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The *Proteocephalus* species-aggregate in freshwater centrarchid and percid fishes of the Neartic Region (North America)

RNDr. Thesis

Bc. Lucie Uhrová

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Annotation: In the present paper, three species of the *Proteocephalus*-aggregate de Chambrier, Zehnder, Vaucher and Mariaux, 2004 (Cestoda: Proteocephalidae) from centrarchid and percid fishes from North America are reviewed and recognized as a valid: (1) *Proteocephalus fluviatilis* Bangham, 1925, (2) *Proteocephalus luciopercae* Wardle, 1932, (3) *Proteocephalus pearsei* La Rue, 1919 and additionaly *Proteocephalus ambloplitis* (Leidy, 1887), which does not belong to this *Proteocephalus* aggregate, is provided for the first time. Moreover, molecular analysis of the contatenated (28S rDNA+COI) dataset of *Proteocephalus*-aggregate species is presented.

Declaration [In Czech]

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THE *PROTEOCEPHALUS* SPECIES-AGGREGATE IN FRESHWATER CENTRARCHID AND PERCID FISHES OF THE NEARCTIC REGION (NORTH AMERICA)

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KEY WORDS

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ABSTRACT

In the present paper, species of the *Proteocephalus*-aggregate de Chambrier, Zehnder, Vaucher, and Mariaux, 2004 (Cestoda: Proteocephalidae) reported from centrarchid and percid fishes in North America are reviewed, and their taxonomic status is critically assessed based on a study of type specimens and new material from Canada and the United States. The following 3 species, supposedly strictly specific to their fish definitive hosts, are recognized as valid: (1) *Proteocephalus fluviatilis* Bangham, 1925 (new synonyms *Proteocephalus osburni* Bangham, 1925 and *Proteocephalus microcephalus* Haderlie, 1953; *Proteocephalus* '*robustus*' nomen nudum) from the smallmouth and largemouth bass, *Micropterus dolomieu* (Lacépède) (type host) and *Micropterus salmoides* (Lacépède) (both Centrarchidae); (2) *Proteocephalus luciopercae* Wardle, 1932 (new synonym *Proteocephalus stizostethi* Hunter and Bangham, 1933) from the walleye, *Sander vitreus* (Mitchill) (type host), and sauger, *Sander canadensis* (Griffith et Smith) (Percidae); and (3) *Proteocephalus pearsei* La Rue, 1919, a parasite of the yellow perch, *Perca flavescens* Mitchill (Percidae). All species are illustrated based on new, properly heat-fixed material. Scanning electron micrographs of the scoleces of percid tapeworms *P. luciopercae* and *P. pearsei*, as well as the bass tapeworms *P. fluviatilis* and *Proteocephalus ambloplitis* (Leidy, 1887), the latter of which does not belong to this *Proteocephalus*-aggregate, are provided for the first time together with a simple key to species identification of proteocephalids from centrarchiform and perciform teleost fishes.

The freshwaters of North America harbor an extraordinarily rich fauna of bony fishes (Warren and Burr, 2014), including some archaic (evolutionarily early branching) groups such as sturgeons (Acipenseridae), bowfin (Amiidae), and gars (Lepisosteidae). North American fishes are also hosts of a great variety of helminth parasites, including tapeworms (see Hoffman, 1999). Some of the cestode groups occurring in North American freshwater fishes were revised at the beginning of the 20th century, such as members of the families Proteocephalidae La Rue, 1911 (formerly the order Proteocephalidea Mola, 1928), Bothriocephalidae Blanchard, 1849, and Caryophyllaeidae Leuckart, 1878 (La Rue, 1911; Cooper, 1919; Essex, 1928; Hunter, 1930). However, since those early works, attention to fish cestodes has waned dramatically over the second half of the 20th century to present, and the current knowledge of the diversity, distribution, host associations, life cycles, and interrelationships of these parasites remains insufficient (Scholz and Choudhury, 2014; de Chambrier et al., 2017; Scholz and Oros, 2017).

Over the 2 last decades, one of the authors (A.C.) was able to collect and accumulate freshly and consistently fixed specimens of tapeworms from a variety of fish hosts in Canada and the United States that were previously unavailable for study. This new material enabled us to critically examine the validity of many of the cestode species reported from North American freshwater fishes. Therefore, the forthcoming series of taxonomic papers focused on fish tapeworms aims to provide a robust baseline for future ecological and evolutionary studies on one of the dominant groups of intestinal helminths parasitizing freshwater fishes in North America (Scholz and Kuchta, 2017).

In the first revision of proteocephalid cestodes published more than a century ago, La Rue (1914) listed only 10 species of the genus *Proteocephalus* Weinland, 1858, including 2 species inquirendae. A few decades later, the number of North American species of *Proteocephalus* increased to 23 (Wardle and McLeod, 1952), whereas Schmidt (1986) and Hoffman (1999) reported as many as 36 and 34 species, respectively. In contrast, de Chambrier et al. (2017) considered only 24 species of the genus as valid, including 6 species from centrarchid and percid fishes, because

Hanzelová and Scholz (1999) and Scholz and Hanzelová (1999) synonymized 11 Nearctic species.

A recent molecular phylogenetic study revealed the genus *Proteocephalus* as an artificial assemblage of at least 7 lineages that are not closely related to one another (de Chambrier et al., 2015). The study also showed that North American species of the genus do not form a monophyletic group. Some species, such as *Proteocephalus fluviatilis* Bangham, 1925 and *Proteocephalus pinguis* La Rue, 1911, are closely related to Palearctic taxa of the *Proteocephalus*-aggregate proposed by de Chambrier et al. (2004). In contrast, other species including *Proteocephalus ambloplitis* (Leidy, 1887) from centrarchids, bowfin (*Amia calva*), and ictalurids (Siluriformes: Ictaluridae), and *Proteocephalus perplexus* La Rue, 1911, from bowfin, are more closely related to Neotropical proteocephalids (de Chambrier et al., 2004, 2009, 2015; Hypša et al., 2005).

The species of the *Proteocephalus*-aggregate de Chambrier, Zehnder, Vaucher, and Mariaux, 2004, which are parasites of Holarctic freshwater fishes, are typified, among others, by the possession of a simple scolex with or without an apical organ (in fact, a muscular, often vestigial/non-functional apical sucker), tightly packed testes, lateral bands of the vitelline follicles not exceeding the anterior or middle part of the ovary, uterine development 2 according to de Chambrier et al. (2004), and uterine diverticula occupying most of the width of gravid proglottids (for a complete diagnosis, see de Chambrier et al., 2004).

In the present study, which is the first article of a planned series focused on North American species of *Proteocephalus* tapeworms, species of the *Proteocephalus*-aggregate from percomorph fishes of the families Centrarchidae (bass) and Percidae (perch, pikeperch) are reviewed on the basis of a critical examination of types and vouchers from museum collections and newly collected material of most nominal species. Original illustrations of all species recognized as valid are provided together with the first scanning electron micrographs (SEM) of the scoleces of 4 species, 2 from percids and 2 from centrarchids. In addition, results of molecular phylogenetic analyses of these tapeworms are presented, and a key to the identification of species of the *Proteocephalus*-aggregate from centrarchiform and perciform fishes in North America is also provided.

The so-called bass tapeworm, *Proteocephalus ambloplitis*, which also occurs in the largemouth and smallmouth bass, is not treated in the present paper (except for SEM micrographs of its scolex for comparison with *P. fluviatilis*) because it is not closely related to species of the *Proteocephalus*-aggregate (de Chambrier et al., 2004, 2015). *Proteocephalus ambloplitis* is closely related to the species that form a big clade composed mainly of parasites of Neotropical siluriforms ('internal Neotropical' clade of Hypša et al., 2005). A more detailed treatment of this parasite will be provided in a forthcoming paper on species of '*Proteocephalus*' from gars and bowfin.

MATERIALS AND METHODS

The present revision is based on the examination of type and voucher specimens from museum collections and newly collected material of *Proteocephalus* spp. from North America (see below). Tapeworms collected by the present authors were detached from the intestinal wall, gently rinsed in PBS or 0.9% NaCl solution, and fixed in hot buffered 10% formalin (~4% formaldehyde solution) or killed in hot, almost boiling, water and immediately fixed in unheated 10% buffered formalin or AFA (FAA). Tapeworms were stored in 70% ethanol after fixation and then stained with acetocarmine or Ehrlich's hematoxylin, dehydrated in an ascending series of ethanol, cleared in methyl salicylate or xylene, and mounted in Canada balsam on slides (for details on methodology, see Scholz et al., 2019). For counts of the testes, data were collected, often using illustrations, from the last mature and the first pregravid proglottids.

For SEM observations, the anterior portions of 2 specimens of *P. fluviatilis*, 2 specimens of *Proteocephalus luciopercae*, and 1 specimen of *Proteocephalus pearsei* were post-fixed in 1.0% osmium tetroxide in 0.15 M phosphate buffer (pH 7.2) for 1–2 hr, washed in 0.15 M phosphate buffer (pH 7.2) for 2 changes of 10 min each and briefly with dH_2O (distilled water), and then dehydrated through a graded ethanol series and infiltrated with hexamethyldisilizane (HMDS). Following infiltration, the HMDS was allowed to evaporate off the specimens; specimens were then mounted on stubs, sputter coated with gold, and scanned using a Hitachi TM3030+ SEM (St. Norbert College; Hitachi, Ltd., Tokyo, Japan). Digital SEM images captured on the Hitachi TM3030+ SEM unit were adjusted for appropriate brightness and contrast using 'Photos' software in Windows 10.

