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Molecular characterization and phylogenetic analysis of cyclopoid copepod *Mesocyclops leuckarti* using mtCOI

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Abstract: Copepods are the most abundant metazoan zooplankton amongst multicellular animals. The present study was performed to establish the sequence variation of partial mitochondrial Cytochrome c Oxidase I gene (COI) from *Mesocyclops leuckarti* collected from the Retteri Lake, Chennai in order to identify and describe their genetic divergence along with the phylogenetic relatedness with other species. DNA of individual *M. leuckarti* was extracted and the partial mitochondrial COI gene was successfully amplified using the universal primers LCO-1490 and HCO-2198. A 576bp partial mitochondrial COI gene sequence was obtained. Analysis of partial COI sequences of *M. leuckarti* exposed 93% similarity amongst all the individual of copepods selected from Genbank. The obtained COI sequences of Cyclopoid copepod was confirmed with BLAST analysis. Phylogenetic analysis of *M. leuckarti along* with selected out groups from different taxa level further supports the clarity and maintained the authentic of taxonomy up to the subclass level: Copepoda. The results showed that, the COI barcoding of cyclopoid copepod species could be distinguished from the others very clearly. Thus, it strongly indicated that COI may be a useful construction of a comprehensive DNA barcode database for copepods inhabiting the freshwater bodies in Chennai.

Key words: Copepod; COI; DNA Barcoding; *Mesocylops leuckarti.*

Introduction

Copepods are numerically abundant and biologically significant tiny aquatic crustaceans. At present, copepods are gaining attention in the aquaculture sector as live feed [1, 2]. Generally, copepods are recognized based on the structure of 5th leg, urosomal segments, length of antenna, observations of the genital segments and caudal setae. Nevertheless these characters show only minor morphological variations and can hold back the correct identification of species [3]. Additional, morphological identification of copepods requires dissection of their body parts and since they are fragile and easily get damaged, they require expertise for the dissection and their careful examination under the microscopes. So, there is a need for a feasible, fast, consistent and precise technique in copepod species differentiation owing to their abundance and morphological ambiguity. Molecular data such as DNA sequences give complementary and revealing data for systematic studies of copepods to determine their evolutionary association, taxonomy and even function of specific genes [4].

In recent times, different kinds of molecular techniques are being employed for the identification and discrimination of intimately related species at their any of their development stage. Between these, DNA barcoding technique is considered more advanced, where different kinds of nuclear and mitochondrial gene markers are being used. As every marker gene has its own merits and demerits, the formation of multiple gene barcode information of each species is more sensible [5]. *Mesocyclops sp.* is one of the leading cyclopoid copepod and it mainly inhabits the freshwaters. The formation of DNA based identifying information in all copepod species over the world is prudent as it has the potential to provide definite species identification, thereby overcoming the taxonomic complexity. Substantial morphology based taxonomic literatures are available on various regional strains of copepod, whereas, the DNA based studies are very scanty. In India, molecular taxonomic studies of copepods are very fewer compared to the other parts of the world [6]. Therefore, the present study pertains to molecular account of cyclopoid and calanoid copepod strains in Indian region using the gene markers mtCOI.

Materials and Methods

Sample collection

Zooplankton samples containing *Mesocyclops* were collected from Retteri Lake (Fig.1) through the plankton net made up of nylon cloth (mesh size 120µm). The collected copepod samples were rinsed with the filtered lake water and preserved in 95% ethanol for further analysis. Identification of the specimens was based on the specific literature keys of [7]. Total DNA was extracted from 5 specimen of *M. leuckarti.*

Genomic DNA and PCR Amplification

Genomic DNA was isolated from copepods by modified standard procedure of Phenol chloroform

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method [8]. The PCR amplification was performed by using standard primers Cop-COI 1498 and Cop-COI-2190. PCR reaction mixtures of 25 µl contained: genomic DNA template 3 µl, deionized water 9.1 µl, Master mixer 12.5 µl and each forward and reverse primer 0.2 µl. In total 40 PCR cycles were carried out with the initial denaturation at 94˚C for 1 min, followed by the annealing at 45˚C for 2mins and extension at 72˚C for 3min and final extension at 72˚C for 7min. PCR products were kept at -20˚C until further use. PCR amplified products were electrophoresed in 1.5% agarose gels containing ethidium bromide (0.5 µg/mL) and then visualized under UV illumination using a gel documentation system (GELSTAN 1312, Mediccare, India).

