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Molecular identification of ascaridoid nematodes from the deep-sea onion-eye grenadier (*Macrourus berglax*) from the East Greenland Sea

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Abstract

Macrourus berglax from the East Greenland Sea was studied for the presence of ascaridoid nematodes in 2001, 2002 and 2003. The fishes were collected between 278 and 413 m water depth using a benthopelagic net. Based on the amplification of the internal transcribed spacer ITS-1, 5.8S, ITS-2 and flanking sequences (= ITS +), three ascaridoid nematode species were identified. The prevalence of infestation during the 3 years ranged from 42.9% to 62.9% and 22.9% to 40.0% for the anisakids *Anisakis simplex* (s.s.) and *Pseudoterranova decipiens* (s.s.), respectively, and from 28.6% to 60.0% for the raphidascarid *Hysterothylacium aduncum*. A total of 18 specimens, two of each species and examination year, revealed no sibling species, suggesting a limited distribution of other ascaridoid siblings into the deep sea. The ITS-1, 5.8S and ITS-2 sequences of *A. simplex* (s.s.) from the East Greenland Sea did not differ from previously published sequence data (GenBank) from other regions in the Atlantic and Pacific oceans. The sequences of *P. decipiens* (s.s.) corresponded most closely to those of specimens from Richardson Bay, western Pacific, and differed in four positions (0.5%). They corresponded least to those of specimens from Japan (1.5%). The sequence data for *H. aduncum* differed in two positions in the ITS-1 (0.2%) and three positions in the ITS-2 (0.3%) from sequences from Japan. A high genetic similarity between the regions can be explained by (a) extensive final host migration in the case of *A. simplex* (s.s.), (b) an overlapping distribution of final host populations along the continental shelves for *P. decipiens* (s.s.) and (c) a low host specificity and large population size in the intermediate and final hosts for *H. aduncum*. The occurrence of the identified species in the macrourid fish underlines the potential of cosmopolitan ascaridoid nematodes to distribute not only horizontally but also vertically in the deep sea.

Keywords: East Greenland Sea; Deep-sea fish; Ascaridoid nematodes; *Anisakis simplex* (s.s.); *Pseudoterranova decipiens* (s.s.); *Hysterothylacium aduncum*; Internal transcribed spacer; Ribosomal DNA; Zoogeography; Parasitic nematode distribution

1. Introduction

Adult nematodes of the superfamily Ascaridoidea are large intestinal roundworms that use vertebrates, especially fish and mammals, as definitive

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hosts. The superfamily contains approximately 52 genera, and many species of these nematodes are highly prevalent within their intermediate and final hosts (e.g. Nadler and Hudspeth, 2000; Marques et al., 2006). The most common species are *Anisakis simplex*, *Pseudoterranova decipiens* and *Hysterothylacium aduncum*. With the first two being zoonotic parasites, they have been studied extensively with respect to morphology (e.g. Køie, 1993; Palm et al., 1994), geographical and host distribution (e.g. Marcogliese, 1995; Klimpel, 2005; Klimpel et al., 2006), genetic structure (e.g. Marques et al., 2006; Mattiucci et al., 1997), host pathogenesis (e.g. Abollo et al., 2001) and life cycles (e.g. Køie, 1993; McClelland, 2002; Klimpel et al., 2004).

Identification and differentiation of the larval stages within the genera *Anisakis*, *Pseudoterranova* and *Hysterothylacium* on morphology alone are neither easy nor always possible. The accurate identification of ascaridoids to species level, however, is the prerequisite for a better understanding of their systematics, geographical distribution and life cycle. Consequently, molecular identification techniques have been developed to provide alternative methods for better identification. Especially, the polymerase chain reaction (PCR) linked to restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacers (ITS-1, ITS-2) and the 5.8S rDNA has been successfully applied for the identification and differentiation of ascaridoid nematodes (D'Amelio et al., 2000; Martin-Sánchez et al., 2005; Pontes et al., 2005). Those authors demonstrated that the morphospecies *A. simplex* and *P. decipiens* are complexes of sibling species (e.g. Abollo et al., 2003) that are morphologically very similar but genetically different, and have distinct host preferences and geographical distribution (e.g. Marques et al., 2006). Molecular analyses of the *A. simplex* complex identified at least three sibling species (*A. simplex* (s.s.), *A. pegreffii*, *A. simplex* C) in the Atlantic Ocean and three siblings of the *P. decipiens* complex (*P. krabbei*, *P. decipiens* (s.s.), *P. bulbosa*) in the North Atlantic, the Norwegian Sea and the Barents Sea (e.g. Nascetti et al., 1986; Zhu et al., 2002). In the case of *H. aduncum*, sibling species have not yet been identified. Congeners such as *H. fabri* consist of at least three siblings with little host specificity (Martin-Sánchez et al., 2003). The fact that *H. aduncum* is also a nematode with remarkably low host specificity and that other *Hysterothylacium* siblings have been recognized leads to the assumption that *H. aduncum* might represent a sibling species complex (Balbuena et al., 1998).

