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Brief Report

Molecular Characterization of *Gymnorhynchus isuri* Robinson, 1959 (Gymnorhynchidae) Infecting the Sharptail Mola *Masturus lanceolatus* (Liénard, 1840) from off the Coast of Kerala, India

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Abstract: The cestode family Gymnorhynchidae (Trypanorhyncha) comprises three genera and six valid species that, as adults, are all intestinal parasites of large pelagic sharks. Their life cycle has not been elucidated yet, but it has been proposed that copepods serve as first, pelagic euphausiids or schooling fish as second, and larger predatory fishes as third intermediate hosts. Molidae fish have been proposed as intermediate hosts for at least two gymnorhynchid species (i.e., *Molicola horridus* and *M. uncinatus*). During a parasitological survey of fish from the coast of Kerala (India), some individuals of a gymnorhynchid species were found in a sharptail mola *Masturus lanceolatus*. Parasites were located on the subcapsular tissue of liver showing a serpiginous route. Based on 28S rDNA molecular and phylogenetic analysis, parasites were identified as *Gymnorhynchus isuri*, which resulted genetically identical to *G. isuri* obtained from the liver of a sun fish *Mola mola* in the Mediterranean Sea.

Keywords: *Gymnorhynchus isuri*; *Masturus lanceolatus*; Molidae; 28S rDNA; Trypanorhyncha; Arabian Sea

Key Contribution: *Masturus lanceolatus* and locality, i.e., Arabian Sea, both represent new records for *Gymnorhynchus isuri*, confirming the importance of Molidae in the life cycle of gymnorhynchid parasites and expanding the geographical range of this cestode.

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1. Introduction

The family Molidae Bonaparte, 1835 comprises three genera of large ray-finned bony fish with currently five recognized species [1,2]. The genus *Masturus* includes a single species, the sharptail mola *M. lanceolatus* (Liénard, 1840), found circumglobally in tropical and sub-tropical waters; however, its occurrence along the coast of India is uncommon [3]. Members of Molidae are known to host rich parasite communities, although most studies have focused on the parasites of *Mola* spp. Consequentially, scarce information on parasites hosted by *M. lanceolatus* is available in literature. According to Bates [4] and the most recent review of parasites occurring in Molidae [5], of the seventy-three taxa listed as parasites in sunfishes, only six have been found in *M. lanceolatus*. Four out of these are ectoparasites (three copepods and one monogenean) and only two are endoparasites, which comprise two trypanorhynch cestodes infecting the liver (i.e., *Molicola horridus* (Goodsir, 1841) Dollfus, 1935, and one unidentified species). Larval forms of these cestodes use Molidae as intermediate hosts to reach the mature stages in pelagic sharks [5,6].

During a parasitological survey of fish from the coast of Kerala (India), some individuals resembling a gymnorhynchid species were found infecting the liver of a *M. lanceolatus*, and they were herein identified based on molecular characters and phylogenetic analysis.

2. Materials and Methods

2.1. Sample Collection and Parasitological Analysis

On 15 January 2024, a sunfish (measuring 132 cm of total length and weighing in total 51 kg) was obtained from professional fishermen at landing at Sakthikulangara (Kollam district, Kerala, India) harbor. The fish was a bycatch from shrimp trawlers, operating at a depth of ~75 m off the coast of Kerala in the Arabian Sea (7°16′36.6″ N–71°57′54.9″ E). The fish, of undetermined sex, was identified as a juvenile *M. lanceolatus* based on the identification key provided by Bray [1]. In particular, the clavus presented a distinct median extension with remaining margin not scalloped.

At landing, the visceral cavity of the fish was cut and viscera were collected and immediately frozen (−20 °C). After thawing, when gross parasitological analysis of viscera was performed in the laboratory, several whitish worms were observed on the liver (Figure 1). Worms were located on the subcapsular tissue showing a serpiginous route grossly resembling larvae of cestodes of the family Gymnorhynchidae (Trypanorhyncha) [5–7]. A total of 13 parasites were removed using scalpel and tweezers; then, they were washed in physiological saline and preserved in 100% ethanol for subsequent molecular analyses.

