Life cycle truncation in Digenea, a case study of *Neophasis* spp. (Acanthocolpidae)

Georgii Kremnev a,*, Anna Gonchar a,b, Vladimir Krapivin a, Alexandra Uryadova a, Aleksei Mirolubov a,b, Darya Krupenko a

a Department of Invertebrate Zoology, Saint Petersburg University, Russia
b Laboratory of Parasitic Worms and Protists, Zoological Institute, Russian Academy of Sciences, Russia

**ARTICLE INFO**

**Keywords:**
- Neophasis oculata
- Neophasis anarrhichae
- Life cycle
- Cercariae
- Metacercariae
- Buccinidae

**ABSTRACT**

Truncated life cycles may emerge in digeneans if the second intermediate host is eliminated, and the first intermediate host, the mollusc, takes up its role. To understand the causes of this type of life cycle truncation, we analyzed closely related species of the genus *Neophasis* (Acanthocolpidae) with three-host and two-host life cycles. The life cycle of *Neophasis anarrhichae* involves two hosts: wolffishes of the genus *Anarhichas* as the definitive host and the common whelk *Buccium undatum* as the intermediate host. *Neophasis oculata*, a closely related species with a three-host life cycle, would be a suitable candidate for the comparison, but some previous data on its life cycle seem to be erroneous. In this study, we aimed to redescribe the life cycle of *N. oculata* and to verify the life cycle of *N. anarrhichae* using molecular and morphological methods. Putative life cycle stages of these two species from intermediate hosts were linked with adult worms from definitive hosts using ribosomal molecular data: 18S, ITS1, 5.8S-ITS2, 28S. These markers did not differ within the species and were only slightly different between them. Intra- and interspecific variability was also estimated using mitochondrial COI gene. In the constructed phylogeny *Neophasis* spp. formed a common clade with two other genera of the Acanthocolpidae, *Tormospolus* and *Pleorchis*. We demonstrated that the first intermediate hosts of *N. oculata* were gastropods *Neptunea despecta* and *B. undatum* (Buccinoidea). Shorthorn sculpins *Myxocephalus scorpius* were shown to act as the second intermediate and definitive hosts of *N. oculata*. The previous reconstruction of the two-host life cycle of *N. anarrhichae* was reaffirmed. We suggest that life cycle truncation in *N. anarrhichae* was initiated by an acquisition of continuous morphogenesis in the hermaphroditic generation and supported by a strong prey-predator relationship between *A. lupus* and *B. undatum*.

1. Introduction

The diversity of complex life cycles is the most compelling feature of the digenetic trematodes (Digenea). One of the repeated events in the digenean evolution is life cycle truncation, which results in fewer hosts and fewer transmission events involved (Poulin and Cribb, 2002). Secondary dixenous (two-host) life cycles in Digenea may evolve in two major ways. One is progenesis, the transfer of the sexual reproduction into the second intermediate host. The benefits and costs of progenesis in digeneans and the factors favoring it have been widely discussed and experimentally tested (Lefebvre and Poulin, 2005; Lagrue and Poulin, 2007, 2009; Villa and Lagrue, 2019; etc.). The second way leading to truncated life cycles is the use of the first intermediate host, the mollusc, as a second intermediate one as well. In this case, the stage of free-swimming cercaria larva is omitted, and the definitive host is infected by consuming the only intermediate host. Such life cycles are known in eleven digenean families (Bartoli et al., 2000; Poulin and Cribb, 2002; Pina et al., 2009). The intriguing question is why most of the digeneans did retain a three-host life cycle even though the use of two hosts and the transmission through the food chain seems to work well enough.

An insight into this question might come from studies of closely related digenean species, some of which have the three-host life cycle and some others, the two-host one. An example can be found in the digenean genus *Neophasis* from the family Acanthocolpidae (Reit, 1973; Bray and Gibson, 1991). Life cycles of acanthocolpids usually involve...
three hosts: marine fish as the definitive host, gastropods of the superfamily Buccinoidea as the first intermediate host and fish or bivalves as the second intermediate host (summarized in Kremnev et al., 2020). *Neophasis anarrhichae* (Nicoll, 1909) Bray, 1987 is an exception. It has a two-host life cycle: wolfishes of the genus *Anarhichas* act as the definitive host and the common whelk *Buccinum undatum* Linnaeus, 1758 is the only intermediate host (Lebour, 1910; Polyansky, 1955; Chubrik, 1966; Keie, 1969). To understand the origin of this condition, we need to examine the three-host life cycles of closely related species, e.g. *Neophasis oculata* (Levinsen, 1881) Miller, 1941. However, it has recently been shown that the life cycle stages from intermediate hosts previously assigned to *N. oculata* (Chubrik, 1966) actually belong to representatives of the family Brachycladiidae, whose sexual adults are parasites of marine mammals (Kremnev et al., 2020). Thus, the range of intermediate hosts of *N. oculata* is unknown, and the drivers of the life cycle truncation in *N. anarrhichae* remain obscure.

The first aim of the present study was to elucidate the life cycle of *N. oculata*, to verify the life cycle of *N. anarrhichae* and, based on these data, to propose a hypothesis about the origin of the truncated life cycle in the latter species. Another aim was to describe the genetic distance between *N. oculata* and *N. anarrhichae*. Our third objective was to establish the phylogenetic position of the genus *Neophasis* and thus gain some insights on the evolution within the clade *Acanthopeloidae* + *Brachycladiidae*.

2. Materials and methods

2.1. Sampling

The definitive hosts of *Neophasis anarrhichae* and *N. oculata*—Atlantic wolfish *Anarhichas lupus* Linnaeus, 1758 and shorthorn sculpin *Myoxocephalus scorpius* (Linnaeus, 1758)—were caught during summer–autumn of 2019 and 2020 in the White Sea (Keret Archipelago, Kandalaksha Bay, Russia). In total, we dissected 22 individuals of *A. lupus* and 61 individuals of *M. scorpius*. Metacercariae of *N. oculata* were obtained from 12 specimens of *M. scorpius* during the same period of time from three distant localities in the White Sea: Keret Archipelago, Velikaya Salma Strait (Kandalaksha Bay, Russia) and Bolshoy Solovetsky Island (Onega Bay, Russia).

To obtain intramolluscan stages of *Neophasis* spp. we collected and dissected gastropods of the family Buccinidae: *Neptuna despecta* (Linnaeus, 1758), *Buccinum scalariforme* Moller, 1842 and *B. undatum*. The sampling was conducted in 2018–2020 at three localities in the White Sea: Keret Archipelago, Velikaya Salma Strait and Bolshoy Solovetsky Island. Overall, we dissected 73 *N. despecta*, 21 *B. scalariforme* and 1393 *B. undatum*.

2.2. Histology and whole mounts

For whole mounts sexual adults of *N. oculata* and *N. anarrhichae* and metacercariae of *N. oculata* were either flat-fixed in 96% ethanol under the pressure of cover glass or heat-killed and then fixed in 96% ethanol. Intramolluscan stages of both species were fixed in 96% ethanol, or in Shaudin’s solution (at 60 °C), or in saturated solution of mercury (II) chloride with acetic acid (100:1). To visualize the general structure, we used staining with acidic carmine (Sigma Aldrich, Germany) followed by destaining in 0.1 M HCl in 70% ethanol, or staining with toluidine blue (for identification of mucoid substances). The stained samples were dehydrated in a graded alcohol series and mounted in BioMount medium (Bio Optica, Italy).

Infected snails and metacercariae of *N. oculata* were fixed in Zenker’s solution with 40% formaldehyde (10:1) for histological study. After 2 h fixation and 2 h washing in water, the specimens were incubated in 70% ethanol with iodine for 1 h, then transferred into 70% ethanol, dehydrated in a graded alcohol series and embedded in Histomix™ medium (BioVitrum, Russia). Sections were cut at 5 µm and stained in four different ways: with Ehrlich’s hematoxylin and eosin; with Heidenhain’s iron hematoxylin, followed by picric acid destaining; with Mallory’s trichrome stain; with Azur II-eosin. The latter stain contains toluidine blue, which is used for visualization of mucoid substances.

