# Molecular Systematics of *Limnodrilus* (Annelida: Clitellata)

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This doctoral thesis in Natural Sciences, specializing in biology, is authorized by the Faculty of Science and will be publicly defended on Monday the 20th March 2017, at 13:00, in the lecture hall at the Department of Biological and Environmental Sciences, Medicinaregatan 18, Gothenburg, Sweden.

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Molecular Systematics of *Limnodrilus* (Annelida: Clitellata)

Cover illustration:

The anterior part of a mounted *Limnodrilus hoffmeisteri* specimen



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## Abstract

The freshwater *Limnodrilus* worms (Clitellata: Naididae: Tubificinae) are segmented hermaphroditic annelids, bearing a unique clitellum ("girdle") during sexual maturity. Some abundant and common taxa of *Limnodrilus* are ecologically and economically important in many respects, but taxonomic controversy, especially regarding the diagnosis of the cosmopolitan *Limnodrilus hoffmeisteri* Claparède, 1862 (the type species of the genus), has lasted for more than a century. In addition, the phylogenetic position of *Limnodrilus* within the subfamily Tubificinae has been uncertain.

Taxonomic studies based on molecular data, e.g., using DNA-barcoding (for animals, the mitochondrial marker COI), have revealed several examples of cryptic speciation among widely distributed clitellate morphospecies. In this thesis, I used both mitochondrial COI barcodes and nuclear ITS data to explore primary species hypotheses from a sample of the morphologically defined *L. hoffmeisteri* collected in the northern hemisphere, and a final conclusion about species boundaries was based on the congruence of the mitochondrial and nuclear phylogenies. Furthermore, the phylogeny of *Limnodrilus* was estimated based on multiple-loci data of several *Limnodrilus* species, including a new one described as *L. sulphurensis* Fend, Liu & Erséus, 2016 from a sulphur cave in North America, and other naidid taxa. Finally, as the commonly used ITS primers are neither efficient nor specific for clitellates, two new pairs of clitellate-specific ITS primers were proposed.

The molecular study showed that the well-known taxon "*L. hoffmeisteri"* actually represents a species complex (with at least ten species) rather than a single, cosmopolitan, species with great morphological variation. This work also showed that DNA barcoding, without using additional nuclear data, is likely to overestimate the number of species. Therefore, the ITS primers specific for clitellates will facilitate future research on species delimitation and the evaluation of mitochondrial DNA barcoding in Clitellata as a whole. In addition, by combining morphological and genetic information, a neotype of *L. hoffmeisteri sensu stricto* was designated, and the new species *L. sulphurensis* was discriminated from the other members of this genus. The neotype of *L. hoffmeisteri* is a baseline for future taxonomic work on the many cryptic species.

The phylogenetic hypothesis presented in this thesis contributes to our understanding of *Limnodrilus sensu stricto,* which is a well-demarcated, monophyletic genus of the naidid subfamily Tubificinae, containing at least three main evolutionary lineages (i.e., three species groups). The sister lineage of *Limnodrilus* in our taxon sample is a group of three genera, *Baltidrilus*, *Lophochaeta* and *Varichaetadrilus*. However, *Limnodrilus rubripenis* Loden, 1977 is phylogenetically closer to *Varichaetadrilus* than to other *Limnodrilus* species.


**Keywords**: Cryptic diversity, Species delimitation, Oligochaeta, Clitellata, Sludge worms, *Limnodrilus*, Integrative taxonomy, Neotype, DNA-barcoding, Phylogeny, Primers.

## List of Papers

This thesis is based on the following four papers (I-IV):

I. Liu Y, Fend SV, Martinsson S, Erséus C (2017). Extensive cryptic diversity in the cosmopolitan sludge worm *Limnodrilus hoffmeisteri* (Clitellata, Naididae). *Organisms Diversity & Evolution*, 1-19, doi: 10.1007/s13127-016-0317-z

II. Fend., S. V., Liu, Y., Steinmann, D., Giere, O., Barton, H. A., Luiszer, F., Erséus, C. (2016). *Limnodrilus sulphurensis* n. sp., from a sulfur cave in Colorado, USA, with notes on the morphologically similar *L. profundicola* (Clitellata, Naididae, Tubificinae). *Zootaxa, 4066*(4), 451-468, doi: dx.doi.org/10.11646/zootaxa.4066.4.6

III. Liu, Y., Martinsson, S., Xu, L., Ohtaka, A., Fend., S. V., Erséus, C. (manuscript). Multi-locus phylogenetic analysis of the genus *Limnodrilus* (Annelida: Clitellata: Naididae).

IV. Liu, Y and Erséus, C. (manuscript). Specific primers developed for amplification of the ITS region in Clitellata (Annelida).

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## 1. Introduction

The work of taxonomy, i.e., naming, describing and classifying organisms as species, has been a continuous process for over 250 years, since Swedish botanist Carolus Linnaeus introduced the binomial system in the 1750s. Linnaeus' system has become a keystone providing continuity across all kinds of biological science research. Names of animal species are regulated by objective rules of the International Code of Zoological Nomenclature (ICZN) (Ride, 1999), but using such systems to classify species based on morphology only is inherently unstable (Tautz et al., 2003). Darwin's theory of evolution gave rise to the integration of traditional taxonomy into modern systematics, leading to, e.g., the development of new species concepts and methods of estimating phylogenetic trees, using various combinations of independent (morphological, ecological and genetic, etc.) evidence.

Molecular analysis has caused revisions of the hypotheses of the phylogeny of Clitellata (also known as Oligochaeta, and the target group in this thesis), which were originally based on morphological cladistics analysis (Brinkhurst, 1991; Erséus et al., 2000; Erséus, 2005; Weigert and Bleidorn, 2016). Moreover, molecular data have proved helpful in the recognition of multiple species within widely distributed nominal taxa of clitellates (Gustafsson et al., 2009; Martinsson et al., 2015b). Systematic studies involving DNA enable us to better understand clitellate species and their relationships, but it is critical to combine such approaches with updated views of the classical, morphology-based taxonomy that still prevail for some clitellate groups, and not the least, for the freshwater genus *Limnodrilus* Claparède, 1862 of the family Naididae (*sensu* Erséus et al., 2008).

The worms of *Limnodrilus* play key roles in freshwater ecosystems (Zhang et al., 2014). These sediment-dwelling deposit feeders are abundant and widely distributed (Kennedy, 1965; Simpson et al., 1993; Matisoff et al., 1999), and therefore, they are an easily available live food source for fish in aquaria and aquaculture in many parts of the world. They are often considered as indicators of organic pollution in environmental monitoring and assessment (Rodriguez and Reynoldson, 2011; Oztetik et al., 2013). However, although some abundant and common taxa of *Limnodrilus* are ecologically and economically important, their great morphological variability has prompted debate. Taxonomic controversy, especially regarding the diagnosis of the type species *Limnodrilus hoffmeisteri* Claparède, 1862, has lasted for more than a century. Since the morphological features used to identify this taxon show great variation, a genetic study to resolve its boundaries has long been warranted.

In addition, although *Limnodrilus* species were often used as outgroups in several phylogenetic studies (Beauchamp et al., 2001; Bely and Wray, 2004; Achurra et al., 2011; Marton and Eszterbauer, 2012), the systematic position of the genus *Limnodrilus* within the subfamily Tubificinae (Annelida: Oligochaeta: Naididae) has been uncertain (Erséus et al., 2002).

## 2. Aims of this thesis

The purposes of this thesis are:

to resolve the morphological taxon *L. hoffmeisteri* into separately evolving lineages (i.e. genetically supported species) using both COI barcoding and nuclear data, and to designate a neotype of *L. hoffmeisteri sensu stricto*. Paper I.

to apply an integrated approach, i.e., combing morphological and genetic information, in the formal description of a new *Limnodrilus* species from a sulphur cave in North America. Paper II.

to estimate the phylogeny of the genus *Limnodrilus,* and to investigate its phylogenetic position within the subfamily Tubificinae (Annelida: Clitellata: Naididae) using multiple loci data. Paper III.

to find new specific ITS primers for clitellates, to facilitate future research on species delimitation and a better evaluation of mitochondrial DNA barcoding in Clitellata as a whole. Paper IV.

## 3. Background

### 3.1 Species concepts

Historically, the typological species concept (TSC) and associated approaches have dominated species delimitation for centuries. TSC defines a species as a group of organisms with some sufficient unchanging characters associated with a type specimen in the Linnaeus system. However, it is often practically difficult to assess morphological character states correctly due to morphological polymorphism (Wiens and Penkrot, 2002), and extensive homoplasy caused by convergence, reversal and hybridization (Garcia et al., 2009; Mott and Vieites, 2009). Furthermore, recently diverged species are likely to share (retain) many ancestral morphological characters, i.e., morphological differences between cryptic species (morphologically indistinguishable but genetically different species) will often only emerge after enough time has passed (Bickford et al., 2007; Harper et al., 2009; Shain, 2009). Therefore, substantial intraspecific morphological variation is likely to be treated as interspecific characters, or *vice versa*, resulting in an overestimation or underestimation of species diversity.

In addition to TSC, there are over 20 different species concepts (Mayden, 1997b), e.g., the well-known biological species concept (BSC) proposed by the German-American biologist Ernst Mayr (Wheeler and Meier, 2000), which defines a species as "groups of interbreeding natural populations that are reproductively isolated from other such groups". However, BSC is not applicable for asexual clitellates (Christensen, 1984; Cosín et al., 2011). Another concept, the phylogenetic species concept (PSC), has become widely applied in the

molecular era, and it regards species as monophyletic groups of organisms (Cracraft, 1983). Approaches based on PSC are especially useful for estimating phylogenetic trees for sexual as well as asexual species, including cryptic species (Taylor et al., 2000). However, relying on phylogenetic trees based on combinations (concatenation) of various genes may lead to erroneous conclusions, due to the common phenomenon of incongruence between separate gene trees and the species phylogeny. Consequently, species delimitation based on any of these species concepts alone is likely to fail (Mayden, 1997a; Carstens et al., 2013).

