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BIODIVERSITY ASSESSMENT OF MARINE SEDIMENTS THROUGH DNA BARCODING

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1 Introduction

1.1 Marine nematode biodiversity

Marine communities occupy one of the largest ecosystems on earth, however, only 1% of the species are estimated to be known (Snelgrove, 1999). Benthic meiofauna represents a major part of marine biodiversity and are made up of 60% of animal phyla (Snelgrove, 1999). Consequently, various groups of researchers have reported that marine benthic metazoan communities display highest diversity on the earth (Hebert *et al.*, 2003; Lambshead & Boucher, 2003; Pereira *et al.*, 2010; Fonseca *et al.*, 2014).

Among these numerous metazoans on earth, nematodes have been described to be the most abundant of all (Decraemer & Hunt, 2006) and are known to perform important roles in marine ecosystem functioning. These include essential roles such as ecological recycling, sediment transport, secondary production, and mineralization (Gage, 1994; Raghukumar *et al.*, 2001; Giere, 2009). They occupy almost every habitat as free living nematodes both terrestrial and marine environments, and as parasitic forms in plants animals and in humans as well (Decraemer & Hunt, 2006).

Nematode abundance in marine and terrestrial domains is surprisingly similar (Lambshead & Schalk, 2001) taking into consideration the total coverage of the world ocean (two thirds of the earth) and the fact that systematics of marine species have received little attention (Lambshead & Schalk, 2001). Penurious dwellings, including the extensive deep-sea abyssal plains, can contain 10^5 nematodes per m^2 while productive habitats such as heterogeneous, fine sand sediments in shallow sea bottoms and interstitial systems that provides enough solute and oxygen transport (Muresan, 2012) have been reported to contain no less than 10^6 or, exceptionally 10^7 with a possible upper limit of 10^8 m^2 nematodes (Lambshead & Boucher, 2003). Free living nematodes have been reported to reach densities of 10^6 individuals per m^2

in marine sediment therefore making them one of the most abundant and diverse groups of meiofauna to inhabit marine sediments (Heip *et al.*, 1985).

Their abundance is dependent on availability of food and most clearly seen in marine sediments where abundance declines with depth (Cook *et al.*, 2005). The highest nematode richness, hence, tends to be found in the rich lowlands, marshes and marine mud around coastlines (Alongi, 1987; Boucher & Clavier, 1990). Exceptions to this general pattern exist in some tropical areas where terrestrial export can be toxic for these organisms (Alongi, 1987; Boucher & Clavier, 1990).

1.2 Dispersal in nematodes

Dispersal is an important life history traits for species evolution and persistence and allows organisms to escape unfavorable environmental conditions, avoid competition and increase their distribution (Derycke *et al.*, 2013). The majority of known species are however based on descriptions of small numbers of specimens from single or just few localities (Coomans, 2002) thereby ignoring the extent of natural variation that may exist among these species (Wilson & Kakouli-Duarte, 2009). The mode and importance of dispersal in generating and maintaining nematode diversity may vary in different enviroments. Costant movement may be the norm in exposed sandy beaches where there nematodes and sediments are constantly remixed (Nicholas & Hodda, 1999; Nicholas, 2001). Competitive exclusion may seldom operate , and similar species will coexist than in other environments hence resulting to higher diversity(Wilson & Kakouli-Duarte, 2009).

A considerable amount of marine nematodes are known to be endobenthic with relatively limited active dispersal capacities (Wetzel *et al.*, 2002). Passive movements through erosion and active emergence into the water columns, sediment including other possible means aids in their dispersal (Wetzel *et al.*, 2002). Nematodes can actively inhabit nearby unoccupied patches (Gallucci *et al.*, 2008; Guilini *et al.*, 2011). It is well known that they utilizes the presence of

receptors to identify suitable spots and to influence where to settle (Ullberg & Ólafsson, 2003). Even though they resuspend passively in the water-column in high hydrodynamic areas, coupled with the fact that they are poor swimmers, their control over the final site of settlement shows that there is an active component to dispersal and settlement (Palmer, 1988; Palmer *et al.*, 1996).

Despite their limited dispersal abilities, many marine meiofauna including marine nematodes are known to have nearly wide to cosmopolitan distributions, which creates a paradox (Jorger *et al.*, 2012) considering earlier reports of low dispersal abilities and that dispersal over large distances is likely to be limited (Derycke *et al.*, 2005). Their migration to distant patches is is substantial at geographical scales of 50 km, but is restricted at larger geographical scales (several 100's of kilometers) (Derycke *et al.*, 2013). *Litoditis marina*, which often is associated with decaying and standing macroalgae (Derycke *et al.*, 2013) has been reported from coastal environments in Europe, along the Mediterranean Sea, on both sides of the Atlantic Ocean (Inglis & Coles, 1961), Vancouver Island, Canada (Sudhaus & Nimrich, 1989), New Zealand, North Africa, Australia and from both the Antarctic and Arctic archipelago (T. Moens. Unpubished). Also *Oncholaimid* species have been reported to be highly cosmopolitan (Bik *et al.*, 2010).

Some mechanisms including water-column processes, natural rafts such as vegetation masses, sea ice, or anthropogenic transports are possible aids in nematode distribution hence accounting for their existence in many parts of the world (Bik *et al.*, 2010). Previous studies have reported an abundance of nematodes in floating mangrove detritus (Arroyo *et al.*, 2006; Faust *et al.*, 2008) thereby promoting the role of raft attachment as a dispersal method that may help maintain cosmopolitan distributions in Oncholaimids. Cosmopolitanism may also be caused by endemic cryptic species and also as a result of the widespread of single species (Derycke *et al.*, 2008b).

1.3 Nematode taxonomy

Despite the huge number of individuals and large number of species found in marine nematode communities, the estimated number of described nematode species is only 27,500 (2, 75%) (Hallan, 2007; Derycke *et al.*, 2008a) out of over 10^6 reported by (Lambshead & Boucher, 2003). This inconsistency between known and estimated diversity is however known among most if not all microscopic eukaryote groups.

