

TAXONOMY AND PHYLOGENY OF *Limnodrilus hoffmeisteri*, Claparède, 1862 BELONGING TO FAMILY TUBIFICIDAE d' Udekem, FROM DIFFERENT WATERBODIES OF JHARKHAND BASED ON DNA BARCODING

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Limnodrilus hoffmeisteri, morphology, DNA barcoding, distance matrix, phylogenetic tree, Jharkhand.

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ABSTRACT

Aquatic Oligochaetes were collected from different habitats located in different districts of Jharkhand. Specimens of *Limnodrilus hoffmeisteri*, Claparède, 1862 was examined in details for ascertaining its taxonomy and phylogeny. Apart from the morphological details, the molecular study based on DNA barcoding was done for the first time for the specimen from this region. Based on the BLASTn results, the specimen was identified as *Limnodrilus hoffmeisteri*, after which the 18s rRNA nucleotide sequence was submitted to DNA databank of Japan and accession ID LC843736 was obtained. The sequence was used to prepare the distance matrix and phylogenetic tree using MEGA X software. The matrix score ranged from a minimum of 0.00000 to a maximum of 0.00329. The Ranchi strain of *Limnodrilus hoffmeisteri* (LC843736.1) differed from that of the other 10 (already available in GenBank with a matrix value of 0.00329 in each case). This indicates that the strain of *Limnodrilus hoffmeisteri* being reported by us from this region may be from an entirely different genetic pool as compared to the other strains selected in this study. From the phylogenetic tree it can be easily seen that the Ranchi strain lies singly in a separate clade from the selected 10 strains which fall in another clade.

INTRODUCTION

Aquatic oligochaetes are important constituent of macrobenthic fauna which play crucial role in nutrient dynamics of water bodies by their activities like mixing deposited matter through burrowing, feeding, respiration and crawling, in the sediments at the mud-water interface may they be ponds, pools, lakes, reservoirs or riverine system (Fischer and Beeton, 1975; Sinha *et al.*, 1989, 1991; Martinez and Levinton 1996; Matisoff *et al.*, 1999; Vorob'ev *et al.*, 2010; Zhang *et al.*, 2012; Liu, 2017). The aquatic oligochaetes play important role in energetics of the system by occupying key place in detritus food chain and food web (Woodward *et al.*, 2008). *Limnodrilus hoffmeisteri* Claparède, 1862, belonging to family Tubificidae of Oligochaeta has long been arbitrarily treated as a single and widely distributed species. *L. hoffmeisteri* has been used as an important tool in biomonitoring of aquatic biotopes (Sinha *et al.*, 1989, 1993, 1993a). Occurrence of *L. hoffmeisteri* with *Chironomus* larvae has been taken as a self-evident case of high organic pollution (Uzunov *et al.*, 1988; Burton 1992; Sinha *et al.*, 1993b, 1998). The

aquatic oligochaete community has been used as tool of deterministic analysis of niche relations (Sinha *et al.*, 1993b, 1994, 1995). This species *L. hoffmeisteri* has also been used as a model organism in both basic and applied science (Liu *et al.*, 2017). In spite of all these facts and importance of the species it is the fact that morphological variability has led to controversy over its taxonomic status for quite long period (Liu *et al.*, 2017). Both *L. hoffmeisteri* and its congener *Limnodrilus udekemianus* Claparède, 1862 were originally collected from a stream near Geneva in Switzerland and were described by Claparède (1862). The inconsistencies in literature and inconclusive results pertaining to taxonomic studies of the species have progressed from splitting to lumping. Brinkhurst and Jamieson (1971) have mentioned more than 40 synonyms, along with three intra-specific nominal forms/varieties, all regarded as a single species, and from an ecological point of view *L. hoffmeisteri* became the commonest, most widely distributed tubificid. In recent years, the notion that the taxon *L. hoffmeisteri* represents a species complex, rather than one species, has gained credibility, along

