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MORPHOLOGICAL AND PHYLOGENETIC CHARACTERIZATION OF DAPHNIA FROM THE HIGH-ALTITUDE BRACKISH (PANGONG) LAKE OF LADAKH USING DNA BARCODING

Zoology

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ABSTRACT

Daphnia on the Tibetan Plateau has been little studied, and information on species diversity and biogeography is lacking. Our study is the pioneer to confirm the presence of the *Daphnia* lineage in Pangong lake situated in eastern Ladakh (UT). We identified the sample specimen both on the basis of morphological and molecular data. Through morphological characteristics using SEM, simple compound microscopy and cellular phone microscope we identified our specimen which showed maximum morphological similarity with the Family Daphniidae using taxonomic keys. Further, using DNA barcoding a fragment of the mitochondrial COI gene and 16S rRNA gene was amplified and phylogenetic analysis was carried out to determine the position of specimen in crustacean classification and the specimen was identified as *Daphnia tibetana* in both the cases.

KEYWORDS

Pangong lake, DNA barcoding, COI, 16S rRNA, Daphnia

INTRODUCTION

Pangong Tso (lake) is one of the geographically and strategically important lake in India situated in the Tibetan plateau as LAC (line of actual control) crosses in between the lake. This lake is exposed anthropogenically in the last few years as it has become a tourist attraction site in Ladakh. Pangong lake in Changthang region of Ladakh is the largest and highest brackish lake situated at a height of almost 4350m.a.s.l in the Trans-Himalayan cold desert ecosystem. This lake is not well explored due to short months of summers and physical isolation. It is characterized by extreme environmental conditions (low temperature, strong radiations, mostly low buffering capacity and low nutrient level) (Bhat et al., 2011; Aftab 2011; Rathour et al., 2017) harboring simple ecosystem with relatively less ecological diversity that react more rapidly to the environmental changes than other lakes (Psenner, 2002; Bhat et al., 2011). Owing to the harsh conditions the productivity is low, because of that majority of lakes in Tibetan plateau support low levels of biodiversity and simple food webs (Rautio et al., 2011; Ren et al., 2022).

Crustaceans are extremely important members of the planktonic and benthic communities in almost all inland water ecosystems. They act as food web linker between primary producers (algae and aquatic weeds) and higher trophic levels and plays a crucial role in nutrient cycling. They have also become an important model in ecology and evolution (Miner et al., 2012; Stollewerk 2010), toxicology (Shaw et al., 2008) and genomics (Colbourne et al., 2011; Liu et al., 2022). *Cladoceran* are monophyletic and one of the key components of microcrustacean zooplanktons but still a great ambiguity was observed in the evolutionary relationships among organisms belonging to different groups of *Cladoceran* (Schmidt-Rhaesa1998; Forró 2008; Deng et al., 2022). One of the important crustaceans observed in Tibetan plateau lakes is water flea (*Daphnia).* It is a branchiopod crustacean genus in the family *Daphniidae* of order *Cladoceran.*

Identification of daphniids have always been complicated due to the wide- range variations created by a combination of phenotypic plasticity i.e., cyclic changes in morphology due to environmental conditions that often leads to the coexistence of morphologically similar species and inter-specific hybridization (Brooks 1957; Ma et al. 2015). Therefore, usage of traditional identification method requires taxonomical expertise and is time consuming so, molecular analysis such as DNA barcoding provide rapid and more precise identification of the species of interest.

With the advancement in scientific techniques, molecular identification of sample specimen using DNA barcoding has become a topic of interest. DNA barcoding is a system for fast and accurate species identification that makes ecological system more accessible by using short DNA sequence instead of whole genome (Hebert et al., 2003; Hebert and Gregory, 2005). The short DNA sequence is generated from standard region of genome known as marker. Most frequently used marker for the identification and phylogeny of *Daphnia* are Cytochrome c oxidase I (COI) (Xu et al., 2018; Hamza et al., 2022), 16S rRNA (Hahn et al., 2011; Wang et al., 2019) and ITS (Weng et al., 2020; Deng et al., 2022). In phylogenetic studies, DNA barcoding can be a starting point for optimal selection of taxa and barcode sequences can be added to the sequence dataset for phylogenetic analysis.

So, keeping the above point in consideration the objective of this study was to identify the specimen with the help of morphological keys as well as construction of phylogenetic tree to determine the position of specimen in crustacean classification.

