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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Identification of co-occurring *Branchinecta* fairy shrimp species from encysted embryos using multiplex polymerase chain reaction

A. G. VANDERGAST,* D. A. WOOD,* M. SIMOVICH† and A. J. BOHONAK‡
**US Geological Survey, Western Ecological Research Center, San Diego Field Station, 4165 Spruance Road, Suite 200, San Diego, CA 92101, USA, †University of San Diego, Department of Biology, 5998 Alacala Park, San Diego, CA 92110, USA, ‡San Diego State University, Department of Biology, 5500 Campanile Drive, San Diego, CA 92182-4614, USA*

Abstract

Morphological identification of many fairy shrimp species is difficult because distinguishing characters are restricted to adults. We developed two multiplex polymerase chain reaction assays that differentiate among three *Branchinecta* fairy shrimp with distributional overlap in southern California vernal pools. Two of the species are federally listed as threatened. Molecular identification of *Branchinecta* from cysts allows for species surveys to be conducted during the dry season, expanding the timeframe for population assessment and providing a less intrusive method of sampling sensitive vernal pool habitats.

Keywords: endangered species, species identification, vernal pools, COI gene

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Accurate identification of rare species is integral to their protection and management. In southern California, USA, vernal pools, the federally endangered *Branchinecta sandiegonensis* and threatened *Branchinecta lynchi* overlap in distribution with nonlisted *Branchinecta lindahli*. These species are morphologically distinguishable only as adults (Eriksen & Belk 1999; Rogers 2002), which can be sampled during infrequent ponding events, or reared from cysts (which has proven difficult; Simovich & Hathaway 1997). There is a pressing need for a species diagnostic that can be used when pools are dry. We developed two multiplexed polymerase chain reactions (PCRs) that amplify species-specific fragments of the mtDNA cytochome oxidase I gene (COI), providing a more reliable alternative to a previously reported random amplified polymorphic DNA diagnostic (Moorad *et al*. 1997).

Primers were developed from a 65 haplotype alignment (5 *B. lynchi*, 5 *B. lindahli*, 55 *B. sandiegonensis*) of 649 bases of COI (GenBank Accession nos FJ439689–FJ439753). Pairwise sequence divergence was 10–14% among species and 0–3% within species. We designed four species-specific primers and one universal primer to be used with a single forward primer (Table 1). Primers were tested on morphologically identified adults, and cysts from pools known to contain a single *Branchinecta* species. Extractions followed Steele *et al*. (in press). Briefly, cysts were decapsulated in a bleach solution, washed in NaCl detergent, and the inner tertiary membrane was torn with a sterile probe. DNA was extracted using Promega DNA IQ System, dried and resuspended in 50 μL of deionized water.

Each species-specific primer (paired with the universal primer) was screened separately against template DNA of all three species, and subsequently tested in multiplex reactions. In preliminary screens, we could not develop one reaction that distinguished all species and also produced a control band to verify reaction success. Therefore, we developed two protocols: (i) a two-step protocol screening for *B. sandiegonensis* in the first step, and for *B. lindahli* or *B. lynchi* in the second step (control bands in both steps); (ii) an alternative single-step protocol that screens for all three species without any control band. Reaction mixtures (25 μL) contained 5–250 ng template DNA, 2.9 mM $MgCl_2$,

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Correspondence: A. J. Bohonak. San Diego State University, Department of Biology, 5500 Campanile Drive, San Diego, CA 92182–4614. Fax: 619-594-5676; E-mail: bohonak@sciences.sdsu.edu

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Table 1 Primers used in the multiplex PCR experiments. F and R indicate forward or reverse orientation. Primer locations relative to the complete mitochondrion sequence of *Drosophila yakuba* (GenBank Accession NC 001322) are indicated in parentheses. Size indicates the length in base pairs of the band generated by the primer together with the universal forward primer, Jyothi-F (Zickovich & Bohonak 2007)

Species	Primer	Sequence (5′–3′)	Size
Universal invertebrate	Jyothi-F (1489)	TTCTCAACAAATCATAAAGATATTGG	
Universal *Branchinecta*	BrUniR (2042)	GCTGTAATACCTACCGATCATACAAATA	553
B. sandiegonensis	SAR1 (1659)	AATAAATGCATGAGCTGTAACAATAACG	170
B. sandiegonensis	SAR2 (2001)	TAATGATATAGAGGAGGGCCGTATRTTTAGGATG	512
B. lindahli	LIR (1830)	TCCTCTTTCTACTATTGATCCTGCTAGG	341
B. lynchi	LYR (2169)	CAAATGTTGATAAAGAATAGGGTCTCCA	680

