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DNA-based phylogeny of the marine genus *Heterodrilus* (Annelida, Clitellata, Naididae)

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Abstract

Heterodrilus is a group of marine Naididae, common worldwide in subtropical and tropical areas, and unique among the oligochaetes by their tridentate chaetae. The phylogenetic relationships within the group are assessed from the nuclear 18S rDNA gene, and the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rDNA genes. Sequence data were obtained from 16 *Heterodrilus* species and 13 out-group taxa; 48 sequences are new for this study. The data were analysed by Bayesian inference. Monophyly of the genus is corroborated by the resulting tree, with *Heterodrilus ersei* (a taxon representing a small group of species with aberrant male genitalia) proposed to be outside all other sampled species. Although earlier regarded as a member of the subfamily Rhyacodrilinae, both molecular and morphological data seem to support that *Heterodrilus* is closely related to Phallodrilinae. However, the results are not conclusive as to whether the genus is the sister group of, or a group nested inside, or separate from this latter subfamily. The studied sample of species suggests at least two major clades in *Heterodrilus* with different geographical distributions, in one of the clades, most species are from the Indo-West Pacific Ocean, while in the other, the majority are from the Western Atlantic Ocean. Morphological characters traditionally used in *Heterodrilus* taxonomy are optimized on the phylogenetic tree, revealing a high degree of homoplasy.

Key words: 16S rDNA gene – 18S rDNA gene – Cytochrome *c* oxidase subunit I – *Heterodrilus* – phylogeny

Introduction

Heterodrilus is a marine group of small clitellates that occurs interstitially in sandy sediments from the intertidal zone down to about 150 m depth. It was traditionally classified as a genus within 'Tubificidae', which is now regarded as a paraphyletic assemblage within Naididae (Erséus et al. 2008). *Heterodrilus* has been recorded from localities in the Mediterranean Sea, the Northwest Atlantic Ocean (including the Caribbean), the Galapagos Islands and the Indo-West Pacific Region. It was one of the first marine oligochaete genera to be described and was established for *H. arenicolus* Pierantoni 1902; found in the Bay of Naples, Italy. Since then, 41 additional species have been described as belonging to, or transferred to, this genus (Erséus 1981, Erséus 1985, 1986, 1988, 1990, 1992a,b, 1993, 1997a,b; Erséus and Wang 2003; Milligan 1987; Sjölin and Erséus 2001; Takashima and Mawatari 1997; Wang and Erséus 2003), and as it is a species-rich and widely distributed genus, it is likely that there are numerous species yet to be described. A majority of the species of *Heterodrilus* are characterized by having trifid (or 'tridentate') anterior chaetae, that is chaetae with three teeth at the distal end (Fig. 1). A few species have bifid anterior chaetae, but Erséus (1990) regarded these taxa to have lost the third tooth secondarily. Different species are morphologically distinguished by details in the form and number of chaetae, and the external and internal features of genital structures. So far, identification has largely depended on the access to sexually mature specimens, and the species have been recognized by their unique character combinations rather than by hierarchical sets of apomorphies.

The systematic position of *Heterodrilus* within Naididae (formerly Tubificidae; see Erséus et al. 2008) was long problematic. The genus was assigned to the subfamily Rhyacodrilinae based on morphological features (Erséus 1981), but using molecular data, it was later suggested to be a member of, or at least close to, Phallodrilinae (Erséus et al. 2000, 2002, 2010; Sidall et al. 2001; Sjölin et al. 2005; Envall et al. 2006). The genus has been taxonomically revised twice. In 1981, Erséus scrutinized all naidid species with trifid chaetae, and intuitively recognized three separate genera: *Heterodrilus* Pierantoni 1902; *Heterodriloides* Erséus 1981 and *Giereidrilus* Erséus 1981. The monotypic *Heterodriloides* was distinguished from *Heterodrilus* by two main features: its spermatheca are located in segment XII, that is in the segment immediately posterior to the one bearing the male gonopores, with a supplementary pair generally located in XI, and its vasa deferentia enter the ectal part of the atrium. In *Heterodrilus* and other naidids, the normal position of the spermatheca is in the segment immediately anterior to the one bearing the male gonopores, and the vasa deferentia enter the apical, ental part of the atrium. *Giereidrilus* was established for two species with unpaired spermathecal and male gonopores, and atria that are not internally ciliated.

