Prevalence, Molecular Identification, and Risk Factors for Cryptosporidium Infection in Edible Marine Fish: A Survey Across Sea Areas Surrounding France

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Cryptosporidium, a zoonotic pathogen, is able to infect a wide range of hosts including wild and domestic animals, and humans. Although it is well known that some parasites are both fish pathogens and recognized agents of zoonosis with a public health impact, little information is available concerning the prevalence of Cryptosporidium in wild aquatic environments. To evaluate the prevalence of Cryptosporidium spp. in commercially important edible marine fish in different European seas (English channel, North sea, Bay of Biscay, Celtic sea and Mediterranean sea), 1,853 specimens were collected as part of two surveys. Nested PCR followed by sequence analysis at the 18S rRNA gene locus was used to identify Cryptosporidium. The overall prevalence of Cryptosporidium spp. reached 2.3% (35 out of 1,508) in a first campaign and 3.2% (11 out of 345) in a second campaign. Sequence and phylogenetic analysis of positive samples identified Cryptosporidium parvum (n = 10) and seven genotypes which exhibited between 7.3 and 10.1% genetic distance from C. molnari, with the exception of one genotype which exhibited only 0.5–0.7% genetic distance from C. molnari. Among 31 analyzed fish species, 11 (35.5%) were identified as potential hosts for Cryptosporidium. A higher prevalence of Cryptosporidium spp. was observed in larger fish, in fish collected during the spring-summer period, and in those caught in the North East Atlantic. Pollachius virens (saithe) was the most frequently Cryptosporidium positive species. In fish infected by other parasites, the risk

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INTRODUCTION

The world fish production increased to 171 million tons in 2016 (compared for instance to a production of 156 million tons in 2012) with European fisheries and aquaculture representing overall 6.4 million tons. Interestingly, within the European Union, around 80% of fish consumed is wild caught with an annual fish consumption of 18.1 kg per capita (FAO, 2018).

Considering that protozoan and metazoan parasites can infect edible fish worldwide, the problem of human health risks due to wild fish ingestion is an important issue. Some of these parasites are both fish pathogens and recognized zoonotic pathogens with public health impacts but uncertainty exists about the zoonotic potential of some fish pathogens. The most important fish species in the fish industries from many countries can be infected with parasites. Furthermore, some fish parasites can affect the appearance, touch, odor, texture, temperature and taste (organoleptic properties) of fish products, negatively impacting the fish industry economy (Agence Française de Sécurité Sanitaire des Aliments [ANSES], 2010).

Even in developed countries, where sanitary infrastructures are usually good, food- or water-borne parasitic infections are frequent (Omarova et al., 2018). Indeed, the persistence of water- or foodborne outbreaks and the occurrence of infections due to emerging or re-emerging pathogens are favored by different factors related to changes in consumer life styles such as: increased consumption of fresh products, exotic food or raw or lightly cooked meat and increasing global demand for protein of animal origin, and home-meal replacement (Collins, 1997; Broglia and Kapel, 2011).

Cryptosporidium is a waterborne and foodborne protozoan parasite responsible for more than 8 million cases of foodborne illness annually (Ryan et al., 2018). The parasite is a cause of severe diarrhea mainly in immunocompromised people and young children but also in a wide range of vertebrates including fish, amphibians, reptiles, birds, and mammals (Ryan et al., 2014). To date, it has been genetically characterized in more than 25 species of both marine and freshwater fish. Three species of Cryptosporidium are recognized in fish: Cryptosporidium molnari (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Palenzuela et al., 2010), C. scophthalmi (Alvarez-Pellitero et al., 2004) and C. kuwi (previously known as piscine genotype I) (Ryan et al., 2015). Additionally, other Cryptosporidium species identified in other groups of vertebrates such as C. parvum, C. hominis, C. scrofarum and C. xiaoi, have also been detected in fish. Furthermore, fifteen Cryptosporidium fish genotypes, and one Cryptosporidium rat III-like genotype, have been reported (Ryan et al., 2014; Yang et al., 2015, 2016; Couso-Pérez et al., 2018).

In fish hosts, Cryptosporidium is found either in the stomach or intestine. Additionally, it has been described that the parasite can cause pathological effects in fish (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Alvarez-Pellitero et al., 2004) as well as an increase in the mortality rate, mainly in juvenile fish (Murphy et al., 2009).

The majority of studies on piscine Cryptosporidium have been carried out on aquarium or farmed fish but scarce data is available concerning the molecular identification of Cryptosporidium genotypes and species in wild marine fish and, in particular, in edible fish. Actually, only two studies have been carried on in Australia and Papua New Guinea on wild marine fish (Reid et al., 2010; Koinari et al., 2013). To our knowledge, no updated information on Cryptosporidium infection in marine fish is available in France. In a previous study from our group, the overall prevalence of Cryptosporidium spp. in freshwater fish sampled from Lake Geneva (France) reached 37% (Certad et al., 2015).

Thus, the main goal of our study was to evaluate the prevalence of Cryptosporidium species and genotypes in selected edible fish species sampled in defined marine geographic areas surrounding France (European fishing waters). Considering that many factors related to the host and to the environment can influence host-parasites relationships (Sitjà-Bobadilla et al., 2005), we also aimed to determine the influence of host and environmental factors on Cryptosporidium prevalence in fish.

