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DNA EXTRACTION FROM RESTING EGGS OF THE CLAM SHRIMP *EULIMNADIA TEXANA* (BRANCHIOPODA: SPINICAUDATA: LIMNADIIDAE)

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ABSTRACT

Large branchiopod crustaceans inhabiting ephemeral ponds are well adapted to their highly unpredictable habitat with a life cycle that includes a short-lived adult stage and a long-lived, desiccation-resistant egg stage. One well studied large branchiopod is the clam shrimp *Eulimnadia texana*, an androdioecious species with populations comprised of males and self-compatible hermaphrodites. Likely due to the harsh environments in which the eggs are found, e.g., dry areas with high heat and high irradiation, extraction of DNA from individual eggs for genetic analyses can be problematic. Here we report a number of modifications that we have tested allowing for increased efficiency and success in extracting high quality DNA from the eggs, nauplii, and adults of *E. texana* that may prove useful for similar studies of other species of large branchiopods.

INTRODUCTION

The clam shrimp *Eulimnadia texana* Packard 1871 inhabits ephemeral ponds in the deserts of the southwestern United States and northern Mexico (Strenth, 1977; Vidrine et al., 1987; Sassaman, 1989). In these unstable environmental conditions, pond duration varies depending on rainfall patterns and air temperature (Marcus and Weeks, 1997). Like most large branchiopod crustaceans, clam shrimp are well adapted to highly unpredictable habitats. This is mainly due to their short-lived adult stage (the complete life cycle occurs in three weeks; Weeks et al., 1997) and resistant resting-eggs. The diapausing eggs undergo desiccation when the ponds dry, and are ready to hatch after rehydration. Exposure to rainy seasons provides them with adequate conditions to reemerge (Brendonck, 1996).

Characterized by an unusual life cycle, *E. texana* is an androdioecious species wherein populations are comprised of self-compatible hermaphrodites and males (Sassaman and Weeks, 1993; Zucker et al., 1997). After fertilization, either through selfing or out-crossing, eggs are moved from the ovotestis to a brood chamber found between the body and the carapace on the back of the hermaphrodite.

Encysted eggs represent a rich source of information for analyses of genotypic structure within a given population. In addition, single clutches of eggs contain the genetic information necessary to analyze the peculiar androdioecious mating system and parental patterns of genetic inheritance found in *E. texana*. The advantages of using eggs instead of adults are numerous (Moorad et al., 1997): 1) once in possession of the eggs, there is no need to hydrate them to raise the nauplii, 2) working with the eggs reduces the early post hatching mortality that can be high for *E. texana* (Weeks et al., 2001), and 3) the observed hatching bias (Brendonck, 1996; Simovich and Hathaway, 1997; Zarattini et al., 2002) is avoided, thus reducing the opportunity for bias in genetic studies of inheritance. Hence, the ability to extract DNA for genetic studies at very early stages of the life cycle, even while eggs are still in the brood chamber of the hermaphrodite, is desired. However, encysted eggs present some challenges for the extraction of DNA necessary for these analyses. Some recent papers have used DNA extracted from resting eggs in different organisms (*Daphnia*: Cousyn et al., 2001; Duffy et al., 2000; Limburg and Weider, 2002; Reid et al., 2002; Rotifers: Gomez et al., 2002), using a number of different methods. These studies met with varying success, but many brachiopod eggs experience more extreme desiccating conditions, and hence might be expected to have DNA protection mechanisms that could make extraction of quality DNA more difficult.

To date encysted eggs of spinicaudata have not been examined with molecular tools. We have attempted to use the anostracan egg extraction method of Moorad et al. (1997) to extract DNA from single resting eggs of *E. texana* with very limited success. Further attempts using standard protocols from a wide variety of commercially available DNA extraction kits were unsuccessful. As a result we explored a number of modifications to typical extraction protocols as well as methods of egg preparation. Here we report protocols for the reproducible extraction of high-quality DNA from eggs, nauplii, and adults of *E. texana* which we believe will be useful in a number of additional clam shrimp species and possibly other large branchiopods in general.

