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Genetic characterisation of four *Lamproglena* spp. (Copepoda, Lernaeidae) from Africa and the first mitochondrial data

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Abstract: Females of species of *Lamproglena* von Nordmann, 1832 are parasitic on the gills of teleost fishes and the 38 nominal species are based on mainly morphological data. Only four of these species have been genetically characterised and no mitochondrial data are available for the genus. The present study aimed to provide representative ribosomal DNA (rDNA) data for two additional species of *Lamproglena* from Africa: *Lamproglena clariae* Fryer, 1956 and *Lamproglena hoi* Dippenaar, Luus-Powell et Roux, 2001, alongside mitochondrial DNA (mtDNA) for these and two other African species, *Lamproglena hemprichii* von Nordmann, 1832 and *Lamproglena monodi* Capart, 1944. The four species were collected from Clariidae, Cyprinidae, Alestidae and Cichlidae, respectively. Representative 18S rDNA and 28S rDNA data were obtained for *L. clariae* and *L. hoi*, while *cox1* mtDNA was obtained for all four species. The respective haplotypes supported the distinctness of all species using all three gene regions investigated. Interestingly, species appeared to be grouped more by geographical origin than host family, with *L. hoi* more closely related to other African species than to Asian species also collected from cyprinid hosts. Even though the results presented here greatly add to the molecular data available for *Lamproglena*, there are still 32 (>80%) species for which no genetic data are available. The interpretation of the results presented here is thus preliminary and much more data are required before the phylogeny of this genus, and other members of the family, such as *Lernaea* Linnaeus, 1758, can be studied appropriately.

Keywords: Arthropoda, Crustacea, Cyclopoida, copepod parasite, DNA barcoding, anchor worms, freshwater fishes, biodiversity

This article contains supporting tables online:
Table S1 at http://folia.paru.cas.cz/suppl/2023-70-014.pdf and Tables S2–S4 online at https://doi.org/10.25415/ujhb.23591700.v1

Adult females of species of the Lernaeidae Cobbold, 1879 are highly transformed cyclopoid copepods mostly parasitic on freshwater teleost fishes (Ho and Kim 1997, Ho 1998). They usually attach to their host by a highly transformed cephalothorax or enlarged maxillae (Ho 1998, Boxshall and Halsey 2004). For the most part, those attached by a modified cephalothorax are highly transformed and mesoparasitic, while those attaching using enlarged maxillae are ectoparasitic (Kabata 1979, Ho 1998, Boxshall and Halsey 2004).

Although the family currently contains 19 genera, two genera have received the most attention and make up more than two thirds of the species in the group (Ho 1998). *Lernaea* Linnaeus, 1758 is the type genus for the family and the most speciose with roughly 50 species (WoRMS Editorial Board 2022). The second largest genus is *Lamproglena* von Nordmann, 1832, with 38 nominal species (Kunutu et al. 2018). The latter genus is historically absent from the Americas and the South Pacific (New Guinea, Australia and New Zealand) (Ho 1998), but an African species (*Lamproglena monodi* Capart, 1944) has been reported from South America (Thatcher 2006, Azevedo et al. 2010, 2012, Tavares-Dias et al. 2015, Garcia et al. 2019), presumably due to the introduction of infected *Oreochromis niloticus* (Linnaeus).

As with many other organisms, the taxonomic study of lernaeids relies heavily on morphology, with sequence data only available for species of the two largest genera. The first genetic data for the family were generated by Song et al. (2008) and included rDNA (18S and 28S) for one *Lernaea* and two *Lamproglena* species. However, the focus of their study was the Ergasilidae von Nordmann, 1832, including data on lernaeids only as outgroup. Even though *Lernaea* is more speciose than *Lamproglena*, sequence data are currently available for four species of each genus. However, more data are available for *Lernaea*, with 244 sequences, including five mitogenomes. In contrast, only 47 sequences are available for *Lamproglena*, all of which are for nuclear ribosomal DNA and no mitochondrial data. Since Song et al. (2008), genetic data for lernaeids have

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been generated mainly for species identification (Stavrescu-Bedivan et al. 2014, Yoshimine et al. 2015, Welicky et al. 2017, Soares et al. 2018, Chakona et al. 2019, Waicheim et al. 2019, Santacruz et al. 2022), but also for expanded taxonomic information (Rindoria et al. 2022, Mabika et al. 2023), whole genome sequencing (Su et al. 2016), and to study the apparent morphological plasticity of species of *Lernaea* (Hua et al. 2019, Zhu et al. 2021). Many available sequences are unpublished (127 sequences, see Table S1), making the study of this group challenging.

Additionally, some unpublished data are for *Lernaea* species which have been considered taxonomically ambiguous. Hua et al. (2019) could not distinguish *Lernaea cyprinacea* Linnaeus, 1758 and *Lernaea cruciata* Lesueur, 1824 genetically using rDNA or mtDNA, concluding that these species are conspecific. Genetic data for *Lernaea ctenopharyngodontis* Yin, 1960 and *Lernaea polymorpha* Yü, 1938 are also available but are currently unpublished.

Contrary to the genetic data for *Lernaea*, published rDNA data for *Lamproglena* appears to support the distinctness of the four species for which genetic data are available. This may be due to the limited number of studies, with most species only studied once, or that all data are for 18S and 28S rDNA. However, some of the data are not clear, with some suggesting the existence of possible subspecies and the need to revise the data for some species (Mabika et al. 2023).

Additionally, just over a tenth of *Lamproglena* species have been genetically characterised (4 of 38) and the lack of mtDNA data for this genus limits comparison with other groups. Internal transcribed spacer (ITS) rDNA is available for one *Lamproglena* species. The use of this marker to study lernaeids has seemingly not gained traction as this is the only ITS rDNA data for the family, and it is unpublished. As such, the aim of the present study was to add rDNA (18S and 28S) data, alongside the first mitochondrial data for the genus, of selected African *Lamproglena*.

There are currently 14 species of *Lamproglena* described from Africa (Kunutu et al. 2018, Scholz et al. 2018). Two of these, *Lamproglena hemprichii* von Nordmann, 1832 and *L. monodi*, have only very recently been genetically characterised using 18S and 28S rDNA (Rindoria et al. 2022, Mabika et al. 2023). During these studies, attempts were made to obtain mitochondrial data from both species using universal primers, but to no avail. Here, cyclopoid specific primers (*cox1* mtDNA) were designed to overcome this. Additionally, two additional species of *Lamproglena* are genetically typed for the first time, *Lamproglena clariae* Fryer, 1956 and *Lamproglena hoi* Dippenaar, Luus-Powell et Roux, 2001, using both rDNA (18S and 28S) and mtDNA (*cox1*).

