African Journal of Biotechnology Vol. 10(3), pp. 312-323, 17 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1473
ISSN 1684–5315 © 2011 Academic Journals

Review

A critique of current methods in nematode taxonomy

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Accepted 8 December, 2010

Morphology based nematode taxonomy and biodiversity studies have historically challenged most biologists. In the past few decades, there have been efforts to integrate molecular methods and digital 3D image-capturing technology in nematode taxonomy, the former to enhance the accuracy of identification of such a taxonomically challenging group and the latter to communicate morphological data. While the employment of these two methods is growing in recent taxonomic, biodiversity and biogeographic studies, a movement to abandon traditional phenotypic identification methods altogether has emerged. Proponents try to justify this trend by citing the challenging gap between the high estimated number of undescribed species and the limited ability of traditional taxonomy to accomplish the task of documenting such diversity. Here we present a review of the various techniques used in the taxonomy of free-living and plant parasitic nematodes and critique those methods in the context of recent developments and trends including their implications in nematode taxonomy, biodiversity and biogeography.

Key words: Species delimitation, taxonomic methods, genetic and molecular methods, traditional taxonomy, nematode biodiversity.

INTRODUCTION

Nematodes are diverse metazoans with an estimated total number of a million species (Lambshead, 2004). They are arguably the most numerous metazoans in soil and aquatic sediments. From an environmental point of view, nematodes are part of nearly all ecosystems in their roles as bacterivores, herbivores, parasites of animals and plants, and consumers of dissolved as well as particulate organic matter. They have critical roles in the flow of energy and cycling of nutrients. From an anthropocentric point of view, they parasitize humans and plants, domestic and wild animals and they can serve as indicators of environmental change. With regard to health, for example, a large proportion (22%) of the human population is parasitized by the intestinal parasite *Ascaris lumbricoides* (Crompton, 1988). Considering their impact on crops, McCarter (2009) estimated a global total loss of $118 billion for 2001, of which nearly half was related to only two crops, rice and maize. This being so, it is remarkable they are among the least studied, with only close to 26,000 (< 3% of estimated) species described to date (Hugot et al., 2001; Hallan, 2007). Despite recent research on the model organism-*Caenorhabditis elegans* -(Wormbook; http://www.wormbook.org/), our knowledge of their diversity, basic nematode biology and ecology remains meager.

Here we review the various methods employed in species delimitation and identification of nematodes. We evaluate the use of those methods in the context of current biodiversity studies and the broader application of morphology in taxonomy.

WHY FOCUS ON TAXONOMY?

Our understanding of the way an ecosystem works not only depends on holistic syntheses of all components but also on our knowledge of the way its individual components function (Kotliar, 2000). Accuracy of identification is, therefore, fundamental to our understanding and communication of the ecological role of any organism. Hugot

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(2002) emphasized the need for correct identification and the role of taxonomy as a science. The current need for a revamped effort to address the gap between estimated and documented nematode species diversity cannot be over emphasized (Hugot, 2002). Traditionally, nematology has its strength in agricultural applications because of its economic implications. As a result, nematode species delimitation methods in the context of agricultural and health-related applications are more refined at the species and below species level than methods employed in nematode biodiversity studies. The biodiversity/ ecological side of nematode taxonomy, which often deals with free-living forms, remains much more wanting in research input. Given these differences, here we will discuss these two aspects of nematode taxonomy separately. We recognize the artificial separation of animal parasitic groups from remaining groups of nematodes. This being so, for brevity this review considers free-living and phytoparasitic groups only.

BIODIVERSITY ASSESSMENT AND TAXONOMY

The predicted number of nematode species is poorly matched with the number of actively working taxonomists. This shortage will not improve as seasoned experts in the field retire without replacement (Hugot, 2002). Therefore, seen in light of the current general trend of students' academic preference and the level of funding to taxonomy, Godfrey's (2002) gloomy prediction for descriptive taxonomy is a realistic assessment. Our understanding of nematode species diversity in the context of species functional redundancy is limited. Therefore, the decline in taxonomy needs to be rectified. In nematology, however, there seems to be a glimmer of hope. Two M.Sc. programs - one funded by the Flemish state in Belgium and the second by the European Union - are currently training (> 15 students per year) nematode taxonomists.

