Morphology and molecular phylogeny of *Heterobothrium lamothei* (Monogenea: Diclidophoridae), a gill parasite infecting the tiger puffer fish *Lagocephalus sceleratus* (Tetraodontidae)

[*Morfologia e filogenia molecular de Heterobothrium lamothei (Monogenea: Diclidophoridae), um parasita das guelras que infecta o peixe tigre pufferLagocephalus sceleratus (Tetraodontidae)*]

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ABSTRACT

Members of the family Diclidophoridae are potentially dangerous species for the puffer fish aquaculture worldwide. They are parasitic polyopisthocotyleans, with a posterior haptor equipped with clamps for attachment to the host's surface, allowing the worm to resist the flow of water to maintain its position on gills. The anterior body of the worm is deformable, allows the worm to feed on blood sucked from fish gills. The present study is the first description of a *Heterobothrium* species from the gills of the tiger puffer *Lagocephalus sceleratus* (Tetraodontidae) from the coasts of the Arabian Gulf at Jubail, Saudi Arabia morphologically by light microscopy as well as by molecular analysis of the parasite partial 28S rRNA through multiple sequence alignments and phylogeny by maximum likelihood analysis which is provided for the first time for the described species. Seventeen tiger puffer fish were captured alive from marine water off Saudi Arabia; gills were separated and further examined for parasitic infection. Nine fish were found infected with a monogenean parasite which was robust, equipped by two buccal organs at the tapered anterior end; the posterior haptor was rectangular with four symmetrically arranged clamps, with no isthmus. Marginal hooks absent. Ovary elongated, U–shaped, testes numerous, irregularly shaped and extended from the posterior part of the ovary to the anterior margin of the haptor. Copulatory organ muscular, as a spherical cup armed with 12 to 15 genital hooks. The molecular analysis of the parasite 28s rRNA and phylogeny revealed a percentage of identities between 87.47–89.09%, with Diclidophoridae species within the monophyletic clade of Mazocraeidea where a maximum percentage of 89.09% were obtained for the morphologically different sister taxon *H. okamotoi*. The results obtained from molecular analysis are consistent with the conclusions drawn from morphological classification where that the parasite recorded was morphologically similar to *H. lamothei* which was not characterized by molecular analysis before. The recovered sequences were deposited into the GeneBank under accession number MT322610.

Keywords: Diclidophoridae, *Heterobothrium*, morphology, molecular study, puffer fish

RESUMO

Os membros da família Diclidophoridae são espécies potencialmente perigosas para a aquicultura de peixes puffer em todo o mundo. Eles são parasitas poliopisthocotyleans, com uma hélice posterior equipada com pinças para fixação na superfície do hospedeiro, permitindo que o verme resista ao fluxo de água para manter sua posição nas brânquias. O corpo anterior do verme é deformável, e permite que o verme se alimente de sangue sugado das guelras dos peixes. O presente estudo é a primeira descrição de uma espécie Heterobothrium *das guelras do tigre* Lagocephalus sceleratus (Tetraodontidae) *das costas do Golfo Arábico em Jubail, Arábia Saudita, usando morfologia por microscopia leve, bem como análise molecular do rRNA parcial do parasita 28S através de alinhamentos de sequências múltiplas e filogenia por análise de máxima verossimilhança que é fornecida pela primeira vez para as espécies descritas. Dezessete peixes tigre puffer foram capturados vivos da água marinha da Arábia Saudita; as brânquias foram separadas e mais tarde examinadas para detecção de infecção parasitária. Nove peixes foram encontrados infectados por um parasita monogênio robusto, equipado por dois órgãos bucais na extremidade anterior afilada; o hortetor posterior era retangular com quatro pinças dispostas simetricamente, sem istmo. Ausência de ganchos marginais. Ovário alongado, em forma de U, testículos numerosos, de forma irregular e estendido desde a parte posterior do ovário até a margem anterior do hortelino. Órgão copulatório muscular, como um copo esférico armado com 12 a 15 ganchos genitais. A análise molecular do parasita 28s rRNA e filogenia revelou uma porcentagem de identidades entre 87,47-89,09%, com espécies Diclidophoridae dentro do clade monofilético de Mazocraeidea onde uma porcentagem máxima de 89,09% foi obtida para o táxon-irmão*

morfologicamente diferente *H.* okamotoi. *Os resultados obtidos da análise molecular são consistentes com as conclusões tiradas da classificação morfológica onde o parasita registrado era morfologicamente semelhante ao H.* lamothei *que não era caracterizado pela análise molecular antes. As sequências recuperadas foram depositadas no GeneBank sob o número de acesso MT322610.*

Palavras-chave: Diclidophoridae, Heterobotrium, *morfologia, estudo molecular, peixe puffer*