The discovery, occurrence and presence of cryptic or hidden species (at least two distinct species identified as a single species based on morphology, but differentiated genetically (Bickford *et al.*, 2007a) in the phylum Nematoda has been well documented (Derycke *et al.*, 2008a; Derycke *et al.*, 2010b; Oliveira *et al.*, 2011; Derycke *et al.*, 2012; Ristau *et al.*, 2013). Despite the presence of cryptic diversity in all metazoan taxa and in all biogeographic regions (Bickford *et al.*, 2007b; Pfenninger & Schwenk, 2007), knowledge about the ecology of cryptic nematode species has only recently been investigated (De Meester *et al.*, 2012; Van Campenhout *et al.*, 2014). These species also differ in ecology hence it is important that they are identified correctly. Therefore nematode species identification would benefit greatly from additional sources of data than only morphological approaches to delineate species.

Identification systems based solely on morphometric and morphological characters is not only time consuming but also very challenging in terms of the fact that there is high phenotypic plasticity among populations and because of the absence of easily observable diagnostic characters for cryptic species. (Avise & Walker, 1999; Derycke *et al.*, 2008a; Fonseca *et al.*, 2008). Consequently, there is still much more taxonomic work to be done than has been done over the last 200 years. (Ristau *et al.*, 2013) hence suggested that, overall diversity of these nematodes have so far been radically underestimated.

1.4 Taxonomy: Traditional and molecular approaches

Lorenzen (1981) and Lorenzen (1994) proposed the classification of the phylum on the basis of cladistic analysis of morphological characters as illustrated in Figure 1. Lorezen‘s framework (Fig.1) is the accepted system for the classification of marine nematodes and has been used as the basis for Platt and Warwick‘s ubiquitous illustrated keys for the identification of marine nematodes (Platt & Warwick, 1983).

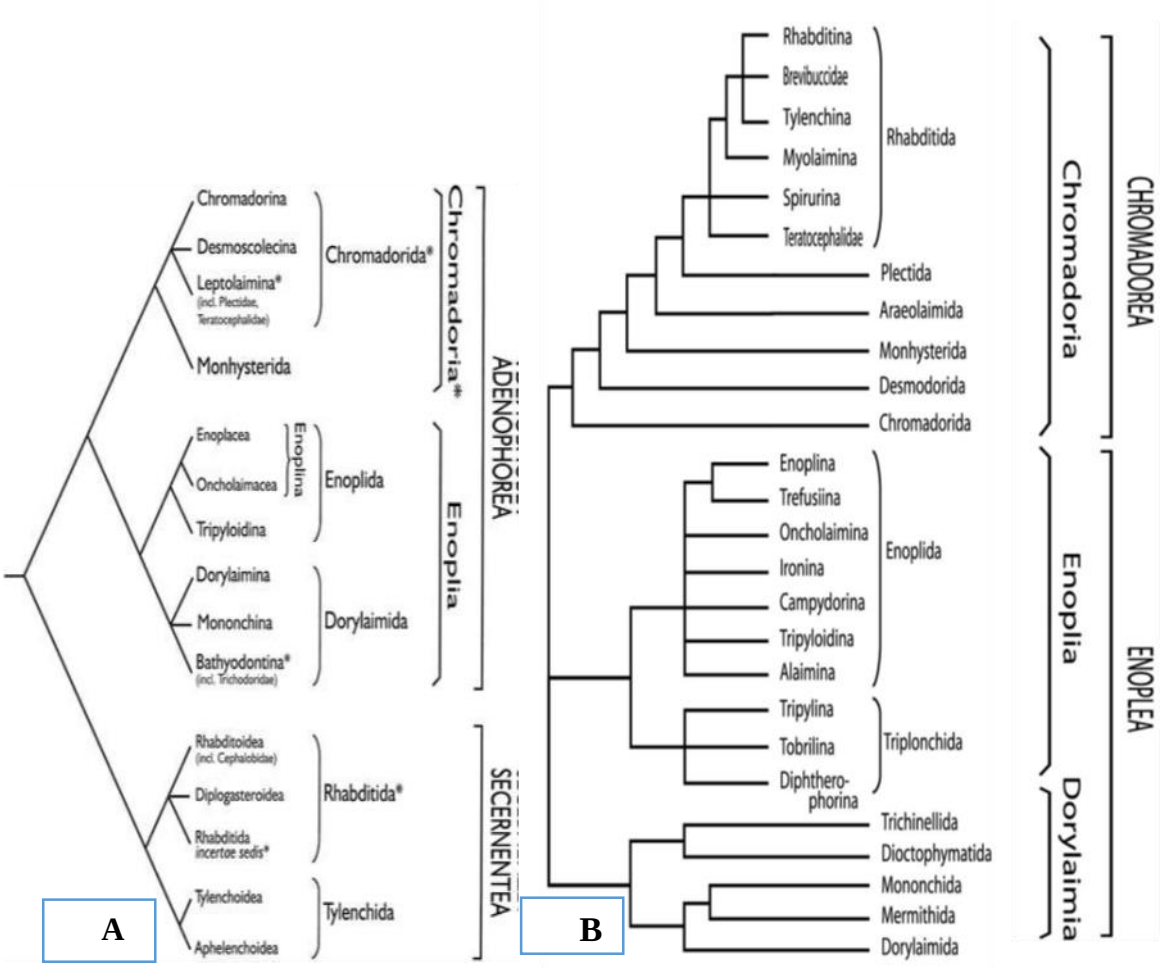

Fig. 1. (Lorenzen, 1981, 1994) on the basis of cladistic analysis of morphological characters with focus on the Adenophorea resolved two basal clades (Enoplids and chromadorids (A), (De Ley & Bert, 2002) phylogenetic relationship based on SSU rDNA sequence data supports three basal clades (Dorylaims, Enoplids and Chromadorids) (B).

The dawn of molecular techniques has opened up new opportunities for research in taxonomy owing to the fact that the vast majority of species are not well differentiated

morphologically (Godfray, 2002) or are simply genetically different but morphologicaly identical (Seberg *et al.*, 2003; Vogler & Monaghan, 2007; De Meester *et al.*, 2012). These new techniques improve taxonomic precision and help in serious investigations of the accuracy afforded by morphological traits that are commonly used in traditional taxonomy (Will & Rubinoff, 2004).

There has been conflicting resolution about the classification and relationships within the phylum nematode based on morphological approaches (Lorenzen, 1981, 1994; Malakhov, 1994). The quest to use molecular tools to further address issues concerning classification, systematics and phylogeny of taxa has been highly sort for.

