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Description of *Pseudobenedeniella johnstoni* sp. n. (Monogenea: Capsalidae) from the gills of Antarctic black rockcod, *Notothenia coriiceps* Richardson in coastal waters of West Antarctica

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Summary

Morphological and metrical analyses of monogeneans from the gills of Antarctic rockcod *Notothenia coriiceps* revealed the presence of a new species, *Pseudobenedeniella johnstoni* sp. n. Fresh specimens of monogeneans collected from *N. coriiceps* from the vicinity of Galindez Island, Argentine Islands, West Antarctica were examined morphologically, by molecular analysis and Energy Dispersive X-ray analysis (EDXA). The new species differs from *Pseudobenedeniella branchialis* Timofeeva, Gaevskaya, Kovaljova, 1987 by the different shapes of anterior and posterior hamuli, the presence of a pronounced sickle-shaped blade of the anterior hamulus and its characteristic wide (wing-like) shaft, serrated on one side, smaller posterior hamulus with a distinct short and broad shaft that widens and is serrated distally, more extensive dimensions of the penis and its pear-like shape, smaller vagina diameter, ovoid egg shape with more pointed anterior pole and blunt posterior pole and long coiled filament as well as different type species of the host in a geographically distant type locality. Genetic data accompanied the description, and phylogenetic analyses inferred that the new species clustered with monogenean species positioned under the family Capsalidae. Molecular data were generated for the 18S and 28S genes of the ribosomal RNA of *P. johnstoni* sp. n. to provide the first molecular analysis for *Pseudobenedeniella* species. EDXA for a species of *Pseudobenedeniella* was offered for the first time.

Keywords: *Pseudobenedeniella johnstoni* sp. n.; *Notothenia coriiceps*; ribosomal RNA; 18S; 28S; EDXA; Capsalidae

Introduction

The fish fauna in the Southern Ocean around Antarctica is dominated by the perciform suborder Notothenioidei, which comprises up to 90 % of the fish biomass and about 77 % of fish species diversity (Near, 2009; Near *et al*., 2012). Antarctic nototheniid fish represent a remarkable example of adaptive radiation, thriving in the icy waters of the Southern Ocean; they account for a significant portion of species diversity, abundance, and biomass. Notothenioidei is a vital fish group for understanding unique evolutionary adaptations, which enable their survival in subzero temperatures and offer insights into biodiversity and climate resilience (Near, 2009). Moreover, Notothenioidei is a food source for various mammals and birds in the Antarctic ecosystems (La Mesa *et al*., 2004)

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and, thus, these teleost fishes are involved in the complex life cycles of different groups of parasites of predatory fish, fish-eating birds, and marine mammals as their intermediate and/or paratenic hosts (Palm *et al*., 1998; Rocka, 2006). Therefore, the parasite fauna of nototheniid fish has high species diversity in all ecoregions of the Southern Ocean (Oğuz *et al*., 2012, 2015; Klimpel *et al*., 2017; Münster *et al*., 2017; Kuzmina *et al*., 2020, 2021, 2022a). Antarctic rockcod *Notothenia coriiceps* Richardson is one of the dominant species in the Southern Ocean (Near, 2009); that is why this fish species can be considered an ideal model organism for studying evolutionary adaptations of teleost fish to extreme environments providing critical insights into the impacts of climate change on Antarctic biodiversity. Because of the high abundance in different parts of the Southern Ocean, *N. coriiceps* has been considered a promising object for long-term monitoring studies of parasite communities (Zdzitowiecki & Laskowski, 2004; Kuzmina *et al*., 2020, 2022a,b; Syrota *et al*., 2023). Parasitic organisms are known as the most sensitive indicators of the state of ecosystems, especially in the marine environment, which is associated with the complex life cycles of various parasites, including various invertebrate and vertebrate animals as definitive, intermediate, and paratenic hosts (Hudson *et al*., 2006; Poulin & Mouritsen, 2006; Poulin, 2011). Therefore, the main groups of endohelminths parasitizing *N. coriiceps* have been studied well (Zdzitowiecki & Laskowski, 2004; Kuzmina *et al*., 2020, 2022a,b; Alt *et al*., 2022; Syrota *et al*., 2023). At the same time, Antarctic rockcod's ectoparasites of the class Monogenea Van Beneden, 1858 have not been thoroughly studied. As host-specific parasites, Antarctic monogeneans are particularly valuable for understanding co-evolutionary relationships and host adaptations in the extreme environmental conditions of Antarctica (Klapper *et al*., 2017). Additionally, together with other groups of parasites, they serve as indicators of ecosystem health, reflecting environmental changes and the impacts of climate shifts on marine biodiversity. Several species of monogeneans have been reported on *N. coriiceps*, including *Pseudobenedenia nototheniae* Johnston, 1931, *Gyrodactylus coriicepsi* Rokicka *et al*., 2009, and *Pseudobenedenia coriicepsi* Rubtsova *et al*., 2023 (Oğuz *et al*., 2012, 2015; Klapper *et al*., 2017; Rubtsova *et al*., 2023). Our recent studies revealed the presence of a new gill monogenean species on *N. coriiceps*, morphologically distinct from all the previously described monogeneans from this host. Detailed examination of the specimens revealed a gill monogenean parasite belonging to the genus *Pseudobenedeniella* Timofeeva, Gaevskaja, Kovaliova, 1987. Until now, this genus was represented only by one species, *Pseudobenedeniella branchialis* Timofeeva, Gaevskaja, Kovaliova, 1987, described from the gills of *Notothenia rossii* Richardson in South Georgia and the Mordvinov Islands, Atlantic sector of Antarctica (Timofeeva *et al*., 1987). Genus *Pseudobenedeniella* differs from the close genus *Pseudobenedenia* Johnston, 1931 by the presence of morphological adaptations for gill parasitism, including a valve-like haptor bent in the anterior-posterior direction with seven radial septae and poorly developed accessory sclerites that have lost their function in this mode of attachment. This study aims to document and describe a new species, *Pseudobenedeniella johnstoni* sp. n., provide its taxonomic identity, and supplement its description with molecular data and Energy Dispersive X-ray Analysis (EDXA). A detailed study of the morphology of sclerotized structures of the haptor and reproductive system, together with different fish host species and geographically separated type localities, strongly supported our discovery documenting reproductive isolation. The study presents a molecular analysis of freshly collected specimens, providing 18S and 28S gene sequences for this new species. It also examines the phylogenetic relationships within the family Capsalidae.

Material and Methods

Field studies and material collection were carried out in 2014 – 2015 and 2022 – 2023 in the coastal waters near the Ukrainian Antarctic Station "Akademik Vernadsky"(Galindez Island, Argentine Islands, West Antarctica; 65°15'S, 64°16'W). In total, 201 Antarctic rockcod *N. coriiceps* individuals were caught off the shore of Galindez Island at depths from 10 to 30 m using a fishing rod (Kuzmina *et al*., 2020). All fish collected were immediately transported to the laboratory, measured, and examined using the standard parasitological techniques (Weber & Govett, 2009). Monogeneans were collected from gills, washed in saline water, and fixed in 70 % ethyl alcohol.

Methods of microscopy and line drawings

A preliminary laboratory examination was performed in the Department of Parasitology of I.I.Schmalhausen Institute of Zoology NAS of Ukraine in Kyiv, Ukraine, using a Zeiss Axio Imager M1 microscope with differential interference contrast optics (Carl Zeiss AG Light Microscopy, Göttingen, Germany). Detailed studies of the inner morphology of the parasites, whole mount preparations, microscopic imaging, drawings, and data analysis of measurements were held at the Institute of Parasitic Diseases, Parasitology Center (PCI), Scottsdale, Arizona, USA. Microscope images were created using 4×, 10×, 20× and 40× objective lenses of Nomarski DIC Phase Contrast Microscope Trinocular (Munich, Germany) and a Canon T3i EOS 600D DSLR Camera (Melville, New York). All measurements are in millimeters; the mean values between parentheses ±SD follow the measurement range; width measurements represent the maximum width of the specimen or organ.

