Redescription of *Aphalloides coelomicola* Dollfus, Chabaud & Golvan, 1957 (Digenea, Opisthorchioidea) based on specimens from *Knipowitschia caucasica* (Berg) (Actinopterygii, Gobionellidae) from a Black Sea lagoon, with comments on the systematic position of the genus

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Abstract The genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957 consists of two species parasitic in the body cavity of sand gobies. Its systematic position in the superfamily Opisthorchioidea Looss, 1899 is unresolved and it has been placed by various authors in three families, i.e. Cryptogonimidae Ward, 1917, Heterophyidae Leiper, 1909 and Opisthorchiidae Looss, 1899. Its type-species, *Aphalloides coelomicola* Dollfus, Chabaud & Golvan, 1957, is here reported from the Caucasian dwarf goby *Knipowitschia caucasica* (Berg) in the lagoon Atanasovsko Lake, Black Sea coast of Bulgaria (new geographical record). The species is redescribed based on light and scanning electron microscopy demonstrating some characters typical for the Cryptogonimidae but also characters distinguishing it from the other genera of the family such as the lack of tegumental spines and the presence of a short excretory vesicle, which does not extend into the forebody. Phylogenetic analysis of the D2-D3 expansion segments of the 28S rRNA gene suggests phylogenetic relationships of *Aphalloides coelomicola* with the cryptogonimid *Centrovarium lobotes* (MacCallum, 1895). These data support the affiliation of the genus *Aphalloides* to the family Cryptogonimidae. The peculiar morphology of the species in the genus is explained by their unusual life-cycles characterised by progenetic development; sand gobies being simultaneously second intermediate and definitive hosts.

Introduction

The digenean genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957 was erected as monotypic for *A. coelomicola* Dollfus, Chabaud & Golvan, 1957 described based on specimens from the common goby *Pomatoschistus microps* (Krøyer) captured in brackish waters at Lake Cane at Banyuls, Mediterranean coast of France. Originally, *Aphalloides* was placed in the family Cryptogonimidae Ward, 1917 (subfamily Siphoderinae Manter, 1934) but this position was considered as "temporary" due to the aberrant morphology of the species resulting in the uncertain systematic position of the genus (Dollfus et al., 1957). Reimer (1970) described the second species of *Aphalloides*, *A. timmi* Reimer, 1970, from the same host species, *P. microps*, in the Baltic Sea off Germany; he disagreed with the placement of the genus in the Siphoderinae and affiliated it with the subfamily Acetodextrinae Morozov, 1952. The two genera originally placed in the latter subfamily, i.e. *Acetodextra* Pearse, 1924 and *Pseudoexorchis*

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Yamaguti, 1938 (see Morozov, 1952), are currently considered as members of the family Heterophyidae Leiper, 1909 (see Pearson, 2008). Naydenova (1970) recorded *A. coelomicola* from three species of gobies, i.e. *Pomatoschistus microps leopardinus* (Nordmann) [=*Pomatoschistus marmoratus* (Risso)], *Knipowitschia longecaudata* (Kessler) and *K. caucasica* (Berg), from the Black Sea and the Sea of Azov; she recognised *Aphalloides* as a member of the family Heterophyidae based on the presence of a genital sinus, a well developed seminal receptacle and the differentiation of the body into an anterior part able to move and an immobile posterior part containing the testes and ovary. At the same time, characters such as the lack of a gonotyl, the dorsal position of the vitellarium, the preovarial seminal receptacle and the strongly developed uterus were considered as characters differentiating *Aphalloides* from representatives of the known subfamilies of the Heterophyidae. For this reason, Naydenova (1970) proposed the new monotypic subfamily Aphalloidinae Naydenova, 1970. Yamaguti (1971) suggested that *Aphalloides* should be placed in the family Opisthorchiidae Looss, 1899 and the subfamily Aphallinae Yamaguti, 1958, proposing the monotypic tribe Aphalloidini Yamaguti, 1971 for it. Since these two family-group taxa (Aphalloidinae Naydenova, 1970 and Aphalloidini Yamaguti, 1971) have the same type-genus, they should be considered as synonymous (ICZN, Article 36.2) with Aphalloidinae Naydenova, 1970 having priority as the older name. Bayssade-Dufour & Maillard (1982) found that the chaetotaxy of the cercariae of *A. coelomicola* significantly differed from that of the representatives of the families Heterophyidae and Opisthorchiidae and was similar to the chaetotaxy of the family Acanthostomidae Poche, 1926, to which they transferred the genus. Brooks (1980) recognised acanthostomids as a subfamily of the Cryptogonimidae. Miller & Cribb (2008a) accepted the synonymy of Acanthostomidae with Cryptogonimidae but did not recognise subfamilies in the latter; they placed the type-genus of the subfamily Aphallinae Yamaguti, 1958, i.e. *Aphallus* Poche, 1926, in the Cryptogonimidae, thus considering Aphallinae as synonymous of the Cryptogonimidae. According to Miller & Cribb (2008a), the two species of the genus *Aphalloides* (*A. coelomicola* and *A. timmi*) are morphologically consistent with the Cryptogonimidae except for the lack of tegumental spines and the short

excretory vesicle; they considered that further study was needed to determine the systematic relationships of *Aphalloides* within the superfamily Opisthorchioidea Looss, 1899.

