Redescription and molecular characterisation of *Comephoronema werestschagini* Layman, 1933 (Nematoda: Cystidicolidae) from the endemic Baikal fish *Cottocomephorus grewingkii* (Dybowski, 1874) (Scorpaeniformes: Cottocomephoridae) with some comments on cystidicolid phylogeny

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Summary. The nematode *Comephoronema werestschagini* (Chromadorea: Cystidocolidae) is redescribed from the Baikal yellowfin *Cottocomephorus grewingkii* (Dybowski, 1874). Important morphological features, such as the presence of four submedian cephalic papillae, four well developed bilobed sublabia, two large lateral pseudolabia, pairs of rounded deirids, as well as the position of phasmids are reported for the first time in this species. The SSU rDNA-based phylogeny of the cystidicolids is defined according to the sequences obtained for *C. werestschagini*, *C. oshmarini* and *Capillospirura ovotrichuria*. The polyphyly of the Cystidocolidae is herein confirmed. All three studied species appear as members of Cystidicolidae *s. str.* clade; however, *C. werestschagini* and *C. oshmarini* are not phylogenetically related.

Key words: 18S rDNA, *Capillospirura ovotrichuria*, Cystidicolidae, nematodes, phylogeny, SEM.

The genus *Comephoronema* Layman, 1933 (Nematoda: Chromadorea: Cystidicolidae) includes five species of nematodes, parasitic in freshwater fish of Eurasia, as well as some marine fish of the Atlantic and Antarctic Oceans (Moravec *et al.*, 2007; Pereira *et al.*, 2014). According to Moravec and Klimpel (2007) and Pereira *et al.* (2014), the genus *Comephoronema* is morphologically close to the genus *Ascarophis* van Beneden, 1871 but differs in the higher number of precloacal papillae in males. Unfortunately, this statement was based mainly on the morphology of *Comephoronema macrochiri* Moravec & Klimpel, 2007, *Comephoronema multipapillatum* Pereira, Pereira & Luque, 2014 and *Comephoronema oshmarini* Trofimenko, 1974, but not the type species *Comephoronema werestschagini* Layman, 1933, whose description remains incomplete (see Layman, 1933).

The geographical range of *C. werestschagini* is limited to Lake Baikal and it most often occurs in scorpaeniform fishes, but a number of authors have noticed this species in some other Baikal fishes, in particular *Brachymystax lenok* (Pallas, 1773) (Salmonidae), *Lota lota* (Linnaeus, 1758) (Lotidae) and *Thymallus arcticus* (Pallas, 1776) (Salmonidae) *s. lato* (Layman, 1933; Oshmarin, 1965; Zaika, 1965; Rusinek & Dzuba, 2002; Dugarov *et al.*, 2016; Rinchinov *et al.*, 2017). However, according to Trofimenko (1974), *C. oshmarini* is typical for *L. lota* from Baikal.

According to present molecular data, the Cystidicolidae is a polyphyletic group (Černotíková *et al.*, 2011; Xiang *et al.*, 2013; Jabbar *et al.*, 2015; Vidal *et al.*, 2016; Choudhury & Nadler, 2018; Pereira *et al.*, 2018) and additional molecular data on their representatives will probably make it possible to obtain a more complete picture of the phylogenetic relationships within this group.

In this paper, we provide a redescription of *C. werestschagini* with the molecular data obtained for the redescribed species and two previously

unsequenced species (*i.e.*, *C. oshmarini* and *Capillospirura ovotrichuria* Skrjabin, 1924).

MATERIAL AND METHODS

Ten specimens of *C. werestschagini* (seven gravid females, two adult males and one incomplete female) were kindly provided by Dr D.R. Baldanova from Institute of General and Experimental Biology, SB RAS. Nematodes were collected during a parasitological examination of adult specimen of *Cottocomephorus grewingkii* (Dybowski, 1874) caught in the Academician Ridge area of Lake Baikal (53°47' N, 108°26' E) in March 2017. Data on the number of fish were published by Rinchinov *et al.* (2017).

One specimen of *C. oshmarini* and one of *Ca. ovotrichuria* were recovered from *Lota lota* caught in Onega Lake near Petrozavodsk city (61°49' N, 34°24' E) and *Acipenser ruthenus marsiglii* (Brandt, 1833) from Irtysh River, near Tobolsk city (58°11' N, 68°13' E).

For light microscopy studies the nematodes were fixed with 70% ethanol and later transferred to anhydrous glycerin *via* slow evaporation method (Seinhorst, 1959) and mounted in the same medium. Morphology was studied with the aid of a light microscope Axio Imager A1 (Zeiss AG, Oberkochen, Germany). Four female specimens of *C. werestschagini* were prepared for SEM study by dehydration through a graded ethanol series and acetone following a critical point drying. After coating with gold, they were examined with a Tescan Vega TS5130MM microscope (s.r.o. TESCAN, Czech Republic).

