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APOPHALLUS MICROSOMA N. SP. FROM CHICKS INFECTED WITH METACERCARIAE FROM COHO SALMON (*ONCORHYNCHUS KISUTCH*) AND REVIEW OF THE TAXONOMY AND PATHOLOGY OF THE GENUS *APOPHALLUS* (HETEROPHYIDAE)

Jayde A. Ferguson*, Sean A. Locke†, William F. Font‡, Michelle L. Steinauer§, David J. Marcogliese†, Calin D. Cojocaru||, and Michael L. Kent

Department of Microbiology, Oregon State University, 220 Nash Hall, Corvallis, Oregon 97331. *email: jayde.ferguson@alaska.gov*

ABSTRACT: Metacercariae of an unidentified species of *Apophallus* Lühe, 1909 are associated with overwinter mortality in coho salmon, *Oncorhynchus kisutch* (Walbaum, 1792), in the West Fork Smith River, Oregon. We infected chicks with these metacercariae in order to identify the species. The average size of adult worms was 197 × 57 μm, which was 2 to 11 times smaller than other described *Apophallus* species. Eggs were also smaller, but larger in proportion to body size, than in other species of *Apophallus*. Based on these morphological differences, we describe *Apophallus microsoma* n. sp. In addition, sequences from the cytochrome *c* oxidase 1 gene from *Apophallus* sp. cercariae collected in the study area, which are likely conspecific with experimentally cultivated *A. microsoma*, differ by >12% from those we obtained from *Apophallus donicus* (Skrjabin and Lindtrop, 1919) and from *Apophallus brevis* Ransom, 1920. The taxonomy and pathology of *Apophallus* species is reviewed.

The identification of parasite species is a critical step in evaluating disease effects in host populations. This is often difficult for parasites with few morphological characteristics or that require further development to reach an identifiable life stage. These problems are common for larval parasites such as digenean metacercariae, which cause important disease and mortality in freshwater and anadromous fishes (e.g., Gordon and Rau, 1982; Lemly and Esch, 1984; Möller and Anders, 1986; Paperna, 1995; Bakke and Harris, 1998; Jacobson et al., 2008; Ferguson et al., 2011).

We have shown that metacercariae of an unidentified species of *Apophallus* (Digenea: Heterophyidae) are associated with mortality of a threatened stock of coho salmon, *Oncorhynchus kisutch* (Walbaum, 1792) in the West Fork Smith River (WFSR) (Ferguson et al., 2011, 2012). Heterophyid metacercariae are notorious for causing pathology in fishes (Paperna, 1995). Among species of *Apophallus* Lühe, 1909, considerable variability has been reported in both pathology and infection site. For example, most species infect skin and cause black spot disease (Lyster, 1940; Miller, 1941, 1942; Timon-David, 1963; Odening, 1970; Sinclair, 1972; Wierzbicka and Wierzbicki, 1973; Niemi and Macy, 1974) whereas some species only target skeletal muscle (Cameron, 1937a, 1945; Rodnick et al., 2008; Ferguson et al., 2010) or vertebrae (Kent et al., 2004). Pathologies can also differ by host species, as *Apophallus brevis* Ransom, 1920, causes ectopic bone formation in muscle (Pike and Burt, 1983; Taylor et al., 1994) of yellow perch, *Perca flavescens* (Mitchill, 1814) but is restricted to the skin, causing black spot disease, in salmonids (Miller, 1941, 1942).

Variations in disease presentation such as those recorded for *Apophallus* spp. in different fishes could reflect several processes. Pathogenicity may vary among parasite species, while host species may vary in their susceptibility to, and tolerance of, a parasite species. To distinguish among these possibilities, it is necessary to identify the parasite to species and determine if cryptic species are involved (Steinauer et al., 2007; Locke et al., 2010). Parasite species identification can also be pertinent in control measures. For example, one way to reduce the exposure of a valued host to parasitic digeneans is to reduce local populations of the snail host in the parasite's life cycle (e.g., Leighton et al., 2000); however, this approach requires an understanding of the parasite's life cycle which is, in turn, dependent on identifying the species of parasite in question.

For these reasons, we undertook to identify the species of *Apophallus* occurring in coho salmon from WFSR using both morphological and molecular methods. To obtain adults, we experimentally infected chicks, *Gallus gallus domesticus* Linnaeus, 1758, with *Apophallus* sp. occurring in coho salmon from WFSR and found that their morphology differed from described species. Here, we describe the new species and identify its first intermediate host; to achieve these goals, we experimentally infected naïve hatchery coho salmon with cercariae from snails collected from the WFSR. Finally, we compared sequences of the barcode region of cytochrome *c* oxidase 1 (COI) in cecariae of the putative species from WFSR, and herein we compare these observations to those for other species of *Apophallus* and Heterophyidae.

MATERIALS AND METHODS

Sample collection

Approximately 20 coho salmon parr (subyearlings) were collected in the lower main stem of WFSR, Oregon (43°48′54.1″N, 123°46′12.5″W) in September 2008 and processed in the Salmon Disease Laboratory of Oregon State University. Fish were caught with a beach seine and killed with an overdose of buffered tricaine methanesulfonate. Fillets obtained from 10 infected parr were fed to chicks (see below) to obtain adult parasites for morphological study. During this time, metacercariae were also collected from these carcasses for use in morphological and molecular analyses. For intermediate snail hosts, approximately 900 *Fluminicola*

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* Current address: Alaska Department of Fish and Game, Commercial Fisheries Division, Fish Pathology Laboratory, 333 Raspberry Road, Anchorage, Alaska 99518.

† Aquatic Biodiversity Section, Watershed Hydrology and Ecology Research Division, Water Science and Technology Directorate, Science and Technology Branch, Environment Canada, St. Lawrence Centre, 105 McGill, 7th Floor, Montreal, Quebec H2Y 2E7, Canada.

‡ Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402.

§ Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, 105 Magruder Hall, Corvallis, Oregon 97331.

|| National Sanitary Veterinary and Food Safety Authority, Sanitary Veterinary and Food Safety Directorate of Timis County, State Sanitary Veterinary and Food Safety Laboratory of Timis County, Aquatic Pathology Laboratory, 4 Surorile Martir Caceu, 300585, Timisoara, Romania.

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virens (Lea, 1838) and 600 *Juga silicula* (Gould, 1847) were collected from June to August 2009 from the same location of WFSR and examined for cercariae. Cercariae from these snails were used for experimental infections in a separate study by Ferguson et al. (2012) and preserved in ethanol for molecular analysis in the current study.

