Molecular Characterization of Clistobothrium sp. Viable Plerocercoids in Fresh Longfin Inshore Squid (Doryteuthis pealeii) and Implications for Cephalopod Inspection

Lisa Guardone 1,*, Alice Giusti 1, Ewa Bilska-Zajac 2, Renato Malandra 3, Miroslaw Różycki 2 and Andrea Armani 1

1 Department of Veterinary Sciences, University of Pisa, 56124 Pisa, Italy; alice.giusti183@outlook.it (A.G.); andrea.armani@unipi.it (A.A.)
2 Department of Parasitology and Invasive Diseases, National Veterinary Research Institute, 24-100 Pula, Poland; ewa.bilska@piwet.pula.pl (E.B.-Z.); mrozycki@piwet.pula.pl (M.R.)
3 Wholesale Fish Market of Milan, ASL of Milan, 20137 Milan, Italy; RMalandra@ats-milano.it

* Correspondence: lisa.guardone@for.unipi.it; Tel.: +39-050-2210206

Received: 23 June 2020; Accepted: 17 July 2020; Published: 21 July 2020

Abstract: Cephalopods, an appreciated seafood product, are common hosts of marine cestodes. The aim of this work is to report visible alive plerocercoids in longfin inshore squid (Doryteuthis pealeii), a cephalopod species commercialized as fresh and whole in Italy. Seventy D. pealeii from the Northwest Atlantic (FAO area 21) were collected and visually inspected. In total, 18 plerocercoid larvae were found in the viscera of 10 host specimens (P: 14.3% 95% CI 7.1–24.7; MI: 1.8, MA: 0.26; range 1–4) and molecularly analyzed targeting the variable D2 region of the large subunit (LSU) rRNA gene and the cytochrome c oxidase subunit I (COI) gene. The molecular characterization allowed to identify all the plerocercoids as Clistobothrium sp., a cestode of the Phyllobothriidae family with Lamnidae sharks as definitive hosts, and cephalopods as second intermediate hosts. These findings represent the first molecular record of Clistobothrium sp. in D. pealeii, thus contributing to elucidate its poorly known life cycle. Even if not affecting consumer’s health, these visible parasites may represent a reason for disgust for consumers. Therefore, the results suggest that Food Business Operators should also check for the presence of these visible parasites during inspection and underline the importance of a correct consumers’ education.

Keywords: cestode; helminths; cephalopods; food hygiene; defect; visual inspection

1. Introduction

Over two-hundred different parasitic species of a variety of taxa are reported in the literature for cephalopods [1,2], mainly as larval and post-larval stages [3]. Although the interest of the scientific community on parasites associated with cephalopods is growing [4], the knowledge on specific aspects, including some parasites’ life cycle and transmission pathway, is still scarce [2].

The cephalopod class includes three worldwide appreciated commercial categories: squid (Myopsida and Oegopsida taxa), octopus (Octopoda) and cuttlefish (Sepiida). Their nutrient composition and the continuously growing worldwide popularity of raw seafood has prompted their demand increase [5]. Spain, Italy and Japan are the main consumers and importers of this kind of seafood [6]. Squid and octopus are particularly requested [5] and their tight supplies [7] are causing a strong rise in trade prices [6,7]. Therefore, products should comply with high-quality and hygienic standards to meet consumers’ requests and expectations. Therefore, Food Business Operators (FBOs)
have to perform regular checks to avoid the commercialization of seafood obviously contaminated by visible parasites that are unfit for human consumption [8–10].

Species of squid, cuttlefish and octopus may act as intermediate or paratenic hosts in the life cycle of cestodes that mature in elasmobranchs and are transferred from host to host through the food chain [2,3]. According to the available literature, different species of cestodes, mainly belonging to the orders Phyllobothriidea, Tetraphyllidea and Trypanorhyncha, were detected as larval stage in almost all the commercial cephalopod species [1] (Table 1). These cestodes represent visible parasites—“a parasite or a group of parasites which has dimension, colour or texture which is clearly distinguishable from fish tissues and can be seen without optical means of magnifying and under good light conditions for human” according to the definition given by [11]. Visible parasites can represent a hazard or a defect depending on their potential zoonotic role and thus require the implementation of specific management measures along the supply chain to reduce their impact on consumer’s health and satisfaction [12,13]. Cephalopod species reaching the market as whole and fresh, in which parasites can be found viable, may be particularly affected, considering that cestodes are commonly found in digestive tracts, buccal mass, mesentery and mantle cavity [1].