INTRODUCTION

Monogeneans are a group of ectoparasitic flatworms commonly found on the skin, gills, or fins of fish. They have a direct life cycle and do not require an intermediate host. The marine forms of these parasites are generally larger than those found on freshwater hosts. Monogenea have an unusual mode of attachment, in which the haptor and the long muscular pre–haptoral stalk of the adult worms are embedded subcutaneously into the gill arch, leaving only the anterior body of the parasite visible externally, causing severe damage to the host gills (Agrawal *et al.,* 2006). The genus *Heterobothrium* Cerfontaine (1895) of family Diclidophoridae Sproston (1946), is highly specific to tetraodontid fishes (Actinopterygii: Tetraodontidae), including 11 blood-feeding species of monogeneans infecting the gills (Williams, 1986; Ogawa, 1997) with high species diversity and host specificity (Boeger and Kritsky, 2001; Desdevises *et al.*, 2002; Šimkova *et al.,* 2006). Immature worms of these parasites attach to the gill filaments of their hosts, feeding on the blood, causing impaired breathing and anemia to their hosts, and then migrate to the buccal cavity wall for maturation (Matsui *et al.,* 2020). They cause serious problems and a high mortality rate in aquaculture of puffer fish worldwide (Yoshinaga *et al.,* 2001; Nakayasu *et al.,* 2002; Nakane *et al.,* 2005). Goto, in 1894, described *Diclidophora tetrodonis* Goto (1894) from *Tetraodon* sp. stating that its peculiar characteristic was the reverse orientation of the anterior most clamp pair compared to the other pairs. Cerfontaine (1895) considered that this species should be allocated to a distinct genus, *Heterobothrium*, with *H. tetrodonis* as the type species (Nagibina 1953). Phylogenetically, diclidophorids are considered an ancient group, and one of the most predominant families of deep–sea monogeneans (Justine *et al.,* 1985; Rohde, 1988). Compared to morphology, phylogeny provides more reliable information regarding evolutionary relationships (Scotland *et al.,* 2003; Pandey and Agarwal, 2008). *Lagocephalus sceleratus* Gmelin (1789), commonly known as the silver–cheeked toadfish,

is an extremely poisonous marine bony fish in the family Tetraodontidae (puffer fishes) distributed in many habitats including the Arabian Gulf of Saudi Arabia. The ancient pharaohs identified puffer fish and draw them on their tombs because of its importance, due to it carrying the very famous alkaloid toxin, Tetrodotoxin (TTX) (Mohammed–Geba *et al.,* 2016). The present study is the first description of a *Heterobothrium* species from the tetraodontid *L. sceleratus* from coasts of the Arabian Gulf at Jubail, Saudi Arabia morphologically by light microscopy as well as molecular analysis of the parasite partial 28S rDNA which is provided for the first time for the recovered species.

MATERIALS AND METHODS

Fish and parasite sampling: A total of 17 specimens of tiger puffer fish *L. sceleratus* (Tetraodontidae) were collected alive from local fishermen along the coasts of the Arabian Gulf at Jubail (26.9598° N, 49.5687° E), Saudi Arabia, between January and May 2019. The fish were morphologically identified according to the guidelines of Randall (1995). Following capture, fish were euthanized by means of percussive stunning followed by severing the spinal cord immediately posterior to the brain. After removing the opercula and exposing the gill arches, the gills were removed carefully from the fish and placed in Petri dishes with tap water, and then examined by a stereomicroscope for the presence of monogeneans (Mendoza–Franco *et al.,* 2018). Worms were collected from the gill filaments with the aid of a fine brush and fixed with hot water (60 °C). Some specimens were stored in a mixture of ammonium picrate glycerin (GAP) (Ergens, 1969), and some in hot 4% formalin for morphological analysis, while some worms were fixed in 70% ethanol for molecular analyses.

Morphological analyses: some worm specimens were stained in Semichon's carmine and dehydrated in series of ethanol (staining for 2 min; cleaning in 70% ethanol for ~ 1 min; destaining in 1% HCl solution for ~ 2 min or

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until the inner structures were differentiated; dehydration in 80, 90, and 100% ethanol for ~2 min; clearing in clove oil for ~2 min) and mounted in Canada Balsam. Unstained specimens were flattened with coverslips on slides with a mixture of ammonium picrate glycerin to study the morphology of the sclerotized structures and remounted in Canada Balsam (Ergens 1969). Terminology and nomenclature of the clamp sclerites were determined according to the key published by Vidal–Martínez and Mendoza–Franco (2008) for Diclidophoridae. Photomicrographs were taken with a BX53 microscope (Olympus Corporation, Japan). Illustrations were made by *camera lucida*. Measurements were expressed in micrometers (µm) as a mean ± Standard deviation followed by the range in parentheses.

Molecular study: From 70% ethanol–preserved samples, genomic DNA (gDNA) was extracted from the recovered worms using a DNeasy tissue kit (Qiagen, Germany), following the manufacturer's instructions. PCR amplification of a partial 28s rRNA sequence was carried out on an MJ Research PTC–150 thermocycler (Marshall Scientific, USA) using the internal primers Halio–F 5´–ACCCGCTGAATTTAAGCAT–3´ and Halio–R 5´–TGGTCCGTGTTTCAAGAC–3´ (García–Vásquez *et al.,* 2015). All PCR reactions were performed in a final volume of 50µL, composed of 5µL 10× PCR buffer, 1.5µL 10 mM deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP, and dTTP, 10µM each), 4.0µL 2.0mM $MgCl_2$, 1.5µL of each primer (10µM), 5µL template DNA, 0.24µL of *Thermus aquaticus* (Taq) DNA polymerase (1.2 units), and 31.26µL of nuclease–free sterile double–distilled water. The following thermocycling profile was used: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94 °C for 30 sec, annealing at 55°C for 30 sec, and a final extension at 72°C for 3 min (García–Vásquez *et al.,* 2015).

Phylogenetic analysis: Sequence identity for the recovered data was checked using the Basic Local Alignment Search Online Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence trimming for the congeneric species recovered was carried out by Bioedit v7.2.5. The 28s rRNA sequences obtained were aligned with those of the other monogenean species using CLUSTAL W v.2.0. The phylogenetic tree was constructed using the MEGA v. 7 program,

based on maximum likelihood analysis. The genetic divergence among species was estimated using the uncorrected "p–distances" method, using the same program.

