

# The application of DNA sequence data for the identification of benthic nematodes from the North Sea

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**Abstract** Nematodes or roundworms represent one of the most diverse and dominant taxon in marine benthic habitats. Whereas a morphological identification of many species is challenging, the application of molecular markers represents a promising approach for species discrimination and identification. In this study, we used an integrative taxonomic approach, combining both molecular and morphological methods, to characterize nematodes of distinct sex and ontogenetic stages from three sampling sites of the North Sea. Morphospecies were discriminated after first visual determination, followed by a molecular analysis of the nuclear 28S rDNA: D2–D3 marker. By linking each sequence to a morphological voucher, discordant morphological identification was subjected to a so-called reverse taxonomic approach. Molecular operational taxonomic units (MOTUs) and morphospecies were compared for all of the three sampling sites to assess concordance of methodology. In total, 32 MOTUs and 26 morphospecies were assigned, of which 12 taxa were identified as described species. Both approaches showed high concordance in taxon assignment (84.4 %) except for a cluster comprising various *Sabatieria* species. Our study revealed the high potential of the analyzed fragment as a useful molecular marker for the identification of the North Sea

nematodes and highlighted the applicability of this combined taxonomic approach in general.

**Keywords** 28S rDNA: D2–D3 · Molecular operational taxonomic units · Molecular species identification · *Sabatieria* · Wadden Sea

## Introduction

Nematodes dominate the marine meiobenthos both in abundance and biomass (Soetaert et al. 1995; Giere 2009). Occupying a broad spectrum of trophic positions, nematodes are of great ecological importance, featuring high production efficiencies (Heip et al. 1990) and representing a crucial component in food webs and nutrient cycles by linking the micro- and macrofauna (e.g. Chardy and Dauvin 1992). Given their high diversity and ubiquitous appearance, their total number of species is still unknown (Hugot et al. 2001; Coomans 2002; Blaxter 2003). As consequence of a conserved and quite simple body plan, morphological diagnostics are difficult and laborious and in most cases restricted to experienced taxonomists. Many species can only be identified as adult males or specific female structures (Floyd et al. 2002), whereas it is almost impossible to identify juveniles or damaged specimens, resulting in a disdain of nematodes in most infaunal studies (Warwick and Robinson 2000). Moreover, natural variation as consequence of phenotypic plasticity (Kiontke and Fitch 2010) as well as cryptic and/or sibling species (Derycke et al. 2008, 2013) complicates a correct species identification based on morphology in many cases.

In order to overcome these limitations, molecular methods may represent a useful alternative or at least

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supplementary approach for the identification of nematodes. In this context, the International Barcode of Life initiative introduced the concept of DNA barcoding (Hebert et al. 2003a, b), using a small specific genomic region as so-called “DNA barcode”. The term “DNA barcoding” sensu stricto is generally used for the application of the small fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) for animals (e.g. Hebert et al. 2003a, b; Blaxter 2004). In the case of nematodes, this marker showed only a low applicability due to high sequence variability in the primer regions (Bhadury et al. 2006a, b; Elsasser et al. 2009). Nevertheless, some newly designed COI primers that promised better amplification quotes have been tested for some selected free-living marine species (Derycke et al. 2010a). Consequently, the application of a number of alternative nuclear DNA sequence markers has become popular for the identification of nematodes. These markers include various hypervariable expansion regions of the nuclear 18S (e.g. Blaxter et al. 1998; Bhadury et al. 2006a, b; Creer et al. 2010) and 28S rRNA genes (e.g. Litvaitis et al. 2000; De Ley et al. 2005; Pereira et al. 2010) as well as internal transcribed spacers (ITS; Hugall et al. 1999; Elbadri et al. 2002; Félix et al. 2014). Here, various studies showed that the D2–D3 region of the 28S rDNA represents one of the most suitable and promising markers for a valid nematode identification, which can be used to delimitate even closely related species (De Ley et al. 2005; Pereira et al. 2010).

However, the combination of molecular and morphological approaches has been shown to be most effective in species identification (De Ley et al. 2005) as well as disentangling species complexes within the Nematoda in general (e.g. Derycke et al. 2008; Gutiérrez-Gutiérrez et al. 2013); for example, Pereira et al. (2010) successfully identified nematodes from Baja California using partial 18S and 28S rDNA sequence data in combination with morphological analysis. Other studies successfully used a variety of markers (partial 28S rDNA, 18S rDNA, ITS and COI) in combination with morphological analysis to reveal the cryptic character and taxonomic status of morphospecies (Derycke et al. 2008, 2010b). An even more integrative approach was performed by Fonseca et al. (2008), combining molecular, multivariate morphometric, typological taxonomic and interbreeding data. All these integrative studies revealed the applicability and, to a certain degree, concordance between morphospecies which rely on morphological species concepts, and so-called sequence-based molecular operational taxonomic units (MOTUs) (Blaxter et al. 2005).

The aim of our study was a combined application of morphological and molecular methods as part of an integrative taxonomic approach to identify free-living benthic

**Table 1** Sampling data and site properties

	Site 1	Site 2	Site 3
Date of sampling	16.04.2012	02.05.2012	03.05.2012
Longitude and latitude	N 53°30'28.58", E 8°7'10.69"	N 53°29'47.85", E 8°11'14.81"	N 53°49'13.86", E 7°54'53.45"
Locality	Wilhelmshaven	Jade Bay	Wangerooge, “Außenjade”
Zonation	Intertidal	Sublittoral	Sublittoral
Tide	Periodical ebb and flow	Permanently flooded	Permanently flooded
Currents	Low physical disturbance, very weak currents	Strong currents of both ebb and flow	Strong currents of both ebb and flow
Sediment properties	60 % medium sand, 40 % mud	50 % medium sand, no mud, no gravel	50 % fine and medium sand

Sediment properties were determined by the project “AufMod” (Adam Kubicki, pers. communication, unpublished)

nematode species of the North Sea. We also tested the concordance of both approaches for the valid identification of male, female, juvenile and/or damaged specimens. Specimens were assigned to morphospecies, followed by a molecular analysis testing the quality of the D2–D3 region of the nuclear 28S rDNA for successful species identification.