MATERIALS AND METHODS

Fish Sampling

Epidemiological studies were conducted during two surveys: For the first survey, commercially important fish species were
collected between 2011 and 2014 through research cruises belonging to the French Ifremer (Institut Français de Recherche pour l’Exploitation de la Mer) in different European seas as follows: the English channel, the North sea, the Bay of Biscay, the Celtic sea and the Mediterranean sea, or through purchases from wholesalers or retailers for commercial catches, and for farmed fish in the case of salmon and sea bass. Fish retailers were chosen by their representativeness of fishing areas in order to complement marine fish species according to range and origin. For the second survey, fish sampling was performed at Boulogne-Sur-Mer, the first France’s fishermen’s port, between 2014 and 2015 targeting the four most frequently caught fish species at this port: saithe, mackerel, herring, and whiting. Sampling surveys allowed catching fresh unfished fish.

Available data about fishing area, fishing vessel, fishing date and kind of storage were recorded. Fish specimens of the selected species were sampled at various times and areas on the basis of fishery season. Fishes were analyzed immediately after being caught in the case of specimens collected through research cruises or after fish landing when obtained from retailers. When fishes were obtained from retailers, it was not always possible to pinpoint the exact location of the fishing area within the North East Atlantic. The weight, size, sex, and sexual maturity were determined for each fish. The presence of ecto and endoparasites other than Cryptosporidium was detected by visual examination of the body of the fish before and after evisceration. In order to classify fish according to weight and size, five groups were defined ranging from 1-smallest fishes to 5-largest fishes (Supplementary Figure S1) using a hierarchical cluster analysis with the R stats package. Fish were dissected, scrapings of the gastrointestinal epithelia were performed for each animal, the cells were preserved in RCL2® (Alphelys, Plaisir, France) and stored at −20°C as previously described (Certad et al., 2015). Sections of the stomach and bowel were fixed in 10% buffered formalin for histological analysis. Ifremer research cruises are carried out with the French Oceanographic Fleet and are under the supervision of the French Ministry of Education and Research. A Steering Committee evaluates and approves the entire scientific campaign program before implementation. The study was performed in accordance with the EU directive 2010/63/EU and followed all the guidelines of the deontology charter of Ifremer’s research.

DNA Extraction
Genomic DNA extraction was performed from scrapings of the gastric and intestinal epithelia on 96-well plates, using the NucleoSpin™ Kit (Macherey-Nagel, GmbH & Co KG, Germany) according to the manufacturer as previously described (Certad et al., 2015). DNA was eluted in 100 μl of elution buffer.

Nested PCR
Fish samples were tested for identification of Cryptosporidium at the 18S rRNA gene locus as previously described (Certad et al., 2015). Nested PCR was performed using a MJ Research PTC-200 Thermal Cycler (Marshall Scientific, Waltham, MA, United States). Secondary PCR products were visualized on a 2% agarose gel stained with Ethidium Bromide fluoresce under ultraviolet light. Although nested PCR may not accurately represent the genetic diversity originally present in the sample, this technique is often appropriate to obtain sufficient DNA copies, considering that environmental samples, and in particular gastric or intestinal tissues from fishes or feces from wildlife, frequently contain low amounts of oocysts and high levels of PCR inhibitors (Paparini et al., 2017).

DNA Sequencing and Analysis
To identify Cryptosporidium species or genotypes, positive secondary nested PCR products were purified using the NucleoFast® 96 PCR kit (Macherey Nagel, GmbH & Co KG, Germany). Purified PCR products were sequenced directly in both directions, using the secondary PCR primers (Genoscreen, Pasteur Institute of Lille, France). Obtained nucleotide sequences were aligned using the BioEdit v7.0.1 package, and compared with available DNA sequences of Cryptosporidium in GenBank database using the NCBI BLAST basic local alignment search tool. Subtyping of C. parvum was based on sequence analysis of the 60 kDa glycoprotein (gp60) gene as previously reported (Gatei et al., 2007). The amplified DNA fragments were purified, sequenced, and analyzed as described above. All of the nucleotide sequences identified in this study were deposited in GenBank under the accession numbers MK236538-MK236548.

Phylogenetic Analysis
The 18S rRNA nucleotide sequences from the present study were aligned with Cryptosporidium sequences retrieved from GenBank (Benson et al., 2004). Sequences were aligned with MUSCLE (Elwakil and Hewedi, 2010) and the most suitable nucleotide substitutions model was selected using jModelTest2 (Darriba et al., 2012). Maximum Likelihood (ML) and Distance trees were constructed using MEGA version 7 using the Kimura 2-parameter model and gaps/missing data treatment set to complete deletion (Kumar et al., 2016). Bootstrap support was based on 1000 replications. Bayesian phylogenetic reconstructions were produced from alignments using MrBayes (Ronquist et al., 2012), with the HK85 substitution model, a MCMC length of 1,100,000, burn-in of 10,000, and subsampling every 200 iterations.

Histological Analysis
Paraffin-embedded tissues were cut to a thickness of 5 μm and stained with hematoxylin and eosin (H & E). A Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID Software, Cambridge, United Kingdom) was used for observation of the histological sections.

Statistical Analysis
Fisher’s exact test was used to analyze the relationship between different categorical variables. A logistic regression model was used to calculate odds ratios (OR) with Cryptosporidium presence as the main outcome. Multiple correspondence analysis (MCA), a data reduction technique similar to factor or principal
## TABLE 1 | Description of campaigns for fish sampling: geographic location, seasonality, taxonomic position of fish species, and prevalence of Cryptosporidium (First survey).

