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Molecular and morphological screening of *Podocotyle* spp. (Trematoda: Opecoelidae) sheds light on their diversity in Northwest Pacific and eastern European Arctic

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Research Paper

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Abstract

Podocotyle is a genus of marine opecoelid digeneans that parasitize a wide variety of fish as adults. We present the first phylogenetic analysis of several *Podocotyle* isolates using nuclear 28S rDNA and mitochondrial *cox*1 DNA regions. New sequences were obtained for *Podocotyle* specimens from fish caught in the Sea of Okhotsk and the White Sea. Based on morphological and molecular data, eight *Podocotyle* lineages of species rank were revealed. However, this diversity is poorly formalized within the current taxonomic model of the genus. As a result, we identified *Podocotyle* cf. *angulata, Podocotyle* cf. *atomon, Podocotyle* cf. *reflexa*, *Podocotyle atomon* of Sokolov et al., 2019, *Podocotyle* sp. of Denisova et al., 2023, *Podocotyle* sp. 1, *Podocotyle* sp. 2 and *Podocotyle* sp. 3. We also highlight the unresolved question of the life cycles of representatives of *Podocotyle* whose intramolluscan stages parasitize the intertidal snails *Littorina* spp.

Introduction

The genus *Podocotyle* Dujardin, 1845 unites marine opecoelid digeneans with a well-developed cirrus sac, long blindly ending ceca, a deeply lobed ovary, unspecialized suckers and eggs, vitelline fields usually restricted to the hindbody and some other features (Cribb 2005; Gibson and Bray 1982). The definitive hosts of *Podocotyle* spp. are fish from various families. The life cycle of these digeneans also includes the first (marine gastropods) and second (marine amphipods and isopods) intermediate hosts (e.g., Hunninen and Cable 1943; Køie 1981; Szuks 1975; Uspenskaya 1963). Martin et al. (2019) classify *Podocotyle* as a member of the subfamily Podocotylinae Dollfus, 1959.

A recent revision of *Podocotyle* recognizes 27 valid species of the genus (Blend et al. 2019). This view, in turn, builds on a number of previously published sources on *Podocotyle* taxonomy (e.g., Blend and Dronen 2015; Blend et al. 2016; Bray and Campbell 1996; Gibson 1986; Gibson and Bray 1982; Martin et al. 2017; Park 1937; Pritchard 1966). However, many species of the genus, and especially three of those known since the 19th century, *Podocotyle angulata* Dujardin, 1845; *Podocotyle atomon* (Rudolphi, 1802) and *Podocotyle reflexa* (Creplin, 1825), require further detailed revision with the mandatory involvement of molecular data (Blend et al. 2019) because they are characterized by a wide range of morphological and ecological variations.

At present, molecular data are available only for sporocysts and/or cercariae of *Podocotyle* sp. of Denisova et al. (2023) and *Podocotyle atomon* of Sokolov et al. (2019) and adults *Podocotyle* cf. *atomon* of Denisova et al. (2023) and *Podocotyle scorpaenae* (Rudolphi, 1819) (Denisova et al. 2023; Jousson et al. 1999; Sokolov et al. 2019). However, data on the genetic marker most appropriate for reliable DNA barcoding, namely the *cox1* mtDNA gene, were obtained only for *Podocotyle* sp. of Denisova et al. (2023) ex *Littorina obtusata* (Linnaeus, 1758) and *Podocotyle* cf. *atomon* of Denisova et al. (2023) ex *Cyclopterus lumpus* Linnaeus, 1758. Hosts of both trematode species were collected in the White Sea.

Due to the urgent need for molecular data on *Podocotyle* spp. we present data on five species from the White Sea and the Sea of Okhotsk with morphological characteristics of the studied adults.

Materials and methods

Sample collection and morphological study

Adult specimens of *Podocotyle* spp. were collected from the intestines of *C. lumpus* Linnaeus, 1758; *Zoarces viviparous* (Linnaeus, 1758); *Pholis gunnellus* (Linnaeus, 1758); *Limanda limanda* (Linnaeus, 1758) and *Platichthys flesus* (Linnaeus, 1758), caught near the Educational and Research

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Station 'Belomorskaia' of St. Petersburg State University (Kandalaksha Bay, White Sea, 66°17'42" N; 33°38'47" E) in 2017–2023, as well as from the intestines of *Pleurogrammus azonus* Jordan and Metz, 1913 from the Sea of Okhotsk off the southwestern coast of Iturup Island, Russia (44°42'4" N; 147°11'7" E) in August and September 2021, and *Rhodymenichthys dolichogaster* (Pallas, 1814), *Pholis picta* (Kner, 1868) and *Pholidapus dybowskii* (Steindachner, 1880) from the same sea off the southeastern coast of Sakhalin Island, Russia (47°54'41" N; 142°31'4" E) in June 2021. All trematodes were initially relaxed in fresh water and fixed in 70% ethanol; after a few minutes, the specimens were transferred to 96% ethanol.

Trematode specimens were studied by morphological and/or molecular methods. For morphological study, samples were stained with acetocarmine, dehydrated in a graded series of ethanol, cleared in dimethyl phthalate, and finally mounted in Canada balsam. All measurements are in micrometers. The drawings were made using a camera lucida. Paragenophores were deposited at the Museum of Helminthological Collections of the Center of Parasitology of the Severtsov Institute of Ecology and Evolution (IPEE RAS; Moscow, Russia).

We did not perform a morphological study of some of the isolates represented by single adults of *Podocotyle* because we could not obtain positive molecular results on body fragments and had to use all material for DNA extraction.

Molecular data and phylogenetic analyses

Total DNA was isolated from individual specimens using a Chelex-100 with Proteinase-K. Forward primer dig12 (5′-AAG CAT ATC ACT AAG CGG-3′) and reverse primer L0 (5′-GCT ATC CTG AGR GAA ACT TCG-3′) (Tkach et al. 2000) were used to amplify partial 28S rDNA, and JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′), JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) (Bowles et al. 1992) were used to amplify partial *cox*1 mtDNA gene. Polymerase chain reactions were performed in a total volume of 20 µl (11.5 µl H_2O, 2.5 µl Taq buffer, 2 µl dNTP's at a concentration of 10 pM, 0.5 µl of each primer at a concentration of 10 pM, 1 µl of Syntol Taq polymerase, 1 µl of the DNA template). The thermal cycler parameters were as follows: initial denaturation at 95°C (3 min); denaturation 20 s, 95°C; annealing 20 s at 53,4°C for dig12/L0 primers, elongation 120 s at 72°C. For JB3/JB4.5 primers, annealing 20 s at 48.9°C and elongation 50 s at 72°C were performed. Final extension 5 min at 72°C for both primer pairs with 35 cycles of polymerase chain reaction was used. All amplicons were sequenced using the equipment of the Research Park of St. Petersburg State University (Centre for Molecular and Cell Technologies). Sequences from both forward and reverse primers were assembled using Chromas Pro 1.7.4 (Technelysium Pty., Ltd.).

