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Field and laboratory methods for DNA studies on deep-sea isopod crustaceans

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Abstract: Field and laboratory protocols that originally led to the success of published studies have previously been only briefly laid out in the methods sections of scientific publications. For the sake of repeatability, we regard the details of the methodology that allowed broad-range DNA studies on deep-sea isopods too valuable to be neglected. Here, a comprehensive summary of protocols for the retrieval of the samples, fixation on board research vessels, PCR amplification and cycle sequencing of altogether six loci (three mitochondrial and three nuclear) is provided. These were adapted from previous protocols and developed especially for asellote Isopoda from deep-sea samples but have been successfully used in some other peracarids as well. In total, about 2300 specimens of isopods, 100 amphipods and 300 tanaids were sequenced mainly for COI and 16S and partly for the other markers. Although we did not set up an experimental design, we were able to analyze amplification and sequencing success of different methods on 16S and compare success rates for COI and 16S. The primer pair 16S SF/SR was generally reliable and led to better results than universal primers in all studied Janiroidea, except Munnopsidae and Dendrotionidae. The widely applied universal primers for the barcoding region of COI are problematic to use in deep-sea isopods with a success rate of 45–79% varying with family. To improve this, we recommend the development of taxon-specific primers.

Key words: Icelandic waters, PCR, DNA sequencing, barcoding, Janiroidea, benthos, bathyal, abyssal.

Introduction

The deep sea harbors an enormous number of species, and it was estimated that the majority is yet undescribed (Mora *et al.* 2011). Isopods are among the most di-

verse taxa, but also represent one of the better known groups inhabiting the deep sea (Rex and Etter 2010). Their correct classification is important for evolutionary, ecological, and biogeographic studies but also for conservation issues as industrial pressures on the deep-sea environment grow (Glasby 2002, Hoagland *et al.* 2010, Barbier et al. 2014). Consequently, there is an urgency to establish standard methods for relatively fast and accurate species identification.

However, the tremendous isopod diversity and the high rate (>90%) of new species discoveries (Hessler *et al.* 1979; Gage 2004; Brandt *et al.* 2007; Wilson 2008) makes their description and classification challenging. Taxonomists working on deep-sea isopods classically (even though usually not explicitly stating so) apply the morphological (genotypic) cluster concept (Mallet 1995) when delimitating and describing new taxa using purely morphological data under the assumption that between-species variability is greater than within-species variability (Sites and Marshall 2004). This operational criterion can be inferred from most of the species concepts (Hausdorf 2011), such as the biological species concept (Mayr 1942, 2000). Comprehending deep-sea isopod diversity is further impaired because in several groups morphologically highly similar or even almost identical – so called "cryptic" – species are being increasingly discovered (Wilson 1982, 1983; Raupach and Wägele 2006; Raupach *et al.* 2007; Brökeland 2010; Riehl and Brandt 2010). In these cases, the (perceived) lack of morphological difference between lineages may disguise true, that is genetic, diversity.

Alternatively, strong dimorphisms hinder allocation of conspecific males and females, for instance where males undergo a metamorphosis during which their appearance is altered beyond variation that is commonly observed in the respective higher taxa (*e.g.* Riehl *et al.* 2012). Testing for the biological species concept is usually impractical as observations of live specimens are generally not feasible as a standard tool and the function of genital copulatory structures are not well enough understood (but see Wilson 1986, 1991) to recognize potential "lock-and-key" patterns.

During the last decade, DNA barcoding and integrative approaches to systematic questions have become standard (Hebert *et al.* 2003; Gibbs 2009; Allcock *et al.* 2011; Schwentner *et al.* 2011; Havermans *et al.* 2013). Various species concepts can be applied when molecular data complement (sparse) morphological information (Schwentner *et al.* 2011). Although molecular methods have been used occasionally for deep-sea isopods (Raupach and Wägele 2006; Raupach *et al.* 2007; Brökeland and Raupach 2008), they are still underdeveloped and lack standardized application, especially in taxonomy. The project *Barcoding Deep-sea Isopoda* (http://www.cedamar.org/en/dna-barcoding.html) was founded to devise such methods.

To date, molecular studies on deep-sea isopods are often not directly comparable because different DNA fragments have been targeted. GenBank (Benson *et al.* 2008) queries for deep-sea isopods (using Isopoda as well as the respective family names as search terms) revealed relatively small numbers of sequences (Table 1) when compared to terrestrial or shallow-water crustaceans. The janiroid isopod

Table 1

Number of sequences per DNA locus present on GenBank (accessed on: 30 May 2014) sorted by family of Asellota (Crustacea) and publication. Only those families are shown that show a partial or exclusive deep-sea distribution. New data presented in this volume are not included.