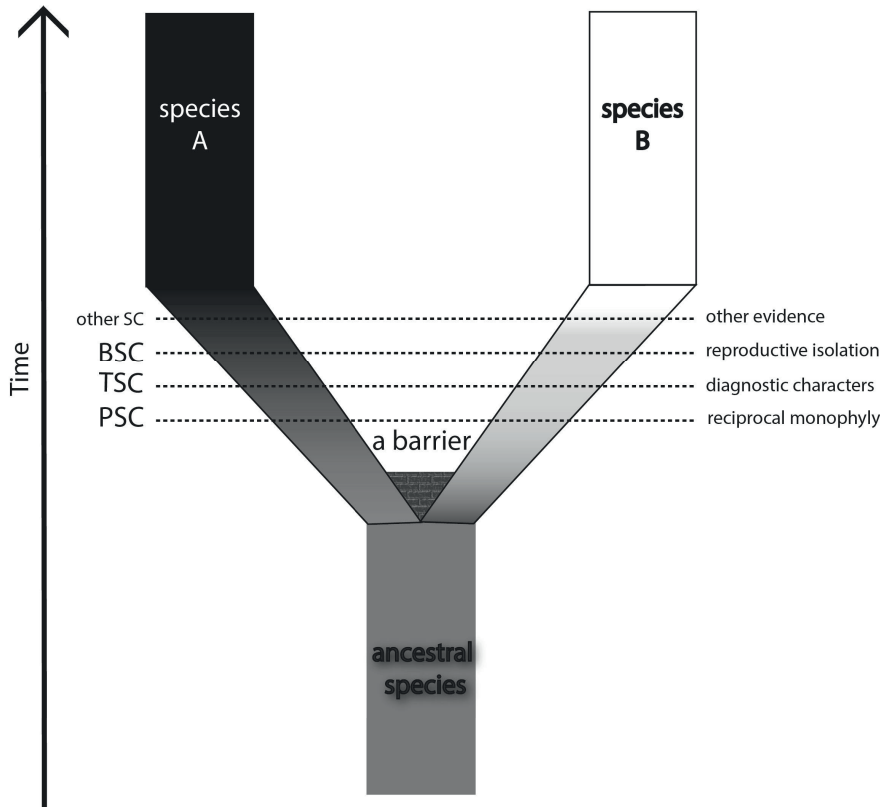



**Figure 1**. The universal lineage species concept (adapted from de Queiroz, 1998, 2007) showing that a barrier of some sort prevents gene flow, or the transfer of alleles of genes, from one population to another within a single ancestral species. After this, two lineages under independent genetic drift or/and selection will eventually lead to two species A and B over time. Two grading grey zones represent the time during which the daughter lineages acquire different properties (shown as dashed lines), which serve as operational criteria for recognizing species boundaries under different concepts (PSC, phylogenetic species concept; TSC, typological species concept; BSC, biological species concept). The four dashed lines of differentiation/evidence of speciation are not meant to be in any fixed order of appearance.

De Queiroz (2007) concluded that the differences among various species concepts only reflect different aspects of a more universal idea of species, where the primary defining property of the species category is "a separately evolving metapopulation lineage", and secondary defining properties are "adopting different properties acquired by lineages during the course of divergence". In other words, two lineages, separated from an ancestor species, will eventually acquire different secondary properties: e.g., they may become morphologically distinct, sexually incompatible, and reciprocally monophyletic. These secondary properties may all serve as cumulative diagnostic evidence relevant for species delimitation (Fig. 1). The conflicts, occurring among other species concepts mentioned above, are caused by the fact that these secondary properties do not necessarily arise at the same time or in a certain order, or may not arise at all during the process of speciation.

### 3.2 Taxonomical issues within *Limnodrilus* species

The type species, *Limnodrilus hoffmeisteri*, and its congener *L. udekemianus* Claparède, 1862 were both described from a small stream near Geneva in Switzerland (Claparède, 1862). These two species were classified in a separate genus (*Limnodrilus*) based on the elongate cuticular penis sheaths and the existence of only bifid chaetae (Fig 3, c), which are not the case in another famous genus (*Tubifex*) known at the time. According to the original description, a live *L. hoffmeisteri* is less vinous, "moins vineuse", in colour, and has a slimmer body with much longer penis sheaths, than *L. udekemianus*. It is also noteworthy that *L. hoffmeisteri* has less robust anterior chaetae than *L. udekemianus*. A third species, *L. claparedianus* Ratzel, 1868, with penis sheaths different from those of the previous two species, was soon after identified and described by Ratzel (1868). Later on, a number of additional species of *Limnodrilus* were formally described (Table 1), but their identification and status were frequently questioned and revised (Grube, 1873; Eisen, 1879; Vejdovský, 1884; Eisen, 1885; Beddard, 1895; Hatai, 1899; Michaelsen, 1900; Bretscher, 1901; Brauer, 1909; Southern, 1909; Nomura, 1913; Chen, 1940; Brinkhurst, 1981). Brinkhurst and Jamieson (1971) lumped numerous morphotypes of *L. hoffmeisteri* into two more or less distinct groups, based on different shapes of their copulatory organs. However, Howmiller (1974) and Stimpson et al. (1982) still regarded them as separate taxa rather than two different intraspecific forms. Yet, others repeatedly concluded that the morphological differences were not consistent enough for the separation of different species in the *L. hoffmeisteri* complex (Barbour et al., 1980; Dzwillo, 1984; Steinlechner, 1988). There are also similar problems with different forms of *L. claparedianus, L. maumeensis* and *L. cervix* (Brinkhurst and Cook, 1966; Hiltunen, 1967; Hiltunen, 1969; Brinkhurst, 1976; Mozley and Howmiller, 1977; Krieger, 1984; Sparks et al., 1986). Taken together, it is obvious that traditional morphological taxonomy has never been able to fully resolve the species in this genus.

Table 1. A list of morphological *Limnodrilus* species, commonly regarded as valid today. Taxa in bold face were studied in this thesis.

***Limnodrilus hoffmeisteri*** Claparède, 1862

***Limnodrilus udekemianus*** Claparède, 1862

***Limnodrilus claparedianus*** Ratzel 1868

***Limnodrilus profundicola*** (Verrill, 1871)

*Limnodrilus dybowskii* Grube, 1873

***Limnodrilus silvani*** Eisen, 1879

***Limnodrilus grandisetosus*** Nomura, 1932

*Limnodrilus neotropicus* Černosvitov, 1939

***Limnodrilus cervix*** Brinkhurst, 1963

***Limnodrilus maumeensis*** Brinkhurst and Cook, 1966

*Limnodrilus variesetosus* Brinkhurst, 1979*

*Limnodrilus bulbiphallus* Block and Goodnight, 1972

***Limnodrilus rubripenis*** Loden, 1977

*Limnodrilus nitens* Semernoy, 1982

*Limnodrilus tendens* Semernoy, 1982

*Limnodrilus tortilipenis* Wetzel, 1987

*Limnodrilus amblysetus* Brinkhurst, Qi &Liang, 1990

*Limnodrilus paramblysetus* Wang and Liang, 2001

*Limnodrilus simplex* He, Cui & Wang, 2010

***Limnodrilus sulphurensis*** Fend, Liu & Erséus, 2016

* A possible synonym of *L. udekemianus* (Brinkhurst and Marchese, 1989)

### 3.3 DNA barcoding and cryptic species

Unlike the morphological descriptions of subjectively selected characters, the strictly heritable DNA contains vital information directly reflecting the speciation process. DNA data are particularly valuable for delimiting species and testing traditional species boundaries, as they are generally much more amenable than morphological data for quantitative alignment, due to the numerous characters (substitutions) at homologous positions. DNA-based analyses thus provide a possibility for systematists to disentangle confusing taxonomic problems (Pons et al., 2006; Bickford et al., 2007), and to re-examine species with wide distributions using large samples of specimens (Bock et al., 2012). For instance, the 5' part of the fast evolving Cytochrome Oxidase subunit I (COI) mitochondrial gene has been proposed as a DNA barcode for animals, and it is increasingly used for species identification, species discovery and delimitation, and for testing traditional species-level taxonomy and revealing cryptic species (Hebert et al., 2003; Hebert et al., 2004; Hajibabaei et al., 2006; Paz and Crawford, 2012; Geiger et al., 2014; Pecnikar and Buzan, 2014). Regarding the *Limnodrilus* species, previous studies based on mitochondrial 16S data suggest that *L. hoffmeisteri* harbours cryptic diversity (Beauchamp et al., 2001; Erséus and Gustafsson, 2009), but few attempts have been made to integrate molecular (barcoding) data with morphological data for a better resolution of this taxon.

### 3.4 Discrepancy between gene and species trees

With the growing access to data of COI barcodes and other loci, the discrepancy of evolutionary histories of species (species trees) and the orthologous genes carried by these species (gene trees, e.g., the COI tree) have increasingly been debated in light of the multispecies coalescent model (Degnan and Rosenberg, 2009). The discordance between individual-gene trees and species trees is a well-documented phenomenon (Degnan and Rosenberg, 2009; Fisher-Reid and Wiens, 2011), and simulation studies have shown that concatenation methods may yield overconfident support for incorrect species trees in the presence of gene tree discordance (Kubatko and Degnan, 2007). In contrast, the coalescent-based approaches are theoretically better than traditional concatenated multi-loci approaches, as they accommodate the topological heterogeneity among gene trees, under the assumption that all combined genes (perhaps with different mutation rates and models for different sites) have evolved into a single evolutionary tree (Kubatko and Degnan, 2007). The coalescent model allows incongruence across gene trees, e.g., incomplete lineage sorting, and thus it can accurately estimate phylogenies from multi-loci data under a variety of conditions (Carstens and Knowles, 2007; Mirarab et al., 2014; Xi et al., 2014). In spite of significant theoretical advancements, coalescent-based software, e.g., *BEAST (Heled and Drummond, 2010) and MP-EST (Liu et al., 2010), have to face computational challenges of increasingly large data sets (Bayzid et al., 2014; Wickett et al., 2014; Zimmermann et al., 2014). Recent simulations, however, show that coalescence may not always provide significantly better performance over concatenation methods (Gatesy and Springer, 2014; Tonini et al., 2015). Thus, incorporating both concatenation and

coalescent-based analyses will help us to estimate a robust phylogenetic tree hypothesis.

