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First Eastern Mediterranean Record of *Xenoligophoroides cobitis*, the Only Dactylogyrid Monogenean Infecting Mediterranean Gobies: Just Arrived or Missed the Boat?

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Abstract: Gobies and their ectoparasitic monogenean flatworms are promising models for species diversification because of their species richness. Recent decades have seen the discovery of several new species of *Gyrodactylus* (Monogenea: Gyrodactylidae) on European gobies, mostly in the sand goby lineage and especially in the eastern Mediterranean. However, the monogenean fauna of other gobies is much less understood. Therefore, we inspected five gobiid species (34 specimens, vouchered, with some representatives sequenced), sampled in Greece, for monogenean ectoparasites. Only specimens of the giant goby, *Gobius cobitis*, were infected; they harbored *Xenoligophoroides cobitis* (Monogenea: Dactylogyridae) on their gills. Here, we provide the first record from Greece, and the first ITS rDNA and COI sequences of the representative of this monotypic genus. Additionally, 28S rDNA was sequenced and compared with published data from across its known distribution, suggesting clinal variation. No sister-group for *Xenoligophoroides* could be proposed, nor could we explain the presence of a single known member of this genus on gobies, due to a lack of sequence data of closely related dactylogyrid monogeneans in public databases. Possible hypotheses include either the ancestral long-term presence on gobiids but "missing the boat" of the diversification events in the "*Gobius*-lineage", or a recent host switch from a non-gobiid host.

Keywords: barcoding; Dactylogyridae; Dactylogyrinae; ectoparasites; giant goby; Gobiidae; *Gobius cobitis*; Greece; Monogenea; Platyhelminthes

1. Introduction

Gobiidae is the most species-rich fish family worldwide, with 1964 valid species as of 28 May 2022 [1], and the most species-rich group of European marine fishes [2]. There are 76 Mediterranean species [3]. Their diversity makes them prime models in evolutionary biology. For example, the occurrence of representatives in a wide variety of salinity conditions, and the often high levels of endemicity of gobies, render them conducive to study biogeographical patterns in aquatic ecosystems [2]. Furthermore, the radiation and local adaptation events in gobies have been fruitfully exploited in speciation research [4–7].

In addition, several goby species are successful invaders, rendering them useful models for the genomics of colonization and invasion [8] or as proof-of-principle of the use of fish parasites to elucidate introduction pathways [9]. Indeed, not only are the gobies valuable targets for biodiversity research, the same goes for their parasites. European sand gobies, with the gyrodactylid monogeneans that infect them [10,11], constitute one of the best studied fish–*Gyrodactylus* host–parasite systems. Since flatworms belonging to *Gyrodactylus* von Nordmann, 1832 are considered "the drosophilids of the parasitic world", sand gobies and their gyrodactylids are, therefore, a promising model in ecological and evolutionary parasitology [12]. Indeed, the parasites of assemblages of closely related host species may reveal important insights in parasite speciation [13]. Goby parasites also hold a lot of potential for biodiversity discovery. The recent description of seven species of *Gyrodactylus* infecting freshwater sand gobies from the Balkan region, the center of endemism of these hosts, underscores that even European species diversity is far from fully inventoried [14]. Despite the recent focus on sand gobies, the discovery of *Gyrodactylus quadratidigitus* Longshaw, Pursglove et Shinn, 2003 on British *Thorogobius ephippiatus* (Lowe, 1839) by Longshaw et al. [15] illustrates that non-sand gobies also hold the promise of undiscovered gyrodactylids. Next to Gyrodactylidae, another species-rich family of Monogenea is represented among goby parasites: Dactylogyridae. In Europe, only one dactylogyrid species is reported from marine gobies: *Xenoligophoroides cobitis* (Ergens, 1963), with its only known host the giant goby, *Gobius cobitis* Pallas, 1814 [16,17]. In general, the discovery of a plethora of marine monogenean species is to be expected: Appeltans et al. [18] estimated that among marine flatworms, Monogenea is the group of which the lowest percentage (7 to 13%) of existing species has been formally described.

Here, we expand our survey of the monogenean parasite diversity of eastern Mediterranean gobies, specifically towards marine gobies outside of the sand goby lineage, with the expectation of retrieving representatives of *Gyrodactylus* and *Xenoligophoroides*.

2. Materials and Methods

2.1. Sampling and Morphological Characterization of Parasites

Gobies outside of the sand goby lineage were collected as bycatch by Vanhove et al. [19]. They were diagnosed by a minimum combination of characters that positively identified the collected specimens among species of the family Gobiidae in the CLOFNAM area ([20,21] and references therein). We focused on larger gobiid species that often may also occur in brackish water. The fish were inspected for monogeneans on their gills, body, and fins; also, the vial and medium were checked.