<table>
<thead>
<tr>
<th>Campaigns for fish sampling</th>
<th>Geographic location</th>
<th>Fishery season</th>
<th>Taxonomic position of fish species (order)</th>
<th>Number of sampled fish specimens</th>
<th>Cryptosporidium-positive fish number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PELGAS 1</td>
<td>Bay of Biscay</td>
<td>Spring</td>
<td>Gadiformes</td>
<td>90</td>
<td>0 (0)</td>
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<td>Perciformes</td>
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<td>Clupeiformes</td>
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<td>Lophiiformes</td>
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<td>Others **</td>
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<tr>
<td>PELMED1</td>
<td>Mediterranean sea</td>
<td>Summer</td>
<td>Gadiformes</td>
<td>112</td>
<td>4 (3.6)</td>
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<td>Perciformes</td>
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<td>Clupeiformes</td>
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<td>Others **</td>
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<tr>
<td>EVHOE 1</td>
<td>Bay of Biscay, Celtic sea</td>
<td>Fall</td>
<td>Gadiformes</td>
<td>119</td>
<td>2 (1.7)</td>
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<td>Pleuronectiformes</td>
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<tr>
<td>IBTS 1</td>
<td>English channel and North sea</td>
<td>Winter</td>
<td>Gadiformes</td>
<td>147</td>
<td>1 (0.7)</td>
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<td>Pleuronectiformes</td>
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<tr>
<td>Retailers</td>
<td>North East Atlantic *, Mediterranean sea, Black sea</td>
<td>Spring</td>
<td>Gadiformes</td>
<td>265</td>
<td>4 (1.5)</td>
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<td>Pleuronectiformes</td>
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<td>Others **</td>
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<td>Others **</td>
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<tr>
<td>PELGAS 2</td>
<td>Bay of Biscay</td>
<td>Spring</td>
<td>Gadiformes</td>
<td>71</td>
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<td>Others **</td>
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<tr>
<td>PELMED 2</td>
<td>Mediterranean sea</td>
<td>Summer</td>
<td>Gadiformes</td>
<td>81</td>
<td>0 (0)</td>
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<td>Others **</td>
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<tr>
<td>EVHOE 2</td>
<td>Bay of Biscay, Celtic sea</td>
<td>Fall</td>
<td>Gadiformes</td>
<td>145</td>
<td>0 (0)</td>
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<td>Pleuronectiformes</td>
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<td>Retailers</td>
<td>North East Atlantic *, Mediterranean sea, Black sea</td>
<td>Summer</td>
<td>Gadiformes</td>
<td>203</td>
<td>21 (10.34)</td>
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<td>Others **</td>
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<tr>
<td>Retailers</td>
<td>North East Atlantic *, Mediterranean sea, Black sea</td>
<td>Fall</td>
<td>Gadiformes</td>
<td>195</td>
<td>3 (1.5)</td>
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<td>Pleuronectiformes</td>
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<tr>
<td>Retailers</td>
<td>North East Atlantic *, Mediterranean sea, Black sea</td>
<td>Winter</td>
<td>Gadiformes</td>
<td>80</td>
<td>0 (0)</td>
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<td>Perciformes</td>
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<td></td>
<td>Pleuronectiformes</td>
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</tbody>
</table>

TOTAL 1508 35 (2.3)

*When fishes were collected from retailers, it was not always possible to pinpoint in detail the exact location of the fishing area within the North East Atlantic. PELGAS (PELagiques GAScone) 1, campaign 2011; PELGAS 2, campaign 2012; PELMED (PELagiques MEDiterranée) 1, campaign 2011; PELMED 2, campaign 2012; EVHOE (EValuation Halieutique de l’Ouest de l’Europe) 1, campaign 2011; EVHOE 2, campaign 2012; IBTS (International Bottom Trawl Survey), campaign 2012. **Beloniformes, Scorpaeniformes, Carcharhiniformes, and Tetraodontiformes.

Component analysis, was applied to identify potential risk factors for Cryptosporidium infection in fishes. This descriptive statistical technique is valuable to analyze data and confirm associations or similarities between quantitative or qualitative variables; then, these variables are categorized without a probabilistic distribution defined a priori (Greenacre and Blasius, 2006). MCA facilitates the examination of different variables simultaneously; the results are represented by a graphic, and a point represents
each category of every variable, and the distance from one point to another and from the center represents the relationship among the categorical variables. Risk factor variables included in the analysis were: localization of the fishing area, campaigns, seasonality, taxonomical position (order) of fish, size and weight grouping. The general significance level was set at a P-value below 0.05. All analyses were performed using Vassarstats software and packages stats from the R statistical computing program.

**RESULTS**

In total, 1,508 fishes were collected in the first survey: 765 onboard Ifremer research vessels and 743 from fish retailers, representing 31 different fish species. The molecular analysis of fish digestive tissues allowed the identification of representing 31 different fish species. The molecular analysis onboard Ifremer research vessels and 743 from fish retailers, in total, 1,508 fishes were collected in the first survey: 765

