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RESEARCH ARTICLE

MOLECULAR IDENTIFICATION OF THE PARASITE *JORYMAHILSAE* FROM *RASTRELLIGER KANAGURTA* (CUVIER, 1816)

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ABSTRACT

The parasitic infestation of isopod parasites in the commercially important fish *Rastrelliger kanagurta* was investigated. Genetic profiling of the *Jorymahilsae* from *Rastrelliger kanagurta* was the main aim of the study.The crustacean parasites along the south-west coast of India is high in *R. kanagurta*. *Jorymahilsae* occupying the entire branchial chamber of the *Rastrelliger kanagurta* may produce pressure on the gill surface and thus affect the efficiency of respiration. Although, the infestation does not cause immediate death, it affects the normal growth and appearance of the host fish. This may lead to economic losses among *Rastrelliger kanagurta*.

Key words: Infestation, Isopod, Parasite, *Rastrelliger kanagurta*, Genetic profiling – Blast Results, *Jorymahilsae*.

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INTRODUCTION

Cymothoids are obligatory parasites infesting many of the commercially important fishes. They are protandric hermaphrodites and bloodsuckers, living on the skin, gill filaments, or in the mouth of the fishes. These parasites retard growth and cause emaciation followed by death. Pathological conditions resulting from parasitic diseases in fishes reaches a high magnitude of epidemics under crowded and other unnatural conditions (Ravichandran *et al.,* 2007). Isopod parasite of the family Cymothoidae has been reported in about 350 species of fishes. Over 80% of these are from tropical and subtropical seas, many are from the Indo-Malaysian archipelago (Lester and Roubal, 1995). Their life cycle involves only one host (holoxenic cycle) (Trilles, 1994) and usually these are large sized parasites, which can cause deleterious effects on the host fishes (Trilles, 1996). The information regarding cymothoid fauna of marine fishes from the Indian coasts is scanty (Pillai, 1954; Bal and Joshi, 1959; Veerapan and Ravichandran 2000). Most of the studies were from the east coast of India (Ravichandran *et al.,* 1999, 2009; Ravichandran and Rameshkumar, 2004; Rajkumar *et al.,* 2004, 2005; Ravichandran 2007; Rameshkumar and Ravichandran, 2010 a, b, Rameshkumar *et al.,* 2011). Ravichandran*et al.* (2009) reported infestation of *Rastrelliger kanagurta* with cymothoid isopod, from Colachel, south-west coast of India.

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Mance (juvenile parasitic stages of cymothoid) feed voraciously and kill fry and fingerlings of several species of fishes.Permanently attached adults parasites stunt the growth of fish and retard reproduction process. Parasites in the gill chamber usually lead to stunted and anaemic gill conditions. Isopod infections can lead to severe economic loss in culture operations (Bragoni, 1984). According to Rameshkumar. (2011) all the species of Joryma are reported from the North-western Indian Ocean. *J. hilsae, J. engraulidis, J. tartoor*and *J. brachysoma,* from the South-western coasts of India and only one species *J. sawayah* from Kuwait. They are often abundant ectoparasites attached to the skin, gills or inside the buccal cavity. Single isopod can cause damage with their biting and sucking mouth parts. Heavy infestations of parasitic juveniles can kill small fish when they first attach (Noga, 2000). These cymothoids have a variety of pathogenic effects, causing direct damage not only to skin, gills and tongue at the site of attachment (Brusca, 1978; Adlard and Lester, 1994), but also indirectly affect host condition, physiological performance and reproductive output (Romestand, 1979; Ostlund-Nilsson *et al.,* 2005). Studies pertaining to pathological effects of isopod parasites on the physiology of host are scanty, and few studies have been made along the Colachel coast environment. (Pillai, 1954; Bal and Joshi, 1959; Veerapan and Ravichandran 2000). Most of the studies were from the east coast of India (Ravichandran *et al.,* 1999, 2009; Ravichandran and Rameshkumar, 2004; Rajkumar *et al.,* 2004, 2005; Ravichandran 2007; Rameshkumar and Ravichandran, 2010 a, b, Rameshkumar *et al.,* 2011). The present study reports the infection of an isopod parasites (*J. hilsae*) in the Indian *Rastrelliger kanagurta which*is a commercial important fish,it

plays an important ecological role and maintains a balance ecosystem. Hence the present attempt was made to study the effect of isopod infestation on such fish. (Ravichandran *et al*., 2009). DNA barcoding aims to provide an efficient method for species-level identifications and, as such, will contribute powerfully to taxonomic and biodiversity research. As the number of DNA barcode sequences accumulates, however, these data will also provide a unique 'horizontal' genomics perspective with broad implications.