During a larger survey on parasitic nematodes in selected species of fresh whole cephalopods [14], visible alive plerocercoids were visually detected in the viscera of some specimens of longfin inshore squid (Doryteuthis pealeii). The present study represents the first molecular record of Clistobothrium sp. larvae in D. pealeii, based on the analysis of DNA fragments from the variable D2 region of the large subunit ( LSU) rRNA gene and from the cytochrome c oxidase subunit I (COI) gene. In addition to update some aspects of the epidemiology of the detected cestode, this work wants to discuss the impact on cephalopods’ quality, considering that D. pealeii is among the cephalopod species most commercialized as fresh and whole on the Italian market.
| Ref. | Samp. Period | Geographical Area | Cephalopod Common Name (Scientific Name), n of Examined Specimens | Species (Family, Order) | Localization | P (%) | Parasite ID |
|------|--------------|-------------------|---------------------------------------------------------------|-------------------------|----------------|-------|-------------|
| Guillén-Hernández et al., (2018) [4] | August, 2009–June, 2010 | Yucatán Peninsula, Mexico (FAO 31) | Mexican four-eyed octopus (Octopus maya), 1202 | Prochristianella sp. (Eutetrarhynchidae, Trypanorhynca) | buccal mass, oesophagus, cecum, intestine | 57.0–98.0 | Morph. |
| | | | | Eutetrarhynchus sp. (Eutetrarhynchidae, Trypanorhynca) | digestive gland, esophagus, intestine, ink sac | 7.0–59.1 | |
| | | | | Nybelinia sp. (Tentaculariidae, Trypanorhynca) | buccal mass, esophagus, intestine | 0.4–51.2 | |
| | | | | Echeneibothrium sp. (Echnebosthridae, Rhinebothriidea) | cecum, intestine | 4.0–21.0 | |
| | | | | Pseudothorium sp. (Prosobothriidea, Onchoproteocephalidea) | digestive gland, ink sac | 16.8–27.0 | |
| | | | | Tetraphyllidea | cecum, intestine | 1.0–7.0 | |
| | | | | Unidentified plerocercoid | digestive gland, ink sac, gills | 10–26.6 | |
| Cavaleiro, (2013) [15] | 2010 | Matosinhos, Portugal, NE Atlantic (FAO 27) | common octopus (Octopus vulgaris), 120 | Nybelinia sp. (Tentaculariidae, Trypanorhynca) | stomach, intestine | 4.2 | Morph. |
| Petrić et al., (2011) [16] | October, 2007–October, 2008 | Central Adriatic Sea (FAO 37.2.1) | shortfin squid (Illex coindetti), 439 | Phyllobothrium sp. (Phyllobothriidae, Phyllobothriidea) | stomach | 2.3 | Morph. |
| Pardo-Gandarillas et al., (2009) [17] | July, 2003–February, 2004 | Central-Southern Chile (FAO 87) | jumbo flying squid (Dosidicus gigas), 124 | Hepatoxylon trichiuri (Sphyriocephalidae, Trypanorhynca) | mantle cavity, gonads, stomach | 70.2 | Morph. |
| | | | | Tentacularia corphacaeae (Tentaculariidae, Trypanorhynca) | mantle cavity, gonads | 5.6 | |
| | | | | Plerocercoid larvae (Tetraphyllidea) | stomach, cecum and intestine | 83.1 | |
| | | | | Pelichnibothrium speciosum (Phyllobothriidae, Phyllobothriidea) | intestine | NR | |
| Nigmatullin et al., (2009) [18] | 1981–1984 | south part of the eastern Pacific (FAO 87) | neon flying squid (Ommastrephes bartramii), 60 | Tentacularia corphacaeae (Tentaculariidae, Trypanorhynca) | whole mantle | 9.1 | Morph. |
| | | | | Scyphophyllidium sp. (Phyllobothriidae, Phyllobothriidea) | stomach cavity and cecum | 4.5 | |
| Ref. | Samp. Period | Geographical Area | Cephalopod Common Name (Scientific Name), n of Examined Specimens | Species (Family, Order) | Localization | P (%) | Parasite ID |
|------|--------------|-------------------|---------------------------------------------------------------|-------------------------|--------------|-------|-------------|
| Brickle et al., (2001) [3] | February, 1999–June, 2000 | Falkland Islands (South Atlantic Ocean) (FAO 41.3.2) | longfin Patagonian squid (Doryteuthis gahi), 1096 | Cistobothrium montaukensis (Phyllobothriidae, Phyllobothriidea) | ceicum, intestine, stomach, mantle | 5.75 | Molec. |
| Shukhgalter and Nigmatullin, (2001) [19] | 1981–1989 | East Pacific Ocean (FAO 77 and 87) | jumbo squid (Dosidicus gigas), 849 | Pelichnibothrium speciosum (Phyllobothriidae, Phyllobothriidea) | rectum, cecum, stomach | 75.2 | Morph. |
| Gonzalez and Kroeck, (2000) [20] | July–November, 1993 | South West Atlantic St. Mattias gulf (FAO 41.3.1) | Argentine short-fin squid (Illex argentinus), 91 | Prosobothrium sp. (Prosobothriidea, Onchoproteocephalidea) | viscera | 100.0 | Morph. |
| Gestal et al., (1998) [21] | December, 1994–December, 1995 | Galician coast, Spain (FAO area 27.9) | Common octopus (Octopus vulgaris), 100 | Phyllobothrium sp. (Phyllobothriidea, Phyllobothriidea) | Intestine, cecum | 3.0 | Morph. |
| Pascual et al., (1996) [22] | 1992–1995 | coast of Galicia, Spain (FAO 27.9) | broadtailed short-fin squid (Illex conedtii), 600 | Phyllobothrium sp. (Phyllobothriidea, Phyllobothriidea) | NR | 48.0 | |
| | | | | Pelichnibothrium speciosum (Phyllobothriidae, Phyllobothriidea) | | 0.3 | |
| | | | | Dinobothrium sp. (Gastrolecithidae, Tetraphyllidea) | | 1.0 | |
| | | | | Nybelinia yamagutii (Tentaculariidae, Trypanorhynca) | | 0.7 | |
| | | | | European squid (Loligo vulgaris), 8 | | 62.5 | |
| | | | | European flying squid (Todarodes sagittatus), 65 | | 20.0 | |
| | | | | European flying squid (Todarodes sagittatus) | | | |
| | | | | European flying squid (Todarodes sagittatus) | | | |
| | | | | European flying squid (Todarodes sagittatus) | | | |
| | | | | European flying squid (Todarodes sagittatus) | | | |
| | | | | European flying squid (Todarodes sagittatus) | | | |
| | | | | European flying squid (Todarodes sagittatus) | | | |
Table 1. Cont.

| Ref. | Samp. Period | Geographical Area          | Cephalopod Common Name (Scientific Name), n of Examined Specimens | Species (Family, Order) | Localization | P (%) | Parasite ID |
|------|--------------|----------------------------|-----------------------------------------------------------------|-------------------------|--------------|-------|-------------|
| Pascual et al., (1996) [22] | 1992–1995 | coast of Galicia, Spain (FAO 27.9) | lesser flying squid *(Todaropsis eblanae)*, 600 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | NR | 31.2 | Morph. |
| | | | common cuttlefish *(Sepia officinalis)*, 38 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | | 2.6 | | |
| | | | pink cuttlefish *(Sepia orbigniana)*, 22 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | | 9.0 | | |
| | | | common octopus *(Octopus vulgaris)*, 70 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | | 4.3 | | |
| | | | lesser octopus *(Eledone cirrhosa)*, 67 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | | 10.4 | | |
| Pascual et al., (1994) [23] | October, 1991–April, 1992 | North Galician Shelf waters (FAO 27.8) | broadtailed short-fin squid *(Illex caprini)*, 70 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | cecum, stomach | 87 | Morph. |
| | | | | *Dinobothrium septaria* (Gastrolecithidae, Tetraphyllidea) | | | | |
| Bower and Margolis (1991) [24] | Summer 1987 | West coast of North America (FAO 21) | flying squid *(Ommastrephes bartramii)*, 68 | *Phyllobothrium* sp. (Phyllobothriidae, Phyllobothriidea) | esophagus, stomach, cecum, intestine, rectum, gills, gonads | 94.1 | Morph. |
| | | | | *Rhadinorhynchus* sp. (Rhadinorhynchidae, Echinorhynchida) | | | | |

Ref: reference; Samp. period: Sampling period; P (%): prevalence expressed as percentage.
2. Results and discussion

