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Genetic Structure of Juvenile Stages of *Phocanema bulbosum* (Nematoda, Chromadorea: Anisakidae) Parasitizing Commercial Fish, Atlantic Cod *Gadus morhua*, and American Plaice *Hippoglossoides platessoides* in the Barents Sea

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Abstract: Atlantic cod *Gadus morhua* and American plaice *Hippoglossoides platessoides* are two of the most commercially valuable species in the Barents Sea (FAO Area 27). They are considered as an important but neglected source of zoonotic risk associated with nematodes from the genus *Phocanema*. The abundance of *Phocanema* spp. in a fish host individual in the Barents Sea may be quite high, which is convenient for studying the genetic structure of its populations. A total of 69 third-stage juveniles of *Phocanema* spp. were isolated from the liver, the mesentery, and the musculature of *G. morhua* and *H. platessoides* and genotyped by the mtDNA *Cox2* gene. Almost all these juveniles (68) were molecularly identified as *P. bulbosum*. The mtDNA *Cox2* gene was also used to reveal the haplotype diversity and the genetic structure of *P. bulbosum*. A comparison of the specimens examined in this study with each other and with the haplotypes previously identified by us in the White Sea showed that there were no significant differences between the groups from different hosts and from different catch areas.

Keywords: nematode; cod; fish infection; helminth; *Phocanema*

1. Introduction

The genus *Phocanema* (Myers, 1959) is a small group of anisakid nematodes comprising five nominal species: *Phocanema azarasi* (Yamaguti and Arima, 1942), *Phocanema bulbosum* (Cobb, 1888), *Phocanema cattani* (George-Nascimento and Urrutia, 2000), *Phocanema decipiens* (Krabbe, 1878) *sensu stricto*, and *Phocanema krabbei* (Paggi, Mattiucci, Gibson, Berland, Nascetti, Cianchi and Bullini, 2000) [1,2].

Phocanema spp. uses pinnipeds as definitive hosts, and marine crustaceans and fishes as intermediate and paratenic hosts, respectively [3]. However, it is hardly appropriate to consider crustaceans in the life cycle of this nematode, as well as other anasakids, as an intermediate host, since the juveniles of these parasites do not have any development in them until the next (higher) stage. The embryonic and initial stages of postembryonic

development of *Phocanema* spp., culminating in the formation of the third-stage juvenile, take place in the egg. The third-stage juvenile emerging from the egg is swallowed by benthic crustaceans and, moving along trophic chains, accumulates in fish [4,5]. Third-stage juveniles of these nematodes cause an important fish-borne zoonosis (e.g., [6–13]).

Phocanema bulbosum was originally described as *Ascaris bulbosa* by Cobb in 1888 [14], but *A. bulbosa* is considered a synonym of *Ascaris decipiens* Krabbe, 1878 (=*Phocanema decipiens*) as recognized later by many authors [1,15,16]. Paggi et al. [17], Deco et al. [18], and Mattiucci et al. [19] showed that *P. decipiens* (as member of *Pseudoterranova* Mozgovoi, 1951 in these authors) is a species complex consisting of at least five species with a clear genetic differentiation. These species were provisionally designated with letters (A, B, C, etc.). Mattiucci et al. [19] resurrected the name *Pseudoterranova bulbosa* for *Pseudoterranova decipiens* C. However, the monophyly of the genus *Pseudoterranova* is not supported by recent research [2,20]. Bao et al. [2] proposed the resurrection of the genus *Phocanema*, with *Ph. decipiens* as the type species, to encompass *P. bulbosum* and several other species. The definitive hosts of *P. bulbosum* are *Erignathus barbatus* (Erxleben, 1777), *Halichoerus grypus*, *Pusa hispida,* and probably *Monachus monachus* (e.g., [16–19,21]. Third-stage juveniles of *P. bulbosum* were discovered in pleuronectid, gadiid, macrourid, sebastid, and cottid fishes (e.g., [16,22,23]. This nematode has a broad geographic distribution in temperate, subarctic, and arctic seas of the Northern Hemisphere (e.g., [17,19,21,23–26]. The data of [27] also indicate that *P. bulbosum* might be present in the Mediterranean Sea. In the Barents Sea, it was recorded in *E. barbatus* and various fishes caught in the northern and western areas [2,17,24,28–30]. There are no records of *P. bulbosum* in the southern and eastern areas of this sea.

