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Mass Culture and Molecular Identification of Zooplankton Species

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Original Research Article

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Abstract: The main aim of this study was to barcode the wild zooplankton using partial sequence of mt-COI gene. Wild zooplankton sample was collected from the Ukkadam lake (Lat. 10.99° N and Long. 76.96° E), Coimbatore, India. The presence of 27 zooplankton species was identified, and each of them was mass cultured by fed with phytoplankton, Baker-*Yeast*, and cow-dung separately. Zooplankton was found to be fairly grown under phytoplankton followed by Baker-*Yeast* and cow-dung. The well grown zooplankton species, such as *Brachionus calyciflorus*, *Brachionus caudatus*, *Brachionus rubens*, *Ceriodaphnia cornuta*, *Eucyclops speratus* and *Macrocyclops albidus* were barcoded by mt-COI gene using universal primers, LCO1490 and HCO2198. The size of genomic DNA in each species was >10kb, and their amplified sequences was >600 bp, which showed 98-100% similarity when matched with NCBI data base. Comparison of amino acid residues among different zooplankton showed more number of variable amino acids, and less number of identical and similar amino acids, which indicated the fact that these species were discriminated. The nucleotide compositions showed >60% AT biases, which indicates the occurrence of less number of NUMTS gene sequences. The phylogenetic tree topology revealed that *C. cornuta* alone sat in one clade and the remaining species aligned in another clade with two clusters. Thus these species are genetically distinct but closely related with each other.
Keywords: Zooplankton, mt-COI gene, AT-GC biases, divergence, phylogeny.

INTRODUCTION

Zooplankton contributes significantly to aquatic productivity. It is an important food item for the young and some adults of many freshwater fishes and prawns which represent a major component of the human diet [1-4]. Zooplankton communities often respond quickly to physico-chemical changes of water due to their short life cycles, they are treated as good indicators of environmental conditions [5].

For fish/prawn culture industry, production of quality seeds with a high survival rate is important. The larval development depends on providing nutrient enriched suitable live feed. Zooplankton plays a vital role as natural food for fishes/prawns, particularly from endo-exogenous to exclusively exogenous feeding stages. Therefore, successful mass cultures of zooplankton using algae and animal wastes have been reported [6-8].

The freshwater zooplankton comprises of various taxonomic groups, rotifera, cladocera, copepoda and ostracoda, so accurate identification often involves the cooperation of specialists. Morphological identification of zooplankton requires experienced specialist, which often creates a bottle neck [9]. Species with different names and sibling species are universal, thereby increasing the difficulty of identification [10]. Therefore, to overcome morphological impediments, many different genetic markers for species identification and phylogeny reconstruction of crustaceans have been considered to complement those conventional approaches [11-13]. Among those, the DNA sequence based identifications, such as the 16S rDNA, the cytochrome c oxidase subunit I (COI) and 18S genes are more popular tools [14-19].

Among various gene regions available for correct and quick discrimination of species, the mitochondrial-COI gene region is unique, because its haplotypes are often used in studies on the molecular ecology/taxonomy of freshwater zooplankton [20, 21]. Actually, mt-COI gene has offered the most efficient and accurate barcoding method for species-level identification of animals including zooplankton regardless of the condition and life history stages [9, 22-25]. Its validity has also been reported in copepods [26-30] and cladocerans [31-34], krill [35]. It also handled morphologically indistinguishable, but genetically distinct, cryptic species complexes, which have frequently been reported in freshwater zooplankton [21, 36, 37].

The present study was dealt with mass culture of 27 endemic species of zooplankton collected from the Ukkadam lake (Lat. 10.99° N and Long. 76.96° E, one of the perennial lakes of Coimbatore city, India), individually fed with phytoplankton, Baker-*Yeast*, and cow-dung separately in order to identify the best feed, and the well grown zooplankton belongs to Rotifera (*Brachionus calyciflorus*, *Brachionus caudatus personatus* and *Brachionus rubens*), Cladocera (*Ceriodaphnia cornuta*) and Copepoda (*Macrocyclops albidus* and *Eucyclops speratus*) were discriminated by DNA barcoding of mt-COI gene. Furthermore, the sequence similarity and divergence, amino acid residues and phylogenetic information, such as synonymous and non-synonymous substitutions, transitional and transvertional substitutions, saturations and phylogenetic tree topology have been assessed.

MATERIALS AND METHODS

The Ukkadam Lake (Lat. 10.99° N and Long. 76.96° E) of Coimbatore city, Tamil Nadu, India, have been described earlier and it contained 27 species of zooplankton, which also have been quantitatively and qualitatively described by us [38].

Rotifera

Rotifers are "wheel-bearer" refers to the crown of rotating cilia around the funnel-shaped mouth, which is used for locomotion and sweeping of food particles towards the mouth, and a specialized pharynx called the mastax, with its cuticular lining differentiated into trophy, a series of pieces that act as jaws.

Order/Family specific features

- Rotifers are with paired generative organs. Rotifers are with single generative organ as well, males present but mostly reduced (Monogononta).
- Marine forms: Corona not with two trochal discs, reduced, males fully developed (Seisonide).
- Freshwater forms: Corona with two trochal discs, latter rarely reduced in some forms; males not known (Bdelloidea).

Species specific features

- Lorica flexible, oval not separated into dorsal and ventral plates; body slightly compressed dorsoventrally, anterior dorsal margin with four broad-based spines of variable length, medians longer than laterals; mental margin flexible, usually somewhat elevated, with shallow V or U shaped notch, unflanked; posterior spines present or absent; posterolateral spines usually absent, lorica smooth or lightly stippled (*Brachionus calyciflorus*) (Figure 1 of Plate 1).
- The characters of main species, lorica heavily stippled, with a pattern of cuticular ridges more or less distinct; lorica moderately compressed dorsoventrally; occipital spines six; lateral occipital spines larger than median spines; at times twice as long as medians; intermediate spines reduced; mental margin wavy; posterior spines not developed in same plane as the axis of body (*Brachionus caudatus personatus*) (Figure 2 of Plate 1).
- Lorica firm, oval, smooth, compressed dorsoventrally and composed of dorsal and ventral plates; anterior dorsal margin with six spines (*Brachionus rubens*) (Figure 3 of Plate 1).

Cladocera

Cladocerans are a primary freshwater monophyletic micro-crustacean (water fleas) with compound eye, usually a carapace covering most of the body, except the head, and at least four pairs of trunk appendages which are in most cases broad lobed and fringed on the inner edges with bristles. No segmentation is visible on the carapace, but in many species the carapace forms a posterior spine. Sometimes there is also a spine on top of the head. The second antennae are very well developed. Their bodies are not divided into a separate thorax and abdomen. The tip of the trunk forms a "post-abdomen", which is bent towards the ventral trunk surface and is equipped with claws and spines for cleaning the carapace.

Order/Family specific features

- Head with a protective head shield. Swimming antennae with less than ten natatory setae (Anomopoda).
- Head without a protective head shield. Swimming antennae with more than ten natatory setae (Ctenopoda).
- Antennules fused with rostrum (Bosminidae).
- Body not laterally compressed. Rostrum absent (Moinidae).

Species specific features

Small species as adult, (<0.5 mm); head with an acute rostrum; Valves distinctly reticulate, head small depressed and separated from body by a distinct ocular depression (*Ceriodaphnia cornuta*) (Figure 4 of Plate 1).

