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. silvani* and *L. grandisetosus*). 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.

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 chaetae 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 newaensis* Michaelsen, 1903 was moved to *Tubifex* by Brinkhurst (1962), *Limnodrilus pseudogaster* Dahl, 1960 was moved to *Tubificoides* by Brinkhurst and Baker (1979), and *Limnodrilus angustipenis* Brinkhurst and Cook, 1966 to *Varichaetadrilus* 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, Achurra 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 neotypification) *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 be 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 Envall 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 H3aF/H3aR (Colgan et al., 1998), and the primer combination for 28S rDNA was 28SC1/28SC2 (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 contigs 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.

Species	Specimen ID	12S	16S	18S	28S	COI	ITS	H3	Voucher ID	Location and habitat	Latitude	Longitude	Collection date	Collector
Limnodrilus hoffmeisteri I	CE1137	**KY636720**	KY369312	**KY636872**	**KY636805**	KY369467	KY369392	**KY636955**	SMNH 158958	U.S.A., Alaska, Juneaux, Glacier Hwy, Peterson Creek above Veteran's Memorial Highway Bridge, creek sediment	58.483 N	134.782 W	23-Aug-2005	Leyla Arsan & Stephen Atkinson
Limnodrilus hoffmeisteri I	CE1171	**KY636721**	KY369314	**KY636873**	**KY636806**	KY369472	KY369394	**KY636956**	SMNH 158960	U.S.A., Alaska, Juneaux, Glacier Hwy, Peterson Creek, Amalga Lake, lake sediment	58.483 N	134.784 W	23-Aug-2005	Leyla Arsan & Stephen Atkinson
Limnodrilus hoffmeisteri I	CE3238	**KY636722**	KY369316	**KY636874**	**KY636807**	KY369529	KY369396	**KY636957**	SMNH 158963	Denmark, Sjaelland, Mölleaa River at Raadvaad, mud	55.806 N	12.561 E	30-Oct-2005	Maria Lindström & Sebastian Kvist
Limnodrilus hoffmeisteri II	CE22814	**KY636723**	**KY636780**	**KY636875**	**KY636808**	KY369489	**KY652931**	**KY636958**	SMNH 158977	Switzerland, Chêne-Bougeries, Chemin de la Montagne 22C, Seymaz River, organic (mostly leaf) matter (10–25 cm)	46.199 N	6.194 E	24-Aug-2014	Yingkui Liu
Limnodrilus hoffmeisteri II	CE22881	-	**KY636781**	**KY636876**	**KY636809**	KY369634	**KY652932**	**KY636959**	SMNH 158980	Switzerland, Chêne-Bourg, Route de Malagnou 251, Seymaz River, fine sand (5–10 cm) with black organic matter	46.191 N	6.188 E	24-Aug-2014	Yingkui Liu
Limnodrilus hoffmeisteri II	CE22884	**KY636724**	KY369320	**KY636877**	**KY636810**	KY369636	KY369400	**KY636960**	SMNH 158981	Switzerland, Chêne-Bourg, Route de Malagnou 251, Seymaz River, fine sand (5–10 cm) with black organic matter	46.191 N	6.188 E	24-Aug-2014	Yingkui Liu
Limnodrilus hoffmeisteri III	CE5201	**KY636725**	KY369323	**KY636878**	**KY636811**	KY369552	KY369403	**KY636961**	SMNH 158997	Belgium, Mechelen, Zenne River, polluted freshwater part of Schelde estuary, intertidal freshwater	51.052 N	4.434 E	28-Aug-2008	Jan Soors
Limnodrilus hoffmeisteri III	CE3779	**KY636726**	KY369322	**KY636879**	**KY636812**	KY369540	KY369402	-	SMNH 158996	USA, Illinois, Piatt County, Robert Allerton Park, spring near Reflecting Pond, spring outflow	40.004 N	88.647 W	20-Mar-2008	Christer Erséus & Mark Wetzel
Limnodrilus hoffmeisteri IV	CE3744	**KY636727**	KY369325	**KY636880**	**KY636813**	KY369537	KY369405	**KY636962**	SMNH 159002	USA, Illinois, Piatt County, Robert Allerton Park, Reflecting Pond, mud	40.004 N	88.647 W	20-Mar-2008	Christer Erséus & Mark Wetzel
Limnodrilus hoffmeisteri V	CE10834	**KY636728**	KY369339	**KY636881**	**KY636814**	KY369604	KY369419	-	SMNH 159004	USA, Louisiana, Tangipahoa Parish, Tangipahoa River at bridge on Rd 10 near Arcola, sandy river bank	30.777 N	90.498 W	16-Jan-2011	Christer Erséus
Limnodrilus hoffmeisteri VI	CNWQ12	**KY636729**	KY369342	**KY636882**	**KY636815**	KY369726	KY369422	**KY636963**	SMNH 159034	China, Shaanxi Province, Xi'an, Wenguobao village, Yuhe river	34.143 N	108.9 E	12-Jun-2013	Yingkui Liu
Limnodrilus hoffmeisteri VI	CNYP25	**KY636730**	KY369344	**KY636883**	**KY636816**	KY369754	KY369424	**KY636964**	SMNH 159054	China, Yunnan Province, Kunming, Panlong River	25.076 N	102.723 E	8-Aug-2013	Yingkui Liu
Limnodrilus hoffmeisteri VII	CE1157	**KY636731**	KY369346	**KY636884**	**KY636817**	KY369469	KY369426	**KY636965**	SMNH 159064	U.S.A., Alaska, Anchorage, Ship Creek 100 m downstream from lowest Dam Near Loop Rd/'A' St. Bridge, creek sediment	61.236 N	149.612 W	31-Aug-2005	Leyla Arsan & Stephen Atkinson
Limnodrilus hoffmeisteri VII	CE604	**KY636732**	KY369345	**KY636885**	**KY636818**	KY369463	KY369425	-	no voucher	Sweden, Västergötland, Lidköping, Lake Vänern, at marina near Northern Harbour, littoral sand	58.514 N	13.153 E	6-Aug-2003	Christer Erséus
Limnodrilus hoffmeisteri VIII	CE2710	**KY636733**	KY369359	**KY636886**	**KY636819**	KY369493	KY369439	**KY636966**	SMNH 159123	Sweden, Alingsås, Lake Anten near Ålanda, littoral zone, gravel and decomposing leaves between boulders	57.991 N	12.467 E	4-Aug-2007	Christer Erséus
Limnodrilus hoffmeisteri VIII	CE3139	**KY636734**	KY369361	**KY636887**	**KY636820**	KY369528	KY369441	**KY636967**	SMNH 159130	Sweden, Västergötland, Göteborg, Vitsippsdalen Stream, sand and gravel	57.682 N	11.955 E	10-Oct-2007	Maria Lindström & Sebastian Kvist