<table>
<thead>
<tr>
<th>Groups of fish</th>
<th>Presence of Cryptosporidium N (%)</th>
<th>Absence of Cryptosporidium N (%)</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male**</td>
<td>11/514 (2.1)</td>
<td>503/514 (97.9)</td>
<td>1.05</td>
<td>(0.50–2.2)</td>
<td>0.89</td>
</tr>
<tr>
<td>Female**</td>
<td>19/863 (2.2)</td>
<td>844/863 (97.8)</td>
<td>4.8</td>
<td>(1.96–10.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>From North East Atlantic</td>
<td>28/713 (3.9)</td>
<td>685/713 (96.1)</td>
<td>3.77</td>
<td>(1.64–8.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>From all other zones</td>
<td>7/795 (0.9)</td>
<td>788/795 (99.1)</td>
<td>5.78</td>
<td>(2.33–13.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sampling from retailers</td>
<td>28/743 (3.8)</td>
<td>714/743 (96.1)</td>
<td>7/765 (0.9)</td>
<td>758/765 (99.1)</td>
<td>4.14 (1.71–10.0)</td>
</tr>
<tr>
<td>Sampling in research campaigns</td>
<td>29/700 (4.1)</td>
<td>671/700 (95.9)</td>
<td>All other orders</td>
<td>6/808 (0.7)</td>
<td>802/808 (99.3)</td>
</tr>
<tr>
<td>Fish order Gadiformes</td>
<td>29/700 (4.1)</td>
<td>671/700 (95.9)</td>
<td>All other orders</td>
<td>6/808 (0.7)</td>
<td>802/808 (99.3)</td>
</tr>
<tr>
<td>Species Pollachius virens</td>
<td>15/80 (18.7)</td>
<td>65/80 (81.2)</td>
<td>Caught in fall-winter</td>
<td>6/686 (0.9)</td>
<td>686/686 (99.1)</td>
</tr>
<tr>
<td>All others species</td>
<td>20/1428 (1.4)</td>
<td>1408/1428 (98.6)</td>
<td>Caught in spring-summer</td>
<td>29/822 (3.5)</td>
<td>793/822 (96.5)</td>
</tr>
<tr>
<td>Infected with other parasites***</td>
<td>33/952 (3.5)</td>
<td>919/952 (96.5)</td>
<td>No detected parasites</td>
<td>2/556 (0.4)</td>
<td>554/556 (99.6)</td>
</tr>
</tbody>
</table>

*, **The sex could not be determined for all fishes. ***952 (63%) fishes out of 1,508 were infected by different parasites. The most frequently detected parasites were nematodes of the Anisakidae family in 56% of fishes. Other parasites were also found such as trematodes, cestodes, microsporidia and copepods. P values in bold are statistically significant.

The MCA (Figure 1) shows the coordinates of each variable on the two dimensions which explains the largest percentage of the variance in the data. Variables which are closest to each other on the scatterplot and far from the center of the plot are the most closely related. Even if these variables are not grouped as perfect clusters, most Cryptosporidium positive fishes are in the zone called cluster 1. In general, this cluster contained larger fishes mainly of size and weight groupings 4 and 5, from the Gadiformes order, caught in the North East Atlantic.

Sequence and phylogenetic analysis (Figure 2) at the 18S rRNA gene locus identified one species of Cryptosporidium and seven genotypes, distributed as follows: 8 (22%) C. parvum, 16 (45.7%) belonged to a novel genotype (#Cryptofish1) that exhibited 7.3–8.5% genetic distance from C. molnari.
### TABLE 3 Cryptosporidium distribution in wild marine fishes identified at the 18S rRNA gene locus (national survey).