To provide context for molecular data obtained from material of interest, we include here sequences from other heterophyids acquired by S.L., D.J.M., and C.C. during ongoing survey work. These include *Cryptocotyle lingua* (Creplin, 1825) from great black-backed gull, *Larus marinus* Linnaeus, 1758 from Kangiqsualujjuaq in Nunavik, Canada; *Ascocotyle* sp. encysted on the heart of pumpkinseed, *Lepomis gibbosus* (Linnaeus, 1758) from Lake Seminole, Tampa, Florida; *Apophallus brevis* from ring-billed gull, *Larus delawarensis* Ord, 1815 from Montreal, Quebec, Canada; and *Apophallus donicus* (Skrjabin and Lindtrop, 1919) encysted on the fins of schneider, *Alburnoides bipunctatus* (Bloch, 1782) and roach, *Rutilus rutilus* (Linnaeus, 1758) from the Bega River between Timisoara and Ghiroda, Romania. *Apophallus donicus* metacercariae were excysted with a pepsin solution, mounted, and photographed as described by Cojocaru (2006).

Experimental culture of adult *Apophallus* sp.

Eight, 1-day-old unfed domestic chicks were obtained from a commercial supplier (Featherland Farms, Eugene, Oregon). Infected muscle tissue from 10 coho parr was made into a slurry and concentrated by centrifugation at 4,500 *g* for 15 min. This concentrated slurry was force-fed with a pipette to 4 chicks. Each chick received approximately 150 metacercariae. The other 4 chicks were untreated negative controls. Chicks were housed on the floor behind a barrier fence with heat lamps and fed a commercial, non-medicated chick starter diet (Nasco, Modesto, California) at the Laboratory Animal Research Center of Oregon State University; formal animal ethics approval was given by Oregon State University's Institutional Animal Care and Use Committee (IACUC 3709). Chicks were killed by CO_2 and necropsied on days 3 ($n = 1$), 6 ($n = 1$), and 8 ($n = 2$) post-infection (PI). Intestines were opened in saline and examined using a stereoscope. All detected worms were removed, heat fixed, and stored in Dietrich's solution (30 ml 95% ethanol, 10 ml formalin, 2 ml glacial acetic acid, and 58 ml distilled water).

Identification of first intermediate host of *Apophallus* sp.

Fluminicola virens and *J. silicula* were identified by recording morphological features and referring to the published list and key provided by Burch (1982). They were used for experimental infection of previously unexposed hatchery coho salmon from the Oregon Department of Fish and Wildlife's Oxbow Hatchery, Oregon in a separate study by Ferguson et al. (2012). In short, snails were transported to the Salmon Disease Laboratory where they were held in flow-through tanks at 20 C under a 12-hr photoperiod produced by 19 W aquaria lamps placed approximately 15 cm from the water surface. Each snail species was screened separately in 12-well plates (3–4 snails per well) for cercariae shedding between 0800–1100 and 1800–2000 for several days using a dissecting microscope at ×50 magnification. We observed amphistomate, echinostomate, and pleurolophocercous-type cercariae from *F. virens* and virgulate-xiphidiocercous, echinostomate, sanguinicolid, pharyngeate-furcocercous (longifercous and distomate) rattenkönig, magnacaudal, microcercous-xiphidiocercous, and pleurolophocercous-type cercariae from *J. silicula*. All the observed pleurolophocercous cercariae were consistent with *Apophallus* species (Niemi and Macy, 1974) and were sampled and fixed in 95% ethanol for molecular analysis.

Pools containing 20–30 infected snails were removed and placed in flow-through tanks with uninfected hatchery fish. *Juga silicula* were fed organic lettuce and *F. virens* were fed algae gathered from WFSR and maintained in the laboratory. Approximately 20–30 coho salmon were maintained with each snail species for 4 mo (July–November, 2009), and infections were eventually enumerated by performing tissue squashes of skeletal muscle. Unexposed control fish were evaluated both prior to ($n = 6$), and after ($n = 28$), the study. Quantification of cercariae exposure to fish was not performed because shedding from snails was highly variable. Instead, fish were periodically evaluated to confirm successful transmission and to estimate infection levels of exposed fish in the tank throughout the study.

Whole mount preparations

Specimens of *Apophallus* sp. (cercariae, metacercariae, and experimentally obtained adults) and *C. lingua* (adults), *A. brevis* (adults), and *Ascocotyle* sp. (metacercariae) were stained with Mayer's carmalum or Semichon's carmine, cleared in accordance with Pritchard and Kruse (1982), and mounted on microscope slides with Permount® or Canada balsam (Fisher Scientific, Pittsburg, Pennsylvania). Vouchers of all specimens except *A. donicus* were deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland (USNPC 105081–105086). Cercariae, metacercariae, and adults of *Apophallus* sp. were deposited. Adults of *C. lingua* and *A. brevis* were large enough to be subsampled for both molecular and morphological analyses, and vouchers include the remains of the sequenced specimens as well as entire specimens also collected from the same tissue and same individual host as the sequenced specimens.

Morphometrics

Specimens were examined with a Leica DMLB microscope and digital images were made using a SPOT Advanced digital camera and software (Diagnostic Instruments Inc., Sterling Heights, Michigan). This software was also used to make measurements of characteristics from digital images. A composite line drawing was produced using a drawing tube and from a series of photographs edited in Corel DRAW 12.0 (Corel Corp., Ottawa, Ontario, Canada).

Molecular analysis

Metacercariae of *Apophallus* sp. from a few coho salmon (both naturally and experimentally infected) and cercariae obtained as described above were fixed in 95% ethanol for molecular analysis. Ethanol-fixed samples of *A. donicus*, *A. brevis*, *C. lingua*, and *Ascocotyle* sp. were also used in molecular analyses. DNA from most specimens was extracted from individual organisms, amplified, and sequenced as described by Moszczynska et al. (2009) using primers MplatCox2dF/R (Integrated DNA Technologies, Coralville, Iowa). The PCR cycling conditions were 94 C for 1 min, 5 cycles of 94 C for 40 sec, 45 C for 40 sec, and 72 C for 1 min followed by 35 cycles of 94 C for 40 sec, 51 C for 40 sec, and 72 C for 1 min, with a final extension at 72 C for 5 min. Sequences were compared to published data using BLAST searches and deposited in GenBank (JQ241151–JQ241166). Sequences, chromatograms, collection data, specimen images, and voucher data are in project HETE on http://www.barcodinglife.org.

A phylogenetic tree of the COI sequences was constructed with MEGA 5.0 (Tamura et al., 2011) using maximum likelihood. Based on the Bayesian information criterion in preliminary model selection, this analysis employed the Hasegawa-Kishino-Yano model of nucleotide evolution and a gamma shape parameter of 0.1612.