About half of the monogeneans recovered were transferred to a water droplet using a dissection needle, fixed in Hoyer's medium, and mounted between slide and coverslip for morphological characterization of haptoral and genital hard parts. Measurements and micrographs were taken under phase contrast with an Olympus BX61 microscope fitted with a DP71 camera and Olympus Stream Motion software. Since only dactylogyrid monogeneans were found (Figure 1a–e), measurements followed Sasal et al. [16], whose study was the most recent publication on dactylogyrid parasites of European gobies at the onset of this work.

The remaining specimens were stored in absolute ethanol for subsequent molecular work. To allow taxonomic identification of these animals, photographic vouchers were made prior to DNA extraction. To this end, flatworms were temporarily mounted in water and photographed under a 100× (oil immersion) phase contrast objective using a Leica DM5000B microscope equipped with a Leica DFC420C camera and LAS imaging software (Figure 1f,g). Host vouchers were deposited in the Natural History Museum Rijeka (Rijeka, Croatia) (PMR), and parasite vouchers in the general invertebrate collection of the Department of Zoology, Swedish Museum of Natural History (Stockholm, Sweden) (SMNH).
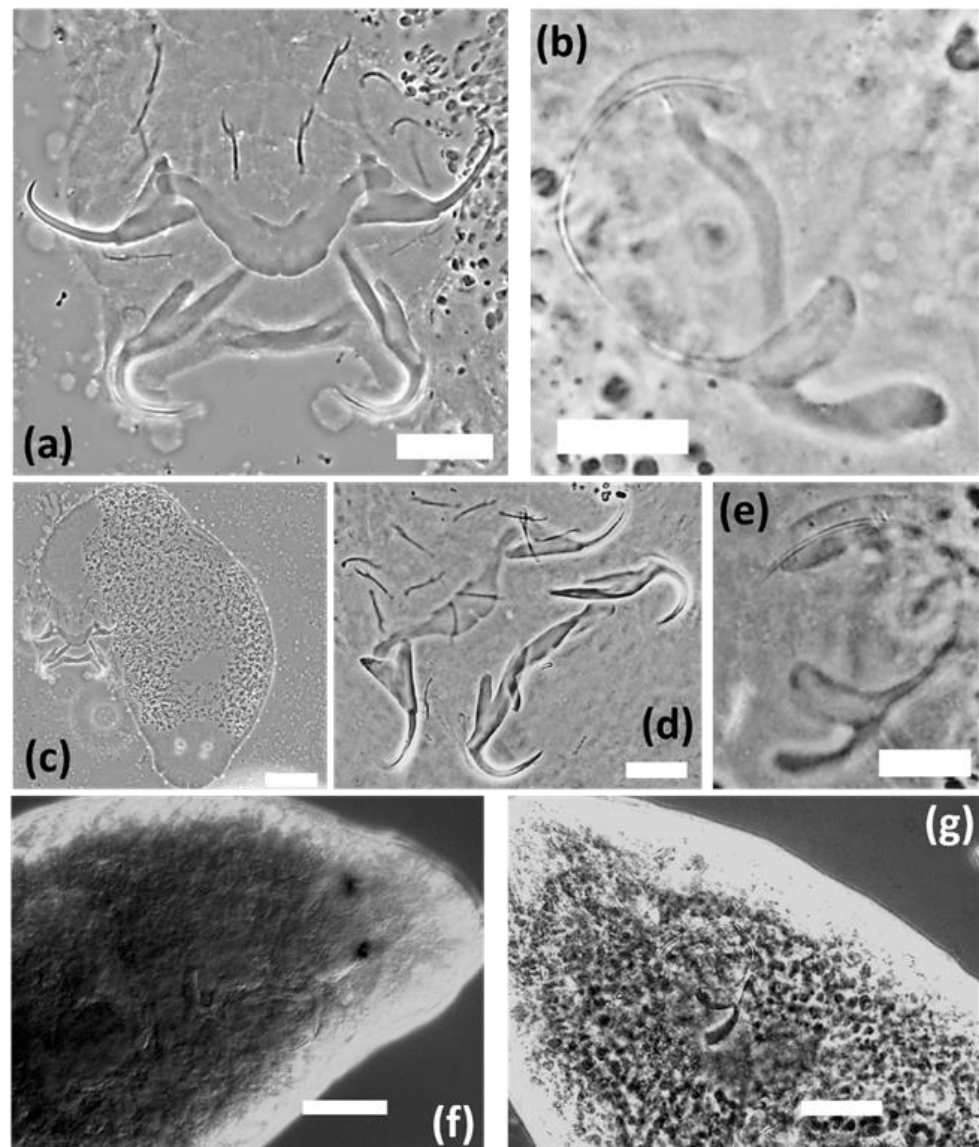
Figure 1. Micrographs of *Xenoligophoroides cobitis*. (**a**) Haptoral hard parts, specimen from Acheloos Delta. (**b**) Male copulatory organ, specimen from Acheloos Delta. (**c**) Whole mount, specimen from Acheloos Delta. (**d**) Haptoral hard parts, specimen from Kryoneri Estuary. (**e**) Male copulatory organ, specimen from Kryoneri Estuary. (**f**) Photo voucher of temporarily water-mounted specimen from Acheloos Delta. (**g**) Photo voucher of temporarily water-mounted specimen from Kryoneri Estuary. Both photo vouchers show the characteristic "bilobed" (sensu Sasal et al. [16]) or "two-chambered" base (sensu Dmitrieva et al. [17]) of the male copulatory organ of *X. cobitis*. Scale bars: 50 μm (**c**), 20 μm (**a**,**d**,**f**,**g**), 10 μm (**b**,**e**).