The aim of the present study is to clarify the family position of the genus *Aphalloides* on the basis of morphological and molecular analysis of its type-species *A. coelomicola*.

Materials and methods

In 2012 and 2013, we examined 186 individuals of *Knipowitschia caucasica* from Atanasovsko Lake for the presence of helminth parasites. Digeneans from the body cavity to be used for light microscopy studies were fixed in hot physiological saline solution and stored in 70% ethanol. They were stained in iron acetocarmine, dehydrated in an ascending ethanol series (70, 80, 90, 96 and 100%), cleared in dimethyl phthalate and mounted in Canada balsam. The metrical data in the description are presented as the range, with the mean and the number of measurements taken (n) in parentheses. The measurements are given in micrometres except where otherwise stated.

Voucher specimens are deposited in the collections of the Natural History Museum, London (NHMUK) and the Natural History Museum of Geneva (MHNG). The remaining specimens are in the helminth collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia.

Specimens for scanning electron microscopy (SEM) were fixed in hot 4% formalin and subsequently transferred to 70% ethanol. In laboratory conditions, worms were transferred to 40% ethanol (10 min), rinsed in 0.1 M cacodylate buffer (10 min), postfixed in 1% OsO_4 (2 h), dehydrated in an ethanol series, with the final dehydration step in hexamethyldesilazane (30 min). They were sputter-coated with gold in a JEOL JFS 1200 fine coater and examined using a JEOL JSM 5510 microscope at 10 kV.

Genomic DNA was extracted from each of four ethanol-fixed worms using a GenJET genomic DNA purification kit (Fermentas, Thermo Scientific, Waltham, USA). The D2-D3 expansion segments of the 28S rRNA gene (*c.*900 bp) was amplified from each specimen using the primers D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA

GGA ACC AGC TAC TA-3′) (De Ley et al., 1999). Polymerase chain reaction (PCR) amplifications were performed in 50 μl volume under the following conditions: initial denaturation at 94°C for 5 min; 40 cycles (denaturation at 94°C for 30 sec; primer annealing at 50°C for 30 sec; extension at 72°C for 1 min) and a final extension step at 72°C for 10 min. PCR products were visualised on 1% agarose gel with GreenSafe (NZYTech, Lisbon, Portugal) under visible and UV light. Fragment size was determined using GeneRuler^{TM} 100 bp Ladder Plus (Fermentas, Thermo Scientific, Waltham, USA). The amplified products were sequenced by Eurofins MWG Operon.

The sequences of the amplified region were manually aligned. The Multiple Sequence Alignments (MSA) of dataset were performed using Clustal Omega tool (Sievers et al., 2011). Subsequently, the MSAs were manually optimised and trimmed using MEGA 5.2 (Tamura et al., 2011). *Fasciola hepatica* Linnaeus, 1758 (GenBank accession no. AY222244) was used as an outgroup taxon.

The phylogenetic reconstructions were based on newly obtained sequences and GenBank data for representatives of the families Cryptogonimidae (mostly associated with studies by Miller & Cribb, 2005, 2007a, b, c, 2008b, 2009, 2013; Miller et al., 2009a, b, 2010). Other published sequences in the GenBank of the Cryptogonimidae, Heterophyidae and Opisthorchiidae were also included. They were performed using Bayesian inference (BI) phylogenetic analysis with MrBayes (Ronquist et al., 2012). Prior to analysis, the best model of nucleotide substitution was selected using MrModeltest2 (Nylander, 2004); this was the general time reversible model, with estimates of invariant sites and gamma distributed among-site rate variation (GTR+G+I). The analysis involved 72 nucleotide sequences and was run for 10×10^6 generations with sampling of every 100th tree and discarding ("burn-in") of the initial 25% of all trees. Otherwise standard MCMC parameters were used. All positions containing gaps and missing data were eliminated. Successful convergence of concurrent runs was assumed by the average standard deviation between split frequencies being consistently below 0.01 near the end of MrBayes analysis.

Family Cryptogonimidae Ward, 1917
Genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957

Aphalloides coelomicola Dollfus, Chabaud & Golvan, 1957

Host: Caucasian dwarf goby, *Knipowitschia caucasica* (Berg) (Actinopterygii, Gobionellidae).
Locality: Atanasovsko Lake, Bulgaria.
Site in host: Body cavity.
Voucher material: NHMUK 2015.2.13.1-2 (2 slides, 3 specimens); MHNG-PLAT-91135 (2 slides, 2 specimens).
Representative sequence: GenBank KJ162159 (28S rDNA).

