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New data on life cycles for three species of Fellodistomidae (Digenea) in the White Sea

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Abstract

Few digeneans of the family Fellodistomidae are known from the Russian Arctic seas. The taxonomic status of these species, their life cycles and host range raised recurrent questions, some of which remain unanswered. To revise the species composition and life cycles of fellodistomids in the White Sea, we searched for them in several known and suspected hosts: wolffish, flatfishes (definitive), gastropods of the family Buccinidae (second intermediate) and protobranch bivalves (first intermediate). Species identification was based both on morphology and 28S ribosomal RNA gene sequences. We found *Fellodistomum agnotum* in the White Sea for the first time. *Buccinum undatum* was proved to be intermediate host of both *F. agnotum* and *Fellodistomum fellis*, and metacercariae of *F. fellis* were registered from two more buccinid species: *Buccinum scalariforme* and *Neptunea despecta*. We also found metacercariae of *F. agnotum* and *F. fellis* producing eggs in the second intermediate host. Two fellodistomids were found in protobranch bivalves: sporocysts and cercariae of *Steringophorus furciger* in *Nuculana pernula*, and sporocysts with large furcocercous cercariae in *Ennucula tenuis*. The latter were identified as *F. agnotum* by molecular analysis; thus, the entire life cycle of this species was reconstructed.

Introduction

Digeneans of the family Fellodistomidae are generally restricted to the marine environment, with sexual adults (maritae) inhabiting fishes, and bivalves acting as first intermediate hosts (Bray & Gibson, 1980). Most fellodistomids are characterized by three- or two-host life cycles (Køie, 1979, 1980; Cribb *et al.*, 2003), though with tendency to progenesis and lifecycle truncation up to one host (Stunkard & Uzmann, 1959).

In the White Sea, the only broad-scale study of fish parasites was held more than 60 years ago (Shulman & Shulman-Albova, 1953). Two fellodistomid species have been recorded in that study: *Fellodistomum fellis* (Olsson, 1868) in wolffish *Anarhichas lupus* Linnaeus, 1758 and *Steringophorus furciger* (Olsson, 1868) Odhner, 1905 in flatfishes (Pleuronectidae). Along with *F. fellis*, *Fellodistomum agnotum* Nicoll, 1909 is highly abundant in wolffish all over the North Atlantic and adjacent Arctic (Polyanski, 1955; Bray & Gibson, 1980; Bray, 1987). *Fellodistomum fellis* inhabits the gall bladder (younger specimens are occasionally found in the bile duct), and *F. agnotum*, the subglobular chamber at the base of the bile duct (Bray, 1987). *Fellodistomum agnotum* exhibits morphological features intermediate between *F. fellis* and *S. furciger* (Bray & Gibson, 1980); thus, the taxonomic status and life cycles of these species have been questioned for a long time.

Among the three listed species, the complete life cycle was only elucidated for *S. furciger* through morphological and experimental data: protobranch bivalves *Nuculana* spp. serve as its only intermediate host (Chubrik, 1966; Køie, 1979). The life cycle of *F. fellis* with *Ennucula tenuis* (Montagu, 1808) as the first intermediate host and *Ophiura sarsi* Lütken, 1855 as the second intermediate host was described by Chubrik (1952, 1966), but later it was rejected (Bray & Rollinson, 1985). Metacercariae of *F. fellis* and *F. agnotum* were found in the stomach of a common whelk *Buccinum undatum* Linnaeus, 1758 (Bray, 1987; Køie & Thulin, 1994). The first intermediate hosts of *F. fellis* and *F. agnotum* remain unknown, though they are suspected to be protobranch (Bray & Rollinson, 1985) or mytilid (Køie & Thulin, 1994) bivalves.

In this paper, we record *F. agnotum* from the White Sea for the first time, provide evidence for the occurrence of *F. agnotum* and *F. fellis* metacercariae in buccinid gastropods, identify *E. tenuis* as the first intermediate host of *F. agnotum* and verify the life cycle of *S. furciger* by molecular analysis.

Material and methods

To obtain fellodistomid sexual adults, we collected wolffish and three species of flatfish (table 1) from the White Sea, Kandalaksha Bay, Keret Archipelago during summer 2019.

