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DNA barcoding and species delimitation: the *Stylodrilus heringianus* case (Annelida: Clitellata: Lumbriculidae)

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Abstract
Individuals of the aquatic oligochaete *Stylodrilus heringianus* Claparède, 1862 were collected across a part of this species' distribution range in Sweden, Estonia, Great Britain and Spain, to test whether they represent a single metapopulation or a number of separately evolving lineages. Using sequences of the barcoding gene cytochrome *c* oxidase subunit I (COI) and two nuclear genes i.e. internal transcribed spacer (ITS) region and histone 3 (H3), three different approaches were conducted: pairwise distance-method, Bayesian inference and network analysis. Both the COI phylogeny and network analyses were concordant in recovering six haplotype clusters, which showed a maximum genetic distance of 7.7% (K2P) among each other. Nevertheless, nuclear genes failed to confirm any lineage separation, and we conclude that the sampled specimens all belong to the same species. A phylogeographic history with allopatric divergence and secondary contact is suggested to explain this intraspecific pattern of mitochondrial divergence and nuclear non-divergence. The study shows that a mitochondrial single-locus approach can be problematic for the accurate delimitation of species, and we emphasize the need for nuclear genes as supplementary markers, when taxonomic resolution is assessed with COI barcodes.

Introduction

DNA barcoding was proposed by Hebert *et al.* (2003a) as a method for assigning unknown individuals to species. A short fragment of the genome of the unidentified specimen is sequenced and compared against a broad database of sequences representing known species. Assuming that intraspecific variation normally is lower than interspecific variation (i.e. the barcoding gap) (Hebert *et al.* 2003b; Hebert and Gregory 2005), it should be possible to use a threshold of sequence similarity when matching a query sequence with already existing sequences in the database.

DNA barcoding provides means for discovering new species and for approaching taxonomic problems difficult to resolve on the basis of morphological characters only. It is especially useful when inter-specific differences are observed only during some life history stages, or in only one gender, or when the human eye simply cannot detect such differences.

Current implementations of the DNA barcoding method for taxonomic analysis have been strongly criticized (e.g. Will and Rubinoff 2004; Will *et al.* 2005; Meier *et al.* 2006; Kwong *et al.* 2012) and subsequently, shortcomings are continually addressed (e.g. Meier *et al.* 2008; Lou and Golding 2012; Srivathsan and Meier 2012). One of those issues is still the small number of suitable markers (mitochondrial and nuclear genes) available (Voyler and Monaghan 2006). The mitochondrial cytochrome *c* oxidase subunit I gene (COI) is the clear favourite candidate for animals (Hebert *et al.* 2003b). It is easily amplified in most taxonomic groups, evolves fast enough to gain differences between closely related species, and yet its amino acid sequence is highly constrained. In contrast, potential problems of using mitochondrial DNA to infer species boundaries include retention of ancestral polymorphism, sex-biased gene flow, hybridisation or introgression, and nuclear copies of mitochondrial genes (Moritz and Cicero 2004; Hebert 2004; Rubinoff *et al*. 2006). Other genes are now being tested as barcode markers e.g. the internal transcribed spacer region (ITS) of the nuclear rDNA (Ben-David *et al.* 2007; Li *et al.* 2010; Yao *et al*. 2010).

To determine species boundaries using gene sequences data, we can follow different approaches. As noted above, a similarity threshold value can be arbitrary established and all specimens yielding sequences below that value will be considered conspecific. For that purpose, pairwise distances (%) are usually chosen, often in the form of Kimura-2-parameter values (K2P); the K2P estimate, however, has been recently criticised (Srivathsan and Meier 2012). Hebert *et al.* (2003a, 2004) proposed a value for intraspecific variation in the COI gene of 2 to 3%, but this seems to vary depending on the taxon (Hajibabaei *et al.* 2006; Lefébure *et al.* 2006; Burton and Davie 2007; Erséus and Gustafsson 2009) and latitude (Martin and McKay 2004). It is also important to sample a species throughout its geographical range, to fully appreciate the amount of its intraspecific variation, which will be crucial when establishing the "barcoding gap" (COI distance) between sister species (Funk and Omland 2003; Moritz and Cicero 2004). However, the hardest criticism to this approach is that it fails to recognize young species, when, e.g., such have arisen through divergent selection in a recently shared ancestral population (e.g., Hickerson *et al.*, 2006) or polyploidization (e.g., Evans *et al.*, 2008). Constructing gene trees using phylogenetic reconstruction is another popular approach for delimitation of species. Nodes in the tree with high branch support values may then be recognized as species. However, as the organismal relationships within a sexually reproducing species are not hierarchical, this may lead to an inadequate resolution, especially with regard to mitochondrial genes, which generally do not account for the paternal contribution to speciation. For solving this and other problems, networking approaches that estimate intraspecific genealogies have been developed (Posada and Crandall 2001), where population phenomena as persistent nodes, multifurcations and reticulations are allowed. Comparing mitochondrial and nuclear gene trees, to look for their least common denominator of topological congruence (the coalescent), is also a straightforward way to find the species tree (e.g., Moore 1995).

Stylodrilus heringianus Claparède, 1862 is a Holarctic lumbriculid annelid (Clitellata) inhabiting freshwater habitats (rivers, lakes, groundwaters) and also oligohaline brackish waters (Laakso 1967); in lakes, it is usually regarded as an indicator of oligotrophic conditions (Milbrink 1973). The worms are yellowish red, 25-60 mm long and consist of 70-110 segments. Diagnostic characters of the species are related to the reproductive system (Cook 1967; Rodriguez 1988), and it can thus be morphologically identified with certainty only on the basis of sexually mature specimens. The most striking feature is its characteristic pair of external, long and pointed penes. In recent time, the conspecificity of various populations of this morphospecies has not been seriously challenged among lumbriculid specialists. However, the ubiquitous lifestyle and our preliminary observations of considerable variation in the COI gene among North European populations of *S. heringianus* raised questions about cryptic speciation within this nominal taxon. This taxon is thus a suitable case for testing species delimitation using DNA data.

