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6. Biogeography of *Anisakis* (Anisakidae) and *Hysterothylacium* (Rhaphidascarididae) nematode species in consumed fish

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Abstract. The presence of ascaridoid nematodes in commonly consumed fish constitutes an important health risk for humans as well as an economic problem for fisheries. Here, information is provided on the taxonomic status of the representative "anisakid-related" species of the families Anisakidae and Raphidascarididae. These parasites have a worldwide marine geographical distribution, mainly related to the presence of the vertebrate hosts involved in their life cycle. Morphological and molecular methods currently used for specific characterization of larval and adult nematode specimens are analysed and discussed. This study is focused on the taxonomy and parasite-host distribution of species of the genera *Anisakis* and *Hysterothylacium* from the North-East Atlantic Ocean and Mediterranean Sea regions.

1. Introduction

In the last four decades fish consumption has nearly doubled worldwide and global fish production, including aquaculture and wild-catch

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fisheries, has increased by many tons to meet the growing market demands [1]. Some of the most habitually consumed fish species are at risk of carrying zoonotic parasites, which can cause economic and sanitary problems [2]. In this context, anisakids that include fish in their life cycle have been ranked by the European Food Safety Authority [3] as a "biological hazard" of the highest importance in seafood products [2]. Species of the genera *Contracaecum* and particularly *Anisakis* and *Pseudoterranova* have been associated with the fishborne disease anisakiosis/anisakidosis, which produces both gastric and allergic reactions [4]. Other "anisakid-related" nematodes, such as *Hysterothylacium* species of the family Rhaphidascarididae, although considered non-pathogenic, are associated with allergic processes in humans [5] and human infection has also been reported [6]. Infection with *Hysterothylacium* can affect the growth rate and health of the fish hosts, making them more vulnerable to diseases and even resulting in mortalities [7,8].

Improving taxonomic descriptions for specific identification will shed light on the life cycle and geographical distribution of these nematodes, and help understand their epidemiological, biological and ecological patterns [9].

1.1. Taxonomical classification

The taxonomic status of fish-associated ascaridoid genera with zoonotical potential is as follows [10,11,12]:

Phylum: Nematoda Rudolphi, 1808
Class: Secernentea Chitwood, 1958
Order: Ascaridida Skrjabin & Schultz, 1940
Superfamily: Ascaridoidea Baird, 1853
Family: Anisakidae Raillet & Henry, 1912
Subfamily: Anisakinae Raillet & Henry, 1912
Genus: *Anisakis* Dujardin, 1845
Genus: *Pseudoterranova* Mozgovoi, 1951
Subfamily: Contracaecinae Mozgovoi & Shakhmatova, 1971
Genus: *Contracaecum* Raillet & Henry, 1912
Family: Raphidascarididae Hartwich, 1954
Subfamily: Raphidascaridinae Hartwich, 1954
Genus: *Hysterothylacium* Ward & Magath, 1917

The evolutionary taxonomy of the superfamily Ascaridoidea is very uncertain, largely because of the great variation in morphological features and life cycle patterns among different species [10,13]. Most evolutionary hypotheses for ascaridoids were developed prior to the widespread use of

molecular techniques and cladistic analysis, and were typically based on the variation in one or a few key morphological structures or life history features [11].

In the last fifty years the systematics and classification of "anisakid-related" species has been much discussed. For example, some authors maintain that the four genera *Anisakis, Pseudoterranova, Contracaecum* and *Hysterothylacium* should be included in the family Anisakidae, with Anisakinae, Contracaecinae and Rhaphidascaridinae reduced to subfamilies [14,15,16,17,18], whereas others consider the subfamily Raphidascaridinae, which includes the *Hysterothylacium* species, to be an independent family taxon, the Raphidascarididae [10,11,12,19,20].

Despite these unresolved issues, no approach integrating both morphological and molecular tools has attempted to assess the specific classification of anisakid nematodes or the systematic importance of their features [12]. However, recent phylogenetic studies based on numerous representatives of anisakid nematodes have revealed three main clades that correspond to two subfamilies of Anisakidae, Anisakinae (which includes the *Anisakis* and *Pseudoterranova* genera among others) and Contracaecinae (which includes the *Contracaecum* among others), and one other clade corresponding to the family Raphidascarididae, which includes the *Hysterothylacium* genus [2,12].

The lack of available molecular and well-presented morphological data for "anisakid-related" nematodes makes it difficult to search for patterns that may resolve their phylogenetic lineages and shed light on their relationships [12].

1.2. Life cycle

Anisakid species mostly parasitize the digestive tract of marine mammals and use teleost fish as paratenic/transfer hosts for their infesting larvae. The most representative life cycle of these nematodes is that of *Anisakis simplex* represented in Fig. 1. The life cycle is as follows:

- L1 eggs are released into water through definitive host faeces, where the larval maturation process L1-L3 takes place in 20-27 days at 5-7ºC.
- Immature L3 hatch and are consumed by the intermediate host, mostly euphasid crustaceans, in which L3 evolve.
- Sea fish and cephalopods ingesting parasitized crustaceans act as paratenic/transfer hosts, harbouring the infesting L3.
- When final hosts feed on parasitized fish or cephalopods, L3 evolves into L4 and finally the adult form, the life cycle ending with egg production by the female.