The first formal phylogenetic assessment of *Heterodrilus* was that of Erséus (1990). It was based on anatomical studies of all 24 species then known, as well as of *Heterodriloides* and *Giereidrilus*. Erséus used parsimony to analyse a data matrix of 15 morphological characters, with a hypothetical ancestor as the out-group, and subjectively weighted some of the characters to reduce the number of equally parsimonious trees. He concluded that *Heterodriloides* and *Gieredrilus* are derived within *Heterodrilus* and therefore synonymized them with the latter. In a molecular systematic study of the Naididae, Sjölin et al. (2005) included eight *Heterodrilus* species and monophyly of the group was corroborated. However, rather than being nested within *Heterodrilus*, *Gieredrilus ersei* (Giere 1979) was placed as the sister taxon to the rest of the group. Still, however, the phylogenetic relationships within *Heterodrilus* have been only tentatively studied.

The aim of this study was to present a DNA-based hypothesis of the phylogeny within *Heterodrilus*, using a larger sample of taxa, and combining data from two rapidly evolving mitochondrial genes, that is the protein-coding cytochrome *c* oxidase subunit I (COI) gene, and the ribosomal 16S rDNA gene, with those of the more slowly evolving nuclear ribosomal 18S rDNA gene.

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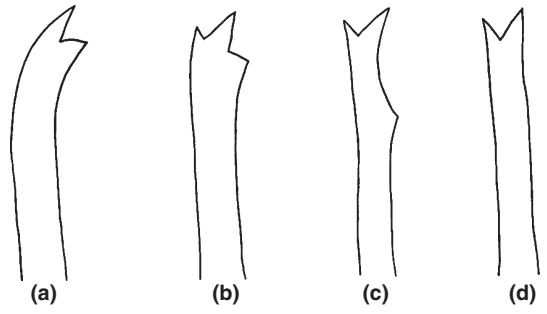

Fig. 1. Different types of preclitellar somatic chaetae within Naididae. (a) Bifid chaeta typical for the majority of species, (b–d) chaetal types found in *Heterodrilus*

Material and Methods

Taxon sampling and collection of new specimens

Sixteen *Heterodrilus* species were designated as the ingroup (Table 1), including one unidentified species from New Caledonia. Unfortunately, the type species (*H. arenicolus)* is not included. *Heterodrilus arenicolus* has not been reported again since the work of its original author (Pierantoni 1902, 1917), but formalin-fixed specimens (i.e. not suitable for DNA analysis) from the Dutch North Sea, and recently identified as this species by the last author (courtesy Ton van Haaren), are in all morphological details 'typical' *Heterodrilus*. Representatives of 11 other naidid genera (most of which belonging to the subfamily Phallodrilinae) and two additional clitellate families (Phreodrilidae and Enchytraeidae) were regarded as out-group. *Buchholzia fallax* was provided by Emilia Rota (University of Siena, Siena, Italy), *Insulodrilus bifidus* by Adrian Pinder (Department of Parks and Wildlife, Kensington, Western Australia), all other specimens were collected by the first or last author. Some of the first worms to be used in this study (10–15 years ago) were collected specifically for DNA work, and to maximize the amount of DNA template, no tissue was saved to serve as a voucher. These specimens were identified using the original or revised descriptions of the respective species in the primary taxonomic literature (see references listed in Introduction above), live in seawater under a coverslip, using a compound microscope, and then preserved whole in 95% ethanol; all tissue was then used for DNA extraction. In the more recent material, each worm was bisected and the posterior part was placed in 95% ethanol (to be used for DNA extraction later on), and the anterior part including the clitellar region was fixed in either ethanol or Bouin's fluid, and later stained in paracarmine and mounted in Canada balsam on a microscope slide (to serve as a voucher specimen). Specimens included in the study, with their taxonomy, locality data and voucher (when present), and GenBank accession numbers are specified in Table 1. Vouchers are deposited in the Swedish Museum of Natural History (SMNH), Stockholm.