To assess the phylogenetic position of *Podocotyle* spp., Bayesian inference analyses were performed on the 28S rDNA and *cox1* gene dataset (Table 1). The general alignment of partial 28S rDNA and *cox*1 gene sequences was generated with the MUSCLE algorithm (Edgar 2004), and trimmed manually in SeaView v. 4 software (Gouy et al. 2010). The final length of alignment was 1203 base pairs (bp) for partial 28S rDNA sequence and 242 bp for *cox*1 gene. The evolutionary model for Bayesian inference analysis was

Table 1. List of species, incorporated into phylogenetic analyses

Parasite species	GenBank number		Host species	Location	Source
	*cox*1 mtDNA	28S rDNA			
Podocotyle cf. *angulata* [available as *Podocotyle* cf. *atomon*]	OQ145418	–	*C. lumpus*	White Sea	Denisova et al. (2023)
Podocotyle cf. *atomon*	OR424374	OR439002	*Pholis gunnellus*	White Sea	This study
Podocotyle cf. *atomon*	OR424375	OR439003	*Z. viviparus*	White Sea	This study
Podocotyle cf. *atomon*	OR424383	–	*Limanda limanda*	White Sea	This study
P. cf. *reflexa*	OR424378	OR439006	*Pleurogrammus azonus*	Sea of Okhotsk	This study
P. cf. *reflexa*	OR424377	–	*Pleurogrammus azonus*	Sea of Okhotsk	This study
Podocotyle sp. 1	OR424376	OR439005	*R. dolichogaster*	Sea of Okhotsk	This study
Podocotyle sp. 2	OR424379	OR439001	*Pholidapus dybowskii*	Sea of Okhotsk	This study
Podocotyle sp. 2	OR424378	–	*Pholis pincta*	Sea of Okhotsk	This study
Podocotyle sp. 3	OR424382	OR439004	*Platichthys flesus*	White Sea	This study
Podocotyle atomon of Sokolov et al. (2019)	–	MH161437	*Littorina saxatilis*	White Sea	Sokolov et al. (2019)
Podocotyle sp. of Denisova et al. (2023)	OQ079535	–	*Littorina obtusata*	White Sea	Denisova et al. (2023)
Podocotyle sp. of Denisova et al. (2023)	OQ079536	–	*Littorina obtusata*	White Sea	Denisova et al. (2023)
Outgroup					
Helicometra. fasciata	MT472179	–	*Symphodus tinca*	Black Sea	Katokhin and Kornyychuk (2020)
H. fasciata	–	OK644194	*Scorpena porcus*	Black Sea	Sokolov et al. (2022)

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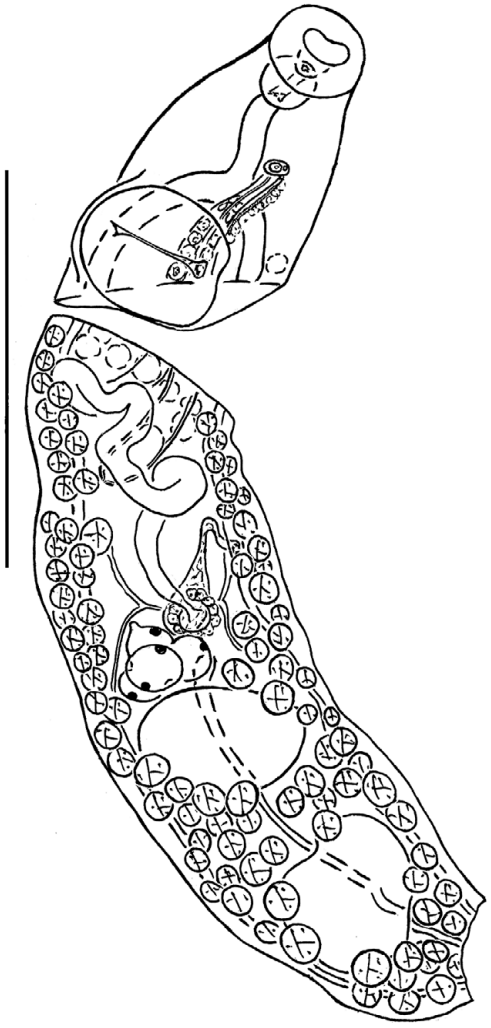

Figure 1. Body fragments of hologenophore of *Podocotyle* cf. *angulata* from intestine of *C. lumpus*, White Sea, ventral view; distance between these fragments is shown out of drawing scale. Scale bar = 1000 μm.

estimated with MrModeltest v. 2.4 (Nylander 2004). The best-fitted model was GTR + G + I. Bayesian analysis was performed using MrBayes v. 3.2.7a at the CIPRES portal (Miller et al. 2010) for 15,000,000 generations. The quality of the chains was estimated using built-in MrBayes tools and additionally estimated with Tracer v. 1.6 package (Rambaut et al. 2018). Based on the estimates by Tracer, the first 5,000 generations were discarded for burn-in in both analyses.

The p-distances were calculated based on partial *cox1* gene sequences with MEGA11 software (Tamura et al. 2021) with standard parameters. We included *Helicometra fasciata* (Rudolphi, 1819) (Opecoelidae, Helicometrinae) as an outgroup in our analysis.

Results

Podocotyle cf. *angulata* Dujardin, 1845

Syn: *Podocotyle* cf. *atomon* of Denisova et al. (2023)

Host: *C. lumpus* Linnaeus, 1758 (Perciformes, Cottoidei: Cyclopteridae).

Site: Intestine.

Locality: Kandalaksha Bay of the White Sea (66°17'42" N; 33° 38'47" E).

Specimens deposited: The hologenophore is stored in the personal collection of the first author.

Description (based on two fragments of one gravid specimen, hologenophore): Body elongate oblong, length according to sum of two fragments 2,758, maximum width 560 (Figure 1). Tegument unarmed. Oral sucker subellipsoid, 208 × 222; mouth opening subterminal. Ventral sucker transversely oval when dorso-ventral orientation, slightly protuberant, 305 × 353. Sucker-width ratio 1 : 1.59. Prepharynx indistinguishable. Pharynx 145 × 142. Oesophagus 249 long. Intestinal bifurcation in posterior third of forebody. Caeca with narrow lumen; terminate blindly posterior to testes.

Testes two, tandem, separated; anterior testis entire, 395 × 270, posterior testis slightly indented, 346 × 367. Cirrus-sac extends well into hindbody. Internal seminal vesicle indistinguishable. Pars prostatica tubular, surrounded by large pars prostatica. Ejaculatory duct distinctly shorter than pars prostatica. Cirrus unarmed. Genital atrium shallow. Common genital pore sinistro-submedian, prebifurcal.

