Parasitology Research (2019) 118:1445–1456
https://doi.org/10.1007/s00436-019-06297-8

GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Transatlantic discovery of *Notocotylus atlanticus* (Digenea: Notocotylidae) based on life cycle data

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Received: 10 October 2018 / Accepted: 18 March 2019 / Published online: 28 March 2019

Abstract

Digenean parasites feature a series of stages with a distinct appearance, reproduction mode, and lifestyle that together constitute their well-known, complex life cycle. Species descriptions of Digenea have always been based on one of these stages—the marita, or sexually reproducing adult in the final host. However, in some cases, data on the life cycle are essential for the differential diagnosis of closely related species. Here, we present the case of *Notocotylus atlanticus*, where different stages of its life cycle were discovered for the first time since the species description, and across the Atlantic. We used a material from a naturally infected intertidal marine snail, *Ecrobia ventrosa*, and several waterfowl species and also carried out infection experiments. For morphological studies, we employed light microscopy, SEM, and CLSM; molecular data obtained include sequences of ITS1 and 28S rRNA gene. We demonstrate that *N. atlanticus* adult worm morphology is barely sufficient to distinguish it from several other species. Cercariae morphology and identity of the first intermediate hosts provide crucial additional information. According to our preliminary phylogenetic reconstructions, two notocotylid lineages are associated with two major gastropod lineages—the Caenogastropoda and the Heterobranchia. The traditional character to identify notocotylid genera (structure of ventral organs) fails to explain the phylogeny and thus requires reassessment. Further reliable morphological, life cycle and molecular data on other species are likely to reveal more patterns in notocotylid systematics, host specificity, and evolution.

Keywords Digenea · Notocotylidae · Life cycle · First intermediate host · Marine parasite · Waterfowl parasite

Introduction

Digeneans with their peculiar life cycles are one of the most fascinating parasite groups. Exciting tasks to study digenean diversity, patterns in their evolution, current distribution features, and interactions with hosts and environment are often hindered by the problem of species identification.

The digenean species has traditionally been represented by a description of an adult of the hermaphroditic generation (marita) based on a whole mount. At the same time, the importance of data on the life cycle has also been widely recognized and was particularly highlighted in major studies on the subject (Ginetsinskaya 1968; Pearson 1972; Cribb et al. 2003; Blasco-Costa and Poulin 2017). Efforts to elucidate digenean life cycles by means of experimental infections can now be supported by molecular genetic analysis. Integrative studies have untangled certain taxonomic stories (e.g., Microphallidae, Galaktionov et al. 2012; Echinostomatidae, Tkach et al. 2016; and Diplostomidae, Blasco-Costa and Locke 2017); many others are not yet resolved.

One of the digenean families with plenty of ambiguities in its systematics is Notocotylidae. Notocotylids are common parasites that may be models for phylogeographic studies and for understanding the establishment of a three-host life cycle in the digenean evolution. Moreover, they are quite pathogenic and at high intensity may be harmful and cause

Section Editor: Sabine Specht

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the death of their hosts, including poultry (reviewed in Kulachkova 1979, Filimonova 1985). Genera in this group seem to be well defined (based on the structure of the ventral organs in adults), but at the species level, there is much controversy. Notocotylid cercariae are routinely identified only to the family level: they are difficult to distinguish because of being quite uniform, and pigmented body impedes observations. Notorious is the case of *Catatropis verrucosa* (Frölich, 1789) that used to be reported from a variety of definitive and first intermediate hosts, with conflicting accounts on adult and cercarial morphology (Kanev et al. 1994).

Our ongoing research on the notocotylid fauna from the marine coasts of the northern Palaearctic has revealed significant diversity, within both bird and molluskan hosts. In one snail species *Ecrobia ventrosa* (Montagu, 1803) (syn. *Hydrobia ventrosa* (Montagu, 1803)), we have discovered three morphologically distinct notocotylid cercariae (Gonchar and Galaktionov 2016). Distinguishing between them largely relies on the structure of the main collecting ducts of the excretory system (Rothschild 1938). These collecting ducts are well visible because they contain refractive excretory granules. The ducts merge in the anterior, and morphotypes differ by the structure of this merging place. If it has a simple rounded shape, the morphotype is Monostomi; if it has a diverticulum, the morphotype is Yenchingensis; if it has a loop extending to the level of the median eyespot, the morphotype is Imbricata.

Here, we present the results of our studies on the Yenchingensis cercariae from *E. ventrosa*, and the corresponding adult stages. Data on the life cycle and morphology indicate that this material belongs *Notocotylus atlanticus* Stunkard, 1966—a species that has not been recorded since its designation. We, for the first time, report its natural definitive hosts, update the morphological description, and supplement it with molecular characteristics. We consider this integrative work a case study which emphasizes the importance of data on life cycles for distinguishing digenean species.

Materials and methods

Sampling

Material presented in this study was collected from definitive and intermediate hosts (birds and mollusks) during 2002–2015 on the White Sea coast (Russia), in Iceland and in France (Table 1). We obtained cercariae from mud snails *E. ventrosa* following a standard procedure (Gonchar and Galaktionov 2016); to obtain rediae, we dissected infected snails. Adults were recovered from birds that were sampled in accordance with local regulations. We preserved material in different media (see below) for further light microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and molecular studies.

Infection experiments

Experimental infections of day-old chicks *Gallus gallus domesticus* Linnaeus, 1758 and domestic ducklings *Anas platyrhynchos domesticus* Linnaeus, 1758 by notocotylid metacercariae were performed at the Institute for Experimental Pathology, University of Iceland, Keldur, in 2002 and 2008. The mollusks *E. ventrosa* were collected in Melabakkar (SW Iceland), and those shedding notocotylid cercariae of Yenchingensis morphotype (Rothschild 1938) were selected for the experiments. These snails were placed into 24-welled plates with sea water, 1–3 pieces of grass, and 1–2 empty mud snail shells. After 24 h, encysted metacercariae (together with small pieces of grass or scraped from the shells) were fed to chicks and ducklings using soft tweezers. The birds swallowed the leaves and cysts after administering small amount of sea water with a pipette into their mouth, each bird receiving 60–80 metacercariae. Chicks were euthanized and dissected 5 days post-infection (pi), and ducklings 4 and 5 days pi.

