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ORIGINAL PAPER

Genetic variability in *Hysterothylacium aduncum*, a raphidascarid nematode isolated from sprat (*Sprattus sprattus*) of different geographical areas of the northeastern Atlantic

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Abstract Species of the genus *Hysterothylacium* are among the most common marine nematode fish parasites in the northern Atlantic. Due to recent findings of cryptic speciation in other parasitic ascaridoid nematodes, a similar pattern of sibling species was hypothesized also for *Hysterothylacium aduncum*. By investigating a 886- to 890-bp-long genomic DNA fragment including ITS-1, 5.8S rDNA and ITS-2 of 40 specimens of *H. aduncum* of sprat (*Sprattus sprattus*) of four different biogeographical regions (North Sea, English Channel, Bay of Biscay, Adriatic Sea), we could not detect significant genetic variability and therefore cryptic speciation. Nevertheless, while ITS-1 and 5.8S rDNA sequences were identical for all analysed specimens, ITS-2 sequences showed a population-specific pattern with the differentiation of an English Channel/Bay of Biscay group from a North Sea/Mediterranean Sea group.

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Introduction

Parasitism, a specific form of symbiosis, is one of the most successful modes of life (Palm and Klimpel 2007). More than half of all plant and animal species on earth are parasites, and there is probably no organism without any parasitic infection during its lifetime (Palm and Klimpel 2007). One of the most widespread groups of parasites are the nematodes, using plants as well as animals and humans as their hosts on global scale (e.g. Blaxter et al. 1998). With approximately 256 families and more than 40,000 species, the phylum Nematoda represents one of the most species-rich and abundant invertebrate groups. They include not only parasitic but also free living forms in various limnic, marine as well as terrestrial habitats (McClelland 2005).

Traditionally, the Nematoda are divided into two classes, the predominantly terrestrial Secernentea and the predominantly marine Adenophorea, but whether this classification reflects the evolutionary history of the group remains highly questionable. Recent phylogenetic studies based on molecular markers divide nematodes into the two classes Enoplea (Adenophorea, Aphasmidea) and Rhabditea (Phasmidea, Secernentea; McClelland 2005). Marine fish parasitic species are found in the enoplean subclass Dorylaimia (e.g. the genera *Capillaria*, *Pseudocapillaria*) and in the rhabditean subclass Rhabditia (e.g. the genera *Anisakis*, *Hysterothylacium*, *Pseudoterranova*; McClelland 2005).

According to Anderson's (1984, 1996) hypothesis, parasitic nematodes first evolved in terrestrial hosts and were only able to invade aquatic environments after the development of heteroxeny (use of intermediate hosts) and paratenesis (use of transport hosts). Heteroxenic life cycles have been frequently described in marine ascaridoid nematodes such as species of the genera *Anisakis*, *Pseudoterranova*

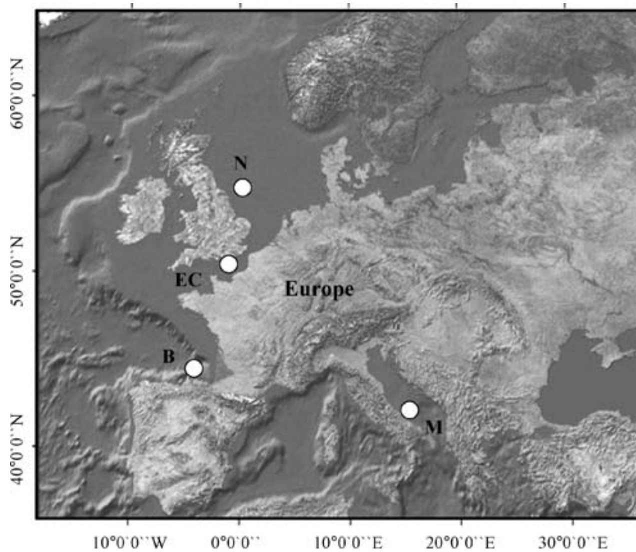

Fig. 1 Area of investigation with the four sampling stations including the North Sea (*N*), English Channel (*EC*), Bay of Biscay (*B*) and Adriatic Sea (*M*)

(both subfamily Anisakinae) and *Hysterothylacium* (subfamily Raphidascaridinae; e.g. Klimpel 2005, Klimpel et al. 2004, Klimpel and Rückert 2005). Ascaridoid nematodes are generally associated with aquatic vertebrates as typically definitive hosts. Transmission pathways are habitat-dependent and usually involve a broad spectrum of invertebrates and intermediate or paratenic fish hosts.