The population genetic structure (the distribution of genetic variation in time and space) affects the response of a species to selection pressures, and so shapes its evolution [31]. Studying the population genetics of parasites provides insights into their infection dynamics [32] and the ways it affects the entire community [33]. Taxonomic issues based on morphology can be elucidated with the help of genetic methods, which advance cladistics to a level inaccessible to morphology, including the allocation of cryptic species, as shown, in particular, for nematodes [34]. Patterns of the population genetic structure of a parasite can provide information on the present and past migrations of their hosts [35–37]. Among the molecular markers in use, the *Cox2* mitochondrial gene is one of the best suited to assess the population genetic structure and the phylogeography of anisakids species (e.g., [36–39]). However, only a few sequences of *Cox2* gene are available for *P. bulbosum* [30,40].

The aims of our study were to genetically identify the third-stage juveniles of *Phocanema* spp. from the Atlantic cod *G. morhua* and the American plaice *H. platessoides* caught in the southern and the eastern parts of the Barents Sea and to assess the genetic diversity and the population genetic structure of *P. bulbosum* in the region. The overall aim of the study was to advance the knowledge of the distribution and population structure of the *Phocanema* species infecting commercial fish in the Barents Sea through the use of genetic analysis.

2. Materials and Methods

2.1. Sampling and Morphological Examination

The host fishes were caught by the bottom trawl in the period from 26 February 2021 to 17 February 2022 in areas of the Barents Sea within the Russian Exclusive Economic Zone (Figure 1). In total, 225 specimens of *Gadus morhua* (TL 22–122 cm, mean 90.46 ± 3.08; weight 178–11, 150 g, mean 7172 ± 471.61; male/female ratio 1:3.91) and 375 specimens of *Hippoglossoides platessoides* (TL 21–47 cm, mean 35.92 ± 0.86; weight 72–1126, mean 490.18 ± 38.10; male/female ratio 1:4.42) were examined. The fishes were caught from RV "Vilnius" under permissions no. 512021030001NI and no. 512022030007NI of 18 February 2021 and 17 January 2022, accordingly, issued by the Federal Agency for Fisheries. Parasitological dissection was performed according to the standard methodology [41,42]. The juveniles of *Phocanema* spp. were fixed with 96% ethanol and stored at −20 °C. Parasito-

logical indices (prevalence, intensity, and mean intensity) were calculated in accordance with [43].

For the purpose of this study, the catch locations were divided into 6 groups (Figure 1, G1–G6), and these groups were compared in order to reveal a geographical trend, if any, in the distribution of the haplotypes in the study area.

