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Research Article

Phylogenetic assessment of Apophallines (Digenea: Opisthorchiidae) with revision of *Apophallus donicus* Skrjabin & Lindtrop, 1919 complex and some taxonomic propositions

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According to the current taxonomic concept, the Apophallinae Ciurea, 1924 is a monotypic subfamily of the Opisthorchiidae that comprises species with a median permanent ventrogenital sac containing a well-developed ventral sucker and two gonotyls. A recently formulated molecular hypothesis suggests polyphyly of this subfamily and the presence of two sibling species within one of the nominal species *A. donicus* Skrjabin & Lindrop, 1919. In this paper, we redescribed *A. donicus s. str.* and renamed *A. donicus auct. non* Skrjabin & Lindrop, 1919 to *A. lari* (Leonov, 1957) based on morphological, biological and molecular (mitochondrial cox1 gene fragments) data. The main morphological differences between species are the length of the distance between the anterior end of the body and the anterior extremity of the vitellarium as a ratio to the forebody length, and the shape of the anterior vitelline follicles. We also aimed to test the monophyly of the Apophallinae through nuclear-ribosomal molecular markers 18S + 28S rDNA. Tree topology showed *Apophallus zalophi* (Price, 1932) clustered closer to *Liliatrema* Gubanov, 1953 than to the other *Apophallus* spp. As a result, we resurrect the genus *Pricetrema* Ciurea, 1933 that earlier was proposed for *A. zalophi* and abolish the Liliatrematinae Gubanov, 1953 with removal of *Liliatrema* into the Apophallinae. Thus, according to the updated concept, the Apophallinae contains three genera, *Apophallus* Lühe, 1909, *Liliatrema* and *Pricetrema*, two of which (*Apophallus*, *Pricetrema*) have the ventrogenital sac with two gonotyls and one (*Liliatrema*) has a genital sac without gonotyls.

Key words: *Apophallus lari*, *Apophallus microtestis*, *Apophallus zalophi*, *Circus aeruginosus*, *Lutra lutra*, Phocidae, species-identification, *Vulpes vulpes*

Introduction

The Apophallinae Ciurea, 1924 is a relatively small monotypic subfamily of opisthorchiids, parasitizing the intestines of fish-eating birds and mammals in adult states (Sokolov et al., 2021). Members of this subfamily are characterized by the presence of a median permanent ventrogenital sac with a well-developed ventral sucker and two gonotyls, which arise dorsally close against the ventral sucker and overhang it ventrally. Apophalline

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metacercariae encyst in various freshwater and anadromous fish (e.g. Cameron, 1937; Ciurea,1928; Ferguson et al., 2012; Lyster, 1940, Odening1970; Sinclair, 1972; Timon-David, 1963; Warren, 1953) and are pathogenic to their hosts (e.g. Kent et al., 2004; Niemi & Macy, 1974; Taylor et al., 1994). Some species are able to infect humans (Niemi & Macy, 1974), which together with the information discussed above, makes the study of apophallines of practical significance.

To date, a type and only genus of this subfamily comprises 20 species (Ferguson et al., 2012; Pearson, 2008; WoRMS, 2022; Yamaguti, 1971), including the type species of the four other nominal genera, *Apohalloides* Yamaguti, 1971 [*Apohalloides pyriformis* (Webster & Wolfgang, 1956) Yamaguti, 1971], *Cotylophallus* Ransom, 1920 [*Cotylophallus venustus* Ransom, 1920], *Pricetrema* Ciurea, 1933 [*Pricetrema zalophi* (Price, 1932) Ciurea, 1933] and *Rossicotrema* Skrjabin & Lindrop, 1919 [*Rossicotrema donicum* Skrjabin & Lindrop, 1919].

According to Sándor et al. (2017), there are two species of *Apophallus* in Europe that possess morphologically similar metacercariae, but differ in relation to host-fish species (cyprinids vs percids) and by molecular data. Metacercariae from European cyprinid fish collected by these authors appeared to be conspecific with those previously identified by Ferguson et al. (2012) as *A. donicus*. However, Sándor et al. (2017), based on experimental data from Ciurea (1928), Mödlinger (1934) and Odening (1973) on cultured *A. donicus* adults from metacercariae infecting percids, disagree with the species identification made by Ferguson et al. (2012). From the viewpoint of Sándor et al. (2017), only metacercariae from percid fish belong to *A. donicus*, whereas those from cyprinids (*A. donicus* of Ferguson et al. (2012)) should be considered *Apophallus* sp. However, this opinion does not take into account the data of Ciurea (1924) on the finding of *A. donicus* (as *R. donicum*) in the intestines of naturally and experimentally infected dogs in Romania. During the experiment, this author fed only cyprinid fish to dogs.

The most recent phylogenetic reconstruction of opisthorchiids based on 28S rRNA gene data, generated using *Apophallus zalophi* Price, 1932 and a type species *Apophallus* Lühe, 1909, *A. muehlingi* (Jägerskiöld, 1899) Lühe, 1909, does not support monophyly of both *Apophallus* and the Apophallinae (Sokolov et al., 2022). Thus, the real interrelationships among species within the Apophallinae remain poorly understood.

In this paper, we redescribed *A. donicus s. str.* (= *Apophallus* sp. of Sándor et al. (2017); *A. donicus* of Ferguson et al. (2012)) based on the study of newly collected specimens and extant type material, and provide results of analysis of the phylogenetic relationships between *Apophallus* species using the cox1 marker. In addition, we used the 18S + 28S rRNA gene sequences data set across *Apophallus* cf. *microtestis* Leonov, 1957, *A. zalophi* and *A. donicus s. str.* for phylogenetic assessment of the Apophallinae.

Material and methods

Sample collection

Two specimens of *Apophallus* cf. *microtestis* were recovered from the intestine of one *Circus aeruginosus* (L.) (Accipitridae) individual taken in hunting grounds along the lower Kuma River (44°47′40″N, 46°52′35″E; Dagestan, Russia), in March 2022. Trematodes were initially relaxed in fresh water and fixed in 70% ethanol, after a few minutes the specimens were transferred into 96% ethanol.

Specimens of *A. donicus s. str*. were discovered during the dissection of the intestines of six red foxes, *Vulpes vulpes* (L.) (Canidae) taken in hunting grounds near the Oka River floodplain (54°48′0″N, 41°36′0″E; Kasimovsky District of Ryazan Oblast, Russia) in January 2022 and from one Eurasian otter, *Lutra lutra* (L.) (Mustelidae) from the River V'yulka (56°51′51″N, 37°56′25″E; Taldomsky District of Moscow Oblast, Russia) in June 2021. Immediately after their shooting, the host individuals were frozen and transported to the laboratory for further study. In total, two and 20 specimens of this parasite species were collected from *L. lutra* and *V. vulpes* respectively. Trematode specimens were washed in fresh water and preserved in 96% ethanol.

Specimens of *A. zalophi* were recovered from the intestine of three seal species from Atlantic Canada in 2018; two grey seals, *Halichoerus grypus* (Fab.) (juvenile, found injured in April and brought to a wildlife rehabilitation centre where it died approximately 9 days later and was subsequently examined via necropsy. Location (approximate): 44°36′36″N, 63°25′12″W and juvenile, found dead but no location provided), one harp seal *Pagophilus groenlandicus* (Erxleben) (juvenile, found injured on a beach in December and euthanized due to severity of injury and poor prognosis. Location (approximate): 46°25′12″N, 63°5′24″W), and one harbour seal *Phoca vitulina* L. (Phocidae) (juvenile, found dead on a beach in Nova Scotia in August. Location (approximate): 44°38′60″N, 63°16′48″W). These trematodes were placed in 95% ethanol for DNA extraction and hot AFA solution

(70% ethanol, 10% formalin, 5% glacial acetic acid) for morphological identification.

Morphology study

Morphological identification of *A. donicus s. str*. and *Apophallus* cf. *microtestis* was carried out using both literary sources (Cameron, 1936; Ciurea, 1928; Leonov, 1957; Odening, 1973; Skrjabin & Lindrop, 1919) and data obtained from re-examination of their type samples. Identification of *A. zalophi* followed Price (1932).

We studied the syntypes of *A. microtestis*, *A. donicus* (originally as *R. donicum*) and *Apophallus lari* (Leonov, 1957) Ferguson et al. 2012 (originally as *Rossicotrema lari*) deposited in the Parasite Collection of the Russian Scientific Research Institute for Fundamental and Applied Parasitology of Animals and Plant, Moscow, Russia (RIPPC):

- Seven syntypes of *A. microtestis;* RIPPC No 11973, host – *Nycticorax nycticorax* (L.), site of infection – intestine, locality – Dnieper liman, the Black Sea;
- Eleven syntypes of *R. donicus;* RIPPC No 13472, 13474, 13476, 13477, host – *Canis familiaris* L., site of infection – intestine, locality – Novocherkassk City, Russia;
- Seven syntypes of *R. lari*; RIPPC No. 11976, host – *Larus argentatus* Pontoppidan (it is probably actually *Larus cachinnans* (Pallas)), site of infection – intestine, locality – Tendra Bay, the Black Sea.

For the morphological study, trematode specimens were stained with acetocarmine (*A. donicus s. str*. and *Apophallus* cf. *microtestis*) or Semichon's carmine (*A. zalophi*), cleared in dimethyl phthalate (*A. donicus s. str*. and *Apophallus* cf. *microtestis*) or clove oil (*A. zalophi*) and mounted in Canada balsam. For morphological descriptions, measurements are reported as the range followed by the mean in brackets (for $n > 1$) and they are all presented in micrometres. Photographs were made using a compound microscope Zeiss Axio Imager Z1 equipped with a camera Zeiss AxioCam HRc. Drawings were made with the aid of a camera lucida.

Hologenophores of *A. donicus s. str*. stored in the personal collection of the first author. Paragenophores of *A. donicus s. str*. and *Apophallus* cf. *microtestis* were deposited in the Museum of Helminthological Collections at the Center of Parasitology of the Severtsov Institute of Ecology and Evolution (IPEE RAS; Moscow, Russia). Paragenophores of *A. zalophi* were deposited in the Canadian Museum of Natural History (Ottawa, Canada). Conspecificity of the genotyped specimens of *Apophallus* cf. *microtestis* and *A. zalophi* with the corresponding paragenophores were assessed based on the similarity of traits available for research without staining, namely, shape and body size, position of ventral sucker and uterus, width of caeca, the arrangement of the vitelline fields and the sizes of the gonads.