In this study, we aim to establish whether a sample of *Stylodrilus heringianus* from different parts of North-west Europe represents a single metapopulation or a number of separately evolving lineages, i.e., cryptic species. We examine sequences of the barcoding gene (COI) and two nuclear markers, the ITS region and histone 3 (H3), using pairwise distances, Bayesian inference and network analysis.

Material and methods

Fifty-one specimens of *Stylodrilus heringianus* were collected during 2000-2011, mostly in Sweden, but also in other countries, plus one individual of each of *S. parvus* Hrabě and Cernosvitov, 1927 and an undescribed *Stylodrilus* sp. (morphologically near *S. parvus*) (Table 1).

Specimens were killed and preserved in 80% ethanol. Posterior parts were cut and transferred into 95% ethanol while most of the anterior parts (especially from mature specimens) were prepared as microscope slides for morphological study. These parts were first stained in paracarmine or Ehrlich's hematoxylin, and then dissected or whole-mounted in

Canada balsam. Identification of mature individuals was based on the reproductive system, while chaetae and other external somatic characters were checked for immature worms. Slides were deposited in the Swedish Museum of Natural History (SMNH).

DNA was extracted from posterior ends using the DNAeasy Tissue Kit (Qiagen) following the protocol supplied by the manufacturer. As specified in Table 1, we successfully amplified 658 bp of the mitochondrial cytochrome *c* oxidase subunit I (COI, 53 specimens), about 1250 bp of the nuclear internal transcriber spacers region (ITS, 23 specimens), and 348 bp of the nuclear histone 3 (H3, 32 specimens). We used primers LCO1490, and HCO2198 (Folmer *et al.*. 1994) or COI-E^{-} (Bely and Wray 2004), for COI, ITS-5 and ITS-4 (White *et al.*. 1990) for the ITS region and H3F, and H3R (Brown *et al.*. 1999) for H3. PCR mixtures contained 21 µl ddH2O, 1 µl of each primer (10µM), 2 µl template DNA and Illustra PuReTaq Ready-To-Go PCR Beads (0.2 ml tubes) (GE Healthcare). The temperature profile was 95ºC/300s, (95ºC/30-40s, 45-55ºC/30-45s, 72ºC/30-60s)* 35 cycles, 72ºC/480s. PCR products were purified with Exonuclease I (Fermentas) and FastAP Thermosensitive Alkaline Phosphatase (Fermentas), and thereafter sent to Macrogen Sequencing System, South Korea for sequencing. Sequences were assembled and corrected using Geneious Pro 5.3.6 (Biomatters Ltd.). For each gene, the alignment was made using Clustal X version 1.8 (Thompson *et al.* 1997) with default settings and then manually corrected in Geneious Pro.

For Bayesian analysis (BA) we used MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003). The three genes were analysed separately. The best-fit models were selected using the Akaike information criterion (AIC) in MrModeltest version 2.3 (Nylander 2004) in conjunction with PAUP* version 4.0b10 (Swofford 2002). For COI a partitioned site specific rate model was used: we applied the Symmetrical model of sequence evolution (SYM) with a proportion of the sites invariable for the COI 1st position, the General Time Reversible model (GTR) for the COI 2nd position and the General Time Reversible model with gamma distributed rates across sites (GTR+G) for the 3rd position. For ITS and H3 the models selected were F81 and HKY respectively. The number of generations was set to one million with four parallel chains (three hot and one cold), sample frequency to every 100th generation, and number of runs to two. The first 2500 samples were discarded as the 'burn-in' of the Markov chain. We checked for stationarity and convergence of the chains with the software TRACER 1.5 (Rambaut and Drummond 2009).

We also constructed trees applying the maximum likelihood (ML) method using the online version of RaxML BlackBox (Stamakis *et al.* 2008), with 100 bootstrap replicates by using the GTRGAMMA model. All model parameters were estimated by the program from its own maximum parsimony starting trees. For the COI the alignment was divided into the same three partitions as used for the Bayesian analysis.

In addition, we estimated haplotype networks using statistical parsimony implemented in the TCS version 1.21 (Clement *et al.* 2000). The software estimates the maximum number of differences among haplotypes as a result of single substitutions with a 95% statistical confidence (parsimony connection limit). For ITS, gaps were treated both as missing data and as a fifth state.

Results

The COI dataset (i.e., 51 specimens of *Stylodrilus heringianus*, one *S. parvus* and one *Stylodrilus* sp.) contains 658 characters, of which 134 are parsimony-informative and 91 are variable but parsimony-uninformative. Both trees (BA and ML) generated with COI sequences produced identical topologies with highly supported clades (Fig 1.A). Clades B, D and F comprise mostly specimens from Scandinavia, but also three specimens from Estonia and Great Britain are in Clade B. Clades C and E consist of specimens from karstic areas in northern Iberian Peninsula and Gotland, Sweden respectively. One specimen from Great Britain (Clade A) is placed separately, as sister to all other clades. The main distribution of the various clades is illustrated in Fig. 1B. At some of the sampled localities in Sweden, two clades were found sympatrically: clades D and B in Lake Lången, and clades B and F both in Lake Sävelången and the swamp at Lake Aspen (see Table 1).

Interspecific uncorrected pairwise (p) distance and Kimura-2-parameter (K2P) distance for *Stylodrilus* are between 19.6 and 23.8% (*p*) and between 22.8 and 28.8% (K2P). Between *S. heringianus* clades, p distance ranges between 5.2 and 7.3% (5.2 and 7.7% K2P). Within them, maximum p distance is 2.7% (2.8% K2P), i.e., in clade F.