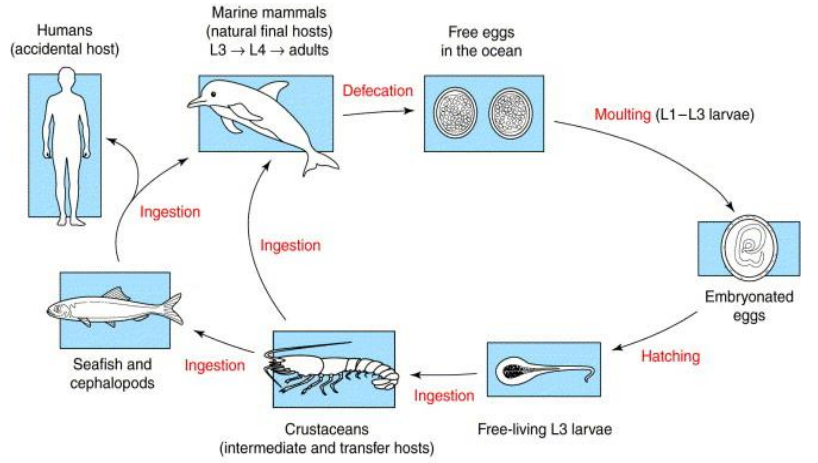


Figure 1. Life cycle of *Anisakis simplex* [4].

These hosts can also be infested by direct consumption of the intermediate crustacean host.

- Humans eating raw parasitized fish can act as an accidental host, in which L3 cannot develop to the adult stage.

In the life cycle of the rhapidascarid *Hysterothylacium* cold-blood organisms like fish, mainly gadiform, act as definitive hosts [21]. Many species of this genus can evolve in marine and freshwater ecosystems in which fish occupying a low place in the food chain, such as anchovy or horse mackerel, usually act as intermediate/paratenic hosts, whereas large predatory fish are the definitive hosts, harbouring the adult forms [22,23].

1.3. Sanitary and commercial interest

The main food-borne zoonoses associated with the consumption of fishery products are mainly attributable to trematodes, cestodes and nematodes. Among the latter, anisakids are the most important parasites from a sanitary point of view, since they are capable of inducing anisakiosis/anisakidosis in humans [24]. Transmission occurs when humans eat raw or marinated fish parasitized with anisakid larvae L3. Most larvae are located in the visceral cavity but can also be present in the flesh surrounding this cavity and even deeper within the dorsal part of the fish, thus representing a major consumer health risk [2].

The disease can evolve with different symptomatology [25]. In gastric anisakidosis, larvae stick to the wall of the stomach and cause abdominal pain, nausea and vomiting 6-12 hours after ingestion. It usually remits spontaneously but sometimes mechanical extraction by endoscopy is necessary. Intestinal anisakidosis occurs when larvae stick to the thin intestinal wall, which usually happens 48-72 hours after ingestion and can provoke serious inflammatory reactions, sometimes requiring surgical extraction. Gastric and intestinal symptoms can be combined in gastro-intestinal anisakidosis.

Anisakidosis can also be manifested by allergic reactions, usually provoking urticaria or angioedema, and in some severe cases causing anaphylactic shock [25]. Some *Anisakis* species may cause a combination of gastric and allergic anisakidosis known as gastro-allergic anisakidosis [2,25].

This fishborne pathology can be an important public health problem in countries where raw fish is habitually consumed, as occurs on the Eastern coast of Asia. The aetiological agents in 90% of documented clinical cases worldwide are *Anisakis simplex* (*sensu stricto*), *Anisakis pegreffii* and *Pseudoterranova decipiens* [26]. Nevertheless, studies on the zoonotic potential of these nematodes should be extended, since human cases of anisakidosis are most likely underreported, probably due to unspecific symptoms associated with acute and chronic infections [2].

Furthermore, "anisakid-related" nematodes can entail economic losses for the fish industry, involving both wild and farmed fish [2]. When present in fish intended for consumption, these parasites have a considerable quality-reducing effect due to their unappealing appearance [27], so heavily infected fish have no commercial value [28].

1.4. Identification methods

Accurate identification at the species level is very important to understand epidemiological, biological, and ecological patterns [2,18]. Morphological methods are useful but are often insufficient for specific identification. New molecular methods have provided solid information for the specific identification of anisakids in the last decades [9].

Morphological criteria

Species identification in Anisakidae and Rhaphidascarididae has traditionally been complicated due to a lack of differentiating morphological features, particularly in larval stages. In adult worms, the morphological characters

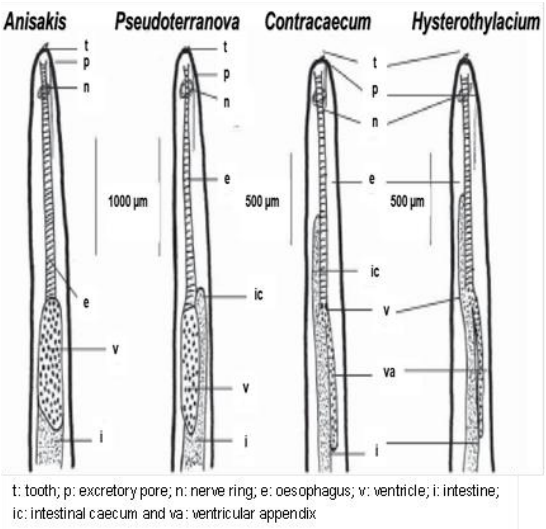


Figure 2. Main morphological differences at the genus level of third stage larvae L3 in "anisakid-related" nematodes [21].

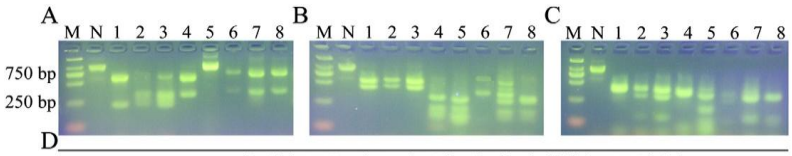
with taxonomic interest are the ventriculus shape; the form of lips; the length and shape of spicules and postanal papillae in males; and the position of the vulva in females [29,30]. The main morphological taxonomic characters of third stage larvae L3 are the structures of the anterior part of digestive tract (oesophagus, ventricle, ventricle appendix intestinal caecum); the anatomical oral tooth; the position of the excretory pore; the distance of the nerve ring to the apical end (Fig. 2), and the caudal morphology, mainly the presence/absence of a caudal spine or mucron [21,31,32]. *Hysterothylacium* species are usually found in fish as fourth stage larvae L4, which can be characterized and differentiated mainly by the presence of labia, the absence of a tooth, and the presence of a cluster of spines at the caudal end [33].

