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Species complexes and life cycles of digenetic trematodes from the family Derogenidae

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Abstract

The best way to study digenean diversity combines molecular genetic methods, life-cycle studies and elaborate morphological descriptions. This approach has been barely used for one of the most widespread digenean taxa parasitizing fish – the superfamily Hemiuroidea. Here, we applied the integrative approach to the hemiuroideans from the family Derogenidae parasitizing fish at the White and Barents Seas. Analysis of 28S, 18S, 5.8S rDNA, ITS2 and *cox1* gene sequences from sexually adult worms (maritae) showed genetic heterogeneity for 2 derogenid species known from this area: *Derogenes varicus* and *Progonus muelleri*. Thus, 2 pairs of genetic lineages were found: DV1 and DV2, PM1 and PM2, respectively. Data from other regions indicate that 2 more lineages of *D. varicus* probably exist. Based on previous records from the White and Barents Seas, we hypothesized that the cercariae found in the moonsnails (family Naticidae) belong to the Derogenidae and may help to differentiate these lineages as species. According to our results, *Cercaria appendiculata* from *Cryptonatica affinis* matched DV1, similar nameless cercariae from *Euspira pallida* and *Amauropsis islandica* matched DV2, and *Cercaria octocauda* from *C. affinis* matched PM1. We provide new data on the structure of these cercariae and discuss the life-cycle pattern of the studied digeneans.

Introduction

The Digenea is the most species-rich group of the parasitic worms, however, their diversity is far from being well studied. The main reasons are: (1) lack of faunistic data in many regions; (2) phenotypic variation; (3) the existence of cryptic or just hardly distinguishable species; and (4) the complex life cycles (Cribb, 2016). Happily, an integrative approach incorporating molecular genetics and modern morphological methods applied to different life-cycle stages gives the opportunity to bring the research on the digenean diversity to a new level (Georgieva *et al.*, 2013; Gilardoni *et al.*, 2020; Gonchar and Galaktionov, 2021; Huston *et al.*, 2021; Faltýnková *et al.*, 2022). This approach has been barely used for one of the most widespread digenean taxa parasitizing fish – the superfamily Hemiuroidea. Species identification of sexual adults (maritae) in certain hemiuroidean groups is problematic due to ambiguous morphological characteristics, apparently low host specificity and worldwide distribution. These problems are particularly true for the family Derogenidae, subfamily Derogeninae, with *Derogenes varicus* (Müller, 1784) Looss, 1901 being reported as one of the most widespread marine digenean species (WoRMS, 2022), but suspected to represent a cryptic species complex (Bray *et al.*, 2016). Molecular data on the derogenids are few, and none of them cover the life-cycle stages from the first intermediate hosts.

Hemiuroideans typically possess 3-host life cycles (Hunninen and Cable, 1943; Køie, 1979, 1989, 1990*a*; Køie *et al.*, 2002). Within the first intermediate host they produce a very special type of cercariae – cystophorous ones. The tail of such cercariae is partially transformed into a caudal cyst, and the body of the infective cercaria is withdrawn inside it. The caudal cyst may bear different types of appendages, some of which are used for locomotion (Køie, 1979, 1990*a*, 1992). The cyst also has a delivery tube – a special structure enabling infection of the next host. The second intermediate hosts of hemiuroideans are small crustaceans, often planktonic copepods. When they bite a caudal cyst, the delivery tube everts and penetrates their foregut, injecting the cercarial body into the haemocoel (Matthews, 1981).

Cystophorous cercariae have been described from marine and freshwater gastropods (Hunninen and Cable, 1943; Madhavi, 1978; Køie, 1979, 1989; Goater *et al.*, 1990; Shameem *et al.*, 1990), and also from bivalves and scaphopods (Wardle, 1975; Køie *et al.*, 2002; Louvard *et al.*, 2022). Among them are 4 different types of cercariae that have been found in the moonsnails (family Naticidae) from the Northern Atlantic and the adjacent Arctic (Barents and White Seas). The first, *Cercaria appendiculata* Pelseneer, 1906 has a furcate locomotory appendage, was primarily described from *Euspira nitida* (Donovan, 1803), and later found in *Cryptonatica affinis* (Gmelin, 1791) (Chubrik, 1966; Timofeeva, 1976; Køie, 1979). Køie (1979) demonstrated experimentally that *C. appendiculata* is the larva of

Derogenes varicus. A similar cercaria was recovered from *Euspira pallida* (Broderip & G. B. Sowerby I, 1829) by Køie (1990*b*). At first, she supposed it belonged to *Hemiurus levinseni* Odhner, 1905, but later reassigned it to the Derogenidae (Køie, 1995, 2000). Another type of cystophorous cercariae described from the moonsnail *C. affinis* from the Barents Sea – *Cercaria octocauda* Tschubrik, 1952 – is characterized by a locomotory appendage with 8 long immotile threads on its end (Chubrik, 1966; Timofeeva, 1976). Finally, *Cercaria saccocaudata* Tschubrik, 1966 was described from *C. affinis* at the Barents and White Seas (Chubrik, 1966; Timofeeva, 1976). It lacks a locomotory appendage, and regarding the morphology it was supposed to belong to the family Lecithasteridae (Chubrik, 1966; Timofeeva, 1976), and finally proved to be *Lecithaster salmonis* (Krupenko *et al*., 2022).

In the present paper we revisit systematics and life cycles of the Derogenidae, subfamily Derogeninae at the White and Barents Seas. Maritae morphologically assigned to *D. varicus* and *Progonus muelleri* (Levinsen, 1881) Looss, 1899 were found to be genetically heterogeneous (in fragments of 28S, 18S, 5.8S rDNA, ITS2 and *cox1* gene), thus possibly representing groups of species challenging to identify. Based on previous records of digenean fauna in the White and Barents Seas (Shulman and Shulman-Albova, 1953; Polyansky, 1955; Chubrik, 1966), we hypothesized that 3 types of cystophorous cercariae infecting the moonsnails in the White and Barents Seas also belong to the Derogeninae. *Cercaria octocauda* was proved to be the larva of *P. muelleri*. Two cercariae with furcate locomotory appendage matched 2 lineages within *D. varicus*.

Materials and methods

Sampling

Intermediate and definitive hosts were sampled at the White Sea (3 sites: Keret Archipelago, Velikaya Salma Strait and Bolshoy Solovetsky Island) in 2018–2021, and at the Barents Sea (Dalniye Zelentsy) in July–August 2021. Three moonsnail species were checked for the digenean infection: *Cryptonatica affinis* and *Euspira pallida* from both the White and Barents Seas, and *Amauropsis islandica* (Gmelin, 1791) from the White Sea. We searched for maritae of the family Derogenidae in 16 fish species from the White Sea and 4 species from the Barents Sea (Table 1). For other purposes we dissected whelks *Buccinum scalariforme* Møller, 1842 ($N = 31$) from the White Sea. A single derogenid marita was found in the stomach of 1 whelk.

Morphological analysis

Rediae and maritae were fixed in 96% ethanol for whole mounts. All specimens of maritae were heat-killed before fixation, except 1 from *B. scalariforme*. Rediae and maritae were stained with acetocarmine (Sigma Aldrich, Germany) followed by destaining in 0.1 M HCl in 70% ethanol, dehydrated in a graded alcohol series, clarified in xylol, and mounted in Canada balsam. Temporary mounts of cercariae, live and fixed in 2.5% glutaraldehyde in sea water, were used to study their gross morphology. Whole mounts were observed under microscopes Leica DM 500 or DM 2500 (Leica Microsystems, Germany); photos and videos were taken either in bright field, or with phase-contrast microscopy, or with differential interference contrast (DIC) using Nikon DS Fi1, or Sony Alpha 7RII, or smartphone camera. Measurements were made using Fiji software (Schindelin *et al*., 2012). Measurements of cercarial body were taken prior to its retraction into the caudal cyst. The delivery tube was measured in live specimens when everted under the cover glass pressure. All measurements are in micrometres.

For scanning electron microscopy (SEM), 2.5% glutaraldehyde-fixed cercariae were rinsed in water, dehydrated in ethanol and acetone, dried in a critical point dryer, coated with platinum, and examined with a Quanta 250 at 15 kV. Visualization of cercarial inner structure was also performed by means of confocal laser scanning microscopy (CLSM). Specimens were fixed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline and stained with tetramethylrhodamine B isothiocyanate (TRITC)-labelled phalloidin and DAPI. The protocol was the same as previously described (Kremnev *et al*., 2020).

Molecular analysis

Ethanol-fixed rediae and maritae were used for molecular studies. To extract DNA, we took 1 redia or fragment of marita (preoral lobe and anterior part of the oral sucker); 32 isolates used for molecular analysis are listed in Table 2. Samples were taken from 96% ethanol and dried completely, incubated in 200 μL of 5% solution of Chelex® 100 resin (Bio-Rad, USA) with 0.2 mg mL^{-1} of proteinase K (Evrogen, Russia) at 56°C for 4 h, then kept for 8 min at 90°C and centrifuged for 10 min at 16,000 g. The supernatant containing DNA was transferred to a new tube and stored at −20°C.

We amplified a fragment of the 28S rDNA (domains D1–D3), a fragment containing 5.8S rDNA and ITS2, a fragment of 18S rDNA, and a fragment of the cytochrome oxidase I gene (*cox1*) with primers and conditions listed in Table 3. The 20 μL reaction mixture contained 4 μL of ScreenMix-HS (Evrogen), 0.5 μL of each primer (10 pmol μL^{-1}), 2 μL of the DNA and 13 μL of PCR grade water (Evrogen). Polymerase chain reactions (PCRs) were run on a Veriti thermal cycler (Applied Biosystems, MA, USA). PCR products were stained with ethidium bromide and visualized through electrophoresis on a 1% agarose gel. Sequencing was performed with PCR primers on an ABI Prism 3500xl genetic analyser (Applied Biosystems, MA, USA). Chromatograms were analysed and edited, and alignments were built in Geneious Prime 2022.0.1 (https://www.geneious.com). Relevant data for alignments were obtained from GenBank (Supplementary Table 1). To annotate 28S and 5.8S + ITS2 regions, we used the sequence of *Didymocystis scomberomori* (KU341979; Schrandt *et al*., 2016) as a reference. Pairwise genetic distances between species (as the number of base differences per site) were calculated in MEGA 7 (Kumar *et al*., 2016). The maximum likelihood (ML) analysis was conducted at the CIPRES Science Gateway (https://www.phylo.org) using RAxML (Stamatakis, 2014) with 1000 bootstrap iterations. The model of nucleotide substitution was estimated as GTR + I for the 28S dataset, TIM2 + G for the 18S dataset, TVM + I for the 5.8S + ITS2 dataset and TIM3 + I + G for the *cox1* dataset using JModelTest (Darriba *et al*., 2012) at the CIPRES Science Gateway. A haplotype network for fragments of *cox1* was constructed in PopART 1.7 (Leigh and Bryant, 2015) by integer neighbour-joining network (reticulation tolerance 0.5).