Table 1 The *N. atlanticus* isolates, their origins, and available sequence data (present study). LSU gene sequences are of D2 domain except for MH818008 (D1–D3 domains)

ID	Stage	Host	Collection site	GenBank accession numbers	
				LSU	ITS1
5	Redia/cercaria	*E. ventrosa*	White Sea	–	MH818012
22	Redia/cercaria	*E. ventrosa*	Iceland	–	–
36	Redia/cercaria	*E. ventrosa*	White Sea	MH818006	MH818013
37	Redia/cercaria	*E. ventrosa*	White Sea	MH818007	MH818014
80	Adult	*A. platyrhynchos*	White Sea	MH818008	MH818015
81	Adult	*A. platyrhynchos*	White Sea	–	–
82	Adult	*A. platyrhynchos*	White Sea	–	–
NOT5	Adult	*A. penelope*	France	MH818009	–
NOT14	Adult	*A. acuta*	France	MH818010	–
NOT15	Adult	*A. acuta*	France	MH818011	–

Morphological analysis

To study rediae and cercariae, we made temporary mounts and examined them under Leitz Dialux 20B and Olympus CH40 microscopes. Cercarial morphotypes were identified and Yenchingensis cercariae were selected. Measurements were made on heat-killed cercariae (only those that had emerged from the molluskan host) and live rediae (only resting specimens, uncontracted and non-elongated) in seawater and under a slight coverslip pressure.

Live adults from wild birds were preliminarily identified under stereo- and compound microscopes and then preserved in 96% ethanol. For this study, we selected individuals that belong to the genus *Notocotylus*. Worms were then stained with carmine or Weigert's iron hematoxylin and mounted in Canada balsam or Mowiol® 4-88 (Sigma-Aldrich) for detailed morphological examinations. Drawings were made using a Leica DM1000 stereomicroscope equipped with a drawing tube. Drawings were processed in Adobe Photoshop CS4 version 11.0 and CorelDraw X3. Measurements were made using an ocular micrometer except for the eggs, which were measured under a Leica DM2500 stereomicroscope equipped with a Nikon DS Fi1 camera using NIS version 5.0 software. All measurements are given in micrometer, with the mean in parentheses and number of observations (*n*).

A digital video image (Z-stack) of the type specimen of *N. atlanticus* was acquired from the National Museum of Natural History, USA (catalog number USNM 1356913), and these data are used with the permission of the National Museum of Natural History, Smithsonian Institution, 10th and Constitution Ave. N.W., Washington, DC 20560-0193. (https://www.nmnh.si.edu/).

To detect additional morphological aspects important for identification, we visualized the musculature and actinous spines using fluorescent phalloidin staining and CLSM. The details of the method have previously been described by Krupenko and Gonchar (2017a).

We used SEM to describe the tegument surface structures. Ethanol-preserved samples were transferred to 100% acetone through a graded series. For sample preparation, we used an automated critical point dryer Leica EM CPD300 (three CO_2 changes for 15 min each) and high vacuum sputter coater Leica EM SCD500 (20-nm gold film). Samples were studied using a Tescan MIRA3 LMU at an accelerating voltage of 7 kV. Spine width was measured using ImageJ 1.51s (Rueden et al. 2017) software. The length of the spines was impossible to determine accurately due to their angle and because their bases were masked by the surrounding spines.

Molecular analysis

Samples for molecular genetic analysis were preserved in 96% ethanol. We removed single rediae or parts of the adult worms from ethanol, let them dry completely, and extracted DNA by either of the two following approaches. The first approach involved incubation in 200 μL 5% Chelex® 100 resin (Bio-Rad, USA) solution with 0.2 mg/mL proteinase K at 56 °C overnight; 8 min at 90 °C; and centrifugation at 20,000*g* for 10 min. Supernatant containing DNA was then transferred to a new tube and stored at − 20 °C. The second approach involved DNeasy® kit (Qiagen, Germany) following the manufacturer's protocol with modifications as described by Gonchar and Galaktionov (2017).

Amplification was performed in 20 μL reaction mixtures containing 12 μL Super-Q® water, 4 μL ScreenMix-HS reaction mix (Evrogen, Russia), 1 μL of both F and R primers, and 2 μL DNA template. Fragments of the ITS1 and the D2 domain of the 28S ribosomal RNA gene (D2 LSU) were amplified using the primers and thermocycling conditions as summarized by Gonchar and Galaktionov (2017). A longer fragment of the LSU (D1–D3 domains) was amplified with LSU5 and 1500R primers (Olson et al. 2003). PCR products were visualized on a 1.5% agarose gel with ethidium bromide, purified with KR-011 kit (Omnix, Russia), and sequenced on ABI PRISM 3130 (Applied Biosystems Inc.) with PCR primers. For the D1–D3 LSU fragment, additional internal sequencing primers were used: 900F, 1200F, and 400R (Olson et al. 2003; Reyda and Olson 2003; Telford et al. 2003). Sequence data were processed using Geneious 7.0.6 (Kearse et al. 2012). Relevant sequences, publically available from GenBank, were used to analyze our results through BLAST, alignments, and phylogenetic reconstructions. The best substitution model was determined as GTR + I + G with BIC in jModelTest 2.1.10 (Darriba et al. 2012). MrBayes v.3.2.6 (Ronquist et al. 2012) was used to build a tree via a Bayesian inference method. The PhyML 3.0 plug-in for Geneious (Guindon et al. 2010) was used to build a tree via a maximum likelihood method. *Diplodiscus subclavatus* (Pallas, 1760) Diesing, 1836 (AY222212) served as an outgroup.

Results

Matching marker DNA sequences of *N. atlanticus* adults from anatids and rediae/cercariae from *E. ventrosa* allowed us to link the two life cycle stages (see "Molecular data" subsection for details). Since distinguishing *N. atlanticus* from several other *Notocotylus* species is complicated, below we provide a detailed morphological description for adults, cercariae, and rediae.