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Fishing area</th>
<th>Mean fish size in cm (SD)</th>
<th>Fish minimum landing size (cm)**</th>
<th>Mean fish weight in g (SD)</th>
<th>Number of Cryptosporidium positive individuals (%)</th>
<th>Number of individuals harboring identified Cryptosporidium species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saithe</td>
<td>Pollachius virens</td>
<td>North East Atlantic*</td>
<td>51.7 ± 9.9</td>
<td>35</td>
<td>1302 ± 835</td>
<td>15/80 (18.8)</td>
<td>15 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Cod</td>
<td>Gadus morhua</td>
<td>North East Atlantic*</td>
<td>43.9 ± 10.3</td>
<td>35</td>
<td>1108 ± 1015</td>
<td>2/132 (1.5)</td>
<td>0 Cr sp 1 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Whiting</td>
<td>Merlangius merlangus</td>
<td>North East Atlantic*</td>
<td>32.7 ± 3.7</td>
<td>27</td>
<td>287.7 ± 68.13</td>
<td>1/128 (0.8)</td>
<td>1 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Blue ling</td>
<td>Molva dypterygia</td>
<td>North East Atlantic*</td>
<td>98.42 ± 9.8</td>
<td>70</td>
<td>3899.4 ± 853.5</td>
<td>369/4 (4.4)</td>
<td>2 Cr sp 1 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Soomber scombrus</td>
<td>North East Atlantic*</td>
<td>30.6 ± 6.1</td>
<td>30</td>
<td>2306 ± 125.7</td>
<td>1/91 (1.1)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Common sardine</td>
<td>Sardina pilchardus</td>
<td>Mediterranean sea</td>
<td>16.7 ± 4.2</td>
<td>11</td>
<td>40 ± 32.9 for 70</td>
<td>1/78 (1.3)</td>
<td>0 Cr sp 1 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Spanish Mackerel</td>
<td>Scomber japonicus</td>
<td>Mediterranean sea</td>
<td>22.5 ± 2.2</td>
<td>None</td>
<td>129.2 ± 12.9</td>
<td>2/31 (6.5)</td>
<td>0 Cr sp 2 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Anchovy</td>
<td>Engraulis encrasicoltus</td>
<td>Mediterranean sea</td>
<td>13.1 ± 2.1</td>
<td>12</td>
<td>15.3 ± 9.5 for 135 out of 146</td>
<td>1/146 (0.7)</td>
<td>0 Cr sp 1 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Hake</td>
<td>Merluccius merluccius</td>
<td>North East Atlantic*</td>
<td>38.5 ± 9.9</td>
<td>27</td>
<td>441.5 ± 452.6 for 139 out of 146</td>
<td>1/146 (0.7)</td>
<td>1 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Herring</td>
<td>Clupea harengus</td>
<td>North East Atlantic*</td>
<td>24.9 ± 5.0</td>
<td>20</td>
<td>151.8 ± 94.6</td>
<td>1/106 (0.9)</td>
<td>0 Cr sp 1 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Ling</td>
<td>Molva molva</td>
<td>North East Atlantic*</td>
<td>78.4 ± 20.0</td>
<td>63</td>
<td>3309.5 ± 3076</td>
<td>7/46 (1)</td>
<td>7 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Angler</td>
<td>Lophius piscatorius</td>
<td>Bay of Biscay, English Channel, North sea</td>
<td>40.8 ± 14.2</td>
<td>None</td>
<td>1323 ± 4723.8</td>
<td>0/53 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>European seabass</td>
<td>Dicentrarchus labrax</td>
<td>Bay of Biscay, English Channel, North sea Aquaculture (Mediterranean, Black sea and Norwegian sea)</td>
<td>36.2 ± 4.5</td>
<td>42</td>
<td>535.5 ± 288.3</td>
<td>0/106 *** (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Haddock</td>
<td>Melanogrammu aeglefinus</td>
<td>North East Atlantic*, Bay of Biscay, English Channel, North sea</td>
<td>28.2 ± 11.0</td>
<td>30</td>
<td>345.3 ± 337.2</td>
<td>0/90 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>European plaice</td>
<td>Pleuronectes platessa</td>
<td>North East Atlantic*, English Channel, North sea</td>
<td>35.7 ± 6.03</td>
<td>27</td>
<td>534.9 ± 302.4</td>
<td>0/32 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Salmon</td>
<td>Salmo salar</td>
<td>Aquaculture (North East Atlantic*)</td>
<td>35.7 ± 6.0</td>
<td>None</td>
<td>2330 ± 517.6</td>
<td>0/40 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Common sole</td>
<td>Solea solea</td>
<td>North East Atlantic*, English Channel, North sea</td>
<td>28.6 ± 3.8</td>
<td>24</td>
<td>240.5 ± 114.0</td>
<td>0/106 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Garfish</td>
<td>Belone belone</td>
<td>Bay of Biscay</td>
<td>83</td>
<td>35</td>
<td>862.5</td>
<td>0/2 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Spotted sea basses</td>
<td>Dicentrarchus punctatus</td>
<td>Bay of Biscay</td>
<td>31</td>
<td>None</td>
<td>250</td>
<td>0/1 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Gurnard</td>
<td>Eutrigla gurnardus</td>
<td>Bay of Biscay</td>
<td>21</td>
<td>None</td>
<td>60</td>
<td>0/1 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>School shark</td>
<td>Galeorhinus galeus</td>
<td>Bay of Biscay</td>
<td>76</td>
<td>None</td>
<td>76</td>
<td>0/1 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Megrim</td>
<td>Lepidorhombus whiffiagonis</td>
<td>Bay of Biscay</td>
<td>39</td>
<td>20</td>
<td>485</td>
<td>0/1 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Common dab</td>
<td>Limanda limanda</td>
<td>English Channel, North sea</td>
<td>26</td>
<td>None</td>
<td>95</td>
<td>0/1 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
<tr>
<td>Blackbelled angler</td>
<td>Lophius budegassa</td>
<td>Bay of Biscay</td>
<td>39.3 ± 11.2</td>
<td>None</td>
<td>482 ± 156.1</td>
<td>0/4 (0)</td>
<td>0 Cr sp 0 Cr p 0 Cr ml</td>
</tr>
</tbody>
</table>

(Continued)
TABLE 3 | Continued

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Fishing area</th>
<th>Mean fish size (cm (SD))</th>
<th>Mean fish weight (g (SD))</th>
<th>Fish minimum landing size (cm)</th>
<th>Number of positive individuals (%)</th>
<th>Cryptosporidium species</th>
<th>Number of individuals harboring identified genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue whiting</td>
<td>Micromesistius poutassou</td>
<td>Bay of Biscay</td>
<td>30</td>
<td>165</td>
<td>None</td>
<td>0.41 (0)</td>
<td>Cr sp</td>
<td>0</td>
</tr>
<tr>
<td>European Pollock</td>
<td>Pollachius pollachius</td>
<td>Bay of Biscay</td>
<td>41</td>
<td>4620</td>
<td>None</td>
<td>0.2 (0)</td>
<td>Cr sp</td>
<td>0</td>
</tr>
<tr>
<td>Atlantic bonito</td>
<td>Sarda sarda</td>
<td>Bay of Biscay</td>
<td>37</td>
<td>537.5</td>
<td>None</td>
<td>0.2 (0)</td>
<td>Cr sp</td>
<td>0</td>
</tr>
<tr>
<td>Black seabream</td>
<td>Spondyliosoma cantharus</td>
<td>Bay of Biscay</td>
<td>28.5</td>
<td>315.5 ± 78.1</td>
<td>None</td>
<td>0.1 (0)</td>
<td>Cr ml</td>
<td>0</td>
</tr>
<tr>
<td>Horse mackerel</td>
<td>Trisopterus luscus</td>
<td>Bay of Biscay</td>
<td>29.6 ± 2.9</td>
<td>3551508 (2.3)</td>
<td>None</td>
<td>0.45 (0)</td>
<td>Cr sp</td>
<td>0</td>
</tr>
<tr>
<td>Total of positive fishes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35/1508 (2.3)</td>
<td></td>
<td>8 (5.7%)</td>
</tr>
</tbody>
</table>

*When fishes were collected from retailers, it was not always possible to pinpoint in detail the location of the fishing area within the North East Atlantic. **The obligation to land all fishes only applies to species for which there is a quota or a total allowable catch.*

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</tr>
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</tbody>
</table>