	COI	12S	16S	18S	28S D1–3	28S D6–8
Acanthaspidiidae	N/A	N/A	**36** (Raupach and Wägele 2006)	**5** (Raupach *et al.* 2004) **5** (Raupach *et al.* 2009)	N/A	**10** (Raupach *et al.* 2009)
Dendrotionidae	N/A	N/A	N/A	**1** (Raupach *et al.* 2004) **4** (Raupach *et al.* 2009)	N/A	**3** (Raupach *et al.* 2009)
Desmosomatidae	**2** (Schnurr and Brix 2012) **16** (Brix *et al.* 2014)	N/A	**1** (Schnurr and Brix 2012) **12** (Brix *et al.* 2014)	**4** (Raupach *et al.* 2004) **9** (Brix *et al.* 2014)	N/A	**4** (Raupach *et al.* 2009)
Echinothambematidae	N/A	N/A	N/A	N/A	N/A	N/A
Haplomunnidae	N/A	N/A	N/A	**1** (Raupach *et al.* 2004) **2** (Raupach *et al.* 2009)	N/A	**2** (Raupach *et al.* 2009)
Haploniscidae	**30** (Brix *et al.* 2011)	N/A	**25** (Brökeland and Raupach 2008)	**5** (Raupach *et al.* 2004) **3** (Brökeland and Raupach 2008) **9** (Raupach *et al.* 2009) **2** (Asmyhr and Cooper 2012)	N/A	**12** (Raupach *et al.* 2009)
Xostylus (inc. sed.)	N/A	N/A	N/A	**1** (Raupach *et al.* 2009)	N/A	**1** (Raupach *et al.* 2009)
Ischnomesidae	N/A	N/A	N/A	**4** (Raupach *et al.* 2004) **1** (Osborn 2009) **2** (Raupach *et al.* 2009)	N/A	**4** (Raupach *et al.* 2009)
Janirellidae	N/A	N/A	N/A	**1** (Raupach *et al.* 2004)	N/A	**1** (Raupach *et al.* 2009)
Janiridae	**2** (Wetzer 2001) **1** (Osborn 2009) **1** (Radulovici *et al.* 2009) **1** (Kilpert *et al.* 2012)	**2** (Michel-Salzat and Bouchon 2000) **2** (Wetzer 2001) **1** (Kilpert *et al.* 2012)	**1** (Kilpert *et al.* 2012)	**1** (Dreyer and Wägele 2001) **3** (Dreyer and Wägele 2002) **1** (Raupach *et al.* 2004) **1** (Osborn 2009) **4** (Raupach *et al.* 2009) **2** (Linse *et al.* 2014) **1** (Wägele *et al.* unpubl. data)	**1** (Osborn 2009)	**4** (Raupach *et al.* 2009)

Table 1 – *continued.*

	COI	12S	16S	18S	28S D1–3	28S D6–8
Joeropsididae	**1** (Wetzer 2001)	**1** (Wetzer 2001)	N/A	**1** (Dreyer and Wägele 2002) 1 (Raupach *et al.* 2009)	N/A	1 (Raupach *et al.* 2009)
Katianiridae	N/A	N/A	N/A	N/A	N/A	N/A
Macrostylidae	**21** (Riehl and Kaiser 2012)	**39** (Riehl and Kaiser 2012) **10** (Riehl and Brandt 2013)	**35** (Riehl and Kaiser 2012) **23** (Riehl and Brandt 2013)	**2** (Raupach *et al.* 2004) **1** (Raupach *et al.* 2009) **3** (Riehl and Kaiser 2012)	N/A	**2** (Raupach *et al.* 2009)
Mesosignidae	N/A	N/A	N/A	1 (Raupach *et al.* 2004) 2 (Raupach *et al.* 2009)	N/A	2 (Raupach *et al.* 2009)
Munnidae	**1** (Fraser *et al.* 2011)	N/A	N/A	N/A	N/A	N/A
Munnopsidae	**34** (Osborn 2009)	**51** (Raupach *et al.* 2007)	N/A	**1** (Dreyer and Wägele 2002) **4** (Wägele *et al.* 2003) **4** (Raupach *et al.* 2004) **8** (Raupach *et al.* 2007) **42** (Osborn 2009) **24** (Raupach *et al.* 2009) **2** (Wägele *et al.* unpubl. data)	**37** (Osborn 2009)	**4** (Raupach *et al.* 2007) **26** (Raupach *et al.* 2009)
Nannoniscidae	N/A	N/A	N/A	**2** (Raupach *et al.* 2009)	N/A	**2** (Raupach *et al.* 2009)
Paramunnidae	N/A	N/A	N/A	N/A	N/A	N/A
Stenetriidae	N/A	N/A	N/A	**1** (Raupach *et al.* 2004) **2** (Raupach *et al.* 2009)	N/A	**2** (Raupach *et al.* 2009)
Thambematidae	N/A	N/A	N/A	N/A	N/A	N/A
Urstylidae	N/A	N/A	N/A	N/A	N/A	N/A

family Munnopsidae is by far the most extensively studied group from the molecular perspective, followed by Macrostylidae, Haploniscidae and Desmosomatidae. For all these taxa, at least four loci have been studied. However, the majority of these data originate from only a few exemplary studies (altogether 18, and some based on shallow-water samples), covering a small range of species; for all other families, molecular data are almost or completely absent (Table 1).

The question is posed: why are there so few molecular studies on deep-sea isopods? One major problem might be the difficulty of obtaining fresh material containing intact DNA due to the remoteness of the habitat and related aspects of sampling. Protocols have been developed for DNA extraction from old and formalin-fixed collections (*e.g.* Schander and Halanych 2003; Boyle *et al.* 2004), but these produce only short DNA fragments (usually <200bp) and require large quantities of tissue as well as an enormous expenditure in terms of time and expense when compared to standard methods. Furthermore, problems with extraction or amplification of DNA from even "fresh" tissue have been frequently reported (Held 2000; Raupach *et al.* 2004; Raupach and Wägele 2006; Brix *et al.* 2011). Raupach *et al.* (2004) and Raupach and Wägele (2006) mention highly active nucleases that may quickly digest DNA, with reference to Dreyer and Wägele (2001, 2002). The latter however, only stated that material "fixed during field trips in warm ethanol yielded less DNA of high quality" compared to specimens that were fixed in ice-cold ethanol. However, finding the exact reason for the patterns observed by Dreyer and Wägele (2001, 2002) as well as the authors of the present paper and others (F. Leese, M.R. Raupach and W. Goodall-Copestake, personal comm.) is beyond the scope of this article.