The haplotype network analysis of the *S. heringianus* specimens exhibited seven different groups, A-F (not connected), when applying the 95% connection limit (Fig 1.A). Five of them correspond to clades recovered by the phylogenetic analyses (A-E). However, a fixed connection limit of 90% was necessary to connect the two networks corresponding to the F clade. The total number of haplotypes is 16, of which eight are private (haplotypes represented by a single sequence in the sample). Networks B and D each comprise four different haplotypes, while E consists of only one haplotype. Each specimen corresponding to clades A and C was placed separately. Translation of the COI fragment resulted in 219 amino acids (aa). Only four (CE285, CE4274, CE6028 and CE2993) of the 51 sequences of *S. heringianus* showed one to two aa differences vis-à-vis the rest. Most substitutions occur in the 3rd codon; 14 substitutions are in the 1st codon and three in the 2nd codon. In contrast, the three *Stylodrilus* morphospecies investigated had between 13 and 17 aa differences among them.

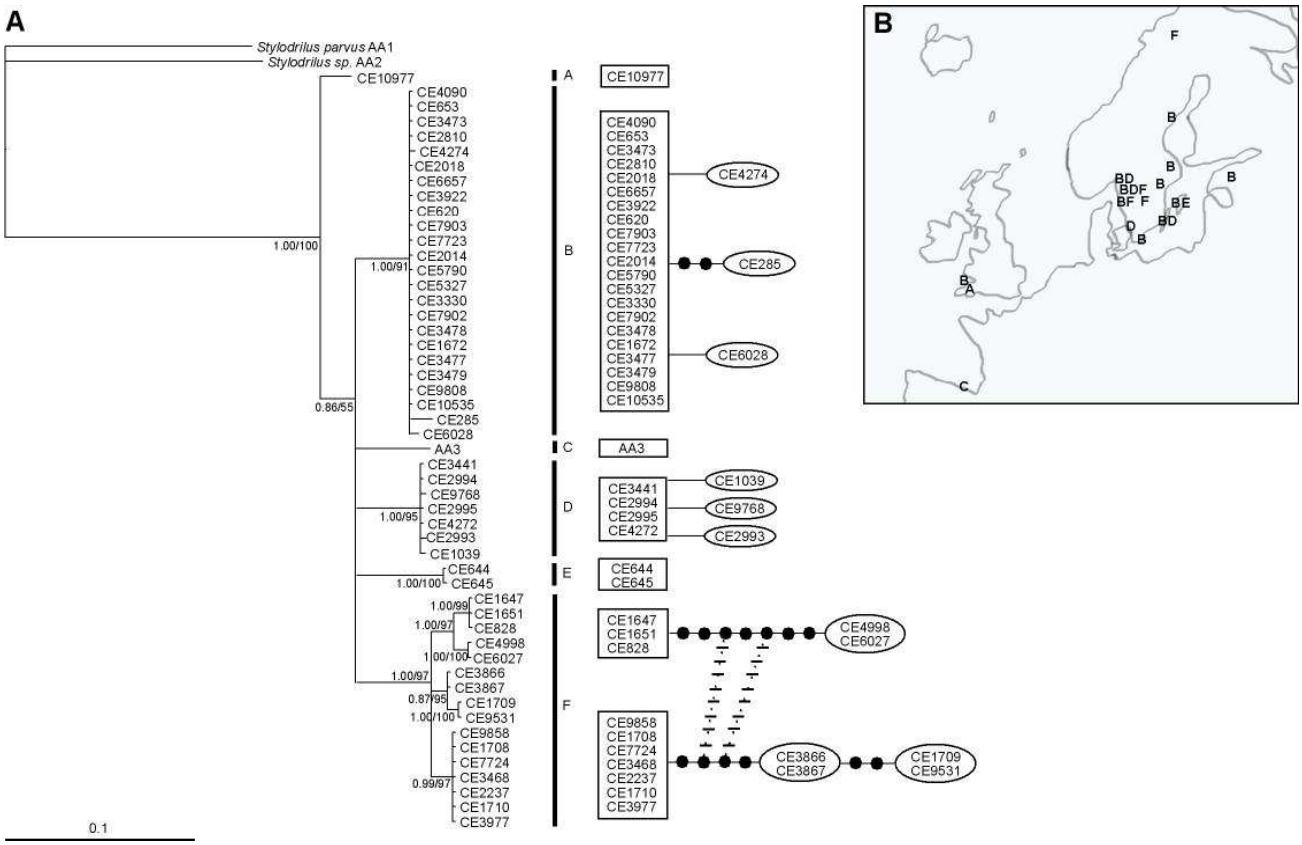

Fig. 1 Results of the COI data set. A: Bayesian consensus tree (left) and statistical parsimony haplotype networks (right). Both methods recovered the same COI groups, which were coded at bars as A-F. Specimens are specified in Table 1. BA posterior probabilities (>0.5) and ML bootstrap values (>50%) are given at the nodes of the tree. In the networks, each oval or rectangle (ancestral haplotype according to TCS) represents a unique haplotype and shows included specimens. An empty line connecting haplotypes represents a single mutational change; each black dot or bar represents one mutational change. Dotted lines indicate that a fixed connection limit of 90% was necessary to connect all haplotypes corresponding to clade F. B: Map of the COI groups in the study area. Some letters represent a group of sites close to each other.

The alignment of the ITS data (i.e., 21 specimens of *S. heringianus,* one *S. parvus* and one *Stylodrilus* sp.) consists of 1246 total characters (1177 when outgroups were excluded), of which 71 are parsimony-informative (two when outgroups were excluded) and 152 are variable but parsimony-uninformative (seven when outgroups were excluded). The resulting ITS topologies showed a polytomy of all the sequences of *S. heringianus* (Fig. 2.A). Uncorrected *p* distances within *S. heringianus* were 0-0.30% (0-0.26%K2P); among the *Stylodrilus* species 11.4-14.1% (12.5-15.7% K2P). A single haplotype network of *S. heringianus* specimens is formed when applying the 95% connection limit, regardless of whether gaps are treated as missing data or as a fifth state (Fig 2.B and 2.C). The total number of haplotypes is six (of which four are private) when gaps are considered missing data and ten (of which seven are singletons) when 5th state. We searched through the alignment (without outgroups) for variable positions that might be concordant with COI and/or H3 groups (Table 2), but there is no evidence of congruent haplotype distribution in the three markers.