MATERIALS AND METHODS

The fishes for the study were collected from the three (Neendakara, Thangassery and Azheekal) main fish landing centersof Kollam District. Altogether 50 specimens of *R. kanagurta* ranging in size from 11 to 14cm (Fig.1) were collected soon after the mechanized and traditional fishing vessels moored at the harbor. The randomly selected fishes were carefully placed in Ice boxes and moved to the laboratory within an hour.The total length of each fish were measured in centimeters (cm) using measuring tape, while the weight of each fish was taken in grams (g) using a weighing balance. Then, the collected fish samples were dissected and the mesenteric cavity examined for parasites. The gastrointestinal tract was then dissected from the rectum to the oesophagus and parasites encountered were carefully detached from the stomach or intestinal mucosa. The internal organs of each fish were also examined for parasites or cysts. The parasites from each fish were then fixed in 70% alcohol. *Rastrelliger kanagurta* belongs to the classification

***Rastrelliger kanagurta* (Cuvier, 1816) (Fig.1)**

Figure 1. Indian mackerel *Rastrelligerkanagurta* (Cuvier 1817)

DNA Isolation

The parasite was ground with a pre-cooled (-20°C) mortar and pestle with liquid nitrogen until a fine powder obtained. Ground insect (2.0g) was resuspended in extraction buffer (12.5 ml) consisting of 200 mMTris – HCl (pH 8.5), 250 mMNaCl, 25 mM EDTA and 0.5 % SDS, after which phenol (pH 7.9) (8.75ml) preheated to 60°C was added followed by the addition of chloroform/isoamylalcohol [24:1 (v/v)] (3.75 ml)]. The suspension was carefully inverted a few times. After centrifugation (12000 rpm in a centrifuge, Sigma-Laboratory Centrifuge, Germany) for 60 min at 4°C the top liquid phase was removed containing the DNA. To remove excess RNA from the liquid phase 500 µl (5 mg/ml) RNase H was added and incubated for 15-20 min at 37°C. Equal volume of phenol was added to the mixture after incubation with the RNase and the mixture was again centrifuged (12000 rpm for 20 min) at 4°C. The liquid phase was removed and the DNA was precipitated with $1/10^{th}$ volumes of isopropanol. The mixture was centrifuged (12000 rpm for 15 min) at 4°C and the resulting pellet was washed with 70 % (v/v) ethanol. The sample was centrifuged (12,000 rpm for 2 min) at 4°C after which the ethanol was aspirated and the pellet was dried under vacuum. The pellet containing the isolated DNA was then dissolved in 1 ml TE buffer 10 mMTris (pH 8.0) and 1 mM EDTA and stored at -20°°C for further manipulation. After complete DNA isolation the presence of DNA was checked by Agarose Gel Electrophoresis.

- **PCR Amplification of CO1 gene Primers Used :**

(Forward Primer) Sequence (5'-3'),
TATTATTAGACAAG AATCTGGTAAA,
(Reverse Primer) Sequence (5'-3')
AGGAAATGTTGAGGGAAGAAAGTAA

The following sets of primers were used for the amplification of CO1 gene. The annealing temperature of the primers was also standardized by running at different temperatures.

PCR Amplification

PCR reactions for CO1 gene amplifications were carried out in Biorad Thermocycler, employing the CO1 gene primers

(Forward Primer) 5'-
TATTATTAGACAAGAATCTGGTAAA -3' and

(Reverse Primer) 5'-
AGGAAATGTTGAGGGAAGAAAGTAA-3'.

PCR amplifications were performed in 25µl reactions containing 1X assay buffer with 1.5mM $MgCl_2$, 5p moles of each primer, 200µM dNTPs, 1.5UTaq DNA polymerase and 20ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. Commercially available 100 base pair ladder was used as standard molecular weight DNA marker to determine the weight of amplified product.