Results

At the White Sea, we obtained maritae morphologically identified as *Progonus muelleri* from *Myoxocephalus scorpius*, *Limanda limanda* and *Triglops murrayi*. The maritae identified as *Derogenes varicus* were found in 10 fish species from the White Sea and 3 fish species from the Barents Sea (Table 1). A marita found in the stomach of the whelk *Buccinum scalariforme* was also identified as *D. varicus*.

Cystophorous cercariae of 4 types were found parasitizing moonsnails. *Cercaria saccocaudata* = *Lecithaster salmonis*

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Table 1. Fish hosts examined, prevalence (%) and intensity data for derogenid maritae from the White and Barents Seas

					Derogenes varicus		*Progonus muelleri*	
		Host family	Host species	*N*	Prevalence (%)	Mean intensity	Prevalence (%)	Mean intensity
White Sea	Keret Archipelago	Agonidae	*Agonus cataphractus* (Linnaeus, 1758)	1	–	–	–	–
		Anarhichadidae	*Anarhichas lupus* Linnaeus, 1758	27	7	1	–	–
		Clupeidae	*Clupea pallasii marisalbi* Berg, 1923	37	5	7	–	–
		Cottidae	*Gymnocanthus tricuspis* (Reinhardt, 1830)	1	–	–	–	–
		Cottidae	*Myoxocephalus quadricornis* (Linnaeus, 1758)	5	20	1	–	–
		Cottidae	*M. scorpius* (Linnaeus, 1758)	90	39	3.5	2	1.5
		Cottidae	*Triglops murrayi* Günther, 1888	5	20	2	20	1
		Gadidae	*Eleginus nawaga* (Walbaum, 1792)	44	14	2	–	–
		Gadidae	*Gadus morhua* Linnaeus, 1758	127	31	4.5	–	–
		Pleuronectidae	*Limanda limanda* (Linnaeus, 1758)	114	10	1.5	1	1
		Pleuronectidae	*Liopsetta glacialis* (Pallas, 1776)	27	–	–	–	–
		Pleuronectidae	*Platichthys flesus* (Linnaeus, 1758)	77	3	3	–	–
		Osmeridae	*Osmerus mordax dentex* Steindachner & Kner, 1870	20	–	–	–	–
		Salmonidae	*Coregonus lavaretus* (Linnaeus, 1758)	4	–	–	–	–
		Salmonidae	*Oncorhynchus gorbuscha* (Walbaum, 1792)	3	67	7	–	–
		Zoarcidae	*Zoarces viviparus* (Linnaeus, 1758)	2	–	–	–	–
	Bolshoy Solovetsky Island	Cottidae	*M. scorpius*	2	50	2	50	5
	Velikaya Salma Strait	Clupeidae	*C. pallasii marisalbi*	1	100	1	–	–
		Cottidae	*M. scorpius*	4	25	1	25	1
		Gadidae	*E. nawaga*	5	–	–	–	–
		Gadidae	*G. morhua*	9	33	1.3	–	–
		Pleuronectidae	*L. limanda*	4	25	5	–	–
Barents Sea	Dalniye Zelentsy	Gadidae	*G. morhua*	11	18	2.5	–	–
		Cottidae	*G. tricuspis*	2	100	1	–	–
		Cottidae	*M. scorpius*	3	100	12.3	–	–
		Pleuronectidae	*P. flesus*	1	100	1	–	–

(family Lecithasteridae) recovered from *Cryptonatica affinis* is not considered further here. *Cercaria appendiculata* was found in *C. affinis* both at the White Sea and Barents Sea. *Cercaria octocauda* was found in *C. affinis* from 1 locality at the White Sea (Velikaya Salma Strait). Finally, rediae with cystophorous cercariae of Derogenidae gen. sp. (according to Køie, 1995, 2000), similar to but much larger than *C. appendiculata*, were found in *Euspira pallida* (White and Barents Seas) and *Amauropsis islandica* (White Sea).

Molecular analysis and linking life-cycle stages

For molecular genetic analysis 32 newly recovered specimens were used: 22 maritae and 10 intramolluscan stages – rediae with

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

					GenBank accession numbers			
ID	Species	Stage	Host species	Locality	28S rDNA	5.8S rDNA + ITS2	18S rDNA	*cox1*
D5.1	*Derogenes varicus*	Marita	*Limanda limanda*	Keret Archipelago (White Sea)	OM761962	OM762002	–	OM807173
D5.2	*Derogenes varicus*	Marita	*Gadus morhua*	Keret Archipelago (White Sea)	OM761963	OM762003	OM761994	OM807174
D5.3	*Derogenes varicus*	Marita	*Myoxocephalus scorpius*	Keret Archipelago (White Sea)	OM761964	OM762004	–	OM807175
D5.4	*Derogenes varicus*	Marita	*Anarhichas lupus*	Keret Archipelago (White Sea)	OM761965	OM762005	–	OM807176
D7.1	*Derogenes varicus*	Marita	*Limanda limanda*	Keret Archipelago (White Sea)	OM761966	OM762006	–	OM807177
D7.2	*Derogenes varicus*	Marita	*Eleginus nawaga*	Keret Archipelago (White Sea)	OM761967	OM762007	–	OM807178
D7.3	*Derogenes varicus*	Marita	*Limanda limanda*	Keret Archipelago (White Sea)	OM761968	OM762008	–	OM807179
D7.5	*Derogenes varicus*	Marita	*Clupea pallasii marisalbi*	Keret Archipelago (White Sea)	OM761969	OM762009	OM761995	OM807180
D7.6	*Derogenes varicus*	Marita	*Clupea pallasii marisalbi*	Keret Archipelago (White Sea)	OM761970	OM762010	–	OM807181
D12.3	*Derogenes varicus*	Marita	*Gadus morhua*	Dalniye Zelentsy (Barents Sea)	OM761971	OM762011	–	OM807182
D12.4	*Derogenes varicus*	Marita	*Myoxocephalus scorpius*	Dalniye Zelentsy (Barents Sea)	OM761972	OM762012	–	OM807183
D12.5	*Derogenes varicus*	Marita	*Myoxocephalus scorpius*	Dalniye Zelentsy (Barents Sea)	OM761973	OM762013	–	OM807184
D14.2	*Derogenes varicus*	Marita	*Gadus morhua*	Velikaya Salma Strait (White Sea)	OM761974	OM762014	–	–
D14.3	*Derogenes varicus*	Marita	*Gadus morhua*	Velikaya Salma Strait (White Sea)	OM761975	OM762015	–	–
D16.2	*Derogenes varicus*	Marita	*Triglops murrayi*	Keret Archipelago (White Sea)	OM761976	OM762016	–	–
A17.20	*Derogenes varicus*	Marita	*Buccinum scalariforme*	Keret Archipelago (White Sea)	OM761977	OM762017	–	–
D4.10	*Progonus muelleri*	Marita	*Myoxocephalus scorpius*	Bolshoy Solovetsky Island (White Sea)	OM761978	OM762018	OM761996	OM807185
D4.11	*Progonus muelleri*	Marita	*Myoxocephalus scorpius*	Keret Archipelago (White Sea)	OM761979	OM762019	OM761997	OM807186
D4.12	*Progonus muelleri*	Marita	*Myoxocephalus scorpius*	Keret Archipelago (White Sea)	OM761980	OM762020	–	OM807187
D14.1	*Progonus muelleri*	Marita	*Myoxocephalus scorpius*	Velikaya Salma Strait (White Sea)	OM761981	OM762021	–	–
D16.1	*Progonus muelleri*	Marita	*Limanda limanda*	Keret Archipelago (White Sea)	OM761982	OM762022	–	–
D16.3	*Progonus muelleri*	Marita	*Triglops murrayi*	Keret Archipelago (White Sea)	OM761983	OM762023	OM761998	–
D4.2	*Cercaria appendiculata*	Rediae and cercariae	*Cryptonatica affinis*	Keret Archipelago (White Sea)	OM761984	OM762024	–	OM807188
D4.3	*Cercaria appendiculata*	Rediae and cercariae	*Cryptonatica affinis*	Keret Archipelago (White Sea)	OM761985	OM762025	–	OM807189
D4.4	*Cercaria appendiculata*	Rediae and cercariae	*Cryptonatica affinis*	Keret Archipelago (White Sea)	OM761986	OM762026	–	OM807190
D12.2	*Cercaria appendiculata*	Rediae and cercariae	*Cryptonatica affinis*	Dalniye Zelentsy (Barents Sea)	OM761987	OM762027	–	OM807191
D12.6	*Cercaria appendiculata*	Rediae and cercariae	*Cryptonatica affinis*	Dalniye Zelentsy (Barents Sea)	OM761988	OM762028	–	OM807192

(*Continued*)

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Table 2. (*Continued.*)

ID	Species	Stage	Host species	Locality	GenBank accession numbers			
					28S rDNA	5.8S rDNA + ITS2	18S rDNA	*cox1*
D4.1	Derogenidae gen. sp.	Rediae and cercariae	*Amauropsis islandica*	Keret Archipelago (White Sea)	OM761989	OM762029	–	OM807193
D11.4	Derogenidae gen. sp.	Rediae and cercariae	*Euspira pallida*	Keret Archipelago (White Sea)	OM761990	OM762030	OM761999	OM807194
D12.1	Derogenidae gen.sp.	Rediae and cercariae	*Euspira pallida*	Dalniye Zelentsy (Barents Sea)	OM761991	OM762031	OM762000	OM807195
D14.4	*Cercaria octocauda*	Rediae and cercariae	*Cryptonatica affinis*	Velikaya Salma Strait (White Sea)	OM761992	OM762032	OM762001	OM807196
D14.5	*Cercaria octocauda*	Rediae and cercariae	*Cryptonatica affinis*	Velikaya Salma Strait (White Sea)	OM761993	OM762033	–	OM807197

Table 3. PCR primers and thermocycling conditions; in all reactions initial denaturation was at 95°C for 5 min and final extension was at 72°C for 10 min