Molecular approaches on the other hand not only allows the rapid and effective identification of most taxa, but also includes those not encountered before (Blaxter *et al.*, 1998; Félix *et al.*, 2000; Floyd *et al.*, 2002; Blaxter, 2003; Blaxter, 2004; De Ley *et al.*, 2005a; Hebert & Gregory, 2005; Holterman *et al.*, 2006; Holterman *et al.*, 2008). (Blaxter *et al.*, 1998) were among the first to exploit the potential of small subunit ribosomal DNA (SSU rDNA) sequence data to resolve phylogenetic relationships among nematodes. A total of five major clades made up of 53 taxa were recognized in their phylogenetic analysis. It was however not surprising when a subdivision of the phylum Nematoda was presented into 12 clades based on a series of mostly well-supported bifurcations in the backbone of the tree (339 taxa)(Fig. 2) (Holterman *et al.*, 2006). More marine nematodes were however included in the classification due to the advancement in the use of molecular tools (Meldal *et al.*, 2007; Holterman *et al.*, 2008).

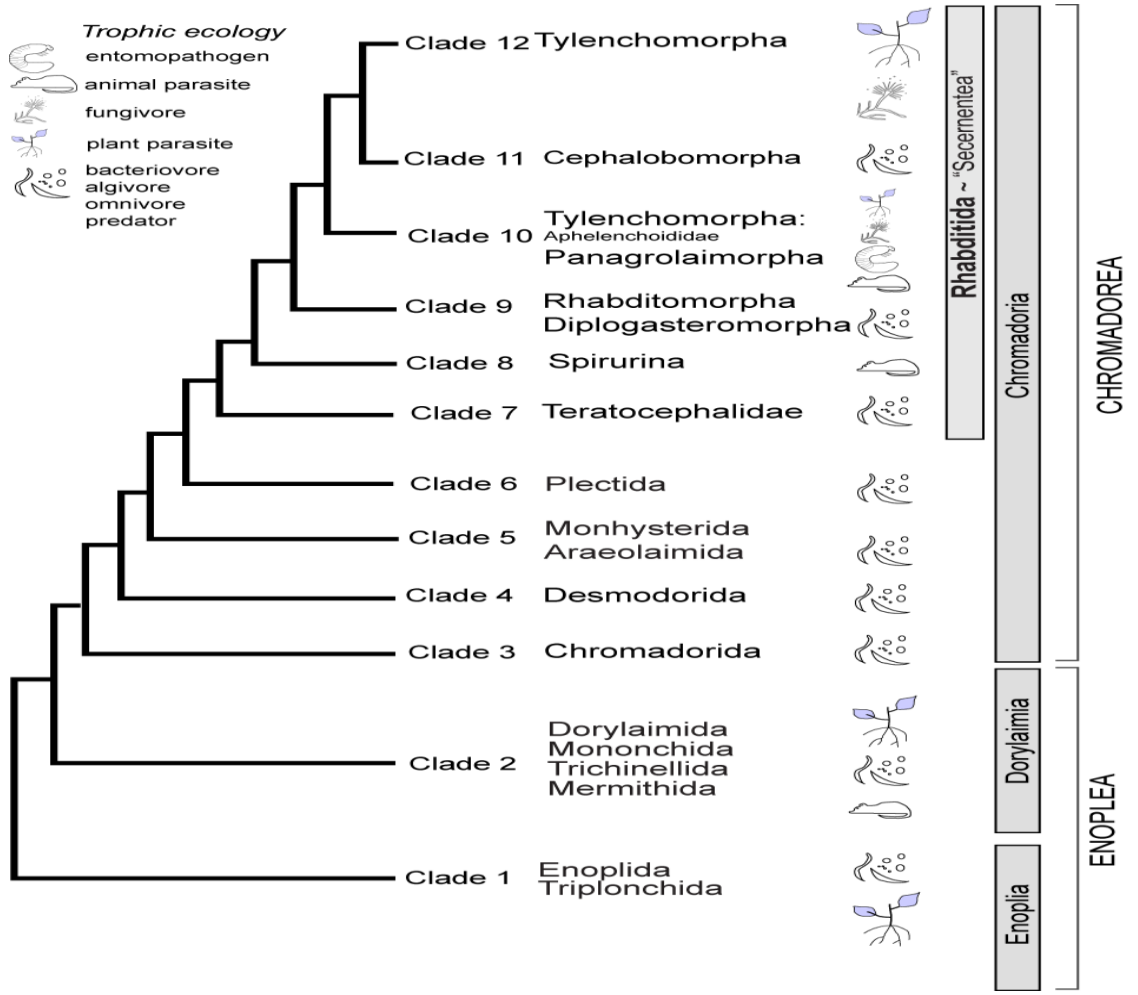

Fig. 2. Classification based on 339 nearly full-length small-subunit rDNA sequences (Holterman *et al.*, 2006; Meldal *et al.*, 2007).

The concept DNA taxonomy proposed by (Tautz *et al.*, 2003) consists of a tissue sample, taken from an individual, from which DNA is extracted. This DNA serves as the reference sample from which one or several gene regions are amplified by PCR and sequenced. The resulting sequence will then serve as an identification tag for the species from which the respective individual was derived (Lipscomb *et al.*, 2003; Mallet & Willmott, 2003; Will & Rubinoff, 2004)

Several studies have already illustrated the advances afforded by the interactive process between morphology and DNA barcoding for efficient species diagnostics in systematics and resolving of relationship in marine nematodes (Blaxter, 2004; Hebert *et al.*, 2004; Lee, 2004; Hebert & Gregory, 2005). The overwhelming task for taxonomy in ecological and biodiversity

research arguably requires entirely new approaches (Godfray, 2002; Da Silva *et al.*, 2010; Derycke *et al.*, 2010b).