The following museum abbreviations were used in the paper: HWML = Harold W. Manter Laboratory, Lincoln, Nebraska; IPCAS = Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic; USNM = National Museum of Natural History, Washington, D.C. (now hosting the previous U.S. National Parasite Collection in Beltsville, Maryland [USNPC]). Fish names follow Froese and Pauly (2019); the orders proposed by Betancur-R. et al. (2017), i.e., Centrarchiformes and Perciformes of the Percomorphaceae, are used.

Genomic DNA was extracted from 96% ethanol-preserved specimens using E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, Norcross, Georgia). Amplification of a partial sequence of the nuclear large subunit ribosomal rRNA gene (*lsr*DNA) was done using the primers LSU5 and 1500R following Waeschenbach et al. (2007). A complete sequence of mitochondrial cytochrome c oxidase subunit 1 gene (*COI*) was amplified with primers B2-TrpF and B2-16SR according to the protocol of de Chambrier et al. (2019). PCR products were gel-checked, purified with Exonuclease I and FastAP alkaline phosphatase enzymes (Thermo Fisher Scientific, Waltham, Massachusetts), and Sanger-sequenced at SeqMe (Dobříš, Czech Republic). Contiguous gene sequences were assembled and checked in Geneious 7.1.9 (http://www.geneious.com; Kearse et al., 2012). *COI* assemblies were trimmed to the protein-coding region. Analysis of both *lsr*DNA and *COI* data partitions followed exactly the strategy described in de Chambrier et al. (2019). The phylogenetic tree was estimated based on the concatenated data set under the maximum likelihood (ML) criterion in IQ-TREE 1.6.5 (Nguyen et al., 2015) using the following partition scheme and evolutionary models selected according to the corrected Akaike information criterion: *lsr*DNA, GTR+F+G4; *COI* 1st, TIM2+F+I; *COI* 2nd, TN+F+I; *COI* 3rd, TIM2+F+I+G4.

DESCRIPTION

Survey of species of the *Proteocephalus*-aggregate from percomorph fishes

(Figs. 1–7)

Proteocephalus fluviatilis Bangham, 1925

(Figs. 1, 2, 3A–C)

Synonyms: Proteocephalus '*robustus*' of G. R. La Rue, nomen nudum; *Proteocephalus osburni* Bangham, 1925, new synonym; *Proteocephalus microcephalus* Haderlie, 1953, new synonym.

Material examined: Three specimens from *Micropterus dolomieu*, Caesar Creek, Ohio, collected by R. V. Bangham on 1 and 2 August 1922 (USNM 1356226; not explicitly labeled as type specimens, but apparently serving for species description); 1 immature (74 mm long) specimen from largemouth bass, *Micropterus salmoides*, from Paul Lake, Michigan, collected in June 2012, and a mature specimen from *M. dolomieu* from Little Trout Lake, Wisconsin, in June 2017; syntypes of *P. microcephalus*, including several fragments on 1 slide, 3 fragments with scoleces, all with withdrawn apical part, from *M. dolomieu*, Putah Creek, near Middletown, Lake County, California, collected by E. C. Haderlie in July 1949 (USNM 1337884/USNPC 37194).

Morphological description: Bangham (1925), Shimazu (1990).

Type host: Micropterus dolomieu Lacépède (Centrarchiformes: Centrarchidae).

Additional definitive hosts: Ambloplites rupestris (Rafinesque), *Micropterus salmoides* Lacépède (all Centrarchiformes: Centrarchidae); record of *P. fluviatilis* from *Lepomis auritus* (Linnaeus) presented by Hoffman (1999) is erroneous (see below).

Type locality: Southern Ohio streams, Ohio.

Type material: USNM 1348663 (syntypes, specimens in vials).

Life cycle: Fischer (1968) studied development of the parasite in copepod intermediate host and seasonality in the occurrence and maturation in the definitive host. He found the copepods *Cyclops bicuspidatus* Claus, *Cyclops scutifer* Sars, *Cyclops vernalis* Fischer, and *Tropocyclops prasinus* (Fischer) as suitable experimental intermediate hosts of *P. fluviatilis*. Development in copepods took 18–21 days at 18 C.

Distribution: Canada (Ontario), United States (Michigan [new geographical record], Ohio, Pennsylvania, South Dakota, Wisconsin); introduced with bass to Japan (Shimazu, 1990).

Representative DNA sequences and phylogenetic relationships: Currently available sequence data originate from 2 specimens: (1) ITS2 (AY551163) and V4 region of *ssr*DNA (AY551126) from *M. salmoides* by Hypša et al. (2005); (2) D1–D3 region *lsr*DNA (KP729390), complete *ssr*DNA (KX768932), mitochondrial *rrn*L (KX768925), and *COI* (KX768945) from *M. dolomieu* from Japan by de Chambrier et al. (2015) and Scholz et al. (2017). Phylogenetic analyses by Hypša et al. (2005) and Scholz et al. (2007) estimated *P. fluviatilis* to form a sister lineage to 2 Holarctic species, *Proteocephalus filicollis* (Rudolphi, 1802) from the 3-spined stickleback, *Gasterosteus aculeatus* Linnaeus, and *Proteocephalus macrocephalus* (Creplin, 1825) from eels (*Anguilla* spp.), and the Palearctic *Proteocephalus gobiorum* Dogiel and Bychowsky, 1939 from gobiid fishes. de Chambrier et al. (2015) found *P. fluviatilis* to form a clade with the former 2 Holarctic species as well as *P. pinguis* from pikes, *Esox* spp., excluding *P. gobiorum* from the group. The current analysis favors sister-group relationship of *P. pinguis* and *P. fluviatilis* but failed to find convincing statistical support for this scenario (Fig. 7).

Two species that commonly occur in the largemouth and smallmouth bass, i.e., *P. fluviatilis* and *P. ambloplitis*, are not closely related. The former species belongs to the Holarctic *Proteocephalus*-aggregate, whereas *P. ambloplitis* is closely related to the species that form a big clade composed mainly of parasites of Neotropical siluriforms (de Chambrier et al., 2004; "internal Neotropical" clade of Hypša et al., 2005). We characterized the *lsr*DNA of a representative specimen of *P. ambloplitis* from smallmouth bass and were able to confirm that it clusters with the remaining representatives of *P. ambloplitis* from other fish hosts characterized previously, forming a clade of unstable position within the highly derived clade of proteocephalids, distant from the relatively more basal lineage of *Proteocephalus*-aggregate (data not shown).

Remarks

The species was described by Bangham (1925) based on tapeworms found in *M. dolomieu* from southern Ohio streams. Shimazu (1990) re-described the species, which was introduced to Japan (Nagano Prefecture, Honshu). He compared tapeworms found in *M. salmoides* with some of the specimens of Bangham (1925) (USNM 1356226) and did not find substantial differences, except for the apical sucker, which was described by Bangham (1925) as functional. Similarly, as observed by Fischer (1968), Shimazu (1990) found that the apical sucker of *P. fluviatilis* was a cellular mass without a cavity. Examination of the newly collected specimens from Michigan and 3 of Bangham's specimens (USNM 1356226) confirms that the sucker is present, but it is non-functional (vestigial, without any cavity) (Figs. 1B, C, 3A–C). The only difference we detected is in the alleged presence of a vaginal sphincter in the species. Bangham (1925) stated "Sphincter vaginæ weak," and Shimazu (1990) stated "weakly developed," but the sphincter was not observed in the studied material (Fig. 2A–D).

Haderlie (1953) described *P. microcephalus* from smallmouth bass, *M. dolomieu*, from Putah Creek near Middletown in California. Even though the morphological description of Haderlie (1953) looks at first glance to be relatively detailed, it was evidently based on poorly fixed specimens that were not fully mature. Examination of syntypes (USNM 1337884) revealed that all mounted scoleces are deformed, and some have the suckers withdrawn (Fig. 1D). Haderlie (1953, p. 341) wrote, "No trace of an apical or fifth sucker," but an apical sucker is in fact present, even though it is non-functional, without a cavity (vestigial) (Fig. 1D). The number of the testes reported by Haderlie (1953), i.e., 40–70 testes, is somewhat lower than we counted in syntypes (75 testes). Illustrations of *P. microcephalus* provided by Haderlie (1953) are schematic (e.g., the ovary was drawn as 2 separated lobes not connected with an isthmus, the cirrus was not differentiated from the internal sperm duct and ejaculatory duct, etc.). The differential diagnosis of *P. microcephalus* is very vague ("A detailed study of this monograph [= La Rue, 1914] and of papers dealing with the genus since 1914 has been made, but none of the species described will accommodate the cestode from the smallmouth bass of California.") and the new species was not differentiated explicitly from any of its congeners, including the species previously reported from *M. dolomieu*, i.e., *P. ambloplitis*,