Experimental infections of chicks did not yield results; in ducklings 4 and 5 days pi, active notocotylid adults were found in intestinal caeca. They were preliminarily

Table 2 Morphometric parameters of *N. atlanticus* adults (present study and data from Stunkard (1966))

Character	Our data	Stunkard 1966, $n = 10$
Body length	1850–3600 (2727), $n = 13$	2200–3650
Body width	515–940 (743), $n = 13$	800–1100
Oral sucker	87–170 (139) × 97–185 (158), $n = 10$	150 × 120–180 × 150
Esophagus length	78–130 (95), $n = 9$	NA
Testes	100–280 (196) × 290–690 (484), $n = 12$	280 × 160–480 × 260
Internal seminal vesicle length	260–390 (336), $n = 7$	250–275
Cirrus	172 × 18, $n = 1$	190 × 28
Cirrus sac length	610–1020 (814), $n = 12$	600–840
Cirrus sac posterior extremity to body length ratio	0.35–0.44 (0.39), $n = 13$	0.375
Ovary	120–375 (246) × 130–345 (241), $n = 11$	190 × 150–50 × 200
Pre-vitelline uterine loops	5–8	2
Uterine loops posterior to anterior margin of vitelline fields	11–15	12–16
Anterior vitelline fields to body length ratio	0.45–0.57 (0.52), $n = 12$	Slightly < 0.5
Metraterm	230–470 (351), $n = 10$	280–370
Metraterm to cirrus sac ratio	0.33–0.51 (0.42), $n = 10$	< 0.5
Eggs	15–20 (18.5) × 10–12.5 (11), $n = 65$	17–19 × 11
Egg filament length	53–151 (85), $n = 25$	NA

identified as *N. atlanticus* based on a set of morphological features. These experimentally obtained specimens were immature, so description below is based on material from wild ducks.

Adult (Table 2, Figs. 1, 2, and 3)

Three species of ducks were found to be naturally infected with *N. atlanticus*: *A. platyrhynchos*, *Anas acuta* Linnaeus, 1758, and *Anas penelope* Linnaeus, 1758. Worms were recovered from caeca or neighboring parts of the hindgut.

Body elongate; ventral concavity pronounced. Oral sucker subterminal; ventral sucker absent. Ventral surface with three rows of papillae, 15–16 in median row and 16–17 in lateral rows. Anteriormost and posteriormost lateral papillae smaller in size than others. Papillae in median row usually 1/2 interval posterior to papillae in lateral rows ($n = 5$), but sometimes 1/2 interval anterior ($n = 1$). Tegumental spines well developed, varying in density and size across the body (Fig. 2). Gradual onset of spines near the anterior extremity of the body (Fig. 2e). Spines largest and densest in anterior-ventral part of the body, becoming smaller posteriorly. Scale-shaped spines occur more anteriorly (Fig. 2f) and laterally (Fig. 2c, 5.9 wide); lanceolate spines occur more posteriorly (Fig. 2g, 0.3 wide; Fig. 2j) and medially (Fig. 2d, 1.9 wide; Fig. 2j). On the dorsal side of the body, spines pronounced only in anterior 1/4 of the body (Figs. 2h, i and 3a).

Pharynx absent; esophagus short; caeca end blindly close to the posterior margin of the body. Testes lobed, lateral to caeca. External seminal vesicle in a form of several loops dorsal to the uterus. Cirrus sac long, containing coiled internal seminal vesicle, pars prostatica and long ejaculatory duct. Cirrus covered with papillae (Fig. 3c). Ovary lobed, medial between testes. Vitelline follicles in two compact groups at both sides of the body, extracaecal, usually extending from the middle of the body to the anterior margin of the testes. Vitelline ducts extend from the posterior region of lateral vitelline fields and pass in the medial direction dorsal to Mehlis' gland. Mehlis' gland median anterior to ovary; initial parts of the uterus ventral to Mehlis' gland. Uterus with 5–8 pre-vitelline transverse loops and 11–15 that go along the vitelline fields. Metraterm[1] with well-developed circular and longitudinal muscle fibers (Fig. 3b). Common genital pore just posterior to the intestinal bifurcation. Eggs with filaments on each pole. Excretory pore on the dorsal side of the body between caeca and posterior to ovary. Secondary excretory system complex; main collecting ducts branch in a dendritic manner.

Cercariae (Fig. 4)

(Measurements based on 48 heat-killed cercariae.) Cercariae of typical notocotylid appearance, dark in color; ventral sucker

[1] The digital image of *N. atlanticus* type specimen (USNM 1356913 at the National Museum of Natural History, USA) indicates that our interpretation of metraterm structure is consistent with that of H. Stunkard. This would have been impossible to ascertain using only the text and figures of his article (Stunkard 1966).

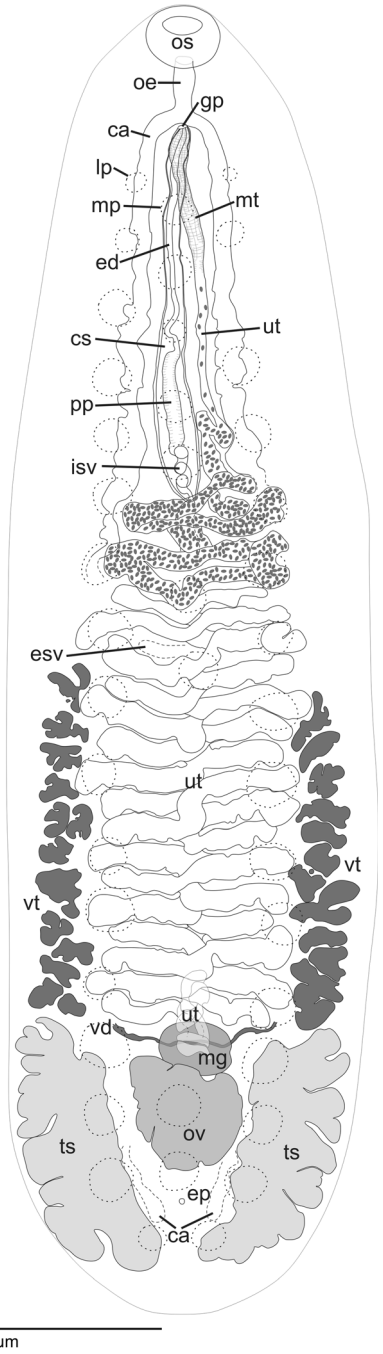

Fig. 1 *N. atlanticus* adult, ventral view. *os* oral sucker; *oe* esophagus; *ca* caecum; *mp* median ventral papilla; *lp* lateral ventral papilla; *ep* excretory pore; *ov* ovary; *mg* Mehlis' gland; *vt* vitellarium; *vd* vitelline duct; *ut* uterus; *mt* metraterm; *gp* genital pore; *ts* testis; *esv* external seminal vesicle; *isv* internal seminal vesicle; *pp* pars prostatica; *ed* ejaculatory duct; *cs* cirrus sac

absent; dorsal adhesive pockets located postero-laterally. Body 257–371 (300) × 114–229 (153); tail 243–400 (305) × 29–43 (34). Three eyespots present. Oral sucker 29–47 (35); pharynx absent; intestine bifurcated. Main excretory ducts fuse anteriorly forming diverticulum that reaches beyond median eyespot; hence, cercariae are of Yenchingensis morphotype. Excretory granules vary in size, 0.88–1.84 (1.32), 4–7 in rows across main excretory ducts. Body filled with cystogenous glands containing uniform secrete—the rod-shaped granules.