The use of molecular markers however, have their own limitations. No standardized molecular marker has been found to be effective in solving phylogeny problems. Most molecular studies in nematodes focuse on the use of 18S rDNA due to the availability of universal nematode primers and ability to resolve at the genus level (De Ley *et al.*, 2005b). Sadly, 18S gene has low resolution and so cannot distinguish between closely related species (Fitch *et al.*, 1995; De Ley *et al.*, 2005b; Derycke *et al.*, 2005; Meldal *et al.*, 2007; Derycke *et al.*, 2010b). In contrast, the mitochondrial COI gene can adequately distinguish between closely related species, but the unavailability of universal primers to amplify the CO1 gene gives poor amplification results (De Ley *et al.*, 2005b; Bhadury *et al.*, 2006b)

No single approach has been reported to be perfect in species diagnostics (Abebe *et al.*, 2013). The combination of various techniques is however appropriate to enable a more holistic and comprehensive approach to solve taxonomic problems (Abebe *et al.*, 2013). DNA sequences alone are not sufficient to characterize species (Lee, 2004), however, their unique reproducibility helps to prevent against duplicate descriptions. Moreover, collection and curation of extracted DNA samples is technically easy

1.5 Molecular markers for systematics and identification of marine nematodes

In nematology, several molecular markers and techniques have been utilized for species diagnosis, example is the use of 18S rRNA in nematode systematics and phylogeny (Aleshin *et al.*, 1998; Blaxter *et al.*, 1998). Moreover, free living marine nematodes have benefited enormously from the use of DNA sequencing for rapid and accurate taxonomic identification including understanding population genetic structure in marine nematodes (De Ley *et al.*, 2005a; Bhadury *et al.*, 2006b; Derycke *et al.*, 2007; Derycke *et al.*, 2008a).

Nuclear and mitochondrial genome regions were studied and their potentials evaluated for use in marine nematode identification (Bhadury *et al.*, 2006b). An overview of the genes employed in DNA-based identification can be classified into two broad groups of genes; nuclear genes and mitochondrial genes.

1.5.1 Nuclear genes

The eukaryotic cell contains ribosomes that are essential for translation of mRNA to proteins. These ribosomes are encoded by the ribosomal subunits which encode ribosomal RNA genes (rRNA) within the nuclear genome as tandem repeat arrays, with each repeat containing one copy of conserved coding regions (28S, 18S, and 5.8S subunit genes). It also has rapidly evolving noncoding regions encompassing the internal and external transcribed spacers (ITS and ETS, respectively) and intergenic spacers (IGS) (Eickbush & Eickbush, 2007). Figure 2 describes the details of the rRNA gene region with it various components.

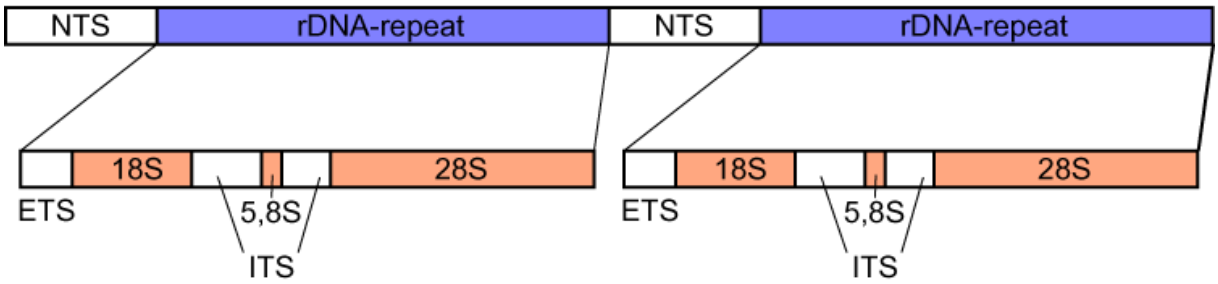

Figure 3: Diagrammatic illustration showing a tandem repeat unit of the various segments of the ribosomal RNA structure. ITS = Internal transcribed spacer; ETS= External transcribed spacer; NTS, non-transcribed spacer.

Ribosomal RNA genes have been used widely as markers for nematodes in general for phylogeny reconstruction (Nadler, 1992; Blaxter *et al.*, 1998; Meldal *et al.*, 2007) diversity analysis and species identification (Meldal *et al.*, 2007; Thornhill *et al.*, 2007).

1.5.1.1 Internal transcribed spacer (ITS)

This region is located between the repeating array of nuclear 18S and 28S ribosomal DNA genes and is a versatile genetic marker (Powers *et al.*, 1997). It is frequently used for molecular phylogenetic analyses estimating genetic population structures, evaluating population level evolutionary processes, and determining taxonomic identity (Ferris *et al.*, 1993; Vrain & McNamara, 1994b; Campbell *et al.*, 1995; Fallas *et al.*, 1996; Cherry *et al.*, 1997; Skantar *et al.*, 2011)

The ITS, intergenic spacer (IGS), and rDNA genes altogether appear to display concerted evolution so that copies of these genes from a single individual tend to be similar to one another, while generally being distinct from those of other species (Elder Jr & Turner, 1995). The availability of universal primers that amplify the ITS region from a wide range of nematode groups (Vrain & McNamara, 1994a) combined with the high variability of ITS renders it a highly suitable phylogenetic marker at species, generic and even family level. Furthermore, it has low functional constraint (Powers *et al.*, 1997). The use of this region for diagnostics is mostly focused on plant parasitic nematode. However it has also been used to detect and quantify cryptic species of the *Litoditis marina* complex (Derycke *et al.*, 2012; Derycke *et al.*, 2013).

1.5.1.2 18 Svedberg ribosomal RNA (18S rRNA)

The first molecular classification of the phylum Nematoda was based on the 18S region (Aleshin *et al.*, 1998; Blaxter *et al.*, 1998; De Ley & Bert, 2002). This gene region is easily accessible because of the fact that it contains highly conserved regions that allow the creation of universal primers (Blaxter, 2003). (Holterman *et al.*, 2008) reported that the small subunit (SSU) rDNA is the most conserved gene region among rRNA encoding genes. Its use in resolving deep phylogenetic relationships between different taxa in the phylum Nematoda has been well documented (Meldal *et al.*, 2007; Fontanilla & Wade, 2008; Van Megen *et al.*, 2009).

The use of 18S rRNA has been so far very successful in species diagnostics but challenges concerning the low rate of polymorphism among species has also been observed for 18S (Félix *et al.*, 2000; Rusin *et al.*, 2003) hence making identification at species level difficult.