Table 1. Fellodistomid adults in wolffish and flatfishes.

Host species	Number of host specimens	*Fellodistomum fellis*		*Fellodistomum agnotum*		*Steringophorus furciger*	
		Prevalence	Intensity	Prevalence	Intensity	Prevalence	Intensity
Anarhichas lupus Linnaeus, 1758	16	87.5%	1–67 (14.9 ± 4.2)	50%	1–10 (4.6 ± 0.8)	–	–
Limanda limanda (Linnaeus, 1758)	30	–	–	–	–	16.7%	2–10 (4.8 ± 0.6)
Liopsetta glacialis (Pallas, 1776)	3	–	–	–	–	–	–
Platichthys flesus (Linnaeus, 1758)	26	–	–	–	–	–	–

For intensity, the range of values is followed by mean and standard error of mean in parenthesis.

Table 2. Isolates, their origin and GenBank accession numbers for 28S rDNA sequences.

Organism	Stage	Host	ID	GenBank accession number
Fellodistomum fellis	Sexual adult	*Anarhichas lupus*	72.48s	MT216735
			137.48s	MT216756
			141.48s	MT216734
			162.48s	MT216733
	Metacercaria	*Buccinum undatum*	42.48s	MT216752
			224.48s	MT216743
			233.48s	MT216738
			258.48s	MT216736
			266.48s	MT216746
			267.48s	MT216739
			314.48s	MT216745
			322.48s	MT216744
		Neptunea despecta	341.48s	MT216741
		Buccinum scalariforme	116.48s	MT216755
Fellodistomum agnotum	Sexual adult	*Anarhichas lupus*	48.48s	MT216737
			140.48s.1	MT216747
			140.48s.2	MT216748
			140.48s.3	MT216749
			140.48s.4	MT216750
			140.48s.5	MT216751
			153.48s	MT216757
	Metacercaria	*Buccinum undatum*	268.48s	MT216742
			269.48s	MT216732
			312.48s	MT216740
	Sporocyst with cercariae	*Ennucula tenuis*	2018-2	MT216758
Steringophorus furciger	Sexual adult	*Limanda limanda*	66.48s	MT216753
	Sporocyst with cercariae	*Nuculana pernula*	111.48s	MT216754

They were dissected and the guts were examined under a stereomicroscope in the physiological solution. Fellodistomids obtained were identified and flat-fixed in 96% ethanol.

Gastropods of the family Buccinidae – namely, *B. undatum*, *Buccinum scalariforme* Møller, 1842 and *Neptunea despecta* (Linnaeus, 1758) – were collected during summer–autumn 2019 at three different locations of the White Sea: around the Keret

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Table 3. Fellodistomid sporocysts and cercariae in protobranch bivalves.

Year	Location	Host species	No. of hosts examined	No. of hosts infected	Prevalence
2018	Bolshoy Solovetsky Island	*Ennucula tenuis*	4	1	25%
		Yoldia hyperborea	5		
2019	Keret Archipelago	*Ennucula tenuis*	1		
		Yoldia hyperborea	48		
		Nuculana pernula	209	2	1%
		Portlandia arctica	1		
	Velikaya Salma Strait	*Ennucula tenuis*	3		
		Yoldia hyperborea	4		
		Nuculana pernula	6		
		Portlandia arctica	7		

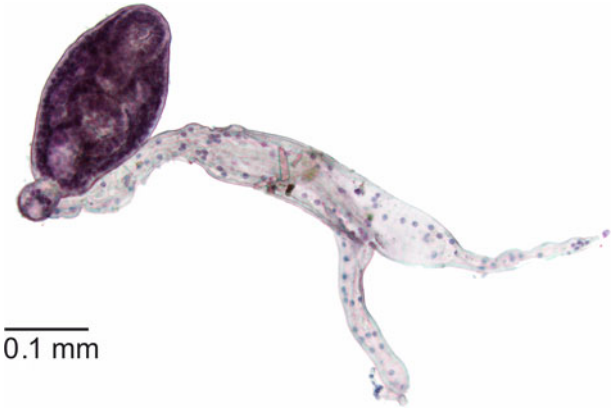

Fig. 1. *Fellodistomum agnotum* cercaria from *Ennucula tenuis*.