RESULTS

Unstained and stained specimens of *Apophallus* spp. from this study are shown in Figures 1 and 2, respectively, and morphologic details of experimentally obtained adult *Apophallus* sp. are illustrated in Figure 3. Coho salmon parr collected from the WFSR were heavily infected, with greater than several hundred heterophyid metacercariae per gram of muscle. For experimental infections, 2–20 worms from each chick were recovered from the posterior 10–15 cm of the intestine. Immature worms were obtained 3 days PI, but sexually mature worms with eggs were recovered at 6 and 8 days PI.

Only 1 morphological type of pleurolophocercous cercariae was found in snails from our experiments; they developed into only 1 morphological type of heterophyid metacercariae in hatchery fish exposed to snails. These metacercariae were identical to the *Apophallus* sp. infecting coho salmon from the WFSR that we used in experimental infections to obtain adults. The pleurolophocercous cercariae had 2 eyespots and dorsal-ventral fin folds

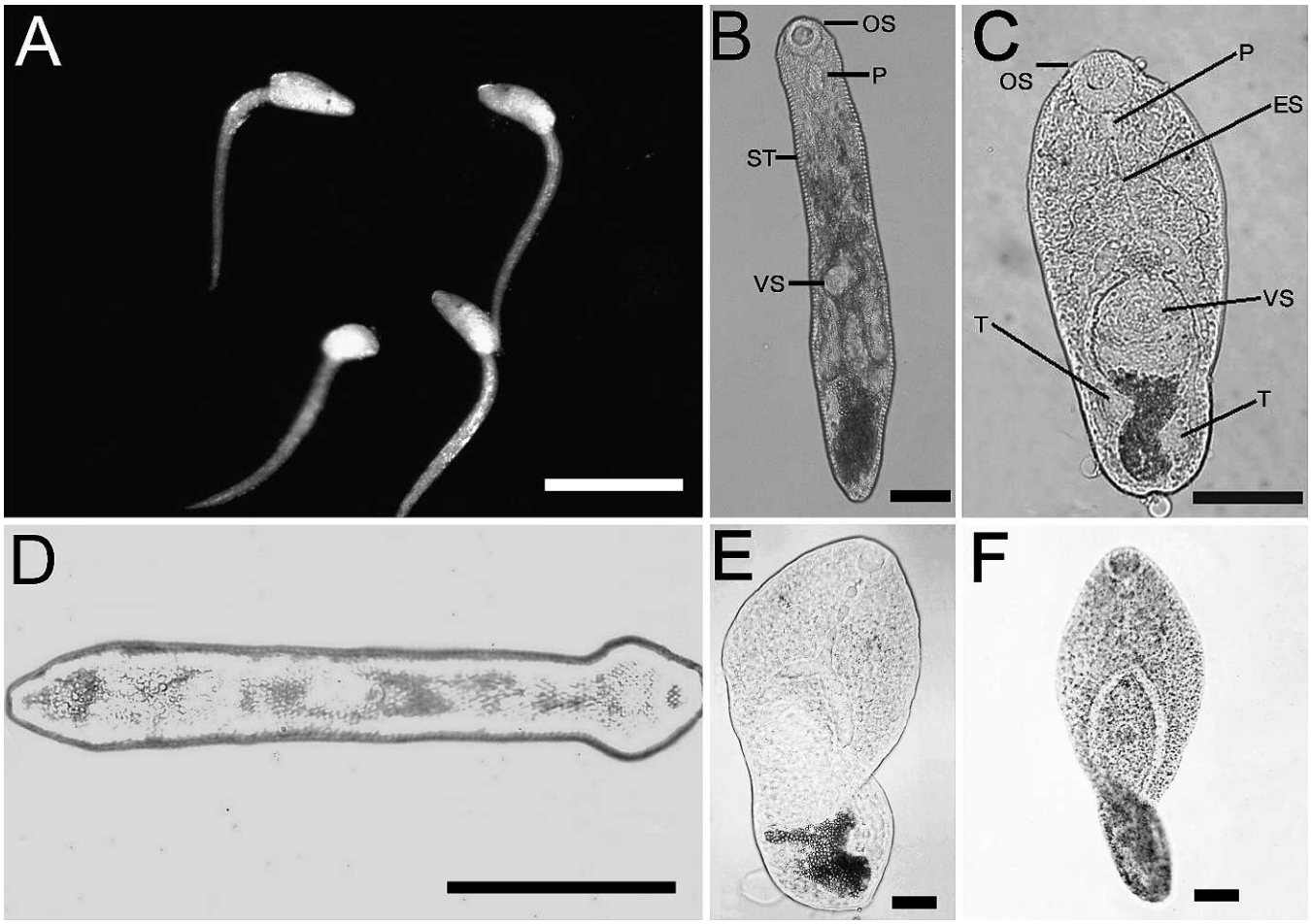

FIGURE 1. Wet whole mounts of *Apophallus* species. (**A–D**) *Apophallus microsoma* from West Fork Smith River, Oregon. (**A**) Putative cercariae released from *Fluminicola virens*, scale bar = 200 μm. (**B–C**) Excysted metacercariae from *Oncorhynchus kisutch* with worm flattened (**B**) to show internal organs, scale bar = 50 μm. (**D**) Adult recovered from the lower intestine of *Gallus gallus domesticus* showing spiny tegument, vitellaria, and oral sucker, scale bar = 100 μm. (**E–F**) Excysted metacercariae of *Apophallus donicus* from *Rutilus rutilus* from Bega River, Romania showing oral sucker and primordial ceca, scale bar = 50 μm. OS = oral sucker, P = pharynx, ES = esophagus, VS = ventral sucker, ST = spiney tegument, T = testis.

(Figs. 1A, 2A). The estimated prevalence of infection in these snails, based on our screening technique, was 2% in *F. virens* and <1% in *J. silicula*. Coho salmon exposed to *F. virens* had a 100% (29/29) prevalence and a mean intensity of 17 *Apophallus* sp. per fish (range = 2–44), whereas coho salmon exposed to *J. silicula* had a prevalence of 34% (11/32) and a mean intensity of 5 (range = 2–14) parasites per fish. Other established metacercariae infections included *Nanophyetus salmincola* (Chapin, 1927) from microcercous-xiphidiocercous cercariae released from *J. silicula* and *Echinochasmus milvi*, Yamaguti, 1939, from echinostomate cercariae released from both snails. No infections were detected in the unexposed control fish.

We were unable to obtain quality sequences of COI from metacercariae of *Apophallus* sp. However, bi-directional sequences requiring little, or no, editing and ranging from 390 to 640 bp in length, were obtained from 2 samples of pleurolophocercous cercariae (1 sample consisting of a single cercaria, the other of 2 cercariae) of *Apophallus* sp. collected from *F. virens* that were used to experimentally obtain metacercariae from previously unexposed hatchery fish. We also obtained sequences from 5 *A. donicus*, 5 *C. lingua*, and 2 *Ascocotyle* sp. Chromatograms from 3 *A. brevis* were of lesser quality and required manual editing and alignment to generate bi-directional sequences.

