Multi-locus phylogenetic analysis of the genus *Limnodrilus* (Annelida: Clitellata: Naididae)

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**A B S T R A C T**

*Limnodrilus* species are annelid worms distributed worldwide in various freshwater sediments. The systematics of *Limnodrilus* has chiefly been based on morphology, but the genus has not been subject to any closer phylogenetic studies over the past two decades. To reconstruct the evolutionary history of *Limnodrilus*, and to assess the monophyly of this genus and its systematic position within the subfamily Tubificinae (Annelida: Clitellata: Naididae). 45 *Limnodrilus* specimens, representing 19 species, and 35 other naidid species (representing 24 genera) were sampled. The data consisted of sequences of three mitochondrial genes (COI, 12S and 16S rDNA) and four nuclear markers (18S and 28S rDNA, Histone 3, and ITS). The phylogeny was estimated, using Maximum Likelihood and Bayesian analyses of concatenated data of seven DNA loci, as well as a multi-locus coalescent-based approach. All analyses strongly suggest that *Limnodrilus* is monophyletic, but only if the morphospecies *L. rubripenis* is removed from it. *Limnodrilus rubripenis* and (at least) *Baltidrilus*, *Lophochaeta* and some species attributed to *Varichaetadrilus* comprise the sister group to the clade *Limnodrilus sensu stricto*, and the latter is further divided into three well-supported groups. One of them contains morphospecies characterized by short cuticular penis sheaths and enlarged chaetae in anterior segments (*L. udekemianus*, *L. silvari* and *L. gran disgosus*). The second is a small group of species with moderately long penis sheaths, i.e., *L. sulphurensis* and *L. profundicola*. The third, and largest group, includes not only the multitude of cryptic species in the *L. hoffmeisteri* complex, but also other, morphologically distinct, species nested within this complex. All studied species in this large group have long penis sheaths, which are exceptionally long in *L. claparedianus*, *L. maumeensis*, and a form morphologically intermediate between *L. claparedianus* and *L. cervix*. The identification and classification of these groups provide a framework for directed sampling in further phylogenetic studies, and for revisionary work on the *L. hoffmeisteri* complex and other unresolved *Limnodrilus* species.

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1. Introduction

Species of *Limnodrilus* Claparède, 1862 are common bioturbator annelids (Clitellata: Naididae: Tubificinae) in freshwater ecosystems throughout the world, except the polar regions (Brinkhurst, 1980; Brinkhurst and Jamieson, 1971). The genus consists of at least 17 currently recognized morphospecies, of which *Limnodrilus hoffmeisteri* Claparède, 1862, *L. udekemianus* Claparède, 1862 and *L. claparedianus* Ratzel, 1868 are the most well-known and indeed cosmopolitan taxa (Brinkhurst and Marchese, 1989; Kathman and Brinkhurst, 1998; Pinder and Brinkhurst, 1994). Two species, *L. neotropicus* Černosvitov, 1939 and *L. bulbiphallus* Block and Goodnight, 1972, are only known from the Neotropics (Brinkhurst and Marchese, 1989), while most other taxa appear more or less restricted to the Holarctic, some being endemic to parts of North America or Asia (Fend et al., 2016; He et al., 2010; Kathman and Brinkhurst, 1998; Semernoy, 2004; Wang and Liang, 2001).

Generally, the *Limnodrilus* species are defined by a few, and often ambiguous, diagnostic morphological features. They lack hair and pectinate chaetae, which are common structures in other genera of the subfamily Tubificinae. The shape of the anterior bifid chaeta has been used for distinguishing a few *Limnodrilus* species (e.g., *L. udekemianus*), but this may still be an insufficiently tapped source of taxonomic information, in particular, within the *L. hoffmeisteri* complex (Liu et al., 2017). The shape of the penis...
sheaths has widely been considered as the most important character to identify Limnodrilus species (Brinkhurst and Jamieson, 1971; Brinkhurst and Marchese, 1989; Kathman and Brinkhurst, 1998), although this feature shows large intra-specific differences, even within the same slide-mounted specimen. A wide and continuous range of penis sheath variation has been observed, especially in the case of L. hoffmeisteri sensu lato (Dzwillo, 1984; Hiltunen, 1967, 1969; Kennedy, 1969), but the occurrence of even reasonably distinctive morphotypes has long been regarded as intra-specific diversity (e.g., Brinkhurst and Jamieson, 1971). Recently, however, it was shown that the taxon L. hoffmeisteri corresponds to a species complex rather than a single species on basis of molecular data (Liu et al., 2017). On the other hand, the phylogenetic relationships, both within Limnodrilus and among related genera, have remained largely unknown, due to the poor resolution of the morphology-based taxonomy and to limited taxon sampling in the few genetic analyses (see below). Some taxa originally assigned to Limnodrilus (mostly due to their possession of cuticular penis sheaths) are today placed in other genera. For instance, Limnodrilus newaeansis Michaelsen, 1903 was moved to Tubifex by Brinkhurst (1962); Limnodrilus pseudogaster Dahl, 1960 was moved to Tubificoides by Brinkhurst and Baker (1979), and Limnodrilus angustipes Brinkhurst and Cook, 1966 to Varichaedrilus by Brinkhurst (1989).