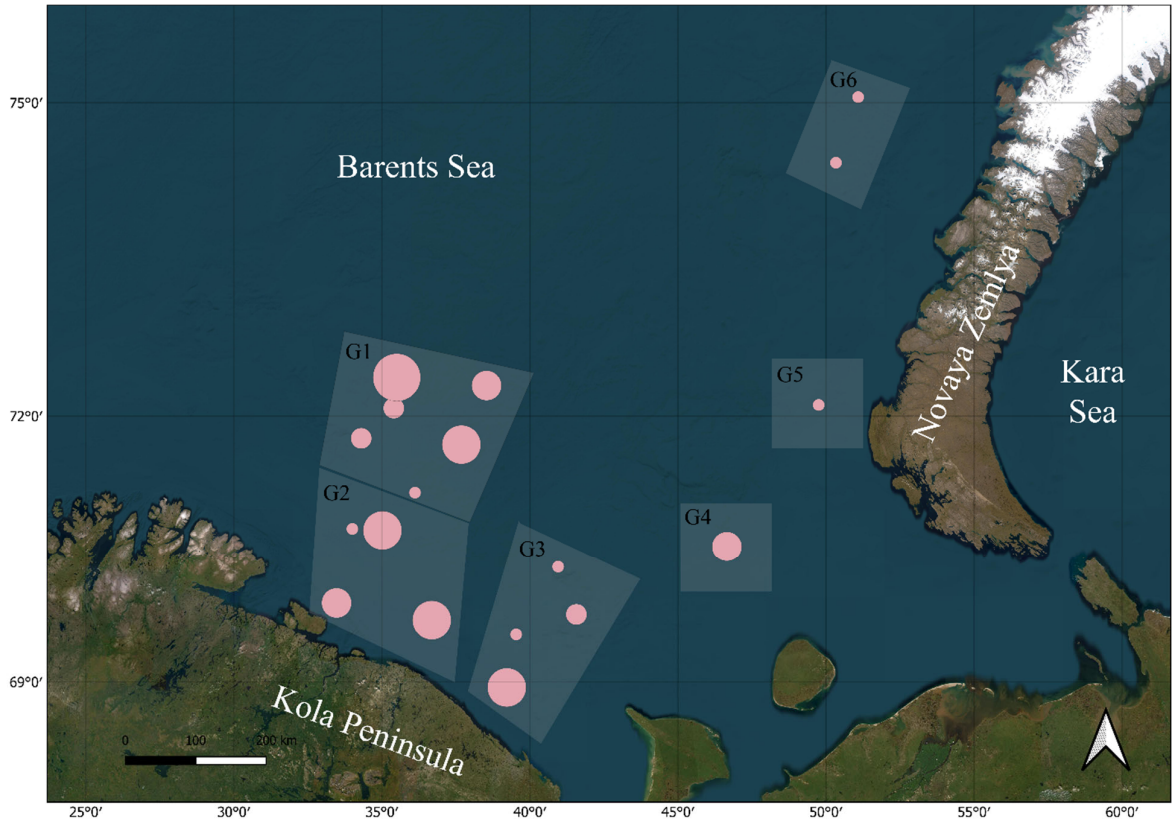

Figure 1. Sampling map. Pink points are sampling locations. Point size represents the number of *Phocanema bulbosum* larvae collected at the location (from 1 to 21). G1–G6 are geographical groups of locations corresponding to the haplotype structure in Figure 2.

2.2. DNA Extraction, Amplification, and Sequencing

Total DNA was extracted separately from each of the 69 third-stage juveniles of *Phocanema* using PALL™ AcroPrep 96-well purification plates by PALL Corp. following a protocol by [44]. The DNA samples were used as a template for amplification of partial cytochrome c oxidase subunit II (*Cox2*) (~671 bp). *Cox2* of *Phocanema* juveniles were amplified by a polymerase chain reaction (PCR) with primers 210 (5′CACCAACTCTTAAAATTATC3′) and 211 (5′TTTTCTAGTTATAGATTGRTTYAT3′) described by [36]. PCRs were carried out in a 25-µL reaction volume (5 µL of 5× HotTaq Red buffer (Eurogen Lab, Moscow, Russia), 0.5 µL of HotTaq polymerase (Eurogen Lab, Moscow, Russia), 0.5 µL of dNTP (50 µM stock), 0.3 µL of each primer (10 µM stock), 1 µL of genomic DNA, and 17.4 µL of sterile water). Amplification was performed according to the following protocol: 3 min denaturation at 94 °C, 34 cycles of 30 s at 94 °C, 60 s at 46 °C, and 1.5 min at 72 °C, and 10 min elongation at 72 °C. The negative control was amplified using both primers.