Monogenean specimens were stained in Mayer's acetocarmine, destained in 4 % hydrochloric acid in 70 % ethanol, dehydrated in ascending concentrations of ethanol (from 70 % to 100 %, 24 hours each), and cleared in 100 % xylene, and then in 50 % Canada balsam and 50 % xylene (24 hours each). Whole worms were mounted after that in Canada balsam. Some specimens that we fixed in glycerin jelly were demounted, washed, dehydrated, and processed with Mayers's acid carmine, as mentioned above, for additional study of details of inner anatomy and measurements. To

Table 1. Primers used for PCR and sequencing

Primer	Sequence (5'–3')	Source
***18S* rDNA**		
WormA		
1270R	GCGAATGGCTCATTAAATCAG	Littlewood & Olson, 2001
930F	GCATGGAATAATGGAATAGG	Littlewood &Olson, 2001
WormB	CTTGTTACGACTTTTACTTCC	Littlewood &Olson, 2001
***28S* rDNA**		
Ancy55F	GAGATTAGCCCATCACCGAAG	Littlewood &Olson, 2001
Ancy1200R	CACCATCTTTCGGGTCTCAACC	Plaisance *et al*., 2005
L300F	CAAGTACCGTGAGGGAAAGTTG	Plaisance *et al*., 2005
ECD2	CCTTGGTCCGTGTTTCAAGACGGG	Littlewood *et al*., 2000

study the armament of the haptor, because of its valve-like shape, we had to cut the haptor and flatten it separately from the rest of the body to see the detailed morphology of sclerotized structures (Gusev, 1983, 1985).

Line drawings were created using a Ken-A-Vision micro projector (Ward's Biological Supply Co., Rochester, N.Y.) with quartz iodine 150W illumination. Images of stained whole mounted specimens were projected vertically on 300 series Bristol draft paper (Strathmore, Westfield, Massachusetts), then traced and inked with India ink.

Specimens

One holotype (collection number HWML-217721), one paratype (HWML-217722), and four vouchers (HWML-217723) of *Pseudobenedeniella johnstoni* sp. n. were accessioned and cataloged at the University of Nebraska's State Museum Harold W. Manter Laboratory (HWML) collection in Lincoln, Nebraska, USA.

Molecular methods of DNA extraction, PCR amplification, and sequencing

Four specimens of *Pseudobenedeniella johnstoni* sp. n. re-fixed in 95 % ethanol were used for DNA extraction using the Qiagen DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions. For the amplification of the 18S and 28S genes, primers used were mentioned in the table (Table 1). For PCR amplification of both genes, 25 µl of the PCR reaction was used that comprised 10× PCR buffer, 0.4 mM dNTP, and 10 pM of each primer pair, 3.5 µl template DNA, 1 U Taq polymerase (Bio tools) and Milli-Q water was added and carried out in an Eppendorf Master Cycler Personal. PCR amplification profile was as follows: denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C (for all primers) for 55 s, extension at 72 °C for 3 min and a final extension at 72 °C for 8 min. PCR products were visualized on 1 % agarose TBE gel and then purified by Purelink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen, Germany) according to the manufacturer's instructions. The PCR products were sequenced in both directions using a Big Dye Terminator vr.3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer by the same PCR primers as for the amplification reaction. Newly generated sequences were assembled and edited using the Geneious Pro v. 5.1.7 platform (Drummond *et al*., 2010) and matched to the GenBank database using BLAST. *Pseudobenedeniella johnstoni* sp. n. sequences were deposited in GenBank; the accession numbers are PP430573–PP430576 (for 18S) and PP430577–PP430580 (for 28S).

Molecular analysis

The phylogenetic tree for both genes (18S and 28S) was built using our newly generated sequences and those of closely related taxa presented in International Nucleotide Sequence Databases (INSD) (https://www.ncbi.nlm.nih.gov/). The sequences of closely related capsalid species available in INSD were included in preliminary phylogenetic analyses (Table 2). Sequences were then aligned separately with MUSCLE in Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura *et al*., 2021). The phylogenetic relationships among species were determined using the maximum likelihood (ML) and Bayesian inference (BI) methods. For choosing the best model for phylogeny, MrModeltest v. 2.3 (Nylander, 2004) substitution model was inferred by resulting in Akaike's information criterion and attaining the GTR + I + G as the best model. Maximum likelihood (ML) analysis was conducted using MEGA version 11 with Nodal support projected by performing 1,000 bootstrap pseudoreplicates. Bayesian inference (BI) analyses were conducted using TOPALi version 2.5 software (Milne *et al*., 2009) with two simultaneous runs of Markov chain Monte Carlo (MCMC) chains, each for 4 million generations and sampling trees every 4,000 generations with a 25 % of the sampled trees were discarded as 'burn-in' for each data set. Pairwise genetic distances (uncorrected p-distance model) were calculated in MEGA v.11. Sequences of representatives of the genus *Polylabris* Euzet and Cauwet, 1967 were included as outgroup taxa.

Table 2. Information on the capsalid monogenean species used for phylogenetic analysis based on the 18S and 28S gene sequences.

Species	Host	Origin	GenBank accession No.	References
18S gene				
Capsala martinieri	*Mola mola*	UK	AJ276423	Littlewood & Olson, 2001
Neobenedenia melleni	*Lutjanus* sp.	Malaysia	KU843502, KU843503, KU843504	Ravi & Yahaya, 2016
Benedenia epinepheli	*Epinephelus* sp.	Vietnam	EU707802	Dang *et al*., 2011*
Benedenia sp.	Perciform teleost	UK	AJ228774	Littlewood & Olson, 2001
Benedenia humboldti	*Seriola lalandi*	USA	MW575871	Baeza & González, 2021
Allobenedenia epinepheli	*Epinephelus* sp.	Vietnam	EU707800	Dang *et al*., 2011*
Neobenedenia melleni	*Epinephelus* sp.	Vietnam	EU707804	Dang *et al*., 2011*
Neobenedenia girellae	*Trachinotus blochii*	South Korea	MT542140	Nam *et al.*, 2020*
Encotyllabe chironemi	*Chironemus marmoratus*	UK	AJ228780	Littlewood & Olson, 2001
Pseudobenedeniella johnstoni sp. n.	*Notothenia coriiceps*	West Antarctica	PP430573, PP430574, PP430575, PP430576	Present study
Pseudobenedenia coriicepsi	*Notothenia coriiceps*	West Antarctica	OR289962, OR289963, OQ803310, Q803312	Rubtsova *et al*., 2023
Benedenia sp.	*Dasyatis pastinaca*	Turkey	MK106094	Turgay, 2018*
28S gene				
Neobenedenia sp.	*Larimichthys polyactis*	South Korea	OM333244	Seo, 2022*
Neobenedenia girellae	*Rachycentron canadum*	Australia	MW690094	Brazenor *et al*., 2018
Neobenedenia girellae	*Lates calcarifer*	Australia	MH843708	Brazenor *et al*., 2018
Neobenedenia girellae	*Trachinotus blochii*	South Korea	MT549677	Nam *et al.*, 2020*
Neobenedenia melleni	*Epinephelus* sp.	Vietnam	EU707805	Dang *et al*., 2011*
Neobenedenia sp.	*Seriola rivoliana*	Ecuador	MK202451	Sepúlveda & González, 2019
Neobenedenia melleni	*Seriola dumerili*	China	JN797596	Ding *et al*., 2011*
Neobenedenia sp.	*Paralabrax humeralis*	Chile	MK202450	Sepúlveda & González, 2019
Neobenedenia sp.	*Cheilodactylus variegatus*	Chile	MT982168	Taborda *et al*., 2023
Neobenedenia sp.	*Aplodactylus punctatus*	Chile	MK202438	Sepúlveda & González, 2019
Neobenedenia sp.	*Anisotremus scapularis*	Chile	MK202439	Sepúlveda & González, 2019
Neobenedenia sp.	*Sphoeroides annulatus*	Mexico	AY486150	Whittington *et al*., 2004
Allobenedenia dischizosepta	*Acanthistius patachonicus*	Argentina	MH929436	Bagnato *et al*., 2022

Allobenedenia epinepheli	*Epinephelus* sp.	Vietnam	EU707801	Dang *et al*., 2011*
Benedenia sciaenae	*Argyrosomus japonicus*	Australia	FJ971970	Perkins *et al*., 2009
Encotyllabe chironemi	*Chironemus marmoratus*	Australia	AF382054	Olson & Littlewood, 2002
Benedenia sekii	*Chrysophrys auratus*	Australia	FJ971971	Perkins *et al*., 2009
Benedenia lutjani	*Gracilobenedenia lutjani*	Japan	AY033939	Whittington *et al*., 2001
Benedenia rohdei	*Seriola quinqueradiata*	Japan	AY033940	Whittington *et al*., 2001
Benedenia seriolae	*Seriola quinqueradiata*	Japan	KC768341	Sepúlveda & González, 2019
Benedenia humboldti	*Seriola lalandi*	USA	MW575871	Baeza & González, 2021
Benedenia epinepheli	*Epinephelus* sp.	Vietnam	EU707803	Dang *et al*., 2011*
Benedenia sargocentron	*Sargocentron spiniferum*	China	JN797597	Ding *et al*., 2011*
Neoentobdella natans	*Pastinachus sephen*	Australia	FJ972009	Perkins *et al*., 2009
Entobdella stenolepis	*Hippoglossus stenolepis*	Canada	FJ971991	Perkins *et al*., 2009
Entobdella hippoglossi	*Hippoglossus hippoglossus*	UK	AY486151	Whittington *et al*., 2004
Capsaloides cristatus	NA	China	JN711434	Yang, 2011*
Nasicola klawei	*Thunnus albacares*	USA	HQ721186	Bullard *et al*., 2011
Capsala martinieri	*Mola mola*	UK	AF382053	Olson & Littlewood, 2002
Capsala laevis	*Istiophorus platypterus*	China	JN980396, JN980397	Yang & Hu, 2011*
Pseudobenedeniella johnstoni sp. n.	*Notothenia coriiceps*	West Antarctica	PP430577, PP430578, PP430579, PP430580	Present study
Pseudobenedenia coriicepsi	*Notothenia coriiceps*	West Antarctica	OR295461, OR295462, OQ820944, OQ820945	Rubtsova *et al*., 2023
Neoentobdella taiwanensis	*Taeniura meyeni*	Taiwan	FJ972010	Perkins *et al*., 2009