Phylogenetic inference based on genetic data has made a significant impact on systematics, and assessments also incorporating morphology will contribute to a better understanding of the evolutionary history of species. Morphological features, however, are increasingly becoming less common as the primary data used to infer evolutionary history and species boundaries. This is especially true when revealing cryptic species, i.e., genetically different species with similar morphological characters (Bickford et al., 2007). Difficulties experienced in morphological identification led to the utilization of some early molecular approaches to identifying and classifying Limnodrilus species. Milbrink and Nyman (1973) combined allozyme data (by electrophoresis) with morphological features to identify four Limnodrilus species, L. hoffmeisteri, L. udekemianus, L. claparedianus, and L. profundicola (Verrill, 1871; in Smith and Verrill, 1871), and allozymes were also used by Weider (1992) to evaluate the monophyletic status of the genus. Weider was even able to conclude that L. udekemianus is a species separate from the clade consisting of L. claparedianus, L. hoffmeisteri, L. cervix Brinkhurst, 1963 and L. maumeensis Brinkhurst and Cook, 1966, and this relationship was also confirmed by Beauchamp et al. (2001) and Marton and Eszterbauer (2012) based on phylogenetic analyses of mitochondrial 16S rDNA. Using a combination of 16S and nuclear 18S rDNA sequences, Sjölin et al. (2005), and with 16S only, Achura et al. (2011), both confirmed that Limnodrilus (although represented by only a few species) is nested within the subfamily Tubificinae of the Naididae sensu Erséus et al. (2008). Molecular data are now gradually being applied in contemporary species delimitation and description, e.g., the new species L. sulphurensis (Fend et al., 2016), and (by nontypification) L. hoffmeisteri sensu stricto (Liu et al., 2017) are partly defined by COI (cytochrome c oxidase subunit I) barcodes. Moreover, a combined analysis of the mitochondrial COI and 16S rDNA datasets, and the nuclear internal transcribed spacer (ITS) region data, refute the hypothesis of a single, euryoecious, and widely distributed L. hoffmeisteri (Liu et al., 2017). This taxon instead represents a large complex of genetically different species, which may not even represent a monophyletic group.

In light of these discoveries, the objectives of the current study are to reconstruct the evolutionary history of Limnodrilus, and to assess the monophyly and systematic position of this genus within the Tubificinae on the basis of a multiple-locus phylogenetic approach. The study is meant to provide a broadened molecular basis for the species-level lineages now recognized within the L. hoffmeisteri complex, and, in particular, to better resolve the phylogeny among them (see above, and Liu et al., 2017).

2. Methods

2.1. Sampling strategy and selection of loci

To assess phylogenetic relationships of Limnodrilus, 45 specimens, representing 19 species including ten species within the L. hoffmeisteri complex (Liu et al., 2017), and the recently described L. sulphurensis Fend et al., 2016, were selected in the current study (Table 1). In light of the study by Enwall et al. (2006), we then sampled 35 other species (24 genera) of the family Naididae to serve as outgroups. A complete list of specimens, including collection information, GenBank accession numbers, and catalog numbers of museum vouchers, is provided in Table 1.

Seven loci, i.e., three mitochondrial genes (Cytochrome oxidase I, COI; 12S and 16S rDNA), one protein coding nuclear gene Histone-3 (H3), and three other nuclear genes (Internal Transcribed Spacer region (ITS), 18S and partial 28S rDNA), were used in the phylogenetic analyses. The three mitochondrial markers and ITS have high evolutionary rates, and thus, they are generally used to resolve molecular phylogenies at shallow taxonomic levels, while H3, 18S and 28S evolve more slowly, providing data that are more useful for high level phylogeny.