Nevertheless, the field and laboratory methods set the base line for any empirical study. In scientific publishing authors are usually encouraged to provide only short methodological protocols and important details may be omitted. In this paper, we provide a comprehensive description of our general methods. The markers employed here are suitable for a wide range of applications as they have strongly contrasting evolutionary rates and comprise mitochondrial as well as nuclear fragments. Detailed descriptions of each relevant step from the collection and fixation of the samples to the cleanup of the PCR product and sequencing allow full reproducibility. We intend to push forward the integrative approach to isopod taxonomy and DNA barcoding in the remote and inaccessible deep-sea ecosystem.

Methods

Protocols for the widely-established markers COI, 16S and 18S are presented in detail. Additionally, protocols are outlined for markers that have only rarely been used for species delimitation in deep-sea isopods, which may be valuable for

taxonomy and systematics: 12S, and two fragments of the nuclear large subunit ribosomal RNA (28SD1–3 and 28SD6–8).

Our molecular methods presented here are not the result of any particular experimental design but rather a trial-and-error approach, and we thus cannot compare many alternative approaches in order to determine specific factors that may have an effect on the outcome of attempts to amplify and sequence DNA. Nevertheless, we are able to compare alternative universal primers for amplification success for the 16S marker.

The described methods were developed and tested during several deep-sea expeditions on the German research vessels *Meteor* (M79/1, DIVA 3, and M85/3, IceAGE), *Polarstern* (ANTXXIV-1, ANDEEP-SYSTCO), and *Sonne* (SO223, KuramBio) to the North Atlantic, South-West Atlantic, North Pacific and Southern Ocean respectively. Subsequent laboratory studies were conducted at the Zoological Museum Hamburg (ZMH), at the Canadian Center for DNA Barcoding (CCDB) and the Smithsonian Laboratories for Analytical Biology (LAB) as well as in the commercial LGC (Laboratory of the Government Chemist) genomics laboratories. General protocols, guidelines and recommendations for DNA Barcoding (Weigt *et al.* 2012) were followed. High-throughput methods were employed at LAB as well as in commercial laboratories. Based on Dreyer and Wägele's (2001, 2002) assumption (see above) and the unsuccessful DNA extractions during initial expeditions (DIVA-1), an undisturbed "cooling chain" was made first priority.

Abbreviations. — 12S, mitochondrial small subunit (mtSSU) rRNA gene; 16S, mitochondrial large subunit (mtLSU) rRNA gene; 18S, nuclear small subunit (nSSU) rRNA gene; 28S, nuclear large subunit (nLSU) rRNA gene; CCDB, Canadian Centre of DNA Barcoding; COI, cytochrome-c-oxidase subunit 1; dNTP, deoxynucleotide triphosphate; LAB, Laboratories of Analytical Biology, Smithsonian Institution, National Museum of Natural History; PCR, polymerase chain reaction.

Sampling. — Samples were collected by means of epibenthic sledges (EBS) as designed by Brenke (2005) and Brandt *et al.* (2013). Both models were equipped with thermally insulated boxes that enclose the cod ends of the nets as well as a spring-lever system that mechanically controls doors at the mouth of the sledge and allows for selectively collecting endo- and suprabenthic organisms only (Fig. 1; Kaiser and Brenke in press). These are designed to have a minimal impact on dynamic pressures of the nets during trawling. During retrieval of the sledge from the ocean bottom, a closing mechanism that is connected to the spring-lever system of the sledges seals off the boxes. Water of the sampling depth is thus locked in and has an insulating effect on the cod ends of the nets. The cod ends themselves are net buckets equipped with an optional rubber flap. The latter is designed to passively seal off the net bucket at its anterior end (opening) whenever the water current is directed against the trawling direction. While the gear with the

Fig. 1. The thermo-boxes on the Camera Epibenthic Sledge (C-EBS; Brandt *et al.* 2013) are able to reduce warming of the samples during retrieval through the water column. **A**. While the C-EBS is lowered and heaved through the water column, the lever is extended (a). The thermo-boxes (b) are closed. **B**. When the C-EBS is at the bottom, the lever is pushed in and the thermo boxes are thus open (c) to reduce dynamic pressure.

samples is heaved through potentially warmer water layers, the samples are thus kept at the temperature of their origin (that is usually between -1.8°C and +4°C), which should reduce the risk of DNA degradation within the samples. The flaps protect the samples from warm-water inflow as well as from being washed out by up-and-down movement of the ship in heavy sea conditions.

Fixation and preservation. — After retrieval of the sample from the gear (*e.g.* net bucket of EBS), samples were sieved (300 µm) using chilled seawater (if required in a cooling room at approx. 2°C) and bulk-fixed in chilled (-30°C to -20°C) 96% ethanol (or higher; preferably non-denatured). Special care was taken to minimize the amount of residual water in the sediment to be fixed, *e.g.* by washing the sample from the sieve into a bucket using pre-cooled ethanol instead of water in the last sieving step. Sample containers were used such that a minimum 5:1 ratio of container volume to sample volume was maintained. Jars of up to 5 L volume were used because larger containers have proven to be difficult to handle during later-on steps of the process. Jars were topped up with ethanol and then stored at -20°C to -30°C. During the first 24 h, the jars were carefully rolled every three to five hours in order to guarantee penetration of the ethanol through the sediment and to avoid separation of a water phase from the ethanol and subsequent freezing of that water phase. After 24 h, the fixation medium was decanted through a 300 µm sieve and exchanged for new 96% non-denatured ethanol. Ethanol concentration in the samples was measured using a portable density meter (Anton Paar: DMA35) and a concentration of at least 70% was ensured.

