

## Research Paper

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# Phylogeny and morphology of some European cyathocotylid digeneans (Trematoda: Diplostomoidea)

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**Abstract**

The Cyathocotylidae Mühling, 1898 is a family of primitive diplostomoid trematodes important for understanding the evolution of the superfamily Diplostomoidea. However, cyathocotylids remain poorly studied with the use of molecular techniques. In this study we sequenced the 5.8S + ITS2 region, 28S rRNA, and *cox1* genes of two cyathocotylid species and obtained new morphological data on them. We propose *Georduboisia* nom. nov. instead of the preoccupied name *Duboisia* Szidat, 1936 (junior homonym of *Duboisia* Stremme, 1911). Adults of *Georduboisia* cf. *teganuma* (Ishii, 1935) and *Paracoenogonimus ovatus* Katsurada, 1914 were collected from fish-eating birds in the south of the European part of Russia. *Georduboisia* cf. *teganuma* was very similar to *G. teganuma* but differed from it in the shape of the testes. The 28S rRNA gene dataset provided the best-resolved phylogeny of the Cyathocotylidae to date. In the phylogram based on partial sequences of this gene, *P. ovatus* was close to members of *Holostephanoides* Dubois, 1983, *Neogogatea* Chandler & Rausch, 1947 and *Gogatea* Szidat, 1936. *Georduboisia* cf. *teganuma* clustered with members of *Cyathocotyle* Mühling, 1896 and *Holostephanus* Szidat, 1936. Phylogenetic analysis based on the 5.8S + ITS2 dataset showed that adults of *P. ovatus* examined in our study were conspecific with the metacercariae from the musculature of fish collected in Hungary and Italy. It also revealed probable misidentifications of larvae and adults of cyathocotylids whose sequences are deposited in GenBank NCBI.

**Introduction**

The family Cyathocotylidae Mühling, 1898 is a compact group of the Diplostomoidea with a complex of plesiomorphic characters such as an undivided or indistinctly divided body and the presence of a true cirrus sac (Niewiadomska 2002). This family has a tumultuous taxonomic history (e.g., Mühling 1896, 1898; Poche 1925; La Rue 1926; Lutz 1935; Szidat 1936; Dubois 1936, 1970, 1987; Sudarikov 1961; Yamaguti 1971). According to the current conceptualisation, the Cyathocotylidae is the senior synonym for the Brauninidae Wolf, 1903 and the Prohemistomidae Lutz, 1935 (Niewiadomska 2002; Achatz *et al.* 2019). Adult cyathocotylids parasitise the intestines of mammals, birds, reptilians, and, exceptionally, fish (Yamaguti 1971).

A recent publication by Achatz *et al.* (2019) is the only modern study deciphering the phylogenetic relationships of the cyathocotylids. Its results indicate that the current system of the Cyathocotylidae based on morphological data (Niewiadomska 2002) should be re-evaluated. However, changes to the system of the Cyathocotylidae cannot be fully implemented due to the lack of molecular data on many cyathocotylid genera, including *Duboisia* Szidat, 1936 and *Paracoenogonimus* Katsurada, 1914.

*Duboisia* Szidat, 1936 is characterised by a pouch-like body with a large holdfast organ enclosed in a deep ventral concavity and a massive vaginal sphincter (Sudarikov 1961; Dubois 1984; Niewiadomska 2002). This genus contains only three species parasitising as adult in Palearctic and African birds – *Duboisia syriaca* Dubois, 1934 (type), *Duboisia skrjabini* Sudarikov & Oshmarin, 1954, and *Duboisia teganuma* (Ishii, 1935) (Dubois 1984). However, the name *Duboisia* Szidat, 1936 is in fact a junior homonym of *Duboisia* Stremme, 1911, the name of a genus of fossil antelopes (Stremme 1911). The life cycle has been studied only for *D. teganuma*, and only partially. Its first intermediate host remains unknown; the role of the second intermediate host is played by fish (Bayanov & Islamov 1977).

The taxonomic boundaries of *Paracoenogonimus* Katsurada, 1914 are assessed differently in the systems of different authors, depending on whether they recognise (e.g., Niewiadomska 2002) or abolish (e.g., Dubois 1983) the genus *Linstowiella* Szidat, 1933. Here we consider *Linstowiella* as a distinct genus following Mishchenko (1974) and Niewiadomska (2002). *Paracoenogonimus sensu stricto* is characterised by an oval to elongate-oval flattened body with shallow ventral

concavity and a ventral sucker with absence of the vaginal sphincter (Niewiadomska 2002). This genus contains two species parasitising as adults various Palearctic fish-eating birds and mammals: *Paracoenogonimus ovatus* Katsurada, 1914 (type) and *Paracoenogonimus ussuriensis* Besprozvannykh & Ermolenko, 2009 (Dubois 1983 with amendments by Niewiadomska 2002 and Besprozvannykh & Ermolenko 2009). The life cycle, which has been studied in both members of the genus, involves gastropods of the Viviparoida as the first intermediate hosts and various fishes as the second intermediate hosts (e.g., Komiya 1938; Ginetsinskaya & Koscheva 1959; Besprozvannykh & Ermolenko 2009).

We collected specimens of cyathocotylids during a parasitological survey of fish-eating birds in the south of the European part of Russia. Based on morphological features they were identified as representatives of *Duboisia* and *Paracoenogonimus*. This study aimed to describe the morphology of these cyathocotylids, obtain molecular data on them, and evaluate their phylogenetic relationships.

## Materials and methods

### Sampling and morphological study

Adult digeneans were collected from the intestines of *Podiceps cristatus* (Linnaeus, 1758) and *Circus aeruginosus* (Linnaeus, 1758). Two individuals of *P. cristatus* were shot by licensed hunters near Kalinino Village, Astrakhan Region, Russia (46°20'9" N; 48°53'29" E), in September–November 2017. In addition, two individuals of *P. cristatus* were found dead in fishing nets in the River Mokraya Chuburka near Leninsky Leskhov Village, Rostov Region, Russia (46°45'38"N; 39°8'1" E) in September 2023. One specimen of *C. aeruginosus* was found dead under power lines near the above-mentioned Kalinino Village in September 2017. Digenean specimens were relaxed in fresh water, fixed with 70% ethanol, and after a few minutes transferred to 96% ethanol.

Adult parasites were studied by morphological and molecular techniques. For the morphological study, the specimens were stained with acetocarmine, dehydrated in a graded ethanol series, cleared with dimethyl phthalate, and finally mounted in Canada balsam. The slides were then observed through a light microscope Olympus BX-53 (Nagano, Japan) equipped with the differential interference contrast (DIC) at the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences, Petrozavodsk, Russia. All the measurements are given in micrometers (µm). In morphological descriptions, the terms prosoma and opisthosoma are used following Achatz *et al.* (2022), and the term forebody is used following Manter (1970). The drawings were made with the help of the camera lucida. Paragenophores were deposited in the Museum of Helminthological Collections of the Center of Parasitology of the Severtsov Institute of Ecology and Evolution (IPEE RAS; Moscow, Russia).

