

—Report—

Comparison of the efficiency of three methods of DNA extraction for deep-sea benthic copepods

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Meiofauna are small animals that are frequently used as indicators of environmental impacts. Among the meiofauna, Copepoda (Crustacea: Maxillopoda) is one of the most sensitive taxa to environmental changes. It is necessary to extract DNA sequences of copepods to obtain precise estimations of their biodiversity. However, the data presently registered are insufficient not only because of the inefficiencies in DNA extraction and PCR amplification, but also because there is a need for morphological reexamination after sequencing them. In the present study, we compared the efficiencies of the following three methods of DNA extraction for copepods—the Squish Buffer, GeneReleaser[®], and InstaGene methods—with respect to extracted DNA concentrations, morphological characteristics following DNA extraction, in addition to the effect of Rose Bengal staining to PCR amplification. The Squish Buffer method yielded more quantity of DNA than the other methods; however, the morphology of their exoskeleton was better preserved with the InstaGene method. Nested PCR using both the universal and specific primer sets amplified a sufficient amount of DNA for sequencing, even when the extracted DNA concentration was <10 ng/μL. Thus, DNA extraction by the InstaGene method followed by nested PCR is currently best way to obtain both morphological and genetic information from a single copepod specimen. A comparison of PCR amplification rates indicated that Rose Bengal inhibited amplification, which is consistent with reports in other taxa. The present results should facilitate both morphological and genetic analyses of deep-sea copepods.

Keywords : meiofauna, *Stygiopontius*, Rose Bengal staining, specific primer

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1. Introduction

Meiofauna are small animals that can pass through a 1-mm sieve but are retained by a 32- μ m sieve. They are useful as indicators of environmental impacts caused by climate change and anthropogenic activities (Zeppilli et al., 2015). Recent application of next-generation sequencing for metagenetic or metabarcoding analyses of sediment-derived DNA have shown tremendous diversity of meiofauna, with variation of broad taxonomic levels. In addition, cryptic species-level diversity can be demonstrated by both Sanger sequencing and morphological observation, and elucidation of the diversity is important to understand functioning of the ecosystem (e.g., Carugati et al., 2015).

Copepoda is the second most abundant meiofaunal taxon, inhabiting a variety of aquatic environments. They are among the most sensitive groups to changing environmental conditions (Zeppilli et al., 2015). In addition, they are integral to the aquatic food web, evolutionary processes, and diversity (Bron et al., 2011). However, only a limited number of DNA sequences of benthic copepods are available in public databases, and morphology-based community analyses have revealed that changes in community composition reflect those in the ecological states of the habitats (Bron et al., 2011, Easton and Thistle, 2014). The existence of cryptic species and the possibility of an underestimation of the meiofaunal biodiversity are now evident (Carugati et al., 2015). Therefore, morphological and genetic data are both required from the same specimen for more precise estimation of biodiversity and environmental impact assessments. However, obtaining such data for meiofauna is difficult, for example, the inefficiency of DNA extraction and amplification, the destruction of very small but important morphological features for identification, and the subsequent challenges encountered in the reexamination and description of the specific features of cryptic or novel species.

Most molecular studies on copepods have extracted a sufficient quantity and quality of DNA either from approximately 100 cultivated individuals derived from a single female copepod or from $>4\text{ mm}^3$ of a collection of environmental samples (e.g., Machida et al., 2002, Huys et al., 2007). Fine-scale ecological studies such as those focused on population genetics require DNA from a single individual. To date, three DNA extraction methods from a single individual is commonly used in genetic analyses

of copepods: 1) the Squish buffer method (Sakaguchi and Ueda, 2010), which has been modified from its primary application in *Drosophila* (Gloor et al., 1993); 2) the GeneReleaser® method, which is a specialized method for small metazoans (Schizas et al., 1997); and 3) the InstaGene method, which is a method being used for deep-sea benthic copepods (Gollner et al., 2011, Easton and Thistle, 2014). Among these methods, only the InstaGene method preserves the morphological characteristics of copepods following DNA extraction (Easton and Thistle, 2014). However, three methods have not been compared with respect to the amount of extracted DNA and the morphological changes induced, as pointed out for many cases (Carugati et al., 2015).