The genus *Hysterothylacium* comprises more than 59 species and is one of the most ubiquitous parasitic nematode species in fishes of the North Atlantic (e.g. Køie 1993; Balbuena et al. 1998, 2000, Navone et al. 1998; Klimpel et al. 2006a). It can be assumed that life cycles within the genus *Hysterothylacium* are principally similar for all species. While sexually mature adults are mainly found in the digestive tract of various marine fish species, their third and fourth larval stages preferably parasitise in organs of the body cavity of their hosts. Over 100 different benthic and planktonic invertebrate species act as intermediate and/or paratenic hosts (Køie 1993; Navone et al. 1998).

In the North Atlantic, the North Sea, the Baltic Sea, the Mediterranean Sea and adjacent temperate and cold waters, the species *Hysterothylacium aduncum* is a very common fish parasite (Margolis and Arthur 1979; Palm et al. 1999; Klimpel et al. 2001), but its taxonomic status is highly ambiguous. Several authors assume *H. aduncum* to be a single species parasitising opportunistically in various marine fish species. In contrast, Hartwich (1975) recognised three distinctive species: *H. aduncum* mainly found in clupeid hosts like sprat (*Sprattus sprattus*) and herring (*Clupea harengus*), *H. gadi* from gadoid fishes and *H. auctum* frequently identified in the eelpout (*Zoarces viviparus*), but also in various flatfishes (Pleuronectiformes). Petter and Cabaret (1995) found variabilities between specimens collected from different fish species, but proposed only two subspecies on the basis of biometrical data. Thus, it would not be surprising if future molecular studies revealed *H. aduncum* as a complex of a yet unknown number of sibling species (Balbuena et al. 1998).

In the present study, we investigated the genetic variability of *H. aduncum* within a single host species (*S. sprattus*) from a phylogeographic perspective to test overall genetic diversity and the influence of geographic distance as well as known biogeographic boundaries like the Gibraltar gateway on reproductive exchange and gene flow of a abundant parasitic fish nematode.

Materials and methods

Sample collection

A total of 160 European sprat (*S. sprattus*), 40 samples each, were collected in 2005 at four sites within the northeastern Atlantic, the North Sea (N), the English Channel (EC), the Bay of Biscay (B) and the northern Adriatic Sea (M; Fig. 1). Immediately after catch with pelagic nets at trawling speeds of roughly four knots, fishes were deep frozen at −20°C. Before examination, fish specimens were defrosted at 0–1°C, and the standard length (SL) and total weight (TW) were recorded to the nearest 0.1 cm and 0.1 g, respectively.

Table 1 Samples of *H. aduncum* isolated from the sprat (*S. sprattus*) used in this study

Parasite species	Stage	Sample code	*n*	ITS-1		5.8S		ITS-2		Geographical origin
				Length	G+C	Length	G+C	Length	G+C	
Hysterothylacium aduncum	Larvae III/IV	H31-H40	10	401	50.6	157	50.9	328	50.9	English Channel (EC)
Hysterothylacium aduncum	Larvae III/IV	H21-H30	10	401	50.6	157	50.9	328	50.9	Bay of Biscay (B)
Hysterothylacium aduncum	Larvae III/IV	H11-H20	10	401	50.6	157	50.9	332	50.3	Adriatic Sea (M)
Hysterothylacium aduncum	Larvae III/IV	H1-H10	10	401	50.6	157	51.3	332	50.3	North Sea (N)

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/ fourth stage larvae of *H. aduncum*

Table 2 Morphometric fish data and prevalence, abundance, intensity and mean intensity of infestation of the sprat (*S. sprattus*) from the four sampling stations

Area of investigation	N	EC	B	M
Morphometric data				
Standard length (cm)	9.9	9.5	9.0	10.1
(Range)	(8.6–10.7)	(8.2–10.2)	(7.3–11.8)	(8.7–12.2)
Total weight (g)	12.3	8.7	8.6	16.4
(Range)	(7.8–15.8)	(5.6–10.9)	(4.1–19.3)	(7.0–21.5)
Parasite species	*Hysterothylacium aduncum*			
Prevalence (%)	100.0	100.0	100.0	100.0
Abundance	20.41	25.39	12.94	23.00
Intensity	2–118	4–85	1–54	1–70
Mean Intensity	19.6	29.8	13.3	23.0