### DNA amplification, sequencing, and phylogenetic analysis

Genomic DNA was isolated individually from nine ethanol-fixed digenean specimens using DNA-Extran kits (Synthol, Moscow, Russia). Partial 28S *rRNA* gene sequences were amplified with dig12 (5'-AAGCATATCACTAAGCGG-3') (Tkach *et al.* 2000) and 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (Tkach *et al.* 2003) primers. A fragment including the 5.8S + ITS2 region was amplified with the primers 3S (5'-GGTACCGGTTACGTTGGC-TAGTG-3') and ITS2.2 (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3') (Bowles *et al.* 1992). The following protocol was used for amplification of both above-mentioned regions: initial denaturation for 1 min at 95°C; 35 cycles for 1 min at 95°C, 1 min at 55°C, and 2 min at

72°C. Partial 28S *rRNA* gene PCR products were sequenced in both directions with dig12 and 1500R primers, alongside inner primers 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3') and ECD2 (5'-CCTTGGTCCGTGTTTCAAGACGGG-3') [Littlewood *et al.* 2000]. Sequencing of the 5.8S + ITS2 region was performed with the same primers as amplification.

Fragments of *cox1* mtDNA was amplified using forward primer JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and reverse primer JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') under the following conditions: 1 min denaturation hold at 94°C, 30 cycles of 15 s at 94°C, 30 s at 50°C, 2 min at 72°C, and a 7 min extension hold at 72°C (Morgan & Blair 1998). Sequencing of these fragments of mtDNA was performed with the same primers as amplification.

Consensus sequences were assembled in MEGA v. 10 (Kumar *et al.* 2018). The newly generated sequences were matched with those of other cyathocotylids in the Basic Local Alignment Search Tool (BLASTn) ([www.ncbi.nih.gov/BLAST/](http://www.ncbi.nih.gov/BLAST/)). To assess the phylogenetic position of the species under study, Bayesian inference analyses were performed for the newly obtained sequences and the previously published Cyathocotylidae sequences of the 28S *rRNA* gene, 5.8S + ITS2 region, and a fragment of the mtDNA *cox1* gene (the full list of the sequences is provided in Supplementary Table 1). The alignments were constructed using the MUSCLE algorithm implemented in MEGA v.10 (Kumar *et al.* 2018). The alignment of 28S *rRNA* gene with a length of 1037 bp included four novel sequences and 21 other sequences of cyathocotylid digeneans available in GenBank NCBI, with a member of the Liolopidae, *Liolope copulans* Cohn, 1902, as the outgroup. According to Pérez-Ponce de León & Hernández-Mena (2019), the Liolopidae is a sister group to the Diplostomoidea. The alignment of the *cox1* gene with a length of 433 bp included five novel sequences and 18 other sequences of cyathocotylid digeneans available in GenBank NCBI, with a member of the Clinostomidae, *Clinostomum tataxumui* Sereno-Urbe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013, as the outgroup. The Clinostomidae is one of the most closely related groups to the Diplostomoidea (Pérez-Ponce de León & Hernández-Mena 2019). The use of a member of this family as the outgroup in this alignment is due to the lack of the *cox1* gene sequence data for liolopids. The alignment of the 5.8S + ITS2 region with a length of 386 bp included four novel sequences and 29 other sequences of cyathocotylid digeneans available in GenBank NCBI, with *Paraharmotrema karinganiense* Dutton & Bullard, 2022 (Liolopidae) and *Clinostomum heluans* Braun, 1899 (Clinostomidae) as the outgroup. The use of only one species as the outgroup did not provide a good resolution.

Prior to the analyses, the best fitting GTR + G + I model for the alignment of the 28S *rRNA* and *cox1* gene sequences and TVM + G + I model for the alignment of the 5.8S + ITS2 region set were identified with the help of jModelTest v2.1.2 (Darriba *et al.* 2012). Bayesian inference analyses were conducted using MrBayes (v3.2.3) (Ronquist *et al.* 2012). Markov chain Monte Carlo simulations were run for 10,000,000 generations, log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus trees. FigTree v1.4 (Rambaut 2012) was used to visualize the trees. Genetic distances were estimated with MEGA v. 10 (Kumar *et al.* 2018).

## Results

### Taxonomy

#### Genus *Georduboisia* nom. nov.

Zoobank number for genus. urn:lsid:zoobank.org:act:3E7FB9AF-F0D1-406F-A0E9-F868A3F9D177

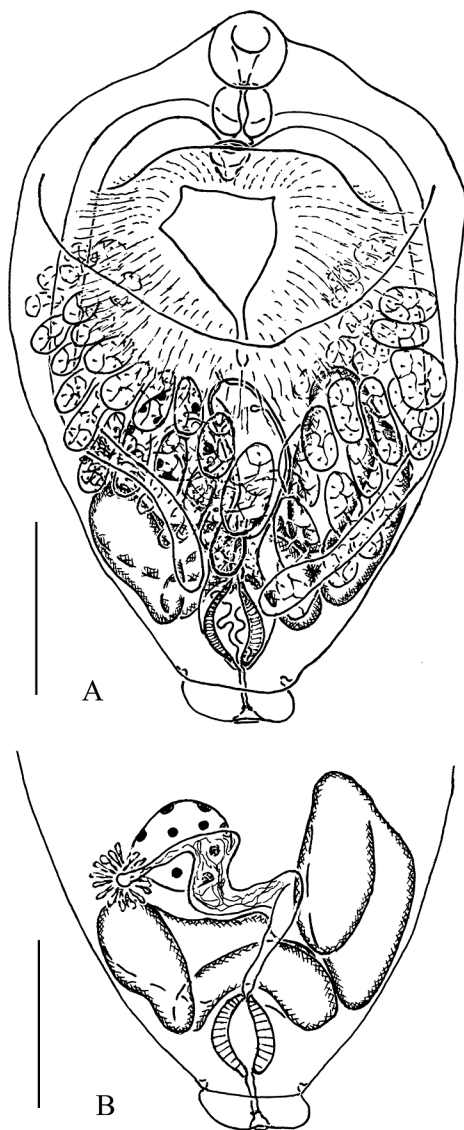
### Remarks

We changed the name *Duboisia* Szidat, 1936 to *Georduboisia* nom. nov. in accordance with the rules of handling homonyms (ICZN 1999, Articles 52–54, 56). Similarly to the name coined by Szidat (1936), *Georduboisia* nom. nov. is an eponym of Georges Dubois (1902–1993), a Swiss parasitologist. The diagnosis of *Georduboisia* nom. nov. corresponds to that of *Duboisia* Szidat, 1936 (see Niewiadomska 2002). Type species: *Georduboisia syriaca* (Dubois, 1934) comb. nov. Other species: *Georduboisia skrjabini* (Sudarikov & Oshmarin, 1954) comb. nov. and *Georduboisia teganuma* (Ishii, 1935) comb. nov.

*Georduboisia* cf. *teganuma* (Ishii, 1935) [Figures 1–2]

### Description

Body unipartite, pouch-like, with deep ventral concavity, 1018–1412 × 692–817, with terminal depression on posterior extremity.



**Figure 1.** Paragenophore of *Georduboisia* cf. *teganuma* from intestine of *Podiceps cristatus*, Astrakhan Region, Russia. (A) whole mount, ventral view; (B) body fragment with elements of female and male (only testes) reproductive systems (cirrus sac, vitellarium, and cecum removed from figure), ventral view. Scale bars: A, B = 300  $\mu$ m.