In a maximum likelihood analysis, the COI sequences from the pleurolophocercous cercariae from *F. virens* in WFSR fell within a well-supported clade with sequences from *A. brevis* and *A. donicus* (Fig. 4). Among all 5 heterophyid species, COI sequences varied by an average of 18.7% (range = 12.3–22.3%) and by 13.5% (range = 12.3–14.7%) among *Apophallus* spp. In translated amino acids, this corresponded to a mean of 18.5 (range = 4–29) differences among the 5 heterophyid species and a mean of 7.7 (range = 4–11) differences among *Apophallus* spp. Sequences from the *Apophallus* sp. cercariae differed by 12.3% and in 4 amino acids from those of *A. brevis* and by 13.5% and 11 amino acids from *A. donicus*. Within species, the mean variation in COI sequences was 0.31% (range = 0–0.8%). All intraspecific variation in COI consisted of synonymous changes at the third codon position except in *A. donicus*. In this species, changes at the second codon position resulted in the replacement of 2 different valine residues by either phenylalanine or isoleucine in 2 different specimens, 1 from *A. bipunctatus* and 1 from *R. rutilus*. Sequences of COI from 4 adult *C. lingua* collected from northern Quebec

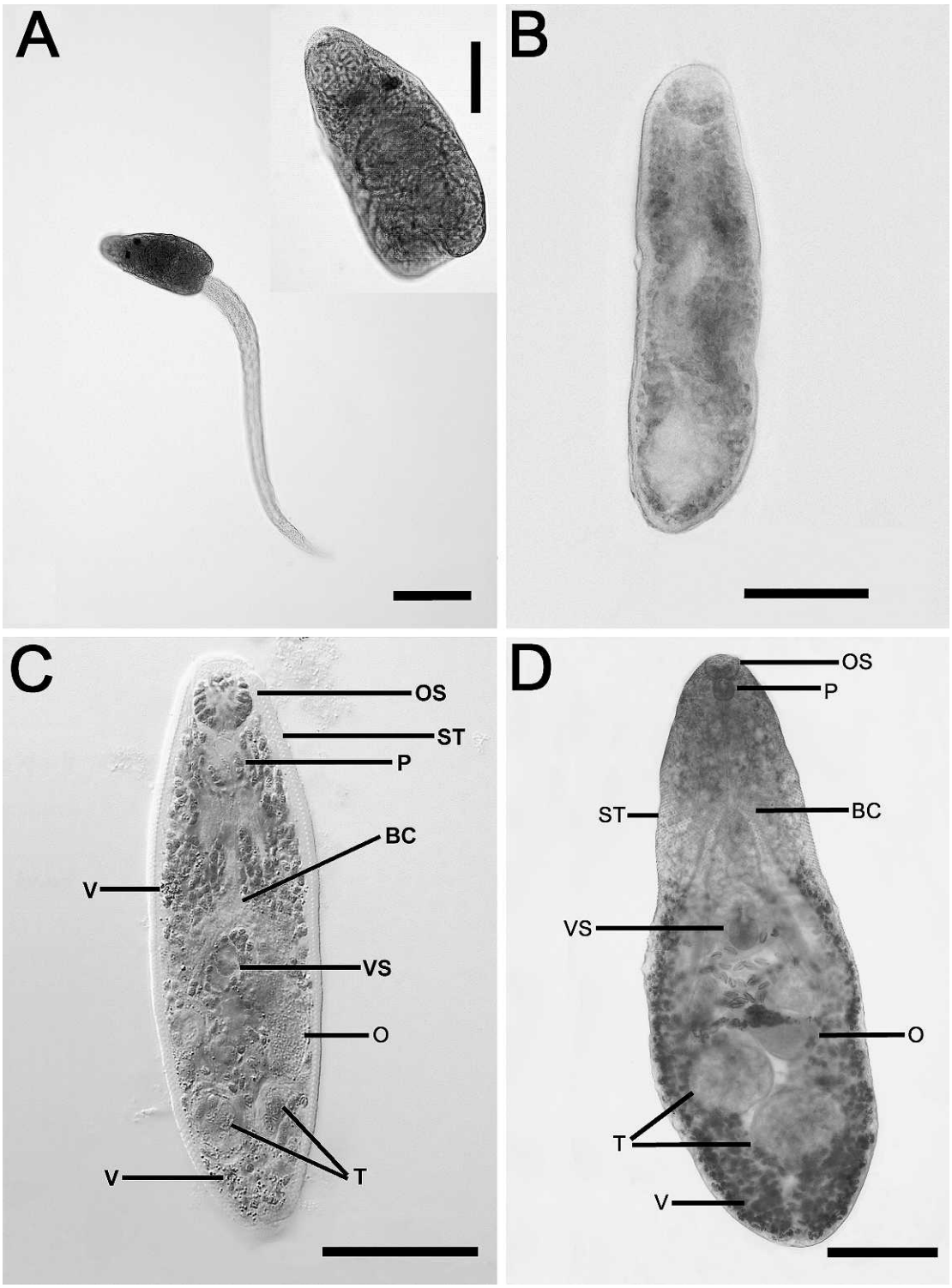

FIGURE 2. Stained whole mounts of *Apophallus* species. (**A–C**) *Apophallus microsoma* from West Fork Smith River, Oregon. (**A**) Putative cercariae released from *Fluminicola virens*, scale bar = 250 μm; inset shows increased detail of parasite body with tail removed, inset scale bar = 100 μm. (**B**) Excysted metacercaria from *Oncorhynchus kisutch*, scale bar = 50 μm. (**C**) Adult recovered from the lower intestine of *Gallus gallus domesticus*, scale bar = 50 μm. (**D**) Adult *Apophallus brevis* from *Larus delawarensis* from Quebec, Canada, scale bar = 250 μm. OS = oral sucker, ST = spiny tegument, P = pharynx, BC = bifurcated cecum, VS = ventral sucker, O = ovary, T = testes, V = vitellaria.