RESULTS

Heterobothrium lamothei Vidal–Martinez and Mendoza–Franco (2008) Monogenea; Diclidophoridae; Mazocraeidea; Parapedocotylinae. *Diagnosis:* Worms were recovered from the gill filaments as well as the wall of the branchial cavity of the host fish. They were always found grasping the gill filaments using their clamps, with the haptor embedded in the host tissue. Most of the infected fish had very pale gills, with massive mucous secretions.

Light microscopy: Body elongate, robust, tapered anteriorly (Fig. 1a, b), with a mouth opening between the two buccal organs (Fig. 1c). Posterior end of the worm was broad, including a sclerotized haptor which was continuous with the rest of the body, no isthmus was observed (Fig. 1d). The total body length including haptor was 3211 (2095–4354) µm, while the greatest width at the ovarian level was 401 (328–530) µm. Paired buccal organs 85±2µm in diameter (75–100; n = 5) were observed, pharynx 91±15 (75–110; n = 5) µm in diameter. Intestinal bifurcation was at the level of the gonopore, while lateral and median diverticula of caeca extended to the anterior margin of the haptor. Haptor was rectangular with four symmetrically arranged clamps (Fig. 1d–f). The diameter of the first pair of clamps (anterior-most) was 175 ± 5 (160–190; n = 5) µm; the second pair was 187 ± 4 (150–220) µm; the third pair was 1080 ± 5 (155–210) µm, while the fourth pair was 168 ± 5 (118–198) µm. Marginal hooks absent. Testes numerous 23–30 in number, they were irregularly shaped, and extended from the posterior part of ovary to the anterior margin of the haptor. The copulatory organ muscular, as a spherical cup and armed with 12 to 15 genital hooks, 80 ± 9 (80–90; n = 5) µm in diameter (Fig. 1g). Gonopore midventral. Ovaries elongated and U–shaped, 310 ± 4 (250–370; n = 5) µm long x 181 ± 3 (150–230; n = 6) µm wide and located in front of the testes. The oviduct ran along the left with the genito–intestinal canal passed from the right intestinal limb, connected posteriorly with a vitelline duct followed by the ootype. The uterus was narrow, with only a few eggs. There was no vagina, but there was a genital pore in front of the male organ. The vitelline reservoir was

situated to the right of the ovary, with follicles scattered from the level of gonopore to the anterior margin of the haptor. Eggs were ovoid, fusiform, and operculated, with long posterior filaments (Figure 1y, j). Figure 2 presents a line diagram illustrating the different morphological diagnostic characteristics of the recovered parasite.

Molecular study: Sequence alignment for the data obtained from 18s rRNA analysis of the monogenean parasite isolated from the host examined yielded 1750 bp aligned with 29 species of Mazocraeidea and Diclybothriidea taxa, recovered after BLAST, with the estimates of evolutionary divergence between sequences calculated (Tables 1 and 2). The constructed phylogenetic tree (Fig. 3) showed that the maximum likelihood analysis of all datasets resulted in Mazocraeidea forming a monophyletic clade. Within this clade, four strongly supported subclades were demonstrated. Sub–clade A includes Diclidophoridae species,

sub–clade B includes the mazocraeid species belonging to the families Protomicrocotylidae, Gastrocotylidae, Thoracocotylidae and Gotocotylidae, sub–clade C includes species of Plectanocotylidae, and sub–clade D includes species belonging to the families Diplozoidae, Discocotylidae, Microcotylidae, Gastrocotylidae and Heteraxinidae. The sequence data of the present parasite was included within the phylogenetic tree between members of the Diclidophoridae species (sub–clade A), with the percentage of identities falling between 87.47–89.09%. The sequence alignment of the parasite recorded showed identities of 86.66%, 86.87%, 87.47%, 87.50%, 87%, 50%, and 88.52% for *Neoheterobothrium hirame*, *Neoheterobothrium* sp., *Choricotyle australiensis*, *Diclidophora minor*, *Diclidophora denticulate*, and *Bravocotyle* sp., respectively, and showed a percentage of 89.09% for the sister taxon *H. okamotoi* isolated from the puffer fish *Takifugu* spp.

Table 1. Parasite species used in the sequence alignment as well as phylogenetic analysis of the *H. lamothei* isolated in the current study. 28S rRNA sequences of the parasite isolated were aligned with 29 species of Mazocraeidea and Diclybothriidea taxa, recovered after BLAST. It was shown that the sequences recorded identities of 87.47–89.09% with species within family Diclidophoridae