2.2. Molecular and Phylogenetic Analyses

Genomic DNA was extracted from a single worm using NucleoSpin® Tissue Kit (Macherey–Nagel, Düren, Germany) according to manufacturer instructions. A segment of the 28S rDNA gene was amplified using the primer set ZX-1 (5′-ACCCGCTGAATTTAAGCAT-3′) and 28sR (5′-GACGATCGATTTGCACGTCA-3′) ([8] and this study). The PCR was performed in 25 µL reactions with 2 µL of DNA sample, 0.6 µL of each primer at 10 mM and 10 µL of MyFi Mix (Bioline Ltd., London, United Kingdom). The thermocycling amplification program included a preliminary denaturation step at 94 °C (3 min) followed by 40 cycles of 94 °C (30 s), 54 °C (30 s), 72 °C (2 min), and a final extension step at 72 °C (10 min). Amplified products were preserved at 4 °C. Amplicons were visualized in a 1% agarose gel with GelRed (Biotium, Fremont, CA, USA) stain on a ~35 min, 95 V electrophoresis. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA). The successful PCR product was purified using the ExoSAP-IT kit (Applied Biosystems, Foster City, CA, USA), following the standard manufacturer recommended protocol. Clean PCR products were Sanger sequenced from both strands, using an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA). The obtained sequences were assembled and edited using Unipro UGENE (v. 50, Unipro, Novosibirsk, Russia) [9]. Sequence identity was verified using the Nucleotide Basic Local Alignment Search Tool (BLASTn) [10].

