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Research Article
Development of an Efficient Method for Producing High Quality Genomic DNA in Crustaceans

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Abstract

Background and Objective: High quality genomic DNA is essential for any Genotyping by sequencing technique (e.g., Restriction Site Associated DNA Sequencing). However, producing high quality genomic DNA from crustacean specimens has been difficult due to rapid degradation of tissue samples. In this study, an effective preservation and subsequent DNA extraction procedure was described for producing high quality genomic DNA in a crustacean shrimp, *Paratya australiensis*. **Materials and Methods:** Tissue samples were preserved following three preservation techniques: freezing (-80°C), 100% ethanol and RNAlater. Then DNA was extracted from each type of preserved sample using three different methods namely Econo spin column extraction, CTAB and salt extraction. **Results:** High quality DNA was produced only through Econo spin column extraction method from tissue samples preserved in 100% ethanol and RNAlater. In this case DNA fragments of >10,000 bp were produced without any smear or sign of degradation. The CTAB and salt extraction methods demonstrated very low quality or degraded DNA for all other types of preserved samples which is unusable for Restriction Site Associated DNA Sequencing (RAD-seq) library preparation. **Conclusion:** High quality genomic DNA was extracted only through Econo spin column extraction process preserving sample in 100% ethanol or RNAlater. The approach described here is easy, simple and does not require impractical cryopreservation methods.

Key words: Genomic DNA, crustaceans, restriction site associated DNA sequencing, Econo spin column, *Paratya australiensis*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For many biological and biomedical applications, DNA extraction is the most important step for subsequent molecular analysis[1,2]. High quality and quantity genomic DNA is the prerequisite for next generation sequencing (NGS) Technology[3]. Genomic DNA with large fragment size (>10,000 bp fragments; approx. 30 ng/5 µL) and without any visible degradation (smears on the gel) should be used for any NGS protocol e.g. Restriction Site Associated DNA Sequencing (RAD-seq)[4]. Preservation method of samples in the field may be a crucial step for producing high quality genomic DNA in the laboratory. Studies on arachnids and insects (Arthropods) have shown that these tissue samples degrade very rapidly and require instant and appropriate preservation immediately after capture[5,6]. Degradation of DNA takes place due to endogenous nucleases and oxidative processes, which cleave DNA strands and cause shearing of DNA, resulting in smaller DNA fragments. Degraded DNA is of limited use because larger DNA fragments are required for RAD-seq[7]. So, a successful preservation method needs to inhibit or prevent denaturation of enzymes and hence prevent DNA degradation.

Cryopreservation is one of the widely used methods for preventing DNA degradation due to nuclease activity. In most cases either dry ice (-78°C) or liquid nitrogen (-196°C) is used for this purpose[8] but the problem with dry ice is that it evaporates very fast and is a useful method of preservation only for short periods. On the other hand, liquid nitrogen is restricted in many parts of the world and is not very convenient to carry in the field. Formalin is widely used to preserve museum specimens but DNA is highly degraded even in neutralized formalin[9]. Again, ethanol (absolute, 95 and 70%) is widely used for preserving tissue samples in different species[1]. It has been suggested that 95% ethanol preserved samples should be kept at -20 or -80°C in arachnids[5].

Several studies have compared methods of tissue preservation for DNA studies in invertebrates[5,6] and vertebrates[10] but there is little or no evidence on tissue preservation and consequent high-quality genomic DNA production methods for crustaceans like shrimps and crabs. Fresh water shrimp *Paratya australiensis* has been found to be highly susceptible to DNA degradation (personal observation). Like many other crustaceans the endonuclease enzyme start degrading the DNA as soon as the shrimp is out of water (personal observation). For population genomics study on this species it is a prerequisite to produce high quality and quantity genomic DNA. So, the aim of the study was to develop an efficient method of preservation of shrimp tissue and subsequent DNA extraction that produces high quality genomic DNA suitable for RAD-seq library preparation.

MATERIALS AND METHODS

Sampling and preservation method: In total, 50 samples of freshwater shrimp *Paratya australiensis* were collected from Booloumba Creek (26°37.960 S, 152°39.124 E) of the Conondale Range, North-West of Brisbane, Australia using a seine net in January-February, 2015. Twenty *Paratya australiensis* samples were brought to the laboratory alive; afterwards they were preserved at -80°C and twenty samples were preserved in 100% ethanol (analytical grade) immediately after capture in the field and were stored at 4°C. Ten samples were preserved in RNAlater immediately after capture.