MATERIALS AND METHODS

Raising of Clam Shrimp

Soil containing encysted eggs of *E. texana* was collected from a cattle tank near Portal, Cochise County, Arizona, during summer 2000. In the laboratory at the University of Akron, soil was hydrated in April 2004 and clam shrimp were reared until maturity by methods previously described (Sassaman and Weeks, 1993; Weeks et al., 1997). Hermaphrodites with a full clutch of eggs in the brood chamber were isolated in individual cups and allowed to drop their eggs. Hermaphrodites were frozen on dry ice and stored at –80°C. Some of the eggs were allowed to dry and were rehydrated to obtain nauplii.

Initially adults, nauplii, and fresh and frozen eggs were extracted using a number of methods but eventually we settled on two methods referred here as the standard and extended lysis method each of which utilize the Qiagen DNeasy tissue extraction kit. The methods differ only by their incubation times, the latter method consisting of a greatly extended incubation. These methods are described for each of the target tissues as follows:

Adults

A frozen adult was placed under the dissecting microscope, the carapace was gently opened and the body of the clam shrimp was removed. The animal was rinsed with distilled water to detach all the eggs, if present. Only the upper portion of the body, in particular the head, was used in the extraction in order to avoid contamination with the digestive and reproductive organs. The tissue was finely ground using sterilized pestles in ceramic spot plates, and then moved to a clean microcentrifuge tube along with 180 μl of buffer ATL (Qiagen, DNeasy tissue kit). Twenty microliters of Proteinase K (600 mAU/ml solution) were added, the sample was mixed by vortexing, and incubated at 55°C. In the standard method, the sample was incubated for 35-40 min. When using the extended lysis method, the samples were incubated overnight (from 12 hours to 24 hours). Four microliters of RNase A (100 mg/ml) were added, mixed by vortexing, and incubated for 2 min at room temperature. After vortexing, 200 μl of buffer AL was added to the sample, mixed again by vortexing, and incubated at 70°C for 10 min. Then, 200 μl ethanol (96-100%) were added to the sample, and again mixed thoroughly by vortexing. The mixture was pipetted into DNeasy Mini Spin Column and centrifuged at 8000 rpm for 1 min, after which 500 μl Buffer AW1 were added and centrifuged at 8000 rpm for 1 min. Finally, 500 μl Buffer AW2 were added and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. The DNeasy Mini Spin Column was then placed in a clean, 1.5 ml microcentrifuge tube, and 20 μl of buffer AE were pipetted directly onto the DNeasy membrane. After incubation at room temperature for 1 min, the sample was centrifuged for 1 min at 8000 rpm to elute. This last step was repeated twice resulting in a final volume of 40 μl of DNA.

Nauplii

Each nauplius was individually isolated in the lid of a clean microcentrifuge tube, ground using a disposable pin, and transferred inside the tube with 90 μl of buffer ATL. The same procedures were followed as above except that half of the amount of all solutions was used. The final elutions were carried out with 15 μl of nuclease-free water to a final volume of 30 μl.

Encysted Eggs

From each single clutch, fresh eggs were observed under a dissecting microscope. Transparent eggs were considered empty and discarded as the yolk of unfertilized eggs degrades in 24-48 h (Weeks et al., 2001; Weeks et al., 2002), leaving an empty shell. The darker eggs were collected with a sterile micropipette tip and then individually placed into the lid of a clean 1.5 mL microcentrifuge tube, with a few microliters of sterilized water. The eggs were crushed in the lid using a disposable sterile pin. To each crushed eggs (inclusive of the exterior coat of the egg) was added 90 μl of buffer ATL, and with a micropipette all the material was transferred to the bottom of the same tube. The remaining steps were the same as those undertaken for the nauplii.

Moorad and colleagues (1997) used a bleach solution to remove adherent microorganisms and the outer protein coat. Our protocol omits the use of bleach to dissolve the outer egg coat because we were less concerned about the possibility of contamination of microorganisms in the extraction as we are using species-specific microsatellite Polymerase Chain Reaction (PCR) primers. Furthermore, standardization of this bleaching step may be difficult to optimize because there is a degree of intra-populational variation in coat thickness.

Upon breaking the eggs, particular attention was directed to whether material was ejected from the eggs after crushing. In this case, the egg was considered fertilized (Weeks et al., 2002), and was used in the extraction. Clutches of frozen eggs that were dropped by the hermaphrodite, eggs still left the brood chamber of frozen hermaphrodites, and eggs laid by the hermaphrodites and preserved in water in tubes in the dark, were also extracted following the same protocol described here for fresh eggs.