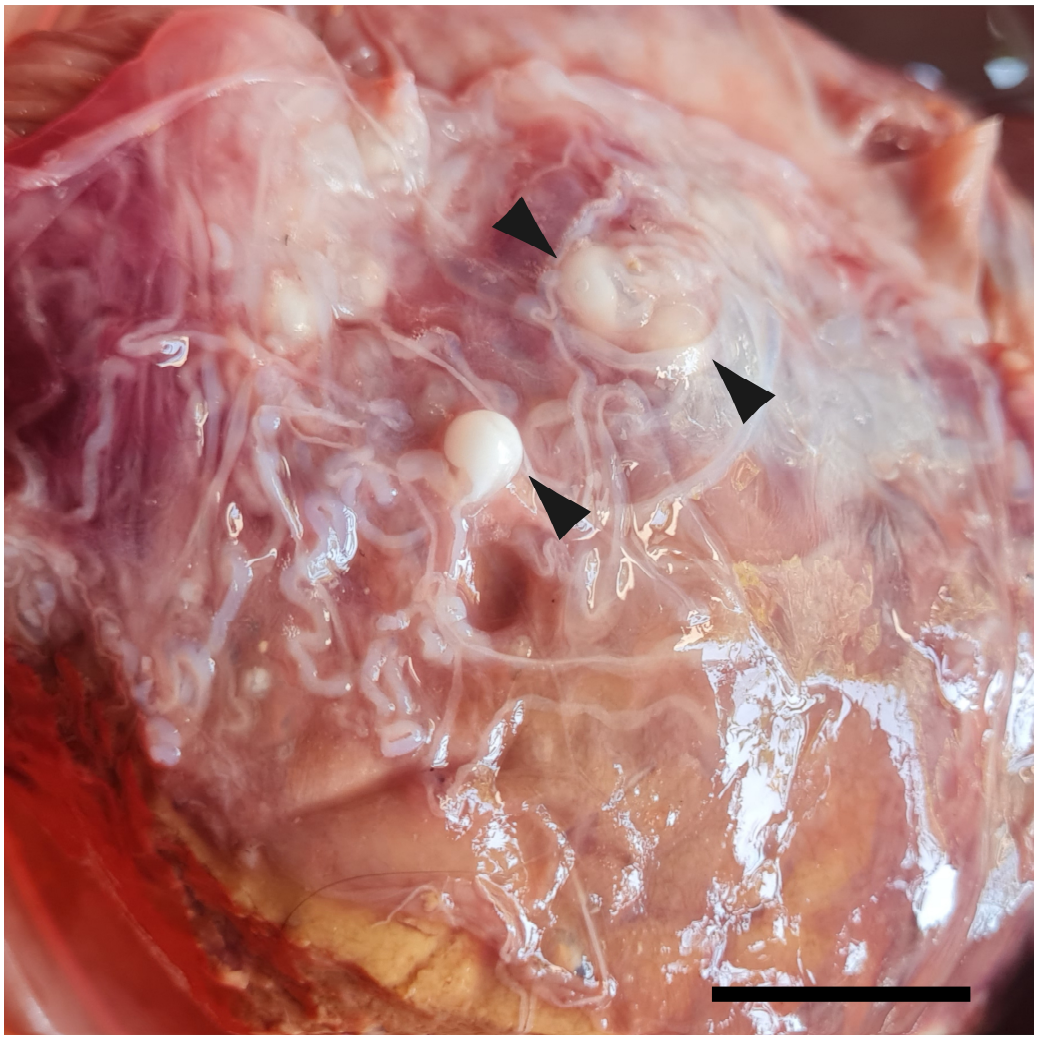

Figure 1. *Gymnorhynchus isuri* infection in the liver of the sharptail mola *Masturus lanceolatus*. Black arrow heads indicate the anterior extremity of larvae. Bar scale: 1 cm.

All available 28S rDNA sequences, representatives of the family Gymnorhynchidae, were retrieved from GenBank (Table 1) and aligned, together with the sequence generated in this study, using the multiple sequence alignment package T-Coffee (CRG, Barcelona, Spain) [11]. The alignment was then submitted to the transitive consistency score (TCS) to verify the reliability of aligned positions and optimize the phylogenetic topology [12]. In total, 25 sequences were analyzed, including the outgroup *Pintneriella musculicola* Yamaguti, 1934 (Trypanorhyncha, Rhopalothylacidae) (Table 1). Phylogenetic analysis was performed using the Maximum likelihood (ML) and the Bayesian inference (BI) approach implemented in IQ-TREE (v. 1.6.12, I, IQ-TREE Development Team, Vienna, Austria) [13] and MrBayes (v 3.2.7 8) [14], respectively. The best fitted evolutionary model was TrN+I, as suggested by jModelTest (v. 2.1.10) [15]. The ML phylogenetic tree was calculated performing 5000 ultrafast bootstrap approximations to test the phylogenetic reliability. Posterior probability distributions for the BI analysis were generated using the Markov Chain Monte Carlo (MCMC) method. MCMC searches were run for 10 million generations on two simultaneous runs of four chains and sampled every 1000 generations; the first 25% of samples from the MCMC algorithm were discarded as burn in. The quality of the Bayesian analysis (parameter densities, ESS [Effective Sample Size] and burn-in) and the chain convergence were examined in Tracer (v. 1.7.2) [16], and trees were visualized using Figtree (v. 1.4.4, Institute of Evolutionary Biology, Edinburgh, UK) [17]. The genetic divergences among taxa for a subset of our dataset, which included the most closely related species to

our specimen, were estimated using absolute nucleotide differences and p-distances using MEGAX (v. 11) [18].

Table 1. Information about specimens used in the phylogenetic analysis obtained from GenBank. These represented all available 28S rDNA sequences of the family Gymnorhynchidae. The sequence generated in this study is shown in bold.

GenBank ID	Parasite Species	Host Species	Host Family	Host Order	Geographic Origin	Ref.
PQ276610	***Gymnorhynchus isuri***	*Masturus lanceolatus*	Molidae	Tetraodontiformes	India	This study
DQ642744	*Chimaerarhynchus rougetae*	*Squalus megalops*	Squalidae	Squaliformes	New Caledonia	[19]
ON197557–ON197561	*Gymnorhynchus gigas*	*Brama brama*	Bramidae	Scombriformes	Mediterranean Sea	[20]
DQ642747	*Gymnorhynchus isuri*	*Isurus oxyrinchus*	Lamnidae	Lamniformes	USA	[19]
MT667258	*Gymnorhynchus isuri*	*Mola mola*	Molidae	Tetraodontiformes	Mediterranean Sea	[7]
FJ572949	*Molicola* sp. HP5	*Taractes rubescens*	Bramidae	Scombriformes	Indonesia	[8]
KX712337–KX712341	*Molicola* sp. SL01–SL05	*Xiphias gladius*	Xiphiidae	Carangiformes	Sri Lanka	[21]
DQ642746	*Molicola uncinatus*	*Thyrsites atun*	Gempylidae	Scombriformes	Australia	[19]
OQ407787	*Molicola uncinatus*	*Thyrsites atun*	Gempylidae	Scombriformes	New Zealand	[22]
MT823197–MT823198	*Molicola uncinatus*	*Lepidopus caudatus*	Trichiuridae	Scombriformes	Mediterranean Sea	[23]
ON197562–ON197566	*Molicola uncinatus*	*Brama brama*	Bramidae	Scombriformes	Mediterranean Sea	[20]
FJ572948	*Pintneriella musculicola* [#]	*Odontaspis ferox*	Odontaspididae	Lamniformes	Indonesia	[8]

[#] Outgroup.

