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1 **Title: Molecular characterization of ‘tubifex worms’ based on 16S rRNA and cytochrome c**
2 **oxidase subunit I**

3

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20 **Abstract**

21 **‘Tubifex worms’** encompass extensive cryptic diversity that requires molecular characterization
22 to confirm the species commonly found in Bangladesh including their lineages. Therefore, this
23 study focused on the molecular characterization and phylogeny analysis at lineages level by
24 sequencing the fragment of 16S rRNA and cytochrome c oxidase subunit I (COI) genes through
25 standard cloning and sequencing techniques followed by phylogenetic characterization. All six
26 isolates, belong to the genus *Limnodrilus* that is confirmed into two different species (*L.*
27 *hoffmeisteri* and *L. claparedianus-cervix*) of the tubifex. The phylogenetic analysis estimated
28 that the resident worms are under the same clade although transmissions from other countries
29 might likely occur through different aquatic routes and birds. The drawbacks were the use of a
30 complex colony rather than a single worm and taking representative isolates instead of all the
31 positive amplicons for sequencing. However, this study is the first report of genetical
32 confirmation of the genus *Limnodrilus* at lineage level from Bangladesh.

33

34 **Keywords:** Tubifex, Phylogeny, Cytochrome Oxidase, 16S rRNA, Bangladesh

35 **Short Title:** Molecular phylogeny of *Limnodrilus* in Bangladesh

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41 1. Introduction

42 Fresh water oligochaetes, usually known as ‘tubifex worms’, denotes a mixture of
43 worms that may comprise the subfamilies of Tubificidae, and three tubificid genera, namely,
44 *Tubifex*, *Limnodrilus*, and *Aulodrilus*, have been identified morphologically in Bangladesh and
45 are widely used as a live feed in fish industry (Mariom et al., 2016). Liu et al. (2017a) reported
46 that hair and pectinate chaetae are the common morphological features in the genera of the
47 subfamily Tubificinae, although may be lacking in many other tubifex worms including the
48 common bioturbator benthic *Limnodrilus*, which is represented by few species (Erséus et al.,
49 2002). Remarkably, the morphology of the hard structure (the anterior bifid chaetae and the
50 shape of the penis sheaths) have extensively deliberated to denote the *Limnodrilus* species which
51 includes at least 17 documented morphospecies, and the penis sheaths show great morphological
52 variation within and among taxa (Erséus et al., 2000; Martin et al., 2007). Therefore, great effort
53 was given to differentiate the interspecies alterations to the intra-species distinction of these
54 features within the genus (Hallett et al., 2005; Vivien et al., 2016a).

55 The recent studies on molecular systematics revealed that the taxon *L. hoffmeisteri*
56 corresponds to a complex of species instead of a single species (Beauchamp et al., 2001; Liu et
57 al., 2017a). Overall, the complexities in morphological studies and the development of molecular
58 genetic assays combinedly provided the basis to utilize the molecular approaches in identifying
59 and classifying morphologically similar worms (Vivien et al., 2016b).

60 Literatures report the morphometric identification of ‘tubifex worms’ commonly found in
61 Bangladesh (Ali and Issaque, 1975; Mariom et al., 2016), information related to molecular-based
62 identification and phylogenetic analysis of these worms is lacking. In addition, there is no report
63 on the species diversity of tubifex in Bangladesh. Therefore, the objective of the present study

64 was to identify and analyze the molecular characterization of the commonly found tubifex genera
65 in Bangladesh through state-of-the-art biological techniques.

66 **2. Materials and Methods**

67 *2.1 Acquisition, handling and preparation of tubifex sample*

68 **'Tubifex worms'** (100 g) were collected from the drains (muddy sediments) of Jashore
69 (Sample JA) and Mymensingh (Sample MM) regions of Bangladesh where most of the catfish
70 hatcheries operate. Specimens of tubifex were also sampled from the muddy area of Manikganj,
71 Dhaka (Sample DK) which were used as ornamental fish feed in ornamental fish market of
72 Katabon, Dhaka. The live worms were sorted out by sieving (0.2 mm sieve mesh size) into fine
73 or sandy sediment, placed in jars with water, and stored on ice for transportation to the laboratory
74 for further study.