Cercariae leave rediae when immature and further develop within the molluskan hemocoel. Only fully formed pigmented larvae are shed into the external environment. In experimental vessels, they swim in the water column for a short time after leaving the molluskan host and encyst mainly on the host shell. A few cercariae encyst on grass and the vessel walls; empty molluskan shells are ignored. The cysts are 170–200 (186) in diameter.

Rediae (Fig. 5)

(Measurements based on 23 living rediae.) Groups of rediae in infected mollusks are heterogeneous and include individuals of variable maturity. Large rediae 770–1570 × 290–370 (1185 × 330); pharynx 60–68 (64) in diameter; contain 3–6 developing cercariae, cercarial embryos, and germinal balls. Smaller (= younger) rediae 270–600 × 114–257 (418 × 176), contain germinal balls and cercarial embryos or only germinal balls. Germinal mass occurs in the posterior region of the body. Cercariae leave rediae via birth pore at the level of the pharynx.

The rediae groups vary in the proportion of individuals at different ages. In mollusks that are already shedding cercariae, the group may be relatively young, with the majority of rediae being small and 2–5 being large. In such groups, there sometimes are 1–2 degenerating mother rediae (or daughter rediae of the first generation), and 1–5 small motile rediae (~ 100) of the next generation with several germinal balls. A mature group of rediae contains up to 50 large individuals and much fewer (~ 5–20) younger ones.

Molecular data

We obtained partial LSU and ITS1 sequences for most of the specimens that were at our disposal. Complete information on the origins of all sequences is presented in Table 1, along with their GenBank accession numbers. All the material, including adults and cercarial samples, proved homogenous.

ITS1 sequences obtained for four isolates were identical. They were 956 base pairs (bp) long after trimming and included a small fragment of 5.8S rRNA gene at the 3′-end. In the 5′-region, they contain two repeats, each 131 bp long, starting at positions 29 and 175. In GenBank, ITS1 sequences are available for three other notocotylid species: Notocotylidae gen. sp. CG-2013 (KF656705), *Notocotylus malhamensis* Boyce et al., 2012 (JQ766940), and *Tristriata anatis* Belopol'skaia, 1953 (seven isolates, e.g., KX833027); none of these three species have repeats in their ITS1 sequences.

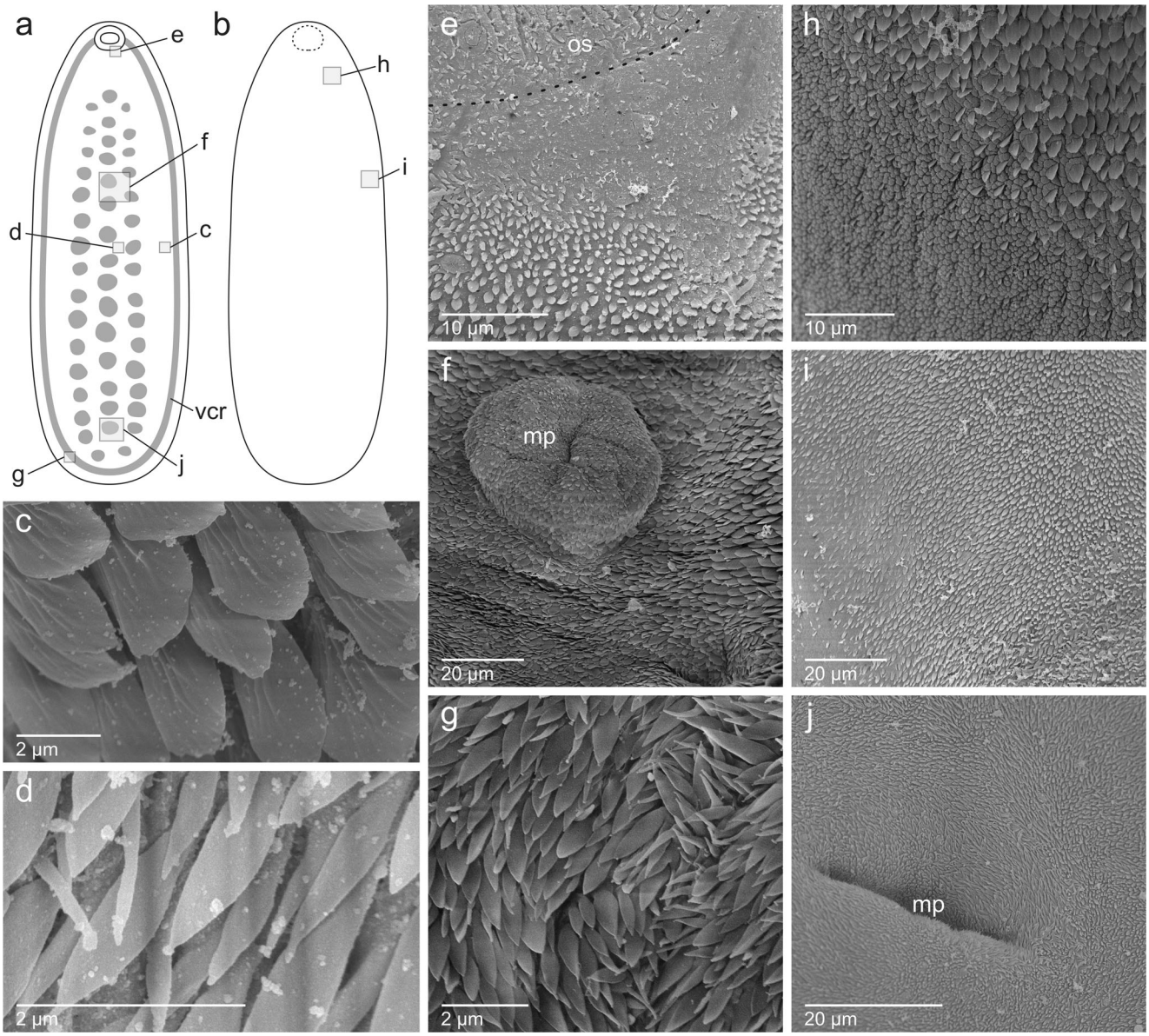

Fig. 2 Distribution of tegumental spines across the body of *N. atlanticus* and their shapes **a**, **b** schemes of the body from the ventral (a) and dorsal (b) sides, indicating regions shown on SEM microphotographs c–j; *vcr* ventral concavity ridge. **c**–**j** SEM microphotographs showing tegumental spines. **c** Scale-shaped spines. **d**, **g** Lanceolate spines. **e** Area free from spines near the oral sucker (*os*). **f** Spines on the everted median papilla (*mp*) and around it. **h** Spines becoming more sparse towards the dorsal side. **i** Spines disappearing towards the dorsal side. **j** Lanceolate spines around the retracted median papilla

