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Classical morphology and DNA barcoding based identification of freshwater ectoparasite *Argulus foliaceus* in rohu *Labeo rohita*

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ABSTRACT

DNA barcoding coupled with classical morphological description have become promising approach for the species level identification. In the present study, 278 numbers of rohu *Labeo rohita* were screened, for *Argulus* infestation, among which 167 fish (60.07%) were found infested with *Argulus*. Morphological attributes of the parasites were studied by light microscope (LM) and Scanning Electron Microscope (SEM) which showed dorsoventrally flattened body comprising of head, thorax, abdomen, a pair of large compound eyes, suctorial organs with sclerotised support structures and two openings of spermatheca at posterior end. Posterior incision of abdomen do not reach the mid-line and the cephalo-thoracic carapace have not extended beyond the beginning of abdomen. Further validation was done using molecular tools for accurate parasite identification. The pair-wise genetic distance value, using Kimura-2 parameter showed a species level variation of 0.001 (1%) with that of *A. foliaceus*, while 0.083 and 0.052 (*i.e.* more than 2%) with that of *A. indicus* and *A. japonicus* respectively. Phylogenetic tree generated using Neighbour-Joining (NJ) and Maximum-Likelihood (ML) methods, with Kimura-2 parameter were also in agreement with pairwise distance values. The mitochondrial cytochrome c oxidase subunit 1 (COI) sequences of *A. foliaceus* formed one cluster with the present studied samples, whereas sequences of *A. japonicus* formed a sister group. Integrating morphological and rapid DNA barcoding tools, the species was delineated as *A. foliaceus.*

Keywords: *Argulus foliaceus,* COI gene, DNA barcoding, *Labeo rohita,* Morphology

Introduction

Intensification, while intended for more production per unit area is also accompanied with disease outbreaks that undermine the productivity of culture systems if unmanaged. Overcrowding in intensive culture systems increases their susceptibility to pathogens, resulting in disease and economic losses (de la Cruz-Cervantes *et al.,* 2020). Parasitism is a complex problem (Saleh *et al.,* 2020) and are often inevitable in fish farms with high stocking densities and poor water quality that aggravates transmission and there by causing adverse impact on the host immune response (Walker *et al.,* 2004). Parasites are widely distributed across the aquatic bodies and cause serious outbreaks of diseases in aquaculture (Roberts, 2012). As per an estimate, the loss due to *Argulus* infestation in aquaculture amounts to approximately ₹29,524 ha^{-1} $year^{-1}$ corresponding to US$615 ha^{-1} $year^{-1}$ and thus, argulosis alone accounts for ₹300 crores (US$62.5 million) loss to the Indian carp farming sector (Sahoo *et al.,* 2021). Further, at any particular point of time, around 48% of aquaculture ponds are found to be infected with this parasite (Sahoo *et al.,* 2013).

Argulus spp. (fish lice) are the most documented macroectoparasites in pisciculture (Mandira *et al.,* 2015). Its infestation and subsequent economic loss of fish is a critical problem which is amplifying globally. Worldwide, more than 100 different species of *Argulus* have been identified and about 15 species are found to infect freshwater fishes (Noaman *et al.,* 2010). Among these, three most studied species, found in the freshwater systems are *A. foliaceus, A. japonicus* and *A. ceregoni*. The parasites have distinctly separated head, thorax and abdomen. The head is covered by a flattened horseshoe-shaped carapace, maxillipeds, preoral sting and basal glands. The thorax has four segments, each having a pair of swimming legs and abdomen has a bilobed segment (Mirzaei *et al.,* 2015). The diverse morphological adaptation of Argulids has taxonomic significance and low host specificity which makes them potent ectoparasites (Feroz *et al.,* 2014) for many important cultivable species. The severity of these ectoparasites is reflected with its direct pathological impacts in host coupled with co-infections, mostly secondary infections. Heavy infestation causes acute haemorrhagic wounds at feeding sites, increased production of mucus, loss of scales and erosion of the fins

