



## Molecular identification of the fish parasite *Lernaea*

B. PALLAVI, K. M. SHANKAR, P. B. ABHIMAN AND IQLAS AHMED

Aquatic Animal Health Laboratory, Department of Aquaculture, College of Fisheries Mangalore - 575 002  
Karnataka, India  
e-mail: kalkulishankar@gmail.com

### ABSTRACT

Isolates of the fish parasitic copepod *Lernaea* from four different freshwater fishes viz., catla (*Catla catla*), guppy (*Poecilia reticulata*), platy (*Xiphophorus maculatus*) and gold fish (*Carassius auratus*) were selected for the study. The isolates had considerably differing anchor shapes and were likely to be grouped as different species. Partial nucleotide sequences of the 18S and 28S rDNA regions of the *Lernaea* isolates were determined. Phylogenetic trees generated by the neighbour joining method revealed that the *Lernaea* isolates examined in this study were of similar genotype and were identified to be *Lernaea cyprinacea*.

Keywords: Crustacean fish parasite, *Lernaea*, Molecular taxonomy, 18S rDNA, 28S rDNA

### Introduction

Till date, 110 species of lernaeids have been systematically placed in 14 different genera (Ho, 1998). Currently 43 valid *Lernaea* species are reported in the World of Copepods database (Avenant, 2012). The taxonomical characteristics used for the identification of species of *Lernaea* often seem ill-defined. The shape of the anchors is selected as the most important characteristic for identification purposes. However, the growth of the anchors is largely influenced by the consistency of the tissue to which the parasite is attached (Hu, 1948; Harding, 1950; Fryer, 1961, 1968). Poddubnaya, (1973, 1978) further complicated the understanding of the species concept of lernaeids when she showed that offsprings with different phenotypes could be obtained from a single maternal specimen. The main contribution of her work is the demonstration that the morphology of the copepod may be substantially influenced by the host and that the influence of the host may be constant and predictable. Furthermore, it became evident from the work of Fratello and Sabatini (1972) that the chromosome of *Lernaea* collected from different fishes were identical and they concluded that all fishes carried the same species of *Lernaea*, i.e., *L. cyprinacea*. A total of nine species of *Lernaea* from India and South-east Asia have been reported so far. They are *L. cyprinacea* (Linnaeus, 1758), *L. oryzophila* (Monod, 1932), *L. polymorpha* (Yu, 1938), *L. lophiara* (Harding, 1950), *L. chackoensis* (Gnanamuthu, 1951), *L. bengalensis* (Gnanamuthu, 1956), *L. arcuata* (Soejanto, 1965), *L. hesarghattensis* (Srinivasachar and Sundarabai, 1974), *L. bhadraensis* (Seenappa *et al.*, 1980) and *L. osphronemi* by Thomas *et al.* (1989). The considerable morphological plasticity of *Lernaea*

species has hindered accuracy in identification (Kabata 1979; Lester and Haywood, 2006). Molecular genetic studies for definitive confirmation of species identity are a basic prerequisite for development of effective control strategies against the parasite.

The traditional taxonomical tools based on external morphology are often laborious, time consuming and require considerable skill. In contrast, molecular methods are relatively recent, reasonably accurate and could be complimented with other taxonomic methods. DNA sequence variations often contain useful information for taxonomic studies (Tautz *et al.*, 2002, 2003; Blaxter and Floyd, 2003; Blaxter, 2004). The nuclear ribosomal DNA (rDNA) in eukaryotes is made up of tandem arrays of a basic unit that contains the transcription units (18S, 5.8S, 28S) and an intervening intergenic spacer (IGS) region with copy numbers up to the order of 10,000 (Schlotterer, 1998). The ribosomal DNA cistron consists of two different regions; one region coding for ribosomal functioning (Raue *et al.*, 1990) and others are non-coding. The different subunits and regions of the ribosomal DNA gene have different degrees of sequence variation and are suitable for comparison at the inter-genetic or interspecies level. The tick (*Boophilus microplus*) antigen Bm86, displays great variability among geographically isolated ticks leading to reduced effectiveness of the anti-tick vaccine (Kaewmongkol *et al.*, 2015). Therefore, while designing a commercially viable vaccine against *Lernaea* it is prudent to ascertain if there is genetic variability among the *Lernaea* populations. Hence the present study was initiated to identify the *Lernaea* isolates using molecular tools.

## Materials and methods

### Collection of *Lernaea* isolates

The *Lernaea* samples were collected from four different species of infected fishes *viz.*, catla (*Catla catla*), guppy (*Poecilia reticulata*), gold fish (*Carassius auratus*) and platy (*Xiphophorus maculatus*) from the fish farm of the College of Fisheries, Mangalore, India. The parasite was pulled out with the help of forceps. Isolates from each host species was washed separately with 0.75% NaCl and preserved in 95% ethanol until isolation of genomic DNA. The anchor shapes were studied under the microscope and confirmed that they were morphologically distinct from one another.