Units of biodiversity assessment

"*The breadth of the concept of biodiversity reflects the interrelatedness of genes, species, and ecosystems*" (World Resources Institute 2003). Although questioned recently, often, species are regarded as the smallest units that interact with the environment (Perry, 2010). Therefore, our understanding of the interaction of nematodes with their environment is affected by what we call a species. Currently, what species are and how we define them is a biological asymptote: despite ongoing discussions on "the species problem" in the literature (Ferris, 1983; Mayr and Ashlock, 1991; Adams, 1998, 2002; Velasko, 2008; Van Regenmortel, 2010), no simple and unifying method of defining a species has been universally accepted beyond recognition of the theoretical difficulties and technical limitations (Adams, 2002; Hey, 2006). Currently, there are many species concepts and we lack one with both the advantages of practicality and theoretical universality. Instead, this search for a panacea has led to a distinction between theoretical species concepts and more operational species recognition methods (Mayden, 1997; Adams, 2002; Van Regenmortel, 2010). In nematology, this distinction helps us acknowledge the limitations of many of these concepts as applied to nematode taxonomy, paving the way for more refined species recognition methods based on a sound theoretical concept (De Ley et al., 1999; Hunt, 1997; Nadler, 2002). The choice of a primary species concept depends upon various factors, including applicability. Mayden (1997) distinguishes between primary (universal) theoretical species concepts and secondary species concepts (practical recognition methods). The consequence of using such practical methods has been the development of group or taxon-specific approaches.

Biodiversity can be assessed at any taxonomic level, and by either molecular or phenotypic characters or a combination of both. The question "What biological level of organization corresponds to what level of naming?" has been difficult for biologists. Whether dealing with economically important forms or attempting to assess nematode diversity, often our yardstick of higher and lower taxon categories is measured using the species scale. Consequently, despite the conceptual and practical problems, we cannot ignore the question of what species really are. Species, however, are testable hypotheses, put forward based on current information but which can be revisited repeatedly as new data becomes available or new approaches of generating data relevant to species delimitation are developed (Adams, 2002).

Traditional taxonomy and its applicability as species delimitation tool

Competent traditional taxonomic work, i.e. based solely on morphological evidence, can be as revealing as any other modern behavioral, biochemical or molecular method as to the identity of a group (Mayr and Ashlock, 1991; Wang et al., 2008). However, the reasoning through which a decision about identity is made inherently involves a certain level of subjectivity, for it is the experience of the taxonomist that matters most in making these decisions. Whether a certain morphologically distinguishable group of nematodes represents a species is indeed a difficult hypothesis to test. With all its limitations, to a large extent morphology is as good as any of the other taxonomic tools in delimiting species (Mayr and Ashlock 1991; De Ley, 2006; Agatha and Strüder-Kypke, 2007). However, its optimal use in microscopic organisms, especially in groups such as nematodes where microscopic structural differences are critical, requires significantly more technical (microscopic) and taxonomic expertise than needed for macroscopic

groups. Communicating morphology also remains a challenge.

RECENT TRENDS IN NEMATODE BIODIVERSITY STUDIES

Currently we observe a shift from the purely phenotypic to using a combination of both phenotypic and molecular methods in nematology (Powers et al., 1997; Powers, 2004). Also, the phylogenetic species concept has gained more support recently (Adams, 1998, 2002) and ways to extend its theoretical appeal into practicality have been evaluated (Nadler, 2002).

Why the shift?

Molecular methods have the following advantages: A) They are applicable in high throughput systems. Genetic information in the form of DNA sequences of a few genes can be acquired for many taxa in a few days. Therefore, these methods offer development of high throughput systems which will make nematodes attractive to environmental studies. Furthermore, the progress in micro-array technology promises the development of faster analysis of bulk environmental samples. Minor technical limitations (e.g., in extraction, finding broadly applicable primers etc.) remain because of the need to adjust methodology to diverse nematode taxa from diverse environments. B) Homology of genes is simpler to predict and test than homology of morphological characters. In addition, both types of data can be used to infer phylogenetic relatedness and thus create a systematic framework in an evolutionary context. However, because of their sheer number and simple methods for predicting homology (Schwarz, 2005), molecular characters rapidly outnumber morphological ones. Inferring phylogeny from DNA sequences, similar to morphology, has its own recognized methodological limitations that may affect the conclusions. Alignment of sequences using computer algorithms may introduce biases, especially when they are subsequently modified by eye. Deciding positional homology of DNA sequences is a more consistently applicable process than similar decisions for morphological characters. C) DNA sequences are already digital and can be communicated across laboratories without interpretation. D) DNA sequences are inherently genetic and therefore overcome many problems of apparent similarity from which morphology suffers, especially with microscopic organisms. DNA sequences have unveiled cryptic species among individuals considered conspecific based on morphology alone (Eyualem and Blaxter, 2003; Hoberg et al., 1999; Chilton et al., 1995; Nadler, 2002; Fonseca et al., 2008; Derycke et al., 2008; Bhadury et al., 2008).