### 3.5 Nuclear loci and species delimitation

Under a universal species concept, the approach of testing the conflicting delineations of species using DNA barcodes in combination with other lines of evidence, e.g., sequences of nuclear loci, may deliver more objective, testable and uniform species units as subjects for a range of studies (Will et al., 2005; Spooner, 2009; Dupuis et al., 2012; Carstens et al., 2013). Maternal mitochondrial DNA (mtDNA), e.g., COI, is used to explore the primary species hypothesis (divergence of closely related maternal lineages), whereas nuclear DNA (nDNA) data provide evidence also of paternal contributions to evolutionary history. The nDNA genes have longer coalescence times due to their larger effective population sizes than mtDNA ones, and thus a single lineage, which has reached fixation at a nuclear locus, will theoretically be at a higher taxonomic level (e.g., at or above the species level) than the one recognized by mitochondrial loci (Heckman et al., 2007; Degnan and Rosenberg, 2009). The congruence among independent estimates of the genealogical history thus provides strong evidence of actual species divergence.

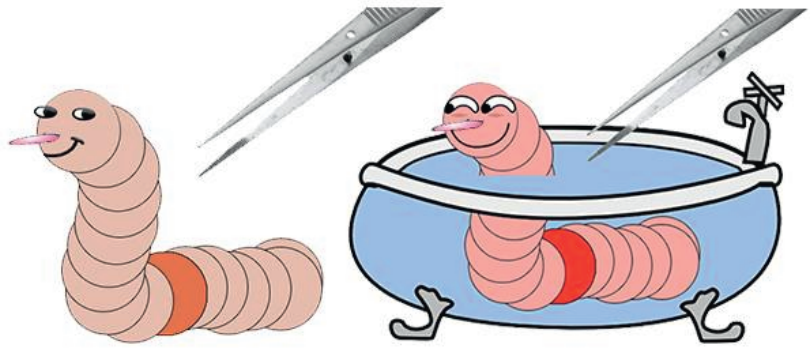
### 3.6 Suitable nuclear loci for clitellate analysis

The variable nuclear ITS region, covering the nuclear internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene and the ITS2 rDNA, has been successfully used, along with COI, to elucidate relationships at the level of species or even species complexes in several annelid (polychaete and clitellate) groups (Kvist et al., 2010; Nygren and Pleijel, 2011; Envall et al., 2012; Achurra and Erséus, 2013; Martinsson et al., 2013; Shekhovtsov et al., 2013; Martinsson and Erséus, 2014). ITS sequences are highly variable among congeneric species due to their high molecular evolutionary rate (Nilsson et al., 2008). Moreover, in analogy with the multiple mitochondrial genome copies in a single cell, the existence of rRNA tandem repeats within a single nuclear genome set provides sufficient ITS copies. Last but not least, the ITS region is flanked by very conservative 18S and 28S rRNA, which theoretically facilitates the design of primers with a broad taxon coverage (Schoch et al., 2012; Wang et al., 2015). Although other commonly used nuclear loci, e.g., 18S, 28S and Histone (H3), are widely used to assess evolutionary relationships among distantly related groups within clitellates (Erséus et al., 2000; Erséus and Källersjö, 2004; Rousset et al., 2007; Marotta et al., 2008; James and Davidson, 2012), they are less adequate than ITS for species resolution, or are as far as known only working for specific species (Halanych and Janosik, 2006). In addition, ITS is also suitable for phylogenetic and phylogeographic studies in generic and infra-generic level classifications (Trontelj and Sket, 2000; Hallett et al., 2005; De Wit and Erséus, 2010; Trontelj and Utevsky, 2012; Porras-Alfaro et al., 2014).

# 4. Methodology

## 4.1 Sampling strategy and physical vouchers

For paper I, we included as many samples of *L. hoffmeisteri* as possible from the northern hemisphere, from where most *Limnodrilus* records are reported in the literature. This large geographic scale strategy was used to cover as many of the major clades of *L. hoffmeisteri* as possible. The species recognized in paper I (see 5.1 below), and other *Limnodrilus* species (including *L. sulphurensis* described in paper II), were used for the phylogeny analysis of the genus *Limnodrilus* (paper III). As the phylogenetic position of *Limnodrilus* within the subfamily Tubificinae (Clitellata: Naididae) was unknown, individuals belonging to 24 other genera of the family Naididae were selected to serve as out-groups in paper III. For paper IV, a broad selection of clitellate species were used to examine the performance of two pairs of newly designed ITS primers.



80%-->95%-->100% ethanol
-->50% ethanol: 50% xylene-->100% xylene

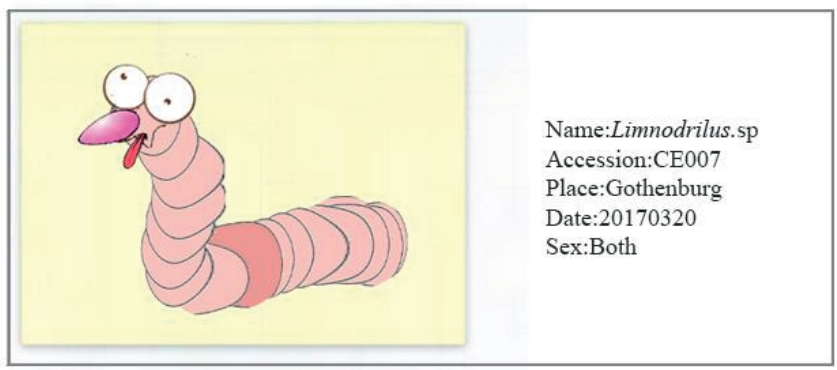



**Figure 2** Steps for the preparation of mounted worm slides for microscopic examination

Sampled specimens were preserved in 80%-95% ethanol. A posterior fragment of each worm was used for DNA extraction, while the remaining anterior part (the physical "voucher") was stained in an alcoholic paracarmine solution and mounted in Canada balsam on a microscope slide (Erséus, 1994) (Fig 2). The mounted vouchers are deposited in the Swedish Museum of Natural History, Stockholm, or the University Museum of Bergen, Norway.

### 4.2 Morphological identification

The body of a *Limnodrilus* worm, as of all clitellates, consists of a head (comprising the prostomium and the peristomium), followed by many segments internally separated by septa, and a terminal pygidium at the posterior end. The numbers of segments (excluding the prostomium and pygidium) are often denoted with roman numerals (segment I, II, III etc.), and each segment typically contains a pair of nephridia, coelomic cavities, ganglia, and four bundles (two ventral and two dorsal) of chitinous chaetae. The mouth is located in the first segment (peristomium) and leads into a digestive tract formed by a large pharynx, a narrow tubular oesophagus, a long intestine covered by chloragogen cells, and an anus terminally located on the pygidium. Nutrition absorbed from the intestine is transported into the blood vessels situated between the intestinal muscles and the coelomic epithelium. The chaetae are hard (easily observed) structures starting from segment II, and they facilitate counting the number of body segments. The more exact shape and number (per bundle) of the chaetae are useful characters for species identification in some cases (Fig. 3, k). In *Limnodrilus*, all chaetae are terminally curved and bifid, i.e., their outer end is forked with one upper and one lower tooth (Fig. 3, c, k). Oxygen is absorbed through the body wall by diffusion, and in organically polluted habitats, some naidid groups (including *Limnodrilus*) may wave the posterior part of body outside of the sediment layer to obtain more oxygen from the water column.

Like all other members of Clitellata, *Limnodrilus* species are hermaphrodites, and they develop an externally visible clitellum, i.e., a thickened but single layer of glandular cells in the epidermis, around some anterior segments at sexual maturity. In the fully mature worm, the internal reproductive organs consist of male and female gonads, male and female ducts, penis sheaths as well as spermathecae (specialized organs for sperm reception and storage, Fig. 3, d).

The genital organs, particularly the male ducts and the spermathecae, are usually crucial morphological features in the systematics of Clitellata, and for species of *Limnodrilus*, the characteristic, cylindrical, shape of their cuticularized penis sheaths have been regarded as particularly important for distinction of species (Fig. 3, g-j). Morphological identification of *Limnodrilus* species partly also relies on the appearance of the anterior chaetae. All *Limnodrilus* species lack (dorsal) hair chaetae, as opposed to the many other naidids commonly bearing hair chaetae in addition to the bifid ones (Brinkhurst and Jamieson, 1971). Some species, e.g., *L. udekemianus* and *L. grandisetosus*, develop bundles of enlarged (or just longer) chaetae in the anterior segments,

and in the new species, *L. sulphurensis* Fend, Liu & Erséus, 2016 (see 5.5 below), many anterior chaetae have unusually long, sharply angled teeth (Fig. 3, k).

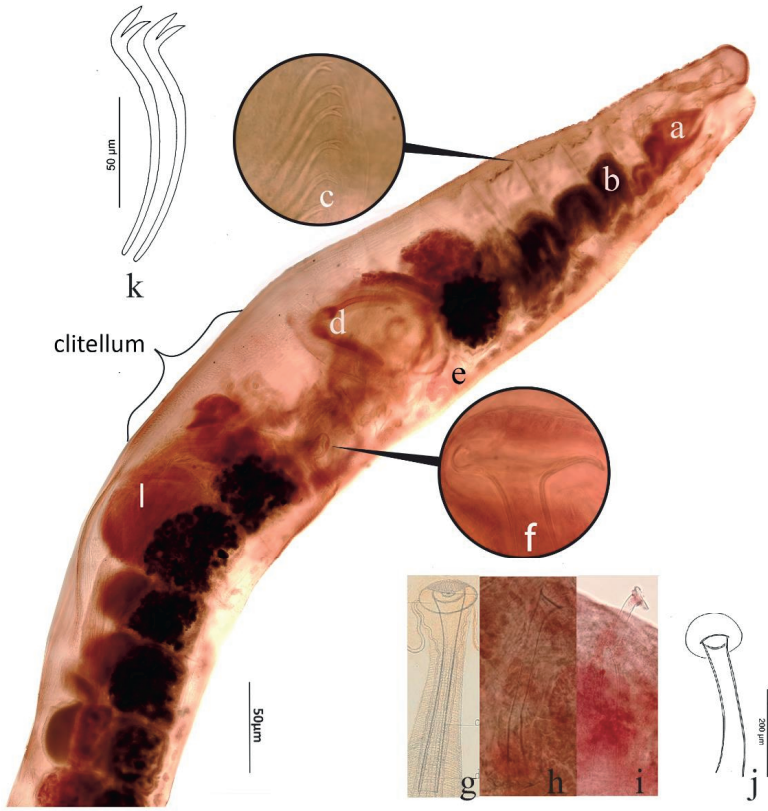



**Figure 3** Illustration of morphological characters of a *Limnodrilus* worm. **a**, pharynx; **b**, starting position of the chloragogen tissue (=the dark parts along the gut); **c**, dorsal chaetae in segment VII; **d**, spermatozeugmata within spermathecae; **e**, testis; **f**, distal part of a penis sheath; **g**, the original illustration of the penis sheath of *L. hoffmeisteri* (Claparède, 1862, plate 1, fig 1); **h**, the "typical" and **i**, "plate-topped" penis sheaths of *L. hoffmeisteri*, as coined by Brinkhurst and Jamieson (1971, page 467); **j**, penis sheath of *L. sulphurensis*; **k**, the (odd) anterior chaetae of *L. sulphurensis*; **l**, egg.