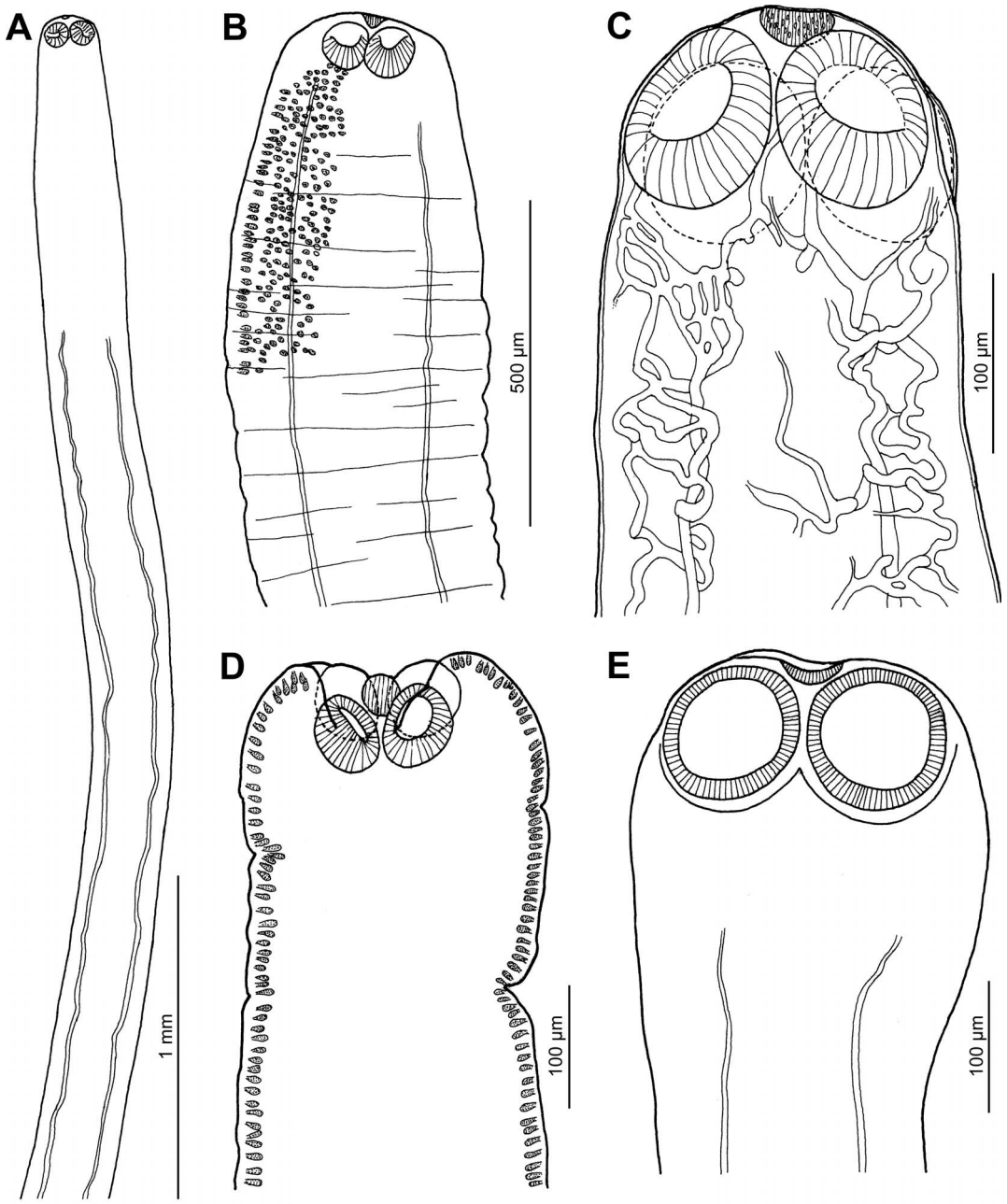

Figure 1. *Proteocephalus fluviatilis* Bangham, 1925 from (**A, C**) *Micropterus salmoides* (Lacépède), Michigan (IPCAS C-364/2) and (**B**) *M. dolomieu* (Lacépède), Ohio (material of R. V. Bangham, USNM 1356226). *Proteocephalus micropterus* Haderlie, 1953 (= new syn. of *P. fluviatilis*) from (**D**) *M. dolomieu*, California; syntype (USNM 1337884). (**E**) *Proteocephalus* '*robustus*' of G. R. La Rue (nomen nudum) from *M. dolomieu* (USNM 1356212). (**A**) Anterior part of the body; (**B–E**) frontal view of the scolex; note the presence of a vestigial apical sucker in all specimens.

P. fluviatilis, *P. osburni*, and *Proteocephalus stizostethi*. Based on examination of its syntypes, *P. microcephalus* is indistinguishable from *P. fluviatilis*, with which it is hereby synonymized.

A specimen (designated 'holotype') labeled by G. R. La Rue '*Proteocephalus robustus*' from *M. dolomieu*, Huron River, Michigan, collected on 6 June 1920, is deposited at USNM (Coll. No. 1356212). However, this species was never described, and thus it is a nomen nudum. The tapeworm is almost indistinguishable from *P. fluviatilis* (which was described 5 yr later), possessing a robust strobila composed of short and wide proglottids with a flask-shaped, short cirrus sac (215–220 µm), representing only about one-tenth of the proglottid length, overlapping proximally (medially) with the lateral-most vitelline follicles only by a small part, and having a vagina opening anterodorsal or dorsal to the cirrus sac (compare Fig. 2B with Fig. 2C).

The only difference between both taxa is the shape of the scolex (claviform in *P. robustus*) with relatively large suckers (diameter 110–117 µm), but the anterior end of specimens of *P. robustus* is unnaturally contracted (Fig. 1E), which may have considerably changed the shape of the scolex. The apical sucker of *P. robustus*

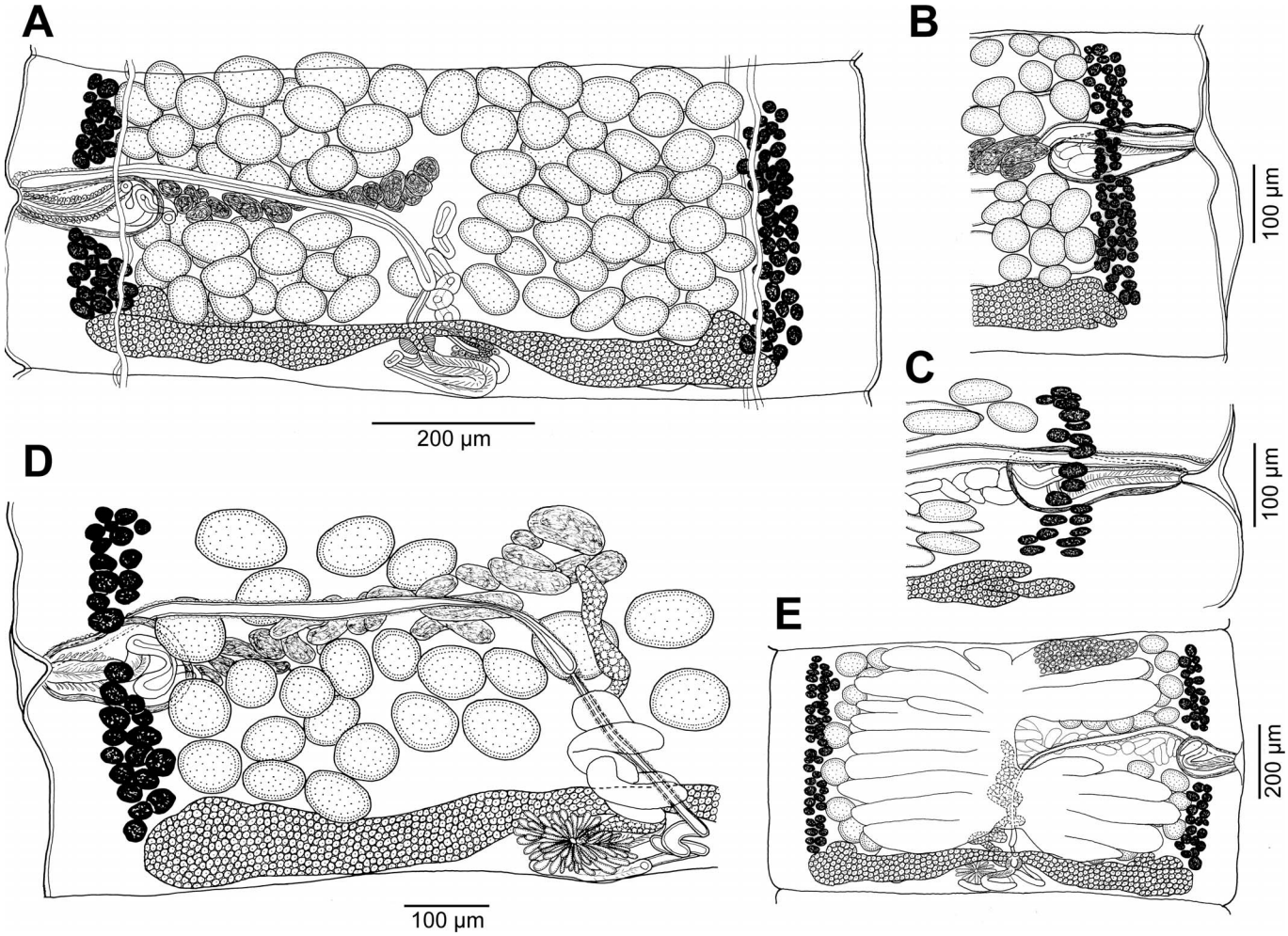

Figure 2. *Proteocephalus fluviatilis* Bangham, 1925 from (**A, D, E**) *Micropterus salmoides*, Michigan (IPCAS C-364/2) and (**B**) *M. dolomieu* (specimens of R. V. Bangham; USNM 1356226). (**C**) *Proteocephalus* '*robustus*' of G. R. La Rue (= nomen nudum) from *M. salmoides* (USNM 1337884). (**A**) Mature proglottid, ventral view; (**B**, **C**) terminal genitalia, dorsal view; note short, pyriform cirrus sac and absence of a vaginal sphincter; (**D**) posterolateral part of a pregravid proglottid, dorsal view; note the anterior position of the seminal receptacle situated at a distance from the ovarian isthmus; (**E**) gravid proglottid, ventral view; eggs illustrated only in the anteriormost uterine diverticulum on the poral side.

is also almost identical to that of *P. fluviatilis*, just being slightly more flattened (diameter 60 µm; Fig. 1E). Therefore, the only existing specimen of '*P. robustus*,' which was never described, is considered conspecific with *P. fluviatilis*.