which invites opportunistic bacteria like *Aeromonas* or *Pseudomonas* leading to skin ulceration (Saurabh *et al.,* 2012; Mandira *et al.,* 2015). In addition, fish lice also act as the vehicle for other fish pathogens, like Rhabdovirus carpio, larval nematodes and the fungus *Saprolegnia* (Avenant-Oldewage, 2001). Earlier studies illustrate that the fish infected with *Argulus* exhibits extreme flicking of the fins (Yildiz and Kumantas, 2002), shoaling behaviour, scale loss and fin damage (Northcott *et al.,* 1997) and reduction in feeding and jumping (Taylor *et al.,* 2006).

Accurate identification of species is fundamental requirement in any studies and traditionally species are identified using morphological characters (Shashank *et al.,* 2014). Even though conventional morphological keys exist for ichthyoparasites, it is very complicated to define such morphological keys for smaller crustaceans like *Argulus* species (Feroz *et al.,* 2014). To address this difficulty, molecular approaches like DNA barcoding offer great promise for precise identification of species of all life stages. Approximately 650 base pair (bp) fragment of the cytochrome c oxidase subunit 1 (CO1) gene of mitochondrial DNA (mtDNA) is among the most widely used genetic markers for species identification (Mausumee *et al.,* 2013). mtDNA are extensively used in species identification, molecular ecology (Galtier *et al.,* 2009) and trophic analysis in fishes (Aguilar, 2017) and moreover, mitochondrial cytochrome c oxidase subunit I has been standardised as a barcode gene for discriminating fishes (Pavan-Kumar *et al.,* 2020). However with a few exceptions (Besansky *et al.,* 2003; Hansen *et al.,* 2007; Ogedengbe *et al.,* 2011) this technique is comparatively a new approach for parasitological studies (Mark *et al.,* 2012). Current study attempted to prescisely identify the ectoparasite *Argulus* sp. infecting rohu, *Labeo rohita*, integrating morphological features including light and electron microscopy, followed by DNA barcoding method for further confirmation.

Materials and methods

Isolation and identification of parasites

The present study was carried out at the ICAR Research Complex for Eastern Region, Patna, India, in October 2016. A total of 278 nos. of rohu, *Labeo rohita* (163.20±7.48 g) samples were collected from aquaculture farms and screened for *Argulus* infestation. Clinical symptoms like external lesion or abnormality of infested fish samples were recorded as described by Mandira *et al.* (2015). The parasites were carefully removed from different body parts (body surface and gills) of infested fish with forceps and a fine brush. Identification and morphological studies were carried out as per the method described by Ali *et al.* (2010). The test organisms were preserved in 70% alcohol and observed later under a light microscope (LM) (Labomed, pixelpro 2.7). Subsequently, samples were analysed using scanning electron microscopy (SEM) as per the methodology described by Everts *et al.* (2009). Briefly the parasites were rehydrated in desending ethanol followed by distilled water immersion. A drop of sodium hypochlorite was added to the immersion medium and the intact mucus was cleaned properly. Following freeze-drying, the parasites were sputter coated with gold and topology was studied by SEM (qCarl Zeiss, EVO 18, Germany) operating at an accelerating voltage of 10-30 kV.