Table 1 (*continued*)

Species	Specimen ID	12S	16S	18S	28S	COI	ITS	H3	Voucher ID	Location and habitat	Latitude	Longitude	Collection date	Collector
Limnodrilus hoffmeisteri VIII	CE608	**KY636735**	KY369358	**KY636888**	**KY636821**	KY369464	KY369438	**KY636968**	no voucher	Sweden, Västergötland, Vårgårda, Lake Lången, littoral sand	57.997 N	12.587 E	26-Jun-2000	Christer Erséus
Limnodrilus hoffmeisteri IX	CE1784	**KY636736**	KY369326	**KY636889**	**KY636822**	KY369477	KY369406	**KY636969**	SMNH 159141	Japan, Akita-ken, Minamiakita-gun, Gojōme-machi, Akita Prefecture, Lake Hachiro-gata	39.933 N	140.082 E	9-Jul-2005	Akifumi Ohtaka
Limnodrilus hoffmeisteri IX	CE3087	**KY636737**	KY369331	**KY636890**	**KY636823**	KY369514	KY369411	**KY636970**	SMNH 159153	Sweden, Västergötland, Göteborg, Mölndalsån River at Liseberg-Ullevi, mud	57.697 N	11.993 E	5-Sep-2007	Maria Lindström & Sebastian Kvist
Limnodrilus hoffmeisteri IX	CE3094	**KY636738**	KY369332	**KY636891**	**KY636824**	KY369521	KY369412	**KY636971**	SMNH 159159	Sweden, Västergötland, Göteborg, Mölndalsån River at Liseberg-Ullevi, mud	57.697 N	11.993 E	5-Sep-2007	Maria Lindström & Sebastian Kvist
Limnodrilus hoffmeisteri IX	CE22811	**KY636739**	KY369336	**KY636892**	**KY636825**	KY369627	KY369416	**KY636972**	SMNH 159152	Switzerland, Chêne-Bougeries, Chemin de la Montagne 22C, Seymaz River, organic (mostly leaf) matter (10–25 cm)	46.199 N	6.194 E	24-Aug-2014	Yingkui Liu
Limnodrilus hoffmeisteri IX	CE22809	**KY636741**	KY369335	**KY636893**	**KY636827**	KY369626	KY369415	**KY636973**	SMNH 159151	Switzerland, Chêne-Bougeries, Chemin de la Montagne 22C, Seymaz River, organic (mostly leaf) matter (10–25 cm)	46.199 N	6.194 E	24-Aug-2014	Yingkui Liu
Limnodrilus hoffmeisteri IX	CE22808	**KY636740**	KY369334	-	**KY636826**	KY369625	KY369414	-	SMNH 159150	Switzerland, Chêne-Bougeries, Chemin de la Montagne 22C, Seymaz River, organic (mostly leaf) matter (10–25 cm)	46.199 N	6.194 E	24-Aug-2014	Yingkui Liu
Limnodrilus hoffmeisteri IX	CNJ8	**KY636742**	KY369338	-	**KY636828**	KY369703	KY369418	**KY636974**	SMNH 159170	China, Shaanxi Province, Xi'an, Jingjia village, Chanhe river	34.019 N	108.765 E	12-Jun-2013	Yingkui Liu
Limnodrilus hoffmeisteri X	CE1990	**KY636743**	KY369364	**KY636894**	**KY636829**	KY369484	KY369445	**KY636975**	SMNH 159180	Sweden, Västergötland, Vårgårda, Lake Lången, 0.3 m, gravel and sand	58.011 N	12.582 E	6-Aug-2006	Christer Erséus