An additional difficulty associated with obtaining sequence data in copepods is the inefficiency of DNA amplification. This appears to be caused by the uptake of a specific stain in the sample. Rose Bengal is a major staining reagent that increases the visibility of meiofauna in sediment samples. However, in nematodes (the most abundant meiofauna), Rose Bengal hinders DNA amplification and sequencing, regardless of the method of fixation (Fonseca and Fehlaue-Ale, 2012). Its inefficiency in copepods has not yet been reported (Easton and Thistle, 2014). In addition, to our knowledge, no comparisons in the efficiency of PCR or DNA sequencing have been made between copepod samples with and without Rose Bengal staining.

We performed the present investigation to answer the following questions: 1) Are there any differences in the concentration of extracted DNA among the methods?, 2) Are there any differences in morphological characteristics following DNA extraction?, and 3) Are there any differences in the processes of DNA amplification in copepods, with or without the use of Rose Bengal staining? A deep-sea benthic copepod of the genus *Stygiopontius* (Copepoda: Siphonostomatoida: Dirivultidae) was used in this study, as this species is abundant in deep-sea hydrothermal vent fields, which are possible targets for deep-sea mining operations (Gollner et al., 2011). Our results will facilitate an informed choice regarding the best method by which copepod DNA can be extracted and amplified for genetic studies.

2. Materials and methods

2.1 Sample collection

Copepods were collected along with other macro-

and meiofauna from bacterial mats, using a suction sampler with a 32 µm-mesh, installed on a remotely operated vehicle (ROV) *Hyper-Dolphin* dive #1647 in the Bayonnaise Knoll hydrothermal vent field, Izu-Ogasawara Arc, northwestern Pacific (31°57.363' N, 139°44.731' E, depth = 778 m). Samples were divided into two parts by a plankton divider (RIGO Corp.): one part was washed with 99.5% ethanol on a 32-µm sieve and was preserved in a 250-mL plastic bottle containing fresh ethanol (99.5%) and stored at room temperature for genetic analysis, and the second part was used for morphology-based analyses (Senokuchi et al., unpublished).

The copepods of the genus *Stygiopontius* were detected using Rose Bengal staining (5–10 mol/L) under a binocular stereoscopic microscope (SMZ800, Nikon) within a terrestrial-based laboratory. Males and females ($N = 14$ each) were used to compare the efficiency of DNA extraction among the three methods. Comparisons were performed individually for males (body length without caudal rami was about 500 µm) and females (about 800 µm), because of the disparity in size. In addition, 4 individuals stained with Rose Bengal and 10 unstained individuals were identified for PCR analysis.

2.2 Morphological observation

All samples were washed with ultrapure water (Milli-Q) before their photographs were taken (Fig. 1A and B). Their morphological characteristics were observed under a compound microscope equipped with differential interference contrast (Eclipse 80i, Nikon) and a digital camera (D5200, Nikon). Samples were identified as an undescribed species of the genus *Stygiopontius* based on the appearance of setae and spine formulae and reference to the identification keys of Gollner et al. (2010) and species descriptions of original articles. Morphology of each of the three specimens was observed following each of the three DNA extraction methods to compare the extent of the damage caused to the specimens. Their entire body was first observed and photographed under the compound microscope. Each specimen was then dissected under the stereoscopic microscope, and the resected appendages were photographed under the compound microscope. Photographs of the resected appendages before DNA extraction were taken from a specimen that was not used for DNA extraction, as the resection appeared to affect the efficiency of DNA extraction and morphological preservation during

the process.

2.3 DNA extraction

DNA was extracted into 10-µL solution from each of three male and three female specimens for comparisons among the Squish buffer (Sakaguchi and Ueda, 2010), GeneReleaser® (BioVentures, Inc.) (Schizas et al., 1997), and InstaGene (Bio-Rad, Inc.) (Easton and Thistle, 2014) methods. The samples were removed from 99.5% ethanol and were washed in nuclease-free water for 10 min prior to DNA extraction. DNA extraction using the InstaGene Matrix was followed according to the procedures outlined by Easton and Thistle (2014), except for the volume of InstaGene Matrix (10 µL instead of 100 µL; described below). The DNA concentrations were measured by the NanoDrop 2000 spectrophotometer (Thermo Scientific, Inc.).

