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DNA Barcoding of a New Aquatic Metazoan, *Paraclepsis vulnifera*

Usha Anilkumar* and Jadhav Anita S.

Department of Zoology, ICLES' MJ College, University of Mumbai, Mumbai, Maharashtra, India

**Corresponding Author*

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Abstract: With the discovery of only one fourth of the aquatic metazoan population so far, accurate identification amongst species at the molecular level has become a daunting task. With the advancement in various molecular diagnostic tools, DNA Barcoding has emerged as a gold standard molecular diagnostic tool across the globe. In this study, a rarely studied leech species was isolated, sequenced at the genetic level and studied for its evolutionary characteristics using phylogenetic analysis. The phylogenetic analysis conducted using the distance-based and character-based methods of Maximum Likelihood (ML), Neighbor-Joining (NJ), and Maximum Parsimony (MP), revealed that the cytochrome C oxidase subunit II (COI) gene of *Paraclepsis vulnifera* was closely related to *Glossiphoniidae* sp. *ZSI TEP3* COI gene. Additionally, the rejection of the null hypothesis for molecular clock analysis, indicated that the species has been evolving at a constant rate with respect to time.

Keywords: Barcoding, DNA, Leech, *Paraclepsis*, Phylogeny, Metazoa, COI gene

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Introduction

The water bodies of our planet comprises of 31 metazoan phyla within which over 230,000 varied species have been discovered so far. Taking into account that water bodies occupy more than double the portion of land on earth, so far only a quarter of the estimated metazoan population has been discovered till date (Bucklin *et al.*, 2011). The International Union for Conservation of Nature (IUCF) reports the increase in ocean temperatures due to the consistent rise in greenhouse gas emissions, thereby leading to endangerment of the aquatic habitat (Li *et al.*, 2018). This issue necessitates the need to uncover the existence of aquatic metazoan species yet to be discovered prior to their extinction. Over the years, DNA Barcoding, a Polymerase Chain Reaction (PCR)-based technique has emerged as a pivotal global standard molecular diagnostic tool for identification of organisms both known and unknown at species level by means of a short biological barcode (Hajibabaei *et al.*, 2006). This highly reliable and quick technique aids in species

identification by recognising the metazoan barcode consisting of 658 base pair long region located at the 5' end of the mitochondrial cytochrome C oxidase subunit II (COI) gene. Although previously many other mitochondrial genes have been proposed and used for species identification, the major reason for the selection of the COI gene arises due to three major reasons. Firstly, the universal Folmer primers for the conserved region of the COI gene have been instrumental in distinguishing an array of metazoan phyla. Secondly, in contrast to other mitochondrial genes, the COI gene acts as a phylogenetic indicator amongst wider levels of taxonomy. Also, it has been observed that the swift evolution of the COI gene enables to separate closely related species even at the geographical level (Bucklin *et al.*, 2011; Kalawate *et al*., 2022).

Leeches are segmented parasites found inhabiting water bodies such as shallow slow-moving freshwater lakes, oceans while some also live on lands and low foliage moistened areas such as wet rain forests. Approximately 650 species of worms with segmented body belong to the phylum Annelida and class Hirudinea and consist of a small sucker which represents the mouth at the anterior whereas a large sucker is situated at the other end of the body. Leeches discovered in India are widely known as the endemic leeches of India as they have not been encountered anywhere else on earth. A zoological survey conducted in the past has reported 32 leech species from different states in India, amongst which 21 species dwell in fresh water, 4 were marine species and 7 were found to be terrestrial (Mandal, 2004). The crucial task of developing the taxonomy of leeches in India was first undertaken by Harding and Moore. Additionally, other important contributions in the past by Baugh, Sanjeev Raj and Gladstone followed by various other contributors to the leech fauna of Indian region has also been reported (Phull *et al*., 2021).