2.2. Molecular and Genetic Analysis

For the host specimens, DNA extraction, PCR amplification of mitochondrial 12S and 16S rDNA, subsequent purification of the PCR product, and Sanger sequencing were performed following the procedures described by Vanhove et al. [19]. Host sequences were deposited in the NCBI GenBank under accession numbers ON847338-45 (16S rDNA) and ON853912-19 (12S rDNA). Parasite DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Three potential monogenean barcoding markers [22] with different mutation rates were amplified for the parasites: a fragment of the large nuclear ribosomal subunit gene (28S rDNA); the region spanning internal transcribed spacer (ITS) 1 and 2 including the 5.8S rDNA; and a fragment of

the mitochondrial cytochrome *c* oxidase subunit 1 (COI). Although the former is widely used in monogenean molecular systematics and in somewhat deeper phylogenetic reconstruction (e.g., [23]), the combination of the two latter markers has recently proven valuable for phylogenetics of closely related monogenean species in the context of host (and parasite) radiation [24]. Sequences of the ITS region are well-established in the molecular taxonomy of gyrodactylid monogeneans and, hence, also in the species assemblage of *Gyrodactylus* on European gobies [10]; this includes the first-ever western Mediterranean record of members of *Gyrodactylus* [25]. Conversely, the broad applicability of COI as a marker for monogeneans and other flatworms has been questioned [22], although recent work on representatives of *Cichlidogyrus* Paperna, 1960 (Monogenea, Dactylogyridae) highlights its potential for barcoding [26]. Hence, taken together, sequence data of COI and of 28S rDNA and ITS rDNA served as versatile genetic resources for work on the monogenean goby parasites retrieved in this study. Primer combinations were C1 (5′-ACCCGCTGAATTTAAGCAT-3′) and D2 (5′-TGGTCCGTGTTTCAAGAC-3′) [27] for 28S rDNA, ITS1A (5′-GTAACAAGGTTTCCGTAGGTG-3′) and ITS2 (5′-TCCTCCGCTTAGTGATA-3′) [28] for ITS rDNA, and ASmit1 (5′–TTTTTTGGGCATCCTGAGGTTTAT–3′) [29] and Schisto3 (5′–TAATGCATMGGAAAAAAACA–3′) [30] for COI. In the latter case, the first polymerase chain reaction (PCR) was followed by a nested PCR, replacing the Schisto3 primer with ASmit2 (5′–TAAAGAAAGAACATAATGAAAATG–3′) [29]. We performed PCR using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare), adding 1 μL of each primer (20 μM) (Sigma Aldrich), 2 μL of template DNA, and 21 μL of double distilled, autoclaved, and filter-sterilized water. A GeneAmp PCR System 9700 (Applied Biosystems) thermocycler was used. Cycling conditions are outlined in Table 1.

Table 1. Polymerase chain reaction protocols for the genetic markers of monogenean parasites; the expected amplicon size is mentioned for each marker. The number of cycles was 39 for the fragment of large subunit rDNA, and 40 for internal transcribed spacer rDNA and the partial cytochrome *c* oxidase subunit 1 gene.

