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# Genetic utility of natural history museum specimens: endangered fairy shrimp (Branchiopoda, Anostraca)

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## Abstract

We examined the potential utility of museum specimens as a source for genetic analysis of fairy shrimp. Because of loss of their vernal pool habitat, some fairy shrimp (including *Branchinecta sandiegonensis* and *B. lynchi*) are listed as threatened or endangered in Southern California by the United States Fish and Wildlife Service. Management of those species requires extensive population genetics studies and the resolution of important genetic complexity (*e.g.* possible hybridization between endangered and non-endangered species). Regulations mandating deposition of specimens of listed species have resulted in thousands of specimens accessioned into the Natural History Museum of Los Angeles County that have been preserved in a variety of solutions. We subsampled those specimens, as well as other Anostraca with known collection and preservation histories, to test their potential for genetic analysis by attempting DNA extraction and amplification for mt16SrDNA. Fixation and preservation in not denatured ethanol had a far greater sequencing success rate than other (and unknown) fixatives and preservatives. To maximize scientific value we recommend field preservation in 95% not denatured ethanol (or, if pure ethanol is unavailable, high-proof drinking spirits, *e.g.* Everclear™, or 151 proof white rum), followed by storage in 95% not denatured ethanol.

## Keywords

Museum specimens, Anostraca, Branchinectidae, *Branchinecta sandiegonensis*, *B. lynchi*, *B. lindahli*, endangered, threatened, vernal pool, California

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## Introduction

The largest collection of endangered Southern Californian fairy shrimp in the United States of America is at the Natural History Museum of Los Angeles County (LACM). The LACM is working closely with the United States Fish and Wildlife Service (USFWS) to increase the scientific value of these specimens for both morphological and molecular studies. Fairy shrimp occur in ephemeral vernal pool habitats worldwide (Keeley and Zedler 1998). In densely human populated areas, their fragile habitats continue to be severely degraded and many have been destroyed by urbanization (Bauder and McMillan 1998, King 1998, Simovich et al. 2013).

At least 15 plant species are recognized as threatened or endangered in California vernal pool habitats, but only a few invertebrates are similarly recognized (USFWS 2005). *Branchinecta conservatio*, *B. longiantenna*, and *B. sandiegonensis* are listed as "Endangered", and *B. lynchi* is listed as "Threatened" by the USFWS. In California, the USFWS issues permits for the collection of fairy shrimp and requires the deposition of endangered and threatened species in one of two repositories: the LACM or the California Academy of Sciences in San Francisco. Traditionally, Southern California specimens come to the LACM and northern California collections go to the California Academy. Since 1995 about 5,000 lots of *B. lindahli, B. lynchi,* and *B. sandiegonensis* have been accessioned into the LACM collections. This represents about 95% of our total anostracan holdings.

Simovich et al. (2013) suggest that human disturbance is increasing the generalist *B. lindahli's* range, which in turn is eroding the native range of *B. sandiegonensis*. Due to increasing sympatric distribution of these species, these authors (and Fugate 1998 before them) claim that the endangered and non-endangered species (*B. sandiegonensis* and *B. lindahli*) are hybridizing, thereby threatening the genetic integrity and persistence of *B. sandiegonensis*. Using a PCR-based screen using mitochondrial DNA to determine maternal lineage, in conjunction with morphological examination, Simovich et al. (2013) claim putative hybrids share their maternal DNA with the more common species at a site. Unfortunately, their claims are not testable or reproducible as the specimens used in their study are unavailable. Aside from this study, only an unpublished master's thesis exists that addresses genetic aspects of putatively hybrid populations of Southern California *Branchinecta sandiegonensis* (Andrews 2013). That study depended on prior researchers' assessments of hybridization in individual pools. These claims of hybridization underscore the need for comprehensive molecular studies to characterize the actual genetic diversity and species boundaries of Southern California fairy shrimp before further management and remediation recommendations are made.