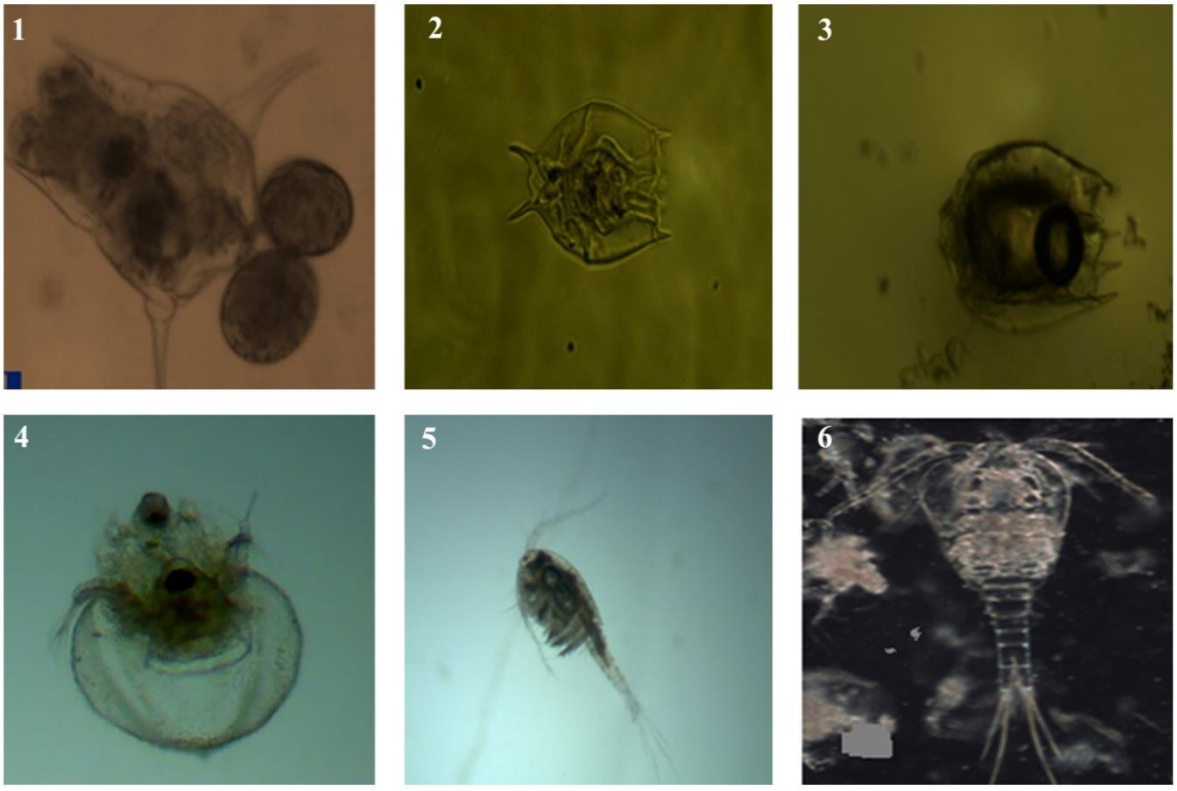

Plate-1: Zooplankton species dominantly grown under mass culture, which were subjected to DNA barcoding
1, *Brachionus calyciflorus*; 2, *Brachionus caudatus personatus*; 3, *Brachionus rubens*;
4, *Cerodaphania carnuta*; 5. *Macrocyclops albidus*; 6, *Eucyclops speratus*

Copepoda

Copepods have short cylindrical bodies clearly divided into a number of segments. The head section is usually rounded and bears prominent, often very long antennae, which when held away from the body, serve to slow sinking rate. There are usually nine free trunk segments. The anterior segments bear the swimming appendages while the posterior segments taper, ending in a pair of caudal rami at the base of the abdomen. On the basis of major articulation of the body, Copepoda is divided into two groups, gymnoplea and podoplea. In gymnoplea (platycopida and calanoida), there are no appendages on the body segments posterior to the major articulation. In podoplea, there are reduced appendages on body segment posterior to the major articulation. Copepods are including three free living groups viz., calanoida, cyclopoida and harpacticoida.

Order/Family specific features

- First antennae very short (<10 segment), do not reach past end of cephalothorax; body cylindrical (Harpacticoida).
- First antennae up to 18 segments, may reach past the posterior end of cephalothorax; body widest behind the head, tapers to urosome (Cyclopoida).
- First antennae long, >20 segments, extend to urosome or past end; body torpedo like (Calanoida).

Species specific features

- Macrocyclops albidus is distinguished by the bare medial surface of the caudal rami and the hyaline membrane on the last segment of the antennule, which is smooth or finely serrated (Figure-5 of Plate 1).
- As in other species under the genus Eucyclops spinules are present (reduced in Eucyclop smacrurus) on the other margin of the comparatively as its caudal rami is longer (more than 5 time) but lateral spinules are very small. The antennules are 12 segmented and reach beyond the cephalothorax (Eucyclops speratus) (Figure 6 of Plate 1).

Mass culture of zooplankton

All the 27 species of zooplankton identified were segregated (100 individual for each species). They were individually subjected to mass culture and fed *ad libitum* with three different types of feeds in separate culture tanks for 60 days. The feeds were mixture of phytoplankton (Spirulina: *Spirulina meneghiniana*, *Arthrospira platensis*, *Arthrospira maxima* and *Labyrinthiformis*; Chlorophyceae: *Pediastrum duplex*, *Pediastrum tetras*, *Spirogyra hyaline*, *Ulothrix zonata* and *Tabellaria fenestrata*; Cyanophyceae: *Aphanocapsa pulchra*, *Chroococcus minutes*, *Oscillatoriasub brevis* and *Phormidium granulatum*), Baker- *Yeast* and Cow-dung respectively. The culture medium maintained under the following conditions: temperature (°C),

24±2.0; pH, 7.0; salinity (ppt), 0.682±0.34; DO(mg/l), 7.63±0.13; TDS(mg/l), 1011±12.8; EC (µS/ cm), 1.112±0.10 with continued aeration. Growth of the zooplankton was determined by using a slide with a counting chamber mounted on a microscope at a magnification of 10 xs and 40 xs. On day 60 of mass culture, the number of species attained growth in each group was counted. There were six species grown dominantly which attained 1000 and above individuals per litter (three species of Rotifera: *Brachionus calyciflorus, Brachionus caudatus personatus* and *Brachionus rubens*; one species of Cladocera: *Ceriodaphnia cornuta;* and two species of Copepoda: *Macrocyclops albidus* and *Eucyclops speratus*) were harvested for molecular identification using mt-COI gene (Table-1).

Table-1: Growth of individual zooplankton species under mass culture for 60-days with different feeds

Zooplankton		Growth (ind./L^{-1}) with different feeds		
		Phytoplankton	**Baker-Yeast**	**Cow-dung**
Rotifera	*Brachionus rotundiformis*	766±40**	489±38**	574±32**
	Brachionus calyciflorus	1465±39***	750±34**	788±31**
	Brachionus caudatus personatus	1176±34***	711±29**	666±25**
	Brachionus rubens	1077±36***	742±32**	612±28**
	Asplanchna intermedia	792±32	610±30**	643±25**
	Asplanchna brightwelli	989±46**	712±24**	619±22**
Cladocera	*Diaphanasoma sarsi*	856±22**	544±31**	465±27*
	Daphnia magna	941±34**	617±32**	566±28**
	Leydigia leydigia	734±26**	512±31**	721±28**
	Ceriodaphnia cornuta	1264±30**	720±33**	663±20**
	Moina micrura	745±31**	489±30*	521±34**
	Moina brachiata	786±32**	448±30*	401±25*
Copepoda	*Heliodiaptomus viduus*	793±29**	589±25**	634±28**
	Cyclops vernalis	735±27**	654±26**	478±26*
	Eucyclops speratus	1256±37***	866±42**	728±32**
	Mesocyclops pehpeiensis	915±46**	826±35**	628±32**
	Thermocyclops hyalinus	845±24**	419±26*	589±27**
	Mesocyclops leuckarti	759±27**	713±30**	587±29**
	Mesocyclops edax	834±31**	536±27**	458±25*
	Macrocyclops albidus	1226±27**	703±27**	666±23**
Ostracoda	*Eucypris bispinosa*	789±28**	478±36*	543±23**
	Cypris decaryi	726±36**	578±32**	534±28**
	Candona candida	790±31**	678±30**	587±26**
	Cyprinotus nudus	847±36**	606±26**	447±29*
	Heterocypris dentatomarginatus	438±29*	447±28*	490±32*
	Prionocypris glacialis	811±35**	645±20**	578±25**
	Cypris protubera	701±38**	528±33**	404±21*

***, Fairly grown; **, Moderately grown; *, Poorly grown.
Each value is mean ± SD of six individual observations.

Molecular analysis

Genomic DNA was isolated from the whole animal (500-1000 numbers) by using Qiagen Dneasy Blood and Tissue Kit (Germany). 1% Agarose Gel Electrophoresis (GENEI, Bangalore, India) was performed and the genomic DNA was detected in a Gel documentation system (Medicare, India). DNA amplification of mt-COI gene was carried out in Applied Biosystem (ABI) Thermo Cycler with universal primers of forward and reverse in nature, LCO1490 and HCO2198 [39]. These primers set were worked well with crustaceans, crabs and prawns [40-44].