Description (Figs. 1, 2)

[Based on 23 specimens; for exact values of n, see Table 1.] Body elongate, 1,209–2,518 × 391–759 (1,961 × 595), with tapering anterior and widely rounded posterior extremity (Fig. 1A) and maximum width in region between ventral sucker and ovary; ratio body length to body width 2.7:4.4 (3.4); ratio forebody length to body length 21.8–30.3% (25.1%). Tegument with transverse corrugations, devoid of spines. Eyespots 2, dark-brown with grainy structure, located at level of pharynx. Oral sucker terminal, 58–100 × 58–92 (81 × 79); slightly transversely elongate, almost spherical; slightly pyriform to almost globular in dorso-ventral view (Fig. 1A). Ventral sucker subspherical, 50–91 × 50–91 (68 × 73), situated at base of tegumental depression (Fig. 1A, B) at border of anterior quarter of body, at level of intestinal bifurcation or slightly posterior to it; typically smaller than oral sucker, rarely slightly larger. Sucker width ratio 1:0.71–1:1.19 (1:0.93; n = 19). Prepharynx absent. Pharynx elongate-oval, thick-walled, muscular, 46–81 × 46–69 (60 × 55). Oesophagus 111–273 (186) long, 33–65 (45) wide, thick-walled, usually winding, rarely straight. Intestinal bifurcation in anterior quarter of body. Caeca elongate-saccular, without lateral diverticula, contiguous, winding, terminate blindly just anterior to anterior testis or slightly overlapping it; sinistral caecum usually slightly longer than dextral; frequently, caeca not distinct along their entire length (masked by uterus and other organs). Excretory pore terminal.
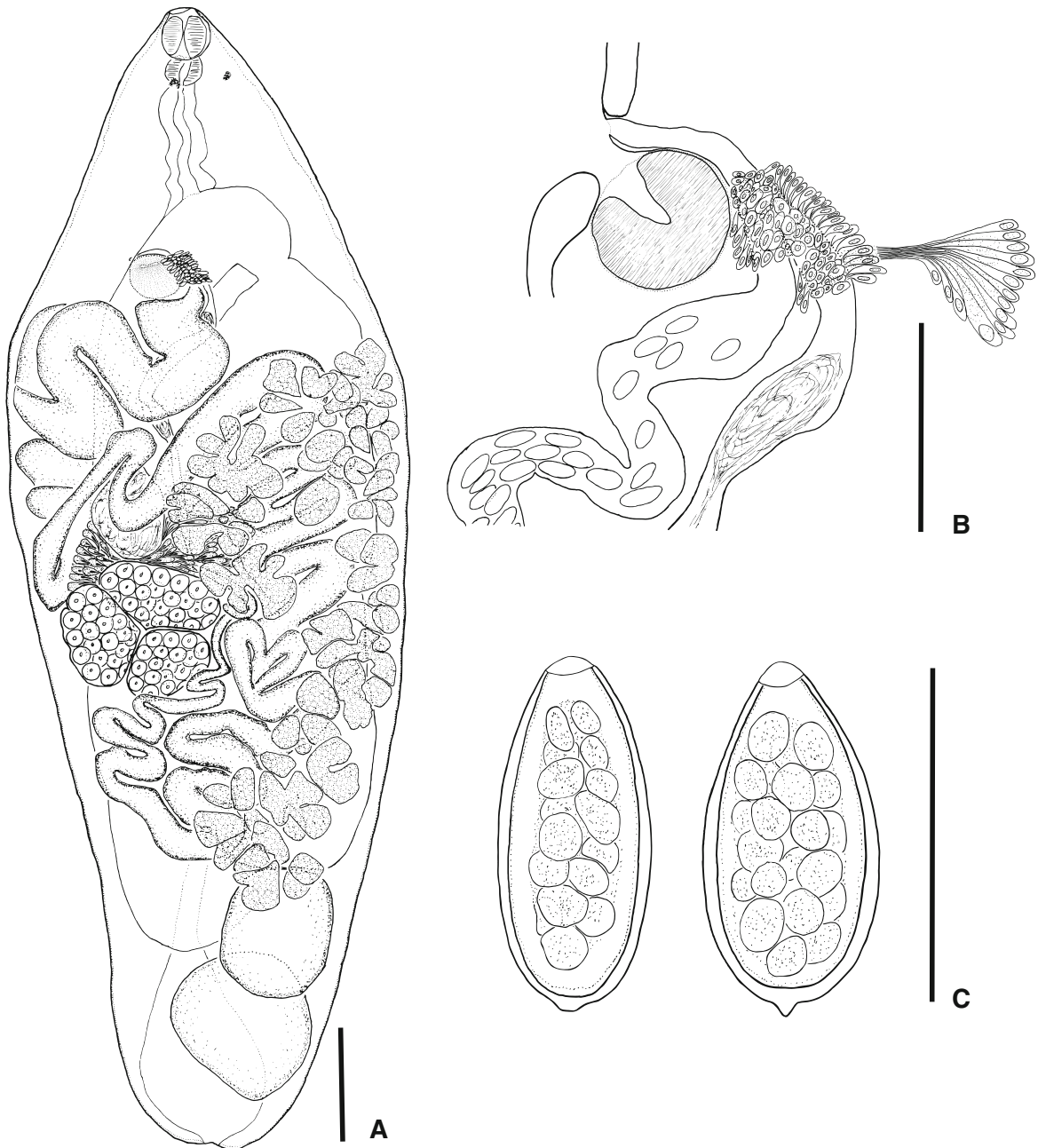
Fig. 1 *Aphalloides coelomicola* Dollfus, Chabaud & Golvan, 1957: A, Entire worm, dorsal view; B, Terminal genitalia, lateral view; C, Eggs isolated from the uterus of mature individuals and examined in physiological saline solution. *Scale-bars*: A, 200 μm; B, 100 μm; C, 30 μm

Excretory vesicle (observed in 7 specimens) Y-shaped, reaches anteriorly to level of posterior margin of ventral sucker or slightly posterior to it.