The term Medicinal Leech Therapy (MLT) has been inculcated in the field of medicine, right from its inception in ancient Egypt. Within the context of Indian medicine, leech therapy has been one of the many essential tools since its origin to address an array of ailments. Although nearly 100 proteins are present in leech secretions only few of them viz; Hirudin, Calin, inhibitors of Kallikrein, Histamine-like substances, Hyaluronidase, and Collagenase are known to exert therapeutic effects (Phull *et al*., 2021). Modern applications of MLT involve treatment of cardiovascular diseases, microsurgery, treatment of cancer and inhibition of metastasis, treatment of Diabetes mellitus complications (DM), infectious diseases and arthritis (Abdualkader *et al.*, 2013). Therefore, more efforts are required to explore new species of leeches and study their potential in medicinal practices. Studies in the past have also depicted the possibility of developing leeches as animal models for scientific investigations in biomedical domain due to their small size, easily detectible responses to external stimuli and their simple anatomical structure (Eguileor *et al.*, 2005). This study attempts to unravel the existence of a new leech species namely *Paraclepsis vulnifera* which has been discovered in the state of Maharashtra in India and belongs to the Glossiphoniidae leech family consisting of freshwater leeches characterised by a proboscis and belonging to the genus *Paraclepsis*. The Zoological Survey of India titled 'Endemic Fauna of India' conducted by Chandra *et al*. in the year 2004 had initially reported the discovery of this species in the state of Tamil Nadu in India. Since then, no other findings of this species in India have been reported till date.

This study aims to investigate the evolutionary relationship amongst the discovered leech *Paraclepsis vulnifera* and other organisms belonging to the leech family. The study of this relationship has been carried out by utilising several distance-based and character-based phylogenetic models along with the molecular clock hypothesis.

Materials and Methods

Collection of leech specimen:

The studied leech sample was retrieved from a rural freshwater pond in Maharashtra. The collected sample was then preserved in ethanol and stored at -20 °C until further analysis.

Isolation of genomic DNA:

For extraction of genomic DNA, leech samples preserved in ethanol were washed twice using distilled water and small fragments of nearly 0.5 mm in length were cut from the anterior end. These fragments were then treated with liquid nitrogen and crushed using mortar and pestle to obtain a fine powder of the leech fragments. The genomic DNA was isolated using the modified Cetyltrimethylammonium bromide (CTAB) protocol as performed by Phull *et al*. (2021). The extracted genomic DNA was then resuspended in 30 µl of TE buffer until further use.

Amplification and sequencing of mitochondrial COI gene and ribosomal subunits:

The isolated genomic DNA was quantified using a NanoDrop spectrophotometer, to adjust the DNA concentration of the sample before initiating the Polymerase Chain Reaction (PCR) process in PCR Thermocycler (Jovanović *et al*., 2021; Phull *et al*., 2021). For amplification of the mitochondrial COI gene, Folmer primers (HCO1490 and LCO2198) were used (Folmer *et al*., 1994). Additionally, for the amplification of the mitochondrial 18S rRNA and 28S rRNA oligonucleotide primers were used. The amplified products obtained from the PCR process were run on 1% agarose gel for visual qualitative analysis. Successful amplified products were then sequenced at Eurofins India Pvt. Ltd.

Phylogenetic analysis of Paraclepsis vulnifera COI gene sequence by NJ and ML method:

Initially FASTA sequences of closely related leeches belonging to different genus were retrieved and aligned along with the resultant COI gene in MEGA 11 using Multiple Sequence Comparison by Log-Expectation (MUSCLE), a multiple sequence alignment method. Phylogenetic analysis of the *Paraclepsis vulnifera* COI gene sequence was carried out using the distance-based methods such as Neighbour Joining (NJ), Maximum Likelihood (ML) and the character-based method of Maximum Parsimony (MP) method along with the Bootstrap Phylogeny test with number of bootstrap replications set at 1000 along with the Kimura 2-parameter model (K-2-P). *Escarpia spicata* (LC413848.1) and *Clymenella torquata* (HQ024010.1) from relatively evolutionarily close taxa were set as an outgroup (Solgi *et al*., 2021).

Molecular Clock analysis using the Maximum Likelihood (ML) Method:

The molecular clock dating analysis is based upon the assumption that molecular evolution occurs at a relatively constant rate within a species (Dos Reis *et al*., 2016). The hypothesis states that protein and DNA sequences undergo evolution at a constant rate with time amongst various organisms belonging to the same species (Ho, 2008). A Maximum Likelihood test of the molecular clock hypothesis was conducted using MEGA 11 software for the selected tree topology in Newick export format and sequence alignment.