Protocol	Large Subunit 28S rDNA (*ca.* 700–900 bp)	Internal Transcribed Spacer rDNA (*ca.* 900–1200 bp)	Cytochrome *c* Oxidase Subunit 1 (*ca.* 445 bp)
initial denaturation	2 min/94 °C	3 min/96 °C	5 min/95 °C
cycle: denaturation	20 s/94 °C	50 s/95 °C	1 min/94 °C
annealing	30 s/56 °C	50 s/52 °C	1 min/50 °C
elongation	1 min 30 s/72 °C	50 s/72 °C	1 min/72 °C
final elongation	10 min/72 °C	7 min/72 °C	7 min/72 °C
cooling	4 °C	4 °C	4 °C

We purified the PCR product using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's guidelines. Bidirectional sequencing was carried out in an Applied Biosystems 3730 DNA analyzer using the BigDye protocol v.1.1. Sequences were validated by eye in MEGA v.7 [31] and aligned in the same software using ClustalW [32]. Pairwise distances were also calculated in MEGA. Sequences were subject to a BLAST search [33] on NCBI GenBank, and deposited there under accession numbers ON847354 (COI), ON853990-96 (28S rDNA) and ON854080-83 (ITS rDNA). In case published sequences of the same markers for conspecifics were found, a median-joining network [34] was inferred in PopART (http://popart.otago.ac.nz/index.shtml, accessed on 5 December 2021). Maps were rendered in QGIS [35].

3. Results

3.1. Host Records

A total of 34 specimens belonging to five goby species were checked for monogenean ectoparasites; species identities and sampling data are provided in Figure 2 and Table 2, rendering the host–parasite relationships traceable (see [36]). Interestingly, for *Gobius couchi* Miller & El-Tawil, 1974, our sample contains the first record in the Corinthian Gulf, quite

geographically distant from surrounding records of this species from Corfu, Crete, and the northeastern Aegean Sea coast [37].
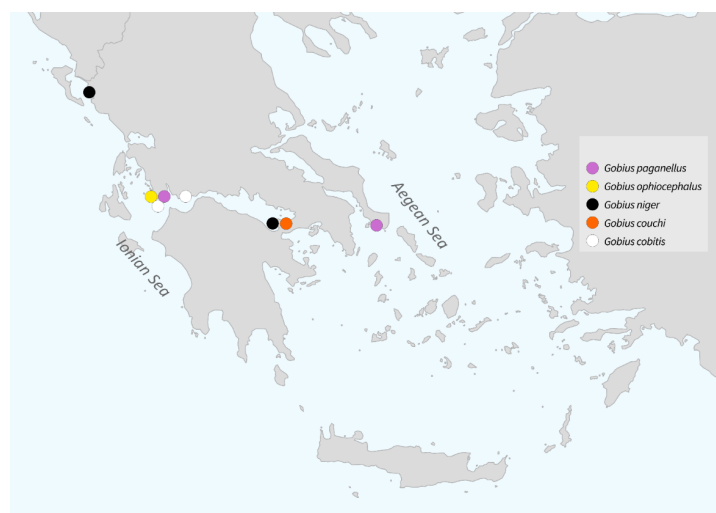

Figure 2. Species and localities sampled for gobies and parasites; see Table 2 for more sampling information.

Table 2. Non-sand gobies inspected for monogenean ectoparasites and hosts sequenced for host identification in this study.

Species	Locality	Sampling Date	Number of Host Specimens Inspected for Parasites/Sequenced	Voucher Specimens	GenBank Accession Numbers
Gobius cobitis Pallas, 1814	Acheloos Delta 38°20′17.6″ N 21°07′39.0″ E	10 June 2008	1/-	PMR VP 3175	/
	Kryoneri Estuary 38°22′23.4″ N 21°51′55.0″ E	7 June 2008	1/1	PMR VP 3215	ON847338 (16S rDNA), ON853912 (12S rDNA)
Gobius couchi [1] Miller & El-Tawil 1974	Lake Heraion 38°01′31.8″ N 22°52′34.6″ E	8 September 2008	1/-	PMR VP 3208	/
Gobius niger Linnaeus, 1758	Drepano Beach 39°30′54.3″ N 20°12′39.4″ E	8 June 2008	16/2	PMR VP 3179 to PMR VP 3194	ON847339-40 (16S rDNA), ON853913-14 (12S rDNA)
	Lake Heraion 38°01′31.8″ N 22°52′34.6″ E	8 September 2008	11/2	PMR VP 3195 to PMR VP 3206, PMR VP 3207, and PMR VP 3209	ON847341-42 (16S rDNA), ON853915-16 (12S rDNA)
Gobius ophiocephalus Pallas, 1814	Acheloos Delta 38°20′17.6″ N 21°07′39.0″ E	10 June 2008	2/1	PMR VP 3176, PMR VP 3178	ON847343 (16S rDNA), ON853917 (12S rDNA)
Gobius paganellus Linnaeus, 1758	Acheloos Delta 38°20′17.6″ N 21°07′39.0″ E	10 June 2008	1/1	PMR VP 3177	ON847344 (16S rDNA), ON853918 (12S rDNA)
	Euboea Island (Livadaki, Karystos) 38°00′15.8″ N 24°23′30.3″ E	3 June 2008	1/1	PMR VP 3210	ON847345 (16S rDNA), ON853919 (12S rDNA)