The life cycles of ascaridoid nematodes and their sibling species in different geographical regions are not fully understood. Ecological surveys, such as in the Norwegian Deep, Baltic Sea and North Atlantic Ocean, and laboratory experiments suggest that they involve three different final hosts. The adults parasitize in the digestive tract of their final hosts, especially cetaceans (toothed and baleen whales) for *A. simplex*, pinnipeds (e.g. seals) for *P. decipiens* and fishes of different trophic levels for *H. aduncum* (e.g. Klimpel et al., 2004; Klimpel and Rückert, 2005). Morphologically, larval stages of these ascaridoids have been recorded from a wide variety of teleost and invertebrate intermediate hosts, and also from the deep sea (Klimpel et al., 2001). Recently *A. simplex* (s.s.) has been identified genetically from *Maurolicus muelleri* from the Mid-Atlantic Ridge in over 1600 m water depth (Klimpel et al., 2007). Palm and Klimpel (in press) reported *A. simplex*, *P. decipiens* and *H. aduncum*, based on morphology, from the deep-sea macrourid *Macrourus berglax* but provided no molecular confirmation. The present study presents the first molecular identification of ascaridoid nematodes from the deep water environment in the East Greenland Sea. Specimens from *M. berglax* that were sampled during 3 consecutive years were studied in order to detect possible (sibling) species variation.

2. Materials and methods

2.1. Sample collection

Fish samples were collected in 2001 (between 24 and 27 October), 2002 (between 23 October and 03 November) and 2003 (between 28 October and 10 November) on board of the German research vessel *Walther Herwig III* during surveys of the International Council for the Exploration of the Sea (ICES) and the Northwest Atlantic Fisheries Organization (NAFO) within the East Greenland Sea (Fig. 1) (Palm and Klimpel, in press). The fishes were caught with a benthopelagic net at a trawling speed of approximately 4 knots. A total of 105 onion-eye grenadiers, *M. berglax*, 35 specimens in each year, were caught at 361 m mean trawling depth (trawling depth range: 329–405 m) in 2001, 334 m (278–387 m) in 2002 and 377 m (340–413 m) in 2003. All fishes were deep frozen at −40 °C immediately after catch