MATERIAL AND METHODS

Sample collection

Parasitic copepods were collected from the gills of four fish species from distinct families in five localities (Table 1): *Lamproglena clariae* from African sharptooth catfish *Clarias gariepinus* (Burchell) in the Vaal Dam (26.8711S, 28.1629E) in the Vaal River system and Lake Heritage (25.9579S, 27.8569E) in the Crocodile River of the Limpopo River Basin, South Africa; *Lamproglena hemprichii* from tigerfish *Hydrocynus vittatus* Castelnau in Lake Kariba, Zimbabwe (see Mabika et al. 2023); *Lamproglena hoi* from bushveld smallscale yellowfish *Labeobarbus polylepis* (Boulenger) in the Komati River (25.8342S, 30.4645E), South Africa; and *Lamproglena monodi* from Nile tilapia *Oreochromis niloticus* from Kibos Fish Farm, Kenya (see Rindoria et al. 2022).

Fish were collected using electrofishing, gillnets, beach seine nets, rod and reel, or obtained from fishermen. Fish were ethically euthanised (South African National Animal Ethics Guidelines), the gills removed, and inspected for the presence of lernaeid copepods. Copepods were removed and stored in 96% or absolute ethanol for molecular analysis or 70% for morphological identification. All specimens used in the present study were confirmed as *Lamproglena* using the key of Boxshall and Halsey (2004) and species identities were determined using the key by Kunutu et al. (2018). Morphological identification was done using light microscopy of lactic acid cleared specimens, or scanning electron microscopy of hexamethyldisilazane dried material (see Rindoria et al. 2022 and Mabika et al. 2023).

PCR and sequencing

Genomic DNA was extracted from either whole copepods, body sections, or isolated egg strands (see Table 1) using a DNeasy® Blood and Tissue kit (Qiagen, Inc., UK) or NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany). Two fragments of the ribosomal genome, 18S and 28S rDNA, were amplified using the protocol and primers of Song et al. (2008). A fragment of the *cox1* mtDNA gene region was also amplified using newly designed primers Cycl_F2 (5' – TGA TCT TGT AAY CAY AAA GAT ATY GG – 3') and Cycl_R3 (5' – CAG CTA AYC CTA AAA AAT GYA TDG G – 3'). Additionally, reverse primer HCO2198 (5' – TAA ACT TCA GGG TGA CCA AAA AAT CA – 3') (Folmer et al. 1994) was used in some instances.

For *cox1* mtDNA PCR, the following conditions were used: 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 45 seconds, 45 °C for 45 seconds and 72 °C for 2 minutes with a final extension at 72 °C for 10 minutes. Successful amplification was verified on a 1% agarose gel impregnated with GelRed™ (Biotium Inc., Fremont City, California). Amplicons were sequenced following Avenant-Oldewage et al. (2014) in both directions, with HCO2198 as an internal primer for *cox1* mtDNA when necessary.

Data analyses

Obtained electropherograms were aligned, inspected, edited when necessary, and merged using Geneious Prime 2020.2.2 (https://www.geneious.com). Haplotypes were analysed using BLAST (Johnson et al. 2008) and confirmed to represent Lernaeidae. All data for Lernaeidae were downloaded from GenBank, alongside other sequences in the BLAST ingroup, and aligned to the respectively obtained haplotypes. Outgroups were not included.

Both 18S rDNA and *cox1* mtDNA data were aligned manually using MEGA 7 (Kumar et al. 2016), while 28S rDNA data were initially aligned with MAFFT (Katoh et al. 2002; Katoh and Standley 2013) via the EMBL-EBI portal, and then manually optimised in MEGA7. Published sequence data with less than 70% cover of rDNA alignments were excluded from analyses. This was not done for mtDNA data, as some of the data generated in the present study covered less than 70% of the analysed alignment.

Table 1. Detail for *Lamproglena* haplotypes included in the present study including hosts species and family, collection site, accession numbers and citation.

Species	Host	Host family	Locality	Isolate	Isolate source	18S	28S	*cox1*	Reference
L. clariae	*Clarias gariepinus*	Clariidae	Vaal Dam, South Africa	LC01	Whole	**OR048797**	**OR048803**	**OR058772**	**Present study**
				LC02	Whole	**OR048798**	**OR048804**	**OR058773**	**Present study**
				GL2	Whole	**OR048799**	**OR048805**	**OR058774**	**Present study**
				GL3	Cephalo-thorax	**OR048800**	**OR048806**	**OR058775**	**Present study**
			Lake Heritage, South Africa	FJ45	Eggs	**OR048801**	**OR048807**	**OR058776**	**Present study**
L. hemprichii	*Hydrocynus vittatus*	Alestidae	Lake Kariba, Zimbabwe	HEMP4	Whole	OP277526	OP277527	**OR058777**	Mabika et al. (2023); **Present study**
L. hoi	*Labeobarbus polylepis*	Cyprinidae	Komati River, South Africa	LH5	Whole	**OR048802**	**OR048808**	**OR058778**	**Present study**
L. monodi	*Oreochromis niloticus*	Cichlidae	Kibos Fish Farm, Kenya	4a	Eggs	ON419439	ON419422	**OR058779**	Rindoria et al. (2022); **Present study**
				4b	Eggs	ON419440	ON419423	**OR058780**	Rindoria et al. (2022); **Present study**
				4c	Eggs	ON419441	ON419424	**OR058781**	Rindoria et al. (2022); **Present study**
				4d	Eggs	ON419438	ON419425	**OR058782**	Rindoria et al. (2022); **Present study**
				4e	Eggs	ON419442	ON419426	**OR058783**	Rindoria et al. (2022); **Present study**
				4f	Eggs	ON419443	ON419427	**OR058784**	Rindoria et al. (2022); **Present study**
				4g	Eggs	ON419444	ON419428	**OR058785**	Rindoria et al. (2022); **Present study**
				4i	Eggs	ON419445	ON419429	**OR058786**	Rindoria et al. (2022); **Present study**
				4j	Eggs	ON419446	ON419430	**OR058787**	Rindoria et al. (2022); **Present study**
				LM1	Abdomen	ON419447	ON419431	**OR058788**	Rindoria et al. (2022); **Present study**
			El-Minia, Egypt	LME1	Eggs	ON419448	ON419432	-	Rindoria et al. (2022)
				LME2	Eggs	ON419449	ON419433	-	Rindoria et al. (2022)
				LME3	Eggs	-	ON419434	-	Rindoria et al. (2022)
			Sharqia, Egypt	LAMP 5	Eggs	ON419450	ON419435	-	Rindoria et al. (2022)
				LAMP 6	Eggs	ON419451	ON419436	-	Rindoria et al. (2022)
				LAMP 7	Eggs	ON419452	ON419437	-	Rindoria et al. (2022)
L. orientalis	*Squaliobarbus curriculus*	Cyprinidae	Dangjiangkou Reservoir, China	LOC	-	DQ107552	DQ107544	-	Song et al. (2008)
	Chanodichthys dabryi	Cyprinidae	Tangxun Lake, China	LOQ	-	DQ107549	DQ107542	-	Song et al. (2008)
	Chanodichthys erythropterus	Cyprinidae	Tangxun Lake, China	LOH	-	DQ107551	DQ107541	-	Song et al. (2008)
	Chanodichthys mongolicus	Cyprinidae	E-zhou farm, China	LOM	-	DQ107550	DQ107543	-	Song et al. (2008)
	-	-	-	DF18S	-	OP076960	-	-	Unpublished
L. chinensis	*Channa argus*	Cyprinidae	-	L1	-	MZ575117	-	-	Unpublished
			Dangjiangkou Reservoir	LCW	-	DQ107553	DQ107545	-	Song et al. (2008)
	-	-	-	XS-18S	-	OP076957	-	-	Unpublished

