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An efficient DNA extraction method for small metazoans

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Abstract

The isolation of total nucleic acids from small metazoan taxa is difficult and often leads to an unacceptably large percentage of unsuccessful polymerase chain reaction (PCR) amplifications. Our work with the evolutionary genetics of harpacticoid copepods was an incentive to refine techniques such that consistent amplifications from minute marine organisms were feasible. We describe these modifications and demonstrate their utility for the amplification of multiple loci from single harpacticoid copepods.

Introduction

Despite the abundance of techniques that have been developed, the isolation of template DNA for PCR (Saiki et al., 1988) amplification from individual, small organisms can be difficult. Metazoan taxa less than 1 mm in length (generally less than 10 μg dry weight) have small amounts of target DNA to start, and generally the entire organism must be consumed in the extraction protocol. When these samples are precious due to difficulties associated with sample acquisition, a low ratio of successful to unsuccessful DNA isolations or PCR amplifications is obviously unacceptable.

Harpacticoid copepods are small (< 500 μm total length) crustaceans associated with marine and aquatic sediments. Isolation and subsequent amplification of the DNA of these organisms has been accomplished (e.g., Burton and Lee, 1994), but can be problematic (Street and Montagna, 1996). We believe this difficulty arises because harpacticoids are small and "dirty"—they live in close contact with sediment, fungi, bacteria, and other contaminants that potentially interfere with standard DNA extraction protocols. Furthermore, many benthic taxa produce mucous substances that accumulate debris on the cuticle during the preservation process. While microscopic, these are adult animals with a relatively thick chitinous cuticle that might make their tissue more difficult to work with than, for example, invertebrate eggs or larvae (Cary, 1996).

Previously, researchers have worked with harpacticoid DNA from large numbers of individuals by establishing isofemale lines (Burton, 1994; Burton and Lee, 1994). The descendants of a single female theoretically share identical cytoplasmic (i.e., mitochondrial) genomes and thus can be pooled for subsequent analysis; however, recombination potentially complicates the analysis of nuclear gene diversity in this manner. Furthermore, collecting the offspring of a single female is labor intensive and requires knowledge of species culturing requirements that can vary widely over the order Harpacticoida (Hicks and Coull, 1983). Alternatively, standard extraction protocols can be used, but the frequency of unsuccessful extractions or amplifications can be as high as 30% to 50% (Street and Montagna, 1996; Street et al., 1997). Similarly, these standard extraction protocols typically produce only minute amounts of template DNA (presumably due to losses associated with precipitation and sample manipulation) so that multiple PCR assays per individual animal are not possible.

We have had consistent success using a modification of the protocol described in a commercially available product (GeneReleaser, BioVentures, Inc.) to extract and subsequently amplify DNA from small marine metazoans. Although originally designed for the extraction of DNA from blood serum and other "dirty" tissues, we suspected that the

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qualities that made the product amenable for working with blood samples would be useful for whole organisms as well. We describe this modified technique and demonstrate its utility for PCR amplification of multiple loci from single harpacticoid copepods.

Results and Discussion

Initially, PCR amplifications of template DNA isolated from pooled samples of 1 to 10 copepods following the manufacturer's protocol did not yield a satisfactory success rate (less than 10%). Template DNA yield was much more consistent when the protocol was modified as follows. A single copepod, (frozen at −80°C or preserved in 95% ethanol) was picked with an Irwin loop while viewed under a dissecting microscope directly into 10 μl of ice-cold PCR Buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3; Perkin-Elmer Cetus) in a 500 μl microcentrifuge tube. The tube was centrifuged briefly, and 1 μl of a10 mg/ml proteinase K (PK) solution was added and mixed by pipetting. The tube was transferred to a water bath and incubated at 55°C for 3 hours (tubes were gently mixed every hour and tap-spun to collect the condensate). Following incubation, each tube was placed in a 100°C heating block for five minutes to ensure complete inactivation of the PK (we have found that this heating step is crucial for successful template isolation and subsequent PCR amplification). Subsequently, 10 μl of GeneReleaser was added to each extraction, and the GeneReleaser cycling protocol was run as per the manufacturer's instructions. Subsequently, each tube was centrifuged for one minute at 13,000 *g*, and the clear supernatant (approximately 20 μl) was transferred to a fresh 500 μl tube. Typically, for a 50-μl reaction, 2 μl of this supernatant was sufficient template to produce an easily visible product on a 1% agarose gel after 40 cycles of the PCR (50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 8.3, 200 μM each dNTP, 5 pmol forward and reverse primer, and 1 unit *Taq* DNA polymerase, Perkin-Elmer Cetus).

Using this extraction method, we have been able to amplify successfully portions of the mitochondrial cytochrome oxidase subunit I gene (COI; forward and reverse primers were modified versions of those described in Folmer et al., 1994) from single specimens of the harpacticoid copepod *Microarthridion littorale* (Figure 1). Direct sequencing of the COI gene product from several individuals

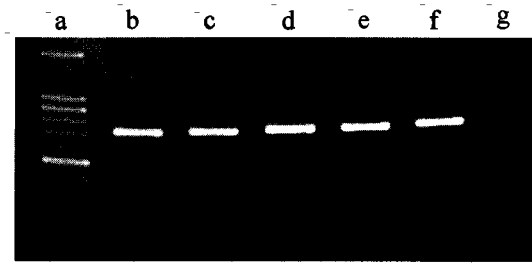

Figure 1. Cytochrome oxidase subunit I PCR products from individual *M. littorale* (using 2 μl template DNA from an approximately 20 μl PK-GeneReleaser extraction). Lane a, Commercial 100 bp ladder (Promega); lanes b–f, PCR products from individual copepods; lane g, Negative control.

and comparison with other sequences in GenBank (Release 100.0) indicated high homology between *M. littorale* COI and COI from the only other harpacticoid copepod in record, *Tigriopus californicus* (Burton and Lee, 1994). This protocol extends to other marine taxa and has been used to amplify the mitochondrial COI locus from other benthic taxa including copepod taxa other than *M. littorale*, polychaete juveniles, barnacles, and amphipods (N.V.S., unpublished data). These results can be generalized beyond the assay of mitochondrial genes to include nuclear gene loci. We have been able to amplify consistently the nuclear gene tubulin (primers were from Palumbi, 1996) from single specimens of *M. littorale* with success rates comparable to that observed with the mitochondrial COI locus (data not shown).

The advantages of this technique include the high rate of successful extraction and amplification from small animals and the ability to assay individual animals for multiple loci. We have performed over 500 PCR reactions using this method with a success rate of greater than 95%. We believe that this success rate can be partly attributed to the fact that precipitation (and associated losses) is unnecessary with our method. Indeed, only a single transfer of an aqueous phase is required, reducing the possibility of template loss and the chance of contamination among samples. Overall, we estimate that the PK-GeneReleaser method provides sufficient DNA template for 8 to 10 PCR amplifications from small metazoans such as *M. littorale*, facilitating the assay of multiple loci per individual. We believe this approach is well suited for isolating and amplifying DNA from a diverse assemblage of small, benthic organisms and might prove amenable to other larger, "problematic" taxa as well.

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