75 *2.2 DNA extraction and Polymerase Chain Reaction (PCR) amplification of mixed sample*

76 The total genomic DNA was extracted from the mixed samples of tubifex by Maxwell®
77 16 automated nucleic acid extraction system (Promega, Madison, WI, USA) using Maxwell® 16
78 tissue DNA purification kit (Promega, Madison, WI, USA; Catalog-AS1030) according to
79 manufacturer's protocol. NanoDrop™ 2000 was used to determine the concentration and optical
80 purity of the extracted DNA. A portion (658 base pairs) of the mitochondrial gene (COI) was
81 amplified using LCO 1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO
82 2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') primers (Folmer et al., 1994). The
83 universal primers (Palumbi et al., 1991) 16sar (5'CGC CTG TTT ATC AAA AAC AT 3') and
84 16sbr (5' CCG GTY TGA ACT CAG ATC AYG T3') were used to amplify the large subunit
85 ribosomal RNA gene fragment (520 base pairs). Each PCR was performed in a total volume of

86 25 µl containing 12.5 µl GoTaq 2× Hot Start Colorless Master Mix (Promega, USA), 1 µl
87 (200nM) of each primer, 2.5 µl of template (extracted genomic DNA) and 8 µl of nuclease free
88 water. The thermal profile comprised an initial denaturation at 95⁰ C for 5 min, followed by 35
89 cycles of 95⁰ C for 40 s, annealing at 45⁰ C for 45 s and extension at 72⁰ C for 60 s, with a final
90 elongation step at 72⁰ C for 8 min. The PCR-amplified products (amplicons) were scrutinized by
91 1% agarose gels (containing ethidium bromide) and purified by using the Wizard® SV Gel and
92 PCR Clean-Up System (Promega, USA).

93 *2.3 Cloning and sequencing*

94 *Escherichia coli* DH5α was used to prepare competent cell by chemical method. The
95 amplified PCR products were cloned into pCR™ 4-TOPO® vector using TOPO® TA Cloning®
96 Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Transformation of the chemically
97 competent *Escherichia coli* DH5α was prepared with recombinant plasmid constructed by
98 cloning reaction. The day after transformation (usually 18 hours after plating transformation
99 reaction on LB agar containing kanamycin), the colonies that appeared on the plates were
100 investigated for transformants by colony PCR by using the primers (T3:
101 5'ATTAACCCTCACTAAAGGGA 3' and T7: 5' TAATACGACTCACTATAGGG 3')
102 provided in the TOPO® TA Cloning® Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA).
103 The amplification profile of the colony PCR was 35 cycles of 94 °C for 1 minute, 48 °C for 1
104 minute and 72 °C for 1 minute 30 seconds with an initial single denaturation step at 94 °C for 10
105 minutes and a single final extension at 72 °C for 25 minutes. To reduce the number of samples
106 having possible redundant sequencing, Restriction Fragment Length Polymorphism (RFLP)
107 technique (9 µl nuclease free water, 1 µl 10× buffer, 1 µl enzyme and 5 µl PCR product) was

108 applied by using the enzyme Pst1 and Hinf1 for amplicons of COI and 16S rRNA gene,
109 respectively.

110 From the representative group based on RFLP, the constructed plasmid from the colony
111 was extracted from the transformants using PureYield™ Plasmid Miniprep System (Promega,
112 Madison, WI, USA) according to producer's guidelines. The extracted plasmid (after being
113 confirmed that is the recombinant one) was separated by gel electrophoresis in 2 % low melting
114 agarose (L.M.T) slab gel (UltraPure™ L.M.P. Agarose, Spain) in a Tris-acetate EDTA (TAE)
115 buffer. The precise band position [Vector (3956 bp) + Insert (658 or 520 bp)] was pooled by gel
116 purification using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).
117 The purified cloned products were subjected to an automated cycle sequencing reaction using
118 BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems®, USA) according to
119 manufacturer's guidelines, analysis of the data was accomplished in ABI Genetic Analyzer
120 (Applied Biosystems®, USA).