## Materials and methods

### Sampling

All samples were taken between April 2012 and May 2012 from an intertidal mudflat in Wilhelmshaven (sampling site 1), a sublittoral site located in the Jade Bay (sampling site 2), and one sublittoral site near the island Wangerooge in the North Sea close to the German coast (sampling site 3; Table 1). For sampling site 1, the topmost 2–3 cm of the sediment was removed with a spoon. All sublittoral samples were taken by a multicorer (MUC,  $\varnothing$  105 mm) where the topmost 5 cm were removed from each tube. All sediment samples were immediately preserved in DESS (Yoder et al. 2006).

In total, 246 nematodes were extracted from the sediment by a procedure of multiple sieving and subsequent centrifugations. A combination of Levasil<sup>®</sup> and Kaolin ( $\text{Al}_2\text{O}_3 \cdot 2\text{SiO}_2 \cdot 2\text{H}_2\text{O}$ ) was added to separate the sediment pellet from the organisms in the liquid phase (see McIntyre and Warwick 1984). Organisms were rinsed with tap water

on a sieve of 40- $\mu\text{m}$  mesh size and transferred into storage containers filled with DESS solution. Nematodes were hand-picked by use of a binocular (Leica M125) and rinsed three times with sterile water to remove traces of DESS. Single specimens were transferred into glycerin solution (79 %  $\text{H}_2\text{O}$ , 20 % absolute ethanol (96 %), 1 % glycerin; modified after Riemann 1988) and followed by the method of slow evaporation used for further preparation.

#### Species identification, vouchering and DNA extraction

Based on successful amplification and sequencing, 151 single nematode specimens were preserved in temporary slides using glycerin and paraffin. All specimens were identified to be morphospecies using a microscope (Leica DM 2500) and appropriate taxonomic literature (Platt and Warwick 1983; 1988; Warwick et al. 1998) as well as with reference to the NeMys data base (Vanaverbeke et al. 2014). For subsequent vouchering and molecular studies, each specimen was removed from its slide and put onto a new slide provided with 5  $\mu\text{l}$  of so-called Worm Lysis Buffer (WLB, composed of 50 mM KCl solution, 10 mM Tris pH 8.3, 2.5 mM  $\text{MgCl}_2$ , 0.45 % Tergitol solution (NP40) and 0.45 % Tween 20, modified after Williams et al. 1992; and International Seabed Authority, 2011). Using two needles, the specimens were cut into several pieces. Anterior and posterior parts were isolated and kept in permanent slides. Additionally, high-quality preparations of whole animals were kept for each morphospecies as vouchers to facilitate a closer subsequent inspection. For this second and more detailed identification, each specimen was sketched and measured using a camera lucida for the identification to species level, using the previously mentioned literature. Gender (male, female) and, in the case of juveniles, the development stage was determined and documented. Total length, excluding the tail, and maximum body diameter were measured for each nematode. For DNA extraction, another 15  $\mu\text{l}$  of WLB and 2  $\mu\text{l}$  of proteinase K (Macherey & Nagel, Düren, Germany) were added to the remaining body fragments. Following an additional centrifugation, extracts were kept at  $-80^\circ\text{C}$  overnight and incubated for 1 h at  $65^\circ\text{C}$ , followed by 10 min at  $95^\circ\text{C}$ . After a final centrifugation, all extracts were stored at  $-20^\circ\text{C}$ .

#### PCR performance and sequencing

The primer pair D2A and D3B (De Ley et al. 2005) was used for the amplification of the 28S rDNA: D2–D3 region. Amplicons were generated using PCR beads (illustra puReTaq Ready-To-Go PCR Beads, GE Healthcare UK Limited, Buckinghamshire, United Kingdom) in 25- $\mu\text{l}$  reactions, filled up with 2  $\mu\text{l}$  of DNA extract, 0.5  $\mu\text{l}$  of

primer D2A (20 pm/ $\mu\text{l}$ ), 0.5  $\mu\text{l}$  of primer D3B (20 pm/ $\mu\text{l}$ ) and 22  $\mu\text{l}$  of dd $\text{H}_2\text{O}$ . Negative and positive controls were provided for each round of reactions. PCRs were performed by providing an initial denaturation at  $95^\circ\text{C}$  (5 min), followed by 40 cycles at  $94^\circ\text{C}$  (denaturation, 30 s),  $55^\circ\text{C}$  (annealing, 1 min),  $72^\circ\text{C}$  (elongation, 2 min) and a final elongation at  $72^\circ\text{C}$  for 10 min. After verification in a 1 % agarose gel stained with GelRed (Biotium Inc., Hayward, USA), PCR products were purified for sequencing using the ExoSap purification kit (Thermo Scientific, Osterode, Germany). Ten  $\mu\text{l}$  of the PCR product was mixed with 2  $\mu\text{l}$  of FastAp (Thermosensitive Alkaline Phosphatase, 1 U/ $\mu\text{l}$ ) and 0.5  $\mu\text{l}$  of Exo I (Exonuclease I, 20 U/ $\mu\text{l}$ ) on ice, and run on a thermocycler ( $37^\circ\text{C}$  for 15 min,  $85^\circ\text{C}$  for 15 min). Purified amplicons were sequenced using the PCR primers in both directions at contract sequencing facilities (Macrogen Europe, Amsterdam, Netherlands, or GATC Biotech AG, Cologne, Germany). All morphological vouchers and DNA extracts were stored as part of the voucher collection of the AG “Molecular Taxonomy of marine Organisms” at the German Center of Marine Biodiversity (DZMB), Senckenberg am Meer, Wilhelmshaven. Chromatograms were assembled, edited and checked manually with the Geneious package (Geneious Pro 5.4.6, Biomatters, Auckland, New Zealand). Ambiguities were corrected according to chromatogram properties, e.g. the presence and quality of peaks.

