


A revision of the genus *Aphalloides* (Digenea: Cryptogonimidae), parasites of European brackish water fishes

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Abstract Trematodes of the genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957 reach maturity in their second intermediate host, small fishes of the family Gobionellidae, genera *Pomatoschistus* and *Knipowitschia*. Two morphologically similar species have been described from European waters: *Aphalloides coelomicola* Dollfus et al., 1957 in the Mediterranean Sea (including the brackish Black Sea region) and *Aphalloides timmi* Reimer, 1970 in the Baltic Sea. There was no difference in morphology and morphometry for specimens corresponding to *A. coelomicola* and *A. timmi* and examination of the 28S rDNA sequence confirmed the similarity. Based on these results, these two species are synonymized and *A. coelomicola* redescribed.

Keywords *Aphalloides* · Taxonomic revision · Zoogeography · Molecular study

Introduction

The Cryptogonimidae Ward, 1917 is a digenean family, which parasitize a large variety of poikilothermic vertebrates, both in fresh and marine waters (Miller and Cribb 2008b). Trematodes of genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957 reach maturity in their second intermediate host such as small fishes of Gobionellidae family (see Thacker 2009), genera *Pomatoschistus* and *Knipowitschia* (Dollfus et al. 1957; Naydenova 1970, 1974; Maillard 1973; Vaes 1978; Zander et al. 2000; Zander and Reimer 2002; Kvach and Winkler 2011; Stoyanov et al. 2015). These fishes are both definitive and second intermediate hosts of these worms, but the first intermediate host are mud-snails of *Hydrobia* group (Maillard 1973; Vaes 1978; Reimer 1970). The adults are located in the coelomic cavity of the host, causing the parasitic castration of the fish (Pampoulie et al. 1999, 2004; Sasal and Pampoulie 2000). The goby hosts are annual fishes (Bouchereau and Guelorget 1998; Mazzoldi et al. 2002), thereafter the parasite life cycle has been shown to require host death (Pampoulie et al. 2000). Apart from gobionellids, only one case of incidental infection of the two-spotted goby (*Gobiusculus flavescens* (Fabricius, 1779) (Actinopterygii: Gobiidae)) has been recorded in the Flensburg Fjord, Germany (Josten et al. 2009).

There are two morphologically very similar parasite species described from the annual gobies from European waters: *Aphalloides coelomicola* Dollfus et al., 1957 in the Mediterranean Sea (including brackish-water in the Black Sea region) and *Aphalloides timmi* Reimer, 1970 in the Baltic Sea (Dollfus et al. 1957; Reimer et al. 1996). *A. coelomicola* was originally described from the Mediterranean fishes, but also occurs in the Black Sea and Sea of Azov (Dollfus et al. 1957; Naydenova 1974). The status of specimens from the North Sea is unclear: in

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Dievengat, Belgium, it was recorded as *A. coelomicola* (Vaes 1978), but in Øra area of Skagerrak, Norway, it was mentioned as *A. timmi* (Bakke 1980).

The Black Sea and the Baltic Sea present some similarities in their water characteristics. For example, both of these water bodies are much desalinated compared to the other seas (average S = 18‰ in the Black Sea, but 6–8‰ in the Baltic Sea) and are both classified as brackish-water basins (Zenkevich 1963; Dethier 1992). The faunas of both seas have the great group of Boreo-Atlantic species, which are relicts of the ancient Tethys Sea (from Upper Miocene) (Zaitsev and Mamaev 1997). Differing from the Baltic Sea, the Black Sea fauna consists of the group of Ponto-Caspian relicts and Mediterranean species (Zaitsev and Mamaev 1997; Zaitsev 1998). The colonization of the Black Sea by Mediterranean species (a process of Mediterraneanization) was started about 7000 years ago and occurs until now (Miller 1965; Zaitsev 1998).

Because of morphological similarity of the representatives of *Aphalloides* genus, the aim of our work was to compare the individuals from different geographical regions (Baltic, Black and Mediterranean seas) to clarify the taxonomic status of the Black Sea samples.