DNA isolation and PCR amplification

Samples preserved in absolute ethanol were used for DNA isolation. Total genomic DNA was isolated from the whole parasite using DNeasy Mini Kit (Qiagen) for pure and intact DNA. The concentration and purity of isolated DNA were estimated using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). The DNA extracted was used as the template for Polymerase Chain Reaction (PCR) and the 650 bp fragment of mitochondrial COI gene was amplified using universal primers, FishF1-5'-TCAACCAACCACAAAGACATTGGCAC-3'and FishR2-5'ACTTCAGGGTGACCGAAGAATCAGAA-3' (Ward *et al.* 2005). A 25 µl PCR mixture was used containing 1.0-2.0 µl of DNA template (25 ng μl^{-1}), 2.5 µl 10X PCR buffer, 1 µl of each primer (10 pm μl^{-1}), 1.5 µl $MgCl_2$ (25 mM), 2 µl dNTP mix (2.5 mM each) and 0.25 U of Taq DNA polymerase. The reactions were performed on a GeneAmp® PCR System (Applied Biosystems Inc., Foster City, CA) for 35 cycles under the conditions of 30 s at 94°C, 45 s at 54°C and 30 s at 72°C, with a final extension at 72°C for 10 min. Following PCR, the amplification product was checked on a 1.5% agarose gel and the PCR amplicons were purified using Qiagen PCR purification kit followed by bidirectional sequencing on ABI 3100 PE automated capillary sequencer.

Sequence analysis

The sequences were edited and aligned using ClustalW program (Thompson *et al.,* 1994) in Bioedit version 7.0.5.3 (Hall, 1999). Closest matches of *Argulus* sp. sequences in the GenBank database were found using BLAST and were used for further analysis. Phylogenetic analyses and pair-wise distance between the species were performed using MEGA version 6.0 (Tamura *et al.,* 2007). Neighbour Joining, Maximum Likelihood and Maximum Parsimony consensus trees were employed for graphical representation of the patterns of COI divergences among

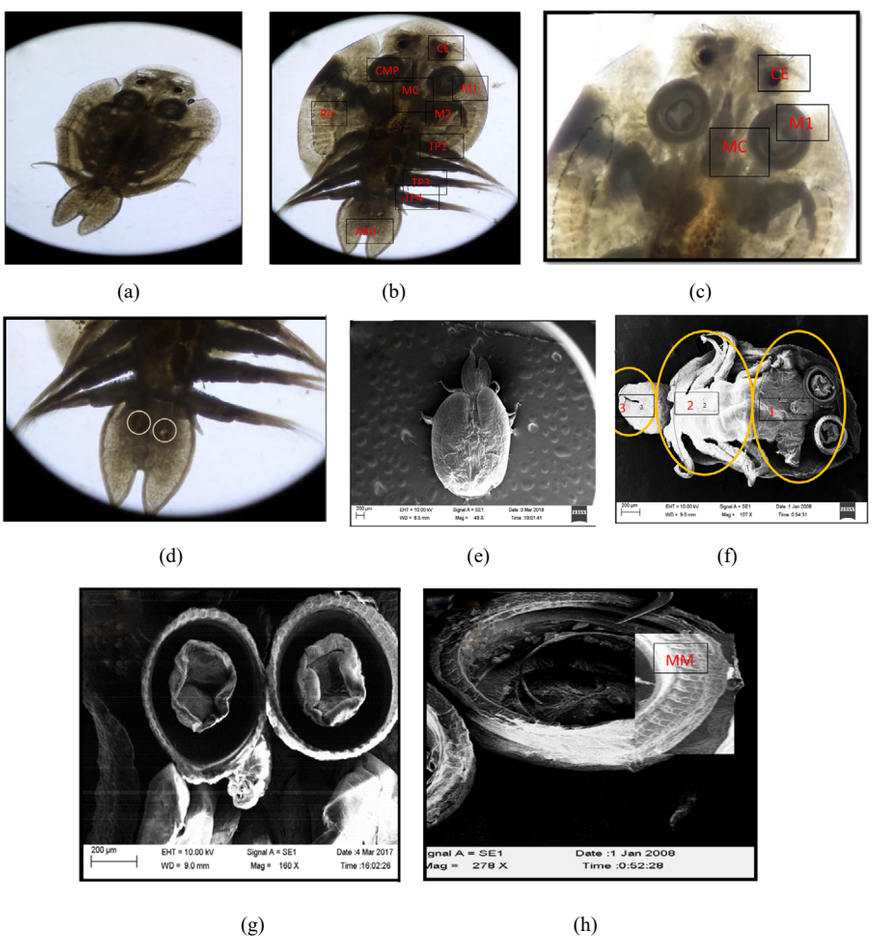
Argulus species using the Kimura-2-parameter (K2P) model with 1000 replicates for bootstrap analysis.