In addition to the aforementioned inter-method comparisons, intra-method comparisons were conducted for the InstaGene method, as the reagent volume originally recommended for this method (100 µL) was greater than that used in the inter-method comparison. We performed DNA extractions with volumes of 100 ($N = 3$, for both male and female), 20 ($N = 2$, for both male and female), and 10 µL ($N = 3$, for both male and female) of the InstaGene Matrix and compared the concentrations and total amounts of extracted DNA. DNA of the samples for comparison of DNA amplification efficiencies with or without Rose Bengal staining was extracted using InstaGene Matrix.

2.4 DNA amplification with PCR

Among the vast genomic sequences currently available, a partial mitochondrial sequence of the cytochrome *c* oxidase subunit I (*COI*) gene is commonly used as a barcode sequence (Savolainen et al., 2005). Previous studies have used the universal primer set designed by Folmer et al. (1994) for PCR. However, DNA extraction using the entire specimen may yield DNA derived from both the target species and from associated fauna or microbial flora, which in turn may lead to the amplification of various DNA fragments by PCR with a universal primer set (e.g., Vrijenhoek, 2009). PCR with a specific primer set is, therefore, more effective in obtaining precise sequence data to avoid amplification of DNA of associated organisms. The specific primer set to amplify a partial *COI* sequence of *Stygiopontius* sp. was designed as follows: st-LCO1: 3'-GGAAGTCTTTACTTAATTAGAGG-5' and st-HCO3:

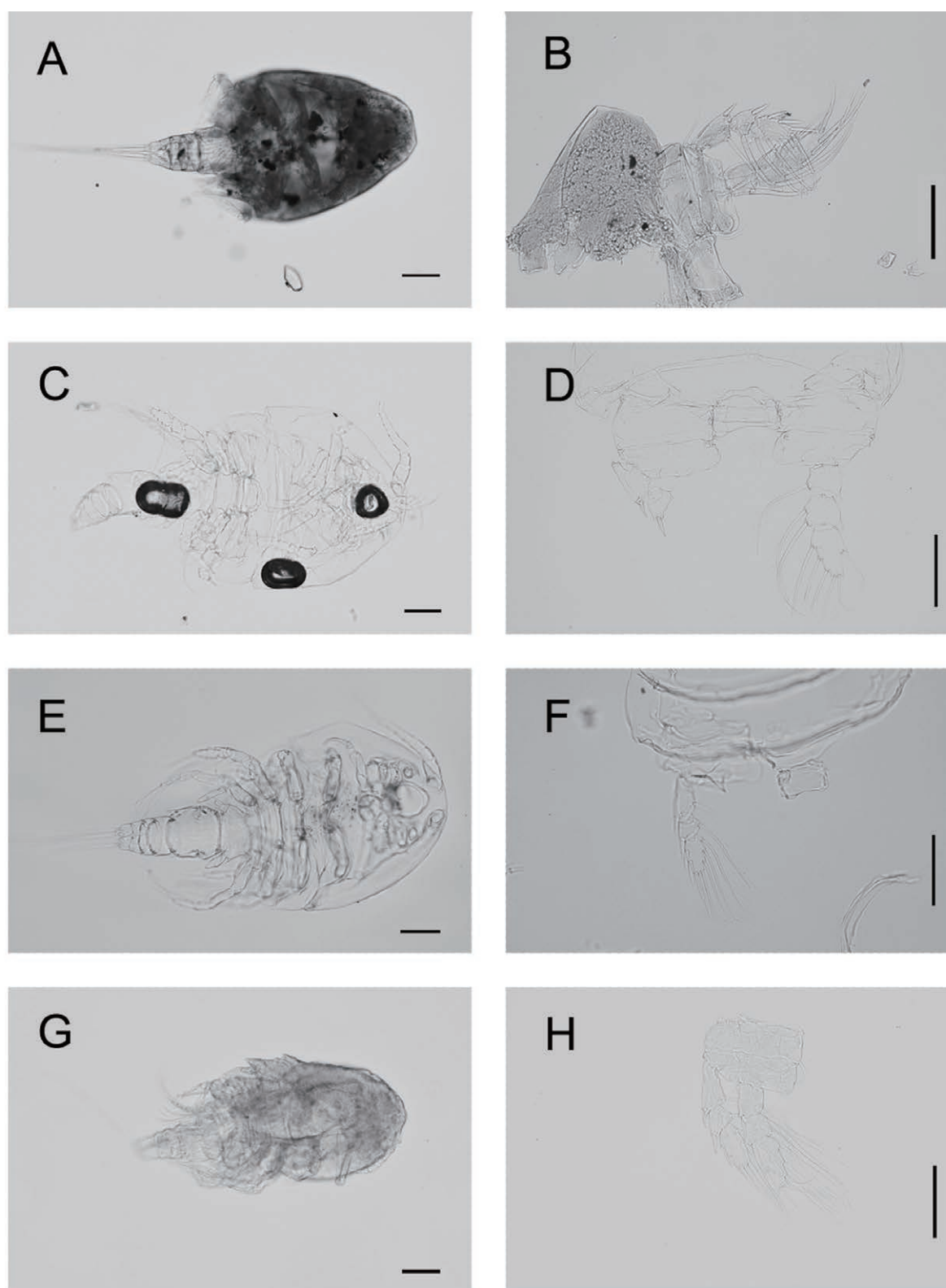


Fig. 1. Morphology of female copepods before and after DNA extraction. A, C, E, and G: entire individual images; B, D, F, and H: magnified images of the second pereopods (P2). A and B: images before DNA extraction; C and D: images after DNA extraction using the Squish Buffer method; E and F: images after DNA extraction using the GeneReleaser® method; and G and H: images after DNA extraction using 100 µL of the InstaGene Matrix. Scale bars: 100 µm.

3'-ATAAGTGTGGAAGAAAGAAATGGG-5', based on the sequence data obtained by Gollner et al. (2011) and new data obtained using a universal primer set designed by Folmer et al. (1994). This primer set is located within a sequence between the priming sites of the universal primers described by Folmer et al. (1994). PCR was conducted with the specific primer. For the samples that were not successfully amplified by the first PCR, nested PCR was conducted; the first run with a universal primer set developed by Folmer et al. (1994) and the second run with the specific primer set. Both PCRs were performed using the Veriti® Thermal Cycler (Applied Biosystems Inc.) and the *ExTaq* Kit (TaKaRa Co.) with the following steps: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s followed by final extension at 72°C for 3 min. The success rate of PCR amplification was compared among the samples comprising 20 individuals stained with Rose Bengal (10 males and 10 females, respectively) and 10 unstained individuals (five males and five females, respectively).

3. Results

3.1 Observation of morphological intactness

The exoskeleton morphology following DNA extraction differed among the three studied methods. Copepods were not shrunken following DNA extraction using the Squish buffer method; however, most appendages were detached (Figs. 1C and D), and the setae on the appendages were mostly dissolved. The soft parts within the exoskeleton were completely dissolved, and no sign of Rose Bengal staining remained. The morphology of the specimens following DNA extraction with GeneReleaser® was similar to that observed with the Squish buffer (Fig. 1E); however, the appendages and some setae remained intact (Fig. 1F). DNA extraction using the InstaGene method had better presentation of the morphology of the specimens; all appendages and setae remained intact, and some soft parts within the exoskeleton retained the Rose Bengal stain (Figs. 1G and H); however, the exoskeleton appeared to be shrunken. In addition, the exoskeleton sometimes became fragile and sometimes collapsed following contact with a fine needle.

3.2 DNA extraction

The inter-method comparison of the concentration of extracted DNA in a 10-μL solution is presented in Fig. 2. The InstaGene method yielded the lowest concentration of DNA, whereas the Squish buffer method yielded the highest. This trend was consistent among both males and females.

The intra-method comparisons with different volumes of the InstaGene Matrix are presented in Fig. 3. The concentration of DNA was highest for both males and females in the 10-μL solution and lowest in the 100-μL solution. However, the total amount of DNA extracted was highest in the 100-μL solution and lowest in the 20-μL solution.