1.5.1.3 28 Svedberg ribosomal RNA (28S rRNA)

This is the structural RNA for the large component of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells. Like the 18S, this gene has also been used for species identification in nematology but its use in marine nematodes is very limited (Blaxter *et al.*, 1998; Nadler *et al.*, 2006; Meldal *et al.*, 2007). Few studies however utilized the gene region to disentangle cryptic species (Derycke *et al.*, 2005; Derycke *et al.*, 2008a; Derycke *et al.*, 2010a; Derycke *et al.*, 2010b)

The 28S gene composed of a mixture of conserved and divergent regions. These have been called "divergence regions D" and are numbered in 5' to 3' direction of mature rRNA (Hassouna *et al.*, 1984). The D1-D2 LSU region of rDNA has been used to infer phylogenetic relationships between members of the Adenophorea (Litvaitis *et al.*, 2000), but the D2 and D3 expansion segments of the 28S gene have been most widely used to determine evolutionary relationships between nematodes (Tenente *et al.*, 2004; Subbotin *et al.*, 2007; Fonseca *et al.*, 2008; Subbotin *et al.*, 2011).

1.5.2 Mitochondrial genes

The mitochondrial gene, cytochrome c oxidase subunit 1 (COI), is currently widely used locus for a ‘universal’ diagnostic barcode (Lorenz *et al.*, 2005; Rach *et al.*, 2008). The marker is highly variable fast and easy to implement but difficult to devlop universal primers(Blaxter *et al.*, 2005; De Ley *et al.*, 2005a; Bhadury *et al.*, 2006b; Creer *et al.*, 2010). The high variability in this gene region renders it problematic making it difficult to develop universal primers thus difficult to apply on marine nematodes (Blaxter *et al.*, 2005; De Ley *et al.*, 2005a; Bhadury *et*

al., 2006b; Bhadury *et al.*, 2008; Creer *et al.*, 2010). COI is known to provide information on gene-flow patterns and also for delineation of species within marine nematodes (Derycke *et al.*, 2005; Derycke *et al.*, 2008a; Derycke *et al.*, 2010b; Derycke *et al.*, 2012; Derycke *et al.*, 2013).

Currently there are no universal primers for CO1 that works across the phylum nematode and PCR success rates are below 50% for most taxa (De Ley *et al.*, 2005a). However, success rate of 98,5% of the COI amplification were reported in *Thoracostoma trachygaster* (Nematoda, Leptosomatidae) (Derycke *et al.*, 2010a) and other marine nematodes (Derycke *et al.*, 2005; Derycke *et al.*, 2006, 2007) thus indicating that there is a high probability of succeful amplification of the gene region in marine nematodes. Some reasons for the poor amplification success are related to the diverse nature of nematode mitochondrial genomes, displaying unusual characteristics such as recombination (Lunt & Hyman, 1997), insertionalediting and multipartitioning (Vanfleteren & Vierstraete, 1999; Armstrong *et al.*, 2000). Also, mitochondrial genes have higher mutation rates, a fourfold smaller size that evolves more rapidly than the nuclear genes (Avise, 2000).

1.6 The DNA barcoding approach

Defining discriminatory morphological characters in very small, morphologically unvarying families that includes species that are well known to be cryptic, in a manner that can be homogeneous across a range of researchers, is quite challenging even to the specialist (Bhadury *et al.*, 2006b).

The use of molecular markers for species delineation has become the new tool for nematode taxonomy. Results from these molecular markers very often do not agree with the traditional or readily available morphological and morphometric data (Abebe *et al.*, 2013). This inconsistency among the two systems raises question marks on the limitations of using light microscopy for nematode taxonomy as well as prior to deposition of sequences to reference databases (Valentini *et al.*, 2009).

DNA barcoding was first mentioned in 1993, in a scientific paper that received little publicity (Arnot *et al.*, 1993). The new age of DNA barcoding began in 2003 by Paul Hebert and his research team and focused on Barcoding animal life (Hebert *et al.*, 2003). The now well established Consortium for the Barcode of Life (CBOL), an international initiative supporting the development of DNA barcoding, aims to both promote global standards and coordinate research in DNA barcoding (Valentini *et al.*, 2009).

For animals, the gene region proposed for the standard barcode is a 658 base pair region in the gene encoding the mitochondrial cytochrome c oxidase 1 (COI) (Hebert *et al.*, 2003). Hebert *et al.* (2003) was quick to indicate that although barcoding has shown prospects in dealing with species diagnosis, some group of organisms could not benefit from it. Divergence in Cnidarians were reported to be far too less as compared to other animal phyla like the fungi and protists, thus the system may not be the ideal to differentiating species in the group. The problem of the Cnidarians was however not surprising since rates of mitochondrial evolution are low in this group of organism (France & Hoover, 2002; Shearer *et al.*, 2002). It has also been reported that species of sponges (congeneric siblings) are difficult to separate with the COI fragment and this is attributed to low variability among species. In Fungi (Seifert *et al.*, 2007), and also in plants (Hollingsworth *et al.*, 2009), COI based DNA barcoding faces problems of insufficient variation to identify up to species level. The internal transcribed spacers of nuclear ribosomal DNA (nrDNA ITS) has been found be the universal DNA barcode marker for fungi and plants respectively (Hollingsworth, 2011; Schoch *et al.*, 2012). It has been shown that COI can provide the information required for identification of individual species with more than 99,99% resolution of animal diversity (Hebert *et al.*, 2003). This has motivated efforts to expand and build a standard sequence reference database for barcoding (Valentini *et al.*, 2009).

The Barcode of Life project initiated in 2004 did not have the ambition to build the tree of life but to focus on producing a simple diagnostic tool which relies greatly on strong taxonomic knowledge that is assembled in the DNA barcode reference library (Schindel & Miller, 2005). The usefulness of this library system cannot be disputed as it enables the acquisition, storage, analysis and publication of DNA barcode records (Frézal & Leblois, 2008). Access to a reference database of taxa, which allows identification of a wide range of species will be useful whenever precise taxonomic identifications are needed (Frézal & Leblois, 2008).

The general application of DNA barcoding to marine nematodes requires first finding a suitable genomic region, or combination of regions, to enable species identification across a variety of taxa. Secondly, it requires building a reference database of sequences (Hebert & Gregory, 2005; Frézal & Leblois, 2008; Valentini *et al.*, 2009) and morphological Vouchers (De Ley & Bert, 2002).