1.0 mм dNTPs, 0.56 μM of each primer, 1× PCR buffer, and 1.25 U polymerase. In the two-step protocol, step 1 contained primers Jyothi-F, BrUni-R, and SAR1, with cycling conditions of 94 °C (2 min), 35 cycles of: 94 °C (30 s) 60 °C (40 s) 72 °C (45 s), and 72 °C (7 min). Step 2 contained primers Jyothi-F, BrUni-R, LIR, LYR with identical conditions except for 59 °C annealing. The single-step protocol contained primers Jyothi-F, SAR2, LIR and LYR, with PCR conditions identical to step 1 above. Products were verified on 1.8–2% agarose gels run at 90 milliamps for 40 min.

Accuracy was estimated by screening 100 cysts per species. False negatives produced the positive control fragment but no species-specific fragment. False positives amplified an incorrect species-specific fragment. Reactions that failed to produce a control or species-specific fragment were considered uninformative. (Failed reactions in the single-step protocol were assessed using a second PCR containing universal primers.) Exact chi-square tests were used to test for differences in accuracy between the two protocols.

We also tested both protocols with mixed DNA to determine their effectiveness on batches of cysts that may contain two species. DNA extracted from individual cysts was pooled for each possible species pair in ratios of 1:1, 1:9 and 1:19, with eight replicate reactions for each possible combination.

The two-step protocol correctly identified 97.9% of individual cysts, outperforming the single-step protocol at 93.7% ($\chi^2 = 6.483$, 1 d.f., exact $P = 0.012$, Table 2). The two-step protocol more accurately identified *B. sandiegonensis* (99% vs. 85.7% for single-step protocol; $\chi^2 = 12.24$, 1 d.f., exact $P = 0.0004$), but there was no difference for the other two species (*B. lynchi* $\chi^2 = 0.1641$, 1 d.f., exact $P = 1.0$; *B. lindahli* $\chi^2 = 0.\ 1949$ 1.0 d.f., exact $P = 1$).

When DNA was combined in mixed-species trials, the single-step assay produced correct results at concentrations of 1:1 and 1:9 (Table 2). However, bands for the rare species were not always detectable at 1:19 (Fig. S1, Supporting Information). The two-step protocol produced correct results for all *B. sandiegonensis*/*B. lindahli* replicates at all concentrations. In contrast, step 2 of the two-step protocol produced faint or no *B. lynchi* bands when *B. lynchi* DNA was present, but in lower concentrations than *B. lindahli*. Because the universal and *B. lindahli* fragments are both smaller than the *B. lynchi* fragment, the universal fragment may create more templates for *B. lindahli* and universal primers during amplification, outcompeting the larger *B. lynchi* fragment for reagents in subsequent cycles. To verify, we repeated the second step for *B. lynchi* and *B. lindahli* in 1:9 and 1:19 concentrations, but excluded universal primers (two replicates each). Both *B. lynchi* and *B. lindahli* bands were present in all reactions. A similar problem was evident in the two-step protocol for *B. lynchi* and *B. sandiegonensis* mixtures, although these two species do not co-occur in nature.

In southern California, *B. lynchi* occurs in only a few locations in Riverside County, where it overlaps with *B. lindahli*. Here, the single-step protocol should correctly identify species > 95% of the time. The single-step protocol also better diagnoses mixed batches of *B. lynchi* and *B. lindahli* cysts at the 1:9 ratio. However, the two-step protocol is preferred within the range of the endangered *B. sandiegonensis*, where it overlaps exclusively with *B. lindahli*. The two-step protocol better distinguished *B. sandiegonensis* than the single-step protocol, and reliably amplified *B. sandiegonensis* in mixed DNA trials at lower concentrations. Identifying pools in which *B. lindahli* and *B. sandiegonensis* coexist is particularly important, since these species may hybridize (Fugate 1992), threatening the genetic integrity of *B. sandiegonensis*.