Photographs were made using a compound microscope Leica DM 2500 (Leica Microsystems, Germany) equipped with a Nikon DS Fi3 camera (Nikon, Japan). Measurements of sexual adults and metacercariae were based on heat-killed worms and conducted in Fiji software (Schindelin et al., 2012). All measurements are in micrometers, the range of values is followed by the mean in parentheses.

2.3. Confocal laser scanning microscopy (CLSM)

Staining with antibodies against acetylated α-tubulin and phospho-tau (phospho Y) along with the application of tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin and DAPI were used for visualization of various organs including nerves, musculature, excretory and reproductive systems. All the protocols were the same as previously described (Kremnev et al., 2020).

2.4. Scanning electron microscopy

For scanning electron microscopy (SEM) study the specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer saline, then rinsed in water, dehydrated in ethanol and acetone, dried in critical point dryer, coated with platinum, and examined with a Quanta 250 SEM at 15 kV.

2.5. Molecular analysis

For molecular analyses, putative and identified life cycle stages of *Neophasis* spp. were fixed in 96% ethanol and stored at 4 °C. Overall, DNA were extracted from 37 isolates (Table 1). For DNA extraction samples were taken from 96% ethanol and dried completely, incubated in 200 µL of 5% Chelex® 100 resin (Bio-Rad, USA) solution with 0.2 mg/mL of proteinase K at 56 °C for 4 h, then kept for 8 min at 90 °C and centrifuged at 16,000 g for 10 min. The supernatant containing DNA was then transferred to a new tube and stored at ~20 °C.

Several primers were used to amplify fragments of ribosomal operon and the mitochondrial COI gene (Table 2). Amplifications were performed in 20 µL reaction mixtures containing 13 µL of Milli-Q® water (Merck Millipore Co., Germany), 4 µL of ScreenMix-HS reaction mix (Evrogen, Russia), 0.5 µL of both F and R primers, and 2 µL of DNA template. PCR products were visualized on a 1% agarose gel stained with ethidium bromide (Helicon, Moscow), and sequenced with PCR primers on an ABI Prism 3500xl genetic analyzer (Applied Biosystems, MA, USA). Sequence data were processed and analyzed using Geneious® 2021.0.2 (https://www.geneious.com). We used the Geneious plugin Repeat Finder 1.0 (Biomatters Ltd.) to detect repeats in the sequences. When the repeat regions hindered the alignment, they were excluded. To estimate the boundaries between all the elements of the ribosomal operon, we used the annotated sequence (KR703279) of *Brachycladium goliath* (van Beneden, 1858) Fraija-Fernández, Aznar, Raga, Gibson & Fernández, 2014, Brachycladiidae (Briscoe et al., 2016). Annotated mitochondrial genome (KR703278) of the same digenean species was used to determine the position of amplified COI fragments. Mean pairwise genetic distance within and between species (as a number of base differences per site) and standard error were calculated in MEGA 7 under the Maximum Composite Likelihood model (Kumar et al., 2016). To assess the quality of the protein-coding nucleotide sequences, we translated them (code 21) to make sure that stop codons were absent and the resulting amino acid sequence corresponded to the expected product. Translated sequences were also used to identify synonymous and non-synonymous substitutions, and to predict the functional effect of the latter in Provean (Choi and Chan, 2015).

Relevant data from GenBank were included into the SSU and LSU alignments (Supplementary Table S1). The best model of nucleotide
Table 1: Isolates of putative and identified Acanthocolpidae from the White Sea, their origin and GenBank accession numbers.

| Isolate number | Stage                                    | Host            | Locality (Russia) | ID            | SSU    | LSU     | ITS1   | 5.8S-ITS2 | COI        |
|----------------|------------------------------------------|-----------------|-------------------|---------------|--------|---------|--------|-----------|------------|
| 1              | Sexual adult                             | Myoxocephalus   | Keret Archipelago | 13.48s        | –      | MW730773| MW750246| MW750294  | MW731655   |
| 2              | Sexual adult                             | Myoxocephalus   | Keret Archipelago | 86.51s.1      | MW730771| MW750247| MW750295| MW731656  |
| 3              | Sexual adult                             | Myoxocephalus   | Keret Archipelago | 86.51s.2      | –       | –       | MW750248| MW750296  | MW731657   |
| 4              | Metacercaria                             | Myoxocephalus   | Keret Archipelago | 56.48s        | –      | MW730775| –       | MW750296  | MW731658   |
| 5              | Metacercaria                             | Myoxocephalus   | Bolshoy Solovetsky Island (Omega Bay) | 386.51s.1 | MW730771| MW750247| MW750295| MW731659  |
| 6              | Metacercaria                             | Myoxocephalus   | Velikaya Salma Strait | 484.51s  | –       | –       | MW750250| MW750299  | MW731660  |
| 7              | Metacercaria                             | Myoxocephalus   | Keret Archipelago | 542.51s       | –       | –       | MW750251| MW750300  | MW731661   |
| 8              | Daughter redia containing embryos and cercariae | Buccinum undatum | Keret Archipelago | 15.45s       | –      | MW730776| MW750252| MW750301  | MW731662   |
| 9              | Daughter redia containing embryos and cercariae | Buccinum undatum | Keret Archipelago | 15.47s       | –       | –       | MW750253| MW750302  | MW731663   |
| 10             | Daughter redia containing embryos and cercariae | Buccinum undatum | Keret Archipelago | 22.47s       | –       | MW730777| –       | MW750303  | MW731664   |
| 11             | Daughter redia containing embryos and cercariae | Buccinum undatum | Keret Archipelago | 291.48s      | –       | MW730778| –       | MW750304  | MW731665   |
| 12             | Daughter redia containing embryos and cercariae | Buccinum undatum | Keret Archipelago | 485.51s      | –       | MW730779| MW750254| MW750305  | MW731666   |
| 13             | Daughter redia containing embryos and cercariae | Neptuna despecta | Keret Archipelago | 172.48s      | –       | MW730780| MW750255| MW750306  | –          |
| 14             | Daughter redia containing embryos and cercariae | Neptuna despecta | Keret Archipelago | 327.48s      | –       | MW730781| MW750256| MW750307  | MW731667   |
| 15             | Daughter redia containing embryos and cercariae | Neptuna despecta | Keret Archipelago | 432.51s      | –       | MW730782| MW750257| MW750308  | MW731668   |
| 16             | Daughter redia containing embryos and cercariae | Neptuna despecta | Keret Archipelago | 534.51s      | –       | –       | MW750258| MW750309  | MW731669   |
| 17             | Daughter redia containing embryos and cercariae | Neptuna despecta | Velikaya Salma Strait | 349.48s | –       | –       | MW750259| MW750310  | –          |
| 18             | Daughter redia containing embryos and cercariae | Neptuna despecta | Velikaya Salma Strait | 352.48s | –       | –       | MW750260| MW750311  | MW731670   |
| Neophasis anarrhicae | Sexual adult                           | Anarhichas lupus | Keret Archipelago | 149.48s      | –       | MW730784| MW750261| MW750312  | –          |
| 20             | Sexual adult                             | Anarhichas lupus | Keret Archipelago | 504.51s.1    | MW730772| MW750285| MW750313| MW731671  |
| 21             | Sexual adult                             | Anarhichas lupus | Keret Archipelago | 504.51s.2    | MW730786| MW750263| MW750314| MW731672  |
| 22             | Sexual adult                             | Anarhichas lupus | Keret Archipelago | 323.51s.1    | MW730787| MW750264| MW750315| MW731673  |
| 23             | Sexual adult                             | Anarhichas lupus | Keret Archipelago | 323.51s.2    | MW730788| MW750265| MW750316| MW731674  |
| 24             | Sexual adult                             | Anarhichas lupus | Keret Archipelago | 146.51s.1    | MW730789| MW750266| MW750317| MW731675  | (continued on next page) |
Table 1 (continued)