In contrast to the lack of work being conducted on endangered Southern Californian fairy shrimp, there has been a large amount of work studying the genetics and phylogeographics of the endangered Californian salamander *Ambystoma tigrinum* (Amphibia: Caudata: Ambystomatidae) (Ryan et al. 2009, Johnson et al. 2010, Johnson et al. 2011). These studies were made possible in large part by a very extensive collection of samples — tail clippings — of *A. tigrinum* that span the salamander's

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geographic range through the last 25 years. Just as important as the breadth of the collection of tail clippings was that these samples were preserved with a method that made them accessible for molecular study decades later. The findings from these studies have already helped the management of *A. tigrinum* by identifying which populations have the greatest genetic diversity and allowing USFWS to target high value populations for increased protection (Johnson et al. 2011). The LACM is working closely with USFWS to assemble a collection of endangered Californian fairy shrimp necessary for similar genetic and phylogeographic studies. Both the LACM and USFWS fully expect that one day such studies will help better inform and shape the management of endangered fairy shrimp.

In this study we test whether preservation in pure not denatured ethanol makes anostracan museum specimens more readily accessible for molecular studies over anostracan museum specimens that had historically been fixed in denatured ethanol, isopropyl, or even acetone, then transferred into pure not denatured ethanol. Our study compares the success rates of amplifying a fragment of mt16SrDNA for specimens preserved in not denatured ethanol and for specimens in other preservatives. Because of their rarity and the difficulty in collecting fresh fairy shrimp specimens, being able to use specimens already in museum collections would be advantageous. To improve the utility of future collections, we suggest improvements in field and post-field preservation and handling based on our findings. If adopted, these improvements will greatly enhance the genetic usefulness of specimens and thereby allow more thorough assessments.

## Methods

### Material examined

We first inventoried, digitized, and georeferenced our entire anostracan collection — approximately 5,000 lots. We selected 50 specimens from across the taxonomic range that had been contributed by different collectors and consulting companies using a range of different field preservatives prior to deposition at the LACM (at the LACM, all specimens are transferred from the field preservative into fresh museum-grade not denatured ethanol). We then attempted to amplify a ~550 bp mt16SrDNA fragment (see Table 1).

### DNA extractions

The starting material for DNA extractions varied among samples, one thoracopod to an entire animal, depending on total animal body size. Tissue samples were placed on paper towel to dry. Precipitation Reagent (Epicentre MMP03750) was added to each sample and vortexed vigorously for 10 sec., then centrifuged at 4 °C for 10 min. at 14,000 rpm. The supernatant (~300 µl) was transferred to a 2 ml tube. Genomic DNA

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**Table 1.** Extractions and amplifications attempted for this publication. Taxa arranged in alphabethical order. Locality, specimen collection date, collector, and preservative are as transcribed from specimen labels. Specimen condition and body part used in extraction are indicated if this information was recorded. Double-stranded DNA concentration in ng/μL. Qubit value indicated as low, *i.e.*, 0<0.05 ng/μL. Asterisk (*) indicates sequence was generated and is listed in Table 2.

| | Taxon | Date of collection | Description of preservative on label | Locality | Collector | Part of specimen used | Extraction number | Outcome | dsDNA ng/μL 0<0.05 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Artemia monica* | 06-Jul-90 | 70% ethanol | California, Mono County, Mono Lake, south Tufa Reserve | H. Kuck | 1 broken specimen | 2013 | contaminated; blasts as *Homo* | 0 |
| 2 | *Artemia monica* | 01-Jan-10 | fixed and preserved in 95% ethanol | California, Mono County, Mono Lake | M. Hauser | 1 whole squished specimen | 2008 | *beautiful sequence | 39.1 |
| 3 | *Branchinecta coloradensis* | 23-Apr-92 | 70% ethanol | California, Lassen County, Hog Flat Reservoir | King, Gluesenkamp, Tritt | 1 broken specimen | 2003 | failed | 0 |
| 4 | *Branchinecta dissimilis* | 23-Mar-92 | 70% ethanol | California, Shasta County, Fall River | King, Gluesenkamp, Kloock | 2 broken pieces | 2017 | failed | 0.2 |
| 5 | *Branchinecta gigas* | unknown | acetone | California, San Bernadino County, Mojave Desert | J. Martin, J. Plum | 2 phyllopods only | 1990 | failed | 0.17 |
| 6 | *Branchinecta gigas* | unknown | not indicated | Washington, Grant County | unknown | 1 small whole specimen | 2006 | failed | 0.13 |
| 7 | *Branchinecta gigas* | unknown | not indicated | Washington, Grant County | unknown | dissected off egg sack with eggs | 2007 | failed | 0 |
| 8 | *Branchinecta lindahli* | 27-Dec-12 | fixed and preserved in 95% ethanol | California, San Diego County, Marine Corps Base Camp Pendleton | L. Woolley | not recorded | 2036 | failed | no data |
| 9 | *Branchinecta lindahli* | 27-Dec-12 | fixed and preserved in 95% ethanol | California, San Diego County, Marine Corps Base Camp Pendleton | A. Fisher | not recorded | 2037 | failed | no data |
| 10 | *Branchinecta lindahli* | 29-Dec-12 | fixed and preserved in 95% ethanol | California, San Diego County, Marine Corps Base Camp Pendleton | A. Fisher | not recorded | 2038 | failed | no data |
| 11 | *Branchinecta lindahli* | 28-Dec-11 | fixed and preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook, et al. | egg sac only | 1992 | *beautiful sequence | 6.62 |