Results and Discussion

Phylogenetic analysis of the COI gene:

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980; Felsenstein, 1985) and are in the units of the number of base substitutions per site (Fig. 1). The analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 672 positions in the final dataset. The average nucleotide composition obtained for the sequences used for phylogenetic analyses is as follows: Thymine (Uracil)=36.8%, Cytosine= 17.8%, Adenine=29.6%, Guanine=15.9%. From Figure 1, it can be concluded that there is no divergence between *Paraclepsis vulnifera* and *Glossiphoniidae* sp. *ZSI TEP3*, whereas some level of divergence was observed between *Paraclepsis vulnifera* and other 19 leech species.

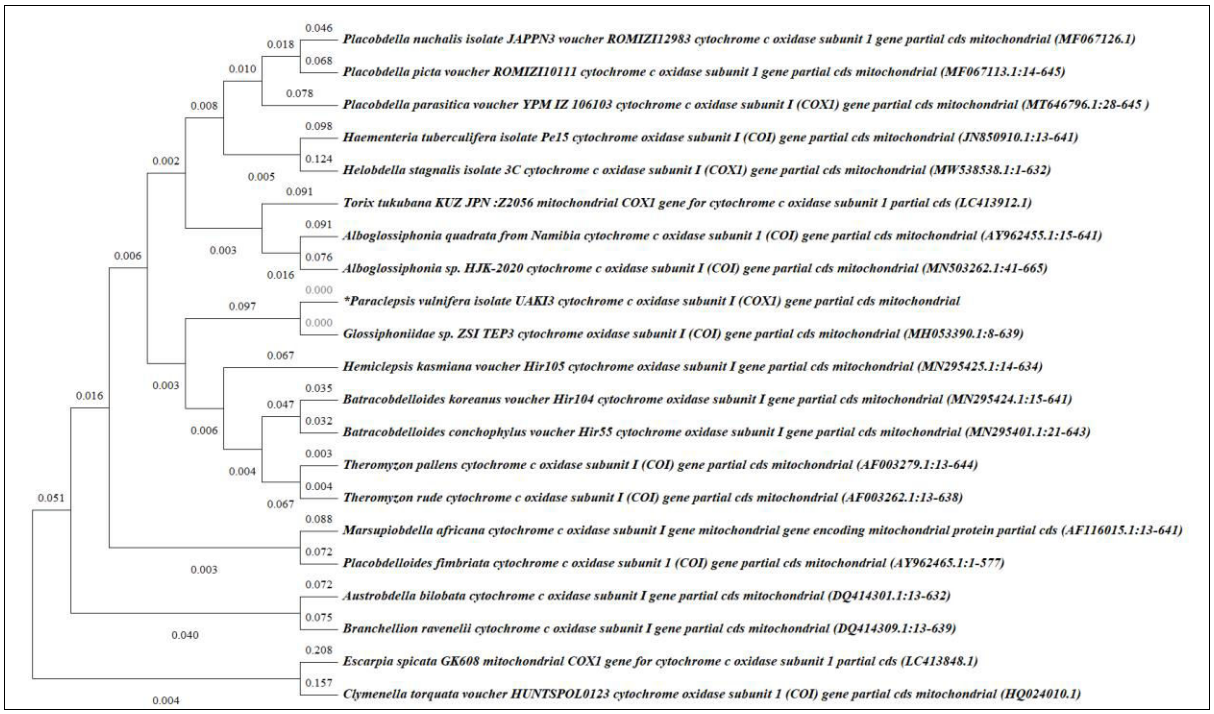

Fig. 1: Phylogenetic tree of *Paraclepsis vulnifera* COI gene sequence obtained by Neighbour-joining (NJ) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses.

