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Nematode taxonomy: from morphology to metabarcoding

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Abstract

Nematodes represent a species rich and morphologically diverse group of metazoans inhabiting both aquatic and terrestrial environments. Their role as biological indicators and as key players in nutrient cycling has been well documented. Some groups of nematodes are also known to cause significant losses to crop production. In spite of this, knowledge of their diversity is still limited due to the difficulty in achieving species identification using morphological characters. Molecular methodology has provided very useful means of circumventing the numerous limitations associated with classical morphology based identification. We discuss herein the history and the progress made within the field of nematode systematics, the limitations of classical taxonomy and how the advent of high throughput sequencing is facilitating advanced ecological and molecular studies.

1 Introduction

The phylum Nematoda is a species rich taxonomic group that has been reported in abundance in a wide range of habitats (Cobb, 1915; Holterman et al., 2009), from aquatic marine and freshwater to terrestrial environments (van Megen, 2009). They represent one of the most dominant metazoans on the surface of the earth in terms of abundance and diversity (Groombridge, 1992; Wilson, 2000), with densities of up to 10^8 individuals per square meter and species richness of up to 60 morphospecies (species delineated based on morphology) per 75 cm^3 of sediment (Lambshead, 2004) in marine environments. Approximately four out of every five metazoans are estimated to be nematodes (Bongers and Bongers, 1998). And in addition to these high abundances, nematodes have been shown to exhibit a remarkable range of feeding guilds (Yeates et al., 1993) and life history strategies (Bongers, 1990). In terms of feeding groups, there are bacterial, fungal and plant feeders, and then omnivores and carnivores. Life strategies span from the small-bodied but highly fecund r-strategists, such

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as the bacterivorous rhabditidae to the large-bodied but less fecund k-strategists, such as the omnivorous dorylaims. Previous studies have shown that prevailing physical characteristics such as soil texture, climate, biogeography, as well as enrichment and disturbance events can be reflected through species composition of the local nematode community (Cobb, 1915; Tietjen, 1989; Yeates, 1984; Neher, 2001). In other words, depending on the state of the environment- for example whether soil is stable or has undergone some recent perturbation, the soil nematode community is likely to differ from one place to another. The contribution of nematodes to nutrient cycling (Bardgett et al., 1999; Blair et al., 1999; Bongers and Ferris, 1999; Wardle et al., 2006) is a very well documented aspect of the role they play in maintaining a balance in the functioning of the ecosystem. And as permanent community members (being unable to escape habitat disturbance), they serve as important biological indicators of sediment quality (Bongers and Ferris, 1999; Sochova et al., 2006; Wilson and Kakouli-Duarte, 2009; Höss et al., 2011). Nematode indices used to assess soil quality are based mostly on grouping, into nematodes feeding guilds, reproductive strategies and general responses to physical and organic disturbances. However, the criteria for allocating individuals into these groupings have often been questioned since even species within the same trophic group are known to sometimes vary in their source of food and response to disturbances (Yeates et al., 1993; De Goede et al., 1993). The need, therefore, for species level identification is vital to accurate and precise computation of nematode indices as determiners of sediment quality. In fact to achieve thorough assessment of soil resilience, species level identification are to be achieved rather than functional group classification needs to be considered (Yeates, 2003). The drawback, however, is that their high abundance, minute size, conserved morphology (Decraemer and Hunt, 2006) as well as the existence of intraspecific variations and cryptic species (valid species species that morphologically indistinguishable) preclude rapid and accurate identification of species. Consequently, this has severely limited the fraction of environmental samples analyzed in ecological studies, leaving ecologists with the only

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option of categorizing nematodes based on higher level classifications such as families and feeding guilds (Porazinska et al., 2010).

In terms of the need for accurate identification to nematode species level, research has largely focused on plant parasitic nematodes, due mainly to the magnitude of direct economic losses they inflict on agriculture – an estimated USD 118 billion in a single year (McCarter, 2009). Their management in field crops has up to now been dependent on the use of nematicides (Hague and Gowen, 1987) which are being gradually phased out following the realization of the impact that these nematicides pose to the environment (Akhtar and Malik, 2000). The EU has recently made some very important modifications to its policy on the use of pesticides to make it more sustainable and to reduce the risk this poses to human health and the environment. This has led to the re-evaluation (Regulation 2009/1107/EC OL and Directive 2009/128/EC) of various synthetic pesticides leaving only a few nematicides available for use by growers (Ntalli and Menkissoglu-Spiroudi, 2011). Alternative non-chemical options have for sometime now been sought to replace the loss of synthetic products (Kerry, 2000). These alternative approaches will undoubtedly rely on our knowledge of the taxonomy and biology of parasitic nematodes in order to devise efficient and taxa-specific control measures.

According to Hussey (1979), the existence of character variation and physiological races within species are some of the problems associated with, but not limited to (Allen and Sher, 1967), the taxonomy of plant parasitic nematodes. These complications have catalyzed the search for alternative approaches devoid of the constraints associated with morphological identifications. Particularly within the genus *Meloidogyne*, a taxon that has received by far more attention than any other group of plant parasitic nematodes (Sasser and Carter, 1982), techniques such as the differential host test (Sasser, 1954), scanning electron microscopy (Eisenback and Hirschmann, 1981; Charchar and Eisenback, 2000; Eisenback and Hunt, 2009), biochemical approaches such as isozyme electrophoresis (Berge and Dalmasso, 1975; Esbenshade and Triantaphyllou, 1985; 1990; Tastet et al., 2001; Carneiro et al., 2000) as well as molecular techniques (Hyman, 1990; Harris et al., 1990; Petersen and Vrain, 1996; Powers et al.,

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2005) have been used to complement the often limited light microscopic approach for identification. Each one of the above mentioned alternatives to light microscope-based approaches have certain constraints that limit their use as quick, accurate and simple tool for nematode identification across the phylum. However, the use of molecular methods has continued to gain recognition for being fast, reliable and an easy diagnostic approach across many taxa within the phylum Nematoda (Floyd et al., 2002; De Ley et al., 2005). It is important to mention that most of the pioneering works on molecular-based nematode detection were developed on plant parasitic nematodes. As evidence of the importance of molecular data in taxonomy, it has become a common practice in recent times that most taxonomic descriptions comprise both morphology and morphometric studies as well as molecular analysis of the taxon's relatedness to other species (Handoo et al., 2004; Vovlas et al., 2011; Cantalapiedra-Navarrete et al., 2013). Over the past two decades there have been a number of published reviews on molecular methods of plant parasitic nematode identification discussing in depth the different markers and DNA target regions used for discriminating species, their future prospects and limitations (Powers et al., 1997; Powers, 2004; Blok, 2004, 2005).