3. Results

A sequence of 1374 bp of the 28S rDNA gene was obtained; it was deposited in GenBank under the accession number PQ276610. Results of the query of the BLASTn tool showed that sequences possessing the highest degree of similarity were *Gymnorhynchus isuri* (MT667258) obtained from the liver of *Mola mola* in the Mediterranean Sea, and *G. isuri* (DQ642747) obtained from the intestine of its definitive type host *Isurus oxyrinchus* (Lamnidae) from USA waters, with 100% and 98.9% of similarity, respectively.

Since BI and ML analyses yielded identical topologies as well as branch support, only the BI tree is shown (Figure 2). The phylogenetic analysis of the alignment, with a final length of 1465 bp, solved the tree clustering species of the Gymnorhynchidae family, for which sequences were available, in five well-supported phylogenetic lineages, corresponding to the relative five species. Indeed, our species clustered with the two above-mentioned *G. isuri*, although it demonstrated higher relatedness with the specimen collected from a more closely related host, as also demonstrated by the difference in absolute number of nucleotides (0 vs. 11), and the p-distance (0% vs. 0.8%) (Table 2). The present *G. isuri* had a p-distance of 3.3% with *G. gigas*, ~2.5% with *Molicola* sp., and ~3.3–3.4% with most of *M. uncinatus* specimens with the exception of *M. uncinatus* from *Lepidopus caudatus*, with which the genetic distance was lower (2%). According to the present analysis, three clades were detected within the family Gymnorhynchidae: the first being represented by *M. uncinatus* and *G. gigas*, the second by the unidentified species of *Molicola*, and the third by *G. isuri*.