2.1. Morphological Identification

In this work, a total of 18 alive plerocercoids were found in the viscera of 10 specimens of longfin inshore squid (P: 14.3%, 95% CI 7.1–24.7; MI: 1.8, MA: 0.26; range 1–4). Overall, D. pealeii specimens had a mean weight of 106.5 g (standard deviation, sd 29.1), a mean total length of 41.2 cm (sd 5.4) and a mean dorsal mantle length of 16.8 (sd 3.5). Details of the size of the positive squids are given in Table 2. A positive correlation was observed between the total weight and the number of parasites per host ($r_s = 0.54$, $p$ (2-tailed) = 0.002), while no statistically significant correlation was found for the dorsal mantle length and the weight. Plerocercoids were 1.5–3.5 cm long, whitish and actively mobile (Figure 1, Video S1). Under optical microscopy the larvae presented an unarmed evaginated scolex, attached to a fusiform larval body, with an apical sucker surrounded by four large bothridia with folded margins, each showing a rounded accessory sucker (Figure 2). The observed characteristics allowed to identify the parasites as a “tetraphyllidean” (or phyllobothridean according to [25]) plerocercoid larvae. This kind of larvae, which should be referred to as Type XV [26], have historically been defined as *Phyllobothrium delphini*, but molecular sequence data have suggested that they may actually belong to *Clistobothrium* sp. [26]. Analogous larval types had been described in squids [27], teleosts [28] and in deepwater sharks [29]. However, considering the morphological uniformity of cestode larvae [3] and the renowned difficulty to reliably identify them [26,30,31], a morphological identification at species level was not achieved, and parasites were submitted to molecular analysis.

| Host Sample Code | Total Weight (g) | Total Length (cm) | Mantle Lengths (cm) | Viscera Weight (g) | Mantle Weight (g) | N Plerocercoid Larvae |
|------------------|------------------|-------------------|---------------------|-------------------|------------------|----------------------|
| D. PEA-23        | 117              | 57                | 22                  | 8                 | 109              | 1                    |
| D. PEA-30        | 87               | 36                | 12                  | 6                 | 45               | 1                    |
| D. PEA-33        | 141              | 41                | 12                  | 6                 | 92               | 2                    |
| D. PEA-34        | 136              | 44                | 12                  | 20                | 70               | 1                    |
| D. PEA-35        | 153              | 52                | 23                  | 26                | 94               | 1                    |
| D. PEA-45        | 175              | 43                | 15                  | 15                | 79               | 2                    |
| D. PEA-58        | 119              | 36                | 24                  | 6                 | 73               | 3                    |
| D. PEA-63        | 163              | 48                | 24                  | 17                | 97               | 1                    |
| D. PEA-65        | 152              | 46                | 23                  | 13                | 89               | 4                    |
| D. PEA-67        | 136              | 44                | 22                  | 13                | 83               | 2                    |
| Overall          | 137.9 (25.5)     | 44.7 (6.5)        | 18.9 (5.4)          | 14.2 (6.4)        | 83.1 (17.8)      | 18                   |

Table 2. Details of the weight and length of the positive longfin inshore squid (*Doryteuthis pealeii*) specimens and the number of larvae found in each specimen.

Figure 1. (a) Macroscopic aspect of the plerocercoid larvae at squid dissection, which were 1.5–3.5 cm long, whitish and actively mobile; (b, c) isolated larvae.
which retrieved 100% identity in the BLAST analysis, as well as with one sequence deposited (ML) phylograms were constructed with the complete dataset created as described in Section 3.4.2. (KT148970), thus not allowing a specific identification. However, a 100% identity value was observed only with 6 sequences deposited as Clistobothrium cf. C. montaukensis, and with one record deposited as Tetraphyllidea sp. (AF382071-72, AF382074, AF382079, AF382081) [3], although with a low interspecies variability has been reported by other authors [35], and other genes, such as COI and ITS, were proposed as alternative markers for distinguishing closely related species of Phyllorchilidae [35,42,43]. Thus, the COI was used as additional target to better assess inter and intra-specific variability. However, reference sequences for this target region are scarce [35], as it can be observed in Table S1, where 427 sequences were retrieved from databases for the LSU, while only 72 sequences were available for the COI gene. A higher taxonomic coverage of LSU in comparison with COI is factually evident (Table S1).

2.2. Molecular Analysis

2.2.1. Molecular Target Selection

The LSU was selected as elective target for the analysis, as it is the most used molecular marker for identifying Cestoda [3,26,32-38], sometimes in combination with the small subunit (SSU) rRNA gene [39-41]. Although Olson et al. [40] indicated that the D2 region of the LSU gene exhibited sufficient variability to be useful for species-level identification, a low interspecies variability has been reported by other authors [35], and other genes, such as COI and ITS, were proposed as alternative markers for distinguishing closely related species of Phyllorchilidae [35,42,43]. Thus, the COI was used as additional target to better assess inter and intra-specific variability. However, reference sequences for this target region are scarce [35], as it can be observed in Table S1, where 427 sequences were retrieved from databases for the LSU, while only 72 sequences were available for the COI gene. A higher taxonomic coverage of LSU in comparison with COI is factually evident (Table S1).

2.2.2. Large Subunit (LSU) rRNA Gene Analysis

LSU sequences were obtained from all the eighteen plerocercoids found. The BLAST analysis showed high similarity (> 99%) with sequences deposited as Clistobothrium cf. montaukensis, Clistobothrium sp., C. montaukensis, Pelichnibothrium speciosum, and with one record deposited as Tetratophyllidea sp. (KT148970), thus not allowing a specific identification. However, a 100% identity value was observed only with 6 sequences deposited as Clistobothrium cf. montaukensis, one deriving from an adult specimen isolated from Lamna nasus (JF436969) [36] and the remaining from plerocercoids found in the Patagonian squid Doryteuthis gahi (AF382071-72, AF382074, AF382079, AF382081) [3], although with a low query coverage (81–91%). Initially, both the LSU Neighbor Joining (NJ) and Maximum Likelihood (ML) phylograms were constructed with the complete dataset created as described in Section 3.4.2. Then, redundant sequences were removed, except for the genus Clistobothrium, for which all the available sequences have been included. Until recently, only three species were reported in the genus Clistobothrium: C. montaukensis, C. carcharodoni and C. tumidum [44]. However, Caira et al. [34] have very recently described two new species (C. amyae and C. gabywalterorum) and suggested an expansion of the total number to six, including the undescribed species C. cf. montaukensis reported by Brickle et al. [3] and Randhawa and Brickle [36].

In both NJ and ML phylograms (Figure 3, only ML shown), the sequences from the plerocercoid larvae produced in this study clustered with the sequences deposited as C. cf. montaukensis, which retrieved 100% identity in the BLAST analysis, as well as with one sequence deposited as Clistobothrium sp. obtained from a cestode larva found in the oarfish Regalecus russelli [45] with

![Figure 2. (a) Microscopic aspect of a plerocercoid larvae scolex, showing four large bothridia with folded margins, each with a rounded accessory sucker; (b) the same microscopic aspect of another plerocercoid.](image-url)
were found in seals, and tentatively attributed to *C. tumidum* 

A (n = 2020) Pathogens sequence variation. As observable in Table S1, a higher number of sequences is available for fragment 2.2.3. Cytochrome c Oxidase Subunit I (COI) Gene Analysis

As already reported, the COI gene was used as an additional target to better assess inter-specific sequence variation. As observable in Table S1, a higher number of sequences is available for fragment A (n = 61) respect to fragment B (n = 11). However, fragment B was amplified to allow the comparison  

Figure 3. Maximum likelihood phylogram created with the large subunit (LSU) sequences of the species belonging to the Phyllobothriidae family retrieved from GenBank, together with 5 of those produced in this study. Redundant sequences were removed.