Product	Primer	Sequence (5′–3′), forward (F) and reverse (R)	Thermocycling profile	Reference
18S rDNA	18S1A	F, GGCGATCGAAAAGATTAAGCCATGCA	94°C – 1 m 52°C – 1 m 72°C – 1 m × 35	Hernández-Mena *et al.* (2017) (primers and conditions)
	32	R, CGAAGTCCTATTCCATTATTC		
28S rDNA	digl2	F, AAGCATATCACTAAGCGG	95°C – 30s 54°C – 30s 72°C – 2 m × 40	Tkach *et al.* (1999) (primers and conditions)
	1500R	R, GCTATCCTGAGGGAAACTTCG		Olson *et al.* (2003) (primers and conditions)
5.8S rDNA + ITS2	3S	F, GTACCGGTGGATCACGTGGCTAGTG	94°C – 30s 55°C – 30s 72°C – 1 m × 30	Morgan and Blair (1995) (primers)
	ITS2.2	R, CCTGGTTAGTTTCTTTTCCTCCGC		
cox1	JB3	F, TTTTTTGGGCATCCTGAGGTTTAT	95°C – 30s 48°C – 40s 72°C – 1 m × 40	Leung *et al.* (2009) (primers and conditions)
	trem.cox1.rrnl	R, AATCATGATGCAAAAGGTA		
cox1	JB3	F, TTTTTTGGGCATCCTGAGGTTTAT	95°C – 30s 45°C – 30s 72°C – 1 m × 35	Urabe *et al.* (2012) (primers and conditions)
	COI R-Trema	R, CAACAAATCATGATGCAAAAGG		

cercariae (Table 2). We obtained 1130–1139 bp sequences of the 28S rDNA fragment (domains D1–D3). The following sequences of the representatives of the Derogeninae from GenBank were also used in the analysis: *D. varicus* AY222189 (Olson *et al.*, 2003) and MW504598–9 (Sokolov *et al.*, 2021); *D. lacustris* LC586089–90 (Tsuchida *et al.*, 2021); *P. muelleri* MW507469–71 (Sokolov *et al.*, 2021). *Prosogonotrema bilabiatum* (AY222191; Olson *et al.*, 2003) served as an outgroup. The alignment was 1052 bp long after trimming the ends to match the shortest sequence. The ML tree was built to visualize the results. Our specimens formed 4 distinct groups (Fig. 1, Supplementary Table 2). The first group included all our *D. varicus* specimens from fish and all rediae of *C. appendiculata* from *Cryptonatica affinis*, with no nucleotide substitutions. The second group comprised the marita of *D. varicus* from the whelk *B. scalariforme* and derogenid rediae from *Euspira pallida* and *Amauropis islandica*; these sequences were identical to one of the *D. varicus* sequences from GenBank (AY222189) from the North Sea. These 2 groups are later referred to as DV1 and DV2. Divergence between them was 0.0068 ± 0.0053 (17 substitutions). The sequences of *D. varicus* from the Pacific (MW504598–9) differed from DV1 in 14 and from DV2 in 11 substitutions; they are labelled in Fig. 1 as DV3. The other 2 closely related groups comprised maritae of *P. muelleri* and rediae of *C. octocauda*; a single variation was in position 501: 3 maritae and 2 *C. octocauda* rediae (T) differed from 3 other maritae (C) (distance = 0.0004 ± 0.0009). These 2 groups of specimens are later referred to as PM1 and PM2, respectively. Previously published sequences of *P. muelleri* from the Pacific and the White Sea (MW507469–71) fell into the PM1 group. Two sequences of *D. lacustris* formed a separate group with intraspecific pairwise distance 0.0005 ± 0.0008 (Fig. 1, Supplementary Table 2).

We also analysed 525–593 bp fragments including partial 5.8S rDNA (110–150 bp), complete ITS2 and the beginning of 28S (31–74 bp). *Accacladocoelium macrocotyle* (KF687303; Ahuir-Baraja *et al.*, 2015) was taken as an outgroup. After trimming the ends, the alignment was 531 bp long, including gaps. In the obtained ML tree our specimens grouped exactly the same way as in the 28S rDNA analysis (Fig. 2). Intragroup variation was absent. Divergence between the groups DV1 and DV2 was 0.0374 ± 0.0101 (18 substitutions and 4 indels) (Supplementary Table 3). The groups PM1 and PM2 differed in a single position (260, A/G) (divergence = 0.0021 ± 0.0019).

A fragment of 18S rDNA 775–778 bp long was sequenced for 8 of our specimens, 2 from each of the groups DV1, DV2, PM1 and

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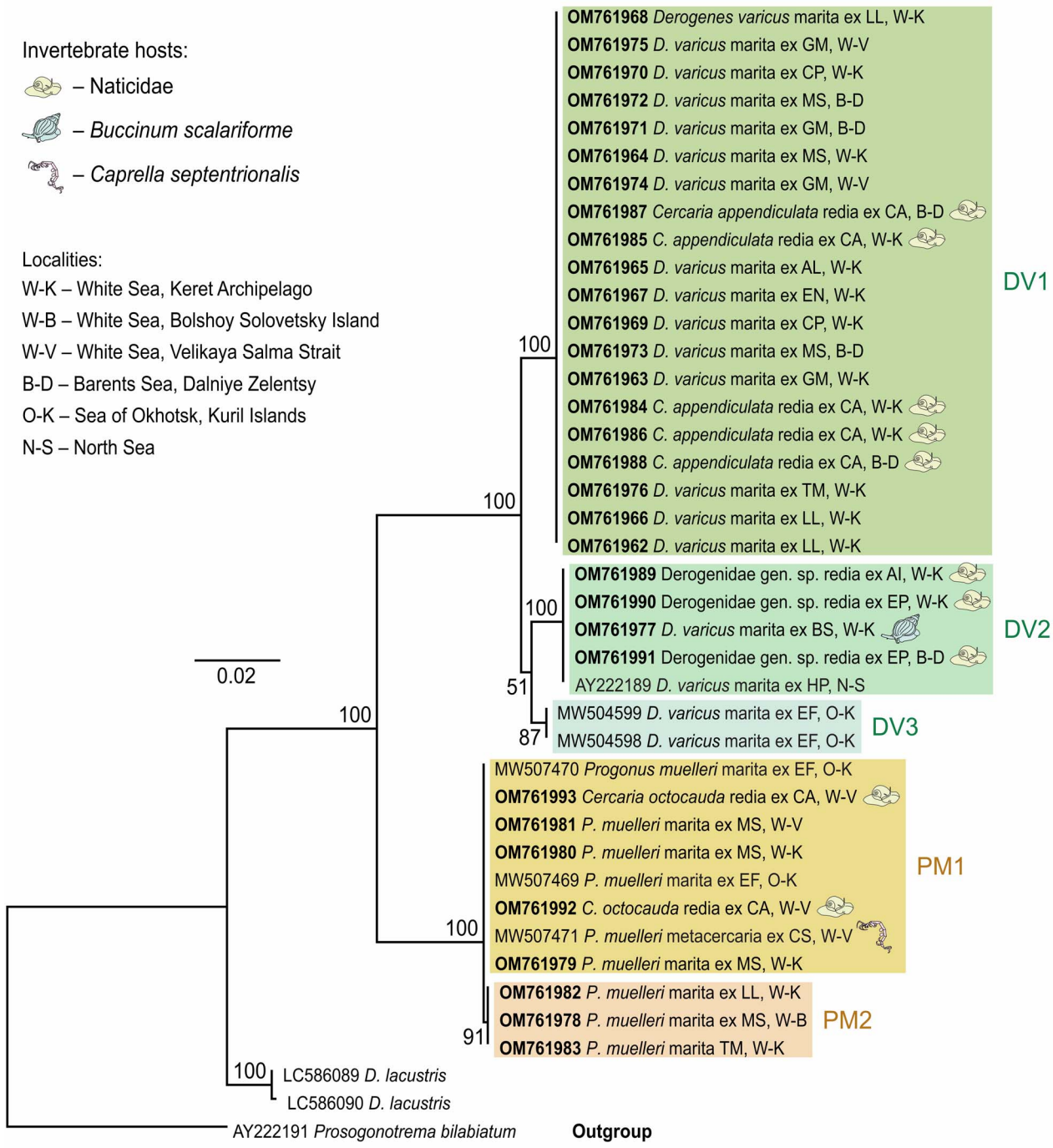

Fig. 1. ML phylogenetic tree based on the 28S rDNA sequence data. Bootstrap values are printed in nodes. Scale bar shows the substitution rate. DV1–3 and PM1–2 mark the separate genetic lineages within *Derogenes varicus* and *Progonus muelleri* species, respectively. Abbreviations: AI – *Amauropsis islandica*; AL – *Anarhichas lupus*; BS – *Buccinum scalariforme*; CA – *Cryptonatica affinis*; CS – *Caprella septentrionalis*; CP – *Clupea pallasii marisalbi*; EF – *Eumicrotremus fedorovi*; EN – *Eleginus nawaga*; EP – *Euspira pallida*; GM – *Gadus morhua*; HP – *Hippoglossoides platessoides*; LL – *Limanda limanda*; MS – *Myoxocephalus scorpius*; TM – *Triglops murrayi*.

PM2 (Table 2). The closest BLAST hit was *D. varicus* (AJ287511; Littlewood and Olson, 2001), which was also included in the analysis. *Prosogonotrema bilabiatum* (AJ287565; Littlewood and Olson, 2001) served as an outgroup. After trimming the ends, the alignment was 779 bp long, including gaps. Our specimens grouped the same way as in 28S and 5.8S + ITS2 analyses (Fig. 3, Supplementary Table 4). PM1 and PM2 differed from each other in 1 position (599, C/T; distance = 0.0013 ± 0.0012). The difference between DV1 and DV2 was in 11 positions (distance = 0.0143 ± 0.005). *Derogenes varicus* from GenBank (AJ287511; Cribb *et al.*, 2001) was closest to DV2, though with 1 substitution and 1 indel (distance = 0.0013 ± 0.0011); this sequence is labelled DV4 on the 18S tree (Fig. 3). We also compared the alignment with another sequence of *D. varicus* from GenBank – AF029816 (Blair *et al.*, 1998). It overlapped our alignment by a rather short fragment (213 bp) and was thus not included in the ML analysis. This fragment was identical in AF29816 and our sequences of DV1, and differed from DV2 and DV4 in 9 positions.