Extraction, gene amplification and sequencing

DNA was extracted from whole specimens or from the posterior part of voucher specimens using the DNAeasy Tissue Kit (Qiagen®) following the protocol supplied by the manufacturer. For other taxa, additional gene amplifications were made from extracted DNA samples already used by Erséus et al. (2000, 2002), and Sjölin et al. (2005). As specified in Table 1, 48 sequences (those set in boldface) are new, 34 are already published. For each taxon with a combination of new and old sequences, all sequences are from the same individual. Amplifications were carried out with Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) as 25 μl reactions.

All PCR and sequencing primers are described in Table 2. For 18S rDNA, about 1800 bp were amplified as two overlapping segments, ca 1100 bp each, in a nested PCR. The entire fragment was first amplified with primers Tim A and Tim B, and two fragments were subsequently amplified from the first PCR with primers Tim A and 1100R and 660F and Tim B, respectively. The thermal cycle profile for the initial PCR was 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s with an initial single denaturing step at 95°C for 5 min and a final single extension step at 72°C for 8 min. In the nested PCR, 30 cycles were used with an annealing temperature of 54°C for Tim A and 1100R and 55°C for 660F and Tim B.

The 16S rDNA amplification was performed using the primers 16SAnnF and 16SAnnR. The thermal profile was as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s; and 72°C for 8 min.

For most taxa, the primers used to amplify COI were LCO1490 and COI-E^{-}; in a few cases, LCO1490 was replaced by primer AnnCOIF, and for one taxon, the 'universal' primers LCO1490 and HCO2198 were used. The amplification profile was as follows: 95°C for 5 min; 40 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s; and 72°C for 8 min.

The PCR products were purified using QIAquick™ PCR Purification Kit (Qiagen®) or with ExoSAP-IT (USB®). Some sequencing reactions were performed with Perkin Elmer Applied BioSystems PRISM terminator cycle sequencing kits with AmpliTaq FS polymerase with BigDye terminators, following the manufacturer's protocol, and sequenced on an ABI PRISM 377 sequencer (Applied BioSystems) or on an ABI PRISM 3100 automated sequencer (Applied BioSystems). In other cases, DNA sequencing was performed by Macrogen (Seoul, Korea). Both strands were sequenced for each gene, and the fragments obtained with different primers were assembled to complete sequences using the STADEN Package (Staden et al. 1998) or GENEIOUS PRO v. 5.5.6 (Biomatters Ltd.). Positions for which the nucleotide could not be determined with certainty were coded with the appropriate IUPAC code.

GenBank data

Several sequences of ingroup and out-group taxa were accessed from GenBank (i.e. sequence numbers not set in boldface in Table 1). However, GenBank data for one additional species, *Heterodrilus keenani* Erséus 1981, were excluded as we suspect its published COI sequence (AY040703) to be a contamination. Moreover, as explained by Kvist et al. (2010), two previously published sequences of *Heterochaeta costata* (i.e. one of our out-groups; see Table 1) were erroneously identified as coming from another marine naidid, *Tubificoides pseudogaster* by the original authors (18S rDNA/AF411873 by Erséus et al. 2002; 16S rDNA/AY885609 by Sjölin et al. 2005). All newly generated DNA sequences were deposited into GenBank (Accession #KJ753848-KJ753898).

Data analysis

For sequence alignment, MAFFT ver. 6 (Katoh and Toh 2008) was used, applying the L-INS-i setting (slow-accurate) to create matrices for the three loci 16S rDNA, 18S rDNA and COI, with 633 [321 parsimony-informative (p-informative)], 1805 (76 p-informative) and 658 characters (302 p-informative), respectively.

Evolutionary models of best fit were chosen using the Akaike information criterion (AIC) implemented by MrModeltest 2.2 (Nylander 2004) within PAUP*4.0 (Swofford 2002). For the COI locus, each codon position was tested for model of best fit independently – the models were determined to be GTR + I + G for the first and second codon positions and GTR + G for the third. For the 16S rDNA and 18S rDNA alignments, MrModeltest determined that GTR + I + G was the most appropriate model. As the mitochondrial loci (16S and COI) evolve together, the two alignments were then joined together into one matrix partitioned by locus and also by codon position for COI. All parameters except topology were unlinked between partitions. An additional matrix was also created by combining the mitochondrial data with data from the nuclear 18S locus, also here partitioning the alignments both after locus and codon position (in the COI region of the alignment).