Da Silva *et al.* (2010) reported the deposition of a total of 600 barcode sequences of marine nematodes in National Center for Biotechnology Information (NCBI) database as of 2010. These barcode sequences were assigned to 150 nominal species from 104 genera. In total, barcoded sequences of 41 species were assigned to Enoplea and 109 species to Chromadorea. With respect to the diversity of the of these species and these low numbers recorded, it is clear that barcode sequences of marine nematodes are not well represented in the phylum (Da Silva *et al.*, 2010). Plant parasitic nematodes on the other hand are better represented in the genetic databases (Da Silva *et al.*, 2010).

This has reinforced the need to produce more sequences in studies with respect to free living taxa in order to increase the number of barcodes in the reference database. Various studies including the successfully amplification of the COI genes from *Litoditis marina* (Derycke *et al.*, 2006), *Halomonhystera disjucta* (Derycke *et al.*, 2007) and *Thoracostoma trachygaster* (Derycke *et al.*, 2010a) have augmented efforts to expand the reference database of marine

nematodes by adding a lot of sequences but with limited number of species. In the quest to increase the number of species in the refrence database, some marine nematodes belonging to at least 33 different genera were identified using CO1(Derycke *et al.,* 2010b).

The deposition of sequences in databases should be accompanied by properly digitized vouchered specimens so that re-examination of reference specimens can be done when the need arises (De Carvalho *et al.,* 2007; Dov, 2007; De Carvalho *et al.,* 2008). This is to ensure that misidentification of species are detected on time and removed from subsequent analyses. Thus, it is important, when considering the storage of barcode data, to also consider long term storage of the morphological characteristics of specimens and their DNA extracts (De Ley & Bert, 2002). The fact that DNA barcoding technique is reliable in species identification, many times quicker to produce, and less reliant on taxonomic expertise, does not mean its 100% efficient.

The main challenges of the approach arise from the fact that it is based on single-locus identification system (Frézal & Leblois, 2008). Even if numerous or several regions from these organelle DNAs are sequenced, it still remains a single-locus approach because different genes of the mitochondrial DNA are always linked. It has been reported by (Ballard & Whitlock, 2004) that identical mitochondrial DNA sequences can be present in different related species due to introgression, or due to incomplete lineage sorting since the time of speciation. Also, nuclear copies of fragments of mitochondrial DNA are common and can be amplified in some situations leading to potential identification errors (Zhang & Hewitt, 1996).

Another limitation of DNA barcoding lies in the length of the sequences used, usually greater than 500 (Hebert *et al.,* 2003) which prevents the amplification of degraded DNA and therefore a shorter sequence will be more appropriate considering its use in ecological studies. In marine nematodes, the use of universal invertebrate M1-M6 primers has been shown to give very poor amplification results (De Ley *et al.,* 2005a; Bhadury *et al.,* 2006a; Derycke *et al.,* 2010b). However diagnostic markers that are located at the ITS- rDNA, D2-D3 expansion

segment of 28S rRNA, 18S rRNA, and heat shock protein 90 (hsp90) have proven to be useful tools for nematode identification, systematics and molecular phylogeny (Al-Banna *et al.*, 1997; Blaxter *et al.*, 1998; Courtright *et al.*, 2000; De Ley & Bert, 2002; Floyd *et al.*, 2002). Also the use of I3-M11 fragment of CO1 has been used to delineate species complex in various studies. (Derycke *et al.*, 2005; Derycke *et al.*, 2007; Derycke *et al.*, 2008a; Da Silva *et al.*, 2010; Derycke *et al.*, 2010b). I3-M11 partition of COI has been used to identify species belonging to families of Monhysteridae (Derycke et al., 2007), Rhabditidae (Derycke et al., 2008) and Leptosomatidae (Derycke *et al.*, 2010a) and many other free living marine nematodes (Derycke *et al.*, 2010b)

Another shortfall is the occurrence of nuclear mitochondrial pseudogenes (NUMTs) which are nonfunctional copies of mtDNA in the nucleus that have been found in major clades of eukaryotic organism (Song *et al.*, 2008). They can be easily coamplified with orthologous mtDNA by using conserved universal primers (Song *et al.*, 2008). Although nonfunctional, disturbance due to NUMTs can be overlooked, it must be taken seriously because it incorrectly overestimates the number of unique species based on the standard metric of 3% sequence divergenc in barcoding analysis. This must be considered in both DNA barcode library construction and further specimen identification (Song *et al.*, 2008). NUMTs have been reported in may eukaryotes including grasshopper (Gellissen *et al.*, 1983) in which a copy of a mitochondrial ribosomal RNA gene was found in the nuclear genome. 82 eukaryotes including nematodes (Gibson *et al.*, 2007; Jacob *et al.*, 2008), honey bees (Pamilo *et al.*, 2007), grasshopper, *Locusta migratoria* (Gellissen *et al.*, 1983), domestic cats (Lopez *et al.*, 1994), mouse and human beings (Richly & Leister, 2004). BLAST search of mitochondrial sequences in the published nuclear genomes suggests that nearly 99% of the mitochondrial sequences were transferred to different parts of the nucleus in both human and mouse (Richly & Leister, 2004).

These studies suggests that NUMTs are very pervasive in nature and that there may be a large number of species with unrealized NUMTs of the COI gene in the nucleus (Song *et al.*, 2008).

Inherent risks due to mitochondrial inheritance has been one of the pitfalls in DNA barcoding due to the fact that diversity of mitochondrial DNA (mtDNA) is strongly linked to the female genetic structure due to maternal inheritance (Frézal & Leblois, 2008). The use of mitochondrial loci can henceforth lead to overestimation of sample divergence and render wrong conclusions on species status. A classic example is the case in *H. mermerodes* (Lepidoptera) where mtDNA polymorphism is structured according to the host plants on which females feed (Hulcr *et al.*, 2007). The two clades produced by phylogenetic analyses were seen to be artefacts of female nutritional choice (Hulcr *et al.*, 2007).