Distances between haplotypes were calculated using uncorrected *p*-distances and the number of base pairs (bp) in MEGA7. Full distance data are provided in the supporting information section (Tables S2–S4), with distances between species presented alongside the results for each marker.

Evolutionary histories were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) approaches. The best nucleotide substitution model was selected for both ML and BI analyses using MEGA7, with discrete Gamma distribution (five categories) and invariant sites included where needed. Bootstrap support was obtained for distance and ML analyses with 1,000 replicates (Felsenstein 1985), and 10 million Markov chain Monte Carlo (MCMC) generations were used for BI analyses. Unrooted topologies for respective gene fragments using both approaches were similar for 18S rDNA analyses. Therefore, a single topology is shown based on BI analysis. For 28S rDNA and *cox1* mtDNA, both topologies are shown. Nodes with less than 50% bootstrap support or 0.5 posterior probability were not annotated. All obtained sequence data were deposited to GenBank (18S - OR048797-802; 28S - OR048803-08; *cox1* - OR058772-88).

RESULTS

18S rDNA

New 18S rDNA data were obtained for *Lamproglena clariae* and *Lamproglena hoi*. The 18S rDNA haplotypes for *Lamproglena monodi* and *Lamproglena hemprichii* specimens studied here have already been presented in Rindoria et al. (2022) and Mabika et al. (2023), respectively. For *L. clariae*, usable sequence data were obtained for five individuals (1392–1399 bp), representing two haplotypes differing only by a single base pair. Usable sequence data (1382 bp) could only be obtained for one individual of *L. hoi*. The alignment of all lernaeid 18S rDNA data (n=120) was 1449 bp, with 1365 conserved, 84 variable, and 61 parsimony informative sites. Based on published data (Table 2), intra- and interspecific distances of up to 0.41% (6 bp) and 0.36–2.35% (5–34 bp), respectively, were calculated for *Lamproglena*. Haplotypes for *L. clariae* were 1.22–2.15% (17–30 bp) from other data for the genus, while *L. hoi* was 0.43–2.24% (6–31 bp) to other *Lamproglena*, indicating the distinctness of these haplotypes from

Table 2. Genetic distances between 18S rDNA data for selected Lernaeidae taxa. Uncorrected *p*-distances below the diagonal, number of base pair differences above the diagonal, and intraspecific variation shaded in the diagonal. Newly generated data in bold.

		Lamproglena						*Lernaea*				
		1	**2**	3	4	5	6	7	8	9	10	11
Lamproglena clariae	**1**	**0.07% (1 bp)**	**18–19 bp**	**17–18 bp**	**17–18 bp**	**24–28 bp**	**27–30 bp**	**23–40 bp**	**35–38 bp**	**34–35 bp**	**35–37 bp**	**24–25 bp**
Lamproglena hoi	**2**	**1.3–1.38%**	**-**	**6 bp**	**7 bp**	**18–22 bp**	**27–31 bp**	**19–38 bp**	**34–36 bp**	**33 bp**	**34–35 bp**	**18 bp**
Lamproglena hemprichii	3	**1.22–1.29%**	**0.43%**	-	5 bp	15–20 bp	24–28 bp	17–38 bp	31–33 bp	30 bp	31–32 bp	15 bp
Lamproglena monodi	4	**1.22–1.29%**	**0.51%**	0.36%	0% (0 bp)	17–20 bp	24–28 bp	17–37 bp	31–33 bp	30 bp	31–32 bp	17 bp
Lamproglena orientalis	5	**1.76–2%**	**1.3–1.59%**	1.1–1.4%	1.22–1.43%	0.41% (6 bp)	27–34 bp	18–43 bp	33–38 bp	33–35 bp	33–36 bp	0–4 bp
Lamproglena chinensis	6	**1.97–2.15%**	**1.97–2.24%**	1.75–1.96%	1.75–2.01%	1.97–2.35%	0.15% (2 bp)	15–38 bp	31–35 bp	30–31 bp	31–33 bp	27–29 bp
Lernaea cyprinacea	7	**2.26–3.3%**	**1.87–3.22%**	1.67–3.26%	1.67–3.12%	1.77–3.69%	1.47–3.26%	0.34% (4 bp)	0–3 bp	0–1 bp	0–2 bp	18–35 bp
Lernaea cruciata	8	**2.53–2.74%**	**2.47–2.62%**	2.24–2.38%	2.24–2.39%	2.42–2.74%	2.26–2.52%	0–0.26%	0.22% (3 bp)	0–2 bp	0–3 bp	33–35 bp
Lernaea ctenopharyngodontis	9	**2.5–2.6%**	**2.43–2.45%**	2.2–2.22%	2.2–2.22%	2.42–2.59%	2.21–2.3%	0–0.09%	0–0.15%	0% (0 bp)	0–1 bp	33 bp
Lernaea polymorpha	10	**2.57–2.74%**	**2.5–2.59%**	2.27–2.37%	2.27–2.37%	2.42–2.67%	2.28–2.44%	0–0.18%	0–0.22%	0–0.07%	0.07% (1 bp)	33–34 bp
Cyclopoida sp.	11	**1.76–1.84%**	**1.32%**	1.1%	1.25%	0–0.29%	1.98–2.13%	1.77–2.99%	2.42–2.57%	2.45%	2.44–2.52%	-

available data. During analyses, an unpublished sequence designated as a "Cyclopoida sp." (MZ575162) was determined to form part of the lernaeid ingroup. No published record is available for this data, but the sequence is identical to sequences OP076960, DQ107549 and DQ107550 for *Lamproglena orientalis* Markevich, 1936 and thus likely represents cyclopoid larvae of this species. Intra- and interspecific distances calculated for *Lernaea* data were inconclusive as similar distances were observed between conspecific (up to 0.34% (4 bp)) and congeneric data at 0–0.26% (0–3 bp). *Lernaea* and *Lamproglena* 18S rDNA data were separated by 1.47–3.69% (15–43 bp).