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| | Taxon | Date of collection | Description of preservative on label | Locality | Collector | Part of specimen used | Extraction number | Outcome | dsDNA ng/µL 0<0.05 |
|---|---|---|---|---|---|---|---|---|---|
| 12 | *Branchinecta lindahli* | 02-Apr-12 | fixed and preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook | 1 gravid female | 2026 | *beautiful sequence | 13 |
| 13 | *Branchinecta lindahli* | 02-Apr-12 | preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook | 1 gravid female | 2027 | *beautiful sequence | 11.3 |
| 14 | *Branchinecta lindahli* | 02-Apr-12 | preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook | 1 gravid female | 2034 | failed | 0 |
| 15 | *Branchinecta lindahli* | 02-Apr-12 | preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook | 1 squished male | 2028 | *beautiful sequence | 29 |
| 16 | *Branchinecta longiantenna* | 23-Mar-10 | preserved in 70% ethanol | California, San Luis Obispo County, California Valley | Chris Powers | posterior half of single broken specimen | 2005 | failed | 39.6 |
| 17 | *Branchinecta lynchi* | 27-Feb-01 | fixed and preserved in 95% ethanol | California, San Luis Obispo County, Paso Robles | M. Dallas | 1 specimen, not gravid, not obviously male | 2032 | contaminated; blasts as cladoceran | 18.7 |
| 18 | *Branchinecta lynchi* | 13-Jan-04 | fixed and preserved in 95% ethanol | California, Santa Barbara Co., Los Padres National Forest | T. Murphey | squished gravid female | 2030 | failed | 0.3 |
| 19 | *Branchinecta lynchi* | 03-Feb-05 | fixed and preserved in 95% ethanol | California, San Luis Obispo County | D. Hacker | posterior half of gravid female | 2033 | failed | 51.4 |
| 20 | *Branchinecta lynchi* | 17-Feb-05 | fixed and preserved in 95% ethanol | California, Santa Barbara Co., Los Padres National Forest, Branch Mountain Quad | T. Murphey | squished gravid female; all animals in this lot are pretty mangled | 2031 | failed | 17 |
| 21 | *Branchinecta mackini* | unknown | 70% ethanol | Washington, Grant County | unknown | 1 specimen | 1991 | failed | 0.254 |
| 22 | *Branchinecta mackini* | 03-Apr-93 | 70% ethanol | California, San Bernadino County, Mojave Desert | C. Cash-Clark, T. Clark | 1 specimen | 2019 | failed | 0.16 |