Species	Accession no.	Order	Family	% Identity
Diclidophora denticulata	AJ228779.1	Mazocraeidea	Diclidophoridae	87.94%
Diclidophora minor	AF382070.1	Mazocraeidea	Diclidophoridae	87.47%
Choricotyle australiensis	AF382069.1	Mazocraeidea	Diclidophoridae	87.50%
Neoheterobothrium sp.	AB162425.1	Mazocraeidea	Diclidophoridae	86.87%
Neoheterobothrium hirame	AB162424.1	Mazocraeidea	Diclidophoridae	86.66%
Bravocotyle sp.	KT267179.1	Mazocraeidea	Diclidophoridae	88.52%
Heterobothrium okamotoi	AB162155.1	Mazocraeidea	Diclidophoridae	89.09%
Bilaterocotyloides carangis	KT267184.1	Mazocraeidea	Protomicrocotylidae	86.27%
Neomicrocotyle pacifica	AJ228787.1	Mazocraeidea	Protomicrocotylidae	86.28%
Neomicrocotyle pacifica	AJ228787.1	Mazocraeidea	Protomicrocotylidae	86.12%
Pseudaxine trachuri	AM157196.1	Mazocraeidea	Gastrocotylidae	86.08%
Paradewesia sp.	AJ287555.1	Mazocraeidea	Thoracocotylidae	86.24%
Mexicotyle sp.	AJ287539.1	Mazocraeidea	Thoracocotylidae	86.36%
Gotocotyla bivagina	AJ276424.1	Mazocraeidea	Gotocotylidae	86.37%
Gotocotyla secunda	KT267178.1	Mazocraeidea	Gotocotylidae	86.27%
Gotocotyla secunda	AJ276425.1	Mazocraeidea	Gotocotylidae	86.10%
Plectanocotyle gurnardi	AJ228790.1	Mazocraeidea	Plectanocotylidae	85.47%
Eudiplozoon nipponicum	AJ287510.1	Mazocraeidea	Diplozoidae	86.26%
Paradiplozoon hemiculteri	KY640614.1	Mazocraeidea	Diplozoidae	85.03%
Discocotyle sagittata	AJ287504.1	Mazocraeidea	Discocotylidae	87.02%
Microcotylidae sp.	JN602094.1	Mazocraeidea	Microcotylidae	86.50%
Microcotyle sp.	KT267180.1	Mazocraeidea	Microcotylidae	86.67%
Gastrocotyle sp.	KT267185.1	Mazocraeidea	Gastrocotylidae	85.61%
Monaxine formionis	KT267181.1	Mazocraeidea	Heteraxinidae	85.83%
Pseudohexabothrium taeniurae	AJ228791.1	Diclybothriidea	Hexabothriidae	83.01%
Diclybothrium armatum	KP796254.1	Diclybothriidea	Diclybothriidae	83.60%
Paradiclybothrium pacificum	KP796243.1	Diclybothriidea	Diclybothriidae	83.55%
Paradiclybothrium pacificum	KP796244.1	Diclybothriidea	Diclybothriidae	83.55%
Paradiclybothrium pacificum	KP796242.1	Diclybothriidea	Diclybothriidae	83.55%

Table 2. Estimates of Evolutionary Divergence between Sequences, the number of base substitutions per site between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model. Evolutionary analyses were conducted in MEGA7. The numbers shown represent the genetic divergence between species, column no. 1 showed the genetic distance variation between the present *H. lamothei* and the comparable species of Mazocraeidea and Diclybothriidea taxa, recovered after BLAST. The closest sequences of species to those of the present species showed genetic distances between 0.0681–0.1516 within family Diclidophoridae (the first 7 species). The most similar species is *H. lamothei* with a divergence of 0.0681 which give rise to the significant morphology between the comparable species

