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PHYLOGENETIC ANALYSIS OF 18S rDNA OF FRESHWATER COPEPODS *NEODIAPTOMUS* SPECIES AND *MESOCYCLOPS* SPECIES

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ABSTRACT: The present work is emphasized on analyzing the molecular characteristics of *Mesocyclops* sp. and *Neodiaptomus* sp. Molecular markers 18s rDNA region was used to resolve the evolutionary relationship between the species. The *Mesocyclops* sp. and *Neodiaptomus* sp. were sequenced and deposited in Gen Bank database. The similarity sequence was searched by BLAST programme and data was retrieved for construction of phylogenetic tree among the group. The percentage of similarity was found in the range of 80-83% and 97-100% in 18s rDNA region of *Neodiaptomus* sp. and *Mesocyclops* sp. to other species respectively. The phylogenetic tree was constructed using Neighbour-Joining method. *Neodiaptomus* sp. 18s rDNA region shows close relationship to *Leptodiaptomus siciloides, Eudiaptomus wolterecki* and *Mastigodiaptomus albuquerquensis*. *Mesocyclops* sp. 18srDNA region shows high similarity to *Thermocyclops crassus* and *Mesocyclops leuckarti* and less similarity to *Mesocyclops edax* clone.

KEYWORDS: *Neodiaptomus*, *Mesocyclops*, NJ method and BLAST

INTRODUCTION

Zooplankton plays an important role in energy and matter transfer through the aquatic ecosystem[1]. Most zooplankton occupies second or third trophic level of the aquatic food web. In zooplankton, copepod and cladoceran are the dominant groups in freshwater[2]. Copepods form a subclass belonging to subphylum crustaceans, and it divided into ten orders. But only three are common in plankton samples: such as Cyclopoida, Poecilostomatida and Calanoid[3,4]. The nutritional quality of copepods is generally accepted to be very good for marine fish larvae, and belived to be of a higher quality than the commonly used live food *Artemia*[5,6]. Cyclopoidae is the mostly found family among copepod species. The joint lies between fourth and fifth segments of the body[7]. Calanoida is another important species in an order of copepods, they include around 40 families, with about 1800 species of both marine and freshwater copepod.

Morphological identification methods provide accurate information about species diversity[8]. Microscopic discrimination of copepods is feasible only at the late copepodite and adult stages[9]. Identification of copepods at egg and larval stages are difficult using microscope and also this method is time consuming and ambiguous. Therefore, it is necessary to develop alternative method for identification of diversity of samples[10.] Molecular techniques have potential to provide species identification even the smaller aquatic organisms[10.] Molecular techniques are useful for identification of the species at early stage level and shown sometimes significant genetic differentiation among species[11,12]. In molecular level, different techniques are available such as RFLP, RAPD, Multiplex PCR and genomic DNA sequences used to discriminate the closely related specie[13]. Recently DNA sequencing provides thousands rather than tens of characters enabling the reconstruction of better resolved and more robust phylogenetic trees[14]. Currently few nuclear and mitochondrial markers have been used to successfully resolve phylogenetic relationship in copepods[15,16]. The main reason for selecting these two markers is highly conserved regions.

The nuclear rRNA is the good molecular marker for the study of copepod biodiversity and it is effective for the study of species level identification of calanoid copepods[17]. 18s rRNA is widely used for diversity research in eukaryotes and also it has been used for resolving phylogenetic relationship among cyclopoid copepods at higher level[18]. Nuclear DNA approximately 166,000 times larger than mtDNA and also provides set of markers that potentially segregate independently and also phylogenetic studies carried at higher systemic level[19]. A molecular phylogenetic study is to recover the order evolutionary events and represent them in evolutionary trees that graphically depict relationship among species or genus over time[20].