with the development of molecular systematics (Liu *et al.*, 2017). Many workers in India have studied and reported the occurrence, ecology and taxonomy of some oligochaetes including *L. hoffmeisteri* (Naidu, 1962, 1963a, 1963b, 1963c, 1964, 1965, 1966; Naidu and Naidu, 1979a, 1979b; Naidu *et al.*, 1979; Dhillon and Kaur, 1991; Kalpana and Naidu, 1979; Rani, 1975; Sharma, 1993; Sinha *et al.*, 1989a; 1989b, 1991, 1993a, 1993b, 1994, 1995, 1998; Neesemann *et al.*, 2004; Singh *et al.*, 2009, Ragi and Jaya, 2014) but no detailed taxonomic study has been done till date. The aquatic oligochaetes from water bodies of Jharkhand have particularly not been studied from taxonomic view point either on morpho-anatomical or molecular basis. Further the use of effectiveness of DNA barcoding in species identification is now a prerequisite for biodiversity conservation. DNA barcoding, however, has been found to be effective (Achurra, 2011; Vivien, *et al.*, 2015; 2020; Zhou *et al.*, 2012; 2021) in aquatic Oligochaete species identification but no such attempt has been made for taxa found in India.

Keeping the gap of knowledge in background the present communication deals with morpho-anatomical as well as the molecular taxonomy and phylogeny of *Limnodrilus hoffmeisteri* Claparede, 1862 based on DNA barcoding.

MATERIALS AND METHODS

The sampling methods of macrobenthic fauna, which predominantly consists of aquatic oligochaetes, depends on the nature and condition of the substratum or sediment. The use of equipment for sampling is decided on the basis of whether it is muddy, sandy, smooth or with pebbles. The presence of weeds or twigs also becomes an important factor. On the basis of the sampling area, both scooping and dredging was done. Sampling was done on regular interval round the year preferably in morning hours from the area with little anthropogenic disturbances (Sinha *et al.*, 1989; 1991). The samples were sieved using sieve of different mesh size and the specimen were transferred to sampling bottles carefully and preserved in 70% alcohol. The specimens were examined and dissected for morpho-anatomical characters.

DNA extraction, PCR amplification and sequencing

DNA was isolated from whole organism. The quality was evaluated on 1.0% agarose gel. A single band of high-molecular weight DNA was observed. Fragments of 18s rRNA gene were amplified by specific forward and reverse primers. A single discrete PCR amplicon band of 623 bp was observed when resolved on agarose gel. The PCR amplicon was then purified to remove contaminants. Sequencing reaction of PCR amplicon was carried out with forward

and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer (ThermoFisher, 2024).

Molecular sequence analysis tools

Following the Sanger sequencing method, a discrete PCR amplicon was obtained. The obtained PCR amplicon was compared with data present in nucleotide databases using BLAST (Basic local alignment and search tool) program based at NCBI (National Center for Biotechnology Information). The nucleotide blast (BLASTn) option was used. BLASTn compares nucleotide query (our sequence) with the subject sequence (data present in databases). BLASTn was performed with parameters selected as Search Set: standard data-base; Program selection: highly similar sequences (megablast). Megablast is used for sequence identification and intra-species comparison (Srivastava *et al.*, 2024; Kumar *et al.*, 2024a; 2024b).

Submission of nucleotide sequence to the GenBank

Based on the BLASTn results, the specimens were identified as *Limnodrilus hoffmeisteri*, after which the accession ID LC843736 was obtained for nucleotide sequence.

Phylogenetic tree and Distance Matrix

The distance matrix and phylogenetic tree were created using the 18s rRNA sequence that was extracted from *Limnodrilus hoffmeisteri* specimens that were gathered from several survey sites in the state of Jharkhand. The Maximum Composite Likelihood method was used to construct the evolutionary distances (matrix), which are given in units of substitutions of bases per site (Tamura *et al.*, 2004). Pairwise deletion was carried out for each pair of sequences for any uncertain spots. There were 624 locations in the final dataset. Using the neighbor-joining approach, an unrooted phylogenetic tree was built to determine the evolutionary relationship between the specimens collected from different locations in Jharkhand (Saitou and Nei, 1987). MEGA X (Molecular Evolutionary Genetics Analysis) version 10.2.6, build 10210527-x86_64 (Windows 11) was used to conduct these analyses (Kumar *et al.*, 2018; Srivastava *et al.*, 2024).

RESULTS AND DISCUSSION

Limnodrilus hoffmeisteri Claparede, 1862, Figs. 109 - 112

Limnodrilus socialis Stephenson 1912; 294 - 301, figs. 9 - 16; Stephenson, 1923: 96 - 98, figs. 36, 37.

Limnodrilus hoffmeisteri, Naidu, 1965: 477 - 479, figs. 6 a - g.