Ovary conical anteriorly and three-lobed posteriorly, slightly dextro-submedian, immediately pretesticular, 187 × 242. Oviduct indistinguishable. Canalicular seminal receptacle saccular, antero-sinistral to ovary. Laurer's canal opens dorsal to left caecum anterior to ovary. Oötype with Mehlis's gland sinistral to anterior part of ovary. Uterus preovarian, intercaecal. Metraterm quite thick-walled, ensheathed in gland-cells, opens to genital atrium antero-sinistrally to male duct. Eggs operculate, deformed in balsam; length of least-deformed eggs 76. Vitellarium follicular; ventral follicles in two lateral fields, overlap caeca, confluent in posttesticular region and almost confluent in intertesticular region, anterior and posterior borders of fields not clear from preserved body fragments; dorsal follicles also in two lateral fields, confluent at level of internal seminal vesicle and in in posttesticular region, anterior border of left dorsal field at level anterior margin of ventral sucker.

Excretory vesicle I-shaped; reaches to ovary.

Remarks

Podocotyle angulata has an intricate taxonomic history, and for a significant period this taxon was considered conspecific with *P. atomon* (e.g., Edmiston 1971; Odhner 1905). In our study, we follow the findings of Blend et al. (2019) and Gibson and Bray (1982) on the validity of *P. angulata*. According to Gibson and Bray (1982), *P. staffordi* Miller, 1941 and *P. atomon* var. *dispar* Nicoll, 1909 are synonyms of *P. angulata*. The most significant morphological differences between *P. angulata* and *P. atomon* are the relative sizes of testes (width of each testis >½ width body at their level versus <½), a ratio of body length and width (5–6 : 1 versus 4 : 1), the cirrus-sac length (extends noticeably posterior to ventral sucker versus short distance from sucker) and a sucker ratio (1 : 2 versus 1 : < 2) (Gibson and Bray 1982; MacKenzie and Gibson 1970). According to Blend et al. (2019), *P. angulata* differs from *P. atomon* in testes separated by a distinct distance filled with vitelline follicles. This finding is consistent with the description of *P. angulata sensu stricto* but contradicts that of *P. staffordi* because this nominal species has an intertesticular space filled with vitelline follicles (compare with Dollfus (1968); Miller (1941)). However, Blend et al. (2019), following Gibson and Bray (1982),

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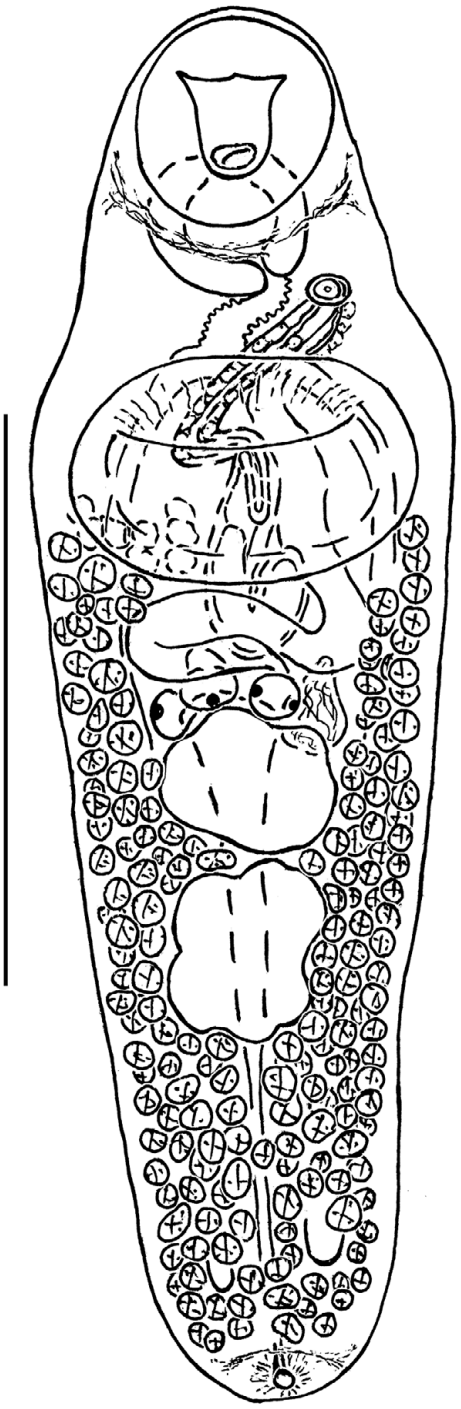

Figure 2. Paragenophore of *Podocotyle* cf. *atomon* from intestine of *Z. viviparus*, White Sea, ventral view. Scale bar = 500 μm.

consider *P. staffordi* to be a junior synonym of *P. angulata*. It is possible that *P. angulata sensu stricto* and *P. staffordi* are actually different species, but we prefer to consider them conspecific for the present.

The trematode specimen from *C. lumpus* studied by us is similar to the one found by Szuks (1975, Figure 17) in same host species from the Baltic Sea and identified by this author within the concept of the *P. atomon* complex. The differences between these specimens are related to a sucker ratio (1 : 1.59 versus 1 : 2). In turn, both trematode specimens are similar to *P. angulata*, more precisely, to its morphological variant, previously described as *P. staffordi*.

The present specimen is a hologenophore for sequence (GenBank acc. number OQ145418) obtained by Denisova et al. (2023), where it appears as *Podocotyle* cf. *atomon*, although a morphological description of the parasite is not presented in their publication.

Podocotyle cf. *atomon* (Rudolphi, 1802)

Host: *Z. viviparus* (Linnaeus, 1758) (Perciformes, Zoarcoidei: Zoarcidae).
Site: Intestine.
Locality: Kandalaksha Bay of the White Sea (66°17'42" N; 33° 38'47" E).
Specimens deposited: Paragenophores, IPEE RAS 14334.

Description (based on four gravid specimens, paragenophores): Body elongate, with ventrally folded anterior end in some specimens, 1,198–1,433 × 367–450; length to width ratio 1:0.27–0.31 (Figure 2). Tegument unarmed. Oral sucker ellipsoid, 204–208 × 152–187; mouth opening subterminal. Ventral sucker transversely oval when dorso-ventral orientation, sessile, 194–228 × 298–350. Sucker-width ratio 1 : 1.87–2.05. Forebody 23.8–26.9% of body length. Prepharynx indistinguishable. Pharynx 132–152 × 132–138. Oesophagus strongly contracted, 53–106 long. Intestinal bifurcation at level of anterior margin of ventral sucker. Caeca narrow; terminate blindly close to posterior extremity.