### 4.3 Molecular methods used in the phylogenetic analysis

In paper I, to explore the number of primary species hypotheses (PSHs) emanating from a large *Limnodrilus* sample, analyses of the single mitochondrial COI sequences (barcodes) were performed using Automatic Barcoding Gap Discovery (ABGD) (Puillandre et al., 2012) and Bayesian General Mixed Yule Coalescent model (bGMYC) (Reid and Carstens, 2012). The PHSs were tested for congruence with the well-supported monophyletic groups in the ITS tree (estimated by BEAST; (Drummond. et al., 2012). These PSHs were also

evaluated with the coalescent-based method BPP using both COI and nuclear ITS data.

In paper III, the phylogeny of the genus *Limnodrilus* was estimated. Analyses of concatenated mitochondrial genes, concatenated nuclear markers and the combined all loci were conducted using MrBayes (Ronquist et al., 2012) and RAxML (Stamatakis et al., 2005). The phylogenetic analyses based on concatenated data were then compared with analyses using a widely implemented multi-locus coalescent method, e.g., *BEAST (Star BEAST; Heled and Drummond, 2010). In *BEAST, the mitochondrial genes were linked in a single partition tree, as they are genetically linked. For the same reason, 18S, ITS and 28S were also combined into a single partition tree as they are all are parts of the ribosomal genome. In addition, each specimen used in paper III was assigned to a species name, including the cryptic lineages identified in the *L. hoffmeisteri* complex (in paper I).

### 4.4 Morphological methods and comparisons with molecular data

The mounted specimens (studied in papers I-III) were examined under an Olympus BX60 compound microscope equipped with a digital camera DXM 1200, using species identification keys by Kathman and Brinkhurst (1998) and van Haaren and Soors (2013). The variation in the length/basal-width ratio of the penis sheaths was quantitatively calculated by cluster analysis in IBM SPSS Statistics. The software BayesTraits V2.0 was used to search the Pagel's lambda value (range 0 to 1) for the best predictive distribution of the given traits on transformation of the COI phylogeny under a Brownian motion model of trait evolution (Pagel and Meade, 2013). The lambda values close to 1 indicate significant phylogenetic signal. In addition, anterior chaetal features (relative thicknesses, and lengths, of distal teeth), together with the shapes of the penis sheaths, were used as supplementary information when evaluating gene trees.

### 4.5 Primer design and primer evaluation *in silico*

The nuclear ITS region was used for both species delimitation and estimation of phylogeny in this thesis (papers I and III). However, the two ITS spacers (ITS1 and ITS2) are highly variable and associated with frequent insertions and deletions (Schoch et al., 2012). Aligning incomplete ITS sequences may thus produce an artificial clustering due to convergent characters resulting from mutational saturation in these highly variable spacers (Kruger et al., 2012), or erroneous alignment with the short residual fragments of 18S and 28S rDNA often associated with, respectively, the ITS1 or ITS2 sequences. During the course of my studies of *Limnodrilus*, I frequently encountered the problem that the ITS primers used for clitellates were neither specific nor universal enough. A little late unfortunately, I therefore decided to finish my thesis (paper IV) with the design of two new pairs of ITS primers, specifically targeting clitellate worms. New clitellate-specific primers for amplifying the whole ITS region (ITS: 29F/1084R) and a part of it (ITS2: 606F/1082R) were developed on the basis of a collection of previously published ITS sequences

with flanking rDNA coding regions. The specificity of these and other primers used for clitellates were then tested (*in silico*) using ecoPCR (Ficetola et al., 2010) by evaluating their mismatches with an entire standard annotated assembled sequence database (version r127) from EMBL, and the new primers were also tested *in vitro* for a taxonomically broad sample of clitellate species.

## 5. Main results

### 5.1 Molecular evidence of cryptic speciation

In paper 1, 295 worms identified as either *L. hoffmeisteri* or other similar (congeneric) morphospecies and collected from 82 locations in the northern hemisphere, were studied. The ABGD analyses of the COI dataset resulted in 31 primary species hypotheses (PSHs), when the initial partitions were used. The classified ABGD groups are more or less the same as those found to be monophyletic in the COI Maximum clade credibility trees derived from BEAST, both in the tree based on the whole dataset (all specimens) and in the tree based on unique haplotypes. The outcome of the bGMYC analysis based on whole COI sequences not only contained a higher number of well supported PSHs than those based on COI haplotypes, it was also largely congruent with the COI PSHs suggested by ABGD, except for one group. In addition, the PSHs based on the phylogenetic analysis of nuclear ITS data were also explored.

The primary species delimitation analyses led to mostly contradictory results. For COI, 31 (ABGD), 32 (all sequences bGMYC), or 25 (haplotypes bGMYC) PSHs were obtained; for ITS, only 16 (ABGD) PSHs. That is, some individuals forming well-supported ITS clades were not classified as monophyletic groups by their mitochondrial genes, or *vice versa*. Therefore, we resorted to use the criterion of reciprocal monophyly to recognize species across all trees and analyses. A consensus among all evidence is that a minimum of 13 species exist in our sample. Ten of these are morphologically identified as *L. hoffmeister*i, and in Fig. 4, they are denoted as lineages I–X. The three other species were identified as *L. claparedianus*, *L. maumeensis* and a species morphologically intermediate between *L. claparedianus* and *L. cervix* (see also 5.2).

This showed that the well-known taxon "*L. hoffmeisteri"* actually represents a species complex rather than a single, cosmopolitan, species with great morphological variation. The smallest uncorrected COI p-distance between our 10 species was 12.1%, and the largest intraspecific p-distance 16.4%, which has serious implications for DNA-barcoding (see Discussion, 5.1).

### 5.2 The limited resolution of morphological characters

In paper I, we confirmed the existence of the two main kinds of distal ends of the penis sheaths in the *L. hoffmeisteri* complex, described by Brinkhurst & Jamieson (1971), i.e., the "typical" form (Fig. 3, h) and the "plate-topped" or "spiralis" form (Fig. 3, i). However, we also found intermediate penes that were

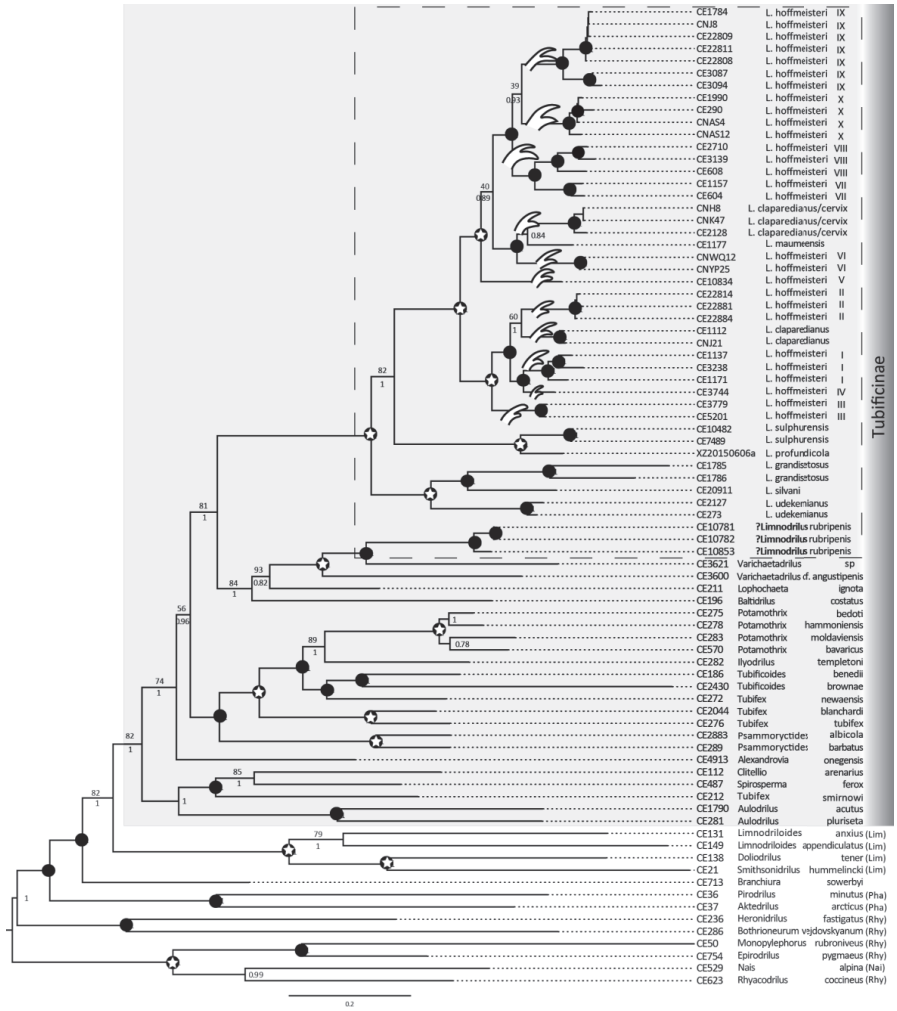



**Figure 4** Phylogeny of *Limnodrilus* estimated using *BEAST based on data of seven markers (COI, 12S, 16S, 18S, 28S, ITS and H3). Statistical values above the clades indicate bootstrap support (BS calculated by RAxML) while values below the clades are Bayesian posterior probabilities (BP calculated by *BEAST). The well-supported nodes (BS > 90 and BP > 0.95) are indicated with black dots; white asterisks on black dots stand for good support (BP > 0.95). In addition, the nodes lacking BS value (only BP value shown, near nodes) indicate the discrepancies between Bayesian and ML 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.

not easily classified into either of these two forms. The other three (congeneric) species in our material had their own distinct penis types: two were easily identified as *L. claparedianus* and *L. maumeensis*, respectively, the third ("*L. claparedianus-cervix"*) has penis sheaths intermediate between those of *L. claparedianus* and *L. cervix* (cf. Hiltunen 1967, figs. 22-24; Ohtaka et al. 2006; Brinkhurst & Cook 1966, fig. 7C).