Heteroplasmy and dual uniparental mitochondrial inheritance as reported in mussels (Terranova *et al.*, 2007) are further misleading processes for mitochondrion based phylogenetic studies (Frézal & Leblois, 2008). Symbiont infection, usually common in some arthropods has also been found to be associated with mitochondrial inheritance. This happens when indirect selection on mitochondrial DNA arises from linkage disequilibria with endosymbionts, either obligate or beneficial micro-organisms, parasitically or maternally inherits symbionts (Funk *et al.*, 2000; Whitworth *et al.*, 2007). Examples of such scenario is Wolbachia infections on at least 20% of Insecta and 50% of spiders, (Hurst & Jiggins, 2005), Cardinium infects around 7% of arthropods, (Weeks *et al.*, 2007). Wolbachia endosymbionts have been found in parasitic nematodes *B. malayi* and *Onchocerca* spp. (Bordenstein *et al.*, 2003) but infections in marine nematodes have so far not been observed.

1.6.1 Application of DNA barcoding: Environmental metagenetics

Owing to the dominance of nematodes in the marine benthic metazoan, in both high abundances and diversity, a more robust approach that utilizes a large scale of taxon richness and the analysis of homologous genes is required to assess concurrently the relative levels of

richness and patterns of diversity of multiple metazoan phyla in an ecosystem (Fonseca *et al.*, 2010).

Also known as metabarcoding, the technique utilizes the next generation sequencing (NGS) of homologous genes (Creer *et al.*, 2010), obtaining long sequence reads of the genes from environmental samples and the result compared to a large sequence library using clustering methods (Creer *et al.*, 2010; Porazinska *et al.*, 2010a; Hajibabaei *et al.*, 2011). The use of Sanger-based DNA sequencing in this technique has contributed enormously to building reference libraries (Hajibabaei *et al.*, 2011). However it inability to handle large environmental samples, coupled with the fact that is relatively expensive makes it use no longer attractive (Kemp & Aller, 2004; Venter *et al.*, 2004; Porazinska *et al.*, 2010a; Hajibabaei *et al.*, 2011).

The new age of using high-throughput sequencing technologies have made it possible to generate large amounts of sequence data within a very short time and at a low cost (Margulies *et al.*, 2005; Hajibabaei *et al.*, 2011). These technologies have paved way for the use of the 18S rRNA for biodiversity studies and analysis on eukaryotes including nematodes (Porazinska *et al.*, 2009; Creer *et al.*, 2010; Porazinska *et al.*, 2010a; Porazinska *et al.*, 2010b; Bik *et al.*, 2012b). The approach was reported to be congruent with detailed morphological assessment of species in an ecosystem that is characterized by many phyla of different microorganisms (Fonseca *et al.*, 2010). Estimators of richness reported the occurrence of about 2500 Operational Clustering of Taxonomic Units (OTUs) of meiobenthic eukaryotes, however nematodes alone constitutes more than 830 (OTUs) from a single habitat (Fonseca *et al.*, 2014). It has also been proven the mitochondrial cytochrome c oxidase 1 (COI) DNA barcode sequence length that can readily and robustly be obtained through 454 pyrosequencing, can provide the information required for identification of individual species with more than 90% species resolution (Hajibabaei *et al.*, 2006; Meusnier *et al.*, 2008).

The problem associated with this metagenic approach however is that many sequences cannot be identified because they are not present in the reference database (Bik *et al.*, 2012b). DNA-based databases are strongly biased towards plant-parasitic nematode taxa (De Ley *et al.*, 2005a). A strong emphasis on morphological and environmental data collection, guide trees and reference sequence databases, and open access repositories for high-throughput datasets are urgently required (Bik *et al.*, 2012a).

1.7 Problem statement and justification

Many marine meiofauna including nematodes are known to have wide to cosmopolitan distributions (Schabetsberger *et al.*, 2013) accompanied by a high degree of gene flow between populations at local scale (Derycke *et al.*, 2013; Ristau *et al.*, 2013). In addition, there is a high taxonomic deficit for meiofauna, partly as a result of low taxonomic efforts and the high number of putative cryptic species (Schabetsberger *et al.*, 2013). The identification of most individuals at the species level using standard techniques is difficult and time-consuming. Also nematode communities are not resolved down to the species level, leaving ecological analysis ambiguous (Porazinska *et al.*, 2009). Adding to this problem is the low number of active taxonomy practitioners in nematology. The worst part of the situation is the fact that experts in this field retire without replacement, probably because of the tedious nature of classical taxonomy and the fast advancement of molecular tools to solving the problem. (Oliveira *et al.*, 2011).

Biodiversity and biogeographical studies have gained from massive molecular techniques to unravel species diversity particularly since the onset of the 'DNA barcoding of life' initiative (Hebert *et al.*, 2003). Presently, these molecular methods are being used practically in various taxonomic groups including marine nematodes (Floyd *et al.*, 2002; Hebert *et al.*, 2004; Ward *et al.*, 2005; Meyer *et al.*, 2010). Genetic surveys have been expanded to include free-living

marine species as well as fresh water species (Derycke *et al.*, 2005; Derycke *et al.*, 2008a; Fonseca *et al.*, 2008; Derycke *et al.*, 2013; Ristau *et al.*, 2013).

With respect to the high diversity of these marine nematode species yet low numbers recorded, it is clear that barcode sequences of marine nematodes are not well represented in the phylum as compared to plant parasitic nematodes (Blaxter *et al.*, 2005; Da Silva *et al.*, 2010). Next generaton sequencing (NGS) provides an opportunity to generate very large amounts of sequence data in a very short time and at low cost (Margulies *et al.*, 2005; Hajibabaei *et al.*, 2011). However, the problem is that using NGS and DNAbarcoding to identify nematodes requires a good reference database with well vouchered and sequences of species (Bik *et al.*, 2012a). The popular 18S rDNA most often used for inferring phylogenetic relationships for the phylum Nematoda (Blaxter *et al.*, 1998; Holterman *et al.*, 2006; Meldal *et al.*, 2007) produces much higher rDNA copies which could lead to overestimation of the relative abundance of species (Porazinska *et al.*, 2009; Gong *et al.*, 2013). This may raise problems in rDNA-based inference of species richness and phylogeny. Therefore it is important that we optimize the use of DNA to solve such problems in future studies. Adding sequences from new areas, will not only include new species to the reference database but will help to unravel the cryptic diversity and possible population structure of marine nematodes. Above all, the meiofauna paradox concerning the wide distribution of nematodes in relation to their dispersal capabilities can be addressed through a meta analyses of all sequnces in MoMentuM.

