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Research paper

Phylogenetic information of freshwater copepod (*Diaptomus sicilis*) with special reference to 18S rRNA

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Abstract

In the present investigation to isolate freshwater calanoid copepods (*Diaptomus sicilis*) was characterized and identify the organisms by 18S rRNA sequencing. Plankton samples containing *D. sicilis* were collected during January 2014 (Post-monsoon) from Madippakkam Lake (12°57'41"N80°11'27"E) Chennai, Tamil Nadu. Immediately after sampling, specimens for genetic analyses were fixed in 95% ethyl alcohol. The total DNA was extracted from the individual copepod *D. sicilis* using Qiagen Blood tissue kit. The nuclear small subunit 18S rRNA gene was amplified using the Universal primer LCO —1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). PCR products were loaded onto a 1% TAE agarose gel. Sequences were carried out an automated sequencer. The nucleotide sequence of 1282 base pair region of 18S rRNA was determined for D. sicilis. The similarity of sequences of *D. sicilis* was retrieved by BLASTn program and maximum identity and E-value was 76% and 0.00, respectively. The PCR products of *D. sicilis* individuals showed 80% similarity with the partial nuclear small subunit 18S rRNA gene region of other calanoid copepods. Based on molecular data the freshwater Calanoid copepods showed different algorithms and similar types of topologies useful for designing molecular analyses using phylogeny tree construction.Present molecular studies on the relationship of D. sicilis with other freshwater calanoid copepods indicate that this species is close to *D. castor* followed by *D. keniraensis.*

Keywords: *Phylogeny; Diaptomus sicilis; 18S rRNA; Calanoid; BLAST.*

1. Introduction

Copepods play a significant role in the food chain of the freshwater ecosystem (Humes 1994). Based on morphological characters, there are 11,500 species of copepods were reported. Copepods well respond to environmental changes and have been recognized as indicators of natural and anthropogenic stress (Hirai et al. 2015). The freshwater diaptomid calanoid Copepods are the most distinct group over 400 species comprised more than 50 genera (Ranga Reddy 1994). In spite of immense taxonomic revision on the basis of detailedminute morphological character, the phylogenetic relationships within and among diaptomid genera remains evasive.In Calanoid copepods, the morphological differences of the male fifth legs play a major role in the classification and identification of species (Ho Young Soh et al. 2012) Molecular taxonomy is an important tool to identify the evolutionary relationship of organisms. Recently, various molecular markers like Internal transcribed spacer (ITS), mitochondrial cytochrome oxidase subunit I (mtCOI) and Restriction fragment length polymorphism [RFLP] has been used to resolve relationships between species and population levels (Sivakumar et al. 2013).

Kanchon et al. (2010) recommended DNA barcoding is a significant and quick method to explore the species its biodiversity assessment. The majority of the researchers used a short section of mitochondrial DNA (mtDNA), the first ~650 bp of the 5' end of the Cytochrome Oxidase. I gene (COI). DNA barcoding has been argued to revolutionize taxonomy allowing rapid species identification without taxonomic expertise at present (Stoeckle et al. 2008). Phylogenetic relationships among calanoid copepods at higher systematic levels (ordinal, familial and generic) have been resolved using the 18S rRNA gene (Bucklin & Steinke 2011). Which can be used to examine the evolution of crustacean orders and families. The lower taxonomic level relationships have been resolved using the ITS2 of the nuclear rDNA gene cluster (Wyngaard et al. 2010).

Combine with mitochondrial genes and nucleic genes would produce more reliable species description than those based on a single gene (Dupuis et al. 2012). One of the candidate nuclear marker is the small nuclear subunit of the ribosomal RNA (rRNA) gene, which is a common molecular marker frequently used in phylogenetic studies (Kruger et al. 2012, Petrov et al. 2014, Fonseca et al. 2014). The nuclear 18S rRNA gene is also favourably used for diversity research in eukaryotes (Tang et al. 2012, Lie et al. 2014 and Zhan et al. 2014). Compared to COI evolution progresses much more slowly in the 18S rRNA gene prospectively making, and it's more valuable marker for discriminating between samples at high taxonomic levels (Thum& Derry 2008). Morphological identification may not effectively capture their inner details (Bucklin 2011). An attempt has been made out of molecular phylogenetic data compared with that of morphological data to clarify copepod Diaptomus sicilis familial relationships of Calanoid.