An asterisk (*) shows the unpublished status of species on the GenBank database. NA shows no information is available about the host species

Energy Dispersive X-Ray analysis (EDXA)

Two specimens of *Pseudobenedeniella johnstoni* sp. n. fixed in 70 % ethanol were processed according to standard procedures (Lee, 1993). The Helios Nanolab 600 was equipped with an energy-dispersive X-ray analysis (EDXA) TEAM Pegasus system (Mahwah, NJ) with an Octane Plus detector. EDXA analyzed the sectioned anterior hamulus cuts and its edge, anterior body end, and haptor. EDXA spectra from the mentioned areas were collected using an accelerating voltage of 15 kV and a probe current of 1.4 nA. Data collected included images of the displayed spectra and the raw collected data. The TEAM software generated relative elemental percentages.

Ethical Approval and/or Informed Consent

The authors declare that they have observed all applicable ethical standards.

Results

Examination of the fresh material collected from *N. coriiceps* revealed morphological differences between our specimens and *P. branchialis* described by Timofeeva *et al*. (1987). After comparing published data, we consider our samples a new species, *Pseudobenedeniella johnstoni* sp. n., as described below.

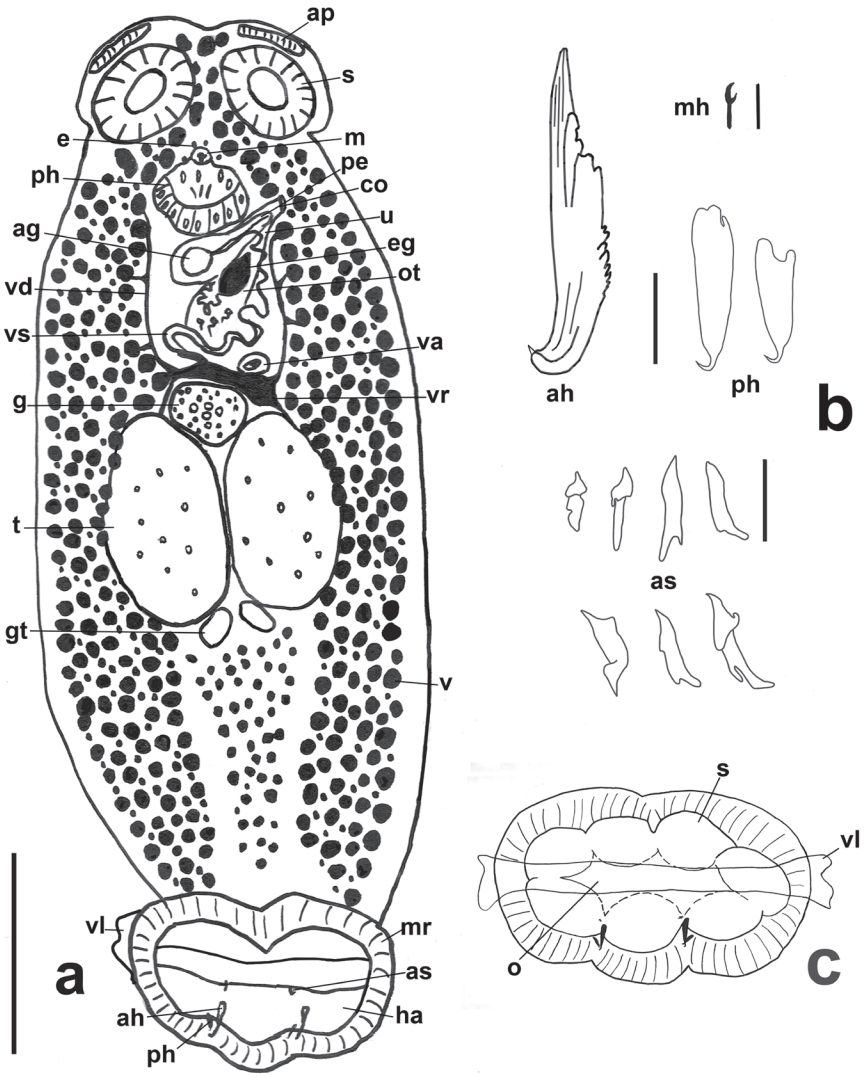

Fig. 1. **a** – The anatomy of an adult specimen of *Pseudobenedeniella johnstoni* sp. n. in ventral view: ah, anterior hamulus; ag, accessory gland; ap, adhesive pad; as, accessory sclerite; co, common genital opening; e, eye; eg, egg; g, germarium; gt, Goto glands; ha, haptor; m, mouth; mr, muscular rim of haptor; ot, ootype; ph, pharynx; pe, penis; ph, posterior hamulus; s, muscular sucker; t, testis; u, uterus, v, vitellarium; va, vagina; vd, vitelline duct; vl, marginal valve; vr, vitelline reservoir; vs, vas deferens. Scale bar: 1 mm. **b** – Sclerotized structures of haptor of *Pseudobenedeniella johnstoni* sp. n.: mh, marginal hooklet (scale bar 0.02 mm); ah, anterior hamulus, ph, posterior hamulus (scale bar 0.1 mm); as, accessory sclerites (variations of shape), scale bar 0.07 mm. **c** – Schematic drawing of clamp-shaped haptor of *Pseudobenedeniella johnstoni* sp. n.: s, septum; o, opening of haptor; vl, marginal valve.

Table 3. Morphometric characteristics of two species of *Pseudobenedeniella* from *notothenid* fish in two localities.

Host	*Notothenia rossi*	*Notothenia coriiceps*
Parasite	*Pseudobenedeniella branchialis* (Timofeeva *et al*., 1987)	*Pseudobenedeniella johnstoni* sp. n.
Authority	Timofeeva et al. (1987)	Present study
Sample size	15	27
Location	gills	gills
Type locality	**South Georgia Island (54°30'28"S, 36°34'32"W)***	**Galindez Island (65˚15'S, 64˚16'W)**
Body length	4.7 – 8.1 (6.3 ± 0.3)	3.85 – 6.75 (5.54 ± 0.71)
Body width	1.8 – 2.6 (2.2 ± 0.1)	1.3 – 3.0 (2.17 ± 0.39)
Haptor diameter	1.4 – 2.0 (1.7 ± 0.07)	0.93 – 2.25 (1.8 ± 0.32)
Body width to haptor diameter ratio	1.29 – 1.37	**1.17**
Accessory sclerite length	0.04 – 0.10 (0.07 ± 0.010)	0.05 – 0.13 (0.07 ± 0.01)
Anterior hamulus length	0.29 – 0.35 (0.32 ± 0.01)	0.23 – 0.47 (0.33 ± 0.041)
Anterior hamulus shape	**Cylindroid shaft, sharply recurved blade (according to Fig. 3 in Timofeeva *et al*. (1987), "without pronounced blade" ****	**Widen (wing-like) shaft, serrated on one side, curved sickle-shaped with pronounced blade**
Posterior hamulus length	0.15 – 0.22 (0.18 ± 0.01)	0.07 – 0.24 (0.18 ± 0.03)
Posterior hamulus shape	Short and cylindroid shaft, no serrations	Short and broad shaft, serrated distally
Haptor marginal valve width	0.15 – 0.18 (0.16 ± 0.01)	0.09 – 0.14 (0.12 ± 0.01)
Sucker diameter/width × length	0.35 – 0.60 (0.43 ± 0.02)	0.25 – 0.88 (0.63 ± 0.12) × 0.31 – 1.00 (0.53 ± 0.13)
Pharynx length × width	0.46 – 0.67 (0.57 ± 0.02) × 0.64 – 0.81 (0.70 ± 0.02)	0.23 – 0.75 (0.47 ± 0.09) × 0.36 – 0.95 (0.65 ± 0.14)
Germarium length x width	0.37 – 0.51 (0.43 ± 0.02) × 0.37 – 0.59 (0.48 ± 0.02)	0.23 – 0.58 (0.43 ± 0.08) × 0.36 – 0.57 (0.52 ± 0.07)
Testis length x width	0.85 – 1.37 (1.03 ± 0.04) × 0.42 – 0.77 (0.66 ± 0.03)	0.62 – 1.20 (0.96 ± 0.12 × 0.40 – 0.83 (0.59 ± 0.07)
Penis length x width	0.48 – 0.71 (0.59 ± 0.03 × 0.20 – 0.30 (0.24 ± 0.02)	**0.46 – 1.43 (0.73 ± 0.12) × 0.16 – 0.62 (0.31 ± 0.05**)
Penis shape	**"Bottle-shaped"****	**Pear-shaped**
Egg length x diameter	0.18 – 0.21 (0.19 ± 0.01)× 0.09 – 0.14 (0.12 ± 0.01)	0.16 – 0.28 (0.22 ± 0.02)× 0.09 – 0.16 (0.13 ± 0.02)
Egg shape	**"Tetrahedral shaped"***, both egg ends of same shape, one end has long coiled filament**	**Ovoid, more pointed anterior pole, posterior pole blunt with long coiled filament**
Vagina outer diameter	0.22***	**0.14 – 0.25 (0.17 ± 0.04)**
Vagina inner diameter	0.11***	**0.05 – 0.12 (0.08 ± 0.02)**
Vagina shape	**"Short, opens near vitelline reservoir"****	**Has wide outer muscular part, and narrow inner part, possibly sclerotized**