Limnodrilus hoffmeisteri X	CE290	DQ459923	AY885613	AF469007	HM460076	HM460271	KY369444	**KY636976**	no voucher	Estonia, Tartu, Emajögi River; kept in Timm's lab culture	58.4 N	26.728 E	1-Dec-2000	Tarmo Timm
Limnodrilus hoffmeisteri X	CNAS4	**KY636744**	KY369374	-	**KY636830**	KY369664	KY369455	**KY636977**	SMNH 159201	China, Anhui Province, Huaining County, Shipai Town, paddy fields	30.41 N	116.64 E	1-Jul-2013	Yingkui Liu
Limnodrilus hoffmeisteri X	CNAS12	**KY636745**	KY369376	-	**KY636831**	KY369668	KY369457	**KY636978**	SMNH 159198	China, Anhui Province, Huaining County, Shipai Town, paddy fields	30.41 N	116.64 E	1-Jul-2013	Yingkui Liu
Limnodrilus maumeensis Brinkhurst and Cook, 1966	CE1177	**KY636746**	KY369378	**KY636895**	**KY636832**	KY369474	KY369459	**KY636979**	SMNH 159221	USA, Louisiana, Turtle-rearing ponds	31 N	93W	8-Oct-2005	Stephen Atkinson
Limnodrilus claparedianus/ cervix	CE2128	**KY636747**	KY369307	**KY636896**	**KY636833**	KY369488	KY369387	-	SMNH 159226	Germany, Osnabrück, lab culture at Zool Dep, Univ Osnabrück	52.283 N	8.033 E	16-Nov-2006	Annette Bergter
Limnodrilus claparedianus/ cervix	CNH8	**KY636748**	KY369308	**KY636897**	**KY636834**	KY369682	KY369388	**KY636980**	SMNH 159235	China, Shaanxi Province, Xi'an, Huxian county, a small river	34.106 N	108.646 E	12-Jun-2013	Yingkui Liu
Limnodrilus claparedianus/ cervix	CNK47	**KY636749**	KY369310	**KY636898**	**KY636835**	KY369709	KY369390	**KY636981**	SMNH 159239	China, Shaanxi Province, Xi'an, Ku Yu valley (1000–1998 m)	34.011 N	109.172 E	12-Jun-2013	Yingkui Liu
Limnodrilus claparedianus Ratzel, 1868	CE1112	**KY636750**	KY369302	**KY636899**	**KY636836**	KY369466	KY369382	**KY636982**	no voucher	Sweden, Småland, Jönköping, Lillån, Kättilstorp, small river	57.751 N	14.149 E	18-Sep-2005	Daniel Gustafsson
Limnodrilus claparedianus Ratzel, 1868	CNJ21	**KY636751**	KY369306	-	**KY636837**	KY369706	KY369386	**KY636983**	SMNH 159220	China, Shaanxi Province, Xi'an, Jingjia village, Chanhe river	34.019 N	108.765 E	12-Jun-2013	Yingkui Liu
Limnodrilus grandisetosus Nomura, 1932	CE1785	**KY636752**	**KY636782**	**KY636900**	**KY636838**	**KY636923**	**KY637016**	**KY636984**	SMNH 160311	Indonesia, Central Kalimantan, Lake Tehang	2.029 S	113.934 E	21-Mar-2005	Akifumi Ohtaka
Limnodrilus grandisetosus Nomura, 1932	CE1786	**KY636753**	**KY636783**	**KY636901**	**KY636839**	**KY636924**	**KY637017**	**KY636985**	SMNH 160312	Japan, Shimosakamoto, south basin of Lake Biwa	35.053 N	135.891 E	13-Feb-2003	Akifumi Ohtaka

Table 1 (continued)