Ten microliter PCRs were carried out with five microsatellite primer combinations and conditions as described by Duff et al. (2004). PCR products were run on 2% agarose gels in TAE (Tris/Acetate/EDTA) buffer stained with ethidium bromide, and on polyacrylamide gels stained with silver staining (Promega). Size was deduced by comparison with a 1 kb ladder (Promega).

Results

Preliminary studies attempted to extract DNA from adults, nauplii, and fresh and frozen eggs using a number of methods including those described by Moorad et al. (1997) and the Qiagen DNAeasy Plant and Animal tissue extraction kits following the manufacturers' specifications. Each of these methods yielded similar results: reproducible amplification of a number of microsatellite loci for adults and nauplii. However, none of the methods resulted in DNAs that could be amplified for all of the target microsatellite loci. Rather, two microsatellite loci (CS5 and CS17, Duff et al., 2004) were consistently amplified for most eggs while other loci rarely resulted in products, with the best results achieved with the Qiagen DNAeasy animal tissue kit. Attempts to improve the microsatellite amplifications, including using differing PCR reagents, changing the length of amplification steps, and modifying the amount of DNA template, all failed.

Speculating that proteins remained bound to the egg DNA may be inhibiting PCR, we employed several modifications to our methods, including an extra phenol-chloroform step, increasing the incubation step, increasing lysis incubation periods, and even extending the denature times (up to 10 minutes) during the first cycle of each PCR. These extra steps resulted in no appreciable difference with the original protocols except for greatly increased lysis incubation periods with the DNeasy Tissue Kit (Qiagen). One difference between this method and the others used is the presence of Proteinase K added to the extraction buffer. The extended lysis incubation with Proteinase K produced no visible effect on the amplification of adult and nauplii DNAs compared to the standard method, but was necessary for amplification of microsatellite loci from the egg DNA (Fig. 1).

Discussion

Resting eggs of *E. texana* and other Branchiopoda represent highly specialized adaptations to extreme environments. Rather than simply protecting the embryos contained within from desiccation, the egg wall protects the embryo from mechanical injury and UV rays (Belk, 1970) and the contents of the egg are allowed to dry down to "virtually complete desiccation" (Clegg, 2001). Several branchiopod species require their eggs to dry and rehydrate in order to hatch (Brendonck, 1996). This ability makes this portion of the clam shrimp life cycle truly desiccation tolerant. Desiccation-tolerance is known from only a few phylogenetically diverse organisms including "water bears" (Tartigrada), insects *Polypedilum* (Blum, 1985) and a number of plants such as the bryophyte *Tortula ruralis* (Oliver and Wood, 1997). In the latter instance, a number of studies have suggested that the desiccation-induced damage to DNA and mRNAs is partially prevented by the presence of a number of DNA and RNA binding proteins that protect the genome in its desiccated state. Upon rehydration these proteins are released enabling the organism to be able to access its genome and mRNAs needed to perform repair functions (Oliver et al., 1998).