Accession no.	Species	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	26	27	28
MT322610	*H. lamothei* (present)																												
AB162155.1	*H. okamotoi*	0.0681																											
KT267179.1	*Bravocotyle* sp.	0.1207	0.0442																										
AJ228779.1	*D. denticulata*	0.1480	0.0691	0.0730																									
AB162425.1	*Neoheterobothrium* sp.	0.1500	0.0694	0.0764	0.0567																								
AB162424.1	*N. hirame*	0.1516	0.0695	0.0765	0.0567	0.0000																							
AF382069.1	*C. australiensis*	0.1504	0.0724	0.0771	0.0504	0.0527	0.0534																						
AF382070.1	*D. minor*	0.1453	0.0654	0.0680	0.0316	0.0535	0.0536	0.0486																					
AJ287504.1	*D. sagittata*	0.1620	0.0846	0.0952	0.0762	0.0770	0.0764	0.0783	0.0725																				
JN602094.1	*Microcotylidae* sp.	0.1633	0.0868	0.0920	0.0758	0.0843	0.0837	0.0772	0.0714	0.0461																			
AJ276424.1	*G. bivagina*	0.1660	0.0867	0.0903	0.0810	0.0817	0.0812	0.0796	0.0764	0.0623	0.0629																		
KT267180.1	*Microcotyle* sp.	0.1612	0.0848	0.0927	0.0757	0.0816	0.0804	0.0796	0.0719	0.0467	0.0121	0.0629																	
KT267178.1	*G. secunda*	0.1663	0.0868	0.0923	0.0849	0.0844	0.0839	0.0823	0.0822	0.0679	0.0679	0.0145	0.0673																
KT267184.1	*B. carangis*	0.1648	0.0856	0.0921	0.0771	0.0762	0.0756	0.0836	0.0759	0.0681	0.0712	0.0358	0.0706	0.0407															
AJ287539.1	*Mexicotyle* sp.	0.1644	0.0841	0.0914	0.0789	0.0803	0.0805	0.0814	0.0763	0.0646	0.0634	0.0180	0.0622	0.0268	0.0311														
AJ287555.1	*Paradewesia* sp.	0.1631	0.0828	0.0901	0.0828	0.0817	0.0805	0.0839	0.0801	0.0684	0.0679	0.0245	0.0654	0.0292	0.0275	0.0186													
AJ228787.1	*N. pacifica*	0.1668	0.0868	0.0900	0.0790	0.0780	0.0775	0.0841	0.0764	0.0686	0.0724	0.0377	0.0730	0.0425	0.0063	0.0335	0.0305												