Tegument with small spines. Oral sucker 93–120 × 103–138, opening terminally or subterminally. Ventral sucker usually with axis inclined anteriorly, 79–103 wide. Sucker-width ratio 1:0.66–0.86. Forebody 13.5–27.4% of body length. Prepharynx absent. Pharynx 79–103 × 83–114. Ratio of oral sucker to pharynx based on mean diameter 1:0.59–0.84. Esophagus very short, 21–28. Ceca terminating blindly in posterior quarter of body, not approaching posterior end of body. Holdfast organ sucker-like, large, with large central cavity, 464–665 × 346–519, posterior to ventral sucker. Two testes, opposite or oblique, very elongate, convoluted or folded on themselves; occasionally oval (one testis in one specimen), entire to variously lobed, contiguous; left testis 692–817 × 138–180, right testis 450–720 × 138–201. Cirrus sac elongate, massive, entirely within posterior half of body or encroaching into anterior half of body, length 369–720 (31.2–53.6% of body length), maximum width 97–125, containing internal seminal vesicle, pars prostatica, ejaculatory duct, and invaginated cirrus. Internal seminal vesicle bipartite; distal reservoir larger than proximal reservoir, tubular, rectilinear, voluminous. Pars prostatica vesicular, with extensive field of prostatic cells. Ejaculatory duct short. Invaginated cirrus massive. Genital atrium tubular, terminates with small sphincter, opens into terminal depression on posterior end of body. Ovary suboval, 111–152 × 104–138, dextro- or sinistro-submedian, pre-equatorial to post-equatorial, at level of proximal end of cirrus sac. Laurer's canal opens dorsally to ovary (visible only in one specimen). Mehlis' gland well developed, lateral or postero-lateral to ovary. Proximal part of uterus acts as uterine seminal vesicle; distal part terminates with massive vaginal sphincter, opens into genital atrium ventrally to male genital pore. Eggs numerous (10–16), 93–96 × 52–55. Vitelline follicles large, in two broad sublateral fields; fields running smoothly from dorsal to ventral side of body, not extending behind ventral sucker anteriorly and testes posteriorly, separate anteriorly, and confluent or separate posteriorly.

*Host.* *Podiceps cristatus* (Linnaeus, 1758) (Podicipedidae).

*Site of infection.* Intestine.

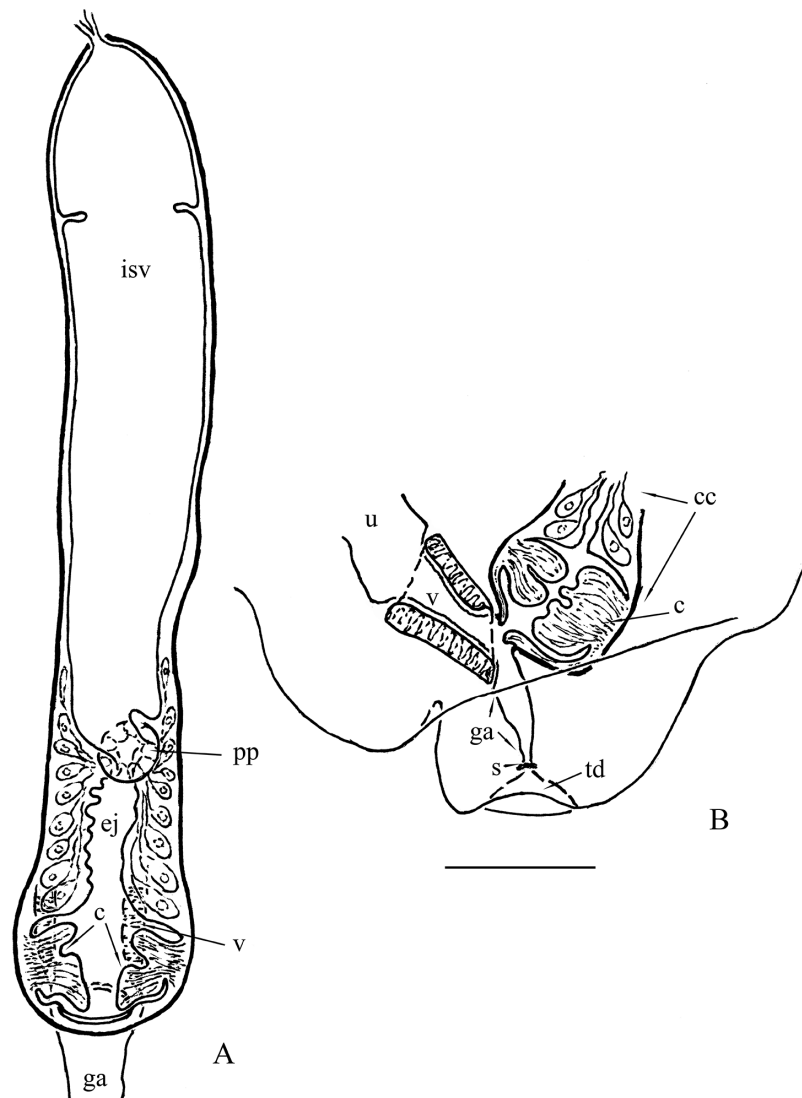
*Material examined.* Five adult specimens (paragenophores), Kalinino Village, Astrakhan Region, Russia (46°20'9" N; 48°53'29" E); IPEE 14345–14349. Two adult specimens (paragenophores), Leninsky Leskhoz Village, Rostov Region, Russia (46°45'38" N; 39°8'1" E); IPEE 14350, 14351.

*Sequences deposited.* PP093046 (28S rRNA gene), PP093047–PP093048 (5.8S + ITS2 region), P092969–PP092970 (*cox1* gene).

### Remarks

The specimens examined in our study should undoubtedly be assigned to *Georduboisia* nom. nov. on the basis of their body shape (pouch-like with a large holdfast organ inside), position of the ventral sucker close to the pharynx, presence of a massive vaginal sphincter, and other morphological characters (Sudarikov 1961; Dubois 1984; Niewiadomska 2002). Only two of the three nominal species of this genus have been recorded in Europe, *G. syriaca* and *G. teganuma* (Dubois 1984). According to Dubois (1984), these digenean species most clearly differ by the taxonomic groups of their definitive hosts (Ciconiidae vs. Podicipedidae). Morphological differences between them are weakly expressed and concern only the width of holdfast organ and the length of the cirrus sac. The minimum values of holdfast organ width and cirrus sac length in *G. syriaca* usually are greater than the corresponding maximum values in *G. teganuma* (Table 1).

Taxonomic affiliation of the hosts, the holdfast organ width, and the cirrus sac length indicate that the digeneans examined in our