| Isolate number | Stage | Host          | Locality ID | SSU     | LSU     | ITS1    | 5.8S-ITS2 | COI               |
|----------------|-------|---------------|-------------|---------|---------|---------|-----------|-------------------|
| 25             | Sexual adult | *Anarhichas lupus* | Keret Archipelago (Kandalaksha Bay, Russia) | 146.51s.2 | –       | –       | –         | MW750267 MW750318 MW731676 |
| **Putative N. anarrhicae** |       |               |             |         |         |         |           |                   |
| 26             | Daughter rediae containing embryos and cercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 319.51s.1 | –       | –       | –         | MW750268 MW750319 MW731677 |
| 27             | Daughter rediae containing cercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 259.48s.1 | –       | –       | –         | MW750269 MW750320 MW731678 |
| 28             | Daughter rediae containing cercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 47.51s.1  | –       | –       | –         | MW750270 MW750321 MW731679 |
| 29             | Daughter rediae containing cercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 240.48s.1 | –       | –       | –         | MW750271 MW750322 MW731680 |
| 30             | Daughter rediae containing cercariae and metacercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 279.51s.1 | –       | –       | –         | MW750272 MW750323 MW731681 |
| 31             | Daughter rediae containing metacercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 491.51s.1 | –       | –       | –         | MW750273 MW750324 MW731682 |
| 32             | Daughter rediae containing metacercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 127.48s.1 | –       | –       | –         | MW750277 MW750325 MW731683 |
| 33             | Daughter rediae containing metacercariae | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 500.51s.1 | –       | –       | –         | MW750275 MW750326 MW731684 |
| **Putative Acanthocolpidae** |       |               |             |         |         |         |           |                   |
| 34             | Mother sporocyst | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 444.51s.1 | –       | –       | –         | MW750276 MW750327 MW731685 |
| 35             | Mother redia | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 445.51s.1 | –       | –       | –         | MW750277 MW750328 MW731686 |
| 36             | Mother redia | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 282.51s.1 | –       | –       | –         | MW750278 MW750329 MW731687 |
| 37             | Mother redia | *Buccinum undatum* | Keret Archipelago (Kandalaksha Bay, Russia) | 292.51s.1 | –       | –       | –         | MW750279 MW750330 MW731688 |

Table 2

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

| Product         | Primer  | Sequence (5'-3'), forward (F) and reverse (R) | Thermocycling profile | Reference |
|-----------------|---------|---------------------------------------------|------------------------|-----------|
| 18S rDNA        | 18S1A   | F, GGCGGACAGAAAAGCTTAAAGCAATCGCA            | 94 °C — 1m             | Hernández-Mena et al. (2017) (primers and conditions) |
|                 | 32      | R, CGAAGGCCTATCTCAATATTC                    | 52 °C — 1m             |           |
|                 | 652     | F, GCAGCCGCTGAATTCGGGTCTGTC                | 72 °C — 1m             |           |
|                 | 28      | R, AGCGAGGGCCGGTGGTCTG                     | × 35                   |           |
| 28S rDNA        | dig12   | F, AAGCATTACATCAAGGGG                      | 95 °C - 30s            | Tkach et al. (1999) (primers and conditions) |
|                 | 1500R   | R, GCTATCTGAGGGAAACCTTG                    | 54 °C - 30s            | Olson et al. (2003) (primers and conditions) |
|                 |         |                                            | 72 °C — 2m             |           |
|                 |         |                                            | × 40                   |           |
| ITS1            | BD1     | F, GTGTTAAAAAATGTTGCTGTA                   | 95 °C - 30s            | Luton et al. (1992) (primers and conditions) |
|                 | 4S      | R, TCTAGATGGTGAATCGTGATG                   | 55 °C - 30s            |           |
|                 |         |                                            | 72 °C — 1m             |           |
|                 |         |                                            | × 35                   |           |
| 5.8S-ITS2       | 38      | F, GTACGGTGGGCTACGCTGGATATGG               | 94 °C - 30s            | Morgan and Blair (1995) (primers) |
|                 | ITS2.2  | R, CCTATGCATTCTGCTGCTGCG                   | 55 °C - 30s            |           |
|                 |         |                                            | 72 °C — 1m             |           |
|                 |         |                                            | × 30                   |           |
| COI             | JB3     | F, TTTTTTGGGCACTCTGAGTTTAT                 | 95 °C - 30s            | Bowles et al. (1993) (primers and conditions) |
|                 | JB4.5   | R, TAAAGAAAGAATGTTGTTA                     | 50 °C - 30s            |           |
|                 |         |                                            | 72 °C — 1m             |           |
|                 |         |                                            | × 35                   |           |
| COI             | JB3     | F, TTTTTTGGGCACTCTGAGTTTAT                 | 95 °C - 30s            | Leung et al. (2009) (primers and conditions) |
|                 | trem.cox1.rnt | R, ATCATGATGCAAAGTTGTA               | 48 °C - 40s            |           |
|                 |         |                                            | 72 °C — 1m             |           |
|                 |         |                                            | × 40                   |           |
substitution was estimated as GTR + G + I using jModelTest (Darriba et al., 2012) at the CIPRES Science Gateway (https://www.phylo.org). The maximum likelihood (ML) analysis was conducted using RAxML (Stamatakis, 2014) at the CIPRES Science Gateway. The stability of clades was assessed using non-parametric bootstrapping with 1000 pseudoreplicates. The Bayesian analysis was conducted using MrBayes v.3.2.6 (Ronquist et al., 2012) with 10,000,000 generations.

3. Results

3.1. Morphological identification of the Neophasis spp. life cycle stages

We found sexual adults of the Acanthocolpidae, which could easily be recognized as Neophasis oculata and N. anarrhicae in Myoxocephalus scorpius and Anarhicas lupus, respectively. We identified the acanthocolpid metacercariae from M. scorpius as N. oculata since this is the only known acanthocolpid species in the White Sea which encysts in fish (Shulman and Shulman-Albova, 1953). Many cases of acanthocolpid infection were found in Buccinum undatum and several, in Neptunea despecta; no specimens of B. scalariforme were infected. Intramolluscan stages of Acanthocolpidae from B. undatum predominantly corresponded to the previously described life cycle stages of N. anarrhicae (Lebour, 1910; Chubrik, 1966; Ksie, 1969). However, in several infected specimens of B. undatum and all infected specimens of N. despecta we found acanthocolpid cercariae with much more prominent eyespots. We suggested that these might belong to N. oculata. Finally, we discovered a mother sporocyst and mother rediae in several B. undatum specimens and supposed that they might belong to the Acanthocolpidae.

3.2. Molecular genetic data and life cycles elucidation

To check our morphological identification and link life cycle stages from intermediate hosts and sexual adults from definitive hosts, we extracted DNA from 37 isolates (Table 1). We sequenced fragments of the ribosomal operon and partial mitochondrial COI gene from this material and submitted these data to GenBank (accession numbers are given in Table 1).

For all the 37 samples, we sequenced a 493 b.p. fragment containing partial 5.8S rDNA, complete ITS2 and the start of the 28S rDNA. The only detected variation was in position 99 (5.8S rDNA). It clearly differentiated the sexual adults of N. oculata (A) and N. anarrhicae (T). A difference between these species was also found in the 18S rDNA sequences. They were obtained for one sexual adult of each species, N. oculata (1707 b.p.) and N. anarrhicae (1636 b.p.), and differed by two
nucleotide substitutions. Neophasis oculata and N. anarrhichae also diverged in the ITS1 region. Out of the 34 ITS1 sequences, 15 were 925 b.p. long and 19 were 831 b.p. long. Their 5′-part contained 47 b.p.-long repeats. The difference in the product length by 94 b.p. was due to the repeat number variation: nine in N. oculata and seven in N. anarrhichae. In addition, these species differed by eight nucleotide substitutions in the ITS1, five within the repeat region and three outside it.

All the genetic differences in the ribosomal operon mentioned above were consistent and made it possible to outline two groups of samples. The first one comprised all the sexual adults of N. anarrhichae (7), putative daughter rediae of N. anarrhichae (8), a mother sporocyst (1) and mother rediae (3). The second group comprised all the sexual adults of N. oculata (3), putative metacercariae of N. oculata (4) and putative daughter rediae of N. oculata (11). No genetic difference was detected within each group. One more ribosomal marker, the partial 28S rDNA (1263–1270 b.p. long), was sequenced for 19 isolates, but did not show any variation. Overall, we outlined subtle but consistent genetic distinctions in rDNA between N. oculata and N. anarrhichae and linked putative life cycle stages from the intermediate hosts and sexual adults from the definitive hosts of both species (Fig. 1).

