DNA barcoding of rotifers along the Cochin backwaters, southwest coast of India

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This study examines the DNA barcoding of rotifers from the Cochin backwaters, Kerala, India, with specimens obtained from the pure culture, using primers LCO1490/ HCO2198 and a recently reported primer 30F/885R which was designed especially for the rotifers. Partial sequences of the mitochondrial COI gene from five species belonging to three genera (*Brachionus*, *Keratella* and *Asplanchna*) and two families (Brachionidae and Asplanchnidae) of the order Ploimida were generated. All the obtained nucleotide sequences were submitted to NCBI (accession numbers: OL477582–OL477584, OL477586 and OL757796).

Keywords: Backwaters, DNA barcoding, partial sequences, pure culture, rotifer.

ROTIFERS are microscopic aquatic invertebrates with high species richness in freshwater ecosystems[1], generally characterized by a corona formed by ciliary bands on the cephalic region[2]. In addition, they possess a complex pharyngeal apparatus called the mastax, consisting of hard parts (trophi), connective musculature and ligaments[3]. The three major clades within the phylum Rotifera are Seisonidae, Bdelloidea and Monogononta[4]. The third clade, Monogononta is thus far the most diverse group with about 1500 described species; it also exhibits prominent morphological inconsistency. Even if some sessile taxa are present, monogononts are mostly free-swimming. Globally above 2000 species of rotifers have been described, including 1571 species from Monogononta and 461 species from Bdelloidea[5,6]. Many monogononts follow a complex parthenogenetic cycle of an amictic phase and a mictic phase, which includes sexual reproduction and the presence of haploid dwarf males[3]. By transferring matter and energy from producers to higher consumers, rotifers which are the major invertebrates in the freshwater community, play an important role in the aquatic food chain[7]. As they are highly susceptible to the environmental changes, rotifers can act as useful indicators of water quality[8]. Some rotifer species are used as model organisms in different fields of biology because of their facultative parthenogenetic cycle and short generation time[9]. Rotifer species show potent variability in morphological patterns. On the contrary, some cryptic species are morphologically similar[10]. Also, the field of rotifer ecology and species distribution has to deal with the issue of inaccurate species identification[11]. DNA barcoding effectively identifies species and can help taxonomists in cryptic species identification. They are supplementary tools for resolving taxonomic ambiguities. For understanding the advancement and heterogeneity of life, molecular phylogeny has become a useful tool when molecular information is available[12]. Kumazaki *et al.*[13] reported the first 5s rRNA sequence from a rotifer and more such DNA sequences have since been reported. For the identification of microfauna such as rotifers, mitochondrial cytochrome oxidase subunit I (COI) barcoding has become popular[11]. COI is the most popular genetic marker used because of its high polymorphism, rapid evolution, easy amplification and sequencing[14]. Though rotifers play an important role in freshwater ecosystems, evolutionary relationships within their phylum are poorly understood. *Brachionus* has always been used for the main molecular studies. Gómez[15] employed allozyme electrophoresis for his study because of the small body size of the rotifers and the difficulty in laboratory culture of many species. According to Gómez[16], mitochondrial DNA is used, because variance at a regional scale can be frequently identified in a species when relatively fast-evolving genes are studied. Haploidy, clonality and uniparental mode of inheritance are the other reasons. HCO12198/LCO11490 are the most common universal primers that help amplify rotifer groups such as *Brachionus* and *Keratella*[17]. These universal primers often fail or amplify relenting practical application[18]. For COI amplification, new universal and highly effective primers, particularly for rotifers, are still under development[14]. A new pair of primers, 30F/885R, to amplify the COI genes of rotifers has been recently developed[19]. It was found to be more efficient (86%) than two pairs of universal primers, namely dgLCO/dgHCO (32%) and Folmer primers (59%)[19]. However, these primers did not significantly improve the amplification efficiency. The COI sequence of *Colurella adriatica* from the Andaman and Nicobar Islands was reported by Madhu *et al.*[20]. A database on the sequence of Indian rotifers is lacking. In this context, the present study aims to barcode the partial mitochondrial COI sequence of certain rotifers in Cochin backwaters, Kerala, India.

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Materials and methods

Algal culture

Prior to rotifer sample collection, algal species such as *Nannochloropsis oculata*, freshwater *Chlorella* sp. and marine *Chlorella* sp. were cultured in the algal laboratory of Central Marine Fisheries Research Institute, Kochi. Then the algal cultures were maintained at 23°C in F/2 medium[21]. Then they were developed in different salinities, according to the site from where the rotifers were proposed to be collected. When the algal growth became optimum, rotifer samples were collected.