This study focuses on Panarea, an Island which is part of Aeoland Islands located in the south-eastern Tyrrhenian Sea, north of the town of Messina, in Sicily, Italy. Panarea Island is close to Stromboli Island with active volcanic eruptions. Therefore it is expected that Panarea Island would be affected by such volcanic disturbance.

Field studies on this Island have identified natural CO_2 vents as natural analougues. However these CO_2 gas emissions, are mostly controlled by the NE-orientated faults

(Gabbianelli *et al.*, 1990; Calanchi *et al.*, 1995). An initial sampling carried out in our studies in Panerea Island in 2011 under the auspices of (ECO_2), identified two CO_2-impacted (High CO_2 (St. B1), Low CO_2 (St. B3) and one reference site (St. B2) for functional long-term investigations of the microbial and meiofaunal community. Preliminary results from the 2012 meiofaunal samples indicated density differences in sediments influenced by the CO_2 seepage. From the sediment samples collected in 2012, nematode densities were seen to be highest in the first two centimeter of sediment of the CO_2 imparted sites, while at the background site, a more gradual decline with depth was observed. Nematode species composition differed between CO_2 impacted sites and a non-impacted background site for 2011, 2012 and 2013. Their composition also differed between the two different seepage sites in 2011 and 2012, but not in 2013. In the most severe CO_2 impacted site there was also a difference detected in nematode species composition between the three consecutive years. Nematode species richness was significantly lower in the CO_2-impacted sites compared to the non-impacted sites, for all three consecutive years. *Calomicrolaimus compridus*, *C. honestus*, *Dracognomus tinae*, *Desmodora schulzi and Paracyatholaimus oistospiculoides* were some of the most dominat species in these sites.

Analyses of marine nematodes to a lower taxonomic level are required to more effectively evaluate impacts of CO_2 seeps on these meiofaunal taxa. Investigating biological impacts of elevated CO_2 is of importance in the context of understanding environmental impacts of not only ocean acidification due to changes in CO_2, but also of potential seepage from sub-seabed geological CO_2 storage, for which no scientific information is currently available (Metz *et al.*, 2005). However, marine nematodes may well be exposed to higher CO_2 although studies have shown that this organism are not really affected by elevated CO_2 but are sublethally affected.

Appropriate experimental protocols must be employed to evaluate the risk of potential seepage from sea-bed geological storage, because the expected hazards are distinctly different

from the impact of CO_2 injection into mid-water depths (Metz *et al.*, 2005). Meiobenthic community structure changes due to differences in CO_2 tolerance among species. Further studies are therefore needed to understand the future impacts of CO_2 seeps on the marine ecosystem. There is therefore the need to utilize a more robust molecular approach in MoMentuM (a new database with the aim of creating libraries of digitized vouchered marine nematode species with their respective 18S and COI sequences) to quickly help in the identification of these nematode assemblages, which could help to understand the diversity and the community structure and the effect of CO_2 on these indices.

The already known contradiction of nematodes having wide to cosmopolitan yet with limited dispersal cannot be overemphasized (Derycke *et al.*, 2013; Ristau *et al.*, 2013). The occurrence of cryptic species, relying on only traditional identification methods may mask their diversity and geographical distribution (De Ley *et al.*, 2005a). With these problems at hand, nematode communities are usually only resolved to genus level (Derycke *et al.*, 2010b). This may be problematic, because functional roles of nematodes may be highly species-specific (De Mesel *et al.*, 2003; 2004) and their population dynamics can be influenced by the presence of closely related species (De Mesel *et al.*, 2003; Postma-Blaauw *et al.*, 2005; dos Santos *et al.*, 2009). It is therefore important that species are vouchered and barcoded in MoMentuM for future ease of identifying nematodes. This will help address unravel problems with species complex, their diversity and biogeographical distribution, that is whether they are endemic to specific localities or are widely distributed.

2 Aims of research

1. Voucher morphological species from Panarea and amplify COI and 18S to further increase the reference database.

Hypothesis:

Based on the morphological characterization of the nematode communities of the seeps, we expect to find species of *Calomicrolaimus compridus C. honestus, Oncholaimus campylocercoides, Dracognomus tinae, Desmodora schulzi Paracyatholaimus oistospiculoides Acanthopharynx micans*, most of which are new for the reference database.

Nematodes preserved in dimethylsulfoxide with EDTA and NaCl salts (DESS) will be temporarily mounted on slides for video capture of important diagnostics features by using LEICA DMR research microscope and LEICA Application Suit (De Ley & Bert, 2002; De Ley *et al.*, 2005a). The vouchered specimen will be transferred into an Eppendorf tube containing 20µL worm lysis buffer for DNA Extraction. DNA extraction and amplication of 18S and CO1 will be carried out as described by Derycke *et al.* (2005) and Derycke *et al.* (2010b) followed by sequencing of reliable bands. This will be done by using G18S4 and 4R as forward and reverse primers respectively to sequence the 18S gene while JB3 and JB5will be used to sequence the CO1 gene respectively.

2. Compare amplification and sequencing success of both markers.

Hypothesis:

Based on the higher variability of the COI gene and the less universal application of the COI primers, we expect a higher amplification and sequencing success in 18S than in COI.

Amplification and sequencing success of both primers will be carried out following the methodology of Thompson *et al.* (1997) and further intra and inter-species level analysis, nucleotide sequence divergences will be computed using the P-distance model in MEGA

v.6.0. Intra - and interspecific genetic distances will be calculated as well for all COI and 18S sequences.

3. Compare identification success of both markers.

Hypothesis:

Because of the lower variability of 18S, we expect to find higher ID success in COI.

ID success will be determined using the program SpeciesIdentifier.

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4 Addendum

4.1 Time schedule

Project Activities	Action plan									
	Oct.	Nov.	Dec.	Jan.	Feb	Mar	April	May	June	July
Extraction of nematodes	●									
Identification and vouchering	●	●	●		●	●				
Literature review		●	●							
DNA extraction		●	●		●	●				
PCR		●	●		●	●				
Sequencing						●	●			
Analysis of data								●		
Thesis writing								●	●	
Thesis submission									●	
Thesis defence										●