* bolded statements are extremes
**quote from Timofeeva *et al.* (1987)
***based on drawing and data in the text in Timofeeva *et al.* (1987) using the scale bar provided

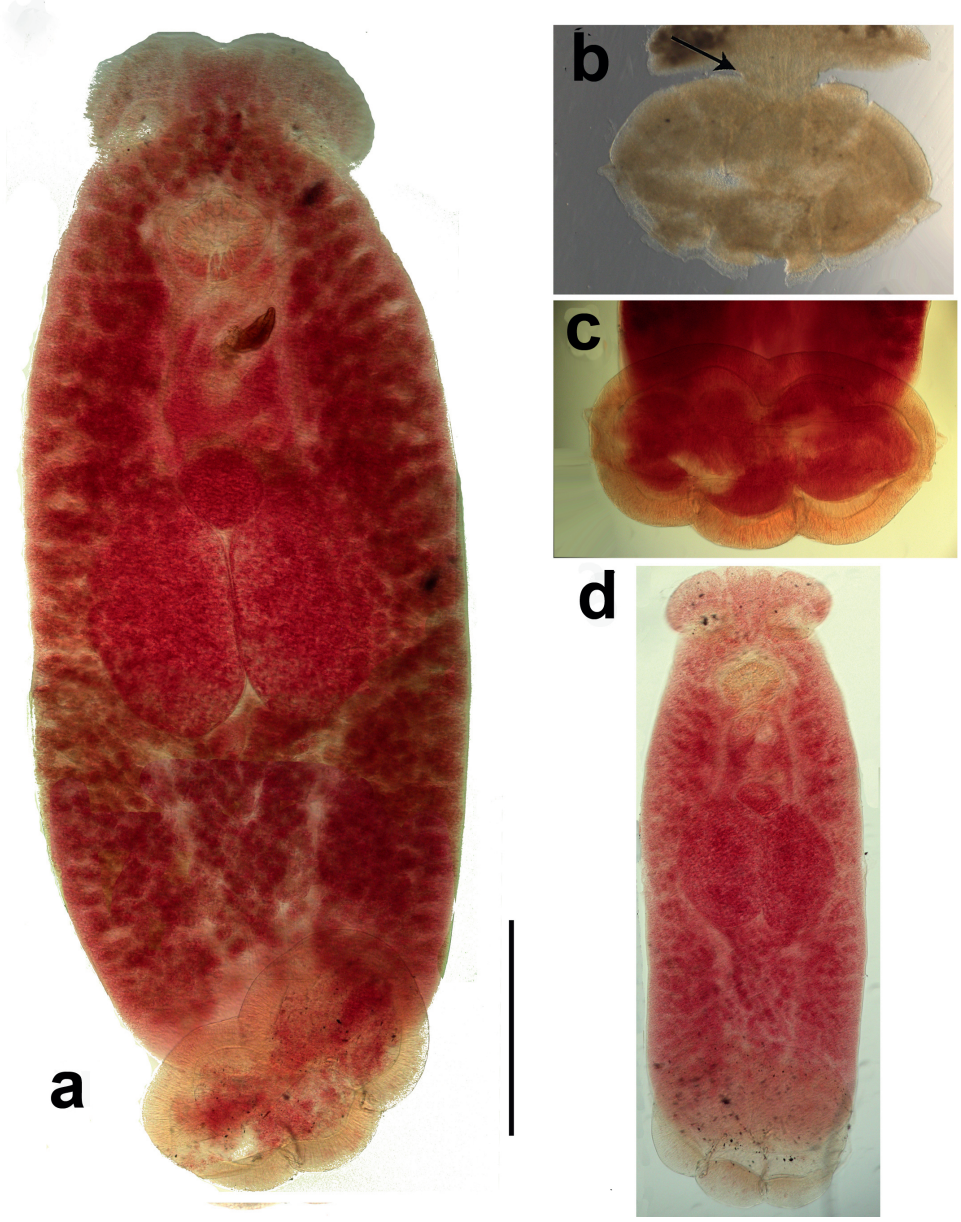

Fig 2. **a** – Microscopic image of carmine-stained adult of *Pseudobenedeniella johnstoni* sp. n. Scale bar: 1 mm. **b** – Haptor of *Pseudobenedeniella johnstoni* sp. n. showed in profile. Note peduncle (arrow). **c** – Haptor of *Pseudobenedeniella johnstoni* sp. n. showed in its closed state. **d** – Juvenile specimen of *Pseudobenedeniella johnstoni* sp. n.

Description of *Pseudobenedeniella johnstoni* sp. n. (Figs. 1a, 2a, Table 3)

Type locality: coastal waters of Galindez Island (Argentine Islands, West Antarctica; 65°15' S, 64°16' W).

Type host: *Notothenia coriiceps* Richardson.

Site of infection: gills.

Etymology: This species is named after Dr. Thomas Harvey Johnston, a leading Australian biologist and parasitologist of the XX century, whose input into the knowledge of Antarctic fauna is priceless.

Material studied for description: 27 specimens stained with acetocarmine, four specimens used for DNA analysis, and two specimens used for EDXA.

Deposited specimens: holotype, paratype, and vouchers HWML-217721, HWML-217722, HWML-217723, University of Nebraska's State Museum, Harold W. Manter Laboratory, Lincoln, Nebraska, USA.

Gene sequences. The 18S and 28S genes of ribosomal RNA sequences were deposited in GenBank under accession numbers PP430573–PP430576 (18S) and PP430577–PP430580 (28S).

ZooBank

Description. Body elongated-oval, total length 3.85 – 6.75 (5.54 ± 0.71), width 1.3 – 3.0 (2.17 ± 0.39) (Table 3, Figs. 1a, 2a). Haptor in its typical (closed) position oval (Figs. 1a, c, 2a, b, c), attached to host tissues by clamping of gill lamellae, diameter 0.93 – 2.25 (1.8 ± 0.32), folded in half at equator and acts like a valve (Figs. 1a, c, 2b, c). Average body width to haptor ratio 1.17. Seven peripheral septae with well-developed muscular peripheral ridges present (Fig 1c, 2c). Main haptor armament includes one pair of well-developed anterior hamuli, adjacent to them smaller pair of posterior hamuli, and one pair of accessory sclerites (Figs. 1b, 3a). Marginal hooklets (0.02) situated on the perimeter of the muscular rim of haptor (Fig. 1b, 3b, c). Accessory sclerites (Fig. 1b, 3a) tiny, irregularly shaped 0.05 – 0.13 (0.07 ± 0.01), appearing degenerate. Anterior hamuli 0.23 – 0.47 (0.33 ± 0.04) slightly curved with sickle-shaped pronounced blade (Fig. 1b, 3a); its shaft widens to middle and then narrows to distal end, serrated on one side. Distal end of anterior hamulus appears thin and soft, while anterior end, with a sickle-shaped blade, is always firm (Figs. 1b, 3a). Posterior hamuli (Figs. 1b, 3a) with shaft that widens distally with serrated uneven end, 0.07 – 0.24 (0.18 ± 0.03). Anterior and posterior hamuli adjacent, in posterolateral septae of haptor (Figs. 1a, c, 3a). Haptor surrounded by thick muscular rim and thin folding marginal valve (Figs. 1a, 1c, 2b, c, d), its width 0.09 – 0.14 (0.12 ± 0.01) (Figs. 1a, 1c). Anterior attachment organ (prohaptor) wide, bladed with slight cleft (Figs. 1a, 2a) and adhesive pads (Fig. 1a) on both sides, along the front-end openings of head glands. Pair of muscular suckers on ventral side 0.25 – 0.88 (0.63 ± 0.12) × 0.31 – 1.00 (0.53 ± 0.13) (Figs. 1a, 2a, d). Muscular mouth leading to glandular pharynx 0.23 – 0.75 (0.47 ± 0.09) × 0.36 – 0.95 (0.65 ± 0.14) (Figs. 1a, 2a). Two pairs of eyes on dorsal side of body in front of pharynx (Figs.1a, 2a). Common genital opening