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| | Taxon | Date of collection | Description of preservative on label | Locality | Collector | Part of specimen used | Extraction number | Outcome | dsDNA ng/µL 0<0.05 |
|---|---|---|---|---|---|---|---|---|---|
| 23 | *Branchinecta orientalis* | 22-Aug-02 | 95% ethanol | Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay) | R. Wetzer, S.L. Boyce, N.D. Pentcheff | 1 whole small specimen | 2004 | failed | 24.4 |
| 24 | *Branchinecta sandiegonensis* | 09-Mar-05 | preserved in 70% denatured ethanol, transferred to 70% ethanol | Mexico, Baja California, Tijuana, Jesus Maria Mesa | K.B. Clark | 1 gravid female | 2024 | failed | 0.7 |
| 25 | *Branchinecta sandiegonensis* | 13-Jan-11 | preserved in 70% denatured ethyl alcohol, transferred to 70% ethanol | California, San Diego County, Brown Field Municipal Airport | D. Wolff | posterior half of gravid female | 2029 | failed | 0 |
| 26 | *Branchinecta sandiegonensis* | 24-Nov-08 | preserved in 95% ethanol | California, San Diego County, Ramona Water District, Ramona Spray Fields | E. Ervin | eggsac + furca from female | 2023 | failed | 4.7 |
| 27 | *Branchinecta sandiegonensis* | 28-Dec-11 | preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook, et al. | anterior portion of female specimen | 1995 | failed | 25.7 |
| 28 | *Branchinecta sandiegonensis* | 17-Dec-07 | transferred to 95% ethanol Feb. 2011 | California, San Diego County, Otay Mesa, Dexstar Property | C. Powers | 1 male | 2025 | failed | 49.2 |
| 29 | *Branchinecta* | 28-Dec-11 | preserved in 95% ethanol | California, San Diego County, San Diego, Carmel Mountain Preserve | J. Snapp-Cook, et al. | 1 specimen | 1993 | failed | 7.5 |
| 30 | *Branchipodopsis affinis* | 22-Aug-02 | 95% ethanol | Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay) | R. Wetzer, S.L. Boyce, N.D. Pentcheff | 1 small specimen | 2001 | failed | 18.8 |
| 31 | *Branchipodopsis affinis* | 22-Aug-02 | 95% ethanol | Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay) | R. Wetzer, S.L. Boyce, N.D. Pentcheff | 1 small specimen | 2002 | failed | 49 |

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| | Taxon | Date of collection | Description of preservative on label | Locality | Collector | Part of specimen used | Extraction number | Outcome | dsDNA ng/µL 0<0.05 |
|---|---|---|---|---|---|---|---|---|---|
| 32 | *Chirocephalus* | 22-Aug-02 | 95% ethanol | Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay) | R. Wetzer, S.L. Boyce, N.D. Pentcheff | 1 whole squished animal | 2018 | *beautiful sequence | 57.9 |
| 33 | *Eubranchipus holmanii* | 07-May-40 | 70% ethanol | Canada, Nova Scotia, Edinberg [sic] | D. Belk | 1 male specimen | 2015 | contaminated; blasts as *Homo* | 0 |
| 34 | *Eubranchipus* | 01-Apr-32 | 70% ethanol | Canada, Ontario, Saint Thomas | M.S. Ferguson | anterior end of broken specimen | 2014 | failed | 0 |
| 35 | *Eubranchipus* | 30-Apr-99 | 70% ethanol | Minnesota, Bloomington | A.B. Forbes | 1 female –– doesn't look well preserved | 2022 | failed | 0 |
| 36 | *Eubranchipus* | 15-May-12 | fixed and preserved in 95% ethanol | California, Lassen County, Poison Lake | M. Hauser, D. Striley | posterior half of the single mushy specimen | 2020 | *beautiful sequence | 27.3 |
| 37 | *Linderiella occidentalis* | 19-Feb-92 | 70% ethanol | California, Tehama County, Tuscan Buttes | King, Mazzucco, Scuderi | 2 pieces broken specimen | 2000 | contaminated; blasts as *Homo* | 0.14 |
| 38 | *Linderiella occidentalis* | 24-Mar-92 | fixative unknown - transferred to 70% ethanol | California, Tehama County, Dale's Plains, Dale's Lake | King, Gluesenkamp, Kloock | 1 whole specimen | 1987 | failed | 0.225 |
| 39 | *Linderiella santarosae* | 26-Mar-04 | 70% ethanol | California, Riverside County, Murrieta, Mesa de Colorado, Santa Rosa Plateau | M. Angelos | 1 small female specimen | 1999 | contaminated; blasts as *Homo* | 0.293 |
| 40 | not identified | 08-Jun-11 | fixed and preserved in 95% ethanol | Utah, Wallsburg, near Provo-Jordan River Pkwy | M. Hauser | 1 female specimen | 2021 | failed | 47.6 |
| 40 | *Phallocryptus* | 22-Aug-02 | 95% ethanol | Mongolia, Dundgovi' aimag, northwest of Delgerhangay (Khashaat/Delger Khanay Uul) | R. Wetzer, S.L. Boyce, N.D. Pentcheff | posterior half of adult specimen | 2009 | *beautiful sequence | 10.3 |
| 42 | *Pristicephalus comptus* | 13-Apr-36 | 70% ethanol | Tennessee, Reelfoot Lake | unknown | 1 specimen, this lot had previously dried and had been realcoholed | 2012 | failed | 0 |