*When fishes were collected from retailers, it was not always possible to pinpoint in detail the location of the fishing area within the North East Atlantic. **The obligation to land all fishes only applies to species for which there is a quota or a total allowable catch.*

The distribution of Cryptosporidium species and genotypes according to the species of fish identified as hosts is shown in Figure 3. Molecular analysis of DNA extracted from each fish stomach or intestine, followed by nested 18S rRNA PCR and sequencing allowed the identification of C. parvum in the intestine of 7 fish and in the stomach of one fish, while the C. molnari-like genotype (#Cryptofish 6) was found in the stomach of one fish. The various novel Cryptosporidium genotypes were detected in the stomach of 20 (76.9%) fish, in the intestine of one (3.8%) fish and in both the intestine and stomach of 5 (19.2%) fish. For the latter case, the same genotypes were found in both organs for each fish. In Figure 4A, the distribution of species/genotypes according to anatomical location is shown. Four gp60 subtypes were identified among the C. parvum positive samples: IIaA13G1R1, IIaA15G2R1, IIaA17G2R1, and IIaA18G3R1.

After examination of histological sections from the digestive tract, the presence of Cryptosporidium-like bodies within the cells of the intestinal epithelium and in apical position was detected in one C. parvum-positive fish (Figure 5). The presence of parasites could not be studied in all positive fishes due to considerable lysis of tissues.

Concerning the second survey, in total, 345 fishes were collected in this regional survey, from fishermen at Boulogne-Sur-Mer. Cryptosporidium exhibited a prevalence of 3.2% (11/345) in edible fish. The parasite was only detected in Pollachius virens and Scomber scombrus, with Pollachius virens the species with the highest prevalence (Table 4), confirming data collected from the national survey. After genotyping, C. parvum was identified in two fishes. Additionally, genotype # Cryptofish 1 was identified in six isolates, genotype # Cryptofish 5 in three and another novel genotype #Cryptofish 7 which exhibited 9.1–10.4% genetic distance from C. molnari was identified in one isolate (Figure 2). Distribution of the different types of Cryptosporidium sequences identified in their corresponding host species is shown in Figure 3. Unfortunately, C. parvum samples from this survey were unsuccessfully subtyped by sequence analysis of the gp60.

The selective extraction of DNA from the organs allowed the identification of C. parvum in the bowel of one fish and the stomach of one fish. The various novel Cryptosporidium genotypes were identified simultaneously in the stomach and bowel of one fish, in the stomach only of four fishes, and in the intestine only of four fishes. In Figure 4B,
the distribution of species/genotypes according to anatomical location is shown.

**DISCUSSION**

This is the first epidemiological and molecular data on the presence of *Cryptosporidium* in edible marine fishes in European waters. Overall, the prevalence of *Cryptosporidium* spp. in sampled fish reached 2.3% in the first campaign and 3.2% in the second campaign. A lower *Cryptosporidium* prevalence in marine fish was found in Western Australia (0.8%) (Reid et al., 2010) and in Papua New Guinea 1.4% (Koinari et al., 2013).

A higher prevalence of *Cryptosporidium* was found in fishes from retailers. As explained before, fish retailers were chosen to complement marine fish species, as the fishing areas covered by them were the same as the research campaigns. One hypothesis to explain the higher prevalence in retail fishes would be that thanks to retailers the collection of all *Pollachius virens* specimens sampled for this study was possible. This fish species had the highest *Cryptosporidium* prevalence contributing to an increase in the overall prevalence of specimens sampled inland. *Pollachius virens* may be more susceptible to this parasite for unknown reasons, further studies need to be done to clarify this aspect. It is not likely that holding the fish prior to processing or the handling itself affected parasite abundance since multiple measures to avoid cross contamination between specimens were taken as a precaution in each experimental step, as described in the materials and methods section.

Previously, other studies described a high prevalence of *Cryptosporidium* spp. in marine fish, but mainly in juveniles. In cultured marine fish, the prevalence of *C. molnari* in *Dicentrarchus labrax* and *Sparus aurata* was 50 and 95% in hatcheries and 58 and 65% in the ongrowing systems, respectively (Sitjà-Bobadilla et al., 2005). Prevalence rates of up to 100% for *C. scophthalmi* were also reported in juvenile turbot (*Psetta maxima*) (Alvarez-Pellitero et al., 2004). Interestingly, in the present study *Cryptosporidium* was not detected in fishes sampled from aquaculture. The absence of parasite species in fishes from aquaculture has been attributed to the strengthened immunity of properly raised fish in aquaculture systems (Mladineo and Poljak, 2014). In addition, farmed fishes are usually fed with fish controlled food. This practice may likely decrease the risk for parasite transmission. In mammals, including calves, pigs and humans, it has been reported that *Cryptosporidium* infections are usually more frequent in neonates and young individuals and...
less prevalent in adults (Ryan et al., 2014). Due to our sampling method, according to size and weight (and sexual maturity when this parameter could be determined) all the analyzed fishes including those from aquaculture were adults (Tables 3, 4).

In addition, the highest prevalence of Cryptosporidium spp. was observed in larger fishes according to the weight and size groupings. A previous study reported a Cryptosporidium prevalence of 5% in adult farmed rainbow trout (Oncorhynchus mykiss), but a higher Cryptosporidium prevalence of 14% was observed in younger animals (Couso-Pérez et al., 2018). As juvenile fish were not analyzed in the present study, the Cryptosporidium prevalence may be underestimated. However, the higher prevalence in larger fish in the present study could be due to parasite accumulation during the lifetime of the fish, as it has been reported for other parasites such as Anisakis (Valero et al., 2000), and longer exposure to contaminated water (Couso-Pérez et al., 2018) or more consumption of prey potentially carrying parasites.

