DNA Barcoding of samples fixed in formalin

Samples collected in Norderney, Lower Saxony and fixed in formalin were used to obtain a DNA barcode. They were first rinsed with water to remove any rest of formalin, and then sorted into higher taxonomic groups.

DNA extraction

We prepared 250 mL of buffer 50 mM BNM 1% SDS (pH 11). Before the DNA extraction, 25 mg of tissue for each sample was taken into a 1.5 mL Eppendorf tube. Then, tissue was rinsed with Milli-Q water to remove traces of formalin.

For each sample, 980 µl of the BNM-SDS lysis buffer and 25 µl of proteinase K were added and incubated for 24 hrs at 60°C. Twelve hours later, other 25 µl of proteinase K were added.

After the tissue lysis step, we proceed with the DNA extraction following the protocol of Monarch DNA purification kit.

DNA reparation and cleaning

We followed the NEBNext FFPE DNA Repair V2 protocol to clean and repair the DNA extractions previously obtained. A master mix of FFPE DNA Repairs Buffer V2 (7 µl) and NEBNext FFPE DNA Repair Mix v2 (2 µl) was added to 51 µl of each DNA extraction template. Samples were incubated for 15 mins at 37°C.

For each sample, 90ul of resuspended SPIR beads were added into a PCR tube and incubated for 5 mins at room temperature. Samples were put on a PCR magnetic plate to separate beads from the supernatant which was removed and discard carefully once the solution was clear.

We added 200 µl of freshly prepared 80% ethanol to each PCR tube, and incubated at room temperature for 30 sec. Supernatant was removed and discard. This step was done twice.

PCR tubes were left open to dry the beads. We removed the PCR tubes from the magnetic plate and added 32 µl of 0.1X TE buffer. Then, the tubes were put back to the magnetic plate and incubated for two mins at room temperature or until the solution was clear. Finally, 30 µl of the supernatant was transferred into a clean PCR tube. In order to concentrate the DNA previously obtained, samples were speeded vac for 15 to 20 mins.

For all extractions, [DNA] was measured using the Invitrogen Qubit 1X dsDNA HS assay kit.

Amplification

COI fragment with a length of ~660 base pairs (bp) was amplified using the forward primer jgLCO1490 and reverse primer jgHCO2198, and the following specific primers for Amphipoda, Actinaria, Annelida and Ophiuroidea (Box 1).

Box 1. Specific primers used for COI amplification.

Taxa	Primer
Amphipoda	jgLCO1490, Pera COI rev
Actinaria	Cni COX1 rev, Cni COX1 for
Annelida	Poly LCO M13, Poly HCO M13
Ophiuroidea	LCOech 1aF1 M13, jgHCO2198

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PCR master mix included 10 µl of Taq DNA polymerase RED, 7 µl of H2O, 0.5 µl of primer forward and 0.5 µl of primer reverse, and 2 µl of the DNA template.

PCR conditions were an initial denaturation step of 2 mins at 95°C, 40 cycles of 15 sec at 95°C, 15 sec at 48°C and 15 sec at 72°C, and a final extension step of 4 mins at 72°C.

An electrophoresis gel 1.5% agarose at 90 volts for 45 mins was run in order to verify that the correct DNA fragment was amplified. We used 15 µl of GelRed, and 5 µl of Quantitas fast ladder and 5 µl every PCR product were loaded. Successful PCR products were sent for purification and sequencing to MACROGEN, Amsterdam, Netherlands.

Results

A total of 14 specimens belonging to six taxa were used for DNA extraction, whose DNA concentrations were lower than 1.5 ng/µl. After the DNA reparation and cleaning up of the above DNA extractions, we also measured their DNA concentrations (Box 2, Fig. 1).

Box 2. Specimens used for DNA extraction.

Taxa	Sample code	[DNA] ng/µl	[DNA] after reparation and cleaning up ng/µl
Annelida	NEY 12(3)_01	0.528	0.280
Annelida	NEY 12(3)_04	0.472	0.178
Annelida	NEY 12(3)_05	0.325	0.135
Gastropoda	NEY 12(3)_08 B	0.127	0.0510
Bivalvia	NEY 12(3)_11	1.13	0.828
Actinaria	NEY 12(3)_20 B	0.728	0.559
Gastropoda	NEY 022	0.0456	Too low
Annelida	NEY 028	0.0684	Too low
Amphipoda	NEY 033	0.178	0.0870
Annelida	NEY 040	0.236	0.126
Annelida	NEY 041	0.0472	0.0810
Ophiuroidea	NEY 042 B	0.054	Too low
Amphipoda	NEY 044	0.201	Too low
Amphipoda	NEY 046	0.0308	Too low

