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SHORT COMMUNICATION

Non-destructive DNA extraction for small pelagic copepods to perform integrative taxonomy

ASTRID CORNILS*

BENTHOPELAGIC PROCESSES, ALFRED-WEGENER-INSTITUT HELMHOLTZ-ZENTRUM FÜR POLAR- UND MEERESFORSCHUNG, AM ALTEN HAFEN 26, BREMERHAVEN 27568, GERMANY

*CORRESPONDING AUTHOR: astrid.cornils@awi.de

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Molecular analyses of small-sized copepods (≤1 mm) generally involve the complete destruction of the specimens. Consequently, incongruences between the molecular and morphological results cannot be investigated since no specimen vouchers remain. The present study provides a modified column-based DNA extraction method to retain the exoskeleton of the specimen and thus, to enable molecular and morphological analysis of the same specimens. The method has been tested on ethanol preserved specimens of nine pelagic copepod genera.

KEYWORDS: specimen voucher; exoskeleton; non-destructive DNA extraction; copepoda

Molecular phylogenetic and phylogeographic studies have revealed a large number of possible cryptic or pseudocryptic species in marine pelagic copepods (e.g. Goetze, 2003; Böttger-Schnack and Machida, 2010; Chen and Hare, 2011; Cornils and Held, 2014). Thus, there is a need for specimen vouchers to carry out detailed morphological analyses to evaluate the molecular results and to correct possible misidentifications. In larger copepod species (>2 mm), it is possible to extract a sufficient amount of DNA for molecular analysis from body parts (e.g. urosome, swimming legs, antennae; Bucklin *et al.*, 2003; Nonomura *et al.*, 2011). The remaining specimens can then be dissected for detailed morphological analysis or archived as specimen vouchers. From smaller sized specimens (≤1 mm) the whole body tissue is needed to obtain a sufficient amount of DNA for molecular analyses. Thus, DNA extraction protocols for these small copepods generally result in the complete destruction of the specimen, leaving only photographs or paratypes as pseudo-specimen vouchers. Morphological

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analysis prior to DNA extraction of these small specimens is limited to a short period of time, since warming and light exposure under the microscope may cause decay of the DNA. Also, dissection of body parts causes loss of DNA and thus, there may not be enough DNA for molecular analyses. The disadvantage of photographs is that they often do not show the necessary morphological characteristics for species identification, such as the ornamentation of the swimming legs. If there is more than one cryptic species in the same region, the paratypes corresponding to stored DNA may not belong to the same cryptic species.

Most of the important morphological characteristics of copepods are found in the segmentation and ornamentation of the chitin exoskeleton (e.g. Bradford-Grieve *et al.*, 2010). Thus, recovering the exoskeletons during the DNA extraction process would enable a detailed morphological and molecular study on the same specimens. For small-sized terrestrial arthropods, several non-destructive DNA extraction methods have been published (e.g. Rowley *et al.*, 2007; Dabert *et al.*, 2008; Hunter *et al.*, 2008; Castalanelli *et al.*, 2010; Porco *et al.*, 2010). Recently, a method for a Chelex®-based exoskeleton recovery procedure for harpacticoid copepods was described (Easton and Thistle, 2014). In the present study, a column-based method (Qiagen) of non-destructive DNA extraction is presented for nine pelagic copepod genera to improve the results of integrative taxonomy.

The copepod specimens used in this study were caught with various plankton nets and were immediately preserved in pure ethanol (96%) and stored at 4°C if possible. The ethanol was exchanged after 24 h to remove excess seawater in the sample. DNA was extracted from specimens of the following genera: *Acrocalanus*, *Microcalanus*, *Paracalanus*, *Spinocalanus*, *Mimocalanus*, *Monacilla*, *Labidocera*, *Oithona* and *Calanus* (Table I). The specimens were transferred individually from the ethanol sample into distilled water to wash the ethanol off. They were identified to at least genus level (without dissecting them) under a stereo microscope with the lowest possible light intensity (LEICA MZ 16). Morphological and morphometric parameters were noted (e.g. body shape, total length and width, length of antennae, prosome:urosome ratio, presence or shape of the rostrum and other noticeable characteristics). Photographs of the whole individual were taken (Fig. 1, left side). The identification process usually took <5 min. Specimens were then transferred individually to a 1.5-mL tube containing 20 μL Proteinase K and 180 μL ATL buffer (QIAamp DNA Mini Kit). They were incubated for 2 h in a thermoshaker at 500 rpm (revolutions per minute) and 56°C. Afterwards the tubes were briefly centrifuged to remove any solution from the caps. The centrifuge was allowed to reach 5000 rpm, and then it was stopped. Under a stereomicroscope, the exoskeletons were removed with either a disposable inoculation loop (volume 1 μL; Fig. 2) or in the case of larger specimens (>1.5 mm) with sterilized featherweight forceps. Finally, the exoskeletons were transferred to a vial with ATL buffer (pH 8.3, containing EDTA and