Specimens for genetic analysis were fixed in 96% ethanol and were kept at –18°C prior to DNA extraction. The DNA was extracted from single nematode specimens using methods detailed by Holterman *et al.* (2006). The worm-lysis solution was prepared immediately before DNA extraction containing 950 µl of a mixture of 2 ml of 1M NaCl, 2 ml of 1M Tris-HCl (pH 8) plus 5.5 ml of deionized water plus 10 µl of mercaptoethanol and 40 µl of proteinase K (20 mg ml^{-1}). Single nematodes were each transferred to 25 µl of sterile water and after addition of 25 µl of worm-lysis solution the tube was incubated at 65°C for 90 min. The tubes with homogenate were then incubated at 99°C for 5 min to deactivate proteinase K and 0.8-1.2 µl of the obtained homogenate was used as a template for PCR reactions.

The PCR reactions were performed using Encyclo Plus PCR kit (Evrogen®, Russia) according to the manufacturer's manual. Partial sequences of SSU (18S rDNA) were amplified with two pairs of primer sets. A pair of nematode-specific primers nem18SF (5'-CGC GAA TRG CTC ATT ACA ACA GC-3') and nem18SR (5'-GGG CGG TAT CTG ATC GCC-3') was used to amplify 5' portion of SSU rDNA (Floyd *et al.*, 2005). PCR cycling parameters included primary denaturation at 95°C for 5 min followed by 5 cycles of 94°C for 30 s, 47°C for 30 s and 72°C for 40 s and 35 cycles of 94°C for 25 s, 54°C for 30 s and 72°C for 40 s, followed by post-amplification extension at 72°C for 5 min. Another pair 24F (5'-AGR GGT GAA ATY CGT GGA CC-3') and Q39 (5'-TAA TGA TCC WTC YGC AGG TTC ACC TAC-3') was used to obtain the remaining 3' end of SSU rDNA (Blaxter *et al.*, 1998). PCR cycling parameters included primary denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 60 s, 53°C for 90 s and 72°C for 90 s, followed by post-amplification extension at 72°C for 6 min.

The PCR reaction products were visualised in agarose gel and bands were excised for DNA extraction with Promega columns (Wizard® SV Gel and PCR Clean-Up System). Samples were directly sequenced using the same primers that were used for the primary PCR reactions.

The nematode sequences were deposited in GenBank NCBI as MN294781 (*C. werestschagini*), MN294782 (*C. oshmarini*) and MN294783 (*Ca. ovotrichuria*).

For comparative purposes and phylogeny construction, SSU rDNA sequences of spiruromorphs from the NCBI GenBank database were also used (GenBank accession numbers are given in the phylograms). Only SSU data exceeding the length of 1500 bp were used to infer the phylogenies. The datasets were aligned separately using Clustal_X (Thompson *et al.*, 1997) under default values for gap opening and gap extension penalties. Phylogenetic trees were inferred using maximum likelihood (ML) method under the GTR+G model of evolution using the programs MEGA 5.2 (Tamura *et al.*, 2011). The model of evolution was chosen under the Akaike criterion using Modeltest 3.7 (Posada & Crandall, 1998) and the fixed parameters were generated with the same program. Bootstrap re-sampling was performed with 1000 non-parametric replications. Phylogenetic trees were rooted on two outgroup species, representing families Philonematidae and Quimperiidae based on previous phylogenies (Sokolov & Malysheva, 2017; Choudhury & Nadler, 2018).

Table 1. Measurements and ratios of *Comephoronema werestschagini*.

Features	Males (n = 2)	Females (n = 3)
Body length, mm	4.79-5.88	9.27-9.73
Maximum body width, mm	0.10-0.12	0.18-0.17
Prostom length, μm	17-19	17-18
Mesostom length, μm	126-131	137-144
Entire stoma length, μm	144-148	156-161
Muscular part of oesophagus length, μm	205-338	334-384
Glandular part of oesophagus length, μm	1084-1112	1300-1356
Muscular to glandular parts of oesophagus length ratio	1 : 5.3-3.3	1 : 3.5-3.9
Length of entire oesophagus and stoma as % of body length	27.2-29.9	19.3-19.5
Distance from anterior extremity to deirids, μm	157-174	160-176
Distance from anterior extremity to nerve ring, μm	210-248	210-253
Distance from anterior extremity to excretory pore, μm	296-331	313-343
Tail length, μm	91-97	56-63
Distance from anterior extremity to vulva, mm	–	5.61-6.31
Distance from anterior extremity to vulva as % of body length	–	60.5-64.8
Left spicule length, μm	369-427	–
Right spicule length, μm	97-102	–
Right to left spicules length ratio	1 : 3.8-4.2	–
Egg sizes, μm	–	55-58 × 25

RESULTS

Family Cystidicolidae Skrjabin, 1946
Comephoronema werestschagini Layman, 1933 (Figs 1–4)

Measurements. See Table 1.

