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RESEARCH ARTICLE

Prevalence, Morphological and Molecular characterization of *Lernaea cyprinacea* isolated from major carps of Southern Punjab, Pakistan

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ABSTRACT

The major carps, including *Labeo rohita*, *Cirrhinus mrigala* and *Catla catla* are popular warm water fish species in Southeast Asia. Parasitic infestation is common among these major freshwater carps, hindering their growth and potentially causing mortality. This study aimed to identify *Lernaea* species morphologically and molecularly across selected fish farms of South Punjab. In total 125 *Lernaea* specimen were collected from 581 three distinct fish species including *L. rohita*, *C. mrigala* and *C. catla* across government and private fish farms located in Rajanpur (29°10´44´N, 70°33´01´E), Hasilpur (29°77´88´N, 72°54´71´E), Bahawalpur (29°38´´71´N, 71°63´40´E), and Multan (30°15´77´N, 71°44´39´E) from January to December 2021. These specimens were meticulously examined for precise morphological characterization by using a stereomicroscope after washing with 0.75% NaCl and identified by using Kabata's identification key. For an accurate understanding of the anatomy of the *Lernaea*, the parasite was subjected to scanning electron microscopy. The specimens were subjected to molecular identification techniques based on 18S rDNA PCR. The results showed that the prevalence of parasite varied in different regions including Rajanpur (68.75%), Hasilpur (25%), Bahawalpur (12.11%) and Multan (22.5%). The fins, gills, skin and eyes were the most common infestation sites. Furthermore, the infestation rate varied among different fish species and regions, suggesting a potential influence of genetic & physico-chemical parameters on the observed differences in infestation levels. The phylogenetic analysis of 18S rDNA showed three distinct clades. Clade 1 included sequences from our study along with those from India, China and Russia, Clade 2 contains sequences from Iran, Australia, Iraq, and Japan while Clade 3 had only two sequences from China. This study offers the initial insight into *Lernaea cyprinacea* in Southern Punjab and sets the groundwork for future research on crustacean fish parasites, aiding in evaluating their economic impact on Pakistani fish farms.

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INTRODUCTION

Today's aquaculture industry exhibits greater diversity, cultivating 45% more fish and shellfish across a wide range of marine, brackish and freshwater environments, worldwide (FAO, 2023). Aquaculture is projected to surge by 62% from 2010 to 2030 to meet rising demands for fish and seafood due to population growth and

shifting consumption patterns. Aquaculture is expected to supply over two thirds of the global consumption of fish and shellfish (Reverter *et al*., 2021). Fishing is the main income source for rural communities in Pakistan particularly along Balochistan and Sindh coastlines and inland near rivers, lakes and dams (Khan and Khan, 2011). The carps including, Thaila (*Catla catla*), Morakhi (*Cirrhinus mrigala*) and Rohu (*Labeo rohita*) are an important food source and are valuable species in Pakistan. Due to their high commercial values and suitability as food items, all of these species are cultured in preferable base (Sheikh *et al*., 2017).

In fish farming, parasitic infestation is a prevalent ailment among freshwater fishes. These parasites can hinder development and lead to fatalities, ultimately reducing fecundity (Prastowo *et al*., 2023). Ectoparasites are a major threat to the fish farming industry particularly in intensive fish farming practice because of high stocking densities (Iqbal *et al*., 2012). Lernaeidae is one of the most common and dangerous families of ectoparasites, most commonly infesting freshwater fish species. Among Lernaeidae, the most frequently occurring parasite is *Lernaea cyprineacea* which is reported to be more dangerous than other sister species. Lernaeid copepods attach to all external as well as internal areas like the mouth, gills, filaments, fins and eyes (Barson *et al*., 2008; Prastowo *et al*., 2023) and induce necrosis making it susceptible to the bacterial, viral and fungal infections (Lester and Hayward, 2006). Inadequate aquaculture management may induce stress, rendering fish vulnerable to parasitic and microbial infections and compromising their immune systems, Combating parasitic infestation outbreaks is critical for farmers to prevent losses (Noga, 2010).

Lernaea or "anchor worms," are crustacean copepod parasites that infect and can kill freshwater wild or pond-bred fish. They have been reported from major carps, *C. catla, L. rohita and C. mirgala*, Chinese carps, *Ctenopharyngodon idella, Hypophthalmichthys molitrix, Cyprinus carpio* (Abbas *et al*., 2014; Bilal *et al*., 2021) and also from various ornamental fish species (Prastowo *et al*., 2023). They are most common during summer, especially in stagnant or slow-moving water. *Lernaea* is worldwide in distribution and prefers temperatures between 26-30°C (Hossain *et al*., 2018).