Figure 1(A): Sampling place of Retteri Lake

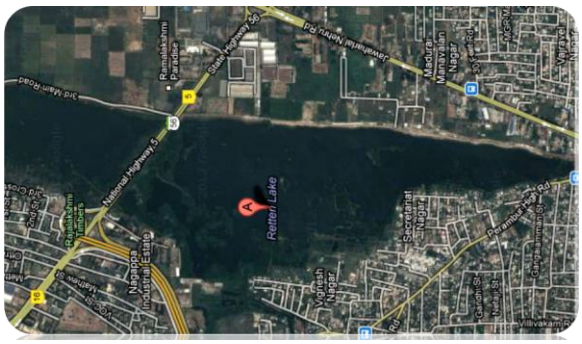

Figure 1(B): Satellite Image

Data processing and sequence analyses

PCR positive samples were sent to Eurofians sequencing Pvt. Ltd., Hyderabad for further sequencing with the forward and reverse primers. The sequences were initially edited with Gene tool and Bio-edit software packages and edited sequences were submitted to NCBI Gen Bank database. Multiple alignments to sequences were done by Clustal X 2.0.11, which is used to determine levels of differentiation within species and between genera. Phylogeny analysis was carried out by Neighbor Joining (NJ) method using molecular evolutionary genetic analysis (MEGA) program version 5. The pairwise nucleotide sequence distance and their divergences between the species in each marker were performed [9].

Results

The isolated genomic DNA and the PCR amplified genomic DNA (583bp) showed in Fig 2 & 3. BLAST analysis was conducted on sequencing data of copepods (*M. leuckarti*) by comparing the partial mitochondrial CO1 gene sequences of *M. leuckarti, M. edax, M. pehpeiensis, T. crassus* with the online database of GenBank (KF357729.1, KF357726.1, KJ020571.1 and HM045300.1). All of the BLAST hits showed significant similarity with the 5 individual of partial CO1 gene sequences. All of the hits retrieved from GenBank database were nucleotide sequences of partial mitochondrial CO1 gene despite of species, hence verifying the CO1 origin of *M. leuckarti* samples.

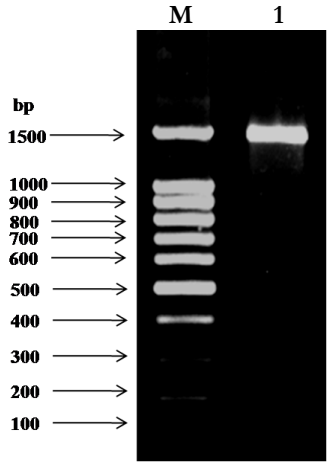

Figure 2: Agarose gel Electrophoresis (0.7%) of genomic DNA isolated from *M. leuckarti*. (M- Marker; 1-Genomic DNA)

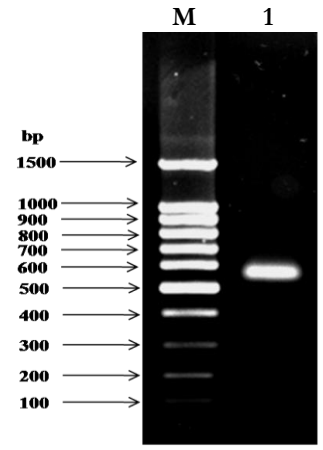

Figure 3: Agarose gel Electrophoresis (1.5%) of PCR amplified DNA product of Copepod (*M. leuckarti*) mtCOI gene (M- Marker; 1-PCR Product)

MtCOI marker gene sequence of *M. leuckarti* was submitted to NCBI (Acc.No. KY412772) and its relatedness were compared with earlier sequences through NCBI-BLAST. The present sequence had 93% similarity with the earlier published conspecific individuals of *M. leuckarti* which showed 89% and 90% similarity with intrageneric species *M. pehpeiensis* and *M. edax*. Further, intrageneric species of (*T. crassus*) cyclopoidae family showed 91% similarity with the present sequence. *C. discaudata* showed 82% similarity with the *M. leuckarti*. In total, five sequences were

retrieved. One was conspecific individual (*M. leuckarti*) and two was congeneric (*M. pehpeiensis, M. edax*) and one was intergeneric species of genera (*T. crassus*) and the other one belong to Candacidae family (*C. discaudata*).

The submitted mtCOI sequence containing 583bp positions were aligned with the retrieved sequences of the NCBI: unaligned sequences were deleted at both sides for all analysis. The Neighbour joining phylogenetic tree dendrogram of *M. leuckarti* with other related species is presented in the Figure 4. The dendrogram shows clear separation between the members of the genera *T. crassus* and out group Canadacidae family at first hierchial level. In the genera Thermocyclops, conspecific individuals are clumped as one group at second hierchial level. Further, inter and intra generic species are delineated from *M. leuckarti* in subsequent levels. It is clearly evident that inter and intra generic species have considerable sequence differences between them. The present mtCOI sequence of *M. leuckarti* showed 4bp differences with earlier published conspecific individuals.

A range between 0.003-1.457 was observed for the pairwise nucleotide distances comparison of *M. leuckarti* COI gene sequences with the COI gene sequences of selected Cyclopoid copepod out groups (*M. pehpeiensis, M. edax, T. crassus*) (Table 1). The inter species divergence rate calculated between different freshwater copepod species was ranged between (0.25- 1.457). The intra species divergence was (0.000-0.003) between *M. leuckarti* of subjected and retrieved species and the species selected from the Genbank (KF357729.1). On the other hand, huge pairwise nucleotide distances of 0.75-2.429 were observed between copepods of the order cyclopoida (including *M. leuckarti*) and the order Calanoida (*C. discaudata*).