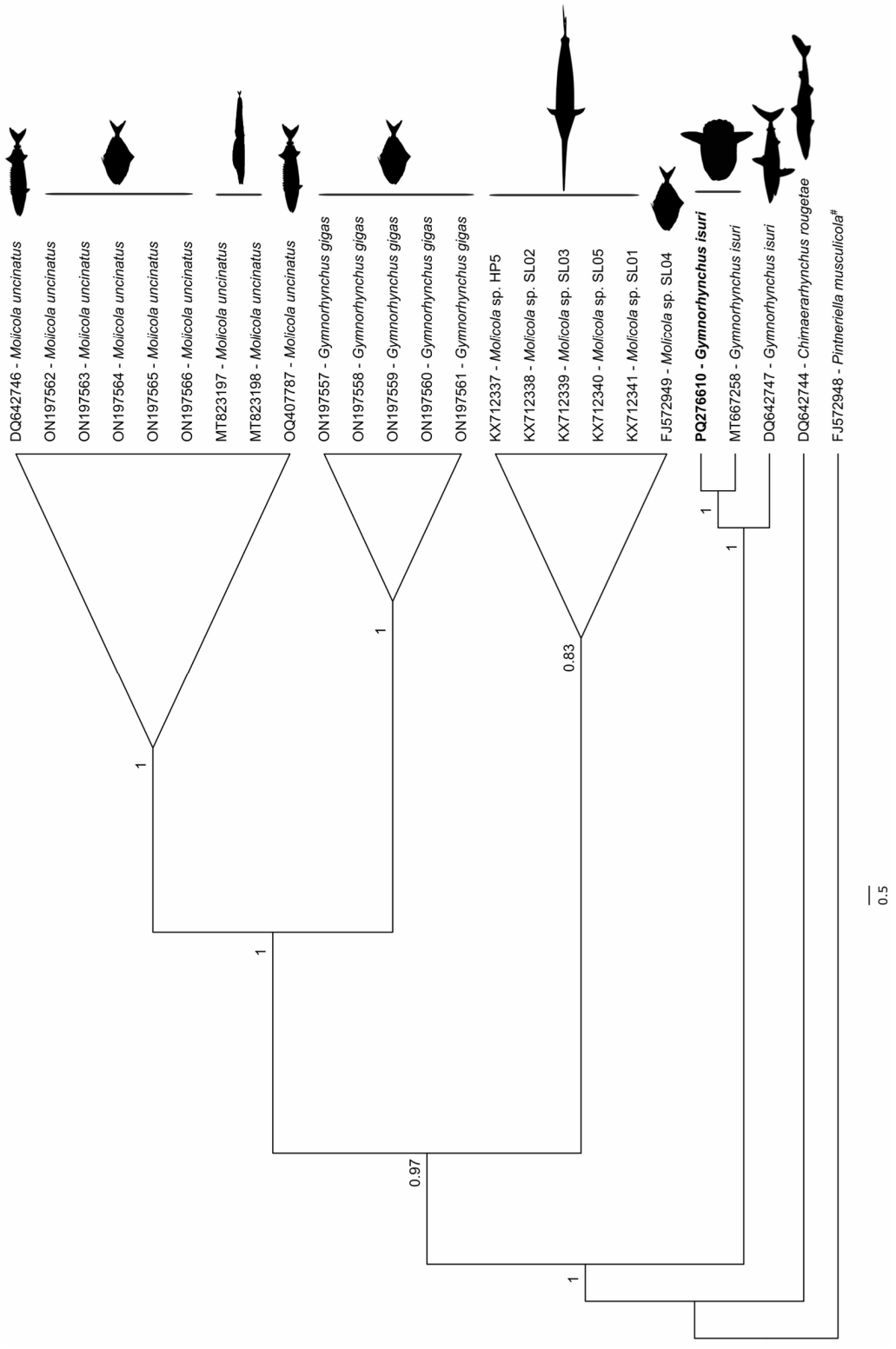

Figure 2. Bayesian inference (BI) tree for the analysis based on the 28S rDNA sequences (alignment of 1465 bp). Nodal support is given as posterior probabilities. The scale bar indicates the expected number of substitutions per site. The sequence generated in this study is shown in bold. Fish silhouettes represent host families. # Outgroup.

Table 2. Differences among all representatives of the family Gymnorhynchidae for which 28S rDNA sequences were available; p-distances (above the diagonal, shown as percentages) and pairwise nucleotide differences (below the diagonal). The sequence generated in this study is shown in bold.