3.3 DNA amplification using PCR

The first PCR cycle amplified *COI* fragments in 1 out of 20 DNA extractions from the specimens stained with Rose Bengal and in 6 out of 10 DNA extractions from unstained specimens. Nested PCR enabled the amplification of sufficient quantities for sequencing from samples with low concentrations of DNA (<10 ng/μL), even if no specific bands were visible following agarose-gel electrophoresis of the products of the first-run PCR. Nested PCR amplified DNA fragments in 1 out of 19 DNA extractions from the specimens stained with Rose Bengal and 2 out of 4 DNA extractions from unstained specimens. In total, the success rate of PCR was 10% in specimens stained with Rose Bengal and 80% in unstained specimens. All PCR products were successfully sequenced (data not shown).

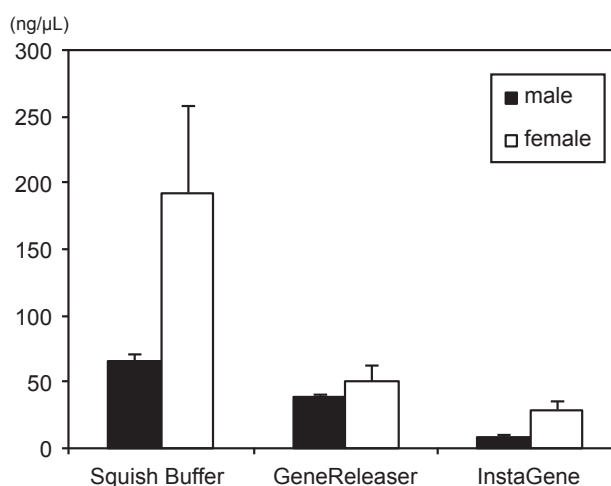


Fig. 2. Differences of DNA concentration in a volume of 10 μL among the three methods. Scale bars denote standard deviation (SD).

4. Discussion

In this study, we demonstrated differences in the efficiency of various methods of DNA extraction and the morphology of specimens following extraction. Each of the three extraction methods was able to extract copepod DNA successfully from a single individual for further sequencing. Although the body length of copepods used has not been mentioned specifically, DNA was extracted from more than 200 *Stygiopontius* specimens using 40 μ L of the InstaGene Matrix to obtain sequence data without multiple or nested PCR (Gollner et al., 2011). Our results show that a sufficient yield in DNA is possible for subsequent sequencing through a single-PCR run if extraction is performed with 40 μ L of the InstaGene Matrix in larger specimens, such as females (ca. 800 μ m in length). DNA extraction from smaller specimens such as males (ca. 500 μ m in length) yielded lower concentrations of DNA (<10 ng/ μ L using the InstaGene method). However, nested PCR facilitated the

amplification of DNA fragments at a very low concentration for sequencing. These findings suggest that appropriate DNA extraction methods, including volume of solution and PCR conditions, should be selected based on the size of the copepod specimens.

Preservation of morphology following DNA extraction differed among the three extraction methods. Some tissues remained intact following DNA extraction using 100 μ L of the InstaGene Matrix. In contrast, a previous study on harpacticoid copepods (which generally tend to be smaller than dirivultid copepods) reported that most tissues had been dissolved (Easton and Thistle, 2014). Nevertheless, in that study (as well as in this study), the morphologies of setae and spines were sufficiently preserved for identification of the specimens. Although Easton and Thistle (2014) did not mention the fragility of harpacticoid exoskeletons following DNA extraction by the InstaGene method, the dirivultid exoskeletons in the present study became fragile following the DNA extraction. This is not favorable for morphological observation; however, the preservation of the setae and spin formulae is more important for species identification. Therefore, the InstaGene method is the most favorable method among the three for the analyses of both the morphology and genetics of a single specimen. The morphology of specimens following DNA extraction using the Squish buffer or GeneReleaser® method was less favorable; most setae were lost from the appendages, and their features (Figs. 1 C–F) were insufficiently preserved for the identification of the species. However, DNA extraction using these two methods was more efficient. Therefore, a trade-off between morphological preservation and efficiency of DNA extraction exists. The Squish buffer method may be more favorable if complete morphological observation has already been performed and a high concentration of DNA is required or if DNA extraction is performed on just one part of the specimen (e.g., an appendage; Sakaguchi and Ueda, 2010). Analyses of DNA sequence data frequently require morphological reexamination of the original sample owing to the possibility of cryptic species (Carugati et al., 2015). The InstaGene method is preferred for copepods, if a review of morphology is required.