General. Cuticle thin with transverse striations distinctly noticeable in middle part of body. Cephalic end rounded. Oral aperture terminal, oval, dorsoventrally elongated, surrounded by four poorly developed submedian labia (Fig. 1). Four submedian sublabia well developed, bilobed (Figs 1A-C & 2B). Two pseudolabia with anteriorly protruding rounded elevations or without them; elevations may differ on left and right sides of same individual (Figs 1 A-C; 2B; 3C, D). Inner margin of each pseudolabium dorsoventrally extended. Four submedian cephalic papillae present (Figs 1 A, B & 2B). Amphids small, located laterally (Fig. 1A, B). Stoma long, with dorsoventrally extended prostom, tubular mesostom and very short collar-like telostom. Oesophagus clearly divided into anterior muscular and posterior glandular parts (Fig. 2A). Nerve ring situated approximately at border of first and second quarter of muscular oesophagus (Fig. 2A). Excretory pore situated slightly posterior to nerve ring level (Fig. 2A). Deirids rounded, situated at or slightly posterior to stoma base level (Figs 2A, D & 3A).

Male. Posterior end of body spirally twisted. Caudal alae wide. Precloacal area with longitudinal rows of cuticular ridges (Fig. 4). Precloacal papillae pedunculate, occurring in combinations 8 + 11 or 8 + 12. Six pairs of postcloacal papillae arranged as five pedunculate, subventral pairs and one pair of minute, ventral, sessile papillae at the same level than posterior subventral pair (Fig. 4). A pair of small phasmids locate ventrally, posterior to ventral papillae. Left spicule elongate with pointed distal end; right spicule smaller, broad, with rounded distal end. Tail conical with rounded distal tip (Fig. 4).

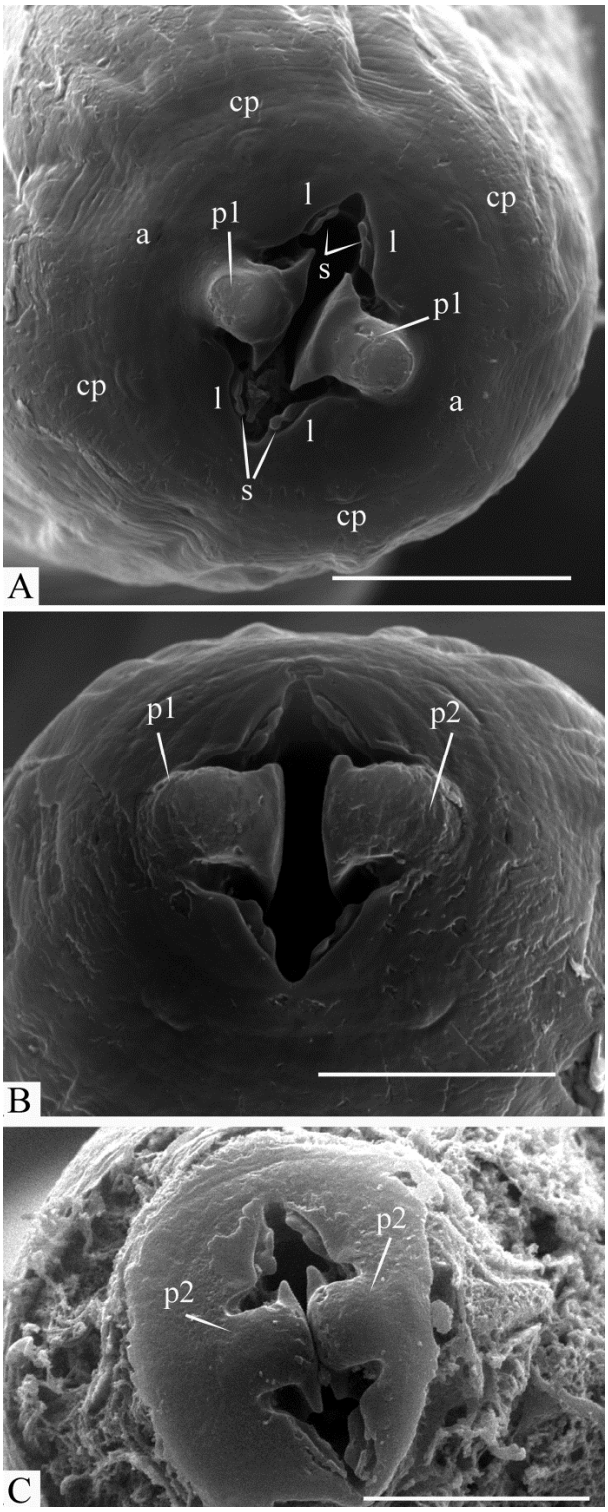

Fig. 1. SEM micrographs of *Comephoronema werestschagini*: A-C – female specimens with different development of apical elevations on pseudolabia; a – amphids; cp – cephalic papillae; l – labia; p1 – pseudolabia with well-developed elevations; p2 – pseudolabia without apical elevations; s – sublabia. Scale bars: A, B – 0.01 mm; C – 0.02 mm.