N North Sea, *EC* English Channel, *B* Bay of Biscay, *M* Adriatic Sea

Raphidascarid nematode isolation and molecular analyses

Nematodes were isolated and identified to a species level based on morphological and biometric characters by the use of existing keys and descriptions before conservation in 96% ethanol. Genomic DNA was isolated and purified from single individuals (Table 1) using genomic DNA extraction kit (Peqlab Biotechnology GmbH, Erlangen, Germany) following manufacturer's instructions. 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′; Shih 2004; Zhu et al. 1998, 2000). The polymerase chain reaction (PCR) mix (26 µl) included 13 µl MasterMix (QIAGEN, Hilden, Germany) containing dNTP, $MgCl_2$, buffer and Taq-polymerase, 3 µl of each primer, 2 µl dest. water and 5 µl genomic DNA. PCR reactions were performed in a thermocycler (Biometra, Germany) under the following conditions: after an initial denaturation at 95°C

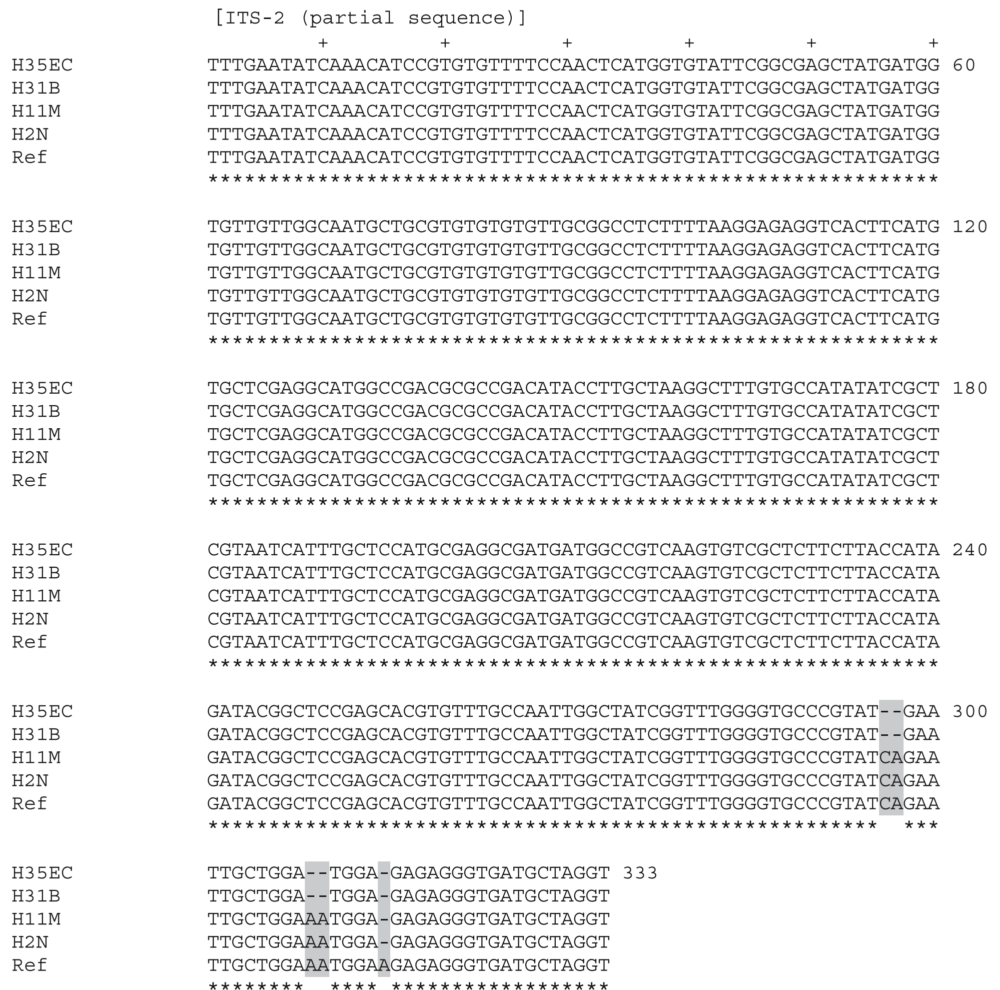

Fig. 2 Alignment of the ITS-2 consensus sequence for third/fourth stage larva of *H. aduncum* (sample code H2, H11, H31, H35) isolated from sprat (*S. sprattus*) from the North Sea (*N*), English Channel (*EC*), Bay of Biscay (*B*), Adriatic Sea (*M*) and reference (*Ref*) of Zhu et al. 1998. The *numbers* refer to the alignment position, and *asterisks* indicate positions with same nucleotides

for 15 min, 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension) were performed, followed by a final extension at 72°C for 5 min. Each PCR run included a sample without DNA (negative control). PCR products were quality-checked on 1% agarose gels. The amplification products were purified with commercial PCR purification kits (E.Z.N.A. cycle-pure kit, Peqlab Biotecnology, Erlangen, Germany) before direct sequencing performed by Seqlab (Goettingen, Germany). Both strands of the two spacer regions and the 5.8S rDNA gene were sequenced for all specimens 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.

