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An effective procedure for DNA isolation and voucher recovery from millimeter-scale copepods and new primers for the *18S* rRNA and *cytb* genes

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ABSTRACT

Many investigators need to determine whether individuals belong to the same species. DNA-sequence data have helped with this task, but current procedures of DNA isolation from millimeter-scale crustaceans, such as harpacticoid copepods, leave little to no voucher material for morphological analysis, and many procedures yield only enough DNA for a single amplification reaction. We therefore developed a DNA-isolation procedure that yielded essentially intact exoskeletons and sufficient DNA for multiple polymerase chain reactions. DNA-amplification success of our DNA-isolation procedure was relatively insensitive to (1) the length of preservation time from sample collection to DNA isolations and (2) the length of time the DNA was stored at −20 °C after isolation. An additional benefit of our procedure is therefore that the DNA isolated is relatively stable. Primers available for the nuclear *18S* rRNA gene and the mitochondrial cytochrome oxidase *b* (*cytb*) gene are known not to work for many harpacticoids. We therefore designed primers that would amplify and sequence an ~750-base-pair fragment of the *18S* rRNA gene and others that would amplify and sequence an ~450-base-pair fragment of the *cytb* gene. Both primer sets worked for at least 12 harpacticoid families.

1. Introduction

Investigators studying ecology, biogeography, or biodiversity must often decide whether individuals are conspecific. Traditionally, these decisions were based on morphological characters, but unfortunately, speciation often occurs without changes in taxonomically important characters (see, e.g., Knowlton, 1993; Wake et al., 1983), causing individuals of different species to be morphologically indistinguishable (i.e., to be cryptic species). Problems also arise if morphological variability within a species is greater than differences ordinarily found between closely related species. In such cases, an investigator using morphological procedures may exclude some individuals that belong to the species in question. Further, the taxonomic importance of some morphological characters may not yet be recognized (see, e.g., Knowlton, 1993; Norris, 2000), so investigators could assign individuals of more than one species to a single species.

As analysis of DNA sequences has become more common, investigators have been able to use them to recognize conspecific individuals (see, e.g., Hajibabaei et al., 2007; Knowlton, 2000; Vogler and Monaghan, 2007). When both morphological and DNA procedures are used on the same individuals and the results agree, an investigator can have much greater confidence in the assignment of individuals to species.

Recent work has demonstrated the need for a combined molecular and morphological approach to the study of copepods (Bron et al., 2011; Garlitska et al., 2012), which are millimeter-scale crustaceans that are abundant and speciose in marine and freshwater environments (Humes, 1994). For example, potential cryptic species have been discovered (Rocha-Olivares et al., 2001; Schizas et al., 1999), and overlooked morphological differences of taxonomic importance have been documented (Easton et al., 2010; Rocha-Olivares et al., 2001; Schizas et al., 1999, 2002; Staton et al., 2005). These issues hinder our ability to understand the biodiversity and the species-specific ecological roles of copepods (Bron et al., 2011; Garlitska et al., 2012) because individuals cannot be assigned to species with confidence. With combined approaches to assigning individuals to species, ecologists can determine their species-level contributions to such issues as carbon cycling and responses to environmental perturbations.

Although combined approaches have been used, current procedures need improvement. First, many of the DNA-isolation procedures in current use destroy the individual (see, e.g., Braga et al., 1999; Burton, 1998; Caudill and Bucklin, 2004; Edmands, 2001; Lindeque et al., 1999; Papadopoulos et al., 2005; Street et al., 1998; Thum, 2004; Vestheim et al., 2005) or leave very little of it (see, e.g., Bucklin et al.,

Abbreviations: 18S, nuclear small-subunit ribosomal RNA; bp, base pair; *cytb*, mitochondrially encoded cytochrome *b*; FSU, Florida State University; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

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2003; Easton et al., 2010; Vestheim et al., 2005) for morphological examination. Because genetic data may reveal the need for additional morphological analysis, as much as possible of each individual should be retained after processing for DNA.

Second, many current DNA-isolation procedures allow only a single DNA-amplification reaction (e.g., those of Bucklin et al., 2003; Burton, 1998; Caudill and Bucklin, 2004; Lindeque et al., 1999; Street et al., 1998), so if in the course of a study, DNA amplification must be repeated or additional target regions amplified, no DNA remains for these reactions. DNA-isolation procedures for millimeter-scale individuals should therefore yield sufficient DNA template for multiple polymerase chain reactions (PCR; Saiki et al., 1988).

These first two problems can be solved with DNA-release procedures. Unfortunately, these methods are not generally known to yield DNA stable enough to permit DNA to be isolated (or PCR amplified) after more than a few weeks of storage (see, e.g., Giraffa et al., 2000; Hajibabaei et al., 2005; Kim et al., 2012). Circumstances can arise under which DNA isolation (or PCR amplification) can only be done months after collection (or DNA isolation), for example, when new primers must be developed.

Fourth, although universal primers are available for the genes (e.g., *COI*, *cytb*, 18S) commonly used for species-level biogeography studies, they tend to be unsatisfactory for use on millimeter-scale copepods (E.E.E., personal observation). In particular, they often fail because complementary sequences do not exist in the target genes for many species of copepods, especially those of the order Harpacticoida. Without primers that will amplify and sequence species belonging to many families, some fundamental studies cannot be done.