DNA amplification and sequencing

Total DNA of *A. donicus s. str*. and *Apophallus* cf. *microtestis* was extracted from adult 96% ethanol-fixed worms with the "hot shot" technique (Truett, 2006). For *A. zalophi*, total DNA was extracted from adult 96% ethanol-fixed specimen using a DNeasy Blood and Tissue kit (Qiagen, Toronto, ON, Canada) as per the manufacturer's instructions.

Complete nuclear 18S rDNA (~2000 bp) was successfully amplified using polymerase chain reaction with the forward primer Worm-A and the two reverse primers Worm B or 18S-F (Littlewood & Olson, 2001). Partial 28S rDNA (~1200 bp) was amplified using primers LSU5 (forward) and 1500 R (reverse) (Tkach et al., 2003). For *A. donicus s. str*. and *Apophallus* cf. *microtestis*, initial PCR reaction was performed in a total volume of 25 µl containing 0.25 mM of each primer pair, 25 ng of total DNA in water and 12.5 µl of PromegaGoTaq Green Master mix (Madison, WI, USA). Amplification was performed in a GeneAmp 9700, Applied Biosystems, Waltham, MA, USA. PCR conditions are described in Sokolov et al. (2022) for 18S rDNA and in Tkach et al. (2003) for 28S rDNA. For *A. zalophi*, each PCR reaction was performed in a 50 µl volume containing 0.50 mM of each primer set separately, 25–50 ng of DNA, 5 µl of 10× PCR buffer (Qiagen), 1 µl of dNTP, 10 µl of Q solution, 3 µl of $MgCl_2$, 0.5 µl (2.5 units) of Qiagen HotStar Taq DNA polymerase and the remaining volume of nuclease-free water. Amplification of the 18S and 28S rDNA for *A. zalophi* was performed in a MyCycler Thermal Cycler, Bio-Rad, with an initial denaturation and activation at 95°C for 1 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C and a final extension at 72 °C for 7 min followed by cooling to 12 °C.

Negative control samples with 2 µl of nuclease-free water were conducted in place of DNA for each primer set for all described PCR procedures.

Amplicons were visualized after 1% agarose gel electrophoresis with SYBR Safe DNA gel stain (Thermo Fisher Scientific) under an ultraviolet light source.

Additionally, a mitochondrial 550-bp cox1 gene fragment of *Apophallus* cf. *microtestis* and *A. donicus s. str*. was amplified and directly sequenced with primers Apom1f (forward) and Apom1r (reverse), described in

Sándor et al. (2017). This primers pair was also used for cox1 gene amplification and sequencing of two *A. muehlingi* specimens, for which ribosomal DNA sequences were generated earlier (Sokolov et al., 2022). Reaction mix was the same as for amplification of rDNA fragments for *A. donicus s. str.* and *Apophallus* cf. *microtestis*. PCR was performed using conditions, described in Sándor et al. (2017). Unfortunately, all attempts to amplify cox1 markers in *A. zalophi* using various primers were unsuccessful (see Supplemental material, Appendix A).

Amplicons were visualized after 1% agarose gel electrophoresis with SYBR Safe DNA gel stain (Thermo Fisher Scientific) under an ultraviolet light source.

PCR products generated for *Apophallus* cf. *microtestis*, *A. donicus s. str.* and *A. muehlingi* were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), as recommended by the manufacturer, with the internal sequencing primers described by Littlewood and Olson (2001) for 18S rDNA, those described by Tkach et al. (2003) for 28S rDNA and those described by Sándor et al. (2017) for cox1 gene. PCR product sequences were analysed using an ABI 3500 genetic analyser at the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. For *A. zalophi*, PCR products were sequenced at Psomagen Inc. Corp., MD, USA. Amplification and bidirectional sequencing were performed at least twice from two independently DNA-extracted trematode samples from each of the three host species. Assembly of the combined corresponding forward and reverse sequences was used to derive the consensus sequence which was aligned using the Clustal W accessory application with the subsequent pairwise similarity calculations determined using BioEdit 7.0 (Hall, 1999).

The sequences were submitted to the GenBank NCBI (Table 1).

Alignments and phylogenetic analysis

Ribosomal 18S and 28S DNA fragments and mitochondrial cox1 gene fragment sequences of *Apophallus* cf. *microtestis*, *A. donicus s. str.* and *A. muehlingi* were assembled with SeqScape v.2.6 software provided by Applied Biosystems. For *A. zalophi*, assembly of the combined corresponding forward and reverse sequences was used to derive the consensus sequence which was aligned using the Clustal W accessory application with the subsequent pairwise similarity calculations determined using BioEdit 7.0 (Hall, 1999). Alignments and estimations of the number of variable sites and sequence differences were performed using MEGA 7.0.26 software (Kumar et al., 2016). Phylogenetic relationships of *Apophallus* spp. with other opisthorchioid digeneans (Table 1) were elucidated using a concatenated data set of complete 18S rRNA gene and partial sequences of 28S rRNA gene. Additionally, phylogenetic relationships of different *Apophallus* species (Table 1) were reconstructed using cox1 gene fragments. These analyses were performed with the help of the Bayesian Inference (BI) and maximum likelihood (ML) algorithms using MrBayes v. 3.1.2 software (Huelsenbeck et al., 2001) and the PhyML v. 3.1 software (Guindon & Gascuel, 2003), respectively. The best nucleotide substitution models, the TVM + I + G and GTR + I + G (Posada, 2003) were estimated with jModeltest v. 2.1.5 software (Darriba et al., 2012) for concatenated 18S + 28S rDNA for Bayesian (BIC criterion) and ML (AIC criterion) algorithms, respectively (Akaike, 1974; Huelsenbeck et al., 2001). The best nucleotide substitution models, TPM1uf + G and TIM1 + I + G (Darriba et al., 2012) for BI (BIC criterion) and ML (AIC criterion) algorithms, respectively, were estimated for partial cox1 gene sequence data set.

BI analyses were performed using 10,000,000 generations with two independent runs. Summary parameters and the phylogenetic tree were calculated with a burn-in of 25% of generations. The significance of the phylogenetic relationships was estimated using posterior probabilities (Huelsenbeck et al., 2001). Estimation of ML phylogenetic relationships significance was performed with the help of the approximate likelihood ratio test with a Bayes support (Guindon & Gascuel, 2003). The phylogenetic trees were rooted based on the findings of Sokolov et al. (2021): the Cryptogonimidae for the tree generated on 18S + 28S rDNA sequences and *Cryptocotyle lingua* (Creplin, 1825) Fischoeder, 1903 (Cryptocotylinae, Opisthorchiidae) for cox1 gene sequences-based tree. Estimates of evolutionary divergence (p-distances) are made with MEGA 7.0.26 software (Kumar et al., 2016).

Additionally, a median-joining network was reconstructed for species of *Apophallus* on the basis of cox1 gene sequence data set with Network v.10.2.0.0 software, developed by Flexus Technology Ltd., Suffolk, UK (https://www.fluxus-engineering.com/sharenet.htm).

Results

Phylogenetic relationships

Both BI and ML analyses based on partial cox1 gene sequences combined *Apophallus* specimens we collected from *L. lutra* and *V. vulpes* into one well-supported clade along with *Apophallus* sp. of Sándor et al. (2017)

Table 1. List of opisthorchioid species involved in current molecular analyses.

	28S rRNA gene		18S rRNA gene		COI mtDNA gene	
Species	**Accession number**	**Reference**	**Accession number**	**Reference**	**Accession number**	**Reference**
Opisthorchiidae						
Apophallinae						
Apophallus brevis	–	–	–	–	JQ241151–JQ241153	Ferguson et al. (2012)
Apophallus donicus s. str. (= *Apophallus* sp. of Sándor et al. (2017) and *A. donicus* of Ferguson et al. (2012))	**OP803125, OP803126** [ex *Lutra lutra*]	**This study**	**OP803128, OP803129**[ex *Lutra lutra*]	**This study**	JQ241154–JQ241158 MF438076, MF4388077, MF438098, MF438099, MF438101 **OP804343, OP804344** [ex *Lutra lutra*] **OP804348-OP804351** [ex *Vulpes vulpes*]	Ferguson et al. (2012) **This study** **This study**
Apophallus donicus of Sándor et al. (2017)	–	–	–	–	MF438081–MF438087, MF438089, MF438093, MF438096, MF438097	Sándor et al. (2017)
Apophallus cf. *microsoma*	–	–	–	–	JQ241159, JQ241160	Ferguson et al. (2012)
Apophallus cf. *microtestis*	**OP803127** [ex *Circus aeruginosus*]	**This study**	**OP803130** [ex *Circus aeruginosus*]	**This study**	**OP804347** [ex *Circus aeruginosus*]	**This study**
Apophallus muehlingi	OK358935, OK358936	Sokolov et al. (2022)	OK384547, OK384548	Sokolov et al. (2022)	MF438078–MF438080, MF438088, MF438090–MF438092, MF438094, MF438090, MF438095, MF438100 **OP804345, OP804346** [ex *Larus cachinnans*]	Sándor et al. (2017) **This study**
Apophallus zalophi	**OM765066** [ex *Halichoerus grypus*]	**This study**	**OM765041** [ex *Halichoerus grypus*]	**This study**	–	–

(*continued*)

Table 1. Continued.