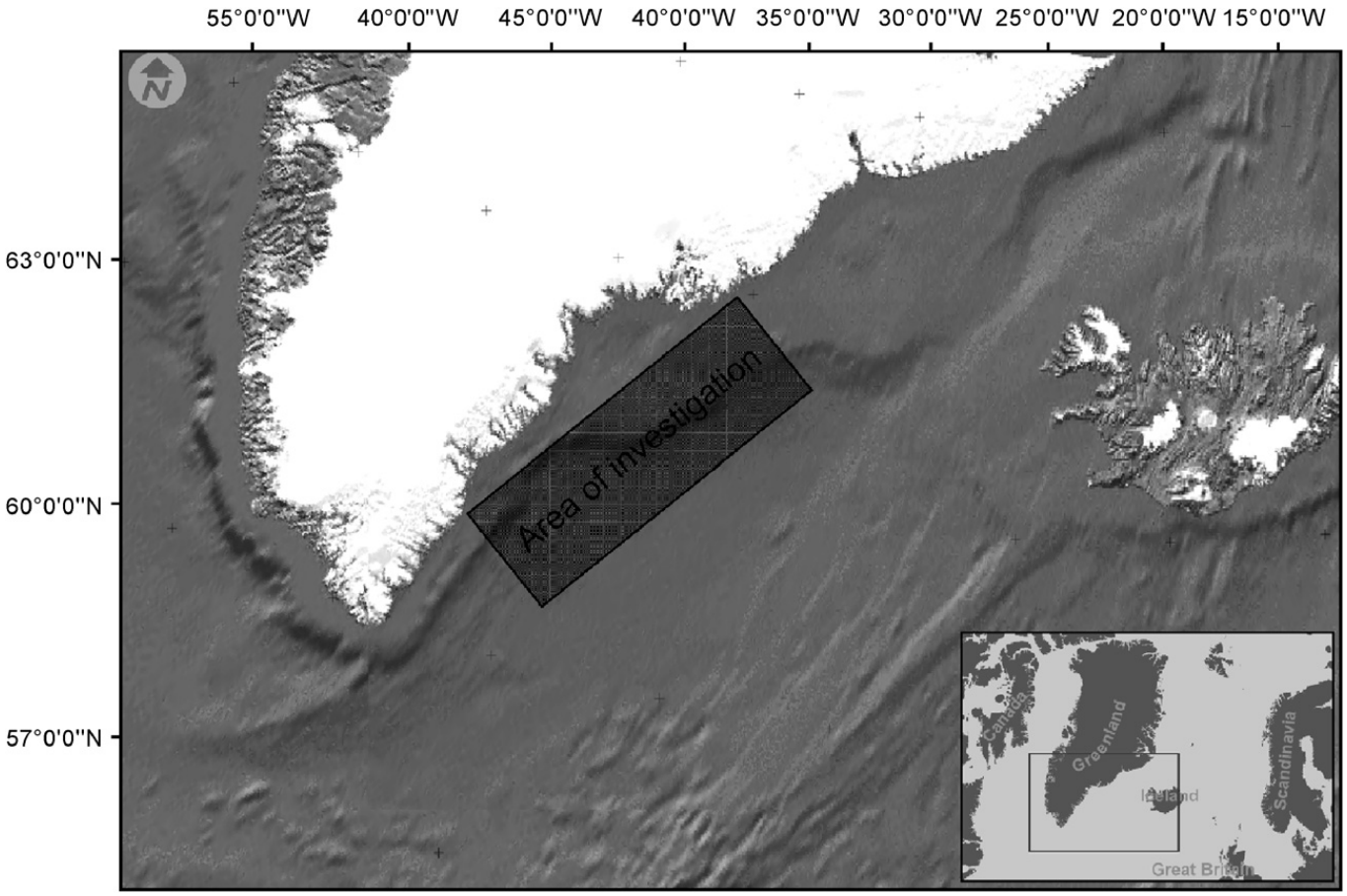

Fig. 1. Map of the area of investigation.

for subsequent examinations. Prior to examination, each fish specimen was defrosted at 0–1 °C.

2.2. Ascaridoid nematode isolation and molecular analyses

Nematodes isolated from the examined *M. berglax* were identified morphologically by existing keys and descriptions (Palm and Klimpel, in press). After isolation and identification, the nematodes were freed from host tissue and stored in 96% ethanol. Genomic DNA was isolated and purified from individual larvae (*A. simplex* (s.s.) and *P. decipiens* (s.s.), third stage larvae; *H. aduncum*, third and fourth stage larvae) with a genomic DNA extraction kit (Peqlab Biotechnology GmbH, Erlangen, Germany) according to the instructions of the manufacturer. The rDNA region comprising the ITS-1, 5.8S, ITS-2 and flanking sequences (=ITS+) was amplified using the previously described primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al., 2000). Each PCR (26 μl) included 13 μl Master-Mix (QIAGEN GmbH, Hilden, Germany) containing dNTP, $MgCl_2$, buffer and taq-polymerase, 3 μl of each primer, 2 μl distilled water and 5 μl genomic DNA. Each PCR was performed in a thermocycler (Biometra, Germany) under the following conditions: after an initial denaturation at 95 °C for 15 min, 30 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 1 min (extension), followed by a final extension at 72 °C for 5 min. Samples without DNA were included in each PCR run. PCR products were checked on 1% agarose gels. A 100-bp ladder marker (peqGOLD, Erlangen, Germany) was used to estimate the size of the PCR products. To identify the ascaridoid nematodes the PCR products were purified with EZNA Cycle-Pure Kit (Peqlab Biotecnology GmbH, Erlangen, Germany). Afterwards a total volume of 7 μl, including 2 μl primer (individually) and 5 μl of the PCR product (250 ng/μl), was sequenced by Seqlab (Goettingen GmbH, Germany). Both spacers and the 5.8S gene from each PCR product were sequenced in both directions,