Reliable PCR identification of cysts expands the time frame for species surveys, and facilitates less intrusive sampling of fragile vernal pool habitats. In southern California, the major threats to fairy shrimp persistence are loss and degradation of habitat. Early detection of listed species on project sites will permit levels of take to be accurately assessed, leading to more efficient allocation of resources for monitoring, restoration and mitigation.

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Table 2 Accuracy of multiplex PCR assays for identifications of southern California *Branchinecta* species from single cysts, and mixed DNA at 1:1, 1:9 and 1:19 cyst ratios

	Dilution	No. of PCR	Correct	False negative	False positive	Failed	Proportion correctly identified (%)
Single species trials							
Single-step protocol							
B. sandiegonensis	*na*	102	78	13	0	11	86
B. lindahli	*na*	100	95	2	0	3	98
B. lynchi	*na*	101	93	3	0	5	97
Total	*na*	**303**	**266**	**18**	**0**	**19**	**94**
Two-step protocol							
B. sandiegonensis	*na*	100	98	0	1	1	99
B. lindahli	*na*	100	95	1	2	2	97
B. lynchi	*na*	100	90	2	0	8	98
Total	*na*	**300**	**283**	**3**	**3**	**11**	**98**
Species combinations							
B. lindahli* and *B. sandiegonensis							
Single-step protocol	1:1, 1:9, 9:1	8 each	8 each	0 each	0 each	0 each	100
	1:19, 19:1	8 each	8, 0	0, 7	0, 0	0, 1	100, 0
Two-step protocol							
Step 1	1:1, 1:9, 9:1, 1:19, 19:1	8 each	8 each	0 each	0 each	0 each	100
Step 2	1:1	8	8	0	0	0	100
	1:9, 9:1	8 each	6,8	0, 0	0, 0	2, 0	100
	1:19, 19:1	8 each	8, 8	0, 0	0, 0	0, 0	100
B. lindahli* and *B. lynchi							
Single-step protocol	1:1, 1:9, 9:1	8 each	8 each	0 each	0 each	0 each	100
	1:19, 19:1	8 each	0, 7	8, 1	0, 0	0, 0	0, 88
Two-step protocol							
Step 1	1:1, 1:9, 9:1, 1:19, 19:1	8 each	8 each	0 each	0 each	0 each	100
Step 2	1:1	8	8	0	0	0	100
	1:9, 9:1	8 each	8, 0	0, 8	0	0	100, 0
	1:19, 19:1	8 each	8, 8	0, 0	0, 0	0, 0	100
B. sandiegonensis* and *B. lynchi *							
Single-step protocol	1:1	8	8	0	0	0	100
	1:9, 9:1	8 each	8, 8	0, 0	0, 0	0, 0	100
	1:19, 19:1	8 each	0, 0	8, 8	0, 0	0, 0	0
Two-step protocol							
Step 1	1:1, 1:9, 9:1, 1:19, 19:1	8 each	8 each	0 each	0 each	0 each	100
Step 2	1:1	8	8	0	0	0	100
	1:9, 9:1	8 each	8, 7	0, 1	0, 0	0, 0	100, 88
	1:19, 19:1	8 each	8, 4	0, 0	0, 0	0, 4	100

*species combination not known to occur in southern California vernal pools. *na* not applicable.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Gel images of the two-step and single-step protocols. (A) Two-step protocol, step 1 showing positive control and *B. sandiegonensis* species-specific bands. The first lane contains 100 bp ladder with fragment sizes (in base pairs) indicated to the left. Samples are as follows: *B. sandiegonensis* (lanes 2–4), *B. lindahli* (lanes 5–7), *B. lynchi* (lanes 8–10), *B. lindahli* samples false positive for *B. sandiegonensis* (lanes 11–12; ghost bands circled). (B) Two-step protocol, step 2 showing the positive control and *B. lindahli* and *B. lynchi* species-specific bands. Samples are as follows: ladder (lane 1), *B. sandiegonensis* (lanes 2–4); *B. lindahli* (lanes 5–7); *B. lynchi* (lanes 8–10); blank (lane 11), *B. sandiegonensis* sample false positive for *B. lindahli* (lane 12; ghost band is circled). (C) Single-step protocol results. Samples are as follows: ladder (lane 1), *B. sandiegonensis* cysts (lanes 2–4); *B. lindahli* cysts (lanes 5–7); *B. lynchi* cysts (lanes 8–10); negative control (lane 11).

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