2 The phylum Nematoda

Although there is a widely acknowledged high taxonomic deficit in our knowledge of nematode diversity, nematodes still outrank all other pseudocoelomates both in terms of survival success as a group and perceived diversity (van Megen et al., 2009). Evidently, they are by far the most species-rich, ubiquitous and abundant group in comparison with related phyla such as Priapulida, the Kinorhyncha as well as their closest sister taxon the Nematomorpha. There are only about 19 known species of Priapulida, 190 of Kinorhyncha and 320 of Nematomorpha (Schmidt-Rhaesa, 2012a; Schmidt-Rhaesa, 2012b; Neuhaus, 2012). And in terms of the number of species estimated to actually exist, the Nematomorpha, which is probably the most diverse of the three rel-

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atives of Nematoda, has only about 2000 species globally (Poinar, 2008) compared to the 1 000 000 estimated for Nematoda (Lamshead, 2004).

The majority of members have throughout their existence maintained a highly conserved general body plan, which one might argue to be the result of their success through time. The idea of nematodes being conversed morphologically is a very popular and long held assertion in nematology, in earlier works, especially those predating the widespread use of scanning electron microscopes (Hegner and Engemann, 1968; Meglitsch, 1972; Fretter and Graham, 1976) and in recent studies too (Powers, 2004). This has however been disputed by De Ley (2000) and De Ley et al. (2005) who argued that this theory simply emanates from the failure of light microscopy to provide enough resolution, thus precluding detailed observation of certain features (De Ley, 2000). It is not unprecedented for a non-expert to conclude nematodes are collectively similar superficially. To a trained eye, however, this assertion as claimed by De Ley (2000), would be deemed overly simplistic.

3 Predicted species diversity leaves so much more to do

To date approximately 27 000 nematode species have been described, which is a relatively small fraction of the predicted number of species of ca. 1 million (Hugot et al., 2001). In other words, close to two centuries of diligent work by taxonomists has only managed to catalogue about 3 % of the predicted diversity, 5–10 % according to Coomans (2002). Some estimates even amount to a controversial maximum of 100 million in true diversity (Lambshead, 1993). Regardless of what estimated figure best represents true diversity, there is no denying that there are still a significant number of nematodes yet to be described.

With the steadily declining number of experienced taxonomists, speculation that we might never be able to obtain a complete catalogue of all nematode species continues to grow, especially with sole reliance on classical morphology. The so-called taxonomic deficit, coupled with the great observational restrictions associated with useful diagnos-

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tic phenotypic characters are probably the reasons why some believe that traditional approaches can never fully describe biological diversity, and that molecular methods in the form of DNA barcodes are probably the only way forward (Blaxter, 2003). DNA barcoding undoubtedly holds a great deal of promise for the future of taxonomy, in terms speed and accuracy of describing biodiversity, but not until sufficient and reliable cataloguing of currently known diversity has been done based on accurate classical morphology-based identification. Therefore, a strong classical taxonomy expertise is still crucial in ensuring the success of any DNA based biodiversity assessment.

4 Classical taxonomy and the vast taxonomic deficit

The need for diagnosticians with the skills for routine identification of taxa based on morphological differences is a problem well acknowledged across many areas of plant pathology, of which nematology is no exception (Blok, 2005). According to Coomans (2002), morphology can still provide useful diagnostic characters, especially if we are able to overcome the limited resolution light microscopy provides. And despite all its limitations, morphology-based study when carried out diligently can be as good as any biochemical or molecular method used in identifying taxa (Mayr and Ashlock, 1991; De Ley, 2006; Agatha and Strüder-Kypke, 2007). What is lacking, however, is the technical and taxonomic expertise required to correctly utilize phenotypic characters and use this to effectively make a decision about the identity of an organism (Abebe et al., 2013).

The continuous decline in the number of taxonomists has serious repercussions to our understanding of life's diversity. According to Coomans (2002) this waning number of specialists is also detrimental even to the quality of taxonomic researches that get published, since less qualified referees have to review such manuscripts. To properly deal with the issue of, De Ley (2000) suggested that reassessment of priorities is the best way to progress. He cited a number of steps to achieve this: (1) focusing on describing taxa with relatively more anthropological and ecological importance rather than

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describing species purely for sake of keeping inventory, (2) making identification and classification easier for non-specialists, and (3) obtaining a better understanding of relationships rather than trying to complete the enormous catalogue of species diversity. The first and last points imply switching efforts from the common practice of specimen by specimen description to a more relevance-based description of species and to move the detailed approach to understanding of broader pattern of nematode diversity. The second point, denotes leaving the routine task of identification to non-specialists to allow time for the experts to execute points 1 and 3. To effectively fulfil this, identification methods requiring very little expert knowledge need to be implemented. An example of this is the use of molecular methods in the form of DNA barcodes, i.e. using a specified DNA sequence to provide taxonomic identification for a specimen (Blaxter et al., 2005). Thus, any technician equipped with adequate training to perform a simple PCR and sequencing can generate sequence data for routine identification of nematode species, providing taxonomists the time to focus more on building a species catalogue (Valentini et al., 2009).