Several records of this genus without species level identification have been reported. Interestingly, Pardo Gandarillas et al. [17] found an unidentified Tetraphyllidea plerocercoid from the jumbo flying squid *Dosidicus gigas* morphologically very similar to the larvae found in this study and stated that the presence of an apical sucker-like structure, accessory sucker on each bothria and the folded and curled bothrial shape resembled *Phyllobothrium tumidum* (former name of *C. tumidum*) described by Stunkard [31]. In the work of Klotz et al. [35], genetically identified *Clistobothrium* sp. merocercoids were found in seals, and tentatively attributed to *C. tumidum* on the basis of bothridial morphology. However, further molecular analysis, ideally investigating also adult specimens, would be needed [35], also considering that the taxonomy of tetraphyllidean and phyllobothridean has undergone major revision [34,38,46]. In fact, the Phyllobothriidae family, which was traditionally included in the Tetraphyllidea order, was recently elevated to ordinal status [25,30].

2.2.3. Cytochrome c Oxidase Subunit I (COI) Gene Analysis

As already reported, the COI gene was used as an additional target to better assess inter-specific sequence variation. As observable in Table S1, a higher number of sequences is available for fragment A (n = 61) respect to fragment B (n = 11). However, fragment B was amplified to allow the comparison...
of our sequences with additional Clistobothrium sp. sequences, considering that most of the 11 fragment B sequences (n = 7) belong to this genus. The BLAST analysis conducted using the fragment A of the COI gene retrieved the highest percentage of identity (85.39–84.32%) with sequences of C. montaukensis, Clistobothrium sp., Paraorygmatobothrium exiguum, P. typicum, P. christopheri and Rhinebothroides scorzai, while the BLAST analysis with the fragment B of the COI gene showed the highest percentage of identity (87–88%) with sequences of C. montaukensis (AN: JQ268541, LC195139, LC195141–43) and also with Pelichnibothrium speciosum (LC195135–38). These results confirm the hypothesis based on the analysis of the LSU gene, also demonstrating that the larvae found in this study are not C. montaukensis. Similarly, no species-specific identification was achieved on BOLD: no match was obtained for both fragments comparing them with the Species Level Barcode Record database, while the comparison with the All Barcode Record database retrieved a highest match of fragment A with C. montaukensis (84.85–85.42%) and with Schyzocotyle nayarensis (82.31–83.08%). The BLAST and BOLD results for the COI gene should be interpreted taking into account the low number of available sequences for Phyllobothriidae and the inter-specific variability. In fact, the results of the pairwise distance analysis on fragment A showed a high inter-specific variability among species of the genus Clistobothrium (16.6–19.3%) (Table S2), and among species of the genus Paraorygmatobothrium (8.5–21.3%), the only genera of the Phyllobothriidae family for which sequences from more than one species were available. Similarly, also the difference between our sequences and C. montaukensis for fragment B was relatively high (13.8–16.7%) (data not shown). A similar inter-specific distance was already observed for the COI gene for Clistobothrium spp. [35] and for Paraorygmatobothrium spp. [38]. Finally, in both the NJ and ML phylogenograms of the fragment A of the COI gene, the sequences produced in this study appeared phylogenetically closer to the clade comprising the sequences from C. montaukensis (JQ268541) and Clistobothrium sp. (KU987913), although they clustered separately with a bootstrap value of 97 (Figure 4, only ML shown). The NJ and ML phylogram of fragment B confirm that our larval specimens belong to the genus Clistobothrium (Figure 5, only ML shown). In general, the low number of sequences and species available for the COI gene does not allow to achieve a species level identification but supports the LSU results.

Figure 4. Maximum likelihood phylogram created with the Cytochrome c Oxidase Subunit I (COI) sequences (fragment A) of the species belonging to the Phyllobothriidae family retrieved from GenBank, together with 5 of those produced in this study. Redundant sequences were removed.
2.3. Viable and Visible Larval Cestodes of Clistobothrium sp: Epidemiology and Implication for Cephalopod Inspection

Tapeworms are ubiquitous residents of the spiral intestine of elasmobranchs, but their life cycles are poorly known. It is generally thought that two or three different intermediate host species are involved before the definitive host infection. Studies are severely hampered by the difficulties associated with identifying cestode larvae [26,30]. Applications of molecular methods have improved the situation, even though the paucity of molecular data for most adult marine tapeworms greatly limits this approach [26,36].

Although the complete life cycle of the species of the genus Clistobothrium is still unclear, the available data support the cycle exhaustively illustrated [35], with sharks as definitive hosts, crustaceans as 1st intermediate hosts of the procercoid larvae, bony fish/cephalopods/sea turtles as 2nd intermediate hosts of plerocercoid larvae and cetacean/pinnipeds as 3rd intermediate hosts of merocercoid larvae. Reliable host reports indicate that species of Clistobothrium are restricted to sharks of the Lamnidae family [44] as definitive hosts. In fact, the adult form of C. carcharodoni was described by [47] in the spiral intestine of the great white shark (Carcharodon carcharias). The great white shark was also identified as definitive host of C. tumidum, originally described as Phyllobothrium tumidum, and transferred to the genus Clistobothrium by [44]. The same author also described for the first time the species C. montaukensis from the spiral intestine of the shortfin mako shark (Isurus oxyrinchus) [44]. Subsequent studies confirmed the occurrence of C. montaukensis in shortfin mako [48] and of C. carcharodoni in the great white shark [37], while, as mentioned, a specific identification could not be achieved for the specimens identified as C. cf. montaukensis found in the porbeagle Lamna nasus [36]. A survey of deeper water sharks from the Azores found tetraphyllidean larval morphologically attributed to Clistobothrium sp. also in the birdbeak dogfish (Deania calcea) and the longnose velvet dogfish (Centroselachus crepidater) [29]. As regards 3rd intermediate hosts, subcutaneous merocercoids were recently found in two Cape fur seals (Arctocephalus pusillus pusillus) [35]. Although cephalopods are described as hosts of this genus, Clistobothrium sp. has not been commonly reported in the most recent parasitological studies (Table 1). In particular, Brickle et al. [3] examined the congeneric longfin Patagonian squid (Doryteuthis gahi) finding plerocercoids provisionally morphologically identified as Phyllobothrium sp. and attributed to Clistobothrium sp. after molecular analysis. The related sequences (AF382071-82) have been deposited as Clistobothrium cf. montaukensis.
Interestingly, heavy infections with metacestodes named as *Phyllobothrium longilinis* have also been reported for *D. pealeii* [31]. Brickle et al. [3] also suggest that previous reports of plerocercoids of *Phyllobothrium* sp. in squid may have been in error, and that identifications have been further complicated by the historical use of the genus *Phyllobothrium* for all non-hooked tetraphyllidean worms with “leaf-like”, marginally crenulated bothridia. The use of the term *Phyllobothrium* with a broad sense can also be observed in some of the studies reported in Table 1. Thus, the presence of the genus *Clisobothrium* might have been underestimated. The current report represents the first molecular description of *Clisobothrium* sp. in *D. pealeii*, confirming the role of squids of the genus *Doryteuthis* as 2nd intermediate hosts [3,31].