Material and methods

The parasites were sampled in different localities in the Mediterranean Sea (St.-Nazaire and Salse-Leucate lagoons), Black Sea (Budaki Lagoon, Sukhyi Estuary) and Baltic Sea (Salzhaff and Unterwarnow) from different species of Gobionellidae: *Pomatoschistus marmoratus* (Risso, 1810) and *Knipowitschia caucasica* (Berg, 1916) from the Black Sea, *Pomatoschistus microps* (Krøyer, 1838) from the Mediterranean and Baltic seas (Table 1). From each infected fish individual, some of the parasites were fixed in pure 96% ethanol for molecular study, and the others were preserved in

hot 4% formaldehyde for morphological study (Cribb and Bray 2010). The formaldehyde-preserved worms were then stained with iron acetic carmine, dehydrated in ethanol of increasing concentration and mounted in Canada balsam as permanent slides (Georgiev et al. 1986). Voucher specimens are deposited in the helminthological collection of the Institute of Parasitology of the Academy of Sciences of the Czech Republic, (České Budějovice, Czech Republic).

The DNA from a worm individual from each sample was extracted using the JetQuick kit (Genomed, Germany). The genomic region of 28S rDNA was amplified by KAPA2G Robust HotStart PCR Kit (Kapabiosystems, USA) using primers and annealing temperature detailed in Table 2. Sanger sequencing of PCR products was commercially performed at GATC Biotech (Germany), and sequences were edited and aligned using Geneious 9.0.5 (Kearse et al. 2012). Phylogenetic analysis was based on 28S sequences of 15 newly sequenced individuals. The data of 28S sequences from previous study of Stoyanov et al. (2015)—*A. coelomicola*, GenBank accession no. KJ162159—were used to compare with 28S sequences from our data.

As out-group, the samples of five other cryptogonimid species from own samples were sequenced (see Table 1). Moreover, we used as out-group the sequences of *Brachycladium goliath* (KR703279), *Acanthostomum burminis* (KC489791; Jayawardena et al. 2013), *Siphoderina jactus* (EU571263) and *Siphoderina poulini* (EU571267; Miller and Cribb 2008a) available online.

The balsam slides were studied under the light microscope Olympus BX50 equipped with phase contrast, differential interference contrast (DIC) and digital image analysis (Olympus MicroImage™ for Windows 95/98/NT 7.0 (Olympus Optical Co.). The length and width of body, oral and ventral suckers, pharynx, ovary, both testes, seminal receptacle, and egg also length of oesophagus were measured to the nearest micrometre.

Table 1 Samples used for sequencing

Parasite taxa	Host	Sample date	Locality	Country
<i>Aphalloides</i> cf. <i>coelomicola</i>	<i>Knipowitschia caucasica</i>	31.05.2005	Black Sea, Sukhyi Estuary	Ukraine
<i>Aphalloides</i> cf. <i>coelomicola</i>	<i>Pomatoschistus marmoratus</i>	28.07.2008	Black Sea, Budaki Lagoon	Ukraine
<i>Aphalloides</i> cf. <i>coelomicola</i>	<i>Pomatoschistus microps</i>	03.03.2008	Mediterranean Sea, Salse-Leucate Lagoon	France
<i>Aphalloides</i> cf. <i>coelomicola</i> (3 samples)	<i>P. microps</i>	06.03.2008	Mediterranean Sea, St.-Nazaire Lagoon	France
<i>Aphalloides</i> cf. <i>timmi</i>	<i>P. microps</i>	19.07.2010	Baltic Sea, Unterwarnow	Germany
<i>Aphalloides</i> cf. <i>timmi</i> (3 samples)	<i>P. microps</i>	10.06.2008	Baltic Sea, Salzhaff	Germany
<i>Timoniella</i> cf. <i>balthica</i>	<i>Syngnathus typhle</i>	10.06.2008	Baltic Sea, Salzhaff	Germany
<i>Allocanthochasmus</i> sp.	<i>Morone chrysops</i>	15.11.2006	Lake Erie, Oregon, OH	USA
<i>Neochasmus umbellus</i>	<i>M. chrysops</i>	15.11.2006	Lake Erie, Oregon, OH	USA
<i>Anisocoelium capitellatus</i>	<i>Uranoscopus scaber</i>	22.10.2011	Black Sea, Gelendzhik Bay	Russia
<i>Anisocladium fallax</i>	<i>U. scaber</i>	22.10.2011	Black Sea, Gelendzhik Bay	Russia