Rotifer collection and culture

Specimens were sampled from various localities of the Cochin backwaters. Rotifers were collected using a 20 µm mesh-sized plankton net and identified morphologically under a compound microscope using standard procedures[2,6]. Next, they were transferred to different test tubes, with water from the same collection area as the medium for development, by filtering with a 17 µm mesh size smaller than the collection net to eliminate ciliates. Also, 4–5 ml of water was initially given as the feed. The isolated specimen was kept in water for 24 h before the addition of algae. Algal feeding started the next day using *N. oculata* and freshwater *Chlorella* sp. alternatively. The cell quantity for freshwater *Chlorella* sp. and *N. oculata* (7 ppt) was 1986.6×10^4 and 643.3×10^4 cells/ml respectively. The algal cell counts were enumerated using a Sedgewick rafter counting cell. The cell quantity of *N. oculata* may change according to salinity. The rotifer culture started developing from the third to fourth day after isolation at a temperature of 32°C. On the seventh day, the culture developed further. The isolated samples were cultured until the required volume of rotifers was obtained.

DNA isolation

For DNA isolation, a minimum of 10–15 rotifers were taken depending on their size. Before DNA extraction, all individuals were washed twice with autoclaved MilliQ water and the specimens were preserved in 95% ethanol. DNA extraction was done using a DNA extraction kit (Origin Diagnostics, India) and quantified using Nanodrop One (Thermo Fisher Scientific, USA). Approximately 650 bp region of the cytochrome C oxidase (CO1) mitochondrial gene were amplified using the universal primers, viz. LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′, HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (ref. 17) and a new set of primers, viz. 30F/885R recently designed for rotifers – 30F F: HACTAATCAYAARGATATTGGWAC and 885R R: RAACATATGATGAGCYCAWACAAT (ref. 19).

PCR was performed using Biorad T100 thermal cycler (Biorad, USA) with a final reaction volume of 20 µl containing 10 µl 2× PCR master mix, 0.4 µl of each oligonucleotide (0.2 µM), 7.2 µl of PCR water and 2 µl of template DNA. PCR amplification was performed with an initial denaturation for 4 min at 94°C followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 30 s, extension at 72°C for 45 s and a final extension at 72°C for 7 min for amplification of the *CO1* gene.

The PCR amplified products were purified and separated on 1.2% agarose gel (1× TBE buffer), stained with ethidium bromide and visualized using a gel documentation system (Vilber Limited, France) under ultraviolet trans-illumination. Sequencing was carried out with an automated sequencer (ABI 3730) using the primers. All the partial sequences of the *COI* gene of the five rotifer species were deposited in GenBank with accession nos OL477582–OL477584, OL477586 and OL757796).

Sequence alignment and analysis

The *COI* gene sequences of the five specimens generated in this study were aligned, edited and analysed with reference sequences from 49 other species belonging to two families (Brachionidae and Asplanchnidae) and three genera (*Brachionus*, *Keratella* and *Asplanchna*) retrieved from Barcode Of Life Data (BOLD) using the ClustalW algorithm in MEGA 10 (ref. 22). The aligned sequences were used for constructing the phylogenetic tree employing that Bayesian inference (BI) method using MrBayes 3.2.7 software[23]. For Bayesian analysis, 100,000 generations were carried out with the Markov chain Monte Carlo (MCMC) method. The Bayesian tree was constructed until the average standard deviation of split frequency was less than 0.01. The subsequent tree from the above software was visualized and modified using FigTree v I.4.3 with customizable settings. The genetic divergence between and within species was estimated using Kimura two-parameter distance values in MEGA 10 (ref. 22). The synonymous (*Ks*) to non-synonymous (*Ka*) substitution ratio was estimated using DnaSP v6 (ref. 24). To evaluate the selective pressures and evolutionary relationships of species, the *Ka* and *Ks* ratios were estimated. $Ka/Ks > 1$ signifies positive (adaptive or diversifying) selection, $Ka/Ks < 1$ denotes negative (purifying) selection, and $Ka/Ks = 1$ shows neutral mutation[25].