Within *Limnodrilus*, the continuous and overlapping variation of the penis sheath length/width ratio, or the length only, has no convincing resolution at the species level. The length and width of the penis sheaths were measured in 91 sexually mature specimens, selected from nine of the ten species in the *L. hoffmeisteri* complex, and the other three recognizable congeneric morphospecies mentioned above. Penis sheath length had a somewhat bimodal distribution, indicating two morpho-groups in our material: one group consists of *L. claparedianus*, "*L. claparedianus-cervix*" and species III of the *L. hoffmeisteri* complex, all with penis sheaths longer than 600 µm. The other group contains *L. maumeensis* and the remaining eight species of *L. hoffmeisteri*, which generally have shorter penis sheaths. The three species/lineages (Species III, "*L. claparedianus-cervix"* and *L. claparedianus*) with penes > 600 µm are not found close to each other on the trees. In the BayesTraits analyses, the lambda values for penis length and the penis sheath length/width ratio were 0.77 and 0.64, respectively, suggesting that these characters carry a phylogenetic signal. However, because the sheath ratio variation is continuous and overlapping, we failed to unequivocally distinguish between nine of the ten species (I-II, IV-X) within the *L. hoffmeisteri* complex, as well as between Species III and "*L. claparedianus-cervix*".

The shape of the chaetae located in the anterior segments (at least in segments II-VI) did not vary much among conspecific individuals, but differences in relative thickness and length of distal teeth were recognized between some species (Fig. 4). This pattern was also found in immature individuals, although their chaetae were smaller. Even though the shape of the anterior chaetae may shed some light on the possible species boundaries in species nested in *L. hoffmeisteri* complex, the power of such a resolution is likely to decrease when considering more congeneric species.

### 5.3 Designation of a neotype of *Limnodrilus hoffmeisteri*

As mentioned above, neither the penis sheaths nor the anterior chaetae were found to be sufficiently suitable for circumscribing species and identifying specimens of *Limnodrilus*. A final conclusion (paper I) about species within the *L. hoffmeisteri* complex was made on the basis of all sources of evidence under a minimum consensus criterion, and the morphological characters used for traditional identification were evaluated among these recognized species.

Based on material collected from the type locality at Geneva (Switzerland) and morphologically conforming to the description by Claparède (1861), we were able to conclude that one of our ten species is very likely to be identical to the original *L. hoffmeisteri*. In paper I, we designated and described a DNA-barcoded neotype from this material. The COI sequence of this specimen thus also genetically verifies its status as the name-bearing type of *L. hoffmeisteri*.

The remaining nine species of the *L. hoffmeisteri* complex, all within the range of the traditional morphology of *L. hoffmeisteri*, were left unnamed. They need to be scrutinized in relation to the many descriptions of taxa considered as

synonyms of *L. hoffmeisteri* in the past. Here also, molecular evidence from old or new type specimens will be crucial to firmly validate their taxonomic status.

### 5.4 A new species, *L. sulphurensis* Fend, Liu & Erséus, 2016

In paper II (Fend. et al., 2016), a new species, *L. sulphurensis* Fend, Liu & Erséus, 2016 was morphologically described, and COI barcodes were supplied as additional evidence for species discrimination. Based on the characters most often used to distinguish *Limnodrilus* species, i.e., proportions and morphology of the penis sheaths (Fig. 3, j), *L. sulphurensis* is most similar to the widespread *L. profundicola* and *L. udekemianus*. However, the phylogenetic analysis based on nuclear and mitochondrial markers (see 5.5 below) showed that *L. sulphurensis* is indeed closely related to *L. profundicola*, but phylogenetically more distant from *L. udekemianus*.

*Limnodrilus sulphurensis* is most easily differentiated from its congeners by the very long, sharply angled teeth of both ventral and dorsal chaetae in anterior segments (Fig. 3, k). The penis sheaths of *L. sulphurensis* also somewhat resemble those of the Jamaican *L. variesetosus*, and the group of endemic Lake Baikal species, *L. dybowskii, L. nitens* and *L. tendens*. Despite our inability to assess any genetic information for these other species, they can all be separated by their chaetal morphology.

### 5.5 Phylogeny of *Limnodrilus* and its position within in Naididae

In paper III, both concatenation and coalescent-based analyses using seven genetic markers showed that *Limnodrilus sensu stricto,* excluding *L. rubripenis*, is a well-demarcated, monophyletic genus of the naidid subfamily Tubificinae, containing at least three main lineages. 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 (*L. profundicola* and *L. sulphurensis*). The third, and largest group, includes not only the multitude of cryptic species in the *L. hoffmeisteri* complex, but also other recognized species nested within this complex. Species in this large group have long penis sheaths, and sheaths are exceptionally long in, e.g., *L. claparedianus, L. maumeensis*, and our 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 revisionary work of the *L. hoffmeisteri* complex and other unresolved species of *Limnodrilus*.

The phylogenetic results presented here have contributed to our understanding of *Limnodrilus*, and the neotype of the type species *L. hoffmeisteri* creates a baseline for future genetic work. In light of our phylogenetic results, however, the genus *Varichaetadrilus* is in great need of a revision before assigning *L. rubripenis* as a member of it. We provisionally regard *Varichaetadrilus cf. angustipenis* and a species referred to as "*V.* sp. 1" in paper III as conspecific lineages, but future work should test for diagnostic morphologically differences that may yet distinguish them.

### 5.6 New clitellate-specific primers for ITS and ITS2

In paper IV, the *in silico* analyses of published data with our new (29F/1084R and 606F/1082R; see 4.5 above) and previously published primer pairs (listed in paper IV) showed that the ITS primers commonly used for clitellates are neither specific nor universal enough for this group. A very high number of non-clitellate amplicons came from fungal groups, in particular, followed by, e.g., chlorophytes (green algae) and some of the more species-rich invertebrate groups, such as Cnidaria, Nematoda, Arthropoda and Plathyhelminthes. Although this result obviously was biased by the limited number of clitellate sequences in the EMBL database, we also observed notable mismatches between the newly amplified complete ITS sequences (using 29F/1084R) and primers targeting 5.8S rDNA. From our 78 genomic samples (representing 11 families), 61 ITS amplicons were successfully amplified using the primer pair 29F/1084R, and 73 ITS2 amplicons were successfully amplified using 606F/1082R. The pair 29F/1084R is likely to be a good option for sequencing the ITS region (i.e., if it is < about 1500 bp) as a whole, in at least some clitellate taxa. The new primer pair 606F/1082R is more suitable than other published primers to amplify the ITS2 regions from a taxonomically broad range of clitellates. Future PCR amplification ITS will hopefully be enhanced by the specific clitellate primers designed in this thesis.

## 6. Future perspectives and implications

### 6.1 The limitation and impact of COI barcoding

The focus of taxonomic endeavours has shifted its emphasis from traditional taxonomy on the basis of morphology to molecular systematics, under the assumption that species boundaries can be more objectively and effectively estimated using genetic rather than morphological information. The concept of a barcoding gap (Meyer and Paulay, 2005) is looking for a set distance threshold to separate intra-specific variation among populations of the same species from inter-specific divergences between different species. The extensive and growing amount of barcoding data highlights the limitations of a taxonomy built purely upon morphological descriptions, but it does not provide sufficient evidence for its application as a single source in species delimitation. Both intrinsic (gene trees and species trees) and extrinsic (methodological issues from sample processing to sequence generation) factors are challenges for barcoding-based species delimitation. The impression of clear barcoding gaps may be artificially caused by insufficient sampling across taxa or the geographic extent of sampling, and different divergence rates within or among lineages may give different threshold values (Wiemers and Fiedler, 2007; Bergsten et al., 2012; Carstens et al., 2013; Luo et al., 2015; Kvist, 2016). There sometimes is deep divergence within COI lineages, but nuclear data show much less variation within species (Dasmahapatra et al., 2010; Darwell et al., 2014; Martinsson et al., 2015a). This disparity between mitochondrial (e.g., COI) and nuclear loci (e.g., ITS) is likely due to their different population genetic properties. Moreover, the nuclear ITS

also maintains a low level of intra-specific and intra-genomic variation due to the concerted evolution mechanism (multiple copies of rDNA tend to homogenize over time).

It is important to keep the universal species concept in mind when we are trying to circumscribe a species. That is, integrating multiple evidence to find species boundaries will reflect the biological reality of the speciation processes much better. The maternally inherited COI barcodes may only provide a bird's view of mitochondria, and the assumption that Barcoding alone is the final solution to taxonomy is too optimistic. However, when COI barcodes are regarded merely as representative markers of a particular vouchered species, within a range of (COI sequence) variation that has been secondarily determined by integrative approaches, they may become useful for practical species identification. In other words, preliminary species hypotheses estimated from barcoding of large samples should lead to the search for additional evidence, e.g., using complementary nuclear marker (e.g., ITS) or data from high throughput sequencing (Kress et al., 2015; Coissac et al., 2016; Hollingsworth et al., 2016), to better identify known species and discover unknown species. Therefore, further refinements in our understanding of cryptic speciation within *Limnodrilus,* as well as other clitellates, may require: 1) a more comprehensive COI reference library linked to nominal species for primary identification; 2) integration of multiple loci (especially nuclear ones) or even genomic datasets; and 3) other additional lines of evidence, e.g., geographical, ecological and/or breeding data other than morphological and molecular data.

### 6.2 Integrating molecular evidence and type material in taxonomy

The taxonomic names of animal species are regulated by the International Code of Zoological Nomenclature (http://www.iczn.org/iczn/index.jsp), and each taxon should be named as an italicized Latinized binomen (a genus name with a capitalized first letter, and a lower-case species epithet. e.g., *Limnodrilus hoffmeisteri*). Species taxonomy is still based on a typological concept, i.e., a species name is attached to a single type specimen, and other specimens can be identified as this species only if it is similar "enough" to this type. Binomial names of type specimens are "anchors" for biological information about species, which has produced a reliable and steadily updated taxonomy based on morphology. However, this system has its pros and cons in the new era of molecular systematics. If the type is genetically characterized, e.g. by a DNA barcode, the taxonomic name has a link also to corresponding molecular data of other specimens. On the other hand, a type without molecular data is basically a dead end for linking traditional taxonomy with molecular evidence.

