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Phylogenetic Analysis of *Thermocyclops decipiens* with Reference to 18S rDNA

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Abstract

Many ecological studies on the diversity and distribution of freshwater planktonic cyclopoid copepods are being published and they depend upon the molecular methods for accurate taxonomic identification. The greater coverage of reference species in the genetic database, GenBank, with the decreasing costs for DNA sequencing, have made large scale plankton identification studies using molecular methods, more feasible. Here, we present a practical molecular approach to identify *Thermocyclops decipiens*, collected from Karapakkam Temple tank, Chennai, India. Molecular identification methods of cyclopoids included amplification of 18S rDNA. The present work on molecular phylogenetic analysis of freshwater cyclopoid copepods deals with the evolutionary relation among 12 species of freshwater cyclopoid copepods. The 18S rDNA sequences were analyzed using ClustalW, Maximum Likelihood method, Distance method and UPGMA method. The Multiple Sequences Alignment showed less score value of 52 between *Thermocylops decipiens* and *Mesocyclops thermocyclopoides*. Among 12 species of freshwater cyclopoid copepods, *Mesocyclops edax* and *Mesocyclops darwini* were single phyletic group in UPGMA method. By Maximum Likelihood analysis of *Mesocyclopos thermocyclopoides*, confident limit was 1.9870–7.30265 with positively significant at $p<0.05$ level and the distance compared with other species was 4.643516–5.873569.

Keywords: Maximum Likelihood, Nuclear 18S rDNA, Phylogeny, *Thermocyclops*, UPGMA.

1. Introduction

The Cyclopida is the most species–rich group among copepod lineages and comprises the largest group within the subclass Copepoda [1, 2]. Molecular taxonomy is an emerging area for identification of species and evolution relationship of the organisms. However, sequencing of a few species of zooplankton (Copepods and Cladocerans) is reported. Recently, ZooGene partnership reported the species identification of calanoid copepods and euphausiids using molecular systematics and gene structures to construct molecular phylogeny and phylogeography.

Members of the copepods are important components of pelagic ecosystems. Proper identification of these species at all life stages is essential for better understanding of early life history characteristics and ecological relationships in the pelagic ecosystem, and to enable effective management. While specific identification of adult is essentially unambiguous [3], identification is problematic in situations where morphological characters are difficult to interpret (early life history stages). Identification of early life history stages of cyclopoids has been challenging. Molecular markers can provide a means for positive identification when morphological identification is uncertain.

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Various molecular markers have been used to identify fish eggs and larvae including Allozymes [4], Polymerase Chain Reaction (PCR)/Restriction Fragment Length Polymorphism (RFLP) analysis [5, 6], Multiplex PCR [7, 8] and Sequencing [9–11]. Many of these techniques have been used to identify copepods and cladocerans.

DNA bar–coding has recently been suggested as a quick method useful for rapid species discovery and biodiversity assessment [12–14]. For animal taxa, the majority of these studies have used a short section of mitochondrial DNA (mtDNA), namely the first ~650 bp of the 5' end of the Cytochrome Oxidase I gene (COI) [15–17]. DNA bar–coding has been argued to revolutionize taxonomy allowing rapid species identification without need for detailed taxonomic expertise with increasing economy [18, 14].

Evolutionary relationships among congeneric copepod and euphausiid species are typically well–resolved by mtCOI sequence variation. mtCOI gene trees were largely concordant with morphological phylogenetic analyses for species of both copepods and euphausiids [19]. Phylogenetic relationships among genera and families of calanoid copepods and euphausiids have been examined using 18S rRNA sequences, which provide accurate resolution [20] and are useful for comparison with morphological analyses. A phylogenetic tree was constructed [38] based on morphological and molecular basis data. Currently, only a few nuclear and mitochondrial markers have been employed to successfully resolve phylogenetic relationships in copepods. The 18S and 28S nuclear ribosomal DNA genes have been used to resolve relationships at the ordinal, familial, or generic levels [20–24], whereas the Internal Transcribed Spacer region II (ITS2) has been used to resolve relationships at the species and population levels [25–28]. Mitochondrial COI and 16S genes appear to contain less phylogenetic signal in copepods relative to other taxa. [29–34, 25, 28]. It is generally accepted that phylogenetic hypothesis is most convincing when supported by data from other sources. Here, we analyse the 18S rDNA of *Thermocyclops decipiens*. We combine molecular phylogenetic data with morphological data to clarify further the familial relationships in cyclopoids.