D2 LSU fragments were obtained for six isolates; an additional longer D1–D3 fragment was sequenced for one isolate. No nucleotide variation was detected among these samples. Within the GenBank data, *Notocotylus attenuatus* (Rudolphi, 1809) Kossack, 1911 (AF184259) with four nucleotide substitutions and *Notocotylus intestinalis* Tubangui, 1932 (JQ890559) with nine are the two taxa closest to *N. atlanticus* based on D1–D3 LSU sequence data. All differences are restricted to the three variable domains; regions between the domains are identical.

Phylogenetic analyses based on the D1–D3 domain LSU sequence (1190-bp alignment of 13 ingroup sequences) with Bayesian and maximum likelihood methods produce trees with the same topology; branch support values are higher for the Bayesian tree (Fig. 6). Representatives of the genus *Notocotylus* are split into two clades. One of them, along with *N. atlanticus* and three other species of *Notocotylus*, includes *Catatropis indicus* Srivastava, 1935 and *Catatropis vietnamensis* Izrailskaia et al., 2019. The second one includes four species of *Notocotylus* and *Pseudocatotropis dvoryadkini*

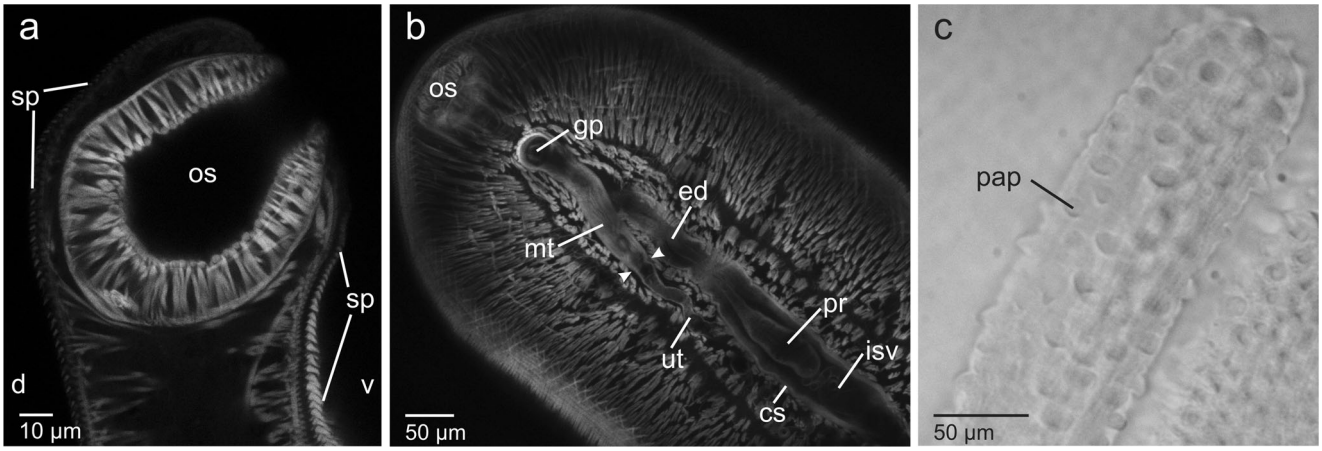

Fig. 3 Anatomic details of *N. atlanticus* adult. **a** CLSM of anterior region, sagittal section; *os* oral sucker; note that spines (*sp*) are present on both ventral (*v*) and dorsal (*d*) sides. **b** CLSM, Z-stack of frontal sections; note thick muscular wall of metraterm (*mt*) and relative length of metraterm and cirrus sac (*cs*); *isv* internal seminal vesicle; *pr* pars prostatica; *ed* ejaculatory duct; *gp* genital pore; arrowheads show the start of metraterm. **c** Microphotograph of cirrus in its distal part, note its papillated surface (*pap*)

Izrailskaia et al., 2019. Attribution of the first intermediate host of these parasites to Caenogastropoda or Heterobranchia is marked in Fig. 6.

Another "larval" notocotylid sample from Thailand is present in the GenBank as KU820968 (Wongsawad et al. 2016). This sequence was not included in the phylogenetic analysis because the fragment is of poor quality at its 3′-end and would have shortened our dataset by approximately 200 bp. However, a 1020-bp alignment shows that it groups closest to *C. indicus* (99.02% identity).

Remarks

We matched intramolluskan and adult stages by comparing marker DNA sequences and by performing infection experiments and proved that we deal with a single species. Adult worms that we have recovered from wild ducks agree in all major identification characteristics, size ranges, and ratios with the original description of *N. atlanticus* given by Stunkard (1966) (Table 2). On the basis of the additional material studied here, we can refine the diagnosis of this taxon.