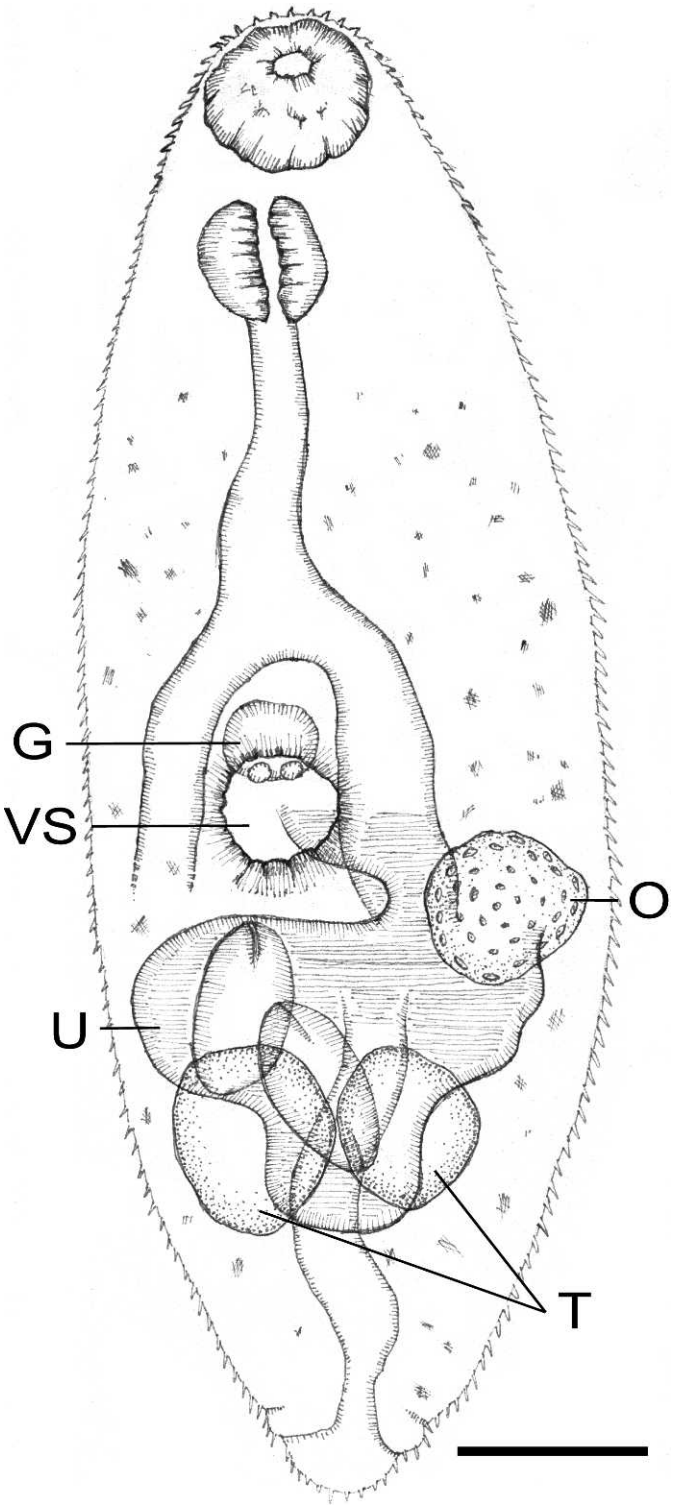

FIGURE 3. Line drawing of experimentally obtained adult *Apophallus microsoma* showing morphologic details. Scale bar = 25 μm. VS = ventral sucker, O = ovary, T = testes, U = uterus, G = gonotyl.

were identical to those of *C. lingua* cercariae collected by Blakeslee et al. (2008) from the Atlantic coast of North America and from northern Europe.

DESCRIPTION

Apophallus microsoma n. sp.

(Figs. 1–3)

Diagnosis: (Adults based on 20 specimens): Digenea: Heterophyidae. Spiny tegument. Long esophagus; pharynx close to oral sucker. Muscular gonotyl (Figs. 1D, 2C, 3) typical of *Apophallus* species. Testes oblique to tandem. Vitellaria extended beyond bifurcation of cecum; anterior to acetabulum. Small body, approximately 197 μm (150–231) length, 57 μm (35–78) maximum width (Table I). Eggs (n = 13) about 23 μm (20–26) length, 12 μm (10–14) maximum width (Table I). Body and egg size smaller than previously described *Apophallus* species; largest egg-to-body ratio in genus (Table I).

Metacercariae (based on 18 excysted worms): Obvious spiny tegument. Long esophagus; pharynx close to oral sucker. Testes oblique to tandem (Figs. 1B, C, 2B).

Taxonomic summary

Type host: Newborn chicks, *Gallus domesticus* Linnaeus (experimental).

Site of infection: Small intestine.

Prevalence: 100% (4 experimentally infected chicks).

Second intermediate host: Coho salmon, *Oncorhynchus kisutch* (Walbaum, 1792).

Site of infection in second intermediate host: Skeletal musculature along the myomeres primarily, but skin infections associated with black spots also occur in rare cases.

Prevalence in second intermediate host: 100% (20 coho salmon).

Type locality: West Fork Smith River, Douglas County, Oregon (43°48′54.1″N, 123°46′12.5″W).

Type specimens: Submitted to the U.S. National Parasite Collection, Beltsville, Maryland. Holotype, adult (USNPC 105778); paratypes, 3 adults (USNPC 105082–105083) and 2 metacercariae (USNPC 105081); voucher specimens, 2 putative cercariae (USNPC 105080).

Etymology: micro, Gr., small; *soma*, Gr., body. The specific epithet is based on the conspicuously small size of this parasite in comparison to congenerics.

Remarks

The morphology of adults of *Apophallus microsoma* differs from known species. Notably, specimens are about 2 to 11 times smaller than in other species of *Apophallus*. The species most similar in size is *A. donicus* as reported by Niemi and Macy (1974), which is an important comparison because their study involved infections in coho salmon also from Oregon. However, Niemi and Macy (1974) reported *A. donicus* to be approximately 2 times longer and 5 times broader, and to have eggs 1.5 times longer and wider, than those of *A. microsoma*. Moreover, the worms identified as *A. donicus* by Niemi and Macy (1974) were about 2 to 4 times shorter and 3 times narrower than the type species described by Skrjabin and Lindtrop (1919).

The morphology of *Apophallus* spp. has been studied in adults obtained from a diverse range of experimentally infected hosts which, along with methods of relaxing and fixing worms, may affect the comparability of characters and morphometrics (Lyster, 1940; Odening, 1970; Meyer and Olsen, 1975). Thus, host-induced variation in size and other morphological features of the worm should be considered. Experimental hosts for *Apophallus* spp. have included cats (*Felis catus* Linnaeus, 1758), chicks of varying age, ducklings, white rats (*Rattus norvegicus* [Berkenhout, 1769]), gerbils, golden hamsters (*Mesocricetus auratus* [Waterhouse, 1839]), and even humans (Lyster, 1940; Timon-David, 1963; Odening, 1970; Niemi and Macy, 1974). Lyster (1940) reported that the body shape of *Apophallus imperator* Lyster, 1940 is pyriform in avian hosts and ovoid in mammals. Odening (1970) found that adult *Apophallus mühlingi* (Jägerskiöld, 1899) Lühe, 1909 varied in shape and size (about 1.5-fold; pictured in Odening [1970]) even within a single experimental host species. Such variability in body size led Witenberg (1929) to place *Apophallus*