Fragments of *cox1* gene were sequenced for 25 specimens (Table 2). After trimming, the alignment was 788 bp long. *Didymocystis wedli* (AB725624) served as an outgroup. In the obtained ML tree, our specimens grouped the same way as in the rDNA analyses (Fig. 4). Minimal intergroup pairwise distance

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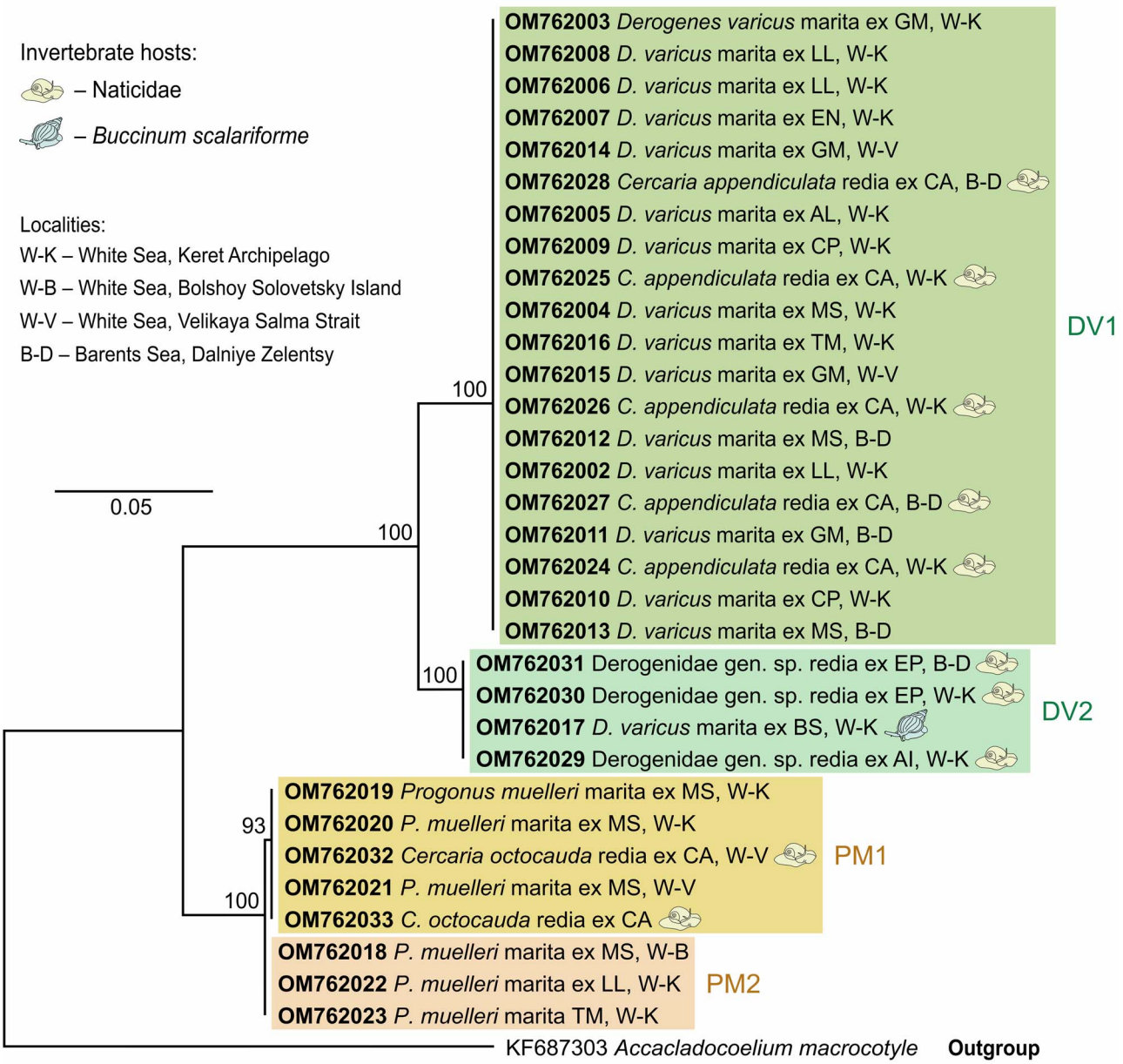

Fig. 2. ML phylogenetic tree based on the ITS2 rDNA sequence data. Bootstrap values are printed in nodes. Scale bar shows the substitution rate. DV1–2 and PM1–2 mark the separate genetic lineages within *Derogenes varicus* and *Progonus muelleri* species, respectively. Abbreviations: AI – *Amauropsis islandica*; AL – *Anarhichas lupus*; BS – *Buccinum scalariforme*; CA – *Cryptonatica affinis*; CP – *Clupea pallasii marisalbi*; EN – *Eleginus nawaga*; EP – *Euspira pallida*; GM – *Gadus morhua*; LL – *Limanda limanda*; MS – *Myoxocephalus scorpius*; TM – *Triglops murrayi*.

was between PM1 and PM2 (0.0905 ± 0.041, 61 positions), and maximal intragroup pairwise distance was in DV1 (0.0155 ± 0.0082, 12 positions) (Supplementary Table 5). In DV1 34 polymorphic sites were present; all haplotypes were unique; overall mean distance was 0.0076 ± 0.0014. We built a haplotype network for DV1, because rediae of this species from the White and Barents Seas clearly differed by size (see below), and thus could represent different lineages. However, geographic origin of isolates was not associated with the position of DV1 samples on the haplotype network (Fig. 5). DV2 had 1 polymorphic site with a synonymous substitution. One variable position was also present within PM1.

Descriptions

All the obtained maritae were identified either as *Derogenes varicus* or as *Progonus muelleri*, based on their morphological characteristics. Measurements are given in Table 4. Considering the genetic heterogeneity between all the specimens of *D. varicus* from fish and the one from *Buccinum scalariforme*, and between maritae of *P. muelleri*, the measurements are shown separately – referred to as DV1 and DV2, PM1 and PM2, respectively. Hologenophores for all maritae isolates and 5 paragenophores of DV1 maritae were deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

The descriptions of 3 types of intramolluscan stages were made *de novo*. Measurements from this and previous studies are summarized in Tables 5 and 6.

Cercaria appendiculata *Pelseneer, 1906 (DV1) (Fig. 6)*

Host: *Cryptonatica affinis*

Localities: White Sea, Keret Archipelago; Barents Sea, Dalniye Zelentsy.

Sites: Reproductive gland and ducts, partially digestive gland, hypobranchial gland.

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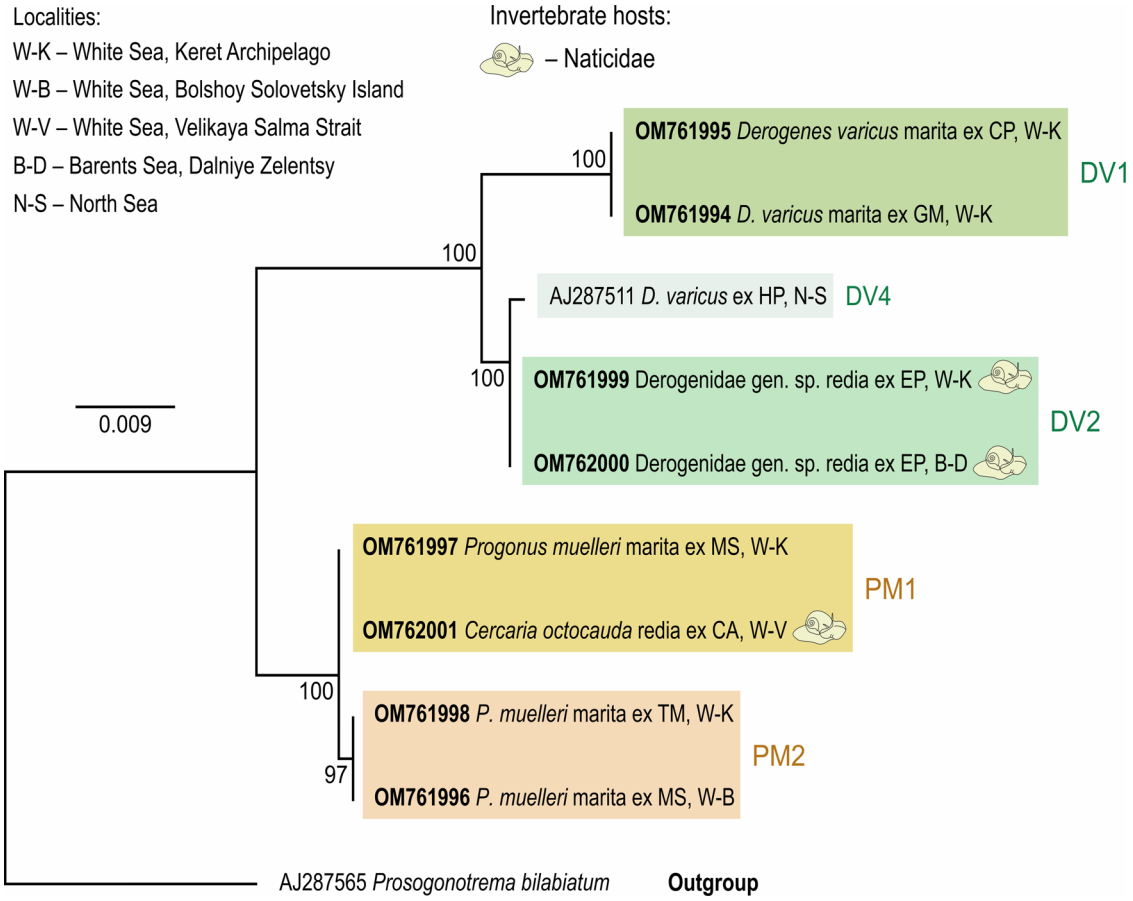

Fig. 3. ML phylogenetic tree based on the 18S rDNA sequence data. Bootstrap values are printed in nodes. Scale bar shows the substitution rate. DV1–2, 4 and PM1–2 mark the separate genetic lineages within *Derogenes varicus* and *Progonus muelleri* species, respectively. Abbreviations: CA – *Cryptonatica affinis*; CP – *Clupea pallasii marisalbi*; EP – *Euspira pallida*; GM – *Gadus morhua*; HP – *Hippoglossoides platessoides*; MS – *Myoxocephalus scorpius*; TM – *Triglops murrayi*.

Prevalence: 5.2% (14 of 270) at Keret Archipelago, White Sea; 5.8% (6 of 104) at Dalniye Zelentsy, Barents Sea.

Rediae measurements based on 29 ethanol-fixed specimens: 16 from White Sea and 13 from Barents Sea. As rediae from White and Barents Seas differed clearly by size, their measurements are given separately (marked WS and BS). WS rediae elongated, 1848 (608–3411) × 174 (141–221). Pharynx small, 47 (28–32) × 34 (25–30). Caecum 548 (318–939) × 50 (27–73). Birth pore close to mouth opening. BS rediae very long, 4024 (3479–5006) × 154 (123–253). Pharynx small, 42 (37–51) × 30 (25–34). Caecum 929 (566–1536) × 30 (20–40). Birth pore close to mouth opening.

Cercariae measurements based on 12 live and 30 glutaraldehyde-fixed specimens: 29 from the White Sea and 13 from the Barents Sea. Cercariae of cystophorous type, tail consisting of caudal cyst, delivery tube and locomotory appendage. Body 170 (124–249) long, 47 (39–56) wide. Oral sucker 27 (25–31) × 27 (25–30), ventral sucker 28 (24–31) × 30 (27–33) and pharynx 13 (12–16) × 16 (14–17). Intestinal caeca reaching slightly behind the ventral sucker. Excretory vesicle Y-shaped, with widened proximal part; excretory ducts unite dorsally near pharynx. Eight flame cells present within the body. Four more flame cells form compact group within caudal cyst close to its aperture. Muscular sphincter present at cyst aperture; retractor muscles connect body hind end with cyst wall.