Material examined: A good number of specimens examined were collected from Dhurwa Dam, Ranchi; Kelaghagh Dam, Simdega; Palna Dam, Jamshedpur; Lotwa Dam, Hazaribagh; Ranital Dam, Palamu; Jamni Dam, Godda and Bundu Lake, Bundu. The sampling sites along with their GPS coordinates have been listed in Table 1.

Table 1: Sampling site locations and their GPS coordinates

Sampling Sites	Locations	GPS Co-ordinates
A	Dhurwa Dam, Ranchi	23° 17'48.31"N & 85° 14'43.79"E
B	Kelaghagh Dam, Simdega	22° 36'1.11"N & 84° 31'14.69"E
C	Palna Dam, Jamshedpur	22° 56'38.80"N & 85° 55'29.07"E
D	Lotwa Dam, Hazaribagh	24° 8'55.25"N & 85° 23'0.97"E
E	Ranital Dam, Palamu	23° 59'51.11"N & 84° 1'40.20"E
F	Jamni Dam, Godda	24° 48'27.18"N & 87° 14'35.05"E
G	Bundu Lake, Bundu	23° 9'52.60"N & 85° 34'57.83"E

Habitat and distribution: *Limnodrilus hoffmeisteri* was collected from habitat with mud, silt, sand, mixed substrate with pebbles and stones from different ponds, pools, lakes, reservoirs and microhabitats like organically polluted drains apart from the above mentioned major habitats. The feeble number or absence of *Limnodrilus hoffmeisteri* from anthropogenically disturbed habitat was noticed. It was found in all the sampling sites showing it to be the most common representative of Tubificidae. As mentioned in literature *Limnodrilus hoffmeisteri* a truly cosmopolitan species, is perhaps the most widely distributed freshwater tubificid in the world.

Morphological Observations. *Limnodrilus hoffmeisteri* is a small (20-35 mm) and, thin worm. The worm appears to be red or reddish brown, imparted by the respiratory pigment hemoglobin.

Prostomium - blunt and conical, setae - both dorsal and ventral commence from II segment, being all bifid and similar, with distal tooth being thinner and equal in length with the proximal. 3- 9 chaetae per bundle in anterior segments. In the succeeding segments, the number of chaetae per bundle decreases showing about 3-5 in the middle segments and 1-2 in the most posterior segments.

Limnodrilus hoffmeisteri Claparede, 1862 (Figure 2, 3 and 4)

Clitellum extends from XI to XII segment. The penis sheath is one of the diagnostic characters used in identification of these worms and can be very clearly seen through the transparent body surface. The penis sheath is longer than broad (length: breadth is about 9) and ends with an asymmetrical, plate-like extremity. Chloragogen tissue in this species starts from the V segment.



Figure 1: Map of Jharkhand, showing the various major sampling sites



Figure 2: Photograph of *Limnodrilus hoffmeisteri*



Figure 3: Diagram of *Limnodrilus hoffmeisteri*

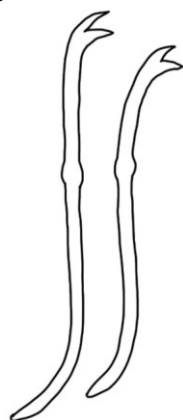


Figure 4: bifid setae



Figure 5: QR code for scanning and accessing the full definition of the 18s rRNA sequence submitted to the GenBank.

Anatomical Observations: Pharynx in segment II-III wide; oesophagus in IV-VII and narrow, stomach absent, intestine starts from VIII. Chloragogues from VI. Blood is yellowish red. Dorsal vessel mid-dorsal in II-V and lateral to left of the gut from VI. Lateral contractile vessels 2 pairs in VIII and IX. Paired testes and ovaries laterally on posterior face of septa 9/10 and 10/11 respectively. Anterior and posterior sperm sacs extend to IX and XVI segments respectively. Ovi-sac extends to XVII. Male funnels laterally on anterior face of septum 10/11, vasa deferentia thin, long and coiled. Atria spindle shaped with a long coiled ejaculatory duct, distal part with chitinous sheath which is about 8-10 times as long as wide. Bifid setae present (figure 4).

Distribution in India: The species has been reported from Kashmir the north of India to Kerala in South. It has been recorded to be cosmopolitan in distribution.

Remark: On the basis of morpho-anatomical observation the description matches with that of monographic details of *Limnodrilus hoffmeisteri* Claparede, 1862.

For getting the identification confirmed and to see the intra-species genetic variation as well as establishing the phylogeny DNA barcoding of the species was done because the above characteristics fail to find out cryptic species and homoplasy.