2.2. DNA extraction, PCR protocol and alignment

Total genomic DNA was extracted from ethanol-preserved tissue using the DNAeasy Tissue kit (Qiagen) or the EZNA Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA). DNA sequences were amplified under reaction conditions given below, in each reaction using a 25 μl volume with 1 μl of each primer, 2 μl of template DNA, and 6 μl of water mixed with 15 μl Red Taq DNA Polymerase Master Mix (VWR, Haasrode, Belgium). The PCR protocols of COI, 12S and 16S rDNA were performed using the primer pairs LCO1490/HCO2198 (Folmer et al., 1994) or COI-E (Bely and Wray, 2004), the primer pair 12SE1/12SH (Jamieson et al., 2002) and the primer pair 16SAR-L/16SBRH (Palumbi et al., 1991), respectively, under the same protocol: after 5 min initial denaturation at 95 °C, denaturing 45 s, annealing at 45 °C for 45 s, and extension at 72 °C for 60 s in 35 PCR cycles with a final extension of 8 min. The whole 18S rDNA was amplified with the TimA and 1100R primers (Norén and Jondelius, 1999) for the 5′ part of 18S, and the 660F (Erséus et al., 2002) or 5f (Giribet et al., 1999) and TimB (Norén and Jondelius, 1999) primers for the 3′ part. PCR conditions here consisted of 5 min initial denaturation at 95 °C, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, elongation at 72 °C for 90 s, and a final extension at 72 °C for 8 min. H3 was amplified with the primer combination H3A-1/H3A-2 (Colgan et al., 1998), and the primer combination for 28S rDNA was 28SCL/28SC2 (Jamieson et al., 2002); PCR conditions differed from the 18S protocol in annealing at 50 °C for 1 min. The ITS sequences were amplified either by the primer pair ITS5/ITS4 (White et al., 1990) or the pair 29F/1084R (Liu and Erséus, unpublished). The primer pair 606F/1082R (Liu and Erséus, unpublished) was used to amplify the ITS2 region when both of the other primer combinations for ITS were unsuccessful.

PCR products were purified using exonuclease I (Fermentas, Burlington, Canada) and FastAP thermosensitive alkaline phosphatase (Fermentas), and amplified fragments were sequenced by Eurofins Genetic Services Ltd. (Germany). Newly generated contaminants were assembled and edited in Geneious V6.1.8, and sequences were submitted to GenBank (Table 1). Sequence alignments for the
## Table 1

List of studied taxa with their specimen ID, GenBank accession numbers for the sequences from analyzed loci (new records in bold fonts, "--" stands for missing data), Voucher ID, Location, Collection date, and Collectors.

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<td>KY636972</td>
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<td>ITS</td>
<td>H3</td>
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<td>Latitude</td>
<td>Longitude</td>
<td>Collection date</td>
<td>Collector</td>
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<td>Elba Italy, Toscana, Elba, off San Andrea, 13 m, sand</td>
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<td>Australia, Western Australia, 5 of Esperance Municipal Museum, man-made freshwater pond</td>
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(continued on next page)
Table 1 (continued)

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<th>28S</th>
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<th>ITS</th>
<th>H3</th>
<th>Voucher ID</th>
<th>Location and habitat</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Collector</th>
<th>Date</th>
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<td>86.6431 W</td>
<td>Sebastian Kent</td>
<td>17-Mar-2008</td>
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</table>

2.3. Phylogenetic analysis

Since mitochondrial and nuclear DNA may have different evolutionary histories, trees based on the corresponding two datasets were separately reconstructed using the maximum likelihood (ML) and Bayesian approaches. In addition, we reconstructed the phylogeny using a combination of mitochondrial and nuclear data to provide an overall view of evolutionary relationships of *Limnodrilus*. The best-fit models for gene partitions (ITS region was split into three partitions: ITS1 spacer, 5.8S rDNA and ITS2 spacer) in all phylogenetic analyses were estimated using the software PartitionFinder (version 1.1.1) under the Bayesian Information Criterion (Lanfear et al., 2012).

The best ML tree was obtained in RAxML (V8.0; Stamatakis, 2014) by optimizing the best parsimony tree out of 1000 random searches and bootstrap values by summarizing tree topologies from 1000 non-parametric replicates. Bayesian phylogenetic analyses were executed in MrBayes V 3.2.3 (Ronquist et al., 2012) utilizing the evolutionary model chosen by PartitionFinder. For the MrBayes analysis, two independent Bayesian runs were initiated from random starting trees, run for 2 × 10^7 generations with 4 incrementally heated Metropolis-coupled Markov chain Monte Carlo (MCMC) chains, and sampled at intervals of 1000 generations, or until the standard deviation of split frequencies stayed below 0.001. Convergence of the runs was assessed by ensuring that the potential scale reduction factors (PSRF) were almost equal to 1 in MrBayes, and the values of effective sample size (ESS) were monitored using Tracer v1.6 (Rambaut et al., 2014). The first 25% of the trees were discarded as burn-in, the remaining trees were used to reconstruct a consensus tree and to estimate Bayesian posterior probabilities.