28S rDNA

New 28S rDNA data were obtained for *L. clariae* and *L. hoi*. The 28S rDNA haplotypes for *L. monodi* and *L. hemprichii* specimens included here have already been presented in Rindoria et al. (2022) and Mabika et al. (2023), respectively. For *L. clariae*, all five individuals displayed a single haplotype (719–735 bp), while the haplotype for the *L. hoi* specimen was 701 bp. The alignment of all lernaeid 28S rDNA data (n=103) was 865 bp, with 512 conserved, 308 variable, and 252 parsimony informative sites. Based on published data (Table 3), intra- and interspecific distances of up to 3.18% (25 bp) and 7.31–18.92% (51–132 bp), respectively, were calculated for *Lamproglena*. Haplotypes for *L. clariae* were 14.93–21.95% (106–155 bp) from other data for the genus, while *L. hoi* was 10.68–18.17% (74–125 bp) to other *Lamproglena*, indicating the distinctness of these haplotypes from available data. Data from *Lamproglena* and *Lernaea* are separated by 19.95–24.15% (128–162 bp). Intra- and interspecific distances calculated for *Lernaea* data were inconclusive as similar distances were observed between conspecific [0–1% (0–7 bp)] and congeneric [0–0.72% (0–5 bp)] data, with the interspecific range falling entirely below the intraspecific limit.

cox1 mtDNA

All four included species could be genetically characterised using *cox1* mtDNA data, but with varying success. Reverse primer Cycl_R3 did not produce ideal results, with reads becoming unstable towards the 3' end of the fragment for some specimens and not producing usable data for others. Forward primer Cycl_F2 and internal reverse primer HCO2198 were more effective, but a much smaller span of the region was obtained with this combination (~700 bp vs ~1200 bp). As such, *cox1* mtDNA haplotypes of varying sizes were obtained. For *L. clariae*, usable sequence data were obtained for all five individuals (672–1071 bp), while for *L. hoi* one (660 bp), for *L. hemprichii* one (1197 bp), and for *L. monodi* ten (1074–1197 bp) specimens could be successfully typed. The alignment of all lernaeid *cox1* mtDNA data (n=77) was 1236 bp, with 738 conserved, 496 variable, and 401 parsimony informative sites. As no comparable *cox1* mtDNA is available for *Lamproglena*, no inter- or intraspecific distances could be calculated from published data. However, using the data obtained here, an intraspecific limit of up to 0.89% (6 bp)

Table 3. Genetic distances between 28S rDNA data for selected Lernaeidae taxa. Uncorrected *p*-distances below the diagonal, number of base pair differences above the diagonal, and intraspecific variation shaded in the diagonal. Newly generated data in bold.

		Lamproglena						*Lernaea*			
		1	**2**	3	4	5	6	7	8	9	10
Lamproglena clariae	**1**	**0% (0 bp)**	**94 bp**	**118 bp**	**106–107 bp**	**152–155 bp**	**139 bp**	**146–164 bp**	**159 bp**	**146 bp**	**145–146 bp**
Lamproglena hoi	**2**	**13.47–13.78%**	**-**	**78 bp**	**74–75 bp**	**119–122 bp**	**125 bp**	**128–144 bp**	**139 bp**	**129 bp**	**128–129 bp**
Lamproglena hemprichii	3	**17–17.05%**	**11.49%**	-	51–52 bp	126–128 bp	130 bp	135–147 bp	145 bp	136 bp	136–137 bp
Lamproglena monodi	4	**14.93–15.4%**	**10.68–10.81%**	7.31–7.44%	-	118–122 bp	129 bp	128–144 bp	138–139 bp	129–130 bp	128–130 bp
Lamproglena orientalis	5	**21.1–21.95%**	**17.2–17.48%**	17.97–18.27%	16.57–17.04%	3.18% (25 bp)	130–132 bp	143–162 bp	153–157 bp	144–148 bp	143–148 bp
Lamproglena chinensis	6	**19.63–20.09%**	**18.17%**	18.92%	18.32–18.35%	18.28–18.57%	-	144–157 bp	152 bp	144 bp	142–143 bp
Lernaea cyprinacea	7	**22.77–23.99%**	**20.32–21.59%**	21.58–22.59%	19.94–21.6%	21.84–24.15%	21.65–23.74%	1% (7 bp)	0–5 bp	0–2 bp	0–4 bp
Lernaea cruciata	8	**22.91–23.45%**	**20.47%**	21.58%	20.09–20.2%	21.98–22.46%	21.93%	0–0.72%	-	0–0 bp	1–2 bp
Lernaea ctenopharyngodontis	9	**23.66%**	**21.43%**	22.26%	21.15–21.28%	23.3–23.83%	23.41%	0–0.32%	0–0%	-	1–2 bp
Lernaea polymorpha	10	**23.5–23.66%**	**21.26–21.43%**	22.26–22.42%	20.98–21.28%	23.14–23.83%	23.09–23.25%	0–0.64%	0.16–0.32%	0.16–0.32%	0.16% (1 bp)

Table 4. Genetic distances between *cox1* mtDNA data for selected Lernaeidae taxa. Uncorrected *p*-distances below the diagonal, number of base pair differences above the diagonal, and intraspecific variation shaded in the diagonal. Newly generated data in bold.

		Lamproglena				*Lernaea*				
		1	**2**	**3**	**4**	5	6	7	8	9
Lamproglena clariae	**1**	**0.89% (6 bp)**	**172–174 bp**	**146–243 bp**	**139–234 bp**	**120–271 bp**	**130–226 bp**	**122–271 bp**	**121–268 bp**	**172–174 bp**
Lamproglena hoi	**2**	**26.18–26.48%**	**-**	**182 bp**	**158–168 bp**	**149–205 bp**	**159–162 bp**	**151–204 bp**	**153–205 bp**	**193 bp**
Lamproglena hemprichii	**3**	**21.73–22.69%**	**27.7%**	**-**	**237–267 bp**	**256–308 bp**	**265 bp**	**257–309 bp**	**180–302 bp**	**180 bp**
Lamproglena monodi	**4**	**20.85–21.96%**	**25.12–25.57%**	**21.79–22.34%**	**0.19% (2 bp)**	**221–304 bp**	**227–262 bp**	**224–304 bp**	**222–303 bp**	**166–169 bp**
Lernaea cyprinacea	5	**23.46–26.04%**	**29.55–31.2%**	**24.37–25.77%**	**23.59–25.4%**	1.96% (21 bp)	1–20 bp	0–20 bp	0–18 bp	10–18 bp
Lernaea cruciata	6	23.55–23.76%	29.55–30.11%	**24.65%**	**23.63–24.33%**	0.09–1.87%	1.62% (18 bp)	10–19 bp	11–17 bp	13–18 bp
Lernaea ctenopharyngodontis	7	**23.84–26.04%**	**30.38–31.05%**	**24.9–25.86%**	**24.45–25.4%**	0–1.87%	0.9–1.77%	0.93% (10 bp)	10–16 bp	11–15 bp
Lernaea polymorpha	8	**23.73–25.74%**	**30.78–31.2%**	**24.52–25.27%**	**24.24–25.31%**	0–1.68%	1.03–1.59%	0.93–1.3%	0.28% (3 bp)	11–15 bp
OM541913 "Lernaeidae gen. n. sp. n."	9	**26.17–26.46%**	**30.39%**	**27.23%**	**25.34–25.57%**	1.88–3.27%	2.31–3.2%	2.12–2.31%	2.12–2.27%	-