Genital pore a transverse slit located immediately anterior to rim of ventral sucker (Fig. 1B). Cirrus and cirrus-sac absent. Hermaphroditic duct short, 30–115 (69; n = 8) long, 10 (10; n = 8) wide; its proximal part covered with prostatic cells. Distal part of seminal vesicle also covered by prostatic cells representing continuation of prostatic cells lining proximal part of
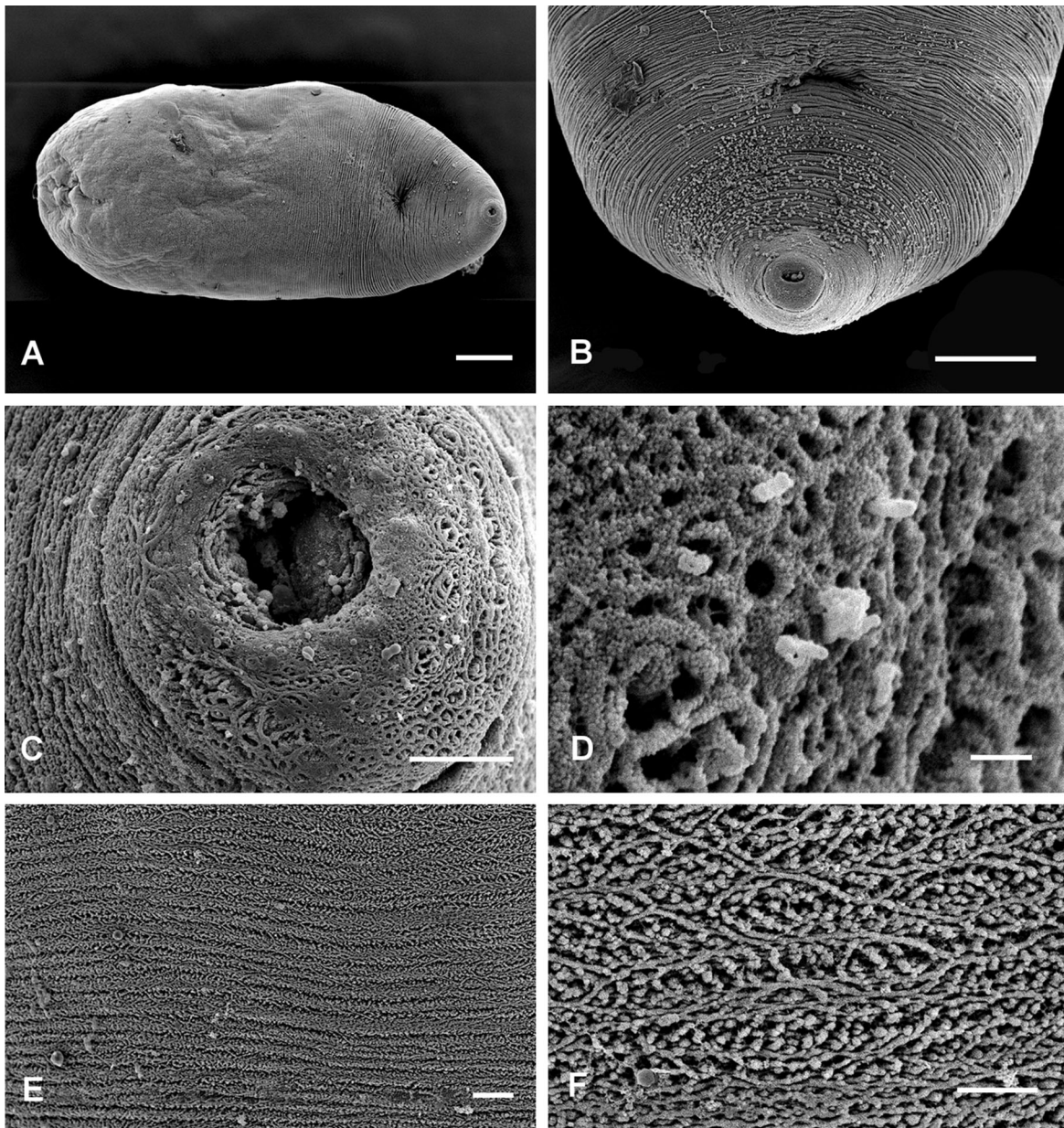
Fig. 2 *Aphalloides coelomicola* Dollfus, Chabaud & Golvan, 1957, scanning electron micrographs. A, Entire worm, ventral view; B, Anterior end with oral and ventral suckers; C, Oral sucker and surrounding papillae; D, Group of ciliated papillae in the region of oral sucker; E, F, Tegumental surface on the ventral side in the region posterior to ventral sucker at different magnifications; note transverse corrugations. *Scale-bars*: A, B, 100 μm; C, E, 10 μm; D, 1 μm; F, 5 μm

hermaphroditic duct; in some specimens, distinct second prostatic complex present, seen as bunch of pedunculate cells (Fig. 1B). Seminal vesicle tubular, curved, extending from junction with uterus to level of anterior end of seminal receptacle (its entire length can rarely be traced). Testes 2, oval, located close to posterior extremity of body (Fig. 1A), at same level (n = 3), one entirely anterior to another (n = 10) or one anterior to another and slightly overlapping it (n = 10), in most cases at angle; anterior testis 138–345 × 150–288 (235 × 191), posterior testis 138–288 × 161–325 (227 × 243).