The longfin inshore squid *D. pealeii* is a high valued species originating from the North West Atlantic, where its commercial catches started in the late 1800s. Still nowadays, *D. pealeii* is both sold internally and, to a lesser extent, exported. In particular, between 1991 and 2012 Italy was the first importer of *D. pealeii*, accounting for 29% of the exports [49].

*D. pealeii*, which is available as whole and fresh on the Italian market usually between May and June, is increasingly appreciated as an alternative for local squid species (authors’ personal communication). At the European level, fishery products must comply with the EU hygiene standards, based on the principles provided by the EC General Food Law [8]. As regards the presence of visible parasites, their possible effect on the quality of the product shall also be taken into consideration [15], and the Regulation (EC) No 853/2004 [10] states that sea-food products that are obviously contaminated with parasites should not be released for human consumption. The visual inspection has become the official method to be included within self-control programs for detecting visible parasites before market release and ensuring seafood quality and safety. In fact, beside the risk posed by zoonotic parasites, visually “un-aesthetic” parasites may decrease the seafood commercial value [50–52]. This might be the case of the visible and alive parasites described in this work. In fact, in case of products eviscerated at home by consumers, these “disgusting” larvae may become clearly visible to the naked eye. Thus, consumer education concerning the possibility that, despite FBOs and Official Veterinarians’ efforts, parasites might be present in wild seafood and information on correctly managing such defects should always be sought. This is in order to avoid excessive and unnecessary alarmism, which may also have negative media impact.

3. Materials and Methods

3.1. Squid Sampling

Overall, 70 *D. pealeii* (superorder Decapodiformes, order Teuthida, family Loliginidae, former name *Loligo pealeii*) specimens morphologically identified by experts according to the FAO morphological keys (http://www.fao.org/3/ac479e/ac479e00.htm) from the Northwest Atlantic (FAO area 21) were collected as whole fresh at the Wholesale fish market of Milan (n = 49) and at the distribution platforms of two leading brands in the organized distribution (n = 21) in June 2019. Three-four specimens were collected in each different sampling day and, overall, the specimens derived from 20 different batches. The squids collected at the Wholesale fish market of Milan were immediately submitted to visual inspection; the parasites were collected and stored separately; then, squids were frozen and transferred to the FishLab for further analysis. Squids collected at the platforms were instead directly transferred on ice to the FishLab where they were visually examined as fresh.

3.2. Parasite Detection

Each squid specimen was measured, registering the total length (TL) and the dorsal mantle length (DML), and weighted (total weight-TW, viscera weight-VW, mantle weight-MW) before visual inspection. The squids were opened longitudinally on their ventral side and a visual inspection under natural light was performed according to Commission Regulation (CE) n. 2074/2005 on both the visceral mass (comprising the digestive, excretory and reproductive organs) and mantle of fresh specimens.
to detect visible parasites. The plerocercoid larvae were counted and washed in 0.9% NaCl solution (Pero, Milano, Italy). After microscopic observations of the key morphological features [26–28,31,53,54], they were preserved in 70% ethanol (Carlo Erba Reagents s.r.l., Barcelona, Spain) and stored at −20 °C until molecular identification. The Spearman correlation coefficient (rho) was used to assess the correlation between the TW, the DML, the TL of the cephalopod specimens and the number of parasites per host.

3.3. DNA Extraction and Evaluation

Total DNA extraction was performed from all the collected plerocercoid larvae, according to [55]. DNA concentration and purity were determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, DE, USA).

3.4. Large Subunit (LSU) rRNA Gene Analysis

3.4.1. PCR Amplification, Sequencing and Sequences Editing

A 780 bp fragment of the variable D2 region of the large subunit (LSU) rRNA gene was selected as elective target and amplified from all the 18 plerocercoid larvae with the primer pair TrypFOR1 (5’-AGTCGGGTGTGTTGAGAATG-3’) and TrypREV (5’-CGTGTTTCAAGACCGGTC-3’), routinely used in FishLab for cestode species identification. PCR amplifications were set up in a 20 µL reaction volume containing 2 µL of a 10× buffer (Biotechrabbit GmbH, Hennigsdorf, Germany), 200 µM of each dNTP (dNTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 250 nM of each primer, 2.5 U PerfectTaq DNA Polymerase (Biotechrabbit GmbH, Hennigsdorf, Germany), 50–100 ng of DNA and DNase free water (Water Mol. Bio. Grade, DNase-RNase and Protease free, 5Prime GmbH, Hamburg, Germany). The following cycling program was used: initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 25 s, 50 °C for 25 s, 72 °C for 35 s; final extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis in 2% agarose gel, and amplicons were subsequently sent for standard forward and reverse Sanger sequencing to an external company. The obtained sequences were analyzed, edited and assembled with the Geneious R7 software (Biomatters Ltd, Auckland, New Zealand) [56]. Five sequences representative of the haplotypes were deposited in GenBank (accession numbers MT584205–MT584209).

3.4.2. Comparison with Databases and Phylogenetic Analysis

The edited sequences were used to run a BLAST analysis on GenBank, selecting the Somewhat similar sequences (blastn) algorithm. Then, genera of the Phyllobothriidae family were searched on the World Register of Marine Species database [57] and also in the most recent works dealing with Phyllobothriidea taxonomy [25,34,38]. Subsequently, for all the retrieved genera, all the available LSU sequences were searched on GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to create a genetic dataset, as detailed in Table S1. Both valid and synonym genus names, as well as taxa inquirenda, were used, to make the collection as exhaustive as possible. All the retrieved sequences, together with those produced in this study, were then aligned with Geneious R7 software (Biomatters Ltd, Auckland, New Zealand) [56] and a Neighbor-Joining (NJ) and Maximum Likelihood (ML) phylograms were constructed using the Kimura 2-parameter model [58] with 1000 bootstrap re-samplings in MEGA-X [59].