Archipelago, in Velikaya Salma Strait (both Kandalaksha Bay), and near Bolshoy Solovetsky Island (Onega Bay). During summer 2019, we collected all available species of protobranch bivalves at the first two locations: *E. tenuis*, *Nuculana pernula* (Müller, 1779), *Portlandia arctica* (Gray, 1824) and *Yoldia hyperborea* (Gould, 1841). Few *E. tenuis* and *Y. hyperborea* were collected in summer 2018 near Bolshoy Solovetsky Island. Molluscs were dissected in sea water and fellodistomid-like larvae were fixed in 96% ethanol. Photos of live worms were made with a Canon EOS 70D (Canon, Inc., Tokyo, Japan) camera and adaptor MFU (LOMO, Saint Petersburg, Russia) for the stereomicroscope MBS-10 (LOMO, Saint Petersburg, Russia).

For the morphological observations, worms were stained with Erlich's haematoxylin for 2–10 min, followed by destaining in 70% ethanol with 0.1 M hydrogen chloride for 1–12 h. Specimens were dehydrated through a series of graded alcohols and mounted in BioMount medium (Bio Optica, Milan, Italy). We photographed whole mounts with the compound microscope Leica DM 2500 (Leica Microsystems, Wetzlar, Germany) and the camera Nikon DS Fi3 (Nikon, Tokyo, Japan). Measurements were made in Fiji software (Schindelin *et al.*, 2012).

Individual sporocysts, metacercariae and maritae were removed from 96% ethanol for DNA extraction (table 2). We then added 200 µl 5% Chelex-100 chelating resin (200–400-mesh particle size) (BioRad, Hercules, California, USA) and 2 µl

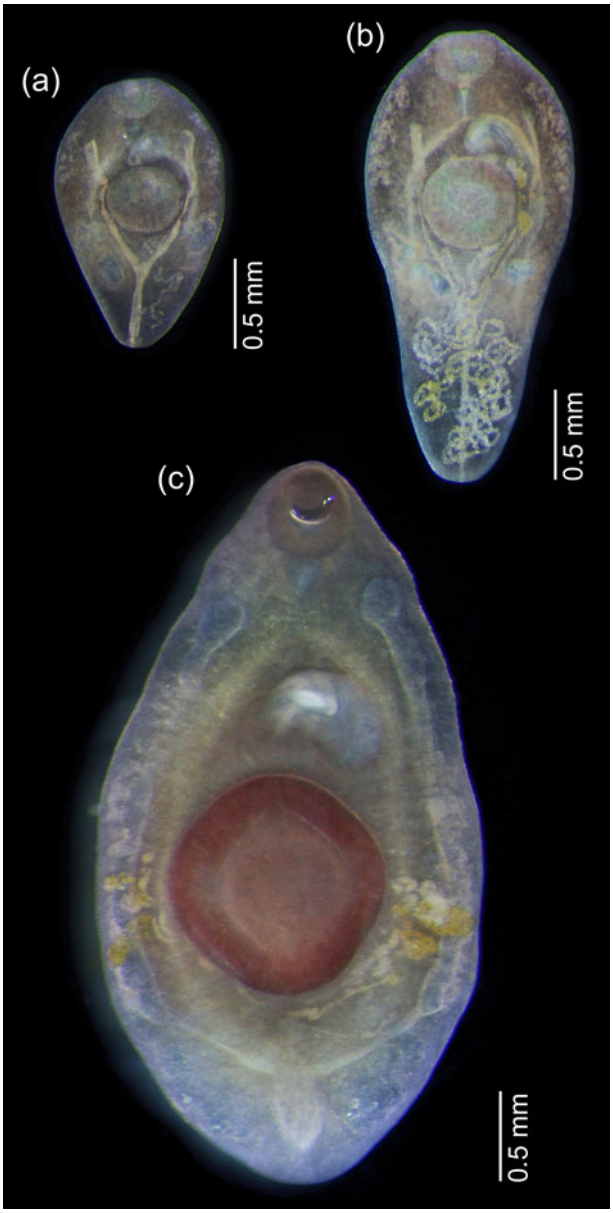

Fig. 2. Live metacercariae from *Buccinum undatum*: (a, b) *Fellodistomum agnotum*, note eggs in uterus; (c) *F. fellis*, the largest specimen. All worms are shown at the same scale.