Testes two, tandem or nearly so, indented, in anterior and middle thirds of hindbody, contiguous; anterior testis 124–159 × 103–180, posterior testis 159–184 × 131–138. Posttesticular region 25.4–33.5% of body length. Cirrus-sac slender, sinuate to looped, 391–595 × 53–71, reaches to posterior margin of ventral sucker (one specimen) or comparatively short distance posterior to ventral sucker up 32–71 (three specimens). Internal seminal vesicle saccular proximally and tubular distally; saccular part rectilinear or with three twists, tubular part forms distinct loop. Pars prostatica tubular, surrounded by large pars prostatica. Ejaculatory duct indistinguishable. Cirrus unarmed. Genital atrium shallow. Common genital pore sinistro-submedian, prebifurcal.

Ovary conical anteriorly and three-lobed posteriorly, median or slightly dextro-submedian, immediately pretesticular, 53–127 × 124–177. Distance from posterior margin of ventral sucker to anterior margin of ovary 3.4–5.6% of body length. Oviduct indistinguishable. Canalicular seminal receptacle saccular, sinistral to ovary. Laurer's canal indistinguishable. Oötype with Mehlis's gland indistinguishable. Uterus comparatively short, intercaecal; proximal uterine loops surround ovary, touching to anterior testis or whole preovarian. Metraterm quite thick-walled, ensheathed in gland-cells, opens to genital atrium antero-sinistrally to male duct. Eggs operculate, deformed in balsam; length of least-deformed eggs 73–82. Vitellarium follicular; ventral follicles in two lateral fields extending from level of posterior quarter or posterior margin of ventral sucker to posterior extremity, overlap caeca, confluent in posttesticular region; dorsal follicles overlap caeca at about level of ventral sucker, then pass into left and right exstracaecal rows and form two posttesticular rows along medial margins of caeca, anterior border of dorsal follicles at same level as ventral follicles (three specimens) or at level of intestinal bifurcation (one specimens).

Excretory vesicle I-shaped; reaches to ovary.

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Figure 3. Body fragments of hologenophore of *P.* cf. *reflexa* from intestine of *Pleurogrammus azonus*, Sea of Okhotsk, ventral view; distance between these fragments is shown out of drawing scale. Scale bar = 1000 μm.

Remarks

The presented specimens of trematodes are characterized by both features corresponding to the description of *P. atomon*, namely a not very elongated body, a cirrus-sac slightly protruding into the hindbody, relatively small testes occupying ≤½ of the body cross section, and by features that have an intermediate manifestation between *P. atomon* and *P. angulata* (sucker ratio 1 : 1.87–2.05). According to Blend et al. (2019), *P. atomon* is characterized by separated testes. However, this thesis at least contradicts the description of *Podocotyle odhneri* Issaitschikov, 1928, the conspecificity of which with *P. atomon* is recognized by this author. Here, we consider the studied specimens from *Z. viviparus* as *Podocotyle cf. atomon*, due to the formal predominance of the specific characters of *P. atomon*.

According to Shulman-Albova (1952), *Z. viviparus* from the White Sea is parasitized by *Podocotyle* specimens, described as *P. atomon* form B. The specimens we studied from this host differ most sharply from *P. atomon* form B of Shulman-Albova (1952) in the sucker ratio (1 : 1.87–2.05 versus 1 : 1.22) and the arrangement of testes (contiguous versus separated).

Podocotyle cf. *reflexa* (Creplin, 1825)

Host: *Pleurogrammus azonus* Jordan and Metz, 1913 (Perciformes, Cottoidei: Hexagrammidae).
Site: Intestine.
Locality: The Sea of Okhotsk off the south-western coast of Iturup Island, Russia (44°42'4" N; 147°11'7" E).
Specimens deposited: The hologenophores are stored in the personal collection of the first author.

Description (based on fragments of two gravid specimens, hologenophores): Body elongate oblong, length according to sum of two fragments 3,967–4,116, maximum width 770–812 (Figure 3). Tegument unarmed. Oral sucker subellipsoid, 228–249 × 242–263; mouth opening subterminal. Ventral sucker with axis inclined anteriorly, protuberant, 485–533 in wide. Sucker-width ratio 1 : 2.00–2.03. Prepharynx indistinguishable. Pharynx 215–222 × 138–152. Oesophagus contracted, 90–104 long. Intestinal bifurcation at level of aperture of inclined ventral sucker. Caeca with narrow lumen; terminate blindly posterior to testes.

Testes two, tandem, indented, separated; anterior testis 532–602 × 420–462, posterior testis 476–504 × 434–476. Cirrus-sac curved, extends well into hindbody, 1,038–1,073 × 194–208. Internal seminal vesicle coiled. Pars prostatica tubular, surrounded by large pars prostatica. Ejaculatory duct distinctly shorter than pars prostatica. Cirrus unarmed. Genital atrium shallow. Common genital pore sinistro-submedian, immediately anterior to aperture of inclined ventral sucker.

Ovary conical anteriorly and three-lobed posteriorly, median or slightly dextro-submedian, pretesticular, separated or contiguous, 280 × 378–392. Oviduct indistinguishable. Canalicular seminal receptacle saccular, dorsal to ovary. Laurer's canal opens sinistral to ovary. Oötype with Mehlis's gland contiguous with sinister or antero-sinister margin of ovary. Uterus preovarian, intercaecal. Metraterm quite thick-walled, ensheathed in gland-cells, opens to genital atrium antero-sinistrally to male duct. Eggs operculate, deformed in balsam; length of least-deformed eggs 76–79. Vitellarium follicular; ventral and dorsal follicles in two lateral fields, extending from nearly or immediately posterior margin of ventral sucker to posterior extremity, overlap caeca, interrupted laterally to testes, confluent in posttesticular and intertesticular regions.

Excretory vesicle I-shaped; reaches to ovary.

Remarks

The presented specimens of trematodes are fully consistent with modern concepts of *P. reflexa*, namely: the body is elongated and relatively narrow, the cirrus-sac is elongated claviform and extends posteriorly from the ventral sucker, the seminal vesicle is coiled, the