3.5. Cytochrome c Oxidase Subunit I (COI) Gene Analysis

3.5.1. Primers Projecting

Primers for the COI gene were ex novo projected in this study by using the Geneious R7 software (Biomatters Ltd, Auckland, New Zealand) [56]. In order to do so, all the available COI sequences from the Phyllobothriidae family, collected as described in Section 3.4.2, were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) database (see Table S1 for details), and aligned
with the Geneious R7 software (Biomatters Lts, Auckland, New Zealand) [56]. After the alignment, two different groups of sequences were observed, corresponding to two distinct regions of the COI gene. Thus, two primer pairs, 55_F/630_R and 734_F/1134_R, were projected for amplifying fragments of 532 bp (fragment A) and 354 bp (fragment B), respectively, from each region (Figure 6).

![Figure 6. Cytochrome c oxidase subunit I (COI) gene primers projected in this study: fragment A and B position in relation to the complete COI complete gene and primers’ sequences.](image)

3.5.2. PCR Amplification, Sequencing and Sequences Editing

PCR amplifications were set up in a 20 µL reaction volume as described in Section 3.4.1 with the following cycling programs: initial denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 25 s, 48 °C (fragment A)/54 °C (fragment B) for 25 s, 72 °C for 30 s; final extension at 72 °C for 5 min. Fragment A and B PCR products were visualized and sequenced as described in Section 3.4.1. The obtained sequences were analyzed, edited and assembled with the Geneious R7 software (Biomatters Lts, Auckland, New Zealand) [56]. Five representative sequences per fragment were deposited in GenBank (accession numbers: MT579473–MT579477 fragment A; MT583827–MT583831 fragment B).

3.5.3. Comparison with Databases, and Phylogenetic Analysis

For both fragment A and B, the BLAST analysis on GenBank was conducted as described for the LSU (Section 3.4.2) and the Identification System (IDs) on BOLD was also used. In addition, a pairwise distance matrix by the use of p-distance model with 1000 nonparametric bootstrap replicates was produced using MEGA-X. NJ and ML phylogenograms were constructed for both fragments using the datasets obtained by database sequences collection (see Section 3.4.2, including the same sequences used for the primer projecting (Section 3.5.1).

4. Conclusions

The life cycles of marine cestodes, especially those maturing in sharks and rays, are poorly known, mainly due to difficulties in larval stages identification [2,26]. Issues have already been highlighted within the Phyllobothriidea order, for example, where the use of molecular methods has often been advocated [32,35]. To our knowledge, in this work the presence of molecularly identified plerocercoid larvae of Clistobothrium sp. in longfin inshore squid (D. pealeii) was assessed for the first time. The results contribute to further elucidate the life cycle of this parasite. Beside an epidemiological relevance, FBOs and official authorities should be aware of the possible presence of live visible plerocercoid larvae in fresh longfin inshore squid sold as fresh on the market. Although not presenting a public health risk, these may present defects affecting cephalopods, constituting a reason of disgust and loss of trust in the control systems for consumers. Thus, consumer education to avoid excessive and unnecessary alarmism is important, particularly for cephalopods sold fresh and whole such as D. pealeii that may contain visible parasites still viable as reported in this study.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/7/596/s1. Table S1: Genetic dataset including all the available sequences of the large subunit (LSU) rRNA and cytochrome c oxidase subunit I (COI) genes of the Phyllobothriidea family. Table S2: Average cytochrome c oxidase subunit I (COI) gene sequences divergences (fragment A). Video S1: Macroscopic aspect of the live plerocercoid larvae at squid dissection.
Author Contributions: Conceptualization, L.G., E.B.-Z. and A.A.; data curation, L.G. and A.G.; formal analysis, L.G. and A.G.; funding acquisition, A.A.; investigation, E.B.-Z.; methodology, L.G., A.G. and A.A.; resources, R.M., M.R. and A.A.; software, A.G.; validation, E.B.-Z., R.M. and M.R.; writing—original draft, L.G. and A.G.; writing—review and editing, E.B.-Z., R.M., M.R. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the 2019 Visiting Fellow Program of the University of Pisa, which financed a period of research of Ewa Bilska-Żajac at the FishLab, Department of Veterinary Science, University of Pisa.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Hochberg, F.G. Diseases of Mollusca: Cephalopoda. In Diseases of Marine Animals; Kinne, O., Ed.; Biologisches Anstalt Helgoland: Helgoland, Germany, 1990; Volume 3, pp. 47–227.
2. Roumbedakis, K.; Krausova, M.; Tyml, T.; Di Cristo, C. A perspective around cephalopods and their parasites, and suggestions on how to increase knowledge in the field. Front. Physiol. 2018, 9, 1573. [CrossRef] [PubMed]
3. Brickle, P.; Olson, P.D.; Littlewood, D.T.J.; Bishop, A.; Arkhipkin, A.I. Parasites of Loligo gahi from waters off the Falkland Islands, with a phylogenetically based identification of their cestode larvae. Can. J. Zool. 2001, 79, 2289–2296. [CrossRef]
4. Guilién-Hernández, S.; López-Struck, A.; González-Salas, C.; Aguirre-Macedo, M.L. Octopus nuyu parasites off the Yucatán Peninsula, Mexico. I. Faunal assemblages. Dis. Aquat. Organ. 2018, 130, 37–43. [CrossRef] [PubMed]
5. Vieites, J.M.; Ruiz, C.S.; Fernández, F.; Alonso, R.C. Importance of cephalopod health and welfare for the commercial sector. In Handbook of Pathogens and Diseases in Cephalopods; Gestal, C., Pascual, S., Guerra, A., Fiorito, G., Vieites, J.M., Eds.; Springer: Berlin, Germany, 2019; pp. 5–7.
6. FAO. The State of World Fisheries and Aquaculture 2018—Meeting the Sustainable Development Goals. Rome 2018. Available online: http://www.fao.org/3/i9540en/i9540en.pdf (accessed on 28 December 2019).
7. FAO. Tight Supply Situation Continues. Information and Analysis on World Fish Trade. Rome 2019. Available online: http://www.fao.org/in-action/globefish-market-reports/resource-detail/en/c/1176219/ (accessed on 28 December 2019).
8. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority, and laying down procedures in matters of food safety. Off. J. Eur. Communities 2002, 31, 1–24.
9. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Off. J. Eur. Communities 2004, 139, 1–54.
10. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Off. J. Eur. Communities 2004, 139, 55–205.
11. Council Regulation (EC) No 2006/96 of 26 November 1996 laying down common marketing standards for certain fishery products. Off. J. Eur. Communities 1996, 334, 1–15.
12. Bao, M.; Pierce, G.J.; Strachan, N.J.; Pascual, S.; González-Muñoz, M.; Levsen, A. Human health, legislative and socioeconomic issues caused by the fish-borne zoonotic parasite Anisakis: Challenges in risk assessment. Trends Food Sci. Technol. 2019, 86, 298–310. [CrossRef]
13. D’Amico, P.; Malandrà, R.; Costanzo, F.; Castiglione, L.; Guidi, A.; Gianfaldoni, D.; Armani, A. Evolution of the Anisakis risk management in the European and Italian context. Food Res. Int. 2014, 64, 348–362. [CrossRef]
14. Guardone, L.; Bilska-Żajac, E.; Castiglione, D.; Giusti, A.; Malandra, R.; Armani, A. Visible parasites in fresh cephalopods sold on the Italian market: Impact on consumers’ perception on safety and quality. In Proceedings of the Annual Scientific Conference and Annual General Meeting of the European College of Veterinary Public Health, Edinburgh, UK, 2–4 October 2019; p. 37.
15. Cavaleiro, M.N.C. Parasite Fauna of Octopus vulgaris (Cephalopoda: Octopodidae) and Platichthys flesus (Actinopterygii: Oeleuronectidae): Morphology, Systematics, Life History Strategies and Ecology. Ph.D. Thesis, Porto University, Porto, Portugal, 2013. Available online: https://core.ac.uk/download/pdf/143398568.pdf (accessed on 28 December 2019).
16. Petrić, M.; Mladineo, I.; Šifner, S.K. Insight into the short-finned squid Illex coindetii (Cephalopoda: Ommastrephidae) feeding ecology: Is there a link between helminth parasites and food composition? J. Parasitol. 2011, 97, 55–62. [CrossRef]
17. Pardo-Gándarillas, M.C.; Lohrmann, K.B.; Valdivia, A.L.; Ibáñez, C.M. First record of parasites of *Dosidicus gigas* (d’Orbigny, 1835) (Cephalopoda: Ommastrephidae) from the Humboldt Current system off Chile. *Rev. Biol. Mar. Oceanogr.* 2009, 44, 397–408. [CrossRef]

