IDENTIFICATION OF SOUTHERN CALIFORNIAN BRANCHINECTID CYSTS (CRUSTACEA, ANOSTRACA) USING RAPD-PCR SPECIES-SPECIFIC MARKERS

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ABSTRACT: Morphology of cysts of fairy shrimp (Crustacea, Anostraca) is often very useful in distinguishing between genera and some morphologically distinct species. However, the cysts of many species are indistinguishable from one another due to high inter-specific morphological similarity or to extensive intra-specific variability. In addition, hybridization between some species is possible and unlikely to be detected by examining cyst morphology. We have developed an approach based on RAPD-PCR molecular markers that allows for the rapid and accurate identification of fairy shrimp species at the cyst stage. This technique can quickly differentiate between three congenic species of potentially co-occurring fairy shrimp in southern California that are known to hybridize and whose cysts are difficult or impossible to identify using morphological characters. This technique has the potential to be expanded to incorporate many more species.

Key words: California, cysts, branchinectids, Brachiopoda, ephemeral wetlands, fairy shrimp, RAPD-PCR analysis

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California's array of ephemeral wetlands (vernal pools, playas, etc.) provide habitat for diverse and largely endemic flora and fauna (Holland and Jain 1988, Simovich 1998). The loss of ephemeral pools in California has caused numerous species of plants and animals associated with these habitats to become federally listed as threatened or endangered, including several species of fairy shrimp (Crustacea: Anostraca) (Federal Register 1988; 1993; 1994*a*, *b*; 1997). Consequentially, documenting the distribution of endangered species, compiling environmental impact reports, and devising conservation and management plans has made accurate identification of species increasingly important.

Two sensitive ephemeral pool animals are the endemic Californian fairy shrimp species, *Branchinecta sandiegonensis* (Fugate 1993) and *B. lynchi* (Eng et al. 1990). Both of these two species may live in the same ephemeral pools or pools in the same area as the more common and unthreatened species *B. lindahli* (Eng et al. 1990, Fugate 1992, Simovich and Fugate 1992, Eriksen and Belk 1999). *Branchinecta sandiegonensis* and *B. lynchi* are endangered and threatened, respectively, and are both subjects of conservation efforts.

Hybridization involving rare species is considered a threat to the persistence of those species for a myriad of reasons, including genetic swamping, out-competition, and increased disease sensitivity (Levin et al. 1996). All three possible combinations of southern Californian branchinectid hybrids have been produced in the laboratory (Fugate 1992). Although no hybrids have yet been observed in natural populations, the possibility of their existence is a conservation concern.

The ephemeral wetlands in which these anostracans are found are characterized by periodic hydration and dehydration events. Hydrations may occur years apart and may last from days to months. Due to the short duration and irregular periodicity of this habitat, the adult forms may be present for only a few weeks out of the year, if at all. Thus, the population usually exists predominately in the soil as encysted embryos. Identification of cysts of genus *Branchinecta* is possible by examining their morphology, but species identification is often problematic (Mura 1991*a*, Hill and Shepard 1997). Previously, the only way to positively identify most branchinectid cysts to the level of species has been to hydrate the cysts, rear the shrimp, and identify the adults based on morphological characteristics (see Belk 1975, Eriksen and Belk 1999). Unfortunately, this process may take weeks to complete. Furthermore, hydration conditions must be suitable to all species present or some may not hatch or survive to adulthood and possibly go undetected.

Efforts have also been made to distinguish the cysts of anostracans using scanning electron microscopy (SEM). Cyst diameter has been abandoned as a diagnostic character due to great variability within species (Belk et al. 1990, Mura 1991*b*) and most attention has focused on detecting species differences in tertiary envelope ap-

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pearance (Mura and Thiéry 1986; Mura 1991*a,b,c*; Hill and Shepard 1997). Unfortunately, this approach requires difficult and often arguably subjective judgment regarding morphology. In addition, tertiary envelope characteristics can be affected by non-genetic factors such as population elevation, maternal stress, preservation conditions, cyst age, and even SEM sample preparation (Mura 1991*c*). These factors, coupled with high within-species morphological variability (Mura 1991*a, c*; Hill and Shepard 1997), compromise confidence in the use of visual identification of the cysts of closely related species.

