SHORT COMMUNICATION

Optimized DNA extraction methods for encysted embryos of the endangered fairy shrimp, *Branchinecta sandiegonensis*

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Abstract The San Diego fairy shrimp *Branchinecta sandiegonensis* is a federally endangered species endemic to vernal pools in southern California, USA. Filling events in these habitats are highly variable, with some pools failing to hold water long enough for reproduction over many successive years. Studies of this species are thus hindered by the relatively rare appearance of aquatically active life history phases. Because diapausing cysts are abundant and present at all times, they provide an under-utilized opportunity for both species identification and genetic studies. However, methods for extracting DNA from cysts are technically challenging because of their structure and size. Here we present a protocol for extracting DNA from *B. sandiegonensis* cysts in sufficient quantities for "DNA Barcoding", microsatellite analysis and other genotyping and sequencing applications. The technique will aid in population genetic studies and species identification (since taxonomic keys only distinguish among adults), and will be applicable to other crustaceans with similar diapausing cysts.

Keywords Fairy shrimp · Diapausing cysts · DNA extraction · Endangered species

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Introduction

The San Diego fairy shrimp *Branchinecta sandiegonensis* (Fugate 1993) (Crustacea: Anostraca) is one of five fairy shrimp of concern in California, and was listed as federally endangered in 1997 (Bauder and McMillan 1998; King 1998; US Fish and Wildlife Service 1997). Although the recovery plan for *B. sandiegonensis* lists genetic structure as a key factor requiring investigation (Bauder et al. 1998), this species has a short lived aquatic stage (often less than 2 weeks), pool filling events only occur seasonally (and not at all in many years), and hatching in the laboratory is difficult (Simovich and Hathaway 1997; A.N. Steele, personal observation). There is also a pressing need for novel techniques for species identification. Character differences between the San Diego fairy shrimp and its congeners are subtle and found only in mature adult males, and only some fairy shrimp in California can be identified with confidence based on adult females or cyst morphology (Rogers 2002; Mura 1991; Hill and Shepard 1997).

Although fairy shrimp have diapausing cysts that can remain dormant in a "cyst bank" for years at a time (Brendonck 1996; Simovich and Hathaway 1997; Philippi et al. 2001), most genetic studies have utilized only adults or juveniles (Davies et al. 1997; Brendonck et al. 2000;

Bohonak 2005). *B. sandiegonensis* cysts have only been used in one study with RAPD markers (Moorad et al. 1997), which have subsequently proven unreliable in this and most other species. This study focused on optimization of techniques for (1) cyst decapsulation and (2) disruption of the internal tertiary membrane followed by PCR and sequencing. Our results facilitate seasonally independent genetic analyses of the San Diego fairy shrimp without the need to rear adults in a laboratory setting.

Materials and methods

Cysts were removed from sediments collected July 2006 by M. Simovich from vernal pool Maddox 2 in Mira Mesa, San Diego, USA (protocol in Simovich and Hathaway 1997). A cyst consists of an embryo within a membrane, surrounded by a cyst wall composed of three layers (Hill and Shepard 1997). We refer to the complete cyst wall as the capsule, and the membrane as the tertiary membrane.

Experiment 1: cyst decapsulation and DNA extraction

Without specifically focusing on the subsequent fate of the tertiary membrane, Experiment 1 tested methods to dissolve or breach the capsule, and two different extraction protocols. Each decapsulation/extraction method combination described below was tested on $n = 20$ embryos. Approximately 50% of cysts did not contain embryos and were not included.

Bleach decapsulation

Motivated by previously described techniques (Spotte and Anderson 1988; Moorad et al. 1997; Velu and Munuswamy 2003), we used undiluted consumer-strength bleach to deteriorate the external capsule. Ten cysts were added to 1 ml of bleach in a 1.7 ml Eppendorf tube, and the solution was agitated by inverting the tube continuously for 15 min. Cysts were removed from the bleach onto a mesh screen, washed in NaCl detergent (96.6% DI water, 3% 4 M NaCl, 0.4% of 10% Triton x-100) for 5 min, and distributed into individual Eppendorf tubes.