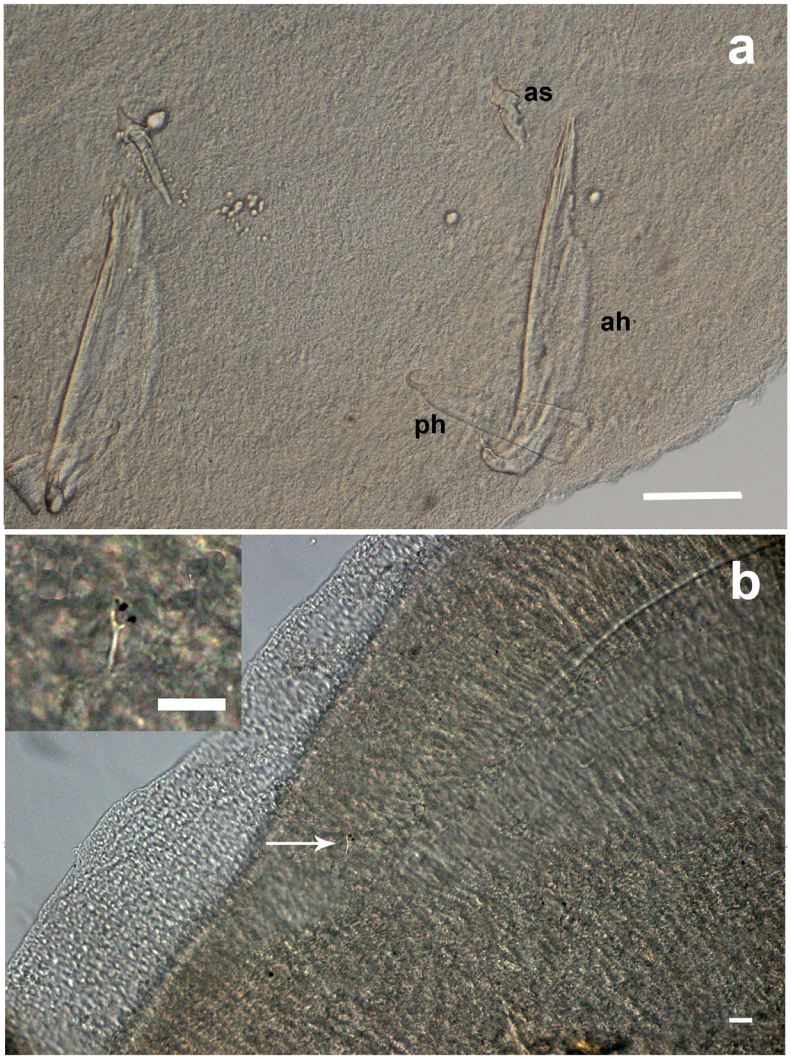

Fig. 3. **a** – Flattened haptor of *Pseudobenedeniella johnstoni* sp. n. with well-seen sclerotized structures in profile (ah, anterior hamulus; as, accessory sclerite; ph, posterior hamulus), close view. Scale bar 0.1 mm. **b** – Marginal hooklet of *Pseudobenedeniella johnstoni* sp. n. (arrow). Inset: close view. Scale bar 0.02 mm.

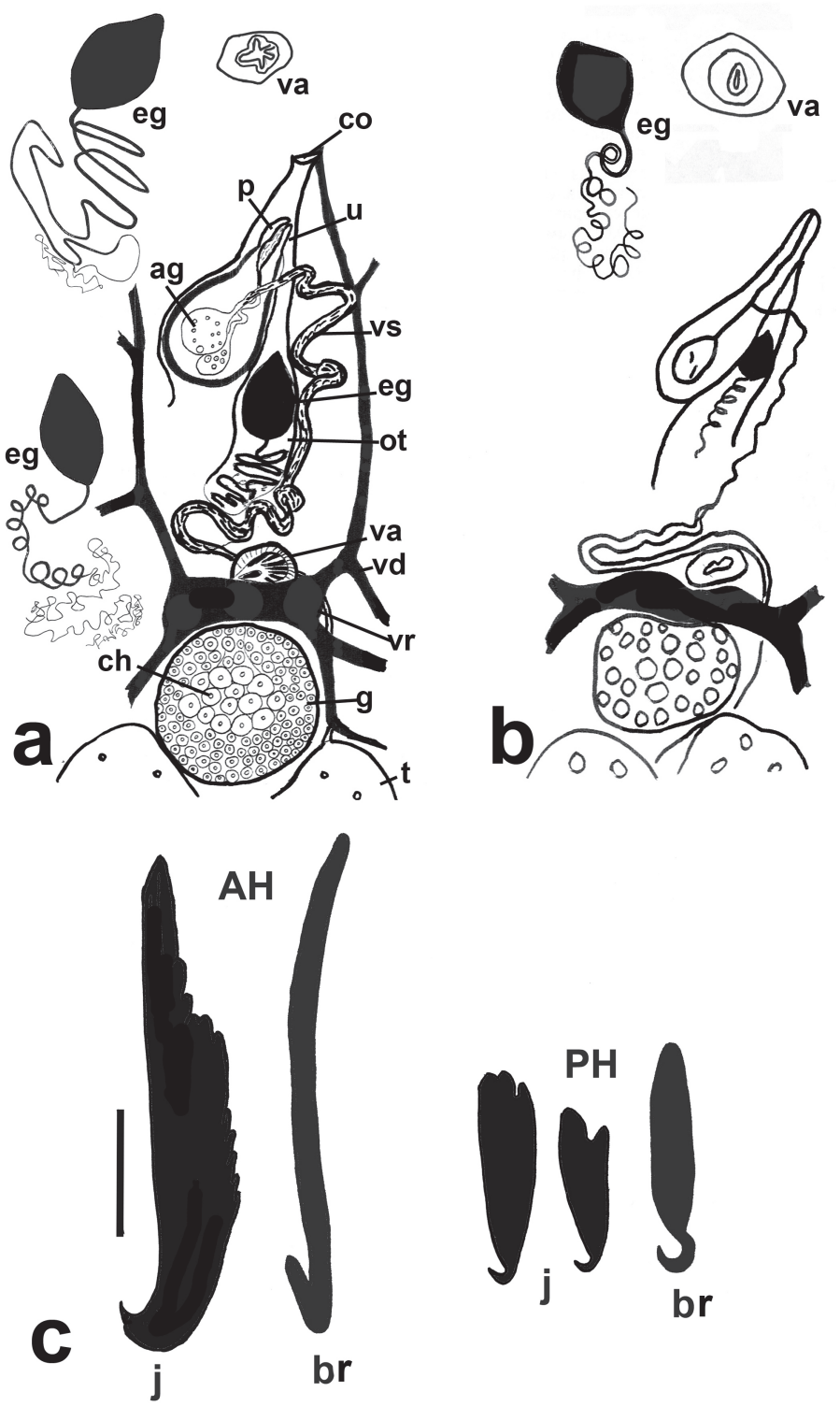

Fig. 4. **a** – Schematic drawing of the reproductive system of *Pseudobenedeniella johnstoni* sp. n.: ag, accessory gland; co, common genital opening; ch, germarium chamber; eg, egg; g, germarium; ot, ootype; p, penis; va, vagina; vd, vitelline duct; vr, vitelline reservoir; vs, vas deferens; t, testis; u, uterus. Insets: variation of eggs (eg); vagina (va). **b** – Modified drawing of the reproductive system of *Pseudobenedeniella branchialis* from Timofeeva *et al*. (1987). **c** – Comparative drawings of silhouettes of anterior hamuli (AH) and posterior hamuli (PH) of *Pseudobenedeniella johnstoni* sp. n. (j) and *Pseudobenedeniella branchialis* (br) [modified from Timofeeva *et al*. (1987)].