Molecular methods

The first molecular method used in the study of anisakid genetics was Multilocus Allozyme Electrophoresis (MAE) (19-24 enzyme loci), which revealed the existence of high genetic heterogeneity within *Anisakis*, *Pseudoterranova* and *Contracaecum* and increased the diversity of species included in these genera. This technique allowed the genetic characterization of several anisakid species: it estimated their genetic differentiation, established their genetic relationships and identified their larval stages without morphological characters [9].

The introduction of polymerase chain reaction (PCR) methods confirmed the taxonomic characterisation obtained through allozyme markers. Among these methods the most used are PCR-RFLP (Restriction Length Polymorphism), a polymorphism study of restriction fragments in the PCR products of the ITS-DNA region (Fig. 3) [34]; PCR-SSCP (Single Strand Conformational Polymorphism), a conformational analysis of simple chain polymorphism of PCR-amplified DNA of ITS regions; direct sequencing of PCR-amplified DNA of the 28S region (LSU) and the complete internal transcribed spacer (ITS-1, 5.8S, ITS-2) of ribosomal DNA; and PCR and sequencing of cytochromoxidase b (mtDNA cytb) and mitochondrial cytochromoxidase 2 (mtDNA cox2) [9]. In recent years the analysis and sequencing of the partial gene of the small subunit of the mitochondrial ribosomal RNA gene (rrnS) and the elongation factor EF1 α-1 of the nuclear DNA gene have also been used after PCR for differentiation [35,36].

The advantage of these PCR techniques is they allow the use of alcohol- or formalin-preserved specimens, whereas MAE is limited to frozen or fresh individuals. Moreover, PCR-DNA methods have also facilitated the study of phylogenetic relationships between anisakid species based on the evolutionary lineage concept and have confirmed the existence of sibling species by establishing their taxonomic status [9].



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Species	Restriction pattern (approximate lengths of major DNA fragments in bp)		
	HinfI	HhaI	TaqI
A. simplex s.s.	**615, 232**, *67, 37*	**530, 421**	**424, 378**, *65, 54, 30*
A. pegreffii	**331, 284, 232**, *67, 37*	**530, 421**	**378, 293, 131**, *65, 54, 30*
A. simplex s.s.×*A. pegreffii*	**615, 331, 284, 232**, *67, 37*	**530, 421**	**424, 378, 293, 131**, *65, 54, 30*
A. typica	**594, 326**, *34*	**308, 212, 180, 153, 101**	**380, 338**, *65, 54, 30*

Figure 3. Molecular identification of *Anisakis* and *Hysterothylacium* larvae by PCR–RFLP with *Hinf*I (A), *Hha*I (B) and *Taq*I (C) restriction enzymes of the ITS PCR products and fragment sizes (D). Fragments in bold might be visible in the gel, while fragments in italics might not. M: the 2000 bp DNA ladder marker; N: ITS PCR products; Pattern 1: *A. simplex* (*s.s.*); Pattern 2: *A. pegreffii*; Pattern 3: Recombinant genotype of *A. simplex* (*s.s.*) and *A. pegreffii*; Pattern 4: *A. typica*; Pattern 5: *Hysterothylacium* spp.; Pattern 6: *H. aduncum*; Pattern 7: *H. fabri*; and Pattern 8: *H. amoyense* [34].

The description of morphospecies, or species complexes, based on previously recognized cosmopolitan species (*sensu lato*), has solved one of the major problems in the systematics of anisakid nematodes, namely the occurrence of parallelism and convergence of morphological features. This can confound the systematic value of morphological criteria and is often associated with a high genetic and ecological divergence between the species [9].

Genetic/molecular markers used to characterize anisakid species have allowed intermediate/paratenic host fish species and definitive host pinnipeds and cetaceans from different geographical marine regions to be screened and identified [2]. Genetic data can also provide information on ecological and evolutionary aspects, such as host preference and host–parasite co-evolutionary adaptations, including host–parasite co-phylogenetic processes [2].

2. Parasite and host geographical distribution

According to a report by the European Food Safety Authority (EFSA) (European Food Safety Authority, Panel on Biological Hazards (BIOHAZ), 2010), no maritime area can be considered free from anisakids. The geographical distribution of different anisakid species, as well as raphidascaridids, depends on the distribution of their definitive hosts. As a wide range of crustaceans, fish and cephalopods can act as intermediary or parathenic hosts, the definitive hosts have more influence on the species distribution [9].

2.1. Family Anisakidae

Most documented and studied species of Anisakidae are included in *Anisakis*, *Pseudoterranova* and *Contracaecum* genera. *Anisakis* species are distributed around the world, parasitizing cetaceans, mainly whales and dolphins. *Pseudoterranova* and *Contracaecum* species usually have pinnipeds as definitive hosts, which tend to live in cold waters and are usually found in the most northern and southern waters of the planet [9].

Genus *Anisakis*

Up to nine different species of the genus *Anisakis* have been described morphologically and molecularly worldwide (Table 1). All these species are characterized by distinct diagnostic genetic markers, possess distinct gene pools and are reproductively isolated [2].

A. simplex (*sensu lato*) is a complex of three sibling species including *A. simplex* (*s.s.*), *A. pegreffii* and *A. berlandi* (= *A. simplex* sp. C), which are morphologically non-differentiable [35]. These species parasitize cetaceans, mainly delphinids: the two first are distributed worldwide and the latter are

Table 1. *Anisakis* species and their geographical distribution based on definitive and paratenic host sampling (following [9]).