Both strands of each amplicon were sequenced with the BigDye Terminator v3.1 sequencing kit (Applied Biosystems. Waltham, MA, USA) and NovaDye Terminator sequencing kit (Thermo Fisher, Waltham, MA, USA). Sequencing reactions were analyzed by capillary electrophoresis on ABI 3500 Genetic Analyser (Thermo Fisher, Waltham, MA, USA) or Nanophore-05 (Syntol, Moscow, Russia) at the Core Centrum of Koltsov Institute of Developmental Biology (Moscow, Russia). All newly obtained sequences were deposited

in GenBank (NCBI) (Table 1). Raw reads for each gene were assembled and checked for improper base-calling using GeneiousPro 10.0.9 (Biomatters, Auckland, New Zealand) and the sites identified in this way were further modified.

The obtained sequences of *Cox2* gene were processed using Geneious 8.1.8 software package [45], ClustalW alignment. Estimates of evolutionary divergence (p-distances) were made with the use of MEGA XI software (ver. 11.0.11) [46].

Table 1. List of the *Cox2* sequences obtained in this study.

GenBank Accession Number	Host	Locality *
OQ731840–OQ731844, OQ731846, OQ731848, OQ731849, OQ731867	Atlantic cod *Gadus morhua*	G1
OQ731832–OQ731839		G2
OQ731845, OQ731850–OQ731866		G3
OQ731847		G5
OQ731868–OQ731874 OQ731882–OQ731887	American plaice *Hippoglossoides platessoides*	G1
OQ731888–OQ731896		G2
OQ731875, OQ731897–OQ731896		G3
OQ731876–OQ731879		G4
OQ731880, OQ731881		G6

* following Figure 1.

2.3. Genetic Diversity and Haplotype Analysis

The nucleotide sequences were translated into the format suitable for constructing a median-joining haplotype network in the PopArt program [47]. The genetic differentiation index (FST) and *p*-value were calculated using the Arlequin 3.5.1.3 program [48], and a FaBox 1.41 converter was used to convert the fasta file into the format required for calculation [49]. The average number of nucleotide substitutions (K), the number of polymorphic sites (S), the number of haplotypes (h), haplotypic diversity (Hd), and nucleotide diversity (Pi) in each sample and across all the samples were analyzed in DnaSP 5.10.01 software package [50]. Tajima's neutrality tests [51] were performed using Arlequin v.3.5.2.2 [48] with 1000 non-parametric permutations ($p = 0.05$). Sequences of mtDNA *Cox2* genes from the waters of Norway OP418114-OP418115 [30] and from the White Sea previously obtained by us (OQ274151-OQ274172) were retrieved from GenBank (NCBI).

3. Results

Third-stage juveniles of *Phocanema* spp. localized in the liver, mesentery, and less commonly, in the muscles of the fish. Parasitological indices are given in Table 2.

Table 2. Occurrence of *Phocanema* spp. third-stage juveniles in two studied fish species.

Localities	Atlantic Cod *Gadus morhua*				American Plaice *Hippoglossoides platessoides*			
	n	Prevalence, %	Intensity, min–max	Mean Intensity	n	Prevalence, %	Intensity, Min–Max	Mean Intensity
G1-G3	150	4.7	1–3	1.57	250	15.6	1–6	1.62
G4-G6	75	4.0	1–2	1.66	125	6.4	1–2	1.25
Total	225	4.4	1–3	1.60	375	12.5	1–6	1.55

The sequences of almost all juveniles (68 out of 69) genotyped in this study were molecularly similar (p-distance $\leq$ 0.021%) with those of *P. bulbosum* available in GenBank (NCBI), namely, with the sequences deposited under numbers HM147280, KU558720, OP418114, and OP418115. At the same time, one juvenile from our material (OQ731831) was molecularly identical with *P. decipiens sensu stricto* (OK338713 and KU558723).