Table I: Species used for the present study and the amplification success

Species	No. of specimens	Region	Total length (mm)	Exoskeleton retrieved	Amplicification success
Acrocalanus gibber	1	Red Sea	0.90	1	1
Calanus spp.	4	Arctic Ocean	3.00–3.18	4	4
Labidocera sp.	2	Papua New Guinea	2.03–2.12	2	2
Microcalanus spp.	15 (2 male)	Southern Ocean	0.65–0.94	14	11[a]
Microcalanus spp.	5	trop. E Atlantic	0.65–0.72	5	5
Mimocalanus spp.	7	trop. E Atlantic	1.25–1.50	7	6
Monacilla typica	2	trop. E Atlantic	2.25–2.38	2	2
Oithona atlantica	3 (1 CV[b])	NW Atlantic	1.05–1.09	3	1
Oithona similis	3	NW Atlantic	0.74–0.75	0	0
Paracalanus sp.	8	Caribbean Sea	0.81–0.89	8	4
Paracalanus sp.	8	Gulf of Panama	0.83–1.10	8	8
Paracalanus sp.	3	Papua New Guinea	0.70–0.75	3	0
Paracalanus sp.	3	Red Sea	0.68–0.71	3	3
Paracalanus aculeatus	3	Papua New Guinea	1.05–1.10	3	2
Paracalanus parvus	4	North Sea	0.94–1.03	4	4
Paracalanus tropicus	4	Red Sea	0.66–0.68	4	2
Spinocalanus spp.	21 (1 CV[b])	trop. E Atlantic	0.88–1.40	21	15
Spinocalanus spp.	8 (2 male)	Southern Ocean	1.06–1.28	8	8
Spinocalanus longicornis	4	Arctic Ocean	1.08–1.20	4	3
Spinocalanus magnus	1	trop. E Atlantic	2.38	0	0
Spinocalanus usitatus	3	trop. E Atlantic	1.75–2.03	3	3

Specimens used were females if not otherwise indicated.
[a]Different reverse primer (C1-N-2191) used.
[b]CV is abbreviation for copepodite stage 5.