Concerning the H3 sequences (i.e., 30 specimens of *S. heringianus,* one *S. parvus* and one *Stylodrilus* sp.), with 348 total characters, 17 are parsimony-informative and 12 are variable but parsimony-uninformative. One clade including 23 specimens was formed, while the other seven specimens clustered separately (Fig. 3). Maximum distance (*p*) within *S. heringianus* was 0.35% (0.34% K2P); distances between *Stylodrilus* species were 3.5-7.4% (3.6-7.7% K2P). For *S. heringianus*, a single haplotype network with only two haplotypes is formed when applying the 95% connection limit (Fig. 3). The only variable character in *S. heringianus* sequences is the nucleotide in position 266. Specimens CE6027, CE645, CE644, AA3, CE653, CE285 and CE10977 here show a T instead of a C (Table 2), which results in an aa change.

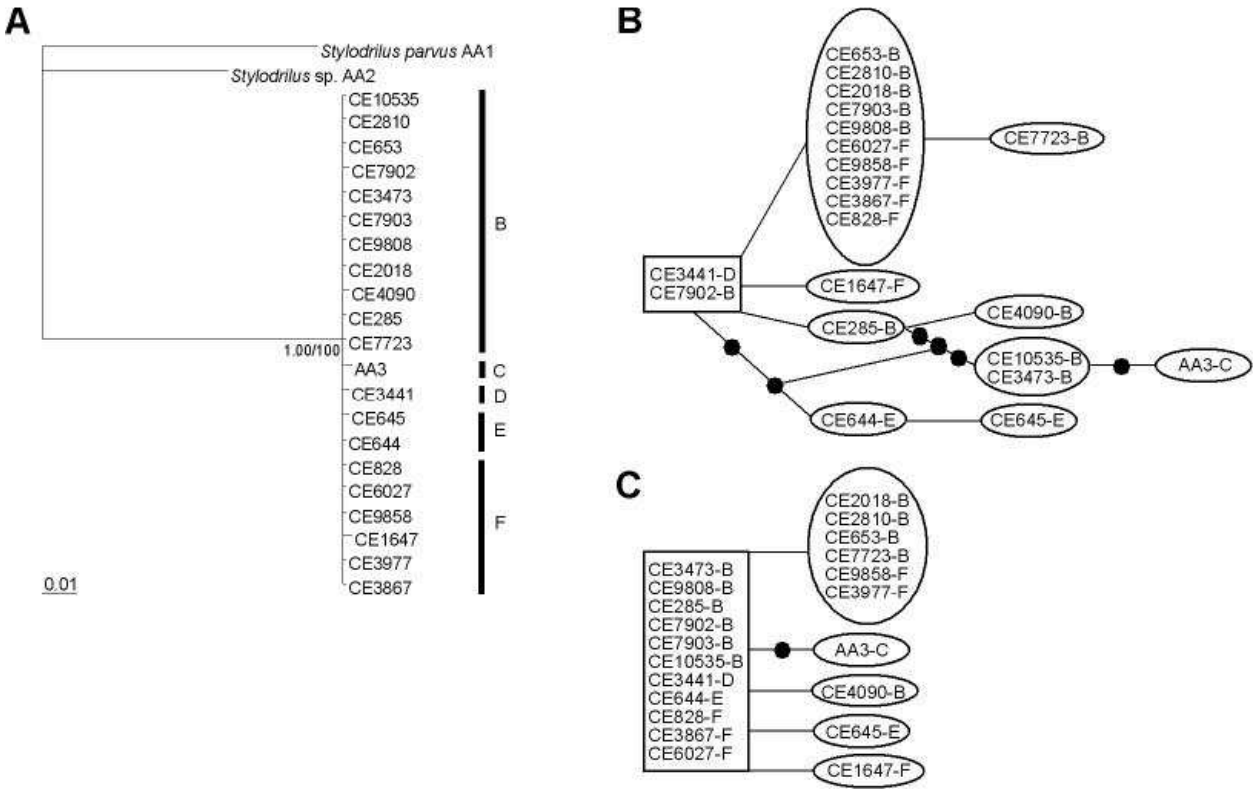

Fig. 2 Results of the ITS data set. A: Bayesian consensus tree. BA posterior probabilities (>0.5) and ML bootstrap values (>50%) are given at the [single] node of the tree. COI based groups (A-F) are identified by their letters at the bars. B: Statistical haplotype networks, gaps treated as 5th state. C: Statistical haplotype networks, gaps treated as missing data. Letter codes of COI based groups (A-F) are added to each specimen's code. For further explanations, see Figure 1.