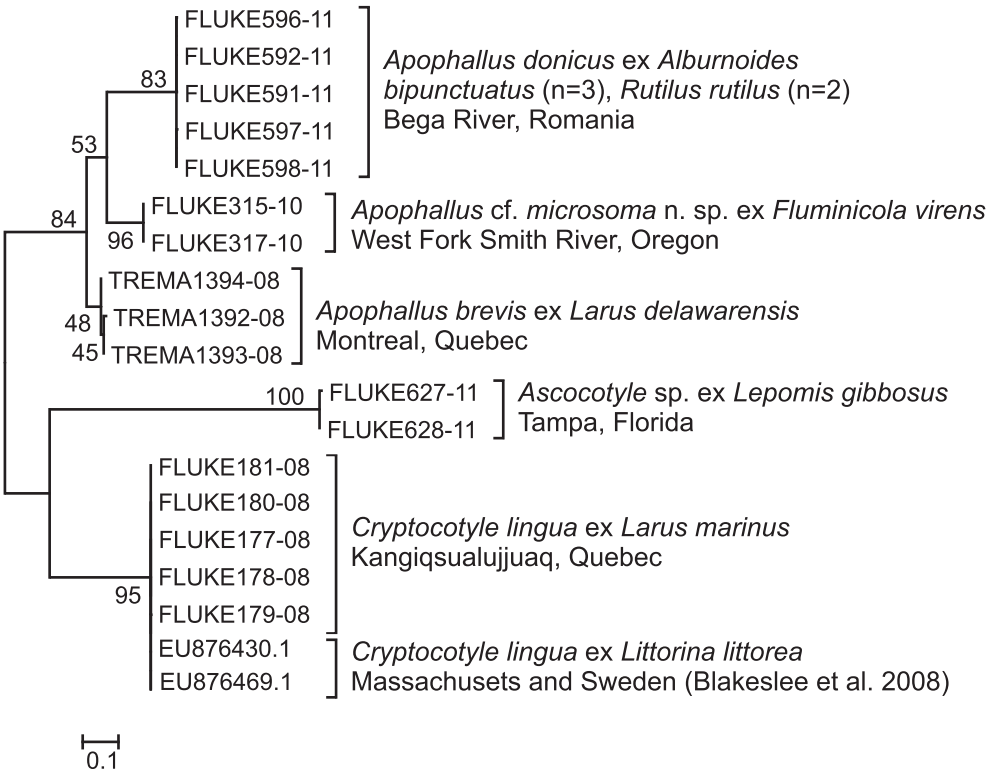

FIGURE 4. Phylogeny reconstruction based on maximum likelihood analysis of a 640-bp alignment of 19 sequences of partial cytochrome *c* oxidase I. Numbers on nodes represent percent bootstrap support (1,000 replicates). Scale units are substitutions per site.

major Szidat, 1924, as a junior synonym of *A. mühlingi*, as these 2 worms only differ by a slight size difference (less than 2-fold).

However, host-induced variation is an implausible explanation for the comparatively large difference in the size of *A. microsoma* and of other described species of *Apophallus*. The eggs of adult *A. microsoma* also differ in size from those of all other species, being closest in size, but still smaller than, those in *Apophallus venustus* (Ransom, 1920). This is significant because egg size tends to vary little in digeneans experimentally reared in different host taxa (Blankespoor, 1974). The egg-to-body ratio of *A. microsoma* was also strikingly larger; approximately double that of other species in the genus.

Sequence data and phylogenetic analysis of putative cercariae

Unfortunately, we were unable to obtain sequences from the metacercariae used to culture the adults that formed the basis of our description of *A. microsoma*. However, the pleurolophocercous cercariae from which we did obtain sequences are most likely conspecific to these metacercariae. These cercariae were shed by *F. virens* snails collected from an area of the WFSR with consistently high *Apophallus* sp. infections in coho salmon (Ferguson et al., 2010, 2011, 2012), and *Apophallus* sp. infections were induced in naïve hatchery coho salmon exposed to these cercariae of interest (Ferguson et al., 2012). Phylogenetic analysis of COI sequences from these cercariae also places them within a strongly supported clade of *Apophallus* spp. rather than among other heterophyids.

The COI sequences from these cercariae were largely differentiated from those of *A. brevis* and *A. donicus*, with 12.3 and 13.5% sequence divergence, respectively. This amount of divergence is greater than the average divergence (11.7%) in COI between 23 other congeneric pairs of digenean species (Vilas et al., 2005). In contrast, there were no differences for COI in the outgroup we used, i.e., *C. lingua* sampled in Europe and North America. This suggests that the divergence in COI between *Apophallus* collected from Quebec, Oregon, and Romania is not likely due to intraspecific variation. The 13.5% divergence between putative *A. microsoma* cercariae in Oregon and *A. donicus* in cyprinids from Romania is particularly interesting because Niemi and Macy (1974) identified metacercariae from coho salmon from the Willamette River, Oregon (near our study site) as *A. donicus* after feeding them to experimental hosts and comparing their morphology to published reports.

DISCUSSION

Taxonomic review

There is considerable debate on the generic status of *Apophallus* and related genera (e.g., *Cotylophallus*, *Rossicotrema*, *Cryptocotyle*, and *Pricetrema*), but greater confusion lies in separating species of *Apophallus*, which has resulted in the synonymy of numerous taxa. For example, *A. donicus* has been synonymized with 3 species (*Apophallus similis* [Ransom, 1920], *A. venustus*, and *A. brevis*) under various generic names (Table I). There is a great deal of similarity in described "species," that is based on characteristics that are highly variable. Cameron (1936) used 3 main features, i.e., body shape, juxtaposition of testes, and placement of vitellaria. He acknowledged the variability of the former 2 characters, thereby placing more emphasis on the latter. He distinguished *A. mühlingi*, *A. donicus*, and *A. brevis* from *A. venustus* (= *Cotylophallus similis*) based on vitellaria not reaching the level of the acetabulum for the former and on these glands reaching the esophageal bifurcation in the latter. Within the first group, *A. mühlingi* is long and narrow with testes almost tandem, *A. donicus* is oval-pyriform with oblique testes, and *A. brevis* is elongate-pyriform with oblique testes. Cameron disagreed with Price (1931) in synonymizing *A. venustus* with *A. donicus*, mainly based on minor differences in the testes and a small difference in

TABLE I. Synopsis of *Apophallus* species. Bold type refers to type description. VS = ventral sucker, NA = not applicable NR = not reported, BS = black spot disease.