In the same paper as *P. fluviatilis*, another species, *P. osburni*, from smallmouth bass in the Akron Hatchery, Ohio, was briefly and apparently erroneously (see below) described by Bangham (1925) based on a single, reportedly immature specimen with only 7 (?!) proglottids found. However, uterine diverticula are reported to be present, and a gravid proglottid is illustrated in his fig. 17 (Bangham, 1925). The species was differentiated from *P. fluviatilis* found in the same fish host by the size of the V-shaped (?) cirrus sac (280–360 µm long × 72–112 µm wide vs. 160–212 × 52–68 µm in *P. fluviatilis*). However, Bangham (1925) apparently misinterpreted a strongly coiled external sperm duct as the proximal part of the cirrus sac, which is obvious from his illustration of the gravid proglottid (fig. 17 of Bangham, 1925). In a mature proglottid of *P. osburni* (fig. 19 of Bangham, 1925), the cirrus sac is small and indistinguishable in size and shape from that of *P. fluviatilis* described earlier in the same paper (figs. 13, 18 of Bangham, 1925). In fact, *P. osburni* does not differ from *P. fluviatilis* in any other morphological and biometrical characters either, such as the shape and size of the scolex, suckers, and apical sucker. Therefore, these taxa are considered conspecific, and *P. osburni* becomes a new synonym of *P. fluviatilis* because it was described later (p. 261) than *P. fluviatilis* (p. 258).

Proteocephalus fluviatilis appears to be a specific parasite of centrarchids, especially smallmouth and largemouth bass, in North America. Hoffman (1999) listed rock bass, *A. rupestris*, and redbreast sunfish, *L. auritus*, as hosts of *P. fluviatilis* based on a single report by Pluto and Rothensbacher (1978) from Pennsylvania. These authors found 11 tapeworms in 3 of 23 rock bass (prevalence 13%, mean intensity of infection 4, range 1–7 specimens), but they did not specify whether the worms were mature or juvenile. In contrast, 24 of 25 smallmouth bass, *M. dolomieu*, harbored 302 tapeworms (prevalence 96%, mean intensity 12, range 1–75). No *Proteocephalus* tapeworms were found in 19 redbreast sunfish, *L. auritus* (only a few specimens of

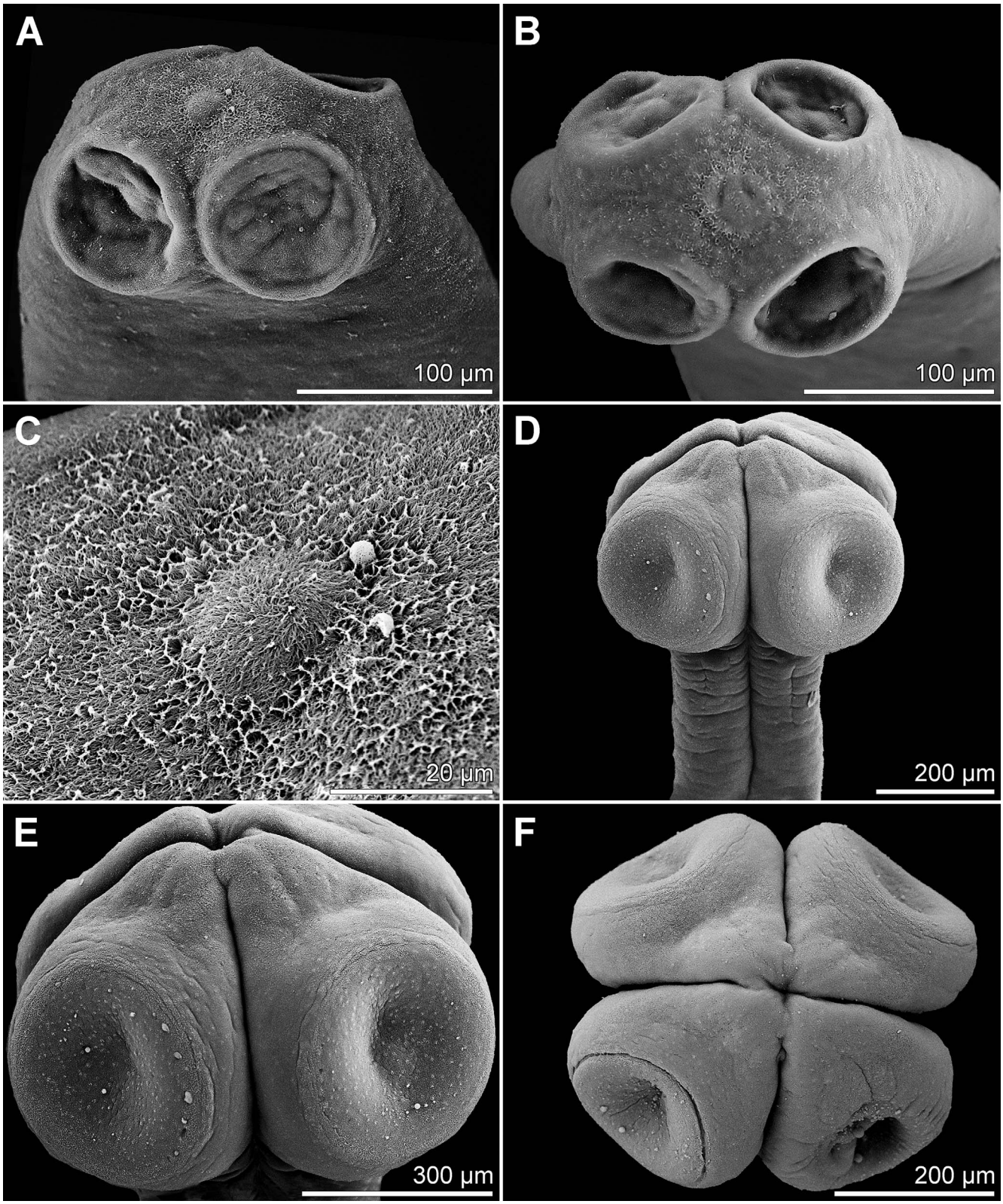

Figure 3. Photomicrographs of the scoleces of *Proteocephalus* species from centrarchids in North America. (**A–C**) *Proteocephalus fluviatilis* Bangham, 1925 from *Micropterus salmoides* (Lacépède), Michigan; (**D–F**) *Proteocephalus ambloplitis* (Leidy, 1887) from *Micropterus salmoides* (Lacépède), Michigan. (**A, D**) frontal view; (**B, F**) apical view; (**C**) detail of apical sucker; (**E**) detail of scolex, frontal view.