Table 2 The DNA sites and primers used for sequencing of region 28S

Primer name	Primer sequence	<i>t</i> °C	Reference
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	57 °C	Littlewood 1994
ECD2	CCTTGGTCCGTGTTTCAAGACGGG		Littlewood et al. 1997
D2A	ACAAGTACCGTGAGGGAAAGTTG	56 °C	Nunn 1992
D3B	TCGGAAGGAACCAGCTACTA		Nunn 1992

Results

Molecular study

Bayesian inference analysis based on sequences of 28S rDNA showed strong support for a monophyletic group encompassing genera *Timoniella* and *Aphalloides* (posterior probability PP = 0.99) (Fig. 1). The genus *Aphalloides* was also monophyletic, included all samples from France, Germany and Ukraine, but the support was only PP = 0.89. Within the genus, only little variation was observed for the 28S rDNA, but it seems that specimens ex. *Knipowitschia caucasica* (samples from Atanasovsko Lake, Bulgaria, and Sukhyi Estuary, Ukraine)

differ from samples ex. *Pomatoschistus* spp. (the latter form supported monophyletic group, PP = 0.99). Sequences from individuals identified previously as *A. cf. timmi* from Germany are identical to those identified as *A. coelomicola* from France.

Morphological study

The morphological study of worms from different region did not show differences (Table 3; Fig. 2). The morphometric parameters overlap between worms from different regions and with data from previous authors or original descriptions (see Table 3; Dollfus et al. 1957; Naydenova 1970; Reimer