MATERIAL AND METHODS:

Collection and morphological identification

Water sample (5L) containing the specimen was collected in summer 2019 from the Pangong lake situated at latitude 33.7595° N and longitude 78.6674° E (Figure1). Filtration of water was carried out on 0.2µ PTFE (polytetrafluorethylene) membrane filter paper with the help of tarsons high voltage vacuum pump. Specimen on filter paper were collected and stored in 10 ml glass vials containing 70% alcohol were shipped via flight (1 hr.) to Department of Zoology, Panjab University Chandigarh. Sorted individuals were transferred into a fresh glass vails and preserved in 95% ethanol at -20°C freezer for further analysis.

Figure 1: (a, b) Collection of sample specimen from pristine Pangong lakes in Changthang region of Ladakh, India, (c) Collection of specimens after filtration of water (d) image of sample using cellular phone microscope.

For morphological identification simple compound microscopy, Scanning Electron Microscope (SEM) and cellular phone microscope were used (Silva-Briano et al., 2015). For SEM analysis, a total of 3 adult specimens were taken in different 1.5mL microcentrifuge tubes. Sample dehydration was done for 20 min in different grades of alcohol from 70%, 90% and 100% concentration followed by mounting of specimen on aluminium stubs using double adhesive tapes and brush under stereomicroscope.

After mounting, samples were subjected to (Critical Point Drying) C.P.D for complete dehydration accompanied by gold coating in

Sputter Coater in argon plasma at 0.1 mbar vacuum. Samples were visualized under JEOL-JSM 6100 SEM at 15-20 kV at low probe current.

Molecular Analysis

a. DNA extraction, PCR amplification

With the advancement in the molecular techniques, an attempt was made to establish phylogenetic relationship to authenticate morphologically identified specimen. Therefore, mitochondrial *16S* rRNA and *COI* gene were used as molecular markers. Total DNA isolation was done using KAPA Express Extraction Kit containing 1U/µL KAPA Express Extract Enzyme, 10X KAPA Express Extract Buffer, PCR-grade water. For amplification of desired ~ 650bp and ~710bp fragment of partial 16S rRNA (16S 1472 and 16Sarc by Cheng et al., 2019) and *COI* mitochondrial gene, degenerate Folmer primers (jgLCO1490 and jgHCO2198 by Gellar et al., 2013) were used. PCR conditions were standardized with different annealing temperature using gradient PCR. For COI gene each 25µL reaction mixture contains 9µL of PCR grade water, 0.5µL DMSO, 1µL of each primer, sample DNA of 1µL and 12.5µL Kappa master mix. Thermal conditions setup for PCR were as follows: one cycle of initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 15 sec, gradient annealing temperature at 42-52°C for 15 sec, extension 72°C for 15 sec and finishing with a single cycle of final extension at 72°C for 5 min. For 16S rRNA each 25µL reaction mixture constitute 4.5µL of PCR grade water, 1µL DMSO, 1µL of each primer, 5µL DNA and 12.5µL Kappa master mix. Thermal conditions setup for PCR were: 95°C for 5 min, 35 cycles of 95°C for 45 sec, 60.5°C for 20 sec, 72°C for 20 sec and 72°C for 10 min. PCR products were subjected to agarose gel electrophoresis of 1.6% concentration, stained using EtBr dye. From the gel, desired fragment was excised and purified using QIAquick Gel extraction kit. After gel elution, results were viewed on the EtBr stained 1.2% agarose gel. The DNA samples were sent for Sanger sequencing.

b. Sequencing and phylogenetic tree Construction

Nucleotides sequence obtained for both 16S and COI gene were retrieved in Bioedit Sequence Alignment Editor version7.2.6.1. The sequences retrieved were provided with the e-score which tells us about the degree of similarity between the sample sequence and retrieved sequences using NCBI BLAST. The selected sequences were downloaded from the NCBI and aligned using MUSCLE algorithm in MEGA 11.0.1 software. To establish an evolutionary relationship of sample with the other species of genus *Daphnia*, nucleotide sequences of already known species were taken based on percent identity and query coverage in NCBI BLAST. *Drosophila melanogaster* was taken as outgroup for 16S rRNA sequence and *Drosophila nigribasis* for COI gene sequence. Phylogenetic tree was reconstructed using Neighbor Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. Along with pairwise deletion in the sequence, pairwise genetic distance was calculated using Tamura 3-Parameter model with gamma distribution as well with Kimura 2-P model. For authentication of our results, bootstrapping was done with 1000 replications.