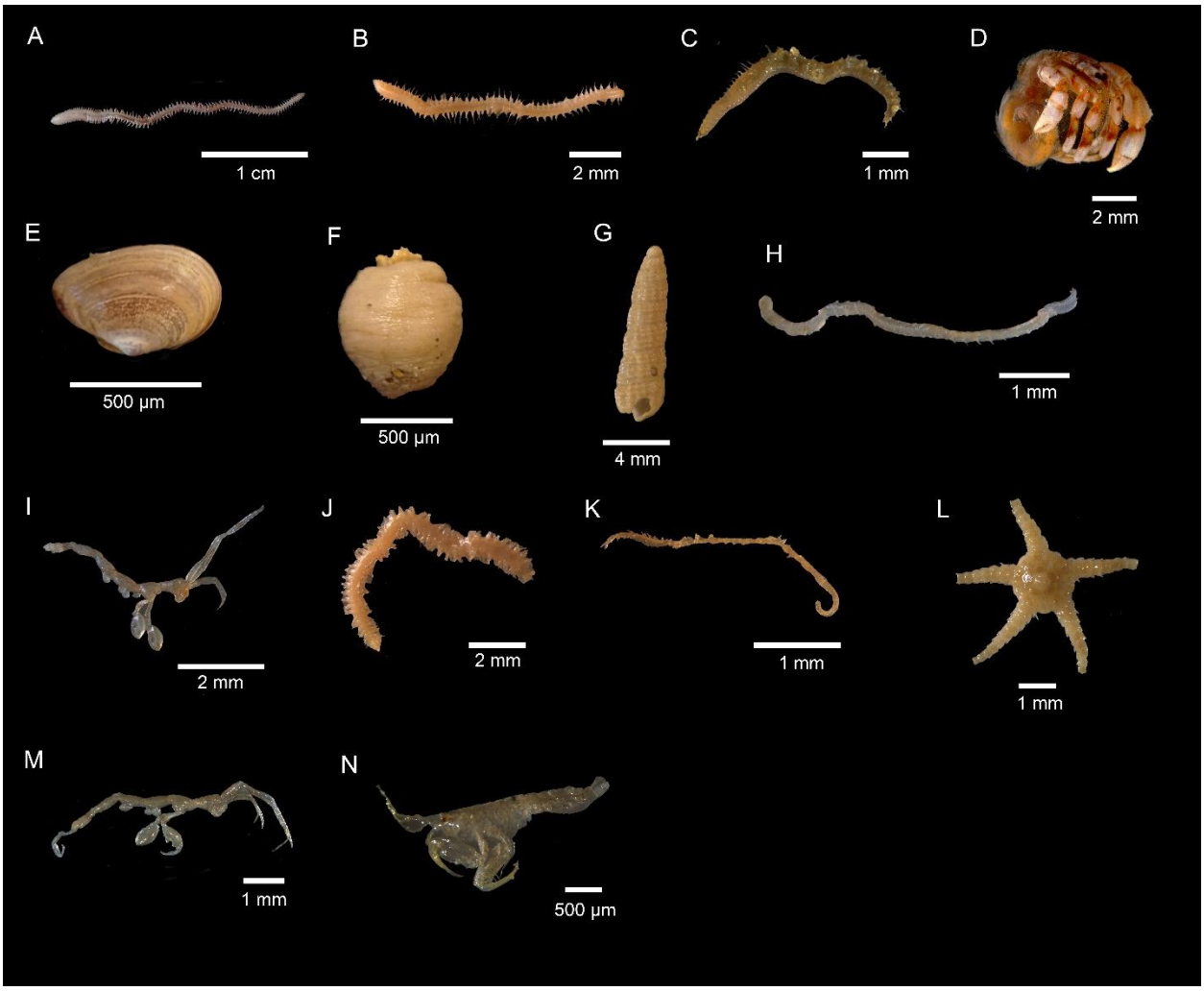

Figure 1. Specimens collected in Norderney, Germany. **A**: NEY 12(3)_01. **B**: NEY 12(3)_04. **C**: NEY 12(3)_05. **D**: NEY 12(3)_08 B. **E**: NEY 12(3)_11. **F**: NEY 12(3)_20 B. **G**: NEY 022. **H**: NEY 028. **I**: NEY 033. **J**: NEY 040. **K**: NEY 041. **L**: NEY 042 B. **M**: NEY 044. **N**: NEY 046.

Only two specimens (NEY 12(3) 08 B and NEY 12(3) 20 B) were supposed to be successfully amplified for COI. Nevertheless, the COI sequence was revealed to correspond to a bacterial sequence.

Neither repairing the above DNA by using the NEBNext FFPE DNA Repair v2 Module nor a cleanup of the extracts by using AMPure XP Beads resulted in a successful amplification of the COI gene region.