Species	Specimen ID	12S	16S	18S	28S	COI	ITS	H3	Voucher ID	Location and habitat	Latitude	Longitude	Collection date	Collector
Limnodrilus profundicola (Verrill, 1871)	XZ20150606a	KY636754	–	–	KY636840	KY636925	–	–	IHB XZ20150606a Hydrobiology, Chinese Academy of Sciences, Wuhan, China.	China, Tibet, Shannan, Comai, a small pond between Ge Co and Zhegu Co Co Lake (4,622 m elevation)	28.74 N	91.67 E	6-Jun-2015	Yu Peng
Limnodrilus rubripenis Loden, 1977	CE10781	KY636755	KY636784	KY636902	KY636841	KY636926	KY637018	–	SMNH 160313	USA, Louisiana, Tangipahoa Parish, Tangipahoa River at bridge on Road 10, near Arcola, sandy river bank	30.777 N	90.498 W	16-Jan-2011	Christer Erséus
Limnodrilus rubripenis Loden, 1977	CE10782	KY636756	KY636785	KY636903	KY636842	KY636927	KY637019	–	SMNH 160314	USA, Louisiana, Tangipahoa Parish, Tangipahoa River at bridge on Road 10, near Arcola, sandy river bank	30.777 N	90.498 W	16-Jan-2011	Christer Erséus
Limnodrilus rubripenis Loden, 1977	CE10853	KY636757	–	KY636904	KY636843	KY636928	KY637020	–	SMNH 160315	USA, Louisiana, Washington Parish, Silver Creek, at bridge near Mount Hermon, muddy sand on banks and in water	30.971 N	90.289 W	17-Jan-2011	Christer Erséus
Limnodrilus silvani Eisen, 1879	CE20911	KY636758	KY636786	KY636905	KY636844	KY636929	–	–	SMNH 160316	USA, Oregon, Klamath County, Upper Klamath Lake	42.396 N	121.872 W	1-May-2013	Steven V Fend
Limnodrilus sulphurensis Fend et al., 2016	CE7489	KY636759	KY636787	KY636906	KY636845	KY636930	KY637021	KY636986	USNM 1283518	USA, Colorado, Routt County, City of Steamboat Springs, Sulphur Cave, high H2S stream in dark zone	40.48 N	106.75 W	20-Sep-2008	David Steinmann & Fred Luiszer
Limnodrilus sulphurensis Fend et al., 2016	CE10482	KY636760	KY636788	KY636907	KY636846	KT692957	KY637022	KY636987	DMNS ZE.46275	USA, Colorado, Routt County, City of Steamboat Springs, Sulphur Cave, high H2S stream in dark zone	40.48 N	106.75 W	11-Apr-2010	David Steinmann & Fred Luiszer
Limnodrilus udekemianus Claparède, 1862	CE2127	KY636761	KY636789	KY636908	KY636847	KY636931	KY637023	KY636988	SMNH 160317	Germany, Osnabrück, lab culture at Zool Dep, Univ Osnabrück	52.283 N	8.033 E	16-Nov-2006	Annette Bergter
Limnodrilus udekemianus Claparède, 1862	CE273	KY636762	AY885612	AF469006	KY636848	KY636932	KY637024	KY636989	no voucher	Originally from Kyrgyzstan Republic (Central Asia), Frunze (Bisjkek); kept in Timm's lab culture	42.85 N	74.37 E	1-Dec-2000	Tarmo Timm
Aktedrilus arcticus (Erséus, 1978)	CE37	KP943902	AY885591	AF209451	KP943838	AF064042	KY637025	KY636990	no voucher	Sweden, Bohuslän, Strömstad, Tjärnö, beach in front of Research Station, intertidal sand	58.8755 N	11.1458 E	1-Aug-1997	Christer Erséus
Alexandrovia onegensis Hrabě, 1962	CE4913	KY636763	KY636790	KY636909	KY636849	KY636933	KY637026	–	SMNH 160318	Canada, Ontario, from ship hull fouling in Great Lakes	43.20 N	79.20 W	1-Sep-2008	Fransisco Sylvester
Aulodrilus acutus Ohtaka and Usman, 1997	CE1790	KY636764	KY636791	KY636910	KY636850	KY636934	KY637027	–	SMNH 160319	Cambodia, Kampong Chnang, Lake Tonle Sap	12.261 N	104.681 E	21-May-2005	Akifumi Ohtaka
Aulodrilus pluriseta Piguet, 1906	CE281	HM459926	HM459991	HM460031	HM460073	HM460268	KY637028	KY636991	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Baltidrilus costatus (Claparède, 1863)	CE196	HM459925	AY340460	AY340432	AY340397	HM460266	KY637029	–	no voucher	Sweden, Bohuslän, Strömstad, Koster archipelago, subtidal sand,	58.875 N	11.080 E	1-Sep-2000	Christer Erséus
Bothrioneurum vejdovskyanum Štolc, 1886	CE286	DQ459879	AY885635	AF411908	KY636851	KY636935	KY637030	KY636992	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Branchiura sowerbyi Beddard, 1892	CE713	KY636765	KY636792	KY636911	KY636852	KY636936	–	KY636993	SMNH 160320	Sweden, Lake Mälaren, Västeråsfjärden, Västerås, Djuphamnen	59.589 N	16.527 E	17-Sep-2003	Tommy Odelström
Clitellio arenarius (Müller, 1776)	CE112	HM459924	AY885615	AF411863	HM460071	HM460265	KY637031	KY636994	no voucher	Sweden, Bohuslän, Strömstad, Tjärnö, Tjärnöviken, subtidal sand	58.876 N	11.145 E	1-Nov-1998	Christer Erséus
Doliodrilus tener Erséus, 1984	CE138	KY636766	AY885626	AF411868	KY636853	KY636937	KY637032	–	no voucher	China, Hainan, E of Sanya City, fish pond at road to Teng Hai, brackish water, coarse sand with black mud	18.28 N	109.73 E	16-Mar-2000	Christer Erséus
Epirodrilus pygmaeus (Hrabě, 1935)	CE754	DQ459890	DQ459936	DQ459963	KY636854	KY636938	–	KY636995	SMNH 82594	Czech Republic, about 60 km W of Brno, Rokytnà village, Rokytnà River (Thay River basin)	49.17 N	15.79 E	1-May-2004	Jana Schenkova