The alignment of all *P. bulbosum* sequences (474 bp) contained 36 variable sites (S), which resulted in 31 haplotypes. Genetic diversity indices for localities and hosts are shown in Table 3. The overall value of haplotype diversity (Hd) was 0.890, that of nucleotide diversity (Pi) was 0.00516, while the value of the average number of nucleotide differences (K) was 2.430. The genetic diversity indices calculated for *P. bulbosum* from each locality showed a similar haplotype diversity, which ranged between 0.801 for G1 and 1.000 for G6. No trends in the changes in Hd and Pi were revealed. Neutrality test, Tajima's D, showed that negative values were statistically significant (p-value < 0.05) for all divisions (Table 3), except for samples G2 and G4. Statistically significant negative values may indicate the effects of the purifying selection on the gene and/or that populations underwent recent expansion.

The value of the FST between *P. bulbosum* samples obtained from the Atlantic cod *G. morhua* and the American plaice *Hippoglossoides platessoides* in the Barents Sea sorted by hosts was negative. The values of the FST between *P. bulbosum* samples sorted by locality were also negative in all cases except pair G1–G2 (FST = 0.033, $p > 0.05$). Thus, in both cases of sorting, the samples are not statistically different.

Table 3. Genetic characteristics of *Phocanema bulbosum* samples in the Barents Sea (N—number of sequences, S—number of polymorphic sites, h—number of haplotypes; Hd—haplotypic diversity, m—error of mean, σ—standard deviation of Hd, K—number of nucleotide substitutions, Pi—nucleotide diversity, D—Tajima's neutrality test, and p-value—Tajima's neutrality test p-value). Significant p-values ($p < 0.05$) are highlighted in bold.

Samples	N	S	h	Hd ± m	σ	K	Pi	D	*p*-Value
G1	22	19	12	0.801 ± 0.007	0.088	2.299	0.005	−2.067	0.010
G2	17	12	9	0.890 ± 0.003	0.054	2.382	0.005	−1.234	0.109
G3	22	19	14	0.922 ± 0.002	0.045	2.861	0.006	−1.668	0.018
G4	4	4	3	0.833 ± 0.049	0.222	2.000	0.004	−0.780	0.187
G5	1	N/A	1	N/A	N/A	N/A	N/A	N/A	N/A
G6	2	1	2	1.000 ± 0.250	0.5	1.000	0.002	0.000	1.000
Cod	36	20	15	0.846 ± 0.002	0.042	1.965	0.004	−1.996	0.011
Flounder	32	29	21	0.921 ± 0.001	0.040	2.972	0.006	−2.092	0.005
All	68	36	31	0.890 ± 0.001	0.027	2.430	0.005	−2.159	<0.00001

Neither the grouping by geographic localities (Figure 2) nor the grouping by the host (Figure 3) revealed any trends in the distribution of *P. bulbosum* juveniles in the Barents Sea. Four haplotypes were present in two or more sampling areas (G1–G6). One of them (OQ731840 and identical ones, Figure 3 No.1) was found in all the six sampling areas (Figure 2). Another one (OQ731832 and identical ones, Figure 3 No. 3) was found in G1–3. The third one (OQ731834 and identical ones, Figure 3 No. 13) was found in G2–4. The fourth one (OQ731867 and identical ones, Figure 3 No. 12) was found in G1 and G3. The network revealed 27 haplotypes that were found only in a single sampling area.