DNA extraction methods: The following DNA extraction protocols were used for the three types of preserved samples i.e., preserved at -80°C, in 100% ethanol and in RNAlater.

Econo-spin column extraction: This extraction protocol and the buffers are modification of the commercially available Qiagen DN-easy blood and Tissue Kit (QIAGEN, GmbH, D-40724, Hilden, Germany). DNA extraction started with adding 200 µL of lysis buffer (0.5% SDS, 250 mM NaCl, 25 mM EDTA, 200 mM Tris HCl ph7.5) and 10 µL of Proteinase K to 10-15 mg of sample and leaving it at 55°C on a dry block heater overnight. The next morning 100 µL of precipitation buffer (4 M ammonium acetate) was added and incubated for 10 min at room temperature. Later on, 300 µL of binding buffer (2 M Gu HCl, 75% ethanol) was added and ~500 µL of the solution was transferred to spin columns. After centrifugation at 6000 rpm for 3 min, the flow through solution was discarded. Then 500 µL of wash buffer (10 mM Tris HCl pH 7.5, ethanol 80%) was added, centrifuged at 6000 rpm for 4 min. After discarding the flow through another 500 µL of wash buffer was added and centrifuged at 20,000 rpm for 5 min. Flow through was discarded, columns were placed in a 1.5 mL Eppendorf tube and 60 µL of Elution buffer (10 mM Tris HCl pH7.5) was added. Two elution steps (30 µL+30 µL) were done and columns were centrifuged for 2 min at 6000 rpm.

CTAB extraction: CTAB extraction method was followed as described by Doyle and Doyle[11].

Salt extraction: The salt extraction method followed here was a modification of the standard salt strip tube method[11]. Sixty microliters of lysis buffer (50 mM Tris buffer, 20 mM EDTA and 2% SDS) and 0.3 µL of Proteinase K (20 µg μL^{-1}) was added to 10 mg tissue and left on the incubator at 55°C over night.

After complete digestion, 20 µL NaCl (5 M) was added and mixed properly. The sample was centrifuged (using Eppendorf AG, 22331, Hamburg, Germany) 4000 rmp for 10 min and the supernatant was carefully transferred to a new 1.5 µL eppendorf tubes. Then 70 µL of cold isopropanol was added to the supernatant, mixed well and kept at -20°C for 1 h to assist in precipitation of DNA. The tubes were then thawed and centrifuged at 4000 rmp for 15 min to form a DNA pellet and liquid was off carefully to avoid pellet loss. Then 100 µL of 70% of ethanol was added to wash the pellet and kept on the bench for 15 min and centrifuged at 4000 rmp for 8 min. The liquid was discarded and this washing step was repeated. In the final step pellet was dried in a vacuum chamber and rehydrated with 60 µL of ddH_2O.

DNA extracted from all 3 methods was kept at 4°C until further use.

DNA quantification: DNA concentration was quantified using a Qubit 3.0 fluorometer from Thermo Fisher Scientific according to the kit instructions.

Gel electrophoresis: Five microliters of the DNA were run at 80 V cm^{-1} for 40 min on a 0.8% agarose gel (consisting of 0.8 g agarose and 80 mL TAE buffer) and photographed under UV illuminator. One kb ladder (250-10,000 bp) was used as a DNA marker for the estimation of the fragment length.

RESULTS

Nine combinations of different DNA extraction methods applied to samples preserved at -80°C, in 100% ethanol and RNAlater are furnished in Table 1. Results showed that samples preserved in -80°C produced DNA of less than 250 bp fragment in Econo spin column, CTAB and salt extraction method. DNA of such small fragments is not suitable for any genotyping by sequencing (GBS) study. In contrast, samples preserved in 100% ethanol roduced DNA of more than 10,000 bp (>30 ng/5 µL concentration) fragments using the Econo-spin column extraction method (Table 1). However, CTAB and salt extraction methods failed to produce high quality DNA despite samples being preserved in 100% ethanol. The quantity of DNA was high (40-50 ng/5 µL, Qubit reading) using the CTAB method but the DNA fragments were not suitable for further study.

Samples preserved in RNAlater showed similar results to 100% ethanol preserved samples. High quality DNA was observed with Econo-spin column extraction method but CTAB and salt extraction failed to produce high quality DNA using samples preserved in RNAlater (Table 1).