			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
1	**PQ276610**	***Gymnorhynchus isuri***	-	7.21	3.31	3.31	3.31	3.31	3.31	0.81	0.00	2.48	2.55	2.63	2.54	2.47	2.47	3.41	3.47	2.03	2.03	3.31	3.31	3.31	3.31	3.31	5.02
2	DQ642744	*C. rougetae*	97	-	7.50	7.50	7.50	7.50	7.50	7.48	7.32	7.49	7.61	7.69	7.59	7.49	7.49	7.55	8.65	5.35	5.35	7.50	7.50	7.50	7.50	7.50	4.97
3	ON197557	*G. gigas*	45	100	-	0.00	0.00	0.00	0.00	3.66	3.50	1.46	1.63	1.71	1.63	1.46	1.46	0.93	1.11	0.76	0.76	0.83	0.83	0.83	0.83	0.83	5.07
4	ON197558	*G. gigas*	45	100	0	-	0.00	0.00	0.00	3.66	3.50	1.46	1.63	1.71	1.63	1.46	1.46	0.93	1.11	0.76	0.76	0.83	0.83	0.83	0.83	0.83	5.07
5	ON197559	*G. gigas*	45	100	0	0	-	0.00	0.00	3.66	3.50	1.46	1.63	1.71	1.63	1.46	1.46	0.93	1.11	0.76	0.76	0.83	0.83	0.83	0.83	0.83	5.07
6	ON197560	*G. gigas*	45	100	0	0	0	-	0.00	3.66	3.50	1.46	1.63	1.71	1.63	1.46	1.46	0.93	1.11	0.76	0.76	0.83	0.83	0.83	0.83	0.83	5.07
7	ON197561	*G. gigas*	45	100	0	0	0	0	-	3.66	3.50	1.46	1.63	1.71	1.63	1.46	1.46	0.93	1.11	0.76	0.76	0.83	0.83	0.83	0.83	0.83	5.07
8	DQ642747	*G. isuri*	11	100	49	49	49	49	49	-	0.62	2.66	2.49	2.56	2.48	2.66	2.66	3.35	3.19	2.04	2.04	3.52	3.52	3.52	3.52	3.52	5.55
9	MT667258	*G. isuri*	0	94	45	45	45	45	45	8	-	2.63	2.55	2.63	2.55	2.63	2.63	3.41	3.47	2.03	2.03	3.50	3.50	3.50	3.50	3.50	5.25
10	FJ572949	*Molicola* sp. HP5	34	101	21	21	21	21	21	36	34	-	0.00	0.00	0.00	0.00	0.00	1.47	1.39	1.14	1.14	1.32	1.32	1.32	1.32	1.32	4.54
11	KX712337	*Molicola* sp. SL01	33	98	21	21	21	21	21	32	33	0	-	0.08	0.00	0.00	0.00	1.47	1.39	1.14	1.14	1.48	1.48	1.48	1.48	1.48	5.01
12	KX712338	*Molicola* sp. SL02	34	99	22	22	22	22	22	33	34	0	1	-	0.00	0.08	0.08	1.55	1.39	1.27	1.27	1.56	1.55	1.56	1.56	1.56	5.09
13	KX712339	*Molicola* sp. SL03	33	98	21	21	21	21	21	32	33	0	0	0	-	0.00	0.00	1.47	1.39	1.14	1.14	1.48	1.48	1.48	1.48	1.48	5.00
14	KX712340	*Molicola* sp. SL04	34	101	21	21	21	21	21	36	34	0	0	1	0	-	0.00	1.47	1.39	1.14	1.14	1.32	1.32	1.32	1.32	1.32	4.53
15	KX712341	*Molicola* sp. SL05	34	101	21	21	21	21	21	36	34	0	0	1	0	0	-	1.47	1.39	1.14	1.14	1.32	1.32	1.32	1.32	1.32	4.53
16	DQ642746	*M. uncinatus*	44	97	12	12	12	12	12	43	44	19	19	20	19	19	19	-	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.56
17	OQ407787	*M. uncinatus*	25	62	8	8	8	8	8	23	25	10	10	10	10	10	10	0	-	0.00	0.00	0.14	0.14	0.14	0.14	0.14	5.26
18	MT823197	*M. uncinatus*	16	42	6	6	6	6	6	16	16	9	9	10	9	9	9	0	0	-	0.00	0.00	0.00	0.00	0.00	0.00	3.30
19	MT823198	*M. uncinatus*	16	42	6	6	6	6	6	16	16	9	9	10	9	9	9	0	0	0	-	0.00	0.00	0.00	0.00	0.00	3.30
20	ON197562	*M. uncinatus*	45	100	12	12	12	12	12	47	45	19	19	20	19	19	19	0	1	0	0	-	0.00	0.00	0.00	0.00	5.07
21	ON197563	*M. uncinatus*	45	100	12	12	12	12	12	47	45	19	19	20	19	19	19	0	1	0	0	0	-	0.00	0.00	0.00	5.07
22	ON197564	*M. uncinatus*	45	100	12	12	12	12	12	47	45	19	19	20	19	19	19	0	1	0	0	0	0	-	0.00	0.00	5.07
23	ON197565	*M. uncinatus*	45	100	12	12	12	12	12	47	45	19	19	20	19	19	19	0	1	0	0	0	0	0	-	0.00	5.07
24	ON197566	*M. uncinatus*	45	100	12	12	12	12	12	47	45	19	19	20	19	19	19	0	1	0	0	0	0	0	0	-	5.07
25	FJ572948	*Pintneriella musculicola* [#]	69	67	73	73	73	73	73	75	68	66	65	66	65	66	66	72	38	26	26	73	73	73	73	73	-

[#] Outgroup.

4. Discussion

Currently, the family Gymnorhynchidae comprises three genera and six valid species that, as adults, are all intestinal parasites of large pelagic sharks. These are *Gymnorhynchus gigas* (Cuvier, 1817) Rudolphi, 1819, *G. isuri* Robinson, 1959, *Molicola horridus*, *M. uncinatus* (Linton, 1924) Palm, 2004, *M. walteri* Palm, 2004, and *Chimaerarhynchus rougetae* Beveridge and Campbell, 1989.