***Anisakis* species**	**Geographical distribution**
A. simplex (*s.s.*)*	North and North-East Atlantic; Bering Sea; South Africa; North-East and North West Pacific
*A. pegreffii**	Mediterranean Sea; North-East Atlantic; South West Atlantic; North West Pacific; New Zealand and South Africa
*A. berlandi**	North-East and South Pacific; South Africa and New Zealand
*A. ziphidarum***	Central Atlantic; South Africa and Mediterranean Sea
*A. nascettii***	Central Atlantic; Iberian Atlantic coasts; South Africa and New Zealand
A. physeteris	Mediterranean Sea; Central and North East Atlantic
A. brevispiculata	South Africa; Central Atlantic and Iberian Atlantic coasts
A. paggiae	South Africa; Central Atlantic and North-East Atlantic
A. typica	Central and South West Atlantic; Mediterranean Sea; China Sea and Somali coast

***Sibling species of the complex *A. simplex* (*sensu lato*); **sibling species**

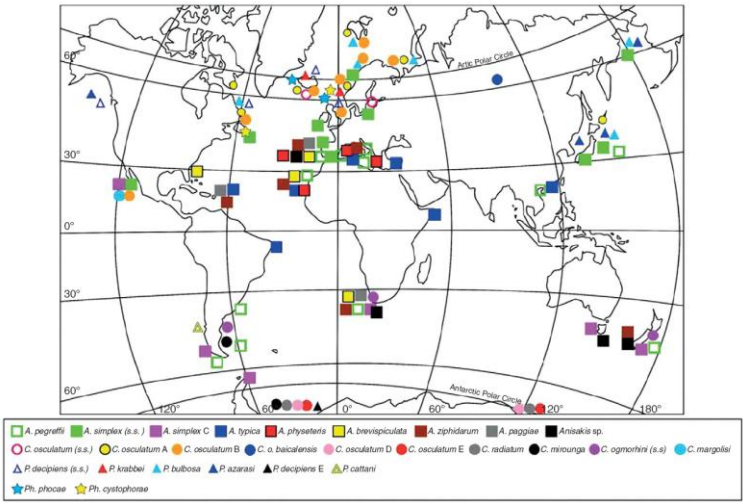

Figure 4. Geographical distribution of *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Phocascaris* species based on definitive and intermediate/paratenic host sampling [9].

more focalized (Fig. 4) [9]. *A. simplex* (*s.s.*) has also been recorded in other cetacean families like Balaenopteridae, Monodontideae and Phocoenidae, and *A. pegreffii* in the family Neobalaenidae. *A. ziphidarum* and *A. nascettii* are sibling species detected in Ziphiidae cetaceans, mainly in warm waters and the southern hemisphere, respectively. *A. physeteris* is a parasite of the kogiidid sperm whale and is typical of Mediterreanean and European Atlantic waters. *A. brevispiculata* and *A. paggiae* have been detected in the pygmy sperm whale in North Atlantic and South African marine waters, and *A. typica* in delphinids from warm waters like the Caribbean Sea [9].

Genus *Pseudoterranova*

Eight distinct species of the genus *Pseudoterranova*, parasitizing a wide range of pinnipeds worldwide, have been molecularly recognised [37]. Adults of *P. decipiens* (*sensu lato*), which are in fact a complex of six biological species, are worldwide-distributed parasites of phocid and otariid seals. *P. decipens* (*s.s.*) has been documented from a wide range of Phocidae species and also some Otariidae, mainly in waters of the northern hemisphere (Fig. 4). *P. krabbei* is typical of the North-East Atlantic and has been recorded in Phocidae species. *P. bulbosa* is habitually found in the bearded seal and has been registered mainly in northern waters. *P. azarasi* parasitizes a wide range of pinnipeds, including sea lions and seals, mainly from northern waters but has also been documented in Japan. *P. cattani* is also a parasite of sea lions but mainly from South Pacific regions. Finally, *P. decipiens E* is a typical parasite of weddell seals and has been reported from the Antarctica [9]. The other two recognised species of *Pseudoterranova* are *P. kogiae* from the pygmy sperm whale, *Kogia breviceps* and *P. ceticola* from the dwarf sperm whale, *K. sima*.

Genus *Contracaecum*

The genus *Contracaecum* comprises at least 50 different species that parasitize mostly pinnipeds and fish-eating birds in their adult form (Fig. 4). The most studied and documented species are those within the *C. osculatum* and *C. ogmorhini* complexes. The former includes five sibling species that usually parasitize Phocidae: *C. osculatum* A, *C. osculatum* B and *C. osculatum* (*s.s.*), documented in Arctic hosts; and *C. osculatum* D and *C. osculatum* E, documented in Antarctic hosts (Fig. 4). The *C. ogmorhini* complex includes two sibling species that mainly parasitize otariid pinnipeds: *C. ogmorhini* (*s.s.*), documented in the Austral region, and

C. margolisi from the Boreal area. Other *Contracaecum* species are *C. osculatum baicalensis*, molecularly differentiated from the *C. osculatum* complex and endemic to the freshwater Lake Baikal (Russia), *C. radiatum*, documented in Antarctic waters, and *C. mirounga*, registered in Antarctic and sub-Antarctic areas [9].

Clustering methods based on allozyme markers showed that the *Phocanema* species, *P. phocae* and *P. cystophorae* (Fig. 4), despite morphological differences with *Contracaecum* species, form a clade with the *Contracaecum* species parasitizing seals, suggesting an evolutionary hypothesis for the systematic status of these species [9].

2.2. Family Raphidascarididae

The family Raphidascarididae includes numerous genera (~13) and their species are distributed worldwide, as are their definitive hosts, which constitute a wide range of marine and freshwater fish species. *Hysterothylacium*, *Raphidascaroides* and *Raphidascaris* are the genera comprising most species, *Hysterothylacium* being the most prevalent in many marine ecosystems [8,17,38].