	28S rRNA gene		18S rRNA gene		COI mtDNA gene	
Species	**Accession number**	**Reference**	**Accession number**	**Reference**	**Accession number**	**Reference**
Apophallus sp.	–	–	–	–	KM538077	Van Steenkiste et al. (2015)
Cryptocotylinae						
Cryptocotyle lingua	MW361240	Gorbushin, A. & Tolstenkov, O. (Direct Submission)	MW361240	Gorbushin, A. & Tolstenkov, O. (Direct Submission)	EU876372 JQ241166 MW361241	Blakeslee et al. (2008) Ferguson et al. (2012) Gorbushin, A. & Tolstenkov, O. (Direct Submission)
Euryhelminthinae						
Euryhelmis costaricensis	AB521799	Sato et al. (2010)	AB521799	Sato et al. (2010)	–	–
Liliatrematinae						
Liliatrema skrjabini	MT303944, MT303945	Sokolov et al. (2021)	MT303881, MT303882	Sokolov et al. (2021)	–	–
Liliatrema sobolevi	OK358933, OK358934	Sokolov et al. (2022)	OK384546	Sokolov et al. (2022)	–	–
Opisthorchiinae						
Amhpimerus ovalis	AY116876	Olson et al. (2003)	AY222121	Olson et al. (2003)	–	–
Clonorchis sinensis	MK450525	Qiu et al. (2020)	MK450527	Qiu et al. (2020)	–	–
Metorchis bilis	OK358937	Sokolov et al. (2022)	OK384551	Sokolov et al. (2022)	–	–
Metorchis orientalis	MK482051–MK482055	Qiu et al. (2020)	MK482051–MK482055	Qiu et al. (2020)	–	–
Metorchis xanthosomus	OK358938	Sokolov et al. (2022)	OK384552	Sokolov et al. (2022)	–	–
Opisthorchis altaevi	OK358941	Sokolov et al. (2022)	OK384553	Sokolov et al. (2022)	–	–
Opisthorchis felineus	MF099790	Le,T., Nguyen,T., Nguyen,T., Rajapakse, R., & Blair, D. (Direct Submission)	MF077357	Le,T., Nguyen,T., Nguyen,T., Rajapakse, R., & Blair, D. (Direct Submission)	–	–
Opisthorchis viverrni	JF823990 HM004188	Thaenkham et al. (2011) Thaenkham,U., Dekumyoy, P., Komalamisra, C., Sato, M., Dung, D., & Waikagul, J.	X55357 HM004211	Korbsrisate et al. (1991) Thaenkham,U., Dekumyoy, P., Komalamisra, C., Sato, M., Dung, D., & Waikagul, J.	–	–

		(Direct Submission)		(Direct Submission)		
Pseudamphistomum truncatum	OK358939, OK358940	Sokolov et al. (2022)	OK384549, OK384550	Sokolov et al. (2022)	–	–
Heterophyide						
Centrocestus formasanus	HQ874609	Thaenkham, U., Nawa, Y., Blair, D., Waikagul, J., & Phuphisut, O. (Direct Submission)	HQ874608	Thaenkham, U., Nawa, Y., Blair, D., Waikagul, J., & Phuphisut, O. (Direct Submission)	–	–
Haplorchis taichui	KX815126	Le et al. (2017)	KX815126	Le et al. (2017)	–	–
Haplorchis pumilio	KX815125	Le et al. (2017)	KX815125	Le et al. (2017)	–	–
Haplorchis yokogawai	HM004178	Thaenkham et al. (2010)	HM004207	Thaenkham et al. (2010)	–	–
Metagonimus miyatai	HQ832635	Pornruseetairatn et al. (2015)	HQ832626	Pornruseetairatn et al. (2015)	–	–
Metagonimus takahashii	HQ832638	Pornruseetairatn et al. (2015)	HQ832629	Pornruseetairatn et al. (2015)	–	–
Metagonimus yokogawai	HQ832641	Pornruseetairatn et al. (2015)	HQ832632	Pornruseetairatn et al. (2015)	–	–
Procerovum cheni	HM004193	Thaenkham et al. (2010)	HM004212	Thaenkham et al. (2010)	–	–
Procerovum varium	HM004182	Thaenkham et al. (2010)	HM004199	Thaenkham et al. (2010)	–	–
Pygidiopsis macrostomum	MF972531	Santos and Borges (2020)	MF972492	Santos and Borges (2020)	–	–
Stellantchasmus falcatus	KY369164	Le et al. (2017)	MF077366	Le, T., Dao, T., Nguyen, T., Nguyen, T., Rajapakse, R., & Dorny, P. (Direct Submission)	–	–
Cryptogonimidae						
Caecincola parvulus	AY222231	Olson et al. (2003)	AY222123	Olson et al. (2003)	–	–
Mitotrema anthostomatum	AY222229	Olson et al. (2003)	AJ287542	Cribb et al. (2001)	–	–
Siphodera vinaledwardsii	AY222230	Olson et al. (2003)	AY222122	Olson et al. (2003)	–	–

(GenBank accession numbers MF438076, MF4388077, MF438098, MF438099, MF438101) and *A. donicus* of Ferguson et al. (2012) (GenBank accession numbers JQ241154–JQ241158). Here and below the members of this clade we recognized as *A. donicus s. str*. (Fig. 1). In turn, *A. donicus s. str*. had a weakly supported sister relationship with the clade including *A. donicus* of Sándor et al. (2017), *Apophallus brevis* Ransom, 1920, *Apophallus* cf. *microsoma* Ferguson et al., 2012 and *Apophallus* sp. of Van Steenkiste et al. (2015). The large clade containing all mentioned species appeared as a well-supported sister group to *Apophallus* cf. *microtestis*. Type species of *Apophallus*, *A. muehlingi*, occupies a basal position to other *Apophallus* spp. mapped to the tree. Mean genetic p-distance value between samples of *A. donicus s. str*. was 0.08 ± 0.05%, whereas those between *A. donicus s. str*. and *A. donicus* of Sándor et al. (2017) was 12.9 ± 1.59%.

Median-joining network, based on partial cox1 gene sequence data set, demonstrated the presence of at least four groups of haplotypes across *Apophallus* spp. related to *A. muehlingi*, *A. brevis* and two species of *A. donicus* complex: *A. donicus s. str.* and *A. donicus* of Sándor et al. (2017) (Fig. 2). The last two species were directly related with each other and differ by 47 mutational steps. *Apophallus donicus s. str*. possesses three haplotypes, including one ancestral (14 sequences) and two unique, whereas *A. donicus* of Sándor et al. (2017) has four haplotypes, including one ancestral (eight sequences) and three unique. *Apophallus* cf. *microtestis* was related with *A. donicus s. str*. through 60 mutational steps. *Apophallus* cf. *microsoma*, *Apophallus* sp. of Van Steenkiste et al. (2015) and *A. brevis* were located between *A. muehlingi* and *A. donicus* of Sándor et al. (2017).

Results of the phylogenetic analysis based on concatenated sequences of complete 18S rRNA and partial 28S rRNA genes overall 2780 bp in length, showed identical tree topology of the Opisthorchiidae tree for ML and BI algorithms (Fig. 3). All species of *Apophallus* used in the study together with liliatrematine digeneans formed one highly supported clade, which was closely related to the Opisthorchiinae. The clade containing *Apophallus* spp. and liliatrematines was subdivided into two highly supported subclades. The first subclade includes *A. muehlingi*, *A. donicus s. str.* and *Apophallus* cf. *microtestis*, the last two species formed a polytomy. The second subclade contained *A. zalophi* and two liliatrematines: *Liliatrema skrjabini* Gubanov, 1953 and *Liliatrema sobolevi* Gubanov, 1953, which also formed a polytomy. Thus, *A. zalophi* has no branching between members of the *Apophallus* clade, and therefore must be differentiated from them at the genus level. Consequently, *Pricetrema* should be resurrected. At the same time, we decided to abolish the subfamily Liliatrematinae and remove *Liliatrema* into the Apophallinae.

Taxonomy

We correct the diagnosis of the Apophallinae and *Apophallus*, redescribe *A. donicus s. str.*, provide a morphological characterization of *Apophallus* cf. *microtestis*, and resurrect the genus *Pricetrema*. Since *P. zalophi* (=*A. zalophi*) can be reliably identified by morphological characters, we do not describe it here. The photos of the specimens of *P. zalophi* examined in our study are provided in Fig. 4.

Family Opisthorchiidae Looss, 1899
Subfamily Apophallinae Ciurea, 1924 *emend*.

Synonymy. Liliatrematinae Gubanov, 1953.

Diagnosis (modified from subfamily diagnosis of Sokolov et al., 2021). Body pyriform to elliptical or more elongate, spined. Oral sucker unspecialized or funnel-shaped, with penta- or heptagonal distal end. Ventral sucker unspecialized. Pharynx present. Caeca blind or forming uroproct. Testes two or one. Cirrus-sac absent. Seminal vesicle bipartite or (?)unipartite. Pars prostatica present. Permanent ventrogenital sac or genital sac present, median. Gonotyls two or absent. Genital pore antero-median to ventral sucker. Ovary, pretesticular. Seminal receptacle canalicular. Uterus pretesticular. Vitellarium follicular; follicles in two lateral fields. Excretory vesicle Y- or T-shaped; stem long, sigmoid, passing between two testes. Intestinal parasites of birds and mammals; Eurasia, North Africa and North America, North Pacific. Type genus *Apophallus* Lühe, 1909.

Other genera: *Pricetrema* Ciurea, 1933, *Liliatrema* Gubanov, 1953.
Genus *Apophallus* Lühe, 1909 *emend*.

Synonymy. *Apohalloides* Yamaguti, 1971, *Cotylophallus* Ransom, 1920, *Rossicotrema* Skrjabin & Lindrop, 1919.

Diagnosis (modified from genus diagnosis of Pearson, 2008). Body pyriform to elliptical or more elongate, spined. Oral sucker unspecialized. Ventral sucker well developed, with axis inclined anteriorly. Pharynx