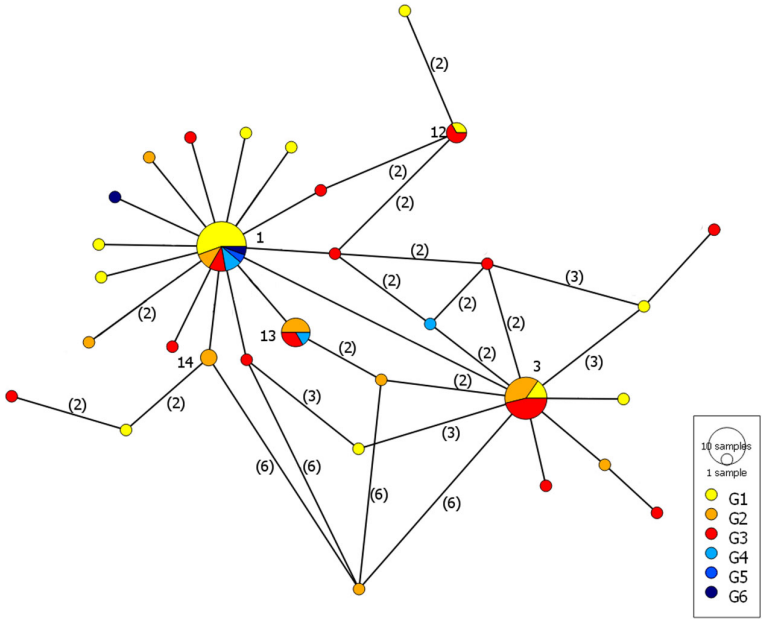

Figure 2. Median-joining haplotype network of *Phocanema bulbosum Cox2* gene sequences obtained from the Atlantic cod *Gadus morhua* and the American plaice *Hippoglossoides platessoides* in the Barents Sea sorted by geographic locations within the boundaries of the Russian Exclusive Economic Zone (Table 1, Figure 1). Circle size represents the frequency of the haplotype. Hatch marks show the number of mutations distinguishing the haplotypes.

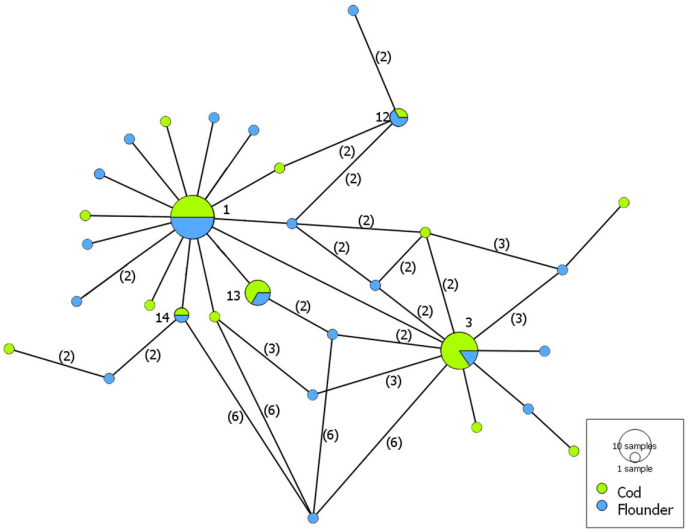

Figure 3. Median-joining haplotype network of *Phocanema bulbosum Cox2* gene sequences obtained from muscular sections of the Atlantic cod *Gadus morhua* and the American plaice *Hippoglossoides platessoides* in the Barents Sea sorted by hosts. Circle size represents the frequency of the haplotype. Hatch marks show the number of mutations distinguishing the haplotypes.

Five haplotypes were shared by both hosts (Figure 3). Haplotype OQ731840 and identical ones (Figure 4, No. 1) together with OQ731832 and identical ones (Figure 4, No. 3) were the most frequent (12 and 9 sequences, respectively). Ten haplotypes were present only in the Atlantic cod, while 16 haplotypes were present only in the American plaice (Figure 4).