Aiming to amplify the mitochondrial COI gene with JB3/JB4.5 primers, we obtained eighteen 387–406 b.p. long sequences of identified N. anarrhichae. These sequences, which appeared to be identical, had a frameshift inside caused by two deletions and thus could not be translated into a functional protein. These were interpreted as a possible nuclear copy of a mitochondrial gene (numt). We submitted one such sequence to GenBank under accession number MW740397 and did not analyze it further. The primer pair JB3/trem.cox1.rrn1 yielded sequences that were 770 b.p. long after trimming in both species, containing no stop codons and were translated into a COI protein. These sequences were used in the alignments.

In N. oculata, the alignment of 16 COI sequences had eight polymorphic sites, four of which were singletons. Mean intraspecific pairwise distance was 0.0030 ± 0.0013 substitutions per site. Seven nucleotide substitutions were synonymous; two amino acid substitutions in positions 217 (Ile → Thr) and 248 (Phe → Leu) were predicted to be neutral. In N. anarrhichae, the alignment of 18 COI sequences had 12 polymorphic sites, three of which were singletons. Mean intraspecific pairwise distance was 0.0040 ± 0.0016 substitutions per site. Eight nucleotide substitutions were synonymous; four amino acid substitutions in positions 107 (Met → Thr), 172 (Ile → Val), 218 (Val → Ala), 248 (Leu → Phe) were predicted to be neutral. The mean interspecific divergence was 0.1573 ± 0.0887 substitutions per site.

3.3. Infection data and morphological descriptions

3.3.1. Intramolluscan stages of Neophasis oculata (Fig. 2)

Localities: White Sea; Keret Archipelago, Velikaya Salma Strait (Kandalaksha Bay).

Hosts: Buccinum undatum, Neptunea despecta.

Sites: Reproductive and digestive gland.

Prevalence: 0.4% (5 of 1376) B. undatum in Keret Archipelago; 7% (5 of 68) N. despecta in Keret Archipelago; 66% (2 of 3) N. despecta in Velikaya Salma Strait.

Vouchers: Isogenophores (NO006–NO011) corresponding to isolates No 9, 10 and 13 deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description:

Daughter rediae (Fig. 2A).

[Measurements based on 20 specimens fixed with saturated solution of mercury (II) chloride with acetic acid: 10 specimens from B. undatum and 10 specimens from N. despecta.]

Rediae elongated, sausage-shaped, 828–1964 (1345) × 133–251 (181). Posterior end usually pointed. Mouth terminal, pharynx small, oval, 35–51 (44) × 18–48 (35). Cecum oval, short, 33–111 (65) × 24–46 (32). Brood cavity occupying almost all inner space of rediae, containing cercarial embryos at different stages of development. Birth pore with distinct birth canal posterior to pharynx. Germinal mass at posterior body end, embedded in parenchyma.

Cercariae (Fig. 2B and C).

[Measurements based on 20 specimens fixed with saturated solution of mercury (II) chloride with acetic acid.]

Distome larvae with pair of pigmented eyespots and simple tail (Fig. 2C). Body 245–504 (356) long, 90–139 (116) wide, bottle-shaped, with narrowed anterior and dilated posterior ends (Fig. 2B; 3A, F).
Spines small, simple; size and density decreasing towards posterior end (Fig. 3F). Tail simple, 260–826 (524) long, 33–52 (46) × 34–48 (40). Ventral sucker spherical, 33–50 (42) × 35–47 (40), near center of body, slightly closer to posterior end (Fig. 2B; 3A, F). Sucker-ratio 1:0.67–1.23 (0.93). Prepharynx long (Fig. 2B). Pharynx pyriform to oval (Figs. 2B and 3A, B, H, I), 21–26 (24) × 11–18 (14). Oesophagus primordium short (Fig. 2B; 3A, H). Ceca primordium as rows of cells, extending to posterior body end, lateral to excretory vesicle (Fig. 2B; 3A, H). Cerebral ganglion dorsal to prepharynx (Fig. 2B; 3A, B, G). Two pigmented eye-spots, large, oval, lateral to oesophagus (Fig. 2B; 3A-E, H, I). Phospho Y positive structure (unpaired unpigmented eyespot) anteromedial to pigmented eyespots (Fig. 2B; 3I); also staining with TRITC-labeled phalloidin (Fig. 3I). Thirteen small unicellular penetration glands in two groups (Fig. 2B; 3A). Anterior group of six glands in forebody, lateral to pharynx (Fig. 2B; 3A); ducts running medially to pigmented eyespots towards anterior body end (Fig. 2B; 3B). Posterior group of seven glands lateral and posterior to ventral sucker (Fig. 2B; 3A); only five ducts present, running lateral to anterior penetration glands and pigmented eyespots towards anterior body end (Figs. 2B and 3B). Each duct opening through individual pore near anterior edge of oral sucker, 11 pores in total (Fig. 2B). Cystogenous glands not detected. Excretory vesicle in hindbody, I-shaped, voluminous, slightly asymmetrical (Fig. 2B; 3A, C); sometimes appearing Y-shaped, due to dilated proximal parts of main collecting ducts. Excretory vesicle wall thick, staining slightly with eosin (Fig. 3C). Excretory system of “Mesostoma” type (Fig. 2B). Main collecting ducts dividing into anterior and posterior parts near anterior edge of ventral sucker. Excretory formula 2[(4 + 4 + 4) + (4 + 4 + 4)] = 48. Caudal excretory duct present in underdeveloped cercariae and absent in infective cercariae (Fig. 2B; 3H). Small testes primordia in hindbody, slightly oblique; posterior testis slightly larger than anterior one (Fig. 2B). Vasa efferentia visible (Fig. 2B). Cirrus-sac primordium as C-shaped group of cells (Fig. 2B). Primordium of female reproductive system crooked, compact (including metraterm, uterus, ootype, Laurer’s canal, oviduct and ovary); from its dorsal part vitellarium primordium passing as two strands of cells laterally, then dividing into short anterior and long posterior part (Fig. 2B).

**Developing cercariae and mucoid structures** (Fig. 3A, D, E).

Cercariae leave rediae underdeveloped, and morphogenesis is completed in the digestive gland of the host. Development of pigmented eyespots begins soon after formation of the tail bud (Fig. 2A). Mucoid glands as described in Xiphidiata and Opisthorchiata are absent. Mucoid substances were found in cytons at the dorsal side of the body, and in the tail of cercaria embryos (Fig. 3D and E). Development of mucoid cytons begins before the larvae leave the rediae (Fig. 3E). During the final stages of cercarial development outside rediae, the mucoid substance is

---

**Fig. 3. Neophasis oculata cercariae.** (A) Infective cercaria, general structure and mucoid in the tegument (toluidine blue), differential interference contrast (DIC). (B) Ducts of the penetration glands in live cercaria. (C) Sagittal histological section of infective cercaria, Erlich’s hematoxylin-eosin. (D–E) Mucoid in underdeveloped cercariae (toluidine blue, whole mount, DIC (D) and Azur II-eosin, histological section (E)). (F) SEM, ventral view. (G–I) CLSM, TRITC-phalloidin, acetylated α-tubulin and phospho Y antibody staining. (G) Infective cercaria, flame cells and nerves. (H–I) Underdeveloped cercariae, excretory ducts (H) and eyespots (I). Scale bars = 50 μm. Abbreviations: ac – anterior collecting duct; cd – caudal excretory duct; ev – excretory vesicle; fc – flame cells; ga – cerebral ganglion; mc – mucoid cytons; os – oral sucker; pe – posterior collecting duct; pg – pigmented eyespots; ph – pharynx; t – tail; ue – unpigmented eyespot; vnc – ventral nerve chords; vs – ventral sucker. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
transferred into the tegument which becomes swollen (Fig. 3A).