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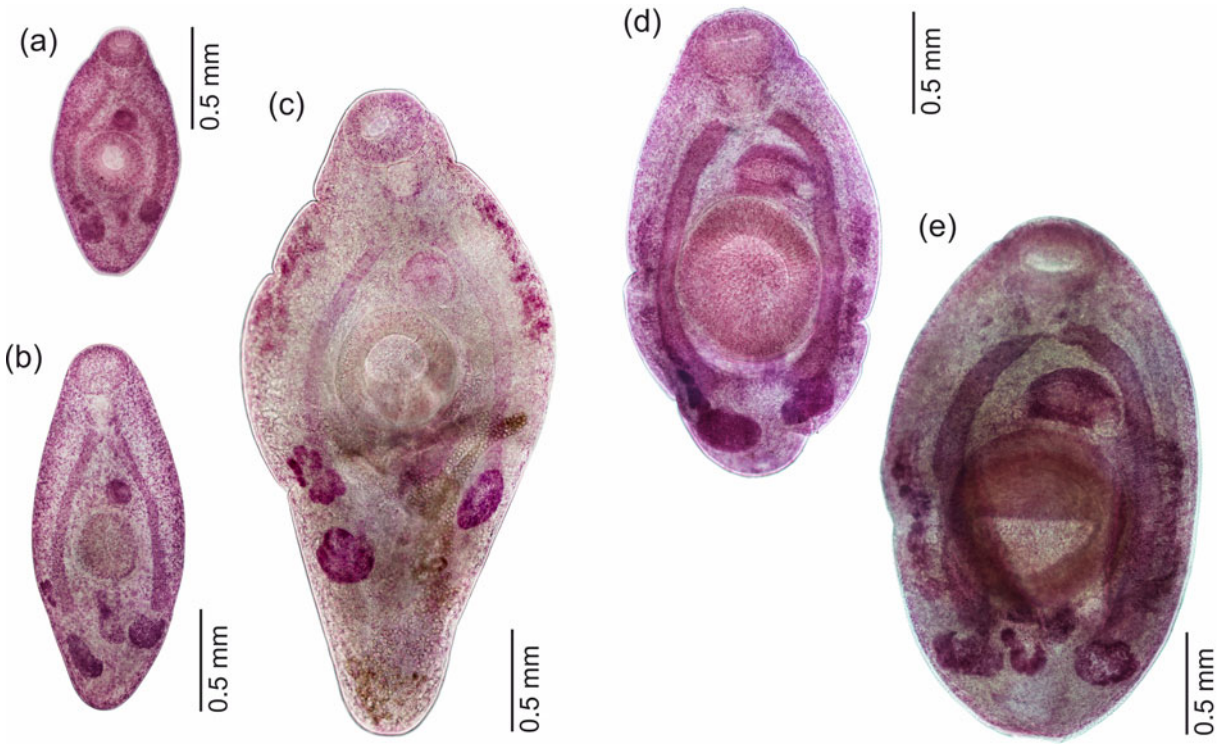

Fig. 3. Whole-mounted metacercariae of *Fellodistomum agnotum* (a–c) and *F. fellis* (d, e). Note eggs on (c). All worms are shown at the same scale.

Table 4. Metacercariae of *Fellodistomum agnotum* and *F. fellis* in buccinid gastropods.

			Fellodistomum fellis				*Fellodistomum agnotum*			
				Localization				Localization		
Location	Host species	No. of hosts examined	Prevalence	% Oes	% St	% Int	Prevalence	% Oes	% St	% Int
Bolshoy Solovetsky Island	*Buccinum undatum*	6	16.7%	0	0	100	33.3%	100	0	0
	Neptunea despecta	2	0	–	–	–	0	–	–	–
Keret Archipelago	*Buccinum undatum*	703	3.7%	38	35	3.8	0.7%	50	0	0
	Buccinum scalariforme	5	20%	100	0	0	0	–	–	–
	Neptunea despecta	31	3.2%	?	?	?	0	–	–	–
Velikaya Salma Strait	*Buccinum undatum*	11	0	–	–	–	0	–	–	–
	Buccinum scalariforme	12	16.7%	0	100	0	0	–	–	–
	Neptunea despecta	3	0	–	–	–	0	–	–	–