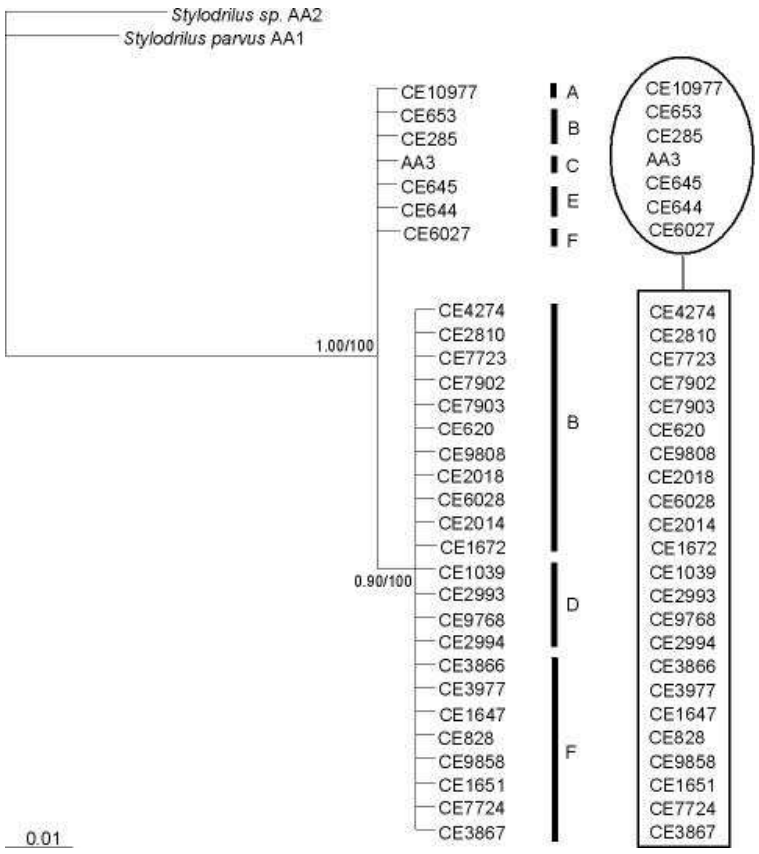

Fig. 3 Bayesian consensus tree (left) and statistical parsimony haplotype network (right) of the H3 data set. BA posterior probabilities (>0.5) and ML bootstrap values (>50%) are given at the nodes of the tree. COI based groups (A-F) are identified by their letters at the bars. For further explanations, see Figure 1.

Discussion

Stylodrilus heringianus, one metapopulation or separately evolving lineages?

As argued by de Queiroz (2007, 2011), there is an emerging unified species concept under which species are considered "separately evolving metapopulation lineages". The use of more than one line of evidence is crucial for inferring lineage separation and, indeed, the case of *S. heringianus* is a good example. Not only genetic distances showed divergences in COI (up to 7.7% K2P genetic distance) within *S. heringianus*; generated trees and networks based on COI data were concordant in revealing 6 groups (Fig 1.A). Therefore, if we had used a one-gene approach, using only the COI barcode, we might have come to the conclusion that *S. heringianus* comprises several different species. However, the analysis of the nuclear data failed to confirm that speciation has occurred within *S. heringianus*. In both ITS and H3 gene data, distances were maximally about 0.3%, and neither clades nor networks (when formed) were concordant with the COI groups (Figs 2 and 3). As species emerge and evolve as the result of recombination of maternal and paternal DNA, a combination of mitochondrial (here COI) and nuclear markers (such as ITS and H3) was needed to establish a reliable species tree from the various gene trees.

The ITS region has already been positively evaluated to be used as a complementary locus to COI for identification of animal species (Källersjö *et al.* 2005, Yao *et al.* 2010) and in fact, recent studies tend to use both COI and ITS data for solving phylogenetic questions between closely related species, species delimitation and population studies (examples for Annelida are Gustafsson *et al.* 2009, De Wit and Erséus 2010, Kvist *et al.* 2010, Nygren *et al.* 2010, Zhou *et al.* 2010, Nygren and Pleijel 2011). The use of H3 for complementing the COI based results is not so common (e.g. Nygren *et al.* 2009, Fernández *et al.* 2011) and still it should be tested whether H3 possesses enough interspecific variation to discriminate between species and whether this gene is sufficiently conserved so that the intraspecific variability is lower than the interspecific one (Kress and Erickson 2008).

Geographic distribution and COI variation

Since variable positions in the nuclear genes are scarce and randomly distributed in the groups suggested by the COI data (Figs 2 and 3; Table 2), and even if the polyploidy status of the individuals is unknown, we can conclude that, at least in the area of mainland Sweden, it is probable that the studied populations are (or have been till recent times) amphimictic, *i.e.* are capable of interbreeding and producing fertile offspring. Moreover, we found specimens at the same locality corresponding to different COI groups, *e.g.* CE7723 (group B) and CE7724 (group F), but sharing the same H3 haplotype (no ITS data). An exception is the case of specimens CE6027 (group F) and CE6028 (group E), which although collected at the same locality, show different H3 haplotypes (Fig. 3, Table 2).

Based on our results, groups B, D and F (12 haplotypes in total) seem to have a random distribution in Sweden (Fig. 1B), inhabiting a variety of habitats (Table 1), while a unique COI haplotype (group E) was found in a karstic area on the island of Gotland. The diverse haplotypes present and randomly distributed in Sweden indicate that dispersal is relatively easy and also rather random, so same haplotypes could be expected in areas directly south of the North and Baltic Seas. Sampling in other geographic areas (*i.e.*, Denmark, Estonia, Great Britain and The Basque Country) added two new COI groups (from Great Britain and The Basque Country) and increased the maximum intraspecific COI distance from 7.2% to 7.7% (K2P). Nevertheless, this variation is still significantly lower than the observed interspecific variation (22.8-28.8% K2P). Although the use of genetic divergence for inferring species has been extensively criticised (Steel *et al.* 1988, Koski and Golding 2001, Ferguson 2002, DeSalle *et al.* 2005, Hickerson *et al.* 2006, Meier *et al.* 2006, Whitworth *et al.* 2007), it is worth mentioning that prior molecular assessments including other lumbriculid species have reported interspecific divergences similar to those found in *Stylodrilus*: 20% (*p*) in *Rhynchelmis* Hoffmeister, 1843 (Zhou *et al.* 2010) and 17.7% (*p*) between two cryptic species in *Lumbriculus variegatus* (Müller, 1774) (Gustafsson *et al.* 2009).

In many cases, two sequences diverging 4% will still code for exactly the same amino acid sequence (Meier 2008). Synonymous substitutions are known to be disproportionately common in mitochondrial DNA (Brown 1983) and are traditionally considered to be neutral (Ballard and Kreitman 1995), meaning no direct link to the fitness of the individual, although they have been shown to be selective in certain cases (Dowling *et al.* 2008). Here, only four specimens showed 1-2 non-synonymous substitutions.