2. Materials and Methods

2.1 Sample Collection

Zooplankton samples were collected from Karapakkam Temple tank, Chennai. The samples were collected during the early hours of the day using plankton net (75 µm) made up of bolten silk. Samples were collected by towing the plankton net horizontally at a depth of 40 cm for about 10 minutes. Live zooplankton samples were transported to the laboratory within an hour in insulated polyethylene container. *T. decipiens* was reared in the laboratory and fixed in 95% ethanol.

2.2 DNA Extraction, PCR Amplification and Sequence

The DNA was isolated by the Saline Citrate Solution (SCS) method. 200 mg of *T. decipiens* were suspended in 800 µl of SCS and homogenized using mortar and pestle. The homogenate was transferred into fresh centrifuge tubes and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in SCS and centrifuged at 3000 rpm for 5min. The pellet was again resuspended in 400 µl of 2M NaCl solution and centrifuged at 10,000 rpm for 15 min at 4°C. Followed by centrifugation, the supernatant was collected in fresh microfuge tubes. To this, double volume of chilled ethanol was added and allowed for few minutes to precipitate the DNA [35].

The region of the ribosomal 18S gene was amplified using the Polymerase Chain Reaction (PCR). Amplification reactions of the 3' end of the 18S rRNA gene were carried out using the primers 18s329 (5':TAATGAT CCTTCCGCAGGTT:3') and 18sI- (5':AACT(C,T)AAAGG AATTGACGG:3') [34]. Amplification conditions consisted of 5 min at 95^{0}C, for initial denaturation and then 40s at 95^{0}C, 25s at 50^{0}C, 1 min at 72^{0}C for denaturation, annealing, extension, respectively of 40 cycles, and final extension for 15 min at 72^{0}C. DNA sequence was carried out on automated sequencer.

2.3 Phylogenetic Analysis

T. decipiens DNA 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 rDNA of all taxa were aligned with ClustalW to create an initial data set. 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 (ML) to resolve phylogenetic relations using Phylip 3.69. DNA 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 bases. The UPGMA method constructs a tree by successive clustering using an average–linkage method of clustering.

3. Results

The nucleotide sequence of 500base pair region of 18S rDNA was determined for *T. decipiens*. Similarity of sequences of *T. decipiens* was retrieved by BLASTn program and maximum identity and E value was 99% and 0.00, respectively. List of accession number and organisms are presented in Table 1. Sequence similarity between *Thermocyclops crassus*, *Mesocyclops darwini*, *Mesocyclops edax* and *Mesocyclops longisetus* when compared to *T. decipiens* were 0, 2, 2 and 0 gaps by Needleman–Wusch method.

4. Genetic Distance

Multiple sequence alignment similarity score value between *T. decipiens* and *M. thermocyclopoides* was 52 and T. decipiens compared with other cyclopoids ranges from 84.00–99.00. (Table 2). Distance matrix of cyclopoid copepods *T. decipiens* compared with *T. crassus* (0.0060) and *M. darwini* (0.0081) showed high similarity and *M. thermocyclopoides* showed high dissimilarity (5.8736) (Table 3).