2. Materials and methods

2.1. Collection and identification of individual

Plankton samples containing Diaptomus siciliswere collected during January 2014 (Post-monsoon) from Madippakkam Lake (12°57'41"N80°11'27"E)Chennai, Tamil Nadu (Fig.1)during the early hours of the day [6-8 am] using 100µm mesh size plankton net at a depth of 40cm for 10 minutes. Immediately after sampling, specimens for genetic analyses were fixed in 95% ethyl

alcohol; those for morphological examination were fixed in 5% formalin-lake water solution. Microscopic examinations and dissections were made in Glycerol using Nikon Microscope. Initial drawings were made with camera lucida interference microscopes. The alcohol was changed after 24 h. The specimens were identified to species level using standard keys (Ranga Reddy 1994).

Fig. 1: Sampling Place of Madipakkam Lake (A), Satellite Image (B), Chennai, India.

2.2. DNA extraction and PCR amplification

The total DNA was extracted from the individual copepod using Qiagen Blood tissue kit. The 18S rDNA gene was amplified using two overlapping fragments. The first fragment was amplified using the forward primer LCO—1490(5'-GGTCAACAAATCATAAAGATATTGG-3') AND HCO-2198(5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The polymerase chain reaction was performed under the following conditions: 25 pmol each primer; 2.0 mM MgCl2; 1× PCR buffer; 2 mM each dNTP; 2.5 units Taq DNA polymerase; and 2–5 µl of DNA extract. The total volume of each PCR reaction was 50 µl. Thermal cycling was performed according to the following: 96˚C for 5 min.; 80˚C while the Taq was added to the reactions; 94˚C for 1 min., 55˚C for 2 min., and 72˚C for 3 minutes for 40 cycles; final extension 72˚C for 8 min. PCR products were loaded onto a 1% TAE agarose gel. Sequences were carried out an automated sequencer.

2.3. Phylogenetic analyses

D. sicilis RNA homology searches were performed using BLASTn 2.2.24 programs at NCBI and similarity sequences were retrieved for phylogenetic analysis. The DNA sequences of the 18S rRNA of all taxa were aligned with ClustalW to create an initial dataset. Likelihood ratio test was performed for determination of substitution model of DNA evolution, and nitrogenous frequency was found out. The data set was analyzed by Distance method UPGMA method and Maximum Likelihood method (Swofford 1998) to resolve phylogenetic relations using Phylip 3.69. The distance method uses nucleotide sequences to compute distance matrix. The distance for each pair of species estimates the total branch length between the two species. ML was implemented to assess substitution basis. The UPGMA method constructs a tree by successive clustering using an average–linkage method of clustering.

3. Results

The nucleotide sequence of 1282 base pair region of 18S rRNA was determined for D. sicilis. The similarity of sequences of D. sicilis was retrieved by BLASTn program and maximum identity and E-value was 76% and 0.00, respectively. Lists of accession number and organisms are presented in Table 1. The PCR products of D.sicilis individuals showed 76% similarity with the partial nuclear small subunit 18S rRNA gene region of other calanoid copepods.

Table 1: Information of Species of Analyzed in this Study.

Accession Number	Description
KM091949	*Diaptomus sicilis*,18S small subunit ribosomal RNA gene, partial sequence
JX945132.1	*Diaptomus cyaneus*, 18S small subunit RNA gene, partial sequence
JX945124.1	*Diaptomus kenitraensis*, 18S small subunit RNA gene, partial sequence
JX945133.1	*Diaptomus mirus*, 18S small subunit RNA gene, partial sequence
JX945123.1	*Diaptomus castor*, 18S small subunit RNA gene, partial sequence
HQ0087591	*Mesocyclops pehpiensis*, 18S small subunit RNA gene, partial sequence

The mean distance between *D. mirus, D. cyaneus, D. kenitraensis, M. pehpeiensis* when compared with that of *D. sicilis* was 3.22.

3.1. Genetic distance

Results were obtained for all 18S rRNA sequence of D. sicilis when compared with selected out-groups in pairwise nucleotide distance analysis (Table 2). Identical results of 0.000were seen in the comparisons between individual of *D. sicilis* 18S rRNA.
A range in between 0.01-0.18 was observed for the pairwise nucleotide distances [Kimura 2-parameter] comparison of *D.sicilis* 18S rRNA gene sequences with the 18S rRNA gene sequences of selected cylcopoid copepods out-group *M.pehpeiensis*. On the other hand, a huge pairwise nucleotide distance of 1.65 – 2.02 was observed between copepods of the order Cyclopoida *M. pehpeiensis* (Table 2).