DISCUSSION

The results of this study described an efficient and simple method of producing high quality genomic DNA from *Paratya australiensis*. Preservation and extraction techniques for high quality DNA vary in plants and animals[12-14]. With the advancement of sequencing technology (RAD-seq), high quality genomic DNA is essential for any genotyping by sequencing method. Our results showed that the efficient method of producing genomic DNA from crustaceans was to preserve the samples in 100% ethanol or in RNAlater immediately after capture and to use an Econo-spin column extraction method for DNA extraction. This study is based on a particular shrimp species, however, findings can be applied for other crustaceans.

Hykin *et al*.[15] extracted DNA from samples preserved in 70% ethanol and in formalin and managed to produce genomic scale DNA for high through put sequencing technology (NGS). So, ethanol has been considered as a good preservative for production of genome quality DNA. Mulcahy *et al*.[16] describes how different tissue preservation method influences production of genome-quality DNA (gDNA) for crustacean (crab) and suggests that 95% ethanol was a better preservative than RNAlater which partly contradicts with the present findings as both was observed to be good preservative for crustacean tissue. On the other hand, PBS glycerol and freezing (at -80°C) showed better results than 100% ethanol in bacteria metabarcoding[17]. So, depending on the species in concern, the preservation method and DNA extraction technique varies.

Preservation of tissue samples in 100% ethanol has been shown to fix the sample DNA and prevent degradation in microcrustaceans[12]. Samples preserved in RNAlater also yielded very high-quality DNA. Similar results were observed by Wang and Wang[14] for mammal tissue. Gorokhova[13] compared microcrustacean samples preserved in ethanol and RNAlater and concluded that RNAlater is the best option for preserving RNA and DNA even left at room temperature. However, RNAlater is very expensive and not a very convenient way to preserve samples when resources are limited. Degradation of shrimp tissue occurs very rapidly and hence DNA shearing was visible although samples were preserved in -80°C. According to Mulcahy *et al*.[16] tissue samples of crab was left at room temperature over a time frame of <10 m, 3 h and 24 h after death and before adding any preservatives. Results showed sign of DNA degradation for all these time frames and hence confirmed that crustacean tissue starts to degrade immediately after

Table 1: Summary results of 9 combinations of preservation and genomic DNA extraction in *Paratya australiensis*

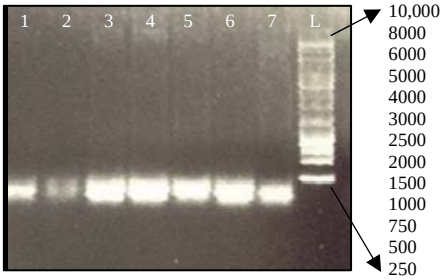
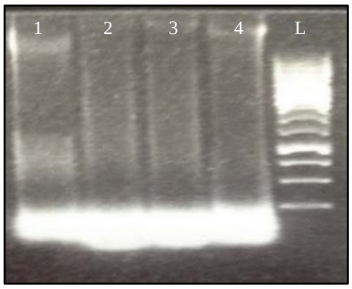
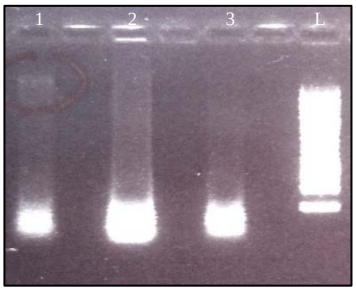
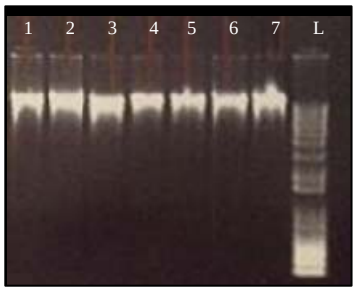
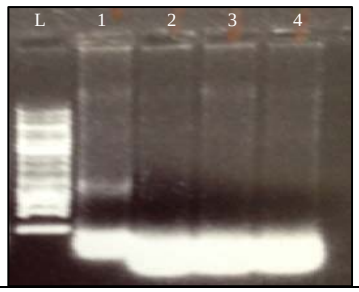
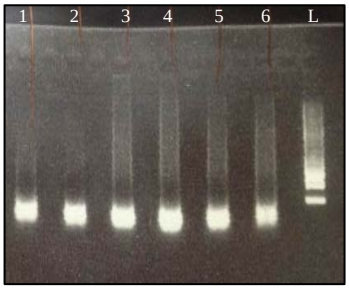
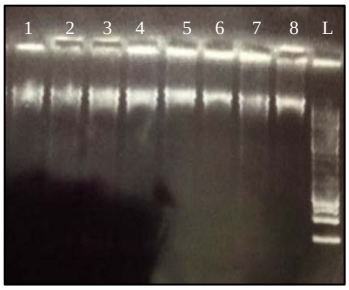
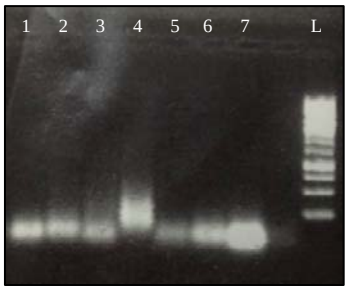
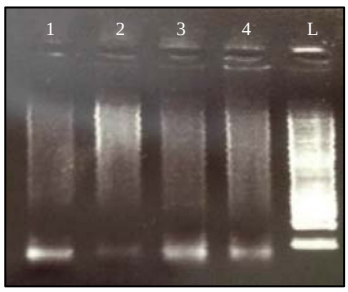
Sample preservation	DNA extraction methods	Gel electrophoresis (1 kb ladder)	Comments
-80°C	A) Econo-spin column		DNA of low fragment size (250 bp fragment)
	B) CTAB extraction		DNA of low fragment size (250 bp fragment)
	C) Salt extraction		DNA of low fragment size (250 bp fragment)
100% ethanol	A) Econo-spin column		DNA of high fragment size (>10,000 bp)
	B) CTAB extraction		DNA of low fragment size (250 bp fragment)