Results

Partial sequences of mitochondrial *COI* gene from five species (Figure 1) belonging to three genera and two families of order Ploimida generated were: *Brachionus calyciflorus* Pallas, 1766; *Brachionus plicatilis* O.F. Muller, 1786; *Brachionus angularis* Gosse, 1851; *Keratella tropica* (Apstein, 1907), and *Asplanchna brightwelli* Gosse, 1850. All the obtained nucleotide sequences were submitted to NCBI.

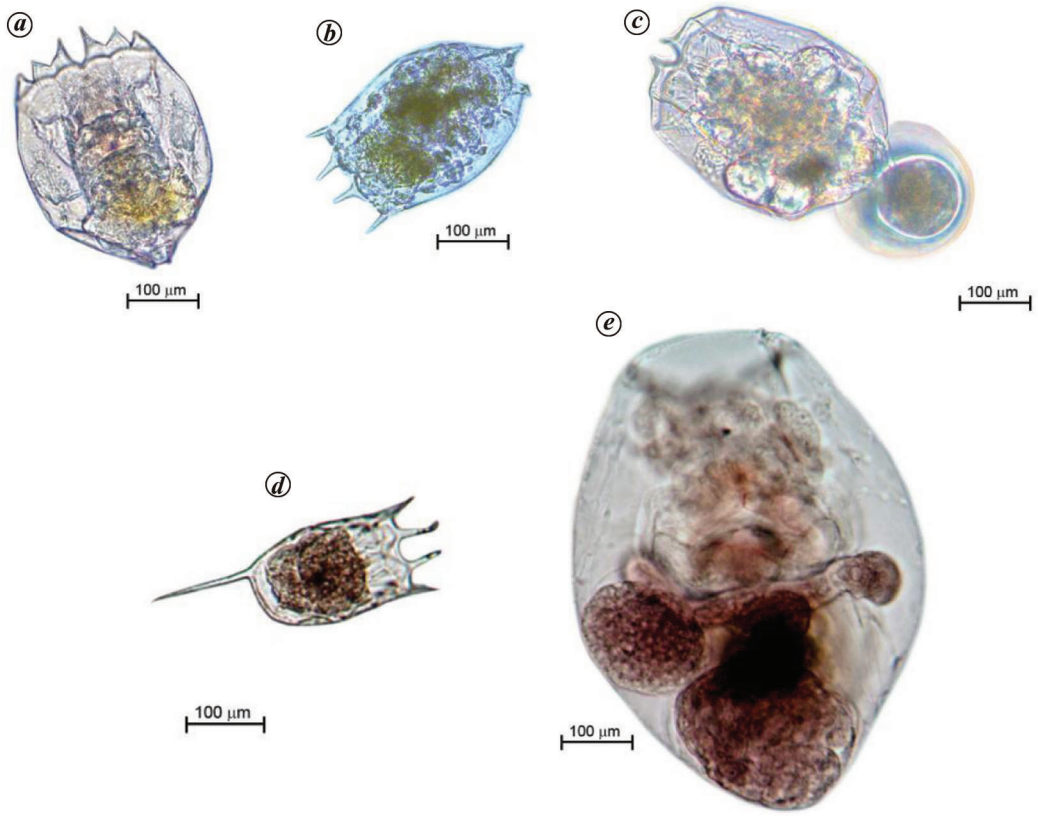

Figure 1. ***a***, *Brachionus plicatilis*; ***b***, *Brachionus calyciflorus*; ***c***, *Brachionus angularis*; ***d***, *Keratella tropica*; ***e***, *Asplanchna brightwelli.*

A total of 54 rotifer sequences were used to construct the phylogenetic tree (Figure 2) and for the Kimura two-parameter calculation. The two families formed distinct clusters in the phylogenetic tree. The sequences generated clustered along with those of the corresponding species retrieved from NCBI, GenBank. The *COI* gene sequence of *Filinia longiseta* and *Filinia opoliensis* retrieved from BOLD were used as outgroups for phylogenetic analysis.

The overall genetic distance was 0.147 (14.7%). The minimum intergenetic distance was observed between *B. plicatilis* (0.109) and *B. calyciflorus* (0.028). The intraspecific distance of the selected rotifers was in the range 1.2–6.6% (Table 1). The highest intraspecific distance was noted among the *B. angularis* (6.6%), followed by *B. calyciflorus*, *K. tropica* (4.4%) and *B. plicatilis* (4.1%). *A. brightwelli* (1.2%) showed the least intraspecific distance. The *Ka*/*Ks* ratio was less than one (0.11459), signifying a strong purifying selection[25].