5 Changes within the classification systems

Nematode systematics has changed constantly through history. There is abundance of contrasting theories within nematological literature on nematode classification and phylogeny. Several proposals to aid the classification of nematodes have been put forward (Micoletzky, 1922; Chitwood, 1937; Pearse, 1942; Chitwood, 1958; Gadea, 1973; Drozdowsky, 1975; Adamson, 1987; Andrassy, 1976; Inglis, 1983; Malakhov, 1986; De Ley and Blaxter, 2002). According to Hodda (2007), the first ever rendition of nematode classification was from Cobb (1919) who used the structure of the buccal cavity to formulate the phylum, two subphyla, three classes, six subclasses and thirteen orders. This system of Cobb's never gained popularity as it was seen as being completely artificial. According to De Ley and Blaxter (2002), however, the first attempt at classification occurred even much earlier when Schneider (1866) endeavoured grouping nematodes

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based on their somatic musculature which much like the Cobb (1919) system, suffered from undue emphasis on few characters with little or no evolutionary traceability. Thus, like the Cobb (1919) classification, this one was also seen as largely artificial (De Ley and Blaxter, 2002). Micoletzky (1922), although not in support of the system set forth by Cobb (1919) (Hodda, 2007) based his classification primarily on stoma structure (De Ley and Blaxter, 2002) and proposed the division of nematodes into 5 families (Rhabditidae, Odontopharyngidae, Tylenchidae, Alaimidae and Tobrilidae). At least four out of the 19 subfamilies within these families now have the ranks of order. Some authors also proposed systems where all zooparasitic nematode taxa were grouped separately from all other nematodes (Perrier, 1897; Stiles and Hassall, 1926), a feat considered as based principally on ecological grounds (De Ley and Blaxter, 2002) rather than any sound phylogenetic grounds. Baylis and Daubney (1926), recognizing the artificial nature of this approach, too put forward a system where both zooparasites and other nematodes were all grouped into five different orders (Ascaroidea, Strongyloidea, Filarioidea, Dioctophymoidea and Trichinelloidea). However, all these orders now contain only zooparasitic nematode taxa.

Chitwood (1937) proposed the division of the nematode phylum into two main classes: Phasmidia and Aphasmidia, based on the presence or absence of phasmids (a pair of secretory structures usually situated in the caudal region). This system was greatly influenced by an earlier grouping proposed by Filipjev (1934) who divided the phylum into at least eleven orders. Subsequent to the proposal of the names Phasmidia and Aphasmidia, the two were replaced by Secernentea and Adenophorea respectively (Chitwood, 1958), since the name Phasmida was already assigned to an insect taxon. This system gained wide acceptance and was adhered to for over four decades of the history of nematode systematics.

Most of the subsequent classifications shifted more and more towards achieving a natural system of classification, one that is based on phylogeny. In the years that followed Chitwood's (1937) proposed system, several nematode higher classifications were published, with some placing nematodes in the rank of phylum and with some

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the rank of class. Some of authors even proposed a tripartite system (division of the phylum/class into three subtaxa) instead of the widely accepted traditional system of dividing the phylum/class in two parts (Table 1). However, to date the tripartite system has not been adopted by taxonomists.

History of nematode taxonomy has shown that the most challenging constraint to achieving a robust and phylogenetically sound classification system has been the deficiency of differentiating characters. For example, the natural classification system proposed by Lorenzen (1981, 1994) suffered from paucity of characters. Here a cladistic approach to classifying free-living and some plant parasitic nematodes was used. Although he included some new characters such as the presence of metanemes, special threadlike sense organs occurring laterally in the epidermis, he admitted to the paucity of characters for a more complete classification system. Another notable challenge to the use of morphological characters for achieving a more natural classification is recognizing characters that are homologous and those that are not. A similar problem has been reported with the use of molecular data where identifying positional homology has been a major hindrance to their use in reconstructing phylogeny among taxa (Abebe et al., 2013).

Although it is evidently much easier to identify and quantify sequence evolution than morphological evolution (De Ley, 2000), DNA data, much like morphological data, when used alone are subject to some amount of noise and artefact (Dorris et al., 1999). In view of this, Dayrat (2005) proposed a more holistic approach to describing biodiversity which involves the integration of as much data about the organism as possible. According to Dayrat (2005), it is better that morphological and molecular approaches are not seen as competing with each other but rather, used to complement one another. For example, Sites and Marshall (2003), in their review of twelve delimitation methods, cautioned against adherence to the use of one method to singly delimit species, since all of the approaches can possibly fail at some point when used in isolation. This integrative approach has been successfully applied in some studies for examining species

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diversity (Boisselier-Dubayle and Gofas, 1999; Shaw and Allen, 2000; Williams, 2000; Drotz and Saura, 2001; Marcussen, 2003, De Ley et al., 2005; Ferri et al., 2009).

Integrative taxonomy is without a doubt an excellent approach to species delimitation, especially with the existence of several species concepts, and the fact that each of the species delineation approaches when used singly only constitutes one of the multiple aspects of life's diversity (Dayrat, 2005). However, a key constraint to the widespread adoption of this method is the time and expertise involved. One of the major goals of modern taxonomy is to find identification methods which are fast, accurate, reliable, affordable and perhaps even capable of characterizing undescribed specimens (Powers, 2004). In the identification of regulated pest species, for example, speed and accuracy are very important (Holterman et al., 2012; Kiewnick et al., 2014). Therefore, although reliable and probably more accurate than any of the individual approaches, integrative taxonomy may lack the speed and simplicity which are equally important in certain situations.

6 Biochemical methods for nematode identification

Several biochemical and molecular approaches have been used for identification of nematodes. Genomic information at all levels have been utilized for identifying nematodes, from DNA sequence, the structure of molecules, genetic mutations to the presence versus absence of genes (Subbotin and Moens, 2006). At the protein level, isozyme analysis (Esbenshade and Triantaphyllou, 1990; Payan and Dickson, 1990), two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D SDS-PAGE) (Ferris et al., 1994), monoclonal or polyclonal antibodies-base serological techniques (Jones et al., 1988; Schots et al., 1990) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Perera et al., 2009) are the methods that have been utilized for distinguishing nematodes at species or subspecific levels.

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The use of molecular data for identification of taxa has been widely accepted, largely because of its inherent ability to overcome most limitations associated with traditional morphology-based nematode identification. Most molecular diagnostic methods are PCR based and rely on DNA sequence divergence. The marker regions often specifically targeted include the nuclear ribosomal DNA, satellite DNAs and various protein coding genes within the mitochondrial genome (Blok, 2005).

Other approaches are based on random amplification of DNA sequences. Examples include the randomly amplified polymorphic DNA (RAPD) (Cenis, 1993 Castagnonesereno et al., 1994), amplified fragment length polymorphism (AFLP) (Semblat et al., 1998; Marche et al., 2001), random fragment length polymorphism (RFLP) (Curran et al., 1986; Carpenter et al., 1992) and sequence characterized amplified DNA regions (SCAR) (Zijlstra, 2000; Zijlstra et al., 2000; Carrasco-Ballesteros et al., 2007). These random DNA target based markers have the advantage of having a higher multiplex ratio, a feature which is particularly useful when there is insufficient sequence divergence in the targeted DNA regions (Blok, 2005).