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| | Taxon | Date of collection | Description of preservative on label | Locality | Collector | Part of specimen used | Extraction number | Outcome | dsDNA ng/µL 0<0.05 |
|---|---|---|---|---|---|---|---|---|---|
| 43 | *Streptocephalus sealli* | 15-Aug-55 | 70% ethanol | California, Tulare County, Yosemite, Tioga Pass | unknown | 1 specimen, these had been previously dried and realcoholed | 1998 | failed | 0.213 |
| 44 | *Streptocephalus sealli* | 15-Aug-55 | 95% ethanol | California, Mariposa County, Yosemite, May Lake Trail | unknown | posterior end of animal | 1989 | failed | 0.18 |
| 45 | *Streptocephalus texanus* | 27-Aug-56 | 70% ethanol | New Mexico, Cain Ranch | S.F. Wood | dissected egg sack | 2010 | failed | 0 |
| 46 | *Streptocephalus woottoni* | 30-Mar-06 | 70% ethanol | California, San Diego County, Camp Pendleton, Marine Corps Base | S. Baldwin | ~5 phyllopods dissected off single specimen (only 1 specimen in the lot) | 1994 | failed | 7.28 |
| 47 | *Streptocephalus woottoni* | 01-Apr-05 | not recorded | California, San Diego County, Carlsbad, Poinsettia Lane Commuter Station Vernal Pools | J. Snapp-Cook | posterior half of male (already broken) | 2016 | *good sequence | 16 |
| 48 | *Streptocephalus woottoni* | 29-Jan-03 | preserved in 70% ethanol | California, Riverside County, Temecula | unknown | 3-4 phyllopods removed from single specimen | 1997 | failed | 6.8 |
| 49 | *Tanymastix stagnalis* | 12-Aug-34 | 70% ethanol | Denmark, Raabjerg Mile | E.W. Kaiser | 3 broken pieces used | 1996 | failed | 0.224 |
| 50 | *Thamnocephalus platyurus* | 01-Aug-56 | 70% ethanol | New Mexico, Gran Quivira | S.F. Wood | posterior portion | 2011 | failed | 0 |

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was extracted and purified with a Quick-gDNA™ MiniPrep Kit (Zymo Research) following the manufacturer's instructions, and eluted in a final volume of 60 µl of distilled water (in two elutions of 30 µl). Double-stranded DNA concentration of extractions was quantified using a Qubit 1.0 Fluorometer (Life Technologies) (see Table 1).

## PCR protocols

The mt16SrDNA fragment was amplified with universal 16Sar and 16Sbr primers (Palumbi et al. 1991) and both strands were sequenced. PCR reactions were done in a final volume of 50 µl. The volume of DNA used in each reaction varied from 2–25 µl depending on the DNA concentration measured on the Qubit. When possible, we tried to use at least 50 ng of DNA. Two different PCR reaction setups were used, as some samples successfully amplified with one, but not with the other. The first setup consisted of 10 µl of GoTaq Promega Buffer 5x, 5 µl of 2.5 mM $MgCl_2$, 4 µl of a 10 mM dNTP mixture, 2 µl of each primer at 20 µM, and 0.3–0.5 µl of GoTaq Polymerase at 5 U/µl (Promega). The second setup consisted of 25 µl of a 2x PCR Master Mix with 1.5 mM $MgCl_2$ (Thermo Scientific), and 1 µl of each primer at 20 µM. Both positive and negative controls were run in each experiment. Amplifications were performed in a BIO-RAD S1000 Thermal Cycler, with the following thermocycler conditions: an initial step of 5 min. at 95 °C, 35 cycles of 30 sec. at 95 °C, 30 sec. at 48 °C, 45 sec. at 72 °C, and a final extension of 10 min. at 72 °C. Amplifications were checked by running 5 µl of the PCR product on a 1.5% agarose gel. All failed amplifications were retried at least twice with different polymerases, buffers, and $MgCl_2$ concentrations. Successful PCR reactions were then purified with a DNA Clean and Concentrator-5 Kit (Zymo Research) and sequenced with both primers at Laragen Inc, Culver City, CA. Chromatograms were visually inspected and edited with 4Peaks (Griekspoor and Groothuis 2014).