Table 1 (*continued*)

Species	Specimen ID	12S	16S	18S	28S	COI	ITS	H3	Voucher ID	Location and habitat	Latitude	Longitude	Collection date	Collector
Heronidrilus fastigatus Erséus and Jamieson, 1981	CE236	**KY636767**	**KY636793**	**KY636912**	**KY636855**	**KY636939**	**KY637033**	**KY636996**	SMNH 160321	New Caledonia, Loyalty Islands, Lifou, Baie de Chataeubriand, Wé 0.5 m, marine, medium sand;	20.55 S	167.17 E	21-Nov-2000	Christer Erséus
Ilyodrilus templetoni (Southern, 1909)	CE282	HM459927	HM459992	HM460032	HM460074	HM460269	KF366654	–	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Limnodriloides anxius Erséus, 1990	CE131	DQ459919	AY885621	AF411866	HM460077	HM460272	**KY637034**	**KY636997**	no voucher	Bahamas, Exuma, Lee Stocking Island, subtidal sand	23.77 N	76.10 W	20-Apr-1999	Christer Erséus
Limnodriloides appendiculatus Pierantoni, 1904	CE149	DQ459918	AY885629	AF411869	**KY636856**	**KY636940**	**KY637035**	**KY636998**	no voucher	Elba Italy, Toscana, Elba, off San Andrea, 13 m, sand	42.81 N	10.14 E	3-May-2000	Christer Erséus
Lophochaeta ignota Štolc, 1886	CE211	DQ459921	AY885610	AF411879	GU902029 /HM460072	GU902114 / HM460267	**KY637036**	**KY636999**	no voucher	Sweden, Västergötland, Vårgårda, Lången Lake	57.997 N	12.887 E	1-Oct-2000	Christer Erséus
Monopylephorus rubroniveus Levinsen, 1884	CE50	DQ459891	AY885637	AF209459	**KY636857**	**KY636941**	**KY637037**	**KY637000**	no voucher	Sweden, Södermanland, Nynäshamn, Torö, seashore	58.84 N	17.87 E	1-Sep-1998	Michael Norén
Nais alpina Sperber, 1948	CE529	DQ459906	DQ459943	DQ459975	GU902020	GU902104	**KY637038**	**KY637001**	no voucher	Sweden, Uppland, Solna, Igelbäcken Stream	59.389 N	18.006 E	10-Oct-2002	Christer Erséus
Pirodrilus minutus (Hrabě, 1973)	CE36	DQ459880	DQ459958 AY885590	DQ459986 AF209463	GU902023	AF064043	**KY637039**	–	no voucher	Sweden, Bohuslän, Strömstad, Tjärnö, beach in front of Research Station, intertidal sand	58.8755 N	11.1458 E	1-Aug-1997	Christer Erséus
Potamothrix bavaricus (Oschmann, 1913)	CE570	**KY636768**	**KY636794**	**KY636913**	**KY636858**	**KY636942**	**KY637040**	**KY637002**	SMNH 160322	Australia, Western Australia, S of Esperance Municipal Museum, man-made freshwater pond	33.8603 S	121.8930 E	9-Feb-2003	Hongzhu Wang
Potamothrix bedoti (Piguet, 1913)	CE275	**KY636769**	**KY636795**	**KY636914**	**KY636859**	KF366636	KF366653	**KY637003**	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by T. Timm, orginally from Lake Võrtsjärv	58.2 N	26.1 E	1-Dec-2000	Tarmo Timm
Potamothrix hammoniensis (Michaelsen, 1901)	CE278	**KY636770**	**KY636796**	**KY636915**	**KY636860**	**KY636943**	**KY637041**	**KY637004**	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Potamothrix moldaviensis Vejdovský and Mrázek, 1903	CE283	**KY636771**	**KY636797**	**KY636916**	**KY636861**	**KY636944**	**KY637042**	–	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Psammoryctides albicola (Michaelsen, 1901)	CE2883	**KY636772**	**KY636798**	**KY636917**	**KY636862**	**KY636945**	**KY637043**	–	SMNH 160323	Sweden, Österåker, Vingåker, Lake Låttern at Valltrand, sand near shore	59.0854 N	16.0426 E	30-Jul-2007	Christer Erséus
Psammoryctides barbatus (Grube, 1861)	CE289	HM459928	HM459993	HM460033	HM460075	HM460270	**KY637044**	**KY637005**	no voucher	Estonia, Rannu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Rhyacodrilus coccineus (Vejdovský, 1875)	CE623	DQ459888	DQ459931	DQ459969	GU902025	GU902110	KF267996	KF267971	no voucher	Sweden, Västergötland, Vårgårda, stream between Iglasjön and Lången Lakes	58.0103 N	012.5836 E	6-Jul-2003	Christer Erséus
Smithsonidrilus hummelincki (Righi and Kanner, 1979)	CE21	DQ459917	AY885628	AF209465	**KY636863**	**KY636946**	–	**KY637006**	no voucher	Belize, Off Dangriga, Carrie Bow Cay	16.803 N	88.082 W	20-Mar-1993	Christer Erséus
Spirosperma ferox Eisen, 1879	CE487	**KY636773**	**KY636799**	**KY636918**	**KY636864**	**KY636947**	**KY637045**	**KY637007**	no voucher	Sweden, Västergötland, Vårgårda, Lången Lake	57.997 N	12.887 E	13-Jul-2002	Christer Erséus
Tubifex blanchardi Vejdovský, 1891	CE2044	**KY636774**	**KY636800**	**KY636919**	**KY636865**	**KY636948**	**KY637046**	**KY637008**	SMNH 160324	Belgium, Oost-Vlaanderen, near Schoonaarde, Paddebeek River	51.02 N	4.05 E	7-Sep-2006	Jan Soors