using primers NC5, NC13 (forward; 5′-ATC GAT GAA GAA CGC AGC-3′), NC13R (reverse; 5′-GCT GCG TTC TTC ATC GAT-3′), XZ1R (reverse; 5′-GGA ATG AAC CCG ATG GCG CAA T-3′) and NC2. The obtained sequences were identified via GenBank (BLAST) and aligned with previously characterized sequences of ascaridoid nematodes using CLUSTAL W (1.83) multiple sequence alignments (Thompson et al., 1994). Pairwise comparison was made on the basis of sequence differences (D) using the formula $D = 1-(M/L)$ (Chilton et al., 1995), where M is the number of alignment positions at which the two species have a base in common, and L is the total number of alignment positions over which the two species are compared. These comparisons were performed independently for the sequence of each spacer, the 5.8S gene and combined sequence data for all three regions. Among the observed ascaridoids the nucleotide sequence of all three regions was compared. Resulting sequence data of ascaridoids from the East Greenland Sea were compared with previously published sequence data in GenBank to analyze intraspecific differences among the specimens.

3. Results

3.1. Ascaridoid nematode parasite fauna

As previously described by Palm and Klimpel (in press) the parasite fauna of *M. berglax* (35 species of each year) was highly diverse. The most abundant parasite taxon were the nematodes of the superfamily Ascaridoidea, especially the anisakids *A. simplex* (s.s.) and *P. decipiens* (s.s.) as well as the raphidascarid *H. aduncum*, which co-occur in the same specimen. The prevalence, intensity and mean intensity (in parentheses) of infestation for the respective nematode species were similar during the 3 years and for the three species (see Palm and Klimpel, in press), ranging from 42.9% to 62.9% and 1–31 (2.6–3.6) for *A. simplex* (s.s.), 22.9% to 40.0% and 1–8 (1.3–2.5) for *P. decipiens* (s.s.) and 28.6% to 60.0% and 1–9 (1.5–4.2) for *H. aduncum*.

3.2. Molecular analysis of the ascaridoid nematodes

A total of 18 specimens, two from each of the three ascaridoid species and examination year, were used for molecular identification. The sequence analyses of the nematodes demonstrated that in all years we identified *A. simplex* (s.s.), *P. decipiens* (s.s.) and *H. aduncum*. For all three species, the amplified PCR product did not vary in size on the agarose gel. The lengths of the PCR product including the three regions with flanking sequences were ~950 bp long, depending on the species (*A. simplex* (s.s.) 910–952 bp, *P. decipiens* (s.s.) 917–950 bp, *H. aduncum* 914–952 bp). The G+C contents for the ITS-1, 5.8S and ITS-2 of rDNA ranged from 42.4% to 51.6% for *A. simplex* (s.s.), 43.6% to 51.6% for *P. decipiens* (s.s.) and 49.8% to 50.9% for *H. aduncum*. The length of the ITS-1 and ITS-2 sequences ranged from 323 to 401 bp and 281 to 307 bp, depending on the individual sequence, whereas all 5.8S sequences were 157 bp long, for all three species and years (Table 1). The alignment of the ITS-1, 5.8S and ITS-2 consensus sequences of one specimen each (from the year 2002) of *A. simplex* (s.s.), *P. decipiens* (s.s.) and *H. adunucm* is given in Fig. 2. Pairwise comparison of the ITS-1, 5.8S and ITS-2 is given in Table 2. Sequence differences between species for the ITS-1 (19.3–47.5%) and ITS-2 (44.1–59.5%) were greater than that for the 5.8S gene (0–3.8%).

Comparison of the ITS-1, 5.8S and ITS-2 sequence data of ascaridoid nematodes from the East Greenland Sea and those of previously published sequences from the Atlantic and Pacific Ocean (GenBank) showed no differences for *A. simplex* (s.s.) (0%, Mid-Atlantic Ridge and Norwegian Deep, Klimpel et al., 2007; 0%, Japan, Abe et al., 2005). The sequences of *P. decipiens* (s.s.) from the East Greenland Sea corresponded most with a sequence published from Richardson Bay, CA, USA (AY825253), and differed in four positions (0.5%). They differed in 11 (1.5%) positions in specimens collected from Monterey, CA, USA (AY821747) and Japan (AB277824). *H. aduncum*