Table 1. Information of species analyzed in this study

Accession	Description
GQ848503.1	Thermocyclops crassus voucher USNM1121776 18S small subunit ribosomal RNA gene, partial sequence
GQ848511.1	Mesocyclops darwini voucher USNM1121764 18S small subunit ribosomal RNA gene, partial sequence
GQ848506.1	Mesocyclops edax voucher USNM1121767 18S small subunit ribosomal RNA gene, partial sequence
GQ848509.1	Mesocyclops longisetus curvatus voucher USNM1121770 18S small subunit ribosomal RNA gene, partial sequence
GQ848510.1	Mesocyclops meridianus voucher USNM1121772 18S small subunit ribosomal RNA gene, partial sequence
GQ848515.1	Mesocyclops aspericornis voucher USNM1121763 18S small subunit ribosomal RNA gene, partial sequence
GQ848513.1	Mesocyclops leuckarti voucher USNM1121768 18S small subunit ribosomal RNA gene, partial sequence
GQ848512.1	Mesocyclops major voucher USNM1121771 18S small subunit ribosomal RNA gene, partial sequence
GQ848514.1	Mesocyclops pehpeiensis voucher USNM1121774 18S small subunit ribosomal RNA gene, partial sequence
EF581894.1	Mesocyclops thermocyclopoides 18S ribosomal RNA gene, partial sequence
GQ848516.1	Mesocyclops ogunnus voucher USNM1121773 18S small subunit ribosomal RNA gene, partial sequence

Table 2. Multiple sequence alignment between *T. decipiens* and other cyclopoid copepods

Seq A	Name	Length	Seq B	Name	Length	Score
1	*T. decipeins*	500	2	*T. crassus*	603	99.0
1	*T. decipeins*	500	3	*M. darwini*	603	99.0
1	*T. decipeins*	500	4	*M. edax*	603	99.0
1	*T. decipeins*	500	5	*M. longisetus curvatus*	603	99.0
1	*T. decipeins*	500	6	*M. meridianus*	533	84.0
1	*T. decipeins*	500	7	*M. aspericornis*	602	98.0
1	*T. decipeins*	500	8	*M. leuckarti*	602	98.0
1	*T. decipeins*	500	9	*M. major*	532	84.0
1	*T. decipeins*	500	10	*M. pehpeiensis*	602	98.0
1	*T. decipeins*	500	11	*M. thermocyclopoides*	597	52.0
1	*T. decipeins*	500	12	*M. ogunnus*	602	98.0

Table 3. Distance matrix between cyclopoid copepods

	TD	TC	MD	ME	ML	MM	MA	ML	MlMR	MP	MT	MO
Thermocyclops decipiens (TD)												
Thermocyclops crassus (TC)	0.0060											
Mesocyclops darwini (MD)	0.0081	0.0134										
Mesocyclops edax (ME)	0.0101	0.0134	0.0168									
Mesocyclops longisetus curvatus (ML)	0.0101	0.0202	0.0151	0.0100								
Mesocyclops meridianus (MM)	0.0121	0.0219	0.0168	0.0117	0.0083							
Mesocyclops aspericornis (MA)	0.0162	0.0834	0.0761	0.0779	0.0780	0.0799						
Mesocyclops leuckarti (ML)	0.0162	0.0797	0.0798	0.0742	0.0799	0.0818	0.0033					
Mesocyclops major (MMR)	0.0162	0.0797	0.0798	0.0742	0.0799	0.0818	0.0033	0.0000				
Mesocyclops pehpeiensis (MP)	0.0182	0.0815	0.0779	0.0761	0.0799	0.0818	0.0017	0.0017	0.0017			
Mesocyclops thermocyclopoides (MT)	5.8736	4.7607	4.6435	4.8883	5.0187	5.1708	5.7543	5.7543	5.6500	5.7543		
Mesocyclops ogunnus (MO)	0.0203	0.0855	0.0856	0.0782	0.0839	0.0858	0.0084	0.0050	0.0050	0.0067	–1.0000	