The economic significance of these lernaeid ectoparasites has risen due to numerous epizootics affecting crucially farmed fish species, worldwide (Tasawar *et al*., 2007). *Lernaea* are typically related with substantial deaths in aquaculture and the outcomes are pretty severe (Soares *et al*., 2018; Zhu *et al*., 2020). Aim of the present study was to gather data on *Lernaea* species infecting fish in Pakistan, with a focus on the Southern Punjab region. Traditionally the parasites are identified morphologically by using identification keys (Abbas *et al*., 2014; Bilal *et al*., 2021), in the present study morphological and molecular identification studies were carried out to ascertain the taxonomic status of this parasite in the area. Additionally, the research aimed to provide an overview of the parasite's distribution and its interaction with the water conditions in the aquaculture environment.

MATERIALS AND METHODS

Study design and sampling of parasites: The study was conducted from January 2021 to December 2021. In total 125 *Lernaea* specimen were collected from 581 three distinct fish species including *L. rohita, C. mrigala* and *C. catla* across government and private fish farms. The samples of *Lernaea* were collected from Rajanpur 29º10′′44′N, 70º33′′01′E, Hasilpur, 29º77′′88′N, 72º54′′71′E including Head Islam, Bahawalpur 29º38′′71′N, 71º63′′40′E, Lodhran and different localities of district Multan 30º15′′77′N, 71º44′′39′E including Jalalpur Pirwala and Shujabad**.** Fish samples were assessed to ascertain the presence or absence of *Lernaea* infestation. The samples of *Lernaea* were collected from fins, gills, tail and oral cavity of infected fish with the help of forceps. Parasites from each fish were preserved in Eppendorf tube separately containing 95% ethanol to avoid the deformities in shape for the purpose of morphological identification (Pallavi *et al*., 2017). In positive samples, the quantity of *Lernaea* on each fish was quantified to assess the extent of infestation. *Lernaea* infestation was categorized as follows: light if 1–5 *Lernaea* were detected on one fish, moderate if 6–10 *Lernaea* were found on one fish and heavy if the fish was infected with more than 10 *Lernaea*.

Analysis of physico-chemical parameters: The parameters of fish ponds such as water temperature (°C), dissolved oxygen (DO), pH, total dissolved solids (TDS), Electric conductivity (EC), salinity, turbidity, hardness, alkalinity and colour was checked at daytime during the survey. The water temperature was recorded with Celsius digital thermometer. TDS, Salinity and EC were measured by smart TDS meter (Model SM-401, CD-43010). DO was measured with DO meter (Model DO-5509). Turbidity was recorded with Secchi disc. Hardness and alkalinity were determined by manual method (Jain *et al*., 2022).

Morphological identification of parasites: Collected parasites were washed in 0.75% solution of NaCl, placed in a petri dish using forceps and observed under the stereomicroscope (Olympus-SZ-1145) having objective lens of 40X. The size of head, tail, horns and body was measured and documented in a separate file for comparative analysis of abdomen, head and tail in the holdfast position. Parasites were identified by using Kabata's identification key (Kabata, 1985).

Scanning electron microscopy: For an accurate understanding of the anatomy of the *Lernaea*, the parasite was subjected to scanning electron microscopy (SEM). The samples were prepared according to the protocol provided by Robinson *et al*. (1996) and sent to the Department of Physics, Lahore University of Management Sciences (LUMS), Lahore. Anatomy of head, abdomen, tail and horn of the *Lernaea* were observed for accurate confirmation of the species by using NAVO NANO SEM. The size of different organs was measured by employing 10KV voltage at 100X.

DNA extraction, Nano-drop quantification, PCR and sequencing: The genomic DNA of the parasites was extracted according to the manufacturer's instructions using GeneJET Genomic DNA extraction kit (Thermo Scientific ®). DNA was stored at -20°C until further use. The purity and concentration of DNA was checked using 2000c spectrophotometer (Thermo Fisher Scientific).