[1] The specimen PMR VP 3208 was identified as *G. couchi* based on the following diagnosis: (1) suborbital papillae of lateral-line system without longitudinal row *a*; (2) all three head canals of lateral-line system present; (3) predorsal area scaled; (4) six suborbital transversal papillae rows; (5) anterior oculoscapular head canal with pore *α* at rear of orbit; (6) oculoscapular papillae row *x1* not extending forwards to head canal pore *β*; (7) scales in lateral series 35–45 (present specimen had 35 and 36); (7) suborbital row *d* divided below between suborbital rows *2* and *3*; (8) pelvic disc complete or no more than 1/8 emarginate (pelvic disc complete in present specimen); (9) pectoral fin count 15–18 (present specimen, for both sides, had 17).

3.2. Parasite Identification

Among the five goby species studied, only representatives of *G. cobitis* were infected by monogenean ectoparasites. A specimen caught in the Acheloos Delta was infected with nine monogenean gill parasites, five of which were used for genetic analyses and four of which were prepared as whole mounts (SMNH 207585-86). For an individual from Kryoneri Estuary, the infection intensity was seven. Three of these worms were subject to molecular analyses and the four others mounted on a slide (SMNH 207587). The two infected individuals were the only representatives of *Gobius cobitis* studied, leading to a

prevalence of 100%. The results of measurements performed on the whole mounts are provided in Table 3. Based on Sasal et al. [16] and Dmitrieva et al. [17], all specimens belong to *X. cobitis* (Figure 1).

Table 3. Morphometric data of the haptoral and genital hard parts of specimens of *Xenoligophoroides cobitis* sampled in Greece. Measurements and their symbols follow Sasal et al. [16] with terminology adapted from Řehulková et al. [38]; measurements, all in μm, are presented as the range, followed by the average and the number of measured structures (n) in parentheses.

Parameter	Acheloos Delta Population	Kryoneri Estuary Population
Body		
Total length	387.6–476.2 (425.7, n = 4)	337.3–408.3 (369.3, n = 4)
Total width	140.1–181.4 (160.0, n = 4)	134.5–203.7 (157.0, n = 4)
Dorsal anchor		
Total length (a)	43.0–46.4 (44.5, n = 4)	49.6–53.7 (51.2, n = 3)
Length to notch (b)	32.3–34.1 (33.2, n = 4)	36.4–40.7 (38.6, n = 3)
Inner root length (c)	18.3–22.9 (20.3, n = 4)	23.7–24.6 (24.2, n = 3)
Outer root length (d)	5.1–9.6 (8.0, n = 4)	9.5–11.5 (10.5, n = 3)
Point length (e)	11.8–16.4 (14.4, n = 4)	13.5–20.0 (17.0, n = 3)
Ventral anchor		
Total length (a)	34.0–40.1 (37.4, n = 4)	36.8–40.1 (38.0, n = 3)
Length to notch (b)	40.4–44.2 (42.0, n = 4)	42.8–46.2 (45.0, n = 3)
Inner root length (c)	10.3–10.9 (10.6, n = 2)	9.8–10.9 (10.4, n = 3)
Outer root length (d)	5.4–6.3 (6.0, n = 3)	6.4–9.3 (8.2, n = 3)
Point length (e)	4.3–5.5 (4.7, n = 4)	5.6–6.3 (6.1, n = 3)
Dorsal bar		
Branch length (h)	35.2–38.4 (36.2, n = 4)	40.1–43.3 (41.7, n = 3)
Thickness at mid-length (w)	8.7–13.5 (11.7, n = 4)	12.8–19.3 (15.4, n = 3)
Total straight width (x)	37.2–59.3 (50.7, n = 4)	56.6–72.4 (67.0, n = 3)
Ventral bar		
Thickness at mid-length (w)	4.6–6.2 (5.5, n = 4)	7.7–9.2 (8.4, n = 3)
Total straight width (x)	41.1–45.0 (43.0, n = 4)	50.6–54.0 (52.3, n = 3)
Hook		
Pair I: total length (o)	15.5–18.1 (16.9, n = 4)	16.9–19.2 (17.7, n = 3)
Pair I: shank length (p)	9.1–12.2 (10.9, n = 4)	10.7–12.7 (11.5, n = 3)
Pair V: total length (o)	13.8–16.0 (14.9, n = 4)	14.6–15.5 (15.1, n = 3)
Pair V: shank length (p)	8.6–9.5 (9.1, n = 4)	8.3–10.2 (9.2, n = 3)
Other pairs: total length (o)	14.2–18.4 (16.4, n = 20)	14.1–20.7 (17.5, n = 18)
Other pairs: shank length (p)	8.7–11.7 (10.4, n = 20)	8.7–14.4 (11.5, n = 18)
Male copulatory organ		
Copulatory tube total straight length (q)	23.3–31.6 (26.7, n = 4)	14.7–35.2 (24.5, n = 4)
Total straight length of the base of the copulatory tube (r)	15.3–19.2 (16.5, n = 4)	13.9–21.0 (17.5, n = 4)
Copulatory tube total curved length (s)	46.7–53.7 (50.4, n = 4)	53.3–60.9 (56.2, n = 4)