Remarks:
In early infections daughter rediae are located only in the reproductive gland while at more advanced stages of infection they move into the digestive gland. We observed numerous infective cercariae only in the two specimens of *B. undatum* captured in April 2018. All the other infected specimens of *B. undatum* and *N. despecta* contained mostly underdeveloped larvae, with few infective cercariae among them.

3.3.2. Metacercariae of *Neophasis oculata* (Fig. 4)

Locality: White Sea; Keret Archipelago, Velikaya Salma Strait (Kandalaksha Bay); Bolshoy Solovetsky Island (Onega Bay).

Host: Shorthorn sculpin *Myoxocephalus scorpius*.

![Fig. 4](image_url)

Fig. 4. *Neophasis oculata* metacercariae, inside cyst (A) and removed from cyst (B).

Other reported hosts: 11 fish species (summarized in Bray and Gibson, 1991).

Sites: Fins, muscles.

Vouchers: Paragenophores (NO004, NO005) corresponding to isolates No 5 and 7 deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description: Measurements in Table 3.

Metacercariae enclosed in round to oval cyst (Figs. 4A), 362–511 (431) × 316–412 (368) (measurements based on 10 ethanol-fixed specimens). Cyst two-layered (Fig. 5A), outer layer opaque and soft, inner layer transparent and elastic. Metacercariae extracted from cyst oval, elongated, with wider hindbody and narrowed ends (Figs. 4B and 5A). Spines in forebody with serrated tips, in hindbody simple, pointed (Fig. 5D–F); size and density decreasing towards posterior end. Oral sucker subterminal, round to oval (Fig. 4B; 5A, D). Ventral sucker round, closer to anterior end (Fig. 4B; 5A, D). Prepharynx long (Fig. 4B; 5A). Pharynx oval (Fig. 4B; 5A). Oesophagus short (Fig. 4B; 5A). Ceca wide, extending to posterior body end, terminating blindly laterally to excretory vesicle; lumen formed (Fig. 4B; 5A). Uroproct absent. Cerebral ganglion dorsal to prepharynx (Fig. 4B). Pigmented eyespots dis-integrated and pigment dispersed (Fig. 4B; 5A). Unpigmented eyespot absent. Excretory vesicle egg-shaped, voluminous (Figs. 4B and 5A), thin-walled (Figs. 4B and 5A, C), filled with opaque globules (Fig. 4A), extending to level of ovary (4B; 5A). Excretory pore terminal (Fig. 4A). Excretory system of “Mesostoma” type. Excretory formula not determined. Reproductive system underdeveloped, primordia of all organs clearly distinguishable (Fig. 4B). Testes large, round, oblique, in hindbody, posterior testis larger than anterior one (Fig. 4B; 5A). *Vasa efferentia* visible (Fig. 4A). Cirrus-sac long, C-shaped, dextral, reaching into hindbody (Fig. 4B; 5A). Developing bipartite seminal vesicle, *pars prostatica* and ejaculatory duct discernible within cirrus-sac (Fig. 4B; 5C). Gametogenesis in testes at initial stages (Fig. 5C). Genital atrium small. Genital pore median, anterior to ventral sucker (Fig. 4B). Ovary round, dextral, closer to ventral side (Fig. 4B; 5A). Oviduct starting at dorsal side of ovary (Fig. 4B; 5A). Laurer’s canal short (Fig. 4B). Ootype prominent, dorso-medial, anterior to excretory vesicle (Fig. 4B; 5A). Uterus short, coiled, ventral to ootype (Fig. 4B). Metraterm sinistral, dorsal to ventral sucker (Fig. 4B). Primordium of vitellarium H-shaped, its middle part close to ootype, longitudinal parts with short lateral claviform outgrowths (developing vitelline follicles) (Fig. 4B; 5C). Lumen in ducts of female reproductive system absent, except in metraterm (Fig. 4B; 5C). Gametogenesis in ovary not started.

Remarks:
Metacercariae of *N. oculata* were found in *M. scorpius* from the White Sea throughout the observation period. We do not provide the data on prevalence and intensity of metacercariae infection since the hosts were examined only to collect material for molecular and morphological studies.

3.3.3. Sexual adults of *Neophasis oculata* (Fig. 5G)

Locality: White Sea; Keret Archipelago (Kandalaksha Bay).

Host: Shorthorn sculpin *Myoxocephalus scorpius*.

Other reported hosts: 40 fish species (summarized in Bray and Gibson, 1991).

Sites: Pyloric ceca, intestine.

Prevalence: 44% (27 of 61).

Intensity (range of values, mean and standard error of the mean): 1–183 (12.9 ± 8.70).

Vouchers: Hologenophore VG5.2 (isolate 2) and paragenophores (NO002, NO003) corresponding to isolates No 2 and 3 deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description: Measurements in Table 3.

Remarks: We found sexual adults of *N. oculata* in *M. scorpius* in the White Sea throughout the observation period. Ovigerous specimens were detected mostly in July. The most common infection site was...
Dimensions and taxonomic characters of the White Sea Neophasis spp.

| Character                  | Neophasis oculata (sexual adults) | Neophasis oculata (metacercariae, n = 10) | Neophasis anarrhichae (sexual adults) |
|----------------------------|-----------------------------------|------------------------------------------|--------------------------------------|
| Length                     | 795-1135 (922)                    | 631-828 (728)                            | 431-653 (558)                        |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| Width                      | 233-326 (272)                     | 175-232 (204)                            | 153-216 (188)                        |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| Forebody, length           | 283-444 (373)                     | 218-332 (284)                            | 149-257 (201)                        |
| (n = 11)                   |                                   | (n = 16)                                 | (n = 16)                             |
| Forebody, % of body length | 26-45 (41)                        | 32-44 (39)                               | 32-39 (36)                           |
| (n = 11)                   |                                   | (n = 16)                                 | (n = 16)                             |
| Oral sucker                | 83-99 × 85-97                     | 57-77 × 62-84 (66 × (91 × 91)            | 73 (83 × 89)                         |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| Ventrail sucker            | 87-110 × 83-113 (98 × 97)         | 68-88 × 55-82 (75 × 69)                  | 73-93 × 73-106 (83 × 94)             |
| (n = 11)                   |                                   | (n = 10)                                 | (n = 16)                             |
| Sucker-ratio               | 1:1.0–1:2.14                      | 1:09–1:24 (1:15)                         | 1:08–1:10                           |
| (n = 10)                   |                                   | (n = 11)                                 | (n = 10)                             |
| Prepharynx                 | 61-207 (125)                      | 59-146 (108)                             | 33-60 (43)                           |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Pharynx                    | 61-87 × 56-79                     | 46-59 × 52-73 (51 × 78 × 72)            | 64-72 × 51-65 (67 × 60)              |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Oesophagus                 | 44-82 (56)                        | 37-56 (49)                               | 15-23 (26)                           |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| IB-VS                      | 19-64 (37)                        | 15-39 (25)                               | 0-24 (10)                            |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Vit-VS                     | 56-118 (85)                       | –                                       | 18-62 (49)                           |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Ovary                      | 62-86 × 48-77                     | 34-54 × 30-48 (41 × 38)                  | 59-74 × 47-62 (66 × 55)              |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 14)                             |
| Testis, anterior           | 102-150 × 66-107                  | 66-107 × 48-96 (84 × 77)                 | 69-104 × 55-84 (85 × 68)             |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Testis, posterior          | 103-158 × 63-142 (136 × 117)      | 69-130 × 53-106 (98 × 85)                | 80-123 × 50-82 (96 × 65)             |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 14)                             |
| Testes overlap, %          | 25-97 (53)                        | 11-79 (43)                               | 0-94 (52)                            |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| Lateral testes overlap, %  | 60-26 (25)                        | 0-18 (4)                                 | 0-37 (22)                            |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| PTR                        | 111-174 (143)                     | 113-163 (137)                            | 58-90 (76)                           |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| PTR, % of body length      | 13-18 (16)                        | 17-22 (19)                               | 10-18 (14)                           |
| (n = 11)                   |                                   | (n = 11)                                 | (n = 16)                             |
| C-PE                       | 25-50 (40)                        | 20-49 (37)                               | 30-77 (49)                           |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Eggs, number               | 1-5 (3)                           | –                                       | 2-6 (4)                              |
| (n = 10)                   |                                   | (n = 10)                                 | (n = 10)                             |
| Egg-size                   | 76-117 × 36-56 (96 × 43)          | –                                       | 64-109 × 32-59 (91 × 43)             |
| (n = 27)                   |                                   | (n = 27)                                 | (n = 27)                             |

IB-VS. Distance from intestinal bifurcation to anterior margin of ventral sucker. Vit-VS. Distance from anterior-most extent of vitelline fields to anterior margin of ventrual sucker.