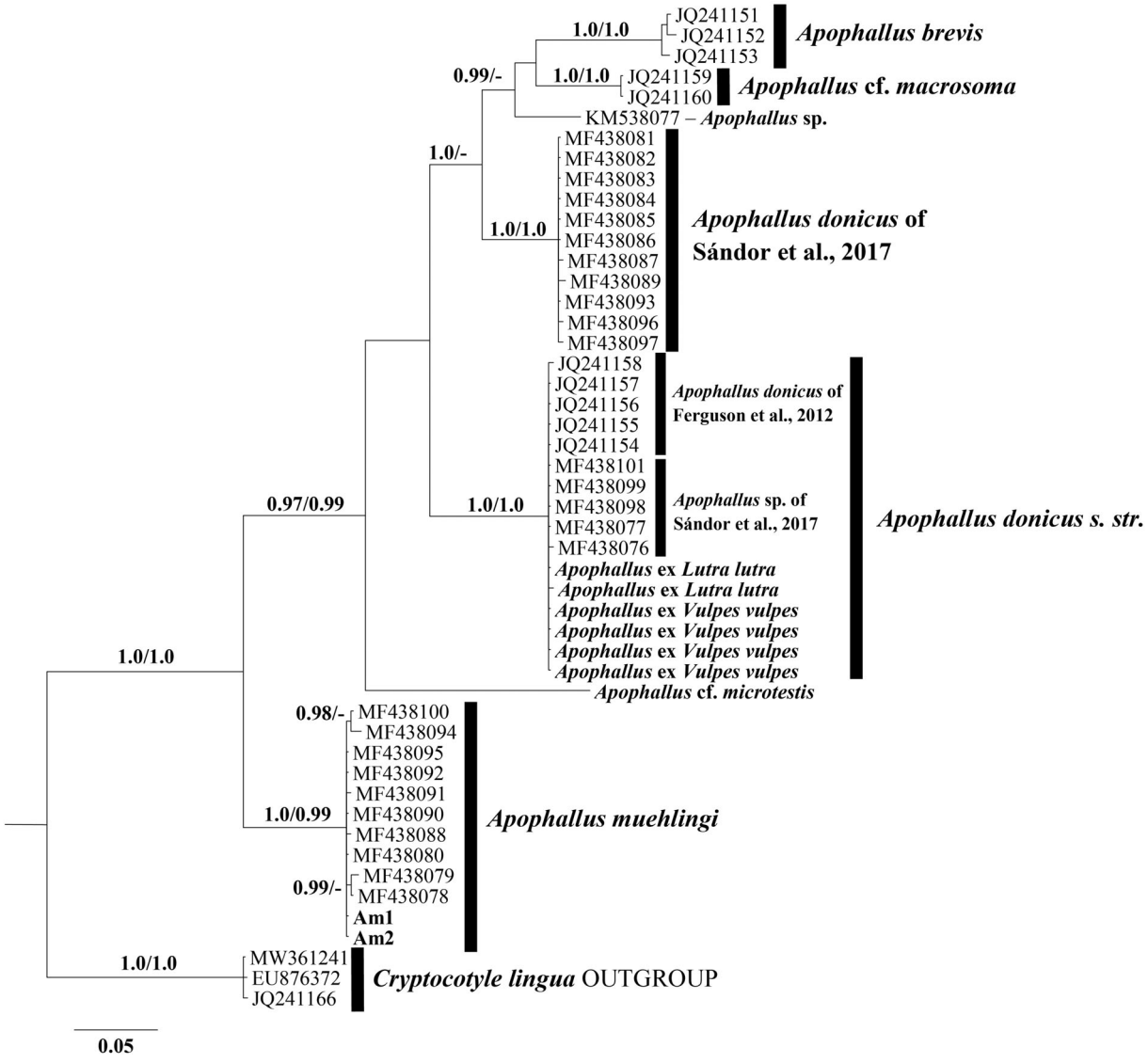

Fig. 1. Phylogenetic relationships of *Apophallus* spp. based on partial sequences of cox1 gene. Nodal support represented for ML/Bayesian algorithms. Only significant values of the posterior probabilities (≥0.9) are indicated. Newly obtained sequences are in bold.

present. Caeca terminating at or near posterior extremity. Testes two or one, entire, tandem or oblique. Cirrus-sac absent. Seminal vesicle elongate, curved or convoluted, bipartite or (?)unipartite; pars prostatica present. Ventrogenital sac permanent, median. Gonotyls two, lateral and opposite, arising dorsally close against ventral sucker, clavate in lateral aspect, rounded in ventral aspect, overhanging ventral sucker ventrally. Genital pore anteromedian to ventral sucker. Ovary entire, submedian, pretesticular. Seminal receptacle canalicular. Uterus pretesticular, not penetrated into forebody. Vitellarium follicular; follicles in two lateral fields penetrated into testes area or post-testicular region. Excretory vesicle Y- or T-shaped; stem long, sigmoid, passing between two testes. Intestinal parasites of birds and mammals; Eurasia, North Africa and North America. Type species *Distoma muehlingi* Jägerskiöld, 1899; valid binomen—*Apophallus muehlingi* (Jägerskiöld, 1899) Lühe, 1909.

Remarks. This genus is morphologically similar to *Pricetrema* (the presence of a median ventrogenital sac with two gonotyls), but different from it in the arrangement of the testes (opposite *vs* oblique or nearly tandem), the distribution of the vitelline follicles (anterior, lateral and, in most species, posterior to testes *vs* only pretesticular) and the position of the distal uterine loops (restricted to hindbody and ventral sucker area *vs*

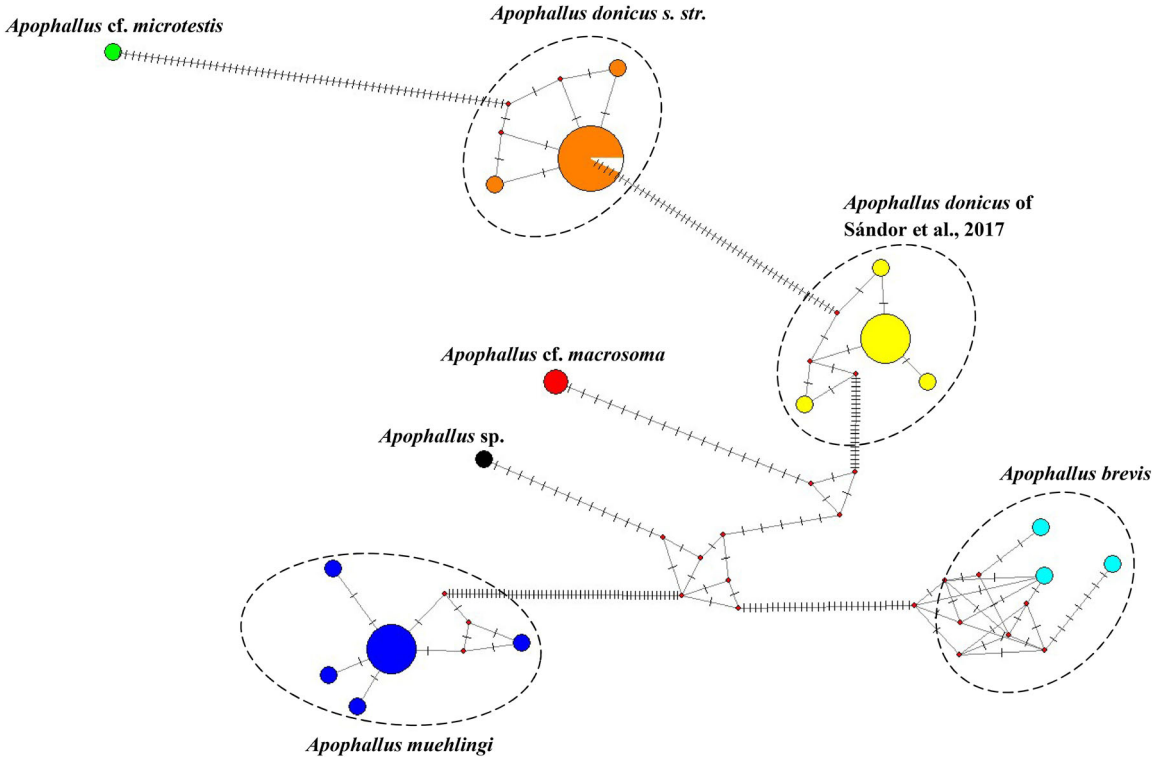

Fig. 2. Median-joining network of *Apophallus* spp. based on partial sequences of cox1 gene. Black stroke is one mutational step.

penetrated into forebody) (Machida et al., 1981; Price, 1932; Shults, 1978; Sokolov et al., 2021; Yurakhno, 1969, 1986). *Apophallus* differs from *Liliatrema* by the morphology of the oral sucker (unspecialized *vs* specialized), the caeca (uroproct absence *vs* presence), the external genital complex (ventrogenital sac with two gonotyls *vs* genital sac without gonotyls) and the distribution of the uterine loops (not penetrated into forebody *vs* penetrated).

Apophallus donicus (Skrjabin & Lindrop, 1919) Price, 1931 *s. str.*
(Figs 5, 6, 7)

Synonymy. *Rossicotrema donicum* Skrjabin & Lindrop, 1919; *Apophallus donicus* of Ferguson et al. (2012); *Apophallus* sp. of Sándor et al. (2017).

Material examined. Eleven syntypes (RIPPC No 13472, 13474, 13476, 13477) and 15 additional adult specimens – 10 paragenophores and two hologenophores collected in *V. vulpes* from Kasimovsky District, Ryazan Oblast, Russia, and one paragenophore and two genotyped trematode specimens collected in *L. lutra* from Taldomsky District, Moscow Oblast, Russia. Non-type specimens deposited: 10 paragenophores, IPEE RAS 14332–14335. Sequences deposited: two complete sequences of the 18S rRNA gene, OP803128 and OP803129, two partial sequences of the 28S rRNA gene, OP803125 and OP803126 (all four obtained from specimens ex *L. lutra*) and six partial sequences of the cox1 gene, OP804343, OP804343 (ex *L. lutra*) and OP804348– OP804351 (ex *V. vulpes*) are deposited in GenBank NCBI.

Description (re-examined syntypes). Body ovoid to elongate-oval, length 1109–1509 (1256), maximum width 512–691 (597) at midlevel of body or posterior half of body, occasionally in anterior half of body; width to length ratio 1: 1.9–2.7 (1: 2.1). Tegument covered with spines.

Oral sucker globular to ellipsoid, 46–72 × 66–87 (59 × 78); mouth subterminal. Ventral sucker globular to ellipsoid, with axis inclined anteriorly, 48–72 × 48–66 (60 × 56), recessed into ventrogenital sac. Ventrogenital sac median, with two gonotyls located just anterior to anterior margin of ventral sucker. Oral sucker to ventral sucker width ratio 1: 0.6–0.8 (1: 0.7). Forebody 28.4–35.1 (31.1)% of body length. Prepharynx absent. Pharynx 36–58 × 47–69 (44–56). Oesophagus 123–213 (157) in length, as 33.3–53.0 (40.3)% of forebody length. Intestinal bifurcation in third quarter or at border of third