Genome Sequence Analysis

DNA extraction, PCR amplification and sequencing

A single discrete PCR amplicon band of 623 bp of 18s rRNA was obtained using specific primer. The resultant nucleotide sequence

(query) was compared with the nucleotide sequence databases using BLASTn (Basic Local Alignment Search Tool) program based at NCBI (National Center for Biotechnology Information). BLASTn was performed with parameters selected as Search Set: Standard database; Program Selection: Highly similar sequences (megablast). Megablast is used for sequence identification and intra-species comparison (Zheng *et al.*, 2000; Aleksandr *et al.*, 2008). Based on the high similarity of our query sequence with the subject sequences belonging to that of 18s rRNA of *Limnodrilus hoffmeisteri*, the annelid worm is confirmed to be member of

species *hoffmeisteri* belonging to genus *Limnodrilus*. Out of all the blast hits top 10 sequences (of *Limnodrilus hoffmeisteri*) were selected (as of October, 2024) for construction distance matrix and phylogenetic tree. These sequences have been listed in Table 2.

Extracted from the BLASTn results, the Table 2 shows maximum score, query coverage, E value, percentage identity and country (region) it belongs to. The sequences used in this analysis have been obtained from GenBank, belonging to various regions

Table 2: Top 10 sequences of *Limnodrilus hoffmeisteri* selected on the basis of BLASTn search result

Species	Total Score	Query Cover	E Value	Percentage Identical	Accession	Country
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636894.1	Sweden
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636893.1	Switzerland
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636891.1	Sweden
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636890.1	Sweden
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636889.1	Japan
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636887.1	Sweden
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636886.1	Sweden
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636883.1	China
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636882.1	China
<i>Limnodrilus hoffmeisteri</i>	1109	97%	0	99.51%	KY636879.1	USA

Submission of data to GenBank

After confirmation of the annelid specimen as *Limnodrilus hoffmeisteri*, the query sequence was submitted to the GenBank and accession IDLC843736.1 was obtained. The complete definition related to the sequence is available online and can be accessed by visiting the link <https://www.ncbi.nlm.nih.gov/nuccore/LC843736.1>.

Additionally, the link has been integrated in a QR (quick response) code presented as Figure 5.

The sequence submitted to the GenBank is also presented below in FASTA format.

>LC843736.1 *Limnodrilus hoffmeisteri* ZOOSXCRAN/AN/2024/01 gene for 18S rRNA, partial sequence

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CGCGGGTTCGCCTCATAGCGTATATTAAGTTGGTGCAGTTAGAAAGCT
CGTAGTTGGATCTCGGGTCCAGGCTCGCGGTTCCGCTCGCGGCGATC
ACTGCCCGTCTGACCTACCTCCCGTTTTCCCTTGGTGCTCTTCGTT
GAGTGCTTCGGGTGGCCGGAACGTTTACTTTGAAAAATTAGAGTGTC
TCAAAGCAGCGGACTGCCTGAATAATCGCGCATGGAATAATGGAAT
AGGACCTCGGTTCTATTTTGTGTTTTCGGAACCTCAGGTAATGATT
AAGAGGGACAGACGGGGCATTCTGATTACGGTGTAGAGGTGAAAT
TCTTGATCGCCGTAAGACGAACTACTGCGAAAGCATTGCAAGAAT
GTTTTTCATTAATCAAGAACGAAAGTCAGAGTTTCAAGACGATCAGAT
ACCGTCGTAGTTCTGACCATAAAGCATGCCAACTGGCGATCCGCGGG
CGTTGATCTAATGACCCCGCGGCGAGCTCCGGGAAACCAAAGTTTTT
GGGTTCCGGGGGAAGTATGTTTGCAAAAGCTGAACTTAAAGGAATTG
ACGGAAGGGCACCACCAGGAGTGAGCCTCGCGCTTAATTTGACTCA
ACACGGA

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Table 3: Distance Matrix prepared using the nucleotide sequences from the 10 sequences presented in table 2 and our query sequence