MATERIALS AND METHODS

Collection and Preservation of the sample:

The Copepods samples were collected from Padalam, Kancheepuram District using plankton net (0.35 mouth diameter) made up of bolting silk cloth (No 10, 158µm for 20-30 mins). The samples were transported to the laboratory immediately and they were fixed in 90% ethanol for taxonomical studies. The copepod had identified on the basis of morphological by using a binocular microscope. Copepod samples are first screened through 500µm mesh to remove fish and prawn larvae. The rinsing is made repeatedly to reduce the

contamination after rinsing the desired copepod is picked from zooplankton samples with the help of fine capillary tubes and needles. Then the copepod is washed with double distilled water and preserved in 90% ethanol to facilitate DNA isolation [21].

DNA Isolation:

DNA was isolated by Saline Citrate Solution method (SCS). 200mg of copepod samples were suspended in 800µl of saline citrate solution (0.14M Nacl and 0.02M Tri Sodium Citrate) and homogenized by morter and pestle. The homogenate is transferred into a fresh centrifuge tube and centrifuged at 3000rpm for 10min and after centrifugation the supernatant will be discarded and the pellet is resuspend in Saline Citrate Solution and then centrifuged at 3000 rpm for 5min. Again the pellet resuspended in 400µl of 2M Nacl solution and centrifuged at 10000rpm for 15min at 4^0C. Followed by centrifugation the supernatant was collected in fresh centrifuge tubes. To this double the volume of ethanol is added and the DNA is allowed to precipitation for 6min [22].

DNA Amplification: Amplification reaction of the 18s rDNA gene will be carried out using the primers 18s rRNA Forward 5'-GCA AGT CTG GTG CCA GCA GCC -3' and 18s rRNA Reverse 5'-TGA TCC TTC CGC AGG TTC AC -3'. Amplification conditions consisted of 5min at 95^0C and then 40sec at 95^0C, 25sec at 50^0C, 3min at 72^0C for 40 cycles, and extension for 15min at 72^0C [23].

Gene Sequencing:

DNA sequencing was carried out on an automated DNA sequencer. Proof reading, editing, and alignments were performed using the program Sequencer 4.1 [24].

Phylogenetic Analysis:

DNA homology searches were performed using BLASTn 2.2.24 programs at NCBI and similarity sequences were retrieved for phylogenetic analysis. Obtained sequences were verified, corrected and assembled with MEGA tool and aligned by ClustalW algorithm. Phylogenetic relationships were evaluated of 18s rRNA nuclear genes by Neighbour Joining method [25].

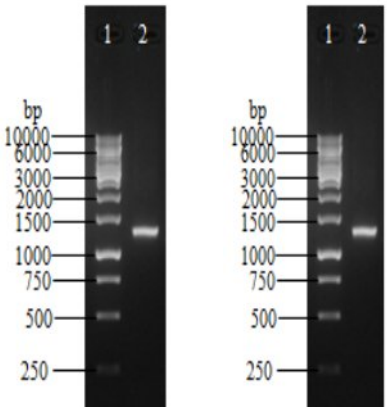
RESULTS AND DISCUSSION

DNA Isolation and amplification:

The DNA was extracted and amplified of 18s rDNA region from *Neodiaptomus* sp. (Fig. 1) and *Mesocyclops* sp. (Fig. 2). The molecular weight of amplified DNA was 1176 (*Neodiaptomus* sp.) and 1363 bp (*Mesocyclops* sp.). DNA Isolation done by using SCS method extracted DNA in the range of 2027 to 23130 bp for both

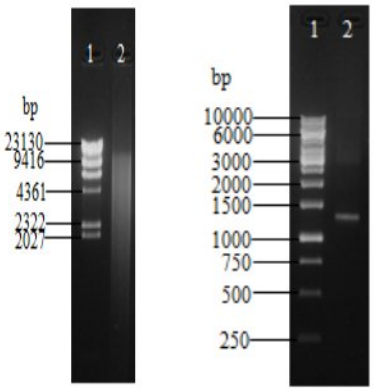
Neodiaptomus sp. and *Mesocyclops* sp. in 18s rDNA region. The extracted DNA was further amplified and nucleotide sequence of 1176 and 1363 base pair of 18s rDNA was determined for *Neodiaptomus* sp. (Fig 1) and *Mesocyclops* sp. (Fig 2), respectively.