Results

Parasite isolation and microscopic observation

Out of 278 fish specimens screened, 167 fishes had *Argulus* infestation. The prevalence of ectoparasite infestation in fish appeared to be 60.07%. The clinical symptoms of infected fish with argulosis were sluggish movements, abnormal swimming, erratic movements, rubbing against the edge and frayed fins. Heavily infested fishes had shown reddish appearance throughout their body with prominent haemorrhages causing lesions at parasite attachment sites.

Morphologically, the *Argulus* sp. collected during the present study, is characterised by dorsoventrally flattened body that comprises, (i) head; (ii) thorax with four biramous thoracopods and (iii) abdomen with short furcal rami (Fig. 1f). Anterolaterally there is a pair of large compound eyes in the carapace (Fig. 1b and c). The first maxillae are modified as powerful suctorial organs and has marginal membrane with the sclerotised support structures (Fig. 1g and h). Posterior incision of abdomen do not reach the mid-line. The posterior lobes of cephalo-thoracic carapace have not extended beyond the beginning of abdomen and openings of spermatheca (circles) are present on the posterior end of the parasite (Fig. 1d). Based on morphologic characteristics, the parasite was preliminary identified as *A. foliaceus*. Futher



(a) (b) (c)

(d) (e) (f)

(g) (h)

Fig. 1. Photomcrographs of *A. foliaceus* under, (a-d): Light microscope (LM) (x100) and (e-h): Scanning Electron Microscopy (SEM). (a and b) Dorsal and ventral view of an adult female (LM); (c) First maxillae, mouth cone and eye spot (LM); (d) Abdominal lobes and spermatheca (marked circles) (LM); (e) and (f) Dorsal and ventral view (1-Head, 2-Thorax, 3-Abdoman) (SEM); (g) First maxillae (SEM) and (h) Supporting sclerites of 1st maxilla (sucker). CE - Compound eye; M1 - First maxilla; M2 - Second maxilla; ABD - Abdomen; CMP - Central movable part; MC - Mouth cone; MM - Marginal membrane; RA - Respiratory area; TP 1-4 - Thoracopodes one-four

DNA barcoding molecular tool was also used to confirm the identity.

Systematic position of the genus Argulus

Class : Crustacea

Subclass : Branchiuran

Order : Arguloidea

Family : Argulidae

Genus : Argulus

Species : Foliaceus

Argulus foliaceus Linnaeus, 1758

Molecular identification using DNA barcoding

Successful amplification of the mitochondrial CO1 gene (Fig. 2) was obtained in PCR using the universal primers, which generated a 650 bp nucleotide sequence. Subsequently, in BLAST analysis, the sequence showed 99.8% similarity to that of *A. foleaceus,* followed by 95% similarity with *A. indicus* and 94% with *A. japonicus*. Clustal W analysis of these species showed that the COI gene is highly conserved within species and the variations are less, leading to be an appropriate gene for barcoding of *Argulus* species. Further analysis revealed a clear clustering of COI sequences of all the samples with *A. foliaceus*. *A. japonicus* formed a sister group with *A. foliaceus,* while separate clade formation noticed with *A. indicus* and *A. japonicus* in both NJ and ML trees using MEGA 6.0 (Fig. 3, since both the figures are similar, only one figure has been provided). The pairwise genetic distance in comparison with the published sequences available in GenBank of the three different species of *Argulus* (*A. foliaceus, A. indicus* and *A. japonicus*) showed that the average K2P intraspecific distance ranged from 0 to 0.1% and that of interspecific distance ranged from 5.2 to 8.3%. A complete pairwise genetic distance of all the five individuals are furnished in Table 1. The nucleotide composition of the *Argulus* species showed that the GC content ranged from 20.06-16.3% and AT content ranged from 38.2-28.2%. This indicative higher range for AT content is in accordance with the typical character of COI sequence. The GC content showed a decreasing trend higher in third position followed by first and then second. The second position showed the least variation in GC content.