on ventral side, sinistrally of pharynx (Figs. 1a, 4a), under sinistral sucker. Compact rounded germarium 0.23 – 0.58 (0.43 ± 0.08) × 0.36 – 0.57 (0.52 ± 0.07) in central part of body, with noticeable chamber in it (Figs. 1a, 2a, d, 4a). Diffuse follicular vitellarium fills all body in adults (Figs. 1a, 2a) and is less developed in juveniles (Fig. 2d), covering intestinal diverticula and leaving uncovered only central part occupied by reproductive system. Vagina outer muscular part diameter 0.14 – 0.25 (0.17 ± 0.04), inner, possibly sclerotized part diameter 0.05 – 0.12 (0.08 ± 0.02), situated on the sinistral side of the body anterior to the vitelline reservoir (Figs. 1a, 4a). Vitelline ducts flow into vitelline reservoir from both sides of the body. Two oval testes 0.62 – 1.20 (0.96 ± 0.12) × 0.40 – 0.83 (0.59 ± 0.07) symmetrically posterior to ovary (Figs. 1a, 2a, d, 4a), slightly postequatorial to the middle part of the body. Penis pear-shaped, 0.46 – 1.43 (0.73 ± 0.12) × 0.16 – 0.62 (0.31 ± 0.05) (Figs. 1a, 4a), contains accessory gland and fleshy penis, which is capable of protrusion. Long coiled vas deference accommodates a large number of sperm cells, making a loop on the dextral side above vitelline reservoir, coiling to the sinistral side, and leading to penis. Goto glands detected posteriorly to testes (Figs. 1a, 2a). The distal part of ootype usually has one mature egg of characteristic ovoid shape, 0.16 – 0.28 (0.22 ± 0.02) × 0.09 – 0.16 (0.13 ± 0.02) anterior pole more pointed, and long coiled filament on its more blunt posterior pole (Figs. 1a, 4a). In immature specimens, a few tiny eggs are sometimes seen.

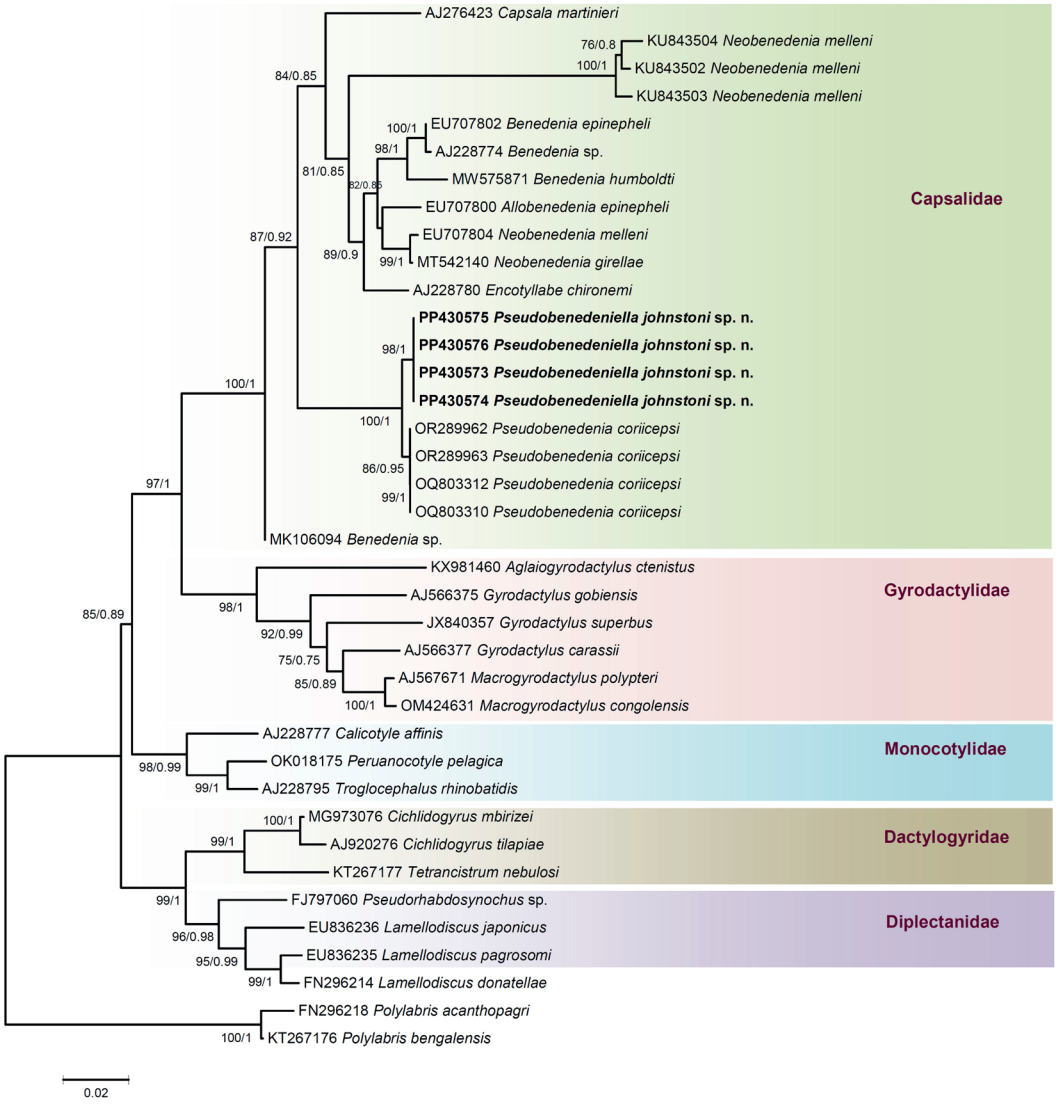

Fig 5. Phylogenetic tree based on the 18S sequence data showing the relationships of *Pseudobenedeniella johnstoni* sp. n. Bootstrap values and Bayesian posterior probabilities shown next to the nodes as ML/BI. Bootstrap support values >70 for ML and >0.70 for Bayesian posterior probabilities are shown. Species sequenced in this study are in bold, and the GenBank accession numbers are listed along with the species names. Scale bars represent the branch length. Families are indicated on the right side.

Differential diagnosis

Pseudobenedeniella johnstoni sp. n. was differentiated from *P. branchialis* described by Timofeeva *et al*. (1987) by different shapes of anterior and posterior hamuli (see Figs. 1b, 3a), namely, by the presence of sickle-shaped blade of anterior hamulus and its unique shape of the shaft, that widens to the middle part and then narrows distally, one side of the shaft is serrated, smaller posterior hamulus with characteristic broad shaft unevenly serrated in the distal end, more extensive parameters of the penis and its pear-like shape, smaller vagina diameter, by body width to haptor diameter ratio, different egg shape, different host species and different type locality in Antarctic waters (Table 3).

Remarks

The type material of *Pseudobenedeniella branchialis* Timofeeva, Gaevskaya, Kovaljova, 1987 is stored in the Zoology Institute (Saint Petersburg, Russia) (Timofeeva *et al.,* 1987). Unfortunately, our repeated attempts to contact the Curator of the Collection and ask for the type of material for examination were ignored. Therefore, the present comparison of *P. johnstoni* sp. n. with *P. branchialis* was based on the published description by Timofeeva *et al*. (1987), using the authors' drawings and morphometrical information data.

To better illustrate our findings, we provided our original drawings of specific taxonomically important hamuli and reproductive sys-