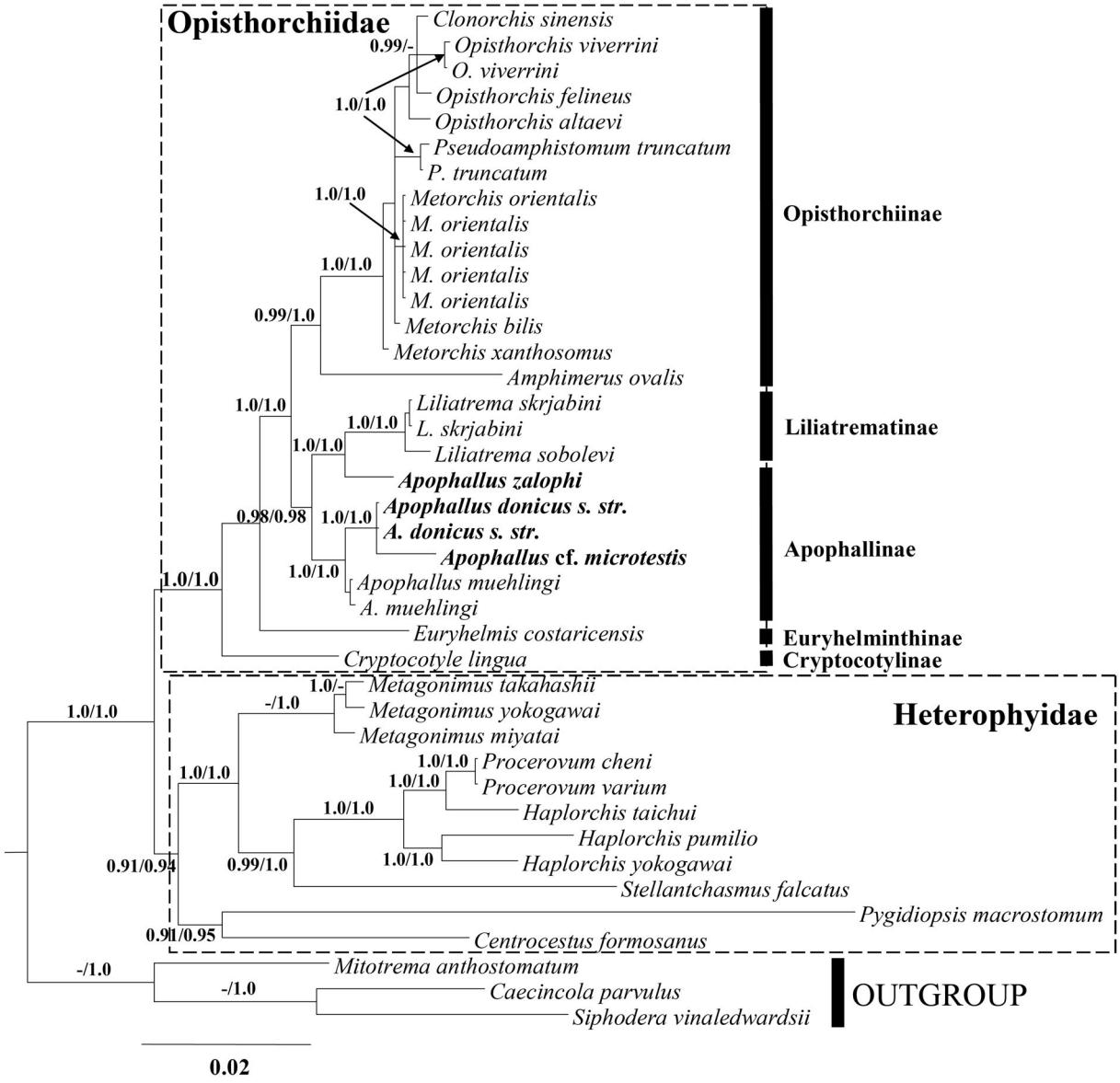

Fig. 3. Phylogenetic relationships of *Apophallus* spp. based on concatenated data set of 18S + 28S rRNA gene sequences. Nodal support represented for ML/Bayesian algorithms. Only significant values of the posterior probabilities (≥0.9) are indicated. Newly obtained sequences are in bold.

and posterior quarters of forebody. Caeca blind, relatively narrow, extending to near posterior margin of body.

Testes two, oblique, in second and posterior third of hindbody, contiguous or so; sinister testes anterior to dexter testes or vice versa; sinister testes subglobular to subtriangular or ellipsoid, 185–332 × 172–258 (250 × 214), dexter testes subglobular to ellipsoid or subsquare, 227–344 × 168–299 (268 × 228). Post-testicular region 5.2–12.7 (9.4)% of body length. Seminal vesicle, sinuous, bipartite with tubular, proximal reservoir and saccular distal reservoir, naked, encroaches distinctly into hindbody. Pars prostatica and ejaculatory duct not visible.

Ovary ellipsoid to subtriangular or ovoid, 80–149 × 97–190 (114 × 141), dextro-submedian or sinistro-submedian, pretesticular, separated. Canalicular seminal receptacle postero-dorsal to ovary. Laurer's canal opens dorso-median at level anterior margin of anterior testes (visible only in one specimen). Oötype with Mehlis' gland median, postero-sinistral or sinistral to ovary. Uterus pretesticular; metraterm not visible. Eggs numerous, operculate, mostly deformed in balsam, size of entire eggs 32–35 × 17–20 (33 × 19); shell with numerous ridges forming ornament in form of honeycombs. Genital atrium distinct, common genital pore between bases of gonotyls. Vitellarium follicular; follicles in

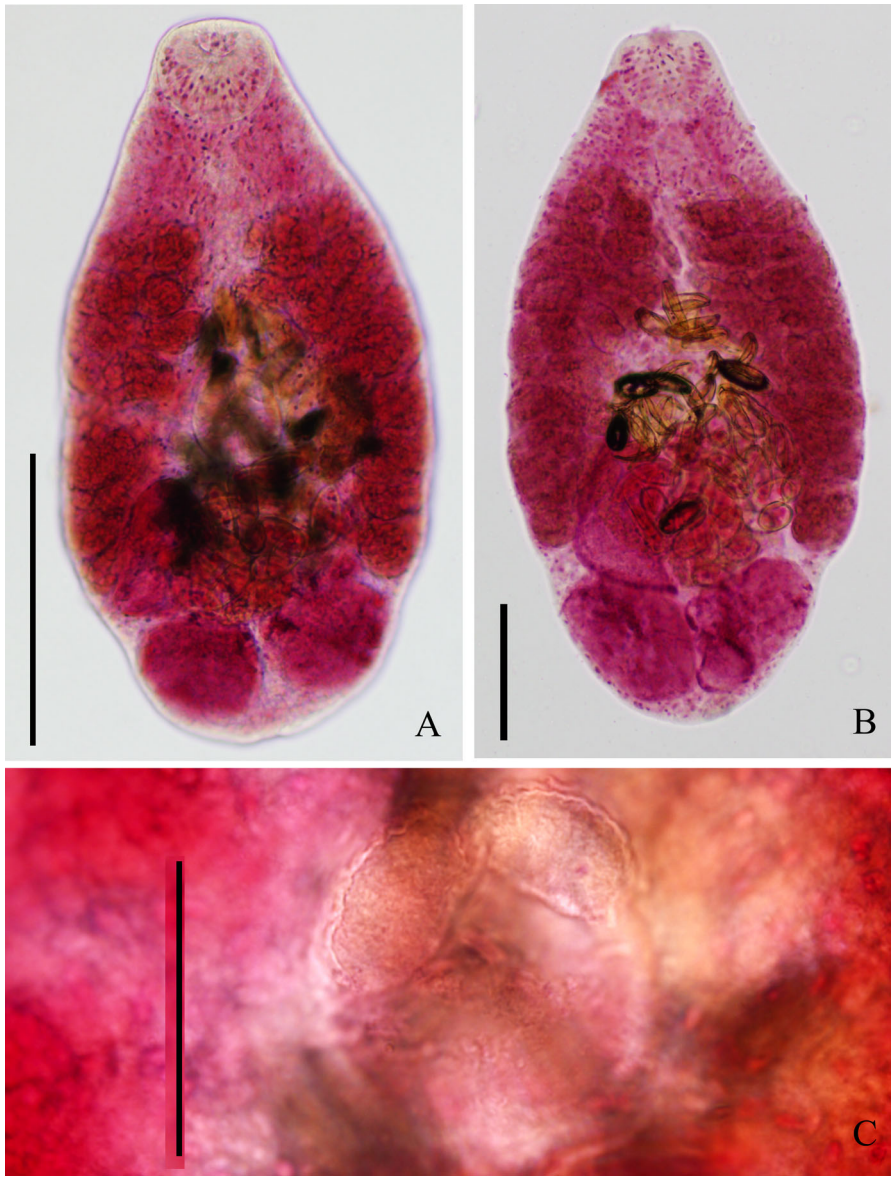

Fig. 4. Voucher specimens (paragenophores) of *Pricetrema zalophi* from intestine of *Halichoerus grypus*. (A), whole ventral view with optical focus on surface body; (B), whole ventral view with optical focus on gonads; (C), two gonotyls projecting from ventrogenital sac. Scale bars: A = 200 μm; B = 100 μm; C = 50 μm.

form of interconnected branching bands anteriorly and more globular posteriorly, in two lateral fields extending from level of intestinal bifurcation or level slightly anterior or posterior to bifurcation to posterior end of body, confluent (as narrow band) or not confluent in forebody and not confluent in post-testicular region. Distance from anterior end of body to anterior extremity of vitellarium 46.2.–72.1 (64.4)% of forebody length. Ventral vitelline follicles extracaecal or encroach over ventral surface of caeca in hindbody and region of ventral sucker, and extracaecal and intracaecal in forebody, dorsal follicles overlap dorsal surface of caeca. Excretory vesicle T-shaped, stem sinuous, reaches to level of anterior margin of anterior testes.

Description (11 paragenophores and two hologenophores). Body elongate-oval, length 672–924 (825), maximum width 238–350 (288) at midlevel of body or posterior half of body; width to length ratio 1: 2.4–3.9 (1: 2.9). Tegumental spines not visible. Oral sucker globular to ellipsoid, 41–55 × 55–62 (49 × 53); mouth subterminal. Ventral sucker globular to ellipsoid, with axis inclined anteriorly, 41–48 × 38–62 (43 × 53),

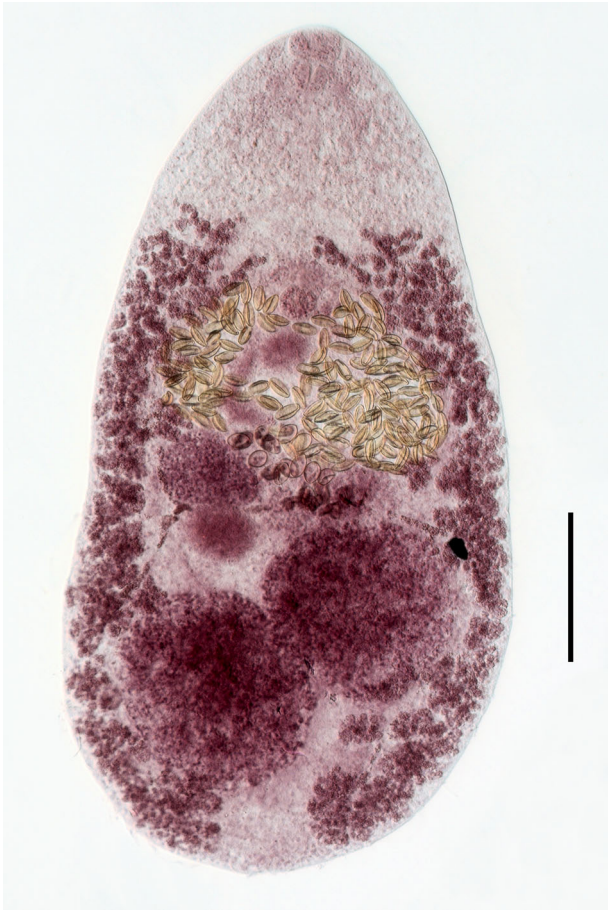

Fig. 5. Syntype of *Apophallus donicus s. str.* (RIPPC No 13472) from intestine of domestic dog, ventral view. Scale bar = 200 μm.