Identification of larvae and adults of Gymnorhynchidae species is historically based on the morphological characterization of hooks on tentacles and, in general, on the pattern of their tentacular armature [6]. However, depending on preservation and subsequent condition of parasite specimens, morphological study may be unable to differentiate among members of Gymnorhynchidae and even among members of Trypanorhyncha, thereby limiting more detailed information on their ecology and geographical distribution [6,20]. When the host tissues containing larvae are frozen, the parasites die and their tentacles remain invaginated, preventing the study of most important morphological characters. Indeed, this is the case presented here. The viscera of the examined *M. lanceolatus* were immediately frozen after landing and, when these were thawed and parasites collected for morphological analysis, it was impossible to obtain parasite larvae with evaginated tentacles to be used for morphological identification. Nevertheless, misidentification of Gymnorhynchidae larvae using morphological criteria alone may occur because the isolation of their scoleces is challenging. Often, the partial or total invagination of their tentacles prevent a correct species identification [6,20]. When this occurs, molecular analysis allows for an unequivocal species identification that would otherwise be problematic or not possible.

The life cycle for members of Gymnorhynchidae has not been elucidated yet, but it has been proposed that copepods serve as first, pelagic euphausiids or schooling fish as second, and larger predatory fishes as third intermediate hosts [6]. According to the parasite-host list in Palm [6], some members of Molidae fish (especially *Mola* spp.) should be considered as the intermediate hosts for *Molicola horridus* and *M. uncinatus*. In literature, it is reported a single case of *M. horridus* larvae recovered from the liver of *M. lanceolatus* from the Gulf of Mannar (India) [4]. Nonetheless, unidentified larval forms of Trypanorhyncha have also been found in the liver of two specimens of *M. lanceolatus* stranded on the southern coast of the State of Pernambuco (Brazil) [24,25]. In addition, larvae of *G. isuri* were found in the liver of a *M. mola* from the Mediterranean Sea, of which identification was confirmed by molecular techniques [7].

Regarding the geographical distribution of *G. isuri* in its final hosts, the species is known infecting the shortfin mako *Isurus oxyrinchus* and the blue shark *Prionace glauca* from north, southwestern, and northeast Atlantic and the Tasmanian Sea in the southwestern Pacific [8,26,27]. Based on the above information, *M. lanceolatus* represents a new host record for *G. isuri*, confirming the importance of Molidae fish in the life cycle of Gymnorhynchidae members. Further, the Arabian Sea represents a new locality record, expanding the geographical range of this cestode.

It has been hypothesized that *G. isuri* could be used as a biological tag to study sunfish movements [7]. The record of this parasite in another member of the Molidae family, in a new geographical area, opens new insights in these host–parasite associations, as well as in potential migration routes of *M. lanceolatus*. However, further information is needed to ascertain whether the present finding of *G. isuri* in the Arabian Sea is a new or casual event, or whether the species is widespread but undetected and/or misidentified (e.g., when only morphological identification is performed).

Finally, the specimen of *G. isuri* reported here was genetically identical to that found in a *Mola mola* from the Mediterranean Sea [7], as shown by molecular and phylogenetic analyses. While, some genetic distance was detected among these two and another specimen collected from its definitive type host from a third location. Unfortunately, it was impossible to include in the analysis (due to difference in the genetic marker examined) another specimen of *G. isuri* collected in intermediate hosts (teleost fishes) from the Mediterranean

Sea [28] to investigate the phylogenetic relationship with the present one. Phylogeny, as well as the genetic distance matrix in Table 2, also confirmed the high similarity of *M. uncinatus* and *G. gigas*, while *G. isuri* represented a separate more basal clade. Although our analysis was performed with a single genetic marker, our results were in agreement with studies including multiple markers (i.e., [7,20,21,23]), supporting this approach as an invaluable method to discriminate among Gymnorhynchidae members. Indeed, it has been suggested that some morphological characters might not have taxonomic value for the genus diagnosis in this family [7]. The 28S rDNA is believed to contribute to a greater proportion in resolving cestode phylogeny, including Trypanorhyncha, than other commonly used markers, such as 18S rDNA, providing good resolution among divergent clades [8,29]. In particular, Olson et al. [29] found that the 28S gene was the most informative compared to 18S and elongation factor-1α, especially when taking into account the sequencing effort, and 28S results indicated a greater degree of hierarchical structure in the data. For this reason, the sequences of cestodes available in freely accessible databases, e.g., GenBank, are represented mostly by the partial region D1-D3 of the 28S rDNA, which can therefore be safely used for phylogenetic analyses.

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Data Availability Statement: No datasets were generated, sequence generated was deposited in the open access GenBank database.

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

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