Fig.1: DNA Isolation and PCR product of 18s rRNA region of *Neodiaptomus* species



Lane 1 – Lambda DNA / Hind III Digest Marker; Lane 2 – Genomic DNA. Lane 1 – 1Kb DNA Ladder; Lane 2 – PCR product.

Fig.2: DNA Isolation and PCR product of 18srRNA region of *Mesocyclops* species.



Lane 1 – Lambda DNA / Hind III Digest Marker; Lane 2 – Genomic DNA.
Lane 1 – 1Kb DNA Ladder; Lane 2 – PCR product.

18s rDNA gene sequence analysis

The 18s rDNA gene is one of the most frequently used molecular marker for eukaryotes in determining generic and specific level relationships and higher order analyses of phylogeny[26]. Similarity of sequences of *Neodiaptomus* sp. and *Mesocyclops* sp. was retrieved by BLASTn program. List of accession numbers and organism are give below in Table 1 and 2. The *Neodiaptomus* sp. and *Mesocyclops* sp. found maximum identity of 98% and 100% for 18s rDNA region, respectively. Blastn program were used to find the similarity of species based on maximum identity and E value. In 18s rDNA region of *Neodiaptomus* species shows high similarity to *Leptodiaptomus siciloides* and *Eudiaptomus wolterecki* (98% and 5e-151) and less similar to *Diaptomus cyaneus* isolate (96% and 0) (Table 1). In *Mesocyclops* species shows (98% and 2e-18) high similarity to Cyclopidae environmental sample clone and low similarity to *Cyclops kolensis* (78% and 1e-110) in 18srRNA region (Table 2). The 18s rDNA had the greatest phylogenetic signal ratio using neighbor joining method[27] and also based on the very limited number of morphological characters present testing of Phylogenetic

hypothesis is useful approach phylogenetic study.

Distance Matrix- In distance matrix *Neodiaptomus* species of 18s rDNA region closely related with *Eudiaptomus graciloides* (0.004) and less related to *Diaptomus cyaneus* (0.516). *Mesocyclops* species 18s rDNA region shows high similarity to *Thermocyclops crassus* and *Mesocyclops* leuckarti (0.000) and less similarity to *Mesocyclops edax* clone (0.667)(Table-3).

Table1: Multiple sequence alignment for *Neodiaptomus* species and other *Calanoid* species in 18s rDNA region

SI. No	Accession Number	Name of the organism	% of similarity	E-Value	Number of residues (bp)
1	KJ959224.1	*Neodiaptomus* Species			
2	AY339149.1	Eudiaptomus graciloides	97%	0	1742
3	JX945135.1	Arctodiaptomus cf. stephanidesi	97%	0	1383
4	JX945130.1	Arctodiaptomus salinus isolate Asal	97%	0	1383
5	JX945129.1	Hemidiaptomus ignatovi isolate	97%	0	1383
6	JX945121.1	Eudiaptomus vulgaris isolate	97%	0	1383
7	GU067947.1	Eudiaptomus environmental sample clone	97%	0	1802
8	AY339148.1	Eudiaptomus gracilis	97%	0	1742
9	AY339145.1	Aglaodiaptomus clavipoides	97%	0	1743
10	AY339144.1	Aglaodiaptomus spatulocrenatus	97%	0	1743
11	JX945122.1	Hemidiaptomus gurneyi canaanita isolate	96%	0	1383
12	JX945134.1	Copidodiaptomus numidicus isolate	96%	0	1383
13	JX945131.1	Arctodiaptomus wierzejskii isolate	96%	0	1383
14	JX945124.1	Diaptomus kenitraensis isolate	96%	0	1383
15	JX945123.1	Diaptomus castor isolate	96%	0	1383
16	AY339160.1	Skistodiaptomus pallidus	96%	0	1743
17	AY339156.1	Mastigodiaptomus nesus	96%	0	1743
18	AY339150.1	Hesperodiaptomus shoshone	96%	0	1726
19	AY339146.1	Aglaodiaptomus leptopus	96%	0	1743
20	JX945127.1	Hemidiaptomus hungaricus isolate	96%	0	1383
21	JX945128.1	Hemidiaptomus maroccanus isolate	96%	0	1383
22	JX945126.1	Hemidiaptomus superbus isolate	96%	0	1383
23	AY339159.1	Skistodiaptomus oregonensis	96%	0	1743
24	AY339155.1	Leptodiaptomus sicilis	96%	0	1743
25	AY339154.1	Leptodiaptomus moorei	96%	0	1743
26	JX945132.1	Diaptomus cyaneus isolate	96%	0	1383
27	AY339157.1	Onychodiaptomus sanguineus	96%	0	1743
28	AY339151.1	Leptodiaptomus ashlandi	96%	0	1727