Clearly, highly dependable identification requires a technique that can: employ discrete characteristics in order to reduce or eliminate subjectivity in scoring; be flexible enough to allow for large amounts of within-species variability; offer enough resolving power to distinguish not only between species but hybrids of closely related species; and examine characters that are immune to environmental modification. Genetic markers have proven useful in identification efforts for diverse systems at morphologically similar taxa (for review see Hadrys et al. 1992). For example, biochemical markers have also been used to investigate phylogenetics and population genetics of branchinectids (Fugate 1992, Davies et al. 1997, Bohonak 1998) but samples have been restricted to adult tissues. This approach works well when dealing with adult forms or otherwise large organisms but it carries substantial tissue requirements. Because branchinectid cysts are several orders of magnitude smaller than the adult forms (4-5 μg each), analysis by either of these techniques is not possible.

The polymerase chain reaction (PCR) offers a solution to the minimal tissue problem. When used in conjunction with randomly amplified polymorphic DNA (RAPD) primers, anonymous DNA fragments (or markers) of different sizes can be amplified and visualized on an agarose gel (Welsh and McClelland 1990, Williams et al. 1990). This technique can provide access to a very large number of genetic markers. These markers can resolve genetic differences between individuals, populations, species, and genera (for review see Hadrys et al. 1992). Recently, RAPD markers have been shown to be very useful in species and subspecies identification of small invertebrates (Ballinger-Crabtree et al. 1992, Coffroth and Mulawka 1995, Humbert and Cabaret 1995). The purpose of this study was to devise an approach for the rapid and accurate identification of cysts of morphologically similar southern Californian branchinectid species (*B.*

Table 1. Sample locations in California for populations of 3 species of fairy shrimp (*Branchinecta* sp.)

Species	Locality	Collectors
B. sandiegonensis	1. Nobel Drive, San Diego County	M. Simovich
	2. Otay Mesa, San Diego County	M. Simovich
	3. Miramar NAS, San Diego County	M. Simovich
B. lynchi	4. Santa Rosa Plateau Reserve, Riverside County	M. Fugate
	5. Corning, Tehama County	M. Simovich
	6. Truckee Creek, Tehama County	M. Simovich
	7. Vina, Tehama County	M. Fugate and K. Kamrath
B. lindahli	8. Banning, Riverside County	M. Simovich
	9. Rogers Dry Lake, Kern County	G. Pratt
	10. San Miguel Island, Channel Islands	B. Arnold
Hybrids		
B. sandiegonensis X *B. lynchi*	11. *B. sandiegonensis* - Del Mar, San Diego County	M. Simovich
B. lindahli X *B. lynchi*	12. *B. lynchi* - Skunk Hollow, Riverside County	M. Simovich
B. lindahli X *B. sandiegonensis*	13. *B. lindahli* - Lakeview, Riverside County	M. Fugate

lindahli, *B. sandiegonensis* and *B. lynchi*) and their respective hybrids using RAPD-PCR markers. This approach should be applicable to other branchiopod species in other areas.

MATERIALS AND METHODS

Cysts of the three species were collected from soil sampled from ephemeral pools or from field-captured specimens bred in the laboratory (Table 1). Intra-specific genetic variability of the representative specimens was maximized by drawing upon geographically diverse populations whenever possible (Figure 1). Samples were collected from sites throughout California.

Genomic DNA from each cyst was extracted using the InstaGene™ matrix (Bio-Rad Laboratories, Hercules, CA; Walsh et al. 1991) as described in Moorad et al. (1997). Each reaction mixture (25µl) consisted of 1X Promega polymerase buffer, 2.0 m*M* $MgCl_2$, 0.15 m*M* deoxynucleotide triphosphates, 1.25 U *Taq* DNA polymerase (Promega), 0.2 pmol random primer (Primer Kit #3, University of British Columbia, Biotechnology Laboratory, Vancouver, Canada) and 150 pg of DNA template. The mixture was overlaid with 40µl light mineral oil and denatured for 5 minutes at 94°C before 40 cycles of 15 seconds at 94°C, 1 minute at 36°C, and 1 minute at 72°C. The PCR was performed in a Perkin-Elmer 480 thermal cycler. One negative control was included in each run for each primer.