"BEAST (Star BEAST) is a Bayesian method, based on coalescent theory, that uses MCMC to co-estimate the gene trees and the species tree given a set of multiple sequence alignments on different loci (Heled and Drummond, 2010). The substitution model for each marker, suggested by PartitionFinder, was used in the analysis of "BEAST V1.8.2. Each marker was given its independent site and clock parameters, and the three mitochondrial genes were linked in a single partition tree. The 18S, ITS and 28S were also linked as a single partition tree since they are genetically linked. Each specimen was assigned to a species name, including cryptic lineages identified in the *L. hoffmeisteri* complex by Liu et al. (2017). The strict clock was used for all partitions, the rate for the COI clock was fixed to 1, and the rate for the other markers was estimated in relationship to the rate of COI. The Yule option was selected as the species tree prior, and an UPGMA starting tree was set for each partition. The effective population sizes of nuclear genomes are expected to be twofold greater than those of mitochondrial ones, as each individual of a hermaphroditic species can theoretically become female and transfer its mitochondrial genome to the next generation (Diaz-Almela et al., 2004). Therefore, the ploidy level of the mitochondrial partition was adjusted manually in the xml file generated by BEAUTi (Drummond et al., 2012) to accommodate this twofold difference. The MCMC chains were run for 400 million generations twice, sampled from the posterior every 40,000 iterations. The first 10% generations were discarded as burn-in by examining effective sample size values (ESS > 200) in Tracer v1.6.
three ITS sequences. Up to 1.4% divergence was observed between the corresponding L. rubripenis to that of CE1785. In contrast, although the COI maximum pairwise distance (uncorrected p distance) among the studied Naididae was 29%. An intra-specific pairwise distance (uncorrected p distance) within the family Naididae, were obtained. The total dataset contains 332 newly generated sequences, 193 sequences downloaded from GenBank, while 35 sequences are missing (1 of 12S; 3 of 16S; 5 of 18S, 4 of ITS; 19 of H3, see Table 1). The COI and H3 alignments were 658 and 328 bp, respectively. Due to intraspecific variation and missing data, the rDNA sequences (12S, 16S, 18S, 28S, and ITS) ranged from 347 to 388 bp, 318 to 490 bp, 1395 to 1795 bp, 314 to 331 bp and 394 to 1664 bp, respectively. The length of the concatenated mitochondrial (COI, 12S and 16S) alignment was 1589 bp, of which 939 nucleotide sites were variable. For the nuclear alignment, with a total of 4838 bp, there were 2076 variable sites, which, however, include gaps. The final combined alignment of all seven markers was 6427 bp long, with 3015 variable sites. The aligned datasets are available in TreeBASE (accession: 20634).

The maximum inter-specific COI variation (uncorrected p distance) among the studied Naididae was 29%. An intra-specific COI distance as large as 20.1% was observed in one morphospecies, L. grandisetas Nomura, 1932, and large differences between the two ITS2 sequences (CE1785 from Indonesia, and CE1786 from Japan) were also observed. The ITS2 sequence of CE1785 was significantly longer (around 150 bp) than that of CE1786, due to duplications and/or inserts/deletions. The ITS1 sequence was 15% longer than that of CE1785 in contrast, although the COI maximum p distance among three L. rubripenis COI sequences was 15.1%, only up to 1.4% divergence was observed between the corresponding three ITS sequences.

3. Results

3.1. General information of datasets

Both mitochondrial genes (COI, 12S and 16S rDNA) and nuclear markers (18S, 28S, ITS and H3) for a total of 80 individuals, representing 25 genera within the family Naididae, were obtained. The total dataset contains 332 newly generated sequences, 193 sequences downloaded from GenBank, while 35 sequences are missing (1 of 12S; 3 of 16S; 5 of 18S, 4 of ITS; 19 of H3, see Table 1). The COI and H3 alignments were 658 and 328 bp, respectively. Due to intraspecific variation and missing data, the rDNA sequences (12S, 16S, 18S, 28S, and ITS) ranged from 347 to 388 bp, 318 to 490 bp, 1395 to 1795 bp, 314 to 331 bp and 394 to 1664 bp, respectively. The length of the concatenated mitochondrial (COI, 12S and 16S) alignment was 1589 bp, of which 939 nucleotide sites were variable. For the nuclear alignment, with a total of 4838 bp, there were 2076 variable sites, which, however, include gaps. The final combined alignment of all seven markers was 6427 bp long, with 3015 variable sites. The aligned datasets are available in TreeBASE (accession: 20634).