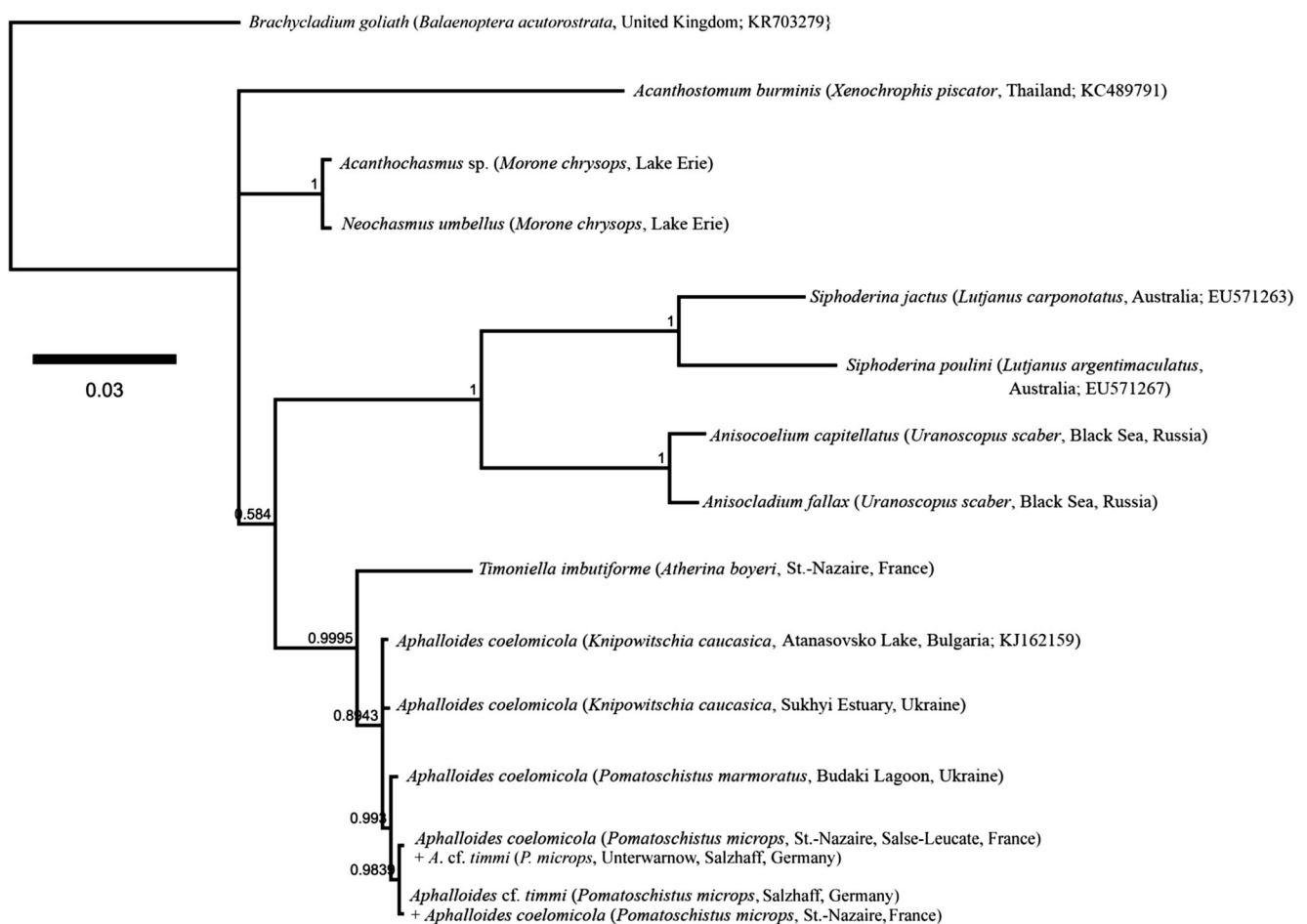
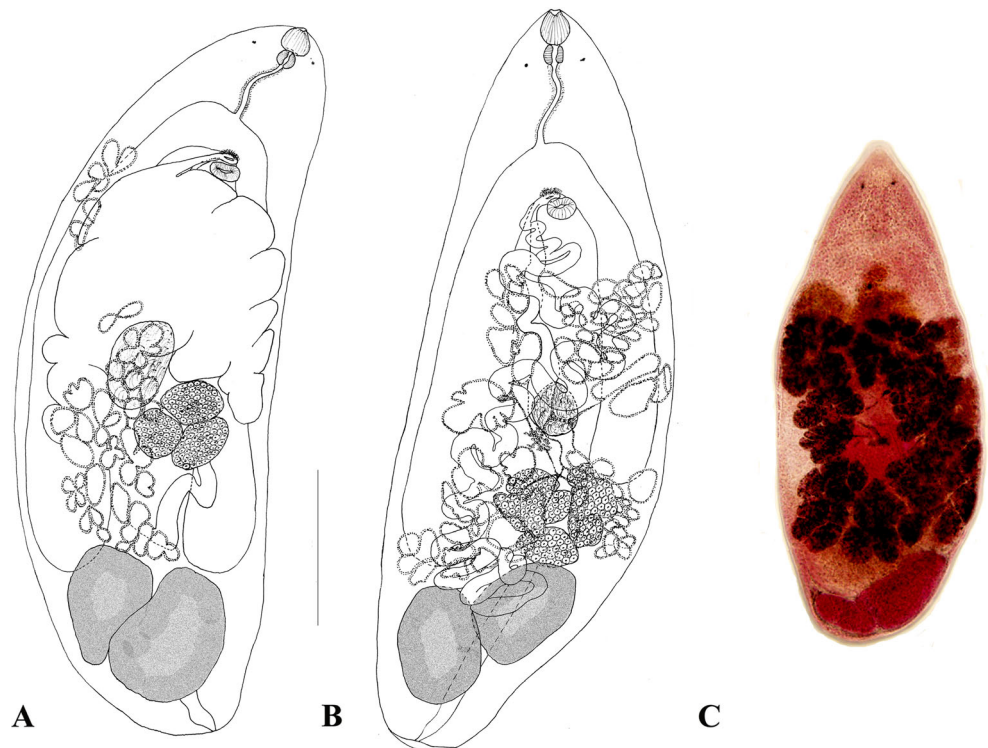
**Fig. 1** Phylogenetic trees of Cryptogonimids based on sequences of 28S rDNA

Table 3 Morphometric parameters of *Aphalloides* sampled from the different localities