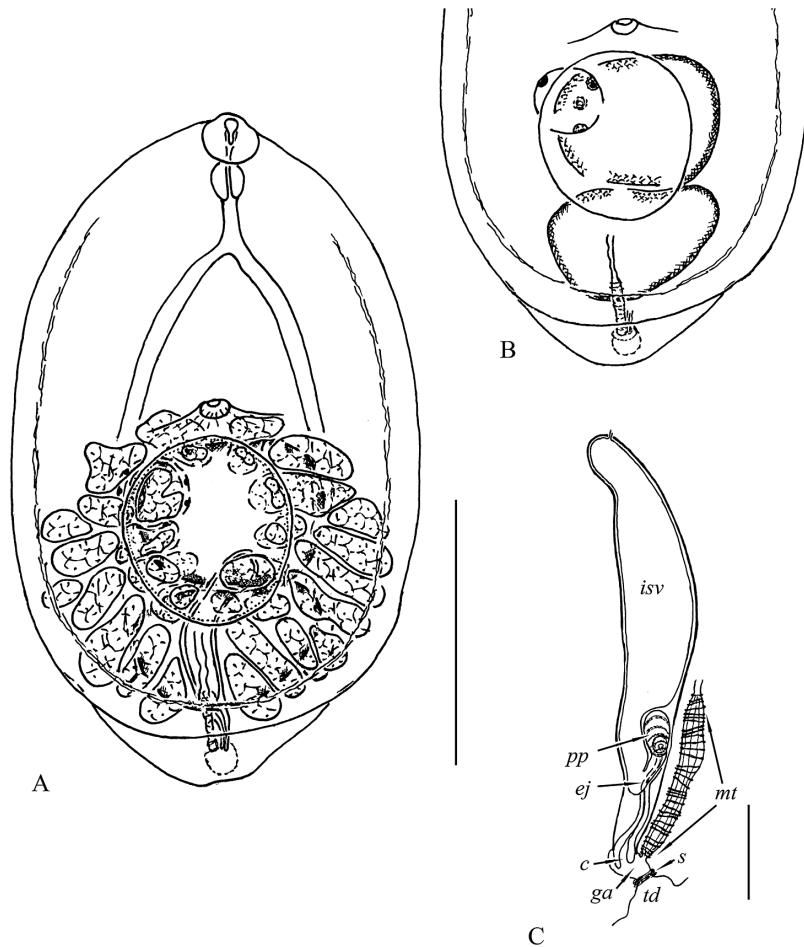


**Figure 2.** Terminal genitalia of *Georduboisia cf. teganuma*. (A) cirrus sac, entire dorsal view; (B) vaginal sphincter, invaginated cirrus and common genital atrium, sinistral view; Abbreviations: c, invaginated cirrus; cc, fragment of cirrus sac; ej, ejaculatory duct; ga, genital atrium; isv, internal seminal vesicle; pp, pars prostatica with field of prostatic cells; s, sphincter of genital atrium; td, terminal depression; u, uterus; v, vaginal sphincter. Scale bars: A = 50  $\mu\text{m}$ ; B = 100  $\mu\text{m}$ .

**Table 1.** Comparative characterisation of *Georduboisia syriaca*, *Georduboisia teganuma*, and *Georduboisia cf. teganuma* based on some metric traits (in  $\mu\text{m}$ )

Characteristic	<i>Georduboisia syriaca</i>	<i>Georduboisia teganuma</i>		<i>Georduboisia cf. teganuma</i>
	Dubois 1938; Janchev 1958; Dubois & Beverley-Burton 1971	Ishii 1935 (with correction of typographical error by Dubois 1938)	Sudarikov <i>et al.</i> (1973)	Our data
Locality	Central Europe, Western Asia, and Southern Africa	Far East	Eastern Europe and Central Asia	Eastern Europe
Body size	1075–2466 $\times$ 660–1651	830–1080 $\times$ 460–620	897–1249 $\times$ 469–772	1018–1412 $\times$ 692–817
Oral sucker size	90–190 $\times$ 114–156	98–103*	69–115 $\times$ 84–120	93–120 $\times$ 103–138
Pharynx size	75–152 $\times$ 53–111	75–94*	61–95 $\times$ 64–105	79–103 $\times$ 83–114
Ventral sucker size	50–152 $\times$ 57–106	–	50–83 $\times$ 50–87	– $\times$ 79–103
Holdfast organ size	– $\times$ 540–840	370–390*	– $\times$ 478	464–665 $\times$ 346–519
Left testis size	270–518 $\times$ 180–484	180–350*	275–425 $\times$ 110–250	692–817 $\times$ 138–180
Right testis size			262–475 $\times$ 120–250	450–720 $\times$ 138–201
Cirrus sac length	700–1056 (often $\geq$ 770)	–	262–500	369–720
Ovary size	114–245 $\times$ 76–210	120 $\times$ 90	55–150 $\times$ 50–155	111–152 $\times$ 104–138
Egg size	87–117 $\times$ 53–68	81 $\times$ 58	90–95 $\times$ 56	93–96 $\times$ 52–55

\*diameter

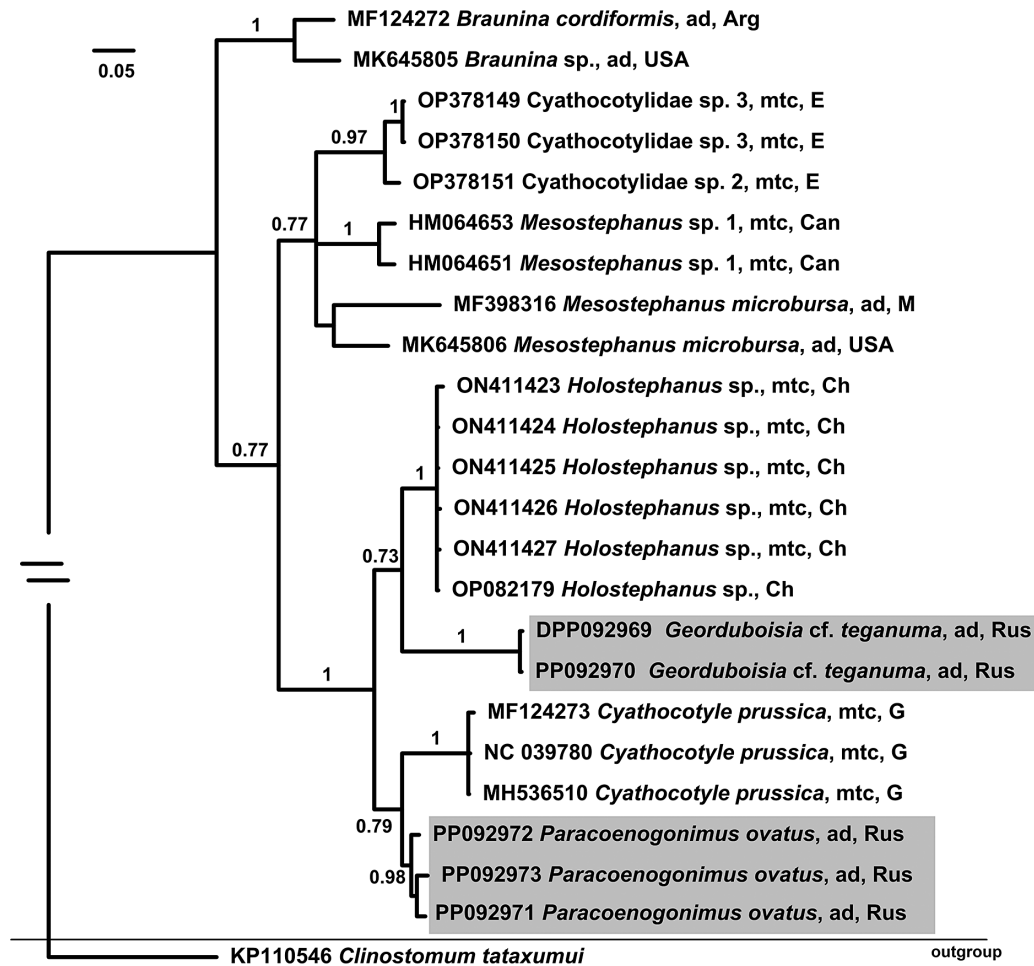


**Figure 3.** Paragenophore of *Paracoenogonimus ovatus*, from intestine of *Circus aeruginosus*, Astrakhan Region, Russia. (A) whole mount, ventral view; (B) body fragment with elements of female and male (only testes) reproductive systems (cirrus sac, vitellarium, and cecum removed from figure), ventral view; (C) terminal genitalia, dextro-lateral view. Abbreviations: *c*, partly evaginated cirrus; *ej*, ejaculatory duct; *ga*, genital atrium; *isv*, internal seminal vesicle; *mt*, metraterm; *pp*, presumptive pars prostatica; *s*, sphincter of genital atrium; *td*, terminal depression. Scale bars: A, B = 300  $\mu$ m; C = 50  $\mu$ m.