29	JX945133.1	Diaptomus mirus isolate	96%	0	1383
30	AY339161.1	Skistodiaptomus pygmaeus	96%	0	1743
31	AY339158.1	Skistodiaptomus mississippiensis	96%	0	1743
32	AY339153.1	Leptodiaptomus minutus	96%	0	1743
33	AY339147.1	Arctodiaptomus dorsalis	96%	0	1728
34	AY339152.1	Leptodiaptomus coloradensis	96%	0	1742
35	GU067992.1	Eudiaptomus environmental sample clone	97%	0	776
36	JX868019.1	Phyllodiaptomus sp.	99%	2e-154	352
37	JX868009.1	Diaptomidae gen. sp.	99%	1e-152	352
38	JX868024.1	Leptodiaptomus siciloides isolate	98%	5e-151	352
39	JX868021.1	Eudiaptomus wolterecki isolate	98%	5e-151	352
40	JX945067.1	Diaptomus cyaneus isolate	96%	0	337

Table 2: Multiple sequence alignment for *Mesocyclops* species and other Cyclopoid species in 18s rDNA region

SI. No	Accession Number	Name of the organism	% of similarity	E-value	Number of residues (bp)
1	KM023644.1	*Mesocyclops* Species			
2	GU066281.2	Diacyclops jasnitskii	80%	2e-126	713
3	GU066285.2	Diacyclops improcerus	80%	2e-126	713
4	FJ825604.1	Acanthocyclops galbinus	80%	2e-126	713
5	JQ315759.1	Apocyclops royi	82%	1e-123	1588
6	GU066288.2	Diacyclops sp.	79%	1e-122	713
7	GU066275.2	Diacyclops incolotaenia	79%	1e-122	713
8	GU066263.2	Diacyclops galbinus	79%	1e-122	713
9	GU066272.2	Diacyclops bicuspidatus	79%	5e-121	713
10	AY626996.1	Euryte sp.	82%	2e-120	1782
11	FJ825598.2	Thermocyclops crassus	79%	6e-120	712
12	FJ825599.1	*Mesocyclops* leuckarti	79%	6e-120	712
13	AJ746334.1	Macrocyclops albidus	79%	2e-119	1808
14	FJ825602.1	Acanthocyclops bicuspidatus	79%	1e-117	713
15	AY626998.1	Cyclops sp.	79%	1e-117	1810
16	GU066264.2	Eucyclops serrulatus	78%	1e-115	710
17	AJ746335.1	Eucyclops dumonti	78%	1e-115	1808
18	AY210814.1	Cyclopidae sp	79%	1e-115	1365
19	AJ746333.1	Eucyclops speratus	78%	6e-114	1809
20	AJ746329.1	Eucyclops macruroides	78%	6e-114	1809
21	DQ107580.1	Thermocyclopssp.	78%	6e-114	1466
22	GU066283.1	Cyclops kolensis	78%	1e-110	707
23	EF532821.2	Cyclops insignis	77%	5e-109	5729
24	AJ746336.1	Ectocyclops polyspinosus	78%	5e-109	1808
25	AY626999.1	Acanthocyclops viridis	79%	6e-101	1816
26	GQ371043.1	Cyclopidae environmental	98%	2e-18	177

		sample clone			
27	JX134314.1	Eucyclops cf. serrulatus	88%	0.041	386
28	AB811994.1	Cyclops vicinus	94%	1.7	5661
29	GQ886040.1	Acanthocyclops vernalis	100%	1.7	567
30	DQ077379.1	*Mesocyclops* edax clone	94%	1.7	504