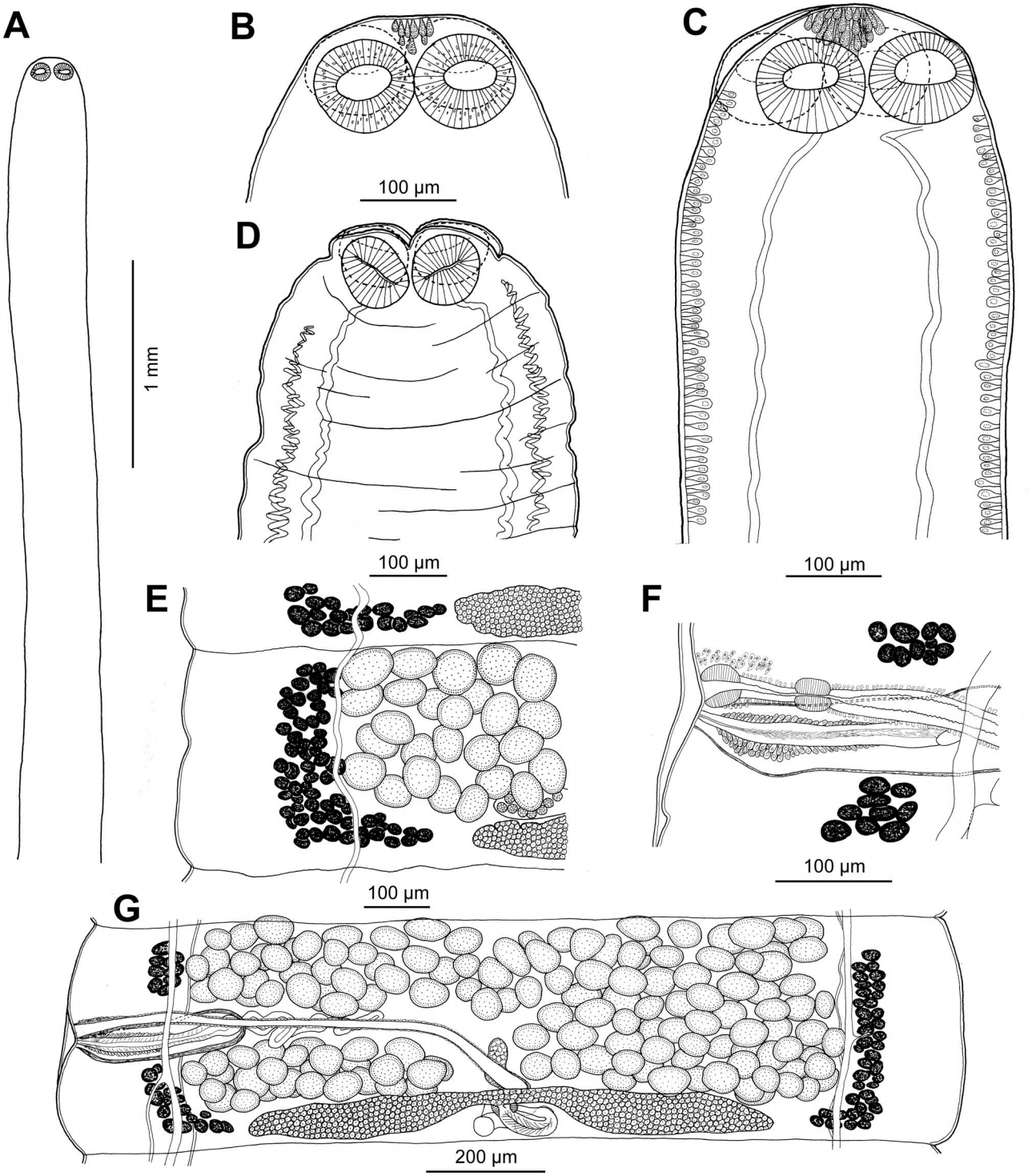

Figure 4. *Proteocephalus luciopercae* Wardle, 1932 from *Sander vitreus* (Mitchill), Wisconsin (**A**, **B**, **G**), Manitoba, Canada (**C**) (IPCAS C-811/1), Ohio (**D**, **E**; paratype of *P. stizostethi* — USNM 1321366), and Minnesota (**F**; USNM 1374926). (**A**) Anterior end of the body; (**B**–**D**) frontal view of the scolex; note absence of an apical sucker (**B–D**) and presence of apically situated, large gland cells (**B, C**) and smaller gland cells beneath the tegument of the scolex and neck region (**C**); (**E**) aporal part of a pregravid proglottid, dorsal view; note L-shaped band of vitelline follicles; (**F**) terminal genitalia, ventral; note an elongate cirrus sac and 2 vaginal sphincters (USNM 1374926); (**G**) last immature proglottid, ventral view.

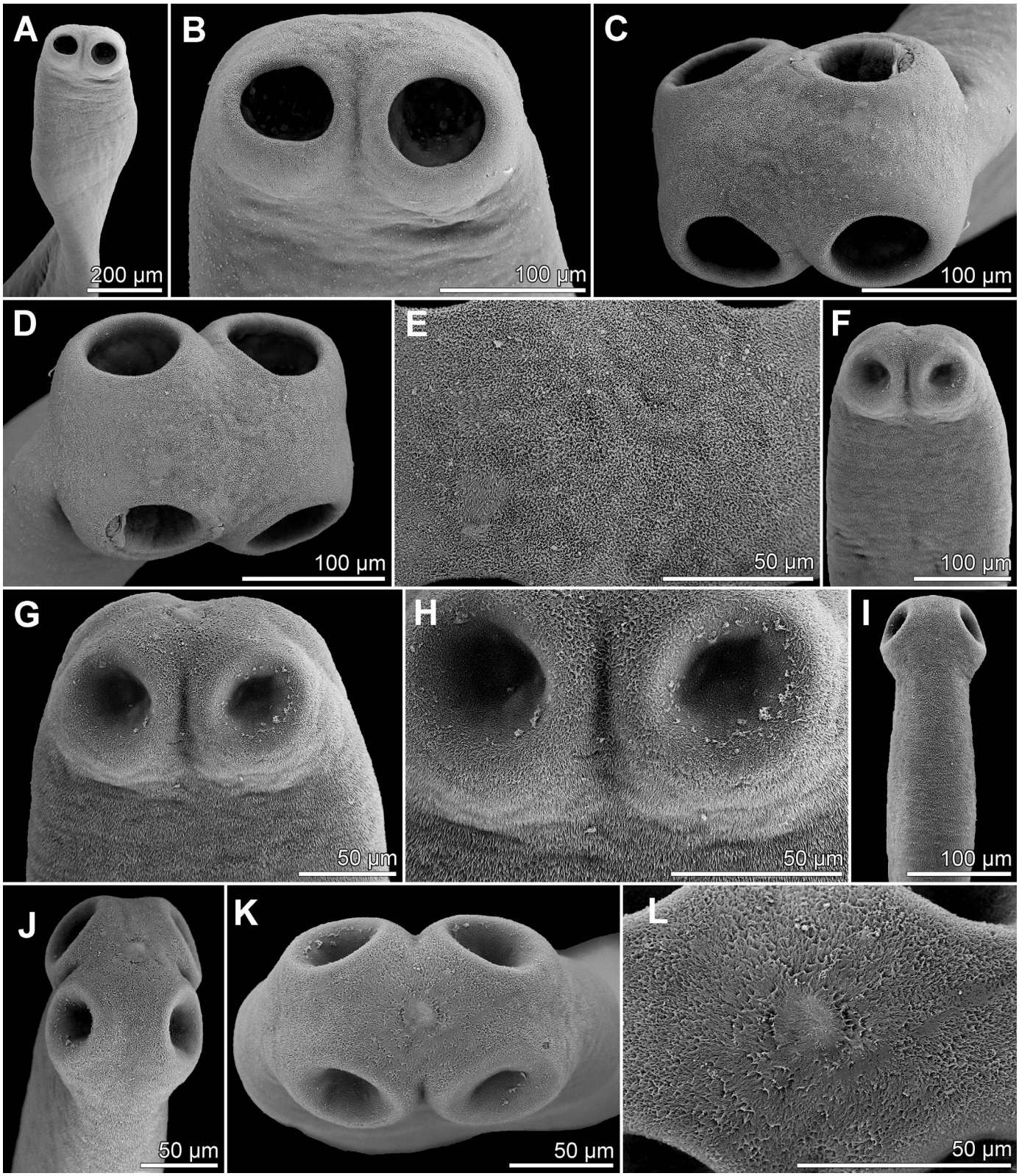

Figure 5. Photomicrographs of the scoleces of *Proteocephalus luciopercae* Wardle, 1932 from *Sander vitreus* (Mitchill), Manitoba, Canada (**A–E**), and *Proteocephalus pearsei* La Rue, 1919 from *Perca flavescens* Mitchill, Manitoba, Canada (**F–L**). **A, B, F, G**: frontal view; **C, D, K**: apical view; **E, L**: detail of apex, note a vestigial apical sucker in L; **H**: detail of suckers; **I**: lateral view; **J**: sublateral view.