**Table 2.** Comparative characterization of *Paracoenogonimus* spp. based on some metric traits (in  $\mu$ m)

Characteristic	<i>Paracoenogonimus ussurensis</i>		<i>Paracoenogonimus ovatus</i>	
	Besprozvannykh & Ermolenko 2009	Komiya 1938; Odening 1963; Niewiadomska 2010	Ginetsinskaya & Koscheva 1959; Golovin 1958; Sudarikov 1961	Our data
Locality	Far East	Western and Central Europe	Eastern Europe	Eastern Europe
Body size	650–750 × 390–430	580–910 × 320–850	580–1000 × 357–770	758–824 × 464–575
Oral sucker size	44–50 × 49–55	46–72 × 49–90	38–88*	55–58 × 58–65
Pharynx size	27–35*	36–56 × 32–52	30–64*	41–52 × 34–40
Ventral sucker size	52 × 55	24–40 × 28–44	30–50*	– × 34–41
Holdfastorgan size	200–240*	140 × –	280–374*	215–284 × 172–242
Anterior testis size	110–130*	80–140 × 83–148	175–186*	145–212 × 186–204
Posterior testis size	110–120 × 130–150	92–160 × 114–172		125–213 × 192–248
Cirrus sac length	240–250	160–260	186–288	203–258
Ovary size	69–70 × 55–83	49–53 × 46–62	42–85*	76–104 × 76–104
Egg size	116–120 × 71–74	120–136 × 72–100	72–133 × 64–120	103–114 × 72–86

\*diameter



**Figure 4.** Phylogenetic relationships of *Georduboisia cf. teganuma* and *Paracoenogonimus ovatus* based on Bayesian inference analysis of partial *cox1* gene sequences. Posterior probability values lower than 0.6 are not shown. Development stages and biogeographical sampling places are indicated for the members of the Cyathocotylidae only. New sequences obtained in this study are highlighted by grey color frames. Abbreviations: *c*, cercaria; *mtc*, metacercaria; *ad*, adult; *Arg*, Argentina; *Can*, Canada; *Ch*, China; *E*, Egypt; *G*, Germany; *M*, Mexico; *Rus*, Russia; *USA*, United States of America.

study are probably conspecific with *G. teganuma*. However, this parasite species is characterised by oval testes (Ishii 1935; Sudarikov et al. 1973), whereas in our specimens the testes are usually very elongate and strongly convoluted or folded on themselves.

#### Genus *Paracoenogonimus* Katsurada, 1914

##### *Paracoenogonimus ovatus* Katsurada, 1914 (Figure 3)

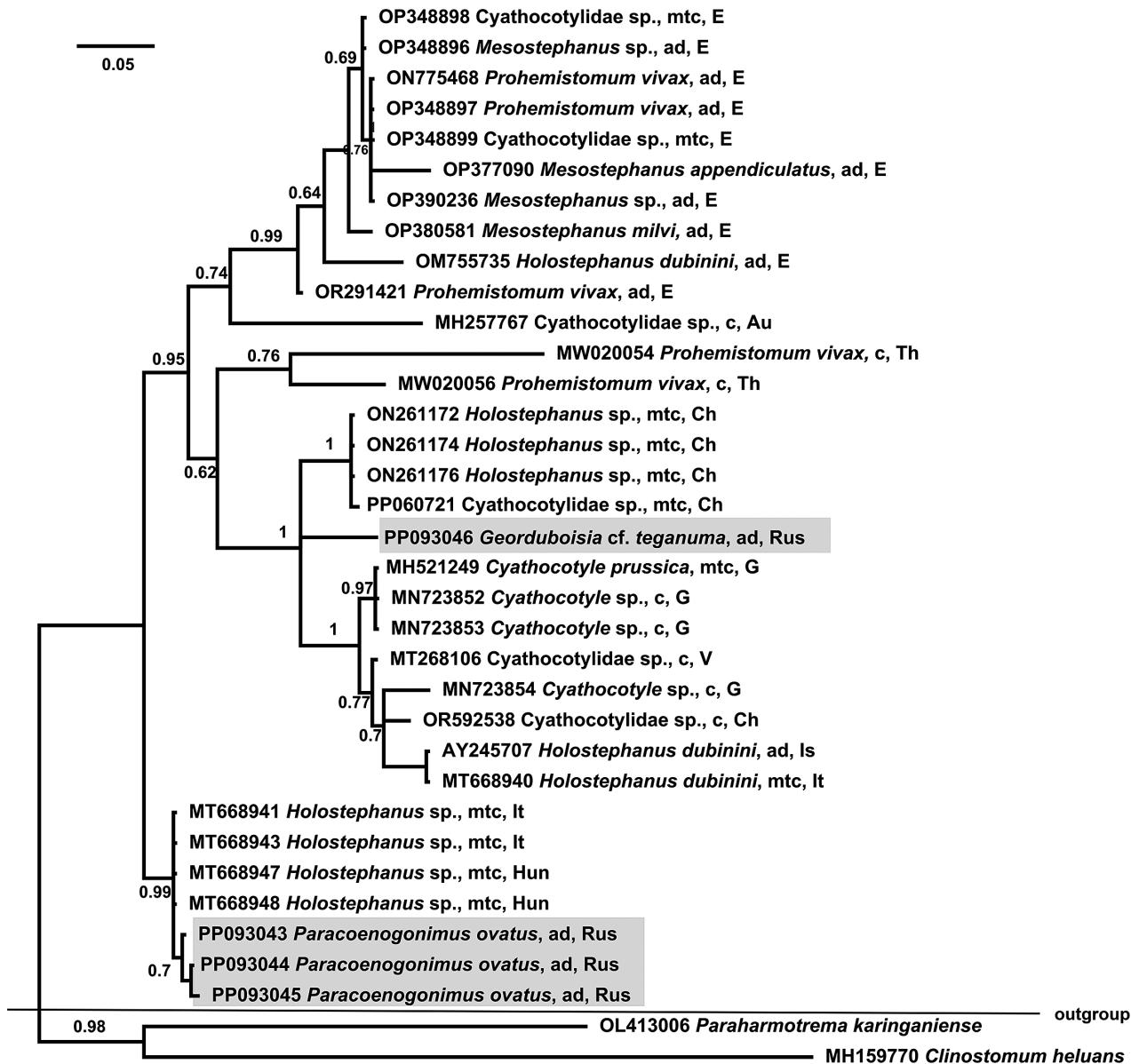
**Description.** Body divided into oval or ovoid concave prosoma and small subtriangular opisthosoma with terminal depression on posterior extremity; total body length 758–824, prosoma length 696–789, prosoma maximum width 464–575, opisthosoma length 35–62. Tegument with small spines. Oral sucker 55–58 × 58–65, opening subterminally. Ventral sucker usually with axis inclined anteriorly, 34–41 wide. Sucker-width ratio 1:0.53–0.71. Forebody 34.5–45.4% of total body length and 36.7–47.6% of prosoma length. Prepharynx absent. Pharynx 41–52 × 34–40. Ratio of oral sucker to pharynx based on mean diameter 1:0.41–0.60. Esophagus 48–69 long. Ceca terminating blindly near posterior extremity of prosoma. Holdfast organ usually everted, 215–284 × 172–242, posterior to ventral sucker. Two testes, slightly oblique, rarely tandem, contiguous, postacetabular; anterior testis oval, 208–228 × 138–166, sinistro-submedian; posterior testis transverse-oval to almost semicircular, 138–208 × 208–277, median, rarely sinistro-submedian. Cirrus sac elongate, in posterior third of body, length 203–258