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3.2. Phylogenetic results

Concatenated mitochondrial (Fig. S1), concatenated nuclear (Fig. S2) and concatenated all-loci phylogenetic trees (Fig. 1) were reconstructed separately using ML and Bayesian methods, but they were topologically similar enough to be shown as one per dataset. The overall level of support for some internal nodes was higher in the concatenated mitochondrial trees (Figs. S1 and 1) than these in the concatenated nuclear trees (Fig. S2). Incongruences between the concatenated mitochondrial and the concatenated nuclear trees included the positions of Branchiura sowerybi Beddard, 1892 and the four species of Limnodriloidae, which were all clustered within the subfamily Tubificinae, but with low support, in the nuclear tree. The tree based on all concatenated mitochondrial and nuclear data (Fig. 1) gave, in this respect, the same result as with the previous two trees. Nevertheless, the group comprising all Tubificinae, Limnodriloidae and Branchiura was highly supported in all phylogenetic analyses. With respect to our focal genus, Limnodrilus, the Bayesian (including MRBayes and ‘BEAST’ and maximum-likelihood analyses recovered very similar topologies, but with discrepancies in the levels of support for some deeper nodes (Fig. S3). Mid-point rooting of the trees yielded very similar topologies, and therefore, only Bayesian trees (from MrBayes analyses), labeled with both Bayesian posterior probability and bootstrap support values estimated from ML analysis and Bayesian posterior probability, are shown and discussed.

Species to date classified within Limnodrilus formed a non-monophyletic group with two separated but well-supported clades (Fig. 1). One of them consists of the three specimens of Limnodrilus rubripenis Loden, 1977, the second contains all other Limnodrilus taxa. The former is nested inside a clade containing also Lophochaeta ignota Štolc (1886), the two Varicathaedrilus species, and Baltidrilus costatus(Claparède, 1863), and this clade was placed as the sister to the remaining Limnodrilus. This topology was supported by all analyses, and the large Limnodrilus “sensu stricto” clade comprises three groups (A–C, in Fig. 1). Group A is composed of L. udekemianus, L. silvanii Eisen, 1879 and L. grandisetas. Group B consists of L. profundicola and L. sulphurensis, and group C encompasses all the species of the L. hoffmeisteri complex recently studied by Liu et al. (2017). In all analyses except the concatenated mitochondrial one (Fig. S1), this complex (C) was divided into two well-supported lineages, one containing L. hoffmeisteri species L-IV and L. claperdanus, the other containing L. hoffmeisteri species V-X (including IX = L. hoffmeisteri sensu stricto), L. maunensis, and “L. claperdanus-cervix”. Relationships within the two lineages of group C were not well resolved in all analyses.

With regard to “outgroup” taxa in our analyses, four genera within the subfamily Tubificinae, i.e., Potamothis Vejdovsky & Mrázek, 1903 (4 species), Tubificoides Lastočkin, 1937 (2 species), Paamoryctides Hrabé, 1964 (2 species), and Aulodrilus Brescher, 1899 (2 species), as well as the two subfamilies Limnodriloidinae (4 species) and Phallodrilinae (2 species) were retrieved as monophyletic entities with good statistical support. Neither the subfamily Rhycodrilinae nor the genus Tubifex Lamarck, 1816 (4 species) were found to be monophyletic.