### DNA extraction and polymerase chain reaction (PCR)

Genomic DNA of the parasite was isolated using the Qiagen DNeasy Blood and Tissue kit as per the manufacturer's protocol. Purity and concentration of the DNA was checked by Nanodrop 2000C spectrophotometer (Thermo Scientific). The 18S rDNA fragments were amplified with primers 18S F (5' - AAG GTG TGM CCT ATC AAC T - 3') and 18S R (5' - TTA CTT CCT CTA AAC GCT C - 3') designed by Song *et al.* (2008). The 28S rDNA fragments were amplified with primers 28S F (5' - ACA ACT GTG ATG CCC TTA G - 3') and 28S R (5' - TGG TCC GTG TTT CAA GAC G - 3') designed by Song *et al.* (2008). PCR reactions were performed in 30 µl mixtures containing 200 ng genomic DNA, 0.2 µMol each of the two primers, 50 µM of each of the dNTPs, 1 × PCR buffer (with 1.5 mM MgCl<sub>2</sub>), 2.5 U of Taq DNA polymerase (Genei, Bangalore, India) in BioRad C 1000 Touch Thermal Cycler under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min with a final extension at 72°C for 5 min. PCR reactions for 28S regions were performed under similar conditions except that the annealing temperature was maintained at 60°C for 25 s, extension at 72°C for 30 s with a final extension at 72°C for 3 min. The amplified fragments were visualised on 1% agarose gel.

### DNA sequencing and assembly

Specific amplified products were eluted from the gel using the Thermo Scientific Gene JET gel extraction and DNA cleanup microkit. Purified products were sent to Chromous Biotech, Bangalore, India for automated sequencing. The sequence was manually checked and edited for accuracy using Bioedit Software. Contigs were assembled using the CAP3 interface in the bioedit software.

### Phylogenetic analysis

*Lernaea cyprinacea* sequences deposited by Song *et al.* (2008) and the *Argulus* sequences to be used as outgroup were downloaded from Genbank (<http://www.ncbi.nih.gov>) in the FASTA format. The sequences were then aligned with homologous sequences of the isolates using 'Clustal W' alignment software (Larkin *et al.*, 2007) in MEGA version 5 (Tamura *et al.*, 2011) (<http://www.megasoftware.net>).

The evolutionary history for both 18S and 28 S sequences from *Lernaea* isolates was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa were clustered together was calculated with the bootstrap test (1000 replicates) (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position. There were a total of 1015 positions in the final dataset for the 18S region and 662 positions for 28S region.

## Results and discussion

The anchor shapes of the *Lernaea* isolates from the four fishes considerably differed from each other (Fig. 1a, b, c and d). Polymerase chain reaction of the 18S rDNA region of *Lernaea* isolates yielded a product of 1370 bp and that of 28S region yielded a product

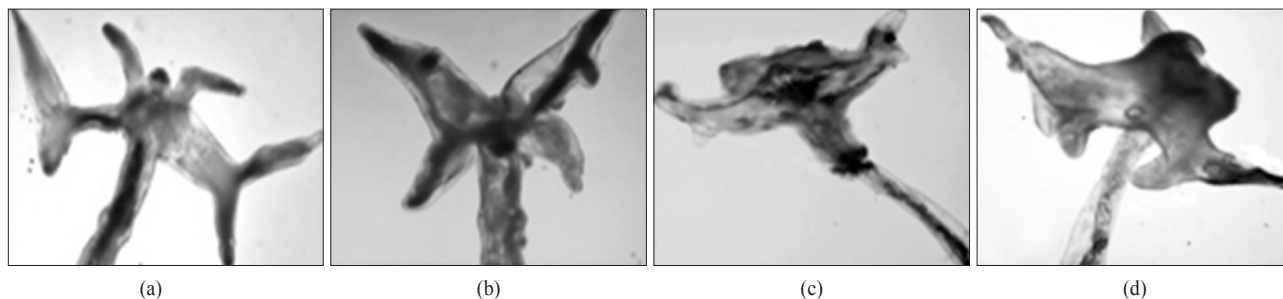


Fig. 1. Anchor of *Lernaea* isolated from a) Catla; b) Guppy; c) Platy; d) Gold Fish (x100)

of 720 bp (Fig. 2 and 3). The BLAST results showed that the sequences for the 18S and 28S rDNA from the *Lernaea* isolates were identical to the *Lernaea cyprinacea* sequences deposited in Genbank (Song *et al.*, 2008). The sequencing results are presented in Fig. 4 and 5. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al.*, 2011). The distance trees generated by neighbor joining method reflect these findings (Fig. 6

and 7). This is reliable as the outgroup species *Argulus* was clearly segregated into a different clade.