In the parallel version of MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003), two separate MCMC analyses were run for each alignment matrix (mtDNA only, 18S only and combined mtDNA+18S), each with 4 Markov chains (one cold and three hot), for 50 million generations, sampling once every 1000 generations. Default MCMC settings for MrBayes were used, except for a change in the branch length prior [Unconstrained: Exponential(100)], to avoid inflation of branch lengths, which has been shown to be an issue, particularly in partitioned Bayesian inference analyses (Brown et al. 2010). The resulting tree files were examined for convergence using the AWTY online software (Wilgenbusch et al. 2004;

Table 1. Taxa used, places of origin, voucher numbers and GenBank accession numbers for the 18S, 16S and COI sequences. New sequences are indicated in boldface. Two asterisked GenBank entries (*) were originally (erroneously) identified as representing *Tubificoides pseudogaster* (see Kvist et al. 2010, p. 695).

Taxon	Spm	Locality	Voucher	18S	16S	COI
INGROUP: Clitellata, Naididae						
Heterodrilus bulbiporus Erséus 1981;	CE935	Fort Pierce, Florida, USA	SMNH136249	**KJ753886**	**KJ753872**	**KJ753850**
Heterodrilus chenianus Wang and Erséus 2003;	CE142	Hainan, China	No voucher	AY885574	AY885601	**KJ753856**
Heterodrilus decipiens Erséus 1997a;	CE10	Rottnest Island, W Australia	No voucher	AF209455	AY885603	–
Hetetodrilus devexus Erséus 1997a;	CE163	Dampier, W Australia	SMNH136250	AY885575	AY885602	–
Heterodrilus ersei (Giere 1979)	CE78	Lee Stocking Isl., Bahamas	No voucher	AY885576	AY885606	**KJ753857**
Heterodrilus flexuosus Erséus 1990;	CE11	Carre Bow Cay, Belize	No voucher	AY885573	AY885600	–
Heterodrilus jamiesoni Erséus 1981;	CE39	Queensland, Australia	No voucher	AF411883	**KJ753875**	**KJ753860**
Heterodrilus minisetosus Erséus 1981;	CE74	Lee Stocking Isl., Bahamas	No voucher	AF411885	AY885599	**KJ753859**
Heterodrilus modestus Erséus 1990;	ES64	Lee Stocking Isl., Bahamas	SMNH136251	**KJ753888**	**KJ753876**	**KJ753855**
Heterodrilus occidentalis Erséus 1981;	CE938	Fort Pierce, Florida, USA	No voucher	**KJ753889**	**KJ753877**	–
Heterodrilus paucifascis Milligan 1987;	CE12	Carrie Bow Cay, Belize	No voucher	AF411865	AY885605	**KJ753858**
Heterodrilus pentcheffi Erséus 1981;	ES22	Lee Stocking Isl., Bahamas	SMNH136252	**KJ753890**	**KJ753878**	**KJ753854**
Heterodrilus perkinsi Erséus 1986;	CE931	Fort Pierce, Florida, USA	No voucher	**KJ753891**	**KJ753879**	**KJ753849**
Heterodrilus queenslandicus (Jamieson, 1977)	CE29	Heron Island, Australia	No voucher	AF411881	AY885604	–
Heterodrilus cf. *virilis* Erséus 1992a	CE1314	Lizard Island, Australia	SMNH136253	**KJ753892**	**KJ753880**	**KJ753853**
Heterodrilus (undescribed species)	CE235	Lifou, New Caledonia	No voucher	**KJ753893**	**KJ753874**	**KJ753852**
OUTGROUPS: Clitellata, Naididae						
Heronidrilus heronae (Erséus & Jamieson, 1981)	CE40	Heron Island, Australia	No voucher	AF209454	AY885616	**KJ753861**
Pirodrilus minutus (Hrabe, 1973)	CE200	Koster area, SW Sweden	SMNH66072	AF209463	AY885590	**KJ753865**
Pectinodrilus rectisetosus (Erséus, 1979)	CE153	Elba, Italy	SMNH63183	AF209462	AY885598	**KJ753864**
Aktedrilus arcticus (Erséus, 1978)	CE37	Koster area, SW Sweden	No voucher	AF209451	AY885591	AF064042
Adelodrilus pusillus Erséus, 1978	CE3258	Koster area, SW Sweden	SMNH137039	**KJ753894**	**KJ753881**	**KJ753867**
Peosidrilus biprostatus (Baker & Erséus, 1979)	CE929	Fort Pierce, Florida, USA	No voucher	**KJ753895**	**KJ753882**	**KJ753870**
Thalassodrilus prostatus (Knöllner, 1935)	CE970	Göteborg, SW Sweden	SMNH137038	**KJ753896**	**KJ753883**	**KJ753871**
Gianius aquadulcis (Hrabe, 1960)	CE654	Ihreviken, Gotland, Sweden	SMNH137037	**KJ753897**	**KJ753884**	**KJ753868**
Heterochaeta costata Claparède, 1863	CE187-1	Koster area, SW Sweden	No voucher	AF411873*	AY885609*	**KJ753863**
Inanidrilus leukodermatus (Giere, 1977)	CE901	Flatts Inlet, Bermuda	No voucher	**KJ753898**	**KJ753885**	**KJ753869**
Bathydrilus rohdei (Jamieson, 1977)	CE30	Heron Island, Australia	No voucher	AF411882	AY885618	**KJ753866**
Clitellata, Enchytraeidae						
Buchholzia fallax Michaelsen, 1887	CE24	Toscana (soil), Italy	No voucher	AF411895	AY885581	**KJ753848**
Clitellata, Phreodrilidae						
Insulodrilus bifidus Pinder and Brinkhurst, 1997	CE271	Bow River, W Australia	No voucher	AF411906	AY885636	**KJ753862**