#### MOTU definition

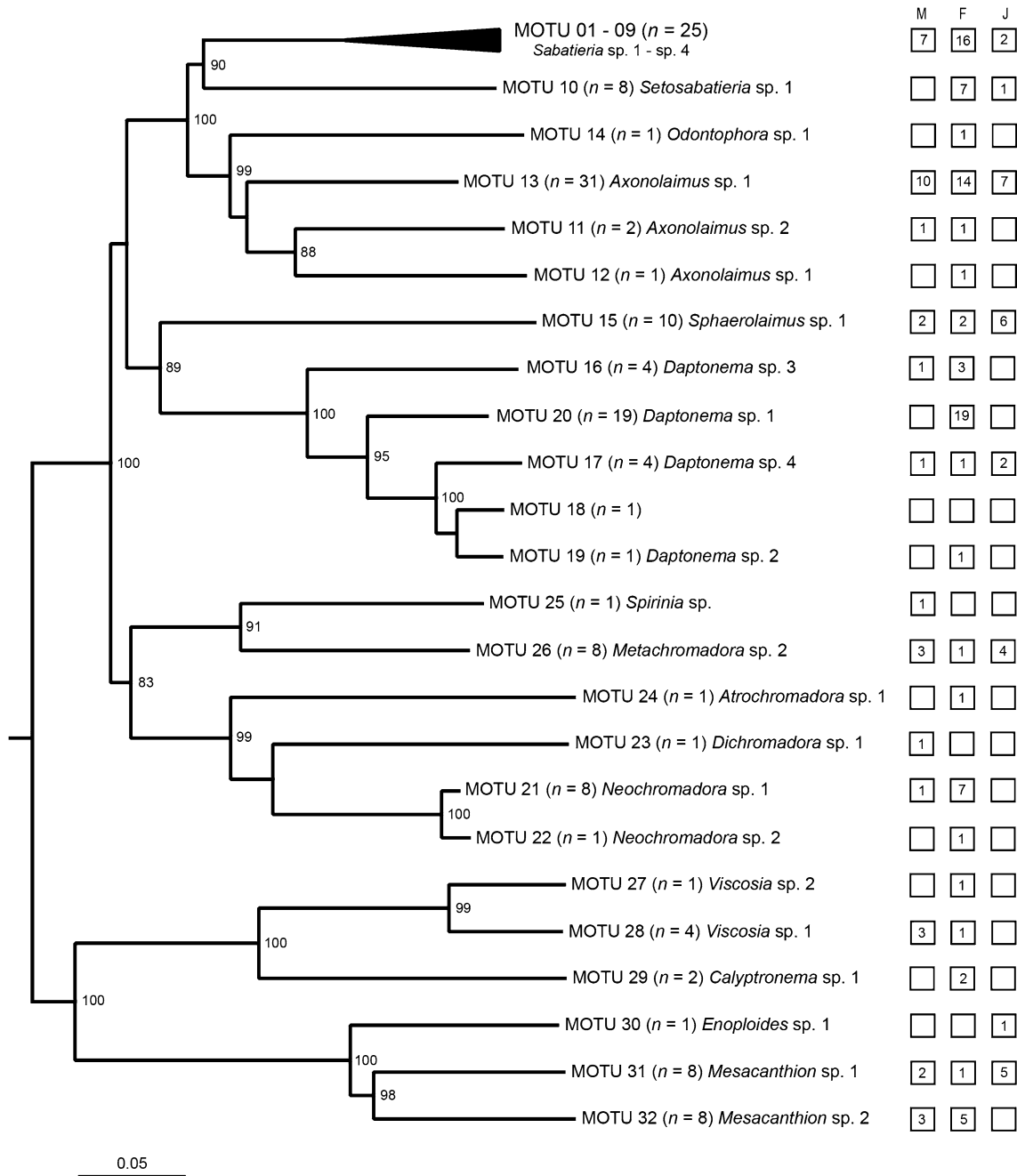
All sequences were aligned in MEGA 5.2.2 (Tamura et al. 2011) using the implemented MUSCLE algorithm (Edgar 2004) with default settings. The alignment was checked manually and by eye for sequence differences as well as ambiguities by comparison with the corresponding chromatograms in Geneious. Following previous studies, sequences differing by  $<3$  base pairs (bp) were designated as a MOTU (see Floyd et al. 2002 for details). A neighbor joining phylogram (Saitou and Nei 1987) with nonparametric bootstrap replicates ( $n = 1,000$ ) (Felsenstein 1985) using default settings ( $p$  distances, pairwise deletions), and calculation of  $p$  distances within and between MOTUs of the same genera (default settings, pairwise deletions) were performed using MEGA. Using BLAST (Zhang et al. 2000; Morgulis et al. 2008), each MOTU was compared with the sequence library of GenBank (NCBI) to exclude contaminations.

#### Data analyses

All obtained MOTUs were compared with the given morphospecies classification in order to analyze the level of

congruence of both approaches. All results apart from congruence were classified as “splittings” and “lumpings” (see Thormann et al. 2011). Whereas “lumping” describes a morphospecies that is represented by two or several MOTUs, “splitting” specifies a MOTU that is represented

by two or more morphospecies (e.g. Thormann et al. 2011). A successful accordance was given if a specific MOTU corresponded to a specific morphospecies, indicating that one MOTU contained all members of one morphospecies, exclusively.



**Fig. 1** Unrooted NJ-phylogram showing congruence of MOTU and morphospecies assignment. The phylogram was created by aligning single representative sequences from each MOTU cluster expect for MOTU 1–9 (see Fig. 2 for a detailed subtree). Morphospecies are shown behind each MOTU. The number of sequences clustered into a

MOTU is given in *brackets*; for details dealing MOTU 18 see paragraph consistency of approaches. *Numbers in boxes* indicate the number of males, females and juveniles. The *scale bar* indicates pairwise differences in percent. Numbers next to internal branches are bootstrap values (in %) which are only given when they had values >75 %

**Table 2** Identified nematode species based on a combination of morphological (morphospecies) and molecular data (MOTU) to species level, with corresponding accession numbers of the 28S rDNA: D2–D3 fragment