COI divergences may be indicative of periods of isolation in the past

High intraspecific mtDNA divergences observed in other invertebrate species have mainly been explained by two nonexclusive hypotheses: fast mtDNA evolution (e.g. Hayashi and Chiba 2000) and allopatric divergence followed by secondary contact (e.g. Verovnik *et al.* 2005). Because rapid evolution has yet not been found in aquatic oligochaetes, the second hypothesis will be discussed. Many organisms currently distributed across Europe seem to have been dramatically influenced by Quaternary climatic oscillations (Hewit 1996). At the last glacial period, most organisms would have been in refugia in the south, many in the peninsulas of Iberia, Italy and the Balkans, and then they would have expanded to the north following distinct routes (see Hewit 1999 for a review of these routes). The final melting of the Scandinavian ice cap, about 9,000 years ago, probably

allowed eastern and western colonists to meet in central Sweden (forming a hybrid zone). Hewit (1999) suggested that new haplotypes that are established during postglacial colonization of Europe would differ by a few substitutions, while higher divergences might have a pre-Pleistocene origin. Thus, Quaternary cold periods are unlikely to be a cause of the observed high COI divergence in *S. heringianus*. One can imagine that populations could have diverged allopatrically before the Pleistocene and then mix; such evolutionary divergences would have been erased from the nuclear DNA by recombination and mutational perturbations and preserved in the mtDNA by maternal inheritance. With the present data we cannot certainly know when populations diverged, and in order to draw conclusions about the evolutionary history of the species, more populations should be analysed. In line with this, however, studies on other freshwater taxa have also rejected that post-glacial events were the main factor for shaping today's population structure and distribution. For instance, a pre-Pleistocene colonization of Europe (during late Miocene) was suggested for populations of the crustacean *Asellus aquaticus* (Linnaeus, 1758) (Verovnik *et al.* 2005); and a Pliocene origin was hypothesized for populations of a teleost, the chub *Leuciscus cephalus* (Linnaeus, 1758), diverging 5.2-7.9% in their COI (Durand *et al.* 1999).

Here we showed that there is no fixed threshold for COI barcode gaps when it comes to delimiting a species. Indeed, the combination with nuclear data was crucial for that purpose. The case of *Stylodrilus heringianus* demonstrates that the evolutionary processes and distributional history responsible for DNA divergences, and ultimately, the source of speciation may be a complex matter, and that species delimitation using COI barcodes is not always straightforward.

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Table 1. List of sequenced specimens, GenBank accession numbers, and voucher data. SMNH, Swedish Museum of Natural History, Stockholm. Country codes: EE, Estonia; GB, Great Britain; SE, Sweden; SP, Spain.

Species	Locality and habitat	Specimen	Genbank Acc. No. COI	Genbank Acc. No. ITS	Genbank Acc. No. H3	Voucher SMNH
Outgroup						
Stylodrilus parvus	Argatxa spring, Ereñozar karst unit, The Basque Country, SP; 43.3585N, 2.6584W, Pilar Rodriguez and Ainara Achurra, 19 July 2006	AA1	JX993900	KC117619	KC117586	SMNH 126472
Stylodrilus sp.	Zubialde spring, Itxina karst unit, The Basque Country, SP; 43.0190N, 2.7555W, Pilar Rodriguez and Ainara Achurra, 30 July 2008.	AA2	JX993901	KC117618	KC117617	SMNH 126473
Ingroup						
S. heringianus	Axpe river cave, Ereñozar karst unit, The Basque Country, SP; 43.3823N, 2.7009W, Pilar Rodriguez and Ainara Achurra, 19 July 2006.	AA3	JX993890	KC117620	KC117601	SMNH 126474
S. heringianus	Lab culture B30, Tarmo Timm's lab, Tartumaa, EE	CE285	FJ639309	KC117636	KC117606	SMNH103927
S. heringianus	Small stream at Lake Lången, 9 km N of Alingsås, Västergötland, sand, SE; 58.0103N, 12.5836E, Christer Erséus, 6 Jul 2003	CE620_1	JX993874		KC117611	SMNH 126475
S. heringianus	Lummelundaån River, Gotland, sand and gravel, SE; 57.7402N, 018.4046E, Christer Erséus, 2 Sep 2003	CE644	JX993898	KC117622	KC117599	SMNH 126476
S. heringianus	Lummelundaån River, Gotland, sand and gravel, SE; 57.7402N, 018.4046E, Christer Erséus, 2 Sep 2003	CE645	KC117641	KC117635	KC117597	SMNH 126477
S. heringianus	Mouth of Ihreån River, Ihre, Gotland, sand, SE; 57.8409N, 018.5988E, Christer Erséus, 2 Sep 2003	CE653	JX993867	KC117624	KC117604	SMNH 126478
S. heringianus	Bank of small stream, Solås, Aspenäs, Lerum, Västergötland, sand, SE; 57.7779N, 012.2541E, Christer Erséus, 1 Jun 2004	CE828_1	JX993863	KC117625	KC117605	SMNH 126479
S. heringianus	Stream at Fladalt, Hallandsåsen, Laholm, Halland, gravel, SE; 56.3687N, 013.1325E, Christer Erséus, 31 May 2005	CE1039	JX993897		KC117588	SMNH 126480
S. heringianus	Stream in Rhododendrondalen, Botanical Garden, Göteborg, Västergötland, sand, SE; 57.6796N, 011.9548E, Daniel Gustafsson, 3 Apr 2006	CE1647	JX993861	KC117637	KC117598	SMNH 126481
S. heringianus	Stream in Rhododendrondalen, Botanical Garden, Göteborg, Västergötland, sand, SE; 57.6796N, 011.9548E, Daniel Gustafsson, 3 Apr 2006	CE1651	JX993862		KC117615	SMNH 126482
S. heringianus	Kvibergsbäcken Stream, Göteborg, Västergötland, sand, SE; 57.737N, 012.043E, Daniel Gustafsson, 6 Apr 2006	CE1672	JX993883		KC117592	SMNH 126483
S. heringianus	Strömsbergsån River, Jönköping, Småland, sand, SE; 57.7529N, 014.1826E, Daniel Gustafsson, 16 Apr 2006	CE1708	JX993851			No voucher
S. heringianus	Strömsbergsån River, Jönköping, Småland, sand, SE; 57.7529N, 014.1826E, Daniel Gustafsson, 16 Apr 2006	CE1709	JX993857			SMNH 126484
S. heringianus	Strömsbergsån River, Jönköping, Småland, sand, SE; 57.7529N, 014.1826E, Daniel Gustafsson, 16 Apr 2006	CE1710	JX993855			SMNH 126485
S. heringianus	Lake Lången, Fly, N of Alingsås, Västergötland, wet peat near shoreline, SE; 57.9970N, 012.5866E, Christer Erséus, 12 Jul 2006	CE2014	JX993877		KC117608	SMNH 126486
S. heringianus	Lake Lången, Fly, N of Alingsås, Västergötland, littoral sand, SE; 57.9970N, 012.5866E, Christer Erséus, 16 Jul 2006	CE2018	JX993871	KC117632	KC117613	SMNH 126487
S. heringianus	Ditch at Medicinaregatan, Göteborg, Västergötland, wet soil with grass, SE; 57.6868N, 011.9616E, Daniel Gustafsson, 12 Jan 2007	CE2237	JX993854			SMNH 126488
S. heringianus	Karstic spring at Drösarp, Mörbylånga, Öland, sand, SE; 56.5749N, 016.6058E, Anna Ansebo, Lisa Matamoros & Christer Erséus, 13 Jun 2007	CE2810	JX993869	KC117623	KC117590	SMNH 126489