and an interspecific range of 20.85–27.7% (139–267 bp) was calculated for the genus (Table 4). Intraspecific variation was observed for both *L. clariae* and *L. monodi*. Most *L. monodi* data shared the same haplotype, with a single specimen differing by 0.19% (2 bp). Three distinct haplotypes were observed in the *cox1* mtDNA data for *L. clariae* with a variation of 0.27–0.89% (2–6 bp) between haplotypes. Similar to the nuclear markers, all *Lernaea* data were closely related, with intra- and interspecific ranges essentially identical, but with the intraspecific limit slightly higher at 1.96% (21 bp) and the interspecific range at 0–1.87% (0–20 bp). The data suggest a clear distinction of *Lamproglena* species based on *cox1* mtDNA, while included *Lernaea* data appear conspecific. Data for an unidentified taxon designated as "Lernaeidae gen. n. sp. n." (OM541913) were also included in the analyses. This data were only 1.88–3.27% (10–18 bp) from *Lernaea* data and thus most likely represents a species of this genus. A clear separation was seen between *Lamproglena* and *Lernaea* based on *cox1* mtDNA, with 23.46–31.2% (120–309 bp) separating the genera. The overlap of the lower end of this intrageneric range and the upper end of the interspecific range of *Lamproglena* needs attention.

Phylogeny

In all produced topologies, *Lernaea* data grouped in well-supported clades, but with no species specific grouping. Thus, clades containing *Lernaea* data were collapsed for simplicity (Figs. 1–3). The evolutionary histories reconstructed using both rDNA fragments showed similar topologies (Figs. 1, 2). In rDNA topologies, *Lamproglena* data formed well-supported (>75%) monophyletic clades at both species and genus levels. However, two significant differences were seen. In the 18S rDNA topology based on BI, data for *Lamproglena chinensis* Yü, 1937 groups basally to all other *Lamproglena* data, with *L. orientalis* data sister to all data for African species. In the 28S rDNA topologies, the species from Asia and Africa form two well-supported sister clades. Nodes based on ML analyses of 18S rDNA were not well-supported. Additionally, in the 18S rDNA topology, the nodes splitting the African species into two clades are not well-supported using either approach. In contrast, in the 28S rDNA ML topology the node grouping *L. monodi* and *L. hemprichii* together is well-supported and the node grouping *L. hoi* and *L. clariae* together is marginally well-supported (>50%; <75%). However, in the 28S rDNA BI topology, *L. clariae* is basal to other African *Lamproglena*, followed by *L. hoi* sister to the well-supported clade of *L. hemprichii* and *L. monodi*. The node placing *L. hoi* sister to the clade of *L. monodi* and *L. hemprichii* is only marginally well-supported. Thus, in most rDNA topologies, there appeared to be a closer relationship between *L. clariae* and *L. hoi*, and *L. hemprichii* and *L. monodi*, mimicking the 28S rDNA distances between these species. Unpublished 18S rDNA sequence MZ575162 ("Cyclopoida sp.") grouped firmly within the ingroup for *L. orientalis* based on 18S rDNA.

The topology based on *cox1* mtDNA also showed all *Lamproglena* data in a monophyletic clade, with all species forming distinct clades (Fig. 3). Contrary to the rDNA results, *L. hoi* was basal to all other *Lamproglena*, followed by *L. clariae* sister to a clade containing *L. monodi* and *L. hemprichii* based on BI analysis. The nodal support was low for interspecific nodes, with the node placing *L. hoi* in the basal position only marginally well-supported and the node grouping *L. monodi* and *L. hemprichii* not well-supported. These nodes were absent in the ML topology, with even lower nodal support placing *L. clariae* basal, followed by *L. hoi* sister to a clade of *L. monodi* and *L. hemprichii*, similar to the 28S rDNA BI topology. The intraspecific topology of the ML analysis for *cox1* mtDNA more closely resembles that of the rDNA analyses, but the nodal support is so low that it is not informative. Intraspecific branching separating the haplotypes of *L. monodi* was only well-supported in *cox1* mtDNA ML analysis. In contrast, the separation of haplotypes of *L. clariae* was well-supported in BI analysis and only limited separation with marginal support in the ML topology. Sequence OM541913 ("Lernaeidae gen. n. sp. n.") grouped within the ingroup of *Lernaea* data, suggesting that it may be a congener of other *Lernaea*.

DISCUSSION

Fragments of the 18S and 28S rDNA for both *Lamproglena clariae* and *Lamproglena hoi* were characterised successfully, presenting the first genetic data for both species. Like other reports for *Lamproglena* based on both rDNA markers (Song et al. 2008, Rindoria et al. 2022, Mabika et al. 2023), these species could be easily distinguished from other data, supporting their taxonomic distinctness. This was based on the distances between the haplotypes for these and other *Lamproglena* data falling within the interspecific range calculated for both markers, as well as

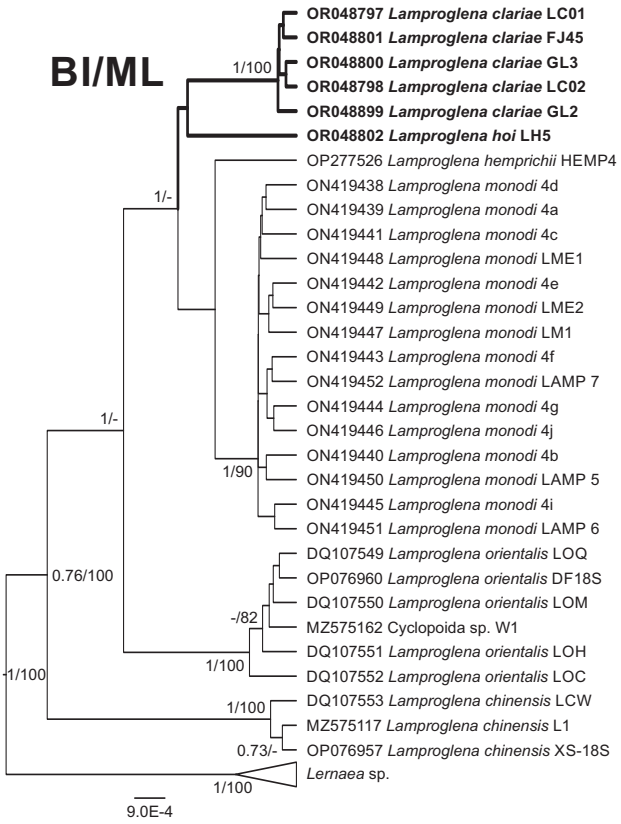

Fig. 1. Unrooted Bayesian inference (BI) topology based on selected 18S rDNA available Lernaeidae data, with data from the present study in bold. Posterior probability (BI) and 1000 bootstrap replicate (maximum likelihood (ML)) support indicated only at nodes with more than 50% support (BI/ML).