Female. Tail conical, with rounded distal tip. Pair of small phasmids situated laterally not far from tail tip (Fig. 2C). Vulval lips slightly elevated; uterus amphidelphic, with numerous eggs. Fully developed eggs elongate-oval; each pole of egg bearing distinct protuberance with numerous fine filaments slightly extended along its axis (Fig. 3B).

Host and locality. Stomach of *Cottocomephorus grewingkii* (Dybowski, 1874) (Cottocomephoridae, Scorpaeniformes) collected at the Academician Ridge area of Lake Baikal, Russian Federation.

Deposited material. Voucher specimens (storage item # 14278) were deposited in the Museum of Helminthological Collections at the Centre of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences (IPEE RAS) in Moscow, Russian Federation.

Molecular phylogenetic analysis. *Comephoronema werestschagini*, *C. oshmarini* and *Ca. ovotrichuria* are integrated into the moderately-supported Cystidicolidae *s. str.* clade, which also includes *Ascarophis arctica* Polyansky, 1952, *Capillospirura* sp. and *Cystidicola farionis* Fischer, 1798 (Fig. 5). In turn, *C. oshmarini*, *A. arctica* and *Cy. farionis* form a weakly supported polytomic subclade, which is sister to *C. werestschagini*; however, this sister connection has no reliable support. *Capillospirura ovotrichuria* appears as a well supported sister to *Capillospirura* sp. ex *Acipenser fulvescens* from the USA. The *Capillospirura* spp. subclade occupies a basal position relative to the other members of the Cystidicolidae *s .str.* clade.

Other cystidicolids were distributed as follows. *Neoascarophis* spp. is a sister group to the *Proleptus* sp. + (Acuariidae + *Ascarophis adioryx* Machida, 1981) clade, while *A. adioryx* in the Acuariidae + *A. adioryx* subclade occupies a terminal position. Nevertheless, *Proleptus* sp. + (Acuariidae + *A. adioryx*) clade and Acuariidae + *A. adioryx* subclade have no reliable support. The *Neoascarophis* spp. + [*Proleptus* sp. + (Acuariidae + *A. adioryx*)] group is sister to the *H. longissimum* + Cystidicolidae *s. str.* clade, but without reliable support. *Metabronema magnum* (Taylor, 1925) unites with group combining *Neoascarophis* spp., *Proleptus* sp., *A. adioryx*, Acuariidae, *H. longissimum* and Cystidicolidae *s. str.* In turn, *Cystidicoloides fischeri* (Travassos, Artigas & Pereira, 1928) is a sister to the large clade of *M. magnum* + above-mentioned nematodes group. However, the sister connection of the both species with listed groups of nematodes is devoid of reliable supports.