Twenty µl of the amplified reaction were electrophoresed in a 1.2% agarose gel in 1X TBE buffer (pH 8) for 3 hours at 50V. Gels were stained with ethidium bromide and photographed under UV light. The photographs were analyzed and the molecular sizes of the markers were determined using PCR Marker (Sigma Chemical Co.).

Three representatives from each species were subjected to preliminary PCR trials using 10 different RAPD primers. Of the 10 primers tested, 4 were selected for more extensive study because they produced (1) many distinct and intense bands, (2) one or more markers exclusive to one species, and (3) no bands in the negative controls. These 4 primers were used to screen 3-4 populations per species of 3-9 individuals each (Table 1). Markers that were present in all individuals of one species and totally absent in all individuals of the other two species were considered species-specific. After species-specific markers were selected, 3 hybrid crosses of 7-9 individuals each were analyzed and compared to the parental marker patterns.

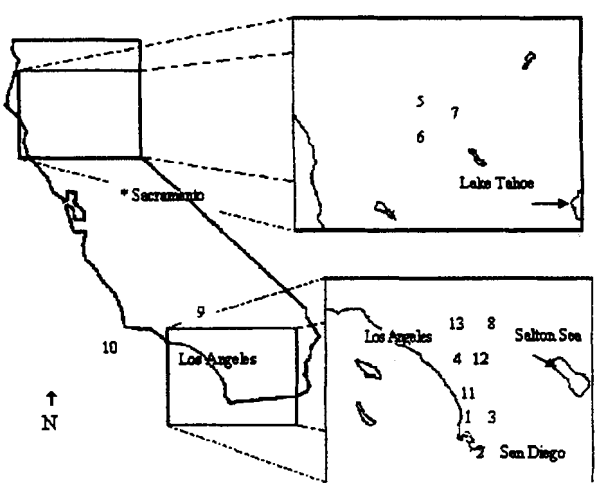

Figure 1. Locations of sampling sites for fairy shrimp in California as described in Table 1.

RESULTS

Of the 10 primers initially screened, primers UBC #212, 226, 246, and 262 satisfied all 3 of the initial set of criteria and were subsequently screened (see Table 2). Of these primers, UBC #262 failed to create markers that were conserved within any of the 3 species. The remaining 3 primers created 7 markers that satisfied the first set of criteria as well as the second with respect to *B. sandiegonensis*, *B. lynchi* and *B. lindahli* (Table 3 and Figure 2).

These seven markers were judged to be species-diagnostic markers. Primers UBC #212 and 246 were individually able to identify species and primer UBC #226 was able to confirm or deny the identity as *B. sandiegonensis*. As expected, each hybrid cyst included the diagnostic markers of both parental species (Table 3 and Figure 2).

DISCUSSION

There has been recent discussion regarding the general repeatability of RAPD marker patterns (Isabel et al. 1999, Rabouam et al. 1999). Our experience with this protocol is that patterns were very stable across multiple trials. This issue is discussed in more detail in Moorad et al. (1997). It should be noted, however, that both of our studies used the same thermocycler and there is the possibility that some of our diagnostic markers may not be conserved across all thermocyclers.

Any coastal southern Californian species of branchinectid fairy shrimp can be identified by the RAPD marker patterns generated from cyst tissue. Ephemeral pool branchinectid diversity can easily be determined by analyzing cysts from soil samples, an operation that can process large numbers of cysts within a few days. This is particularly valuable for determining presence of species and relative frequencies.

Inter-specific hybrids can also be detected using this technique. Hybridization is a potential problem in disturbed areas because tolerant generalists (such as *B. lindahli*) could out-compete and eliminate rare endemics (such as *B. sandiegonensis* and *B. lynchi*). Due to ex-

Table 2. Selection of primers for species-specific markers for distinguishing among 3 species of fairy shrimp from California.