Amplification was performed in a total volume of 100 µl containing 1 µl of DNA template, 400 ng of each primer (Forward primer, 400 ng (0.5 µl); Riverse primer, 400 ng (0.5 µl)), 4 µl dNTPs (10mM each), 10 µl of 10X ChromTaq DNA Polymerase Assay Buffer, 1 µl of ChromTaq DNA Polymerase Enzyme (3U/µl) and Water of 83 µl. The thermo cycler condition was as follows: 5 min at 95°C for pre-running, 35 cycles of 30 s each at 95°C for denaturation, 45 s at 57°C for annealing, and 1 min at 72°C for extension, followed by 7 min at 72°C for a final extension. The amplified product was resolved with 2% AGE (GENEI, Bangalore, India). Sequencing was performed with total volume of 20 µl containing 3 µl of template DNA, 3.2 pM/µl of primers (forward, 0.50 µl and reverse, 0.50 µl), 2 µl of 5X big dye sequencing buffer and 4 µl of 2.5X ready reaction premix (Tris-HCL, pH 9.0 and $MgCl_2$) and 10 µl of

DNase-RNase free water. The PCR sequencing cycling condition was as follows: 25 cycles for 1 s each at 96°C for pre running, 25 cycles at 96°C for 10 sec for denaturation, followed by 25 cycles for 5 sec each at 50ºC for annealing, 30 cycles of 4 minutes each at 60ºC for elongation. After completion of the PCR program, the sample was processed for ethanolic precipitation. From the PCR tubes, the samples were transferred to 96 well microlitre plates and 5 µl of 125 mM EDTA was added to each well. 60 µl of ice cold 100% ethanol (stored at -20ºC) was added to each reaction, the plate was sealed and mixed by vortexing for 20-30 sec and incubated at room temperature for 15 minutes. The sample plate was spined at 3,000 × g at 4ºC for 30 minutes. The supernatant was carefully removed by inverting the plate, spined up to 180 × g for 1 min and then removed from the centrifuge. The pellet was rinsed once with 60 µl of ice cold 70% ethanol (stored at -20ºC) by centrifugation at 1650 × g at 4ºC for 15 minutes. Again the plate was inverted and spined up to 180 × g for 1 minute, and then removed from the centrifuge. The sample was re-suspended in 10 µl of Hi-Di Formamide and incubated for 15 min at room temperature. The re-suspended samples were transferred to the appropriate wells of the sample plate. Ensured each sample was positioned at the bottom of its tube or well. The samples were denatured at 95ºC for 5 minutes with snap chill and the plate was loaded into Sequencer, after completion of run the data was analyzed (ABI 3500 XL Genetic Analyzer, Chromous Biotech, Bangalore, India).

The forward and reverse sequences were aligned pair wise by using CAP3. The sequence similarity available with NCBI database was identified and the internal stop codon was removed by BLAST. The reading frame shift was deducted by ORF finder. The trimmed sequence was authenticated with GenBank. The multiple sequence alignment was done by using T-Coffee and the aligned sequence was highlighted with multiple align show (MAS) as identical, similar and variable sites of amino acids. The nucleotide composition (AT and GC biases), nucleotide divergence (K2P model; [45]) and some phylogenetic information were calculated by using MEGA v. 6.01. Assessment of synonymous (Ks) and non-synonymous (Ka) substitutions for 3rd codon positions was calculated by Li93 method using DAMBE [46]. The transitional (Ts) and transvertional (Tv) substitutions of nucleotides were determined [47]. Analysis of sequence saturation, index of substitutional saturation (Iss) and critical value of index of substitutional saturation (Iss.c) was done by Xia method using DAMBE [48, 49]. Finally the phylogenetic tree was reconstructed by Maximum Likelihood model [50, 51].

RESULTS AND DISCUSSION

Mass Cultured Zooplankton

Among the 27 species of zooplankton under four groups subjected to mass culture for 60 days with phytoplankton, Baker-*Yeast* and cow-dung, six species were found to be grown well and attained >1000 int/L (for DNA isolation, more number of individual zooplankton is required). They were, three species of Rotifera: *B. calyciflorus, B. caudatus personatus and B. rubens*; one species of Cladocera: *C. cornuta*; and two species of Copepoda: *E. speratus* and *M. albidus.* None of the species of Ostrocoda was grown to reach 1000 int/L. Among the three feeds used, the zooplankton was found to be fairly grown in mixed phytoplankton fed category followed by Baker-*Yeast* and cow-dung. The actual number of individuals observed in these six species are given in Table 1, *B. calyciflorus* (1465 ind./L), *B. caudatus personatus* (1176 ind./L), *B. rubens* (1077 ind./L), *C. cornuta* (1264 ind./L), *E. speratus* (1256 ind./L) and *M. albidus* (1226 ind./L).

Mass culture of zooplankton, like *Brachionus*, *Daphnia*, *Ceriodaphnia* and *Moina* with different feeds, such as chlorella, Yeast, condensed phytoplankton products, cow-dung, pulse bran water, poultry manure and snail faeces have been reported [52-58].

Genomic DNA and its amplification

The size of isolated genomic DNA from the selected six zooplankton species was >10 kb nucleotides each (Figure 1) and its PCR amplified product was >600 bp each (Figure 2). Actually the size of each species aligned sequence was 659 bp, 648 bp, 609 bp, 673 bp, 628 bp and 646 bp for *B. calyciflorus*, *B. caudatus personatus*, *B. rubens*, *C. cornuta*, *M. albidus* and *E. speratus* respectively.

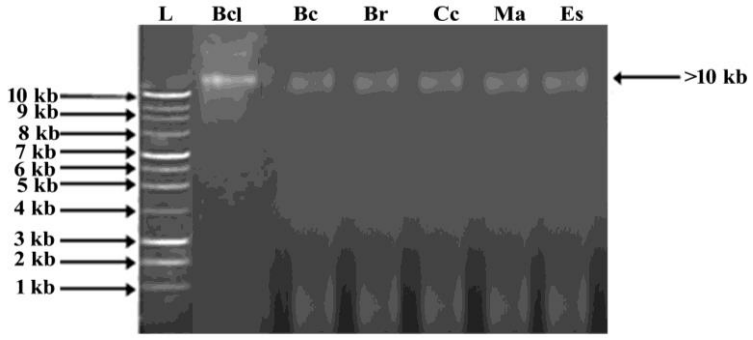

Fig-1: AGE (1%) of zooplankton shows >10 kb genomic DNA

L, Ladder (1 kb); Bcl, *B. calyciflorous*; Bc, *B. caudatus personatus*; Br, *B. ruben*; Ma, *M. albidus*; Es, *E. speratus*

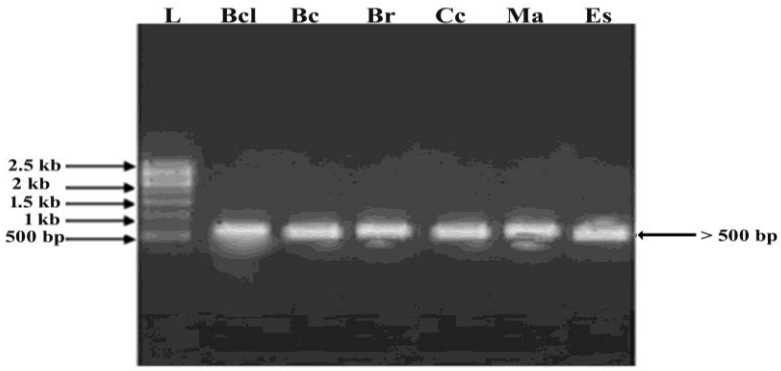

Fig-2: AGE (2%) of PCR amplified DNA products of zooplankton species shows >500 bp
L, ladder (500 bp); **Bcl**, *B. calyciflorous*; **Bc**, *B. caudatus personatus*; **Br**, *B. ruben*; **Ma**, *M. albidus*; **Es**, *E. speratus*

The BLAST similarity of each subjected and its respective matched sequence revealed 98-100%. *B. caudatus personatus*, *C. cornuta* and *M. albidus* showed 100% similarity, *B. calyciflorus* and *B. rubens* showed 99%, and *E. speratus*, 98% with their respective matched sequences of NCBI data base (Table 2).