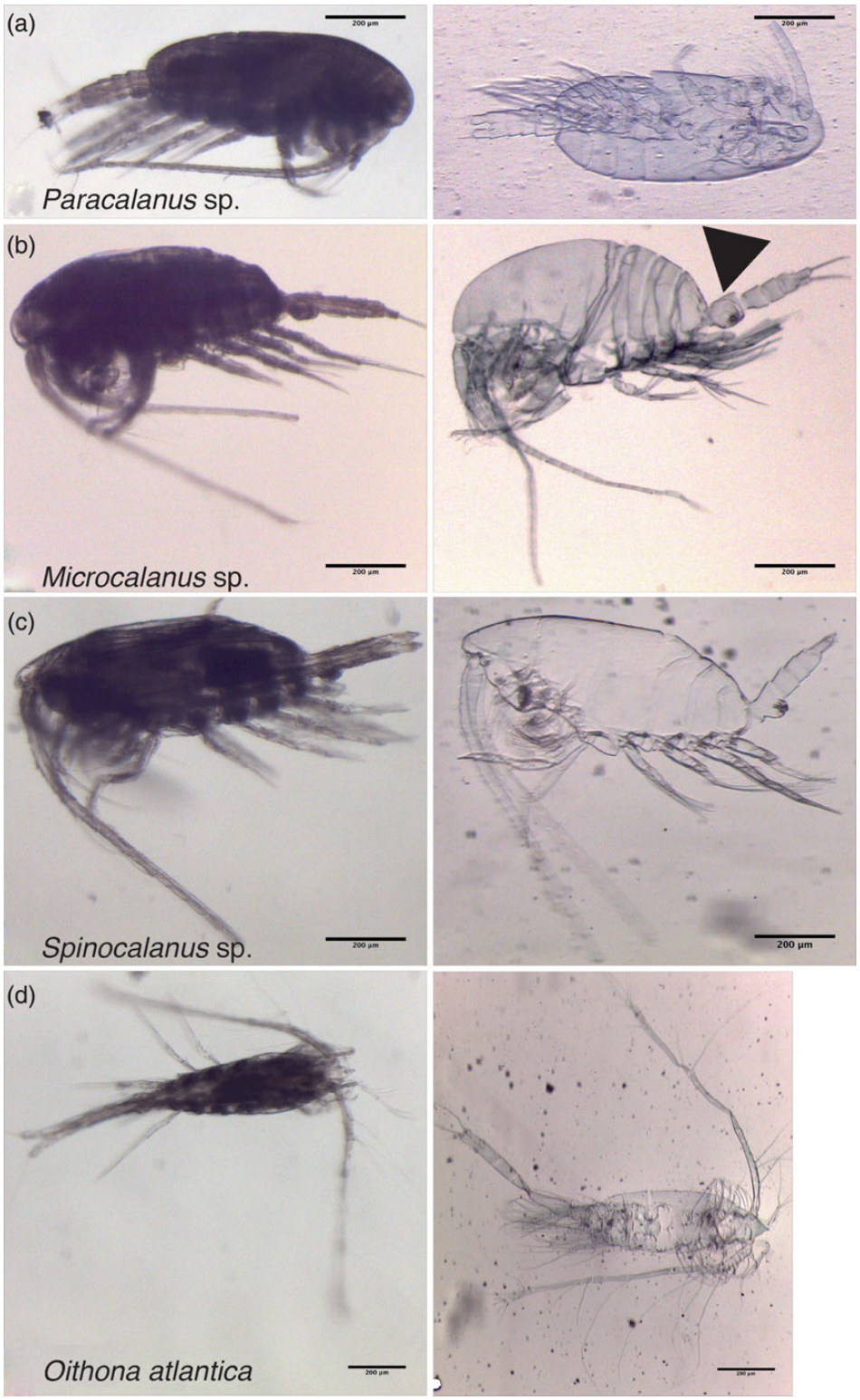

Fig. 1. Specimens before (left) and after (right) tissue lysis. (**a**) *Paracalanus* sp., (**b**) *Microcalanus* sp., (**c**) *Spinocalanus* sp., (**d**) *Oithona atlantica*. In *Microcalanus* sp., the spermatheca is still visible (indicated by arrow). The scale bar in all photographs: 200 μm.

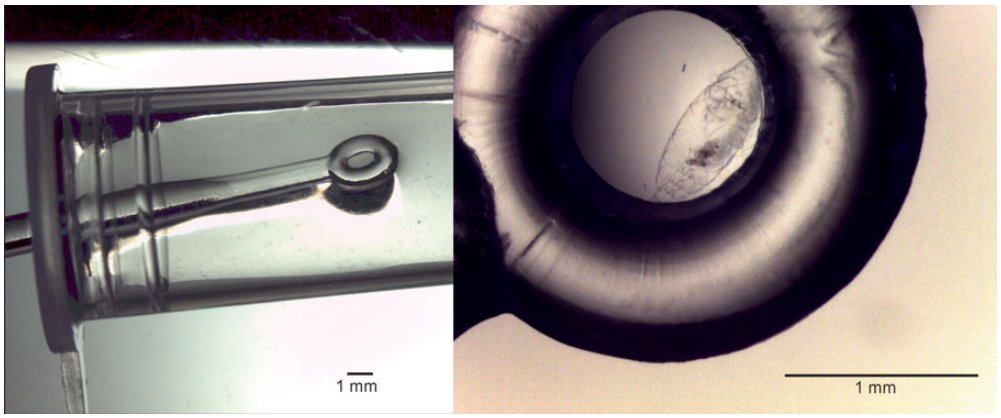

Fig. 2. Left side: inoculation loop (volume: 1 μL) in the 1.5 mL tube containing the lysed tissue in the ATL buffer and Proteinase K solution. Right side: exoskeleton of a copepod specimen within the inoculation loop; Scale bar: 1 mm.

sodium dodecyl sulphate. They were stored at room temperature for up to 5 months for further morphological analysis. The buffer is an aid in the tissue lysis process, so it may gradually decompose the chitinose exoskeleton of the copepods. However, the possibilities of long-term storage in ATL buffer have not yet been tested. Several media were tried before choosing ATL buffer for mid-term storage for the exoskeletons. In ethanol (96%) and glycerine, the exoskeleton shrank and it was not possible to view or dissect the specimens.