Genus *Hysterothylacium*

The genus *Hysterothylacium*, currently consisting of ~67 species, is considered one of the largest of the fish-parasitising ascaridoid genera, with worldwide distribution [33,39]. *Hysterothylacium* species have been documented in an extensive range of marine and freshwater fish, which act as paratenic or definitive hosts [17].

Among the five most widely distributed species, *H. aduncum* has been detected in many geographical areas, including the Mediterranean Sea, North-East Atlantic, North-East Pacific and the Yellow Sea, as well as Antarctic waters and New Zealand coasts. *H. corrugatum* has been recorded along North American Atlantic coasts and also the coasts of Ecuador. *H. cornutum* has been reported in the Adriatic Sea as well as the North Atlantic and Pacific Oceans. *H. fortalezae* is found in the Mediterranean Sea, the Brazilian Atlantic coasts and the Gulf of Mexico. *H. reliquens* has been registered in Brazil, Canada and Central America Atlantic coasts, Colombian Pacific coasts and the Persian Gulf. Finally, *H. zenis*h has been detected from the East and South China Sea to the Java Sea, the North-East Australian shelf and Namibia coasts [40].

The genetic study of *Hysterothylacium* species is still ongoing and their taxonomical status is not clear. Martín-Sanchez et al. [41] suggest *H. fabri*, frequently detected in the Mediterranean Sea, is a complex of three sibling species. As more work is carried out analysing the possible existence of sibling species, the distribution of identified species may change.

3. *Anisakis* spp.

3.1. Morphological and molecular specific identification

To date, nine species belonging to the genus *Anisakis* have been identified worldwide [35]. The need to correctly identify *Anisakis* species is especially important at the larval level because they are the causative agents of anisakidosis, mainly *A. simplex* (*s.s.*) and *A. pegreffii*. Morphological taxonomy of *Anisakis* species has traditionally relied on adult specimens, but in the absence of these forms third stage larvae can be distinguished in the morphological types I and II, following the criteria of Berland [31], which is based mainly on the length of the ventricle and the presence/absence of a spine or mucron at the caudal end. *Anisakis* type I, characterized by a long ventricle and the presence of a mucron, includes the *A. simplex* (*s.l.*) complex, with an oblique ventricle-intestine union, and the species *A. ziphidarum*, *A. nascettii* and *A. typica*, with a blunt ventricle-intestine union (Table 2). Species included in type II are *A. physeteris*, *A. brevispiculata* and *A. paggiae*, whose larvae lack a mucron and have a short ventricle; they also tend to be bigger than species of type I.

In many cases these morphological differences are insufficient for species identification, and molecular approaches are needed. Discriminatory morphometric analysis of the main morphological characters of larvae of non-differentiable species of the *A. simplex* complex, *A. simplex* (*s.s.*) and *A. pegreffii*, has been suggested as a possible method of species differentiation [42]. Ventricle length and the oesophagus/ventricle length ratio have been proposed as discriminating parameters in both L3 and L4, after measuring the total body length, the maximum body width, the distance of the nerve ring from the anterior end, the length of the oesophagus, the ventricle length and width, the ratio between the oesophagus and ventricle length, the tail length and the mucron. More morphometric studies of the two sibling species larvae from different geographical areas are required to find more discriminatory functions of morphological parameters.

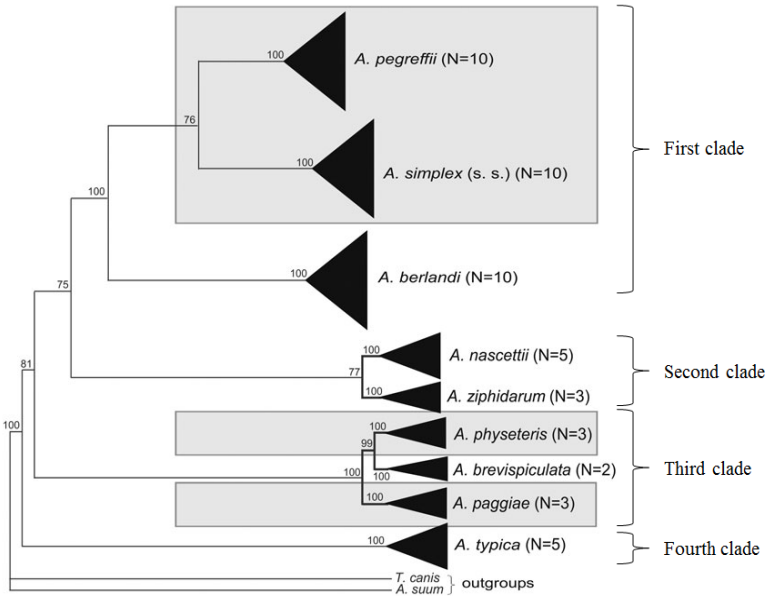

Figure 5. Phylogenetic clades based on the combined mtDNA cox-2, rrnS rRNA and ITS rDNA from sequence data of all characterized species of the genus *Anisakis* (modified from [2]).

In the specific genetic characterisation of *Anisakis* species several molecular methods have been used, principally allozyme markers, sequence analysis of mtDNA *cox*2 and *rrnS*, and direct sequencing of nuclear DNA such as EF1 α-1, ITS rDNA and PCR-RFLP. Four different phylogenetic clades comprising different *Anisakis* species have been detected by these methods [2] (Fig. 5). The first and the second clades include two groups of sibling species: *A. simplex* (s.s.), *A. pegreffii* and *A. berlandi* (= *A. simplex* sp. C); and *A. ziphidarum* and *A. nascettii*, respectively. The third clade is formed by the species *A. physeteris*, *A. brevispiculata* and *A. paggiae*; and the last clade, as a separate lineage, includes *A. typica* [2].

The phylogenetic classification of *Anisakis* species shows that the six species with larvae morphologically characterized as type I are distributed in the first, second and fourth clades, whereas the three species whose larvae belong to type II are all in the third clade (Table 2).