Table-2: BLAST identification of COI partial gene sequences of subjected and retrieved zooplankton species and their GenBank accession numbers

Queried sequences	Author, Country and Accession Number	I (%)	G (%)	M.S	Retrieved/ Matched species	Author, Country and Accession Number
Brachionus calyciflorus	Paper authors, India KX822034	99	0	Plus	*Brachionus calyciflorus*	Xiang *et al*., 2016 China GU232714
Brachionus caudatus personatus	Paper authors, India KX822035	100	0	Plus	*Brachionus caudatus*	Garcia-Morales *et al*., 2013 Mexico JX216524
Brachionus rubens	Paper authors, India KY231380	99	0	Plus	*Brachionus rubens*	Proios *et al*., 2014 Finland KM051938
Ceriodaphnia cornuta	Paper authors, India KY231381	100	0	Plus	*Ceriodaphnia cornuta*	Wang *et al*., 2015 China KP148261
Macrocyclops albidus	Paper authors, India KX822033	100	0	Plus	*Macrocyclops albidus*	Prosser *et al*., 2013 Mexico
Eucyclops speratus	Paper authors, India KY231382	98	0	Plus	*Eucyclops speratus*	Sukhikh, 2014 Russia KC627338

>*Brachionus calyciflorus* (648bp) KX822034
AAAGATATTGGAACGCTTTACTTTATTTTCGGAATTTGAGCCGGCTTAATTGGTCTTAGCATAAGATTCCTT
ATCCGCCTAGAACTAGGTGTAGTGGGGTCTTATCTTGGAGATGAGCATTTATACAATGTACTCGTCACAGC
TCATGCATTTGTAATGATTTTCTTTATAGTTATGCCAGTCTCTATGGGCGGCTTCGGTAATTGACTTATTCCA
CTTATGTTAGGGGTAGCTGATATGGCTTTCCCTCGTATGAATAATTTATCTTTCTGGCTTTTAGTCCCTGCAT
TTATGTTTTTACTTCTGTCTTCCGCTATTGATGCTGGAGCCGGTACAGGGTGGACTGTTTACCCTCCCCTTTC
AGATTCGAGATACCATAGTGGTATTTCGGTTGATTTAGCGATTTTTAGTCTTCACTTATCTGGGGTCTCTTCT
ATCTTAGGTAGGATTAACTTCTTGACCACTATTATTTGCTCACGTACTACAAAAAGAATCTCGTTAGACCGT
CTTCCTCTCTTCTTATGGGCTATTGCTGTAACAGCAGTGCTCTTGATTACAAGGCTTCCCGTGTTAGCTGGG
GCTATTACTATGTTACTTACCGATCGTAATTTTAATACCTCTTTCTTTGACCCTGCTGGTGGAGGGAACCCA
GTTCTCTA

>*Brachionus caudatus personatus* (648 bp) KX822035
TATTTCATTTTTGGTATTTGAGCTGGTCTTATTGGTTTAAGAATAAGATTCTTAATTCGTTTAGAATTAGGTG
TTGTTGGTTCATATTTAGGTGATGAGCATCTTTATAATGTTTTAGTTACTGCTCATGCTTTTGTTATAATTTTT
TTTATAGTTATGCCTGTCTCTATGGGTGGTTTTGGTAATTGATTAATCCCACTTATGCTTGGTGTTGCTGATA
TGGCTTTCCCTCGTATGAATAACTTATCGTTTTGATTGTTAGTTCCTGCTTTTGTTTTCCTTCTTTTATCTTCT

GTTCTTGATGCTGGTGTTGGTACAGGTTGAACTGTTTATCCTCCTTTATCTGATTCTATTTACCATAGAGGTG
TGTCAGTCGATCTTGCTATTTTTAGTCTTCATTTATCTGGTGTTTCTTCTATTCTTGGTAGTATTAATTTTTTA
ACTACTATTATCTGTTCACGTACTACTAAAAGTGTGTCTTTAGATCGTCTTCCTTTAATGTTGTGAGCTATTG
CTGTCACAGCTATTCTTTTAATTACAAGTCTTCCGGTTTTAGCAGGTGCTATTACTATGTTACTTACTGATCG
TAATTTTAATACATCTTTCTTCGATCCTTCTGGTGGTGGTAATCCTGTGTTATACCAACAT

>*Brachionus rubens* (609 bp) KY231380
AATCATAAAGATATTGGTACTCTTTATTTTATCTTCGGTATTTGAGCCGGCTTAATCGGGTTAAGAATAAGG
TTCTTAATTCGCCTAGAGCTTGGTGTTGTAGGTTCGTATCTTGGTGACGAACACCTTTACAATGTATTGGTT
ACTGCTCATGCATTTGTAATGATTTTCTTTATAGTTATGCCTGTTTCTATGGGTGGTTTTGGTAATTGATTAA
TTCCTTTAATAGTAGGTGTTGCAGATATAGCCTTCCCTCGAATGAATAATCTTTCCTTCTGATTGTTAGTCCC
AGCTTTTTTCTTTTTACTTTTATCTTCTATTTTAGATGCAGGTGTAGGTACTGGTTGAACAGTCTACCCTCCT
TTATCTGATTCTACTTATCATAGAGGGGTTTCTGTTGATTTAGCTATTTTTAGTTTACATGTTTCTGGTGTTTC
TCTATTTTAGGTAGAATTAACTTTTTAACTACTATTATTTGCTCTCGTACAACTAAAAGAATCTCTTTAGATC
GCATGCCTTTAATGTTGTGAGCTATCGCTGTTACAGCTATTCTTCTAATTACTAGGCTTCCTGTTTTAGCTGG
TGCTATTACTATGCTTTTAACTGATC

>*Ceriodaphnia cornuta* (673 bp) KY231381
TCAAAATAAATGCTGGTATAAAATTGGATCCCCCCCTCCAGCTGGGTCAAAAAATGAGGTGTTTAAATTAC
GATCTGTAAGTAATATAGTAATAGCCCCAGCTAAGACTGGTAAACTTAATAGAAGTAATAAAGCAGTGAT
ACCAACAGCTCAAACAAATAAAGGAATTCGATCTAACGTTATTCCTTGAGATCGTATATTAATAATAGTAG
TAATAAAATTAACCGCCCCTAAGATTGAGGAAATCCCAGCTAAATGTAATGAAAAAATACTAAGATCTAC
AGAGGCCCCAGAGTGAGCAATTCCAGCAGATAGAGGAGGATAAACAGTTCAACCAGTCCCGGCACCTCTT
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AGGAAAAGCCATGTCAGGGGCTCCTAACATCAAAGGCACTAATCAGTTTCCAAATCCCCCAATTATAATAG
GTATAACCATAAAAAAAATTATAATAAAAGCGTGAGCAGTAACAATAACATTATAAATCTGGTCATCCCCA
ATCAATCTACCAGACTGGCCAAGTTCTGCTCGAATAAGTATACTTAAAGCAGTTCCTACCATCCCAGATCA
AACCCCAAAAATAAAATATAAAGTACCAATATCTTTA

>*Macrocyclops albidus* (628 bp) KX822033
AACTTTATATTTATTAGCAGGTGCTTGAGCCGGATTAGTTGGAACTGGTTTAAGTATAATTATTCGATTGGA
ATTGGGACAACCTGGAAGTTTATTGGGGGATGACCAGATTTATAATGTTGTAGTAATAGCTCATGCTTTTGT
AATAATTTTTTTTATAGTTATACCTATTTTAATTGGGGGGTTTGGAAACTGATTAGTTCCTCTAATATTAGGA
TCCCCGGATATGGCTTTTCCTCGTATAAATAATATAAGGTTTTGGTTTTTATTACCAGCTTTAATCCTTTTAC
TAGCTAGAGCCTTAGTGAGTCTGGTGCCGGGACTGGGTGAACAGTTTACCCTCCCCTAAGAAGTAATTTGG
CTCACTCTGGAGCCTCGGTAGATTATGCTATTTTTTCTTTACATTTGGCTGGTGTTTCTTCTATTTTAGGAGC
TGTAAATTTTATTAGCACAATGGGAAATTTACGAACTTTTGGTATAACCGGAGATCGGGTTCCCCTATTTGC
ATGAGCTGTTTTAATAACAGCCATTCTTTTACTTCTTTCACTGCCTGTTTTAGCAGGGGCAATTACCATATTA
TTAACTGACCGTAATTTAAATACAACTTTTTATGATCCAAGTGGAGGA

>*Eucyclops speratus* (646 bp) KY231382
TATTTGCTTGCGGGGGCTTGAGCGGGACTGATCGGGACAGGGCTAAGGGTATTAATTCGTCTAGAATTAGG
CTCTCCAGGTAGTTTAATAGGAGATGATCAGCTTTATAATGTCATTGTGACAGCCCATGCTTTTAATATAAT
TTTTTTTATAGTTATACCTATTTTAATTGGGGGGTTTGGAAATTGACTTGTTCCGTTAATATTAGGATCTCCA
GATATAGCGTTTCCACGAATAAACAATATAAGGTTTTGGTTTTTAATACCTGCTTTAGTAATACTATTAATA
AGGGCTCTGGTGGAAAGAGGGGCAGGAACAGGGTGAAGAGTTTATCCTCCTCTAAGAAGTAATTTAGCTC
ACGGGGGAGCATCTGTTGATTTTGCAATTTTCTCCTTACATTTGGCAGGAGTCTCTTCTATTTTAGGTGCGG
TAAATTTTATTAGCACACTAGGAAACCTTCGTTCTCTAGGACTTTCCATAGACCGGGTTCCGTTATTTGGGT
GGGCTGTTTTGGTGACCGCAGTTTTGCTTCTACTTTCTTTACCAGTCTTAGCAGGGGCCATTACTATATTATT
AACTGATCGAAATTTAAACACTAGATTTTATGATGTTAGAGGTGGGGGTGATCCGGTTTTGTACCAGCACT

I, Identity; **G**, Gap; **M.S**, Matched strand; **COI**, Cytochrome C oxidase subunit I gene

The results of multiple sequence alignment revealed less numbers of identical and similar amino acid residues (171 and 66 respectively), and more number of variable amino acid sites (444) among the subjected sequences (Table 3; Figure 3).