Table 1 Metrical data for the species of the genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957 from various hosts and localities

Species	*Aphalloides coelomicola* Dollfus, Chabaud & Golvan, 1957					*Aphalloides timmi* Reimer, 1970	
Source	Dollfus et al. (1957)	Naydenova (1970)	Present study			Reimer (1970)	
Host	*Pomatoschistus microps* (Krøyer)	*Pomatoschistus microps leopardinus* (Nordmann), *Knipowitschia longecaudata* (Kessler), *K. caucasica* (Berg)	*K. caucasica*			*P. microps*	
Locality	France (Mediterranean coast)	Ukraine (Black Sea coast)	Bulgaria (Atanasovsko Lake)			Germany (Baltic Sea)	
	Range	Range	Range	Mean	n	Range	Mean
Body length	2,500	840–3,750	1,209–2,518	1,961	23	2,170–3,620	2,970
Body width	1,000	256–1,200	391–759	595	20	535–950	720
Oral sucker length	120	37–83	58–100	81	23	38–70	58
Oral sucker width	120	37–93	58–92	79	20	61–94	–
Ventral sucker length	95	40–96	50–91	68	22	47–94	68
Ventral sucker width	130	45–109	50–91	73	19	59–90	76
Pharynx length	95	34–76	46–81	60	23	47–66	55
Pharynx width	55	31–47	46–69	55	20	26–52	38
Oesophagus length	165	93–202	111–273	186	23	108–264	201
Oesophagus width	30	–	33–65	45	20		–
Ovary length	210	150–265	150–368	237	23	247–318	245
Ovary width	390	78–210	173–311	245	20	159–365	277
Seminal receptacle length	680	239–555	92–184	147	3	224–517	349
Seminal receptacle width	600	120–405	104–207	160	3	99–282	196
Hermaphroditic duct length	–	–	30–115	69	8	–	–
Hermaphroditic duct width	–	–	10–10	10	8	–	–
Anterior testis length	500	109–450	138–345	235	23	247–472	337
Anterior testis width	230	68–225	150–288	191	20	118–460	229
Posterior testis length	500	112–525	138–288	227	23	294–610	410
Posterior testis width	230	81–265	161–325	243	20	141–318	189
Egg length	28	26–31	23–33	27	230	28–34	30
Egg width	12	16–17	12–13	13	230	13–15	14

Ovary tri-lobed, 150–368 × 173–311 (237 × 245), anterior and separated from testes, almost equatorial, slightly shifted to left. Vitellarium follicular, forming clusters of follicles arranged in groups, extending from some distance posterior to ventral sucker to level of testes, sometimes slightly overlapping anterior testis (Fig. 1A). Mehlis' gland just anterior to and comparable in size with ovary.