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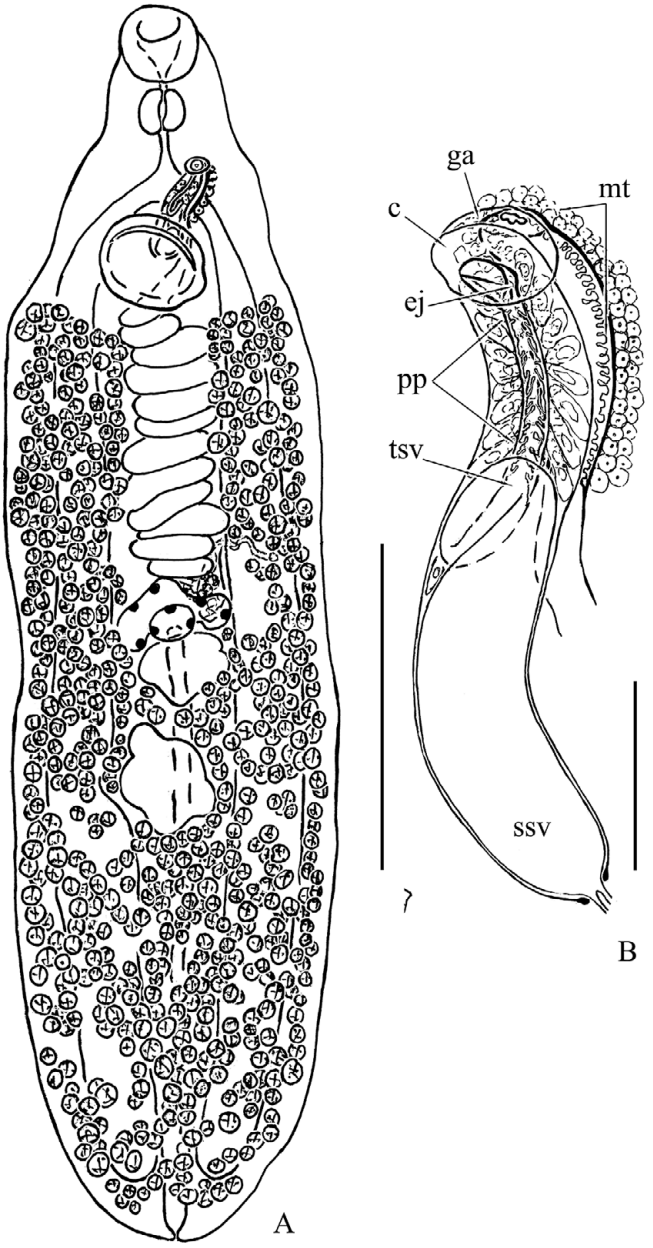

Figure 4. *Podocotyle* sp. 1 from intestine of *R. dolichogaster*, Sea of Okhotsk. **A** – paragenophore, whole ventral view; **B** – terminal genitalia of hologenophore, ventral view. c — cirrus partially everted through genital atrium; ej — ejaculatory duct; ga — genital atrium; mt — metraterm; pp — pars prostatica; ssv — saccular part of internal seminal vesicle; tsv — tubular part of internal seminal vesicle. Scale bar; A = 1000 μm; B = 100 μm.

fields of vitelline follicles are interrupted at the testicular level and not penetrated into the forebody and the ventral sucker is twice as wide as the oral sucker (Blend et al. 2019 with addition by Edmiston 1971). The type locality of *P. reflexa* is the Baltic Sea (Northern Atlantic) (Creplin 1825). In this regard, we leave some doubt about the identification and designate our specimens as *Podocotyle* cf. *reflexa.*

Podocotyle sp. 1

Host: *R. dolichogaster* (Pallas, 1814) (Perciformes, Zoarcoidei: Pholidae).
Site: Intestine.
Locality: The Sea of Okhotsk off the southeastern coast of Sakhalin Island, Russia (47°54'41" N; 142°31'4" E).
Specimens deposited: Paragenophore and hologenophore, IPEE RAS 14335.

Description (based on two gravid specimens from *R. dolichogaster*, paragenophore and hologenophore; measurements based on paragenophore only): Body elongate oblong, 3,773 × 966; length to width ratio 1 : 0.26 (Figure 4A). Tegument unarmed. Oral sucker subspaerical, 208 × 215; mouth opening subterminal. Ventral sucker transversely oval when dorso-ventral orientation, sessile, 284 × 339. Sucker-width ratio 1 : 1.58. Forebody 16.7% of body length. Prepharynx 28 long. Pharynx 138 × 132. Oesophagus 187 long. Intestinal bifurcation in posterior third of forebody. Caeca with wide lumen; terminate blindly close to posterior extremity.

Testes two, tandem, strongly indented, in mid-third of hindbody, separated; anterior testis 228 × 272, posterior testis 312 × 284. Posttesticular region 33.0% of body length. Cirrus-sac curved, extends posteriorly from anterior margin of ventral sucker by 34.2–41.7% sucker length, 340 × 92. Internal seminal vesicle saccular proximally and tubular distally; saccular part rectilinear or with one twist, tubular part forms distinct loop which overlaps distal quarter or third of saccular part (Figure 4B). Pars prostatica tubular, surrounded by large pars prostatica. Ejaculatory duct distinctly shorter than pars prostatica. Cirrus unarmed. Genital atrium shallow. Common genital pore sinistro-submedian, prebifurcal.

Ovary transversely elongate, conical anteriorly and three-lobed posteriorly, median, immediately pretesticular, 194 × 360. Distance from posterior margin of ventral sucker to anterior margin of ovary 22.3% of body length. Oviduct leaves from anterior conical region of ovary. Canalicular seminal receptacle saccular, sinistral to ovary. Laurer's canal opens dorsal to left caecum some anterior to ovary. Oötype with Mehlis's gland sinistral to anterior margin of ovary. Uterus extensive, preovarian, intercaecal. Metraterm quite thick-walled, ensheathed in gland-cells, opens to genital atrium antero-sinistrally to male duct. Eggs operculate, deformed in balsam; length of least-deformed eggs 79–85. Vitellarium follicular; ventral follicles in two lateral fields extending from posterior margin of ventral sucker to posterior extremity, overlap caeca, confluent in posttesticular and intertesticular regions, dorsal follicle along medial and lateral margins of ceca only.

Excretory vesicle I-shaped; reaches to ovary.

Remarks

The presented specimens are very similar to *P. apodichthysi* Price, 1937 *sensu stricto*, as indicated by the position of the loop of the tubular part of the internal seminal vesicle along the distal portion of the main saccular region of the seminal vesicle, the short oesophagus, the strongly indented testes, narrower than the ovary, and the fields of vitelline follicles extending anteriorly to the level of the posterior margin of the ventral sucker (compare with Edmiston 1971 and Price 1937).