18. Nigmatullin, C.M.; Shchetinnikov, A.S.; Shukhgalter, O.A. On feeding and helminth fauna of neon flying squid *Ommastrephes bartramii* (Lesueur, 1821) (Cephalopoda: Ommastrephidae) in the southeastern Pacific. *Rev. Biol. Mar. Oceanogr.* 2009, 44, 227–235. [CrossRef]

19. Shukhgalter, O.A.; Nigmatullin, C.M. Parasitic helminths of jumbo squid *Dosidicus gigas* (Cephalopoda: Ommastrephidae) in open waters of the central east Pacific. *Fish. Res.* 2001, 54, 95–110. [CrossRef]

20. González, R.A.; Kroecz, M.A. Enteric helminths of the shortfin squid *Illex argentinus* in San Matias Gulf (Argentina) as stock discriminants. *Acta Parasitol.* 2000, 45, 89–93.

21. Gestal, C.; Abollo, E.; Ablas, C.; Pascual, S. Estudio al MEB de larvas plerocercoides de *Phyllobothrium* sp. (Tetraphyllidea, Phyllobotriidae) y *Nybelinia lingualis* (Trypanorhyncha, Tentaculolidae), cestodos parásitos de *Octopus vulgaris* (Mollusca, Cephalopoda) en la Ría de Vigo. *Iberus* 1998, 16, 125–132.

22. Pascual, S.; Gestal, C.; Estévez, J.M.; Rodriguez, H.; Soto, M.; Abollo, E.; Arias, C. Parasites in commercially-exploited cephalopods (Mollusca, Cephalopoda) in Spain: An updated perspective. *Aquaculture* 1996, 142, 1–10. [CrossRef]

23. Pascual, S.; López, B.; Arias, C.; González, Á.F.; Guerra, Á. Helminth parasites of *Illex coindetii* (Cephalopoda: Ommastrephidae) off the Galician coast (NE Atlantic). *Sci. Mar.* 1994, 58, 269–272.

24. Bower, S.M.; Margolis, L. Potential use of helminth parasites in stock identification of flying squid, *Ommastrephes bartramii*, in North Pacific waters. *Can. J. Zool.* 1991, 69, 1124–1126. [CrossRef]

25. Caira, J.N.; Jensen, K.; Waaschenbach, A.; Olson, P.D.; Littlewood, D.T. Orders out of chaos—Molecular phylogenetics reveals the complexity of shark and stingray tapeworm relationships. *Int. J. Parasitol.* 2014, 44, 55–73. [CrossRef]

26. Jensen, K.; Bullard, S.A. Characterization of a diversity of tetraphyllidean and rhinebothriidean cestode larval types, with comments on host associations and life-cycles. *Int. J. Parasitol.* 2010, 40, 889–910. [CrossRef] [PubMed]

27. Dollfus, R.-P. Énumération des cestodes du plancton et des invertébrés marins (6e contribution). *Ann. Parasitol. Hum. Comp.* 1964, 39, 329–379. [CrossRef] [PubMed]

28. Scholz, T.; Euzet, L.; Moravec, F. Taxonomic status of *Pelcichnibothrium speciosum* Monticelli, 1889 (Cestoda: Tetraphyllidea), a mysterious parasite of *Alepisaurus ferox* Lowe (Teleostei: Alepisauridae) and Prionace glauca (L.) (Euselachii: Carcharinidae). *Syst. Parasitol.* 1998, 41, 1–8. [CrossRef]

29. Caira, J.N.; Pickering, M. Cestodes from deep-water squalliform sharks in the Azores. *Deep Sea Res. II* 2013, 98, 170–177. [CrossRef]

30. Caira, J.N.; Jensen, K. A digest of elasmobranch tapeworms. *J. Parasitol.* 2014, 100, 373–391. [CrossRef]

31. Stunkard, H.W. Studies on Tetraphyllidean and Tetrarhynchidean metacestodes from squids taken on the New England coast. *Bio. Bull.* 1977, 153, 387–412. [CrossRef]

32. Agustí, C.; Aznar, F.J.; Olson, P.D.; Littlewood, D.T.J.; Kostadinova, A.; Raga, J.A. Morphological and molecular characterization of tetraphyllidean merocercoids (Platyhelminthes: Cestoda) of striped dolphins (*Stenella coeruleoalba*) from the Western Mediterranean. *Parasitology* 2005, 130, 461–474. [CrossRef]

33. Aznar, F.J.; Agustí, C.; Littlewood, D.T.J.; Raga, J.A.; Olson, P.D. Insight into the role of ceteceans in the life cycle of the tetraphyllideans (Platyhelminthes: Cestoda). *Int. J. Parasitol.* 2007, 37, 243–255. [CrossRef]