		France			Ukraine			Germany			
		Our data (<i>n</i> = 16)		Dollfus et al. 1957 (<i>n</i> = 7)	Our data (<i>n</i> = 17)		Naydenova 1970	Our data (<i>n</i> = 20)		Reimer 1970	
		Mean	Min-max		Mean	Min-max		Min-max	Mean	Min-max	Mean
Body	L	1456	347.5–3466	2500	1951	1040–3441	840–3750	2243	707.5–4367	2970	2170–3620
	W	540	160–1516	1000	757	399–1367	256–1200	853	192–1291	720	535–950
Oral sucker	L	70	43–114	120	72	55–99	37–83	86	53.5–122	58	38–70
	W	69.5	44–115	120	68	50.5–82	37–93	88	50–129.5	58	61–94
Ventral sucker	L	69	34–200	95	72.5	53–100	40–96	77	43–115	68	47–94
	W	70	33.5–140	130	87	63–107.5	45–109	91	46–126.5	76	59–90
Pharynx	L	50	27–92	95	56	45–68	34–76	58	33–85	55	47–66
	W	49	31–73.5	55	48	41.5–59	31–47	54	32–85	38	26–52
Oesophagus		167	66–311	165	163	139–192	93–202	186	13–323	201	108–264
Ovary	L	140	46–237	210	197	131–346	150–265	243	68–316	245	247–318
	W	154	77–247.5	390	232	171–344	78–210	243	56–342	277	159–365
Anterior testis	L	287	66–677	500	279	121–429	109–450	368	67–601.5	337	247–475
	W	204	55–498	230	162	79.5–282	68–225	253	60–583	229	118–460
Posterior testis	L	259.5	59–500	500	305	223–348.5	112–525	301	88–451	410	294–610
	W	188	36.5–464	230	227	127.5–293	81–265	238	87–579	189	141–318
Seminal receptaculum	L	56	44–68	680	101	50–183	239–555	145	44–392	349	224–517
	W	54	44–64	600	80	53–123	120–405	94.5	46–206	196	99–282
Egg	L	27	24–30	28	30	26–35	26–31	29	24–32	30	28–34
	W	13	11.5–14.5	12	13	10–16	16–17	13	11–15.5	14	13–15

Fig. 2 *Aphalloides coelomicola*. **a** ex. *Pomatoschistus microps*, St.-Nazaire Lagoon. **b** ex. *P. microps*, Salzhaff. **c** Photograph of a specimen ex. *Knipowitschia caucasica* with vitellin ducts visible. Scale bar 500 μ m



1970). Based on our observations, we provide a redescription of *A. coelomicola* as follows:

Family Cryptogonimidae Ward, 1917.

Genus *Aphalloides* Dollfus, Chabaud & Golvan, 1957.

Aphalloides coelomicola Dollfus, Chabaud & Golvan, 1957 (Fig. 2).

Synonym: *Aphalloides timmi* Reimer, 1970

Type host: *Pomatoschistus microps* (Krøyer, 1838) (Actinopterygii, Gobionellidae).

Other hosts: *Pomatoschistus minutus* (Pallas, 1770), *Pomatoschistus marmoratus* (Risso, 1810), *Knipowitschia caucasica* (Berg, 1916), and *Knipowitschia longicaudata* (Berg, 1916) (Actinopterygii: Gobionellidae); Incidentally: *Gobiusculus flavescens* (Fabricius, 1779) (Actinopterygii: Gobiidae).

Type locality: Mediterranean Sea near Banyuls-sur-Mer, France.

Other localities: Mediterranean Sea (Camargue, Salse-Leucate Lagoon, St.-Nazaire Lagoon), Black Sea (Atanasovsko Lake, gulfs of Odessa and Yagorlyk, lagoons and estuaries of the North-Western Black Sea, Sea of Azov), Baltic Sea (Rügen Island, lagoons and bights of South-Western Baltic Sea) and North Sea (Dievengat, Øra area of Skagerrak) (see Fig. 3).

Site in host: body cavity, gonads.

Voucher material: D-640 (Helminthological collection of the Institute of Parasitology of Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic).

Representative sequence: KY978885, KY978886, KY978887, and KY978888.