RESULTS AND DISCUSSION

The present study aims at the identification of the fresh water cladoceran crustacean belonging to class Branchiopoda found in the hypersaline lake through morphological as well as molecular analysis.

I. Morphological Analysis:

For morphological identification, along with simple compound microscopy, SEM studies were used for the taxonomic identification of the collected specimen (Elías-Gutiérrez et al., 1999; Silva-Briano et al., 2015).

The morphological characters used for the identification are listed as under (Figure 2 and 3).

1. Body enclosed in carapace having spines on posterior region
2. Head shape may vary
3. Presence of compound eye (position may vary)
4. Absence of helmet over head
5. Absence of rostrum on ventral side
6. Presence of cervical sinus on dorsal side
7. Gut was clearly visible
8. Second antenna having non-plumose swimming setae (length almost equal to body length)
9. Emergence of two branches (dorsal and ventral ramus) from second antennae
10. Presence of reticulations on second antennae
11. First antennule was not clearly visible
12. Females were having egg in their brood chamber

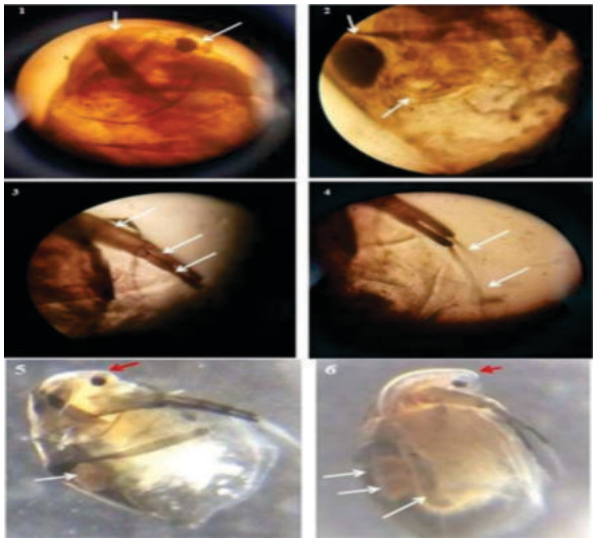

Figure 2: Images of Daphnia obtained from compound microscope showing (1) compound eye and head with no helmet (2) one egg in a brood chamber and portion of hindgut (3) second antenna and two branches arising from it representing dorsal and ventral ramus (4) swimming setae arising from second antenna (5) female with one egg in brood chamber (6) Gut of female, 2 eggs in brood chamber of female. Both the female individuals have variation in head shape indicated by red arrow.

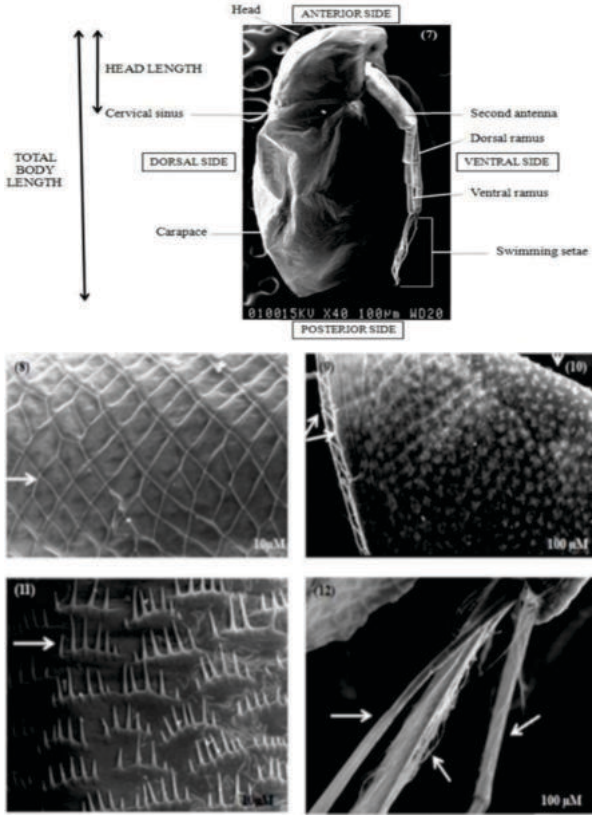

Figure 3: 7) SEM microphotograph showing single specimen of whole Daphnia (8) carapace surface pattern (9) ventral ridge of posterior region having two rows of spines (10) smooth surface of carapace on dorsal region without spines (11) reticulations present over surface of second antenna (12) swimming setae present on second antenna.