Table 1 (continued)

Species	Specimen ID	12S	16S	18S	28S	COI	ITS	H3	Voucher ID	Location and habitat	Latitude	Longitude	Collection date	Collector
Tubifex newaensis (Michaelsen, 1903)	CE272	KY636775	KY636801	KY636920	KY636866	KY636949	KY637047	KY637009	no voucher	Estonia, Rannu, Vörtsjärv Limnological Station, lab culture kept by Tarmo Timm	58.212 N	26.110 E	1-Dec-2000	Tarmo Timm
Tubifex smirnowi Lastockin, 1927	CE212	KY636776	AY885620	AF411880	KY636867	KY636950	KY637048	KY637010	no voucher	Sweden, Västergötland, Vårgårda, Längen Lake	57.997 N	12.887 E	13-Jul-2002	Christer Erséus
Tubifex tubifex (Müller, 1774)	CE276	KY636777	KY636802	EU126846	KY636868	KY636951	KY637049	KY637011	no voucher	Originally from Kyrgyzstan Republic (Central Asia), Frunze (Bisjkek); kept in Timm's lab culture	42.85 N	74.37 E	1-Dec-2000	Tarmo Timm
Tubificoides benedii (Udekem, 1855)	CE186	DQ459925	AY885611	AF411872	KY636869	KY636952	KY637050	KY637012	no voucher	Sweden, Bohuslän, Strömstad, Tjärnö, at Research Station, intertidal sand	58.876 N	11.146 E	1-Sep-2000	Christer Erséus
Tubificoides brownae Brinkhurst and Baker, 1979	CE2430	HM459916	HM459977	HM460021	HM460061	HM460230	HM460321	-	SMNH 109013	USA, Virginia, Gloucester County, York River, Clay Bank, 8 m, brackish water	37.35 N	76.61 W	13-May-2007	Sebastian Kvist
Varichaetadrilus cf. angustipenis (Brinkhurst and Cook, 1966)	CE3600	KY636778	KY636803	KY636921	KY636870	KY636953	-	KY637013	SMNH 160325	USA, Alabama, Madison County, Huntsville, WEUP Radio Station Pond	34.7603 N	86.6431 W	17-Mar-2008	Christer Erséus & Mark Wetzel
Varichaetadrilus sp	CE3621	KY636779	KY636804	KY636922	KY636871	KY636954	KY637051	-	SMNH 160236	USA, Alabama, Madison County, Huntsville, WEUP Radio Station Pond	34.7603 N	86.6431 W	17-Mar-2008	Christer Erséus & Mark Wetzel

protein-coding COI and H3, as well as the four (12S, 16S, 18S and 28S) rDNAs, were performed visually using the MAFFT plugin (version 7.017; (Katoh and Standley, 2013) in Geneious. The two ITS spacers were annotated using ITSx (version 1.0.11; Bengtsson-Palme et al., 2013), which facilitated the alignment's success in MAFFT alignment, using the L-INS-i algorithm with 1 PAM/k = 2. The uncorrected p distance was calculated using MEGA 6 (Tamura et al., 2013).

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 analysis 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

(Rambaut et al., 2014). Resulting trees were combined in LogCombiner V1.8.3, and 2000 trees were discarded as burn-in in TreeAnnotator to generate a maximum clade credibility tree. Bayesian Posterior Probabilities (BP) were plotted on this tree using FigTree 1.4.2 (Rambaut, 2014).

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).

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. grandisetosus* 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 for CE1786 was unsuccessfully amplified and could not be compared to 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.