After the removal of the exoskeleton the tubes with the ATL buffer, Proteinase K and the lysed tissue were vortexed for 15 s and briefly centrifuged (see above). The DNA isolation process was continued according to the protocols of the QIAamp DNA Mini Kit, excluding the incubation step for 10 min at 70°C. DNA samples were eluted in 200 μL elution buffer for 20 min. To test the DNA extraction success, PCR amplifications were performed for cytochrome c oxidase subunit I (COI) using the primer pair LCO1490 and HCO2198 (Folmer *et al.*, 1994), or a different reverse primer C1-N-2191 (Simon *et al.*, 1994). For detailed amplification and sequencing procedures see Cornils and Held (Cornils and Held, 2014).

For the morphological analysis, the exoskeletons were stained with chlorazol black and photographed. Subsequently, they were either mounted directly on glass slides in Faure's solution (Pantin, 1964) or were dissected beforehand. Except for *Paracalanus* spp. and *Calanus* spp., the copepod exoskeletons were so soft that they collapsed during the transfer in Faure's solution. Therefore, most of the specimens were dissected for a better view of the diagnostic morphological characters on swimming legs (P), mouthparts or urosome.

In total, 112 specimens from nine copepod genera were used in this study (Table I). Except for *Oithona similis* and one specimen each of *Microcalanus* spp. and *Spinocalanus magnus*, all exoskeletons could be removed with an inoculation loop or sterilized forceps. The *O. similis* specimens were too small to be recovered with the inoculation loop (Prosome length: 0.46 mm) and too fragile to be retrieved in one piece with the forceps.

Generally, there were no tissue remains left in the exoskeleton (Fig. 1). In some cases, the spermatheca in the female genital segment was still visible (Fig. 1b). Contrary to similar methods, the present approach to exoskeleton recovery is rather fast and includes only a 2-h thermoincubation instead of overnight or 72-h incubations (Easton and Thistle, 2014; Dabert *et al.*, 2008). Castalanelli *et al.* (Castalanelli *et al.* 2010) also provide a very fast method [ANDE (accelerated nuclear DNA equipment)], but only sequences up to about 800 bp could be amplified from the extracted DNA. Compared with Chelex®-based methods, spin-column-based methods using, e.g. Qiagen kits produce a DNA isolate of higher purity (Casquet *et al.*, 2011). It has to be taken into account however, that the costs for Chelex®-based DNA extraction are clearly lower as for the extraction with Qiagen kits (e.g. Casquet *et al.*, 2011).

In most other non-destructive DNA extraction methods for arthropods, the supernatant is transferred to a new tube after leaving the exoskeleton with some solution in the original tube. In the present study, however, the exoskeleton was removed from the tube with only a minimal amount of liquid (1 μL). Both procedures of isolating the exoskeleton involve the use of a stereo microscope to either remove or retain it.

For calanoid or oithonid copepods, the method presented in this paper may be preferred. The specimens are often already damaged due to sampling with plankton nets. Exposing them to the Chelex® resins and vortexing them during the tissue lysis procedure proposed in the method of Easton and Thistle (Easton and Thistle, 2014) may result in even further damage to the appendages. For the fragile *O. similis*, it may be more effective in the

future to transfer the supernatant instead of the exoskeleton to a new tube after DNA isolation as described in Easton and Thistle (Easton and Thistle, 2014). However, this also causes a higher loss of the DNA isolate as the exoskeleton has to remain submerged in the solution.

During DNA extraction method described the specimens are only incubated for a short period at 56°C and exposed to a short spin in a centrifuge without any vortexing. Comparison of specimens before and after tissue lysis revealed that the exoskeletons remain mostly unharmed (Fig. 1). In some cases, the first antennae were broken further, but most of the damage was done prior to the DNA extraction due to the sampling process.

Of the specimens used in this study 75% could be amplified for COI (Table I), which is in the range of previous molecular studies on planktonic copepods (e.g. Cornils and Held, 2014) but, in some copepod species, the PCR success rates were much lower (e.g. Hirai *et al.*, 2013; Cepeda *et al.*, 2012). The small size of the copepods (and thus low DNA content of the DNA isolate [e.g. for *Paracalanus* cf. *indicus* 4.9–7.3 ng/μL or *Spinocalanus* cf. *abyssalis* 2.3–8.3 ng/μL (unpublished data)] and the preservation condition of the specimens prior to DNA extraction play an important role in the success of amplification. Some of the specimens might have been dead already during sampling with plankton nets and, therefore, the DNA might have been destroyed. Possibly, also the universal primer used across taxa decreases the overall PCR success.

In summary, this modified protocol of the Qiagen Mini Kit provides the possibility to perform molecular and morphological studies on small-sized pelagic copepod species. Thus, incongruences between morphological results and molecular analysis can be investigated.

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