Morphospecies	Identified species	MOTU	Accession number
<i>Sabatieria</i> sp. 3	<i>Sabatieria celtica</i> Southern, 1914	MOTU 8	KC755203
<i>Setosabatieria</i> sp. 1	<i>Setosabatieria hilarula</i> de Man, 1922	MOTU 10	KC755205
<i>Metachromadora</i> sp. 2	<i>Chromadoropsis vivipara</i> de Man, 1907	MOTU 26	KC755217
<i>Spirinia</i> sp.	<i>Spirinia parasitifera</i> Bastian, 1865	MOTU 25	KC755216
<i>Mesacanthion</i> sp. 1	<i>Mesacanthion diplochma</i> Southern, 1914	MOTU 31	KC755226
<i>Sphaerolaimus</i> sp. 1	<i>Sphaerolaimus hirsutus</i> Bastian, 1865	MOTU 15	KC755210
<i>Viscosia</i> sp. 1	<i>Viscosia elegans</i> Filipjev, 1922	MOTU 28	KC755223
<i>Calyptronema</i> sp. 1	<i>Calyptronema maxweberi</i> de Man, 1922	MOTU 29	KC755224
<i>Axonolaimus</i> sp. 1	<i>Axonolaimus paraspinosus</i> Schuurmans, Stekhoven & Adam, 1931	MOTU 12	KC755209
<i>Ascolaimus</i> sp. 1	<i>Ascolaimus elongatus</i> Bütschli, 1874	MOTU 13	KC755207
<i>Neochromadora</i> sp. 1	<i>Neochromadora</i> aff. <i>poecilosoma</i> de Man, 1893	MOTU 21	KC755218
<i>Atrochromadora</i> sp. 1	<i>Atrochromadora</i> aff. <i>microlaima</i> de Man, 1889	MOTU 24	KC755221

## Results

### Morphological assignment and species identifications

The morphological identification of 151 specimens revealed 26 morphospecies. Out of these, 86 (57 %) were female, 36 male (23.8 %) and 28 juvenile (18.5 %). One of the vouchers was lost, so that no morphological assignment was performed. Each morphospecies comprised between one and 31 individuals (Fig. 1). It should be noted that the classification of some morphospecies was based on females or juveniles only, lacking important traits for a valid identification. In this context, the classification of *Odontophora* sp. 1, *Axonolaimus* sp. 1, *Daptonema* sp. 2, *Atrochromadora* sp. 1, *Neochromadora* sp. 2 and *Viscosia* sp. 2 was only based on one female specimen, whereas *Calyptronema* sp. 1 was represented by two females, and *Daptonema* sp. 1 by 19 females. In the case of *Enoploides*, only one juvenile was given. Furthermore, most specimens of the found *Sabatieria* morphospecies were females: Four individuals classified as *Sabatieria* sp. 1 were females, one was a male and one was a juvenile. The morphospecies *Sabatieria* sp. 2 included six females whereas *Sabatieria* sp. 3 was assigned by six female specimens and three males, and *Sabatieria* sp. 4 by three males and one juvenile.

Twelve morphospecies were determined as described species by morphological identification. The assignment of morphospecies to species is summarized in Table 2. It should be noted that two species were found to be *species affinis*, indicating that species were related to the binomial species name indicated and showed a similar morphological appearance but were not identical with it.

### Consistency of approaches

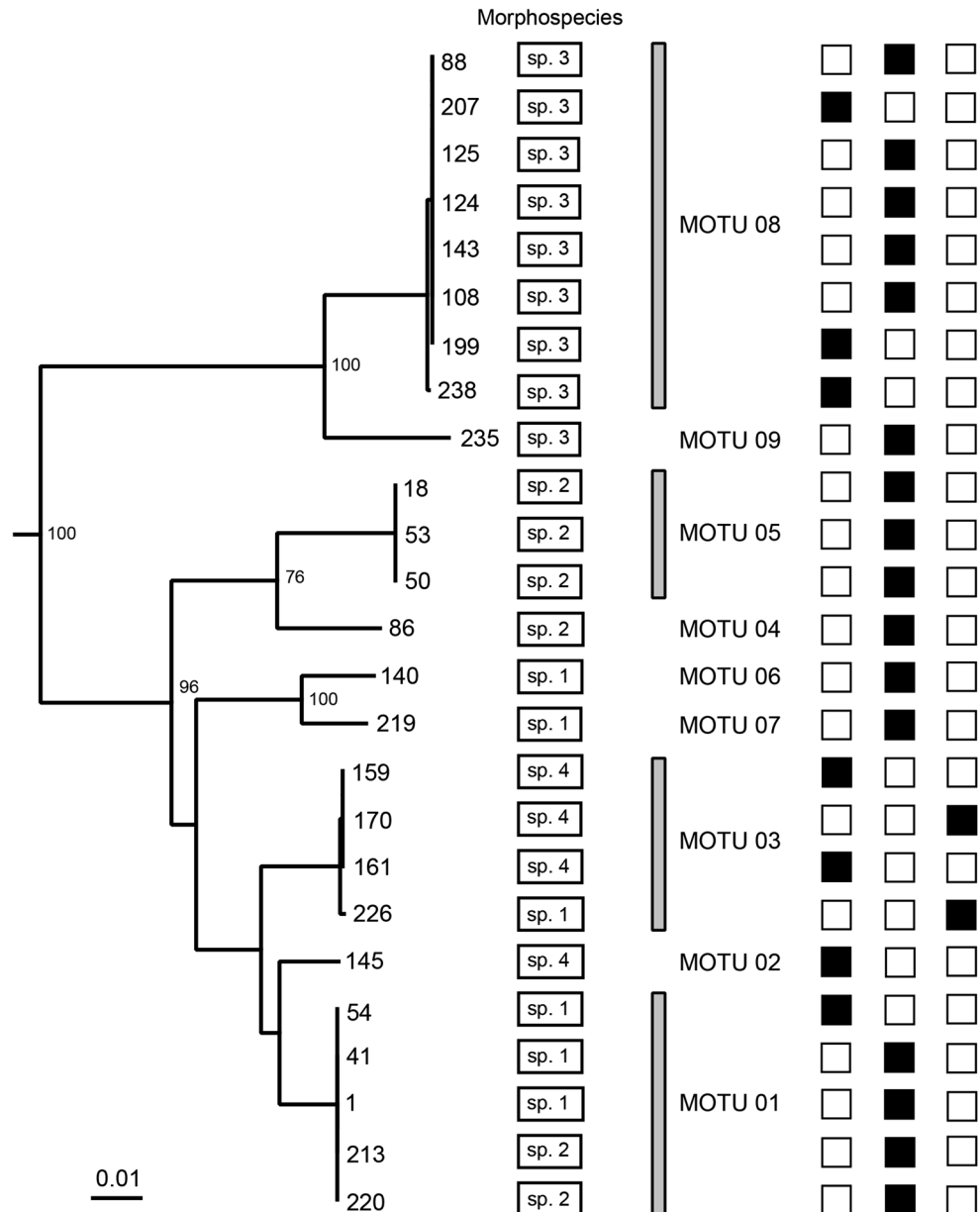
The majority of the analyzed specimens showed congruence in MOTU and morphospecies assignment ( $n = 126$  or 80.8 %, Fig. 1). The genus *Daptonema* Cobb, 1920 was represented by four morphospecies and corresponding MOTUS. Due to the loss of the corresponding voucher, it was not possible to correlate a fifth MOTU (MOTU 18) to a morphospecies. Consequently, this MOTU was classified as a member of the *Daptonema* morphospecies cluster on the basis of sequence properties only. However, it was possible to differentiate all remaining four morphospecies by a corresponding sequence (Fig. 1). This was also true for the morphospecies of *Axonolaimus* de Man, 1889, *Viscosia* de Man, 1890, *Neochromadora* Micoletzky, 1924 and *Mesacanthion* Filipjev, 1927, which were represented by two morphospecies (Fig. 1). In total, this study covered nine different families of five different orders of the Nematoda.