Established taxonomic keys (Balcer et al., 1984; Doan-Dang et al., 2015) were used to carry out further species identification.

Following characters were found to be in confirmation with that of the taxonomic keys:

1. Segmented body, presence of distinct head, and presence of a pair of antennae…… **Phylum Arthropoda**
2. Compressed body, all except head usually enclosed within a bivalve carapace, carapace is single folded plate, longest head appendage with usually two branches, single compound eye present…… **Subclass Branchiopoda, Order Cladocera**
3. Rostrum absent, head small and curved ventrally, antennules not situated at apex of rostrum, head 1/6 of the length of carapace ventral margins of valves without the fringe of the plumose setae…... **Family Daphniidae**

After morphological analysis with the support of following characters i.e., body shape and size, presence of compound eye, carapace covering the whole body, second antennae extending almost equal to body length, presence of swimming setae on second antenna, two branches (dorsal and ventral ramus arising from second antenna), round shaped head with no helmet present over it and presence of cervical sinus on dorsal side were same as genus *Daphnia* (Balcer et al., 1984; Doan Dang et al., 2015). It could be inferred that our specimen shows maximum morphological similarity with the Family Daphniidae hence, we can say that our specimen may belong to the genus *Daphnia.*

II. Molecular analysis:

Further to validate the results molecular analysis were done with the barcoding fragment of the mitochondrial (COI) and 16S rRNA gene and each of these gene were amplified from total genomic DNA using polymerase chain reaction (PCR) (Hajibabaei et al., 2005). The PCR of each gene showed a sharp prominent band at the expected amplicon size as shown in Figure 4. The PCR products were sequenced and the sequences were assembled and edited in BioEdit (Hall, 1999) and aligned using MEGA.

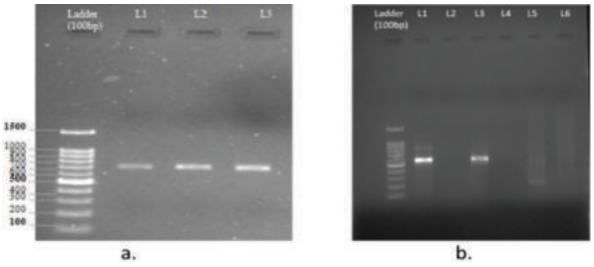

Figure 4: Representative image showing (a.) purified COI gene amplicon (b.) lane L1 showing the amplicon of 16S on 1.2% agarose gel.

(A) COI gene

The obtained sequences were of good quality as nucleotide peaks are easily distinguishable from the background noise but was found to be lacking in length of 239bp.

Phylogenetic tree construction and analysis using *COI* gene

Out of 239 positions, 149 were conserved, 84 variable, 62 Parsimony informative sites (PIS) and 21 singleton sites. For comparative phylogenetic analysis, 14 nucleotide sequences of different organism of the phylum Arthropoda were selected from GenBank database. This included MG544016 *Daphnia tibetana*, KX890199 *Daphniopsis tibetana*, KX890201 *Daphniopsis tibetana*, AY921421 *Daphniopsis tibetana*, MG544021 *Daphnia tibetana*, JN294914 *Anomopoda sp*., (Fly) GU803819 *Solva pallipes*, MF744210 *Simocephalus sp.*, (Fungus gnat) KM961605 *Gnoristinae sp.*, (Mayfly) MW459563 *Ecdyonurus dispar*, (Flies) JF874823 *Hadroneura sp.,* (Cricket) MK130869 *Mecopoda elongate*, (Beetle) HG793316 *Deronectes brannanii,* KM956054 *Gnoristinaesp.* Outgroup chosen for the study was *Drosophila nigribasis* (GenBank MK277192). Phylogenetic trees were constructed using NJ, MP and ML methods.