Discussion

In the present study, morphological attributes studied under LM and SEM, preliminarily points towards

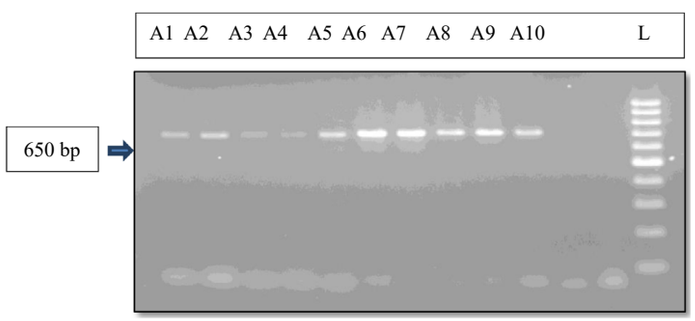


Fig. 2. Gel images of PCR products on 1% agarose gel (L: Ladder, A1-A10: *Argulus* test samples)

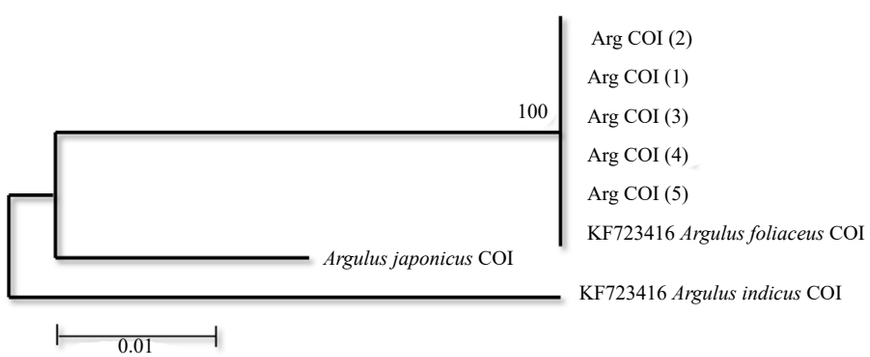


Fig. 3. Phylogenetic tree generated using Kimura-2-parameter model with 1000 replicates for bootstrap analysis. Phylogenetic analyses were performed using MEGA version 5.1 (Tamura *et al.,* 2007)

Table 1. Pairwise genetic distance computed using Kimura-2-parameter using MEGA software

	KF723419.1_ *A. foliaceus*_COI	KF723416.1_ *A. indicus*_COI	*A. japonicus*_ COI	Arg_ COI(5)	Arg_ COI(4)	Arg_ COI(3)	Arg_ COI(2)	Arg_ COI
KF723419.1_*A. foliaceus*_COI								
KF723416.1_*A. indicus*_COI	0.083							
*A. japonicus*_COI	0.052	0.057						
Arg_COI(5)	0.001	0.083	0.052					
Arg_COI(4)	0.000	0.083	0.052	0.000				
Arg_COI(3)	0.000	0.083	0.052	0.000	0.000			
Arg_COI(2)	0.000	0.083	0.052	0.001	0.000	0.001		
Arg_COI(1)	0.000	0.083	0.052	0.000	0.000	0.000	0.000	0.000