Nylander et al. 2008) and were subsequently summarized using a burn-in of 10 million generations to calculate statistical support values for the clades. Support values higher than 0.8 were plotted on the majority-rule consensus trees, which were extracted from the tree files. All trees were rooted with *Buchholzia* and *Insulodrilus*.

To examine the robustness of the results of the model-based analysis, the three-alignment matrices were also analysed using a parsimony optimality criterion and a bootstrap resampling scheme. This was performed in PAUP*4.0 (Swofford 2002), using 1000 pseudoreplicates with 10 random addition-sequences each and a TBR branch-swapping algorithm, saving the shortest tree after each pseudoreplicate. Bootstrap proportions > 70 were added to the Bayesian consensus trees for comparison.

Results

Gene tree concordance

The gene trees based on the two loci provide statistical support at different levels, with no topological conflicts whatsoever, indicating that the loci can be combined without violating the assumption of identical gene tree topologies (Figs S1–2). In the 18S rDNA tree (Fig. S1), monophyly of *Heterodrilus* is strongly supported (pp = 0.99, bootstrap support 92), and its close relationship to the Phallodrilinae (including *Bathydrilus rohdei*) has maximal support; Phallodrilinae as such is supported by pp = 0.96. Otherwise, relationships within *Heterodrilus* are unresolved. The mtDNA tree (Fig. S2) gives maximal support for *Heterodrilus* (with *H. ersei* as sister group of all other species) and for a group containing all Phallodrilinae except *Bathydrilus rohdei*. Further, it places *B. rohdei* in an unresolved group together with *Heterodrilus, Heronidrilus heronae* and *Heterochaeta costata*; this group, however, have moderate support only (pp = 0.93). The Phallodrilinae (i.e. here excluding *B. rohdei*) is the sister group of this group, supported by pp = 1. The parsimony bootstrap analysis also supports *Heterodrilus* (87) and some relationships within the genus, but could not resolve relationships within the Phallodrilinae.