Table 1
Average length (in bp) and G+C content (in %) of the first internal spacer (ITS-1), 5.8S and second internal spacer (ITS-2) rDNA sequences of the third stage larvae of *Anisakis simplex* (s.s.) and *Pseudoterranova decipiens* (s.s.) and the third/ fourth stage larvae of *Hysterothylacium aduncum*

Species	ITS-1		5.8S		ITS-2	
	Length	G+C	Length	G+C	Length	G+C
A. simplex (s.s.)	362	46.9	157	51.6	307	42.4
P. decipiens (s.s.)	323	48.3	157	51.6	300	43.6
H. aduncum	401	50.6	157	50.9	281	49.8

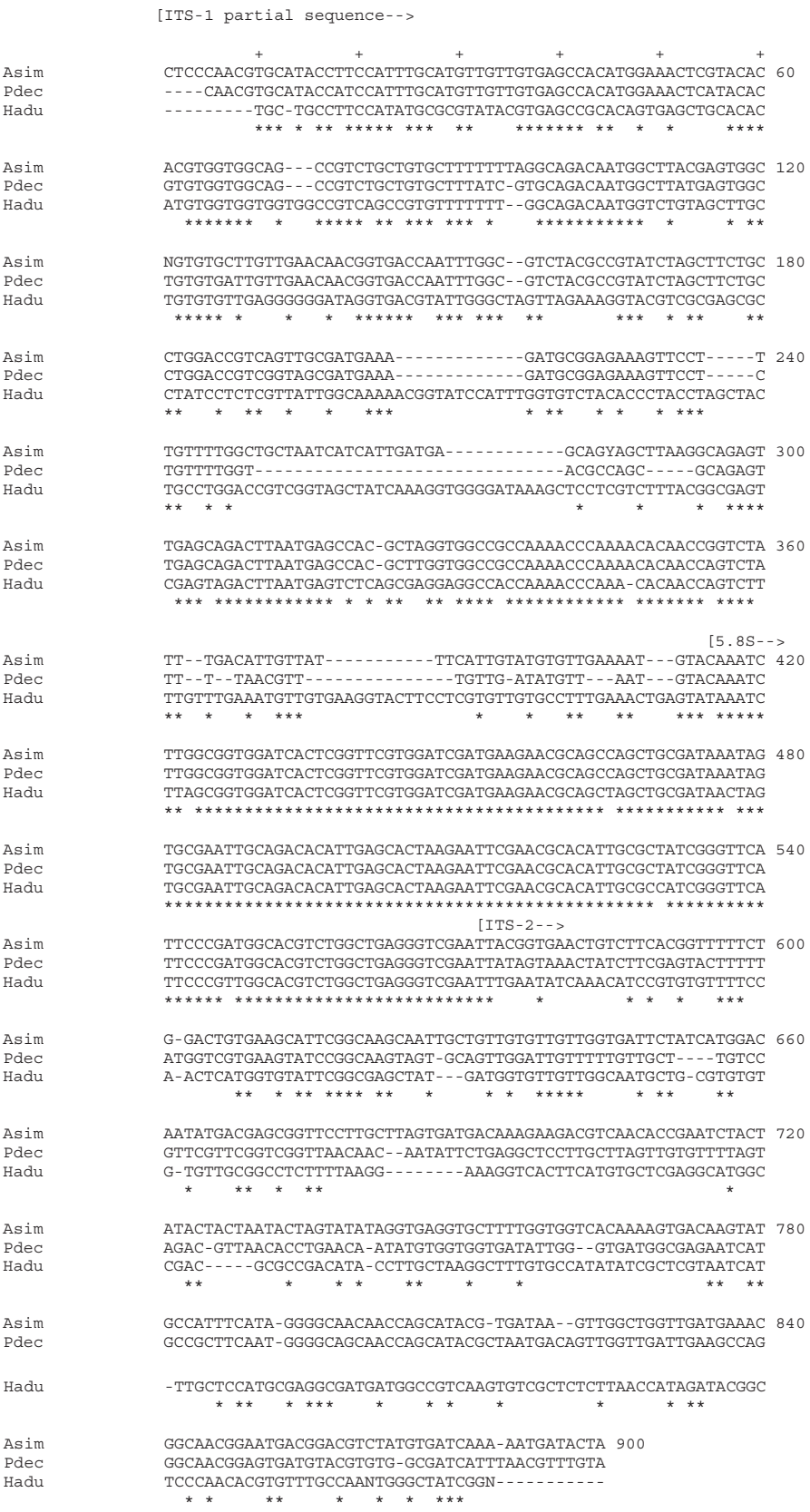

Fig. 2. Alignment of the first internal spacer (ITS-1), 5.8S and second internal transcript spacer (ITS-2) for *Anisakis simplex* (s.s.) (Asim), *Pseudoterranova decipiens* (s.s.) (Pdec) and *Hysterothylacium aduncum* (Hadu). The numbers refer to the alignment position, and asterisks indicate positions with the same nucleotides.

Table 2
Pairwise comparison of the sequence differences (%) among *Anisakis simplex* (s.s.), *Pseudoterranova decipiens* (s.s.) and *Hysterothylacium aduncum* for the first internal transcript spacer, 5.8S and second internal transcript spacer and for all rDNA sequences combined

rDNA region	Species	*A. simplex* (s.s.)	*P. decipiens (s.s.)*	*H. aduncum*
ITS-1	*A. simplex* (s.s.)	–	19.3	47.5
	P. decipiens (s.s.)	19.3	–	44.9
	H. aduncum	47.5	44.9	–
5.8S	*A. simplex* (s.s.)	–	–	3.8
	P. decipiens (s.s.)	–	–	3.8
	H. aduncum	3.8	3.8	–
ITS-2	*A. simplex* (s.s.)	–	44.1	56.3
	P. decipiens (s.s.)	44.1	–	59.5
	H. aduncum	56.3	60	–
ITS-1, 5.8S, ITS-2	*A. simplex* (s.s.)	–	25.5	43.0
	P. decipiens (s.s.)	25.5	–	43.3
	H. aduncum	43.0	43.4	–

was compared with a sequence from Japan (AB277826). Only two positions in the ITS-1 (0.2%) and three positions in the ITS-2 (0.3%) differed from the sequences of *H. aduncum* from the East Greenland Sea. These differences were within an acceptable range (Zhu et al., 2000, 2002), so that the sequence of *P. decipiens* corresponded to those of the Richardson Bay and the sequence of *H. aduncum* to the one from Japan.

4. Discussion