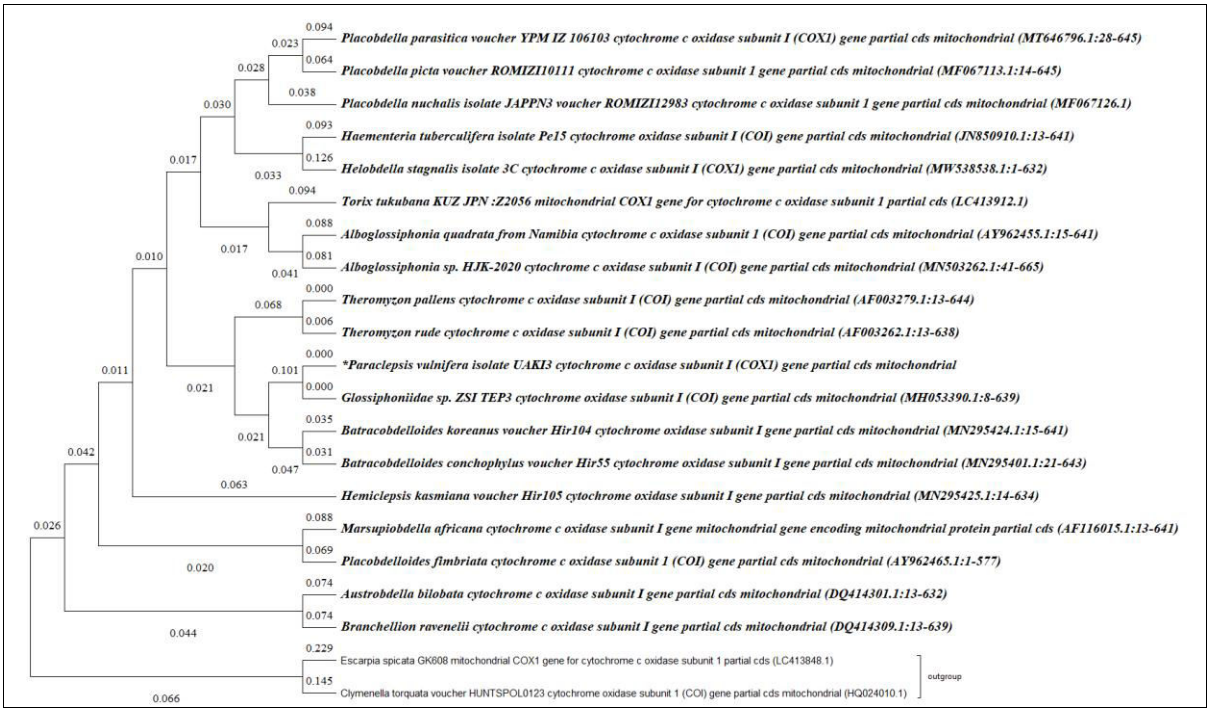

Fig. 2: Phylogenetic tree of *Paraclepsis vulnifera* COI gene sequence obtained by Maximum Likelihood (ML) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses.