Freeze/thaw decapsulation

Five freeze/thaw cycles were performed in an attempt to create enough microfissures in the capsule to allow penetration of lysis buffer. Cysts were placed into individual Eppendorf tubes with 200 μl of prepared lysis buffer. Each tube was placed in liquid nitrogen for 5 min and then immediately moved to a heat bath at 56°C for 5 min.

Freeze/thaw decapsulation with SDS

Following the freeze/thaw method described above, a 10% SDS solution was added to each tube for a final concentration of 2% SDS, followed by 1 h incubation at 56°C.

Standard DNA extraction

The protocol began by placing each embryo in an Eppendorf tube with 250 μl of prepared lysis buffer (100 parts Promega DNA IQ kit lysis buffer: 1 part 1 M DTT). The Promega DNA IQ small sample casework protocol (Promega, Madison, WI, USA) was used for extraction with two modifications: samples were incubated at 95°C for 30 min after adding the initial aliquot of prepared lysis buffer, and we omitted use of a spin basket.

Extraction with proteinase K

Proteinase K extractions started with each embryo in 20 μl PBS, 2 μl of 20 mg/ml Proteinase K, 2 μl of 1 M DTT, and 250 μl prepared lysis buffer. Samples were incubated at 56°C overnight, followed by the DNA IQ kit extraction protocol described above (after the initial incubation step).

DNA yield was estimated using a Nanodrop ND-1000 spectrophotometer (v 3.1.0, NanoDrop Technologies, Wilmington, DE, USA). We amplified a 640 bp region (excluding primers) of the mitochondrial gene cytochrome *c* oxidase subunit 1 (CO1). Primers were developed using known fairy shrimp sequences, with modifications from those described in Zickovich and Bohonak (2007). Reactions contained up to 15.2 μl of DNA per sample (as necessary to reach the target of 50 ng), 2.5 μl of 10× Invitrogen buffer, 2.5 μl of 8 mM dNTPs, 1.2 μl of 15 μM J-2 primer (5′TTCTCAACAAATCATAAAGAT ATTGGAACACTCTA), 1.2 μl of 15 μM 2198-2 primer (5′TAAACTTCAGGGTGACCAAAAAATCAAAACAA GTG), 1.2 μl of 50 mM $MgCl_2$, 1 μl of 10 μg/μl BSA, 0.2 μl Platinum Taq Polymerase (Invitrogen), and water to reach a final volume of 25 μl. Negative and positive controls (adult fairy shrimp DNA) were included with each set of reactions. Thermocycling was performed on an Eppendorf Mastercycler EP at 94°C (2 min), followed by 32 cycles of 94°C (30 s), 51°C (30 s), 72°C (1 min); final extension was 72°C for 7 min.

PCR products were verified on 1.8–2% agarose gels with 1× TBE. Success was determined by the presence and intensity of a band at the expected size range, compared to the standardized ladder. Samples with no band were scored a 0, a faint band (<3.95 ng/μl) scored a 1, and a bright band (>3.95 ng/μl) was scored a 2.

Samples that scored a 1 or 2 for PCR product quality were purified with the MoBio Ultraclean kit (final elution

of 50 µl) and sequenced by the Microchemical CORE facility at San Diego State University using BigDye (ABI). Chromatograms were analyzed manually using Sequencher 4.1 (Gene Codes Corp.), and nucleotides with multiple peaks were characterized as ambiguous if the intensity of the tallest peak was less than twice that of the others. Sequence quality was scored as 0 for a failed sequence, 1 for more than 10 ambiguities and 15 edits in the final sequence, and 2 for fewer than 10 ambiguities and 15 edits.

Experiment 2: tertiary membrane disruption

A second experiment focused on determining the best method of disrupting the tertiary membrane, which was not explicitly disrupted in the first experiment. After the bleach decapsulation protocol described above, each embryo was placed into 20 µl PBS and 2 µl of 20 mg/ml Proteinase K, and then underwent one of the four tertiary membrane disruption treatments described below.

Friction pipet (n = 25)

Embryos were vigorously pipetted for 2–5 min using a pipet with a 200 µl aerosol-barrier tip, until the membrane ruptured (as verified under a dissecting microscope).