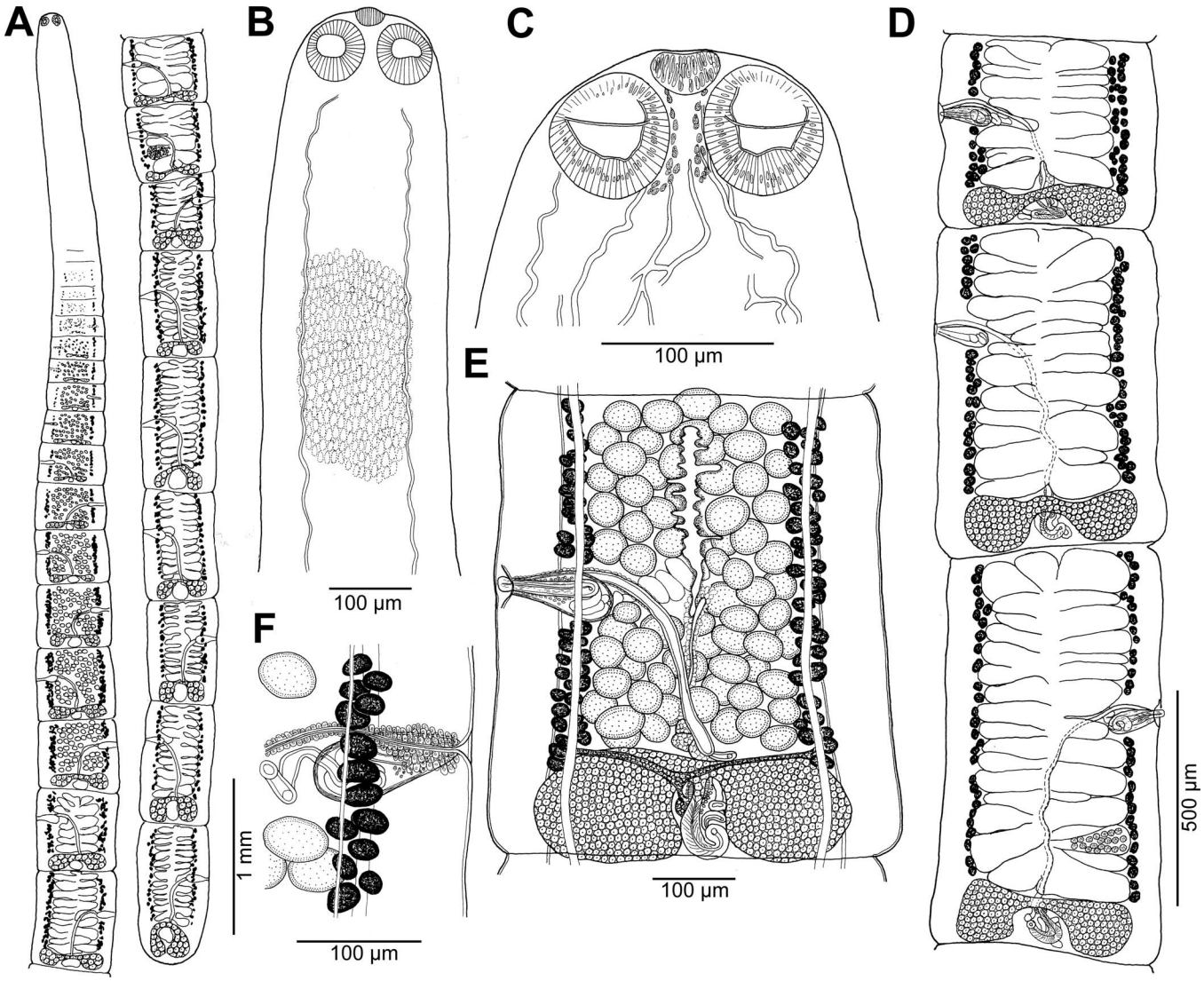

Figure 6. *Proteocephalus pearsei* La Rue, 1919 from *Perca flavescens* Mitchill, Manitoba, Canada (IPCAS C-772/1): (**A**) whole worm, dorsal view; (**B**) anterior end of the body, note median concentration of gland cells posterior to the scolex; (**C**) scolex, note a vestigial, rather high (thick) apical sucker and dense network of osmoregulatory canals; (**D**) gravid proglottids of different shape (rectangular and elongate), ventral view; (**E**) mature proglottid, ventral view; (**F**) terminal genitalia, dorsal view, note a pyriform, short cirrus sac and absence of a vaginal sphincter.

Bothriocephalus sp.), and thus Hoffman (1999) erroneously reported this fish as the host of *P. fluviatilis*.

Proteocephalus fluviatilis has also been translocated along with its bass hosts, e.g., in California (Haderlie, 1953) and Japan (Shimazu, 1990). The species is characterized by a large strobila (more than 200 mm long) composed of acraspedote proglottids, which are much wider than long. The anterior end of the body is almost parallel up to the scolex, which is slightly narrower than the indistinct neck region (Figs. 1, 3A). The suckers are sublateral and large in relation to the scolex width (Fig. 3B). The apical sucker is vestigial, muscular, and flattened (Fig. 1C), 30–47 µm wide, and 18–27 µm thick (high) (32–45 × 30 µm according to Bangham, 1925; 30–60 × 20–40 µm according to Shimazu, 1990).

Osmoregulatory canals form a dense network of strongly convoluted, anastomosed canals posterior to the suckers (Fig. 1C). The testes are densely packed, in two or more layers, numbering 79–95 (mean 85; n = 5; counted from illustrations of mature proglottids; 73–98 according to Bangham, 1925; 60–96 according to Shimazu, 1990). The vagina is anterior (Fig. 2A) or anterodorsal (Fig. 2B–D) to the cirrus sac, similar to observations by Bangham (1925) and Shimazu (1990).

Two species of *Proteocephalus*, namely, *P. ambloplitis* and *P. fluviatilis*, are typical parasites of the largemouth and smallmouth bass. Both species are medium- to large-sized species, but they differ conspicuously from each other in several characters, such as the shape of the scolex (4-lobed feature, much wider than the neck region in *P. ambloplitis*; compare Fig. 3A–C with Fig. 3D–F) and proglottids, and proglottid anatomy, especially the presence of a huge vaginal sphincter and a large cirrus sac that contains a strongly coiled, very long internal sperm duct in *P. ambloplitis*, rather than no vaginal sphincter and a small cirrus sac with a short internal sperm duct in *P. fluviatilis* (Fig. 2). In addition, the species are also not closely related (see above).

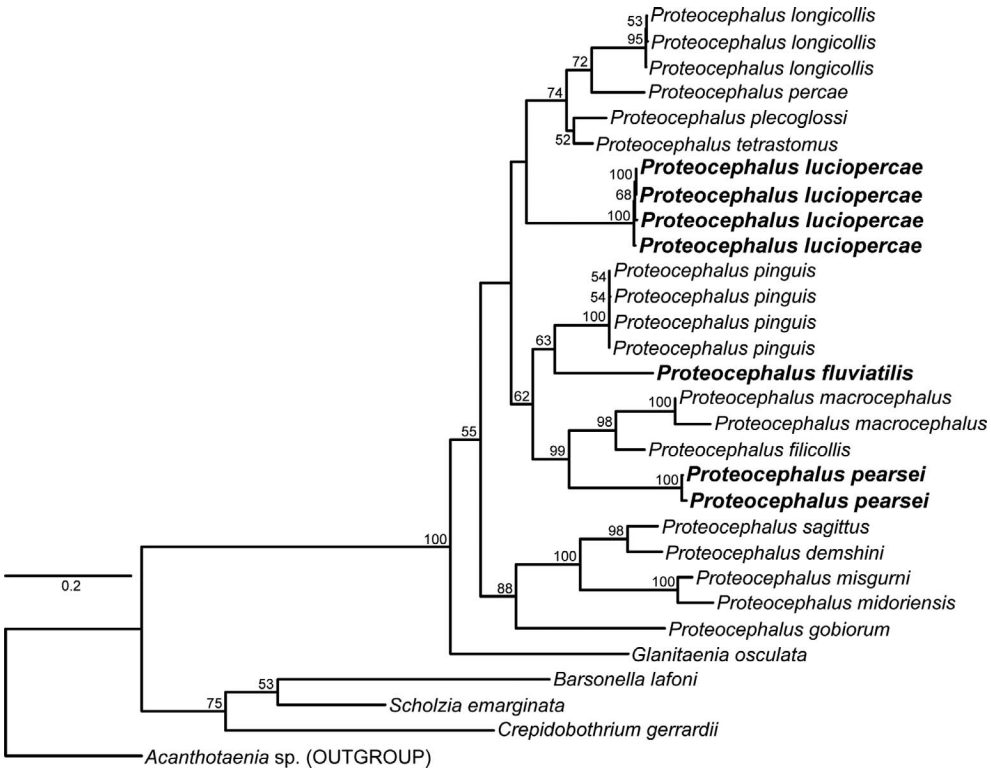

Figure 7. Maximum likelihood analysis of the concatenated (*lsr*DNA, *COI*) data set estimated in IQ-TREE. Nodal supports depict standard bootstrap supports estimated over 1,000 repetitions; only values above 50 are shown. Branch length scale bar indicates number of substitutions per site. Species of the *Proteocephalus*-aggregate from centrarchid and perciform fishes from Nearctic region are shown in bold.

***Proteocephalus luciopercae* Wardle, 1932**

(Figs. 4, 5A–E)

New synonym: Proteocephalus stizostethi Hunter and Bangham, 1933.

Material examined: Holotype and paratype of *P. stizostethi* (USNM 1321365 and 1321366); voucher of *P. luciopercae*, Whitefish Lake, Minnesota, collected in April 1984 (USNM 1374926); 1 mature specimen and 1 immature specimen used for SEM, and 1 immature (37 mm long) specimen stained and mounted, all from *Sander vitreus*, Turtle-Flambeau Flowage, Wisconsin, collected by A. Choudhury on 20 January 2007.

Morphological description: Wardle (1932), Hunter and Bangham (1933).

Type host: Sander vitreus (Mitchill) (Perciformes: Percidae).

Additional definitive host: Sander canadensis (Griffith and Smith).

Other fish hosts of uncertain status: The following fish have been reported as hosts of *P. stizostethi* (= *P. luciopercae*), but they most likely represent accidental, paratenic, or postcyclic hosts: *Ambloplites rupestris*, *Lepomis macrochirus* Rafinesque, *Micropterus dolomieu*, *Pomoxis nigromaculatus* (Centrarchidae), *Esox lucius* Linnaeus (Esocidae).

Type locality: Lake Winnipeg, Manitoba, Canada.

Type material: Not known to exist for *P. luciopercae*; holotype of *P. stizostethi* (USNM 1321365; formerly USNPC 8618); paratype of *P. stizostethi* (USNM 1321366; USNPC 8619).