4. Discussion

4.1. Congruence between concatenation and coalescence-based phylogenies

To evaluate the robustness of our reconstruction of the phylogenetic tree of Limnodrilus, we used both traditional concatenation and coalescence-based approaches. One reason for this is that there is a great controversy over whether coalescent-based species tree estimation methods or the standard approach of concatenation should be used (Lambert et al., 2015; Roch and Warnow, 2015; Simmons and Gatesy, 2015; Xi et al., 2014; Zhang et al., 2015); the performance of the two kinds of methods may not be the same as they are based on completely different assumptions. Assuming that all combined genes (perhaps with different mutation rates and models for different sites) have evolved into a single evolutionary tree, the concatenation approach may result in over-confident support for incorrect species trees in the presence of gene tree discordance (Kubatko and Degnan, 2007). The discordance between individual gene trees and species trees is a well-documented phenomenon (Degnan and Rosenberg, 2009), and incongruity between gene trees from concatenated mitochondrial DNA and concatenated nuclear DNA data are observed in some studies (Fisher-Reid and Wiens, 2011). In contrast, causes of gene tree discordance, such as deep coalescence (incomplete lineage sorting) and hybridization, can be investigated using a coalescent-based method (Mirarab et al., 2014). However, recent simulations show that coalescence may not provide significantly better performance over concatenation methods (Gatesy and Springer, 2014; Tonini et al., 2015). Therefore, using both approaches in the present study was a way to test whether they give contradictory results in our case.
As discussed further below, the placement of "L. rubripens" as a terminal group within the sister lineage to the remaining Limnodrilus was substantially supported by all our phylogenetic analyses. The resolution of this relationship is notable due to the concordance across both concatenated mitochondrial and nuclear trees using Bayesian and ML methods. Furthermore, this relationship was consistent with concatenated trees and the coalescence-based Bayesian tree estimated from all seven loci (summarized in Fig. 1). Collectively, the results seen in all trees are therefore likely based Bayesian tree estimated from all seven loci (summarized in Fig. 1). Collectively, the results seen in all trees are therefore likely to represent a good estimate of the underlying phylogeny of Limnodrilus. In contrast, the level of support for the resolution of relationships among some other Tubificinae, especially for Branchiura sowerbyi, was less convincing. Although the Bayesian and ML analyses of concatenated mitochondrial and all-loci datasets consis-
tently gave support for the placement of *Branchiura* as the sister to the clade consisting of *Limnodriloidinae* and *Tubificinae* (Fig. 1), this topology was not supported in the coalescence species tree (Fig. S3) and the BI and ML trees inferred from concatenated nuclear data (Fig. S2). The ambiguous phylogenetic positions of these lineages are obviously explained by differences between the mitochondrial and nuclear gene trees, in consistency with conclusions of other studies (Paczesniak et al., 2013; Papakostas et al., 2016; Toews and Brelsford, 2012).

### 4.2. Phylogeny and taxonomy

The primary focus of our study is to shed light on the phylogenetic position of the genus *Limnodrilus* within the subfamily *Tubificinae*, and the evolutionary relationships among its many species. Previous molecular phylogenetic analyses included only much smaller subsets of *Limnodrilus* taxa, and too few other members of the subfamily *Tubificinae*, to find the most likely sister group of *Limnodrilus* (Beauchamp et al., 2001; Erös et al., 2000; Siddall et al., 2001; Sjölin et al., 2005; Achura et al., 2011).

Based on a more exhaustive sampling, our multi-locus analyses recovered a strongly supported monophyletic group, hereafter referred to as *Limnodrilus* sensu stricto, which comprises a vast majority of the sampled *Limnodrilus* specimens (Fig. 1). It contains the type species *Limnodrilus hoffmeisteri sensu stricto* (i.e., species IX notypified by Liu et al., 2017), plus the nine other species of the “*L. hoffmeisteri complex*”, which were all genetically delimited by congruence between mitochondrial and nuclear data (Liu et al., 2017). Three *Limnodrilus* groups A–C (shown in Fig. 1) were well supported in all analyses. The first one (A), comprising *L. udekemi anus*, *L. silvani* and *L. grandisetusos*, is also supported morphologically by a rather stiff integument (body wall) and a very slender thread-like posterior body, short penis sheaths (often only 150–200 µm, but also up to 360 µm, unpublished information from A. Ohtaka), and short atria (Brinkhurst, 1963, 1965, 1971; Brinkhurst et al., 1990; Eisen, 1879; Hiltunen, 1967; Howmiller, 1974a; Kathman and Brinkhurst, 1998; Ohtaka, 1985; Ohtaka et al., 2006; Pignut, 1913; Pinder and Brinkhurst, 2000; Wang and Liang, 2001). Group B consists of *L. profundicola* and *L. sulphurensis*, both characterized by moderately long (150–410 µm) penis sheaths without spiral muscles, and short atria (Cui et al., 2015; Fend et al., 2016; Kathman and Brinkhurst, 1998; Kennedy, 1969; Lee and Jung, 2014; Ohtaka, 1992; Semernoy, 2004; van Haaren and Soors, 2013). Group C contains all the remaining *Limnodrilus* sensu stricto taxa, i.e., the large *L. hoffmeisteri* complex with its many siblings (spp. I-X), *L. claparedianus*, *L. maumeensis* and the unidentified species “*Limnodrilus claparedianus-cervix*”. As the latter is morphologically intermediate between *L. claparedianus* and *L. cervix*, we suggest that *L. cervix sensu stricto* also is a group C species. All species of this group have long (>400 µm) or very long penis sheaths and relatively elongate atria (Brinkhurst, 1971; Cernosvitov, 1939; Hiltunen, 1967, 1969; Howmiller, 1974b; Ohtaka et al., 1990; Pinder and Brinkhurst, 2000; Southern, 1909). It is likely that *L. tortilipenis* Wetzel, 1987, with penis sheaths up to 4 mm long and considered by Kathman and Brinkhurst (1998, p. 162) as a “monster” (polyploid?) variant of *L. claparedianus*, belongs here too. [What is said here assumes that *L. cervix sensu stricto* and *L. tortilipenis* indeed belong to *Limnodrilus*; compare with *L. rubripenis*, below.]