6.1 Protein based approach

Isozyme analysis also referred to as the protein electrophoresis was the first biochemical method to be applied in nematology (Subbotin and Moens 2006). By this method, isoenzymes (enzymes present in almost all species but exhibit slight variations between species) from nematodes are gel-separated on the basis of their molecular weight under electric field. This is then followed by selective staining of bands corresponding to specific proteins and the resulting patterns of the samples compared. Technological advancement has made it possible to carry this out in a miniaturized system such as the PhastSystem® where protein extract from a single sedentary female is sufficient for electrophoretic separation. This method has been mostly used for studying species of cyst and root-knot (Esbenshade and Triantaphyllou, 1990; Karssen et al., 1995). Although there are a few reports of intraspecific variation in isozyme patterns within some species of *Meloidogyne* (Esbenshade and Triantaphyllou, 1985;

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Hernandez et al., 2004), the method is still being widely used (Blok and Powers 2009) due to its relative stability among most species of this genus (De Waele and Elsen, 2007).

Another variant of the electrophoretic protein-based nematode identification method is 2-D PAGE. This approach gives a better resolution, since it separates soluble proteins using both their charges and masses on the first and second dimension respectively. It has been used to distinguish between some species and populations of *Meloidogyne* and *Globodera* (Bossis and Mugniéry 1993; Navas et al., 2002).

The use of antibody-based serological techniques generally referred to as ELISA (Enzyme Linked Immunosorbant Assay) for distinguishing species of nematodes has also been studied (Ibrahim et al., 1996, Ibrahim et al., 2001). This technique requires the use of monoclonal or polyclonal antibodies for detecting some species plant parasitic nematodes. Polyclonal antibodies have high sensitivity, but give less specificity due to their occasional cross-reactive nature. Monoclonal antibodies on the other hand give better specificity but are expensive to produce. Schots et al. (1990), used monoclonal antibodies to successfully differentiate between the two potato cyst nematodes (PCN) *Globodera pallida* and *G. rostochiensis*. However, Ibrahim et al. (2001) discovered cross-reactivity to be the main reason for failure of this method to detect some PCN populations, and purported that further antibodies would need to be raised against *Globodera pallida*, in particular, to distinguish and quantify all species in a sample.

Perera et al. (2009) used matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOFMS) to differentiate between two races of stem and bulb nematodes, *Ditylenchus dipsaci*. Using this approach, they identified three protein biomarkers associated with the lucerne race that were almost absent in the oat race.

Although many of these protein-based methods have proven very useful in nematode diagnostics, they still do not match the level of resolution that can be achieved using DNA based methods. Moreover, DNA-based approaches are faster, cheaper and unaffected by any environmental or developmental conditions (Subbotin and Moens, 2006).

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6.2 DNA based approach

Molecular diagnostics of nematodes has over the years seen enormous progress, and technological advancements, particularly in the areas of DNA amplification and sequencing have been the main driving forces towards achieving this. They have made it possible to accumulate substantial amounts of genetic data with sufficient information on sequence divergence that can aid in reliable and easy identification of nematodes (Blok, 2005). A study of nematological publications from the past two decades will show a steady increase in the use of molecular data for nematode diagnostics. Data provided by molecular diagnostics have also enhanced our understanding of nematode systematics and biology in general, by demonstrating whether or not a targeted DNA region will be suitable for species identification (Holterman et al., 2009). Molecular approaches have enabled the validation of most of the classically delineated nematode taxa (Powers and Fleming, 1998) while providing clarification in areas where the classical approach has failed. For example, molecular approaches may provide the only practical means of discriminating between cryptic species (Powers, 2004). They are also fast, relatively simple, applicable to all nematode life stages, provide highly specific means of identifying taxa, (Powers, 2004) and most of all provide substantial amount of differential characteristics in the form of sequence divergence (Blok, 2005).

Most molecular diagnostics have routinely targeted two main genomic regions for sequence divergence: the nuclear ribosomal RNA genes with their transcribed and untranscribed spaces and the mitochondrial cytochrome oxidase I gene. The nuclear ribosomal RNA genes constitute a highly conserved but sufficiently divergent region of the genome that has proven very useful for species discrimination among many groups of nematodes. These genes occur in multiple copies in the genome, thus making them easily amplifiable by Polymerase Chain Reaction (PCR). These tandemly repeating units may also occur in variable number of copies between different taxa and even between closely related individuals in nematodes. Basically, rRNA genes consist of 18S, 5.8S and the 28S genes separated by the non-coding internal transcribed spacers

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1 and 2 (ITS 1 and 2) positioned between 18S and 5.8S and between 5.8S and 28S respectively.

The internal transcribed spacer (ITS) regions between the rDNA genes have been used for species discrimination within a number of genera either based on size polymorphism of their amplified PCR products or fragments of their amplified products resulting from digestion with restriction endonucleases. Due to its low evolutionary constraints (Powers et al., 1997), it is subject to a higher mutation rate than the highly conserved flanking 18S and 28S rRNA genes. The conserved flanking regions serve as suitable templates for primer design. The two ITS regions have been used in the past both as phylogenetic and diagnostic markers. For example, Subbotin et al. (2004) using sequences of ITS1, ITS2 and the 5.8S gene studied the phylogeny of the gall-forming Anguinids to reveal possible cospeciation events occurring between the grass parasitizing members and their host plants. Additionally, based on the two ITS regions, Subbotin et al. (2001) reconstructed the phylogeny of 40 cyst forming nematode species of the family Heteroderidae. Spiridonov et al. (2004) also combined the use of morphology and the molecular information of the region spanning ITS1, 5.8S and the ITS2 to infer phylogenetic relationship between species of the genus *Steinernema*. Maafi et al. (2003) used RFLP of the ITS to analyze and identify 45 populations of Iranian cyst-forming nematodes representing 21 species. They also used full-length sequence of this region to infer the phylogenetic relationship between these populations. There have been several earlier studies using this region for identifying the important genera of parasitic nematodes (Campbell et al., 1995; Gasser and Hoste, 1995; Hoste et al., 1995; Cherry et al., 1997; Orui 1996; Thiery and Mugniery, 1996). Much like other repetitive regions, the ITS is amplifiable by PCR even from a single cell. This region has, however, been shown to have high incidence of intra-individual sequence variations which make sequence alignment very difficult (Blaxter, 2004), thus making this region unsuitable for inferring phylogeny. This is because insertions and deletions, rather than substitution, are the main sources of mutations in this region (Blaxter, 2004)

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resulting in sequences with high size variations as in Trichodorids and *Pratylenchus* (Boutsika, 2002; Powers, 2004) as well as poor sequence homology.