The highest genetic distance between all cyclopoids taxa was caused by extreme variation in the *Mesocyclops thermocyclopoides* and *Mesocyclops darwini* 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 between the *Mesocyclop aspericornis* was 6th node of tree. Distance matrices are used to compare genetic distances within and among taxa and to determine whether a given group of cyclopoids has diverged, on average, more or less than others. molecular systematic and phylogenetic assessment between the species. Also, the more slowly evolving nuclear subunit 18s rDNA gene proved to be appropriate to resolve phylogenetic relationship because of the low level divergence and lack of saturation which allowed statistically sound resolution of familial relationships [16]. 18s rDNA region of Mesocyclops species shows close relationship to *Mesocyclops leukarti* based on distance and and 18s rDNA region of, *Neodiaptomus* species 18s rDNA shows close relationship to diaptomus group based on distance and E value particularly *Eudiaptomus gracilodies*. In *Neodiaptomus* and *Mesocyclops* species both region do not show the close relationship to the common species, because of the lack of database. *Mesocyclops* species shows close

PHYLOGENETIC ANALYSIS

Phylogenetic tree was constructed using neighbor joining method. The tree constructed based on 18s rDNA shows *Neodiaptomus* species and *Eudiaptomus graciloides* are the same group and have a shortest neighbor distance similarly *Mesocyclops* species and *Mesocyclops leuckarti* share the same branch. (Fig 1 and 2). Phylogenetic relationship among 11 copepod genera were reconstructed using a 660bp region of nuclear small subunit 18s rRNA, molecular phylogeny was consistent with the accepted limits of the *calanidae* and *clausocalanidae* and clearly resolved relationships among genera within each family and shows no variability within species. 18s rDNA are used as a marker to find the relation between taxa. 18s rDNA sequence variation proved to be useful for both

relationship to cyclopidae family particularly under cyclopinae subfamily.

CONCLUSION

Tree based on different regions and different methods all yielding similar topology. In this study *Neodiaptomus* species and *Eudiaptomus graciloides* are the same group , then in *Mesocyclops* species and *Mesocyclops leuckarti*_ are the same group in 18srDNA region based on distance method.