AJ287510.1	*E. nipponicum*	0.1689	0.0926	0.1018	0.0895	0.0868	0.0875	0.0882	0.0868	0.0705	0.0706	0.0848	0.0698	0.0868	0.0805	0.0828	0.0873	0.0804											
AJ276425.1	*G. secunda*	0.1685	0.0882	0.0956	0.0882	0.0871	0.0865	0.0817	0.0842	0.0686	0.0699	0.0139	0.0693	0.0063	0.0414	0.0263	0.0322	0.0432	0.0862										
AM157196.1	*P. trachuri*	0.1668	0.0874	0.0921	0.0822	0.0857	0.0852	0.0862	0.0816	0.0787	0.0821	0.0535	0.0814	0.0536	0.0353	0.0499	0.0443	0.0395	0.0921	0.0555									
KP796254.1	*D. armatum*	0.2060	0.1279	0.1365	0.1264	0.1302	0.1289	0.1225	0.1229	0.1072	0.1116	0.1166	0.1127	0.1174	0.1134	0.1131	0.1166	0.1148	0.1173	0.1168	0.1174								
KT267185.1	*Gastrocotyle* sp.	0.1739	0.0938	0.1019	0.0880	0.0947	0.0948	0.0881	0.0862	0.0608	0.0492	0.0737	0.0461	0.0788	0.0834	0.0749	0.0749	0.0852	0.0789	0.0808	0.0918	0.1171							
KT267181.1	*M. formionis*	0.1777	0.1011	0.1026	0.0860	0.0907	0.0902	0.0875	0.0867	0.0614	0.0535	0.0713	0.0503	0.0727	0.0808	0.0724	0.0776	0.0808	0.0819	0.0759	0.0912	0.1188	0.0431						
KY640614.1	*P. hemiculteri*	0.1824	0.1015	0.1088	0.1022	0.1071	0.1059	0.0984	0.0988	0.0823	0.0848	0.0947	0.0836	0.0936	0.0891	0.0927	0.0946	0.0891	0.0565	0.0943	0.1027	0.1313	0.0896	0.0913					
AJ228790.1	*P. gurnardi*	0.1706	0.0940	0.1029	0.0898	0.0923	0.0918	0.0883	0.0862	0.0672	0.0731	0.0720	0.0757	0.0740	0.0714	0.0694	0.0739	0.0701	0.0877	0.0773	0.0785	0.1153	0.0783	0.0783	0.0986				
KP796244.1	*P. pacificum*	0.2068	0.1266	0.1364	0.1217	0.1262	0.1249	0.1198	0.1169	0.1059	0.1081	0.1132	0.1080	0.1140	0.1099	0.1091	0.1125	0.1126	0.1145	0.1127	0.1139	0.0139	0.1110	0.1120	0.1296	0.1165			
KP796243.1	*P. pacificum*	0.2068	0.1266	0.1364	0.1217	0.1262	0.1249	0.1198	0.1169	0.1059	0.1081	0.1132	0.1080	0.1140	0.1099	0.1091	0.1125	0.1126	0.1145	0.1127	0.1139	0.0139	0.1110	0.1120	0.1296	0.1165	0.0000		
KP796242.1	*P. pacificum*	0.2068	0.1266	0.1364	0.1217	0.1262	0.1249	0.1198	0.1169	0.1059	0.1081	0.1132	0.1080	0.1140	0.1099	0.1091	0.1125	0.1126	0.1145	0.1127	0.1139	0.0139	0.1110	0.1120	0.1296	0.1165	0.0000	0.0000	