VS-Ovary. Distance from posterior margin of ventral sucker to anterior margin of ovary.

PTR. Length of post-testicular region.

C-PE. Distance from posterior-most extent of the intestinal caeca to posterior extremity of worm.

In the metacercariae of Neophasis anarrhichae only nine ducts of penetration glands are evident (Fig. 7F). Glandular cells just behind the oral sucker, which were mentioned by Lebour (1910), are in fact a closely packed bunch of ducts. These ducts open at the bottom of the buccal cavity, whereas the glands itself are located laterally to the pharynx. The ceca never reach the posterior body end. Excretory vesicle is egg-shaped (Fig. 7E, I). The metacercariae often retain a short tail, in which the caudal excretory duct is absent. All elements of the reproductive system are clearly distinguishable (Fig. 7E, G, H). There is a lumen in the female and male reproductive ducts (Fig. 7H) and early stages of gametogenesis are commonly observed in the ovary and the testes (Fig. 7G). Formed sperm is found regularly in male gonads. The

pyloric ceca. In heavily infected fish, the worms were also found in the other regions of the digestive tract such as the stomach, the midgut and the hindgut.
metacercariae can start egg production while they are still within the daughter rediae (Fig. 7 I), which was observed in 15 host specimens in 2019–2020.

Remarks: We found a mother sporocyst just once during the observation period. Alongside with it, several mother rediae were present in the kidney of that whelk, while its other organs were free from digenean infection. It seems that the mother rediae are initially located only in the kidney and later move into the digestive and the reproductive gland, where they can be found besides the daughter rediae. The key distinction between them is the content of the brood cavity: mother rediae produce rediae of the next generation whereas daughter rediae produce cercarial embryos.

3.3.5. Sexual adults of Neophasis anarrhichae (Fig. 7 J)

Locality: White Sea; Keret Archipelago (Kandalaksha Bay).
Host: Atlantic wolffish Anarhichas lupus.
Other reported hosts: A. denticulatus, A. minor (summarized in Bray and Gibson, 1991).
Site: Intestine.

Prevalence: 77% (17 of 22).

Vouchers: Paragenophores (NA001-NA003) corresponding to isolates No 20 and 21 deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description: Measurements in Table 3.

Remarks: The number of parasites per fish ranged from less than ten to more than 5000 individuals. We found ovigerous sexual adults of Neophasis anarrhichae in A. lupus throughout the observation period. The most common infection site was the upper midgut. The number of parasites diminished towards the end of the midgut. In heavily infected fish sexual adults were also found in the hindgut, though in lesser numbers than in the midgut. A few immature specimens were sometimes seen in the stomach.

3.4. Phylogenetic position of Neophasis spp

Concatenated SSU and LSU dataset comprised 24 sequences and yielded 2977 characters, including gaps. Phylogenetic trees inferred with ML and Bayesian approaches revealed the same topology. The
Bayesian tree is presented in Fig. 8; ML bootstrap support values are additionally mapped onto it next to the posterior probabilities (PP) values for all the ML-supported nodes.

There are three well-supported major groups on the tree: Brachycladiidae + Acanthocolpidae clade A and Acanthocolpidae clade B. This is in agreement with previous studies (Bray et al., 2005; Curran and Pulis, 2014; Fraija-Fernández et al., 2015; Kremnev et al., 2020). Two Neophasis spp. group together, with high PP and bootstrap support values, within the Acanthocolpidae clade A. This clade also includes Tormopsolus orientalis Yamaguti, 1934 and Pleorchis spp., but the branching order of these three lineages is unresolved.

4. Discussion

In this study we re-elucidated the three-host life cycle of Neophasis oculata, and found that its first intermediate hosts were gastropods Neptuna despecta and Buccinum undatum. We also verified the two-host life-cycle of N. anarrhicaeh. Genetic differences between the two species of Neophasis were shown in ribosomal markers and in mitochondrial COI gene. We provided the first detailed descriptions of N. oculata cercariae and metacercariae, and N. anarrhicaeh mother sporocyst and mother rediae. Previous morphological data on the daughter rediae, cercariae and metacercariae of N. anarrhicaeh were supplemented. The closest relatives of the genus Neophasis were shown to be representatives of Acanthocolpidae clade A, genera Tormopsolus and Pleorchis.

The genus Neophasis comprise six species restricted to temperate and cold seas of the Northern Hemisphere (WoRMS, 2021). Only two of them, N. oculata and N. anarrhicaeh, have been reported from the North-eastern Atlantic and the adjacent Arctic (Bray and Gibson, 1991). The morphological identification of N. oculata from our material is unambiguous, but the specimens of N. anarrhicaeh do not entirely match the morphometric characteristic of this species given by Bray and Gibson (1991), and even resemble another Neophasis species, N. pusilla Stafford, 1904 (Table 3). However, N. pusilla has been recorded only in North-western Atlantic and inhabits not only the intestine but also the urinary and the gall bladder of Anarhichas lupus. Actually, N. pusilla and N. anarrhicaeh might be synonymous, as suggested by Bray and Gibson (1991), but this hypothesis has to be checked by molecular methods.
Genetic differences between *N. oculata* and *N. anarrhichae* are clearly seen in our data on 18S rDNA (two substitutions), ITS1 (eight substitutions), and 5.8S-ITS2 (one substitution). The D1–D3 domains of 28S rDNA showed no interspecific variation, which is, to our knowledge, the first such case in Digenea. In addition to the sequence divergence, the ITS1 region also includes an unequal number of repeats: seven in *N. anarrhichae* and nine in *N. oculata*. As the result, the PCR products differ in length by about 90 bp and this molecular “signature” can be detected on the gel prior to sequencing. In general, the genetic distance between *N. oculata* and *N. anarrhichae* in the fragments of the ribosomal operon is rather small.

The COI fragments demonstrate a degree of variability consistent with that in other digeneans studied in this respect. The gap between the intra- and interspecific distances is clear and allows an unambiguous delimitation of the boundaries between the two species. The detection of what we consider as numt in *N. anarrhichae* is something that the researchers should be aware of when interpreting mitochondrial (mt) DNA sequence data. In contrast to the actual mt COI gene, the numt sequences of *N. anarrhichae* lack intraspecific variability. Had this numt not been mistaken it for mt COI, which would have led to erroneous conclusions. This highlights the importance of testing the quality of mt DNA sequences and to elucidate the life cycle of *N. oculata* and *N. anarrhichae* in the fragments of the ribosomal operon.

The affinity of the genus *Neophasis* to the Acanthocolpidae was predicted by Bray and Gibson (1991) based on the morphology and life cycle data. Here we established the phylogenetic position of this genus by molecular methods for the first time and showed that *Neophasis* spp. formed a branch within the Acanthocolpidae clade A. Based on our and previous 28S rDNA data, the other members of the clade are *Tormopsolus, Pleorchis* and a group of species known only from first intermediate host, *Cercaria capricornia* group 1–3 (Barnett and Miller, 2018; Kremnev et al., 2020). The latter were uninvolved into our analysis as their 18S rDNA sequences are lacking in GenBank®.