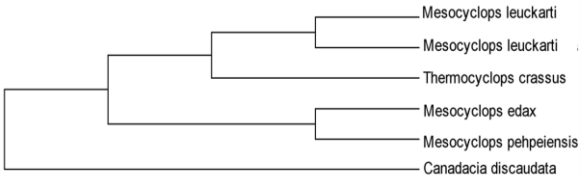

Figure 4: Dendrogram of partial mitochondrial COI gene region of *M. leuckarti* and selected out groups. Unrooted mitochondrial COI gene tree was reconstructed by Neighbour-Joining (Saitou and Nei, 1987) using Kimura 2-parameter; tree was bootstrapped 1000x.

Table 1: Pair wise nucleotide distance (Kimura 2-parameter) for partial CO1 gene sequences between *M. leuckarti* and out groups.

Species Name	*M. leuckarti*	*M. leuckarti*	*M. pehpeiensis*	*T. crassus*	*C. discaudata*
Mesocyclops leuckarti	0.000				
Mesocyclops leuckarti	0.003				
Mesocyclops edax	0.809	0.255			
Mesocyclops pehpeiensis	0.656	1.457	0.203		
Thermocyclops crassus	0.595	0.665	0.757	1.102	
Candacia discaudata	1.127	1.776	2.493	2.429	0.75

Discussion

The recognition of cryptic species is necessary for conservation planning and research on this theme has improved exponentially over the past two decades mostly because of the rising accessibility of DNA sequencing technology. In this study, the recovered 583bp partial mitochondrial CO1 gene sequence was confirmed with Waiho *et al.,* [10]. Where partial CO1 gene sequences of 576bp was recovered using the same universal primers Waiho *et al.,* [10]. Bucklin *et al.,* [11] reported in their study that primers LCO-1490 and HCO-2198 were able to amplify partial mitochondrial CO1 gene sequence of 710bp and readable sequences of approximately 651bp were obtainable. This perfectly matched with the 583bp CO1 sequence implied that *M. leuckarti* samples were tested. This obtained CO1 gene sequence can serve as reference partial CO1 gene sequences of *M. leuckarti* for any future studies such as identification, population studies, intraspecific and interspecific discrimination of *M. leuckarti.*

The high similarity (82%) was observed between a species of Cyclopoid copepod (*M. leuckarti*) and the calanoid studied in this research (*C. discaudata*) further verifies that the partial CO1 gene sequences obtained in this study belongs to subclass Copepoda. Waugh, [12] also experienced similar situations whereby 88% similarity (closest match) to a *Murex troscheli* (sea snail) CO1 sequence was found when they compare the obtained CO1 gene sequence of *Calanus finmarchicus* with CO1 gene sequences in GenBank database.

According to Udayasuryan *et al.,* [13] the genetic distances of the mitochondrial COI gene sequence between various animals taxa (Vertebrate & invertebrate) have been reported and the general ranges for the intra and interspecies distances are 0.0001-0.05 and 0.04-0.21 respectively the most commonly identified divergence rates in crustaceans for mitochondrial COI genes are 1.4% and 2.6% per million years [13]. This study says that a divergence rate of 0.3% between two copepod species of *M. leuckarti* and suggested that they have been separated relatively recently. Patarnello *et al.,* [14] Reported interspecies divergence of 0.500-2.697 between different freshwater prawn species.

Phylogenetic analysis on the partial mitochondrial CO1 gene sequences of *M. leuckarti* and selected out groups (*M. pehpeiensis, M. edax, T. crassus* and *C. discaudata)* using Neighbour-Joining method confirmed the taxonomic hierarchy of *M. leuckarti* up to order level (Copepoda) (Figure 1). Based on this phenogram, it is deducible that all the samples were equally identical in terms of genetic distance and as Cyclopoids *M. leuckarti* is more closely related to other cyclopoid copepods compared to the calanoid copepod (*C. discaudata)*. This confirmation is important, because studies done on copepods (especially CO1 gene) revealed extreme genetic divergence even over short geographical distances [11]. Studies have also shown that some freshwater invertebrates (including copepods) will undergo cryptic speciation, diverging at molecular level but remains morphologically similar [13].

Conclusion

This study, successfully established the DNA barcoding system for *M. leuckarti*. The identification of cryptic species is a major challenge for the understanding of the processes that establish biodiversity patterns throughout the World and the planning of conservation strategies. In the present study, a cryptic *M. leuckarti* species was identified at a site in Retteri Lake, Chennai. Further studies are required in Chennai and other parts of Tamil Nadu, in order to find out the distribution of new species and its phylogeography and genetic variation in the region.

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