Podocotyle apodichthysi sensu stricto was originally described by specimens collected from *Apodichthys flavidus* Girard, 1854 (Zoarcoidei, Pholidae), California (Park 1937). This parasite species was further discovered by Edmiston (1971) in the same host species and in the same locality. Tsimbaliuk et al. (1979) record *P. apodichthysi* in gadiid, pleuronectid and cottid fish of the intertidal zone of Iturup Island. However, in fact, these authors were dealing with another species of *Podocotyle* (see below, *Podocotyle*

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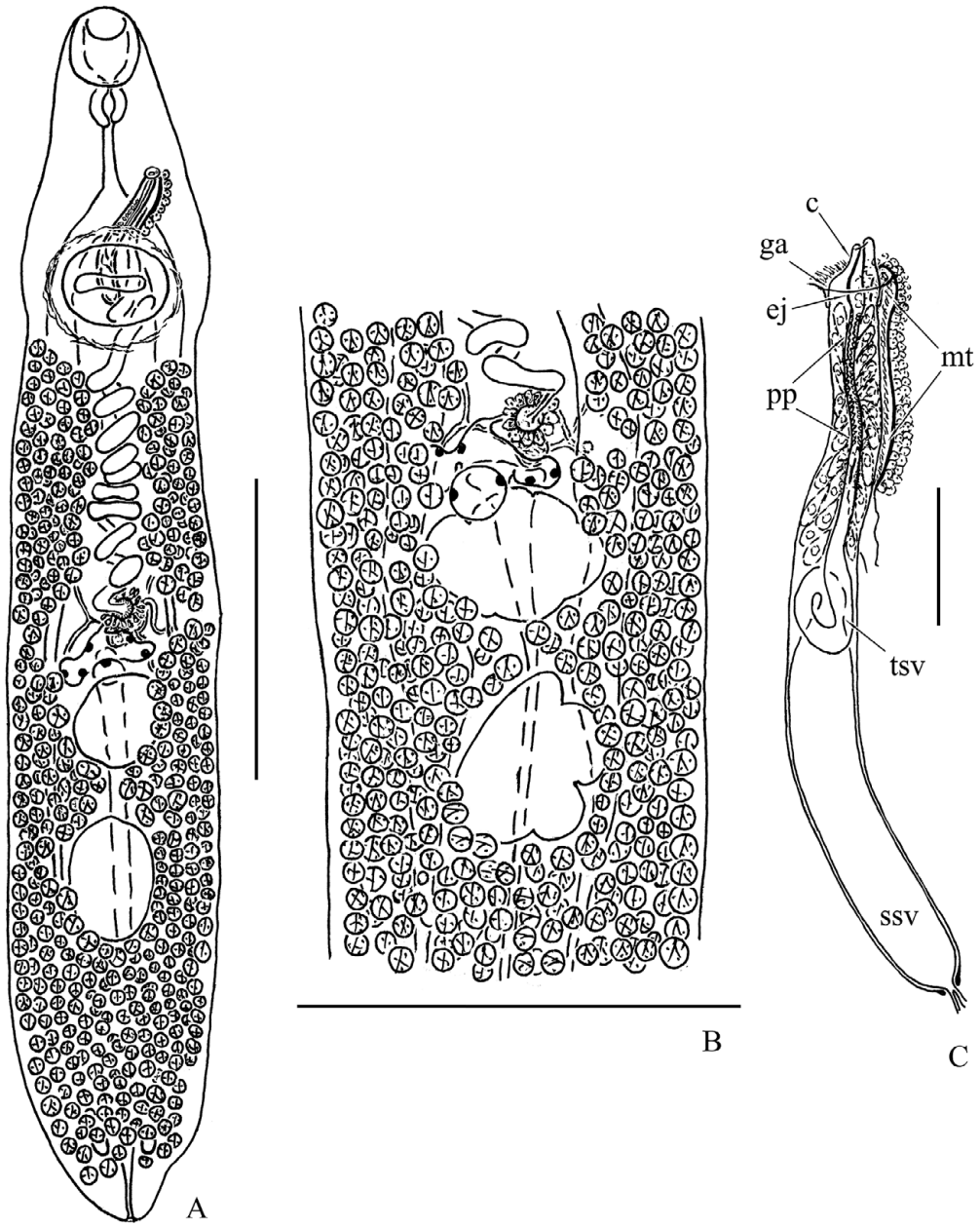

Figure 5. Paragenophores of *Podocotyle* sp. 2 from intestine of *Pholidapus dybowskii*, Sea of Okhotsk. **A** – specimen with entire testes, whole ventral view; **B** – specimen with indented testes, fragment of body, ventral view; **C**– terminal genitalia. c — cirrus partially everted through genital atrium; ej — ejaculatory duct; ga — genital atrium; mt — metraterm; pp — pars prostatica; ssv — saccular part of internal seminal vesicle; tsv — tubular part of internal seminal vesicle. Scale bar; A, B = 1000 μm; C = 100 μm.

sp. 2). *Podocotyle* sp.1 differs from *P. apodichthysi sensu stricto* in the position of the cirrus-sac relative to the ventral sucker (extends backward almost to the ventral sucker midlevel versus not further than the anterior quarter of the sucker), the ratio of suckers (1 : 1.58 versus 1 : 1.20–1.48), morphology of the saccular part of the seminal vesicle (rectilinear or with one twist versus exceptionally rectilinear), caeca morphology (comparatively wide versus relatively narrow), and eggs size (79–85 versus 60–76 μm in length). The taxonomic significance of these differences cannot be adequately assessed based on the available number of *Podocotyle* sp. 1 specimens.

Podocotyle sp. 2

Host: *Pholidapus dybowskii* (Steindachner, 1880) (Perciformes, Zoarcoidei: Opisthocentridae).

Site: Intestine.

Locality: The Sea of Okhotsk off the southeastern coast of Sakhalin Island, Russia (47°54'41" N; 142°31'4" E).

Specimens deposited: Paragenophores, IPEE RAS 14336.

Description (based on three gravid specimens, paragenophores): Body elongate oblong, 3,430–3,948 × 630–868; length to width ratio 1 : 0.17–0.25 (Figure 5A). Tegument unarmed. Oral sucker subellipsoid, 242 × 215–222; mouth opening subterminal. Ventral sucker transversely oval when dorso-ventral orientation, slightly protuberant, 277–284 × 318–332. Sucker-width ratio 1 : 1.44–1.55. Forebody 18.1–20.0% of body length. Prepharynx 14 long or indistinguishable. Pharynx 132–138 × 125–135. Oesophagus 173–242 long. Intestinal bifurcation in posterior third of forebody. Caeca comparatively broad in anterior two thirds

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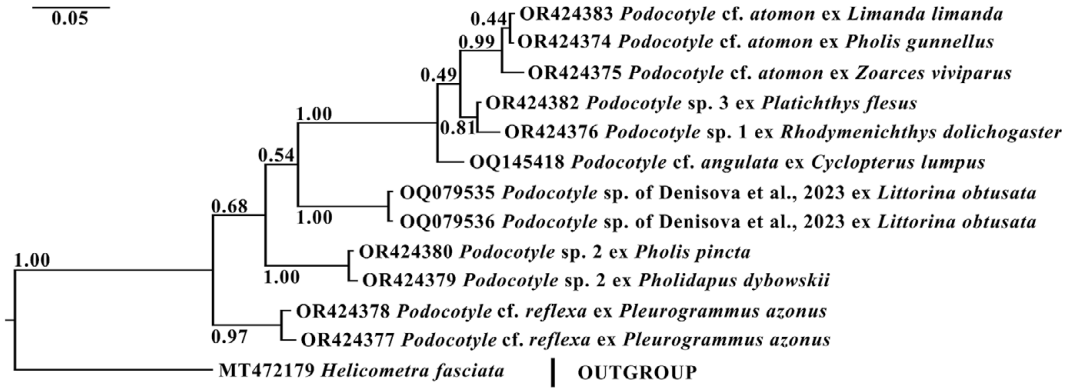

Figure 6. Phylogenetic relationships of *Podocotyle* spp. reconstructed by Bayesian inference analysis of *cox1* gene sequences. Nodal support represents values of posterior probabilities.

and narrowed posteriorly; terminate blindly close to posterior extremity.