For the genus *Sabatieria* Rouville, 1903, our molecular data revealed more MOTUs than previously defined four morphospecies (Fig. 2). This is the case for MOTU 8 and MOTU 9 as well as MOTU 4 and MOTU 5. Both latter ones featured genetically distinct but closely related sequences and exhibited identical morphological assignment (Fig. 2). In contrast to this, a lumping was found for morphospecies 3, designated as *Sabatieria celtica* (Southern, 1914), whose sequences were differentiated as MOTU 8 and MOTU 9. Morphospecies 2 was represented within MOTU 1, MOTU 4 and MOTU 5. Furthermore, MOTU 1, MOTU 3, MOTU 6 and MOTU 7 contained sequences labeled as morphospecies 1, whereas morphospecies 4 was differentiated as MOTU 2 and MOTU 3 (Fig. 2). Moreover, MOTU 1 contained sequences labeled as morphospecies 1

**Fig. 2** Subtree of the analyzed *Sabatieria* specimens correlated with morphospecies information and corresponding MOTUs.

Numbers at the tips of the branches indicate specific sequence numbers.

Corresponding morphospecies assignment of each sequence is given in rectangles (sp. 1–4). Bars indicate specimens of the corresponding MOTU. Black boxes reveal the gender of the adult specimens (left males, central females) or juveniles (right) with unspecific gender. The scale bar shows pairwise differences in percent. Numbers next to internal branches are bootstrap values (in %) which are only given when they had values >75 %



and 2 and MOTU 3 included so defined morphospecies 4 and 1.

## Discussion

### Choice of marker and implications

Our results clearly demonstrated a successful species discrimination using the 28S rDNA: D2–D3 marker for the analyzed nematode species. The amplification success of the used marker (about 62 %) is comparable to previous studies (i.e. 67 % in Pereira et al. 2010). Earlier studies using the same fragment reported “unreliable” amplification results

(Bhadury et al. 2006a) as well as very high success rates (De Ley et al. 2005). Due to the fact that a marker for molecular species identification should be easy to amplify, PCR optimization represents an essential task for further studies. Problems in sample and specimen preservation and thus the degradation of DNA cannot be excluded as reasons for limited PCR success and should be optimized also. Furthermore, sequencing problems may be caused by the putative forming of primer dimers when using the PCR primer pair D2A and D3B for direct sequencing (GATC Biotech AG, European Custom Sequencing Centre, Cologne, Germany; personal communication).

A special characteristic of the D2–D3 region and ribosomal expansion segments in general is given by the high

proportion of variable sites as consequence of insertions and deletions, which are recognizable when observing the secondary structure of the complete 28S rRNA (Bae et al. 2010). Variation in secondary structures as stems, loops, single and double strands can affect the probability for mutations and therefore the variability within sites of the fragment significantly (Subbotin et al. 2007). Moreover, homology of expansion segments cannot be assumed between far-related species (e.g. Vogler et al. 1997; Gillespie et al. 2004; Raupach et al. 2010), making alignments of these fragments difficult to impossible. Whereas this putative disadvantage of the rDNA genes limits their use in phylogeny drastically (e.g. De Ley et al. 2005; Schmidt et al. 2006; Xie et al. 2009), this fact can be interpreted as beneficial according for the identification of even closely related species (e.g. Sonnenberg et al. 2007; Raupach et al. 2010; Khalaji-Pirbalouty and Raupach 2014). In terms of marine nematodes, some studies proposed that the D2–D3 domain can be used for DNA species identification as well as phylogenetic analyses (e.g. Derycke et al. 2008).

Based on sequence data, specimens can be identified and assigned to a known species when a close sequence match in a public sequence data base is given. For our data, only one match was found in GenBank [*Calyptronema maxweberi* (de Man 1922), Acc. No. AF210399], revealing the relatively low number of published nematode LSU sequences ( $n = 11,300$ ; NCBI, 21.08.2014) including many sequences of the same species (e.g. *Caenorhabditis elegans* Maupas, 1900 = 1,027, or *Unicaria lucasi* Stiles, 1901 = 141) in comparison with the total number of described nematode species (>25,000; Zhang 2013). Even morphologically well-studied and common species of the intertidal and sublittoral areas of the North Sea are still seriously underrepresented.