A comparison of the success rate of PCR between samples that had been stained using Rose Bengal and those that remained unstained (10% and 80%, respectively) suggests that staining results in a less efficient reaction. Similar evidence has been reported in nematodes (Fonseca

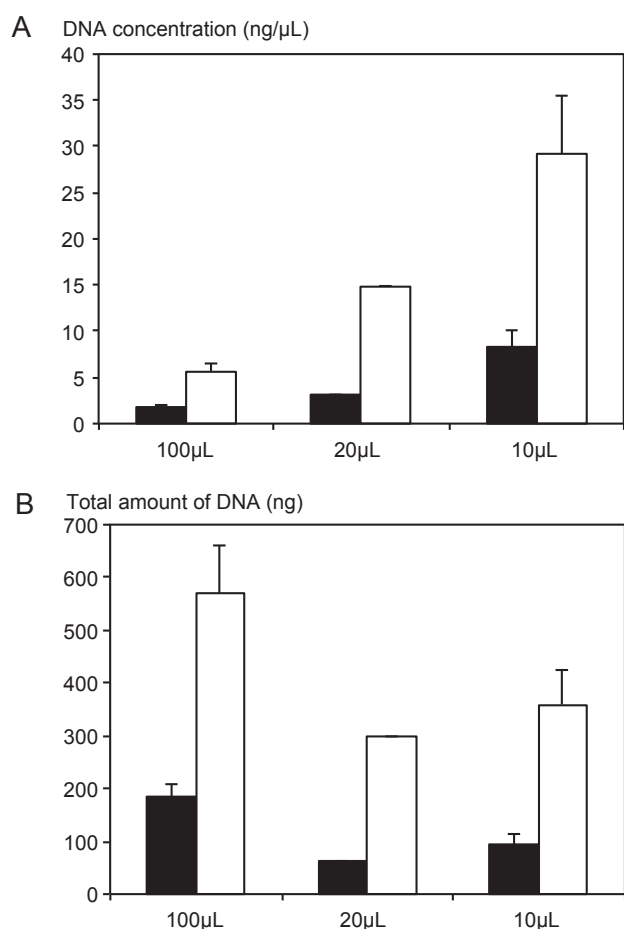


Fig. 3. Comparison of DNA concentration (A) and total amount of extracted DNA (B) following extraction using different volumes of the InstaGene Matrix. Scale bars denote standard deviation (SD).

and Fehlaue-Ale, 2012); however, comparable findings have not been reported in deep-sea harpacticoid copepods (Easton and Thistle, 2014). Staining is a common and effective procedure that aids in the microscopic identification of meiofauna in sediment, and the loss of PCR efficiency induced by Rose Bengal staining appears to be common among various taxa (Fonseca and Fehlaue-Ale, 2012). Our results show that Rose Bengal staining of dirivultid copepods as well as nematodes should be avoided if genetic analyses are to be performed. There may be differences among taxa in the effects of Rose Bengal staining, even among copepod taxa. The effects of staining that have been used for meiofaunal investigation should be investigated to facilitate further application of meiofauna as an indicator of environmental change.

This study suggests the following to obtain both morphological and genetic data from a single copepod specimen: 1) It is best to avoid Rose Bengal staining because of its unfavorable effects, 2) The exoskeleton can be used for morphological observations following DNA extraction using the InstaGene method, and 3) Nested PCR using both universal and specific primer sets facilitates the amplification of specific DNA fragments for subsequent sequencing, even for low concentrations of DNA-extracted solution. These suggestions should be able to guide the effective collection of both morphological and genetic data to elucidate the biodiversity of copepods, which are effective indicators of environmental impacts.

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