5. Phylogenetic Analysis

The molecule based tree constructed using ML method, illustrated that, it forms two out groups, one is *Thermocyclops* and another is *Mesocyclops*. Substitution frequency of A–0.21967, C–0.24523, G–0.28110, T–0.25400 was calculated between cyclopoid species. Out of 21 clusters, 10 were significantly different at P <0.01 level. Cladostic of *M. thermocyclopoides* and 9 cluster distance level range is 1.9870–7.30265 (Table 4). In the ML method, *M. thermocyclopoides* had high level of substitution to form the cluster with *M. darwini*. In the tree constructed in the present study, *T. decipiens* and *T. crassus* (0.00301), *M. thermocyclopoids* and *M. ogunnus* (–0.5000), *M. aspericornis* and *M. pehpeiensis* (0.00803), *M. leuckarti* and *M. major* (0.0000), *Mesocyclops longisetus curvatus* and *M. meridians* (0.00417). *M. darwini* is either nested or in sister of clade containing *M. major*, *M. leuckarti*, *M. pehpeiensis*, *M. aspericornis*, *M. meridianus*, *M. longisetus curvatus*, *M. edax*. *M. thermocyclopoids* and *M. ogunnus* cluster with other cyclopoids distance was 1.84332. (Figure 1), while in UPGMA method, *T. decipiens* cluster with *T. crassus* and *M. thermocyclopoides* and *M. ogunnus* (1.84332) cluster with other species of cyclopoids (1.30985) (Figure 2).

6. Discussion

The 18S rDNA molecule was evolved slowly within group. This molecule has proven to be molecular markers in eukaryotes, and its use in resolving generic and species level relationships [36] and also in higher order analyses of copepod phylogeny [20, 23, 24]. No inter-population variability in the 3' end fragment of the 18S rDNA molecule was observed in *T. decipiens*, *T. crassus*, *M. darwini*, *M. edax*, *M. logngicetus curvates*, *M. meridianus*,

Table 4. Maximum likelihood phylogenetic analysis

Between	And	Approx. confidence limit
2	*Thermocyclops decipiens*	(0.0000–infinity)
2	1	(0.0000–0.00642)*
1	9	(0.0000–0.01055)**
9	*Mesocyclops thermocyclopoides*	(1.9870–7.30265)**
9	*Mesocyclops darwini*	(0.0000–0.00577)
1	5	(0.0000–0.00807)**
5	6	(0.04830–0.09298)**
6	8	(0.0000–0.00492)*
8	*Mesocyclops pehpeiensis*	(0.0000–infinity)
8	7	(0.0000–0.00493)*
7	*Mesocyclops major*	(0.0000–infinity)
7	10	(0.0000–infinity)
10	*Mesocyclops ogunnus*	(0.0000–0.01070)**
10	*Mesocyclops leuckarti*	(0.0000–infinity)
6	*Mesocyclops aspericornis*	(0.0000–0.00342)
5	3	(0.0000–0.00839)**
3	4	(0.0000–0.00498)*
4	*Mesocyclops meridianus*	(0.0000–0.01067)**
4	*Mesocyclops longisetus curvatus*	(0.0000–0.00794)**
3	*Mesocyclops edax*	(0.0000–0.01081)**
2	*Thermocyclops crassus*	(0.0000–0.01270)**

*= significantly positive, $P < 0.05$
**= significantly positive, $P < 0.01$

M. aspericornis, *M. leuckarti*, *M. major*, *M. pehpeiensis* and *M. ogunnus*.

It is interesting to compare genetic distances within and among taxa to determine whether a given group of cyclopoids has diverged, on average, more or less than others. The divergence values among members of the cyclopoids vary up to 7.30 substitutions per site. The highest genetic distance between all cyclopoids taxa was caused by extreme variation in the *M. thermocyclopoides* and *M. darwini* (1.9870–7.30265 substitutions per site), which was more than twice as high as for the other cyclopoids. They form two basic clades which, based on their difference in genetic distances, should be raised to suborder level. The lowest variation in genetic distance was between *M. aspericornis* 6th node of tree (0.0000–0.00342 substitutions per site).