Uterine seminal receptacle anterior to ovary, oval to circular, 92–184 × 104–207 (147 × 160), partially overlaps ovary in some worms. Uterus tubular, strongly developed, forms numerous loops filling space between ventral sucker and testes; metraterm absent, distal part of uterus with poorly developed muscles (Fig. 1A, B). Eggs small, elongate-oval, yellowish-brown, not embryonated, 23–33 × 12–13 (27 × 13, n = 230);
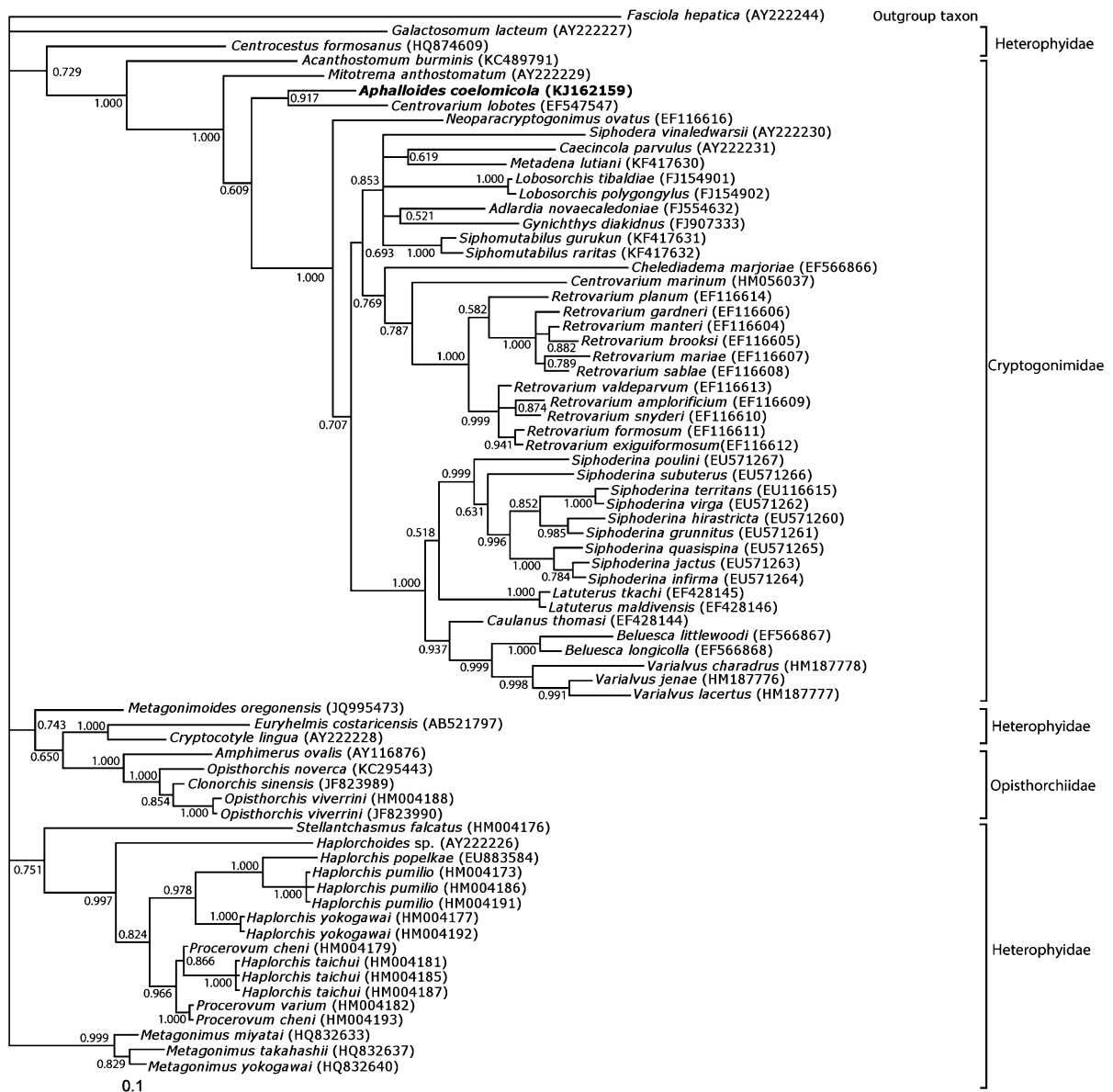

Fig. 3 Position of the genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957 in the superfamily Opisthorchioidea Looss, 1899 as revealed by Bayesian inference analysis using D2-D3 region of the 28S rRNA gene under the model GTR+G+I. Nodal support is given by posterior probabilities. GenBank accession numbers are given in parentheses

operculum present at one pole of egg; pole opposite to operculum rounded, often with distinct rounded or mucron-shaped thickening (Fig. 1C).

SEM observations

SEM observations confirmed that the body shape was elongate, with a rounded posterior extremity and a gradually tapering anterior part (Fig. 2A). The oral sucker was terminal (Fig. 2B), surrounded by numerous papillae (Fig. 2C). Most of the papillae were clearly ciliated (Fig. 2D). The ventral sucker was located in a deep depression and only the orifice of the depression was distinct on the ventral surface (Fig. 2A, B). Transverse corrugations were distinct along the entire body length (Fig. 2B, E); these consisted of transverse parallel ridges (Fig. 2E), which, at higher magnification, had complicated texture of fibrilar and granular elements (Fig. 2F). No spines were observed on the tegument.

28S rRNA gene sequence analysis

Sequences of the 28S rDNA fragments obtained from each of the four worms used were identical and were registered as one entry (GenBank KJ162159). Nucleotide sequence included 747 bp starting from 303 position of the 28S rRNA gene (see *Fasciola hepatica* GenBank AY222244). BLAST analysis revealed high similarity to digeneans of the family Cryptogonimidae, especially to *Mitotrema anthostomatum* Manter, 1963, a parasite of serranid fishes from Australian waters (GenBank AY222229; a sequence published by Olson et al., 2003) and to an unidentified species of the genus *Centrovarium* Stafford, 1904 isolated from the freshwater yellow perch *Perca flavescens* Mitchill from Minnesota, USA ("*Centrovarium* sp. VT-2007a", a sequence published in GenBank by Feigal, Tkach and Bell, EF547547). The latter material has subsequently been identified as *Centrovarium lobotes* (MacCallum, 1895) (V.V. Tkach, personal communication, 26 January 2014). The genetic divergence between *A. coelomicola* and the above-mentioned two digenean species is *c.*6% (702/748 bp and 537/573 bp, respectively).

The phylogenetic analysis did not resolve the relationships among the three families of the superfamily Opisthorchioidea and the consensus tree contained a basal polytomy; furthermore, the Heterophyidae was not revealed as a monophyletic group (Fig. 3). However, the family Cryptogonimidae was demonstrated as a monophyletic group, with a representative of *Acanthostomum* Looss, 1899 being basal to the remaining members of the family (Fig. 3). *Aphalloides coelomicola* formed a well-supported clade with *Centrovarium lobotes*, a parasite of freshwater fish that had a position relatively basal to the remaining members of the family.

Discussion

The morphology of the specimens studied corresponds well to the generic diagnosis of *Aphalloides* by Miller & Cribb (2008a). The generic identification is also supported by the specific host group (gobies) as well as the unusual site of infection, i.e. body cavity, which is characteristic for this genus (Dollfus et al., 1957; Naydenova, 1970; Reimer, 1970; Miller & Cribb, 2008a).