- **PCR-Product Electrophoresis:** Loaded 10µl of PCR product with 4µl loading dye in 1.5% Agarose gel. Ran the gel at constant voltage of 100V and current of 45A for a period of 1 hour 20 minutes till the bromophenol blue has travelled 6cm from the wells. Viewed the gels on UV transilluminator and Photograph of the gels was taken.
- **Purification and DNA Sequencing of Samples:** The amplified products along with forward primer were sent for purification and DNA sequencing.
- **Analysis of Data:** The sequenced data obtained were again confirmed with NCBI-BLAST program, the species identification

RESULTS

Infestation of Rastrelliger kanagurta

Out of 50 specimen of *R. kanagurta* collected from the Kollam coastal environment, and examined of which 20 fish were infested. Isopods, *Jorymahilsae* (Fig.2) inhabiting the buccal cavity and branchial chamber were identified. In *Rastrelliger kanagurta* the site of infection in the buccal cavity of the

parasite. The site of attachment of the parasite, indicative of mucus and blood feeding were found at the time of observation. The parasites were normally seen protruding through the mouth opening of the host. The studies have found that parasitic infection may reduced or interfere with the ability of the host. Gross lesions observed in the buccal cavity of infested fish showed small pin-holes in the tongue region, through which dactyls of pereopod's penetrating claws dig into the host tissues.

The isolated parasite belonged to family cymothoidae.
The parasite belongs to the following classification:

Kingdom : Animalia
Phylum : Arthropoda
Subphylum :Crustacea
Class : Malacostraca
Order : Isopoda
Family :Cymothoidae
Genus :*Joryma*
Species :*hilsae*

(a)

(b)

Figure 2. Isopod parasite *J.hilsae*the (a) branchial region and (b) buccal cavity of *R. kanagurta*

(c)

(d)

Figure 3. Dorsal and ventral view of parasite Larvae of *J.hilsae*

Isolation of parasite DNA

To confirm the species, the DNA of the parasite was isolated. After this, the DNA isolated are undergone Agarose Gel Electrophoresis with 0.8% Agarose. The result is shown in the figure (5). After the DNA isolation, the sample is undergone Polymerase Chain Reaction for the amplification of DNA sample isolated. The result of PCR is shown in the figure.

>SR873-CO
CACCTTATATTTTTTATTTGGGATCTGAGCTGGATTTC
TAGGAGTAGCATTTAGGGTAATTATCCGAGCTGAATT
AGCTCAACCGGGTTCATTCATTGGTAGAGATCAAACC
TATAATGCTATCGTAACAGCCCACGCATTTATTATAA
TTTTCTTTATAGTTATACCTATTATAATTGGAGGTTTT
GGTAATTGACTTGTCCCCCTTATAATCAGAGCACCAG
ATATAGCCTTCCCACGAATAAACAATATAAGATTTTG
ACTTTTACCCCCAGCTCTCACTCTTCTTGTTGTAGGAG
CTTTTGTAGAAGAAGGTGCAGGCACAGGATGAACCG
TCTATCCGCCATTATCCGGAAGATCAGCCCATAGAGG
AGCTTCTGTCGATTTTTCAATTTTCTCCCTTCATTTAG
CAGGAGTTTCATCCATCTTAGGAGCCGTAAATTTTAT
TACAACAATTATCAATATACGACCTTCTACAATATTT
TTACCACGAATGCCTCTTTTCGTTTGATCTATCTTTAT
CACAGCTATCCTACTTCTTCTCTCCCTTCCAGTTCTAG
CAGGAGCTATTACTATATTATTAACAGACCGAAATCT
TAATACATCCTTTTTTGATCCTAGAGGAGGAGGTGAT
CCAATTTTATTTCAACATTTATTT