However, we found, with strong support, that the morphospecies “*Limnodrilus rubripenis*” is not a part of *Limnodrilus*, but instead a terminal lineage within the latter’s sister group, i.e., the clade containing *Baltidrilus*, *Lophochaeta*, and the two forms identified as *Varichaetadrilus* species (Fig. 1). Both specimens CE3621 and CE3800 have long atria lacking distinct ejaculatory ducts (Fig. S4), suggesting that they belong to *Varichaetadrilus sensu Brinkhurst and Kathman, 1983*. Moreover, both of our *Varichaetadrilus* specimens have variable anterior chaetae (Fig. 2), similar to those of *V. angustipenis* (Brinkhurst and Cook, 1966). The visible parts of the penis sheaths in CE3621 are somewhat crumpled distally, while the sheaths of CE3600 are cylindrically tube-like, and short (Fig. 3). Thus, here we refer to CE3600 as *V. cf. angustipenis*, although the penis sheath is short and uniformly narrow, lacking the basal expansion of typical *V. angustipenis* (Brinkhurst and Cook, 1966). CE3621 remains named as “*Varichaetadrilus sp.*” ([with only a distal part of the penis sheath visible in our slide-mounted individual]. Admittedly, the long penis sheaths of *L. rubripenis* (compare with those of *Limnodrilus* group C, Fig. 4), as well as its bifid chaetae – which are modified in some segments (e.g., as in *L. grandisetusos*) – are superficially similar to features found also in *Limnodrilus*. *Loden* (1977) evidently assigned his *L. rubripenis* to this genus based on these similarities. However, regarding male duct and chaetal morphology, *L. rubripenis* is in fact a typical member of *Varichaetadrilus*, the most obvious shared character being the very long atria (Fig. S5); in *Limnodrilus*, atria are much shorter (Kathman and Brinkhurst, 1998). The cuticular penis sheaths of *Varichaetadrilus* species vary in length and shape, but those of *L. rubripenis* (Fig. 4) are basically similar to those of *V. psammophilus* (Loden, 1977), *V. angustipenis* (Brinkhurst and Cook, 1966), *V. harmani* (Loden, 1979), and our own *Varichaetadrilus* cf. *angustipenis* and *Varichaetadrilus* sp. Finally, although *Varichaetadrilus* (as *Varichaetadrilus*) was originally defined as having having ha`ir and pectinate chaetae in addition to bifids (Brinkhurst, 1981), *L. rubripenis* resembles *Varichaetadrilus fulleri* (Brinkhurst and Kathman, 1983), *V. vestibulatus* (Cui and Wang, 2009), *V. psammophilus*, *V. angustipenis*, and our *V. sp.*, in having bifid chaetae only. It should be noted that *V. psammophilus* and *V. angustipenis* were also originally described as *Limnodrilus*, largely based on penis sheaths, and these species, plus *V. harmani*, were transferred to *Varichaetadrilus* using similar morphological arguments (Brinkhurst, 1989: Timm, 2006). We thus conclude that “*L. rubripenis*” should be reclassified as a member of *Varichaetadrilus*. Nevertheless, this is not entirely trivial as the genetic data suggest that our three *rubripenis* specimens represent two different species, with a maximum COI p-distance of about 15%, and a ITS p-distance of about 1.4%. This complicates the taxonomy of this taxon, in particular with regard to the identity of *Loden’s* (1977) original material, but its final revision and nomenclature are beyond the scope of this paper.

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**Fig. 2. Varichaetadrilus cf. angustipenis, anterior ventral chaetae of segments II–V (specimen ID: CE3600).**
It can be noted here that, as suggested by the 16S-based analysis by Achurra et al. (2011), we consider Varicaetadrilus bizkaiensis Rodriguez and Giani, 1984, as more closely related to the genus Potamothrix than to Varicaetadrilus.

The morphospecies L. hoffmeisteri has long been known as one of the most widely distributed tubificine taxa in the world (Brinkhurst and Jamieson, 1971), and its taxonomy has been a matter of scientific debate for about a century. Now there is both molecular and morphological evidence for the nominal “L. hoffmeisteri” being a species complex rather than a single species. Intra- and inter-specific variation within this complex was investigated by Liu et al. (2017) largely on the basis of two genetic markers (mitochondrial COI and nuclear ITS), with support also from 16S data. The topologies of that study are generally consistent with the concatenated and coalescence-based seven-locus trees obtained in the present study, and now we can make conclusions also about the greater picture of the phylogenetic relationships within Limnodrilus and it position within Tubificinae as a whole. The take-home messages are summarized here:

(1) Limnodrilus is a well-demarcated, monophyletic genus of Tubificinae, and although there still may be some nominal species that are erroneously classified within it, new genetic information in the future will likely aid in the identification of these errors.