Sample sorting and determination. — The subset of the samples to be used for DNA extraction was sorted directly onboard the research vessels. The EBS models used (Brenke 2005; Brandt *et al.* 2013) contain two separate samplers which are arranged on top of each other. The upper (supra) net was usually best suited because it has proven to be frequently the cleanest and thus fastest to sort. Other fractions of the samples were either fixed in formaldehyde and subsequently preserved in 80% denatured ethanol or fixed and preserved in 70–96% denatured ethanol. Sample sorting started after 48 hours of fixation. Stereo microscopes were used for sorting, which was conducted at room temperature. However, all jars, vials and sorting dishes as well as squeeze bottles with extra ethanol were kept on ice at all times using ice baths, chilled metal racks and the like (Fig. 2).

Isopods were identified to species level wherever possible using original scientific literature, identification keys and expert knowledge (family level minimum). They were individually separated as vouchers to allow for more exact determination in the lab. Individual numbers allow tracing each DNA sequence back to the specimen it originated from. Specimens are deposited and stored in freezers at the Senckenberg German Center for Marine Biodiversity Research (Deutsches Zentrum für Marine Biodiversitätsforschung, DZMB) in Hamburg and given DZMB numbers using the local Access 2010 database (Brix *et al.* 2012) or at the ZMH.

Tissue dissection. — Tissue was dissected under sterile conditions and on ice. This was conducted on board immediately after sorting and identification, whenever ship time and sea state allowed. Otherwise, this step was conducted in the home laboratory. To minimize the morphological damage, only small amounts of limb tissue were dissected (one to three walking legs from one side from janiroidean isopods, de-

Fig. 2. "Sorting on ice" set-up. Keeping the samples cool throughout the whole range of processes, from sampling until the extraction, seems to be beneficial for generating high-quality DNA sequences. **A**. Ice dish used to cool the samples during the sorting process. **B**. Ice bath with metal racks holding taxon vials.

pending on the size of the specimen). Otherwise, specimens were kept intact for vouchering and to allow further morphological studies and identification. Tissue was preserved until extraction in a minimal volume of ethanol (one drop from a 20 µL pipette) or extraction buffer (150 µL) and kept frozen (-20°C) whenever possible.

Transport and shipping. — Samples and tissue were transported under cold conditions whenever possible. For domestic land-based transport, dry ice was preferred. International sea-shipping was conducted using freezing containers. For international priority air shipping of the tissue, Styrofoam boxes and cooling packs were used that guaranteed 4°C or less for more than 48 h (tested in laboratory).

Total DNA extraction. — Residual ethanol was removed from the tissue by evaporation at room temperature. At LAB, extractions were done on an AutoGenPrep 965 following the manufacturer's protocol for animal tissue. Tissue digestion was performed overnight in a shaking bath at 56°C and 50 rpm using the AutoGen buffers and proteinase K. The suspension volume of extracted total DNA was 50 µL.

At LGC Genomics, the samples where homogenized with steel beads and extracted using the sbeadex forensic kit according to the manufacturer's protocol.

PCR at LGC Genomics. — The complete 18S sequence as well as fragments of 16S and COI were separately amplified using 20 µL reaction volumes contain-

ing 1.0 µL DNA, 2 µL 5xBiostab PCR Optimizer, 4 µL Reaction Buffer (MyTaq Bioline 5x, containing dNTP and $MgCl_2$), 0.2 µL MyTaq Polymerase (5 µ/µL), 1 µL of each primer (5 pmol/µL), and 10.8 µL nuclease-free H_2O.

COI. — For COI, the universal primers of Folmer *et al.* (1994) were used (LCO1490/HCO2198, Table 2). The PCR temperature profile consisted of an initial denaturation at 94°C (10 min), followed by 5 cycles of denaturation at 96°C (1 min), annealing at 45°C (45 s) and extension at 72°C (1 min). These cycles were followed by another 35 cycles of denaturation at 93°C (1 min), annealing at 50°C (45 s) and extension at 72°C (1 min) followed by a final extension at 72°C (5 min). Cycle sequencing was performed using the same primers as used for PCR.

16S. — For 16S, the SF/SR primer pair was employed (Tsang *et al.* 2009). The PCR temperature profile consisted of an initial denaturation at 95°C (10 min), followed by 36 cycles of denaturation at 95°C (30 s), annealing at 48°C (30 s) and extension at 72°C (45 s). These cycles were followed by a final extension at 72°C (5 min). Cycle sequencing was performed using the same primers as used for PCR (Table 2).

Table 2

12S, 16S and COI primers used for amplification and sequencing of deep-sea isopod DNA.

Primer name	Sequence [5'–3']	Reference
16S AR	CGCCTGTTTATCAAAAACAT	Palumbi *et al.* 1991
16S BR	CCGGTCTGAACTCAGATCACG	Palumbi *et al.* 1991
16S SF	GACCGTGCTAAGGTAGCATAATC	L.M. Tsang, pers. comm.
16S SR	CCGGTCTGAACTCAAATCGTG	Tsang *et al.* 2009
H13842-12S	TGTGCCAGCASCTGCGGTTAKAC	Ryuji Machida, pers. comm.
L13337-12S	YCTWTGYTACGACTTATCTC	Ryuji Machida, pers. comm.
dgLCO1490 (COI)	GGTCAACAAATCATAAAGAYATYGG	Meyer *et al.* 2005
dgHCO2198 (COI)	TAAACTTCAGGGTGACCAAARAAYCA	Meyer *et al.* 2005
LCO1490 (COI)	TCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
HCO2198 (COI)	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* 1994
CrustCOIF (COI)	TCAACAAATCAYAAAGAYATTGG	Teske *et al.* 2006
DecapCOIR (COI)	AATTAAAATRTAWACTTCTGG	Teske *et al.* 2006

18S. — The sequences were amplified in partially overlapping fragments using three primer pairs (18 A1 and 700 R; 400 F and 1155 R; 1000 F and 1800). The PCR profile comprised an initial denaturation at 95°C (10 min), followed by 36 cycles of denaturation at 94°C (30 s), annealing at 54°C (45 s) and extension at 72°C (3 min 12 s) followed by a final extension at 72°C (10 min). Cycle sequencing was performed using the same primers as used for PCR (Table 3).