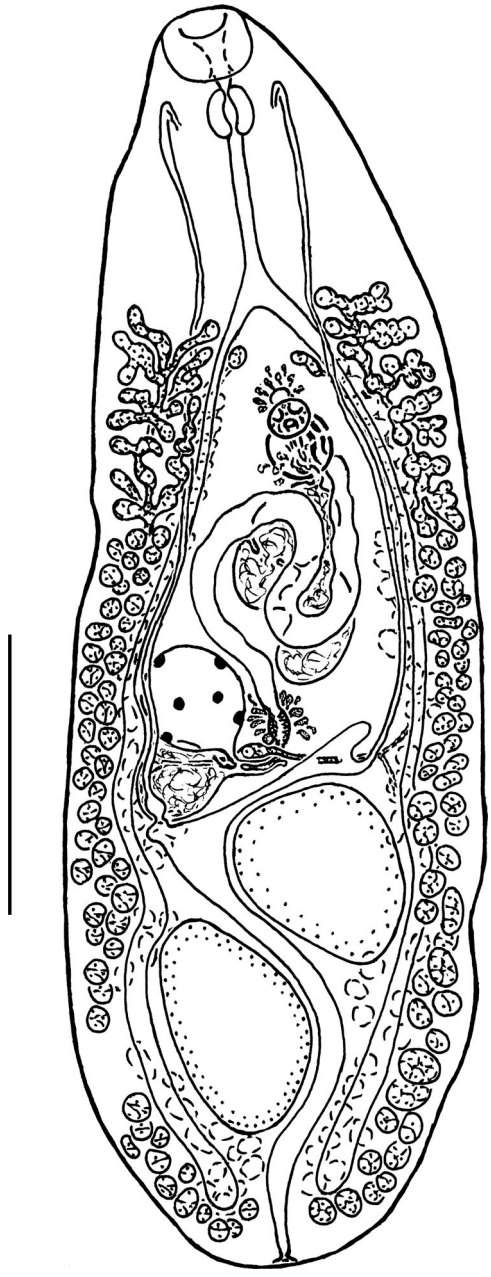

Fig. 6. Voucher specimen (paragenophore) of *Apophallus donicus s. str.* from intestine of *Vulpes vulpes*, ventral view. Scale bar = 200 μm.

recessed into ventrogenital sac. Ventrogenital sac median, with two gonotyls located just anterior to anterior margin of ventral sucker. Oral sucker to ventral sucker width ratio 1: 0.6–1.5 (1: 0.8). Forebody 32.5–39.2 (35.9)% of body length. Prepharynx 3–10 (7) in long. Pharynx 31–38 × 28–34 (33 × 31). Oesophagus 69–118 (99) in length as 27.0–39.5 (33.5)% of forebody length. Intestinal bifurcation in third quarter or at border of third and posterior quarters of forebody. Caeca blind, narrow, extending to near posterior margin of body.

Testes two, oblique, in second and posterior third of hindbody, separated; anterior testes subglobular to subtriangular or crescent-shaped, strongly sinistro-submedian, 90–159 × 97–159 (118 × 116), posterior testes subglobular to subtriangular or ellipsoid, strongly dextro-submedian 111–159 × 83–145 (133 × 110). Post-testicular region 9.7–16.0 (11.9)% of body length. Seminal vesicle, bipartite with tubular, sinuous proximal reservoir and saccular distal reservoir, naked, encroaches distinctly into hindbody. Pars prostatica saccular, surrounded by field of prostatic cells. Ejaculatory duct opens into genital atrium postero-ventral to female genital pore.

Ovary ellipsoid to subtriangular, 41–72 × 58–89 (54 × 83), dextro-submedian, pretesticular, separated. Canalicular seminal receptacle postero-dorsal to ovary. Laurer's canal opens dorso-median at level of ovary. Oötype with Mehlis' gland median, sinistrally to ovary. Uterus pretesticular; metraterm, opens into genital atrium dorsally. Eggs deformed in balsam, length of

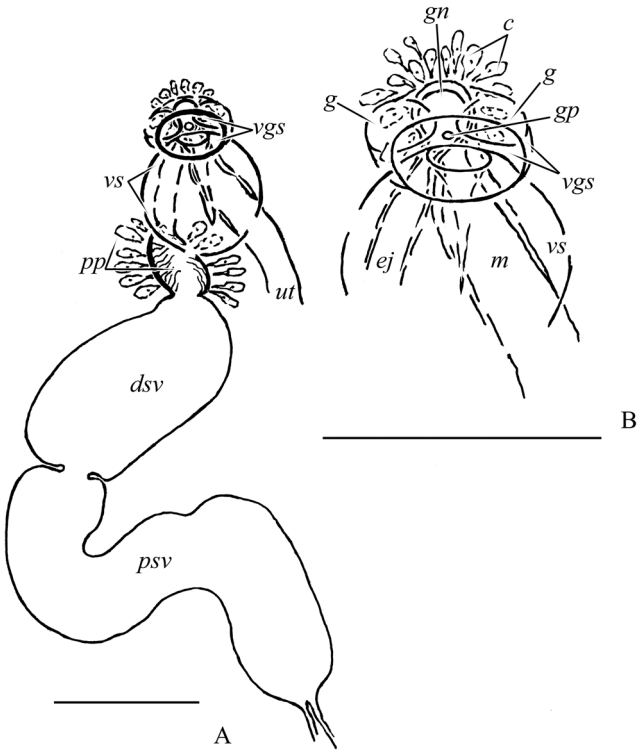

Fig. 7. Terminal genitalia and ventrogenital sac of voucher specimen (paragenophore) of *Apophallus donicus s. str.* (A), whole ventral view; (B), distal portions of terminal genitalia, ventral view. Abbreviations: c, gland cells around genital atrium; dsv, distal reservoir of seminal vesicle; ej, ejaculatory duct; g, gonotyls, gn, genital atrium, gp, genital pore; m, metraterm; pp, pars prostatica with field of prostatic cells; psv, proximal reservoir of seminal vesicle; ut, uterus; vgs, ventrogenital sac; vs, ventral sucker. Scale bars (A, B) = 50 μm.

least deformed eggs 32–39 (35); shell with numerous ridges ornamented in form of honeycombs. Genital atrium distinct, common genital pore between bases of gonotyls. Vitellarium follicular; follicles in form of interconnected branching bands anteriorly and more globular posteriorly, in two lateral fields extending from level of intestinal bifurcation or level slightly anterior to or just posterior to bifurcation to posterior end of body, confluent (as narrow band) or not confluent in forebody and not confluent in post-testicular region. Distance from anterior end of body to anterior extremity of vitellarium 51.7–73.9 (65.7)% of forebody length. Ventral vitelline follicles extracaecal or encroach over ventral surface of caeca in hindbody and region of ventral sucker, and extracaecal and intracaecal in forebody, dorsal follicles overlap dorsal surface of caeca.

Excretory vesicle T-shaped, occasionally with one reduced transverse branch, stem sinuous, reaches to level of anterior margin of anterior testes. Two main lateral collecting ducts reach to pharynx level.

Remarks. *Apophallus donicus* was originally described based on specimens collected from a domestic dog from Novocherkassk City, Russia (Skrjabin & Lindrop, 1919). We re-examined the syntypes and newly collected specimens of this trematode species. Conspecificity of our samples ex *V. vulpes* and *L. lutra* with the type specimens of *A. donicus* (originally as *Rossicotrema donicum*) is proven by similarity of body form, the position of the ventral sucker and the gonads, the morphology and the arrangement of the fields of the vitelline follicles. Newly collected specimens have lesser body and organ sizes in comparison to syntypes which is not essential for our taxonomic finding, because these metric parameters of *Apophallus* are variable depending on its host species (e.g. Odening, 1973).

Apophallus cf. *microtestis* Leonov, 1957 (Fig. 8)

Material examined. Adult specimen, paragenophore, collected in *C. aeruginosus* from hunting grounds along the lower Kuma River, Dagestan, Russia. Specimen deposited: one paragenophore, IPEE RAS 14331. Sequence deposited: complete sequence of the 18S rRNA gene and partial sequences of the 28S rRNA and cox1 genes are deposited in GenBank NCBI, OP803130, OP803127 and OP804347, respectively.

Description (paragenophore). Body elongate-oval, length 633, maximum width 253 at midlevel of body; width to length ratio 1: 2.5. Tegument of anterior half of body covered with spines. Oral sucker subglobular 52 × 65; mouth subterminal. Ventral sucker globular, with axis inclined anteriorly, 49 × 45, recessed into ventrogenital sac. Ventrogenital sac median, with two gonotyls located just anterior to anterior margin of ventral sucker. Oral sucker to ventral sucker width ratio 1: 0.7. Forebody 57.4% of body length. Prepharynx absent. Pharynx 39 × 36. Oesophagus 159 in length, as 43.6% of forebody length. Intestinal bifurcation at border of middle and posterior thirds of forebody. Caeca blind, very wide in proximal two-thirds of their length and narrow in the distal third, extending to near posterior margin of body.