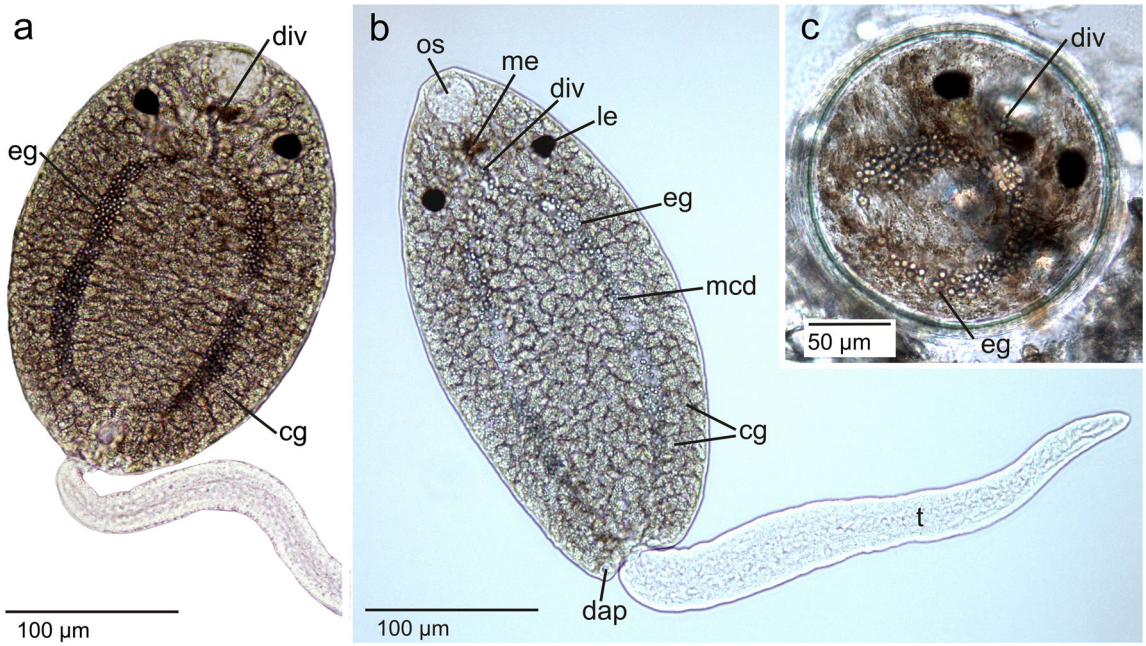

Fig. 4 Microphotographs of cercariae and metacercaria of *N. atlanticus*. Cercaria changes shape when moving under cover slide, contracting (**a**) and elongating (**b**). *os* oral sucker; *dap* dorsal adhesive pocket; *le* lateral eye; *me* median eye; *mcd* main collecting duct of the excretory system with excretory granules (*eg*), and its anterior diverticulum (*div*); *cg* cystogenous glands; *t* tail. **c** Metacercaria, note that anterior diverticulum is still visible

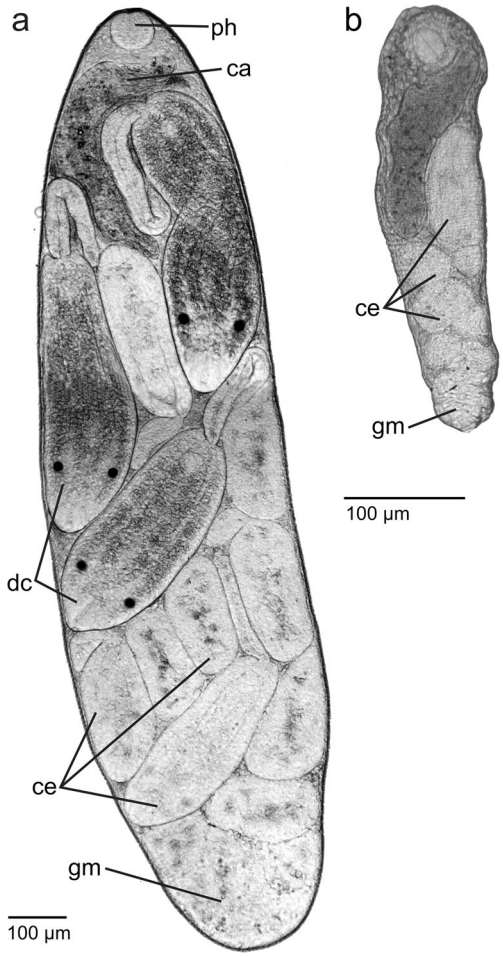

Fig. 5 Microphotographs of *N. atlanticus* rediae. *ph* pharynx; *ca* caeca; *dc* developing cercaria; *ce* cercarial embryo; *gm* germinal mass. **a** Mature redia. **b** Young redia

The number of ventral papillae is an important feature in keys to species of *Notocotylus*. The ventral organs may be small and indistinct in whole mounts, so we recommend to assess their type and number in unmounted material (live or fixed), or by using SEM/CLSM[2] photographs. According to Stunkard (1966), *N. atlanticus* adults bear 16 papillae in each of the three rows; we found 15–16 papillae in the median row and 16–17 in the lateral rows. Such ranges instead of precise numbers are common in descriptions of other notocotylids (e.g., Dubois 1951) and may be due to both intraspecific and ontogenetic variability (Radlett 1979; Filimonova 1985). So, we should not delimit notocotylid species basing only on the papillae count. Our data on the position of the front papilla of the median row agree with those of Stunkard: it can be either 1/2 interval anterior or 1/2 posterior to the front papillae of the lateral rows.

[2] The musculature of *N. atlanticus* with emphasis on how the ventral concavity is sustained and how the ventral papillae move was described by means of CLSM in our previous work (Krupenko and Gonchar 2017b).

We used SEM to demonstrate that spines cover the body of the *N. atlanticus* adult. They are more prominent on the ventral side but also extend to the dorsal side. Stunkard examined whole mounts and claimed that the body is "unarmed, except for the ventral surface" (Stunkard 1966). We find that spines, readily visible on some whole mounts, appear to be completely absent on others. They may have fallen off during manipulation or may be masked by the thick body. Spines on the dorsal side were not visible on any of our whole mounts. We recommend using SEM to obtain a complete picture of the spination pattern, and additional visualization using CLSM is also informative. Tegumental spines similar to those we observed in *N. atlanticus* (scale-shaped and lanceolate) occur in other notocotylids that have been studied using SEM (Wittrock 1978; Radlett 1980; Tandon and Roy 1996; Bayssade-Dufour et al. 1996; Naem and Smythe 2015).

Although adult worms studied here fit the description of *N. atlanticus*, there can be confusion in distinguishing them from several other species, most notably *N. attenuatus*, *Notocotylus imbricatus* (Loos, 1893) Szidat, 1935, *Notocotylus magniovatus* (Yamaguti, 1934) and *N. intestinalis*. Adult worms of these species agree in almost all diagnostic characters. Exceptions are the length of egg filaments and metraterm to cirrus sac ratio: these may help differentiate adult worms of *N. atlanticus*, but both characters are variable and should be assessed critically.

Cercariae that we have studied here match cercariae of *N. atlanticus* described by Stunkard (1966) in size and morphology. They are also the same as cercariae that we have earlier found in Iceland and at the White Sea and identified as *Cercaria Notocotylidae* sp. no. 13 Deblock, 1980 (Skírnisson et al. 2004; Gonchar and Galaktionov 2016). Deblock in his solid review (1980) also suspects conspecificity of his *C. Notocotylidae* sp. no. 13 and *N. atlanticus*. Several other species of *Notocotylus* have similar cercariae, but they differ by snail hosts and have freshwater life cycles (Table 3). Of the five species discussed above, *N. attenuatus* clearly stands out, with cercariae of Monostomi morphotype; and *N. magniovatus* is the only one where rediae have locomotory appendages.