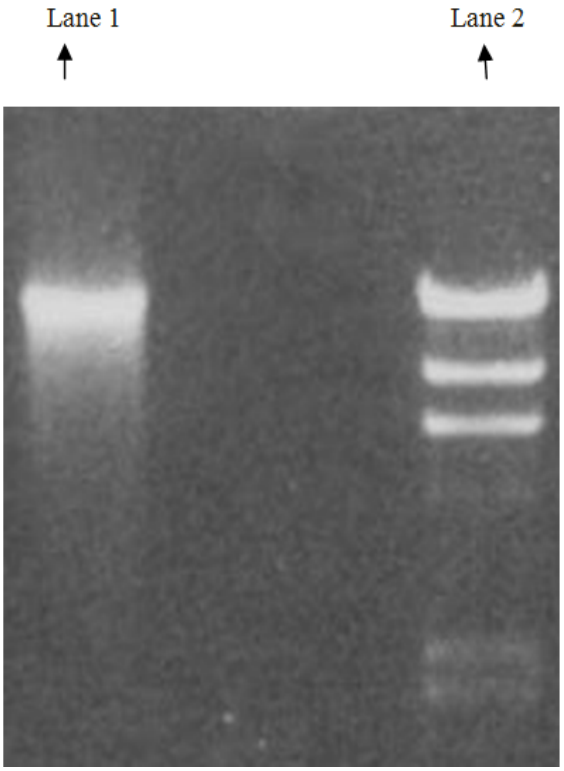

Figure 5. Agarose Gel Electrophoresis of isolated DNA sample of insect (Lane 1: Genomic DNA-*approx.*10, 000kb, Lane 2: Marker –lambda phage Hind III ECORI doubledigest)

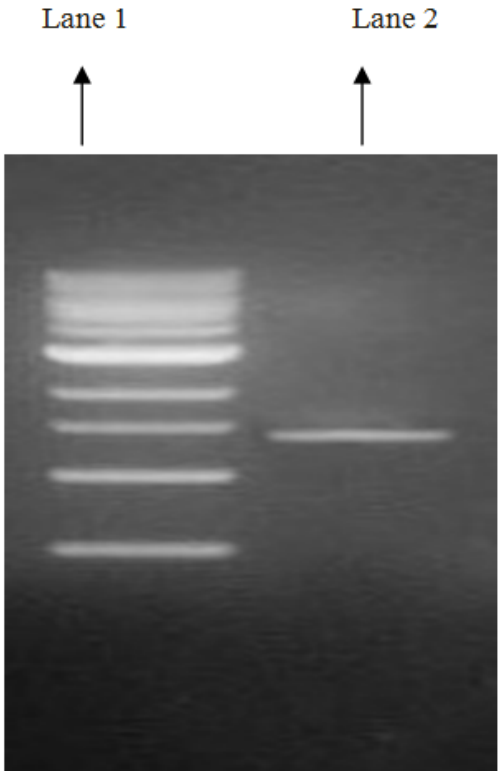

Figure 6. Agarose Gel Electrophoresis of amplified PCR products (Lane 1: 2-1kb marker, Lane 2: Amplicon)

NCBI Blast:Nucleotide Sequence (658 letters)

https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_511106484

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Joryma hilsae voucher CASMBAUTRLC8 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	819	819	70%	0.0	99%	KC896399.1
Alydus eurinus voucher UAM:Ento:142520 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	455	455	99%	8e-124	79%	KU874066.1
Alydus eurinus voucher CNC#HEM300461 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	455	455	99%	8e-124	79%	KR038096.1
Alydus calcaratus voucher 10BBCHEM-1341 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	453	453	99%	3e-123	79%	KR579184.1
Alydus conspersus voucher CNC#HEM301331 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	453	453	99%	3e-123	79%	KR039616.1
Alydus eurinus voucher CNC#HEM301334 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	453	453	99%	3e-123	79%	KR034868.1
Endoxyla sp. ANIC3 voucher 10ANIC-09223 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	449	449	91%	4e-122	80%	HQ951902.1
Alydus conspersus voucher CNC#HEM301333 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	448	448	99%	1e-121	79%	KR040556.1
Tropidophlebia costalis voucher BC_ZSM_HETA_0885 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	446	446	91%	5e-121	80%	KM022512.1
Antiopala ebenospila voucher CCDB-15837-D09 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	446	446	99%	5e-121	79%	KF400960.1