Description of the sequenced species

Figure 1 *a*–*e* shows images of the five sequenced species[26].

Brachionus plicatilis, Muller, 1786 (accession no. OL477-586)

Lorica flexible, oval, greater width of about two-thirds lengths of lorica from anterior end, anteriorly narrows, not sharply separated into dorsal and ventral plates but slightly compressed dorso-ventrally; antero-dorsal margin with six broad-based saw-toothed spines; nearly equal in length; posterior spines wanting; mental margin four-lobed; foot opening with small sub-square aperture dorsally and a longer *V*-shaped aperture ventrally.

Brachionus calyciflorus, Pallas, 1776 (accession no. OL4-77584)

Lorica flexible, not separated into dorsal and ventral plates; slightly dorso-ventrally compressed body, anterior–dorsal margin with four broad-based spines of variable length, median spines longer than laterals. Flexible mental margin, somewhat elevated with shallow *V*- or *U*-shaped notch, unflanked; posterior spines present or absent; lorica smooth.

Brachionus angularis (Gosse, 1851) (accession no. OL47-7583)

Firm lorica, lightly or heavily stippled, divided into dorsal and ventral plate, antero-dorsal margin with two median spines; lateral and intermediate spines absent; larger foot aperture in ventral plate.

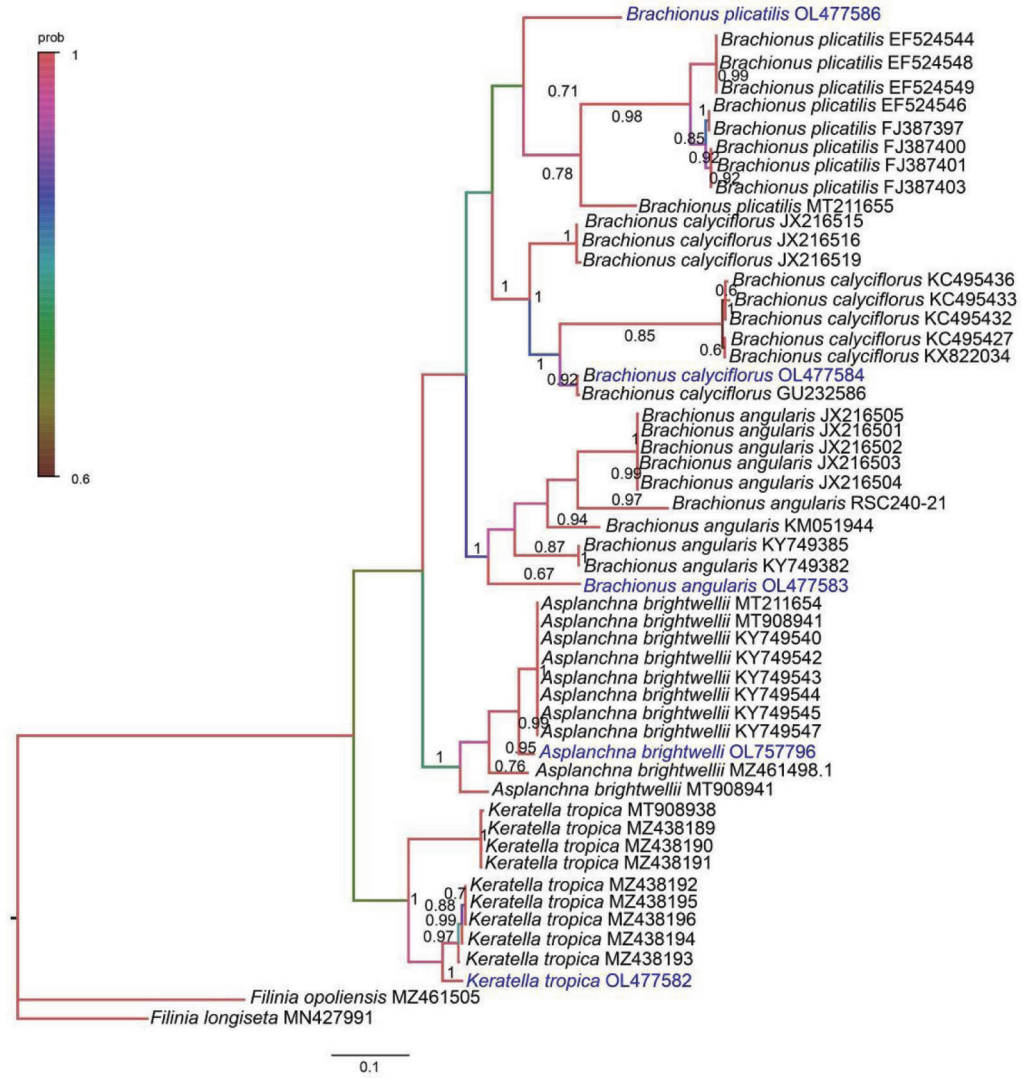

Figure 2. Bayesian inference tree inferred from the nucleotide sequences of COI sequences of different rotifer species.