Testes two, oblique, in second and third quarters of hindbody, contiguous; anterior testis sinistro-submedian, ellipsoid, 78 × 65, posterior testis dextro-submedian, subglobular, 81 × 88. Post-testicular region 12% of body length. Seminal vesicle convoluted, constriction not visible, because distal portion bent dorsally, naked, encroaches distinctly into hindbody. Pars prostatica not

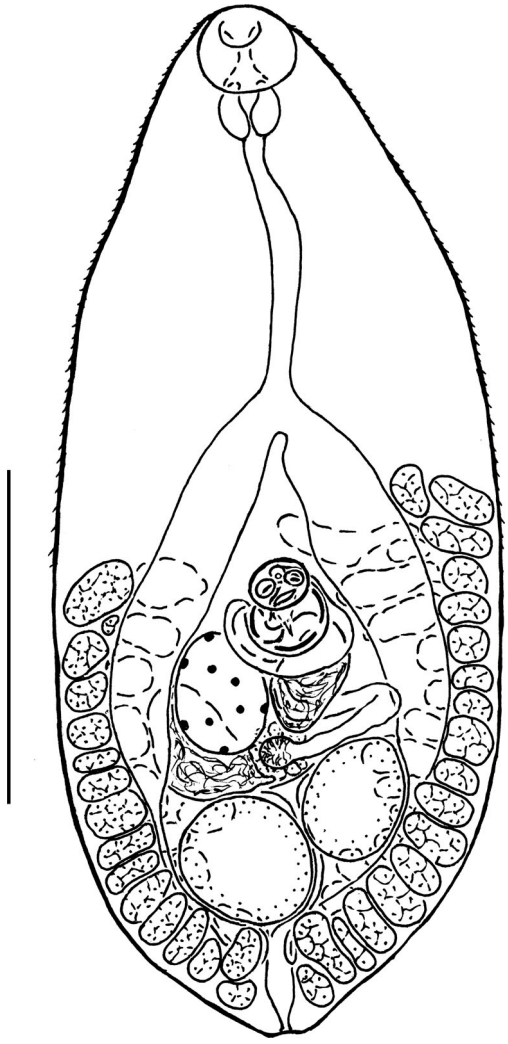

Fig. 8. Voucher specimen (paragenophore) of *Apophallus* cf. *microtestis* from intestine of *Circus aeruginosus*, ventral view. Scale bar = 200 μm.

visible. Ejaculatory duct opens into genital atrium postero-ventral to female genital pore.

Ovary ellipsoid, 75 × 65, dextro-submedian, pretesticular, separated. Canalicular seminal receptacle postero-dorsal to ovary. Distal portion of Laurer's canal not visible. Oötype with Mehlis' gland median, between ovary and testes. Uterus pretesticular; metraterm not clearly separated from uterus, opens into genital atrium dorsally. Eggs deformed in balsam, length of least deformed eggs 36; details of shell ornamentation not visible. Genital atrium distinct, common genital pore between bases of gonotyls. Vitellarium follicular; follicles large, numerous, densely packed, in two slightly asymmetrical lateral fields extending from midlevel distance between intestinal bifurcation and anterior margin of ventral sucker (sinistro-dorsal field) and level slightly anterior to anterior margin of ventral sucker (dextro-lateral field) to posterior end of body, almost confluent in post-testicular region but distinctly separated over excretory vesicle. Distance from anterior end of body to anterior extremity of vitellarium 83.0% of forebody length. Ventral vitelline follicles lie extracaecally anterior to level of testes and encroach over ventral surface of caeca posterior to so, dorsal follicles overlap dorsal surface of caeca or encroach over so.

Excretory vesicle T-shaped with sinuous stem, reaches to level of oötype.

Remarks. *Apophallus microtestis* was originally described based on specimens collected from *Nycticorax nycticorax* (Linnaeus, 1758) (Ardeidae) caught on the islands of Dneprovsky Liman (Leonov, 1957). Syntypes of this species (seven specimens RIPPC No 11973) studied by us are of poor quality; in particular, ventrogenital sac, gonotyls and seminal vesicle (except for a small part of this vesicle in one specimen) are not visible (Fig. 9). Meanwhile, the syntypes of *A. microtestis* differ slightly from the individual depicted in the original publication (Leonov, 1957, Fig. 2), namely, they have broader caeca and a symmetrical constriction in the midlevel of the body or just anterior to it. In addition, re-examined syntypes lack vitelline follicles on the ventral side of the median area of the post-testicular region, but single follicles are present in this area on the dorsal side. Probably Leonov (1957, Fig. 2) in his drawing mistakenly transferred the dorsally lying follicles to the foreground.

Our specimen is similar to *A. microtestis* by a number of key morphological characteristics, namely, the post-equatorial position of ventral sucker, the testes to body (at level of testes) width ratio ≥1: 3, the ovary to testes width ratio ≤1: 2 and broad caeca (at least in anterior part). However, it differs from this species in body size (633 × 253 *vs* 489–546 × 158–200), length of oesophagus as a ratio of the forebody length (43.6% *vs* 19.9–33.7%), length of the post-testicular region as proportion of the body length (12% *vs* 14.3–17.6%) and arrangement of vitelline follicles (densely packed *vs* loosely packed). Possibly, these differences are related to host-induced morphological variability or age of trematode specimens.

Genus *Pricetrema* Ciurea, 1933 *emend.*

Diagnosis (based on description of *Pricetrema* spp. in Price, 1932; Machida et al., 1981; Shults, 1978; Yurakhno, 1969, 1986). Body ovoid to elliptical or more elongate, spined. Oral sucker unspecialized.

Ventral sucker well developed, with axis inclined anteriorly. Pharynx present. Caeca terminating at or near posterior extremity. Testes two, entire, opposite. Cirrus-sac absent. Seminal vesicle elongate, curved, probably bipartite, naked; pars prostatica present. Ventrogenital sac permanent, median. Gonotyls two, lateral and opposite, arising dorsally close against ventral sucker, clavate in lateral aspect, rounded in ventral aspect, overhanging ventral sucker ventrally. Genital pore antero-median to ventral sucker. Ovary entire, submedian, pretesticular. Seminal receptacle canalicular. Uterus pretesticular, distal loops anterior to ventral sucker. Vitellarium follicular; follicles in two lateral pretesticular fields. Intestinal parasites of marine mammals; North Pacific. Type species *Apophallus zalophi* Price, 1932; valid binomen – *Pricetrema zalophi* (Price, 1932) Ciurea, 1933.

Remarks. The morphological differences of *Pricetrema* from the type genus of the Apophallinae were discussed above. *Pricetrema* differs from their sister genus *Liliatrema* by the morphology of the oral sucker (unspecialized *vs* specialized), the caeca (uroproct absence *vs* presence) and the external genital complex (ventrogenital sac with two gonotyls *vs* genital sac without gonotyls), the distribution of the vitelline follicles (present in post-testicular region *vs* absent), the arrangement of the testes (opposite *vs* tandem or nearly so).

Discussion

Apophallus donicus s. str. (=*R. donicum* of Skrjabin & Lindrop, 1919, *Apophallus* sp. of Sándor et al. (2017) and *A. donicus* of Ferguson et al. (2012)) has perfunctory similarity with *A. donicus auct. non* Skrjabin & Lindrop, 1919 (= *R. donicum* of Ciurea (1928), *A. donicus* of Mödlinger (1934), *A. donicus* of Odening (1973), *A. donicus* of Sándor et al. (2017)) described on the basis of experimental material from European percid fish (Ciurea, 1928; Mödlinger, 1934; Odening, 1973; Sándor et al., 2017). However, according to phylogenetic reconstructions for apophallines, *A. donicus auct. non* Skrjabin & Lindrop, 1919 and *A. donicus s. str.* are independent species (Sándor et al., 2017; this study).

These two species differ in the distance between the anterior end of the body and the anterior extremity of the vitellarium as a ratio to the forebody length: 51.7–73.9 (65.7)% *vs* 73.2–97.6 (83.1)% (values for *R. donicum* of Ciurea (1928), *A. donicus* of Mödlinger (1934) and *A. donicus* of Odening (1973) obtained from original figures). At the same time, most specimens of *A. donicus auct. non* Skrjabin & Lindrop, 1919 have values of this feature in the range 75.0–97.6%. This proportion is below 75% (namely 73.2%) only in one specimen of this digenean species from Odening (1973, Fig. 12i), however, the anterior extremity of vitellarium in this specimen lies clearly posterior to the intestinal bifurcation. Moreover, *A. donicus s. str.* differs from *A. donicus auct. non* Skrjabin & Lindrop, 1919 in morphology of anterior vitelline follicles: branching bands *vs* globular (compare with Cameron, 1936, 1937; Ciurea, 1928; Mödlinger, 1934; Odening, 1973; Figs 5, 6 in this study).

In turn, *A. donicus auct. non* Skrjabin & Lindrop, 1919 is very similar to *A. lari* described by Leonov (1957) (Fig. 10). Odening (1973) rightly considered *A. lari* and his specimens identified as *A. donicus* to be conspecific trematodes. Indeed, none of the key

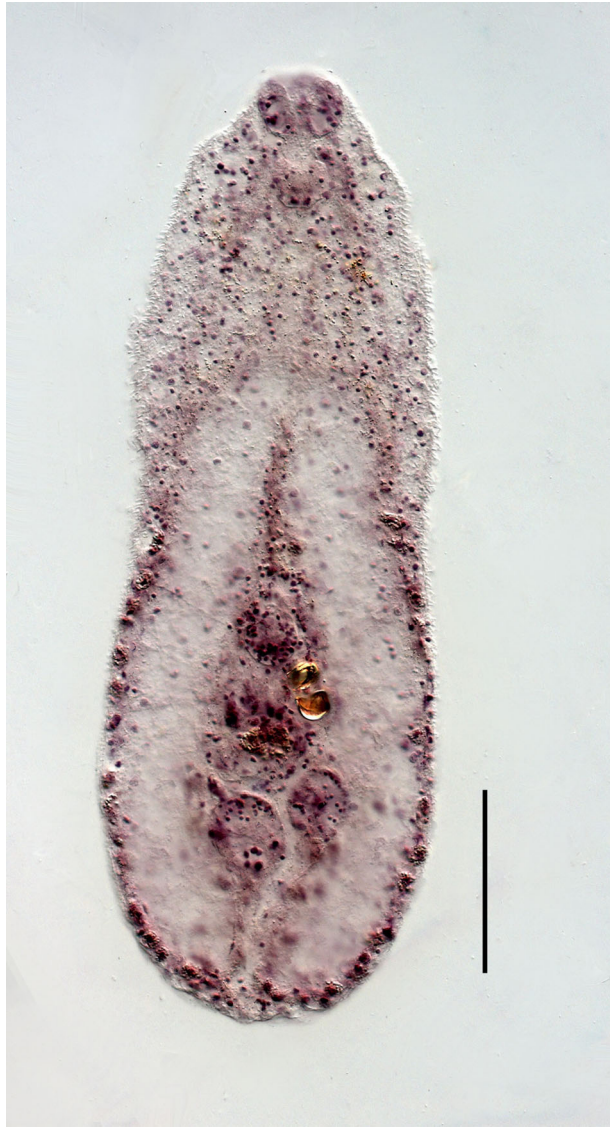

Fig. 9. Syntype of *Apophallus microtestis* (RIPPC No 11973) from intestine of *Nycticorax nycticorax*, ventral view. Scale bar = 100 µm.

characteristics, including body size, the position of the ventral sucker and the gonads, the arrangement of the field of vitelline follicles, globular shape of anterior vitelline follicles, and eggs size do not delimitate *A. donicus auct. non* Skrjabin & Lindrop, 1919 and *A. lari* from each other (compare with Cameron, 1936; Leonov, 1957; Odening, 1973). Thus, we establish the name *A. lari* for *A. donicus auct. non* Skrjabin & Lindrop, 1919 (= *R. donicum* of Ciurea (1928), *A. donicus* of Mödlinger (1934), *A. donicus* of Odening (1973), *A. donicus* of Sándor et al. (2017)). It is unknown which specimen of the *A. donicus* complex was represented in the figure in Ciurea (1924) – from an experimentally (cyprinids as second intermediate host) or naturally (unclear second intermediate host) infected dog? Meanwhile, that specimen is similar to *A. donicus s. str.* in distribution of vitelline follicles in the anterior part of the body.