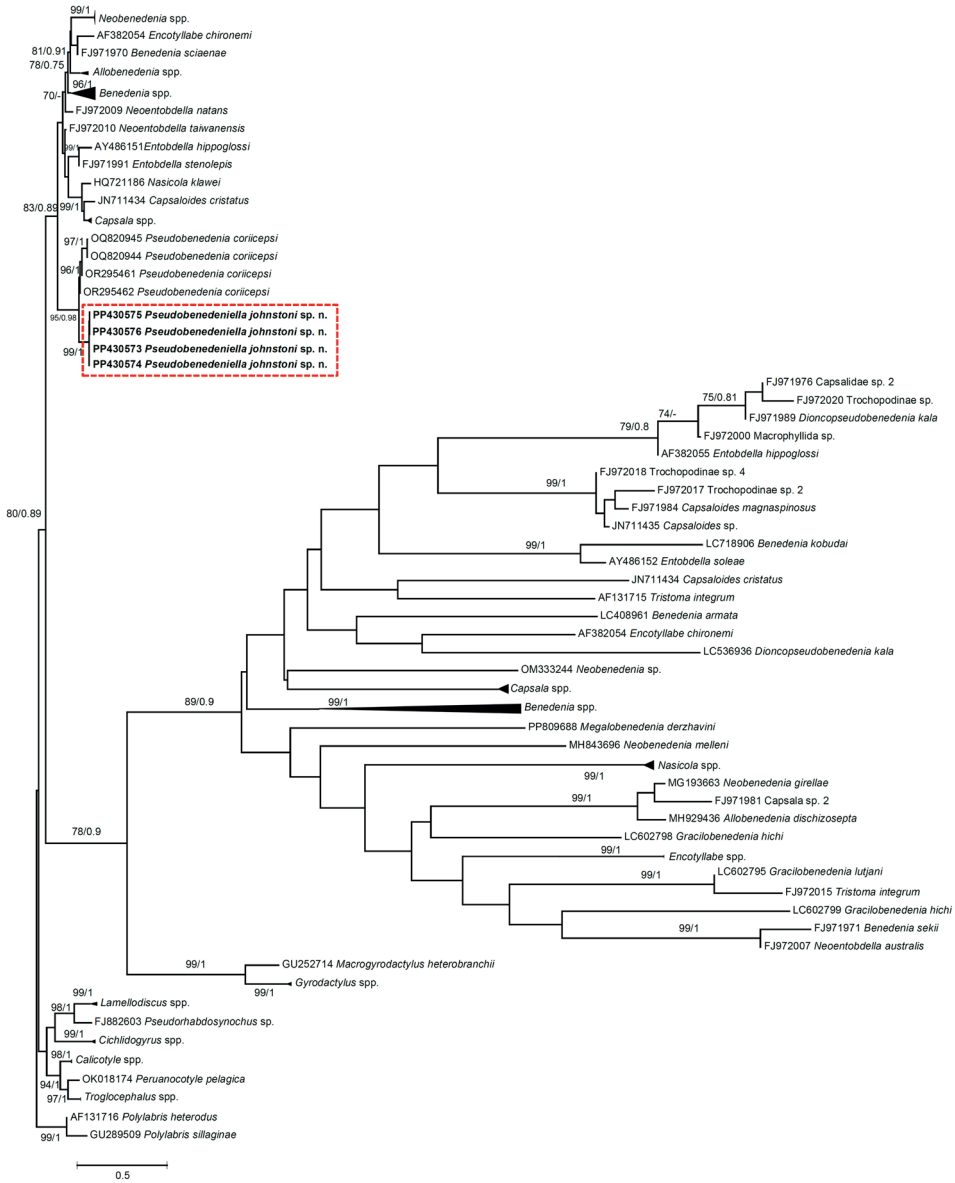

Fig. 6. A phylogenetic tree of capsalid taxa produced from maximum likelihood and Bayesian inference analyses of the 28S nuclear sequence data for the Capsalidae with outgroup taxa. ML bootstrap and PP values are indicated with each node as ML/BI. Species in the red box are sequenced during the present study.

tem characteristics and modified drawings by Timofeeva *et al*. (1987) (Figs. 4a-c).

Molecular analysis

ML and BI analyses of 18S and 28S gene sequences produced almost identical trees (Figs. 5 and 7). Nucleotide sequence data supported the position of *Pseudobenedeniella johnstoni* sp. n. and formed a well-supported clade in the family Capsalidae.

The alignment of the 18S rDNA dataset for the *Pseudobenedeniella johnstoni* sp. n. from two isolates along with sequences for representatives of the families Capsalidae, Gyrodactylidae Cobbold, 1864, Monocotylidae Taschenberg, 1879, Dactylogyridae Bychowsky, 1933, and Diplectanidae Monticelli, 1903 retrieved from ISND was used for phylogenetic analysis. Some taxa were not included in analyses because GenBank's 18S sequences were unavailable. *Pseudobenedeniella johnstoni* sp. n. isolates showed only 0.006 % intraspecific pairwise genetic distance. Both ML and BI phylogeny based on the 18S dataset (Fig. 5) represented firmly resolved grouping at the family level. *Pseudobenedeniella johnstoni* sp. n. received strong branch support by both ML and BI (BP=100 and PP=1) (Fig. 5). In the 18S tree, the Capsalidae family represented by species of the genera *Capsala* Bosc 1811, *Encotyllabe* Diesing 1850, *Benedenia* Diesing 1858, *Pseudobenedenia* Johnston, 1931, *Neobenedenia* Yamaguti 1963, and *Allobenedenia* Yamaguti 1963 that resolved in a separate clades. Finally, a strong connotation between *P. johnstoni* sp. n. and *P. coriicepsi* showing the sister-group relationship situated on the same clade (Fig. 5). The pairwise genetic distance among *P. johnstoni* sp. n. and *P. coriicepsi* showed 1.57 – 1.46 % nucleotide variability.

Newly generated *Pseudobenedeniella johnstoni* sp. n. sequences

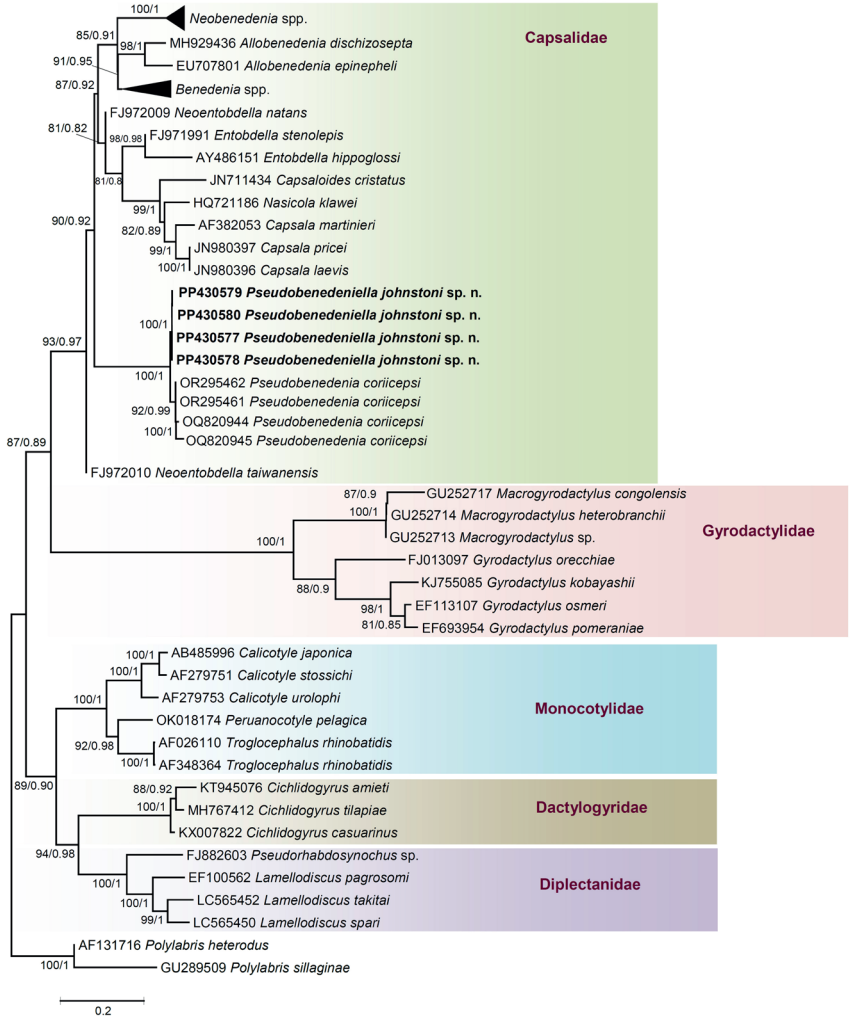

Fig. 7. A phylogenetic tree was constructed using data from 28S rDNA sequences of *Pseudobenedeniella johnstoni* sp. n. and other monogeneans. Values shown at the nodes indicate posterior probabilities from ML analysis (>70) and BI posterior probabilities (>0.70). GenBank accession numbers precede species names. The scale bar indicates the expected number of substitutions per site. Species sequenced in the current study are shown in bold. Families are displayed on the right side.

showed low overall intraspecific genetic divergence for the 28S gene of 0 – 0.005 %. We have generated two 28S trees for the new species; the first tree (Fig. 6) corroborated a far-related capsalid species to show and support the distinct species status of *P. johnstoni;* the second tree (Fig. 7) showed a comparison with closely related capsalid species in the family Diplectanidae. The main clade of the family Capsalidae represented the following genera: *Capsala*, *Entobdella* Blainville et Lamarck 1818, *Encotyllabe, Benedenia, Pseudobenedenia*, *Capsaloides* Price 1936, *Neobenedenia, Allobenedenia*, *Nasicola* Yamaguti 1968, and *Neoentobdella* Kearn et Whittington 2005 (Fig. 7). The 28S analysis of *P. johnstoni* sp. n. shows formed a strongly supported clade, sister to a clade representing *P. coriicepsi* (BP=100 and PP=1) (Fig. 7).

EDXA results

The percent weights of phosphorus (P), sulfur (S), and calcium (Ca) of three different parts of the body of *Pseudobenedeniella johnstoni* sp. n. obtained by the EDXA are shown in Table 4. The highest level of P was observed at the anterior end of the body and prominent S – at the anterior hamulus edge.