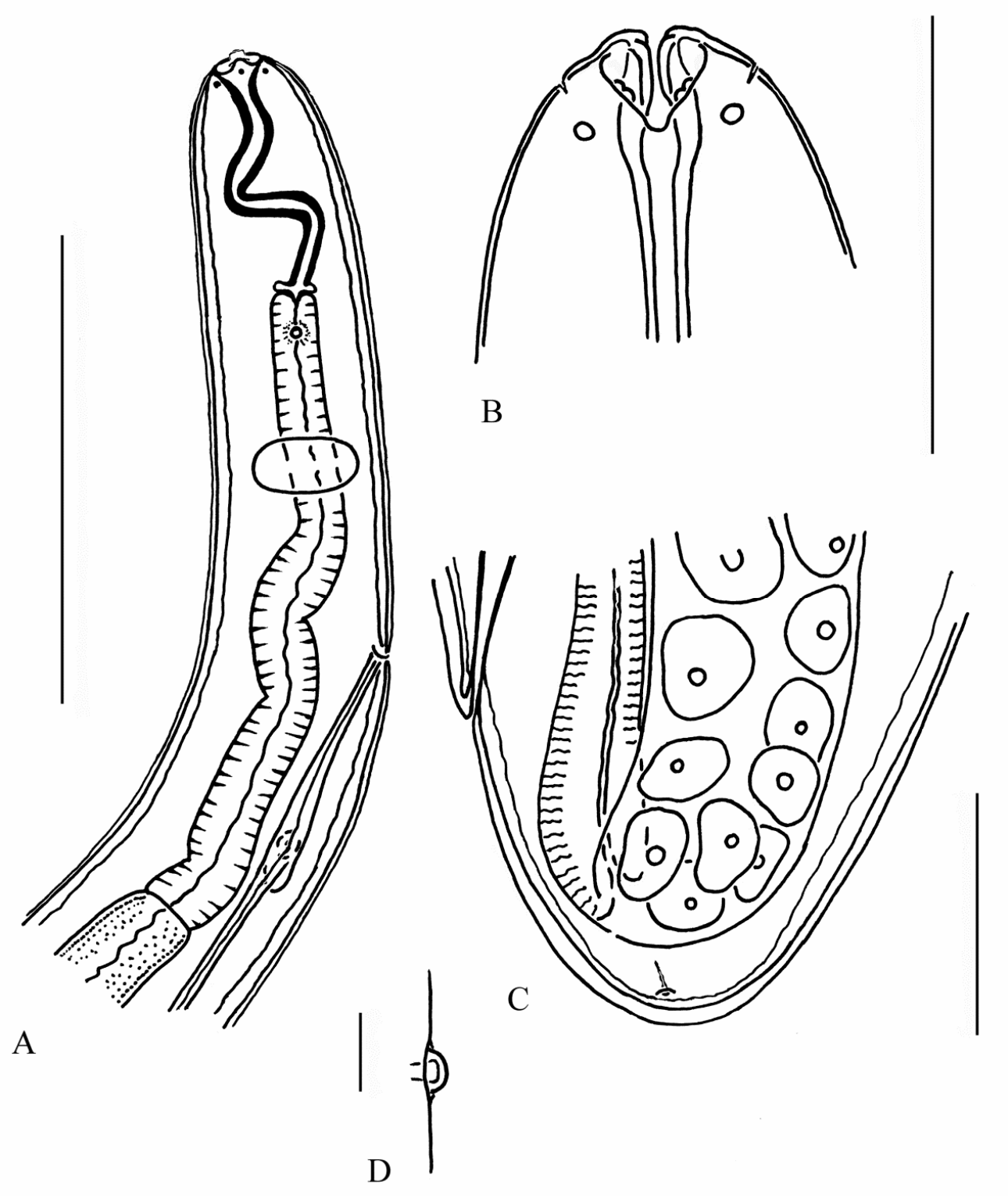

Fig. 2. Female of *Comephoronema werestschagini*: A – anterior end, lateral view; B – cephalic end, dorsoventral view; C – caudal end, lateral view; D – deirid, dorsoventral view. Scale bars: A – 0.2 mm; B, C – 0.05 mm; D – 0.01 mm.

Members of *Salmonema* Moravec, Santos & Brasil-Sato, 2008 are associated with poorly supported sister relationships with rhabdochonid nematodes. At the same time, *Spinitectus* spp. forms a well-supported group that occupies a basal position in relation to all aforementioned spiruromorph nematodes.

DISCUSSION

The body size of males, length and shape of left spicule, total number of precloacal papillae, asymmetry in the number of these papillae of the left and right body sides and egg structure are consistent with those of *C. werestschagini*, as described by Layman (1933). However, the body length of females (9.27-9.73 mm *vs* 6.80-7.41 mm) and length of right spicule in males (97-102 μm *vs* 84-97 μm) were slightly larger than those in the original description (see Layman, 1933). Moreover, the presence of amphids, four submedian cephalic papillae, four submedian labia and sublabia, two large lateral pseudolabia, as well as the position of deirids, phasmids and the sixth pair of ventral sessile postcloacal papillae is herein reported for the first time since they were overlooked in the original description. Aforementioned morphological features confirm the presence of the typical head and tail structures in *C. werestschagini* as in other cystidicolids (see Moravec, 2007).

Polymorphism in the organisation of pseudolabia found in our specimens of *C. werestschagini* has not been reported previously. We cannot exclude that such morphological differences could be caused by fixation process. However, the similarity of the studied individuals on the number of morphological characters, including the number of precloacal papillae (8-12 on each body side) and the rounded shape of deirids does not allow us to doubt their conspecificity. It is worth noting that most of the congeners excepting *C. beatriceinsleyae* (Holloway & Klewer, 1969) from McMurdo Sound, Antarctica,