S. heringianus	Dry stream on limestone at Drösarp, Mörbylånga, Öland, grassroots and sand, SE; 56.5749N, 016.6058E, Anna Ansebo, Lisa Matamoros & Christer Erséus, 13 Jun 2007	CE2993	JX993896		KC117603	SMNH 126490
S. heringianus	Dry stream on limestone at Drösarp, Mörbylånga, Öland, grassroots and sand, SE; 56.5749N, 016.6058E, Anna Ansebo, Lisa Matamoros & Christer Erséus, 13 Jun 2007	CE2994	JX993892		KC117614	SMNH 126491
S. heringianus	Dry stream on limestone at Drösarp, Mörbylånga, Öland, grassroots and sand, SE; 56.5749N, 016.6058E, Anna Ansebo, Lisa Matamoros & Christer Erséus, 13 Jun 2007	CE2995	JX993894			SMNH 126492
S. heringianus	Swamp at Lake Aspen, Aspenäs, Lerum, Västergötland, wet soil, SE; 57.7759N, 012.2405, Christer Erséus, 21 Oct 2007	CE3330	JX993880			No voucher
S. heringianus	Kvennebäcken Stream, Tjärnö, near Lovén Center, Strömstad, Bohuslän, sand, SE; 58.8813N, 011.1433, Christer Erséus, Oct 2007	CE3441	JX993891	KC117631		SMNH 126493
S. heringianus	Sävelången Lake, Tollered, Lerum, Västergötland, 18 m, SE; 57.825N, 012.418E, Anita Sjöstrand, David Karlsson & Inger Elfman Börjesson, 29 Nov 2007	CE3468	JX993853			SMNH 126494
S. heringianus	Sävelången Lake, Tollered, Lerum, Västergötland, 30.5 m, SE; 57.838N, 012.431E, Anita Sjöstrand, David Karlsson & Inger Elfman Börjesson, 29 Nov 2007	CE3473	JX993868	KC117627		SMNH 126495
S. heringianus	Sävelången Lake, Tollered, Lerum, Västergötland, 27 m, SE; 57.831N, 012.426E, Anita Sjöstrand, David Karlsson & Inger Elfman Börjesson, 29 Nov 2007	CE3477	JX993884			SMNH 126496
S. heringianus	Sävelången Lake, Tollered, Lerum, Västergötland, 27 m, SE; 57.831N, 012.426E, Anita Sjöstrand, David Karlsson & Inger Elfman Börjesson, 29 Nov 2007	CE3478	JX993882			SMNH 126497
S. heringianus	Sävelången Lake, Tollered, Lerum, Västergötland, 27 m, SE; 57.831N, 012.426E, Anita Sjöstrand, David Karlsson & Inger Elfman Börjesson, 29 Nov 2007	CE3479	JX993885			SMNH 126498
S. heringianus	Swamp at Lake Aspen, Aspenäs, Lerum, Västergötland, wet soil, SE; 57.7761N, 012.2411E, Christer Erséus, 28 Apr 2008	CE3866	JX993859		KC117591	SMNH 126499
S. heringianus	Swamp at Lake Aspen, Aspenäs, Lerum, Västergötland, wet soil, SE; 57.7761N, 012.2411E, Christer Erséus, 28 Apr 2008	CE3867	JX993860	KC117640	KC117593	SMNH 126500
S. heringianus	Swamp at Lake Aspen, Aspenäs, Lerum, Västergötland, wet soil, SE; 57.7761N, 012.2411E, Christer Erséus, 28 Apr 2008	CE3922	JX993873			SMNH 126501
S. heringianus	Särö Västerskog, Kungsbacka, Halland, wet soil in rich deciduous forest, SE; 57.505N, 011.926E, Pierre De Wit, 3 Dec 2007	CE3977	JX993856	KC117639	KC117596	SMNH 126502
S. heringianus	Stream at Charlottenlund, Ystad, Skåne, sand, SE; 55.4236N, 013.6981E, Christer Erséus, 31 May 2008	CE4090	JX993866	KC117633		SMNH 126503
S. heringianus	Lake Lången, Fly, N of Alingsås, Västergötland, detritus at water line, SE; 57.9972N, 012.5866E, Christer Erséus, 26 Jun 2008	CE4272	JX993895			SMNH 126504
S. heringianus	Lake Lången, Fly, N of Alingsås, Västergötland, detritus at water line, SE; 57.9972N, 012.5866E, Christer Erséus, 26 Jun 2008	CE4274	JX993870		KC117589	SMNH 126505
S. heringianus	Banks of Ormån Stream, Ingalsröd, Lysekil, Bohuslän, fine sand, SE; 58.4327N, 011.5744E, Anna Ansebo, 21 Sep 2008	CE4998	JX993864			SMNH 126506
S. heringianus	Spruce forest, Krusenberg, Uppsala, Uppland, wet soil, SE; 59.736N, 017.647E, Tryggve Persson, 15 Apr 2008	CE5327	JX993879			SMNH 126507
S. heringianus	Vitsippsdalen, Botanical Garden, Göteborg, Västergötland, wet sandy soil, SE; 57.6817N, 011.9567E, Christer Erséus, 8 Apr 2009	CE5790	JX993878			SMNH 126508
S. heringianus	Ditch at Långesjö Road, Fly, N of Alingsås, Västergötland, wet sand, SE; 57.995N, 012.588E, Christer Erséus, 15 May 2009	CE6027	JX993865	KC117630	KC117594	SMNH 126509
S. heringianus	Ditch at Långesjö Road, Fly, N of Alingsås,	CE6028	JX993887		KC117616	SMNH