The best fitting substitution model was selected in MEGA11.0 based on likelihood score of 24 nucleotide substitution models, Akaike information criterion (AIC) and the Bayesian information criterion (BIC) was T92+G substitution model which was further used in ML method for tree construction. Substitution rates were set to be gamma distributed. 1000 bootstrap replications were done. Overall the transition/ transversion bias R=1.65 and Pairwise distance was calculated assuming pairwise deletion of gaps.

Analysis:

Pairwise genetic distance was found to be maximum (0.31) between KM956054 *Gnoristinae sp.* and sample sequence and minimum (0.088) between MG544016 *Daphnia tibetana* and sample sequence followed by KX890199 *Daphniopsis tibetana* (0.083), KX890201 *Daphniopsis tibetana*, AY921421 *Daphniopsis tibetana* (0.094) and MG544021 *Daphnia tibetana* (0.10). All trees (Figure 5) from three methods were identical in representing the paraphyletic relation between the sample sequence, MG544021 *Daphnia tibetana* and MG544016 *Daphnia tibetana* along with *Daphniopsis tibetana* irrespective of the species thus, indicating their common ancestry with higher bootstrap value except in ML tree with low bootstrap values at two branches. As *Daphnia tibetana* is endemic to the Tibetan Plateau, previously researcher had recorded it as *Daphniopsis tibetana (Sars*, 1903; Chiang and Du, 1979; Benzie 2005) but now *Daphniopsis* is regarded as a junior synonym of Daphnia (Xu et al., 2018). Therefore, our sample sequence showed highest similarity with MG544021 *Daphnia tibetana* (86%) in NJ based phylogenetic tree. Similarly with ML and MP trees, sample sequence showed higher similarity with *Daphniopsis tibetana* (42-99%) and *Daphnia tibetana*. The other cluster closest to our sample includes *Anomopoda sp*. and *Simocephalus sp.* of class Branchiopoda. This also implies that specimen in question is more related to the organism belonging to the class Branchiopoda. These results were in agreement with the Blastn analysis that showed maximum similarity with the *Daphnia tibetana*. The evolutionary distance between our sample sequence and *Drosophila nigribasis* was found to be lesser than other species which were included in the tree indicating their potential to be used as outgroup in further phylogenetic studies related to genus *Daphnia* of family Daphniidae.

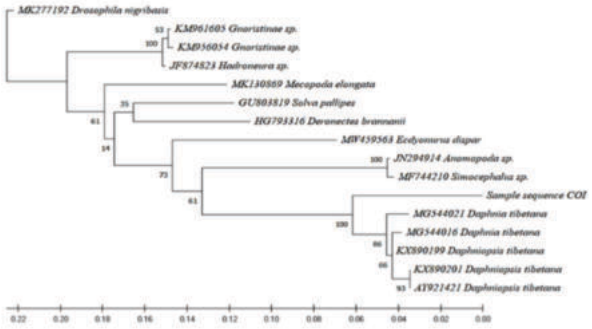

Figure 5: NJ (Neighbour joining) method based Phylogenetic tree using partial mitochondrial COI gene sequences of sample sequence and related species from GenBank database. Number above the branches represents the bootstrap confidence limit (1000 replicates)

B. 16S sequence:

Phylogenetic tree construction and analysis using *16S* gene

We obtained 477bp long consensus sequence with total of 564 positions in the final dataset. Out of them, 216 sites were parsimony informative sites (PIS), 239 were conserved sites, 298 were variable sites and 78 single ton sites.

For comparative phylogenetic analysis, 14 nucleotide sequences of genus *Daphnia* from GenBank database were included in the study based on their percent identity with sample sequence. This includes following species: NC064987 *Daphnia tibetana*, AY921437 *Daphniopsis tibetana*, LS991489 *Daphnia atkinsoni*, AY921456 *Daphnia exilis*, AY921439 *Daphniopsis ephemeralis*, FJ427461 *Daphnia spinulata*, AY921447 *Daphnia muddensis*, AF064189 *Daphnia longicephala*, *LS991487 Daphnia similis*, AY921438 *Daphniopsis studeri*, LS991493 *Daphnia hispanica*, LS991503 *Daphnia magna*, LS991498 *Daphnia lumholtzi*, LS991490 *Daphnia barbata*. *Drosophila melanogaster* (GenBank KP730808) was chosen as outgroup. The phylogenetic trees were built using NJ, MP and ML methods (Figure 6,7).