Figure 2: Phylogenetic tree of *Neodiaptomus* species for 18s rDNA region

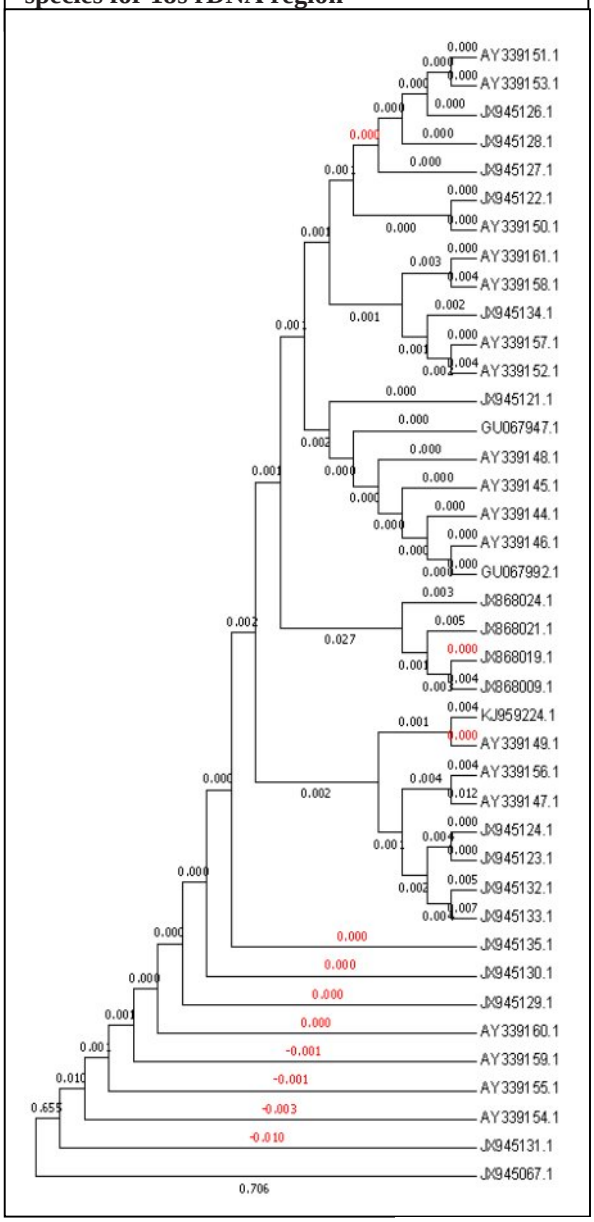
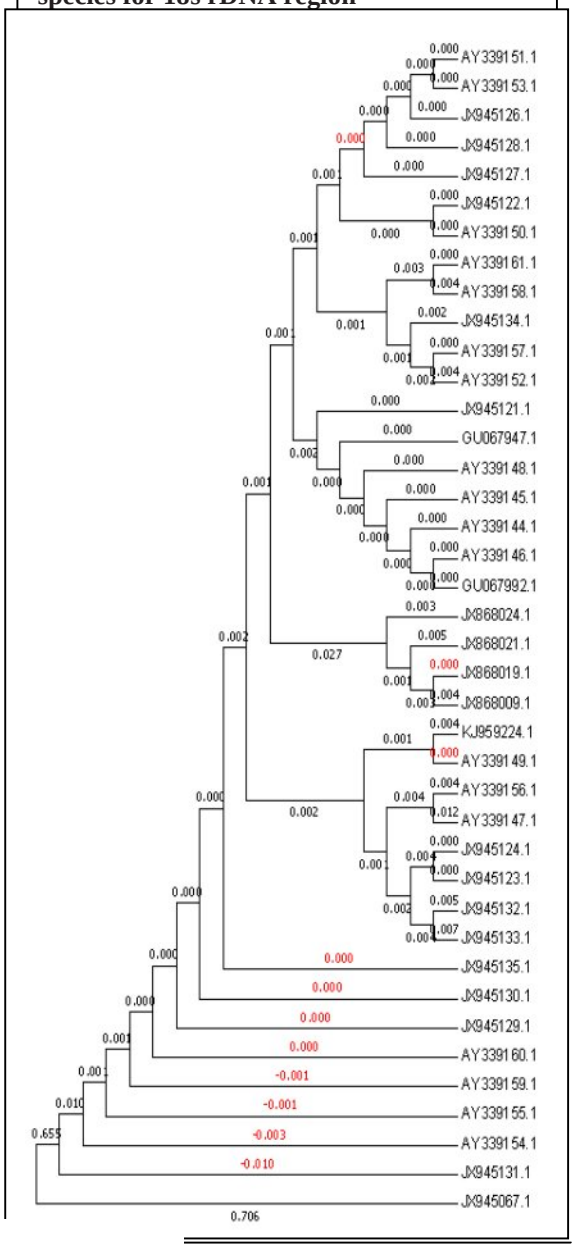


Figure 2: Phylogenetic tree of *Mesocyclops* species for 18s rDNA region



Molecular marker mtCOI region shows less similarity or larger distance to other taxa. It concludes Phylogenetic tree constructed using two different regions are the best method remove for to find the evolutionary relationship between the species and also its used for creating database as a tool for identification of the species. In Addition morphological key character also gives the additional information towards constructing topologies.

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