.7175
PcLG-32	11	0.7063	0.9070	0.7502	0.2090

Table 3—The mean number of alleles (N_a), mean number of effective alleles (N_e), mean polymorphism information content (*PIC*), mean observed heterozygosity (H_o) and mean expected heterozygosity (*He*) of 7 microsatellite loci in the red swamp crayfish population

Number of alleles(N_a)	Number of effective alleles (N_e)	Polymorphism information content (*PIC*)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)
9.5714	5.0190	0.6769	0.4638	0.7214

already documented[10]. The PCR amplification was demonstrated successfully in the CE samples using the same primer pair (PPO-F, PPO-R). Despite the primer dimers were visible which may be due to the problem of primer design, however, the same fragment of target (about 1.7 kb) was obtained with the PCR amplification in CE and tail muscles (Fig. 2). Results showed that CE DNA isolates did not yield DNA with more amplification problems than tail muscle DNA isolates, and showed that the CE sample DNA harbored the same genetic information as that contained by muscle sample.

The genetic diversity of the RSC population of Hengjin village showed high level of genetic diversity in RSC. The results were basically in accordance with the observation of Liu[1] and Wang *et al.*[2], and led us to the conclusion that the CE samples harbored the same genetic information as that contained by muscle samples, and the results showed that the CE samples could be used for the population genetic research as well.

In conclusion, the modified DNA extraction protocol is efficient and the CE is a useful and alternative DNA source for PCR-based genetic research.

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