The individual base composition of the COI gene fragment varied among the species. The variation for AT biases was ranged between 59.0-66.8% (*B. calyciflorus* and *B. caudatus personatus*) and for in GC biases between 33.2-41.0% (*B. caudatus personatus* and *B. calyciflorus*) (Table 4). The more AT biases recorded indicates the lower abundance of nuclear copies of mt-DNA (NUMTs) known as pseudogenes, homologs or paralogs. The higher AT biases have been reported in crabs and prawns [41, 42, 59], and in freshwater zooplankton as well [43, 44]. The higher A+T and lower G+C contents have also been reported by Wang *et al*., [60].

Table-3: Number of identical and similar amino acid residues, and number of variable amino acid sites of the COI gene partial sequences generated for subjected zooplankton species

Comparison of zooplankton species	Number identical amino acid residues	Number of similar amino acid residues	Number of variable amino acid sites
Zooplankton species	171	66	444

COI, Cytochrome C oxidase subunit I gene

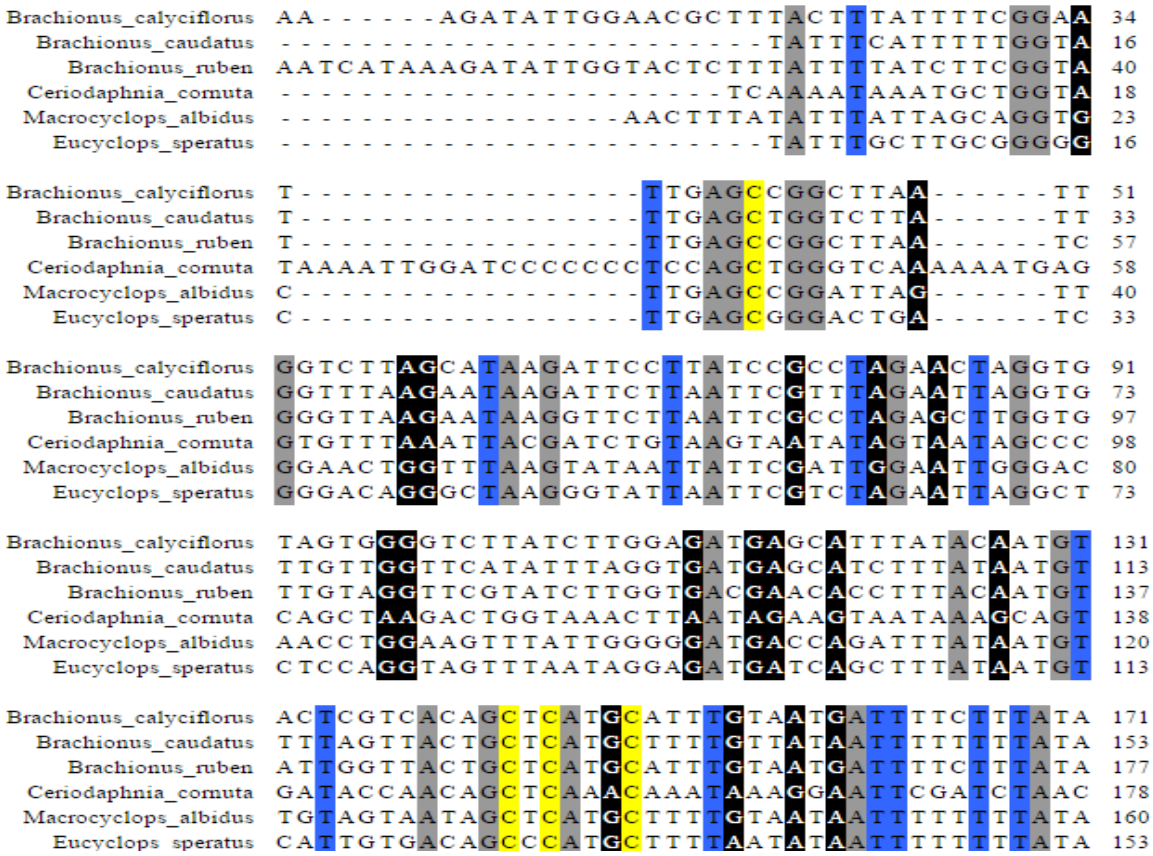

Fig-3: Multiple sequence alignment of COI gene sequences generated for subjected zooplankton. An alignment is formatted by using multiple align show (MAS) with coloured background and a consensus setting of 100%. Identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background

Table-4: Nucleotide composition percentage in COI gene partial sequences for subjected zooplankton species

Species Name	Nucleotide %					
	T	C	A	G	AT	GC
Brachionus calyciflorus	38.1	20.5	20.9	20.5	59.0	41.0
Brachionus caudatus personatus	47.2	15.3	19.6	17.9	66.8	33.2
Brachionus rubens	43.5	16.4	22.0	18.1	65.5	34.5
Ceriodaphnia cornuta	23.9	20.5	39.8	15.8	63.7	36.3
Macrocyclops albidus	38.5	15.6	25.3	20.5	63.9	36.1
Eucyclops speratus	35.8	15.9	24.6	23.7	60.4	39.6
Average	**37.7**	**17.4**	**25.5**	**19.4**	**63.2**	**36.8**

COI, Cytochrome C oxidase subunit I gene; **A**, Adenine; **T**, Thymine; **G**, Guanine; **C**, Cytosine

Inter species nucleotide divergence

In the subjected category (6 species: 3 Rotifers; 1 Cladoceran; 2 Copepods), among the fifteen combinations of different zooplankton, the mean divergent rate was 2.633 with a maximum of 8.33 (between *B. calyciflorus* vs. *C. cornuta*) and minimum of 0.138 (between *B. rubens* vs. *B. caudatus personatus*). However, the divergent value was >3% in following five combinations, *B. calyciflorus* vs. *C. cornuta* (8.333); *B. rubens* vs. *C. cornuta* (7.763); *B. caudatus personatus* vs. *C. cornuta* (6.248); *C. cornuta* vs. *M. albidus* (5.521) and *C. cornuta* vs. *E. speratus* (6.889) (Table 5).