The morphological results of the present study resemble previous descriptions of adults of *Aphalloides coelomicola* (see Dollfus et al., 1957; Naydenova, 1970), including the morphometric data (Table 1). The only exception is the length of the seminal receptacle, which is smaller in our specimens than in previous data (Table 1); however, we have been able to measure this organ in only a few specimens because it is largely overlapping with the uterus and our data about its size are not representative. We note that our specimens exhibit the characters used by Reimer (1970) to distinguish *A. coelomicola* from *A. timmi*. These are the different sucker ratio (in *A. coelomicola*, the oral sucker is usually larger than the ventral sucker whereas in *A. timmi*, the ventral sucker is larger than the oral sucker) and the formation of a ring of vitelline ducts surrounding the seminal receptacle in *A. timmi*, which is lacking in *A. coelomicola* (see Reimer, 1970). We did not observe a ring of vitelline ducts surrounding the seminal receptacle in any of the specimens studied. Therefore, we identify our specimens as *A. coelomicola*. The metrical data provided by our study widen the known range of variation for the width of the pharynx, the length and width of the oesophagus, the size of the ovary, the size of the seminal receptacle, the size of the testes and the length of the eggs (Table 1).

Until now, there have only been SEM observations on the second species of the genus *Aphalloides*, *A. timmi* (see Bakke, 1980). The present study provides the first SEM data on *A. coelomicola*. Our results confirm the absence of tegumental spines in the species of *Aphalloides* and exhibit the relative uniformity of their surface ultrastructure. The only previous study reporting the presence of tegumental spines in a species of *Aphalloides* is the brief description presented by Gaevskaya (2012). Similarly to *A. timmi* as described by Bakke (1980), the rim of the oral sucker of *A. coelomicola* is encircled by ciliated papillae (Fig. 2C) but, in addition, some of them form groups of several papillae (Fig. 2D). In both species, the ventral sucker is located in a deep depression and the body surface consists of numerous transverse corrugations. We have not observed the presence of dome papillae surrounding the ventral sucker as well as the cobblestone-like appearance of the tegument posteriorly to the ventral sucker and dorsally on the anterior part of the body as described by Bakke (1980). Currently, it is not clear if these are differences between the two species or if they reflect different age of specimens studied, or they are a result of the fixation and preparation of specimens.

According to the generic diagnosis of Miller & Cribb (2008a), the Y-shaped excretory vesicle extends to mid-way between the ovary and ventral sucker. None of the previous descriptions of *Aphalloides* spp. (see Dollfus et al., 1957; Naydenova, 1970; Reimer, 1970) has specified the exact level to which the branches of excretory vesicle reach in anterior direction. This character has been difficult to observe in the present material. However, it has been distinct in three individuals, in all of them the anterior branches of the excretory vesicle reach to the level of the posterior margin of the ventral sucker. In addition, according to Miller & Cribb (2008a), the oral sucker is subterminal (while it is rather terminal in our specimens, Figs. 1A, 2A, B), and the prepharynx is very short (while it is entirely lacking in specimens studied by us or in the previous descriptions of *Aphalloides* spp.).

According to the generic diagnosis (Miller & Cribb, 2008a), a gonotyl is absent. Reimer (1970) described the genital pore of *A. timmi* as a gonotyl; however, his excellent illustration of the terminal genital ducts does not show any muscular formations surrounding the genital pore. Our observations on the present material are in agreement with the generic diagnosis of Miller

& Cribb (2008a) in relation to the absence of gonotyl in this genus.

As mentioned above, the systematic relationships of *Aphalloides* within the superfamily Opisthorchioidea have been controversial. The present study identified several morphological characteristics consistent with the diagnosis of the family Cryptogonimidae: pigmented eye-spots in adults, terminal oral sucker, ventral sucker in anterior half of body, intestinal bifurcation in anterior quarter of body, elongate caeca ending blindly, genital pore a transverse slit located just anterior to the rim of the ventral sucker, cirrus and cirrus-sac absent, replaced by hermaphroditic duct. In contrast to the remaining cryptogonimids, the tegument lacks spines (confirmed by the present SEM examination) and the excretory vesicle is relatively short, not entering the forebody (in other cryptogonimids, arms of excretory vesicle usually extend well into the forebody, see Miller & Cribb, 2008a). Nevertheless, the 28S rDNA data clearly show the relationship of *Aphalloides* within the Cryptogonimidae. The closest relationship detected is to the freshwater *C. lobotes*, a species with obviously differing morphology (see Miller & Cribb, 2008a) from that of *A. coelomicola*. However, Hoffman (1999) reported that progenetic metacecariae of *C. lobotes*, a species with predatory fish as definitive hosts in North America, may start producing eggs within metacercarial cysts in the musculature of the fathead minnow *Pimephales promelas* (Rafinesque) (Cyprinidae). This is a facultative life-cycle feature, which resembles, to some extent, the life-cycle of *A. coelomicola*. Interestingly, we have found most of the specimens of *A. coelomicola* free in the body cavity of their fish host; however, in several cases, one or several gravid individuals have been found located in a cyst, which frequently contained eggs released by the digeneans. We interpret such cysts containing adult oviparous individuals of *A. coelomicola* as vestigial metacercarial cysts, similar to those of *C. lobotes*. Nevertheless, phylogenetic studies involving more cryptogonimid genera are needed to reveal other similar forms related to the genus *Aphalloides*.