The original description of *N. atlanticus* lacks data on the natural definitive hosts: adult worms were experimentally grown in ducklings of the common eider *Somateria mollissima* (Linnaeus, 1758) (Stunkard 1966). We found adults of *N. atlanticus* in wild mallard, pintail, and widgeon and for the first time describe the natural host range and morphology of adults excluding any possible bias due to rearing worms in an experimental host.

Discussion

Evidence from molecular, morphological, and life cycle data indicates that the material we deal with in this study belongs

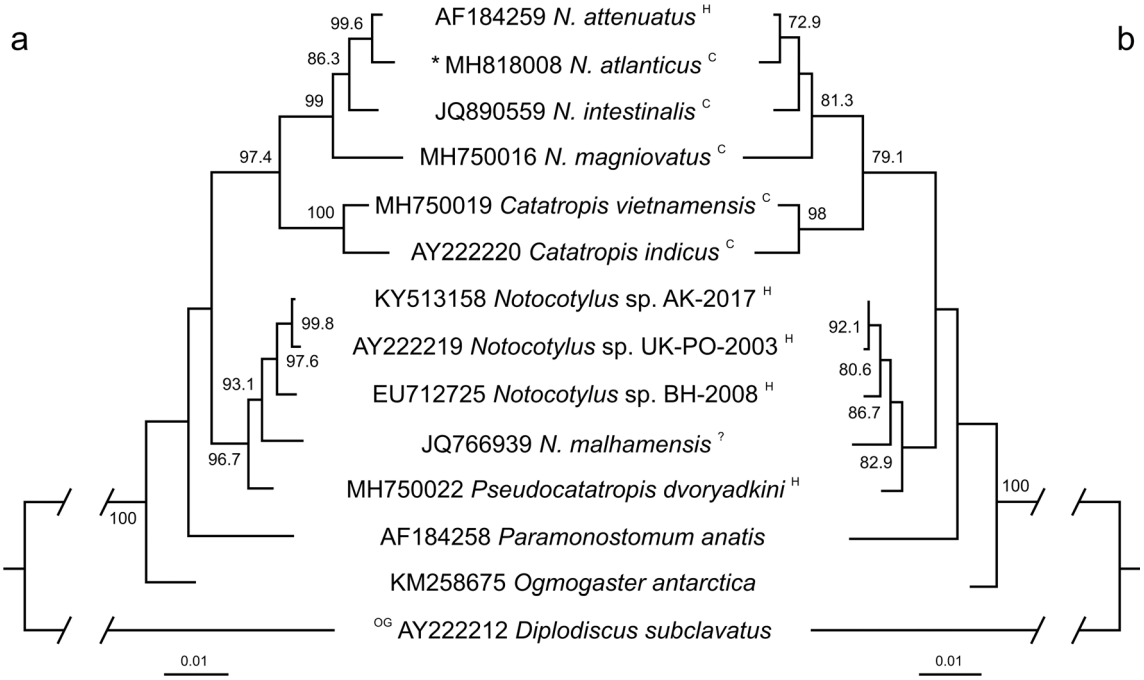

Fig. 6 Phylogenetic position of *N. atlanticus* within Notocotylidae based on D1–D3 LSU sequence data. Asterisk indicates sequence obtained in this study; for GenBank sequences the accession number and published name are provided; [OG] indicates an outgroup; taxonomic affiliation of the first intermediate host (snail) is marked: [C], Caenogastropoda; [H], Heterobranchia; [?], unknown; *scale bar* shows substitutions per site. **a** Bayesian inference, posterior probabilities above 70% are shown. **b** Maximum likelihood method, bootstrap support values above 70% are shown

to one species that likely is *N. atlanticus*. This species has not been reported since its description in 1966, but Yenchingensis cercariae of Notocotylidae were found in hydrobiid snails in Europe (Rothschild 1941; Deblock 1980; Skírnisson et al. 2004; Gonchar and Galaktionov 2016). One of them (*Cercaria Notocotylidae* sp. no. 13 Deblock, 1980), as demonstrated in the "Remarks" section, in fact belongs to *N. atlanticus*. We also provide the first molecular characterization of *N. atlanticus*; this will ensure easier identification in the future and more accurate data on the distribution of this species. In fairness, it must be said that—considering the high morphological similarity of several species of *Notocotylus* (see "Remarks" section)—there remains a chance that North American isolates of *N. atlanticus* are not conspecific to our material from Europe. To ultimately test this, molecular data on samples from the type location in New England are necessary.

The first intermediate host of *N. atlanticus* in North America is *Spurwinkia salsa* (Pilsbry, 1905), and in Europe—*E. ventrosa.* These two species were previously classified as a single genus, *Hydrobia*; now, they belong to different families within one superfamily (Wilke et al. 2013). This is not a unique case among notocotylids: *Paramonostomum alveatum* was discovered in the same two species of mudsnails as *N. atlanticus* (Kulachkova 1954; Stunkard 1967); *N. magniovatus* develops in snails from two families within Cerithioidea (see Table 3). Many other notocotylids were considered specific to one species or genus of snails, but advances in gastropod taxonomy challenge this idea. For example, Filimonova (1985) summarizes ten species of *Lymnaea* as hosts of *N. attenuatus*; now, these species belong to seven different genera. So, host specificity of notocotylids to their first intermediate hosts (or species boundaries) will probably be reassessed when more data are available. Meanwhile, we think that a single species hypothesis for North American and European *N. atlanticus* is preferable. Dispersal of this parasite between Europe and North America may happen as their bird hosts migrate across the ocean, even though this is not a major flyway (Bruun 1971; Newton 2007).

The first intermediate hosts of *N. atlanticus* and *N. attenuatus* are gastropods from two distant clades: Caenogastropoda ("prosobranchs", gill-breathing snails descended from marine ancestors) and Heterobranchia ("pulmonates", lung-breathing snails descended from terrestrial ancestors). This raises questions about close grouping of these two notocotylid species in a phylogenetic tree and their small (four base pairs) divergence. One possible explanation is that GenBank entry AF184259 was assigned to *N. attenuatus* erroneously. This piece of molecular data was obtained as part of a large-scale phylogenetic reconstruction and is not accompanied by any figures or descriptions of a hologenophore (Tkach et al. 2001). In such a study, it was important to be sure of dealing with the genus *Notocotylus* as an adequate representation of the family. Identifying a particular species within this genus might have been done superficially.