#### Integrated taxonomy

Except for the *Sabatieria* cluster it was possible to link all obtained MOTUs to a specific morphospecies of five different orders. In case of the genus *Daptonema*, we were able to demonstrate the value of combining molecular results with a revision of corresponding vouchers: The most analyzed specimens were female and showed almost no diagnostic traits, e.g. spicules or a gubernaculum, and many specimens were also found in a bad preservation state. A comparison of these specimens with vouchers of a better condition and quality as result of identical sequences allowed assignment of these nematodes to the corresponding morphospecies. For our data, MOTUs have been shown not to be artificial units but to correspond to taxon assignment by morphological manners in most cases. Based on these considerations, referenced sequence libraries or

stepping-stone sequences (Blaxter 2004) will improve the quality of public databases and overcome the gap between large scale studies, which are restricted to molecular data only. Therefore, DNA sequence data should become part of new species descriptions. Such data can allow ecologists and taxonomists a more efficient handling of the overwhelming abundance and variety of nematode specimen (Fonseca et al. 2010) and may significantly advance knowledge and progress in meiofaunal research (Blaxter and Floyd 2003; Markmann and Tautz 2005; Creer et al. 2010) by revealing new perspectives to the understanding of ecological and taxonomic identity, rank abundance as well as concomitant trophic dynamics (Fonseca et al. 2010).

#### The *Sabatieria* species complex

The molecular and morphological analysis of the analyzed *Sabatieria* specimens revealed a discrepancy between both approaches. MOTUs comprised sequences of individuals which were assigned to different morphospecies. Even an extensive morphological revision of the voucher specimens was not able to establish congruence between both approaches. This might be due to the variable character of many traits or difficulties in identifying them. Most analyzed specimens of the genus *Sabatieria* were females (66.6 %) or juveniles (8.3 %). Furthermore, a majority of these individuals were in bad condition, complicating a closer inspection of taxonomically important morphological traits. In case that the given morphospecies classification was correct, our results clearly revealed the lack of diagnostic traits of many morphospecies. Many species of this genus are classified primarily by anatomical structures found in males, e.g. the gubernaculum or the precloacal supplements (Platt 1985; Platt and Warwick 1988), making the identification of juveniles or females impossible. Interestingly, the genus *Sabatieria* itself is defined by a combination of plesiomorphic traits as well as the lack of other characteristics that are present within other genera of the family of the Comesomatidae (Platt 1985). The genus itself is very diverse, including over 100 described species (WoRMS 2014). Specimens of *Sabatieria* represent some of the most common free-living nematodes in marine benthic habitats (Platt 1985). Therefore it is not surprising that most species suffer from insufficient descriptions and diverse synonymizations (Platt 1985). As a consequence, this genus has been subjected to various more or less comprehensive revisions (Jensen 1979; Platt 1984, 1985; Vincx 1986). Furthermore, Sharma et al. (2006) found in a study combining morphological and molecular methods that several characters used for classification of the Comesomatidae in general have to be reevaluated.

It is obvious that a combination of morphological and molecular approaches clearly enhances the efficiency of

species delimitation and identification within this enigmatic genus. Specimens can be collected and identified to morphospecies on first sight. Subsequently, morphological vouchers of diagnostically important traits must be kept. In addition, digital vouchers can be made according to methods already described (De Ley et al. 2005). This method allows extensive revision of the available vouchers after molecular analysis of corresponding specimen.

## Summary

Our results confirmed the effectiveness of integrative taxonomic approaches for nematode species differentiation using a single molecular marker. The sequence analysis of distinct MOTUs facilitated the concept of reverse taxonomy with corresponding vouchers, allowing the identification of juvenile specimen, females or damaged specimens. Furthermore, the given amplification and sequencing results of the 28S rDNA: D2–D3 marker underlined the need for PCR and sequencing optimization.