(2) Limnodrilus appears to be sister to a group of genera, including at least Baitridrilus, Lophochaeta and Varicaetadrilus. Some species within this group, now classified within Varicaetadrilus, resemble Limnodrilus in terms of chaetae and penial sheaths. Otherwise, this group contains great variation in, e.g., morphology and arrangements of chaetae (including species with hair and pectinate chaetae), and length and shape of the penis sheaths.

(3) One monophyletic group of Limnodrilus (group A), which may be the sister group of the rest of the genus, is characterized by penis sheaths of rather short or moderate length (150–360 μm); at least L. udekemianus, L. silvani and L. grandisetosus belong to this group.

(4) A second group (B), so far with L. profundicola and L. sulphurensis as its only (established) members, has tubular penis sheaths of short or, more generally, moderate length (150–410 μm). This group may be the sister to group C.

(5) A third group (C) contains the many species with long (>400 μm) or very long penis sheaths, i.e., the vast radiation of species within the L. hoffmeisteri complex. This complex is not even monophyletic using the established specific criteria, as some species with apomorphic elaboration of, in particular, the length and shape of the penis sheaths (at least L. claparedianus, L. maumeensis and “L. claparedianus-cervix”) are nested among the hoffmeisteri morphotypes.

(6) Limnodrilus is a group of Tubificinae prone to cryptic speciation, not only in the obvious case of L. hoffmeisteri, but also as suggested by great genetic variation in our two L. grandisetosus samples. Such large variation suggests that cryptic species also exist in the morphospecies L. grandisetosus.

(7) Our present study neither corroborates nor refutes the hypothesis, proposed by Timm (2012), that the genus Limnodrilus originated in North America. It may be possible to test...
this with a more exhaustive taxonomic sampling, e.g., including several endemic species of parts of North America and Asia, but it may still be difficult considering the great evidence of global dispersal of many species (see Liu et al., 2017).

(8) As for phylogenetic relationships of other genera and subfamilies of Naididae sampled as outgroups in the present study, it would be premature to draw any far-reaching conclusions, as we only included a small fraction of the many naidid genera known. Besides, our trees were deliberately not rooted with outgroups outside the family, as it would cause alignment problems, in particular, with ITS. Nevertheless, it can be noted that both Tubificinae (with or without Branchiura; see above) and Limnordriloidinae came out as monophyletic and as sister groups in our BI, ML and ‘BEAST’ trees based on all genes together (Fig. 1), as also suggested by previous studies (Erséus, 1987; Erséus et al., 2002, 2000; Ferraguti and Erséus, 1999; Marotta et al., 2008; Sjölin et al., 2005).

Many taxa of *Limnodrilus* have still not been sufficiently sampled for a fully resolved reconstruction of the evolutionary history of this common and widely distributed genus. Continued systematic research, including the further use of coalescent-based Bayesian analyses to model gene tree discordance within a species tree framework, will be necessary to further elucidate the relationships among these poorly resolved lineages. Adding more taxa and genetic information (such as broadening the selection of loci), and exploring more of the possibilities of multi-locus coalescent methods, will contribute to a comprehensive and robust phylogenetic reconstruction of *Limnodrilus*.

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Loden, M.S., 1977. Two new species of
Liu, Y., Fend, S.V., Martinsson, S., Erséus, C., 2017. Extensive cryptic diversity in the
Fisher-Reid, M.C., Wiens, J.J., 2011. What are the consequences of combining nuclear
and mitochondrial data for phylogenetic analysis? Lessons from
Erséus, C., Wetzel, M.J., Gustavsson, L., 2008. ICZN rules - a farewell to Tubificidae
Katoh, K., Standley, D.M., 2013. MAFFT Multiple Sequence Alignment Software
Jamieson, B.G., Tillier, S., Tillier, A., Justine, J.-L., Ling, E., James, S., McDonald, K.,
Hrabeš, S., 1973. A contribution to the knowledge of marine Oligochaeta, mainly
Grube, A.E., 1861. Ein Ausflug nach Trieste und dem Quarnero. Beiträge zur
Hrabeš, S., 1972. A contribution to the knowledge of marine Oligochaeta, mainly
Zhang, L., Wu, W., Yan, H.-F., Ge, X.-J., 2015. Phylotranscriptomic analysis based on coalescence was less influenced by the evolving rates and the number of genes: a case study in Ericales. Evol. Bioinform. 11, 81–91 (Online).