34. Caira, J.N.; Jensen, K.; Hayes, C.; Ruhnke, T.R. Insights from new cestodes of the crocodile shark, *Pseudocarcharias kamoharai* (Lamniformes: Pseudocarchariidae), prompt expansion of *Scyphophyllidium* and formal synonymization of seven phyllobothriidean genera—at last! *J. Helminthol.* 2020, 94, 1–25. [CrossRef]

35. Klotz, D.; Hirzmann, J.; Bauer, C.; Schöne, J.; Iserringhausen, M.; Wohlsin, P.; Baumgärtner, W.; Herder, V. Subcutaneous merocercoids of *Clistobothrium* sp. in two Cape fur seals (*Arctocephalus pusillus pusillus*). *Int. J. Parasitol. Parasites Wildlife* 2018, 7, 99–105. [CrossRef]

36. Randhawa, H.S.; Brickle, P. Larval parasite gene sequence data reveal cryptic trophic links in life cycles of porbeagle shark tapeworms. *Mar. Ecol. Prog. Ser.* 2011, 431, 215–222. [CrossRef] [PubMed]

37. Randhawa, H.S. Insights using a molecular approach into the life cycle of a tapeworm infecting great white sharks. *J. Parasitol.* 2011, 97, 275–281. [CrossRef] [PubMed]

38. Ruhnke, T.R.; Daniel, V.; Jensen, K. Four new species of *Paraorygmatobothrium* (Eucestoda: Phyllobothriidea) from sharks of the Gulf of Mexico and the Atlantic Ocean, with comments on their host specificity. *J. Parasitol.* 2020, 106, 133–156. [CrossRef] [PubMed]
39. Littlewood, D.T.J.; Curini-Galletti, M.; Herniou, E.A. The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Mol. Phylogenetics Evol.* 2000, 16, 449–466. [CrossRef] [PubMed]
40. Olson, P.D.; Littlewood, D.T.J.; Bray, R.A.; Mariaux, J. Interrelationships and evolution of the tapeworms (Platyhelminthes: Cestoda). *Mol. Phylogenetics Evol.* 2001, 19, 443–467. [CrossRef]
41. Waeschenbach, A.; Webster, B.L.; Bray, R.A.; Littlewood, D.T.J. Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. *Mol. Phylogenetics Evol.* 2007, 45, 311–325. [CrossRef]
42. Trevisan, B.; Primon, J.F.; Marques, F.P. Systematics and diversification of *Anidobothrium* Marques, Brooks & Lasso, 2001 (Euccestoidea: Rhinebothriidea). *PLoS ONE* 2017, 12, e0184632.
43. Van Steenkiste, N.; Locke, S.A.; Castelin, M.; Marcogliese, D.J.; Abbott, C.L. New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes). *Mol. Ecol. Resour.* 2015, 15, 945–952. [CrossRef]
44. Ruhnke, T.R. A new species of *Clistobothrium* (Cestoda: Tetraphyllidea), with an evaluation of the systematic status of the genus. *J. Parasitol.* 1993, 79, 37–43. [CrossRef]
45. Kuris, A.M.; Jaramillo, A.G.; McLaughlin, J.P.; Weinstein, S.B.; Garcia-Vedrenne, A.E.; Poinar, G.O.; Pickering, M.; Steinauer, M.L.; Espinoza, M.; Ashford, J.E.; et al. Monsters of the sea serpent: Parasites of an oarfish, *Regalecus russelli*. *J. Parasitol.* 2015, 101, 41–45. [CrossRef]
46. Ruhnke, T.R. A Monograph on the Phyllobothriidae (Platyhelminthes, Cestoda). *Bull. Univ. Neb. State Museum* 2011, 25, 1–208. Available online: https://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=10411&view=collections=museumbulletin (accessed on 13 June 2020).
47. Dailey, M.D.; Vogelbein, W. *Clistobothrium carcharodoni* gen. et sp. n. (Cestoda: Tetraphyllidea) from the spiral valve of the great white shark (*Carcharodon carcharias*). *Proc. Helminthol. Soc. Wash.* 1990, 57, 108–112.
48. Penadés-Suay, J.; Tomás, J.; Merchán, M.; Aznar, F.J. Intestinal helminth fauna of the shortfin mako (*Isurus oxyrinchus*) (Elasmobranchii: Lamnidae) in the northeast Atlantic Ocean. *Dis. Aquat. Organ.* 2017, 123, 45–54. [CrossRef] [PubMed]
49. Arkhipkin, A.I.; Rodhouse, P.G.K.; Pierce, G.J.; Sauer, W.; Sakai, M.; Allcock, L.; Arguelles, J.; Bower, J.R.; Castillo, G.; Ceriola, L. World Squid Fisheries. *Rev. Fish. Sci. Aquac.* 2015, 23, 92–252. [CrossRef] [PubMed]
50. Giarratana, F.; Ziino, G.; D’Andrea, V.; Panebianco, A.; Giuffrida, A. Quality assessment of *Zeus faber* (Peter’s fish) ovaries regularly commercialized for human consumption. *Ital. J. Food Saf.* 2018, 7, 28–33. [CrossRef] [PubMed]
51. Rodriguez, H.; Abollo, E.; González, Á.F.; Pascual, S. Scoring the parasite risk in highly-valuable fish species from southern ICES areas. *Fish. Res.* 2018, 202, 134–139. [CrossRef]
52. Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2005 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off. J. Eur. Communities* 2005, 338, 27–59.
53. Agustí, C.; Aznar, F.J.; Raga, J.A. Tetraphyllidean plerocercoids from western Mediterranean cetaceans and other marine mammals around the world: A comprehensive morphological analysis. *J. Parasitol.* 2005, 91, 83–92. [CrossRef]
54. Williams, H.H. The taxonomy, ecology and host specificity of some Phyllobothriidae (Cestoda: Tetraphyllidea), a critical revision of *Phyllobothrium* Beneden, 1849 and comments on some allied genera. *Philos. Trans. R. Soc. B* 1968, 253, 231–307.
55. Guardone, L.; Malandra, R.; Costanzo, F.; Castiglione, L.; Tinacci, L.; Gianfaldoni, D.; Guidi, A.; Armani, A. Assessment of a sampling plan based on visual inspection for the detection of anisakid larvae in fresh anchovies (*Engraulis encrasicolus*). A first step towards official validation? *Food Anal. Methods* 2016, 9, 1418–1427. [CrossRef]
56. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012, 28, 1647–1649. [CrossRef]
57. WoRMS. Phyllobothriidae Braun, 1900. 2020. Available online: http://www.marinespecies.org/aphia.php?p=taxdetails&id=104946 (accessed on 29 May 2020).
58. Kimura, M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]

59. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).