(26.4–32.1% of total body length), maximum width 34–45, containing internal seminal vesicle, pars prostatica (presumably, as not clearly discernible), ejaculatory duct and cirrus. Internal seminal vesicle saccular over most of length and tubular distally, tubular part forms distinct loop. Presumptive pars prostatica vesicular, with muscular wall of uneven thickness (thickest wall on side facing proximal end of cirrus sac); prostatic cells indistinguishable. Cirrus invaginated or partly evaginated, poorly differentiated from ejaculatory duct. Genital atrium small, terminates with small sphincter, opens into terminal depression on posterior end of body. Ovary rounded, 76–104 × 76–104, strongly dextro-submedian, at level of anterior testis or intertesticular, partly overlapped by anterior or both testes dorsally. Ovarian complex indistinguishable. Distal part of uterus forms metraterm, 89–113 long; wall of metraterm with distinct circular and longitudinal musculature. Eggs few (1–2), 103–114 × 72–86. Vitelline follicles in two broad sublateral fields surrounding holdfast organ; fields separate posteriorly and confluent or separate anteriorly.

**Host.** *Circus aeruginosus* (Linnaeus, 1758) (Accipitridae).

**Site of infection.** Intestine.

**Material examined.** Five adult specimens (paragenophores), Kalinino Village, Astrakhan Region, Russia (46°20'9" N; 48°53'29" E); IPEE 14352.



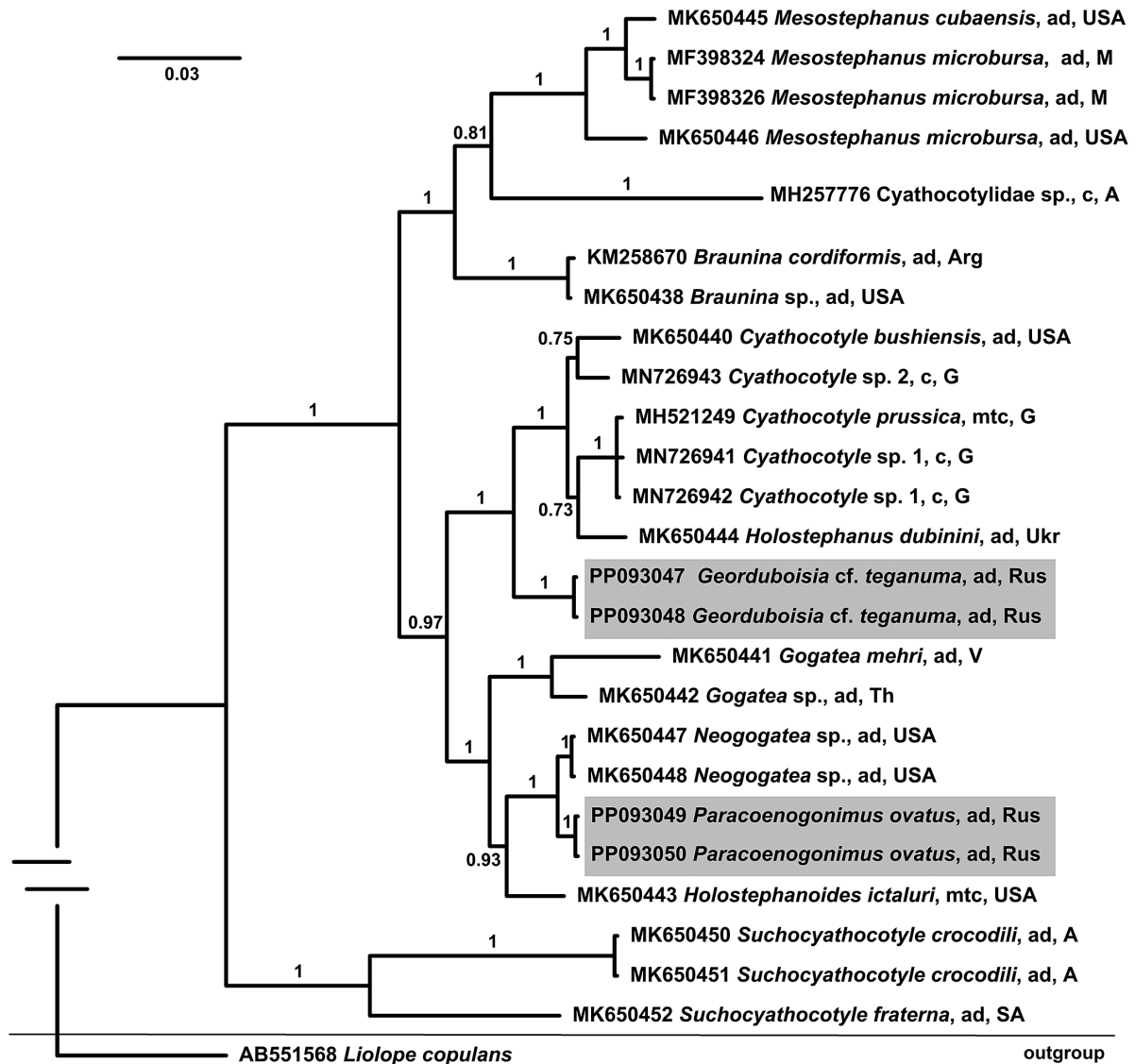
**Figure 5.** Phylogenetic relationships of *Georduboisia cf. teganuma* and *Paracoenogonimus ovatus* based on Bayesian inference analysis of partial 5.8S+ITS2 region sequences. Posterior probability values lower than 0.6 are not shown. Development stages and biogeographical sampling places are indicated for the members of the Cyathocotylidae only. New sequences obtained in this study are highlighted by grey color frames. Abbreviations: c, cercaria; mtc, metacercaria; ad, adult; Au, Australia; Ch, China; E, Egypt; G, Germany; Hun, Hungary; Is, Israel; It, Italy; Rus, Russia; Th, Thailand; V, Vietnam.

**Sequences deposited.** PP093043–PP093045 (5.8 + ITS2 region), PP093049–PP093050 (28S rRNA gene), PP092971–PP092973 (*cox1* gene).

### Remarks

The specimens examined in our study can be assigned to the genus *Paracoenogonimus* based on morphological characters such as the body divided into the oval or ovoid concave prosoma and the small subtriangular opisthosoma, the presence of the ventral sucker, the absence of the vaginal sphincter, the position of the relatively small holdfast organ in the posterior half of the prosoma, a non-massive cirrus sac and the vitellarium surrounding holdfast organ and gonads (Komiya 1938; Niewiadomska 2002).