Interestingly, 35.5% of selected sampled species were positive for Cryptosporidium, and eleven new species of fish were identified as potential hosts for Cryptosporidium (Tables 3, 4) increasing the host range for this waterborne parasite. Among these fish hosts the most commonly Cryptosporidium positive were: Pollachius virens (saithe),
Cryptosporidium in Edible Marine Fish

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FIGURE 3 | Global distribution of different types of Cryptosporidium sequences identified at the 18S rRNA gene locus according to fish species found as hosts in both surveys (n = 46). Eleven new species of fish were identified as potential hosts for Cryptosporidium. Cryptofish 1 was the most frequently identified novel genotype. Cryptosporidium genotypes had less host diversity when compared to C. parvum which was found in seven different fish species.

Gadus morhua (cod) and Molva dypterygia (blue ling) belonging to the order Gadiformes and Scomber scombrus (mackerel) belonging to the order Perciformes. As already mentioned above, Pollachius virens was the species in which the parasite was most frequently detected in both national and regional campaigns, with a 16-fold higher risk of being Cryptosporidium positive. Interestingly, in these hosts, one type of sequence (#Cryptofish1) was recorded in the majority of animals either in the national or the regional campaign. The behavior of these fish species could influence parasite transmission. As gadid fishes are social and gregarious (Anders et al., 2017), parasite transmission could occur during cohabitation in dense groupings. This type of transmission was reported in a population of reared fishes experimentally infected with C. molnari (Sitjà-Bobadilla and Alvarez-Pellitero, 2003). Moreover, as the fish species included in our survey were predators, Cryptosporidium could be transmitted to other fishes through the food chain. For example, Mendez-Hermida (Méndez-Hermida et al., 2007) reported the potential role of the live microcrustacean Artemia salina as a Cryptosporidium vehicle for piscine fishes. As fishes in the present study were sampled mainly off shore, contamination through coastal water would appear to be less significant. However, in some fish species, particularly those caught in the Mediterranean sea, and in the case of intertidal fish (moving in and out of the seashore), this kind of transmission cannot be excluded.

One species of Cryptosporidium (C. parvum) and seven genotypes were detected in the present report. The 18S rRNA gene sequences of all C. parvum isolates identified in the present study in either the national or the regional campaign were 99–100% identical to those of C. parvum GenBank reference sequences. The genotypes, however, exhibited substantial genetic distances from C. molnari (7.3–10.1%), with the exception of one genotype (#Cryptofish6) which exhibited 0.5–0.7% genetic distance from C. molnari and was 100% identical to C. molnari-like genotypes from Peach anthias (Pseudanthias dispar) (KR610356) and Murray Cod (HQ585890). This “C. molnari-like genotype” has been previously described (Zanguee et al., 2010), and more recently has also been characterized at the actin locus (Yang et al., 2015), where it exhibited 7.3–8.7% genetic distance from C. molnari and is therefore likely to be a novel species. Similarly, the large genetic distances of genotypes #1–5 and #7 from C. molnari also indicate that they are representative of novel species, even if further analysis at the actin locus is required for confirmation. The high diversity of Cryptosporidium species and genotypes identified in fish in the present and in other studies indicate a long-term association of Cryptosporidium to their fish hosts. In addition, when considering the spectrum of fish hosts, C. parvum seems to have a broad host range (Figure 3).

Seasonality of Cryptosporidium spp distribution was observed in the present study, with maximal prevalence occurring in spring and summer. Consistently, a seasonal distribution of C. molnari was also described in farmed gilthead seabream in Spain, with maximal parasite intensity and prevalence during
FIGURE 4 | Distribution of different types of Cryptosporidium sequences identified at the 18S rRNA gene locus according to the anatomical location. (A) First survey (n = 35). C. parvum was identified in the intestine of 7 fishes and in the stomach of one fish, while the C. molnari-like genotype (#Cryptofish 6) was found in the stomach of one fish. The various novel Cryptosporidium genotypes were detected in the stomach of 20 fish, in the intestine of one fish and in both the intestine and stomach of 5 fish. (B) Second survey (n = 11). C. parvum was identified in the bowel of one fish and the stomach of one fish. The 3 novel Cryptosporidium genotypes were identified simultaneously in the stomach and bowel of one fish, in the stomach only of four fishes, and in the intestine only of four fishes.

the same seasons (Sitjà-Bobadilla et al., 2005). Variations in the intensity of parasitic infections linked to seasonality have been described in marine ecosystems (Wilson et al., 2001) and seasonal changes may influence the physiology of the host including the immune function or the intensity of feeding. This correlation has been also reported in natural infections of different fishes (Sitjà-Bobadilla et al., 2005) and in other Cryptosporidium infected non-piscine hosts including humans (Jagai et al., 2009; Wells et al., 2015), however, it is difficult to know if the seasonality found in the present study could be attributed to the infection or more likely to different fish species caught at each season.