Life cycle: Not known.

Distribution: Canada (Manitoba, Québec, Saskatchewan), United States (Minnesota, Wisconsin; Lakes Huron and Erie, St. Lawrence River).

Representative DNA sequences and phylogenetic relationships: Four specimens from *S. vitreus* (see Table I): Partial *lsr*DNA (MN061853–MN061856) identical in sequence and complete *COI* (MN061841–MN061844, nucleotide divergence 0–1.2% of 1,626 bp). Phylogenetic position of *P. luciopercae* within the *Proteocephalus*-aggregate, here analyzed for the first time, remains to be resolved confidently (Fig. 7).

Remarks

Proteocephalus luciopercae was briefly described (and illustrated in 2 rather schematic figures) from walleye, *S. vitreus* (reported as *Lucioperca vitreum*), and sauger, *S. canadensis*, from the Canadian lakes Winnipeg (Manitoba) and Waskesiu (Saskatchewan) by Wardle (1932). One year later, Hunter and Bangham (1933), who were apparently unaware of Wardle's (1932) description of *P. luciopercae*, described *P. stizostethi* from the same host, *Stizostedion glaucum* Hubbs (synonym of *Sander vitreus*; see Froese and Pauly, 2019), as well as from *Sander canadensis* and *Micropterus dolomieu* from the western end of Lake Erie in Ohio.

Proteocephalus luciopercae and *P. stizostethi* share the following morphological characters: a large-sized body (total length of 250 and 186 mm, respectively) with a small scolex that lacks any apical sucker, short neck region, proglottids always wider to much wider (three and more times) than long, prominent suckers with

Table I. List of molecular data utilized within this study and their sources.

Species	Host	Location*	*lsr*DNA	*COI*
Acanthotaenia sp.	*Varanus indicus*	Australia	MK328918	MK328926
Barsonella lafoni	*Clarias gariepinus*	Brazil	KC786015	KC785980
Crepidobothrium gerrardii	*Boa constrictor*	Peru	KC786018	n/a†
Glanitaenia osculata	*Silurus glanis*	Switzerland	KX768937	KX768943
Proteocephalus demshini	*Barbatula toni*	Russia	KX768942	KX768950
Proteocephalus filicollis	*Gasterosteus aculeatus*	United Kingdom	AJ388636	n/a
Proteocephalus fluviatilis	*Micropterus dolomieu*	Japan	KP729390	KX768945
Proteocephalus gobiorum	*Neogobius fluviatilis*	Ukraine	KP729393	KX768944
Proteocephalus longicollis	*Micropterus dolomieu*	Lac Coeur, Quebec, Canada	MN061862	MN061850
Proteocephalus longicollis	*Coregonus clupeaformis*	Turtle Flambeau Flowage, Wisconsin	MN061863	MN061851
Proteocephalus longicollis	*Sander vitreus*	USA	MN061864	MN061852
Proteocephalus luciopercae	*Sander vitreus*	Turtle Flambeau Flowage, Wisconsin	MN061853	MN061841
Proteocephalus luciopercae	*Sander vitreus*	Turtle Flambeau Flowage, Wisconsin	MN061854	MN061842
Proteocephalus luciopercae	*Sander vitreus*	Hook Lake, Ontario, Canada	MN061855	MN061843
Proteocephalus luciopercae	*Sander vitreus*	Ontario, Canada	MN061856	MN061844
Proteocephalus macrocephalus	*Anguilla anguilla*	Czech Republic	AJ388609	n/a
Proteocephalus macrocephalus	*Anguilla anguilla*	United Kingdom	EF095261	JQ268552
Proteocephalus midoriensis	*Lefua echigonia*	Japan	AJ388610	n/a
Proteocephalus misgurni	*Misgurnus anguillicaudatus*	Russia	KX768941	KX768949
Proteocephalus percae	*Perca fluviatilis*	Switzerland	AJ388594	KX768947
Proteocephalus pearsei	*Perca flavescens*	Otsego Lake, New York	MN061857	MN061845
Proteocephalus pearsei	*Esox niger*	Otsego Lake, New York	MN061858	MN061846
Proteocephalus pinguis	*Esox lucius*	USA	KP729395	n/a
Proteocephalus pinguis	*Esox lucius*	Minnesota	MN061859	MN061847
Proteocephalus pinguis	*Esox lucius*	Minnesota	MN061860	MN061848
Proteocephalus pinguis	*Esox lucius*	Minnesota	MN061861	MN061849
Proteocephalus plecoglossi	*Plecoglossus altivelis*	Japan	AJ388606	KX768946
Proteocephalus sagittus	*Barbatula barbatula*	Czech Republic	KP729391	KX768948
Proteocephalus tetrastromus	*Hypomesus nipponensis*	Japan	AJ388635	n/a
Scholzia emarginata	*Phractocephalus hemioliopterus*	Brazil	KC786016	KC785981

* More precise locality provided only in newly sequenced samples.
† Data not available = n/a.

deep cavities, and numerous testes (80–100 and 90–125, respectively [118–155 in the present material]). A study of the holotype and paratype of *P. stizostethi* confirmed the presence of the above-mentioned characters (Fig. 4).

Unlike Wardle (1932), Hunter and Bangham (1933) described precisely one of the most characteristic features of the species, i.e., the posteriormost vitelline follicles expanded medially, thus forming L-shaped bands (Fig. 4E, G). According to Wardle and McLeod (1952), such L-shaped bands are present only in this species and in *P. perplexus*.

Both nominal species, *P. luciopercae* and *P. stizostethi*, occur in the same fish hosts (walleye and sauger) and are apparently conspecific. Even though the description of *P. stizostethi*, including its illustrations, is more detailed, and the type specimens of the species are preserved in USNM, *P. luciopercae* has priority. Therefore, *P. stizostethi* is hereby synonymized with *P. luciopercae*.

Proteocephalus luciopercae differs from the remaining North American species of *Proteocephalus* by several characters, such as (1) L-shaped lateral bands of the vitelline follicles (Fig. 4E, G; present only in *P. perplexus*); (2) no apical sucker (the apical part of the scolex contains a concentration of large, unicellular glands; Fig. 4B, C); (3) elongate cirrus sac with the straight, uncoiled internal sperm duct (Fig. 4F, G); (4) very short and wide (more than three times wider than long) proglottids of a large strobila (Fig. 4G); (5) and the vagina alternating in position (anterior to the cirrus sac in 68% proglottids, including paratype of *P. stizostethi*, or posterior; n = 243) and possessing a vaginal sphincter near the opening of the vagina into a shallow vaginal atrium. In a somewhat contracted voucher of *P. luciopercae*, another sphincter is situated at a short distance from the terminal one (Fig. 4F), but this sphincter is difficult to see in the type specimens of *P. stizostethi*, most likely due to decomposition of their tissues.

Proteocephalus pearsei **La Rue, 1919**

(Figs. 5F–L, 6)

Material examined: Holotype from *Perca flavescens*, Lake Monona, Wisconsin (USNM 1355985); 2 paratypes from *P. flavescens*, Lake Hubbard, Michigan, collected by M. Kemper on 25 July 1912 (USNM 1349991); 1 voucher from *P. flavescens* (USNM 1370322); 8 gravid specimens (8–15 mm long) from *P. flavescens*, The Narrows, Lake Manitoba, Manitoba, Canada, collected by A. Choudhury and P. Nelson on 18 and 19 July 1997; 7 specimens (2 gravid, 9.5 mm long) from Lake Falcon, Manitoba, Canada, collected on 27 July 1997 (IPCAS C-772).

Morphological description: La Rue (1919).

Type host: Perca flavescens Mitchill (Perciformes: Percidae).

Additional fish hosts (all most likely represent postcyclic, paratenic, or accidental hosts): Ambloplites rupestris (reported in the original description), *Micropterus dolomieu*, *Pomoxis nigromaculatus* (Lesueur) (all Centrarchidae), *Esox niger* (Esocidae), *Morone americana* (Gmelin), *Morone chrysops* (Rafinesque) (Moronidae), *Aplodinotus grunniens* Rafinesque (Sciaenidae), and *Oncorhynchus mykiss* (Walbaum) (Salmonidae).

Type locality: Lake Monona, Wisconsin.

Type specimens: Holotype USNM 1355985; paratypes USNM 1348670 and 1349991.

Life cycle: Bangham (1925, 1927) studied the life cycle of the parasite and reported the following copepods as its intermediate hosts: *Cyclops prasinus* Fischer (Cyclopidae), *Epischura lacustris* Forbes (Temoridae), and *Eurycercus lamellatus* Müller (Eurycercidae).

Distribution: Canada (Manitoba — new geographical record, Ontario), United States (Michigan, New York, Pennsylvania, Wisconsin).

Representative DNA sequences and phylogenetic relationships: Two specimens from *P. flavescens* and *E. niger* (Table I): Partial *lsr*DNA (MN061857, MN061858) identical in sequence and complete *COI* (MN061845, MN061845, nucleotide divergence 1.7%, sequence length 1,644 bp and 1,677 bp, respectively). The first sequence data for the species place *P. pearsei* as a sister lineage to the clade consisting of *P. filicollis* + *P. macrocephalus*, parasites of three-spined sticklebacks and eels, respectively, with Holarctic distribution (Fig. 7).