The best fitting substitution model was selected in MEGA11.0 based on likelihood score of 24 nucleotide substitution models, BIC and AIC criterion suggested T92+G substitution model which was further used in ML method for tree construction. Max-mini-Branch and bound approach was followed for MP method. Substitution rates were assumed to be gamma distributed. The number of bootstrap replications was set as 1000. Overall transition/transversion comes out

to be R= 1.33. T92+G model was used to estimate pairwise genetic distance with an assumption of pairwise deletion of gap sites.

Analysis:

Pairwise genetic distance of sample sequence was found to be maximum (0.96) with *Drosophila melanogaster* and minimum (0.62) with *Daphnia tibetana.* NJ, MP and ML trees revealed concordant tree topologies. All tree topology showed 100% bootstrap support for the cluster of *Daphnia tibetana* and sample sequence. This result was in agreement with the blastn analysis showing maximum percent identity with *Daphnia tibetana*. All trees were found to be rooted with *Drosophila melanogaster* justifying it as outgroup. NJ and ML consensus trees discovered *Daphnia barbata* to be sister clades showing monophyly with the sample sequence having low bootstrap value unlike MP tree topology which did not support this relation at all. *Daphniopsis tibetana* was found to be polyphyletic with the same group in all tree topologies. It was also observed that three clusters of *Daphnia exilis* with *Daphnia spinulata*, *Daphnia exilis* with *Daphnia.spinulata* and *Daphnia nuddensis* with *Daphnia longicephala* displayed higher bootstrap values (77-100%) in all topologies.

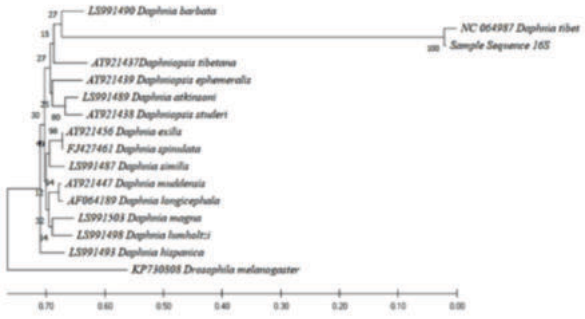

Figure 6: NJ (Neighbour joining) method based Phylogenetic tree using partial mitochondrial 16S rRNA gene sequences of sample sequence and related species from GenBank database. Number above the branches represents the bootstrap confidence limit (1000 replicates).

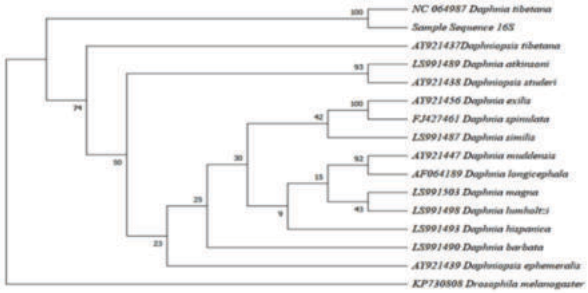

Figure 7: MP (Maximum Parsimony) method based Phylogenetic tree using partial mitochondrial 16S rRNA gene sequences of sample sequence and related species from GenBank database. Number above the branches represents the bootstrap confidence limit (1000 replicates)

Relatedness between different species of Daphnia from various Geographical regions.

Since the data analyses of COI and 16S rRNA clearly showed that our sample was closest to *Daphnia tibetana* or *Daphniopsis tibetana* thus a separate dataset was generated to identify the *Daphnia* from different geographical regions of the world (Figure 8). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). Different species of the *Daphnia* clustered as per their sampling region. *Daphnia tibetana* and *Daphniopsis tibetana* showed 100% similarity, representing them as homological pair thereby showing same ancestry in phylogenetic analysis. The Asian species formed a monophyletic group where as our sample sequence was much closer to the *Daphniopsis tibetana* (Tibet) or *Daphnia tibetana* (China). Thus, the phylogenetic tree inferred clearly that the species which are clustered together, belonged to the Palearctic geographical realms. Our sample was clustered with Tibet and China species which was acceptable as our sampling region was near to the LAC (border of India and Tibet (occupied by China). The northwestern European-North American *Daphnia sp.* formed a separate clade at the base of the tree. It included different species of *Daphnia* from northwestern group, with its distribution ranging from Antarctica to North America. Within this group the *Daphnia angulata* species from Australia formed a separate node and could be distinctly recognized from that the other species from American and Antarctica.