3.2. 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 sowerbyi* Beddard, 1892 and the four species of Limnodriloidinae, 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, Limnodriloidinae 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 *Varichaetadrilus* 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. silvani* Eisen, 1879 and *L. grandisetosus*. 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 I-IV and *L. claparedianus*, the other containing *L. hoffmeisteri* species V-X (including IX = *L. hoffmeisteri sensu stricto*), *L. maumeensis*, and "*L. claparedianus-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., *Potamothrix* Vejdovský & Mrázek, 1903 (4 species), *Tubificoides* Lastočkin, 1937 (2 species), *Psammoryctides* Hrabě, 1964 (2 species), and *Aulodrilus* Bretscher, 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 Rhyacodrilinae 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 overconfident 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.
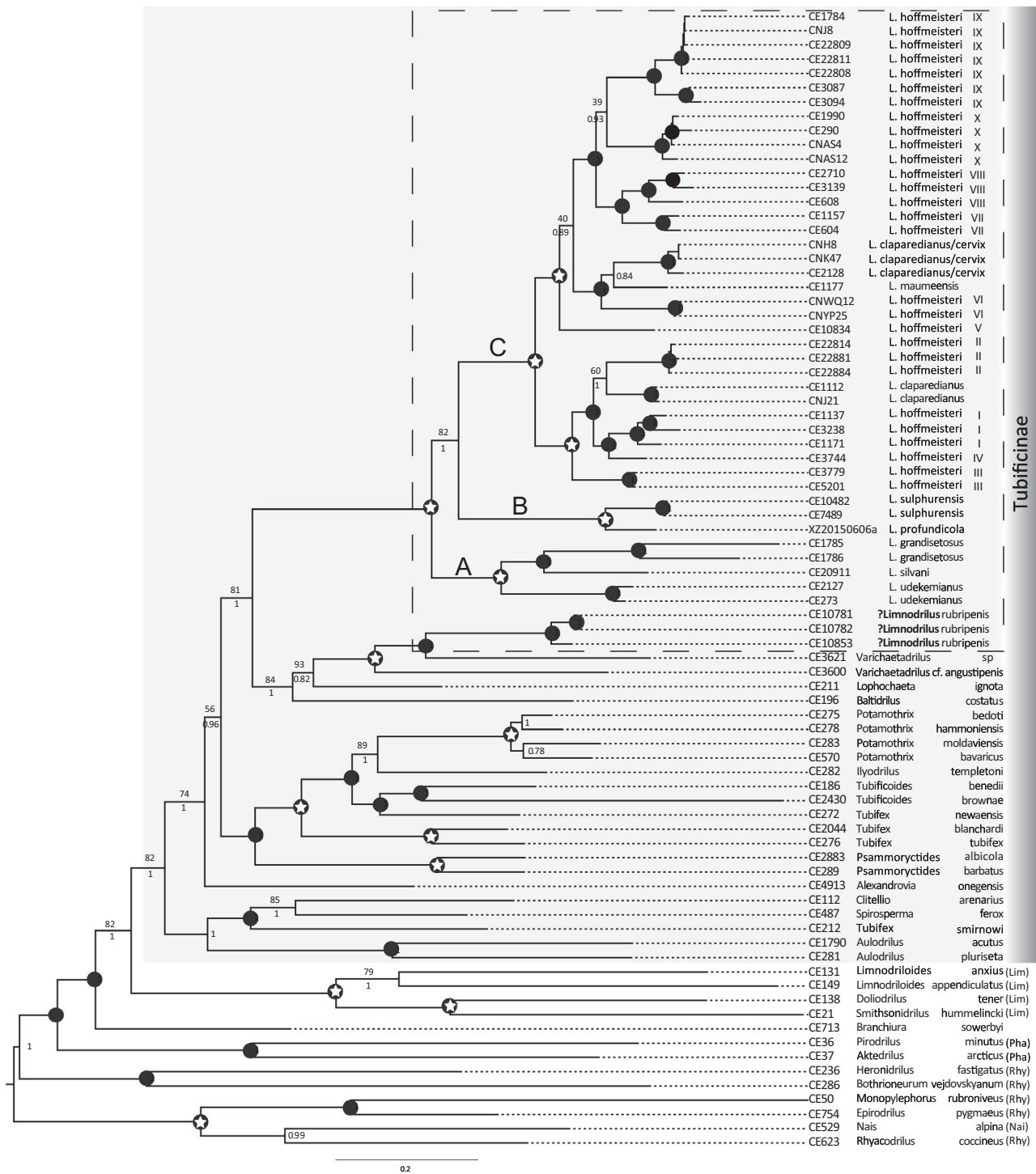
Fig. 1. Phylogeny of *Limnodrilus* was estimated using MrBayes based on data of seven markers (COI, 12S, 16S, 18S, 28S, ITS and H3). Statistical values above the clades indicate bootstrap support (BS, calculated by RAxML) while values below the clades are Bayesian posterior probabilities (BP, calculated by MrBayes). The well-supported nodes (BS > 90 and BP > 0.95) are indicated with black dots. In addition, the nodes lacking BS value (only BP value shown near nodes) indicate the discrepancies between Bayesian and ML analysis. White asterisks on black dots stand for good support (BP > 0.95) estimated in the *BEAST analysis. Clades belonging to the subfamily Tubificinae are shown with the background in gray. Three letter acronyms represent subfamily names: Lim, Limnodriloidinae; Nai, Naidinae; Pha, Phallodrilinae; Phy, Rhyacodrilinae; Tub, Tubificinae. The scale bar indicates the number of substitutions per site.