Our molecular data made it possible to verify the life cycle of *N. anarrhichae* and to elucidate the life cycle of *N. oculata* (Fig 9). The first hypothesis on the life cycle of *N. oculata* was proposed by Chubrik (1966). She found oculate cercariae in the naticid caenogastropod *Cryptonatica affinis* (Gmelin, 1791) and oculate metacercariae in bivalves *Cerastoderma edule* (Linnaeus, 1758) and *Astarte crenata* (Gray, 1824). Based on the superficial morphological similarities shared by the discovered larvae and sexual adults of *N. oculata*, she claimed that three-host life cycle of this species was elucidated. Recently, we have shown that these life cycle stages belong to the family Brachycladiidae (Kremnev et al., 2020). Here we demonstrate that the first intermediate hosts of *N. oculata* are buccinid gastropods *N. despecta* and *B. undatum* (Neogastropoda: Buccinidae). Cercariae ensure the transmission of infection towards the second intermediate host, *Myxoecephalus scorpius* and other fish species (summarized in Bray and Gibson, 1991). Encysted metacercariae undergo further development but never reach sexual maturity. Definitive fish hosts (mostly members of the Cottidae—see Bray and Gibson, 1991) become infected by feeding on the second intermediate hosts. In the White Sea, we found sexual adults of *N. oculata* only in *M. scorpius*, but it was also reported from *M. quadricornis* (Linnaeus, 1758) (Shulman and Shulman-Albova, 1953). The presence of ovigerous sexual adults mostly in July and infective cercariae mainly in April may indicate the seasonal dynamics in the life cycle.

The only intermediate host of *N. anarrhichae* is the common whelk *B. undatum*. The development of all intramolluscan stages was documented.
in this mollusc. The mother sporocyst was found in the kidney where it produces mother rediae. Koie (1971) observed a mother sporocyst of \textit{N. anarrhichae} once in a serially sectioned \textit{B. undatum}, but she described it as a small spherical object filled with \textasciitilde{}100 rediae and located under the epithelium at the base of the siphon. We think that this mother sporocyst belongs to another digenean species. Mother rediae of \textit{N. anarrhichae} initially accumulate in the kidney but later migrate into the reproductive and the digestive gland, where they begin to produce daughter rediae. As the infection proceeds, daughter rediae can also spread into the kidney, gall, and mantle. Daughter rediae produce cercarial embryos which develop directly into metacercariae without encystment. Metacercariae can achieve sexual maturity and start producing eggs while still within rediae. The definitive host (in the White Sea—only \textit{A. lupus}) becomes infected via ingestion of the whelks with metacercariae.

Early infection stages of \textit{N. oculata} and \textit{N. anarrhichae} in their shared gastropod host, \textit{B. undatum}, may be easily misidentified. To avoid misidentification, one should pay attention to the pigmented eyespots, which are larger in the cercariae of \textit{N. oculata} and appear earlier in the development, and to the mucoid cytons, which are present in \textit{N. oculata} and absent in \textit{N. anarrhichae}. Reexamination of whole mounts stored at the Department of Invertebrate Zoology of Saint Petersburg University (Russia) showed that the cercariae of \textit{N. anarrhichae} described by 

![Diagram A](image1)

![Diagram B](image2)

**Fig. 9.** Proposed life cycle scheme of \textit{Neophasis oculata} (A) and \textit{N. anarrhichae} (B).

The studied cercariae of \textit{N. oculata} and the cercariae from the closely related Brachycladiidae share several common morphological features: long ceca primordia as rows of cells; presence of three eyespots (two pigmented and one unpigmented); thick-walled I-shaped excretory vesicle; excretory system of the “Mesostoma” type (Kremnev et al., 2020). The cercariae of \textit{N. oculata} and those of brachycladiids can be told apart by the number and arrangement of the penetration glands and the excretory formula, as well as the absence of mucoid glands in the former.

Within the Acanthocotilidae clade A putative cercariae of \textit{Tormospolus} and \textit{Cercaria capricornia} group 1–3 have been described (Bartoli and Gibson, 1998; Barnett et al., 2008). They all have an expanded hindbody, \textit{Cercaria capricornia} group 2 and 3 also possess lateral outgrowths, presumably to attract attention of microphagous fish, which possibly serve as the second intermediate hosts. In contrast, the cercariae of \textit{N. oculata} have a modest appearance, which is probably plesiomorphic for the Acanthocotilidae clade A. Evolutionary changes in the cercarial morphology within the clade may reflect the transition from benthic to planktonic fish as the second intermediate host.

Though the cercariae of \textit{N. anarrhichae} do not leave the molluscan host, they retain most of the provisional organs such as penetration glands, eyespots, tail. This supports the idea that \textit{N. anarrhichae} evolved from a species with free-swimming cercariae (Koie, 1973; Bray and Gibson, 1991). However, smaller eyespots and the absence of mucoid cytons in \textit{N. anarrhichae} betrays a loss of adaptations to the transmission of infection from the first to the second intermediate host. Successive development of the digestive and the excretory systems in \textit{N. anarrhichae} and \textit{N. oculata} is also generally similar although ceca in \textit{N. anarrhichae} never reach the posterior region of the body. Nevertheless, unlike \textit{N. oculata}, \textit{N. anarrhichae} continue to grow and mature in the intermediate host: cercariae develop without encystment into metacercariae, infective for the definitive host. The morphogenesis of \textit{N. anarrhichae} may proceed even further: its metacercariae can become sexually mature and start egg production while still in the intermediate host. Thus, its life cycle may become a facultative one-host life cycle, as we described and discussed before (Krupenko et al., 2019).

In our opinion, the transition to continuous morphogenesis of the hermaphroditic generation was crucial for the life cycle truncation within the genus \textit{Neophasis}. In typical three-host digenean life cycles cercariae cannot infect the definitive host, and a second intermediate host is essential for additional morphogenetic changes of the larvae. However, when the cercariae acquire the ability to develop into metacercariae within the mollusc, the second intermediate host becomes superfluous, and its role is taken up by the first intermediate one.

The switch of \textit{N. anarrhichae} to \textit{Anarrhichas} spp. as new definitive hosts was probably due to the diet of these fish, which feed on hard-shelled invertebrates such as molluscs, echinoderms and crustaceans (Bray, 1987; Falk-Petersen et al., 2010). It is noteworthy that only \textit{Buccinum} is the intermediate host of \textit{N. anarrhichae} while another whelk, \textit{Neptunea}, is not. The reason may be associated with a higher accessibility of \textit{Buccinum} to wolfish due to their higher abundance, smaller size and thinner shell.

Secondary dixenous life cycles, with the historical second intermediate host being eliminated and the first intermediate host, the mollusc, taking up its role, are known in eleven digenean families: some species of Microphallidae, Monorchidiidae, Lissorhidiidae, Felodistomidae, Zoonogidae, Gymnothallidae, Gorgoderiidae, and all members of Cyclocoelidae, Eucotylidiidae, Leucoclerididae and Hasistesidae studied in this respect (Bartoli et al., 2000; Poulin and Cribb, 2002; Pina et al., 2009). Secondary dixenous life cycles in the latter four families seem to be associated with life in the terrestrial environment. Evolutionary expansion of the two-host life cycles in the Microphallidae is related to migratory coastal birds in high latitudes (Galaktionov, 2017; Galaktionov and Blasco-Costa, 2018; Galaktionov et al., 2012). \textit{Gymnothallus choledochus} Odhner, 1900 switches from the three-host to the
two-host life cycle in winter (Loos-Frank, 1969). Unlike all these cases, the life cycle truncation in *N. anarrhichae* is apparently not associated with existence under stressful conditions. The seasonal changes are present in the subtidal of the White Sea but are not pronounced in most other parts of *N. anarrhicae* distribution (the North-eastern Atlantic and the southern Barents Sea).

The origin of the two-host life cycle in *N. anarrhicae* seems to have been supported by a strong prey-predator relationship between *A. lupus* and *B. undatum*. Feeding on hard-shelled invertebrates (durophagy) is thought to entail considerable fitness costs, according to the optimal foraging theory (MacArthur and Pianka, 1966; Pyke et al., 1977). Though even non-specialist animals may consume hard-shelled prey under certain conditions (Langerhans et al., 2020), vertebrates generally seem to prefer feeding on arthropods, annelids and other vertebrates rather than gastropods. This may be one of the restrictions standing in the way of life-cycle truncation in digeneans.

**Declaration of competing interest**

None.