Analysis of the combined data set

The majority-rule consensus tree from the Bayesian inference (BI) analysis is shown in Fig. 2. Fourteen of its nodes are supported by a posterior probability (pp) ≥ 0.95, and 13 of them have pp = 1. Monophyly of *Heterodrilus* (pp = 1) is corroborated, as is monophyly of a group containing all Phallodrilinae except *Bathydrilus rohdei*; *Bathydrilus rohdei* is virtually unresolved from the other two groups. Within the *Heterodrilus* clade, the Caribbean *H. ersei* is the sister group of all remaining species (pp = 1, bootstrap support 80), and the latter form three clades (see Fig. 2): clade A (pp = 1), clade B (pp = 0.98) and *H. paucifascis*. Clades A+B are suggested as sister group of *H. paucifascis*, a Caribbean species, but this is weakly supported (pp = 0.92). Within clade A, six Indo-Pacific species (*H.* cf. *viri-*

Table 2. Primers used for PCR and sequencing in this study

Primer name	Used for	Primer sequence	Reference
18S Primers			
Tim A	PCR, sequencing	5′-AMCTGGTTGATCCTGCCAG-3′	Tim Littlewood (pers.comm. in Norén and Jondelius 1999)
Tim B	PCR, sequencing	5′-TGATCCATCTGCAGGTTCACCT-3′	Tim Littlewood (pers.comm. in Norén and Jondelius 1999)
660F	PCR, sequencing	5′-GATCTCGGGTCCAGGCT-3′	Erséus et al. (2002)
1100R	PCR, sequencing	5′-GATCGTCTTCGAACCTCTG-3′	Norén and Jondelius (1999)
4FB	Sequencing	5′-CCAGCAGCCGCGGTAATTCCAG-3′	Norén and Jondelius (1999)
4FBK	Sequencing	5′-CTGGAATTACCGCGGCTGCTGG-3′	Norén and Jondelius (1999)
7FK	Sequencing	5′-GCATCACAGACCTGTTATTGC-3′	Norén and Jondelius (1999)
16S Primers			
16S AnnF	PCR, sequencing	5′-GCGGTATCCTGACCGTRCWAAGGTA-3′	Sjölin et al. (2005)
16S AnnR	PCR, sequencing	5′-TCCTAAGCCAACATCGAGGTGCCAA-3′	Sjölin et al. (2005)
COI Primers			
LCO1490	PCR, sequencing	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994)
HCO2198yy	PCR, sequencing	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer et al. (1994)
AnnCOIF	PCR, sequencing	5′-TATGAGCNGGAATAGTTGGTACMGG-3′	Bodil Cronholm pers. comm
COI-E¯	PCR, sequencing	5′-TATACTTCTGGGTGTCCGAAGAATCA-3′	Bely and Wray (2004)

lis, H. queenslandicus, Heterodrilus (undescribed species), *H. decipiens, H. chenianus* and *H. devexus*) are strongly supported (pp = 1) and appear as a sister group of the Caribbean *H. modestus*. *Heterodrilus* cf. *virilis* + *H. queenslandicus* and *H. chenianus* + *H. devexus* are also supported by posterior probabilities of 1 (bootstrap support 88 and 93, respectively).

Clade B contains a strongly supported group (pp = 1 and bootstrap support 85) of four species (*H. perkinsi* through *H. flexuosus*, all Caribbean), in which *H. perkinsi* and *H. bulbiporus* are proposed as closely related sister species with pp = 1. The bootstrap analysis also supports *H. minisetosus* + *H. flexuosus* (82; indicated by a red line in Fig. 2). Otherwise, clade B, which also contains *H. jamiesoni* (Great Barrier Reef), and *H. occidentalis* and *H. pentcheffi* (both Caribbean), is resolved with low support.