Table 2. Morphological differences of L3 of *Anisakis* species, related to larval type and cladistic classification.

Species	**Main larval morphological differences**	**Larval type** (Berland, 1961**)** [31]	**Cladistics** (Mattiucci *et al.* 2017) [2]
A. simplex (*s.s.*)* *A. pegreffii** *A. berlandi**	Presence of mucron, long ventricle. Oblique ventricle-intestine union	I	First clade
*A. ziphidarum*** *A. nascettii***	Presence of mucron, long ventricle. Blunt ventricle-intestine union	I	Second clade
A. typica	Presence of mucron, long ventricle. Blunt ventricle-intestine union	I	Fourth clade
A. physeteris *A. brevispiculata* *A. paggiae*	Absence of mucron, short ventricle	II	Third clade

***Sibling species of the complex *A. simplex (sensu lato);* ****sibling species**

3.2. Presence of *Anisakis* species in vertebrate hosts from the North-East Atlantic Ocean and Mediterranean Sea

Regarding fish consumption and anisakidosis risk in the Iberian Peninsula, two marine geographical areas are of interest, the North-East Atlantic Ocean, corresponding to FAO (Food and Agriculture Organization) zones 27.8 and 27.9, and the Mediterranean Sea, corresponding to FAO zone 37. Focusing on the *Anisakis* species distribution in these two maritime zones, *A. simplex* (*s.s.*) and *A. pegreffii* are the most detected species, and also the most associated with human cases of anisakidosis. *A. simplex* (*s.s.*) is the most documented species in the North-East Atlantic, its southern limit being the Spanish Atlantic coast near Gibraltar and the Alboran Sea, and the northern limit the Arctic Sea. This species has not been detected in the Mediterranean although it has been registered in the Alboran Sea, oceanographically considered part of the Atlantic Ocean. On the other hand, *A. pegreffii* is widely distributed in the Mediterranean Sea and is also present, but with less prevalence, in the North-East Atlantic. *A. pegreffii* shares a southern limit with *A. simplex* (*s.s.*) of the Spanish coasts, whereas its northern limit is the Bay of Biscay, although it has been detected in some migratory fish species from more northern waters [2].

Several cetacean species have been documented as definitive hosts for *A. simplex* (*s.s.*) and *A. pegreffii* (see Table 3). Although both sibling species

Table 3. List of definitive hosts recorded for the species *A. simplex* (*s.s.*) and *A. pegreffii* from the North-East Atlantic and Mediterranean Sea (modified from [2,9]).

Definitive host	*A. simplex* (*s.s.*)	*A. pegreffii*
Cetaceans		
Balenopteridae		
Balaenoptera acutorostrata	NEA	-
Delphinidae		
Delphinus delphis	NEA	M
Globicephala melaena	NEA	NEA, M
Lagenorhynchus albirostris	NEA	-
Stenella coeruleoalba	NEA	M
Tursiops truncatus	-	M
Phocoenidae		
Phocoena phocoena	NEA	-

NEA: North-East Atlantic; M: Mediterranean Sea

can share the same definitive hosts, in the North-East Atlantic *A. pegreffii* has only been documented in one cetacean species, *Globicephala melaena*, while in the Mediterranean it has been reported in other species like *Delphinus delphis* and *Stenella coeruleoalba*, which are also hosts of *A. simplex* (*s.s.*) in the North-East Atlantic [2].

A. simplex (*s.s.*) and *A. pegreffii* share and even co-infect a wide range of teleost fish species of several families, which act as paratenic hosts (see Table 4). Some of these species are habitually consumed fish such as hake (*Merlucius merlucius*), horse mackerel (*Trachurus trachurus*), blue whiting (*Micromesistius poutassou*), cod (*Gadus morhua*), anchovy (*Engraulis encrasicolus*), Atlantic mackerel (*Scomber scombrus*) and squid (e.g. *Todarodes sagittatus*) [2]. *A. simplex* (*s.s.*) has also been recorded in three squid species of the family Ommastrephidae [2].

In sympatric areas where the sibling species *A. simplex* (*s.s.*) and *A. pegreffii* share cetacean and fish hosts, hybrid specimens between these species have been reported [43,44,45,46]. However, the large recovery of larval hybrid forms in fish and the rare observation of hybrid adults in marine mammals has induced controversy in the taxonomical interpretation of these hybrids, becoming an important unresolved issue in *Anisakis* taxonomy [36,47,48].

Table 4. List of paratenic/fish hosts recorded for the species *A. simplex* (*s.s.*) and *A. pegreffii* from the North-East Atlantic and Mediterranean Sea [2,9].