Resulting sequence data of raphidascarids were aligned using CLUSTAL W (1.83) multiple sequence alignments (Thompson et al. 1994) and compared with previously published sequence data in GenBank to analyse intraspecific differences.

Results

Morphometric measurements (standard length, total weight) did not reveal any significant differences between sprat (*S. sprattus*) populations from the four sampling sites (Table 2). The predominant parasite species in all analysed *S. sprattus* was the raphidascarid nematode *H. aduncum*, with high values of prevalence (*P*%), abundance (*A*), intensity (*I*), as well as mean intensity (mI) of infestation (Table 2).

Multiple sequence analyses of *H. aduncum* indicated low genetic diversity only for the ITS-2 region, while ITS-1 and 5.8S rDNA were identical for all investigated 40 specimens. The total lengths of the PCR products varied between 886 and 890 bp, consisting of ITS-1 with a constant length of 401 bp, ITS-2 ranging from 328 to 332 bp and the 5.8S sequence with an again constant length of 157 bp. G+C contents of the three regions varied between 50.3 to 51.3% (Table 1). Sequence divergence within ITS-2 follows a population-specific pattern with no intra- but low interpopulation differentiation based on indels separating an English Channel/Bay of Biscay group from a North Sea/Mediterranean group (Fig. 2, Table 1). The only accessible reference sequence published by Zhu et al. (1998) further differs by a single polymorphic site in ITS-1 (alignment position 61) and an additional indel not shared by any of our the here investigated specimens in ITS-2. The alignment of the ITS-1, 5.7S rDNA and ITS-2 consensus sequence representing *H. aduncum* from one specimen each of the different geographical areas is given in Fig. 2.

Discussion

The present study is the first molecular characterization of raphidascarid nematodes isolated from European sprat (*S. sprattus*) of different biogeographic regions. The an-

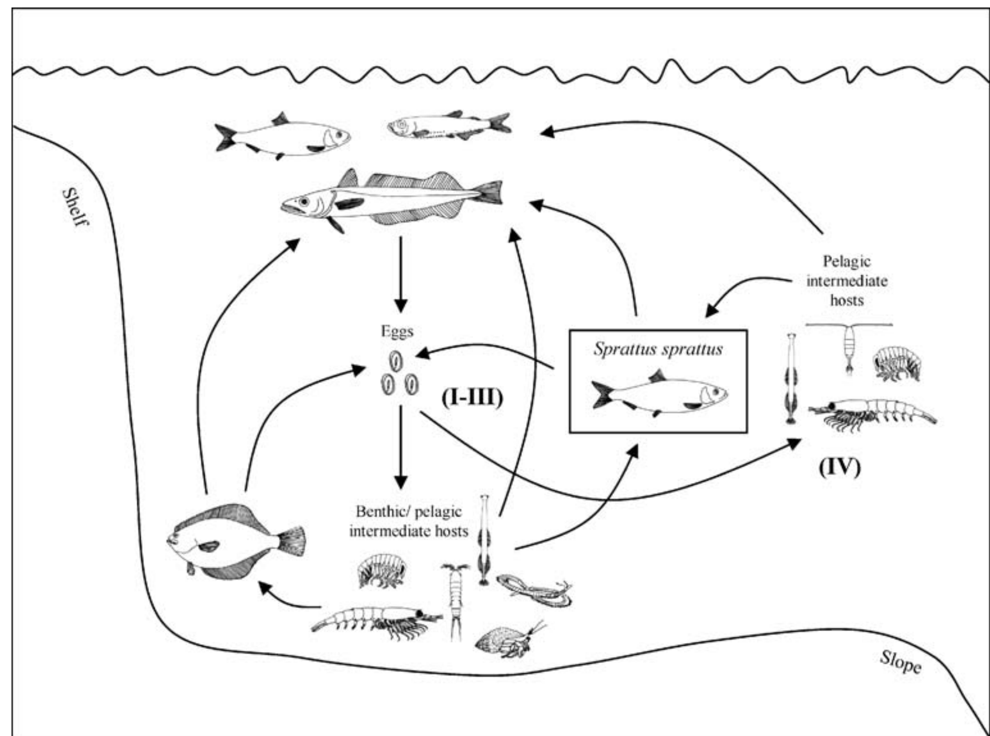

Fig. 3 Schematic illustration of the main life cycle of *H. aduncum* isolated from the sprat (*S. sprattus*) in waters of the investigated areas: the North Sea, English Channel and Adriatic Sea (I–III), and the Bay of Biscay (IV). The nematode utilizes similar pathways to reach the final benthic or pelagic fish hosts. In the areas I to III, the nematode use generally benthic and pelagic intermediate hosts, while in the area IV, pelagic intermediate host plays a more important role in the life cycle pattern

alysed nematodes could be identified morphologically as well as genetically as *H. aduncum*, and no cryptic sibling species could be detected.