Also, *A. donicus s. str.* is similar to *Apophallus venustus* (Ranson, 1920) Cameron, 1936 and *Apophallus similis* (Ranson, 1920) Lyster, 1940 from North America. Each of these three species is characterized by a relatively short distance between the anterior end of the body and the anterior extremity of the vitellarium as a ratio to the forebody length: 51.7–73.9%, 61.1–71.2%, 51.4% respectively (Cameron, 1936, Fig. 1; Cameron, 1937, Plate III, Fig. 4; Niemi, 1973, Plate IV, Fig. 5; Ranson, 1920, Figs. 22, 24, 25, 26; this study). Assumptions about the conspecificity of *A. venustus* and *A. similis* both in relation to each other (Cameron, 1936; Morozov, 1952; Price, 1932; Witenberg, 1929) and to *A. donicus sensu lato* (*A. donicus s. str.* + *A. lari*) (Price, 1932; Witenberg, 1929). However, *A. donicus s. str.* differs from *A. venustus* and *A. similis* in morphology of the anterior vitelline follicles: branching bands *vs* globular (Cameron, 1936, 1937; Niemi, 1973; Ranson, 1920; this study). Moreover, the eggs of *A. venustus* are slightly smaller than those of *A. donicus s. str.*: 32–39 μm *vs* 26–32 μm in length. *Apophallus lari* in comparison with *A. venustus* and *A. similis* possess shorter fields of vitelline follicles within the forebody (Cameron, 1936, 1937; Odening, 1973). Until molecular data on *A. venustus* and *A. similis* are obtained, we prefer to consider them as independent species, which agrees with the opinion recorded in Yamaguti (1971), Ferguson et al. (2012) and WoRMS (2022).

A question of true species membership of *A. donicus* of Niemi & Macy, 1974 from Oregon is still unclear. Metacercariae of this parasite encysted beneath the skin of cyprinid catostomid and salmonid fishes (Niemi & Macy, 1974). Relative to *A. donicus s. str.*, *A. donicus* of Niemi & Macy, 1974 is characterized by more extensive and densely packed vitelline follicles within the forebody (Niemi, 1973; Niemi & Macy, 1974).

The current study has confirmed our previous finding (Sokolov et al., 2022) on the paraphyly of *Apophallus sensu* Pearson, 2008 and the *Apophallinae sensu* Sokolov et al. (2021). It was surprising that *A. zalophi*, despite a greater similarity to *Apophallus* was phylogenetically closer to *Liliatrema*. We therefore resurrected *Pricetrema* and removed *Liliatrema* into the Apophallinae. The only morphological feature that united *Pricetrema* and *Liliatrema* is the position of the distal uterine loops within the forebody. Perhaps missing information on life cycles of these trematode groups, namely, the composition of the first intermediate hosts and the morphology of cercariae can add clarity to the relationship described.

At the same time, our study did not support the validity of *Rossicotrema*. As mentioned above, *A. donicus* is a type species of the nominal genus *Rossicotrema.* The validity of this genus has been discussed many times because of its close morphological similarity with

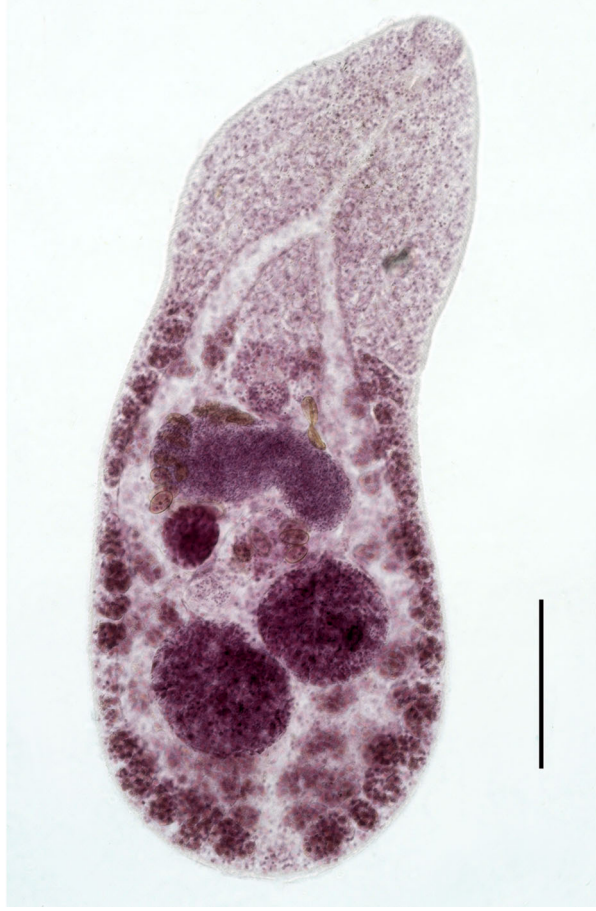

Fig. 10. Syntype of *Apophallus lari* (RIPPC No 11976) from intestine of *Larus argentatus*, ventral view. Scale bar = 200 μm.

Apophallus (Cameron, 1936; Ciurea, 1924, 1928, 1933; Lyster, 1940, Morozov, 1952; Pearson, 2008; Price, 1931; Witenberg, 1929; Yamaguti, 1971). According to Morozov (1952), two genera differ by the position of the vitellarium anterior border: at the level of the ventral sucker or posterior to it (*Apophallus*) *vs* at the level of the intestinal bifurcation (*Rossicotrema*). However, there are species described under *Apophallus* or *Rossicotrema* which show an intermediate state of the mentioned diagnostic feature (anteriorly to the ventral sucker level, but posteriorly to the bifurcation level), in particular, *Apophallus brevis* Ranson, 1920 and *A. lari* (originally as *R. lari*) (see Leonov, 1957; Ranson, 1920). In this regard, most authors synonymize *Rossicotrema* with *Apophallus* (see Cameron, 1936; Lyster, 1940, Pearson, 2008; Yamaguti, 1971). However, in the Russian-language literature, the validity of *Rossicotrema* has been recognized until recently (e.g. Ivanov & Semenova, 2004). Data obtained in our study, as well as results of previous studies (Ferguson et al., 2012; Sándor et al., 2017), show that phylogenetic relationships of the type genus of *Rossicotrema* resolved within the *Apophallus* clade.

In general, the current phylogenetic analysis based on the concatenated sequences of 18S and 28S rDNA support our previous conclusion that a united group of *Apophallus*, *Liliatrema*, the Opisthorchiinae, and the recently formalized *Pricetrema* are monophyletic (Sokolov et al., 2022). However, newly obtained data, unlike the previous reconstruction, show the existence of a nearest common ancestor in *Apophallus*, *Liliatrema* and *Pricetrema*. Structure of the Opisthorchiidae clade allows us to hypothesize the presence of ventrogenital sac as a plesiomorphic feature of the opisthorchiid digeneans and the reduction of this structure in the terminal groups. The ventrogenital sac with or without 1–2 gonotyls is present in cryptocotylines, euryhelminthines and basal apophallines (*Apophallus*, *Pricetrema*) but completely absent in opisthorchiines or reduced to the genital sac in *Liliatrema* (Sokolov et al., 2021; present study).

Phylogenetic reconstructions based on ribosomal 18S + 28S rRNA genes and partial sequences of the cox1 gene show similar topology for *A. muehlingi*, *A. donicus s. str.* and *Apophallus* cf. *microtestis*. The first of them appears as the basal species in relation to *A. donicus s. str.* and *Apophallus* cf. *microtestis* on both trees. Results of median-joining analysis demonstrate detailed relationships between species of *Apophallus* and show that they have different molecular heterogeneity, which cannot be seen on the BI and ML trees (Fig. 1, 2).

Conclusions

The molecular and morphological data we obtained allow us to consider *A. donicus s. str.* and *A. lari* (=*A. donicus auct. non* Skrjabin & Lindrop, 1919) as distinct species. These species differ in their specificity to a second intermediate host. In light of the new data, it is clear that metacercariae of *A. donicus s. str.*, associated with cyprinid fishes, and metacercariae of *A. lari*, with percid fishes (Ciurea, 1928; Ferguson et al., 2012; Mödlinger, 1934; Odening, 1973; Sándor et al., 2017). The first intermediate host in *A. donicus s. str.* is unknown, while that in *A. lari* is freshwater snails *Lithoglyphus naticoides* (Pfeiffer, 1828) and *Lithoglyphus pyramidatus* Möllendorf, 1873 (Ivanov & Semenova, 2004; Odening, 1973). To date, *A. donicus s. str.* has been reliably recovered only from mammals, while *A. lari* parasitizes birds (spontaneous and experimental data) and mammals (experimental data) (Skrjabin & Lindrop, 1919; Ciurea, 1933; Mödlinger, 1934; Leonov, 1957; Odening, 1973; present data). It remains unknown whether *A. donicus s. str.* can parasitize birds and to what extent *A. lari* are obligate hosts for mammals. The segregation of these species by ecological features is a matter for future research.

Despite the success in solving the problem of the *A. donicus* complex achieved in this study, there are still a number of issues that require further clarification namely, what is the true taxonomic status of the North American forms, *A. venustus*, *A. similis* and *A. donicus* of Niemi & Macy, 1974. The phylogenetic position of *A. zalophi* revealed in the current study points to the need to separate it and related species from *Apophallus* as a distinct genus, and to abolish the Liliatrematinae.

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No potential conflict of interest was reported by the author(s).

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