Mitochondrial Cytochrome Oxidase I (mtCOI) gene has been shown to be very useful to resolve evolutionary relationships among closely related species groups for a wide range of taxa [36], especially for calanoid copepods and euphausiids [37]. For most species, variation of mtCOI within a species is far less than variation between species, making the gene a diagnostic molecular systematic character. While intraspecific mtCOI sequence variation ranges from 0.5% to 2%, interspecific variation generally ranges from 10% to 20% [20].

The reconstruction of *Mesocyclops* phylogeny using the combined character set and limited taxon sampling generally yielded higher levels of support when compared with analyses based on limited taxon sampling and only one type of character [38]. The present study on the construction of phylogeny of freshwater cyclopoid copepods, *Mesocyclops* and *Thermocyclops* species based on 18S rDNA reveals that *T. decipiens* is close to *T. crassus*, with common node of this group cluster with *M. darwini*. The methods used in the present study show two slightly different tree topologies (Figures 1 and 2). However, all methods agree that there are well-separated clades, confirmed by high bootstrap support for cyclopoid species. The support value for a few nodes connecting families is weak and their true phylogenetic relationship remains unsettled.

7. Conclusion

Phylogeny tree construction of freshwater cyclopoid copepods based on molecular data in different algorithm showed similar types of topologies and is useful in designing molecular studies. Present molecular studies on relationship of *T. decipiens* with other freshwater cyclopoid copepods indicate that this species is close to *T. crassus* followed by *M. darwini* in both the trees.

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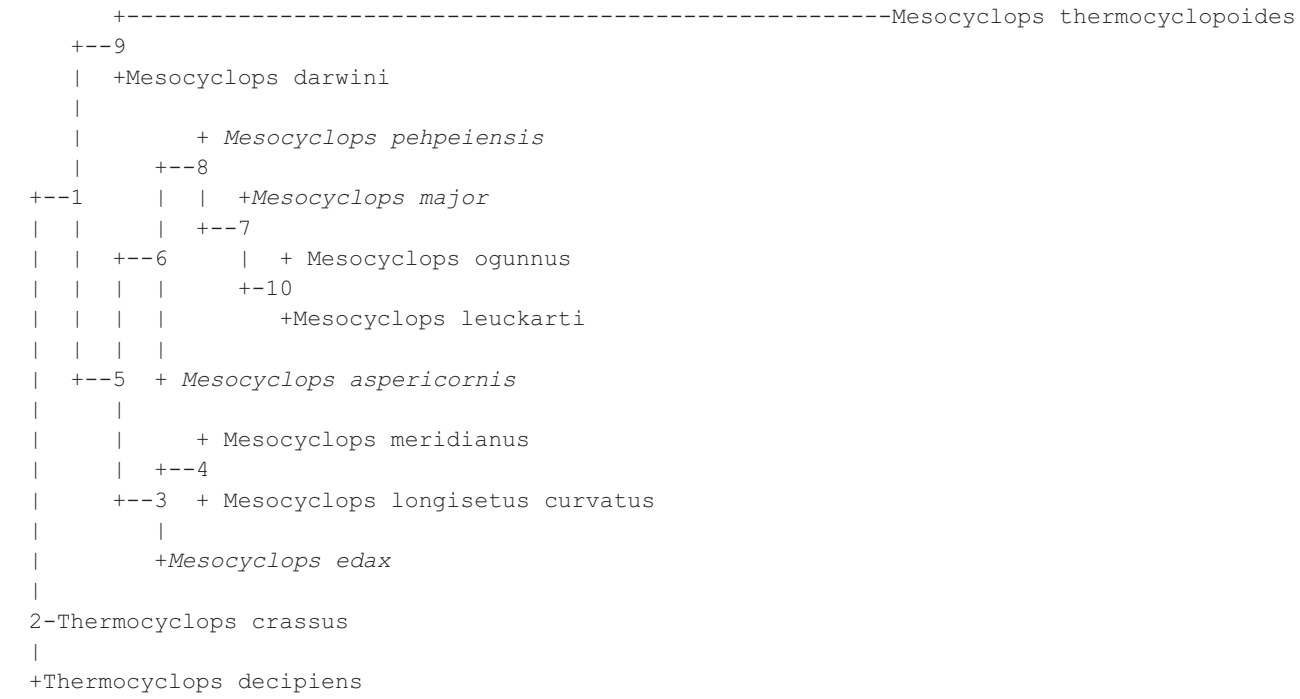