3.2. Phylogenetic analysis

The molecule based tree constructed using ML method illustrated that it forms one out group M. pehpeiensis. Substitution frequency of A–0.000, T/U–8.86, C–8.86-18.43 and G–0.00-7.96 was calculated between Calanoid species. The 18S rRNA sequences of D.sicilis and selected outgroups were used to create a gene tree using UPGMA method [Fig. 2]. The tree branched out into two main clusters according to different orders as expected withOrder Calanoida on one cluster while the rest of the copepods from the order Cyclopoida on other main clusters.The cyclopoid copepod out groups was resolved with a high bootstrap value of 76%, thus differentiating *D.sicilis* as well.

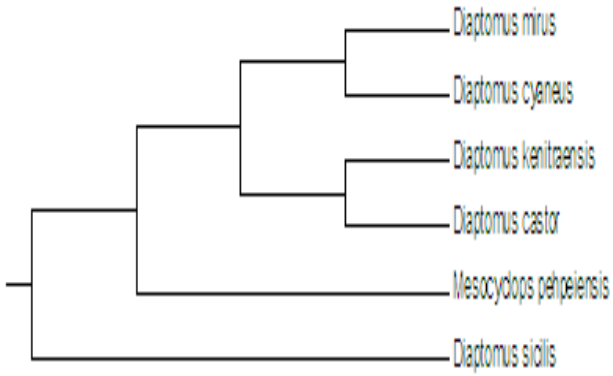

Fig. 2: Phylogeny Relationships of Calanoid Copepods on Molecular-Based Analysis by UPGMA Method..

4. Discussion

The 18S rRNA gene proved to be effective for identifying the calanoid copepod upto species level (Shu et al. 2015). We found that 76% similarity between the species when to use the 18S rRNA which is unfeasible when attempted to achieve a high rate of successful identification due to potential PCR or sequencing errors. The recovered 1282bp nuclear small subunit 18S rRNA gene sequence, this study was in conformation with [23].This This conformation also reflects the ability and effectiveness of Universal Primers LCO-1490 and HCO-2198 described by Folmer et al. (1994) to target partial nuclear small subunit 18S rRNA region in most invertebrates, including copepods.

Table 2: Distance Matrix between Calanoid Copepods

Species	DS	DM	DC	DK	DC	MP
Diaptomus sicilis(DS)	0.00					
Diaptomus mirus(DM)	0.16					
Diaptomus cyaneus(DC)	0.16	0				
Diaptomus kenitraensis (DK)	0.15	0	0.00			
Diaptomus castor (DC)	0.18	0	0.01	0.00		
Mesocylops pehpeiensis (MP)	1.65	1.92	1.93	2.01	2.02	

The 18S rRNA sequences resolve relationships among genera but are not likely to resolve relationships within genera because of the overall slow rate of molecular evolution of ribosomal RNA genes (Hirai et al. 2015) which is reflected by generally low bootstrap values for species relationships within genera. Faster evolving molecules such as mitochondrial DNA genes are generally preferred for resolving such relationships.

This molecule proved to be the molecular markers for eukaryotes used in resolving generic and species-level relationships (Sivakumar et al. 2013). To compare genetic distances within and among taxa used to determine whether a given group of calanoids have diverged on an average more or less than others. The divergence values among members of the calanoids were varying up to 2.02 substitutions per site (Table 2). The highest genetic distance between all calanoid taxa caused by extreme variation in *M.pehpeiensis* (0.01-0.18 substitution per site), which was more than twice as high as for the other order also for higher-orderanalyses of copepod phylogeny (Lie et al. 2014). The lowest variation in Pairwise distance was between *D.castor* [0.00-0.01substitutions per site] (Table 2). In Maximum likelihood method, the variation between species is 7.21-18.43 (Table 2).Thum (2004) claimed that 18S rRNA could be used to evaluate the monophyly of recognized genera and their inter-relationships. Even rapidly-evolving mitochondrial genes often show support for deep notes. Cameron et al. (2004) reported that 18S rRNA had the best phylogenetic signal ratio using Neighbour-joining tree. Mitochondrial Cytochrome Oxidase I (mtCOI) gene proved to be useful to resolve evolutionary relationships among closely related species for a wide range of taxa, especially for calanoid copepods and euphausiids (Bucklin 2011). In most of the species variation of mtCOI within a species is far less than variation between species making the gene as a diagnostic molecular systematic character. While intraspecific mtCOI sequence variation ranged from 0.5% to 2% and interspecific variation generally ranged from 10% to 20% (Bucklin 2011).

Based on molecular data the freshwater calanoid copepods showed different algorithms and similar types of topologies useful for designing molecular analyses using phylogeny tree construction. Present molecular studies on relationship of *D.sicilis* with other freshwater calanoid copepods indicate that this species is close to *D.castor* followed by *D.keniraensis*.

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