Species and synonyms	Reference	Size	Shape	Vitellaria
***Apophallus bacalloti* Morozov, 1952**	Morozov, 1952 (*Apophallus* sp. of Balozet and Callot, 1939)	600 × 150	NR	posterior VS*
Apophallus bacalloti†	Timon-David, 1963	(520–820) × NR	elongate, attenuated anteriorly	posterior VS
***Apophallus brevis* Ransom, 1920** *Rossicotrema donicum*—Witenberg (1929); *Apophallus imperator*—Miller (1941)	Ransom, 1920	(600–900) × (120–260)	posteriorly broader than *Apophallus mühlingi*	anterior VS
A. brevis	Sinclair, 1972	(910–1,330) × (230–320)	elongate	gonotyl
A. brevis	Present study	(1,280–1,340) × (400–500)	oval	gonotyl
***Apophallus crami* Price, 1931**	Price, 1931	(1,500–1,900) × (279–341)	slender	posterior VS
***Apophallus donicus* (Skrjabin and Lindtrop, 1919) Price, 1931** *Rossicotrema donicum*, *Rossicotrema simile*, *Cotylophallus venustus*-*Cotylophallus similis*—Price (1931); *Rossicotrema simile*, *Rossicotrema venustus*, *A. brevis* [for *Rossicotrema donicum*]—Witenberg (1929)	Skrjabin and Lindtrop, 1919	(1,120–1,300) × (580–720)	oval	intestinal fork
Apophallus donicus†	Niemi and Macy, 1974	311 (298–554) × 262 (186–303)	oval, pyriform-linguiform	intestinal fork
***Apophallus eccentricus* Africa and Garcia, 1935**‡	Africa and Garcia, 1935	2,150 × 350	elongate	ovary
***Apophallus imperator* Lyster, 1940**†	Lyster, 1940	NR	linguiform, pyriform, or discoid	gonotyl
A. imperator	Sinclair, 1972	(745–941) × (157–287)	NR	gonotyl
***Apophallus lari* (Leonov, 1957)—** *Rossicotrema lari*	Leonov, 1957	NR	pyriform	posterior intestinal fork
***Apophallus lerouxi*, Rayski and Fahmy, 1962**§	Rayski and Fahmy, 1962	(850–930) × (168–172)	elongate, attenuated anteriorly	between intestinal fork and VS
***Apophallus majori* Szidat, 1924**	Szidat, 1924	(1,800–2,900) × (240–350)	biscuit-shaped	intestinal fork
***Apophallus microtestis* (Leonov, 1957)**	Leonov, 1957	(530–546) × (172–187)	pyriform	anterior VS
***Apophallus mühlingi* (Jägerskiöld, 1899) Lühe, 1909** *Distoma mühlingi*—Lühe (1909); *A. major*—Witenberg (1929)	Jägerskiöld, 1899	(1,200–1,600) × (190–230)	biscuit-shaped	intestinal fork
A. mühlingi†	Odening, 1970	881 × 257	NR	VS*
***Apophallus similis* (Ransom, 1920) Price, 1931** *Cotylophallus similis*—Price (1931)	Ransom, 1920	(500–1,140) × (220–390)	NR	anterior VS
***Apophallus venustus* (Ransom, 1920) Price, 1931** *Cotylophallus venustus*—Price (1931)	Ransom, 1920	up to 1,300 × (230–650)	NR	anterior VS
A. venustus	Cameron, 1936	(950–1,400) × (250–550)	elongate-oval; few pyriform	intestinal fork
***Apophallus microsoma* n. sp. Ferguson et al., 2012**†	Present study	197 (150–231) × 57 (35–78)	elongate-linguiform or oval-pyriform	intestinal fork

* Pictured in reference cited.

† Data from experimental infections.

‡ May be assigned to the wrong genus because the uterus extends to the posterior region of the body (Cameron, 1936).

§ If a cirrus pouch is actually present like the authors described, then this species does not belong to *Apophallus* (Yamaguti, 1971).

TABLE I. Extended.

Testes	Egg	Egg:body	Fish species and tropism	Geographic location
mostly tandem*	30 × 18	0.05 × 0.12	NA	Tunisia
mostly tandem*	(30–31) × 18.9	(0.04–0.06) × NA	*Gasterosteus aculeatus* *Gambusia affinis* skin (BS)	St. Chamas, France
oblique-tandem	(36–40) × (16–22)	(0.04—0.06) × (0.08–0.13)	NA	Washington D.C., USA
oblique	37 (32–41) × 20 (16–21)	(0.03–0.04) × (0.06–0.07)	*Perca flavescens* muscle	New York, USA; Ontario, Canada
oblique	37 (35–37.5) × 18 (17.5–18.5)	0.03 × 0.04	NA	Quebec, Canada
oblique	33 × 25	0.02 × (0.07–0.09)	NA	Oregon, USA
oblique	35 × 25	0.03 × (0.03–0.04)	NR	Russia
oblique	32 (21–33) × 18 (17–20)	0.10 (0.06–0.07) × 0.07 (0.07–0.09)	*Oncorhynchus kisutch* skin (BS*)	Oregon, USA
oblique	22 × 12	0.01 × 0.03	NA	Philippines
oblique-tandem	(28–34) × (16–17)	NA	*Salvelinus fontinalis* skin (BS)	Quebec, Canada
oblique	33 (27–37) × 18 (15–20)	0.04 × (0.07–0.09)	*S. fontinalis* skin (BS)	New York, USA; Ontario, Canada
oblique-tandem	(34–37) × 18	NA	NA	Eastern Europe
diagonal	29 × 17	0.03 × 0.10	NA	East Scotland
oblique-tandem	(32–36) × (18–21)	(0.01–0.02) × (0.06–0.08)	NA	Europe (East Prussia)
tandem	(31–34) × 18	0.06 × 0.10	NA	Europe
oblique-tandem*	32 × 18	(0.02–0.03) × (0.08–0.09)	NA	Eastern Europe
usually tandem	NR	NA	*Carassius carassius* muscle, skin (BS)	Germany
oblique-tandem*	(30–35) × (16–20)	(0.03–0.06) × (0.05–0.07)	NA	Washington D.C., USA
oblique-tandem*	(25–35) × (15–20)	up to (0.02–0.03) × (0.03–0.06)	NA	Washington D.C., USA
oblique	(26–32) × (18–22)	(0.02–0.03) × (0.04–0.07)	NA	Quebec, Canada
oblique- tandem	23 (20–26) × 12 (10–14)	0.12 (0.11–0.13) × 0.21 (0.18–0.29)	*O. kisutch* muscle	Oregon, USA

egg size. Cameron (1937a) also argued that these species differ for biological reasons, based on host specificity, and noted that *A. mühlingi* sensu stricto is a European parasite.