Table-5: Inter-species divergence of subjected zooplankton species

Subjected species	Divergence (%)
Inter species divergence (subjected)	
Brachionus calyciflorus vs. *Brachionus rubens*	0.156
Brachionus calyciflorus vs. *Brachionus caudatus personatus*	0.145
Brachionus calyciflorus vs. *Ceriodaphnia cornuta*	8.333
Brachionus calyciflorus vs. *Macrocyclops albidus*	0.558
Brachionus calyciflorus vs. *Eucyclops speratus*	0.755
Brachionus rubens vs. *Brachionus caudatus personatus*	0.138
Brachionus rubens vs. *Ceriodaphnia cornuta*	7.763
Brachionus rubens vs. *Macrocyclops albidus*	0.653
Brachionus rubens vs. *Eucyclops speratus*	0.698
Brachionus caudatus personatus vs. *Ceriodaphnia cornuta*	6.248
Brachionus caudatus personatus vs. *Macrocyclops albidus*	0.632
Brachionus caudatus personatus vs. *Eucyclops speratus*	0.746
Ceriodaphnia cornuta vs. *Macrocyclops albidus*	5.521
Ceriodaphnia cornuta vs. *Eucyclops speratus*	6.889
Macrocyclops albidus vs. *Eucyclops speratus*	0.253
Average	2.633
Inter species divergence (subjected and retrieved)	
Brachionus calyciflorus vs. *Brachionus rubens* of Finland	0.157
Brachionus calyciflorus vs. *Brachionus rubens* of Canada	0.157
Brachionus rubens vs. *Brachionus calyciflorus* of China	0.174
Brachionus rubens vs. *Brachionus calyciflorus* of Mexico	8.598
Brachionus rubens vs. *Brachionus calyciflorus* of Finland	0.197
Brachionus rubens vs. *Brachionus calyciflorus* of Italy	0.137
Brachionus rubens vs. *Brachionus calyciflorus* of Russia	0.156
Brachionus rubens vs. *Brachionus calyciflorus* of Spain	0.137
Brachionus caudatus personatus vs. *Brachionus rubens* of Finland	0.133
Brachionus caudatus personatus vs. *Brachionus rubens* of Canada	0.133
Brachionus caudatus personatus vs. *Brachionus calyciflorus* of China	0.162
Brachionus caudatus personatus vs. *Brachionus calyciflorus* of Finland	0.135
Brachionus caudatus personatus vs. *Brachionus calyciflorus* of Italy	0.118
Brachionus caudatus personatus vs. *Brachionus calyciflorus* of Russia	0.145
Brachionus caudatus personatus vs. *Brachionus calyciflorus* of Spain	0.118
Brachionus rubens vs. *Brachionus caudatus personatus* of Mexico	0.138
Brachionus calyciflorus vs. *Brachionus caudatus personatus* of Mexico	0.145
Ceriodaphnia cornuta vs. *Brachionus rubens* of Finland	7.299
Ceriodaphnia cornuta vs. *Brachionus rubens* of Canada	7.299
Ceriodaphnia cornuta vs. *Brachionus calyciflorus* of China	6.587
Ceriodaphnia cornuta vs. *Brachionus calyciflorus* of Finland	0.211
Ceriodaphnia cornuta vs. *Brachionus calyciflorus* of Mexico	5.574
Ceriodaphnia cornuta vs. *Brachionus calyciflorus* of Italy	6.248
Ceriodaphnia cornuta vs. *Brachionus calyciflorus* of Russia	8.333
Ceriodaphnia cornuta vs. *Brachionus calyciflorus* of Spain	6.248
Ceriodaphnia cornuta vs. *Brachionus caudatus personatus* of Mexico	6.248
Brachionus rubens vs. *Ceriodaphnia cornuta* of China	7.856
Brachionus calyciflorus vs. *Ceriodaphnia cornuta* of China	8.455
Brachionus caudatus personatus vs. *Ceriodaphnia cornuta* of China	6.333
Brachionus rubens vs. *Ceriodaphnia cornuta* of Australia	0.616
Brachionus calyciflorus vs. *Ceriodaphnia cornuta* of Australia	0.696
Brachionus caudatus personatus vs. *Ceriodaphnia cornuta* of Australia	0.640
Macrocyclops albidus vs. *Brachionus rubens* of Finland	0.651
Macrocyclops albidus vs. *Brachionus rubens* of Canada	0.651
Macrocyclops albidus vs. *Brachionus calyciflorus* of China	0.559
Macrocyclops albidus vs. *Brachionus calyciflorus* of Mexico	5.947
Macrocyclops albidus vs. *Brachionus calyciflorus* of Finland	0.595
Macrocyclops albidus vs. *Brachionus calyciflorus* of Italy	0.525

Macrocyclops albidus vs. *Brachionus calyciflorus* of Russia	0.558
Macrocyclops albidus vs. *Brachionus calyciflorus* of Spain	0.525
Macrocyclops albidus vs. *Brachionus caudatus personatus* of Mexico	0.632
Macrocyclops albidus vs. *Ceriodaphnia cornuta* of China	5.612
Macrocyclops albidus vs. *Ceriodaphnia cornuta* of Australia	0.391
Brachionus rubens vs. *Macrocyclops albidus* of Mexico	0.613
Brachionus calyciflorus vs. *Macrocyclops albidus* of Mexico	0.655
Brachionus caudatus personatus vs. *Macrocyclops albidus* of Mexico	0.652
Ceriodaphnia cornuta vs. *Macrocyclops albidus* of Mexico	5.521
Brachionus rubens vs. *Macrocyclops albidus* of Korea	0.613
Brachionus caudatus personatus vs. *Macrocyclops albidus* of Korea	0.632
Ceriodaphnia cornuta vs. *Macrocyclops albidus* of Korea	4.924
Brachionus rubens vs. *Macrocyclops albidus* of Russia	0.701
Brachionus calyciflorus vs. *Macrocyclops albidus* of Russia	0.755
Brachionus caudatus personatus vs. *Macrocyclops albidus* of Russia	0.653
Ceriodaphnia cornuta vs. *Macrocyclops albidus* of Russia	4.280
Eucyclops speratus vs. *Brachionus rubens* of Finland	0.694
Eucyclops speratus vs. *Brachionus rubens* of Canada	0.694
Eucyclops speratus vs. *Brachionus calyciflorus* of China	0.760
Eucyclops speratus vs. *Brachionus calyciflorus* of Mexico	8.388
Eucyclops speratus vs. *Brachionus calyciflorus* of Finland	0.704
Eucyclops speratus vs. *Brachionus calyciflorus* of Italy	0.681
Eucyclops speratus vs. *Brachionus calyciflorus* of Russia	0.755
Eucyclops speratus vs. *Brachionus calyciflorus* of Spain	0.681
Eucyclops speratus vs. *Brachionus caudatus personatus* of Mexico	0.746
Eucyclops speratus vs. *Ceriodaphnia cornuta* of China	6.998
Eucyclops speratus vs. *Ceriodaphnia cornuta* of Australia	0.343
Eucyclops speratus vs. *Macrocyclops albidus* of Mexico	0.230
Eucyclops speratus vs. *Macrocyclops albidus* of Korea	0.253
Eucyclops speratus vs. *Macrocyclops albidus* of Russia	0.476
Brachionus rubens vs. *Eucyclops speratus* of Russia	0.698
Brachionus calyciflorus vs. *Eucyclops speratus* of Russia	0.755
Brachionus caudatus personatus vs. *Eucyclops speratus* of Russia	0.746
Ceriodaphnia cornuta vs. *Eucyclops speratus* of Russia	6.889
Macrocyclops albidus vs. *Eucyclops speratus* of Russia	0.253
Average	2.164
Intra species divergence (subjected and retrieved)	
Brachionus calyciflorus vs. *Brachionus calyciflorus* of Spain	0.103
Brachionus calyciflorus vs. *Brachionus calyciflorus* of Itali	0.103
Brachionus calyciflorus vs. *Brachionus calyciflorus* of Mexico	9.598
Brachionus calyciflorus vs. *Brachionus calyciflorus* of Finland	0.121
Brachionus calyciflorus vs. *Brachionus calyciflorus* of China	0.012
Brachionus calyciflorus vs. *Brachionus calyciflorus* of Russia	0.000
Brachionus caudatus personatus vs. *Brachionus caudatus personatus* of Mexico	0.000
Brachionus rubens vs. *Brachionus rubens* of Finland	0.000
Brachionus rubens vs. *Brachionus rubens* of Canada	0.000
Ceriodaphnia cornuta vs. *Ceriodaphnia cornuta* of China	0.000
Ceriodaphnia cornuta vs. *Ceriodaphnia cornuta* of Australia	3.189
Macrocyclops albidus vs. *Macrocyclops albidus* of Korea	0.057
Macrocyclops albidus vs. *Macrocyclops albidus* of Mexico	0.072
Macrocyclops albidus vs. *Macrocyclops albidus* of Russia	0.351
Eucyclops speratus vs. *Eucyclops speratus* of Russia	0.000
Average	0.907

When retrieved zooplankton species were included, the mean inter species divergence value was 2.164 with a maximum of 8.598 (between *B. rubens* vs. *B. calyciflorus* of Mexico) and minimum of 0.118 (*B. caudatus personatus* vs. *B. calyciflorus* of Italy and *B. caudatus personatus* vs. *B. calyciflorus* of Spain). In twenty combinations, the divergence value was >3%. These including four combinations of Rotifer species

(*B. rubens* vs. *B. calyciflorus* of Mexico, 8.598; *B. rubens* vs. *C. cornuta* of China, 7.856; *B. calyciflorus* vs. *C. cornuta* China, 8.455; *B. caudatus personatus* vs. *cornuta* of China, 6.333), twelve combinations of Cladoceran species (*C. cornuta* vs. *B. rubens* of Finland, 7.299; *C. cornuta* vs. *B. rubens* of Canada, 7.299; *C. cornuta* vs. *B. calyciflorus* of China, 6.587; *C. cornuta* vs. *B. calyciflorus* of Mexico, 5.574; *C. cornuta* vs. *B. calyciflorus* of Italy, 6.248; *C. cornuta* vs. *B. calyciflorus* of Russia, 8.333; *C. cornuta* vs. *B. calyciflorus* of Spain, 6.248; *C. cornuta* vs. *B. caudatus personatus* of Mexico, 6.248; *C. cornuta* vs. *C. cornuta* of Australia, 5.521; *C. cornuta* vs. *M. albidus* of Korea, 4.924; *C. cornuta* vs. *M. albidus* of Russia, 4.280; *C. cornuta* vs. *E. speratus* of Russia, 6.889) and four combinations of Copepod species (*M. albidus* vs. *B. calyciflorus* of Mexico, 5.947; *M. albidus* vs. *C. cornuta* of China, 5.612; *E. speratus* vs. *B. calyciflorus* of Mexico, 8.388; *E. speratus* vs. *C. cornuta* of China, 6.998) (Table 5).