Remarks

The species was described by La Rue (1919) based on a few specimens (some immature) found in yellow perch, *P. flavescens*. A study of the new material from Manitoba and its comparison with the holotype of *P. pearsei*, which is slightly contracted and curved, revealed the following differences:

(1) The apical sucker was described by La Rue (1919), apparently based on the holotype, as follows: "It has a shallow cup, is muscular and has the appearance of being functional"; however, the 'cup-like' (concave) appearance of the apical sucker was undoubtedly caused by contraction of the scolex and withdrawal of the suckers, including the apical one, which is thus flattened and as much as 43 μm in diameter (vs. 34 μm in other specimens; La Rue, 1919); in fact, the apical sucker is pad-like, vestigial, without any functional cavity, 34–40 μm in diameter, and 23–28 μm thick (high) (Fig. 5G, J–L; Fig. 6B, C).

(2) La Rue (1919) reported 60–90 testes per proglottid (but only 60–64 testes in holotype); in the new specimens from Canada, fewer testes (34–67; n = 18; Fig. 6E) were counted; it is possible that La Rue (1919) provided numbers of testes also of another species of *Proteocephalus* (see below).

(3) The present study did not reveal the presence of a vaginal sphincter (Fig. 6F); La Rue (1919, p. 4) stated, "A sphincter vaginae seems to be lacking or if present is very weakly developed."

(4) No details of anastomosed osmoregulatory canals and gland cells in the scolex were provided in the original description, even though they are conspicuous (see Fig. 6C).

(5) The specimen from Ward's collection (no. 12.148), illustrated by La Rue (1919, his fig. 5), is most likely not *P. pearsei*, as indicated by a much longer, elongate cirrus sac crossing the vitelline follicles by more than one third of its length (vs. much shorter, pyriform in *P. pearsei*, with only a small part of the sac median to the vitelline follicles), the vagina anterior to the cirrus sac (usually dorsal in *P. pearsei*), and many more testes (94 in fig. 5 of La Rue [1919] vs. less than 70 in *P. pearsei*).

Proteocephalus pearsei is a common parasite of yellow perch; records from other fish hosts need verification (pike may serve as a postcyclic host, as it is in *Proteocephalus percae* in Europe; see Scholz and Hanzelová, 1998). In the Palearctic Region, European perch (*Perca fluviatilis* Linnaeus) harbors another species of the *Proteocephalus*-aggregate, *P. percae* (Müller, 1780). This species differs from *P. pearsei* most conspicuously by the presence of a well-developed, ring-like vaginal sphincter, a long, elongate cirrus sac, a more robust and much longer body (up to 150 mm in total length vs. only 24 mm in *P. pearsei*), and the scolex more tapered towards the anterior extremity compared to that of *P. pearsei* (see also Scholz and Hanzelová, 1998; Scholz et al., 1998; Hanzelová et al., 1999).

Key to identification of the species of the *Proteocephalus*-aggregate from centrarchiform and perciform fishes in the Nearctic Region (see also Table II)

1(2) Apical sucker absent, replaced by a few gland cells; vitelline follicles form L-shaped lateral bands, i.e., bent inwards posteriorly toward ovarian lobes; in walleye and sauger (*Sander* spp.) ..... *P. luciopercae*

2(1) Apical sucker present; vitelline follicles lateral but do not form L-shaped bands 3

3(4) Small worms (total length of gravid worms <25 mm), with only a few proglottids wider than long and other proglottids quadrate to longer than wide; in yellow perch (*Perca flavescens*) ..... *P. pearsei*

4(3) Long worms (total length up to 180 mm), with all proglottids including many (>300) immature ones wider than long; in bass (*Micropterus* spp.) *P. fluviatilis*

DISCUSSION

The present revision recognizes 3 species of the *Proteocephalus*-aggregate that occur in North American centrarchiform and perciform fishes, rather than as many as 11 species reported previously (Schmidt, 1986; Hoffman, 1999). Three species, *P. microcephalus*, *P. osburni*, and *P. stizostethi*, are newly synonymized with previously described species. Doubtful reports by Amin (1977) and Pearse (1924) of another 2 species, *Proteocephalus buplanensis* Mayes, 1976 and *P. perplexus* La Rue, 1911, which are typical parasites of other, more distantly related groups of fishes (cyprinids, gars, and bowfin), are considered to be misidentifications.

The North American species of the genus *Proteocephalus* were described superficially, and intraspecific variability was usually not considered (see La Rue, 1914; Freze, 1965). In addition, original descriptions were in many cases based on poor-quality specimens, as was confirmed by examination of the type specimens.

Table II. Selected morphological and biometrical characters of species of the *Proteocephalus*-aggregate from centrarchid and perciform fishes in the Nearctic Region, and *Proteocephalus percae*. Diagnostic characters in bold.

Species	*Proteocephalus fluviatilis**	*Proteocephalus luciopercae*†	*Proteocephalus pearsei*‡	*P. percae*§
Host	*Micropterus* spp.	*Sander* spp.	*Perca flavescens*	*Perca fluviatilis*
Distribution	North America, Japan (introduced)	North America	North America	Europe
Total length (mm)	>200	up to 250	**<25 (<15‖)**	Up to 150
Proglottid shape#	Wider than long	Wider than long	**Quadrate to longer than wide**	Wider than long
Proglottid number	Dozens to hundreds	Dozens to hundreds	**A few (23–36)**	Many dozens
Scolex width (μm)	160–200 (208–223)¶	165–340 (303–320)	228–310 (174–198)	136–291
Apical sucker (diameter; μm)	32–60 (30–43)	**Absent**	34–43 (34–40)	23–48
Testis number	60–98 (79–95)	80–125 (118–155)	60–90 (34–67)	25–98
Cirrus sac shape	Pyriform	Elongate	Pyriform	Elongate
Vaginal sphincter	Absent	Absent	Absent	**Present**
Band of vitelline follicles	I-shaped	**L-shaped**	I-shaped	I-shaped

* Bangham (1925), Shimazu (1990).
† Wardle (1932), Hunter and Bangham (1933).
‡ La Rue (1919).
§ Scholz and Hanzelová (1998).
‖ Present data in parentheses.
Mature, pregravid and gravid proglottids.
¶ Width at the level of the posterior margin of the suckers.

Since the monograph of La Rue (1914), the species composition of *Proteocephalus* tapeworms in North America has not been critically revised, and the literature has been littered with doubtful host records and apparent misidentifications (see Hoffman, 1999). In addition, the actual status of some fish hosts that may serve as postcyclic (e.g., pikes) and paratenic (sunfishes) hosts has not been considered. This also concerns several records from centrarchiform and perciform fishes, which likely harbor their specific fauna of tapeworms. Based on reliable records, it is assumed that each host group, i.e., bass (centrarchids), pikeperch, and perch (percids), has its own specific parasite, i.e., *P. fluviatilis*, *P. luciopercae*, and *P. pearsei*, respectively.

Based on their morphology, these 3 studied species can be distinguished easily from one another (Table II). Molecular sequence data support the recognition of the 3 species, but the phylogenetic placement of these taxa, and their interrelationships within the entire *Proteocephalus*-aggregate clade, remain largely unresolved, with the exception of *P. pearsei*, which forms a well-supported clade with *P. filicollis* and *P. macrocephalus* (Fig. 7). Better understanding of the evolutionary history of the group will require broader sampling of individual parasite species in the region, as well as complementing the current data set with partially missing (*ssr*DNA, *COI*) and ideally additional sequence data. Interestingly, both the previously published and the current phylogenetic estimates suggest that the Palearctic *P. percae*, a parasite of the European perch, is not closely related to *P. pearsei* from yellow perch, but most probably to *Proteocephalus longicollis* (Zeder, 1800), a parasite of salmoniform fishes, which also occurs in North America; however, this scenario receives only low nodal support (Scholz et al., 2007, 2017; Fig. 7).

Proteocephalid tapeworms represent one of the dominant groups of endoparasitic helminths in North American freshwater fishes (Hoffman, 1999), yet little is known about their species diversity, actual host specificity, geographical distribution, and ecology, including their effect on host populations. In fact, little attention has been paid to all but a few pathogenic parasites of freshwater fishes in North America (Scholz and Choudhury, 2014).

The main obstacles to a better understanding of fish tapeworms in North America are shortages of taxonomic expertise and adequate funding. In addition, the quality of museum material is often poor, which does not allow for detailed morphological or molecular studies. Improper methods, such as using unheated fixatives, flattening tapeworms, and apparently holding them for excessive periods of time before fixing them, have resulted in irreversible deterioration of valuable material including type and voucher specimens. The present study profited from applying simple, but verified methods of processing, especially fast heat-fixing of freshly collected, live specimens, which made it possible to obtain uniformly fixed material suitable for comparative taxonomic studies.

It is hoped that this article and forthcoming taxonomic accounts on other North American proteocephalids, as well as other fish tapeworms, will stimulate more intensive research on this common and remarkable group of freshwater parasites in the Nearctic region. Finally, we propose that future collaborative efforts should focus on filling the existing gaps in the present knowledge of the parasite fauna in North American freshwaters, using methods of integrative taxonomy and molecular phylogenetics.

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