As discussed further below, the placement of "*L. rubripenis*" 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 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; Erséus et al., 2000; Siddall et al., 2001; Sjölin et al., 2005; Achurra 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 neotypified 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. udekemianus*, *L. silvani* and *L. grandisetosus*, 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; Piguet, 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; Černosvitov, 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 CE3600 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. grandisetosus*) – 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 *Varichaeta*) was originally defined as having hair 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.
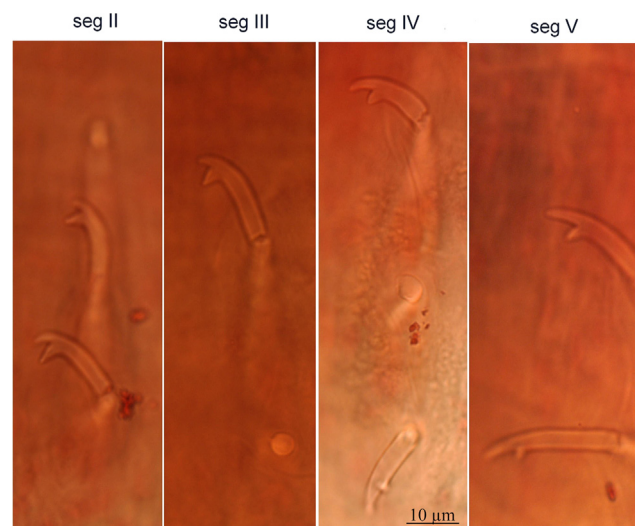

Fig. 2. *Varichaetadrilus* cf. *angustipenis*, anterior ventral chaetae of segments II–V (specimen ID: CE3600).

Fig. 3. Penis sheath of *Varichaetadrilus* cf. *angustipenis* (specimen ID: CE3600).
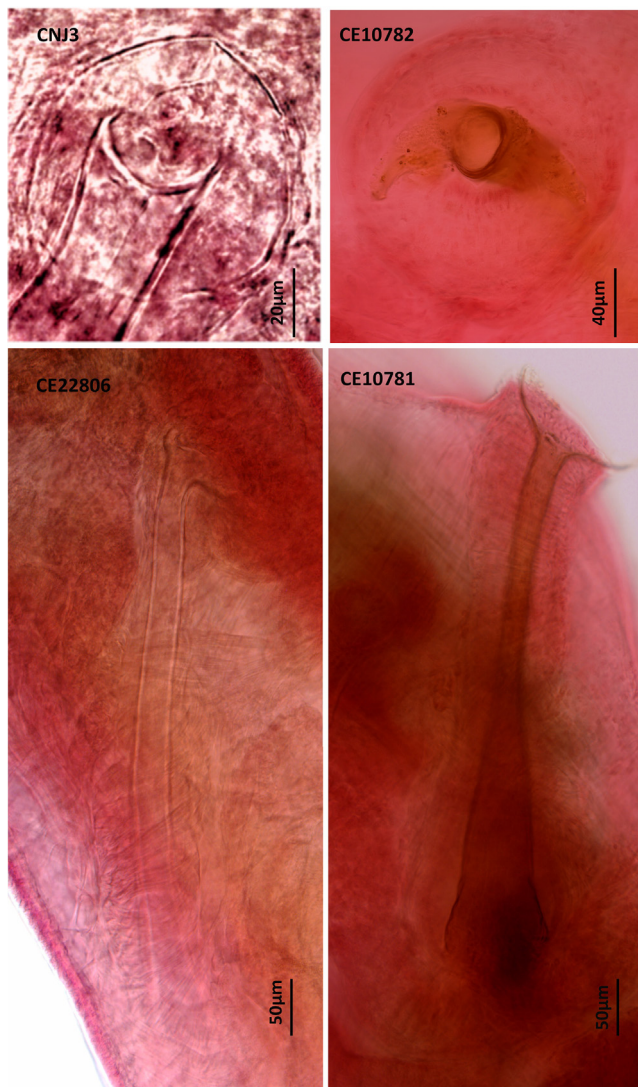

Fig. 4. Micrographs of penis sheaths from slide-mounted specimens of the examined *Limnodrilus hoffmeisteri sensu stricto* (specimen ID: CE22806 (neotype), and CNJ3) and *Limnodrilus rubripenis* (specimen ID: CE10781 and CE10782). Upper pictures showing distal end, lower pictures the full length of the sheath.

It can be noted here that, as suggested by the 16S-based analysis by Achurra et al. (2011), we consider *Varichaetadrilus bizkaiensis* Rodriguez and Giani, 1984, as more closely related to the genus *Potamothrix* than to *Varichaetadrilus*.

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 mat-

ter 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 *Baltidrilus*, *Lophochaeta* and *Varichaetadrilus*. Some species within this group, now classified within *Varichaetadrilus*, 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 sub-families 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 Limnodriloidinae 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*.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.04.019.

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