For the molecular identification of the parasite, 20µl volume of PCR mixtures was prepared containing 2µl of genomic DNA, 2µl of each forward and reverse primer, 4µl of ddH_2O and 10µl GoTaq® Green Master Mix (Madison, Wisconsin). During PCRs *L. cruscuta* was used as a positive control while double distilled water was included as a negative control in each PCR run. Primers L-F (5´-CACCGGAAGGATTGACGAT-3´) and L-R (5´-ACTCGCCAGGCAGTAGAAAAT-3´) were designed and used to amplify partial 18S rDNA region of 196 bp. Thermal protocol used for the PCR included initial heating of 95°C for 5 min, followed by 35 cycles of 95°C for 1 minute, 54.7°C for 30 seconds, and 72°C for 1 minute, with final extension at 72°C for 7 minutes using Touch Thermal, Bio-Rad, USA, model C1000 (Song *et al*., 2008). Following PCR, aliquots (5µl) of individual amplicons were observed by agarose gel electrophoresis (1.5% gels, 100ml TAE buffer, 7µl SYBR™ Safe). The electrophoresis tank was connected with the Power Pac (Bio-Rad, USA), gels were transferred to UV Illuminator (LED Product, Taiwan) for imaging. Amplicons were separately purified using the Gel/PCR Purification Mini Kit (WizPrep™) as per manufacturer instructions. Six samples were randomly sequenced with the reverse primer L-R using Applied Biosystems BigDYE® terminator v3.1 Kit on an automated sequencer (ABI Applied Biosystems Model 3730xl).

Phylogenetic analysis: Obtained sequences were aligned by Muscle V 3.8.31 software and then adjusted manually by using the software mesquite V 3.03 while polymorphic sites were designated by International Union of Pure and Applied Chemistry codes. Phylogenetic analysis was performed by using neighbor-joining (NJ) model with previously reported *Lernaea* spp. sequences available on GenBank. The NJ analysis was performed by using the software MEGA 11.0.13 (Kumar *et al*., 2016) and topologies were evaluated with 2000 bootstrap values.

RESULTS

The data from the fish farms of different regions of Southern Punjab showed that most of the time the fish farmers used underground water pumped out with tube-wells for aquaculture. However, some of the farms also relied upon canal and river water as water resources like Head Islam (Personal communications). These farms showed varying prevalence of *L. cyprinacea* with relation to body weight of different fish species as shown in Table 1.

Table 1: Prevalence of *L. cyprinacea* in major carps' fish farms with relation to body weight. Infestation intensity: (+) =≤5, (++) =6-10, (+++) =≥11

	Average Weight (gm)			
City	*L. rohita*	*C. mrigala*	*C. catla*	Infestation intensity
Rajanpur	1600	1400	800	+++
Hasilpur	1200	750	900	++
Bahawalpur	1500	1000	800	++
Lodhran	1300	1100	700	+
JPPW A*	800	1600	1100	++
Shujabad A	700	900	600	+
JPPW B*	500	700	900	+
Shujabad B	900	1000	1300	+
JPPW C*	700	1200	800	++
Head Islam	1200	1500	1600	+

*Jalalpur pirwala

Physico-chemical parameters of fish ponds: Water quality of fish ponds was determined based on temperature, pH, DO, TDS, TH, TA, EC, turbidity, salinity and water colour that are recorded in Table 2 & 3. These data show that the water quality in the fishponds was within the normal range and could be tolerated by the fish and parasites. Infestation of parasites was maximum on Rajanpur sites as temperature conditions were comparatively higher than other fishponds and the owner of fish farm was not taking care of the hygienic conditions of the fish feed (Personal observation/communication).

Morphological identification and prevalence of *Lernaea* spp.: Area and species-wise prevalence/infestation rates of *L. cyprinacea* was calculated in major carps (*L. rohita, C. mrigala, C. catla*) as listed in Table 4. Total length and size of the body parts like head, abdomen, tail and horns were measured and recorded as in Table 5; Fig. 1 & 2.

Fig. 1: A & B showed the total length of parasites while image C & D represents the horns and tail of the parasites under stereomicroscope (Olympus SZ51) at 40X.