Fish species	*A.simplex* (*s.s.*)	*A. pegreffii*	Fish species	*A.simplex* (*s.s.*)	*A. pegreffii*
Belonidae			**Muraenidae**		
Belone belone	NEA	NEA	*Muraena helena*	-	M
Bothidae			**Phycidae**		
Arnoglossus laterna	NEA	-	*Phycis phycis*	-	M
Arnoglossus imperialis	-	NEA	**Pleuronectidae**		
Carangidae			*Pleuronectes platessa*	NEA	-
Trachurus trachurus	NEA	NEA, M	**Schophtalmidae**		
Citharidae			*Lepidorhombus boscii*	NEA	NEA, M
Citharus linguatula	NEA	NEA	**Scombridae**		
Clupeidae			*Scomber scombrus*	NEA	NEA, M
Alosa alosa	NEA	-	*Thunnus thynnus*	-	M
Clupea harengus	NEA	-	**Scorpaenidae**		
Congridae			*Scorpena scrofa*	NEA	NEA
Conger conger	NEA	M	**Sebastidae**		
Engraulidae			*Helicolenus dactylopterus*	-	M
Engraulis encrasicolus	-	M	**Soleidae**		
Gadidae			*Dicologoglossa cuneata*	-	NEA
Boreogadus saida	NEA	-	*Solea senegalensis*	NEA	-
Gadus morhua	NEA	-	**Sparidae**		
Melanogrammus aeglefinus	NEA	-	*Spondyliosoma cantharus*	NEA	-
Micromesistius poutassou	NEA	NEA, M	**Sternoptychidae**		
Pollachius virens	NEA	-	*Maurolicus muelleri*	NEA	-
Trisopterus luscus	NEA	-	**Trachichthyidae**		
Hexagrammidae			*Haplostethus mediterraneus*	-	M
Pleurogrammus azonus	NEA	-	**Trachinidae**		
Lophiidae			*Echiichthys vipera*	-	M
Lophius piscatorius	NEA	M	**Trichiuridae**		
Lotidae			*Lepidopus caudatus*	-	M
Brosme brosme	NEA	-	*Trichiurus lepturus*	-	M
Molva dypterygia	NEA	-	**Triglidae**		
Merluccidae			*Eutrigla gurnardus*	NEA	-
Dicentrarchus labrax	NEA	-	**Xiphiidae**		
Merluccius merluccius	NEA	NEA, M	*Xiphias gladius*	NEA	M

NEA: North-East Atlantic; M: Mediterranean Sea

Regarding other *Anisakis* species, according to Mattiucci's review, three species have been detected in the North-East Atlantic and the Mediterranean [2,9]. *A. physeteris* has been documented in the North-East Atlantic from the sperm whale *Physeter macrocephalus* (Physeteridae) and in the Mediterranean Sea from *Physeter catodon. A. typica* has been registered in the Mediterranean delphinid *Stenella coeruleoalba*, and *A. paggiae*, although not recorded in the North-East Atlantic, has been associated with Kogiid whales (*Kogia breviceps* and *K. sima*) from this area, due to the presence of larvae in the deep-sea fish *Anoplogaster cornuta*, which supports an oceanic deep-water life cycle for this species [49]. These three *Anisakis* species have also been detected in different paratenic/fish hosts from the same zones: *A. physeteris* in *Trachurus trachurus*, *Merlucius merlucius*, *Phycis phycis*, *Physcis blenoides*, *Scomber scombrus* and *Xiphias gladius*; *A. typica* in *Trachurus trachurus*, *Merlucius merlucius*, *Phycis phycis* and *Scomber scombrus*; and *A. paggiae* in *Merlucius merlucius* [2,9].

4. *Hysterothylacium* spp.

4.1. Morphological and molecular specific identification

Hysterothylacium species are potential zoonotic parasites and are the most common species of Raphidascarididae, having been reported in a wide range of fish [13,50]. The study of adult worms in their fish final hosts is essential for a correct specific identity, but is not always available.

Morphological larval type description is based on the main morphological parameters: the presence/absence of a tooth for L3 or labia morphology for L4, the position of the excretory pore, the ventricular appendix, the intestinal caecum and the morphology of the tail, with the presence/absence of a mucron or a cluster of spines (also called a cactus) as shown in Fig. 6. Morphometric analysis of these parameters is also important for the larval classification [33].

The attempt to characterize and classify these larvae has been extensive in marine teleost fish from the South Pacific (Australia and New Caledonia) and the Persian Gulf. Up to sixteen different larval morphotypes have been described in these areas, most of them with both a morphological and molecular characterization [33,51,52]. Shamsi et al. [33] proposed a key to differentiate the several morphotypes present in Australian waters. This key needs to be extended to include the new morphotypes described in other regions.

Each larval morphotype cannot be associated with a single species because sometimes the same morphotype presents different genotypes [33],

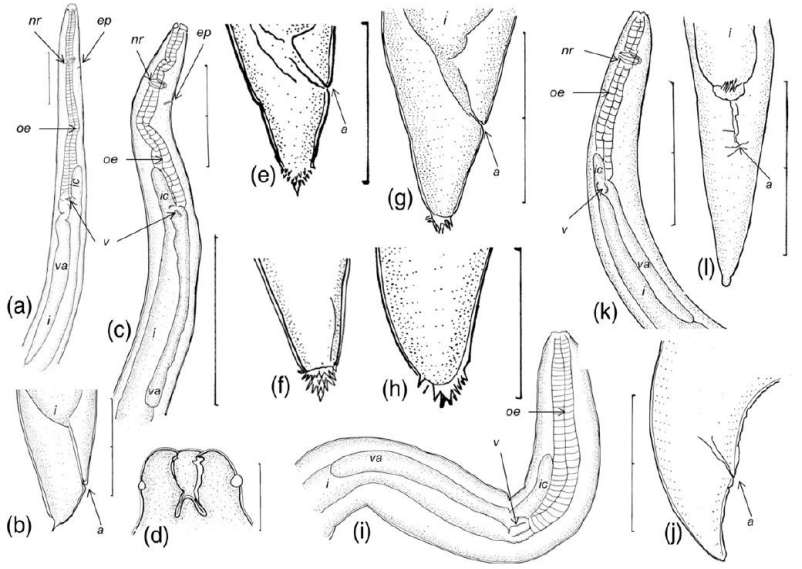

Figure 6. *Hysterothylacium* morphotypes. Larval type III: a) and b) anterior and posterior ends, respectively (scale-bars=0.4 and 0.2 mm, respectively). Larval type IV: c) anterior end (scale-bar=0.4 mm), d) labia (scale-bar=0.3 mm) and e–h) posterior ends (scale-bar=0.2 mm in e and f and 0.1 mm in g and h). Larval type V: i) and j) anterior and posterior ends (scale-bars=0.2 mm). Larval type VI: k) and l) anterior and posterior ends (scale-bars=0.4 and 0.2 mm, respectively), excretory pore was not visible in this specimen (modified from [33]).

meaning that different species can have similar larval morphology. Moreover, larvae can exhibit rather uniform morphology, which is completely different from their adult forms [18]. A comparison between larval morphology and genetics is needed to specifically identify larval morphotypes, the sequencing of ITS-1 and ITS-2 of rDNA after PCR amplification of these regions being the most used molecular method for this purpose [18,33].