The small subunit ribosomal RNA gene (also called 18S or SSU rRNA) is the most widely used marker region for nematode species delimitation. It is a gene with mosaic sequence of highly conserved regions (useful for easy primer annealing, and stability within species) and fairly variable regions providing sufficient sequence divergence between species. As a barcode marker region, these and other qualities like its universality across all Eukaryotes as well as availability of substantial number of sequences in databases from identified taxa (Creer et al., 2010) make it the most suitable choice. Indeed, SSU rDNA satisfies most of the features outlined by Valentini et al. (2009) of an ideal barcode marker in being variable (having regions with sufficient sequence divergence), standardized (widely used as marker region), phylogenetically informative (good marker for deep phylogeny eg. Aguinaldo et al., 1997; Blaxter et al., 1998; Aleshin et al., 1998; Holterman et al., 2006; van Megen et al., 2009) and extremely robust (having conserved annealing sites for universal primers). There are, however, groups within which species discrimination using SSU rDNA has been unsuccessful, for example, the tropical root-knot nematode species, as well as the two EU quarantine root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax* (Holterman et al., 2009). In fact, comparison of the resolutions provided by the commonly used barcode markers (mitochondrial COI and COII; nuclear SSU and LSU rDNAs) indicated that none of them was singly suitable for distinguishing between these tropical root-knot species (Kiewnick et al., 2014).

The large subunit ribosomal rRNA gene (also called the 28S or LSU rDNA) is another universal genomic region with effective and well tested primers for a reliable amplification across most taxa (Blaxter, 2004). Sequence divergence regions within this part of the rDNA gene are often longer than they are in the SSU. These divergent regions number between D1–D12 (Hassouna et al., 1984). In comparison with the SSU, the LSU region has been shown to provide better resolution among closely related taxa due to the higher degree of sequence divergence it contains (Markmann and Tautz,

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2005; Subbotin et al., 2008). Although evidently more useful for detecting diagnostic signatures at the species level, its use over the SSU rDNA as an identification marker has been limited due to the considerably lower number of sequences available in public databases.

The mitochondrial cytochrome oxidase I gene (COI gene) is the gene that has so far been most widely applied as barcode region in animals. It has been considered as the primary barcoding marker for all members of the animal kingdom (Hebert et al., 2003). It has also been demonstrated to be a suitable target for molecular phylogenetic studies in mammals (Saccone et al., 2000). The COI like the nuclear rDNA genes is present in all animals, and could perhaps be the most universal barcoding gene in animals (Ratnasingham and Hebert, 2007). This gene, however, has not received extensive application in nematology (Kiewnick et al., 2014), albeit it has been successfully used in identifying various species of marine nematodes (Derycke et al., 2010). Due to the frequent rate of mutation in mitochondrial DNA, finding a conserved region for the design of a universal primer often becomes a constraint. Therefore, primer sets designed for amplifying COI fragments are often likely to be less universal (Blaxter, 2004) compared to any of the nuclear rRNA genes. Studies have also demonstrated that through nuclear integration of mtDNA, a phenomenon by which parts of the mitochondrial genome are transferred to the nuclear genome (Bensasson et al., 2001; Richly and Leister, 2004), there is a possibility of overestimating diversity particularly when mitochondrial COI genes are coamplified with these so called COI nuclear mitochondrial pseudogenes (Song et al., 2008).

Like all DNA based identification methods, DNA barcoding was designed for situations where the morphology-based approach proved problematic. It is defined as the use of standardized DNA regions as markers for rapid and accurate species identification (Hebert et al., 2005; Blaxter, 2005). The key distinguishing feature between DNA barcoding and other molecular diagnostic methods is the use of standardized markers in the former. Therefore, one of the aims of the barcoding consortium is to build taxonomic reference libraries with sequences of standardized markers from different

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organisms (Taberlet et al., 2012). Thus, by comparing the sequences of such markers from unidentified organisms with these reference sequences, their identities can be determined. Indeed, the barcoding approach can be helpful in instances where classical taxonomy prove inconclusive.

It has proven particularly useful in understanding the degree of variation there is between certain species and how these variations can obscure identification. For example the concept of cryptic species shows how morphology alone cannot be relied on for discriminating phenotypically identical but valid species. Studies have shown that there are several examples of cryptic species (e.g. *Tobrilus gracilis* (Ristau et al., 2013)) within the phylum Nematoda that were previously considered to be the same species (Chilton et al., 1995; Derycke et al., 2005; Fonseca et al., 2008). Barcoding also provides a means of identifying rare species or specimens with limited availability. It also offers the only option available for identifying an organism when the required life stage or specific sex for morphological identification is lacking or the morphology of the specimen being studied is badly distorted. And finally on the control of pest movement within trade where speed and accuracy of species identification is critical, barcoding offers a quick and reliable means of detecting quarantine nematode species (Powers, 2004).

Hebert et al. (2003), in their heavily cited study on biological identifications through DNA barcoding, proposed the use of COI of the mitochondrial DNA as a molecular marker for DNA barcoding. As a result, COI has been widely used as standard barcode marker for metazoans (Ferri et al., 2009). Different markers have been proposed for other groups of cellular organisms. Markmann and Tautz, (2005) used the nuclear rRNA gene to study the diversity of meiobenthos (small meiofauna that live in marine and freshwater sediments). Applying the environmental metabarcoding approach, Fonseca et al. (2010) used the nuclear SSU gene of the rRNA to study marine metazoan biodiversity. In plants, on the other hand, the preferred barcode markers are ones found within the chloroplast genome, and identification often entails the use of combination of two or more regions of this genome (Lahaye et al., 2008; Hollingsworth et al., 2009)

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or with other nuclear genes (Tripathi et al., 2013). The nuclear small subunit ribosomal RNA gene has also been successfully used as marker for studies involving nematodes (Floyd et al., 2002; Porazinska et al., 2010).