This is the first molecular identification of ascaridoid nematodes from a deep-sea environment in the East Greenland Sea. The analyzed specimens from *M. berglax* could be identified as *A. simplex* (s.s.), *P. decipiens* (s.s.) and *H. aduncum*, and, based on our molecular data, no further sibling species could be detected. The ITS-1, 5.8S and ITS-2 sequences of *A. simplex* (s.s.) did not differ from previously published sequence data (GenBank), and the sequences of *P. decipiens* (s.s.) and *H. aduncum* were very similar to specimens from the USA and Japan. We thus detected a high similarity of the East Greenland Sea specimens to those from other geographical regions.

The length of the PCR product was similar to the results of Zhu et al. (1998; 2002). Sequence differences in the ITS-2 region were greater than that for the ITS-1 and 5.8S region. Differences in the ITS-1 and ITS-2 were highest between *A. simplex* (s.s.) and *H. aduncum* followed by differences between *H. aduncum* and *P. decipiens* (s.s.). This result is different from those of Shih (2004), who reported a higher similarity between *H. aduncum* and *P. decipiens* (s.s.) in the analyzed regions. The 5.8S gene of *A. simplex* (s.s.) and *P. decipiens* (s.s.) was identical, whereas *H. aduncum* differed by 3.8%. This underlines the ITS-1 to ITS-2 region as a highly conserved region and suggests a low genetic variation in each *A. simplex* (s.s.), each *P. decipiens* (s.s.) and each *H. aduncum*.

A. simplex is a complex of sibling species with differences in their distribution and life-cycle strategies. *A. simplex* (s.s.) occurs predominantly in the North Atlantic and Pacific Oceans and has a pelagic life cycle (e.g. Klimpel et al., 2004), whereas *A. pegreffii* occurs in the Mediterranean Sea, northeast Atlantic and Southern Hemisphere, and similarly follows a pelagic life cycle (Mattiucci et al., 1997; Mattiucci and Nascetti, 2006). *A. simplex* C is also common in the Pacific Ocean (Pacific coast of Canada) and in the Southern Hemisphere (Mattiucci et al., 1997; Mattiucci and Nascetti, 2006). Our findings correspond to those of Klimpel et al. (2007), who examined *M. muelleri* (pearlside) from the Mid-Atlantic Ridge (1630–1650 m water depth) and the Norwegian Deep (175–215 m). The authors also identified *A. simplex* (s.s.) in both regions, and the comparison of the specimens from the East Greenland Sea, Mid-Atlantic Ridge, the Norwegian Deep and Japan showed no sequence difference in the ITS-1, 5.8S and ITS-2 regions.