The identification of C. parvum among edible fish hosts is of public health significance, as this species is the most common source of zoonotic infections (Ryan et al., 2014). Previously, studies in Papua New Guinea, Australia and Spain also described the detection of C. parvum in fishes (Reid et al., 2010; Koinari et al., 2013; Certad et al., 2015; Couso-Pérez et al., 2018). The presence of C. parvum in fish samples, and in particular the IIA subtype (IiaA13G1R1, IiaA15G2R1, IiaA17G2R1, and IiaA18G3R1) is maybe related to water contamination by animal and human wastes. Indeed, the zoonotic C. parvum IIA subtype family has mainly been reported in humans and calves in Europe, North America, and Australia (Xiao, 2010; Follet et al., 2011).
Subtypes IIA17G2R1 and IIA15G2R1 were identified in North Atlantic fishes and are the same subtypes that were previously identified in the Geneva lake, France (Certad et al., 2015). Cryptosporidium parvum subtypes IIA18G3R1 and IIA15G2R1 have been detected in foals in Brazil (Inácio et al., 2017) and are also common subtypes in both humans and cattle worldwide including Australia (Zahedi et al., 2016a). The subtype IIA13G1R1 has been identified in lambs, goats, and wild boars in Spain (Díaz et al., 2018).

In a previous study from our group, the presence of C. molnari was detected by nested 18S rRNA PCR and sequencing in freshwater European perch (Perca fluviatilis) filets (Certad et al., 2015). We suggested that filet contamination with the parasite could occur after handling the fish. Although there is no evidence of transmission of Cryptosporidium from fish hosts to mammals (Certad et al., 2010), the presence of the parasite in filets highlighted the risk of Cryptosporidium infection to humans, either during the preparation process of fish or when consuming uncooked or undercooked fish carrying zoonotic species of this parasite.

The findings of the present study are important from a public health point of view considering that some of these fish species are hosts for C. parvum including pilchards, anchovies, and herrings which are commonly eaten after only a slight preparation (for example salted or marinated), and even without gutting (Sánchez-Monsalvez et al., 2005; Mladineo and Poljak, 2014).

In addition, it was reported that fishermen were at risk of cryptosporidiosis after fishing and consuming captured fish (Roberts et al., 2007) and that C. parvum oocysts could be transferred to persons handling blue crabs (Gracey et al., 2007). Moreover, immunosuppressed patients are also at risk of Cryptosporidium infection, either by consumption of raw or undercooked fish or by contact with fish during preparation and handling (McOliver et al., 2009).

Since C. parvum is a zoonotic species, fish carrying this species are a potential source of infection for other animals and in particular for humans, and may also contribute to the

TABLE 4 | Cryptosporidium distribution in wild marine fishes identified at the 18S rRNA gene locus (regional survey).

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Fishing area</th>
<th>Size in cm (SD)</th>
<th>Mean fish weight in g (SD)</th>
<th>Fish minimum landing size (cm)</th>
<th>Number of individuals</th>
<th>Number of Cryptosporidium positive individuals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saithe</td>
<td>Pollachius virens</td>
<td>Eastern English channel, Norwegian sea</td>
<td>49.42 ± 8.8</td>
<td>114.8 ± 53.8</td>
<td>85</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Scomber scombrus</td>
<td>Eastern English channel</td>
<td>30.21 ± 3.5</td>
<td>231.8 ± 99.1</td>
<td>30</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Herring</td>
<td>Clupea harengus</td>
<td>Eastern English channel, Southern North sea</td>
<td>28.32 ± 3.8</td>
<td>202.0 ± 79.4</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Whiting</td>
<td>Merlangius merlangus</td>
<td>Central North sea</td>
<td>31.25 ± 4.4</td>
<td>283.3 ± 116.2</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>11/345 (3.2)</td>
<td>8/0</td>
<td>3/0</td>
<td></td>
<td>0/0</td>
</tr>
</tbody>
</table>

Cr sp, Cryptosporidium sp; Cr p, C. parvum; Cr ml, C. molnari-like genotype.
contamination of the aquatic ecosystem. Nevertheless, it is not still clear if C. parvum can cause a true infection and multiply in fish hosts. The analysis of digestive histological sections from a C. parvum-positive marine fish however, allowed the identification of intracellular round bodies in apical position suggestive of C. parvum developmental stages on epithelial cells. No signal was detected when immunofluorescence using an anti-Cryptosporidium antibody (Crypto Cel immunofluorescence test, Cellabs, Brookvale, New South Wales, and Australia) was performed to confirm the presence of the parasite in fish tissues. This failure was probably due to the fact that formalin progressively cross-links proteins of the parasite, particularly after long time of formalin fixation (Barugahare et al., 2011). Further studies have to be done to confirm this aspect.

In conclusion, this study provides the first epidemiological data regarding the presence of Cryptosporidium in marine edible fish in European waters. New fish species were identified as hosts for this parasite. In addition, the influence of host factors such as the species, the weight and size groupings and environmental factors such as geographical localization or seasonality were shown to have an impact on parasite infection. Finally, the detection of zoonotic Cryptosporidium in fish suggests that the parasite may represent a sentinel for environmental contamination. Since wildlife can potentially contribute to Cryptosporidium contamination of water systems, the identification of the sources/carriers of zoonotic strains is needed for accurate risk assessment (Zahedi et al., 2016b). In addition, as the consumption of raw or thermally inadequately treated fishery products represents novel trends in human eating habits, strategic research in the field is extremely important. Further studies are required to confirm the species status of the seven novel genotypes identified.

**AUTHOR CONTRIBUTIONS**

GC, JF, KG, EF, VV-B, MG, CA-D, and EV conceived and designed the experiments. NG, OH-G, SB-V, YS, BD, and CC performed the experiments. GC, JF, NG, GE, UR, and EV analyzed the data. GE, VV-B, MG, and CA-D contributed to reagents, materials, and analysis tools. GC and UR wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01037/full#supplementary-material

**FIGURE S1** | Hierarchical cluster analysis was used to define variables (R stats package). Five groups were defined ranging from 1-smallest fishes to 5-largest fishes.

**FIGURE S2** | The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Tamura 3-parameter model. The analysis involved 29 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 371 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

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Cryptosporidium

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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