In infective cercariae, body and delivery tube pulled into caudal cyst, aperture closed. Body lies with anterior end near folded delivery tube. Caudal cyst roundish, 94 (85–103) × 91 (81–100) in diameter, 61 (57–65) thick, 2-layered, space between layers narrow. One side with depression, and furcate locomotory appendage attached at its bottom; outer layer forms membranous outgrowth. Locomotory appendage 145 (109–178) long, 18 (16–21) × 8 (7–10) in diameter, furca 58 (53–66) long. Everted delivery tube 409 (306–456) long, 15 (12–20) wide in middle region, narrower in distal part, wider at tip; roundish extension present in proximal region.

Derogenidae gen. sp. (DV2) (*Fig. 7*)

Hosts: *Euspira pallida*, *Amauropsis islandica*

Localities: White Sea, Keret Archipelago; Barents Sea, Dalniye Zelentsy.

Sites: Reproductive gland and ducts, partially digestive gland.

Prevalence: 4.2% (1 of 24) *E. pallida*, 33.4% (1 of 3) *A. islandica* at Keret Archipelago, White Sea; 12.5% (1 of 8) *E. pallida* at Dalniye Zelentsy, Barents Sea.

Rediae measurements based on 22 ethanol-fixed specimens: 6 from Barents Sea and 16 from White Sea. Rediae elongated, 2265 (1313–3961) × 219 (145–308). Pharynx small, 55 (42–68) × 42 (31–51). Caecum 1063 (805–1366) × 45 (33–56). Birth pore close to mouth opening.

Cercariae measurements based on 18 live and 9 glutaraldehyde-fixed specimens: 25 from the White Sea and 2 from the Barents Sea. Cercariae of cystophorous type, tail consisting of caudal cyst, delivery tube and locomotory appendage. Body 238 (187–283) long, 77 (59–109) wide. Oral sucker 32 (26–37) × 38 (32–47), ventral sucker 39 (37–45) × 42 (35–53), pharynx 17 (14–21) × 22 (19–24). Intestinal caeca almost reaching posterior body end. Excretory vesicle Y-shaped, with

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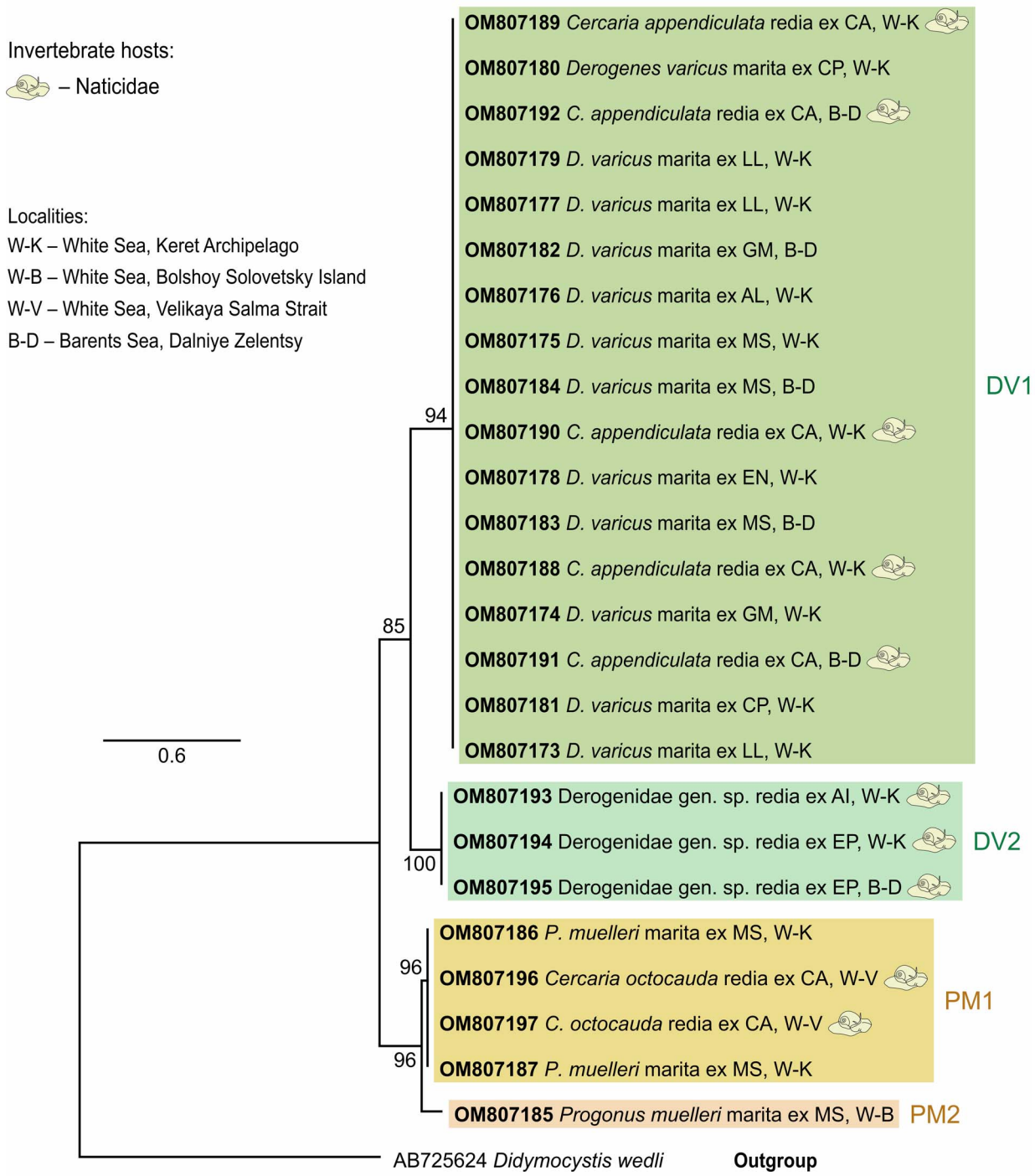

Fig. 4. ML phylogenetic tree based on partial *cox1* gene sequences. Bootstrap values are printed in nodes. Scale bar shows the substitution rate. DV1–2 and PM1–2 mark the separate genetic lineages within *Derogenes varicus* and *Progonus muelleri* species, respectively. Branches within the DV1 clade are collapsed. Abbreviations: AI – *Amauropsis islandica*; AL – *Anarhichas lupus*; CA – *Cryptonatica affinis*; CP – *Clupea pallasii marisalbi*; EN – *Eleginus nawaga*; EP – *Euspira pallida*; GM – *Gadus morhua*; LL – *Limanda limanda*; MS – *Myoxocephalus scorpius*.

widened proximal part; excretory ducts uniting dorsally near pharynx. Eight flame cells within body, 4 more lying loosely within caudal cyst close to the aperture. Muscular sphincter at cyst aperture; retractor muscles connecting body hind end with the cyst wall.

In infective cercariae, body and delivery tube inside caudal cyst, aperture closed, anterior body end close to tightly packed delivery tube. Caudal cyst oval, 195 (170–211) × 155 (138–169) in diameter, 102 (90–123) thick, 2 layered, space between layers broad. Locomotory appendage 600 (529–688) long, 36 (30–41) × 17 (14–21) in diameter, attached at depression on caudal cyst side; furca 72 (62–85) long. Everted delivery tube 446 (430–471) long, 35 (30–44) wide at base, proximal part inflated, maximal width 79 (71–94).

Cercaria octocauda *Tschubrik, 1952 (PM1)* (*Fig. 8*)

Host: *Cryptonatica affinis*

Locality: White Sea, Velikaya Salma Strait.

Sites: Reproductive gland and ducts, partially digestive gland.

Prevalence: 26.7% (4 of 15)

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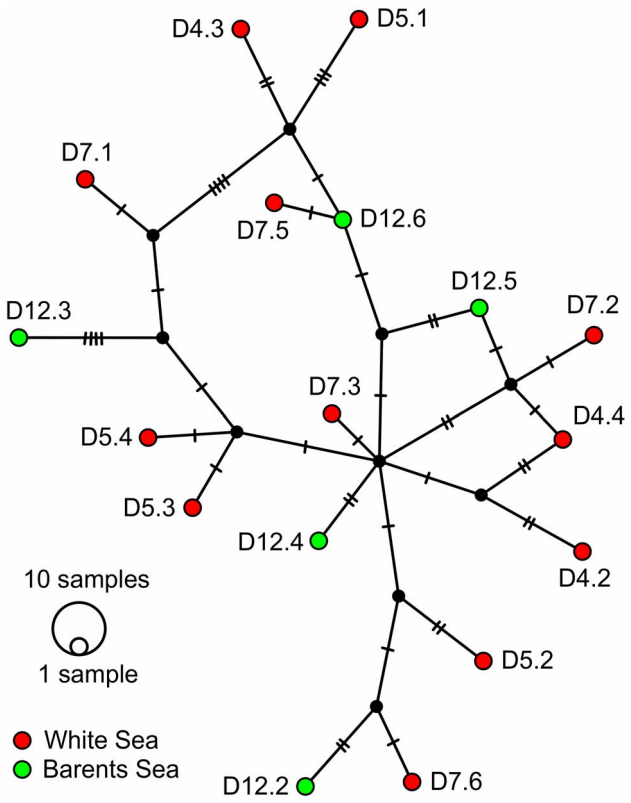

Fig. 5. Haplotype network for DV1 isolates based on partial *cox1* gene sequences. Circle size represents the haplotype frequency. Black dots illustrate missing haplotypes. Number of hatch marks corresponds to the number of substitutions between haplotypes.

Rediae measurements based on 14 ethanol-fixed specimens. Rediae elongated, 1897 (1018–2906) × 182 (138–228). Pharynx small, 43 (36–48) × 33 (29–38). Caecum 422 (309–512) × 27 (20–32). Birth pore close to mouth opening.

Cercariae measurements based on 24 live and 11 glutaraldehyde-fixed specimens. Cercariae of cystophorous type, tail consisting of caudal cyst, delivery tube and locomotory appendage. Body 282 (205–373) long, 57 (32–84) wide. Oral sucker 29 (23–36) × 33 (21–44), ventral sucker 33 (23–38) × 33 (22–41), pharynx 15 (13–18) × 17 (13–22). Intestinal caeca reaching middle of ventral sucker. Excretory vesicle Y-shaped, with widened proximal part; excretory ducts uniting dorsally near pharynx. Eight flame cells within body, 4 more lying within caudal cyst close to aperture, opposite to locomotory appendage attachment spot. Muscular sphincter at cyst aperture; retractor muscles connecting body hind end with anterior part of cyst wall.