PCR at LAB. — Amplification and cycle sequencing reactions were mostly carried out on Peltier PTC200 and PTC225 Thermal Cyclers (MJ Research) and 2720 Thermal Cyclers (Applied Biosystems).

Taable 3

18S sequencing primers used for deep-sea isopods.

	Primer name	Sequence [5'–3']	Reference
Forward	18A1mod	CTGGTTGATCCTGCCAGTCATATGC	Raupach *et al.* 2009
	A700Fmod	GCCGCGGTAATTCCAGC	Raupach *et al.* 2009
	1155F	GTGAAACTTAAAGGAATTGACGG	Dreyer and Wägele 2001
	1250FNmod	GGCCGTTCTTAGTTGGTGGAG	Raupach *et al.* 2009
Reverse	1800mod	GATCCTTCCGCAGGTTCACCTACG	Raupach *et al.* 2009
	700R	CGCGGCTGCTGGCACCAGAC	Dreyer and Wägele 2001
	1155R	CCGTCAATTCCTTTAAGTTTCAG	Dreyer and Wägele 2001
	1500mod	CATCTAGGGCATCACAGACC	Raupach *et al.* 2009
Previous studies	1000F	CGATCAGATACCGCCCTAGTTC	Dreyer and Wägele 2001

Mitochondrial genes. — Three mitochondrial genes were partially amplified and sequenced. The approximately 450–500 bp fragment of 16S rRNA, an approximately 650 bp fragment of COI and an approximately 550 bp fragment of 12S were amplified separately using reaction volumes of 10 µL containing 0.25 µL BSA, 0.5 µL dNTP [2.5 mM each], 1 µL Bioline 10xNH_4 reaction buffer, 0.3 µL of each primer [10 µM], 0.5 µL Biolase $MgCl_2$ [50 mM], 0.1 µL Biolase DNA Pol [5 u/µL], 2 µL of template DNA and nuclease-free H_2O. For 16S and 12S, the same primers were used for PCR amplification and cycle sequencing. Primers are listed in Table 2.

In most cases for COI, PCR amplification was carried out using the primers dgLCO1490/dgHCO2198 which had been tagged with M13 primers. In these cases, M13 primers were then used for subsequent cycle sequencing. For several specimens, the primer pair LCO1490/HCO2198 was successfully used to amplify COI where dgLCO1490/dgHCO2198 failed. The PCR temperature profile for both sets of primers consisted of an initial denaturation at 95°C (5 min), followed by 34–36 cycles of denaturation at 95°C (30 s), annealing at 48°C (30 s) and extension at 72°C (45 s) followed by a final extension at 72°C (5 min). Sequencing and PCR primers were identical for specimens amplified with LCO1490. For cycle sequencing 30 cycles of 95°C (30 s), 48°C (30 s) and 60°C (4 min) were employed.

18S. — At LAB, the complete 18S gene was amplified in a 20 µL reaction volume containing 0.5 µL BSA, 1.0 µL dNTP [2.5 mM each], 2.0 µL Bioline 10xNH_4 reaction buffer, 0.6 µL of each primer [10 µM], 1.0 µL Biolase $MgCl_2$ [50 mM], 0.2 µL Biolase DNA Pol [5 u/ µL], 4.0 µL of template DNA and nuclease-free H_2O. PCR primers used were 18SA1mod/1800mod (Table 3). The PCR temperature profile consisted of an initial denaturation at 95°C (5 min), followed by 34 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min) and extension at 72°C (3 min), and a final extension at 72°C (7 min). Cycle sequencing was performed using the PCR primers plus additional primers (altogether five forward and five reverse; Table 3). For cycle sequencing, 30 cycles of 95°C (30 s), 50°C (30 s) and 60°C (4 min) were employed. This protocol is based on Raupach *et al.* (2009).

28S. — The 28S D1–D3 fragment was amplified in a 10 µL reaction volume containing 0.13 µL BSA, 0.5 µL dNTP [2.5mM each], 1 µL Bioline 10xNH_4 reaction buffer, 0.3 µL of each primer [10µM], 0.5 µL Biolase $MgCl_2$ [50 mM], 0.1 µL Biolase DNA Pol [5 u/µL], 2 µL of template DNA and nuclease-free H_2O. PCR and cycle sequencing primers used were LSUD1F/D3AR (Table 3). Amplification and cycle sequencing reactions were mostly carried out on Peltier Thermal Cyclers PTC200 and PTC225 (MJ Research) and 2720 Thermal Cyclers (Applied Biosystems). The PCR temperature profile consisted of an initial denaturation at 95°C (5 min), followed by 40 cycles of denaturation at 95°C (1 min), annealing at 60°C (1 min) and extension at 72°C (3 min), and a final extension at 72°C (7 min). Cycle sequencing was performed using the same primers as used for PCR. For cycle sequencing 30 cycles of 95°C (30 s), 50°C (30 s) and 60°C (4 min) were employed. This protocol was adapted from Osborn (2009) and primers are listed in Table 4.