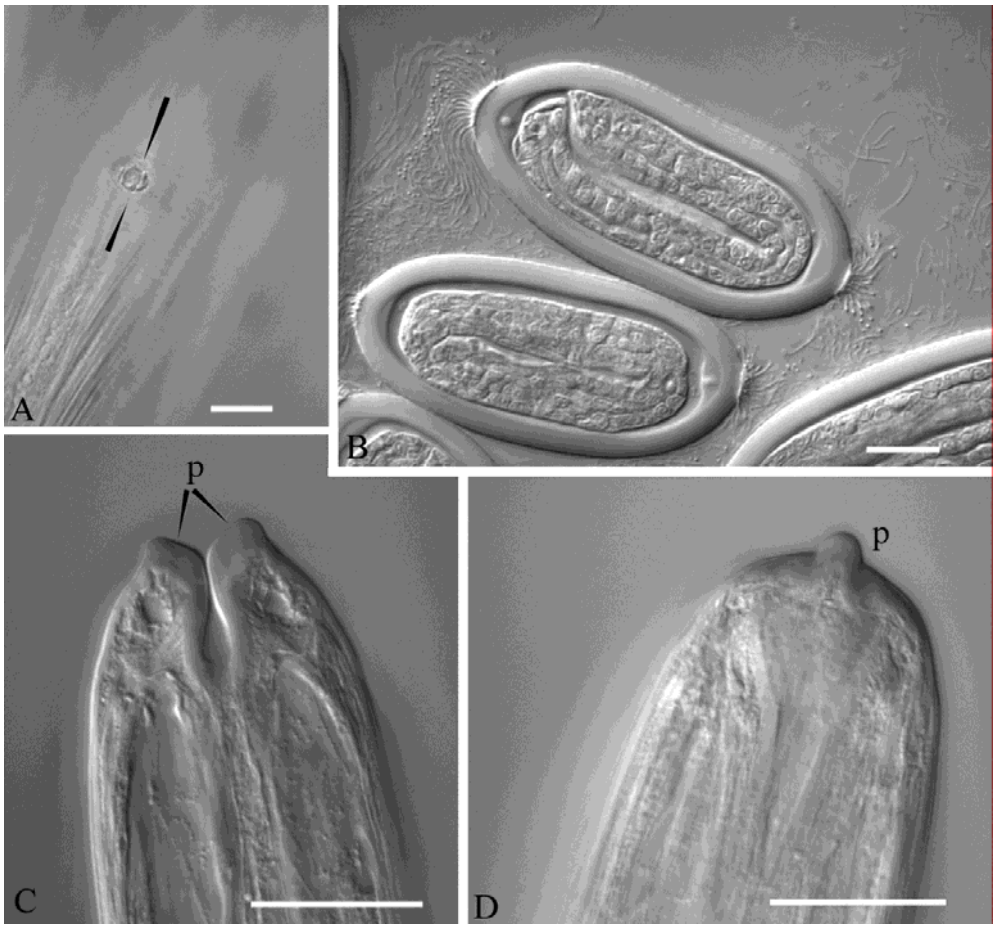

Fig. 3. Light micrographs of *Comephoronema werestschagini*: A – deirid of female, lateral view; B – eggs with filaments; C, D – cephalic end of male with elevations on pseudolabia, dorsoventral and lateral views, respectively; p – pseudolabia. Scale bars: A, B – 0.01 mm; C, D – 0.02 mm.

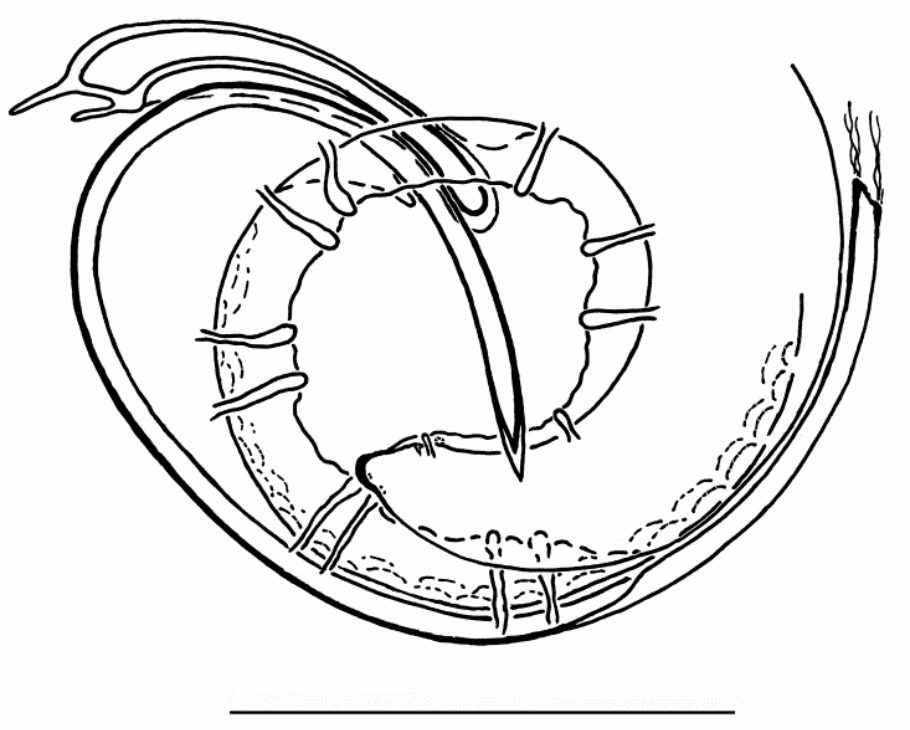

Fig. 4. Caudal end of male of *Comephoronema werestschagini*, lateral view. Scale bar: 0.09 mm.

for which these data are missing, possess Y-, I- or T-shaped deirids (Moravec & Klimpel, 2007; Moravec *et al.*, 2007; Pereira *et al.*, 2014).