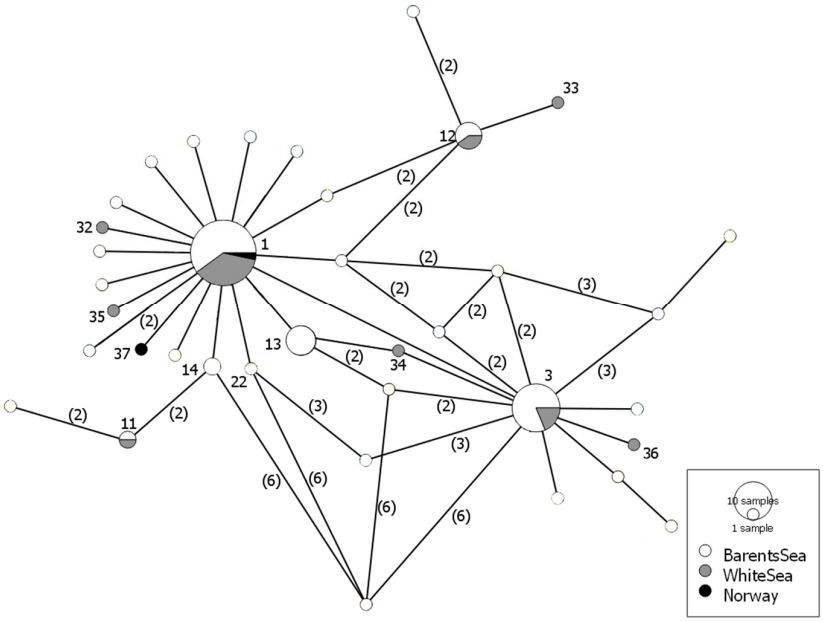

Figure 4. Median-joining haplotype network of *Phocanema bulbosum* sorted by the Barents Sea (Russian waters, this study), the White Sea (our previous studies), and the waters of Norway [30].

Only two sequences of the *Cox2* gene of *P. bulbosum* specimens from the Barents Sea were available in GenBank before this study: OP418114 and OP418115. They belong to specimens collected from the Atlantic cod. Sequence OP418114 matches our haplotype number 1 (OQ731840 and identical ones) (Figure 4, 'Norway'). Sequence OP418115 has no match and forms a separate haplotype (one substitution).

4. Discussion

There were no records of *P. bulbosum* juveniles in fish from the Russian waters of the Barents Sea before our study, although *Phocanema* spp. was the dominant group of helminths in terms of occurrence in the Atlantic cod and the American plaice in all previous parasitological research in this area. Our finding of this species was undoubtedly due to the use of improved identification methods. It is obvious that *P. bulbosum* was previously reported from the Russian waters of the Barents Sea as *P. decipiens sensu lato* [52–56].

The Atlantic cod and the American plaice are common and widespread fishes in the Barents Sea, occurring, on average, in 78–93% of bottom trawl catches in most of this area all year round [57]. The cod makes an extended autumn–winter migration from the southern, eastern, central, and northwestern regions to the southwestern part of the sea, where most of its mature individuals spawn off the northwestern coast of Norway in March–April. During the summer months, it returns to the feeding areas [58]. The American plaice, on the contrary, makes only minor local migrations, being distributed all year round over most of the Barents Sea.

The diet of cod juveniles (age 1–2 years, TL up to 24 cm) is dominated by Hyperiidae, Euphausiidae, and the northern prawn (shrimp) *Pandalus borealis*. As it grows, the cod starts to consume more small pelagic fish such as capelin, *Mallotus villosus* (Müller, 1776), polar cod *Boreogadus saida* (Lepechin, 1774), and herring *Clupea* spp. and juvenile bottom fish such as the Atlantic cod, haddock *Melanogrammus aeglefinus* (Linnaeus, 1758), *Sebastes* spp., American plaice, etc. [57].

The diet of juvenile *H. platessoides* (TL up to 15 cm) consists of benthic organisms such as echinoderms (mainly Ophiuroidea) and annelids, and less often, Euphausiidae, *Parathemisto* sp. and Mysidacea. Larger individuals switch to the predominant consumption of various fish (mainly capelin, polar cod, and Atlantic cod fry), and less often, shrimps (Pandalidae) and bivalve molluscs. At an older age (length more than 30 cm), in addition to the food objects mentioned above, *H. platessoides* also consumes fishery waste such as

trimmings and homogenized residues from the processing of fish and invertebrates on fishing and factory vessels [57].

Juveniles of the genus *Phocanema* commonly parasitize bottom fish, which become infected while feeding on bottom crustaceans (amphipods, copepods, mysids, and isopods), polychaetes, and molluscs as well as other fish infected with this nematode (its intermediate or paratenic hosts) [59–63]. This explains the fact that *H. platessoides* and *G. morhua* are paratenic hosts in the life cycle of these nematodes. Significantly higher infection with *P. bulbosum* in the American plaice in the southwestern part of the Barents Sea (prevalence 15.60% vs. 6.40% in the north-east) may be due to its active consumption of fishing waste in this area. The absence of geographical differences in the infection of cod with this nematode is due to its migratory activity: Russian and Norwegian sampling sites are located on the cod spawning migration routes from the southern part of the Barents Sea.