Species-strains		1	2	3	4	5	6	7	8	9	10	11
LC843736.1:Ranchi	1		0.00329	0.00329	0.00329	0.00329	0.00329	0.00329	0.00329	0.00329	0.00329	0.00329
KY636894.1:Sweden	2	0.00329		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
KY636893.1:Switzerland	3	0.00329	0.00000		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
KY636891.1:Sweden	4	0.00329	0.00000	0.00000		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
KY636890.1:Sweden	5	0.00329	0.00000	0.00000	0.00000		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
KY636889.1:Japan	6	0.00329	0.00000	0.00000	0.00000	0.00000		0.00000	0.00000	0.00000	0.00000	0.00000
KY636887.1:Sweden	7	0.00329	0.00000	0.00000	0.00000	0.00000	0.00000		0.00000	0.00000	0.00000	0.00000
KY636886.1:Sweden	8	0.00329	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000		0.00000	0.00000	0.00000
KY636883.1:China	9	0.00329	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000		0.00000	0.00000
KY636882.1:China	10	0.00329	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000		0.00000
KY636879.1:USA	11	0.00329	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	

Distance matrix and Phylogenetic tree construction

All the ten 18s rRNA sequences listed in Table 2 and our query sequence from *Limnodrilus hoffmeisteri* collected from Ranchi were used to prepare a distance matrix using MEGA X software. Distance matrices are used in phylogeny as a non-parametric distance method. These distances are then reconciled to construct phylogenetic tree. Distance matrix methods of phylogenetic analysis explicitly rely on a measure of genetic distance between the sequences being studied(Mount, 2004).

FASTA-aligned sequence of all 10 sequence and our query sequence were obtained from BLAST search results page, which was used for construction of distance matrix and phylogenetic tree using neighbor-joining statistical method. Bootstrap method

was employed for the test of phylogeny (Sautou and Nei, 1987). The distance matrix is presented as Table 3. The phylogenetic tree has been presented as Figure 6.

The matrix score ranged from a minimum of 0.00000 to a maximum of 0.00329. From Table 3 it is clear that the Ranchi strain of *Limnodrilus hoffmeisteri* (LC843736.1) differs from the other 10 with a matrix value of 0.00329 in each case. This indicates that the strain of *Limnodrilus hoffmeisteri* being reported by us is from an entirely different genetic pool as compared to the other strains selected in this study. This feature is further exhibited by the phylogenetic tree presented as Figure 6. The phylogenetic tree shows evolutionary relationships between organisms(Mount, 2004).Kimura-2 parameter model and

the maximum likelihood approach were used to derive the evolutionary history (Kimura, 1980). From the phylogenetic tree it can be easily seen that the Ranchi strain belongs singly in a separate clade from the selected 10 strains which fall in another clade. From the phylogenetic tree it can clearly be seen that the branch length of Ranchi strain is longest as compared to other,

this indicates higher variation in the Ranchi strain as compared to the other 10 sequences from their ancestor. A DNA barcode sequence 18s rRNA obtained from a mature specimen of *Limnodrilus hoffmeisteri* is closest to lineage VII and X of the *L. hoffmeisteri* complex sensu Liu *et al.*, (2017).

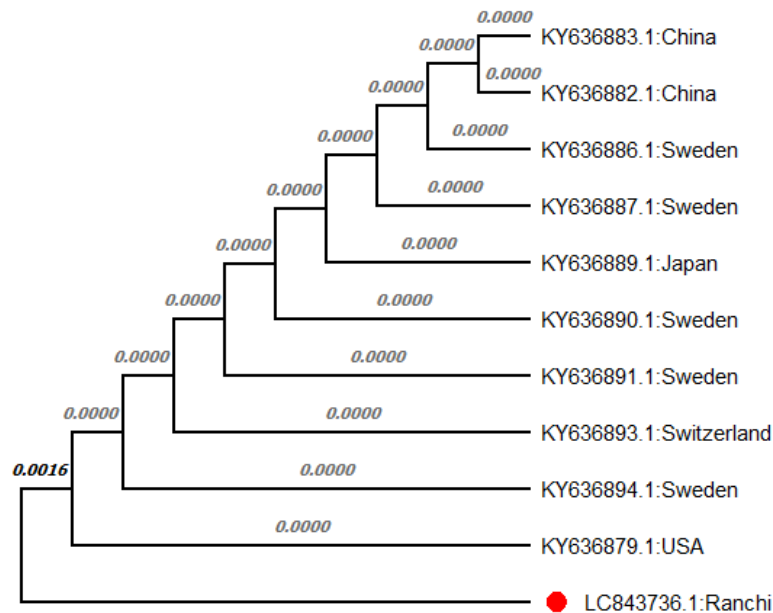


Figure 6: Phylogenetic tree

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