Adding DNA data to the information of morphological described species will be very valuable and time-saving, by providing more confidence to its identity so as to avoid synonymous or questionable species descriptions. Even if only a single gene sequence (e.g. COI) is available, it will still be sufficient for an initial hypothesis of phylogenetic relationships, in particular at the species level. The new species *L. sulphurensis* from USA, formally described in the

paper II, thus is published with its barcoding sequences and several deposited vouchers/type specimens. In the case of the "*L. claparedianus/cervix*" form nested in the *L. hoffmeisteri* complex (paper I), morphological evidence is insufficient to resolve whether this form is a new taxon, or *L. claparedianus,* or *L. cervix*, but molecular data from other nominal species indeed give a hint of its systematic position in the genus.

DNA-based species delimitation, however, is still not generally adopted for Clitellata. In a recent meta-barcoding study it was found that the corresponding sequences of nominal species in the COI reference library are far from satisfying (Trebitz et al., 2015). This is also partially reflected in that many contemporary taxonomists still are describing new species on morphological features only. There is no remaining original type specimen of *L. hoffmeisteri* and we have no possibility to know anything about the genetic characteristics of Claparède's (1862) material. We found specimens at the Swiss type locality closely matching morphological description of the type species. From this material, we designated and described a COI-barcoded neotype of *L. hoffmeisteri*, which corresponds to one of the ten genetically delimited species in paper I. Naming the remaining nine *Limnodrilus* species with proper Linnaean binominal names is a demanding process, involving scrutiny of the taxonomic literature and the validation of physical vouchers. Nevertheless, *L. hoffmeisteri sensu stricto* provides a baseline for further revisions of the taxonomy of the whole species complex.

### 6.3 Potential implications of cryptic species for ecological studies

The presence of cryptic species among biological indicators, e.g., *L. hoffmeisteri,* also has ramifications for the assessment of biodiversity and ecological function of these taxa. Although there are not yet clearly recognised morphological differences between the many species within the *L. hoffmeisteri* complex, these species may vary in other respects, e.g., in their life history strategies, dispersal abilities, and habitat and/or food preferences. That is, maintaining an inappropriate taxonomic resolution of these cryptic species would lead to confusion in the ecological interpretations. For future studies, therefore, the need for correlating genetic differences also with evidence of ecological and biological properties should be emphasised. Our species delimitation results provided an appropriate barcoding reference for future studies within the *L. hoffmeisteri* complex, and it may also shed a light on other clitellates studies.

## 7. Acknowledgements


*If winter comes with falling snowflake, can the blossom of the cherry trees near our Zoology building be far behind?*

(Modified from Percy Bysshe Shelley "Ode to the West Wind")

The pronunciation of the Swedish word "zoologi" in Chinese sounds like Jurassic; various colleagues work on different "dinosaurs". I work on the aquatic dragon, which refers to the elegant name of earthworm (earth dragon). Within this dinosaur zoo, I have learned to study, appreciate and think in a scientific way; hereby, I would like to express my deepest gratitude to my supervisor, Christer Erséus, for his expertise, understanding, patience, and engagement throughout my PhD studies. Christer gave me the full freedom to do whatever I wanted, at the same time continuing to contribute valuable feedback and advice. I appreciate his encyclopedic knowledge in many areas other than worms, and his help with the English grammar in my manuscripts.

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## 8. References


Achurra, A., Elejalde, M.A., Rodriguez, P., 2011. Phylogenetic analysis of oligochaete Tubificinae (Annelida: Clitellata) based on mitochondrial sequence data. Invertebr. Syst. 25, 208-218.

Achurra, A., Erséus, C., 2013. DNA barcoding and species delimitation: the *Stylodrilus heringianus* case (Annelida: Clitellata: Lumbriculidae). Invertebr. Syst. 27, 118-128.

Barbour, M., Cook, D., Pomerantz, R., 1980. On the question of hybridization and variation in the oligochaete genus *Limnodrilus*. In: Brinkhurst, R., Cook, D. (Eds.), Aquatic Oligochaete Biology. Plenum Press, New York, pp. 41-53.

Bayzid, M.S., Hunt, T., Warnow, T., 2014. Disk covering methods improve phylogenomic analyses. BMC Genomics 15, (Suppl 6):S7.

Beauchamp, K.A., Kathman, R.D., McDowell, T.S., Hedrick, R.P., 2001. Molecular phylogeny of tubificid oligochaetes with special emphasis on *Tubifex tubifex* (Tubificidae). Mol. Phylogen. Evol. 19, 216-224.

Beddard, F.E., 1895. A monograph of the order of Oligochaeta. Clarendon Press.

Bely, A.E., Wray, G.A., 2004. Molecular phylogeny of naidid worms (Annelida: Clitellata) based on cytochrome oxidase I. Mol. Phylogen. Evol. 30, 50-63.

Bergsten, J., Bilton, D.T., Fujisawa, T., Elliott, M., Monaghan, M.T., Balke, M., . . . Vogler, A.P., 2012. The Effect of Geographical Scale of Sampling on DNA Barcoding. Syst. Biol. 61, 851-869.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., . . . Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends Ecol. Evol. 22, 148-155.

Block, E.M., Goodnight, C.J., 1972. A new species of tubificid oligochaete from Central America, *Limnodrilus bulbiphallus* n. sp. Trans. Am. Microsc. Soc. 91, 579-585.

Bock, D.G., MacIsaac, H.J., Cristescu, M.E., 2012. Multilocus genetic analyses differentiate between widespread and spatially restricted cryptic species in a model ascidian. Proceedings of the Royal Society B-Biological Sciences 279, 2377-2385.

Brauer, A., 1909. Die Süsswasserfauna Deutschlands, Heft 13: Oligochaeta und Hirudinea. G. Fischer, Jena.

Bretscher, K., 1901. Beobachtungen uber Oligochæten der Schweiz. Rev. Suisse Zool. 9.

Brinkhurst, R.O., 1963. Taxonomical studies on the Tubificidae (Annelida, Oligochaeta). Internat. rev. hydrobiol. Syste. Beih 2, 1-89.

Brinkhurst, R.O., 1976. Aquatic Oligochaeta recorded from Canada and the St. Lawrence Great Lakes / by R. O. Brinkhurst. Institute of Ocean Sciences, Patricia Bay, Victoria, B.C.

Brinkhurst, R.O., 1979. A New Species Of *Limnodrilus* (Oligochaeta: Tubificidae) From Jamaica. Proc. Biol. Soc. Wash. 92, 42-44.

Brinkhurst, R.O., 1981. A Contribution To The Taxonomy Of The Tubificinae Oligochaeta Tubificidae. Proc. Biol. Soc. Wash. 94.

Brinkhurst, R.O., 1991. A Phylogenetic Analysis of the Tubificinae (Oligochaeta, Tubificidae). Canadian Journal of Zoology-Revue Canadienne De Zoologie 69, 392-397.

Brinkhurst, R.O., Cook, D.G., 1966. Studies on the North American aquatic Oligochaeta III: Lumbriculidae and additional notes and records of other families. Proc. Acad. Nat. Sci. Phila. 118, 1-33.

Brinkhurst, R.O., Jamieson, B.G.M., 1971. Aquatic Oligochaeta of the world. Oliver and Boyd, Edinburgh, Great Britain.

Brinkhurst, R.O., Marchese, M., 1989. Guide to the freshwater aquatic Oligochaeta of South and Central America. Asociación de Ciencias Naturales del Litoral, Santo Tomé.

Brinkhurst, R.O., Qi, S., Liang, Y., 1990. The aquatic Oligochaeta from the People's Republic of China. Can. J. Zool. 68, 901-916.

Carstens, B.C., Knowles, L.L., 2007. Estimating species phylogeny from gene-tree probabilities despite incomplete lineage sorting: An example from Melanoplus grasshoppers. Syst. Biol. 56, 400-411.

Carstens, B.C., Pelletier, T.A., Reid, N.M., Satler, J.D., 2013. How to fail at species delimitation. Mol. Ecol. 22, 4369-4383.

Černosvitov, L., 1939. VI. Oligochaeta. Trans.Linn.Soc.Lond. Ser 3 1, 81-116.

Chen, Y., 1940. Taxonomy and faunal relations of the limnitic Oligochaeta of China. Contributions from the Biological Laboratory of the Science Society of China. Zoological series 14.

Christensen, B., 1984. Asexual propagation and reproductive strategies in aquatic Oligochaeta. Hydrobiologia 115, 91-95.