Figure 1. Phylogeny relationships of cyclopoid copepods on molecular based analysis by ML method.

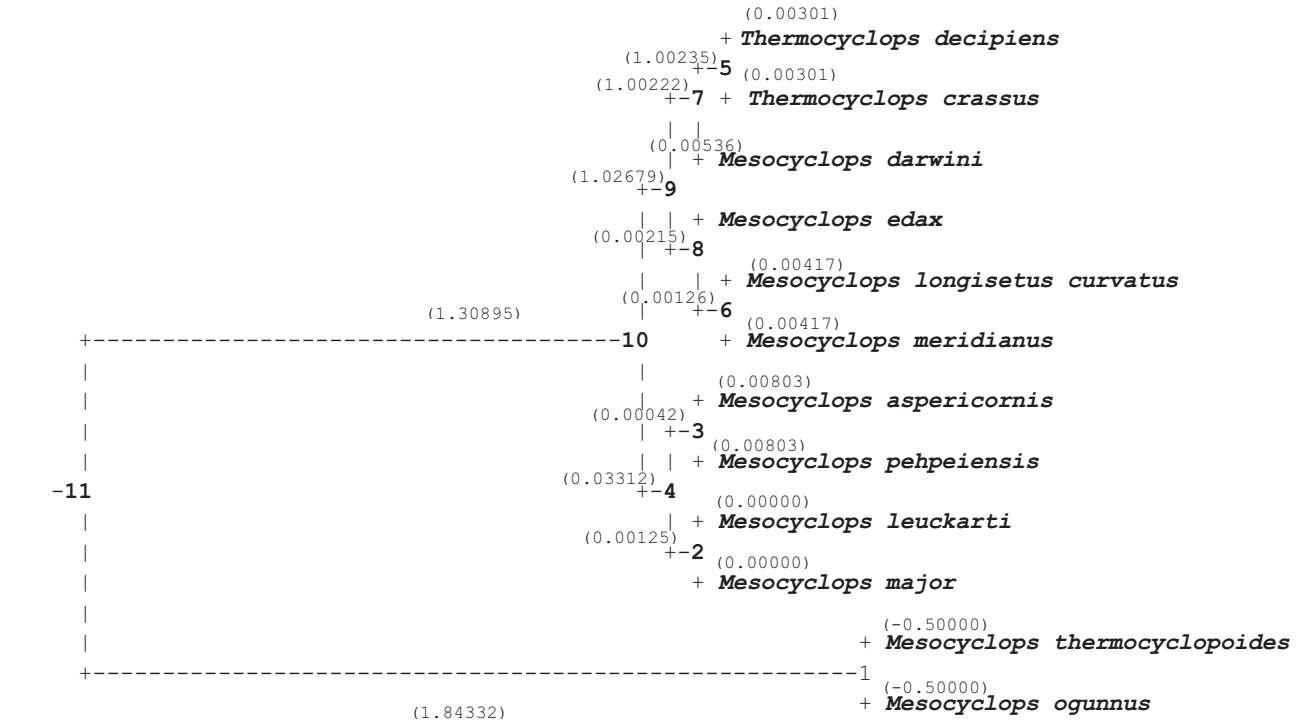

Figure 2. Phylogeny relationships of cyclopoid copepods on molecular based analysis by UPGMA method.

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