As mentioned earlier, the rRNA genes (SSU and LSU) are preferred over the mitochondrial COI gene in most nematological studies due to the availability of more conserved regions for universal primer design. Moreover, the abundance of sequences of these two genes from described taxa in public databases make matching sequences for identification an easier job than when using COI. In terms of resolution, however, COI is capable of discriminating between species more than either of the rRNA genes. But a combination of the SSU and LSU genes has been shown to be able to significantly improve the resolution, thereby achieving better detection levels (Porazinska et al., 2009). With current advancements in sequencing technology resulting in increasingly wide usage of next generation sequencing, a form of barcoding which has recently gained much popularity is DNA metabarcoding. Taberlet et al. (2012) defined metabarcoding as the automated identification of several species from a single bulk sample containing multiples of different taxa. Using this approach, it is possible to carry out high throughput identification of several species in a parallel fashion. DNA metabarcoding typically involves the analysis bulk DNA derived from environmental samples (Taberlet et al., 2012). It should, however, not be confused with metagenomics, a term often used to refer to the genomic analysis of organisms from environmental samples (Handelsman, 2004; Tringe et al., 2005; Hugenholtz and Tyson, 2008). Another form of environmental DNA analysis that is just as common as, and often albeit wrongly used as synonym of, metagenomics is metagenetics. The two terms have one common attribute, in that they both involve the analysis of multi-genome units or community (Handelsman, 2009). Creer et al. (2010) described metagenomics as the functional analysis of environmentally derived DNA from unculturable organisms and metagenetics as the large-scale analysis of taxon richness via the analysis of homologous genes.

A typical metabarcoding approach proceeds as follows (i) extracting bulk DNA from the organisms or directly from the environment (ii) amplifying a selected DNA bar-

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code marker region using universal primers (iii) sequencing all the amplified regions in parallel via a next generation sequencing platform (iv) clustering of sequences into molecular operational taxonomic units (MOTU) and (v) matching each MOTU against sequences of identified organisms in a reference database (Valentini et al., 2009). Metabarcoding like standard barcoding is based on the assumption that with appropriate barcode markers, each molecular operational taxonomic unit can be assigned to a described species through its DNA sequence (Orgiazzi et al., 2015) or identified as unknown if not yet described to assist with the discovery of unknown biodiversity.

Almost all DNA metabarcoding applications in nematology have mainly been based on the analysis of bulk samples of entire organisms already isolated from the containing substrates such as soil, water, plant material etc. (Porazinska et al., 2009; Porazinska et al., 2010; Creer et al., 2010; Bik et al., 2012). Beyond multispecies identification from bulk samples of entire extracted organisms, metabarcoding also comprises the use of total and typically degraded DNA extracted directly from environmental samples without prior isolation of organisms (Taberlet et al., 2012). This approach, if successfully applied in nematology, can help overcome the inconsistencies and poor recovery rates associated with various nematode extraction methods (see, den Nijs and van den Berg, 2013). This method was applied for community profiling of nematodes from European soils using the 18S rDNA (Waite et al., 2003), although detailed studies into the efficiency of DNA recovery from the soil are generally lacking. Moreover, since most meiofaunal organisms are often found in substrates with volumes profoundly larger than the total biomass of the organisms themselves, it becomes eminent that they are separated first before DNA can successfully be extracted (Creer et al., 2010).

7 Limitations of high throughput DNA barcoding

There are a number of challenges associated with DNA metabarcoding analysis of environmental DNA. The most notable of these is the identification of a suitable marker to provide the required taxonomic coverage and species resolution. This problem is

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not unique to only metabarcoding but is shared by the single species standard barcoding as well. As mentioned in earlier paragraphs, the SSU rRNA gene has been the most commonly used marker in nematode barcoding due to the availability of extensive database resources and the possibility of using conserved regions for designing versatile primers. It has however, been shown to have limited taxonomic resolution among certain taxa within the phylum Nematoda. The COI on the other hand, is the designated marker for animals as a result of the degree of sequence divergence associated with it, thus permitting species-level delimitation (Deagle et al., 2014). In the case of nematodes, there appears to be a challenge finding a suitable primer that can amplify this marker across distant taxa due to the extreme sequence divergence within the mitochondrial genome within this phylum (Taberlet et al., 2012). Hence, the challenge still remains as to where the most suitable barcode marker(s) might be found within the nuclear and mitochondrial genome.

Another issue with DNA metabarcoding is its reliance on PCR (Taberlet et al., 2012). Significant amount of errors have been shown to accrue during amplification (Haas et al., 2011; Porazinska et al., 2012). These errors often lead to misinterpretation of diversity within samples, mainly due to the formation of chimeras (Huber et al., 2004; Edgar et al., 2011). Fonseca et al. (2012) defined chimeras as artefacts of PCR consisting of sequence fragments from two or more phylogenetically distinct sequence origins. They are produced when an incompletely extended DNA fragment from one cycle anneals to a template of an unrelated taxon and gets copied to completion in the subsequent cycles. Their formation has been shown to be higher in samples that are specios and genetically diverse (Fonseca et al., 2012). Several bioinformatic tools are available designed to identify and discard such hybrid sequences from the reads generated from high throughput sequencing platforms (Beccuti et al., 2013). For biodiversity studies, the most commonly used ones are CHIMERA_CHECK, Pintail, Mallard, Bellerophon, ChimeraChecker, ChimeraSlayer, Perseus and UCHIME. Persues and UCHIME, operate on the assumption that chimeric sequences should be less frequent than the parental sequences (Edgar et al., 2011; Bik et al., 2012). In other words, the assump-

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tion is that chimeras are less abundant than their parents because they have undergone fewer cycles of amplification compared to their parents.

One other constraint to DNA barcode-based identification is the need for a huge repository of sequences of characterized species. This data generation process is arguably the most important step, as the success of any future identification will depend on how accurate sequence information in the database is. Without any sequence from described taxa to match the obtained sequences with, they may convey limited biological or taxonomic meaning to the investigator. This need for existing sequence information for specific applications has been the main hindrance to many efforts to widen the choices of potential barcode markers, since that would mean channeling a substantial amount of effort into building databases with sequence information from as many characterized species as possible. It also explains why almost all metabarcoding studies involving nematodes tend to use only the SSU rDNA as barcode (Porazinska et al., 2009, Creer et al., 2010, Bik et al., 2012).

