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

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Keywords: specimen voucher, exoskeleton, non-destructive DNA extraction, Copepoda

Abstract

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 at the same specimens. The method has been tested on ethanol preserved specimens of nine pelagic copepod genera.

Short communication

Molecular phylogenetic and phylogeographic studies have revealed a high amount 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) (e.g. 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 (≤1mm) the whole body tissue is needed to gain 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 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 cause 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 charactistics 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 of 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 hours to remove the excess seawater in the sample. DNA was extracted of specimens of the following genera: *Acrocalanus*, *Microcalanus*, *Paracalanus*, *Spinocalanus*, *Mimocalanus*, *Monacilla*, *Labidocera*, *Oithona* and *Calanus* (Table 1). 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 noticable characteristics). Photographs of the whole individual were taken (Fig. 1, left side). The identification process usually took less than five minutes. 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 (500 rpm) at 56°C and 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, contains EDTA and SDS (Sodium dodecyl sulfate)). They were stored at room temperature for up to five months for further morphological analysis. The buffer is an aid in the tissue lysis process, so it may gradually decompose the chtinose exoskeleton of the

copepods. However, the possibilities of longterm storage in ATL buffer have not been tested yet. Several media were tried before choosing ATL buffer for midterm storage for the exoskeletons. In ethanol (96%) and glycerine the exoskeleton shrunk 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 sec and shortly centrifuged (see above). The DNA isolation process was continued according to the protocols of the QIAamp DNAMini Kit, excluding the incubation step for 10 min at 70°C. DNA samples were eluted in 200 μL elution buffer (AE) for 20 minutes. 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 (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 1). 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 with the forceps in one piece.

Generally, there were no remains of tissue in the exoskeleton left (Fig. 1). In some case the spermatheca in the female genital segment were 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 hours incubations (Easton and Thistle, 2014; Dabert et al., 2008). Castalanelli et al. (2011) 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 to Chelex® based methods, spin-column based methods using e.g. Qiagen kits produce an 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 usage of a stereo microscope to either remove or retain it.

For calanoid or oithonid copepods the here presented method may be preferred. The specimens are often already damaged due to the 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 (2014) may result in even further damage of 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 (2014). However, this causes also a higher loss of the DNA isolate as the exoskeleton has to remain submerged in the solution.

During the described DNA extraction method the specimens are only incubated for a short period at 56°C and exposed to a short spin in a centrifuge without any vortexing. The comparison of the specimens before tissue lysis and after 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 1), which is in 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 extractions play an important role in the amplification success. Some of the specimens might have been dead already during the 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 the pelagic small-sized copepod species. Thus, incongruences between the morphological results and the molecular analysis can be investigated.

Acknowledgements

The author is grateful to the following colleagues who took samples from various regions: Benjamin Kürten, Joy Smith, Sigrid Schnack-Schiel, Barbara Niehoff, Nancy Copley, Jasmin Renz, Ann Bucklin. The manuscript was improved by the comments of two anonymous reviewers. The author was supported by the DFG founded project: CO706/2-1.

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1 Table 1: Species used for the present study and the amplification success. Specimens used
2 were females if not otherwise indicated.

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**
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*)	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*)	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

3 *CV is abbreviation for copepodite stage 5. **different reverse primer (C1-N-2191) used.

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Figure 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 mm.

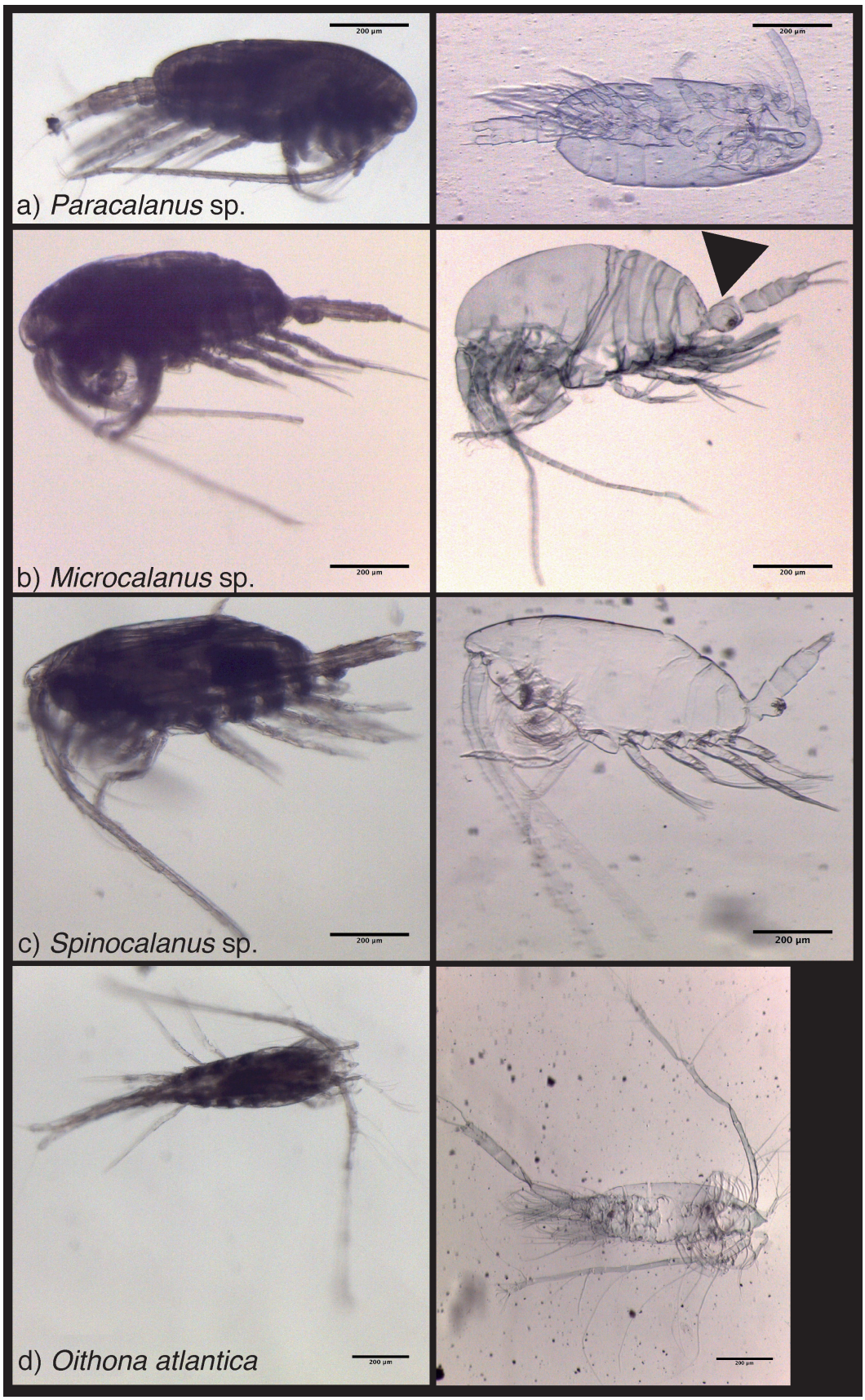

Figure 2: Left side: inoculation loop (volume: 1 mL) in the 1.5 mL tube containing the lysed tissue in the ATL buffer and Proteinase K solution. Right side: exoskeleton of a copepod specimen within the inoculation loop; Scale bar: 1 mm.