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## References

- Bae CH, Robbins RT, Szalanski AL (2010) Secondary structure models of D2–D3 expansion segments of 28S rRNA for Hoplolaiminae species. *J Nematol* 42:218–229
- Bhadury P, Austen MC, Bilton DT, Lamshead PD, Rogers AD, Smerdon GR (2006a) Development and evaluation of a DNA-barcoding approach for the rapid identification of nematodes. *Mar Ecol Prog Ser* 320:1–9. doi:10.3354/meps320001
- Bhadury P, Austen MC, Bilton DT, Lamshead PD, Rogers AD, Smerdon GR (2006b) Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. *Aquat Microb Ecol* 44:97–103. doi:10.3354/ame044097
- Blaxter M (2003) Counting angels with DNA. *Nature* 421:122–124. doi:10.1038/421122a
- Blaxter ML (2004) The promise of a DNA taxonomy. *Philos Trans R Soc B* 359:669–679. doi:10.1098/rstb.2003.1447
- Blaxter M, Floyd R (2003) Molecular taxonomies for biodiversity surveys: already a reality. *Trends Ecol Evol* 18:268–269. doi:10.1016/S0169-5347(03)00102-2
- Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas WK (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* 392:71–75. doi:10.1038/32160
- Blaxter M, Mann J, Chapman T, Thomas F, Whitton C, Floyd R, Abebe E (2005) Defining operational taxonomic units using DNA barcode data. *Philos Trans R Soc B* 360:1935–1943. doi:10.1098/rstb.2005.1725
- Chardy P, Dauvin J-C (1992) Carbon flows in a subtidal fine sand community from the western English channel: a simulation analysis. *Mar Ecol Prog Ser* 81:147–161. doi:10.3354/meps081147
- Coomans A (2002) Present status and future of nematode systematics. *Nematology* 4:573–582
- Creer S, Fonseca VG, Porazinska DL, Giblin-Davis RM, Sung W, Power DM, Packer M, Carvalho GR, Blaxter ML, Lamshead PJD, Thomas WK (2010) Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. *Mol Ecol* 19:4–20. doi:10.1111/j.1365-294X.2009.04473.x
- De Ley P, De Ley IT, Morris K, Abebe E, Mundo-Ocampo M, Yoder M, Heras J, Waumann D, Rocha-Olivares A, Jay Burr AH, Baldwin JG, Thomas WK (2005) An integrated approach to fast and informative morphological vouchers of nematodes for applications in molecular barcoding. *Philos Trans R Soc B* 360:1945–1958. doi:10.1098/rstb.2005.1726
- Derycke S, Fonseca G, Vierstraete A, Vanfleteren J, Vincx M, Moens T (2008) Disentangling taxonomy within the *Rhabditis (Pelioditis) marina* (Nematoda, Rhabditidae) species complex using molecular and morphological tools. *Zool J Linn Soc Lond* 152:1–15. doi:10.1111/j.1096-3642.2007.00365.x
- Derycke S, Vanaverbeke J, Rigaux A, Backeljau T, Moens T (2010a) Exploring the use of cytochrome oxidase c subunit I (COI) for DNA barcoding of free-living marine nematodes. *PLoS One* 5:e13716. doi:10.1371/journal.pone.0013716
- Derycke S, De Ley P, De Ley IT, Holovachov O, Rigaux A, Moens T (2010b) Linking DNA sequences to morphology: cryptic diversity and population genetic structure in the marine nematode *Thoracostoma trachygaster* (Nematoda, Leptosomatidae). *Zool Scr* 39:276–289. doi:10.1111/j.1463-6409.2009.00420.x
- Derycke S, Backeljau T, Moens T (2013) Dispersal and gene flow in free-living marine nematodes. *Front Zool* 10:1. doi:10.1186/1742-9994-10-1
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797. doi:10.1093/nar/gkh340
- Elbadri GAA, De Ley P, Waeyenberge L, Vierstraete A, Moens M, Vanfleteren J (2002) Intraspecific variation in *Radopholus similis* isolates assessed with RFLP and DNA sequencing of the internal transcribed spacer region of the ribosomal RNA cistron. *Int J Parasitol Res* 32:199–205. doi:10.1016/S0020-7519(01)00319-8
- Elsasser SC, Floyd R, Hebert PDN, Schulte-Hostedde AI (2009) Species identification of North American guinea worms (Nematoda: *Dracunculus*) with DNA barcoding. *Mol Ecol Res* 9:707–712. doi:10.1111/j.1755-0998.2008.02393.x
- Félix M-A, Braendle C, Cutter AD (2014) A streamlined system for species diagnosis in *Caenorhabditis* (Nematoda: Rhabditidae) with name designations for 15 distinct biological species. *PLoS ONE* 9:e94723. doi:10.1371/journal.pone.0094723
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Floyd R, Eyualem A, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. *Mol Ecol* 11:839–850. doi:10.1046/j.1365-294X.2002.01485.x
- Fonseca G, Derycke S, Moens T (2008) Integrative taxonomy in two free-living nematode species complexes. *Biol J Linn Soc* 94:737–753. doi:10.1111/j.1095-8312.2008.01015.x