Our phylogenetic analysis revealed that the Heterophyidae is not a monophyletic group and that some heterophyids group together with the opisthorchiid taxa included in the study. These results are congruent with previous analyses demonstrating paraphyletic relationship between the families Heterophyidae and Opisthorchidae (Thaenkham et al., 2011, 2012).

The present results support the placement of *Aphalloides* in the family Cryptogonimidae and the suppression of the family-group taxa based on this genus, i.e. the subfamily Aphalloidinae Naydenova, 1970 and the tribe Aphalloidini Yamaguti, 1971. Therefore, although being a member of the Cryptogonimidae, *Aphalloides* exhibits aberrant morphology, distinguishing it from the remaining members of the family.

A possible explanation of the unusual morphological features of *Aphalloides* spp. is their unusual life-cycle. Typically, the digeneans of the family Cryptogonimidae have three-host life-cycles that include gastropods as first intermediate hosts, fish as second intermediate hosts and fish or other vertebrates as definitive hosts (Miller & Cribb, 2008a). The first intermediate hosts of *Aphalloides* spp. are hydrobiid snails, i.e. *Hydrobia ventrosa* (Montagu) along the Mediterranean coast of France (Maillard, 1973) and *Hydrobia stagnorum* (Gmelin) at the North Sea coast of Belgium (Vaes, 1978) for *A. coelomicola*, and *Hydrobia ulvae* (Pennant) for *A. timmi* at the Baltic Sea coast of Germany (Reimer, 1970). Cercariae penetrate into gobies and transform into metacercariae encysting in the musculature or mesenteries (Naydenova, 1970; Maillard, 1973; Vaes, 1978). After 8–10 days (Maillard, 1973; Vaes, 1978), metacercariae are released from the cysts and pass into the body cavity where they reach maturity. The death of the host seems to be a necessary condition for the transmission of the eggs into the environment and the infection of the first intermediate host (Maillard, 1973; Vaes, 1978; Pampoulie et al., 2000). Thus, gobies are both second intermediate and definitive hosts for *Aphalloides* spp. Their life-cycle can be considered as secondarily simplified from the general three-host cryptogonimid life-cycle; previous opinions suggest that the sexually-mature generation of these species consists of progenetic metacercariae (Naydenova, 1970; Maillard, 1973; Bakke, 1980; Lefebvre & Poulin, 2005). Some unusual (for cryptogonimids) morphological characters of adults of *Aphalloides*, such as the relatively short arms of the excretory vesicle reaching to the level of the ventral sucker, can be considered as vestigial from the metacercarial stage. Another unusual character, i.e. the lack of spines in the tegument, which typically may have attachment functions, might be considered as an adaptation to the uncommon site of infection in the body cavity where adults are moving (not being attached). It is interesting to mention that the presence of tegumental spines has been described for cercariae (see Maillard, 1973) and metacercariae (Naydenova, 1970) of *A. coelomicola*, which is consistent with the interpretation of the lack of spines in adults as strongly adaptive. Therefore, the two-host life-cycle of *A. coelomicola* should be considered as a derived state of the general life-cycles of cryptognimids, as an adaptation to the short life span of the fish host (Lefebvre & Poulin, 2005).

Hitherto, *A. coelomicola* has been reported from *Pomatoschistus microps* from wetlands at the Mediterranean coast of France (Dollfus et al., 1957; Pampoulie et al., 1999, 2000, 2004; Pampoulie & Morand, 2002) and the North Sea coast of Belgium (Vaes, 1978). This digenean has been recorded also from *Pomatoschistus marmoratus* (Risso) along the Black Sea and the Sea of Azov shores of Ukraine (Naydenova, 1970, 1974; Kvach, 2004a, b, 2005, 2010; Krasnovyd et al., 2012), and from *Knipowitschia caucasica* from the Black Sea coast of Ukraine (Naydenova, 1970, 1974; Krasnovyd et al., 2012). This parasite is also known from *K. longecaudata* from the Black Sea coast of Ukraine (Naydenova, 1970, 1974). *Aphalloides timmi* is known from *Pomatoshistus microps* along the Baltic Sea coasts of Germany (Reimer, 1970; Zander et al., 1999, 2000; Zander, 2003, 2004, 2005a, b; Kvach & Winkler, 2011) and from the estuary of River Glomma, Norway (Bakke, 1980). Thus, the geographical range includes brackish wetlands along the shores of the European seas. The host range consists of four species of gobies of the genera *Pomatoschistus* T.N. Gill and *Knipowitschia* Iljin. Recent phylogenetic studies suggest that these genera belong to the family Gobionellidae (sand gobies), which is considered as a sister group of the true gobies, the family Gobiidae (see Thacker, 2013). This family contains 93 genera divided into four phylogenetic lineages (Thacker, 2013), most of which are poorly studied from a parasitological point of view. Further studies are needed in order to reveal if other gobionellid genera are also hosts of digeneans of the genus *Aphalloides*.

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