8 Next generation sequencing technology

Almost all DNA sequence analyses predating the advent of next generation sequencing have relied in one way or the other on the Sanger method (Sanger et al., 1977). Following this milestone discovery, several improvements were made to the method (Smith et al., 1986; Prober et al., 1987; Mandabhushi, 1998). Basically, the Sanger method involves the random incorporation of one of the four 2′, 3′-dideoxynucleotide (which are analogues of the normal deoxynucleotides) to a growing strand, essentially leading to the termination of extension process, hence their name chain terminators. In the end, this reaction produces several differently sized fragments with each terminating in either G, C, A or T terminators. These fragments are then separated via capillary electrophoresis to enable the sequence to be deciphered.

In spite of the immense improvements made to the capillary electrophoresis sequencing method, cost of sequencing, time and labour needed were still too high for

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the growing demands for DNA sequence information (Metzker, 2005) – it was so until the introduction of the various next generation sequencing (NGS) platforms. These platforms have reduced the cost and run time for sequencing significantly (Zhou et al., 2013). The run time for these sequencers can range from just minutes to weeks (Glenn, 2011). There are currently a number of platforms available, all based on some common basic principles, such as their streamlined library preparation steps, and the simultaneity of sequencing and detection processes. They each employ complex interactions of enzymology, chemistry, high-resolution optics, hardware, and software engineering (Mardis, 2008). The following are some of the next generation sequencing platforms that surfaced into the market some years ago: the Roche 454 genome sequencer, the Illumina Solexa technology, the SMRT sequencing technology by Pacific Biosciences, the Ion Torrent and the ABI SOLiD platform. Other platforms included the Polonator and the HeliScope single molecule sequencer technology. Both the Polonator and the HeliScope are single molecule (shotgun) sequencing platforms; hence no amplification step is needed. These have the advantage of eliminating biodiversity inflation or artifacts often associated with PCR-based sequencing methods. The absence of PCR in their sequencing pipelines also means abundant information of taxa in samples, which are often obscured by amplification, can be revealed (Zhou et al., 2013). There have been several review articles that have covered in detail how each of these platforms operate including the chemistry and the instrumentations involved (Mardis, 2008; Metzker, 2009). This review will, therefore, only touch on a few basic and key features of these platforms.

The Roche 454 pyrosequencer was the first next generation sequencing platform to become commercially available. It was introduced into the market in 2004 (Mardis, 2008). This method is based on the pyrosequencing approach which was first described by Hyman (1988). In pyrosequencing, no chain terminators are utilized, instead, incorporation of nucleotide into a growing DNA strand is registered by the emission of light. Only one type of nucleotide is introduced into the reaction per cycle. DNA templates to be sequenced are first sheared into fragments. Each fragment then gets

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immobilized on a bead surface with help of special oligonucleotides where they are each amplified through emulsion PCR (emPCR). The main advantage to using this platform is the relatively long read lengths of the sequences, thus making assembly of contigs easier even in the absence of reference genomes. On the other hand, it has shallow sequencing coverage due to the few reads it generates per run (~1 million sequences). It also has higher errors rates, especially when it encounters homopolymer repeats within the sequence (Ekblom and Galindo, 2011). These characteristics are some of the reasons why the technology has since been superseded by other approaches described below. Most published nematode community studies have used the Roche 454 platform.

The 454 technology was soon followed by the Solexa/Illumina technology as the second NGS platform to be available commercially. As for sequencing by synthesis, DNA is first fragmented and each fragment ligated with an adapter- a short single strand DNA fragment complementary to oligonucleotides attached to the surface of a flow cell. Solexa sequencing has a far more superior sequencing output and depth of coverage than the 454 pyrosequencer. It records fewer incidences of errors in homopolymer regions compared to its 454 predecessor. It currently can produce read lengths of up to 2 × 300 bp (www.illumina.com/systems/miseq.html) which is an improvement over the 35 bp read lengths of the early Solexa platforms. Nonetheless, Illumina has its own unique base calling errors. For instance, it has been observed that accumulation of errors tend to be higher towards the 3′ end than at the 5′ end (Schroder et al., 2010). There has also been an observed association between increase single-base errors and GGC sequence motifs (Nakamura et al., 2011).

The SOLiD platform from Applied Biosystems employs a similar library preparation as the previously mentioned NGS platforms. But unlike the other platforms, it uses ligation to determine sequences. Because each base pair is essentially sequenced twice, the error rates encountered tends to be less in this platform (Ekblom and Galindo, 2011).

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The HeliScope was the first NGS platform to introduce the single-molecule sequencing approach. Although this platform has the advantage of being less prone to errors especially those related to amplification artifacts, it produced read lengths that are short compared to any of the previous technologies. For this reason and the high cost of the instrument, the HeliScope is no longer being sold (Glenn, 2011).

The Ion Torrent platform operates in a similar fashion as the 454 technology in that they both involve similar library preparation steps and sequential introduction of each of the four bases. However, instead of registering base incorporation by fluorescent emission, H^+ are released and a signal in proportion to the number of incorporated bases is detected (Rothberg et al., 2011). The PGM (Personal Genome Machine) of Ion Torrent was evaluated together with other platforms such as Illumina and Pacific Biosystem by Quail et al. (2012). The results indicated that the PGM gave an excellent coverage for those sequences with high GC content to moderate AT richness. However, sequencing of AT-rich genomes resulted in substantial amount of bias with coverage for only about 70 % of the genome. On its ability to detect variants, it slightly outperformed the MiSeq, but in doing so recorded significant amount of false positives as well.

The SMRT sequencing technology by Pacific Biosciences is based on the natural process of DNA replication by DNA polymerase for real time sequencing of individual DNA molecules (Eid et al., 2009). Each dNTP has a specific fluorescence label attached to its terminal phosphate, which upon incorporation of a nucleotide gets detected immediately before it is cleaved off (www.pacificbiosciences.com/products/smrt-technology/). Features such as high speed, long read lengths, high fidelity and low cost per experiment have made this technology a desirable investment (Glenn, 2011, https://genohub.com/ngs-instrument-guide/). However, in comparison with the Ion Torrent and MiSeq sequencers, higher depth of coverage is required for calling of variants (Quail et al., 2011).