3.3. Sequence Analyses

After trimming, sequence fragments of a maximal length of 858 bp (28S rDNA), 880 bp (ITS rDNA), and 171 bp (COI) were retained. Although only a single COI sequence was retrieved (from the Acheloos Delta), the four obtained ITS sequences (two from each locality) yielded a maximal uncorrected pairwise distance of 0.9% between specimens collected from the two different sites.

After searching nucleotide BLAST for the ITS fragment of *X. cobitis*, the highest identity score (93.2%) was found for two species of *Dactylogyrus* Diesing, 1850 (unpublished sequences KX369215 and KX369219), followed by a score of 91.2% for several representatives of *Cichlidogyrus* (sequences of [24,39]). These high scores were only found for a fragment that covered 23–25% of the total query (ca. 220 bp), or more specifically, in the region spanning 5.8S rDNA and internal transcribed spacer 2. For the COI fragment, the sequences with the highest pairwise similarity score belonged to *Kapentagyrus tanganicanus* Kmentová, Gelnar et Vanhove, 2018 (between 79.5% and 80.2% identity, sequences of [40]), followed by *Euryhaliotrema pirulum* (Plaisance et Kritsky, 2004) (identity 84.5%, sequence of [41]) and by species of *Cichlidogyrus* (maximal similarity of 82.0%, sequences of [24]) and *Sciadicleithrum* Kritsky, Thatcher et Boeger, 1989 (maximal similarity of 81.4%, sequences of [42]).

Only a single 28S rDNA genotype was found for all seven specimens successfully sequenced for this marker. Since other 28S rDNA sequences of *X. cobitis* are available [17], we only carried out intraspecific comparisons for this marker. The Greek genotype differed 0.7 to 3.1% (uncorrected p-distance) from those from the Black Sea, and between 0.6 and 1.3% from Sardinian conspecifics. The median-joining haplotype network, including all other published sequences of *X. cobitis* for this marker, situated the Greek population in between the Sardinian and Black Sea ones (Figure 3). In contrast to the Greek specimens all sharing a genotype, all genotypes from the Sardinian and Black Sea populations were unique.
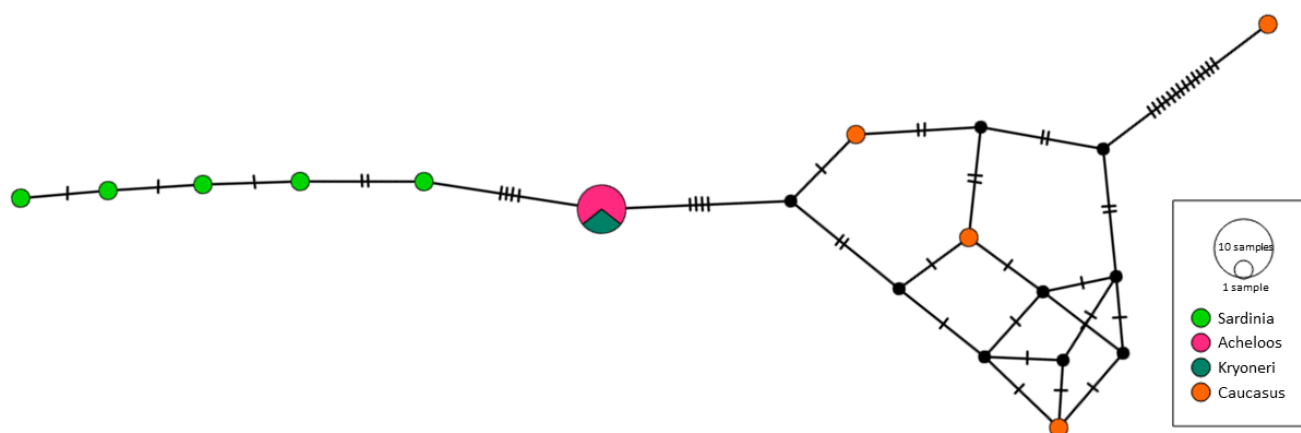