Testes two, tandem, entire or strongly indented, in about of mid-third of hindbody, separated; anterior testis 284–325 × 291–408, posterior testis 388–402 × 284–353 (Figure 5A, B). Post-testicular region 23.0–26.1% of body length. Cirrus-sac slender, rectilinear to curved, overlaps 66–100% of ventral sucker length, not reaches into hindbody, 464–495 × 60–74. Internal seminal vesicle saccular proximally and tubular distally; saccular part rectilinear or with three twists, tubular part forms distinct loop which overlaps distal fifth of saccular region (Figure 5C). Pars prostatica tubular, surrounded by large pars prostatica. Ejaculatory duct distinctly shorter than pars prostatica. Cirrus unarmed. Genital atrium shallow. Common genital pore sinistro-submedian, prebifurcal.

Ovary transversely elongate, conical anteriorly and 3-lobed posteriorly, median or slightly dextro-submedian, immediately pretesticular, 166–208 × 312–325. Distance from posterior margin of ventral sucker to anterior margin of ovary 18.8–25.2% of body length. Oviduct leaves from anterior conical region of ovary. Canalicular seminal receptacle saccular, sinistral or antero-sinistral to ovary. Laurer's canal opens dorsal to left caecum, at level of ovary. Oötype with Mehlis's gland sinistral to anterior margin of ovary. Uterus extensive, preovarian, intercaecal. Metraterm quite thick-walled, ensheathed in gland-cells, opens to genital atrium antero-sinistrally to male duct. Eggs operculate, deformed in balsam; length of least-deformed eggs 76–82. Vitellarium follicular; ventral follicles in two lateral fields extending from nearly or immediately posterior margin of ventral sucker to posterior extremity, overlap caeca, confluent in posttesticular and intertesticular regions, dorsal follicle along medial and lateral margins of ceca only.

Excretory vesicle I-shaped; reaches to ovary.

Remarks

Podocotyle sp. 2 is very similar to *P. apodichthysi* of Tsimbaliuk et al. (1979) from gadiid, pleuronectid and cottid fish of the intertidal zone of Iturup Island in many key morphological characteristics, namely body shape, distribution of vitelline follicles, sucker ratio, length and anatomy of the cirrus-sac, shape and ratio of gonads, and eggs size (compare with Tsimbaliuk et al. 1979). In turn, *P. apodichthysi* of Tsimbaliuk et al. (1979) most strikingly differs from *P. apodichthysi sensu stricto* in the position of the cirrus-sac relative to the ventral sucker (extends backward almost to the posterior margin of the ventral sucker versus no further than the anterior quarter of the sucker), and eggs size (70–80 versus 60–76 μm in length) (compare with Edmiston 1971; Park 1937; Tsimbaliuk et al. 1979). We note an unfortunate mistake in Edmiston's (1971) description of the position of the cirrus-sac in *P. apodichthysi sensu stricto*. Indeed, as can be seen from the context and the drawings given by this author, the sinus-sac in *P. apodichthysi sensu stricto* extends no more than one-fourth of the length of the ventral sucker relative to its anterior margin (in the author, relative to the posterior margin). The only difference between *Podocotyle* sp. 2 and *P. apodichthysi* of Tsimbaliuk et al. (1979) consists in the ratio of the lengths of the oesophagus and pharynx. In *P. apodichthysi* of Tsimbaliuk et al. (1979), the oesophagus is three times longer than the pharynx, and in *Podocotyle* sp. 2 it is only1.31–1.75 times longer. According to Manter (1940), the oesophagus length is one of the fairly reliable species characteristics of *Podocotyle*. In this regard, we currently prefer to consider *Podocotyle* sp. 2 and *P. apodichthysi* of Tsimbaliuk et al. (1979) as a separate species.

Apart from *Podocotyle* sp. 2 and *P. apodichthysi* of Tsimbaliuk et al. (1979), only *Podocotyle californica* Park, 1937 has a cirrus-sac, which posteriorly reaches the posterior half of the ventral sucker and does not cross its posterior margin (Edmiston 1971; Park 1937). However, the first two listed species differ from *P. californica* in the distribution of the fields of vitelline follicles (lateral gaps absent versus present), morphology of the internal seminal vesicle (loop of the distal tubular region present versus absent) and eggs size (70–82 *versus* 57–73 μm in length) (compare with Edmiston 1971; Park 1937; Tsimbaliuk et al. 1979; present data). Additional material is required to clarify the taxonomic status of *Podocotyle* sp. 2.

Phylogenetic analyses

We obtained partial *cox1* gene sequences from four isolates identified by morphological characters of holo- or paragenophores, namely *Podocotyle* cf. *reflexa* (two specimens), *Podocotyle* cf. *atomon* (one specimen), *Podocotyle* sp.1 (one specimen) and *Podocotyle* sp. 2 (one specimen), as well as from four morphologically unstudied isolates (three specimens from the White Sea and one from the Sea of Okhotsk). Partial 28S rDNA sequences were obtained from the same four previously identified isolates (one specimen from each) and only two morphologically unstudied isolates (two specimens from the White Sea).

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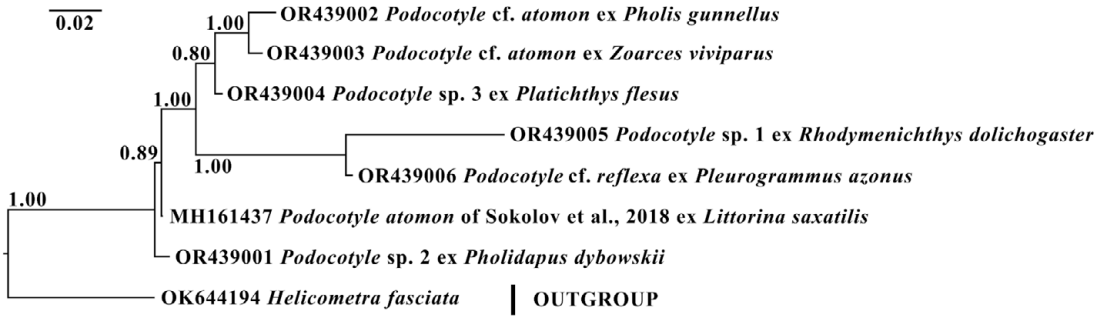

Figure 7. Phylogenetic relationships of *Podocotyle* spp. reconstructed by Bayesian inference analysis of 28S rDNA sequences. Nodal support represents values of posterior probabilities.