Description:

Body large, elongate, variable in size: 350–3750 × 160–1516. Maximum body width in central part, in second quarter or at the level of ovary. Anterior part tapering, posterior part rounded. Tegument slightly rugose transversally, devoid of spines. Two brown eyespots at level of pharynx.

Oral sucker terminal, slightly elongate transversely, different in shape (from pyriform to globular): 37–122 × 37–130. Ventral sucker subspherical in shape, typically smaller than oral sucker, rarely slightly larger, 34–200 × 34–140. Positioned at level of intestinal bifurcation or slightly posterior, at base of tegumental depression.

Prepharynx absent. Pharynx muscular, thick-walled, elongate-oval, 27–95 × 31–59. Oesophagus different in length, thick-walled, 13–323, 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 sometime slightly longer than dextral. Excretory pore terminal.

Genital pore located immediately anterior to ventral sucker. Gonotyle absent, cirrus and cirrus-sac absent. Excretory vesicle Y-shaped, reaches posterior margin of ventral sucker or

slightly posterior to it. Distal part of seminal vesicle covered by prostatic cells. Seminal vesicle tubular, curved, positioned at middle of body.

Testes two, oval, located at same level, close to posterior extremity of body, one anterior to another, sometime slightly overlapping. Anterior testis 66–677 × 55–583, posterior 59–610 × 37–579. Ovary deeply tri-lobed, 46–346 × 59–365, anterior to testes. Ovary positioned sometime immediately at anterior margin of anterior testis, sometime distanced, almost equatorial. Follicular vitellarium in clusters of follicles arranged in groups, positioned from level of posterior end of ventral sucker to level of testes. Vitelline ducts can form ring surrounding seminal receptacle and/or anterior part of ovary. Uterine seminal receptacle anterior to ovary, oval to circular, usually 44–395 × 44–206, sometime gigantic, 500–680 × 282–600, can partially overlap ovary. Space between ventral sucker and testes usually overfill by uterus with eggs. Uterus tubular, strongly developed, forms numerous loops. Metratem absent. Eggs small, elongate-oval, 24–35 × 12–17, yellowish-brown, not embryonated.

Discussion

In this work, based on both morphological and molecular data, we consider *A. coelomicola* and *A. timmi* as a single species in the monotypic genus *Aphalloides*. The species has a wide geographical range, which includes brackish waters of the Mediterranean region (including the Black Sea) and the Baltic Sea. The host range is limited by the group of annual gobies of Gobionellidae family. The genus was placed in Siphoderinae Manter, 1934, a subfamily of Cryptogonimidae (Dollfus et al. 1957). Naydenova (1970) placed this genus to Heterophyidae, which was later disputed by Bayssade-Dufour and Maillard (1982). Miller and Cribb (2008b) do not recognize subfamilies in Cryptogonimidae, confirming *Aphalloides* inside this family.

The parasite was first described from the common goby (*P. microps*) from a small brackish-water canal in Banyuls-sur-Mer in Southern France, leading to the creation of a new genus and a new species (Dollfus et al. 1957). Later, this parasite was confirmed in the lagoons of the Rhone delta (Maillard 1973 and more recently Pampoulie et al. 1999). Maillard (1973) showed that the life cycle of *A. coelomicola* includes two hosts: a snail host (*Hydrobia* spp.) as first intermediate host with parthenogenetic stages, and the annual gobies, where metacercariae develop into adults. A progenetic life cycle has been characterized for this species, and the worms start to produce eggs at the metacercarial stage (see Lefebvre and Poulin 2005; Stoyanov et al. 2015).