The above organism showed 99% similarity to *Jorymahilsae* a new species of parasite reported in 2011. From the blast results we find that the CO1 sequence shows 99% similarity to *Jorymahilsae*

DISCUSSION

The study based on examination of 50 specimens of *Rastrelliger kanagurta* collected from Neendakara, Kollam revealed the isopod parasite of the Indian mackerel to be fairly rich comprising *Cymothoaexigua.* The Indian Mackerel *Rastrelliger kanagurta* is an important food fish commonly consumed in South and Southeast Asian countries. Though the trans-boundary species is harvested by different nations, its population genetics is relatively unknown to the scientific world. It is of such importance that it contributed an average 8.8 per cent of the total marine fish production in the country. Its average annual catch was estimated to be 0.27 million tonnes, according to available data. Unambiguous identification of the parasites helps to assess the stock, evolve fisheries management methods and protective measures to sustain the regional fisheries; hence DNA bar coding of the parasites was done. Parasitic crustaceans are the largest fish parasites, which cause considerable damage to their hosts. Isopods inhabiting the buccal cavity and branchial chamber of the fish, inflict damage to gills through attachment and feeding and that the extent of damage is directly proportional to the size of the parasites and duration of settlement. *Jorymahilsae*, or the tongue-eating louse, is a parasitic isopod of the family Cymothoidae. This parasite enters fish through the gills, and then attaches itself to the fish's tongue. The female attaches to the tongue and the male attaches on the gill arches beneath and behind the female. Females are 8–29 millimetres (0.3–1.1 in) long and 4–14 mm (0.16–0.55 in) in maximum width. Males are approximately 7.5–15 mm (0.3–0.6 in) long and 3–7 mm (0.12–0.28 in) wide *(RichardC. Brusca, 1981).*. The parasite severs the blood vessels in the fish's tongue, causing the tongue to fall off. It then attaches itself to the stub of what was once its tongue and becomes the fish's new tongue.

Jorymahilsae extracts blood through the claws on its front causing the tongue to atrophy from lack of blood. It appears that the parasite does not cause much other damage to the host fish(*R. C. Brusca; M. R. Gilligan,1983),* but it has been reported by Lanzing and O'Connor (1975) that infested fish with two or more of the parasites are usually underweight (*Ruiz-Luna, Arturo, March 1992).* Once *Jorymahilsae* replaces the tongue, some feed on the host's blood and many others feed on fish mucus. This is the only known case of a parasite assumed to be functionally replacing a host organ. When a host fish dies, *Jorymahilsae* will detach itself from the tongue stub after some time, leave the fish's mouth cavity, and can then be seen clinging to its head or body externally. It is not fully known what then happens to the parasite in the wild. Infestation causes serious problems to host animals either directly or indirectly affecting the physiological status of host. The main factors determining the fish parasite fauna as well is intensity and prevalence of infestation in marine environments. In the parasitized host, fish to the bodyweight was found reduced. The loss of weight can be, attributed to more than one cause and the most obvious one is the loss of food reserve drawn from the variousdepots and other tissues to help in coping with the range of infestation. The parasite occupies the entire branchial chamber of the host may produce pressure on the gill surface and thus affecting the efficiency of respiration. Although, the infestation did not cause immediate death, it had affected the normal growth of the host fish. They may lead to economic loses among the fish species. From the 50 Indian Mackerals collected for the study, at least 20 of them were infected with the isopod parasite. The 40% of infected fish from such a small sample indicates that this is not a negligible factor to be ignored and it highlights the need to undertake much detailed advanced studies. DNA barcoding of the parasites was utilized to aid in the precise identification of the parasites. The study employed DNA barcoding to provide a standardized measure of sequence, specifically examining the extent of sequence diversity in the 648-bp region of the cytochrome *c* oxidase 1 (COI) gene that has been adopted as the standard barcode for members of the animal kingdom. From the blast results for the DNA barcoding, we find that the CO1 sequence shows only 92% similarity to *Cymothoa*so it was interpreted to belong to *Cymothoa*sp. But phenotypically and based on peculiarities of growth on fish it was tenatatively identified as *Cymothoaexigua*. However, due to lack of CO1 sequence of *C.exigua*sp in NCBI database we cannot compare the obtained sequence.

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