Analysis based on the *cox1* gene sequences showed that two morphologically unstudied isolates from two White Sea fish species, *Pholis gunnellus* and *Limanda limanda,* together with *Podocotyle* cf. *atomon* (ex *Z. viviparus*) formed one well-supported clade (Figure 6), all members of which had relatively low genetic segregation among themselves (p-distance 0–2%). A morphologically unstudied isolate ex *Pholis pincta* from the Sea of Okhotsk was clustered with *Podocotyle* sp. 2 (ex *Pholidapus dybowskii*) with high support. The p-distance between these isolates was 0%. Both above-mentioned isolates from *Pholis gunnellus* and *Limanda limanda* are hereafter referred to as *Podocotyle* cf. *atomon* and the isolate from *Pholis pincta* as *Podocotyle* sp. 2. A morphologically unstudied isolate ex *P. flesus* from the White Sea turned out to be a poorly supported sister taxon to *Podocotyle* sp. 1 (ex *R. dolichogaster*). This isolate ex *P. flesus* is hereafter referred to as *Podocotyle* sp. 3.

In turn, the *Podocotyle* cf. *atomon* clade was a poorly supported sister group to the *Podocotyle* sp. 1 + *Podocotyle* sp. 3 clade, and all of them together formed a large sister group to *Podocotyle* cf. *angulata* (ex *C. lumpus*) with a well support. The *Podocotyle* cf. *angulata* + (*Podocotyle* cf. *atomon* + (*Podocotyle* sp. 1 + *Podocotyle* sp. 3)) appeared as a poorly supported sister clade to *Podocotyle* sp. of Denisova et al. (2023) (ex *Littorina obtusata* (Linnaeus, 1758)), and all of them together also formed a poorly supported sister clade to *Podocotyle* sp. 2. *Podocotyle* cf. *reflexa* (ex *Pleurogrammus azonus*) occupied a basal position relative to all mentioned species (Figure 6).

Podocotyle cf. *reflexa, Podocotyle* cf. *atomon, Podocotyle* sp. 1, *Podocotyle* sp. 2 and *Podocotyle* sp. 3 are also supported as separate species by 28S rDNA analysis. However, the tree topology obtained from the analysis was somewhat different from the topology based on mitochondrial DNA data. Thus, *Podocotyle* cf. *reflexa* appeared as a well-supported sister clade to *Podocotyle* sp. 1. The group of these species in turn formed a well-supported sister clade to a poorly supported one containing *Podocotyle* sp. 3 and *Podocotyle* cf. *atomon*. The clade uniting all listed isolates was sisterly related to *P. atomon* of Sokolov et al. (2019) with high support. *Podocotyle* sp. 2 occupied a basal position relative to the rest of the sample of *Podocotyle* (Figure 7).

Discussion

The present genetic and morphological study allowed us to identify three species of *Podocotyle* in the Sea of Okhotsk and five in the White Sea. However, this diversity of *Podocotyle* is poorly formalized within the current taxonomic model of the genus. In fact, based on morphological characteristics, we reliably identified only one nominal species, namely *P. reflexa*. Nevertheless, we prefer to recognize our specimens from the Northwestern Pacific only as *Podocotyle* cf. *reflexa*. In the Northwestern Pacific, *P. reflexa* has been repeatedly recorded by various authors (e.g., Layman 1930; Tsimbaliuk et al. 1979; Zhukov 1960). However, Gibson and Bray (1982) were dubious about reports of *P. reflexa* from this region. The only available description of *P. reflexa* specimens from the Northwestern Pacific (Tsimbaliuk et al. 1979) does not provide unequivocal evidence of their conspecificity to the relevant species. For example, the sucker ratio in the specimen drawn by these authors (Tsimbaliuk et al. 1979, Figure 5) is only 1 : 1.83. At the same time, *P. reflexa* is characterized by a ratio equal to 1 : 2 (e.g., Blend et al. 2019). A final conclusion about the presence of *P. reflexa* in the Northwestern Pacific requires molecular comparison of Atlantic isolates of this species with the specimens we studied.

Most of the morphologically unstudied isolates presented in our study are probably conspecific to one or another isolate identified by morphological characters, namely specimens from *Pholis gunnellus* and *Limanda limanda* to *Podocotyle* cf. *atomon,* and a specimen from *Pholis pincta* to *Podocotyle* sp. 2. This is evidenced by both high support for clades that include morphologically described isolates and their genetically corresponding morphologically unstudied isolates, as well as a low level of intragroup differentiation between them. An exception is the morphologically unstudied isolate from *P. flesus* caught in the White Sea (=*Podocotyle* sp. 3). Lack of reliable support for a node connecting it to *Podocotyle* sp. 1 from the Sea of Okhotsk in reconstruction based on the *cox*1 gene, as well as the absence of a direct phylogenetic relationship between them based on the analysis of 28S rDNA, does not yet allow us to conclude that these isolates are conspecific. The low level of differences between *Podocotyle* sp. 3 and *Podocotyle* sp. 1 in the studied fragments of the *cox1* gene (p-distance 1%) compared with that of 28S rDNA (p-distance 11%) contradicts modern ideas about the variability ratio of these loci. We cannot yet explain the reasons for this phenomenon.

Intramolluscan stages of *Podocotyle* sp. of Denisova et al. (2023) and *P. atomon* of Sokolov et al. (2019), parasitizing *Littorina* spp., are a particular taxonomic problem. Their belonging to the genus *Podocotyle* is undoubted (Novotný 2019; this study), but the species affiliation remains enigmatic. Cercariae of two nominal species of *Podocotyle* are known from *Littorina* spp., *P. atomon* and *P. staffordi* (=*P. angulata*) (e.g., Chubrik 1966; Gibson 1974; Granovitch and Johannesson 2000; Hunninen and Cable 1943; James 1969; Kaliberdina and Granovich, 2003; Køie 1981; Levakin et al. 2012; Szuks 1975; Uspenskaya 1963). The adults of *Podocotyle* studied in the present work bear some degree of similarity to these nominal species but are not conspecific with the cercariae and sporocysts listed above. It is surprising that for the species of *Podocotyle*, common for mollusks of the intertidal zone of the White Sea, we have not yet been able to detect conspecific adults inhabiting fish.

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Thus, our research makes an additional contribution to the study of *Podocotyle* spp. from marine fish and mollusks. Obviously, the revealed differences in the level of interspecific variability between the two genes used raise a serious problem in choosing a genetic marker that adequately characterizes the biodiversity of these parasites. It is possible that further data on more isolates from other hosts and localities will help resolve this problem.

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Competing interest. All authors declare that they have no conflict of interest.

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

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