their separation from other species in phylogenetic topologies. Additionally, all *L. clariae* data form well-supported monophyletic clades in all rDNA topologies, which, alongside the distances between haplotypes falling within the intraspecific limits, support the conspecificity of all specimens included.

Fragments of *cox1* mtDNA gene were successfully obtained for *L. clariae*, *L. hoi*, *Lamproglena monodi* and *Lamproglena hemprichii*. As mentioned, the material used to generate the *cox1* mtDNA for the latter two species are the same as those used to obtain 18S and 28S rDNA in Rindoria et al. (2022) and Mabika et al. (2023), respectively. This allows for a direct correlation between all sequence data for the specimens of *Lamproglena* generated here. Based on *cox1* mtDNA, all four included species could be easily distinguished. As no other data are currently available for this genus, no intra- or interspecific ranges could be used to support the distinctness of the included species. Still, the distances between the included species did not suggest any irregularities as they differed by more than the 1% intraspecific limit generally accepted for *cox1* mtDNA. This was further supported by the close relation of conspecific haplotypes of *L. clariae* and *L. monodi*, which are both below 1%.

As discussed in Mabika et al. (2023), the overlap in the intra- and intraspecific ranges for *Lamproglena* based on 18S rDNA is due to the large distances between *L. orientalis* haplotypes and the high similarity between *L. monodi* and *L. hemprichii* data. The authors attribute the high variation in *L. orientalis* data to the possible presence of misidentified specimens or cryptic species, as the specimens of this species studied by Song et al. (2008) were all collected from different hosts and different localities. Furthermore, the morphology, hosts and localities of the specimens studied by Rindoria et al. (2022) and Mabika et al. (2023) of *L. monodi* and *L. hemprichii*, respectively, were distinct enough to negate their genetic conspecificity. This was supported by 28S rDNA data, for which intra- and interspecific distances do not overlap, prompting Mabika et al. (2023) to suspect that available *L. orientalis* data represent very closely related species, or even sub-species, but not a single taxon. Considering the data from the present study, a similar situation can be seen. While the 18S rDNA data for *L. clariae* are sufficiently distant from other *Lamproglena* by 1.22–2.15% (17–30 bp), the distance between *L. hoi* and both *L. hemprichii* (0.43%; 6pb) and *L monodi* (0.51%; 7 bp) are relatively close to the border between intra- and interspecific distances. However, the 28S rDNA separates *Lamproglena* spp., including *L. hoi*, easily. This may suggest that 28S rDNA is a superior taxonomic marker for Lernaeidae.

Furthermore, considering the data generated here, there appears to be great conservedness in the markers used to study African *Lamproglena*. As per Rindoria et al. (2022), all rDNA data for *L. monodi* were identical, even when comparing data collected in Kenya and different localities in Egypt. A similar observation was made here as the rDNA

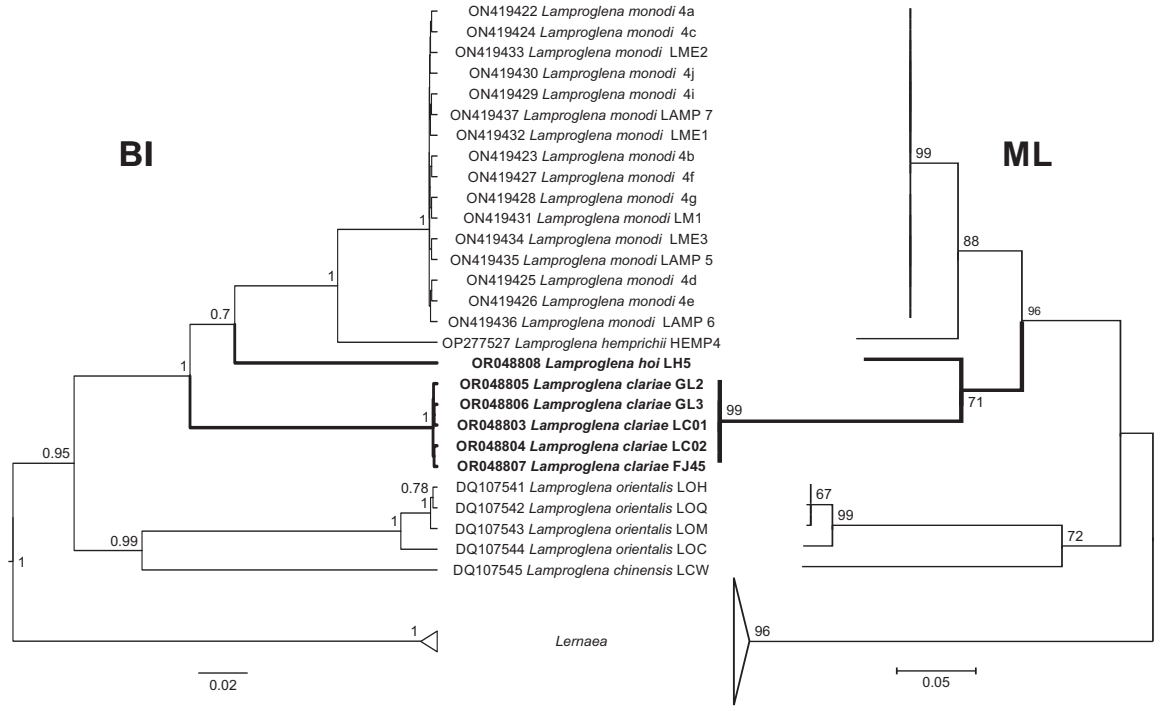

Fig. 2. Unrooted Bayesian inference (BI) and maximum likelihood (ML) topologies based on selected 28S rDNA available Lernaeidae data, with data from the present study in bold. Posterior probability (BI) and 1000 bootstrap replicate (ML) support indicated only at nodes with more than 50% support.

data for *L. clariae* were highly similar between specimens, irrespective of their origin. While all *L. clariae* material was collected in South Africa, they were collected in two separate catchments. The Vaal Dam is in the Vaal River system and forms part of the Orange River Basin, which is west-flowing and mouths out into the Atlantic Ocean. Lake Heritage is located along the Crocodile River and forms part of the Limpopo River Basin, which is east-flowing and mouths out into the Indian Ocean. However, the similarity in haplotypes may be due to the sampling localities being relatively near one another (~150 km), the catchments bordering one another in some areas, and historic transfer of water between the Vaal and Olifants rivers (Avenant-Oldewage 2001), as the latter also forms part of the greater Limpopo River Basin. Additionally, the partially air-breathing host of *L. clariae*, *Clarias gariepinus*, can move across land between water bodies during rainy seasons (Skelton 2001), potentially facilitating the distribution of their parasites and increasing gene flow between parasite populations.