The 28S D6–D8 fragment was amplified in a 10 µL reaction volume containing 0.13 µL BSA, 0.5 µL dNTP [2.5 mM each], 1 µL Bioline 10xNH_4 reaction buffer, 0.3 µL of each primer [10 µM], 0.5 µL Biolase $MgCl_2$ [50 mM], 0.1 µL Biolase DNA Pol [5 u/µL], 2 µL of template DNA and nuclease-free H_2O. PCR and cycle sequencing primers used were 28EE/D8R (Table 3). Amplification and cycle sequencing reactions were mostly carried out on Peltier PTC200 and PTC225 Thermal Cyclers (MJ Research) and 2720 Thermal Cyclers (Applied Biosystems). The PCR temperature profile consisted of an initial denaturation at 95°C (5 min), followed by 35 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min) and extension at 72°C (2 min), and a final extension at 72°C (7 min). Cycle sequencing was performed using the primers listed in Table 3. For cycle sequencing 30 cycles of 95°C (30 s), 50°C (30 s) and 60°C (4 min) were applied. This protocol was adapted from Raupach *et al.* (2009) and primers are listed in Table 5.

For cycle sequencing, 2.0 µL of PCR product was analyzed for purity and size conformity by electrophoresis in a 1.5% agarose gel with ethidium bromide. The re-

Table 4

28S D1-3 PCR and cycle-sequencing (CS) primers used for deep-sea isopods.

Primer name		Sequence [5'–3']	Reference
Forward	LSUD1F	ACCCGCTGAATTTAAGCATA	Lenaers *et al.* 1989
Reverse	D3AR	ACGAACGATTTGCACGTCAG	Lenaers *et al.* 1989

Table 5

28S D3-8 sequencing primers used for deep-sea isopods.

Primer name		Sequence [5'–3']	Reference
Forward	28EE	ATCCGCTAAGGAGTGTGTAACAACTCACC	Hillis and Dixon 1991
	28F3	GACGACAGCCCGGGGA	Raupach *et al.* 2009
Reverse	D8R	GAGTCAAGCTCAACAGGGTCTTCTTTCCC	Omilian and Taylor 2001
	28R1	GGGTTCGCTAGACAGTAGA	Raupach *et al.* 2009
	28R2	GGCCATCGCGATGCTTT	Raupach *et al.* 2009

maining PCR product was purified using ExoSap-IT (USB). A 5x dilution of the enzyme was used and 2 µL of that solution were added to 8 µL PCR product (or 4 µL were added to 18 µL PCR product). Samples were incubated at 37°C (30 min) and the enzyme was deactivated at 80°C (20 min). Cycle sequencing was performed in 10 µL volume containing 1 µL purified PCR product, 0.5 µL BigDye Terminator, 1.75 µL Big Dye Terminator reaction buffer, 0.5 µL primer and nuclease-free water. Cycle sequencing products were cleaned up with the Sephadex G-50 (Sigma S-5897) method, dried and stored at -20°C until run on a 3730xl DNA Analyzer.

Multiple sequence alignment was conducted to analyze divergence within and between taxa. The widely applied alignment programs ClustalW (Thompson *et al.* 1994) and MAFFT (Katoh *et al.* 2002) were used and subsequently alignments were checked and corrected by hand where necessary.

Results

Our first attempts to extract DNA from deep-sea isopods were performed in the year 2000 with the beginning of the DIVA project (*Latitudinal Gradients of deep-sea BioDIVersity in the Atlantic Ocean*) and the initial expedition DIVA-1 (M48-1) to the Southeast Atlantic Ocean. Unfortunately, all extractions were unsuccessful (Brix *et al.* 2014). Based on the observations of Dreyer and Wägele (2001, 2002) an undisturbed "cooling chain" was made our first priority during subsequent expeditions. While DIVA-2 (M63-2, 2005) was a first attempt and resulted in sample-by-sample extractions of about 280 single isopod specimens, during DIVA-3 (M79-1, 2009), standardized protocols as described above were applied. As these seemed to increase the success rate from around 40–60% (DIVA-2) to over 70%, this approach was followed further with additional stepwise modifications.

Through the above-mentioned protocols, we were able to obtain sequences for 15 families of Janiroidea (Dendrotionidae, Desmosomatidae, Echinothambematidae, Haplomunnidae, Haploniscidae, Ischnomesidae, Joeropsidae, Katianiridae, Macrostylidae, Munnidae, Munnopsidae, Nannoniscidae, Paramunnidae, Stenetriidae, Thambematidae) and *Xostylus* (*incertae sedis*). Furthermore, sequences could be obtained for Valvifera (Arcturidae and Idoteidae), Cymothoida (Cirolanidae, Gnathiidae, Leptanthuridae), Sphaeromatidea (Serolidae, Brandt *et al.* in press) which are rather rare in the deep sea and thus limited in numbers in our samples. The first pioneer studies on Desmosomatidae (Schnurr and Brix 2012; Brix *et al.* 2014, in press), Haploniscidae (Brix *et al.* 2011) and Macrostylidae (Riehl and Kaiser 2012; Riehl and Brandt 2013) have been published and other taxonomic and phylogenetic studies are in progress.

Due to financial restrictions, we concentrated on COI and 16S. For these markers, in total about 2300 specimens of isopods, 100 amphipods and 300 tanaids were amplified and sequenced. The other markers were sequenced for only a subset of the