Species of the genus *Hysterothylacium* are found in the adult stage predominantly in the digestive tract of teleost fishes and rarely in elasmobranches (Køie 1993; Klimpel et al. 2003). Larvae are also widely distributed and have been reported in the organs and body cavities of a variety of marine fishes and pelagic and benthic invertebrates (e.g. Palm et al. 1999; Klimpel and Rückert 2005). *H. aduncum* is a parasite that is not very host-specific in either its adult or its larval stages. The adults are found in the digestive tract of marine fishes; several marine invertebrates act as intermediate hosts. In general, *H. aduncum* is one of the most ubiquitous helminth species in the North Atlantic that was also detected in the Pacific Ocean (Shih 2004). In the North Atlantic and the Baltic Sea, *H. aduncum* is the only recognised species, but its taxonomy is still unresolved. The two other important ascaridoid nematodes in the North Atlantic, *A. simplex* and *P. decipiens*, use mammals as definitive hosts, while *H. aduncum* uses only fishes as final hosts (Balbuena et al. 1998; Klimpel and Rückert 2005). Eggs of the nematode *H. aduncum* pass out in the faeces of the fish final host (see Fig. 3) and develop to the second stage larvae (L2). Larvae are ingested by various benthic and/or pelagic invertebrates, hatch in their intestine and attain into their haemocoel (Køie 1993; Klimpel et al. 2006b). Development to the third stage larvae (L3) takes place in invertebrates, and the nematode may then transfer to the definitive fish host. Larval size seems important in determining their fate. Only, third stage larvae (L3) longer than 0.3 cm moult into the fourth stage larvae (L4) and subsequently into adult stages in the digestive tract lumen of the fish host (Køie 1993). Different fish species heavily acquire and accumulate different stages of *H. aduncum* by ingesting crustaceans, chaetognaths and small fishes that are infested and serve as carriers (Klimpel 2005; Klimpel and Rückert 2005).

Morphological differentiation of nematode species belonging to the genus *Hysterothylacium* is extremely difficult. One of the most important criteria for nematode species identification is the position and anatomy of the foregut, which often is hardly visible and therefore not useful for an unambiguous identification. Molecular genetic techniques are sometimes more reliable for a proper species identification of larval and adult ascaridoid nematodes, such as of the genera *Anisakis*, *Contracaecum* and *Pseudoterranova*. Different authors could demonstrate that morphospecies such as *Anisakis simplex* and *Pseudoterranova decipiens* are actually complexes of sibling species (e.g. Abollo et al. 2003) that are morphologically extremely similar but genetically distinct. At least three sibling species could be identified within the *A. simplex* complex in the Atlantic Ocean and three siblings of the *P. decipiens* complex in the North Atlantic, Norwegian and the Barents Sea (e.g. Nascetti et al. 1986; Zhu et al. 2002). Each of these cryptic sibling species was found to have much more specific host preferences and a geographically much more confided distribution than known from the original morphospecies (e.g. Marques et al. 2006). These studies show that the region covering ITS-1, 5.8S rDNA and ITS-2 is subject to moderate to fast evolutionary changes and can be successfully used for an unambiguous distinction of closely related nematode species (e.g. D'Amelio et al. 2000).

The fact that *H. aduncum* is also a nematode parasite with remarkably low host specificity and that siblings have been detected in congeners like *Hysterothylacium fabri* (Martin-Sánchez et al. 2005) led to the assumption that *H. aduncum* might represent a sibling species complex as well (Balbuena et al. 1998). Nevertheless, intraspecific genetic diversity in our investigation was unexpectedly low with three indels only in ITS-2, while ITS-1 and 5.8S rDNA from specimens of regions as widely separated as the North Sea and the northern part of the Mediterranean Adriatic Sea did not differ at all. Despite that, the low levels of sequence divergence within ITS-2 follow a population-specific pattern. With no intra- but low interpopulation differentiation, an English Channel/Bay of Biscay group can be separated from a North Sea/Mediterranean Sea group. This pattern can hardly be explained from a phylogeographic point of view and has to be confirmed by higher sample sizes. But it gives a hint towards a possible loss of reproductive exchange and therefore gene flow, which has to be tested by the use of more polymorphic markers.

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