It is interesting to note that, while there was no variation in the rDNA data for *L. monodi* from Kenya and Egypt, a slight variation of the 18S rDNA data for *L. clariae* were observed. This variation was not related to the locality of *L. clariae* as both haplotypes occurred in the Vaal Dam and the haplotype for the individual from Lake Heritage was identical to those of some specimens from the Vaal Dam. Interestingly, this variation was not present in the 28S rDNA data. Even *cox1* mtDNA did not show geographical variation in *L. clariae* as the haplotype from the specimen in Lake Heritage was identical to one of the three haplotypes generated for material from the Vaal Dam. There was also slight variation in *cox1* mtDNA of *L. monodi* from Kenya with two observed haplotypes. The intraspecific stability of specific markers needs to be investigated further, but it appears that all three gene regions used here have the potential to differentiate *Lamproglena* spp., with 28S rDNA superior. This supports the notion that the *L. orientalis* data generated by Song et al. (2008) may represent cryptic species as both rDNA topologies had high support for the separation of specimens collected from a *Squaliobarbus* Günther and those from *Chanodichthys* hosts, with well-supported separation of 28S rDNA haplotypes from different *Chanodichthys* species.

The proficiency of 28S rDNA data are not surprising as Song et al. (2008) remarked that the transitions and transversions of 28S rDNA data in their study became saturated, while 18S rDNA data did not. Additionally, these authors indicated that the evolutionary history constructed using 28S rDNA may be more informative than that based on 18S rDNA, although they still suggest retaining the use of both markers. In the topologies generated here, it appears that most interspecific branching based on 18S rDNA and *cox1* mtDNA was not well supported, with only those in 28S rDNA topologies well-supported. However, variation depending on the phylogenetic approaches used may indicate that the presently selected markers or approaches are not ideal for the phylogenetic study of this group, or that the topologies are inaccurate due to the extremely limited number of species included. The *cox1* mtDNA topologies had virtually no well-supported interspecific nodes, possibly indicating that the phylogenetic study of lernaeids using this marker may not be informative for relationships above the intraspecific level, as has also been noted for other parasitic groups like monogeneans (Joveline and Justine

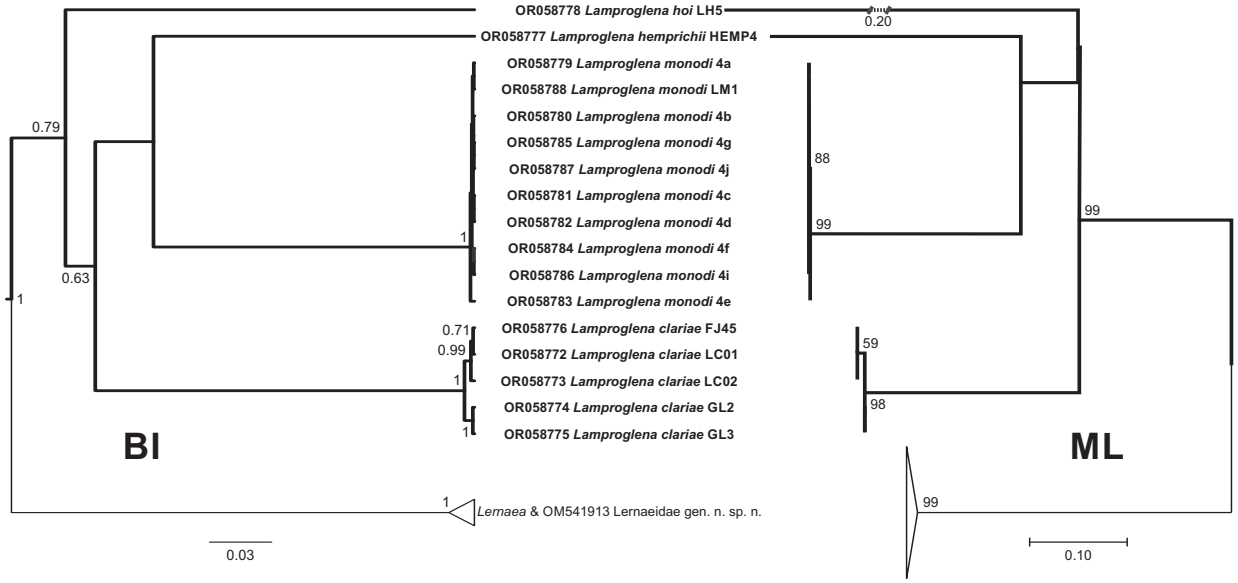

Fig. 3. Unrooted Bayesian inference (BI) and maximum likelihood (ML) topologies based on selected *cox1* mtDNA available Lernaeidae data, with data from the present study in bold. Posterior probability (BI) and 1000 bootstrap replicate (ML) support indicated only at nodes with more than 50% support.

2001). Song et al. (2008) also noted no benefit in combining 18S and 28S rDNA data based on the result of an Incongruence Length Difference test. This, and the lack of multiple gene region data for some isolates, precluded the combination of markers in the present study.

The success of using three gene regions to differentiate *Lamproglena*, even indicating possible cryptic species, may contribute to the study of other lernaeids. Hua et al. (2019), confirmed that *L. cruciata* is a synonym of the cosmopolitan *Lernaea cyprinacea*. These authors, and later Zhu et al. (2021), supported the long-standing suspicion that the taxonomic reliance on the anchor of *Lernaea* spp. is not ideal due to their morphological variation linked to host species and the site of attachment. This is not the case for *Lamproglena*, as morphologic and genetic profiles of species support their distinctness.