**Acknowledgements**

This study was supported by the Russian Science Foundation (Russia), grant no. 19-74-10029. The fieldwork was carried out at the Educational and Research Station “Belomorskaia Zelenskaia” of St. Petersburg University (SPBU), Russia, the White Sea Biological Station (WSBS) “Kartesh” of the Zoological Institute of the Russian Academy of Sciences (IZ RAS), Russia, and the N.A. Pertsov White Sea Biological Station of Moscow State University, Russia. The work at the WSBS “Kartesh” was supported by a research program of ZIN RAS (project no. AAAA-A19–119020690109-2). Our sincerest thanks for the help with the sampling are due to Sergei Bagrov, Stanislav Ilyutkin, Olga Knyazeva, Anastasiia Nikitenko, Anna Mikhlina and Irina Ekimova. The authors are particularly grateful to Drs. Anna Romanovich and Aleksey Masharsky for excellent sequencing support. We thank two anonymous reviewers for their helpful comments on the initial version of the manuscript and Natalia Lentsman for linguistic assistance. During research we utilized the equipment of the resource centers “Molecular and Cell Technologies” and “Center for Microscopy and Microanalysis” of the Research park of SPBU and “Taxon” Research Resource Center (http://www.kcp-rf.ru/kcp/3038/) of ZIN RAS.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpaw.2021.05.001.

**References**

Barnett, L.J., Smales, L.R., Cribb, T.H., 2008. A complex of putative acanthocolpid cercariae (Digenaea) from *Nassarius olivaceus* and *N. dorsatus* (Gastropoda: Nassariidae) in central Queensland, Australia. Zootaxa 1705 (1), 21–28.

Chubrik, G.K., 1966. Fauna and ecology of trematode larvae from molluscs in the Barents and White Seas. Tr. Murn. Morsk. Biol. Inst. 10 (14), 78–166 (in Russian).

Curren, S.S., Pulis, K.E., 2014. Confirmation of *Pseudolipophyllum* ballisti in the *Acanthocolpidae* (Digenaea) based on phylogenetic analysis of ribosomal DNA. J. Parasitol. 100 (6), 856–859.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. *JModelTest* 2: more models, new heuristics and parallel computing. Nat. Methods 9 (8), 772.

Fajn-Jernandez, N., Olson, P.D., Crespo, E.A., Raga, J.A., Armar, F.J., Fernandez, M., 2015. Independent host switching events by digenetic parasites of ceteceans inferred from ribosomal DNA. Int. J. Parasitol. 45, 167–173.

Galaktionov, K.V., 2017. Patterns and processes influencing helminth parasites of Arctic coastal communities during climate change. J. Helminthol. 91 (4), 387.

Galaktionov, K.V., Blasco-Costa, I., Olson, P.D., 2012. Life cycles, molecular phylogeny and historical biogeography of the *pygmaeus* microphallids (Digenaea: Microphallidae): widespread parasites of marine and coastal birds in the Holarctic. Parasitology 139 (10), 1346.

Galaktionov, K.V., Blasco-Costa, I., 2018. *Microphallus ochotensis* sp. nov. (Digenaea: Microphallidae) and relative merits of two-host microphallid life cycles. Parasitol. Res. 117 (4), 1051–1066.

Hernández-Mena, D.I., García-Varela, M., de León, G.P.P., 2017. Filling the gaps in the classification of the *Digenaea Carus*, 1863: systematic position of the *Proterodiplostomidae* Dubois, 1936 within the superfamily Diplostomotoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences. Syst. Parasitol. 94 (8), 833–848.

Koie, M., 1969. On the endoparasites of *Buccinum undatum* L with special reference to the trematodes. Ophelia 6 (1), 251–279.

Koie, M., 1971. On the histochemistry and ultrastructure of the redia of *Neophasis lagomena* (Lebour, 1910) (Trematoda, *Acanthocolpidae*). Ophelia 9 (1), 113–143.

Koie, M., 1973. The host-parasite interface and associated structures of the cercaria and adult *Neophasis lagomena* (Lebour, 1910). Ophelia 12 (1–2), 205–219.

Kremenov, G., Gonchar, A., Krapivin, V., Krayenzova, O., Krupenkov, D., 2020. First elucidation of the life cycle in the family Brachycladiidae (Digenaea), parasites of marine mammals. Int. J. Parasitol. 50 (12), 997–1009.

Kremenov, G., Krapivin, G., Krapivin, V., 2019. Possible progenesis in *Neophasis anarrhicae* (Nicolli, 1909) Bray, 1987 in the White Sea Parasites. Int. J. Parasitol. 70, 82–85.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874.

Lagrue, C., Poulin, R., 2007. Life cycle abbreviation in the trematode *Coicusmum pygmaeus* can parasites adjust to variable conditions? J. Parasitol. 93 (3), 1189–1195.

Lagrue, C., Poulin, R., 2009. Life cycle abbreviation in trematode parasites and the developmental time hypothesis is the clock ticking? J. Evol. Biol. 22 (8), 1727–1738.

Langerhans, R.B., 2019. Evolutionary implications of a trematode with special reference to the cercaria of *Diphtherostomum brusinae* (Diphtherostomidae) from north-Norwegian waters. Mar. Biol. Res. 6 (2), 201–212.

Langerhans, R.B., Goins, T.R., Stemp, K.M., Riesch, R., Araujo, M.S., Layman, C.A., 2020. The mitochondrial genome of the *Diplostomum sp. nov.* (Digenea: Procercomorphidae) from a stranded minke whale. Parasitol. Int. 65 (3), 271–275.

Løvlie, M., 1973. The host-parasite interface and associated structures of the cercaria and adult *Neophasis lagomena* (Lebour, 1910). Ophelia 106 (3), 75–85.

Løs, T.L., 1962. Zur Kenntnis der gymnophalliden Trematoden des Nordseeraumes. I. Die Alternativzyklen von *Gymnophallus choledochus* L. with special reference to the 37-collar-spine Atlantic forms. Syst. Parasitol. 19 (2), 95–106.

Morgan, J.A.T., Blair, D., 1995. Nuclear rDNA ITS sequence variation in the trematode *Diplostomum sp. nov.* and the historical biogeography of the 'diplostomids'. J. Helminthol. 69 (4), 281–291.

Morgan, J.M., 2005. Relationships within the superfamilies Paragonimidea and Microphallidea. Parasitol. Res. 99 (6), 105–112.

Morgan, J.M., 2008. Classification and historical biogeography of the 'diplostomids'. J. Helminthol. 82 (3), 156–156 (in German).

Morgan, J.M., Thorne, B., 2010. From North America to the southern Barents Sea). J. Helminthol. 84 (2), 279–282.

Morgan, J., 1968. Fauna and ecology of trematode larvae from molluscs in the Barents and White Seas. Tr. Murn. Morsk. Biol. Inst. 10 (14), 78–166 (in Russian).

Morgan, J.A.T., Blair, D., 1995. Nuclear rDNA ITS sequence variation in the trematode *Diplostomum sp. nov.* and the historical biogeography of the 'diplostomids'. J. Helminthol. 69 (4), 281–291.

Morgan, J.M., 2005. Relationships within the superfamilies Paragonimidea and Microphallidea. Parasitol. Res. 99 (6), 105–112.

Morgan, J.M., 2008. Classification and historical biogeography of the 'diplostomids'. J. Helminthol. 82 (3), 156–156 (in German).

Morgan, J.M., Thorne, B., 2010. From North America to the southern Barents Sea). J. Helminthol. 84 (2), 279–282.
Polyansky, Y.I., 1955. Studies on the Parasitology of the Fish in the Northern Seas of the USSR. Parasites of Fish of the Barents Sea, 19, 5–170. Trudy Zoologicheskogo Instituta [In Russian: English translation (1966) Israel Program for Scientific Translations, Cat. No. 1655, 158 pp.].

Poulin, R., Cribb, T.H., 2002. Trematode life cycles: short is sweet? Trends Parasitol. 18 (4), 176–183.

Pyke, G.H., Polliam, H.R., Charnov, E.L., 1977. Optimal foraging: a selective review of theory and tests. Q. Rev. Biol. 52 (2), 137–154.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61 (3), 539–542.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, S., Saalfeld, S., Schmid, B., Schmied, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

WoRMS, 2021. Neophasis Stafford, 1904. http://www.marinespecies.org/aphia.php?p=taxdetails&id=108549 on 2021-04-21.

Further reading

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30 (9), 1312–1313.