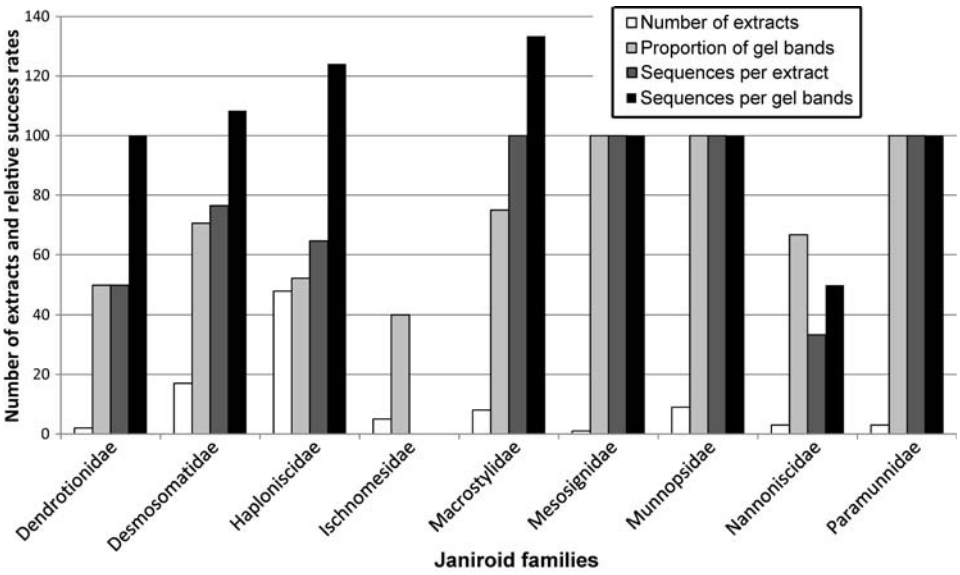

Fig. 3. Success rates of amplification and sequencing using the universal 16S AR/BR primer pair. The success rates above 100% in case of the number of sequences per gel band are due to cases where sequences were successfully generated from the PCR product despite no bands could be detected on the gel. PCR products might contain too low concentrations of DNA to show up on an ethidium-bromide stained agarose gel. It might still contain sufficient DNA for successful sequencing. The graph shown is based on 96 samples belonging to nine janiroid (Isopoda: Asellota) families. Samples were collected during the expedition DIVA-3 with R/V *Meteor* in the South Atlantic.

samples. We failed to find a set of primers for the mitochondrial markers that were targeted that would consistently amplify DNA from all isopod taxa. Variability in success was apparent even within families. PCR were conducted in 96-well plates and whenever at least 50% of the wells showed distinct gel bands, the whole 96-well plate was carried further for cycle sequencing. Due to this approach, we observed that even when the amplification product was too low in concentration to be detected on an agarose gel, it often was a suitable template for cycle sequencing (Fig. 3). Regarding 16S, the 16S SR/16S SF primers generally led to better amplification success than the universal primers 16S AR/BR (Fig. 3). Application of 16S AR in combination with 16S SR was also successful and led to a slightly longer fragment. We observed that universal primers for COI (Folmer *et al.* 1994) were for some taxa not as reliable as primers for 16S (Tsang *et al.* 2009) resulting in incomplete datasets (Fig. 4).

Discussion

During the last decade and in the context of the *Barcoding Deep-sea Isopoda* project, the protocols presented in this paper have been evolving gradually and were applied to a wide range of isopods as well as other peracarids (Amphipoda

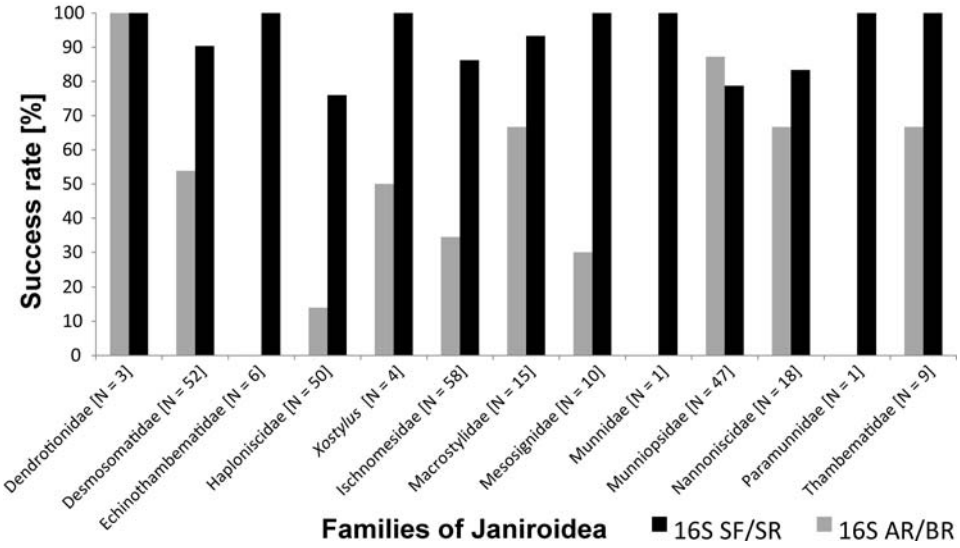

Fig. 4. Amplification success using the universal primer pairs 16S AR/BR *versus* 16S SF/SR sorted by family of Janiroidea (Isopoda). The graph is based upon a dataset comprising 13 janiroid families and altogether 274 species. The same extracts were used as templates for both PCRs. Samples were collected during the DIVA-3 expedition on R/V *Meteor*. Lab work took place at the Smithsonian Laboratories of Analytical Biology. Except for Dendrotionidae and Munnopsidae, the 16S SF/SR primer pair consistently provided higher success rates compared to the 16S AR/BR primer pair.

and Tanaidacea). Due to space restrictions, only the state of the art is presented in this paper but the yield of high-quality sequences grew from around 40% to substantially more than 80% in certain taxa (see Fig. 4). Despite the apparent usefulness of genetic data to address systematic questions in biological studies (Hebert *et al.* 2003; Pons *et al.* 2006), these data have rarely been applied for deep-sea Isopoda so far. By closing a methodological information gap that might be partly responsible for this situation, the present paper aims to promote the application of standardized and field-tested molecular methods on deep-sea isopods.