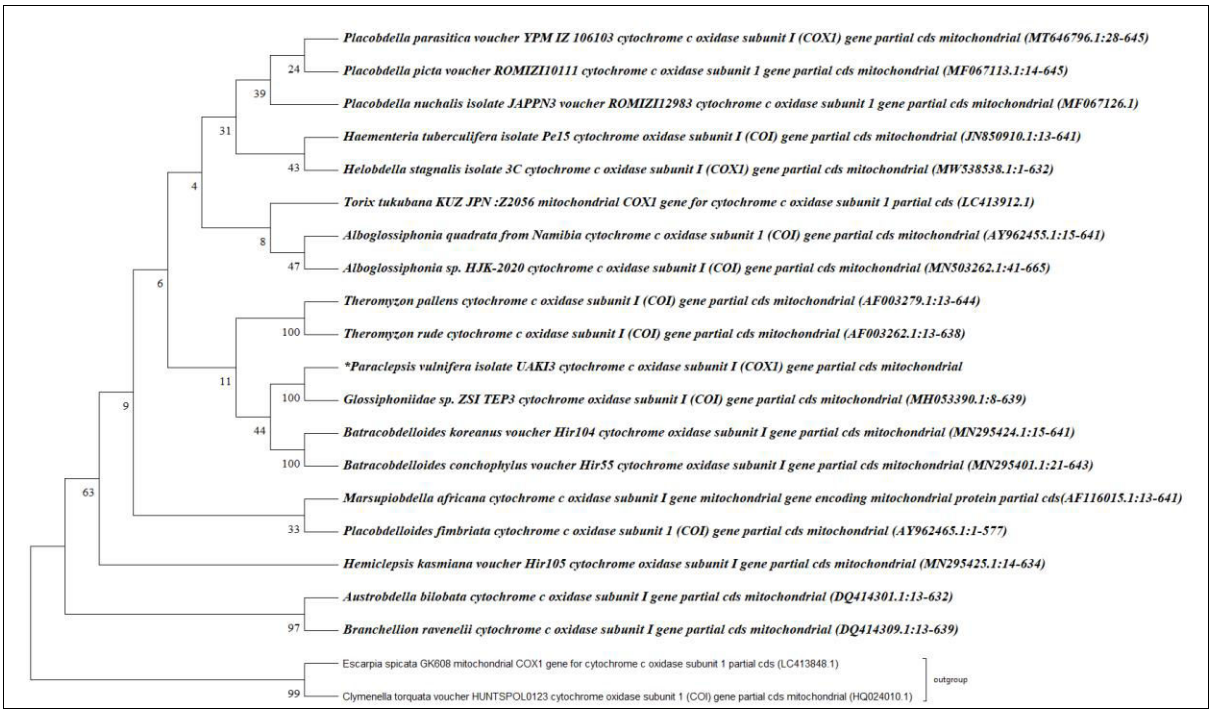

Fig. 3: Phylogenetic tree of *Paraclepsis vulnifera* COI gene sequence obtained by Maximum parsimony (MP) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses

Another phylogenetic analysis was performed using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-6322.52) is shown (Fig. 2). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. The analysis indicated that the COI gene of *Paraclepsis vulnifera* was almost similar to that of *Glossiphoniidae* sp. *ZSI TEP3* with a percentage similarity of 100%. This clade was also observed to share a common ancestor with its sister clade consisting organisms from the *Batracobdelloides* sp. The sister clades consisting of COI genes from *Paraclepsis vulnifera* and *Batracobdelloides* sp., respectively had branches of lengths 0.101 and 0.047 indicating the occurrence of more genetic changes occurring in the *Batracobdelloides* sp. after descending from the common ancestor.

The character-based phylogenetic analysis of the Maximum Parsimony method was used to conduct evolutionary analysis. The most parsimonious tree with length = 1245 is shown (Fig. 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 21 nucleotide sequences. There were a total of 672 positions in the final dataset and evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021). The result obtained from this analysis also concluded that *Paraclepsis vulnifera* COI gene was closely related to that of *Glossiphoniidae* sp. *ZSI TEP3*.

Molecular Clock Analysis:

The molecular clock test was performed by comparing the ML value for the given topology

Table 1: Results from a test of molecular clocks using the Maximum Likelihood method

	lnL	Parameters	(+G)	(+I)
With Clock	-6691.144	21	n/a	n/a
Without Clock	-6659.115	40	n/a	n/a

with and without the molecular clock (Table 1) constraints under 2-parameter model (Kimura, 1980). The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P = 1.648E-006). Thus, the proposed molecular clock hypothesis that constant evolution occurs with respect to time amongst organisms of the same species is accepted. This analysis involved 21 nucleotide sequences. There were a total of 672 positions in the final dataset.

Conclusion

With respect to the rate of discovery of new species and a fast rate of extinction of already existing species on earth, the need to correctly identify a unique organism and conduct its evolutionary analysis has become a complicated task. However, as new tools and techniques are emerging in the field of phylogeny analysis, DNA Barcoding technique has emerged as a gold standard to accurately identify both known and unknown organisms at the molecular level. In this study, a rarely encountered and studied leech species was identified by means of this tool. Various phylogenetic analyses were conducted by Maximum Likelihood (ML), Neighbor-Joining (NJ), and Maximum Parsimony (MP) methods in order to determine the evolutionary behavior of the discovered leech species *Paraclepsis vulnifera* as compared to the other organisms of the same family. The results from all these analyses thereby confirmed a close resemblance between the studied leech organism and *Glossiphoniidae* sp. *ZSI TEP3*. The molecular clock analysis result proved that the species has been evolving at a constant rate with respect to time.

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