This is further supported by the presence of *L. orientalis* and *L. chinensis* from different hosts (*Squaliobarbus curriculus* (Richardson) and *Channa argus* (Cantor); both Cyprinidae), in the same system (Dangjiangkou Reservoir, China) showing distinct haplotypes (Song et al. 2008). Similarly, both *L. monodi* and *L. hemprichii*, which appear genetically distinct, have been recorded from Lake Kariba, Zimbabwe (Douëllou and Erlwanger 1994), even though they have not been genetically characterized from the same locality. Douëllou and Erlwanger (1994) noted that it is possible that *L. clariae* also occurs in Lake Kariba, but this was not confirmed. Similarly, in Lake Victoria, the Malagarasi region, and Lake Albert and the Lower Nile, three, two, and eight distinct *Lamproglena* species co-occur in each respective system (Fryer 1968). This suggests that *Lamproglena* in the same system does not necessarily represent the same species and that host specificity, above that of host family level, may be narrow. Fryer (1968) already noted that species of *Lamproglena* exhibit greater host specificity compared to other lernaeids, but there is still much taxonomic uncertainty regarding *Lamproglena* spp. due to limited and incomplete morphological records and descriptions of some species (Dippenaar et al. 2001, Uyeno et al. 2021).

Data for two other *Lernaea* species, *L. ctenopharyngodontis* and *L. polymorpha*, are available, but as none of these data could be related to published records, their interpretation is limited. When applying the intra- and interspecific ranges calculated for the three gene regions of *Lamproglena* spp. (in the present study) to those for *Lernaea* spp., it is clear that the available *Lernaea* data need revision. There is virtually no separation in intra- and interspecific data and no identifiable molecular operational taxonomic units (MOTUs).

It may even be possible that the intraspecific limits for *Lamproglena*, some of which are above the upper interspecific limit of *Lernaea* data (rDNA), may be used to confirm the conspecificity of available *Lernaea* data. Inversely, if all *Lernaea* data are considered conspecific, the low variation of 28S rDNA data may support the separation of *L. orientalis* data into different species. Additionally, this conclusion would further support the remarkable geographic conserveness of rDNA markers, as *Lernaea* specimens from South America (Soares et al. 2018, Waicheim et al. 2019), Central America (Santacruz et al. 2022), South Africa (Welicky et al. 2017, Chakona et al. 2019), Europe (Stavrescu-Bedivan et al. 2014), Asia (Song et al. 2008, Yoshimine et al. 2015, Su et al. 2016, Hua et al. 2019), and Australia (Zhu et al. 2021) are highly similar. Only the *cox1* mtDNA for *Lernaea* is more diverse than the intraspecific limit of *Lamproglena* data, higher than the generally accepted 1% intraspecific limit for this marker. However, there is still a lack of meaningful phylogenetic separation of *cox1* mtDNA data for *Lernaea* into MOTUs, without correlation to specific morphotypes or species identities.

The evolutionary histories presented here lack a concise narrative. Although both topologies based on rDNA sug-

gest a closer relationship between *L. clariae* and *L. hoi*, and *L. hemprichii* and *L. monodi*, respectively, in some cases, these were generally not well-supported. The *cox1* mtDNA BI topology may be more accurate as it places *L. hoi* closer to *Lernaea* taxa, which were all collected from cyprinids. However, the phylogenetic signal between the host group and copepod phylogeny may be more complex as the 18S rDNA topology suggests that *Lamproglena* has oriental origins, likely from cyprinid hosts, diversifying to other host groups after dispersal to Africa. Four of the 12 African *Lamproglena* sp. have been recorded from cyprinid hosts, while the other eight species are spread across eight other fish families (Scholz et al. 2008). This proclivity to cyprinid hosts is also reported in other lernaeid genera (Ho 1998).

Additionally, except for *L hoi* and *Lamproglena cleopatra* Humes, 1957, African *Lamproglena* from cyprinids have been collected from a single host species, with the two exceptions collected from congeneric hosts. In contrast, some African *Lamproglena* occurring on other host families have been recorded from more than one host genus and even family. This may suggest that some *Lamproglena* lineages became less specific to allow colonisation of non-cyprinid hosts, like *L. monodi* which has been collected from a plethora of cichlid host.

It has been speculated that many Lernaeidae taxa originated in Gondwanaland, with radiation into Asia via the Indian subcontinent (Ho 1998), contradicting what is suspected here in terms of the oriental origin of *Lamproglena*. Ho (1998) further speculates that the colonisation of cyprinid hosts may have occurred after the separation of the African and South American continents due to the lack of cyprinids and Lernaeidae in South America. However, all phylogenetic interpretations are likely premature due to the extremely limited molecular data for the Lernaeidae. It is thus required to characterise more lernaeid taxa to study the phylogeny of the group.

Even though all the rDNA data generated here could easily be obtained with high-quality reads using the primers and protocol of Song et al. (2008), the generation of *cox1* mtDNA was more challenging. It appears that, similar to other parasitic invertebrates, *cox1* mtDNA of *Lamproglena* is highly variable, and the success of universal primers is sporadic. The primers used in the present study were designed based on *Lernaea* mitogenomes. But, this only resulted in limited success, and shorter spans of *cox1* mtDNA could be achieved by substituting the designed reverse primer for universal primer HCO2198. Additionally, some *cox1* mtDNA reads had particularly low-quality scores and thus may contain errors. This is especially possible for the *cox1* mtDNA data of *L. hoi* as it contained insertions and deletions in comparison to other lernaeid data, as well as an internal stop codon, which is troublesome. This data have been noted as "Unverified" on GenBank (OR058778). This will hopefully be resolved in future studies should more viable material become available for this species, but unfortunately this has not yet been possible.

Additionally, the generation of *Lamproglena* mitogenomes would be most helpful in designing more effective primers. Considering the *cox1* mtDNA available for *Lernaea*, many of the sequences available do not cover a portion of the 5' end of the standard barcoding region. This, along with the limited span of some of the data obtained here, also limits the comparison of all mtDNA. The variation in the *cox1* mtDNA data of *L. clariae* and *L. monodi* might hint at variation within the populations sampled, indicating the possibility of informative population genetics studies. This may be especially useful in studying the translocation of *C. gariepinus* and *Oreochromis niloticus*, both of which are internationally important aquaculture species. As mentioned, *L. monodi* has already been recorded from South America (Thatcher 2006, Azevedo et al. 2010, 2012, Tavares-Dias et al. 2015, Garcia et al. 2019) and the Philippines (Yamabot and Lopez 1997) due to the introduction of *O. niloticus*.

Even though the present study adds ribosomal DNA data for two *Lamproglena* species not previously genetically characterised and the first mitochondrial sequence data for the genus, there are still 32 species for which no molecular information is available. Additionally, the currently available data suggest the presence of cryptic species which need further attention, using both morphological and genetic approaches.

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Author contributions. QMDS – designed research, collected *Lamproglena hoi*, performed research, analysed data, wrote manuscript. NMR – access to *Lamproglena monodi* genomic DNA. AAO – designed research, supervised project, wrote funding proposals, secured funding, and contributed to every draft of the manuscript.

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