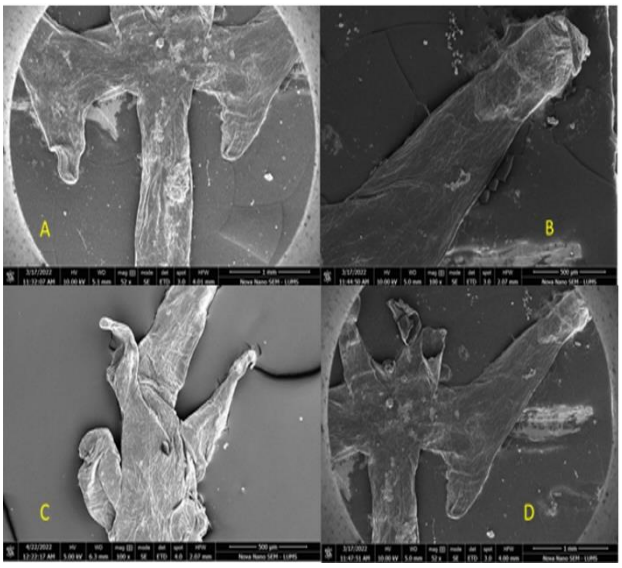

Fig. 2: A, B, C & D depict the shape of Head, tail, abdomen and horn of *Lernaea* spp, respectively.

Molecular and phylogenetic analysis: Molecular analysis of the samples was done through PCR using validated primers & positive/negative controls and expected amplicon of 196 bp was obtained. Amplified DNA

Table 2: Records of the selected physico-chemical parameters at surveyed fish farms

Name of fish farm	TM°C	DO mg/L	TDS (ppm)	pH	EC (ms/cm)	TH mg/L	TA mg/L	TUR cm	Salinity	Colour
Rajanpur	29.6	8.1	3.02	8.1	2.9	400	290	39	1.45	Green
Hasilpur	27	7.9	1.4	8.7	2.3	820	380	40	1.15	Green
Bahawalpur	25.5	7.2	1.6	8.4	2.7	620	270	36	1.35	Light Green
Shujabad A	24	8	3.8	8	2.2	540	270	39	1.1	Light Green
JPPW A	12	8.4	4.1	7.9	2.51	820	370	39	1.25	Green
Shujabad B	14.2	8.2	2.05	8.2	2.6	500	370	39	1.3	Light Green
Lodhran	15.4	7.6	4	8.5	2.91	1000	620	36	1.455	Green
JPPW B	14.8	7.4	3.2	7.3	2.6	900	480	39	1.3	Green
JPPW C	16	7.9	9.1	7.2	3.1	560	430	35	1.7	Light Green
Head Islam	15	5.5	6.5	7.2	4	700	360	33	2.2	Green

(TM; temperature: DO; dissolved Oxygen: TDS; total dissolved salt: pH; power of Hydrogen: EC; electrical conductivity: TH; total hardness: TA; Total alkalinity: TUR; Turbidity)

Table 3: Pearson correlation significance analysis of physico-chemical parameters of fish farms

Sr. No	1	2	3	4	5	6	7	8	9
Parameters	TM°C	DO mg/L	TDS (ppm)	pH	EC (ms/cm)	TH mg/L	TA mg/L	TUR cm	Salinity
Pearson Correlation Sig.(2-tailed)	1	.144	-.445	.503	-.299	-.435	-.562	.303	-.310
		.692	.197	.138	.401	.209	.091	.395	.384
N	10	10	10	10	10	10	10	10	10
Infected	.660*	.303	-.416	-.416	-.185	-.300	-.470	.372	-.234
Fishes	.038	.395	.232	.232	.609	.400	.170	.289	.516

(TM; temperature: DO; dissolved Oxygen: TDS; total dissolved salt: pH; power of Hydrogen: EC; electrical conductivity: TH; total hardness: TA; Total alkalinity: TUR; Turbidity).