Discussion

This study is a more comprehensive phylogenetic analysis of *Heterodrilus* based on molecular sequence than those published earlier (see Introduction), and yet only 16 of the 43 known species are included; one of these is a hitherto undescribed species from New Caledonia. Unfortunately, this species can only be described when more material is collected. The Bayesian analyses of 18S rDNA, mtDNA and the concatenated combined data set support monophyly of *Heterodrilus*. Further, both gene trees

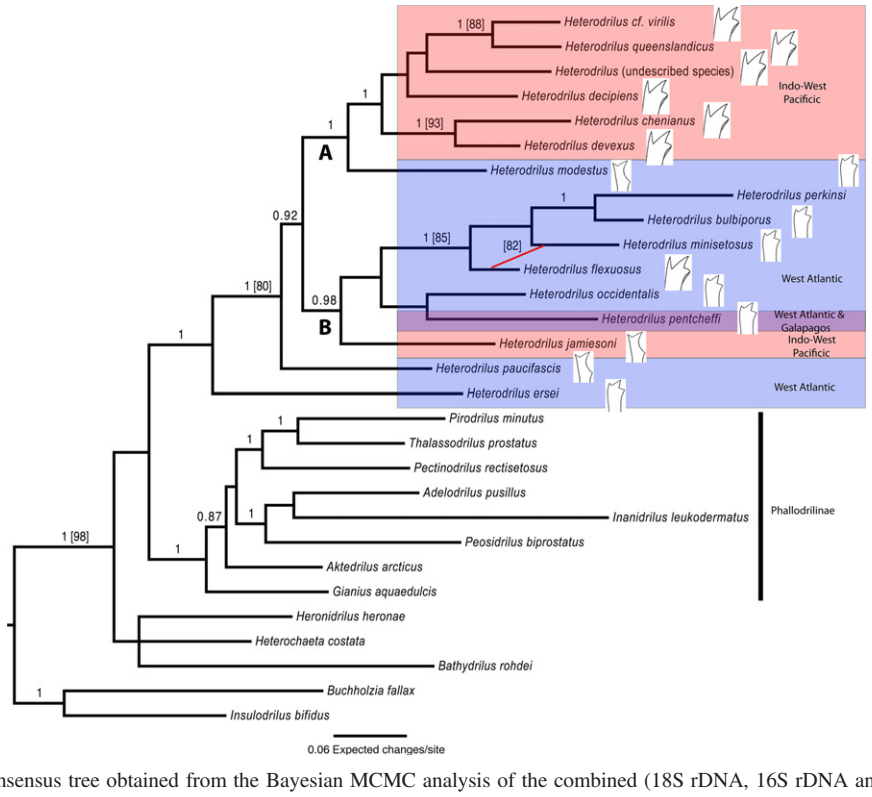

Fig. 2. Majority-rule consensus tree obtained from the Bayesian MCMC analysis of the combined (18S rDNA, 16S rDNA and COI) data set. Posterior probabilities > 0.80 are indicated. Parsimony bootstrap proportions > 70 are marked in brackets, when applicable. The red line indicates a clade supported only by the bootstrap analysis. Inset images depict the chaetal tip shape of the *Heterodrilus* species (see Fig. 1 for explanation), and geographic species distributions are colour coded with purple for West Atlantic species and pink for Indo-West Pacific species

and the combined data also place *H. ersei* outside all other sampled species of the genus.

The monophyly of *Heterodrilus* is morphologically supported by the presence of trifid anterior chaetae, a feature that is found only in this genus among all naidids. A few species assigned to *Heterodrilus,* but not studied herein, have bifid chaetae only (Fig. 1D)**,** and others, in this study represented by *H. modestus*, *H. paucifascis* and *H. jamiesoni*, are intermediate in the sense that they have some clearly bifid anterior chaetae as well as other anterior chaetae with a subdistal third tooth (Fig. 1C). All the three species with intermediate chaetae appear to be phylogenetically nested within *Heterodrilus*, and it is possible that the third tooth has become secondarily reduced in some lineages (Erséus 1990), although the bifid species within *Heterodrilus* (*H. hispidus, H. subtilis* and *H. tripartitus*) should be included in a more extensive molecular phylogenetic study to test this hypothesis.

The analysis of the combined data set places *H. ersei* outside the rest of *Heterodrilus*; *H. ersei* is the type species of *Giereidrilus*, a genus once proposed by Erséus (1981).