121 *2.4 Sequence Submission and Phylogenetic Reconstruction*

122 The raw sequence data from the six different colonies of different samples were
123 assembled using SeqMan version 7.0 (DNASTAR, Inc., Madison, WI, USA) and the assembled
124 sequences were compared with other entries from NCBI GenBank (Benson et al., 2005) using
125 BLAST (Altschul et al., 1990) search to reveal the identification of tubifex. Using the sequence
126 data, we tested for model selection and reconstructed phylogenetic trees for 16S rRNA genes and
127 COI gene fragments with maximum likelihood methods using generalized time-reversible model
128 by bootstrapping 1000 times.

129 **3. Results and Discussion**

130 The morphological identification of aquatic oligochaete often misguided the taxonomist
131 for the two main reason. Firstly, a greater portion of specimens is immature and cannot be
132 identified. Secondly, some common morphospecies encompasses a high level of cryptic
133 diversity. In addition, a cluster of ‘tubifex worms’ forms a small colony (Baldo and Ferraguti
134 2005; Vivien et al., 2017; Vivien et al., 2015). Moreover, it is difficult to detach one worm from
135 a colony, and there is a chance to lost the tiny body fragment which may misguide in
136 morphological identification. As a result, the molecular technique was applied as a suitable
137 approach to identify the tubifex genera and their lineages. Hence, we proceed with the cloning
138 method to divulge the different genera/lineages randomly distributed in the samples by targeting
139 two genes (16S rRNA and COI) of the organisms.

140 RFLP of the maximum possible colonies was performed to differentiate strains after
141 successful cloning reaction. For COI gene-based identification randomly selected three isolates
142 (from two RFLP groups) were sequenced (BAN/DK/KB-9/2017, BAN/MM/MS-9/2017 and
143 BAN/ JA/JS-8/2017; GenBank Accession no: MN294954-MN294956) and another three isolates
144 (from one RFLP groups) namely BAN/JA/JS-1/2017, BAN/DK/KB-1/2017 and BAN/MM/MS-
145 1/2019 (GenBank Accession no: MN267043-MN267045) were sequenced for 16S rRNA gene.

146 Cytochrome c oxidase subunit I gene-based identification found that while BAN/JA/JS-
147 8/2017 strain had G, BAN/MM/MS-9/2017 and BAN/DK/KB-9/2017 had A in 470 nucleotide
148 position. However, the predicted organism, using both BLAST and phylogenetic reconstruction
149 tools in all three cases, is *Limnodrilus hoffmeisteri* complex unlabelled lineage (Figure 1). This
150 tree confirmed that most groups (A, B and C) were under the genus *Limnodrilus*, and within
151 group C, the unlabeled lineage resides in between other lineages. Another barcode (16S rRNA
152 gene) typically used for identification of invertebrates, predicted the organism as- *Limnodrilus*

153 *hoffmeisteri* complex lineage IX (isolate- BAN/DK/KB-1/2017) and *Limnodrilus sp.*
154 *claparedianus-cervix* (isolate- BAN/JA/JS-1/2017 and BAN/MM/MS-1/2017) by BLASTing
155 and molecular phylogeny (Figure 2). The phylogenetic tree showed the position of the isolates
156 within group C, in two different clades- one within lineage IX of *L. hoffmeisteri* complex and
157 another belongs to *Limnodrilus sp. claparedianus-cervix*. Recently, to overcome the difficulties
158 related to morphology-based identification of oligochaetes, standard cytochrome c oxidase I
159 (COI) barcode, including other barcoding markers, has been used as a genetic tool for rapid
160 identification of oligochaete diversity based on mixed specimen samples (Vivien et al., 2016b).
161 However, species delimitation based on only mitochondrial COI gene may not reveal species
162 margins as precisely as an integrative tactic exploring a combination of numerous data sources,
163 hence mitochondrial 16S ribosomal DNA and mitochondrial marker (COI), in combination, have
164 been applied in contemporary species identification and description.