## Contamination screening

Sequences were edited and contigs assembled in the software program Sequencher (Gene Codes Corporation 2004), and all contigs were BLAST searched in the NCBI database to verify they were not contaminants (*i.e.,* that sequence was indeed from the taxon of interest).

## Statistical testing

A Fisher's exact test (two-tailed, $\alpha$=0.05) was used to determine whether there was a statistically significant difference in sequencing success between the ethanol-preserved and other samples (Zar 1999). A Qubit 1.0 Fluorometer (Life Technologies) was used

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to quantify double-stranded DNA (Table 1). A one-tailed Mann-Whitney U test (Zar 1999) was used to assess statistical significance between double-stranded DNA concentration and amplification success.

## Results

Of the 50 individual anostracan samples on which we attempted PCR amplification, 13 were known to have been fixed and preserved in pure 95% ethanol, and 37 samples had unknown preservation histories but were suspected of being fixed and stored in denatured ethanol sometimes for years, until they were incorporated into the LACM collection. Of the samples fixed and preserved in 95% ethanol, 62% (8 out of 13) yielded useable mt16SrDNA sequences. In contrast, of the samples with unknown fixative and preservative history, only 3% (1 out of 37) yielded useable mt16SrDNA. The nine sequences generated here are available on GenBank (see Table 2). Sequencing success between samples fixed and preserved in ethanol and other samples was significantly different (Fisher's exact test, two-tailed, $P < 0.0007$).

The one-tailed Mann-Whitney U Test showed that there was a difference (at the $\alpha = 0.05$ level) between Qubit measurements of double-stranded DNA concentration for successful sequences vs. failed sequences, when amplifications of contaminants were considered as failed amplifications. However, direct examination of the data (see Table 1) showed that DNA concentration was a very poor predictor of sequencing success (except for the case of 0 or near-0 readings, which invariably failed).

## Discussion

### Existing museum specimens

Specimens known to be collected and preserved in 95% ethanol were successfully extracted, amplified and sequenced at a much higher success rate than those with unknown preservation history (probably denatured alcohol). Although some specimens enumerated in Table 1 indicate that they were preserved in 95% ethanol, label data does not distinguish denatured from not denatured ethanol, and the additional collector information provides only hints of the actual preservative in most cases. Specimens preserved in 70% denatured ethanol in the field and subsequently transferred to 95% not denatured ethanol failed. Based on previous experimentation, neither acetone nor isopropyl alcohol preservation resulted in successful amplification, so these preservatives were excluded from this analysis. Similarly, specimens known to have been exposed to formalin were excluded, as all previous attempts have failed for these types of broad taxonomic, spatial, and temporal studies using Sanger sequencing approaches (RW, pers. obs.). The interactions of formalin with specimens result in denaturation of the DNA and a variety of other reactions (Tang 2006). Additionally, over time, oxida-

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**Table 2.** Nine new mt16SrDNA Anostraca sequences: taxonomy, Genbank number, and locality information. All specimens and DNA are deposited in the collections of the Natural History Museum of Los Angeles County. Required permits are on file at USFWS and/or LACM.