Table 1: Continue

Sample preservation	DNA extraction method	Gel electrophoresis (1 kb ladder)	Comments
	C) Salt extraction		DNA of low fragment size (250 bp fragment)
RNAlater	A) Econo-spin column		DNA of high fragment size (>10,000 bp fragment)
	B) CTAB extraction		DNA of low fragment size (250 bp fragment and shearing)
	C) Salt extraction		DNA of low fragment size (250 bp) and shearing

death. Besides, freezers have constant temperature cycling which also causes DNA shearing if left in the freezer for longer periods[18].

The possible reason for such rapid degradation is believed to be hemocyte degranulation in crustaceans[19]. Crustaceans live in an environment enriched with bacteria and viruses. The external cuticle is the first line of defense against pathogens. When a pathogen gets entry in to the invertebrate system there is a cellular immune response that involves different types of hemocytes. These hemocytes participate in pathogen clearance by different mechanisms, one of which is cytotoxic reaction known as degranulation[20]. The circulating hemocytes are well known in invertebrates to degranulate in the presence of foreign particles[19].

Preservation of samples prior to DNA extraction is very important, because depending on the storage condition DNA

degrades over time and becomes unsuitable for molecular studies[18]. In this study CTAB and salt extraction did not yield high quality genomic DNA despite being preserved in ethanol and RNAlater. The possible reason could be the precipitation step that caused DNA shearing. Athanasio *et al.*[21] tested different DNA extraction methods (Agencourt, DNAdvance, Master Pure DNA, ZR genomic DNA and CTAB method) for samples preserved in liquid nitrogen and RNAlater and similar results were observed with CTAB extraction method. It was suggested that precipitation step consisting of isopropanol facilitated co-precipitation of salts from RNAlater solution and interfered with production of genomic DNA without any smears[21].

Similar findings were reported by Devi *et al.*[22] where they observed shearing of DNA following the traditional CTAB extraction method. Furthermore, preserving samples in RNAlater yields good result but it is rather expensive (US$ 240/500 mL)[23]. Besides, using RNAlater for tissue preservation is more suitable when projects require RNA extraction[22]. So, ethanol is preferable for preservation of tissue samples. When high quality DNA is required Vink *et al.*[6] reported that ethanol preserved samples were better preserved if kept at -20°C which was not done in this study but could be applied if the samples need to be preserved for longer periods. They also suggested that samples preserved in 95% ethanol would start to degrade after 5 days if kept at room temperature[6], hence in the current study ethanol preserved samples were kept at 4°C.

CONCLUSION

It is concluded, for crustacean samples especially for *Paratya australiensis*, the efficient method of producing high quality genomic DNA was to preserve the samples in 100% ethanol or in RNAlater immediately after capture and to use an Econo-spin column extraction protocol described here. This study is based on a particular shrimp species, however, findings can be applied for other crustaceans. With the advancement of sequencing technology, high quality genomic DNA is essential for RAD-seq technology.

SIGNIFICANCE STATEMENTS

This study has discussed an efficient method producing genomic DNA. It would benefit studies focused on genomics of crustaceans.

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