Most NGS-based nematode community studies have used the pyrosequencing method of the Roche 454 platform (Porazinska et al., 2009, 2010; Creer et al., 2010; Bik et al., 2012). The relatively longer read lengths generated with this platform made it

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more suitable for metagenetic analysis. Moreover, the Roche 454 was the most widely used platform at this time. Porazinska et al. (2009) carried out one of the early studies to evaluate the suitability of NGS for nematode metagenetic analysis. Using a combination of the SSU and the LSU markers, up to 97 % of the species in the tested community were detected in this study. Using either of these markers alone could only not provide this high coverage of the diversity in the sample. Later, Creer et al. (2010) reported a case study of meiofaunal diversity in marine littoral benthos and tropical rainforest habitats. Out of eleven classified taxonomic groups recovered from each of the case studies, nematodes emerged as the most dominant taxonomic group in both environments through the proportion of the total number of molecular operational taxonomic units (MOTUs) that matched sequences of nematodes.

High throughput Next Generation Sequencing (NGS) methods have also been applied in sequencing of complete mitochondrial genomes (Jex et al., 2008a, 2009). The process involved an initial amplification step referred to as Long PCR which is important to provide enough copies of the mitochondrial genome for sequencing. This step basically amplifies the entire mitochondrial genome as two overlapping fragments of approximately 5 and 10 kb sizes (Hu et al., 2002) which are subsequently bulked and then sequenced using the Roche 454 platform. Prior to the use of NGS for whole mitochondrial genome sequencing, the sequencing step was carried out by "primer walking" on capillary sequencers (Jex et al., 2008b). However, the mitochondrial genome has not been sufficiently exploited for barcoding. Almost all marker-based identification of nematodes has targeted the COI gene. The utility of the complete mitochondrial genome for inferring phylogeny between related taxa also remains to be properly tested. Currently, the widely accepted phylogenic relationship derived from molecular data is based on the small subunit ribosomal RNA gene (Blaxter et al., 1998; Holterman et al., 2006; van Megen et al., 2009). Until a recent study by Kim et al. (2015), phylogeny based on complete mitochondrial genome has been studied mainly in nematode parasites of vertebrates (Kim et al., 2006; Lui et al., 2014; Mohandas et al., 2014). Kim et al. (2015) used translated amino acids of 12 protein coding genes of 100

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nematode species to infer phylogenetic relationships across the phylum with special emphasis on the suborder Tylenchina. The mtDNA-based trees were rather inconsistent with the established nuclear rDNA-based trees, mostly in terms of support for the monophyly of a number of infraorders within Tylenchina. By aligning the mitochondrial genomes of different taxa, regions of highly conserved sequences can be located and utilized for designing primers with broad coverage across a diversity of taxa. These primers could be designed such that the intervening sequence contains sufficient divergence between species (Jex et al., 2010). The challenge using the complete mitochondrial genome is difference in order of arrangement of genes in the linearized genomes of some members of the Enoplean class (Hyman et al., 2011). Although this difference in order of gene arrangements could potentially be useful for diagnostic purposes (Jex et al., 2010), alignment is only possible with genomic sequences displaying identical order of gene arrangement. A possible solution to this will be to annotate the genomes, compare them gene-by-gene or region-by-region and then concatenated the genes back with a common order of arrangement (Jex et al., 2010).

9 Concluding remarks

The major determining factor for the success or otherwise of any marker-based molecular identification method, whether it is standard DNA barcoding or metabarcoding, is finding the most suitable marker or a combination of markers. Several markers have been tested on different nematode groups and these have exhibited varying degrees of performances, but there still seem to be no known marker that can demonstrate all the key qualities required of an ideal marker- to contain a region of very low substitution rate for ease of amplification with a universal primer, to have regions of sufficient mutations to allow for inter-species delimitation while still maintaining sufficient within species similarity across the entire phylum.

DNA barcoding is a tool with numerous potentials in the field of taxonomy. It can serve as a rapid identifying feature of organisms written simply as sequence of four

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distinct bases, thus providing an unambiguous reference for rapid identification. The application of this tool will allow non-experts to carry out some of the routine tasks of identifying species, thus equipping scientists with tools for identifying known organisms and recognition of new species. It can facilitate the recognition and discrimination of cryptic species. This is especially useful when distinguishing invasive species from closely resembling but harmless species. Unlike classical taxonomy, DNA barcoding makes it possible to determine the identity of a species from any life stage available. And this becomes particularly useful when analyzing samples intercepted in trade, where diagnosticians are often confronted with the problem of having very limited material to work with.

Although the ultimate goal in DNA barcoding is the development of molecular tool(s) capable of profiling as wide diversity of the phylum as possible, for now, at least in nematology, both the classical and molecular fields are needed for a better understanding of the biology and diversity of nematodes. With the speed and higher output that the molecular approaches introduce, nematode community analysis will be less laborious. This, for example, will facilitate the use of nematodes as indicators.

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Table 1. Historical review of nematode higher classification. Nematoda ranked as either a phylum or class. (Revised from Adamson, 1987 (Courtesy Decraemer 2011: Systematics and molecular Phylogeny lecture notes)).

Proposed higer level classifications			Authors
Ph: Nemata			Cobb (1919)
Subph: Alaimia	Subph: Laimia		
Cl: Alaimia	Cl: Anonchia	Cl: Onchia	
Cl: Nematoda			Chitwood (1937)
Subcl: Phasmidia	Subcl: Aphasmidia		
Ph: Nematoda			Chitwood (1937, 1950)
Phasmidia: Cl	Cl Aphasmidia:		
Ph: Nematoda			Chitwood (1958)
Cl: Adenophorea	Cl: Secernentea		
Ph: Nematoda			Gadea (1973)
Cl: Enoplinomorpha	Cl: Chromadorimorpha		
Cl: Nematoda			Drozdowsky (1975, 1978, 1980)
Subcl: Enoplia	Subcl: Chromadoria		
Ph: Nematoda			Andrassy (1976)
Cl: Penetrantia	Cl: Torquentia	Secernentia	
Ph: Nematoda			Inglis (1983)
Cl: Enoplea	Cl: Chromadorea	Cl: Rhabditea	
Cl: Nematoda			Malakhov (1986)
Subcl: Enoplia	Subcl: Chromadoria	Subcl: Rhabditia	
Ph: Nematoda			Adamson (1987)
Cl: Enoplea	Cl: Rhabditea		
Ph: Nematoda			Present: De Ley and Blaxter (2002)
Cl: Enoplea	Cl: Chromadorea		

Ranks: Ph = Phylum, Subph = Subphylum, Cl = Class, Subcl = Subclass.

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