Localization shows % of worms in oesophagus (Oes), stomach (St) and intestine (Int); % sum may be not equal to 100 as we do not have localization data for some of the samples.

proteinase K (20 mg/ml), incubated overnight at 56°C while mixing at 750 rpm (Eppendorf Comfort thermomixer; Eppendorf, Hamburg, Germany) and 8 min at 90°C. After centrifugation at 16,000 g for 10 min at −4°C, DNA was in supernatant. Polymerase chain reaction (PCR) amplification was carried out in 25 µl reaction mixtures, which contained 17 µl Milli-Q® water (Merck Millipore Co., Darmstadt, Germany), 5 µl ScreenMix-HS reaction mix (Evrogen, Moscow, Russia), 1 µl of each forward

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and reverse primers and 2 µl DNA template. Two primer pairs for 28S ribosomal DNA (LSU) variable regions were used: digl2, AAGCATATCACTAAGCGG (Tkach *et al.*, 1999) and 1500R, GCTATCCTGAGGGAAACTTCG (Olson *et al.*, 2003) for ~1200 base pairs (bp) D1–D3 domains; C2′B, GAAAAGTAC-TTTGRARAGAGA (Bayssade-Dufour *et al.*, 2000) and D2, TCCGTGTTTCAAGACGGG (Vân Le *et al.*, 1993) for a ~600 bp D2 domain. The PCR thermal profile included initial denaturation at 95°C for 5 min; amplification cycles (35 for D2 or 40 for D1–D3) with 30 s at 95°C, 30 s at 53°C (D2) or 54°C (D1–D3), and 1 min (D2) or 2 min (D1–D3) at 72°C; and final elongation at 72°C for 10 min. PCR products were visualized with SYBR Green (Invitrogen, Carlsbad, California, USA) following electrophoresis in 1% agarose gel. PCR products were sequenced by automated Sanger method using an ABI Prism 3500xl (Applied Biosystems, Foster City, California, USA) with PCR primers. We evaluated chromatogram quality, assembled sequences from forward and reverse reads and aligned all sequences in Geneious Prime® 2019.2.1 (www.geneious.com). Species identification was confirmed by matching our sequences to those available in GenBank for *F. agnotum*, *F. fellis* and *S. furciger* from the North Sea (AJ405289, AJ405290 and AJ405292; Bray *et al.*, 1999). To estimate sequence divergence, we built an illustrative alignment covering all combinations of studied species and lifecycle stages, as well as GenBank voucher samples. To compute pairwise distances and standard error estimates (1000 bootstrap replicates) for this alignment, we used a maximum composite likelihood model in MEGA version 7 (Kumar *et al.*, 2016); all positions containing gaps and missing data were eliminated. The accession numbers for the newly generated sequences are MT216732–MT216758 (table 2).

Results

Two species of the genus *Fellodistomum* – *F. fellis* and *F. agnotum* – were found in wolffish (16 examined). *Fellodistomum fellis* was found in the gall bladder at a prevalence of 87.5% and an intensity of 1–67 (14.9 ± 4.2). *Fellodistomum agnotum* was found in the subglobular chamber at the base of the bile duct at a prevalence of 50% and an intensity of 1–10 (4.6 ± 0.8) (table 1). Among three studied flatfish species, *S. furciger* maritae were present only in *Limanda limanda* at a prevalence of 16.7% and an intensity of 1–10 (4.8 ± 0.6). Worms were found mostly in the intestine, once in the stomach (two specimens) and once in the rectum (two specimens).

Two fellodistomids were found in bivalves (table 3). Sporocysts and cercariae similar to those previously described for *S. furciger* (Chubrik, 1966; Køie, 1979) were recovered from two *N. pernula* collected near Keret Archipelago. A single infected specimen of *E. tenuis* collected near Bolshoy Solovetsky Island contained sporocysts and large furcocercous cercariae, which were similar to ones described by Chubrik (1952, 1966) as *F. fellis*, though an important difference was that in these cercariae the excretory vesicle was Y-shaped, not V-shaped (fig. 1). None of the *Y. hyperborea* ($n = 57$) and *P. arctica* ($n = 8$) were infected.