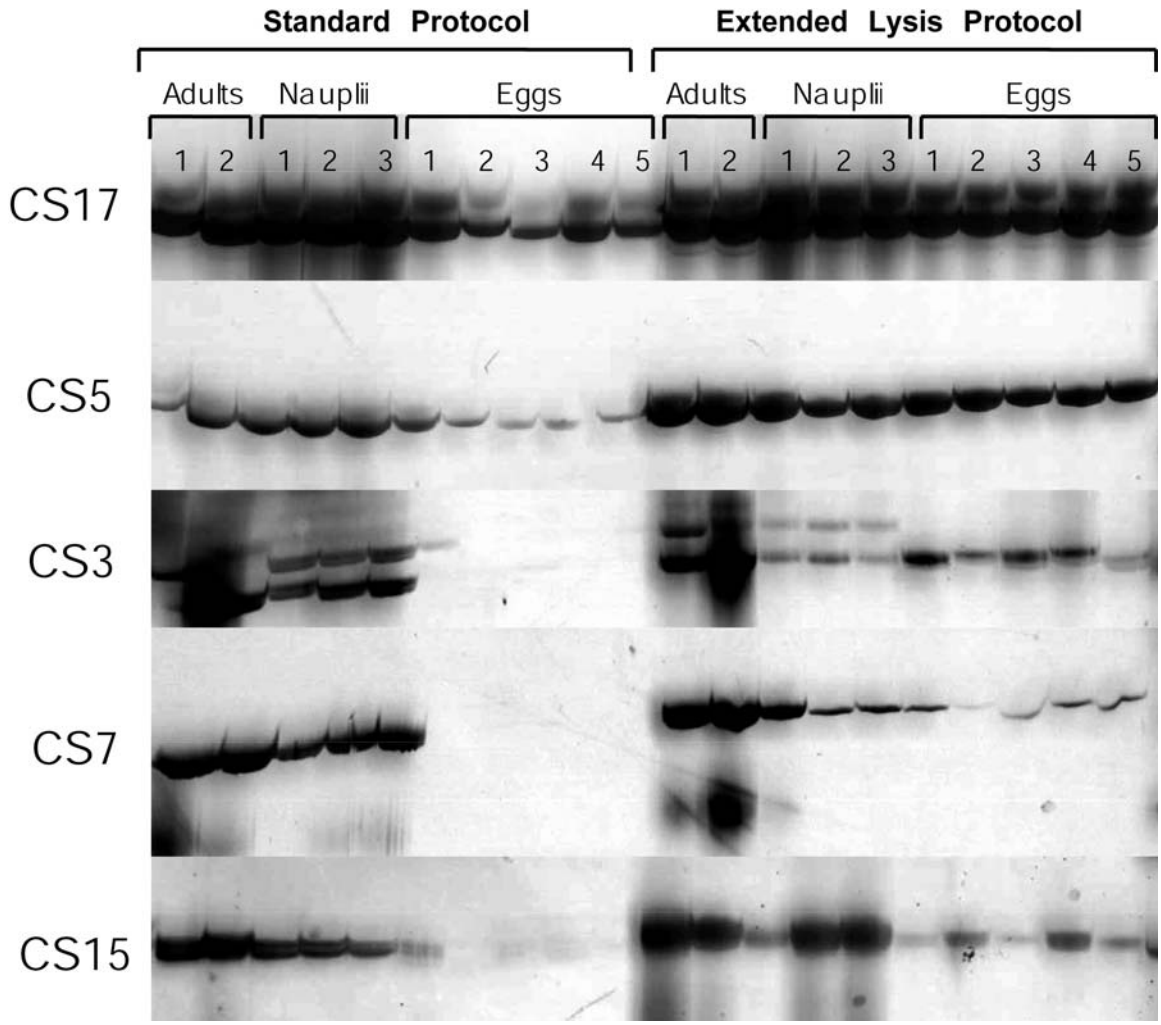

Fig. 1. Comparison of amplification of microsatellite loci from adults, nauplii, and eggs from *Eulimnadia texana*. Microsatellite primers (Duff et al., 2004) used for amplifications are indicated to the left of the figure. Microsatellites were run on 6% polyacrylamide gels and visualized with silver staining (Promega).

Given the observed difficulty in amplifying egg DNA versus adult and nauplii DNAs, we propose that similar DNA binding proteins are especially abundant in the eggs of clam shrimp. Hence, the amplification of only a few microsatellite loci may be attributed to the availability of some portions of the DNA genome to PCR primers while other primer binding sites are blocked by strongly bound proteins that have not been removed with standard protocols. The efforts to remove these bound proteins were almost completely ineffective, attesting to the potential strength of these interactions. However, the addition of Proteinase K with an extended incubation does have a clear affect on the quality of DNA for PCR, as seen in Fig. 1. Based on these results we suggest that the addition of these steps may be necessary in extracting DNA from organisms in which the cells have entered into a desiccation-tolerant state.

Zarattini and colleagues (2002) compared RAPD markers in 3 successive hydrations of the same clutch of eggs and suggested a variation in hatching linked to genetic variability. However, their attempts to use the Moorad et al. (1997) protocol on anostracan *Chirocephalus diaphanus* eggs were unsuccessful. That protocol does not include Proteinase K but rather a period of sample boiling. Our results suggest that Zarattini et al.'s method, as well as others, would be used to greater success with eggs by the simple addition of Proteinase K and an extension of the lysis incubation period.

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