Since the start of the *Barcoding Deep-sea Isopoda* project, the focus lay on gathering samples as well as developing and testing molecular methods. As a next step, reference databases need to be filled with quality-tested data. We are using the *Barcode of Life Database* (BoLD) for data storage and projects will soon be made publicly available with continuing publication of our research.

One major problem that we face at the current stage stems from the lack of similar sequences on GenBank (Benson *et al.* 2008). Another major concern is potentially related to primer mismatches. It is due to the pioneering nature of current molecular investigations on deep-sea isopods that a publicly available database does not exist to compare the new results against. Already within deep-sea isopod families, such as Macrostylidae (Riehl and Brandt 2013), Desmosomatidae (Brix *et al.*

2014), Haploniscidae (Brix *et al.* 2011) or Munnopsidae (Osborn 2009), relatively fast-evolving markers, such as COI and 16S show variation clearly above 20% uncorrected p-distance (Brix *et al.* 2011, in press; Riehl and Brandt 2013). These values reach levels that are strongly influenced by saturation effects. Consequently, within families sequence divergence can be similar to that between any isopod and other peracarid crustaceans or even hexapods. As a result, using the megablast search (Altschul *et al.* 1990; Zhang *et al.* 2000) in the context of contamination checking, the most frequent results comprise insects. The risk of missing contaminations, especially those caused by other deep-sea crustaceans, for example during the sample handling, is consequently relatively high. By continuously publishing new data, we are working to overcome this situation.

While the methods described in this article are specifically designed to work on deep-sea isopods, Asellota in particular, to some extent they provide a first step for molecular research on other peracarid groups as well. The methods for COI have been tested extensively and successfully on Amphipoda (Havermans *et al.* 2013) and Tanaidacea (Błażewicz-Paszkowycz *et al.* 2014). Most effort was spent on developing the protocols for the faster evolving DNA markers (COI, 16S, 12S) and especially the first two were most widely applied. However, due to their slower evolutionary rates and consequently more conserved priming regions, the 18S and 28S protocols can likely be regarded as more universal.

The statement that nucleases in isopods are particularly active cannot be substantiated here. However, our experience shows that an immediate transfer of the sample upon arrival on deck into cold (-20°C) conditions is preferable. This is in accordance with previous assumptions (Dreyer and Wägele 2002; Raupach *et al.* 2004). We therefore suggest that in cases where the sample retrieved from the gear contains only negligible amounts of sediment, it should be fixed with chilled high-grade ethanol immediately and without sieving.

We further recommend dissecting tissue for DNA extraction directly on board and after an over-all fixation period of 48 hours. Although we cannot prove this statistically, the cooling chain and fresh tissue may be regarded as essential for a high success rate. However, there is evidence (not shown here) suggesting that acceptable results might be possible even after two years of storage as long as the samples were constantly kept in chilled conditions as recommended for various other taxa (Quicke *et al.* 1999; Gemeinholzer *et al.* 2010; Nagy 2010).

Recommended next steps. — The protocols presented in this paper allowed sequencing of about 2300 specimens of isopods, 100 amphipods, and 300 tanaids and provide the first large-scale approach to sequencing DNA from deep-sea isopods. We were able to make family-specific suggestions regarding 16S primer choice. However, our results indicate that further optimization is required: sequencing the barcoding marker COI was prone to a high rate of contamination and failed sequencing runs compared to *e.g.* 16S (Fig. 5). The alignments across families revealed variability beyond 30% uncorrected p-distance and only a very lim-

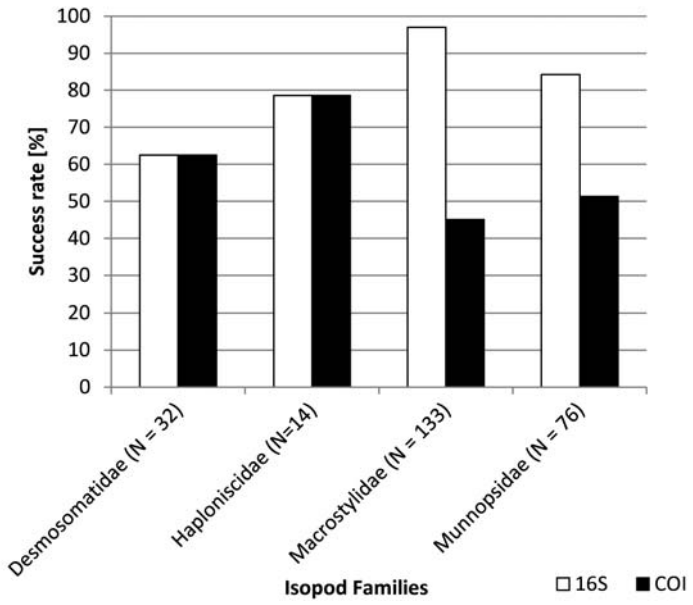

Fig. 5. Success rates compared for sequencing cytochrome-c-oxidase subunit I (COI) and the mitochondrial large ribosomal RNA subunit (16S). Data are predominantly shown for those isopod families which were used for tissue dissection during the KuramBio expedition. Success rates for Desmosomatidae and Haploniscidae were similar (below 80%) for both markers. For Macrostylidae and Munnopsidae, 16S could be sequenced much more reliably (96% and 84% success) using universal primers than COI (45% and 51% success).

ited number of conserved sites. We assume that the primer region might be variable in other Janiroidea as well. Taxon-specific primers may need to be created in order to achieve a higher yield (compare Derycke *et al.* 2010; Zeale *et al.* 2011).

The methods presented here were not tested using an experimental design. To evaluate further and more qualitatively the effects that sampling devices, storage and fixation temperature, working speed and laboratory methods have on the quality of the DNA, we recommend a thoroughly designed experimental setup. Too many variables might have influenced DNA degradation for us to distinguish the most crucial variables at the present time.

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