	Västergötland, wet sand, SE; 57.995N, 012.588E, Christer Erséus, 15 May 2009					126510
S. heringianus	Forest at Krokek, Norrköping, Östergötland, wet soil, SE; 58.67N, 016.32E, Christer Erséus, 7 Jun 2009	CE6657	JX993872			SMNH 126511
S. heringianus	Bank of stream in Södra Guldheden, Göteborg, Västergötland, wet soil, SE; 57.6827N, 011.9708E, Christer Erséus, 28 May 2010	CE7723	JX993876	KC117638	KC117595	SMNH 126512
S. heringianus	Bank of stream in Södra Guldheden, Göteborg, Västergötland, wet soil, SE; 57.6827N, 011.9708E, Christer Erséus, 28 May 2010	CE7724	JX993852		KC117587	SMNH 126513
S. heringianus	Stream at Bönhamn, Kramfors, Ångermanland, sand, SE; 62.8797N, 018.4314E, Christer Erséus, 9 Jun 2010	CE7902	JX993881	KC117626	KC117600	SMNH 126514
S. heringianus	Stream at Bönhamn, Kramfors, Ångermanland, sand, SE; 62.8797N, 018.4314E, Christer Erséus, 9 Jun 2010	CE7903	JX993875	KC117628	KC117602	SMNH 126515
S. heringianus	Small river between Björkliden and Riksgränsen, Kiruna, Lappland, sand, SE; 68.4277N, 018.4448E, Christer Erséus, 12 Jun 2010	CE9531	JX993858			SMNH 126516
S. heringianus	Alnus forest, Hälle, Strömstad, Bohuslän, wet soil, SE; 59.079N, 011.370E, Christer Erséus, 8 Sep 2010	CE9768	JX993893		KC117610	SMNH 126517
S. heringianus	Strömsvattnet Lake, Strömstad, Bohuslän, sand near water's edge, SE; 58.9451N, 011.1933E, Christer Erséus, 8 Sep 2010	CE9808	JX993886	KC117629	KC117612	SMNH 126518
S. heringianus	Groundwater seepage at Bålerödsbäcken Stream, Strömstad, Bohuslän, forest litter, SE: 58.9003N, 011.2261E, Christer Erséus, 8 Sep 2010	CE9858	JX993850	KC117634	KC117609	SMNH 126519
S. heringianus	Spring at Youldon Farm, South Hams, Devon, GB; 50.4N, 003.8W, Lee Knight, 12 Mar 2010	CE10535	JX993888	KC117621		SMNH 126520
S. heringianus	Higher Walreddon Farm, S of Tavistock, Dartmoor, Devon, old water tank, GB; 50.521N, 004.472W, Tim Jones, 20 Apr 2010	CE10977	JX993889		KC117607	SMNH 126521

Table 2. Main variable nucleotide positions observed in alignments of ITS and H3 sequences (gaps and outgroups not considered). Specimens are ordered according to COI based groups. A: Adenine; T: Thymine; G: Guanine; C: Cytosine; Y: Cytosine or Thymine. Hyphens denote gaps and question marks denote missing nucleotide data.

Specimen/Nucleotide position	ITS-339	ITS-340	ITS-546	ITS-989	H3-266	COI-based group
CE653	-	-	-	C	T	**B**
CE2810	-	-	-	?	C	**B**
CE2018	-	-	-	C	C	**B**
CE7903	-	-	-	Y	C	**B**
CE7723	-	-	-	C	C	**B**
CE7902	-	-	-	T	C	**B**
CE9808	-	-	-	?	C	**B**
CE285	-	-	T	T	T	**B**
AA3	A	T	T	T	T	**C**
CE3441	-	-	-	T	?	**D**
CE2994	A	T	T	?	C	**D**
CE2993	A	T	T	?	C	**D**
CE644	-	-	-	T	T	**E**
CE645	-	-	-	T	T	**E**
CE1647	-	-	-	T	C	**F**
CE828	-	-	-	?	C	**F**
CE6027	-	-	-	?	C	**F**
CE3867	-	-	-	Y	C	**F**
CE9858	-	-	-	C	C	**F**
CE7724	A	T	T	T	C	**F**
CE3977	-	-	-	C	C	**F**

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