- Fonseca VG, Carvalho GR, Sung W, Johnson HF, Power DM, Neill SP, Packer M, Blaxter ML, Lambhead JD, Thomas WK, Creer S (2010) Second-generation environmental sequencing unmasks marine metazoan biodiversity. *Nat Commun* 1:98. doi:10.1038/ncomms1095
- Giere O (2009) *Meiobenthology: the microscopic motile fauna of aquatic sediments*. Springer, Berlin
- Gillespie J, Cannone JJ, Gutell RR, Cognato A (2004) A secondary structure model of the 28S rRNA expansion segments D2 and D3 from rootworms and related leaf beetles (Coleoptera: Chrysomelidae; Galerucidae). *Insect Mol Biol* 13:495–518. doi:10.1111/j.0962-1075.2004.00509.x
- Gutiérrez-Gutiérrez C, Cantalapiedra-Navarrete ER, Remesal E, Palomares-Rius JE, Navas-Cortés JA, Castillo P (2013) New insight into the identification and molecular phylogeny of dagger nematodes of the genus *Xiphinema* (Nematoda: Longidoridae) with description of two new species. *Zool J Linn Soc Lond* 169:548–579. doi:10.1111/zooj.12071
- Hebert PDN, Cywinska A, Ball SL (2003a) Biological identifications through DNA barcodes. *Proc R Soc Lond B Biol* 270:313–321. doi:10.1098/rspb.2002.2218
- Hebert PDN, Ratnasingham S, de Waard JR (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc R Soc Lond B Biol* 270(Suppl.):S96–S99. doi:10.1098/rsbl.2003.0025
- Heip CHR, Vincx M, Vanreusel A, Smol N, Herman R, Herman PMJ (1990) Composition, distribution, biomass and production of North Sea meiofauna. *Neth J Sea Res* 26:333–342
- Hugall A, Stanton J, Moritz C (1999) Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic Meloidogyne. *Mol Biol Evol* 16:157–164
- Hugot JP, Baujard P, Morand S (2001) Biodiversity in helminths and nematodes as a field of study: an overview. *Nematology* 3:199–208
- International Seabed Authority (2011) *Marine benthic nematode molecular protocol handbook (nematode barcoding)*. International Seabed Authority, Kingston
- Jensen P (1979) Nematodes from the brackish waters of the southern archipelago of Finland. Benthic species. *Ann Zool Fenn* 16:151–168
- Khalaji-Pirbalouty V, Raupach MJ (2014) A new species of *Cymodoce* Leach, 1814 (Crustacea: Isopoda: Sphaeromatidae) from the Persian Gulf based on morphological and molecular characteristics, with a redescription of *Cymodoce tribullis* from Queensland. *Zootaxa* 3826:230–254. doi:10.11646/zootaxa.3826.1.7
- Kiontke K, Fitch DHA (2010) Phenotypic plasticity: different teeth for different feasts. *Curr Biol* 20:R710–R712. doi:10.1016/j.cub.2010.07.009
- Litvaitis MK, Bates JW, Hope WD, Moens T (2000) Inferring a classification of the Adenophorea (Nematoda) from nucleotide sequences of the D3 expansion segment (26/28S rDNA). *Can J Zool* 78:911–922. doi:10.1139/z00-039
- Markmann M, Tautz D (2005) Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. *Philos Trans R Soc B* 360:1917–1924. doi:10.1098/rstb.2005.1723
- McIntyre AD, Warwick RM (1984) Meiofauna techniques. In: Holme NA, McIntyre AD (eds) *Methods for the study of marine benthos*, 2nd edn. Blackwell, Oxford, pp 217–244
- Morgulis M, Coulouris G, Rayseis Y, Madden TL, Agarwala R, Schäffer AA (2008) Database indexing for production MegaBlast searches. *Bioinformatics* 24:17157–17164. doi:10.1093/bioinformatics/btn322
- Pereira TJ, Fonseca G, Mundo-Ocampo M, Guilherme BC, Rocha-Olivares A (2010) Diversity of free-living marine nematodes (Enoplida) from Baja California assessed by integrative taxonomy. *Mar Biol* 157:1665–1678. doi:10.1007/s00227-010-1439-z
- Platt HM (1984) The freeliving marine nematode genus *Sabatieria* (Nematoda: Comesomatidae). II. Redescriptions of five European species. *Bull Br Mus Nat Hist Zool* 46:355–375
- Platt HM (1985) The freeliving marine nematode genus *Sabatieria* (Nematoda: Comesomatidae). Taxonomic revision and pictorial keys. *Zool J Linn Soc Lond* 83:27–78. doi:10.1111/j.1096-3642.1985.tb00872.x
- Platt HM, Warwick RM (1983) *Free-living marine nematodes Part I: British Enoplids*. Cambridge University, Cambridge
- Platt HM, Warwick RM (1988) *A synopsis of the free-living marine nematodes (Part II. British chromadorids)*. Brill and Backhuys, Leiden
- Raupach MJ, Astrin JJ, Hannig K, Peters MK, Stoeckle MY, Wägele JW (2010) Molecular species identification of Central European ground beetles (Coleoptera: Carabidae) using nuclear rDNA expansion segments and DNA barcodes. *Front Zool* 7:26. doi:10.1186/1742-9994-7-26
- Riemann F (1988) Nematoda. In: Higgins RP, Thiel H (eds) *Introduction to the study of Meiofauna*. Smithsonian Institution Press, Washington, DC, pp 293–301
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schmidt S, Driver F, De Barro P (2006) The phylogenetic characteristics of three different 28S rRNA gene regions in *Encarsia* (Insecta, Hymenoptera, Aphelinidae). *Org Divers Evol* 6:127–139. doi:10.1016/j.ode.2005.07.002
- Sharma J, Sun L, Hope WD, Ferris VR (2006) Phylogenetic relationships of the marine nematode family Comesomatidae. *J Nematol* 38:229–232
- Soetaert K, Vincx M, Wittoeck J, Tulkens M (1995) Meiobenthic distribution and nematode community structure in five European estuaries. *Hydrobiologia* 311:185–206
- Sonnenberg R, Nolte AW, Tautz D (2007) An evaluation of LSU rDNA D1–D2 sequences for their use in species identification. *Front Zool* 4:6. doi:10.1186/1742-9994-4-6
- Subbotin SA, Sturhan D, Vovlas N, Castillo P, Tambe JT, Moens M, Baldwin JG (2007) Application of the secondary structure model of rRNA for phylogeny: D2–D3 expansion segments of the LSU gene of plant-parasitic nematodes from the family Hoplolaimidae Filipjev, 1934. *Mol Phylogenet Evol* 43:881–890. doi:10.1016/j.ympev.2006.09.019
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739. doi:10.1093/molbev/msr121
- Thormann B, Raupach MJ, Wagner T, Wägele JW, Peters MK (2011) Testing a short nuclear marker for inferring staphylinid beetle diversity in an African tropical rain forest. *PLoS One* 6:e18101. doi:10.1371/journal.pone.0018101
- Vanaverbeke J, Bezerra TN, Braeckman U, De Groote A, De Meester N, Deprez T, Derycke S, Guilini K, Hauquier F, Lins L, Maria T, Moens T, Pape E, Smol N, Taheri M, Van Campenhout J, Vanreusel A, Wu X, Vincx M (2014) NeMys: world database of free-living marine nematodes. <http://nemys.ugent.be>. (Accessed Aug 2014)
- Vincx M (1986) Free-living marine nematodes from the Southern Bight of the North Sea. I. Notes on species of the genera *Gonionchus* Cobb, 1920, *Neochromadora* Micoletzky, 1924 and *Sabatieria* Rouville, 1903. *Hydrobiologia* 140:255–286
- Vogler AP, Welsh A, Hancock JM (1997) Phylogenetic analysis of slippage-like sequence variation in the V4 rRNA expansion segment in tiger beetles (Cicindelidae). *Mol Biol Evol* 14:6–19
- Warwick RM, Robinson J (2000) Sibling species in the marine pollution indicator genus *Pontonema* Leidy (Nematoda:

- Oncholaimidae), with a description of *P. mediterranea* sp. nov. J Nat Hist 34:641–662. doi:[10.1080/002229300299327](https://doi.org/10.1080/002229300299327)
- Warwick RM, Platt HM, Somerfield PJ (1998) Free-living marine nematodes Part III: Monhysterids. Field Studies Council Press, Shrewsbury
- Williams BD, Schrank B, Huynh C, Shownkeen R, Waterston RH (1992) A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics 131:609–624
- WoRMS Editorial Board (2014) World register of marine species. <http://www.marinespecies.org>. (Accessed 1 Feb 2014)
- Xie Q, Tian X, Qin Y, Bu W (2009) Phylogenetic comparison of local length plasticity of the small subunit of nuclear rDNAs among all Hexapoda orders and the impact of hyper-length-variation on alignment. Mol Phylogenet Evol 50:310–316. doi:[10.1016/j.ympev.2008.10.025](https://doi.org/10.1016/j.ympev.2008.10.025)
- Yoder M, De Ley IT, King IW, Mundo-Ocampo M, Mann J, Blaxter M, Poiras L, De Ley P (2006) DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. Nematology 8:367–376
- Zhang Z (2013) Animal biodiversity: an update of classification and diversity in 2010. Zootaxa 3703:5–11. doi:[10.11646/zootaxa.3703.1.3](https://doi.org/10.11646/zootaxa.3703.1.3)
- Zhang Z, Schwatz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214. doi:[10.1089/10665270050081478](https://doi.org/10.1089/10665270050081478)