The inter-species divergence of 0.613–1.142 between different species of freshwater zooplankton has been reported by us previously [43]. Similarly, the distance of 0.08–0.46 has been reported between different Rotifer species [61]. According to Lefebure *et al.*, [62], the divergences between species in both Cladocera and Copepoda are comparatively high.

Intra-species nucleotide divergence

The sequences of 6 subjected zooplanktons were matched with sequences of the same species available from all over the world revealed no intra-species divergence was seen in six different combinations (*B. calyciflorus* vs. *B. calyciflorus* of Russia; *B. caudatus personatus* vs. *B. caudatus personatus* of Mexico; *B. rubens* vs. *B. rubens* of Finland; *B. rubens* vs. *B. rubens* of Canada; *C. cornuta* vs. *C. cornuta* of China and *E. speratus* vs. *E. speratus* of Russia). However, the intra species divergence was >3% in two combinations (*B. calyciflorus* vs. *B. calyciflorus* of Mexico (9.598) and *C. cornuta* vs. *C. cornuta* of Australia (3.189) (Table 5).

In *Daphnia magna*, little and clear intra-specific divergence have been reported within populations of Europe and North America respectively [63]. In *Daphnia lumholtzi*, a clear intra-specific divergence has been reported between African and Australian populations [63]. These reports indicated the fact that these genetically divergent allopatric populations were reproductively isolated. In the same continent, significant divergence within the same species is based on their adaptation to different environmental conditions existed, and thus, different populations of the same species may evolve independently. The members of such populations can no longer breed with each other which prevent the gene flow between the populations. Penton *et al.* [34] discriminated two cryptic species within the *Daphnia obtusa* complex in North America using COI sequences. Adamowicz *et al*., [33] showed that 15 species of *Daphnia* from Argentina by the same gene. Generally, deep genetic divergence exists among allopatric populations of a single species. For example, five phylogroups of *Daphnia ambigua* (four in North America and one in South America) had been reported with >3% divergence [32]. In a study with six phylogroups of *Sida crystallina*, >5% divergence has also been reported [31].

In the present study, the retrieved species of *B. calyciflorus* from Mexico and *C. cornuta* of Australia showed higher level of intra-species divergence, 9.598 and 3.189 with respective subjected species when compared with species taken from other countries. This may be due to long geographical barrier and reproductive isolation between these populations.

Phylogenetic information

The predicted phylogenetic information, such as synonymous (Ks) and non-synonymous (Ka) substitutions, transitional (Ts) and transvertional (Tv) substitutions, and saturation, index of substitutional saturation (Iss) and critical value of index of substitutional saturation (Iss.c) are presented in Table 6; Plates 2 and 3. In the subjected category, the Ka was higher (2.206) than that of Ks (0.704), which indicates the possibility of occurrence of more deleterious mutation and less silent mutation. Similarly, the Tv was higher (0.37) than that of Ts (0.22), which indicates the fact that these sequences have more phylogenetic information. However, saturation might have not been occurred in these sequences, which was confirmed by the predicted higher Iss.c value (0.776) than that of the Iss (0.640) and more phylogenetic differences existed between sequences (Table 6; Figures 1 and 2 of Plate 2). The similar trend was also recorded when the retrieved and subjected species are pooled and analyzed together. The Ka, Tv and Iss.c was higher (2.195, 0.44 and 0.709, respectively) than that of Ks, Ts and Iss (0.671, 0.23 and 0.646, respectively) (Table-6; Figure-1 and 2 of Plate 3). The phylogenetic information have also been studied by us in species of crab, prawn, shrimp and plankton [40-44]. Saturation of substitutions in sequences decreases phylogenetic information [48, 64]. In the extreme case, when the sequences have experienced full substitutional saturation, the similarities between the sequences depend entirely on the similarity in nucleotide frequencies [46, 48, 65] which often does not reflect phylogenetic relationships.

Table-6: Overall average phylogenetic information of subjected and retrieved zooplankton species

Phylogenetic information	Ks	Ka	Ks-Ka	Ts	Tv	Tv-Ts	Iss	Iss.c	Iss.c-Iss
Subjected species	0.704	2.206	1.502	0.22	0.37	0.15	0.640	0.776	0.136
Subjected and retrieved species	0.671	2.195	1.524	0.23	0.44	0.21	0.646	0.709	0.06

Ks, Synonymous substitution; **Ka**, Non-synonymous substitution; **Ts**, Transitional substitution; **Tv**, Transversional substitution; **Iss**, Index of substitution saturation; **Iss.c**, Critical value of index of substitution saturation

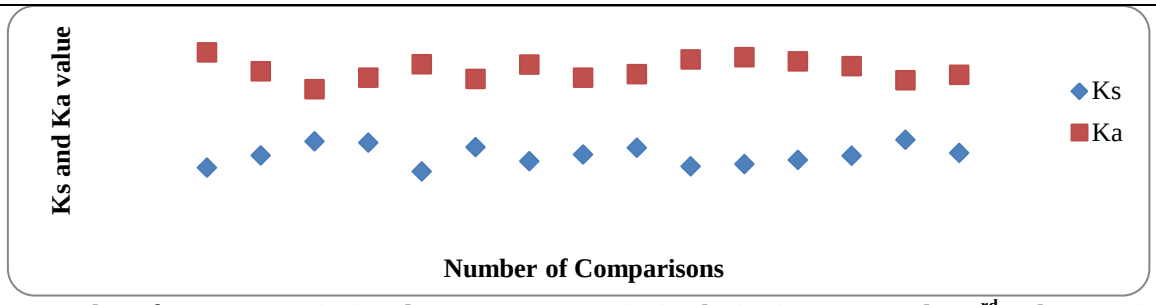

Fig-1: Number of synonymous (Ks) and non-synonymous (Ka) substitutions occurred at 3rd codon position in nucleotides of COI gene partial sequences within subjected zooplankton species

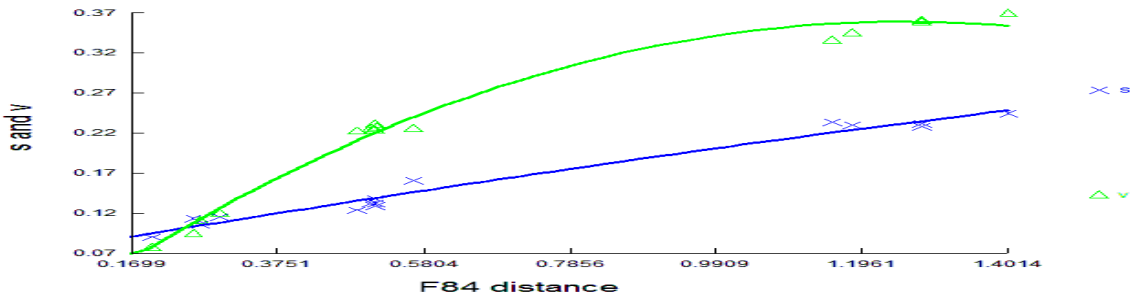

Fig-2: Scattergram of transitional (X, blue) and transversional (Δ, green) type substitutions occurred in COI gene partial sequences within subjected zooplankton species

Plate-2: Number of synonymous (Ks) and non-synonymous (Ka), transitional (X, blue) and transversional (Δ, green) substitutions of COI gene partial sequences within subjected zooplankton species