Fellodistomid metacercariae were recovered from the digestive tract of *B. undatum*, *B. scalariforme* and *N. despecta*. All of the metacercariae could be clearly assigned to either *F. fellis* or *F. agnotum* based on morphology. The most prominent difference was in the excretory vesicle, which was V-shaped in *F. fellis* and Y-shaped in *F. agnotum*. It was most clearly visible in live worms (fig. 2), easily seen in fixed ones, but became scarcely

Table 5. 28S rDNA sequence divergence between isolates of three fellodistomid species, featuring a subset of different developmental stages and host species.

				Fellodistomum agnotum				*Fellodistomum fellis*				*Steringophorus furciger*			
				1	2	3	4	5	6	7	8	9	10		
1	ad	*AJ405289	*Al*		0.0000	0.0000	0.0000	0.0017	0.0017	0.0017	0.0531	0.0531	0.0531		
2	ad	MT216757	*Al*	**0.0000**		0.0000	0.0000	0.0017	0.0017	0.0017	0.0531	0.0531	0.0531		
3	sp	MT216758	*Et*	**0.0000**	**0.0000**		0.0000	0.0017	0.0017	0.0017	0.0531	0.0531	0.0531		
4	mc	MT216740	*Bu*	**0.0000**	**0.0000**	**0.0000**		0.0017	0.0017	0.0017	0.0531	0.0531	0.0531		
5	ad	*AJ405290	*Al*	**0.0036**	**0.0036**	**0.0036**	**0.0036**		0.0000	0.0000	0.0539	0.0539	0.0539		
6	ad	MT216756	*Al*	**0.0036**	**0.0036**	**0.0036**	**0.0036**	**0.0000**		0.0000	0.0539	0.0539	0.0539		
7	mc	MT216745	*Bu*	**0.0036**	**0.0036**	**0.0036**	**0.0036**	**0.0000**	**0.0000**		0.0539	0.0539	0.0539		
8	ad	*AJ405292	*Ll*	**0.1359**	**0.1359**	**0.1359**	**0.1359**	**0.1392**	**0.1392**	**0.1392**		0.0000	0.0000		
9	ad	MT216753	*Ll*	**0.1359**	**0.1359**	**0.1359**	**0.1359**	**0.1392**	**0.1392**	**0.1392**	**0.0000**		0.0000		
10	sp	MT216754	*Np*	**0.1359**	**0.1359**	**0.1359**	**0.1359**	**0.1392**	**0.1392**	**0.1392**	**0.0000**	**0.0000**			

Developmental stages: ad, sexual adult; sp, sporocyst; mc, metacercaria. Host species: *Al*, *Anarhichas lupus*; *Et*, *Ennucula tenuis*; *Bu*, *Buccinum undatum*; *Ll*, *Limanda limanda*; *Np*, *Nuculana pernula*. Asterisks mark sequences from Bray *et al.* (1999) (isolates from the North Sea). All other sequences were generated in this study (isolates from the White Sea). Base substitutions per site are given in bold below the diagonal; Standard error values are given above the diagonal.

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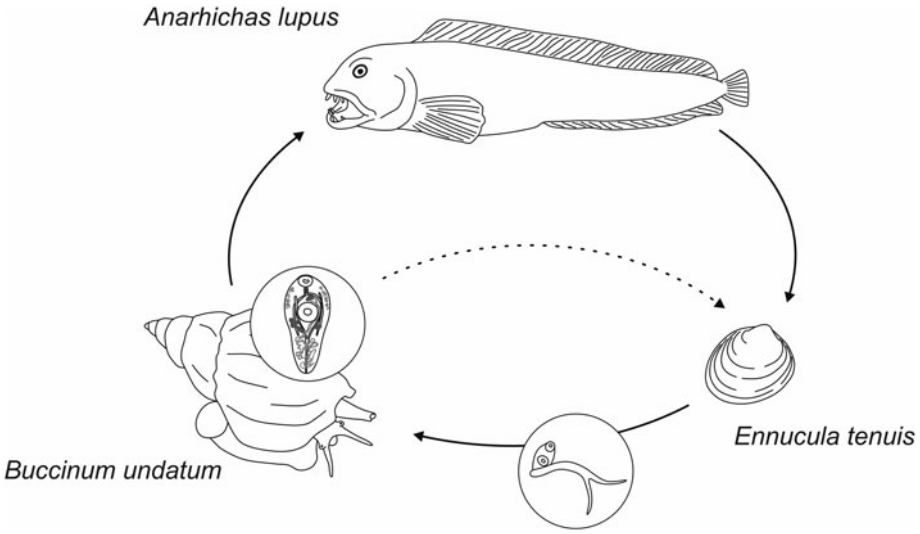