There are a number of morphological features that distinguish *H. ersei*, as well as its close relatives *H. inermis* (Erséus 1981), *H. apparatus* Erséus 1993 and *H. rapidensis* Erséus 1997a,b; from other species of *Heterodrilus*. For instance, all these species have unpaired spermathecal and male gonopores as opposed to the paired structures in other *Heterodrilus* species. Further, and perhaps even more significantly, their prostate glands are divided into two distinct bodies on each atrium, that is a feature typical of most phallodrilines (Erséus 1992*c*), whereas the rest of the *Heterodrilus* species have their prostate glands diffusely spread along the atrial surfaces (Erséus 1981, 1993, 1997a,b). Thus, the basal position of *H. ersei* in our tree strengthens the support for *Heterodrilus* being a phallodriline; that is, the biprostate condition is possibly an ancestral feature in *Heterodrilus*. Further, when optimized on our tree, several morphological features commonly used in naidid taxonomy are more or less homoplasious. For example, all species except *H. modestus* in clade A (Fig. 2) have trifid chaetae with a ligament, but this is also the case for *H. flexuosus* in clade B. Some species in our study lack spermatheca (*H. flexuosus*, *H. virilis*, *H. chenianus*, *H. modestus*), but they are scattered in the tree (Fig. 2), indicating that the spermatheca have been lost several times. Most species in our tree have long vasa deferentia (sperm ducts), except for *H. perkinsi* and *H. minisetosus* in clade B, which have short ducts. The molecular data (as inferred from Fig. 2) suggest that the latter condition has evolved convergently in these two taxa.

The shape and arrangement of the penial chaetae, which are fundamental characters in naidid taxonomy in general (Erséus 1980), and in the taxonomy of the subfamily Phallodrilinae in particular (Erséus 1992*c*), also exhibits convergence in our tree. Many *Heterodrilus* species have two large penial chaetae per bundle, and these chaetae are arranged in a 'V-shaped' formation (i.e. tips closer together than inner ends). However, although not closely related, *H. modestus* and *H. chenianus* both lack penial chaetae. On the other hand, *H. flexuosus* and *H. minisetosus*, which are closely related (see Fig. 2), both have minute penial chaetae in unisetal 'bundles', a condition thus likely to be synapomorphic. In clade A (Fig. 1), most species have penial chaetae that are tightly parallel within each pair/bundle, the exceptions being *H. decipiens* with its 'V-shaped' bundles, and *H. modestus* and *H. chenianus* with their lack of penial chaetae. The tightly parallel arrangement of the penial chaetae is not found in any species outside clade A.

To summarize, the topology indicated in this study (Fig. 2) is in great conflict with the topology based on morphological data (see Erséus 1990), although some of the species in the latter study are not included in this study and vice versa. Thus, the results are not fully comparable. Nevertheless, our study suggests that the two major clades in our tree are largely congruent with the geographical distributions of their respective members. The species in clade A (except *H. modestus*) are from the Indo-West Pacific (Australia, New Caledonia and China), while the species in clade B (except *H. jamiesoni*) are all from the warmer parts of the NW Atlantic Ocean (Florida, Bahamas and Belize). Moreover, the basal positions of *H. ersei*, *H. paucifascis* and *H. modestus*, all Caribbean taxa, seem to suggest that the genus originated in the Atlantic Ocean. However, it should be noted that, while *H. ersei* is a NW Atlantic species, the other three species in the putative monophyletic taxon earlier referred to as '*Giereidrilus*' (see Erséus 1981; and above) are all Indo-West Pacific. This means that both '*Giereidrilus*' and its putative sister group ('*Heterodrilus' sensu stricto*) are circum-tropical in their distribution, but also that the group as a whole is characterized by regional species radiation in the different parts of the world. The phylogenetic position of the monotypic genus *Heterodriloides* Erséus 1981; proposed for the NW Atlantic species *H. quadrithecatus* Erséus 1981, remains to be clarified. This, as well as establishing a more complete phylogeny of *Heterodrilus* and its position among the Naididae, will become a future task based on a much broader sampling.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Majority-rule consensus gene tree based on Bayesian inference of the 18S rDNA locus. Posterior probabilities > 0.80 are indicated and parsimony bootstrap proportions > 70 are marked in brackets, when applicable.

Figure S2. Majority-rule consensus gene tree based on Bayesian inference of the mtDNA locus. Posterior probabilities > 0.80 are indicated, and parsimony bootstrap proportions > 70 are marked in brackets, when applicable.