Table 1. Kimura two-parameter intraspecific genetic distances based on COI sequences and standard error values

Rotifer species	*d*	*e*
Brachionus plicatilis	0.041	0.012
Brachionus angularis	0.066	0.016
Brachionus calyciflorus	0.044	0.015
Keratella tropica	0.044	0.015
Asplanchna brightwelli	0.012	0.006

d, Intra-genetic distance; *e*, Standard error.

Keratella tropica (Apstein, 1907) (accession no. OL477582)

Dorso-ventrally compressed lorica; dorsal plate sculptured, with flat or slightly concave ventral plate; antero-dorsal margin mostly with six spines, with medians being the longest; one or two posterior spines often present, head retractile and illoricate.

Asplanchna brightwelli Gosse, 1850 (accession no. OL757796)

Large form with a transparent sacciform body, extremely contractile, lorica absent, a large body cavity, and a stomach lying separated from the body wall. Stomach lobed, *U*-shaped vitellarium and foot not present.

Discussion

The present study deals with DNA barcoding of rotifers with specimens obtained from the pure culture using primers LCO1490/HCO2198 and 30F/885R. According to Zhang *et al.*[19], rotifer species differ largely in body weight, which may affect successive mtDNA amplification and sequencing of rotifers. This determines the amount of DNA for extraction. The smaller body size may not be the main reason for failure of amplification and sequencing of certain rotifers.

COI sequences show great variation among different rotifer species[27].

Rooting was done with *Filinia*, as these belong to the Gnesiotrocha, the sister group of Ploimids, and the relation between the groups is well established. The Kimura two-parameter distance values and phylogenetic tree helped understand the genetic divergence within and between the rotifers of different geographical areas. DNA barcoding covered five species of rotifers in the present study area, and the obtained barcode database will benefit future surveys of rotifers in similar regions.

Table 1 reveals that a certain inter- and intraspecific genetic distance exists between rotifer species like *B. angularis*, *K. tropica*, *B. calyciflorus* and *B. plicatilis*, and the result is concordant with the following mentioned works. Zhang *et al.*[19] also reported high intraspecific genetic distance in 15 rotifer species, including *B. calyciflorus* and *K. tropica*. Rotifers are among the most prevalent zooplankton in freshwater and brackishwater ecosystems, with the presence of cryptic species in *B. plicatilis*[28], *B. calyciflorus*[29] and *K. tropica*[18]. According to Fontaneto *et al.*[30], *B. plicatilis* species complex comprises at least 14 cryptic species. Gilbert and Walsh[31] have reported that *B. calyciflorus* constitutes eight cryptic species. Campillo *et al.*[32] mentioned the deep genetic divergences in spite of morphological similarities between cryptic *B. plicatilis* species.

According to Serra *et al.*[33], the genetic differentiation across geographical areas may be due to the effect of rotifer occupancy in transitory changing environments, which in turn impacts the timing of sexual reproduction in cyclic parthenogenetic organisms.

Conclusion

In conclusion, pure cultures of rotifers were obtained for DNA isolation and sequencing. They were washed and preserved in 95% ethanol and DNA was isolated using the DNA extraction kit. The amplified PCR product was sequenced and the six nucleotide sequences obtained were submitted to NCBI. Using these and other sequences retrieved from BOLD, a phylogenetic tree was constructed and genetic divergence was estimated using Kimura two-parameter distance values in MEGA 10. Also, *Ka*/*Ks* ratio was estimated to evaluate the selective pressure and evolutionary relationships of the species. This molecular approach can analyse the regional genetic diversity of rotifers and help uncover rotifer species diversity. It can open up avenues for further molecular studies of Indian rotifers, especially in the Cochin backwaters. Our attempt of initiating barcoding studies and creating a database of Indian rotifers in the Cochin backwaters will pave the way for more advanced molecular studies in this area.

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