Levsen et al. [30] studied infection of the Atlantic cod, the saithe, *Pollachius virens* (Linnaeus, 1758), and the haddock *M. aeglefinus* with *P. bulbosum* in the Barents Sea, namely, at 'Helmsøybanken' bank (approximately 71°N 25°E) off West-Finnmark, Norway. According to these authors, the mean prevalence of this parasite with the Atlantic cod infection in 2019 was 37.7%, while the maximal intensity never exceeded 14, being 1.3 ± 2.7 on average. These values are much greater than those recorded in our study (Table 2). Though these differences could be due to methodology, e.g., the use of a hydraulic pressing device and 366 nm UV-light by Levsen et al. [30], it cannot be ruled out that they reflect real differences in infection between sampling sites. Najda et al. [24] also presented data on the infection of *G. morhua* by *P. bulbosum* in the Barents Sea with comparable mean intensity of infection (1.2 ind.), but without genetic data on *P. bulbosum.*

Sequences of *P. bulbosum* that we collected earlier from the White Sea cod, *Gadus morhua marisalbi* (Derjugin, 1920), the navaga *Eleginus navaga* (Walbaum, 1792), and the shorthorn sculpin, *Myoxocephalus scorpius* (Linnaeus, 1758) in the White Sea (Velikaya Salma Straight, Lomonosov Moscow State University White Sea Biological Station) were deposed in GenBank with accession numbers OQ274151-OQ274172. Despite the obvious isolation caused by a low depth of the White Sea Throat (app. 30 m) and freshening of the White Sea, especially in the Dvina Bay and the Onega Bay, where salinity could be 8–12‰, we see no clear differentiation between the seas (Figure 4). Three haplotypes were present in both seas. The network revealed five haplotypes that were found only in the White Sea, twenty four haplotypes found only in the Barents Sea, and one only in Norwegian waters.

The genetic diversity indices calculated for mtDNA *Cox2* sequences of *P. bulbosum* (Table 3) from all sampling localities in the Barents Sea within the boundaries of the Russian Exclusive Economic Zone (G1-G6) showed a high haplotype diversity (Hd) but a low nucleotide diversity (Pi). A single haplotype (OQ731840 and identical ones, Figure 4, No. 1) clearly dominated, being distributed in all six localities in the Barents Sea (Figure 1) as well as in the White Sea (Figure 4, No. 1). It indicates a clear connection between the samples of *P. bulbosum* from the Barents Sea and from the White Sea. Moreover, the distribution of this haplotype seems even wider since it corresponds to the haplotype (OP418114) of *P. bulbosum* from the waters of Norway (Figure 4). A high frequency of this haplotype and its wide geographic and host range might mean that it is the ancestral haplotype of this species.

5. Conclusions

This is the first study to reveal the genetic diversity of *P. bulbosum*. Our molecular data on the *Cox2* gene and the comparison of the available sequences from the White Sea and from the waters of Norway showed the genetic homogeneity of third-stage juveniles of *P. bulbosum* and the absence of differences between geographic locations and host species. Using different genetic markers to assess genetic variation of *Phocanema* spp. led to the discovery of a similar picture of low genetic distances between conspecific populations, even though they were collected thousands of kilometers apart, indicating high levels of gene flow. Further studies involving more sequenced specimens and host species

might reveal some patterns in the distribution of *P. bulbosum* larvae in Russian waters. However, given the unity and scale of the marine ecosystem, the current picture is quite consistent with the modern concepts of the distribution of generalist helminths in marine ichthyocenoses. This study provides useful information about the genetic diversity of these parasites in the Barents Sea.

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