Fig. 4. Life cycle of *Fellodistomum agnotum*. Sporocysts and cercariae develop in protobranch bivalve *Ennucula tenuis*. Metacercariae inhabit digestive tract of whelk *Buccinum undatum*. Wolffish probably gets infected through eating whelks. Metacercariae may produce eggs within the second intermediate host, which makes lifecycle truncation possible.

discernible in mounted and stained specimens because they were too transparent (fig. 3). The size of ventral sucker (relative to oral sucker and body length) may be a good differential character (larger in *F. fellis*), but does not work so well for younger specimens when allometric growth is not yet prominent. The position of reproductive organs primordia differed in mature metacercariae of *F. fellis* and *F. agnotum*, but was also similar in younger specimens.

Three of the seven *F. agnotum* metacercariae contained eggs in the uterus, and two of them did not differ from full-grown adults from *A. lupus*; the largest specimen was 3.2 mm long (figs 2 and 3). The largest *F. fellis* metacercaria was 4.0 mm long (fig. 2). Several *F. fellis* metacercariae had fully formed reproductive systems and had started producing a few eggs.

The majority of metacercariae were found in the stomach and oesophagus, with a few in the beginning of the intestine (table 4). For *F. agnotum* the intensity was 1, for *F. fellis* it was 1–3. A double infection was found thrice.

We obtained partial LSU sequences for the sporocyst from *E. tenuis* and for 24 *Fellodistomum* specimens, 11 maritae and 13 metacercariae, originating from 20 different host individuals (table 2). After trimming and quality control, six sequences were 1237–1257 bp long, corresponding to the D1–D3 variable LSU domains. Eight sequences were 546 bp long (D2 LSU) and 13 were 757–866 bp long (D1–D2 LSU). We also obtained two D1–D3 LSU sequences for *S. furciger* (sporocyst and marita).

All sequences from metacercariae and maritae of *F. fellis* (including AJ405290) were identical. The same was true for sequences from metacercariae and maritae of *F. agnotum* (including AJ405289) and sporocyst from *E. tenuis*, except for two ambiguous positions in MT216758. All *F. fellis* and *F. agnotum* differed consistently by three nucleotide substitutions restricted to the D2 LSU. When comparing six available longer (D1–D3) sequences – two for *F. agnotum* and four for *F. fellis* – one more segregating site was detected in position 956 of the 1257-bp-long alignment. Finally, there were also no differences between *S. furciger* sequences from sporocyst and maritae (including AJ405292). The alignment used to illustrate genetic distances within and between studied species included ten sequences and, trimmed to the shortest sequence, was 848 bp long (table 5).

Discussion

Adult worms of *F. agnotum* were recorded in the White Sea for the first time basing both on morphological and molecular characteristics. We suggest that previous data on *S. furciger* from *A. lupus* (Shulman & Shulman-Albova, 1953) may actually refer to *F. agnotum*. Bray (1987) discovered the same mistake in the records on the Barents Sea wolffish by Polyanski (1955).

The gastropods *B. undatum*, *B. scalariforme* and *N. despecta* were proved to be the second intermediate hosts for *F. fellis*, and *B. undatum* for *F. agnotum*, based on morphology and molecules, which complies with previous observations (Bray, 1987). *Anarhichas lupus* probably gets infected by eating whelks, its common prey (Templeman, 1985; Bray, 1987). We routinely found buccinid shells in the digestive tract of the White Sea wolffish.