165 Before the molecular genetics era, the combination of allozyme data (by electrophoresis)
166 with morphometric characteristics isolated four *Limnodrilus* species, *L. hoffmeisteri*, *L.*
167 *udekemianus*, *L. claparedianus*, and *L. profundicola* by Schmelz (1996). The monophyletic
168 status of the genus *Limnodrilus* has confirmed that *L. udekemianus* is a species distinct from the
169 clade comprising of *L. claparedianus*, *L. hoffmeisteri*, *L. cervix* and *L. maumeensis* (Weider
170 1992; Liu et al., 2017a). Although the *L. hoffmeisteri* sensu stricto was partly defined and
171 characterized by COI (cytochrome c oxidase subunit I) barcoding (Liu et al., 2017b; Sjolín et al.,
172 2005) , the small mitochondrial 16S rDNA data sets in earlier molecular studies advocated that
173 the common and broadly dispersed clitellate morphospecies are complexes in cryptic species
174 (Erséus and Gustafsson 2009; James et al., 2010; Martinsson and Erséus 2014; Martinsson and
175 Erséus 2017), and the morpho-taxon *L. hoffmeisteri* is likely to encompass cryptic lineages

176 (Marton and Eszterbauer 2012). The large *Limnodrilus* sensu stricto clade entails three groups
177 (A-C), in which, group C covers all species of *L. hoffmeisteri* complex (Liu et al., 2017a) with its
178 numerous siblings (spp. I-X). The complex C is alienated into two well-supported lineages, one
179 enclosing *L. hoffmeisteri* species I-IV and *L. claparedianus*, the other covering *L. hoffmeisteri*
180 species V-X (including IX= *L. hoffmeisteri sensu stricto*), *L. maumeensis* and *L. claparedianus-*
181 *cervix* which is the morphologically intermediate between *L. claparedianus* and *L. cervix* (Liu et
182 al., 2017a). The lineage identified as *L. claparedianus*, *L. maumeensis* and *L. claparedianus-*
183 *cervix* are species (Liu et al., 2017b). The morphospecies *L. hoffmeisteri* (group C) is the most
184 broadly circulated cosmopolitan taxon (Brinkhurst and Jamieson 1971). Based on both molecular
185 characterization using genetic markers (mitochondrial COI, nuclear ITS, and 16S rDNA) and
186 morphological evidence, *L. hoffmeisteri* has been designated as a species complex rather than a
187 single species (Liu et al., 2017a).

188 The genus *Limnodrilus* includes 17 morphospecies and 10 genospecies (nine separate
189 species and a single complex) (Pinder and Brinkhurst 1994; Liu et al., 2017a). According to Liu
190 et al. (2017b), species to date categorized with *Limnodrilus* formed a non-monophyletic group
191 with two detached clades (one of them consist of three specimens of *L. rubripenis* and other
192 comprise all other *Limnodrilus spp.*). The *Limnodrilus* individuals were morphologically
193 indistinguishable and different clades suggest the existence a of genetic barrier for gene flow
194 (Crottini et al., 2008; Gustafsson et al. 2009).

195 While two different species of *Limnodrilus* have been identified within samples JA and
196 MM, we have identified only one distinct lineage of *L. hoffmeisteri* for sample DK. However, all
197 of the isolates are under Group C which is quite distinct from other groups of *Limnodrilus*.

198 According to Timm (2009) asexual mode of reproduction of 'tubifex worms' dominates in
199 tropical water which might be the possible cause of the absence of other tubifex species.