A. foliaceus. The same parasite has been earlier characterised using various morphological characters (Moller, 2006; Ali *et al.,* 2010; Mandira *et al.,* 2015; Blazhekovik *et al.,* 2017) and by several other workers. The morphological features, observed in the present study, such as wide, oval dorsoventrally flattened body, features of head, thorax, abdomen, compound eyes, thoracopodes, first maxilla with sclerites, abdominal lobes and spermatheca are consistent with previous studies. Acute haemorrhagic skin wounds, increased production of mucus, erosion of scales, and frayed fins were observed in heavily infested rohu. It is reported that the immune response of fish is weakened by the first infection with the parasite making it more susceptible to bacterial infections (Everts, 2009). Pathology in host includes haemorrhages, disintegration of fins and high mucus production. The same kind of abnormalities accompanied by secondary infection and anaemia in host have been previously witnessed (Noaman *et al.,* 2010; Aalberg *et al.,* 2016; Blazhekovik *et al.,* 2017).

Morphological identification of crustaceans is time-consuming and very often requires highly trained taxonomists (Adriana *et al.,* 2009). Previously, morphological characterisation keys were the only means employed for the identification of organisms. However, these traditional methods of identification pose problems in case of closely related species. In this context, DNA barcoding is a practical tool for species identification, when morphological classification of an organism is difficult (Bjorn *et al.,* 2014). Species-level characterisation can explain more about its pathogenicity, host specificity, life cycle and so on. Understanding the limitations to demarcate boundaries between species, DNA barcoding is becoming the standard tool for species identification (Bucklin *et al.,* 2007; Costa *et al.,* 2007). Here, based on morphological characters, the parasites were preliminarily identified as *A. foliaceus.* Nevertheless, the described morphological attributes may not be adequate to confirm the species level identity of the parasite. Hence mitochondrial CO1 gene was used to resolve the taxonomic ambiguity (Ruber *et al.,* 2006; Erguden *et al.,* 2010) and thereby facilitating the discrimination of morphologically similar species (Mehnus *et al.,* 2016).

During the past several years, DNA barcoding has been more extensively used in crustaceans (Michael *et al.,* 2015), mainly because DNA sequences can be used to identify species at all developmental stages, especially for the parasites with complex life cycles (Criscione *et al.,* 2005). COI sequences are more superior than other markers for delineating species of important and taxonomically difficult pathogens (Sean *et al.,* 2010). The ratio of interspecific to intraspecific variation was much higher than the threshold (10×) proposed by Hebert *et al.* (2004) as a species boundary. It was clear from the genetic distance study, upon comparing with the available *Argulus* sequences, the test species could be *A. foliaceus*. To further confirm this result, phylogenetic analysis was done using Kimura-2-parameter (K2P). In previous studies COI sequences along with other mitochondrial and nuclear markers have been frequently used to reconstruct the phylogeny of various taxa of Crustacea (Blanco-Bercial *et al.,* 2011; Matzen *et al.,* 2011; Klaus *et al.,* 2013). The current study does not reconstruct the phylogeny of *Argulus* species, but an endeavour to differentiate the species using phylogenetic clustering pattern, resulted in a clear clustering of the test organism with *A. foliaceus* and thereby confirmed the species as *A. foliaceus*. Appropriate morphological and molecular identification can generate knowledge on species diversity and biology which will help in devising better immunoprophylactic measures (Saravanan *et al.,* 2017; Tandel *et al.,* 2021). The present study employed species delineation by DNA based molecular method to complement the finding of classical morphological taxonomy.

Precise species identification is a prerequisite for development of control measures against any pathogen. Our study following, both morphological characteristics and molecular investigation, confirmed the test species to be *A. foliaceus*. These ectoparasites are creating widespread

problem in the aquaculture ponds of the Eastern region of India, specifically around the state of Bihar and imparting huge loss to the farmers in terms of time and money. This scientific output invites future research efforts to link pathogenicity, population dynamics and life cycle of the parasite so as to have a better understanding about the host-parasite relationship and critical environmental issues regulating the transmission of this parasite. In a nut shell, comprehensive identification of *A. foliaceus* through this study not only alarms about its emergence but also adds knowledge on biology of this species to develop better management strategies in the context of fish health management.

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