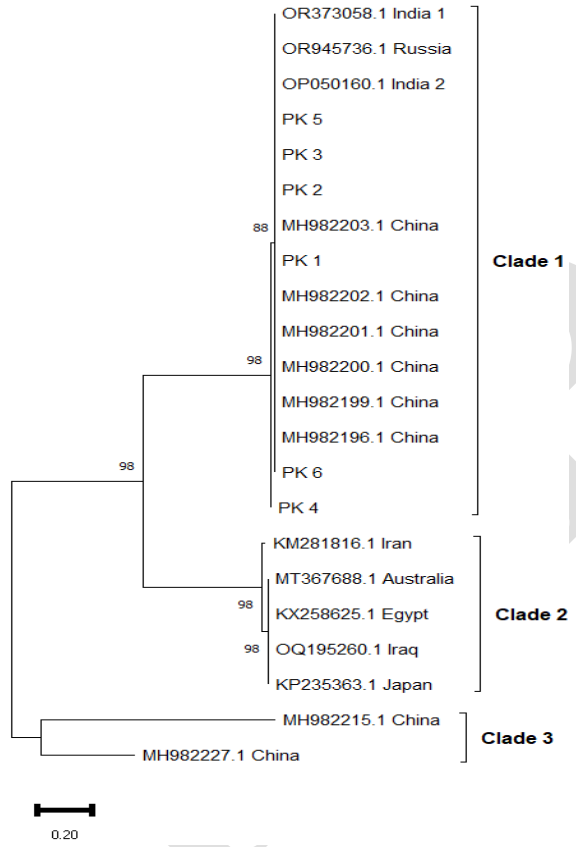

Fig. 3: Phylogenetic tree of 18S rDNA generated by using MEGA 11.0.13 adjusting the bootstrap values of 2000. All the topologies were adjusted and *L. crusita* was used as out group.

sequences were aligned over 196bp and along with previously reported 18S rDNA seq of *Lernaea* spp (Fig. 3). Alignment of the sequences revealed that all the sequences were most similar with 95-99% similarity with reported sequences. The polymorphic sites were found at position 101bp, 147 and 185bp sites and were adjusted according to International Union of pure and Applied Chemistry codes. The phylogenetic analysis of 18S rDNA indicated that tree consists of three clades. Clade 1 included sequences from our study along with those from India, China and Russia, Clade 2 contains sequences from Iran, Australia, Iraq, Japan and Japan while Clade 3 had only two sequences from China (Fig. 3).

Table 5: The size of head, abdomen, tail, horns and total length of *Lernaea* spp. (Ranging between 0.58 cm to 0.78 cm with an average ±0.73cm)

Fish	No of samples	Head (cm)	Abdomen (cm)	Tail (cm)	Horn(cm)
L. rohita	3	0.23	0.18	0.015	0.1
		0.20	0.17	0.013	0.075
		0.16	0.18	0.014	0.1
C. mrigala	3	0.13	0.18	0.016	0.063
		0.1	0.15	0.014	0.055
		0.175	0.16	0.012	0.05
C. catla	3	0.2	0.18	0.014	0.07
		0.22	0.15	0.016	0.11
		0.18	0.15	0.017	0.055

DISCUSSION

There is scarcity of evidence on the prevalence of infestation and intensities of *L. cyprinacea* in the fish farming facilities of Southern Punjab. Diagnosis of *L. cyprinacea* is normally based on microscopic examination that is problematic in accurate identification due to substantial morphological flexibility of *Lernaea* spp. (Pallavi *et al*., 2017; Raja *et al*., 2023). Traditional taxonomical techniques depending on morphology are often hectic, time taking and involve significant expertise (Rahmati-Holasoo *et al*., 2023). We identified the parasites through microscope and SEM and it was observed that the result of SEM was more accurate and reliable. In this study we used SEM for morphological identification and PCR for molecular identification of *L. cyprinacea* for conclusive confirmation of species. The present study offers the first understandings of genetic characterization of *Lernaea* spp. from major carps (*C. marigala*, *L. rohita*, *C. catla*) of different localities of Southern Punjab. *L. cyprinacea* is an ectoparasite of fish that cause significant pathological problems in states of tropics and subtropics around the

World (Toksen *et al*., 2014). In this study we have found that fins (caudal and pectoral) gills and anal region are the most infected regions and similar findings were reported by Nur *et al*. (2022).

In the present study, the number of *Lernaea* parasites ranging from 1 to 7 per fish. Infestation rate was higher in the samples collected from Rajanpur where prevalence of infestation of the parasite was found to be 68.75%. This may be related to the higher temperature and poor management practices as were observed during the sampling. Parasitic infestation was higher in the present study as compared with the previous studies in other regions of Punjab (Abbas *et al*. 2014; Aslam *et al*., 2016). These differences in the prevalence of *L. cyprinacea* could be due to (i) ecological location of fish farms; in previous study samples were collected from Lahore, Gujranwala and Kasur, (ii) effect of environmental temperature; higher temperature in South Punjab *vs* comparatively low temperature in Northern regions of Punjab, & (iii) difference in farm management practices.

In this study, it was observed that the infestation of *L. cyprinacea* was higher in the fish an increased body weight and *C. catla* was the most infected fish species. The same trend of infestation was reported by Abbas *et al*. (2014) and Aslam *et al*. (2016). Tufail *et al*. (2014) reported that in *C. catla*, the parasitic load increased with an increase in water temperature being highest in the months of July and August while Abbas *et al*. (2014) reported highest infestation of *L. cyprinacea* in the month of June in *C. catla.* Contrary to this, it was found that the parasites were most prevalent in late winter (December to April) when the water temperature was between 13 and 23℃ (Hossain *et al*., 2018).