Claparède, R.-É., 1862. Recherches anatomiques sur les Oligochètes. Mèmoires de la Société de physique et d'histoire naturelle de Genève 16, 217-291.
Coissac, E., Hollingsworth, P.M., Lavergne, S., Taberlet, P., 2016. From barcodes to genomes: extending the concept of DNA barcoding. Mol. Ecol. 25, 1423-1428.
Cosín, D.J.D., Novo, M., Fernández, R., 2011. Reproduction of Earthworms: Sexual Selection and Parthenogenesis. In: Karaca, A. (Ed.), Biology of Earthworms. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 69-86.
Cracraft, J., 1983. Species Concepts and Speciation Analysis. In: Johnston, R.F. (Ed.), Current Ornithology. Springer US, Boston, MA, pp. 159-187.
Darwell, C.T., al-Beidh, S., Cook, J.M., 2014. Molecular species delimitation of a symbiotic fig-pollinating wasp species complex reveals extreme deviation from reciprocal partner specificity. BMC Evol. Biol. 14, 189.
Dasmahapatra, K.K., Elias, M., Hill, R.I., Hoffman, J.I., Mallet, J., 2010. Mitochondrial DNA barcoding detects some species that are real, and some that are not. Molecular Ecology Resources 10, 264-273.
De Queiroz, K., 2007. Species concepts and species delimitation. Syst. Biol. 56, 879-886.
De Wit, P., Erséus, C., 2010. Genetic variation and phylogeny of Scandinavian species of Grania (Annelida: Clitellata: Enchytraeidae), with the discovery of a cryptic species. J Zool Syst Evol Res 48, 285-293.
Degnan, J.H., Rosenberg, N.A., 2009. Gene tree discordance, phylogenetic inference and the multispecies coalescent. Trends Ecol. Evol. 24, 332-340.
Drummond., A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969-1973.
Dupuis, J.R., Roe, A.D., Sperling, F.A.H., 2012. Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. Mol. Ecol. 21, 4422-4436.
Dzwillo, M., 1984. Phylogenetic and taxonomic problems in freshwater Oligochaeta with special emphasis on chitinous structures in Tubificinae. Hydrobiologia 115, 19-23.
Eisen, G.A., 1879. Preliminary report on genera and species of Tubificidae. Bihang till Kungl. Svenska Vetenskaps-Akademiens Handlingar 5, 1-25.
Eisen, G.A., 1885. Oligochaetological researches. United States Fish and Wildlife Service. Report of the U.S. Commissioner of Fisheries 11, 879–964
Envall, I.D.A., Gustavsson, L.M., Erséus, C., 2012. Genetic and chaetal variation in *Nais* worms (Annelida, Clitellata, Naididae). Zool. J. Linn. Soc. 165, 495-520.
Erséus, C., 1994. Oligochaeta and Polychaeta: Phyllodocida (Phyllodocidae to Paralacydoniidae). Taxonomic Atlas of the Benthic Fauna of the Santa Maria Basin and Western Santa Barbara Channel. Santa Barbara Museum of Natural History Santa Barbara, California.
Erséus, C., 2005. Phylogeny of oligochaetous Clitellata. Hydrobiologia 535, 357-372.
Erséus, C., Gustafsson, D., 2009. Cryptic speciation in clitellate model organisms. In: Shain, D.H. (Ed.), Annelids in Modern Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 31-46.
Erséus, C., Källersjö, M., 2004. 18S rDNA phylogeny of Clitellata (Annelida). Zool. Scr. 33, 187-196.
Erséus, C., Källersjö, M., Ekman, M., Hovmoller, R., 2002. 18S rDNA phylogeny of the Tubificidae (Clitellata) and its constituent taxa: dismissal of the Naididae. Mol. Phylogen. Evol. 22, 414-422.
Erséus, C., Prestegaard, T., Källersjö, M., 2000. Phylogenetic analysis of Tubificidae (Annelida, Clitellata) based on 18S rDNA sequences. Mol. Phylogen. Evol. 15, 381-389.
Erséus, C., Wetzel, M.J., Gustavsson, L., 2008. ICZN rules - a farewell to Tubificidae (Annelida, Clitellata). Zootaxa 1744, 66-68.
Fend., S.V., Liu, Y., Steinmann, D., Giere, O., Barton, H.A., Luiszer, F., Erséus, C., 2016. *Limnodrilus sulphurensis* n. sp., from a sulfur cave in Colorado, USA, with notes on the morphologically similar *L. profundicola* (Clitellata, Naididae, Tubificinae). Zootaxa 4066, 451-468.
Ficetola, G.F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessiere, J., . . . Pompanon, F., 2010. An in silico approach for the evaluation of DNA barcodes. BMC Genomics 11, 434.
Fisher-Reid, M.C., Wiens, J.J., 2011. What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades. BMC Evol. Biol. 11, 1-20.
Garcia, P.E., Schonswetter, P., Aguilar, J.F., Feliner, G.N., Schneeweiss, G.M., 2009. Five molecular markers reveal extensive morphological homoplasy and reticulate evolution in the Malva alliance (Malvaceae). Mol. Phylogen. Evol. 50, 226-239.

Gatesy, J., Springer, M.S., 2014. Phylogenetic analysis at deep timescales: Unreliable gene trees, bypassed hidden support, and the coalescence/concatalescence conundrum. Mol. Phylogen. Evol. 80, 231-266.
Geiger, M.F., Herder, F., Monaghan, M.T., Almada, V., Barbieri, R., Bariche, M., . . . Freyhof, J., 2014. Spatial heterogeneity in the Mediterranean biodiversity hotspot affects barcoding accuracy of its freshwater fishes. Molecular Ecology Resources 14, 1210-1221.
Grube, E., 1873. über einige bisher noch unbekannte Bewohner des Baikalsees. Jahres-Bericht der Schlesischen Gesellschaft für Vaterländische Cultur Bd.50 (1872), 66-68.
Gustafsson, D.R., Price, D.A., Erséus, C., 2009. Genetic variation in the popular lab worm *Lumbriculus variegatus* (Annelida: Clitellata: Lumbriculidae) reveals cryptic speciation. Mol. Phylogen. Evol. 51, 182-189.
Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W., Hebert, P.D.N., 2006. DNA barcodes distinguish species of tropical Lepidoptera. Proc. Natl. Acad. Sci. U. S. A. 103, 968-971.
Halanych, K.M., Janosik, A.M., 2006. A review of molecular markers used for annelid phylogenetics. Integr. Comp. Biol. 46, 533-543.
Hallett, S., Atkinson, S., Bartholomew, J., 2005. Countering morphological ambiguities: development of a PCR assay to assist the identification of *Tubifex tubifex* oligochaetes. Hydrobiologia 543, 305-309.
Harper, J.T., Gile, G.H., James, E.R., Carpenter, K.J., Keeling, P.J., 2009. The Inadequacy of Morphology for Species and Genus Delineation in Microbial Eukaryotes: An Example from the Parabasalian Termite Symbiont Coronympha. PLoS One 4.
Hatai, S., 1899. On *Limnodrilus gotoi,* n. sp. Annotationes zoologicae Japonenses 3, 5-12.
He, X., Cui, Y., Wang, H., 2010. *Limnodrilus simplex* sp. nov. (Oligochaeta: Naididae: Tubificinae) from Changjiang River, China. Zool. Sci. 27, 768-770.
Hebert, P.D., Penton, E.H., Burns, J.M., Janzen, D.H., Hallwachs, W., 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc. Natl. Acad. Sci. U. S. A. 101, 14812-14817.
Hebert, P.D.N., Cywinska, A., Ball, S.L., DeWaard, J.R., 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B-Biological Sciences 270, 313-321.
Heckman, K.L., Mariani, C.L., Rasoloarison, R., Yoder, A.D., 2007. Multiple nuclear loci reveal patterns of incomplete lineage sorting and complex species history within western mouse lemurs (Microcebus). Mol. Phylogen. Evol. 43, 353-367.
Heled, J., Drummond, A.J., 2010. Bayesian inference of species trees from multilocus data. Mol. Biol. Evol. 27, 570-580.
Hiltunen, J.K., 1967. Some oligochaetes from lake Michigan. Trans. Am. Microsc. Soc. 86, 433-454.
Hiltunen, J.K., 1969. Distribution of oligochaetes in western Lake Erie, 1961. Limnol. Oceanogr. 14, 260-264.
Hollingsworth, P.M., Li, D.Z., van der Bank, M., Twyford, A.D., 2016. Telling plant species apart with DNA: from barcodes to genomes. Philosophical Transactions of the Royal Society B-Biological Sciences 371.
Howmiller, R.P., 1974. Studies on aquatic Oligochaeta in inland waters of Wisconsin. Trans. Wisc. Acad. Sci. Arts Lett. 62, 337-356.
James, S.W., Davidson, S.K., 2012. Molecular phylogeny of earthworms (Annelida: Crassiclitellata) based on 28S, 18S and 16S gene sequences. Invertebr. Syst. 26, 213-229.
Kathman, R.D., Brinkhurst, R.O., 1998. Guide to the freshwater oligochaetes of North America. Aquatic Resources Center, Tennessee
Kennedy, C.R., 1965. The distribution and habitat of *Limnodrilus* Claparède (Oligochaeta: Tubificidae). Oikos 16, 26-38.
Kress, W.J., Garcia-Robledo, C., Uriarte, M., Erickson, D.L., 2015. DNA barcodes for ecology, evolution, and conservation. Trends Ecol. Evol. 30, 25-35.
Krieger, K.A., 1984. Benthic Macroinvertebrates as Indicators of Environmental Degradation in the Southern Nearshore Zone of the Central Basin of Lake Erie. J. Great Lakes Res. 10, 197-209.
Kruger, M., Kruger, C., Walker, C., Stockinger, H., Schussler, A., 2012. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. New Phytol. 193, 970-984.
Kubatko, L.S., Degnan, J.H., 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. Syst. Biol. 56, 17-24.
Kvist, S., 2016. Does a global DNA barcoding gap exist in Annelida? Mitochondrial DNA 27, 2241-2252.

Kvist, S., Sarkar, I.N., Erséus, C., 2010. Genetic variation and phylogeny of the cosmopolitan marine genus *Tubificoides* (Annelida: Clitellata: Naididae: Tubificinae). Mol. Phylogen. Evol. 57, 687-702.

Liu, L.A., Yu, L.L., Edwards, S.V., 2010. A maximum pseudo-likelihood approach for estimating species trees under the coalescent model. BMC Evol. Biol. 10.

Loden, M.S., 1977. Two New Species of *Limnodrilus* (Oligochaeta-Tubificidae) from Southeastern United-States. Trans. Am. Microsc. Soc. 96, 321-326.

Luo, A.R., Lan, H.Q., Ling, C., Zhang, A.B., Shi, L., Ho, S.Y.W., Zhu, C.D., 2015. A simulation study of sample size for DNA barcoding. Ecology and Evolution 5, S869-S879.

Marotta, R., Ferraguti, M., Erséus, C., Gustavsson, L.M., 2008. Combined ‐ data phylogenetics and character evolution of Clitellata (Annelida) using 18S rDNA and morphology. Zool. J. Linn. Soc. 154, 1-26.

Martinsson, S., Achurra, A., Svensson, M., Erséus, C., 2013. Integrative taxonomy of the freshwater worm *Rhyacodrilus falciformis* s.l. (Clitellata: Naididae), with the description of a new species. Zool. Scr. 42, 612-622.

Martinsson, S., Erséus, C., 2014. Cryptic diversity in the well-studied terrestrial worm *Cognettia sphagnetorum* (Clitellata: Enchytraeidae). Pedobiologia 57, 27-35.

Martinsson, S., Rhodén, C., Erséus, C., 2015a. Barcoding gap, but no support for cryptic speciation in the earthworm *Aporrectodea longa* (Clitellata: Lumbricidae). Mitochondrial DNA, 1-9.

Martinsson, S., Rota, E., Erséus, C., 2015b. Revision of Cognettia (Clitellata, Enchytraeidae): re-establishment of Chamaedrilus and description of cryptic species in the sphagnetorum complex. Syst. Biodivers. 13, 257-277.

Marton, S., Eszterbauer, E., 2012. The susceptibility of diverse species of cultured oligochaetes to the fish parasite *Myxobolus pseudodispar* Gorbunova (Myxozoa). J. Fish Dis. 35, 303-314.

Matisoff, G., Wang, X., McCall, P.L., 1999. Biological redistribution of lake sediments by tubificid oligochaetes: *Branchiura sowerbyi* and *Limnodrilus hoffmeisteri/Tubifex tubifex*. J. Great Lakes Res. 25, 205-219.