Comephoronema is apparently a paraphyletic taxon. Additional data on a wide range of cystidicolids, in particular *Ascarophis*-like nematodes should be involved in future studies to clarify the taxonomic status of *Comephoronema*. For the time being *C. beatriceinsleyae*, *C. macrochiri*, *C. multipapillatum*, *C. oshmarini* and *C. werestschagini* should remain within *Comephoronema s. lato* until more molecular data are obtained.

Our data confirm the polyphyly of the Cystidicolidae as stated by Černotíková *et al.* (2011), Xiang *et al.* (2013), Jabbar *et al.* (2015), Vidal *et al.* (2016), Choudhury & Nadler (2018) and Pereira *et al.* (2018); however, due to insufficient data on other members of the family, we refrain from taxonomic decisions on the restructuring of Cystidicolidae, limiting ourselves to some assumptions on this topic.

To date, molecular data showed that genera *Cystidicola* Fisher, 1798, *Capillospirura* Skrjabin, 1924 and *Comephoronema s. lato*, as well as one representative of the genus *Ascarophis* – *A. arctica*, can be referred to the family Cystidicolidae *s. str.* Morphological synapomorphies of Cystidicolidae *s. str.* at the moment are difficult to determine, since we have only preliminary data on the composition of this family. Note that Cystidicolidae *s. str.* combines species that differ greatly in the structures of the cephalic end, such as the shape of pseudolabia, the presence of sublabia, buccal teeth and other appendages (*e.g.*, Moravec, 2007). According to Pereira *et al.* (2018), *C. vaucheri* can be included in the Cystidicolidae *s. str.*, although, the results of our analysis do not confirm the direct phylogenetic relationship between these nematodes. Thus, the family affiliation of *C. vaucheri* remains unclear.

Apparently, the subgenus *Dentiascarophis* Moravec & Justine, 2009 proposed for the species *A. (D.) adioryx* (see Moravec & Justine, 2009) should be raised to the generic rank. *Ascarophis adioryx* differs from many members of the nominative subgenus of the genus *Ascarophis*, including *A. arctica* and *Ascarophis morrhuae* van Beneden, 1871 (type species) in the head end and left spicule morphology bearing a large ventral triangular outgrowth at its distal end (Gordon, 1951; Ko, 1986; Appy, 1981; Fagerholm & Berland, 1988; Moravec & Justine, 2009). The family affiliation of *A. adioryx* is not yet clear. Černotíková *et al.* (2011), Xiang *et al.* (2013), Vidal *et al.* (2016) and Pereira *et al.* (2018) combined *A. adioryx* in the same clade with representatives of Acuariidae.