Table 3 Morphological features of adult worms, rediae, and cercariae and identity of the first intermediate host may help discriminate between five species of *Notocotylus*

Species	Egg filament length, μm	Metraterm to cirrus sac ratio	Locomotory appendages in rediae	Cercariae	First intermediate host		
					Species	Family, superfamily	High-level clade
N. atlanticus	53–151	Below 1/2	Absent	Yen	*Spurwinkia salsa*, *Ecrobia ventrosa*	Cochliopidae, Hydrobiidae (Rissoidea)	Caenogastropoda
N. intestinalis	NA	Above 1/2	Absent	Yen	*Parafossarulus manchouricus*, *P. spiridonovi*	Bithyniidae (Rissoidea)	Caenogastropoda
N. magniovatus	Up to 500	Below 1/2	Present	Yen	*Semisulcospira* spp., *Parajuga subtegulata*, *Melanoides obliquigranosa*	Semisulcospiridae, Thiaridae (Cerithioidea)	Caenogastropoda
N. imbricatus	185–240	1/4–3/4	Absent	Yen	*Bithynia tentaculata*, *B. inflata*	Bithyniidae (Rissoidea)	Caenogastropoda
N. attenuatus	300–464	2/7–4/5	Absent	Mon	*Lymnaea* spp.	Lymnaeidae	Heterobranchia

Souces of data: N. atlanticus — our study; Stunkard 1966. *N. intestinalis* — Besprozvannykh 2010. *N. magniovatus* — Yamaguti 1938; Dubois 1951; Odening 1964. *N. imbricatus* — Dubois 1951; Skrjabin 1953. *N. attenuatus* — Szidat and Szidat 1935; Pike 1969; Filimonova 1985. *Yen*, Yenchingensis morphotype; *Mon*, Monostomi morphotype

Considering the morphological similarity of several species, misidentification was not unlikely.

Such mistakes have happened in the pre-molecular era. Usually, they are hard to detect and result in taxonomic problems, but some cases are quite clear. For example, Yamaguti (1938), in an experimental life cycle study, recovered what he identified as *N. attenuatus* after an infection of ducks with material from a bythiniid snail (*Bulimus striatulus japonicus*). He himself called this result "almost incomprehensible," considering that lymnaeids and planorbids are known as first intermediate hosts of this parasite in Europe. A similar conflict exists in our phylogenetic tree.

We assume that *N. attenuatus* (AF184259) could in reality be another, innominate, species with another, "prosobranch," first intermediate host. This assumption provides a more consistent interpretation of the phylogenetic tree: two clades of parasites correspond to two groups of gastropods, Caenogastropoda and Heterobranchia, that serve as their first intermediate hosts. *C. indicus*, *C. vietnamensis*, and *P. dvoryadkini* also fit this interpretation. One more specimen KU820968 (not shown on a tree) groups close to the two *Catatropis* species. These sequence data come from a study where more than a thousand snails belonging to 14 species were batch-tested for trematodes. There was no link between the trematode sequence and host identity, but 11 of the 14 snail species in this study were members of the Caenogastropoda (Wongsawad et al. 2016).

Dubois (1951) noticed a similar pattern, distinguishing two "biological groups" within genus *Notocotylus*: the first one confined to "pulmonate gastropods" and the second one—to "prosobranch gastropods." Our findings echo this idea and extend it—adding genera *Catatropis* and *Pseudocatatropis*. The identification and first intermediate hosts of *C. indicus*, *C. vietnamensis*, and *P. dvoryadkini* are reliable (Koch 2002; Olson et al. 2003; Izrailskaia et al. 2019), but these species appear among representatives of another genus, *Notocotylus*, in the tree. To resolve this conflict between phylogeny and taxonomy, we should accept that ventral organs are not a key feature in the genus level classification of Notocotylidae, as previously thought (Barton and Blair 2005[3]).

Conclusion

N. atlanticus is, for the first time, recorded in the Palaearctic in naturally infected first intermediate (*E. ventrosa*) and definitive (anatid bird) hosts. Morphologically, it is similar to several species of *Notocotylus*, and probably it used to be misidentified previously. Genetically, it is close to other species of *Notocotylus* that develop in "prosobranch" snails, the Caenogastropoda.

The identity of the first intermediate host (Caenogastropoda or Heterobranchia) defines two clades on a preliminary phylogenetic tree of Notocotylidae. This finding highlights the potential for further studies on the evolution of Notocotylids and their gastropod hosts.

The character traditionally (and almost solely) used to delineate genera of Notocotylidae—the structure of ventral organs—is not supported by our phylogenetic findings. This

[3] Barton and Blair 2005, p. 391: "10a. Median continuous solid ridge on ventral surface normally flanked on each slide by row of papillae — *Catatropis* Odhner, 1905"; p. 393: "10b. Median and two submedian rows of discrete papillae on ventral surface — *Notocotylus* Diesing, 1839."

indicates that the classification of Notocotylidae may have to change significantly in the future. New studies integrating morphological, life cycle, and molecular data would help to succeed with this responsible task.

Acknowledgements Molecular genetic and light microscopy studies were performed with funding from the Russian Science Foundation grant no. 18-14-00170 by K.G. and A.G. The authors acknowledge Saint Petersburg State University (SPbU) for a grant no. 1.42.963.2016 to A.G. that covered travel expenses during her visit to the University of Reims, France. The life cycle experiments in Iceland were supported by the Research Fund of the University of Iceland. We are grateful to Dr. Anna J. Phillips and Freya E. Goetz (Smithsonian Institution, National Museum of Natural History) for kindly providing a digital image of the type specimen; to ONCFS and Matthieu Kaltenbach for the French birds; and to Dr. Hubert Ferté for providing sample NOT5. The authors thank the Educational and Research Station "Belomorskaia" of SPbU and the White Sea Biological Station of the Zoological Institute of the Russian Academy of Sciences (ZIN RAS) for providing fieldwork resources. Parts of the study were carried out using the equipment of research resource center "Molecular and Cell Technologies" of SPbU and the Laboratory of Molecular Systematics of ZIN RAS. Authors would like to thank two anonymous reviewers for their helpful comments and efforts to improve the language.

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