Lyster (1940) studied specimens of *A. brevis* and *A. donicus*, collected by Cameron and Ciurea respectively, and concluded that the distribution of vitellaria is not sufficient to differentiate these species because of its variability within species. He proposed that the arrangement of the acetabulo-genital complex can separate *Apophallus* species. He noted 2 types of gonotyls: (1) "papillae-like, far removed from progenital type" and (2) "non papillae-like, showing affinity to a true genital sucker." He assigned *A. brevis* to the former and *A. venustus* and *A. donicus* to the latter, which could be separated further with these structures. He also noted that *A. mühlingi* and his newly described *A. imperator* had similar gonotyls but differed in size and shape. However, Miller (1941) re-studied the morphology of *A. imperator* and *A. brevis* and concluded that the use of the gonotyl was only sufficient for generic separation. Perhaps egg size or egg-to-body ratio are important characters for comparing species, as these may be less influenced by methodologies. *Apophallus microsoma* had smaller eggs and a distinctly larger egg-to-body ratio than any other described species.

Further complicating matters is the description of 2 species based on immature or larval forms. *Apophallus americanus* Van Cleave and Mueller, 1932, was described from the intestines of piscivorous fish using only 2 immature worms, and which the authors concluded was likely an accidental infection in aberrant hosts. Metacercariae of *Apophallus itascensis* Warren, 1953, were described from yellow perch with an unusual cyst of "hard and glassy" composition, which undoubtedly represents the unique infection of *A. brevis* in yellow perch. Additional confusion arises from Miller (1941) in reducing *A. imperator*, the causative agent of black spot in brook charr, *Salvelinus fontinalis* (Mitchill, 1814) to a junior synonym of *A. brevis* based on life cycle studies. However, Sinclair (1972) found that although adult *A. brevis* and *A. imperator* are nearly identical morphologically, there are other differences in metacercariae (host specificity, sexual development, cyst type, and location) and adult (maturation time and longevity) stages. Thus, *A. brevis* is most likely synonymous with 2 species and confused with a third. Therefore, other phenotypic traits are likely needed to differentiate *Apophallus* species. The importance of egg size, egg-to-body ratio, host specificity, and disease presentation is discussed below.

Host specificity

Metacercariae are often regarded to have low host specificity (Paperna, 1995), but experimental and molecular studies have shown metacercariae of some digeneans are specific to different families or species of fish (Hoffman, 1958; Locke et al., 2010). Reports of host specificity for *Apophallus* species are varied and conflicting. Cameron (1945) contended that *A. brevis* was specific to brook charr and that even rainbow trout (*Oncorhynchus mykiss*) were refractory to infection. Conversely, *A. venustus* occurs in 8 different families of fish (Cameron, 1937a, b). A few authors contend that *A. donicus* exclusively infects percids and *A. mühlingi* mainly infects cyprinids (Bykhovskaya-Pavlovskaya et al., 1964; Chiriac and Udrescu, 1973; Wierzbicka and Wierzbicki, 1973). However, Yamaguti (1971) and more recent literature (Bauer, 1987; Moravec, 2001; Cojocaru, 2006) lists *A. donicus* as infecting cyprinids in addition to percids. Odening (1970) concluded that the mixed accounts of specificity for these 2 Eurasian *Apophallus* species are likely due to the difficulty of accurately identifying their metacercariae. Ciurea (1924) experimentally infected dogs (type host) with *A. donicus* from cyprinids collected from Somova Lake, Danube Delta, Romania (about 2,000 km from the type location of Novocherkassk, Russia). This supports records of *A. donicus* in cyprinids in more contemporary literature, including the specimens we obtained from cyprinids within 1,000 km from the type locality. To our knowledge, *A. donicus* has never been reported from salmonids in Eurasia (type region). Along with previously mentioned morphological differences (see Remarks), this suggests that Niemi and Macy (1974) may have misidentified the *Apophallus* species they observed in coho salmon from Oregon.

The host specificity of digeneans in the molluscan hosts tends to be more restricted (Paperna, 1995). *Apophallus venustus* utilizes the pleuroceriid snail *Goniobasis livescens* (Cameron, 1937a), whereas *A. brevis* (and *A. imperator*) have been shown to use the hydrobiid snail *Amnicola limosa* (Say) (Cameron, 1945). *Apophallus* sp. from our studies infects snails from both these families (Ferguson et al., 2012), and heterophyids are somewhat unique in their adaptive use of molluscan hosts (Malek 1980).

Disease presentation

Reports on the pathology elicited by different *Apophallus* species also vary greatly. For example, even though *A. brevis* causes black spot disease in many fish species, this parasite causes ectopic bone formation only in yellow perch (Pike and Burt, 1983; Taylor et al., 1994) and is associated with both lethal and sub-lethal effects (Johnson and Dick, 2001; Marcogliese et al., 2005, 2010). Another unusual lesion associated with *Apophallus* sp. is skeletal deformity in cyprinids from the Willamette River, Oregon (Kent et al., 2004), which was more severe in younger fish. Cameron (1937a, 1945) argued that *A. venustus* predominantly infects musculature, rarely skin, and is not associated with black spot. Similarly, *A. mühlingi* is thought to primarily infect muscle and only occasionally skin (Odening, 1970; Wierzbicka and Wierzbicki, 1973), and it causes a larger fibroblastic host cyst than does *A. donicus* (Wierzbicka and Wierzbicki, 1973). *Apophallus microsoma* mainly infects skeletal muscle along the myomeres, but skin infections associated with black spot also occur in rare cases. Niemi and Macy (1974) studied black spot disease in Oregon fishes and identified their specimens as *A. donicus*. They observed mortality in small coho salmon infected with as few as 35 cercariae released from *Fluminicola* spp. The low cercarial dose reported to induce mortality in this study likely represents an underlying health problem for these fish.

In conclusion, morphologic data from metacercariae and adults, and molecular data from the putative cercariae, both support that the *Apophallus* sp. described here is a novel species. *Apophallus microsoma* is the smallest member of this genus reported to date. The dramatic size difference is not likely to be a fixation artifact or a result of using an unnatural definitive host. Furthermore, egg size, which is considered a relatively fixed characteristic, was also smaller than previously described species and the egg-to-body ratio was the largest on record. Host specificity and disease presentation may also be important phenotypic traits of different *Apophallus* species that could be

used as identifying characters. *Apophallus microsoma* metacercariae infecting coho salmon in our system do not typically infect skin or cause black spot. Given that many *Apophallus* species cause black spot disease and that *A. donicus* has been regarded as a parasite of percids and cyprinids from Eurasia, infection site and host specificity may help distinguish this latter species of *Apophallus* from *A. microsoma*. Finally, the putative cercariae of *A. microsoma* from an endemic area of WFSR used to infect naïve hatchery coho salmon differed genetically from the other *Apophallus* species in our study. Clearly the entire genus is in need of revision, and information on traits other than adult morphology, such as disease presentation and DNA sequence comparison, will likely be important. If possible, such data should be derived from type hosts and localities to resolve the taxonomy of members of this important genus affecting fishes.

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