AJ228791.1	*P. taeniurae*	0.2130	0.1316	0.1378	0.1394	0.1384	0.1385	0.1346	0.1320	0.1201	0.1180	0.1203	0.1213	0.1205	0.1221	0.1200	0.1263	0.1233	0.1250	0.1199	0.1267	0.0997	0.1281	0.1339	0.1366	0.1223	0.1050	0.1050	0.1050
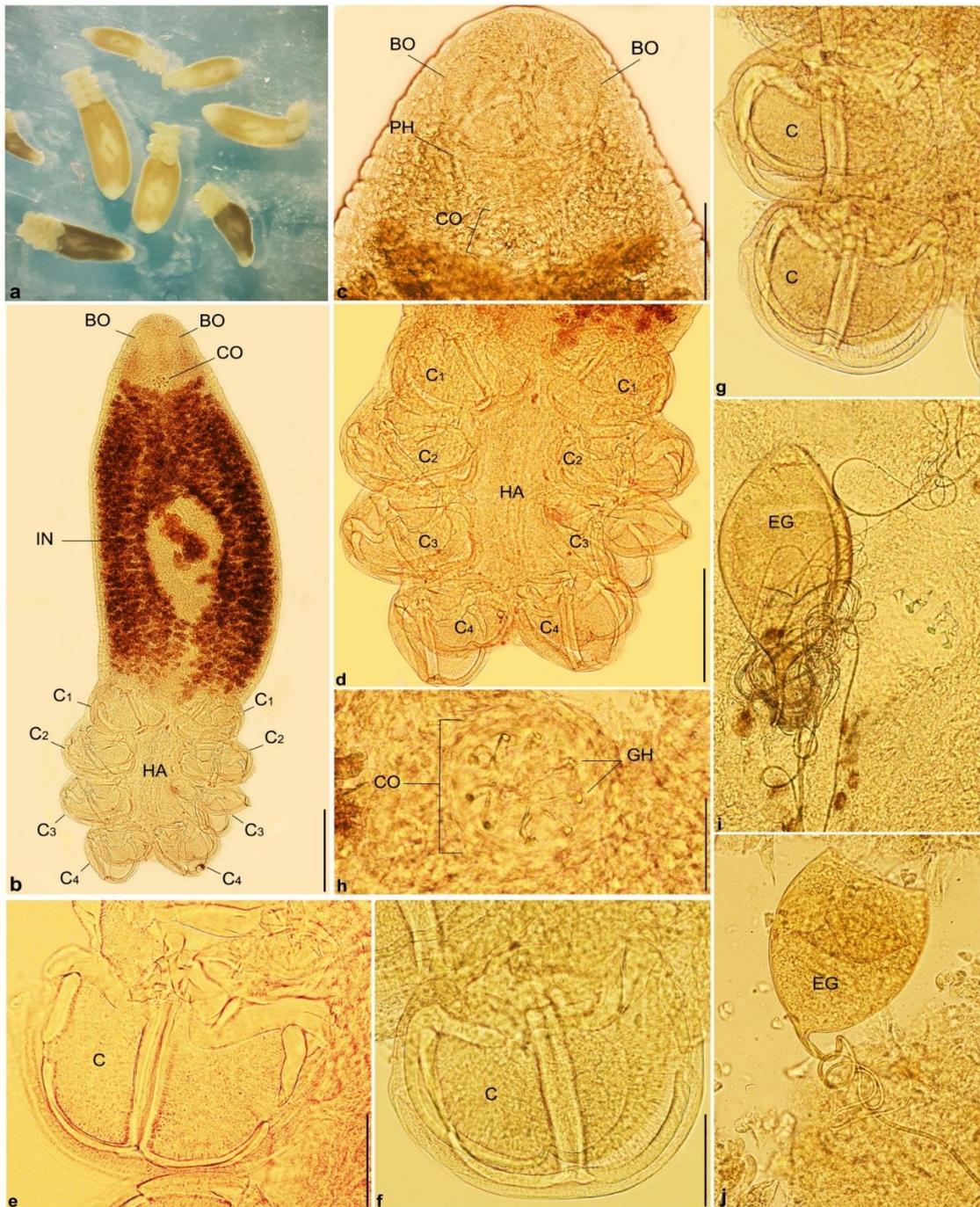
Figure 1. Photomicrographs of *H. lamothei* from *Lagocephalus sceleratus* off the Arabian Gulf. (a) A paragraph showing the freshly isolated worms from the host gills, (b) Whole−mount of a paratype adult specimen, dorsal view, BO buccal organ, HA haptor, IN intestinal caeca, C clamps, CO copulatory organ, (c) Anterior end of the body, PH pharynx, (d) Haptor (HA) with clamps (C), (e) Anterior clamp, (f) Posterior clamp, (g) Median clamps, (h) Copulatory organ (CO) armed with genital hooks (GH), (i,j) Operculated eggs (EG). Scale bars: b = 500 µm; c = 130 µm; d = 80 µm; e = 100 µm; f,g = 95 µm; h= 20 µm; i = 40 µm; j =50 µm.
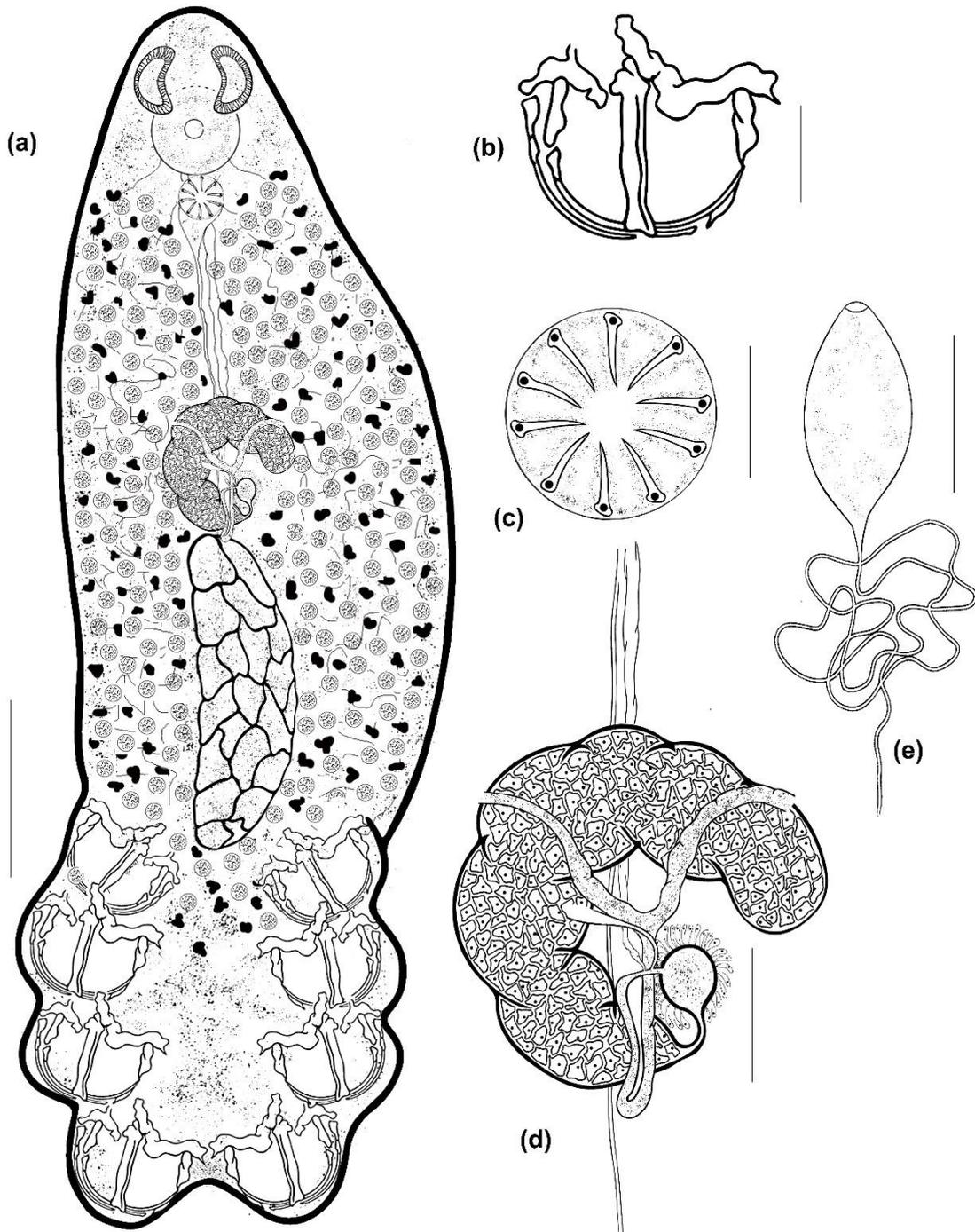
Figure 2. *H. lamothei*, (a) Total view, (b) Clamp, isolated median jaw, dorsal view, (c) Armed copulatory organ, (d) Reproductive system, (e) Egg. Scale bars: a = 400 μm; b = 20 μm; c = 45 μm; d = 117 μm; e = 150 μm.
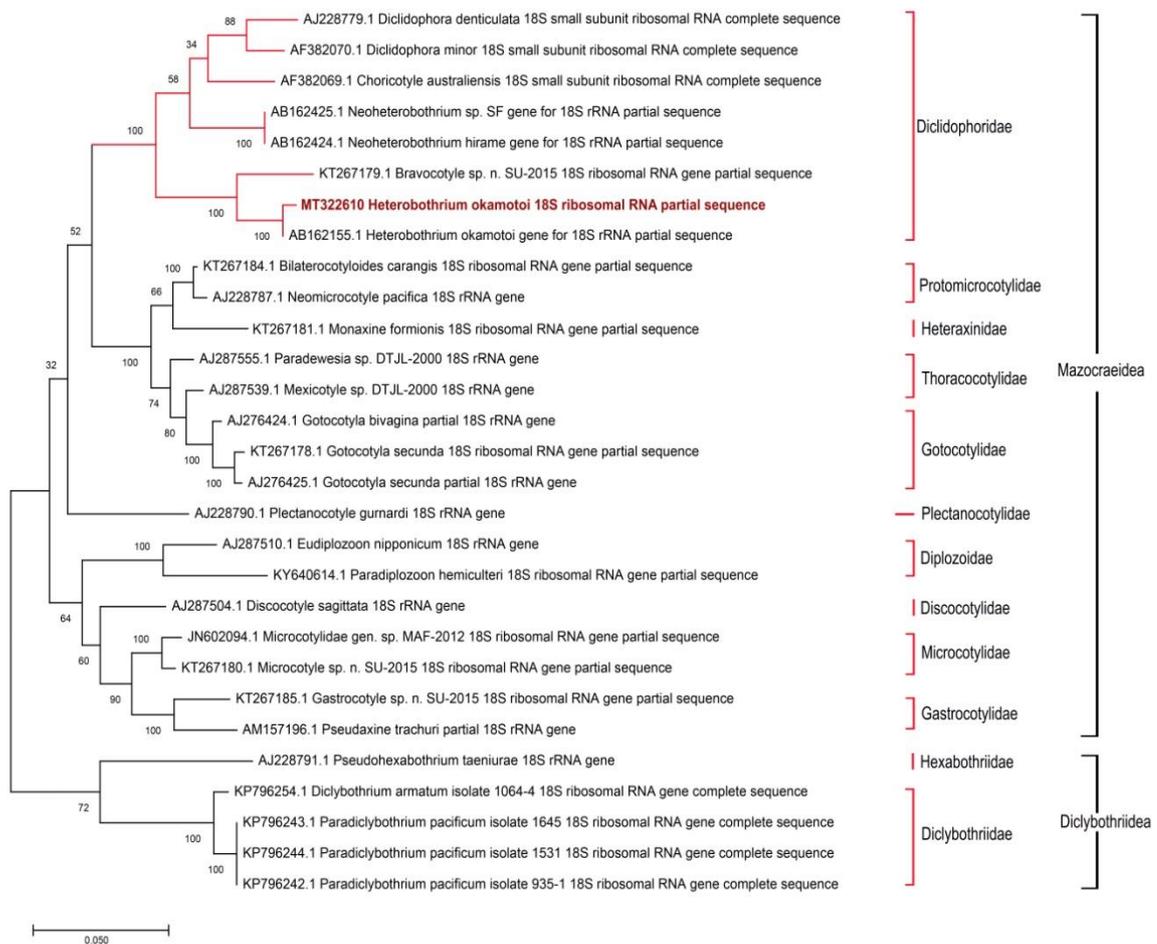
Figure 3. Phylogenetic tree constructed with the maximum likelihood method and Tamura 3–parameter model. The analysis involved 29 nucleotide sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1750 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Type–host: Tiger puffer fish *Lagocephalus sceleratus* (Tetraodontidae).
Type–locality: Coasts of Arabian Gulf at Jubail (26.9598° N, 49.5687° E), Saudi Arabia.
Site of infection: Gills.
Voucher material: Eleven specimens: 6 holotype and 5 paratypes (acc. no. KKU. BIO20.1–11) deposited in the parasite collection of the parasites section, Biology department museum, College of Science, King Khalid University, Saudi Arabia.