Phylogenetic analyses further confirmed that the Daphnia is an extensive monophyletic group consisting of a series of subclades, i.e., of taxonomically informal aggregates (Colbourne et al., 2006; Xu et al., 2018). The present study also supports the fact that genus Daphnia had evolved as a monophyletic group. The separation of the northwestern and southeastern groups is possibly associated with the uplift of the Tibetan Plateau (Hou et al., 2007). Placement of our sample with *Daphnia tibetana* supports the view that it is capable of surviving in harsh conditions of hypersaline lake where there is a least possibility to find animal fauna such as fishes and other organisms.

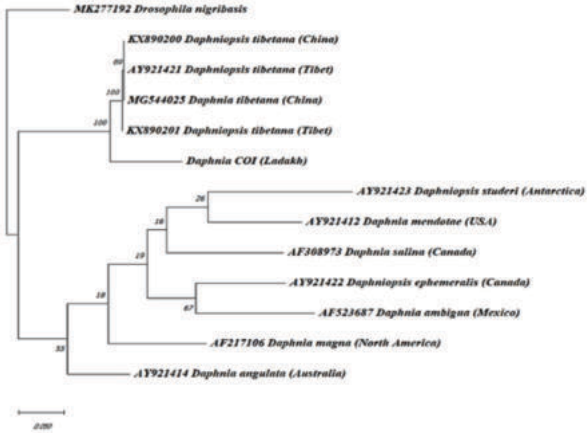

Figure 8: ML (Maximum Likelihood) method based Phylogenetic tree using partial mitochondrial COI gene sequences of sample sequence and other species sequences from NCBI database

CONCLUSION:

Our study is the first to confirm the presence of the *Daphnia tibetana* lineage in Pangong lake in Ladakh using DNA barcoding as a tool. Morphological analysis of our specimen was in agreement with the finding using molecular tools signifying that specimen belonged to genus *Daphnia.* Morphological analyses were helpful in resolving the phylogenetic relationship upto family level suggesting the need of more specific and detailed taxonomic keys for identification upto genus/ species level. It also highlighted the use and need of molecular markers to identify and authenticate the sample upto the species level.

Both mitochondrial molecular markers (16S rRNA and COI gene) pointed towards the closest relationship of our sample specimen with the *Daphnia tibetana* followed by *Daphniopsis tibetana* which are synonyms to each other (Sars 1903; Chiang and Du 1979; Benzie 2005; Xu et al., 2018). In *COI* gene analysis, some species of *Daphnia* other than outgroup exhibited more genetic distance as compared to the former discovering their outgroup potential for further analysis. *Daphnia tibetana* (MG544021) which was the closest to our sample in COI gene analysis showed more genetic divergence than *Daphnia tibetana* (MG544016) which had low bootstrap relation with the sample. 16S rRNA and COI gene showed monophyletic and paraphyletic relation with *Daphnia tibetana* and our sample again providing ambiguity in the evolutionary relationship.

Therefore, it can be concluded that the specimens collected from the Pangong Lake were *Daphnia tibetana*. However, further studies would be required to resolve the position of Daphniids at genus and species level in the hypersaline lakes. For this more marker regions and highly specific primers are needed to be designed for species identification. It is also advisable to use a greater number of related species for reconstruction of phylogenetic trees of family Daphniidae to clear the doubtfulness present in this study. And more comprehensive coverage and more number of sample is required to phylogenetically delineate this species in the ecologically important region of the world.

Data Availability

Nucleotide sequences were submitted to NCBI database under accession number: Daphnia_16S OQ518677 and Daphnia_COI OQ644522.

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Competing Of Interest
The authors declare no competing/conflict of the relevant financial or nonfinancial interest to disclose.

Authorship Contribution Statement:
Phuntsog Dolma:
Samples collection, Conducted the molecular experiments, Analysed the data, Writing of manuscript.

Pallavi Narania:
Conducted the morphological experiments, Data curation, Writing of manuscript.

Dr. Archana Chauhan:
Conceptualization, Designed and supervised the experiment, Provided the laboratory facilities and funds from the projects, Review and editing of manuscript.

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