Caudal cyst 311 (277–365) long, rounded in cross section, maximal diameter 81 (67–99), anterior end spherical, opposite end pointed. Cyst 2-layered, space between layers broad; outer layer forming arrow-shaped fin at pointed end, 122 (100–145) long, 77 (67–92) wide. Locomotory appendage attached near fin base, 213 (173–254) long, 17 (14–21) in diameter, with 8 immotile threads 631 (547–734) long. In infective cercariae, delivery tube and body drawn into caudal cyst through aperture at its wide end, aperture closed. Tube everting at pointed end of cyst. Delivery tube 1155 (1045–1272) long, width uniform (27 (25–30) – middle region) except wider tip – 39 (36–41).

Discussion

According to our data based on morphological identification, 2 species of the family Derogenidae, subfamily Derogeninae parasitize fishes in the White Sea: *Derogenes varicus* and *Progonus muelleri*. The former was found in a wide range of fishes (10 of 16 examined species), and the latter – predominantly in *Myoxocephalus scorpius*. Such results are consistent with the published data on White Sea fish parasites (Shulman and Shulman-Albova, 1953). We also recovered 1 marita of *D. varicus* from the stomach of the whelk *Buccinum scalariforme* from the White Sea. Such a non-typical host probably received the worm through feeding on an infected dead fish – a post-cyclic transmission event which had been presumed for a long time for various Hemiuroidea (Køie, 1979; Gibson and Bray, 1986) and recently proved to occur in *D. lacustris* (Tsuchida *et al*., 2022). In the Barents Sea we found only *D. varicus*, obviously due to the small number of fish dissected.

Previously, *D. varicus* and *P. muelleri* were recorded as the only derogenid parasites in the Barents Sea (Polyansky, 1955; Issaitschikov, 1933; Mitenev, 1993; Køie, 2009; Kuklin *et al*., 2012). Shulman and Shulman-Albova (1953) claimed to have found 1 more species of the genus *Derogenes* in the White Sea – *D. crassus*. The key difference between *D. varicus* and *D. crassus* is the egg size: 50–66 × 26–39 (Odhner, 1905; Manter, 1926; Lloyd, 1938) and 63–75 × 33–39 (Manter, 1934; Yamaguti, 1938), respectively. Shulman and Shulman-Albova (1953) did not provide morphometric data, and thus there is no convincing evidence that they actually found *D. crassus*. Worldwide, *D. crassus* has otherwise been recovered from warm seas (Manter, 1934; Yamaguti, 1938; McCauley, 1960; Dyer *et al*., 1992; Kuramochi, 2014), and this distribution also raises doubts about the presence of this species in the White Sea.

Genetically our specimens of *D. varicus* split into 2 groups: all the maritae from fish *vs* the marita from the whelk *B. scalariforme*. The former matched the *D. varicus* of Blair *et al*. (1998) (AF029816) from an unspecified locality in 18S rDNA sequence. The latter had the sequence of 28S rDNA fragment identical to the sequence of *D. varicus* from the North Sea (AY222189; Olson *et al*., 2003). Morphological reexamination and morphometry of our specimens identified only 1 difference between maritae from the fish and that from the whelk – average egg size was 51 × 31 and 60 × 35, respectively. However, the ranges overlapped (44–60 × 25–37 and 57–63 × 32–38, respectively), and generally complied with the egg size in the previous descriptions of *D. varicus*: 50–66 × 26–39 (Odhner, 1905; Manter, 1926; Lloyd, 1938). It should be also taken into account that the marita from *B. scalariforme* may be morphologically deviant because it was recovered from a non-typical host. Nonetheless, based on the present data and the sequences of *D. varicus* from GenBank, we conclude that there are 2 clearly distinct lineages of *D. varicus* which we marked as DV1 and DV2. Unfortunately, we have not found maritae of DV2 in fish to compare with DV1, so we cannot diagnose these groups as 2 species through maritae morphology. We may speculate that DV2 is the species that Shulman and Shulman-Albova (1953) called *D. crassus*, but this is yet to be proved. The 18S rDNA sequence AJ287511 of *D. varicus* (Littlewood and Olson, 2001) from the North Sea differed from both DV1 and DV2, and thus represents another lineage of *D. varicus* (labelled DV4 in Fig. 3). Finally, one more lineage is represented by 28S rDNA sequences MW504598–9 of *D. varicus* from the Kuril Islands, Sea of Okhotsk (Sokolov *et al*., 2021), labelled DV3 in Fig. 1. As DV3 and DV4 have been recognized by different markers, it cannot be ruled out that they are one and the same lineage. However, the geographic origin (Pacific *vs* Atlantic) may be an argument that they are not.

In the moonsnails from the White and Barents Seas we found digenean life cycle stages which matched maritae of DV1 and DV2 in the analysed rDNA fragments. Thus, DV1 utilizes *Cryptonatica affinis* as the first intermediate host, and its rediae

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Table 4. Measurements of *Derogenes varicus* (DV1 and DV2) and *Progonus muelleri* (PM1 and PM2) maritae

	DV1 (based on 15 hologenophores and 5 paragenophores)1	DV2 (based on 1 hologenophore)1	PM1 (based on 3 hologenophores)	PM2 (based on 2 hologenophores)
Body length	1291 (873–1885)	1161	1211 (745–1827)	1389 (1351–1427)
Body maximum width	371 (257–498)	469	326 (242–471)	378 (359–397)
Forebody	527 (349–786)	473	564 (300–828)	562 (548–575)
FO/BL	41 (35–47) %	41 %	45 (40–48) %	40 (40–41) %
Post-caecal region	104 (41–165)	37	158 (115–206)	234 (206–262)
PC/BL	8 (5–11) %	3 %	14 (11–20) %	17 (14–19) %
Preoral lobe length	28 (20–40) (based on 4 paragenophores)	–	Not prominent	Not prominent
Oral sucker	152 (89–214) × 167 (117–224)	188 × 217	–	125 (104–145) × 133 (115–151)
Ventral sucker	304 (224–426) × 313 (219–437)	352 × 354	275 (174–351) × 248 (176–362)	314 (291–337) × 313 (294–332)
Sucker-length ratio	2.07 (1.58 –2.88)	1.87	–	2.56 (2.32–2.80)
Sucker-width ratio	1.93 (1.52–2.43)	1.63	–	2.38 (2.20–2.56)
Pharynx	65 (41–87) × 77 (52–104)	81 × 87	60 (55–64) × 64 (62–65)	66 (60–72) × 76 (73–79)
Sinus sac	84 (64–112) × 87 (60–106)	98 × 85	68 (48–91) × 78 (48–109)	66 (65–67) × 83 (77–89)
Pars prostatica length	230 (176–337)	132	125 (65–184)	130 (116–144)
Seminal vesicle	75 (39–225) × 46 (23–81)	93 × 52	90 (53–148) × 49 (28–62)	142 (111–173) × 71 (65–76)
Left testis	105 (70–157) × 105 (62–144)	142 × 88	82 (66–110) × 83 (67–113)	145 (125–164) × 125 (115–134)
Right testis	105 (75–142) × 102 (61–160)	158 × 77	105 (79–147) × 84 (71–107)	154 (140–167) × 131 (120–142)
Ovary	103 (77–153) × 109 (69–158)	146 × 127	143 (114–186) × 124 (92–177)	121 (111–131) × 116 (112–120)
Left vitelline mass	152 (109–203) × 116 (63–201)	173 × 121	146 (103–203) × 113 (76–173)	132 (124–139) × 95 (92–98)
Right vitelline mass	144 (97–211) × 114 (69–173)	167 × 117	153 (113–206) × 99 (76–118)	134 (131–137) × 100 (99–101)
Left vitelline mass position	Anterior, symmetrical, posterior	Anterior	Anterior	Anterior, symmetrical
Eggs	51 (44–60) × 31 (25–37)	60 (57–63) × 35 (32–38)	52 (44–63) × 27 (23–31)	47 (43–54) × 26 (23–29)

FO/BL, forebody to body length ratio; PC/BL, postcaecal region to body length ratio.
1Body length and oral sucker were measured from some of hologenophores before taking a piece for DNA extraction.

and cercariae were described as *Cercaria appendiculata* (Chubrik, 1966; Timofeeva, 1976; Køie, 1979). The first intermediate hosts of DV2 are *Euspira pallida* and *Amauropsis islandica*, and its rediae and cercariae were described by Køie (1990*b*). Cercariae of DV1 and DV2 have similar furcate locomotory appendages, but clearly differ in size and shape of the caudal cyst and delivery tube. Isolates from the first intermediate hosts allowed us to analyse variability in *cox1* gene for DV1 and DV2. The monophyly of these groups was supported, and the gap between intra- and intergroup variation was evident. It is curious that DV1 rediae from the White and Barents Seas differed dramatically in length, but these differences were not consistent with molecular diversity in *cox1*. Such phenotypic variation may arise from different environmental conditions, but currently there are not enough data to discuss it.

To sum up, DV1 and DV2 differ in several genetic markers, both rather conservative (rRNA) and variable (*cox1*), as well as in the first intermediate hosts and in cercaria structure. Thus, the current criteria of species delineation are accomplished (Huston *et al.*, 2021; Bray *et al.*, 2022) and DV1 and DV2 deserve to be recognized as 2 species.

The maritae of *P. muelleri* were genetically heterogeneous, too. Three substitutions were present in the analysed fragments of rDNA: 1 in 28S rDNA, 1 in ITS2 and 1 in 18S rDNA. The divergence in *cox1* gene between these groups was quite high (61–62 substitutions). As genetically different lineages occurred in sympatry, a question on their conspecificity came up, so we refer to them as PM1 and PM2. However, the genetic distance between these lineages is much smaller than that between DV1 and DV2, and in 28S it is similar to the distance between 2 sequences of *D. lacustris* from Tsuchida *et al.* (2021). No morphological differences were found between maritae of *P. muelleri*. *Cercaria octocauda* from *C. affinis* matched 3 maritae of PM1 by all the fragments of rDNA analysed and by *cox1*. Unfortunately, we did not find cercariae matching PM2 to compare them with *C. octocauda*. Therefore, for now we lack morphological or biological evidence to recognize PM1 and PM2 as 2 species.