| Genus/species | Genbank No. | Locality |
|---|---|---|
| Artemiidae: *Artemia monica* | KF790567 | USA, California, Mono County, Mono Lake, ~38.011°N ~119.012°W, hypersaline lake, 95% ethanol. 1 Jan 2010. Coll. M. Hauser. RW12.244.2008 |
| Branchinectidae: *Branchinecta lindahli* | KF790568 | USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.929°N, 117.22°W, vernal pool 4 in. deep, 8 ft. wide, 28 ft. long, water slightly murky, 63 µm net, 95% ethanol. 28 Dec 2011. JS pool #21, MBPC 11637. Coll. J. Snapp-Cook, C. Lieberman, A. Wall, P. Sun, R. Wetzer. RW13.042.1992 |
| Branchinectidae: *Branchinecta lindahli* | KF790569 | USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.933°N, 117.215°W, vernal pool in dirt road, 95% ethanol. 2 Apr 2012. City ID # 22, js_fs_37, MBPC13258. Coll. J. Snapp-Cook. RW13.047.2026 |
| Branchinectidae: *Branchinecta lindahli* | KF790570 | USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.932°N, 117.215°W, vernal pool in dirt road, 95% ethanol. 2 Apr 2012. City ID # 20, js_fs_38, MBPC13259. Coll. J. Snapp-Cook. RW13.048.2027 |
| Branchinectidae: *Branchinecta lindahli* | KF790571 | USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.928°N, 117.22°W, vernal pool in dirt road, 95% ethanol. 2 Apr 2012. City ID # 26, js_fs_35, MPBC13256. Coll. J. Snapp-Cook. RW13.046.2028 |
| Chirocephalidae: *Chirocephalus* sp. | KF790572 | Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay), 46.135°N, 105.106°E, 2 acre pond, 0-1 ppt, 23.2°C, 63 µm mesh net, 95% ethanol. 22 Aug 2002. GPS#016, Mongolia Expedition 2002, MBPC 431. Coll. R. Wetzer, S.L. Boyce, N.D. Pentcheff. RW13.034.2018 |
| Chirocephalidae: *Eubranchipus sp.* | KF790573 | USA, California, Lassen County, Poison Lake, 40.659°N, 121.197°W, temporary lake, hand, 95% ethanol. 15 May 2012. Coll. M. Hauser and D. Striley. RW12.242.2020 |
| Steptocephalidae: *Streptocephalus woottoni* | KF790574 | USA, California, San Diego County, Carlsbad, Poinsettia Lane Commuter Station Vernal Pools, large pool at southern end of complex, 33.108°N, 117.318°W, vernal pool 15 m x 30 m, 12-24 inches deep, murky water, 1 Apr 2005. MBPC 10061. Coll. J. Snapp-Cook. RW13.007.2016 |
| Thamnocephalidae: *Phallocryptus sp.* | KF790575 | Mongolia, Dundgovi' aimag, northwest of Delgerhangay (Khashaat/Delger Khanay Uul), 45.424°N, 104.481°E, large lake reduced to tiny watering hole, 11 ppt, 28°C, 63 µm mesh net, 95% ethanol. 22 Aug 2002. GPS#020, Mongolia Expedition 2002, MBPC 435. Coll. R. Wetzer, S. L. Boyce, N. D. Pentcheff. RW13.036.2009 |

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tion of formaldehyde in formalin to formic acid produces an acidic solution resulting in the scission of DNA. The smaller the specimen, the greater the effect, and the lower the likelihood of success of long strand DNA extraction. The Tang (2006) study, commissioned by the National Academy of Sciences, provides a detailed (and discouraging) review of DNA extraction and sequencing from formalin-fixed biological samples.

## Collecting recommendations

Our aim was to maximize the scientific value of specimens and their biological usefulness for future studies. First, the results of our study make a very compelling case that initial specimen fixation and preservation in the field should use 95% ethanol — *not* denatured ethanol or other alcohols. If not denatured ethanol is unavailable, we recommend fixation and preservation in 100 proof (or higher) vodka, rum, Everclear™, or similar drinking alcohol, rather than any sort of denatured alcohol. This method, although the next best choice, has been successfully used during expeditionary work by one of us (RW) since the mid-1980s. Although 100 proof spirits are only 50% ethanol by volume, the quality of the alcohol matters more than the concentration — if you cannot drink it, it's *not* good for specimens. Second, specimens should always be in a volume ratio of at least 3:1 alcohol:specimens to avoid degradation from dilution of preservative by body fluids. Third, once specimens are returned from the field, ethanol should be replaced with fresh 95% not denatured ethanol to compensate for dilution of the preservative by water extracted from specimen tissue.

In addition to the changes we suggest for the fixation and preservation, we also suggest changes to the type and number of voucher specimens being deposited after an environmental impact report is completed. We recommend accessioning specimens of all species, whether listed or not (*e.g.* whether endangered or threatened, or not). For example, simply accessioning both the listed and non-listed species will make it possible to definitively address questions about hybridization between *B. sandiegonensis* and *B. lindahli*. Furthermore, depositing all specimens collected for a survey, not just a single voucher specimen for each species, will increase sample sizes to enable population level molecular studies.

These small improvements to collecting protocols will make it possible to derive high-quality data for future biodiversity and phylogeographic research. Since the sacrifice of endangered and non-endangered crustaceans is necessary to evaluate their presence and abundance in the wild, they can become a valuable historic resource if properly curated and deposited.

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