Mayden, R.L., 1997a. A hierarchy of species concepts: the denouement in the saga of the species problem. Species: The units of biodiversity.

Mayden, R.L., 1997b. A hierarchy of species concepts: the denouement in the saga of the species problem. In: Claridge, M.F., Dawah, H.A., Wilson, M.R. (Eds.), Species: The units of diversity. Chapman and Hall, pp. 381–423.

Meyer, C.P., Paulay, G., 2005. DNA Barcoding: Error Rates Based on Comprehensive Sampling. PLoS Biol. 3, e422.

Michaelsen, W., 1900. Oligochaeta R. Friedländer und Sohn, Berlin.

Mirarab, S., Bayzid, M.S., Warnow, T., 2014. Evaluating summary methods for multilocus species tree estimation in the presence of incomplete lineage sorting. Syst. Biol., 1-15.

Mott, T., Vieites, D.R., 2009. Molecular phylogenetics reveals extreme morphological homoplasy in Brazilian worm lizards challenging current taxonomy. Mol. Phylogen. Evol. 51, 190-200.

Mozley, S., Howmiller, R.P., 1977. Environmental status of the Lake Michigan region. Volume 6. Zoobenthos of Lake Michigan. Argonne National Lab., Ill.(USA).

Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., Larsson, K.H., 2008. Intraspecific ITS Variability in the Kingdom Fungi as Expressed in the International Sequence Databases and Its Implications for Molecular Species Identification. Evolutionary Bioinformatics 4, 193-201.

Nomura, E., 1913. On two species of aquatic Oligochaeta, *Limnodrilus gotoi* HATAI and *Limnodrilus willeyi*, n. sp. Imperial University of Tokyo 35, 1-49.

Nomura, E., 1932. *Limnodrilus grandisetosus* nov. sp., a freshwater oligochaete. Science Reports of the Tohoku Imperial University. Series IV, Biology. 7, 511-527.

Nygren, A., Pleijel, F., 2011. From one to ten in a single stroke–resolving the European Eumidasanguinea (Phyllodocidae, Annelida) species complex. Mol. Phylogen. Evol. 58, 132-141.

Oztetik, E., Cicek, A., Arslan, N., 2013. Early antioxidative defence responses in the aquatic worms (*Limnodrilus* sp.) in Porsuk Creek in Eskisehir (Turkey). Toxicol. Ind. Health 29, 541-554.

Pagel, M., Meade, A., 2013. BayesTraits v. 2.0. http://www.evolution.rdg.ac.uk/BayesTraits.html

Paz, A., Crawford, A.J., 2012. Molecular-based rapid inventories of sympatric diversity: a comparison of DNA barcode clustering methods applied to geography-based vs clade-based sampling of amphibians. J. Biosci. 37, 887-896.

Pecnikar, Z.F., Buzan, E.V., 2014. 20 years since the introduction of DNA barcoding: from theory to application. J. Appl. Genet. 55, 43-52.

Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., . . . Vogler, A.P., 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol. 55, 595-609.

Porras-Alfaro, A., Liu, K.L., Kuske, C.R., Xie, G., 2014. From Genus to Phylum: Large-Subunit and Internal Transcribed Spacer rRNA Operon Regions Show Similar Classification Accuracies Influenced by Database Composition. Appl. Environ. Microbiol. 80, 829-840.

Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol. Ecol. 21, 1864-1877.

Ratzel, V.F., 1868. Beiträge zur anatomischen und systematischen Kenntniss der Oligochaeten. Z. Wiss. Zool. 18, 563-591

Reid, N.M., Carstens, B.C., 2012. Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. BMC Evol. Biol. 12: 196.

Ride, W., 1999. International code of zoological nomenclature. International Trust for Zoological Nomenclature.

Rodriguez, P., Reynoldson, T.B., 2011. The pollution biology of aquatic oligochaetes. Springer, Netherlands.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., . . . Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539-542.

Rousset, V., Pleijel, F., Rouse, G.W., Erséus, C., Siddall, M.E., 2007. A molecular phylogeny of annelids. Cladistics 23, 41-63.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., . . . Consortium, F.B., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. U. S. A. 109, 6241-6246.

Semernoy, 1982. Novye vidy oligokhet iz ozera Baikal [New oligochaete species from Lake Baikal]. In: G.I.Galazii (Ed.), Novoe o faune Baikala [New on fauna of Baikal]. Novosibirsk, USSR, Canadian Translation of Fisheries and Aquatic Sciences No 5023 (1983). pp. 58-85.

Shain, D.H., 2009. Annelids in Modern Biology. John Wiley & Sons, Inc., Hoboken, New Jersey.

Shekhovtsov, S.V., Golovanova, E.V., Peltek, S.E., 2013. Cryptic diversity within the Nordenskiold's earthworm, *Eisenia nordenskioldi* subsp. *nordenskioldi* (Lumbricidae, Annelida). Eur. J. Soil Biol. 58, 13-18.

Simpson, I.C., Roger, P.A., Oficial, R., Grant, I.F., 1993. Density and composition of aquatic oligochaete populations in different farmers' ricefields. Biol. Fertil. Soils 16, 34-40.

Southern, R., 1909. Contributions towards a monograph of the British and Irish Oligochæta. Proc. R. Ir. Acad. B 27, 119-182.

Sparks, R.E., Tazik, P.P., Blodgett, K.D., Warren, G.L., Wetzel, M.J., 1986. Des Plaines River Long-Term Monitoring Program: Phase 1. INHS Aquatic Biology Section 1986 (6).

Spooner, D.M., 2009. DNA Barcoding will frequently fail in complicated groups: an example in wild potatoes. Am. J. Bot. 96, 1177-1189.

Stamatakis, A., Ludwig, T., Meier, H., 2005. RAxML-II: a program for sequential, parallel and distributed inference of large phylogenetic. Concurrency and Computation-Practice & Experience 17, 1705-1723.

Steinlechner, R., 1988. Identification of immature tubificids (Oligochaeta) of Lake Constance and its influence on the evaluation of species distribution. Hydrobiologia 155, 57-63.

Stimpson, K.S., Klemm, D.J., Hiltunen, J.K., Monitoring, E., Laboratory, S., 1982. A Guide to the Freshwater Tubificidae (Annelida: Clitellata: Oligochaeta) of North America. Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H., Vogler, A.P., 2003. A plea for DNA taxonomy. Trends Ecol. Evol. 18, 70-74.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31, 21-32.

Tonini, J., Moore, A., Stern, D., Shcheglovitova, M., Orti, G., 2015. Concatenation and species tree methods exhibit statistically indistinguishable accuracy under a range of simulated conditions. PLoS Currents 7.

Trebitz, A.S., Hoffman, J.C., Grant, G.W., Billehus, T.M., Pilgrim, E.M., 2015. Potential for DNA-based identification of Great Lakes fauna: match and mismatch between taxa inventories and DNA barcode libraries. Sci. Rep. 5, :12162.

Trontelj, P., Sket, B., 2000. Molecular re-assessment of some phylogenetic, taxonomic and biogeographic relationships between the leech genera *Dina* and *Trocheta* (Hirudinea: Erpobdellidae). Hydrobiologia 438, 227-235.
Trontelj, P., Utevsky, S.Y., 2012. Phylogeny and phylogeography of medicinal leeches (genus *Hirudo*): Fast dispersal and shallow genetic structure. Mol. Phylogen. Evol. 63, 475-485.
van Haaren, T., Soors, J., 2013. Aquatic oligochaetes of the Netherlands and Belgium. KNNV Publishing, Zeist, The Netherlands.
Vejdovský, F.e., 1884. System und Morphologie der Oligochaeten. Franz Řivnáč, Prag.
Verrill, A.E., 1871. Notice of the Invertebrata Dredged in Lake Superior in 1871, by the U. S. Lake Survey, under the direction of Gen. G. B. Gomstock, S. I. Smith, naturalist;. Am. j. sci. arts 2, 448–454.
Wang, H.Z., Liang, Y.L., 2001. A preliminary study of oligochaetes in Poyang Lake, the largest freshwater lake of China, and its vicinity, with description of a new species of Limnodrilus. Hydrobiologia 463, 29-38.
Wang, X.-C., Liu, C., Huang, L., Bengtsson-Palme, J., Chen, H., Zhang, J.-H., . . . Li, J.-Q., 2015. ITS1: a DNA barcode better than ITS2 in eukaryotes? Molecular Ecology Resources 15, 573-586.
Weigert, A., Bleidorn, C., 2016. Current status of annelid phylogeny. Organisms Diversity & Evolution 16, 345-362.
Wetzel, M.J., 1987. *Limnodrilus tortilipenis*, A New North american Species Of Fresh water Tubificidae (Annelida clitellata-Oligochaeta). Proc. Biol. Soc. Wash. 100.
Wheeler, Q., Meier, R., 2000. Species Concepts and Phylogenetic Theory: A Debate. Columbia University Press.
Wickett, N.J., Mirarab, S., Nguyen, N., Warnow, T., Carpenter, E., Matasci, N., . . . Leebens-Mack, J., 2014. Phylotranscriptomic analysis of the origin and early diversification of land plants. Proc. Natl. Acad. Sci. U. S. A. 111, E4859-E4868.
Wiemers, M., Fiedler, K., 2007. Does the DNA barcoding gap exist? - a case study in blue butterflies (Lepidoptera: Lycaenidae). Frontiers in Zoology 4, :8.
Wiens, J.J., Penkrot, T.A., 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (Sceloporus). Syst. Biol. 51, 69-91.
Will, K.W., Mishler, B.D., Wheeler, Q.D., 2005. The perils of DNA barcoding and the need for integrative taxonomy. Syst. Biol. 54, 844-851.
Xi, Z.X., Liu, L., Rest, J.S., Davis, C.C., 2014. Coalescent versus concatenation methods and the placement of *amborella* as sister to water lilies. Syst. Biol. 63, 919-932.
Zhang, L., Shang, J., He, W., You, B., Fan, C., 2014. The role of tubificid worms (*Limnodrilus hoffmeisteri*) in sediment resuspension: a microcosm study. Annales de Limnologie - International Journal of Limnology 50, 253-260.
Zimmermann, T., Mirarab, S., Warnow, T., 2014. BBCA: Improving the scalability of *BEAST using random binning. BMC Genomics 15, (Suppl 6):S11.

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