The goals of our study were to develop a DNA-isolation procedure for single, millimeter-scale copepods that (1) yields an essentially complete voucher for morphological analysis, (2) yields sufficient DNA template for multiple PCR amplifications, and (3) yields DNA that is stable for more than one year. We also developed (4) amplification and sequencing primers for a target region in the nuclear *18S* ribosomal RNA (rRNA) gene and one in the mitochondrial cytochrome oxidase *b* (*cytb*) gene that work for individuals from many harpacticoid families.

2. Material and methods

2.1. Sample collection and preparation

2.1.1. Cultured individuals

We used cultures of a shallow-water harpacticoid species, *Amphiascoides atopus* Lotufo and Fleeger 1995 (Miraciidae), to develop and test our procedure. We maintained them in 1-L flasks with 550 ml of artificial seawater (salinity = 30) made with Instant Ocean® Aquarium Sea Salt (Spectrum Brands, Madison, WI) and deionized water. We fed the cultures ~0.01 g of crushed TetraMin® Tropical Crisps (Tetra Holding, Blacksburg, VA) every two to four weeks. (Cultures of this species are also maintained by Adelaide Rhodes, Harte Institute for Gulf of Mexico Studies, Corpus Christi, TX.) Living individuals were placed in 100% ethanol at −20 °C for at least 24 h before DNA isolation.

2.1.2. Deep-sea individuals

We tested our procedure on benthic deep-sea copepods, mostly members of the Harpacticoida. We collected sediment samples with a version of the Barnett et al. (1984) multiple corer (Ocean Instruments MC 800 Multi Core, San Diego, CA) from stations on the continental rise off the west coast of the United States of America (Table 1). The overlying water and the top centimeter of sediment from a given core were collected, combined, preserved with cold 95% ethanol, and stored at −20 °C.

In the laboratory, we used sieves to separate the 300-μm fraction from the 30-μm fraction for each sample. Organisms in the 300-μm fraction were stained overnight in a solution of 200 ml of 100% ethanol and 0.25 g of rose bengal. For the 30-μm fraction, we used Ludox® HS-40 (E.I. du Pont de Nemours, Wilmington, DE) to separate most organisms from most of the sediment (see Burgess, 2001). Organisms in the 30-μm fraction were stained overnight in a solution of 200 ml of 100% ethanol and 0.3 g of Congo red. We used a stainless-steel loop to remove copepods from both size fractions under a dissecting microscope. Copepods were stored in 100% ethanol at −20 °C.

Table 1
Average depth, latitude, and longitude of stations from which we collected copepods.

Station	Depth (m)	Latitude (decimal degree)	Longitude (decimal degree)
1	3247	44.00	−130.39
2	3593	42.56	−131.92
3	3673	39.99	−125.88
4	2733	40.00	−125.45
5	3682	36.80	−123.70
6	2717	36.68	−122.82
7	3854	32.87	−120.62
8	2699	32.80	−120.37

2.2. DNA isolation

2.2.1. InstaGene™ Matrix

To isolate DNA, we used the DNA-releasing solution InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA). It contained a Chelex®-based ionic resin thought to bind to PCR-inhibiting and DNA-degrading compounds (see Walsh et al., 1991, and references therein).

2.2.2. DNA isolation from individuals

2.2.2.1. Pre-DNA-isolation imaging. In studies of single individuals, we used a stainless-steel loop to transfer each individual from ethanol to 100 μl of nuclease-free water (Integrated DNA Technologies, Coralville, IA) in a well of a custom-made, acrylic, depression-well plate (~0.5-ml volume wells with a diameter of ~15 mm). After the individual rehydrated for at least 10 min, we transferred it to a drop of glycerin on a single-concavity slide (Fisher Scientific, Pittsburgh, PA), placed the individual under a #1.5 cover slip, and made lateral and dorsal images of it (at 5 × to 20 × magnification, as appropriate) with a Moticam 2500 camera (Motic, Richmond, British Columbia, Canada) mounted on an Axioskop compound microscope (Carl Zeiss Microscopy, Oberkochen, Germany). After imaging, we returned the individual to the original well for at least 10 min to remove glycerin and ethanol and then transferred it to a new well that contained 100 μl of nuclease-free water.

2.2.2.2. DNA isolation. After ~10 min, we isolated DNA with InstaGene™ Matrix following a modified version of the manufacturer's protocol. The InstaGene™ Matrix was mixed at room temperature for a minimum of 60 s at a moderate speed (set at 4.5 on a 6-point scale) on a Thermix® Stirring Hot Plate 310 T (Fisher Scientific). We transferred 100 μl of the material to a sterile, 0.5-ml, Axygen® MAXYMum Recovery™ microcentrifuge tube (Corning, Tewksbury, MA) using a 1000-μl pipette. (Note that a large-bore pipet tip is required for maintenance of the original concentration of 6% InstaGene™ Matrix.)

We transferred the ethanol-free (see Section 2.2.2.1.) individual to this tube of InstaGene™ Matrix with a stainless-steel loop that had been sterilized in 5% hydrogen peroxide. After we confirmed visually that the individual was submerged, we incubated the tube in a water bath at 56 °C for 30 min, vortexed it on a Maxi Mix II (Thermolyne, Dubuque, IA) at maximum speed for 10 s, shook the solution to the bottom of the tube by hand, confirmed that the copepod was submerged, and placed the tube back in the water bath overnight. We then vortexed the tube for 10 s at maximum speed, shook the solution to the bottom of the tube by hand, and confirmed that the copepod was submerged. The