Studies on *Hysterothylacium* morphotypes from fishes in different European marine waters are scarce. In this area *Hysterothylacium* larvae are usually identified based solely on morphological parameters and very few studies compare the larval morphology with a proper molecular analysis [38,53]. Therefore, more studies are needed to ascertain the possible morphotypes present in European marine waters.

4.2. Presence of *Hysterothylacium* species in vertebrate hosts from the North-East Atlantic Ocean and Mediterranean Sea

Within *Hysterothylacium* species in Mediterranean and North-East Atlantic regions, *H. aduncum* is the most frequently reported in a wide range of teleost fish [22,54]. However, *H. fabri* is typically reported in many Mediterranean fish species, sometimes with a higher prevalence than *H. aduncum* [38,41,55]. As mentioned in section 2.2, while *H. aduncum* has been detected worldwide, for example, in the North-East Pacific and the Yellow Sea as well as Antarctica and New Zealand waters, *H. fabri* has only been documented in the South and East China Sea [40].

H. aduncum and *H. fabri* specimens from the Mediterranean and the North-East Atlantic have been mostly detected in their larval forms (see Table 5) and very few studies have documented their adult form in final fish hosts in these regions. Sanmartin-Duran et al. [56] detected adult specimens of *H. aduncum* in *Scophthalmus maximus* and *Conger conger*, while Mackenzie et al. [54] and Carreras-Aubets et al. [57] reported the adult form in *Trachurus trachurus* and *Mullus barbatus*, respectively. Adult forms of *H. fabri* have been documented [58] in *Mullus surmulentus*.

Other *Hysterothylacium* species, including *H. corrugatum*, *H. incurvum* and *H. petteri*, have been recorded in swordfish (*Xiphias gladius*) from the Mediterranean Ionic and Tyrrhenian Sea, and the North-East Atlantic Ocean [35]. Moreover, some authors have also found *H. auctum* in the Baltic Sea [68], and Gibson [69] lists 13 different *Hysterothylacium* species in European marine waters, including *H. aduncum* and *H. fabri* but without specifying the region. Regarding the Mediterranean Sea, Bruce et al. [39] detected *H. fortalezae*, without specifying the region, *H. cornutum* and *H. increscens* in the Adriatic Sea, *H. bifidalatum* in the Algerian part of the Mediterranean and *H. rhacodes* in the East Mediterranean.

5. Conclusion

The present review highlights the importance of improving taxonomic descriptions of "anisakid-related" nematode species. Accurate species identification and knowledge of their geographical distribution would shed light on the epidemiological, biological and ecological patterns of these parasites, which are of sanitary and commercial concern. Among Anisakidae, *Anisakis* spp. are the main causative agents of anisakidosis and the most widely detected in cetacean definitive hosts worldwide, while *Pseudoterranova* and *Contracaecum* species have a more reduced distribution, mainly in the most northern and southern areas of the planet, pinnipeds being their main definitive hosts.

Table 5. List of intermediate fish hosts recorded for the species *H. aduncum* and *H. fabri* from the North-East Atlantic and Mediterranean Sea [55,56,58,59,60,61,62,63,64,65,66,67].

Fish species	*H. aduncum*	*H. fabri*
Carangidae		
Trachurus mediterraneus	M	M
Trachurus trachurus	NEA, M	-
Clupeidae		
Alosa alosa	NEA	-
Alosa fallax	NEA	-
Sardina pilchardus	M	-
Congridae		
Conger conger	NEA	-
Engraulidae		
Engraulis encrasicolus	NEA, M	-
Gadidae		
Micromesistius poutassou	NEA, M	-
Labridae		
Symphodus tinca	-	M
Merluccidae		
Merluccius merluccius	M	M
Mullidae		
Mullus barbatus	M	M
Mullus surmulentus	M	M
Phycidae		
Phycis phycis	M	M
Phycis blennoides	M	M
Pomatomidae		
Pomatomus saltatrix	M	-
Schophtalmidae		
Lepidorhombus whiffiagonis	NEA	-
Lepidorhombus boscii	NEA	-
Scophthalmus maximus	NEA	-
Serranidae		
Serranus scriba	-	M
Scorpaenidae		
Scorpaena porcus	-	M
Scorpaena scrofa	-	M
Soleidae		
Microchirus variegatus	NEA	-
Solea solea	M	-
Sparidae		
Sparus aurata	M	-
Diplodus vulgaris	M	-
Pagellus erythrinus	M	M
Boops boops	M	-
Triglidae		
Trigla lucerna	NEA	-

NEA: North-East Atlantic; M: Mediterranean Sea

Classification of the genus *Hysterothylacium* at the family level remains controversial, and its inclusion in the family Raphidascarididae is not unanimously accepted. In their larval stages, *A. simplex* (*s.l.*) and *H. aduncum* are the most frequently detected species in a wide range of commonly consumed fish from European and Spanish marine waters, including the North-East Atlantic and Mediterranean. Specific identification of these nematodes at larval stages, combining morphological and molecular methods, is crucial from an epidemiological point of view, due to the existence of morphologically non-differentiable sibling species, such as *A. simplex* (*s.s.*) and *A. pegreffii*, both of sanitary importance. The detection of hybrids of these two species needs to be followed up by genetic characterization studies to ascertain if they are viable hybrids giving rise to hybrid adults. Although molecular methods are effective in many cases, morphological knowledge of larvae and adults is still important for correct identification. It is therefore necessary to undertake studies on *Hysterothylacium* morphotypes in fish from marine European waters for which data remain quite scarce.

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