In the Black Sea, the parasite was first recorded in the 1960s from the marbled goby (*P. marmoratus*) in the Gulf of Odessa and Tyligul Estuary (Chernyshenko and

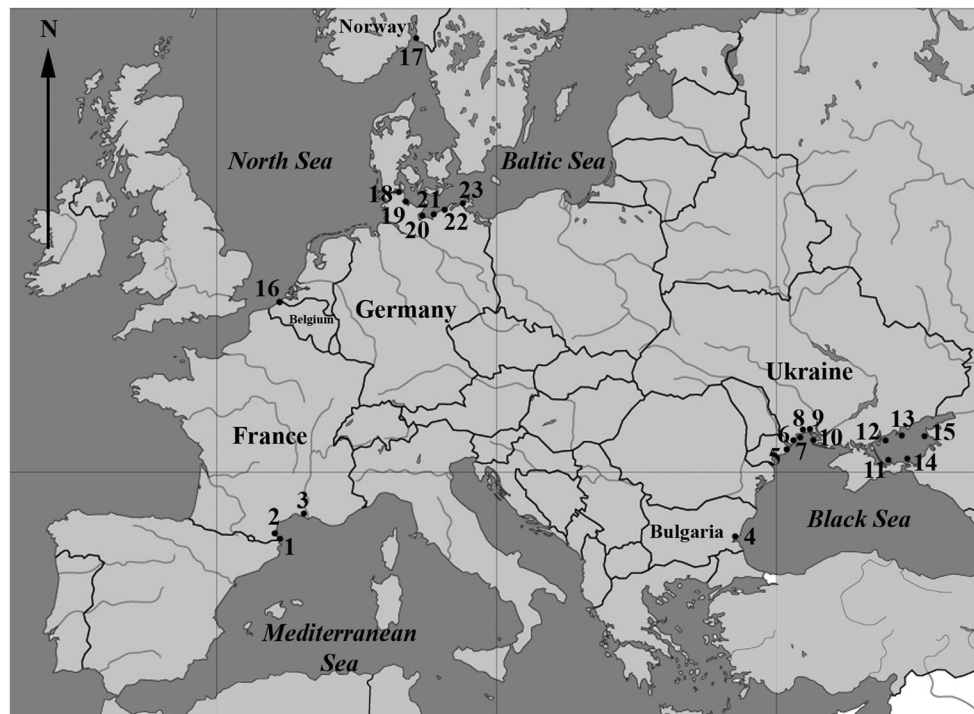


Fig. 3 Confirmed findings of *Aphalloides coelomicola* in Europe. Mediterranean Sea: 1 Banyuls-sur-Mer (Dollfus et al. 1957), 2 Salse-Leucate and St.-Nazaire lagoons (this study), 3 Vaccarès and Malagroy lagoons, Camargue (Maillard 1973; Bayssade-Dufour and Maillard 1982; Pampoulie et al. 1999, 2000, 2004; Sasal and Pampoulie 2000); Black Sea: 4 Atanasovsko Lake (Stoyanov et al. 2015), 5 Budaki Lagoon (Kvach 2010; this study), 6 Sukhyi Estuary (Kvach 2010; Krasnovyd et al. 2012; this study), 7 Gulf of Odessa (Chernyshenko and Svetsytskaya 1970), 8 Hryhorivsky and Tyligul estuaries (Chernyshenko and Svetsytskaya 1970; Kvach 2010), 9 Berezan

Estuary (Chernyshenko and Svetsytskaya 1970), 10 Gulf of Yagorlyk (Naydenova 1974; Parukhin et al. 1983); Sea of Azov (Naydenova 1974): 11 Gulf of Arabat, 12 Obytichna Split, 13 Biryuchy Island, 14 Taman, 15 Gulf of Yeysk; North Sea: 16 Dievengat Pond (Vaes 1978), 17 Øra area, Glomma River Estuary (Bakke 1980); Baltic Sea: 18 Flensburg Fjord (Josten et al. 2009), 19 Kiel Bight (Zander et al. 2002; Zander 2005), 20 Lübeck Bight (Zander 2003), 21 Salzhaff (Zander et al. 1999, 2000; Zander and Reimer 2002; this study), 22 Unterwarnow (Kvach and Winkler 2011; this study), 23 Rügen Island (Reimer 1970; Reimer et al. 1996)

Svetsytskaya 1970). Naydenova (1970, 1974) found both metacercariae and adults in three goby species (*P. marmoratus*, *Knipowitchia longicaudata* and *K. caucasica*) from the northern Sea of Azov. Also, the adults occurred in *Pomatoschistus minutus*, *P. marmoratus* and *K. caucasica* from different parts of the North-Western Black Sea (Parukhin et al. 1983; Kvach 2010; Krasnovyd et al. 2012). Recently, it was recorded in *K. caucasica* from brackish Atanasovsko Lake in Bulgaria (Stoyanov et al. 2015).