Køie (1969) had observed *F. agnotum* with immature eggs in whelks (she described it as *S. furciger*). We also found metacercariae of *F. agnotum* with eggs three times, and two of them were as developed as full-grown adults from the definitive host. Several *F. fellis* metacercariae also produced eggs, but not many. It seems that *F. agnotum* is more predisposed to progenesis than *F. fellis*. As for the other fellodistomids, egg production in the intermediate host had been previously reported in *Steringotrema ovacutum* (Lebour, 1908) Yamaguti, 1954 by Køie (1980), though that was just three larger metacercaria specimens with few eggs. Also, progenetic development is very typical for two *Proctoeces* species (Stunkard & Uzmann, 1959; Oliva & Huaquin, 2000). Thus, egg production by metacercariae appears to be frequent among Fellodistomidae. Viability and infectivity of eggs from *Fellodistomum* metacercariae is a matter for further studies.

Our LSU sequences of sporocysts from *N. pernula* matched those of *S. furciger* adults from *L. limanda*, as well as data from GenBank on this species. Thus, the two-host life cycle of *S. furciger* proposed by Chubrik (1966) and Køie (1979) was verified.

Sporocysts and furcocercous cercariae that we found in *E. tenuis* near Bolshoy Solovetsky Island were shown to belong to *F. agnotum* by evidence from LSU sequences. Thus, the life cycle of this species was resolved (fig. 4). Chubrik (1952, 1966) reported large furcocercous cercariae from *E. tenuis* and considered them to be the larvae of *F. fellis*. Køie (1980) doubted this assumption and, according to her own results of experimental

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infection, decided that *F. fellis* is a synonym of *S. ovacutum*. This was rejected by enzyme electrophoresis analysis by Bray & Rollinson (1985), who attributed lifecycle data of Chubrik and Køie to *S. ovacutum* but not *F. fellis*. They also suggested that *Cercaria megalocerca* Chubrik, 1966 from bivalves *P. arctica* may be the larva of *F. fellis*. However, this is not likely as *P. arctica* is a high-arctic species and its distribution does not correspond to that of *F. fellis* (Warén, 1989). We suspect that the first intermediate host of *F. fellis* may be *E. tenuis*, just like for *F. agnotum*, and the morphological differences between these species are inconspicuous at the cercaria and sporocyst stages. In this case, *F. fellis* and *F. agnotum* would have completely identical life histories except for the site of infection in the definitive host. Unfortunately, low accessibility of *E. tenuis* in the research areas confines our sample sizes, and we have not found a *F. fellis* infection yet.

The close relationship between *F. fellis* and *F. agnotum* is highlighted by the small genetic distance in the LSU fragment (0.36%; table 5). Among fellodistomids, a similar distance in LSU was observed between the supposed intraspecific groups of *Proctoeces sicyases* (Oliva *et al*., 2018) and between possible cryptic species within *Proctoeces maculatus* (Antar & Gargouri, 2016; Wee *et al*., 2017). The interpretation of such similarity requires additional data (several genetic markers, lifecycle data, geographic range, etc.). The distinctness of *F. fellis* and *F. agnotum* is supported by evidence of various natures: morphology and location within the definitive host (Bray & Gibson, 1980), enzyme electrophoresis (Bray & Rollinson, 1985), 18S rDNA (small subunit) (Lumb *et al*., 1993) and LSU (Bray *et al*., 1999) sequences.

Conclusion

To sum up, our study has contributed to clarifying the life cycles of three species of White Sea fellodistomids: *S. furciger*, *F. agnotum* and (except for the first intermediate host) *F. fellis*. Many of the previous accounts on the life cycles of these species were controversial. Here, for the first time, we provided DNA sequence data for material from intermediate hosts, which served to ultimately resolve some of the disputes. However, many questions remain. One of them concerns other possible fellodistomid species from those White Sea fishes that have not yet been thoroughly surveyed. Another issue is the identity of fellodistomid cercariae from *P. arctica* (Chubrik, 1966). Further study of the nature of divergence between two very close species, *F. fellis* and *F. agnotum*, is also worth attention.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

Conflicts of interest. None.

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