Primer no.	Markers Present	Exclusive Markers	Conserved Markers
201	no		
204	yes	no	
212	yes	yes	yes
220	yes	no	
226	yes	yes	yes
228	yes	no	
246	yes	yes	yes
262	yes	yes	no
280	yes	no	
289	yes	no	

Table 3. Diagnostic marker size (in base pairs) for 3 species of branchinectids (*Branchinecta* sp.) from California.

Species and Inter-Specific Hybrids	UBC-212 (GCT GCG TGA C)	UBC-226 (GGG CCT CTA T)	UBC-246 (TAT GGT CCG G)
B. sandiegonensis	1230	940	670
	1465		
B. lynchi	1140		1830
B. lindahli			600
B. sandiegonensis	1140	940	670
X	1230		1830
B. lynchi	1465		
B. lynchi	1140		600
X			1830
B. lindahli			
B. lindahli	1230	940	600
X	1465		670
B. sandiegonensis			

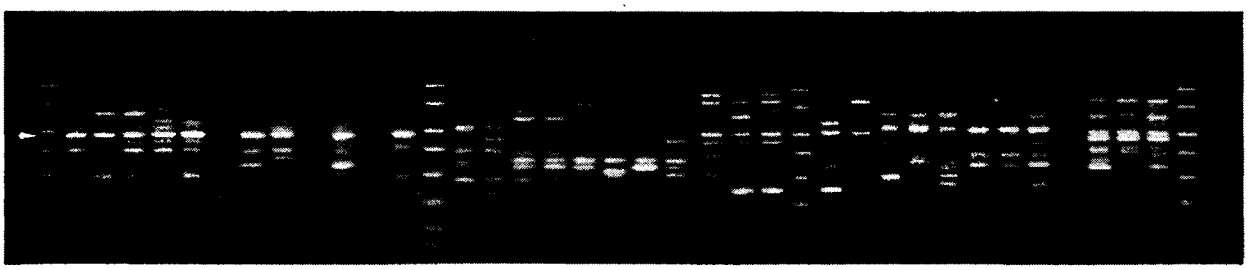

Figure 2. Example of a diagnostic marker for *Branchinecta sandiegonensis*. The primer UBC#226 amplifies a constant RAPD marker of 940 bp (arrow) present in *B. sandiegonensis* (lanes 2-6,8-9) but not in *B. lynchi* (lanes 15-22) or *B. lindahli* (27-34). In addition, note that this marker is conserved in the *B. sandiegonensis / B. lynchi* and *B. sandiegonensis / B. lindahli* hybrids (lanes 10, 12 and 36-38, respectively). RAPD weight marker lanes (1,14,26, and 39) consist of 2000, 15000, 1000, 750, 500, 300, 150, and 50 bp fragments) and are designated by 'M'. Blank lanes are negative controls in which no DNA template was added to the PCR.

treme habitat degradation and threats of development, most habitats containing these endemic species are already at risk.

Adult hybrid morphologies are intermediate between parental species and can be identified using morphological characters (Fugate, pers. comm.). However, because the tertiary envelope is maternally produced (Linder 1960), it follows that hybrid cyst morphology might more closely resemble the maternal species. As the parental species examined in this study produced visually indistinguishable cysts, it is not surprising that hybrid cysts of reciprocal species crosses were also indistinguishable. Studies using more dissimilar parental species and SEM may be used to test this hypothesis of tertiary envelope inheritance but it is clear that species-specific genetic markers are better able to resolve differences between the cysts of some closely related branchinectid species and their hybrids than are morphological analyses.

This is the first study to exploit branchiopod cysts as subjects for direct genetic investigation. Analyses using RAPD species-specific markers can quickly and easily differentiate between the 3 congeneric species in southern California. This allows occurrence or co-occurrence to be established at any time of the year if cysts are available. Extension of this technique to include all California species could be an important tool in providing for the protection, management and recovery of several listed anostracan species.

Future work will focus on expanding the library of known markers to include northern and central California species. We also plan to apply these techniques to the analysis of population genetic structure.

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