In the Baltic Sea, the distribution of this species is limited to German coastal waters from the Flensburg Fjord (Josten et al. 2009) to Rügen Island (Reimer et al. 1996), where it is common in two gobiids (*P. microps*, *P. minutus*) in Kiel Bight (Zander et al. 2002; Zander 2005), Lübeck Bight (Zander 2003), Salzhaff (Zander et al. 1999) and Unterwarnow (Kvach and Winkler 2011).

Describing the new species *A. timmi*, Reimer (1970) reported differences relative to *A. coelomicola* as the ratio of suckers (the ventral sucker is larger than the oral sucker in *A. timmi*), the size of seminal receptaculum (smaller in *A. timmi*), and

vitelline ducts surrounding the seminal receptacle (absent in *A. coelomicola*). Studying the specimens from the Øra area of Skagerrak, Norway, Bakke (1980) confirmed the absence of tegumental spines. Later, Miller and Cribb (2008b) mentioned this item as one of the characteristic of the genus *Aphalloides*.

Stoyanov et al. (2015) provided redescription of this species, based on specimens from *K. caucasica*. They confirmed the relation of Black Sea specimens to *A. coelomicola*, noting the smaller sizes of seminal receptacle in comparing to previously described (Dollfus et al. 1957; Naydenova 1970). Also, they noted the morphometrical variability in studied specimens. According to Stoyanov et al. (2015), only the presence of vitellin ducts surrounding the seminal receptacle is discriminating between the two species of *Aphalloides*. Our study confirmed the presence of these ducts, but they were observed not only in specimens from the Baltic Sea, but also in many specimens from France and Ukraine (see Fig. 2c).

Zander and Reimer (2002) considered this species (noted as *A. timmi*) to be a brackish-water/marine endemic of the Baltic Sea. But, the type-host of this parasite, the common goby (*P. microps*), is a Boreal-Atlantic fish, widely distributed

from Norway in the north to the Canary Islands in the south, including the Mediterranean and Baltic seas (Miller 1986). Another host of this parasite, the sand goby (*P. minutus*), has similar range, also inhabiting the Black Sea region (Miller 1986). Taking into account the host specificity of *Aphalloides*, the presence of endemic parasites in widely distributed host seems unlikely.

In the Black Sea (including the Sea of Azov), *A. coelomicola* was considered as Mediterranean species (Naydenova 1974; Kvach 2010). The marbled goby (*P. marmoratus*) is a Mediterranean immigrant, entering the Black Sea fauna about 7000–12,000 years ago (Miller 1965; Zaitsev and Mamaev 1997). So, it seems probable that the parasite migrated with its host. But, taking into account the presence of *A. coelomicola* in the Baltic and North seas, we can consider *A. coelomicola* as a Boreal-Atlantic species, which is probably part of a relict fauna (from Upper Miocene) in the Black and Baltic seas. The parasite can use different species of mud-snail of *Hydrobia* group as first intermediate host. So, for the Mediterranean Sea, *Ecrobia ventrosa* (Montagu, 1803) is recorded as the snail host (Maillard 1973), but *Semisalsa stagnorum* (Gmelin, 1791) is mentioned for the Baltic Sea (Vaes 1978; Reimer 1970). According to Zander et al. (2002), in the Baltic Sea, both *Peringia ulvae* (Pennant, 1777) and *E. ventrosa* are used by *Aphalloides* as first intermediate host. Two snails of the *Hydrobia* group, *E. ventrosa* and *Hydrobia acuta* (Draparnaud, 1805), are common in the Black Sea fauna (Butenko 2000; Osikowski et al. 2016). These molluscs are related to a group of the European lagoonal species, distribution today in the region of the old Tethys Sea, including eastwards into the Ponto-Caspian basin, in all European coasts, also to the eastern seaboard of North America (Barnes 1989). The wide distribution of snails of *Hydrobia* group, especially of *E. ventrosa* as main host, can support the Boreal-Atlantic status of *A. coelomicola*.

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