Comparison of morphological data on the two known species of this genus, *P. ovatus* and *P. ussuriensis*, shows that they are differentiated from each other only by the size of the ventral sucker: 24–50 × 28–50 vs. 52 × 55 (Table 2). According to Besprozvannykh & Ermolenko (2009), adults of *P. ovatus* differ from those of *P. ussuriensis* also in the distribution of vitelline follicles: the follicles form two sublateral groups clearly separated from each other at the level of the anterior and posterior testes in the former species and are assembled in a semicircle with a gap at the level of the posterior testis in the latter species. However, these authors did not take into account the data of Golovin (1958) on the distribution of vitelline follicles in specimens of *P. ovatus* ex *Haliaeetus albicilla* (Linnaeus, 1758) from European part of Russia. The follicles in these specimens were arranged in a semicircle with a gap at the level



**Figure 6.** Phylogenetic relationships of *Georduboisia cf. teganuma* and *Paracoenogonimus ovatus* based on Bayesian inference analysis of partial 28S rRNA gene sequences. Posterior probability values lower than 0.6 are not shown. Development stages and biogeographical sampling places are indicated for the members of the Cyathocotylidae only. New sequences obtained in this study are highlighted by grey color frames. Abbreviations: c, cercaria; mtc, metacercaria; ad, adult; Arg, Argentina; Au, Australia; G, Germany; M, Mexico; Rus, Russia; SA, South Africa; Th, Thailand; Ukr, Ukraine; USA, United States of America; V, Vietnam.

of the posterior testis. The measurements of the ventral sucker of the specimens in our study correspond to those of *P. ovatus*.

### Phylogeny

We obtained partial sequences of the *cox1* gene from two adult specimens of *Georduboisia cf. teganuma* (one from the Astrakhan Region and one from the Rostov Region) and three adult specimens of *P. ovatus* (Astrakhan Region). The sequences of *Georduboisia cf. teganuma* were very similar to each other, differing by only one nucleotide. The sequences of *P. ovatus* were also similar to each other (*p*-distance 1.6–2.1%). Partial sequences of the 5.8S + ITS2 region were obtained from one adult specimen of *Georduboisia cf. teganuma* collected in the Astrakhan Region and from three adult specimens of *P. ovatus* from the same region. The three newly obtained sequences of this fragment of rDNA from adults of *P. ovatus* from the Astrakhan Region were similar to each other (*p*-distance 0.1–0.3%). Partial sequences of the 28S rRNA gene

were obtained from two adult specimens of *Georduboisia cf. teganuma* and two adult specimens of *P. ovatus*, all collected in the Astrakhan Region. These two sequences of *Georduboisia cf. teganuma* were identical, and so were the two sequences of *P. ovatus*.

Phylogenetic analysis based on partial *cox1* gene sequences (Figure 4) placed *Georduboisia cf. teganuma* as a poorly supported sister to the clade of metacercariae of *Holostephanus* sp. from *Carassius auratus* (Linnaeus, 1758) from China, whereas *P. ovatus* was a poor sister to the clade of metacercariae of *Cyathocotyle prussica* Mühling, 1896. In turn, the *Georduboisia cf. teganuma* + *Holostephanus* sp. appeared to be a strongly supported sister to the *P. ovatus* + *C. prussica* clade. The group containing all these species had a poorly supported sister relationship with the clade including *Mesostephanus* spp. and unidentified cyathocotylids. The other cyathocotylid digeneans involved in this analysis, namely *Braunina* spp., occupied a basal position to all the species mentioned above.



Phylogenetic analysis based on the sequences of the partial 5.8S + ITS2 region of nuclear DNA (Figure 5) placed *Georduboisia* cf. *teganuma* into the strongly supported polytomic clade that also contained the clade of metacercaria of *Holostephanus* spp. ex *C. auratus* (China) and *Cyprinus carpio* Linnaeus, 1758 (Hungary) and the clade formed by the adult specimen and metacercaria of *Holostephanus dubinini* Vojtek & Vojtkova, 1968, metacercaria of *C. prussica*, and cyathocotylid cercariae from molluscs of Europe, China, and Vietnam, unidentified with respect to genus or species. The above-mentioned metacercariae of *Holostephanus* sp. ex *C. auratus* of China were conspecific with those in the *cox1*-tree. The clade containing *Georduboisia* cf. *teganuma*, *H. dubinini*, *C. prussica*, and unidentified cyathocotylid cercariae appeared as a poorly supported sister to the also poorly supported group of cercariae obtained from the Thailand mollusk *Filopaludina polygramma* (von Martens, 1860) and identified as *Prohemistomum vivax* (Sonsino, 1892). The clade containing all above-mentioned species was a moderately supported sister to the poorly supported clade including *Mesostephanus* spp., specimens originally identified as *H. dubinini* and *P. vivax*, and some unidentified cyathocotylid cercariae and metacercariae. Adult specimens of *P. ovatus* examined in our study clustered in one strongly supported clade with the metacercariae from Hungarian and Italian fishes originally classified by Cech *et al.* (2021) as *Cyathocotylidae* gen. sp. (available in GenBank NCBI as *Holostephanus* sp.). P-distance between adults of *P. ovatus* and the metacercariae from Hungarian and Italian fishes are 0.4–0.5%. Therefore, we consider these metacercariae from Hungary and Italy as probably conspecific with *P. ovatus*. In turn, the clade of adult specimens of *P. ovatus* and metacercariae probably conspecific with them had a sister relationship with the large clade represented by all cyathocotylids mentioned above.

Phylogenetic analyses based on the partial 28S rRNA gene sequences (Figure 6) revealed *Georduboisia* cf. *teganuma* to be a strongly supported sister to the *H. dubinini* + *Cyathocotyle* spp. clade. *Cyathocotyle* spp. within the *H. dubinini* + *Cyathocotyle* spp. clade appeared as a paraphyletic assemblage, as one of the groups of *Cyathocotyle* spp. (metacercaria of *C. prussica* and two unidentified cercariae *Cyathocotyle* sp.) appeared to be sister to *H. dubinini* and not to the other congeners; however, this sister relationship was poorly supported. This analysis supported *P. ovatus* as sister to unidentified specimens of *Neogogatea* Chandler & Rausch, 1947. The *P. ovatus* + *Neogogatea* group was nested into the strongly supported clade also containing *Gogatea* spp. and *Holostephanoides ictaluri* Vernberg, 1952. This clade had a moderately supported sister relationship with the *Georduboisia* cf. *teganuma* + (*H. dubinini* + *Cyathocotyle* spp.) clade. The group of *Mesostephanus* spp., *Braunina* spp., and unidentified cyathocotylid cercariae from Australia appeared as a strongly supported sister to the large clade including all the above-mentioned cyathocotylids. In turn, the entire set of the species just mentioned was a sister to the *Suchocotylidae* spp. clade.