The traditional taxonomic method considers the anchor shape of *Lernaea* as the most distinguishing characteristic to delineate the species. Using anchor shape as the basis for classification, the *Lernaea* isolates in the present study were likely to be grouped into four different species. The anchor shape is not a good characteristic for

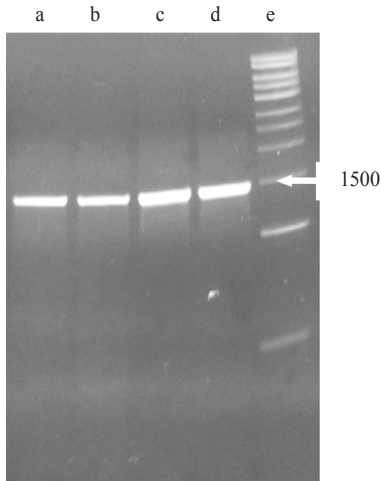


Fig. 2. Photograph of agarose gel containing PCR products of the 18S rDNA region of *Lernaea* isolates  
Lane a: Catla, b: Guppy, c: Platy, d: Gold fish, e: Marker

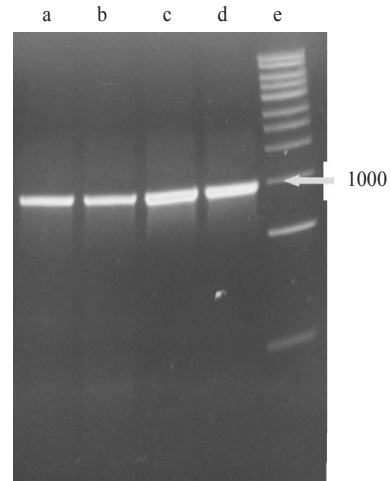


Fig. 3. Photograph of agarose gel containing PCR products of the 28S rDNA region of *Lernaea* isolates  
Lane a: Catla, b: Guppy, c: Platy, d: Gold fish

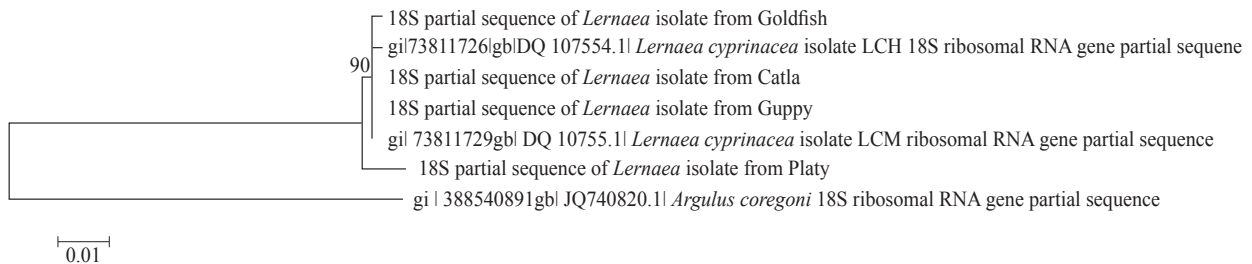


Fig. 6. Neighbor joining tree inferred from the analysis of partial 18S rDNA sequences of four *Lernaea* isolates. Numbers along branches indicate the percentages of support values. The optimal tree with the sum of branch length = 0.15845141. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

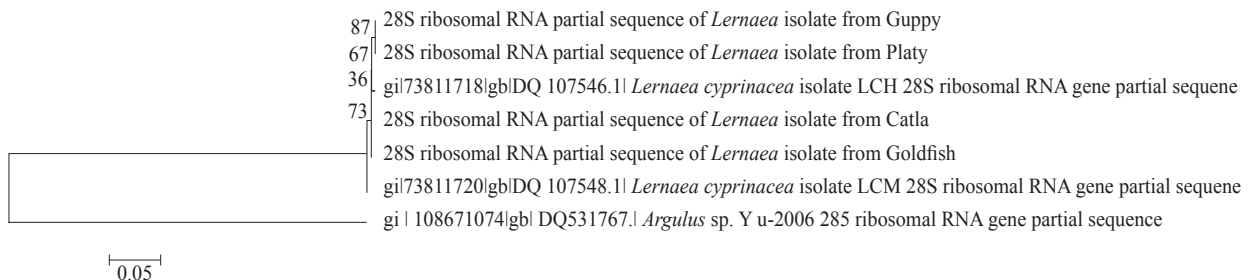


Fig. 7. Neighbor joining tree inferred from the analysis of partial 28S rDNA sequences of four *Lernaea* isolates. Numbers along branches indicate the percentages of support values. The optimal tree with the sum of branch length = 0.63954580. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree





Fig. 5. Clustal W alignment of nucleotide sequences of the 28S rDNA regions of the *Lernaea* isolates from catla, guppy, platy and goldfish

identification of species as it is usually influenced by the type of fish tissue within which it developed. Very little sequence variability in the 18S and 28S rDNA regions supports that *Lernaea* parasitising the fishes in this study are monophyletic and might be the cosmopolitan species *Lernaea cyprinacea* itself. However, further analyses of genes of these isolates will be required before final conclusion. Other genes and a much diverse range of host fish infected with *Lernaea* need to be included. Further studies would help to understand whether the isolates share similar biological and epidemiological properties. Accidental introduction of *L. cyprinacea* might have occurred in the water bodies of Mangalore most probably as a result of the import of tropical ornamental fish from different parts of Asia. The host fishes of *Lernaea* are prevalent throughout the country and further surveys in the rivers of India might lead to the discovery of *L. cyprinacea* in those areas.

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