Discussion

This study documents the presence of a new monogenean species that parasitizes Antarctic black rockcod *N. coriiceps*; therefore, three specific monogenean species were found to parasitize this fish host – *Gyrodactylus coriicepsi* infecting the gills (Rokicka *et al*., 2009), *Pseudobenedenia coriicepsi* (Rubtsova *et al*., 2023) infecting body surface and *Pseudobenedeniella johnstoni* sp. n. infecting the gills. Our new species, *Pseudobenedeniella johnstoni* sp. n., having basic features of the genus Pseudobenedeniella infecting the gills and having valve-like haptor with seven septae, showed several morphological and morphometrical differences with *P. branchialis*. A thorough investigation of the morphology of sclerotized structures in the haptor and reproductive system, combined with data on different fish host species and geographically separated type localities, strongly supports the conclusion of reproductive isolation for this species.

The discovery of this new species was possible because of our close attention to the localization of the parasite on the body of the fish host (gills) and the detailed examination of the fine morphology of haptor attachment structures. Gill monogeneans have a different mode of attachment to the host tissues (Gusev, 1983, 1985). To the best of our knowledge, only representatives of the family Capsalidae possess the accessory sclerites; moreover, for a long time, Capsalidae were considered to have three pairs of hamuli. Llewellyn (1963) suggested calling these structures "accessory sclerites" despite the presence of two other pairs of true hamuli. Kearn (1963) indicated that these structures originated from marginal hooks in the early stages of embryogenesis. Cases of full or partial reduction of accessory sclerites and anterior hamuli in Capsalids are connected to changes in their mode of attachment to the host's tissues. The original type of attachment in capsalids is the suction of a saucer-like haptor to a relatively smooth body surface of their hosts with simultaneous clamping tissues between anterior hamuli and accessory sclerites. Kearn (1964) and Willams *et al*. (1973) studied the mechanism of this type of attachment. Accessory sclerites are well-developed in the close to *Pseudobenedeniella* genus *Pseudobenedenia* and play an essential role in attachment on the skin of saucer-like haptor (Williams *et al*., 1973). The well-developed muscular rim of the haptor (Figs 1a, c, 2a) in *Pseudobenedeniella* is another feature that indicates that the haptor is being used as a clamping device to hold on to a host gill lamellae.

The morphology of the haptor's attachment structures and peculiarities of the reproductive system are characteristic features of differentiation between monogenean species (Gusev, 1983, 1985; Bykhovsky,1957; Poulin, 2001). To make possible the detailed examination of the haptor attachment structures, we performed a microsurgical action on these monogeneans: cutting the haptors and pressing them between slide and cover glass in the drop of glycerin jelly following Gusev (1983). The only way to correctly see the sclerotized structures of the haptor after such manipulation was by using differential interference contrast; light microscopy was found to be uninformative (Rubtsova, 2009). In the original description of *P. branchialis* (Timofeeva *et al*., 1987), the authors performed the same procedure, explicitly indicating that they "cut haptor from unpressed fixed specimens and studied it separately – to study its morphology" and some of the haptors they "even

Table 4. The percent weights of phosphorus (P), sulfur (S), and calcium (Ca) in the attachment body parts of *Pseudobenedeniella johnstoni* sp. n. and *Pseudobenedenia coriicepsi* from Antarctic rockcod *Notothenia coriiceps* from the coastal waters of Galindez Island, West Antarctica obtained by the EDXA

	Pseudobenedeniella johnstoni sp. n. Present study			*Pseudobenedenia coriicepsi* (from Rubtsova *et al.*, 2023)		
Location on fish body	gills			skin		
Element, %	**P**	**S**	**Ca**	**P**	**S**	**Ca**
Anterior body end	0.87	1.54	1.59	0.25	0	1.45
Haptor	0	0.51	1.12	0	1.3	2.38
Anterior hamulus edge	0.04	8.06	1.54	0.01	0.01	0.67
Anterior hamulus center	0	3.97	0.68	0.44	8.83	1.95

opened up" [see p. 85 in Timofeeva *et al*. (1987)]. That means that authors observed sclerotized structures of *P. branchialis* in profile; nevertheless, they stated that "anterior hamuli have no expressed blade." While looking at our specimens of *Pseudobenedeniella johnstoni* sp. n., we observed the characteristic shape of the blade that somewhat resembled the head of a bird with a beak (Figs. 1b, 4c [AH, j]). Timofeeva *et al*. (1987) depicted a hamulus with a hook-shaped, sharply recurved blade (Fig. 4c [AH, br]). Also, the base of the anterior hamulus in *P. johnstoni* sp. n. specimens widened in the middle. Then, it narrowed to the distal part, which somewhat resembled the shape of the stretched bird's wing, with serrations on one side (Figs. 1b, 3a, 4c [AH, j]). In the 15 specimens examined by Timofeeva *et al*. (1987), such details were not marked either graphically (Fig. 4c [AH, br]) or verbally; the distal part of the anterior hamulus in *P. branchialis* was depicted as narrow, cylindrical along all lengths from the base to the blade (Fig. 4c [AH, br]). Also, some differences were detected in the shape of the posterior hamulus. The shape of the posterior hamulus in *P. branchialis* has a cylindrical shaft of equal width along its length from the proximal to the distal part (Fig. 4c [PH, br]). In contrast, our *P. johnstoni* sp. n. specimens have a characteristically widened serrated distal end in their shaft (Fig. 4c [PH, j], Table 3).

The reproductive system of *Pseudobenedeniella johnstoni* sp. n. differed from the reproductive system of *P. branchialis* by the larger average size of the penis and by its shape (Figs. 4a, b, Table 3), by the average inner and outer diameters of the vagina (Fig. 4a, b, Table 3), egg shape and size of the eggs (Figs. 4a, b, Table 3). All the above differences verify the reproductive isolation of these two species. Different fish host species are usually parasitized by specific monogenean species (Scheifler *et al*., 2022). The distance between the type location of the habitat of *N. rossii*, the host of *P. branchialis*, and the type location of *N. coriiceps*, the host of our new species, is about 2000 km and is also a significant factor of geographical isolation. According to Dewitt *et al*. (1990), *N. coriiceps* inhabits mainly shallow water shelf areas of West Antarctica. Therefore, different shapes of anterior and posterior hamuli, eggs, and habitats on the gills of varying host species from geographically distant parts of Antarctica support the new species described herein.

We utilized EDXA to analyze attachment structures, presenting new data on the composition and functionality of these adaptive structures. New data on the percent weights P, S, and Ca in different parts of the body obtained with EDXA for *Pseudobenedeniella johnstoni* sp. n. in this study are only known for *P. coriicepsi* within the Capsalidea family (Rubtsova *et al*., 2023). Therefore, here we provided a comparison of these parameters (Table 4) as critical, descriptive characters, the same as morphometric measurements or gene-sequencing data analyzed for monogeneans (Rubtsova *et al*., 2018; Rubtsova & Heckmann, 2019) and for other groups of helminths before (Amin *et al*., 2019, 2023). Inclusively, our study generated the first molecular data for any species of the genus *Pseudobenedeniella*; also, we performed comparative sequence analyses of two genes (18S and 28S) and made phylogenetic reconstructions for *P. johnstoni* n. sp. According to 18S and 28S rDNA phylogenetic inference, the ML and Bayesian trees derived both placed *Pseudobenedeniella johnstoni* n. sp. as sister taxa to a skin monogenean *P. coriicepsi*, both parasitizing the same host – Antarctic rockcod *N. coriiceps* in coastal waters of West Antarctica. The phylogenetic tree shows that *P. johnstoni* sp. n. is positioned close to *P. coriicepsi*, which perfectly agrees with the likely site switching on the same host. Apparently, *Pseudobenedenia coriicepsi* adapted to a new location in gill chambers and evolved into a new form adjusted to a new habitat on the gills. This new form must have modified the attachment apparatus from a sucker-like to a clamp-like haptor. Another example of a comparably unique case of clamp-like morphology of haptor is known for another gill monogenean, *Pseudallobenedenia opakapaka* Yamaguti 1966 (Yamaguti, 1968).

Thus, the integrated approach we took in this study of a new monogenean species highlights the significance of documenting monogenean diversity, contributing to increased knowledge of Antarctic biodiversity, and helping to conserve these unique ecosystems in the face of global warming and human activities.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

All authors contributed to the study's conception. Conceptualization: N. Y. Rubtsova, A. Chaudhary, T. A. Kuzmina T. A. Material collection: Kuzmina T. A., S. Glotov. Helminth identifications and data processing: N. Y. Rubtsova, A. Chaudhary. Data analysis: N. Y. Rubtsova, A. Chaudhary. N. Y. Rubtsova wrote the initial

manuscript draft, while all the other authors provided comments and feedback. All authors approved the final manuscript.

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