Redescription of 'true' *D. varicus* and *P. muelleri* also cannot be provided as long as there is no molecular data from the type localities and type hosts. For *D. varicus* these are Danish Exclusive Economic Zone and *Salmo salar* Linnaeus, 1758

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Table 5. Measurements of *C. appendiculata* (DV1) and Derogenidae gen. sp. (DV2) cercariae compared with previous descriptions

	C. appendiculata (DV1)				Derogenidae gen. sp. (DV2)	
	This study (based on 11 live and 30 fixed specimens)	Køie (1979)	Chubrik (1966)	Timofeeva (1976)	This study (based on 18 live and 9 fixed specimens)	Køie (1990*b*)
Caudal cyst Ø1	94 (85–103)	100–110	60–120	80–120	195 (170–211)	180–200
Caudal cyst Ø2	91 (81–100)	–	50–90	–	155 (138–169)	150
Caudal cyst thick	61 (57–65)	–	–	–	102 (90–123)	–
Motile appendage length	145 (109–178)	140–200	90–170	140–210	600 (529–688)	500–600
Motile appendage Ø1	18 (16–21)	20	20	20	36 (30–41)	–
Motile appendage Ø2	8 (7–10)	8	4	10	17 (14–21)	–
Furca length	58 (53–66)	40–60	20–60	40–60	72 (62–85)	80
Delivery tube length	409 (306–456)	500–600	–	300–400	446 (430–471)	500
Delivery tube width (middle)	15 (12–20)	–1	–	10	–	–
Delivery tube width (base)	–	–	–	–	35 (30–44)	30
Delivery tube maximum width	–	–	–	–	79 (71–94)	100
Body length	170 (124–249)	210	150–190	160–210	238 (187–283)	230
Body maximum width	47 (39–56)	55	30–50	30–50	77 (59–109)	80
Oral sucker	27 (25–31) × 27 (25–30)	26 × 34	20	26–28	32 (26–37) × 38 (32–47)	40
Ventral sucker	28 (24–31) × 30 (27–33)	30 × 35	20–30	29–32	39 (37–45) × 42 (35–53)	40
Pharynx	13 (12–16) × 16 (14–17)	14	8–10	10 × 14	17 (14–21) × 22 (19–24)	15

1Only narrow distal part width is mentioned – 10 μm.

Table 6. Measurements of *Cercaria octocauda* (PM1) cercariae compared with previous descriptions

	This study (based on 24 live and 11 fixed specimens)	Chubrik (1966)	Timofeeva (1976)
Caudal cyst length	311 (277–365)	210–280	210–310
Caudal cyst width	81 (67–99)	40–60	40–60
Fin length	122 (100–145)	–	–
Fin maximum width	77 (67–92)	–	–
Motile appendage length	213 (173–254)	120–210	150–210
Motile appendage Ø	17 (14–21)	–	–
Immotile threads length	631 (547–734)	360–430	320–510
Delivery tube length	1155 (1045–1272)	–	600–650
Delivery tube width (middle)	27 (25–30)	–	20–30
Delivery tube width (end)	39 (36–41)	–	–
Body length	282 (205–373)	110–300	140–240
Body maximum width	57 (32–84)	20–50	40–60
Oral sucker	29 (23–36) × 33 (21–44)	20–30	21 × 31
Ventral sucker	33 (23–38) × 33 (22–41)	20–30	36
Pharynx	15 (13–18) × 17 (13–22)	10	–

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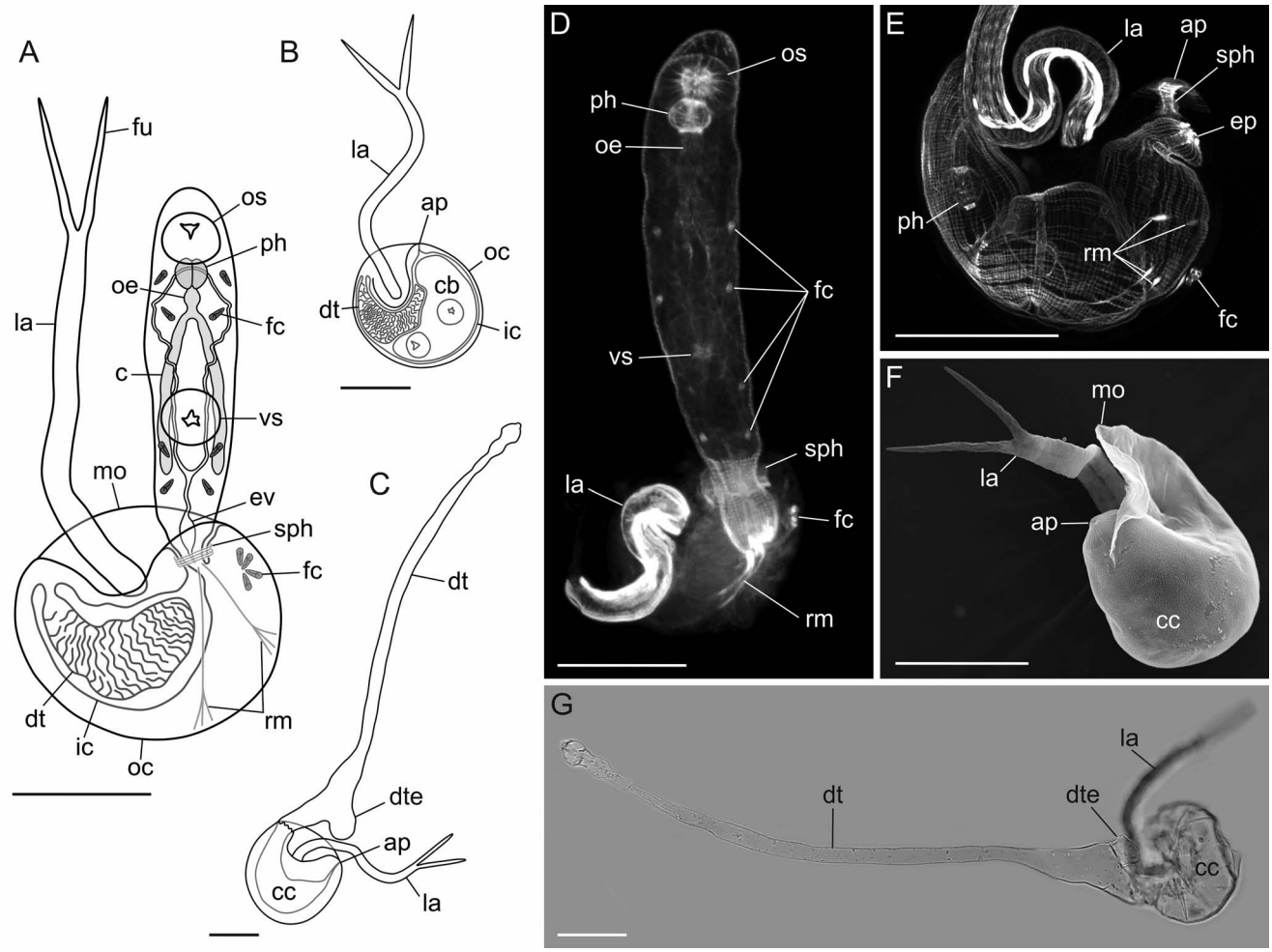

Fig. 6. *Cercaria appendiculata* (DV1). Schemes of pre-infective (A), infective (B) cercariae, and caudal cyst with everted delivery tube (C). (D, E) CLSM, TRITC-phalloidin staining; muscular, digestive and excretory systems in pre-infective (D) and infective (E) cercaria. (F) SEM, infective cercaria, lateral view. (G) Bright field, caudal cyst with everted delivery tube. Scale bars – 50 μm. Abbreviations: ap – aperture; c – caecum; cb – cercarial body; cc – caudal cyst; dt – delivery tube; dte – delivery tube extension; oe – oesophagus; ep – excretory pore; ev – excretory vesicle; fc – flame cell; fu – furca; ic – inner cyst layer; la – locomotory appendage; mo – membranous outgrowth; oc – outer cyst layer; os – oral sucker; ph – pharynx; rm – retractor muscles; sph – sphincter; vs – ventral sucker.

(Müller, 1784). Type locality of *P. muelleri* is West Greenland Shelf, but type host is not specified, that is either *Myoxocephalus scorpius* or *Gadus macrocephalus* Tilesius, 1810 (Levinsen, 1881).

Our results for the life cycles of DV1, DV2 and PM1 comply with the previous data on the life cycles of the Hemiuroidea in general. Moonsnails serve as the first intermediate hosts for all these 3 species. This may be characteristic of the subfamily Derogeninae, but not the Derogenidae as a whole: representatives of the other subfamilies infect pulmonates and different groups of Littorinimorpha (Madhavi, 1978; Goater *et al.*, 1990; Køie and Gibson, 1991). Metacercariae of *D. varicus* had been described from various planktonic copepods (Wright, 1907; Weinstein, 1972). They probably serve as the most common second intermediate hosts, at least for DV1, according to the experiments of Køie (1979). Experimental data on DV2 cercariae are ambiguous: Køie (1990*b*) succeeded in infecting 3 copepod species by cystophorous cercariae from *E. pallida*, but those copepods did not survive for more than several weeks. Metacercariae identified as *D. varicus* also had been found multiple times in Chaetognatha (Kulachkova, 1972; Weinstein, 1972; Øresland, 1986; Rolbiecki and Waskusz, 2005), and it is probable that they were infected by eating copepods (Dollfus, 1960; Køie, 1979). Unlike *D. varicus*, *P. muelleri* had never been recorded in the second intermediate host until recently when its metacercaria was found in a skeleton shrimp *Caprella septentrionalis* Krøyer, 1838 (Caprellidae, Amphipoda) from the White Sea (Sokolov *et al.*, 2021). The 28S rDNA sequence of this metacercaria fell into the PM1 group in our analysis. Thus, all the 3 hosts of PM1 are revealed now.

The hemiuroidean cercariae are quite diverse in tail structure, though some patterns with respect to phylogeny may be derived. For example, the vast majority of the Hemiuridae with known larval stages are characterized by cercariae with an oar-shaped locomotory appendage of the tail (Køie, 1990*a*, 1990*b*, 1992, 1995; Krupenko *et al.*, 2020). Cercariae of the Lecithasteridae are immotile and possess roundish caudal cyst (Hunninen and Cable, 1943; Køie, 1989; Køie *et al.*, 2002; Krupenko *et al.*, 2022). Within the family Derogenidae cercariae are not uniform (Madhavi, 1978; Køie, 1979; Goater *et al.*, 1990; Køie and Gibson, 1991). Our data showed that cercariae noticeably differ even within the subfamily Derogeninae. Both *Derogenes* and *Progonus* have locomotory appendages, but in the former it is furcate and in the latter it has 8 immotile threads. Differences in the cystophorous cercariae structure must be strongly dependent on the second intermediate host biology. A case of *Halipegus occidualis* Stafford, 1905 is particularly illustrative in this respect: evolutionary switch to ostracod second intermediate hosts resulted in the modification of cercariae tail and infection mechanism (Zelmer and Esch, 1998). Close phylogenetic relationship of *Derogenes* and *Progonus* contrasts with the difference in their tail morphology, and this brings us back to their life cycles. Structure of *Progonus* cercariae may be interpreted as adaptive to infect *C. septentrionalis* which is a filter-