Figure 3. Median-joining haplotype network based on 702 bp of 28S rDNA from the newly sequenced individuals of *Xenoligophoroides cobitis* from Greece, and the sequences from Dmitrieva et al. [17]. Genotypes are represented by circles with the size of the circle correlating with the number of specimens displaying the respective genotype. Colors denote sampling localities; genotypes are connected with lines, indicating the number of mutations between them. Colors correspond to the sampling localities in Figure 4.

Figure 4. Distribution of *Xenoligophoroides cobitis*. Star: type locality, Gulf of Vlorë, Albania. Triangles: previously published records without accompanying genetic data (overview: see [17] and references therein). Green: population from northwestern Sardinia, Italy; orange: population from Gelendzhik, Russia, both sequenced by Dmitrieva et al. [17]. Pink: population from the Acheloos Delta, Greece; turquoise: population from Kryoneri Estuary, Greece, both sequenced in the present study. Colors correspond to those in the haplotype network (Figure 3).

4. Discussion

To further our understanding of the monogenean fauna of eastern Mediterranean gobies, we screened a number of Greek gobies belonging to five species for monogenean ectoparasites. The 2 individuals of the giant goby *G. cobitis* harbored a total of 16 dactylogyrid flatworms on their gills, morphologically and genetically identified as *X. cobitis*; no other monogeneans were found in any of the studied individuals. Given these infection parameters, and since *X. cobitis* is, here and in other studies (e.g., [16,17,43]), consistently reported on *G. cobitis* from different localities, an accidental infection is unlikely.

Ergens [43] described this parasite species as *Ancyrocephalus cobitis*. It was synonymized with *Haliotrema cupensis* Sasal, Pages et Euzet, 1998 and assigned to *Haliotrema* Johnston et Tiegs, 1922 by Merella et al. [44] as *Haliotrema cobitis* (Ergens, 1963). Dmitrieva et al. [17] erected the new monospecific genus *Xenoligophoroides* Dmitrieva, Sanna, Piras, Garippa et Merella, 2018 for it. With its type locality in the Adriatic Sea, and earlier observations from the western Mediterranean and the Black Sea, we here report *X. cobitis* (and hence any representative of *Xenoligophoroides*) for the first time in Greece, in the Ionian Sea and the eastern Mediterranean (Figure 4).

Dmitrieva et al. [17] provided an overview of morphometric data of the various populations of *X. cobitis* hitherto studied. In addition to the similarity these authors observed in overall body morphology, in shape of the hard parts, and in soft-part anatomy, they also mentioned considerable size ranges. For example, the (inner) length of the copulatory tube varies from minimally 25 μm in France to maximally 63 μm in the Black Sea; of the dorsal anchor, from 37 μm to 57 μm, and of the ventral anchor, from 25 μm to 45 μm (each time the minimal size in the French population and the maximal size in that of the Black Sea). Dmitrieva et al. [17] mentioned the geographical origin of the parasite populations, and the different sizes of the hosts studied from the Mediterranean compared to the Black Sea localities, as potential explanations for the size difference in the parasites. Measurements performed on the specimens from Greece fit within the ranges mentioned by Dmitrieva et al. [17]. It is noteworthy that the average

value for most hard-part measurements is higher for the parasites collected at Kryoneri Estuary in comparison to their conspecifics in the Acheloos Delta (Table 3). The Kryoneri host specimen (total length = 88.9 mm, standard length = 72.1 mm + caudal fin length 16.8 mm) was larger than the one from the Acheloos (total length = 35.6 mm, standard length = 28.1 mm + caudal fin length 7.5 mm). Despite the limitations of our sample size, given the fact that these two Greek localities are only about 65 km apart, this could suggest an influence of host size on the size of the hard parts of *X. cobitis*. Morphological differences in various haptoral structures correlating with host size have been reported in other dactylogyrid monogeneans (e.g., [40]) though other studies did not find such correlations (e.g., [45] for a gill-infecting polyopisthocotylean monogenean). An increasing size of the gill lamellae in larger host specimens, potentially rendering larger haptoral hard parts an advantage for attachment to bigger-sized hosts, has been cited as a potential explanation for the link between host size and haptor morphology in monogeneans ([46] and references therein).