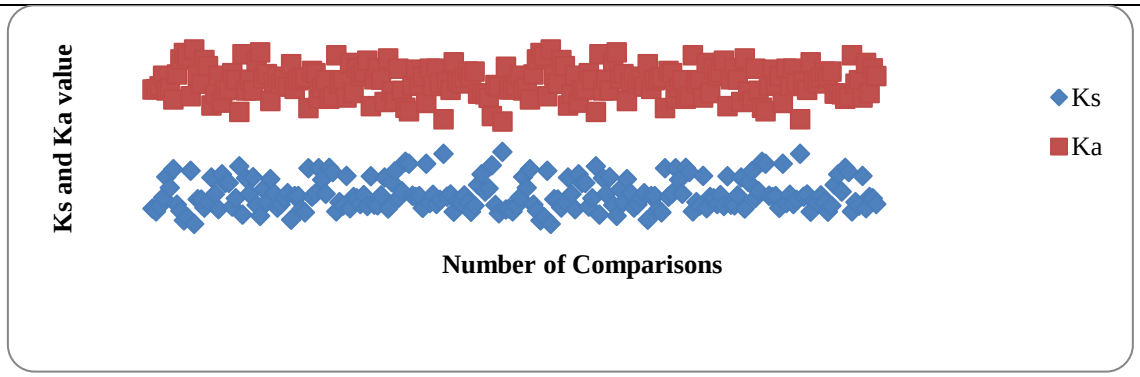

Fig-1: Number of synonymous (Ks) and non-synonymous (Ka) substitutions occurred at 3rd codon position in nucleotides of COI gene partial sequences of subjected and retrieved zooplankton species

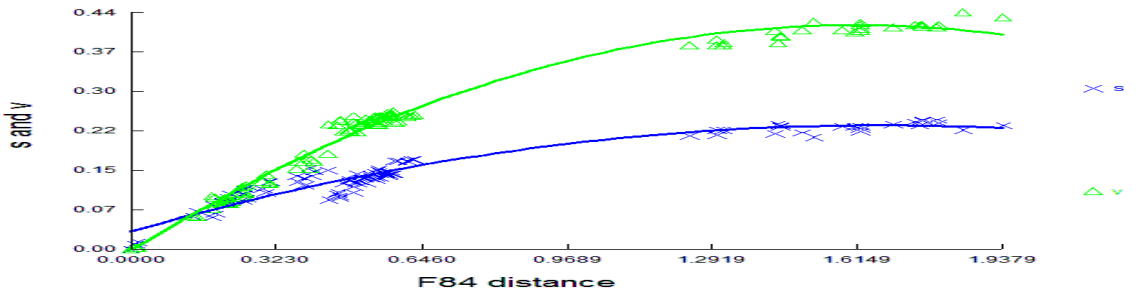

Fig-2: Scattergram of transitional (X, blue) and transversional (∆, green) type substitutions occurred in COI gene partial sequences of subjected and retrieved zooplankton species

Plate-3: Number of synonymous (Ks) and non-synonymous (Ka), transitional (X, blue) and transversional (∆, green) substitutions of COI gene partial sequences of subjected and retrieved zooplankton species

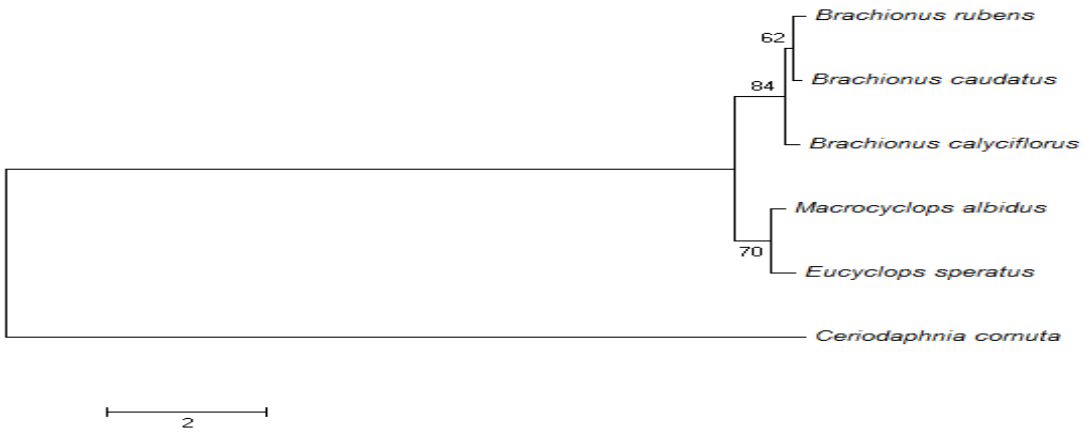

Fig-4: Phylogenetic tree topology of subjected zooplankton

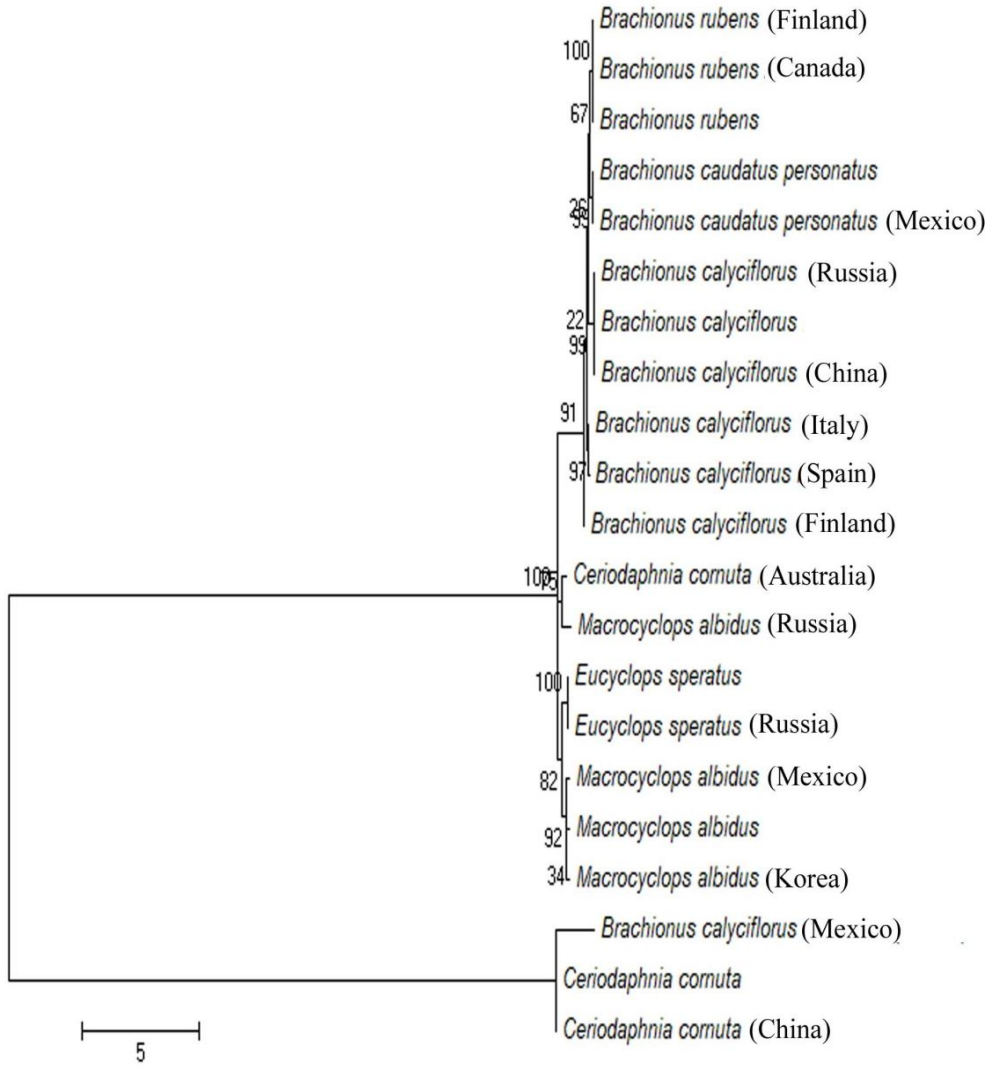

Fig-5: Phylogenetic tree topology of the subjected and retrieved zooplankton species

CONCLUSIONS

In this study, the mixed phytoplankton was served as the best feed for mass culture of zooplankton. The molecular phylogeny of studied species of Rotifera (*B. calyciflorus*, *B. caudatus personatus* and *B. ruben*), Cladocera (*C. cornuta*) and Copepoda (*M. albidus* and *E. speratus*) revealed that these groups are genetically distinct and highly conserved. However, species of each group is less conserved within themselves. Therefore, they would subject to evolutionary forces in due course.

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