200 Active immigration is a tremendously deliberate process, hence, passive transport may
201 play a vital role in the pan-distribution of *L. hoffmeisteri* complex species and other *Limnodrilus*
202 species (Koel et al., 2010). Several transport mechanisms are known to cause large scale massive
203 dispersal of aquatic fauna, among this anemochorous dispersal of cocoon or cyst, zoochorous
204 transport (most likely exploiting bird migration) or antropic use of the species (Milbrink and
205 Timm 2001; Crottini et al., 2008). Naveed (2012) demonstrated that the cosmopolitan species *L.*
206 *hoffmeisteri* is collected by aquarium owners and is widely used as live feed for ornamental
207 fishes worldwide. Therefore, the use of these worms as aquaria food in different firms may have
208 played a key role in the distribution of these species complex.

209 The route of entry of *L. hoffmeisteri* complex lineage IX, *L. hoffmeisteri* complex
210 unlabelled lineage, and *L. claparedianus-cervix* into Bangladesh would be from Japan, China,
211 Malaysia, and Thailand; India and China, respectively as other countries related to those groups
212 are related to European countries, USA and Canada. (Liu et al., 2017a).

213 The main drawback of this study might be grouping of the isolates from transformants
214 using RFLP wherein some of the isolates might get missed. Nevertheless, it is an established
215 technique to choose the representative from many samples. Another drawback was using a mixed
216 colony instead of a single worm which would help us avoid the cloning step also. Nevertheless,
217 this study is successful as fulfilling the main objective of the study characterizing the tubifex at
218 the lineage level using gene data.

219 **4. Conclusion**

220 The findings of this study, for the first time, confirmed the existence of two genetically
221 different, unnamed taxa (species) of *Limnodrilus* in Bangladesh. However, within a single
222 sample, multiple species were spotted which refers to the complex colony formation. However,
223 our key goal was to identify tubifex by using molecular data in this study, and in the future work,
224 the single colony will be isolated before sorting out from the core sample.

225 **Authorship contribution statement**

226 Md. Inja-Mamun Haque **collected** the worm samples and did molecular works including
227 manuscript preparation. A. S. M. Rubayet Ul Alam carried out the studies (molecular and
228 bioinformatics analysis) and **participated in the preparation of the manuscript**. Nafia Akter
229 partially did molecular works on bench. Mohammad Anwar Siddique helped to develop the
230 molecular methodology. Munawar Sultana and M. Anwar Hossain investigated the whole work
231 and helped to review and prepare the manuscript. Mahmud Hasan designed the study, acquire
232 fund and participated in preparing and revising the manuscript. All authors read and approved
233 the final manuscript.

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336 **Caption of the Figure**

337 **Fig. 1.** Phylogenetic tree showing the relationships among different *Limnodrilus* spp. based on
338 cytochrome c oxidase subunit I gene. Three groups (A, B, and C) were clustered separately than
339 the outgroup *Limnodrilus* (*L. appendiculatus* and *L. anxius*). Within outgroup C, along with
340 other lineages, *L. hoffmeisteri* complex, the unlabelled lineage resides, and all of the isolates of
341 the study fell under the same lineage (unlabelled) of *L. hoffmeisteri* complex. The value in the
342 nodes represents the bootstrap percentage where >70 % was considered statistically significant.
343 For a better representation of the tree, we compressed the branches.

344

345 **Fig. 2.** Phylogenetic tree showing the relationships among different *Limnodrilus* spp. based on
346 16S gene. Three isolates from RFLP groups falling under group C showed that *Limnodrilus*
347 *hoffmeisteri* complex lineage IX and *L. sp. claparendianus-cervix* were present in our samples.
348 The outgroup of the tree contains *L. anxius* and *L. appendiculatus*, and other two sister groups, A
349 and B consist of *L. sulphurensis*, *L. silvani*, and *L. grandisetosus*. The bootstrap value showing in
350 percentage can be spotted under the node where we considered >70 % as statistically significant.

351

352

353 **Caption of the supplementary figure**

354 Supplementary Figure 1

355 Phylogenetic tree showing the relationships among different *Limnodrilus* spp. based on
356 cytochrome c oxidase subunit I gene (uncompressed).