## Discussion

In this study, we provided morphological descriptions of adults of *Georduboisia* cf. *teganuma* and *P. ovatus*. *Georduboisia* cf. *teganuma* differed from *G. teganuma* studied by Ishii (1935) and Sudarikov *et al.* (1973) in the shape of the testes. Unfortunately, the collection material of these authors has not been preserved. We assume that Ishii (1935) and Sudarikov *et al.* (1973) examined

strongly pressured specimens, whose very elongate, convoluted testes could be perceived as oval. Our assumption that the testes of the specimens of *G. teganuma* studied by V. Sudarikov with coauthors could indeed be elongate and at the same time folded on itself or convoluted is supported by the following words of these authors: 'Forms with hypertrophied testes are sometimes found among normally developed specimens. In such forms large, rounded areas of the same histological structure as the testes themselves become separated from the body of the testes. In these cases, a regular symmetric arrangement of the testes is broken and one of them descends closer to the posterior end of the body' (Sudarikov *et al.* 1973, p. 59). However, due to this discrepancy in the testes morphology, we cannot be entirely sure about the species of the digeneans examined in our study. Therefore, we identify them as *Georduboisia* cf. *teganuma*. In addition, we re-examined the worms found in *P. cristatus* from the Novosibirsk Region (Russia) and identified as *G. teganuma* (IPEE 951/Tr) by Filimonova & Shalyapina (1975). The shape of the testes in them was the same as in our specimens.

We demonstrated the presence of a muscular metraterm in *P. ovatus*, the type species of *Paracoenogonimus*. This is the first report of a metraterm in this genus.

The results of our phylogenetic analyses based on the three genetic markers were apparently different, the differences being associated both with the sets of species involved in them and with the strength of the phylogenetic signal for deep or external nodules. The best-resolved phylogenetic relationships of *Georduboisia* cf. *teganuma* and *P. ovatus* were obtained using the 28S rRNA gene sequences. According to the latest variants of the system of cyathocotylid digeneans based on morphological data, *Georduboisia* nom. nov. (= *Duboisia* Szidat, 1936) is a member of the *Prosostephaninae*, which also includes *Prosostephanus* Lutz, 1935 and either *Neogogatea* (see Dubois 1987) or *Serpentostephanus* Sudarikov, 1961 (see Niewiadomska 2002). The absence of molecular data on *Prosostephanus* does not allow us to test the hypothesis about the proximity of *Georduboisia* nom. nov. to this genus. At the same time, our data do not support the conclusion of Dubois (1987) regarding the affinity of *Georduboisia* nom. nov. to *Neogogatea*. However, it should be emphasised that our recent conclusion about the absence of a close relationship between *Georduboisia* nom. nov. and *Neogogatea* is largely conditional because the genus affiliation of the specimens deposited in GenBank as *Neogogatea* sp. (MK650447, MK650448) was not confirmed morphologically. On the other hand, our data agree with the hypothesis of Sudarikov (1961) that *Cyathocotyle* Mühling, 1896 and *Holostephanus* Szidat, 1936 are related to *Georduboisia* nom. nov. Morphological evidence substantiating the phylogenetic position of *Georduboisia* nom. nov. based on molecular data is the undoubted similarity between its adult specimens and *Holostephanus* in body shape (e.g., Sudarikov 1961). The paraphyly of *Cyathocotyle* spp. revealed in the present study cannot yet be discussed in detail. This issue requires accumulation of molecular data on adult specimens of *Cyathocotyle* and *Holostephanus*.

Our data did not support the traditional hypotheses about the phylogenetic affinity of *Paracoenogonimus* to *Mesostephanus* (e.g., Dubois 1938, 1953, 1987; Sudarikov 1961; Niewiadomska 2002). *Paracoenogonimus* appeared in our 28S-tree within a strongly supported clade together with *Holostephanoides* Dubois, 1983 and the members of the *Szidatiinae* (*sensu* Niewiadomska 2002), *Neogogatea* (but see above), and *Gogatea* Lutz, 1935. Previously Achatz *et al.* (2019) demonstrated the clustering of *Holostephanoides* with these two szidatiine genera. *Paracoenogonimus* and

*Holostephanoides* are similar to *Neogogatea* and *Gogatea* only in general features characteristic of all cyathocotyliids. This means that the phylogenetic position revealed in our study has no obvious morphological confirmation. In general, it can be noted that the current classification of the Cyathocotyliidae at the subfamily level is inadequate for describing the phylogenetic relationships of its genera. In addition, it should be noted that our phylogenetic analyses revealed a number of controversial points in the identification of cyathocotyliids (in particular *H. dubinini* and *P. vivax*) whose sequences are represented in GenBank NCBI (Figure 5).

This is a new report of *Georduboisia* nom. nov. (= *Duboisia* Szidat, 1936) from podicipedid birds. Specimens of *Georduboisia* cf. *teganuma* described in this study have been found in the Rostov, Astrakhan, and Novosibirsk regions of Russia. *Georduboisia teganuma* (= *Cyathocotyle teganuma*), to which these digeneans are very similar, has been recorded in podicipedids in Japan, Kazakhstan, Buryatia, Bashkiria, and the Astrakhan Region (Sudarikov et al. 1973; Bayanov & Islamov 1977; Dorzhiev et al. 2021). Zhatkanbaeva (1964) found specimens of *Georduboisia* nom. nov. in *Podiceps grisegena* (Boddaert, 1783) and *Podiceps nigricollis* (Brehm, 1831) from Turgay lakes (Kazakhstan), identifying them as *G. syriaca*. These specimens are indeed similar to *G. syriaca* at least in cirrus sac length (785 according to the drawing of Zhatkanbaeva 1964). A rather high morphological similarity between *G. syriaca*, *G. teganuma*, and *Georduboisia* cf. *teganuma* raises the question of their possible synonymy. However, to address this question, it is necessary to involve molecular data on *Georduboisia* nom. nov. isolates from other localities and hosts, particularly from storks (Ciconiidae).

*Paracoenogonimus ovatus* has been repeatedly recorded in Western and Central Europe (e.g., Komiyama 1938; Edelényi 1974; Niewiadomska 2010) and Eastern Europe, including the Astrakhan Region (e.g., Golovin 1958; Ginetsinskaya & Koscheva 1959). In most locations only metacercariae of this parasite were recorded, which can be explained by limited data on the parasite fauna of fish-eating birds in these regions. Our phylogenetic analysis based on the 5.8S + ITS2 dataset showed that the metacercariae from the musculature of fish from Hungary and Italy were probably conspecific with adults of *P. ovatus*. Metacercariae of *P. ovatus* exhibit a weak specificity to fish hosts (Ostrowska et al. 2019). Wierzbicka & Einszporn-Orecka (1972) observed strong emaciation in *A. brama* when the density of these metacercariae reached 95 ind.·g<sup>-1</sup> of host muscle. However, Ostrowska et al. (2019) did not detect any pathological changes in *R. rutilus* even when the density of infection with metacercariae of this digenean species was very high (66 ind.·g<sup>-1</sup>).

It has been suggested that metacercariae of *P. ovatus* may potentially be harmful to humans (Sudarikov 1961). This suggestion was based on reports that mammals could be experimentally infected with *P. ovatus* (e.g., Komiyama 1938; Ginetsinskaya & Koscheva 1959). Interestingly, however, the zoonotic potential of metacercariae of *P. ovatus* (incorrectly identified as *Holostephanus* sp.) was not confirmed in the experiments of Sándor et al. (2020). A possible explanation of these results is that the parasites from birds and from mammals identified as *P. ovatus* belong, in fact, to different species, as has been demonstrated to be the case with the *Apophallus donicus* Skrjabin & Lindtrop, 1919 species complex (Sokolov et al. 2023).

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**Ethical standard.** This article does not include any studies involving animal experiments. The birds were shot by licensed hunters during the hunting seasons under permission of Department of Environmental Management and Environmental Protection of Astrakhan Oblast (#000003 dated 20 February 2017).

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