Manual disruption (n = 25)

A fine point dissection probe was used to manually tear the tertiary membrane under a dissecting microscope (60× magnification). Clean probes were used for each cyst to avoid cross-contamination.

Freeze/thaw (n = 25)

Eppendorf tubes containing embryos went through five cycles of liquid nitrogen for 5 min, and a 56°C water bath for 5 min.

Control (n = 20)

The bleach decapsulation protocol was applied to 20 embryos (not used in Experiment 1), without disruption of the tertiary membrane.

Each cyst's membrane was examined under a microscope. Prepared lysis buffer of 250 µl was added to each sample, and they were incubated at 56°C overnight. Resin of 7 µl from the Promega DNA IQ kit was added and samples were incubated at room temperature for 5 min. The remaining steps of the extraction with proteinase K were followed. PCR amplifications for Experiment 2 used a 50 µl reaction with a target amount of DNA between 100–175 ng, and each reagent volume was doubled from

the previous experiment. PCR and sequencing methods were identical to Experiment 1.

Analysis

Response variables for each experiment were (1) DNA yield (log transformed), (2) PCR success (agarose gel staining score), (3) sequence quality (sequence chromatogram score). ANOVA was used to test for differences in DNA yield, with Tukey's HSD Post Hoc test for pairwise comparisons. PCR success and sequence quality were analyzed using Kruskal–Wallis tests. Sequencing success rates were high enough in two treatments from Experiment 2 to compare sequence quality using a two sample *t*-test, with the number of ambiguities and the number of edits as continuous variables ($n = 41$ samples scoring 1 or 2). Both the number of ambiguities and the number of edits were transformed using the 4th root to reduce heteroscedasticity. All analyses were completed using Systat 12 (Systat Software, Inc.) or DataDesk v. 6.1.1 (Velleman 1997).

Results

Experiment 1: cyst decapsulation and DNA extraction

There was a significant difference among methods in DNA yield ($F_{5,114} = 20.113$, $P < 0.001$, Fig. 1). However, only 4 of 120 samples yielded successful PCR products (Table 1). Visual verification that the outer shell had been removed was easiest with bleach decapsulation, and it was much more difficult to determine whether the capsule had cracked in the freeze/thaw method. Because the bleach method with proteinase K also provided the best of the four sequences from Experiment 1, we chose this method to further refine in Experiment 2.

Experiment 2: tertiary membrane disruption

DNA yield differed significantly among the membrane disruption methods ($F = 5.098$, df = 3, $P = 0.003$, Fig. 1). Manually puncturing the membrane and friction pipetting yielded more DNA than freeze/thaw or control methods (Tukey's test, $\alpha = 0.05$). Membrane disruption methods also differed in PCR success (Kruskal–Wallis test statistic = 51.331, df = 3, 91, $P < 0.001$). Friction pipetting and manually tearing the membrane had the highest success rates ($\approx 95\%$), while freeze/thaw and control methods performed poorly (Table 1). Sequence quality did not differ significantly among treatments (Kruskal–Wallis test statistic = 0.489, df = 3, $P = 0.92$). When analyzed as continuous variables, sequence quality did not differ significantly in terms of ambiguities ($t = -0.396$, df = 39,