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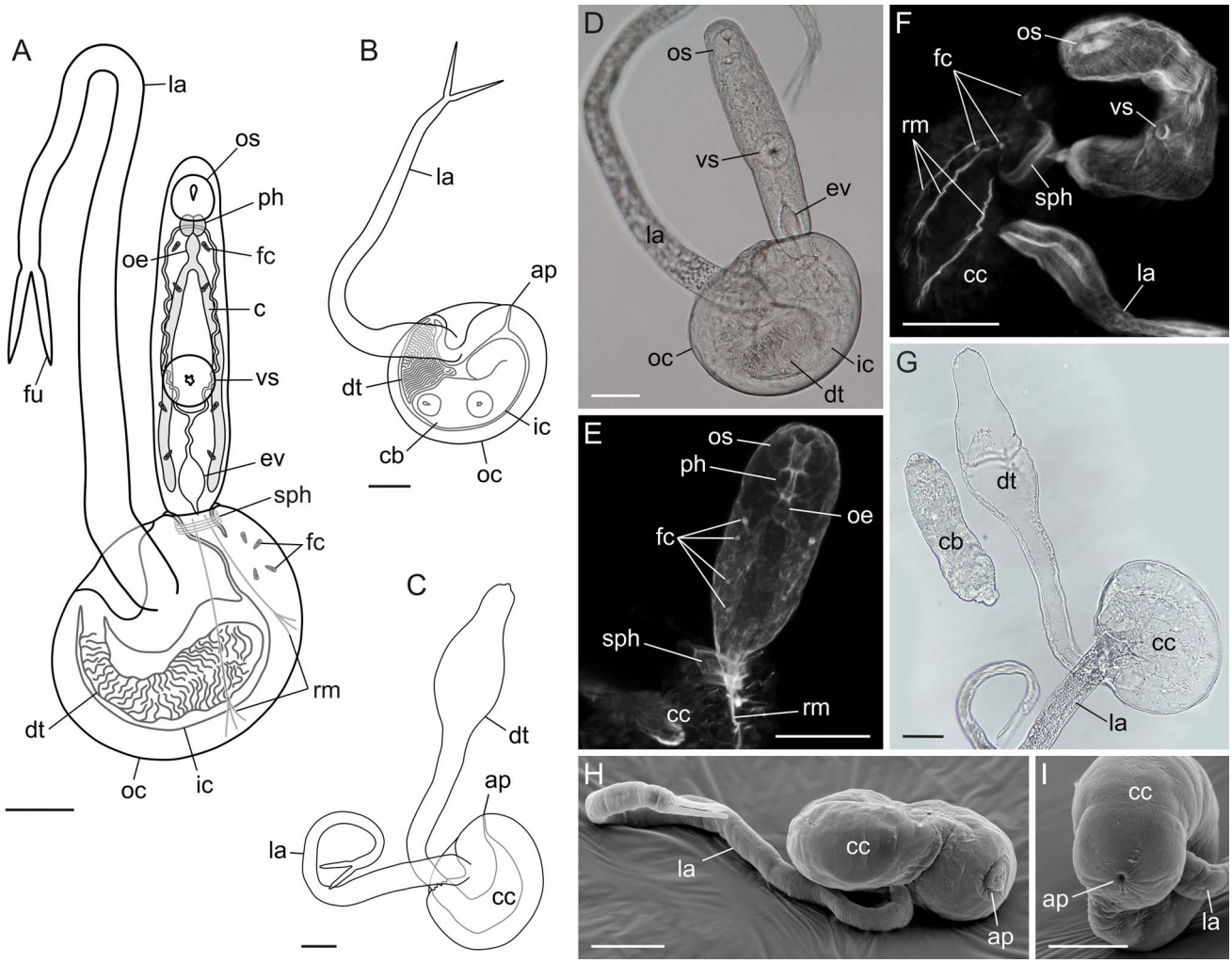

Fig. 7. Derogenidae gen. sp. (DV2). Schemes of pre-infective (A), infective (B) cercaria and caudal cyst with everted delivery tube (C). (D) Bright field, live pre-infective cercaria, general view. (E, F) Pre-infective cercariae, CLSM, TRITC-phalloidin staining; muscular, digestive and excretory systems. (G) Bright field, caudal cyst with everted delivery tube. (H, I) SEM, infective cercaria. Scale bars – 50 μm. Abbreviations: ap – aperture; c – caecum; cb – cercarial body; cc – caudal cyst; dt – delivery tube; oe – oesophagus; ev – excretory vesicle; fc – flame cell; fu – furca; ic – inner cyst layer; la – locomotory appendage; oc – outer cyst layer; os – oral sucker; ph – pharynx; rm – retractor muscles; sph – sphincter; vs – ventral sucker.

feeder (Legeżyńska *et al.*, 2012): threads on the locomotory appendage of cercaria let it be easily trapped in the setose antennae 2 of *C. septentrionalis*. Switch to skeleton shrimps also might have resulted in narrowing the definitive host range of *Progonus*: demersal fish are favoured, and the highest infection rates are registered for the European sculpin (Shulman and Shulman-Albova, 1953; Polyansky, 1955; our data). Thus, the life cycle of *Progonus* seems to be closely associated with benthic communities, rather than with the planktonic ones as in *Derogenes*.

Conclusion

Marine digeneans with wide geographical distribution and low specificity towards the definitive host are obvious candidates to be species complexes. *Derogenes varicus* was proved to be the one, and *Progonus muelleri* is under suspicion now. To untangle these complexes, a worldwide sampling is needed, and primarily – specimens from the type hosts and type localities. At the same time, quite a number of marine digeneans had been described based on a single or few specimens, and do not have reliable diagnostic features. For instance, it is doubtful that *D. robustus* Brinkmann, 1967, *D. chelidonichthydis* Shen, 1989, *D. minoi* Shen, 1990, *D. magnus* Wang, 1991, *D. bohaiensis* Qiu & Liang in Shen & Qiu, 1995 can be distinguished from *D. varicus*. Molecular methods are essential to check whether these species are valid, and life-cycle studies are likely to be equally valuable.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S003118202200110X.

Data availability. The aligned 28S, 18S, 5.8 rDNA, ITS2 and *cox1* datasets used in phylogenetic analysis are freely accessible at http://dx.doi.org/10.13140/RG.2.2.19523.27683/1.

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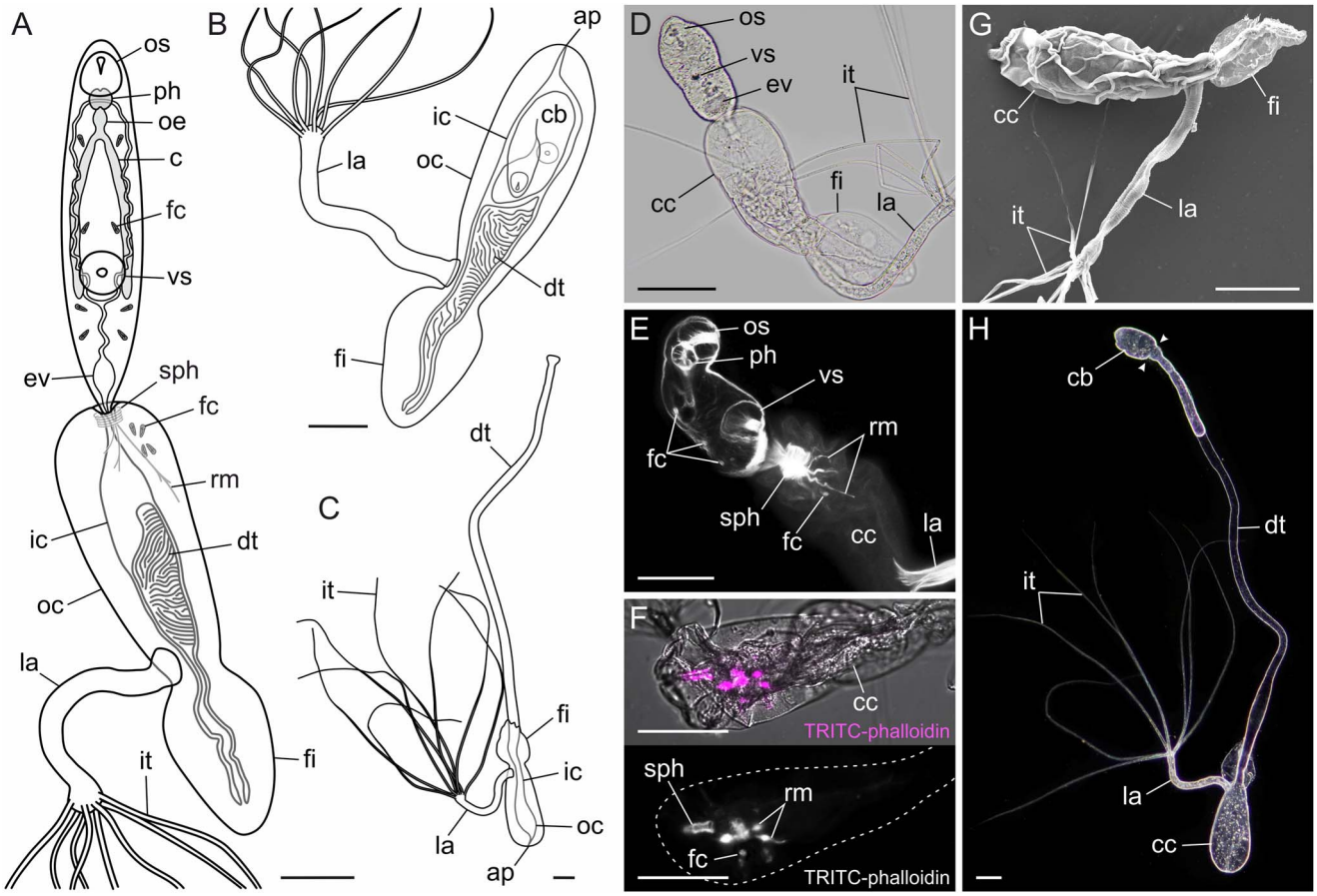

Fig. 8. *Cercaria octocauda* (PM1). Schemes of pre-infective (A), infective (B) cercaria and caudal cyst with everted delivery tube (C). (D) Bright field, live pre-infective cercaria, general view. (E, F) CLSM, TRITC-phalloidin staining; muscular, digestive and excretory systems in pre-infective cercaria (E) and in caudal cyst (F). (G) SEM, infective cercaria. (H) Phase-contrast microscopy, cercaria leaving caudal cyst through delivery tube; arrowheads indicate tip of delivery tube. Scale bars – 50 μm. Abbreviations: ap – aperture; c – caecum; cb – cercarial body; cc – caudal cyst; dt – delivery tube; oe – oesophagus; ev – excretory vesicle; fc – flame cell; fi – arrow-shaped fin; ic – inner cyst layer; it – immotile threads; la – locomotory appendage; oc – outer cyst layer; os – oral sucker; ph – pharynx; rm – retractor muscles; sph – sphincter; vs – ventral sucker.

Author's contributions. DK conceived and designed the study. DK, GK, AU, VK, OS, AGu and OK conducted data gathering. DK, GK and AGo performed molecular analyses. AM, DK and GK performed morphological analyses. DK, GK and AGo wrote the article.

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Conflicts of interest. The authors declare there are no conflicts of interest.

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