P. decipiens is a cosmopolitan nematode that occurs in many hosts, mainly pinnipeds, from the boreal to the Antarctic regions. Likewise *A. simplex*,

the sealworm *P. decipiens* is a complex of sibling species. *P. decipiens* (s.s.) and *P. bulbosa* are distributed in the North Atlantic, Canadian Atlantic and the Norwegian and Barents Seas, whereas *P. krabbei* occurs in the North Atlantic and the Norwegian and Barents Seas. *P. decipiens* E has been identified in Antarctic waters, *P. cattani* in the Pacific Ocean and *P. azarasi* in Arctic–Boreal and Japanese waters (Zhu et al., 2002). In the East Greenland Sea, only minor differences could be detected between the sequences of our examples and those from California and Japan, verifying the occurrence of *P. decipiens* (s.s.) also in deep waters of the East Greenland Sea. Palm et al. (1994) and Palm (1999) likewise recorded *P. decipiens* E down to 710 m water depth in the eastern Weddell Sea and 820 m off the South Shetland Islands.

H. aduncum is one of the most ubiquitous helminth species in the North Atlantic that was also detected from the Pacific Ocean (Shih, 2004). In the North Atlantic and the Baltic Sea, *H. aduncum* is the only recognized species, but the taxonomy is still unresolved (e.g. Klimpel and Rückert, 2005). Until now most authors have believed that there is a single species of *H. aduncum* parasitizing marine teleosts, while other authors, such as Hartwich (1975), recognized three species: *H. aduncum* mainly from clupeids, *H. gadi* from gadoid fishes and *H. auctum* from *Zoarces viviparous* (eelpout) and several flatfishes of the Pleuronectiformes (Balbuena et al., 1998; Klimpel and Rückert, 2005). Likewise, Petter and Cabaret (1995) recorded differences between specimens collected from different fish species and proposed two subspecies (*H. aduncum aduncum*, *H. aduncum gadi*). As in *P. decipiens* (s.s.) there exists a high genetic similarity between the sequences of *H. aduncum* from the East Greenland Sea and the sequence data from Japan, suggesting the wide range of distribution for this fish parasitic nematode.

5. Conclusion

All three identified ascaridoid nematodes show wide distribution patterns while maintaining a high degree of genetic similarity. The studied ITS-1, 5.8S and ITS-2 sequences were highly similar to those of the specimens from other geographical regions and suggest high genetic homogeneity. This might be caused by (a) extensive final host migration in the case of *A. simplex* (s.s.), (b) an overlapping distribution of final host populations along the continental shelves for *P. decipiens* (s.s.) and (c) a low host specificity and large population size in the intermediate and final hosts for *H. aduncum*. We cannot explain, however, why no further sibling species that occur commonly in North Atlantic fish could be recorded within the present study. This warrants further investigation, involving more hosts, regions and annual comparison.

Macrourids are one of the most important deep water fish families and are distributed in all oceans from the Arctic to Antarctic regions (Carrassón and Matallanas, 2002). They are entirely marine and have their greatest diversity on the upper continental and insular slopes, down to at least 4000 m (e.g. Jørgensen, 1996; Froese and Pauly, 2007). Our results confirm the dispersal of the generalistic ascaridoids *A. simplex* (s.s.), *P. decipiens* (s.s.) and *H. aduncum* into the deep-sea environment in the North Atlantic, as suggested by Palm and Klimpel (in press). Though having very different life cycles, pelagic (*A. simplex* (s.s.)), benthopelagic (*H. aduncum*) and benthic (*P. decipiens* (s.s.)), all three nematode species infect *M. berglax* at the studied deep water locality. This suggests the ability of the benthodemersal *M. berglax* to either feed in the upper pelagic environment, or to have access to infected pelagic invertebrates during their regular vertical migration.

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