Fig. 1 DNA yield per cyst for Experiment 1 (decapsulation and extraction methods) and Experiment 2 (tertiary membrane disruption). *No* and *Yes* refer to the inclusion of proteinase K in the extraction. *Freeze/thaw* has been denoted as F/T, and *Freeze/thaw/SDS* is denoted by F/T/SDS. Box plots depict the median, 25th and 75th percentiles (box hinges), whiskers extending to the minimum and maximum data values occurring within 1.5× range above and below the box hinges, outliers (circles), and extreme outliers (stars) (Velleman 1997). A, B, C denote non significance amongst the log transformed DNA extraction data (log ng/μl) treatments for the post hoc test after ANOVA
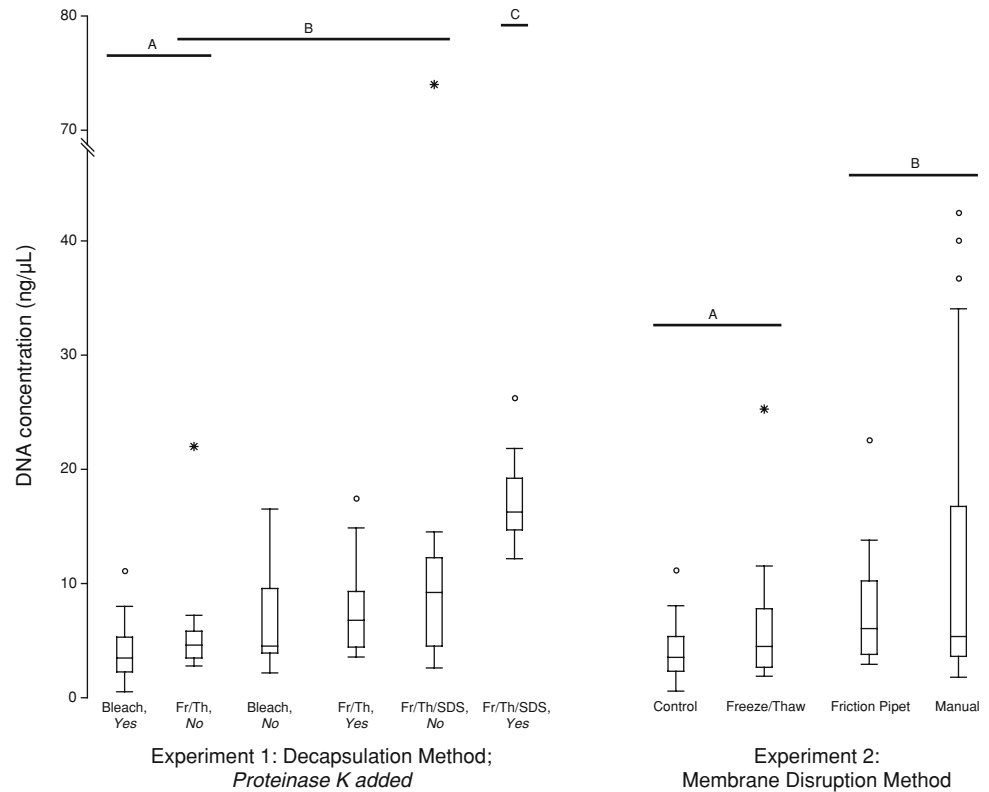

Table 1 Number of cysts for each PCR success score and sequence quality score, divided by experiment

Experiment 1: Decapsulation method

	Bleach		Freeze/thaw/SDS		Freeze/thaw	
Proteinase K addition	Yes	No	Yes	No	Yes	No
PCR success						
0	19	19	20	20	19	18
1	0	0	0	0	1	0
2	1	1	0	0	0	2

Experiment 2: Disruption method

	Friction pipet	Freeze/thaw	Manual	Control
PCR success				
0	2	20	5	19
1	3	2	1	0
2	20	3	19	1
Sequence quality				
0	1	2	1	0
1	11	0	9	0
2	11	3	10	1

Cysts that scored a 0 for PCR success were not sequenced

$P = 0.69$) or number of manual edits ($t = 0.788$, df = 39, $P = 0.44$).

Discussion

DNA can be successfully extracted from cysts of *B. sandiegonensis* using bleach to decapsulate, and a fine dissection probe to tear the embryonic membrane. Although other decapsulation methods may provide larger DNA yields, the bleach protocol has an added benefit of eliminating foreign DNA on the outside of the cyst capsule. Our protocol improves on that described by Moorad et al. (1997) for analysis of RAPDs, since it requires approximately half as much time for decapsulation, is likely to be more reliable, and yields sufficient DNA for microsatellite analysis (A.J. Bohonak, unpublished data).

The opportunities provided by these methods include genetic studies of fairy shrimp at any time, and the opportunity to identify cysts to species level without laboratory hatching and rearing (Vandergast et al. in press). Ultimately, studies that work directly with cyst DNA should have higher sample sizes and potentially be less biased than those which involve laboratory hatching, since

only a fraction of *B. sandiegonensis* cysts hatch under laboratory conditions (Simovich and Hathaway 1997). The ability to provide improved genetic information on this endangered fairy shrimp may aid in its management and recovery, and facilitate rapid, cost-effective species identifications.

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