From universal perception, the molecular prevalence of *L. cyprinacea* (68.75%) of this study are comparable with those previous studies around the world such as Iran 61.1% (Rahnama *et al*., 2016); Turkey 52.36% (Koyun *et al*., 2015); Turkey 5.3% (Innal *et al*., 2019); Australia 12.3% (Zhu *et al*., 2020) and Philippines 65% (Balagtas *et al*., 2023). The difference in the prevalence could be due to several contributing factor like sample size, fish species, environmental condition, prophylactic measures and management (Tasawar *et al*., 2007).

The incidence of fish infected with *L. cyprinacea* was variable in different localities of Southern Punjab with an average rate of 21.51% (125/581). The highest prevalence (68.75%) was found in Rajanpur followed by Hasilpur (25%), Jalalpur pirwala district Multan (22%), Head Islam (13%), and (14.29%) in Lodhran, respectively. These disparities in prevalence rate could be due to differences in temperature, average rain fall and geographical location of the farms. Major carps in a polyculture, semi-intensive system of Southern Punjab was found to have *L. cyprinacea* exposure, occurrence, and dissemination. The findings indicated that *L. rohita, C. mrigala,* and *C. catla* had substantial infestation levels. (Hemaprasanth *et al*. (2017) found that *Ctenopharyngodon. idella, Hypothalmichthys molitrix, C. catla,* and *L. fimbriatus* were at risk in both types of culture systems due to their low resistance to parasitic infestation. *Cyprinus carpio* and *L. calbasu* showed low or no Lernaeid infestation under monoculture and polyculture. However, *L. rohita* shown resistance in monoculture but was unable to continue exhibiting resistance in polyculture techniques.

In the present study, it was observed that the most affected areas were the fins, gills and skin. *Lernaea* species seem to adhere more often to the fish's abdomen and ventral side. In addition to providing better protection from the parasite, these locations are also more permeable to it. *L. cyprinacea* is known to favor a position that is more protected from water currents. In spite of this, the nature of *Lernaea* assault makes this specific type of attachment very pathogenic (Avenant-Oldewage, 2011).

In Punjab, Pakistan occurrences of mass fish mortality owing to *Lernaea* infestation are uncommon, but chronic and long-term consequences are fairly widespread, significantly impacting the productive capacity of fisheries (Tufail *et al*., 2014). Larvae of *L. cyprinacea* are treated with baths, dips and pond water. Chemical pond treatment is more successful than the first two options since it is quicker and less taxing on the body of labor involved. It's unusual for mass fish deaths to occur due to *Lernaea* infestation in Punjab, but the long-term impacts have a significant impact on fish output (Hemaprasanth *et al*., 2017).

The phylogenetic analysis of 18S rDNA sequences of *L. cyprinacea* from Southern Punjab and those from other geographic origins showed three distinct clades. Clade 1 included sequences from our study along with those from China, India, Iran, Russia and regardless of where they were from different geographic localities, while Clade 2 shows sequences from Japan, Iran, Australia, Egypt and Clade 3 shows two sequences from China. The haplotypes of our study based on 18S rDNA gene showed 100% similarity among the specimens. Prastowo *et al*. (2023) Santos *et al*. (2023) reported genetic similarity between 28S rRNA sequences of *L. cyprinacea* from Yogyakarta and those from other topographical origins including India, Russia, Australia, China, Egypt and Iran. Results suggest cosmopolitan distribution of parasites along the globe.

Conclusions: In conclusion, *L. cyprinacea* is common parasite in freshwater aquaculture in tropical and sub-tropical regions hampering the growth of fish. The present study provided useful insights into the lernaeid infestation and its prevalence in Southern Punjab. The fins, gills, skin and eyes were the most common infestation sites for this parasite. The infestation rate varied among different fish species and regions, suggesting a potential influence of genetic & physico-chemical parameters on the observed differences in infestation levels. Appropriate measures are required to minimize the economic losses of the fish farmers caused by this copepode.

Authors contribution: SQAS; KM: designed study, executed experiment, analyzed data, involved in writing manuscript MIR; SA: executed experiment, analyzed data, involved in reviewed manuscript HN, MLS, ON: analyzed data, involved in reviewed manuscript ASA; SS: designed study, executed experiment, analyzed data, involved in review & submission of manuscript.

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