Infection details: Of the seventeen caught fishes, nine were infected by monogeneans, each parasitized by 2–5 (mean 3 ± 1) worms.

Etymology: the generic name of the parasite is derived from the presence of anterior most pair of skeletons orientated inversely while the specific name of the parasite is derived from the specific name of the fish where the parasite was recorded for the first time.

DISCUSSION

Fifteen species of *Heterobothrium* are currently recognized worldwide: *H. minimum* Sproston (1916), *H. affinis* Linton (1898), *H. ecuadori* Meserve (1938), *H. galapagense* Meserve (1938), *H. elongatum* Williams (1986), *H. tetrodonis* Goto (1894), *H. tonkinense* Bychowsky *et al.* (1976), *H. torquigeneri* Williams (1986), *H. praeorchis* Bychowsky *et*

al. (1976), *H. exilis* Mamaev (1987), *H. okamotoi* Ogawa and Inouye (1991), *H. shinagawai* Ogawa and Inouye (1991), *H. yamagutii* Ogawa and Inouye (1991), *H. bychowskyi* Ogawa and Inouye (1991), and *H. lamothei* Vidal–Martinez and Mendoza–Franco (2008). Several research papers have been published recently on *Heterobothrium* spp. such as those of Igarashi *et al.* (2017) and Matsui *et al.* (2020), who concluded that these parasites represent a serious problem for the aquaculture of puffer fish. Morphologically, the recorded parasite in the present study belongs to the genus *Heterobothrium* according to the key published by Llewellyn (1941); it is a polyopisthocotylean parasite with a penis armed with a coronet of pointed hooks, 8–15 in number, testes posterior to the ovary with no vagina, and skeletons of the anterior most pair of posterior suckers orientated inversely in comparison with those of the other three posterior pairs. Generally, polyopisthocotylean monogenean classification is mainly based on the clamp structure, copulatory organ, number and location of testes (Llewellyn, 1941). The present parasite resembles *H. ecuadori* Meserve (1938)*, H. okamotoi* Ogawa and Inouye (1991) and *H. yamagutii* Ogawa and Inouye (1991) in their general appearance; however, *H. ecuadori* differs from it by having a smaller copulatory organ (80–95) armed with 12–15 genital hooks, arranged in a circle, comparable to (114–116) armed with 9–11 hooks in *H. ecuadori*. Additionally, the number of testes of *H. ecuadori* (27–40) varies from the parasite assessed in this study, which was 23–30. *H. okamotoi* has been found to be a problem for Japanese aquaculture of the puffer fish *Takifugu* spp., it is morphologically different from the present species; it is longer (45–67.7), with a haptor separated from the body proper by a slender isthmus. The number of testes is relatively similar to that of *H. yamagutii* Ogawa and Inouye (1991), but this parasite has a copulatory organ armed with 10 genital hooks, and has clamps that are similar but longer in size (7400–14,400) than the parasite recorded herein. *H. lamothei* isolated from the gills of the checkered puffer fish *Sphoeroides testudineus* off México by Vidal–Martinez and Mendoza–Franco (2008) which is the most morphologically similar species to the present parasite: both have a posterior sucker equipped by clamps with no isthmus, copulatory organ consisting of muscular subspherical cup armed with 12 to 15 genital hooks arranged in a circle, number and

dimensions of testes as well as size shape of the ovary are also similar. The degree of variation in the morphology between the present parasite and other monogenean species should be supported by molecular studies to justify the taxonomy of this species (Tambireddy *et al.* 2016). Construction of phylogenetic trees is important for monogenean taxonomy through the expression of 28s rRNA, which is known to allow better phylogenetic resolution among monogenean families (Mollaret *et al.,* 2000; Tambireddy *et al.,* 2016). The sequence alignment and phylogenetic tree in the present study showed that the position of family Diclidophoridae is unambiguous and constructed in a separate clade. Similar observations have been reported in previous studies concerning the partial 28s rRNA of parasitic monogeneans (Mollaret *et al.,* 2000; Tambireddy *et al.,* 2016). Additionally, the current sequence analysis showed that the partial 28s rRNA gene has a sufficient phylogenetic signal, making it useful for phylogenetic tree reconstruction in accordance with Tambireddy *et al.* (2016). Since that the percentage of sequence identities between the present parasite and the aligned sequences showed a maximum identity of 89.09% with *H. okamotoi* which is morphologically different, the recorded parasite cannot be assigned to any of the aligned sequences. The parasite is mostly similar to *H. lamothei* which was not phylogenetically analyzed before. Thus, the present study should be considered as the first molecular study analyzing 28s rRNA gene of *H. lamothei* isolated from the tiger puffer fish *Lagocephalus sceleratus* (Tetraodontidae).

CONCLUSION

The present study is the first to provide morphological description as well as molecular analysis of the partial sequences of the 28S rDNA of *H. lamothei* from the tetraodontid *L. sceleratus* from the coasts of Arabian Gulf at Jubail, Saudi Arabia which is included in the establishment of a data base for the genus that will aid future studies and species circumscription. The results obtained from molecular analysis are consistent with the conclusions drawn from morphological classification. Detailed studies relating the morphology of attachment organs have provided valuable data on the mechanical processes accounting for the high host specificity of species and deserve further attention. We also

suggest that future studies studying in vivo morphological and physiological changes during the development of monogeneans attached on unusual hosts, especially with phylogenetically close host species, are needed.

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