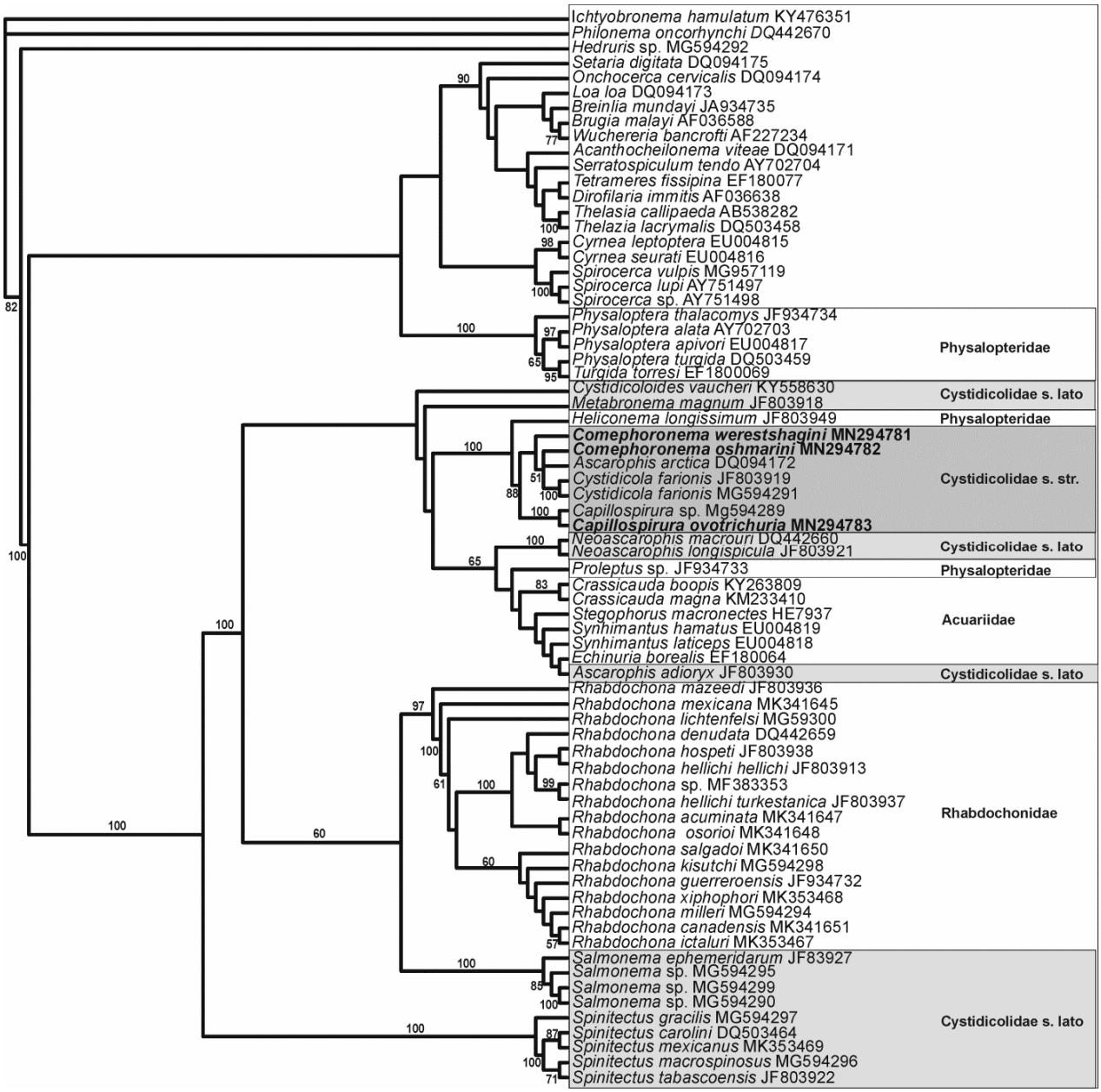

Fig. 5. Phylogenetic relationships of cystidicolid nematodes inferred from ML analysis of SSU rDNA (1650 bp long alignment). The bootstrap values are given near nodes. Newly obtained sequences are marked in bold.

However, in our tree, it took the terminal position in contrast to the basal position as given by mentioned authors.

We also assume that the genus *Spinitectus* Fourment, 1883 should probably be demarcated as a separate family, as proposed by Skrjabin *et al.* (1967). Presumably, both genera *Metabronema* Yorke & Maplestone, 1926 and *Salmonema* (we prefer *Sterliadochona* Skrjabin, 1948, see Sokolov *et al.*, 2012) should be raised to family level. The phylogenetic position of the genus *Neoascarophis* Machida, 1976 is variable in the phylograms of different authors (*e.g.*, Černotíková *et al.*, 2011; Choudhury & Nadler, 2018; Pereira *et al.*, 2018; this study) and still remains unclear.

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С.Г. Соколов, Е.Л. Воропаева и С.В. Малышева. Переописание *Comephoronema werestschagini* Layman, 1933 (Nematoda: Cystidicolidae) из эндемичной рыбы озера Байкал *Cottocomephorus grewingkii* (Dybowski, 1874) (Scorpaeniformes: Cottocomephoridae), с некоторыми комментариями по филогении цистидиколид.

Резюме. Цистидиколидный вид нематод *Comephoronema werestschagini* переописан по материалу, собранному из байкальской рыбы *Cottocomephorus grewingkii*, и изученному с применением светового и сканирующего электронного микроскопов. Сообщается о новых важных морфологических признаках этого вида: наличие четырех субмедианных головных папилл, четырех хорошо развитых двулопастных сублябий (sublabia), двух крупных латеральных псевдолябий (pseudolabia), пары округлых дейридов, а также наличие фазмид. Для *C. werestschagini* и двух других видов цистидиколид – *Comephoronema oshmarini* и *Capillospirura ovotrichuria* – получены и проанализированы частичные последовательности 18S рДНК. Семейство Cystidicolidae проявляет себя как полифилетический таксон. Все три рассматриваемые вида нематод интегрированы в кладу Cystidicolidae *s. str.*, однако *C. werestschagini* с *C. oshmarini* не имеют прямых филогенетических связей друг с другом.