We consider the variation found in the ITS rDNA sequences of our specimens of *X. cobitis* to be intraspecific, as it remains below the threshold of 1% divergence, for this marker often associated with a difference between species (in *Gyrodactylus*: [47]). In contrast to the Sardinian and Black Sea populations sequenced by Dmitrieva et al. [17], all parasites in our sample sequenced for 28S rDNA yielded a single identical genotype. This contrasts with the diversity found by Dmitrieva et al. [17], where all five specimens from a single site in Sardinia and all four specimens from a single site in the Black Sea had a unique genotype (Figure 3).

Based on phylogenetic analyses by Dmitrieva et al. [17] using 28S rDNA, the species most similar to *X. cobitis* all seemed to share a marine lifestyle, belonging to *Ergenstrema* Paperna, 1964, *Ligophorus* Euzet et Suriano, 1977, *Euryhaliotrema* Kritsky et Boeger, 2002, and *Haliotrematoides* Kritsky, Yang et Sun, 2009. Our BLAST analyses based on the ITS and COI markers pointed towards similarities with both marine (e.g., *Euryhaliotrema*) and freshwater (e.g., *Kapentagyrus* Kmentová, Gelnar et Vanhove, 2018) genera. We consider our BLAST results a consequence of the scarcity of barcoding data for monogenean flatworms. Hopefully, the advent of mitochondrial genomes of an ever-increasing phylogenetic range of monogeneans (e.g., [48,49]) will help alleviate this important data gap in the near future.

The most recent and much more comprehensive phylogeny of dactylogyrids [23] placed all above-mentioned genera under Dactylogyrinae, but did not confirm a close relationship between any of them and *X. cobitis*. This dactylogyrid phylogeny also positions the members of *Gobioecetes* Ogawa et Itoh, 2017, infecting freshwater and diadromous gobies, in the Palearctic Far East [50], in Dactylogyrinae. *Gobioecetes*, with its sister taxon *Ancyrocephalus mogurndae* (Yamaguti, 1940) that is known to infect the Asian freshwater goby *Gymnogobius urotaenia* (Hilgendorf, 1879) among other hosts (see [51]), belonged to an entirely different clade than *X. cobitis*, indicating that gobies have been colonized by members of Dactylogyrinae at least twice independently.

Kmentová et al. [23] could not suggest a lineage closely related to *Xenoligophoroides*. Hence, it is impossible to propose a scenario of how (and when) this monogenean, currently the only known dactylogyrid from European gobies, colonized its host. Additionally, the host phylogeny adds little information: as the "*Gobius*-lineage" also includes eastern Atlantic genera from Norway to South Africa, Ponto–Caspian freshwater representatives, and even has affinities with tropical Pacific and neotropical gobies [4,52], we cannot reliably hypothesize where this lineage first infected gobies, and whether this happened in a marine or freshwater environment.

In contrast to the species-rich assemblage of *Gyrodactylus* on European (sand) gobies, only a single species of *Xenoligophoroides* is known. Although one has to caution against overinterpretation as many more goby species should be studied for parasites, this difference in species richness between goby-infecting monogenean lineages could be due to, for example, a recent colonization of the "*Gobius*-lineage" (long after it diversified) from another host lineage, or to "missing the boat" of diversification of these gobies, i.e., the

absence on the founder populations of diverging goby lineages (see [53]). In order to favor any of these scenarios, identifying the sister group relationships of *Xenoligophoroides* would be key, as would inspecting fish species occurring in sympatry with *G. cobitis*. It would perhaps allow the inference of host-switching events such as those proposed by Huyse et al. [54], who studied sand goby-infecting members of *Gyrodactylus*. These authors suggested recent host-switches between sand gobies and sticklebacks, and between sand gobies and eels. In this respect, it may be useful to reflect on the former taxonomic affinities of *X. cobitis*. This species was previously assigned to *Haliotrema* and to *Ancyrocephalus* Creplin, 1839. Although no other Mediterranean monogeneans are classified under *Haliotrema*, other monogeneans occurring in the Mediterranean (e.g., *Ancyrocephalus salinus* Paperna, 1964 infecting *Aphaniops dispar* (Rüppell, 1829)) currently belong to *Ancyrocephalus*, which can safely be assumed to be a catch-all taxon (see [51,55]). Therefore, we concur with Dmitrieva et al. [17] that it will be fruitful to verify their systematic position in general, and their potential affinity to *X. cobitis* in particular. Apart from, e.g., the killifish host of *A. salinus*, further screening of gobiids and fishes occurring sympatrically with gobiids seems a fruitful approach to increase our understanding of the Mediterranean gyrodactylid and dactylogyrid fauna.

Conflicts of Interest: The authors declare no conflict of interest.

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