Despite the advantages of molecular data in collection, analysis and communication compared to morphology, like morphological characters, molecular data can violate the assumptions of phylogenetic analysis. For example, sequences from different taxa can change at very different rates confounding their use in phylogenetic inference (Britten, 1986; Mallatt et al., 2010). Furthermore, the selection of loci can be critical: some such as the genes of animal mtDNA evolve rapidly and are useful for intraspecific analysis (Lazarova et al., 2006). These same loci, however, suffer from extreme homoplasy when compared across divergent taxa.

The inference of relatedness among taxa from their DNA sequences is constrained by one major step-alignment (i.e. identifying positional homologies in sequences). This topic has been discussed at length by Lee (2004). Automation of alignment in a hands-off way could eliminate biases from the alignment process by excluding the current step of fine-tuning by eye, a factor which may be a cause for discrepancies between hypothesis that can be generated from the same sequence, and its much unwanted aspect - subjectivity. Clustering of aligned DNA sequences also suffers from inconsistency, a serious drawback to the definition of Molecular Operational Taxonomic Units (MOTUs) and interpretation of their implications (Blaxter, 2004).

The question of integrating cryptic taxa delimited by molecular methods into current taxonomy remains unsolved, i.e. those studies that revealed cryptic species have not led to formal proposals of named new taxa (Eyualem and Blaxter, 2003; Hoberg et al., 1999; Chilton et al., 1995; Nadler, 2002; Fonseca et al., 2008; Derycke et al., 2008; Bhadury et al., 2008). There is a general inability of various genes/markers to delimit populations corresponding to “species” determined by phenotypic means. However, unquestionably each marker will be consistent and testable at the level on which the marker is employed (taxon category higher or lower than species). In other words, phenotypic diversity may not always correspond to genetic diversity and vice versa. For the moment we should recognize the limitations of single markers at hand and make use of varying genetic markers that can resolve diversity at various levels in the different major nematode groups.

Digital Multifocal Images (DMI) enhance the communication of morphology. Like the methodology of recognizing phylogenetic affinity, the way we communicate morphological information is also changing rapidly (De Ley and Bert, 2002; Eyualem et al., 2004). The recognition of the seriousness of this problem led researchers to consider the exploitation of current technologies for the documentation and communication of morphological information not only with unsurpassed completeness but also with speed (De Ley and Bert, 2002). A marriage between such data capturing technology and the Internet creates unsurpassed accessibility of morphological information to scientists globally (Eyualem et al., 2006). Despite many current practical limitations

that need to be addressed and agreed upon by nematologists, the use of video imaging (De Ley and Bert, 2002; Eyualem et al., 2004) will augment voucher type material collections in museums and foster the communication of morphological vouchers. Imaging of critical voucher specimens (e.g. types) will be important for linking the taxonomy of known nematodes with future species discoveries. Ongoing efforts to integrate these images in dynamic, online and accessible keys will open new possibilities in nematode taxonomy and the communication of morphology.

Molecular barcoding: Is it a panacea?

The reaction of the scientific community to the proposal to use a universal DNA barcode for species identification was either idealistic enthusiasm or extreme reservation and resistance. The idealistic optimism and passionate appeal from ecologists (Janzen, 2004) was not based on sound justification of the methodology, instead it was a plea for change that resulted from frustration with traditional taxonomy, where only a few experts can identify specific taxa. Traditional taxonomy is slow to document biological diversity, a problem for most ecologists. But, to try to change traditional taxonomy and stop using century-old morphological information is not necessarily the same as replacing it with the ultimate-species-identification-tool. Janzen (2004) states '*The answer lies in a process that will for the first time connect the collective species-level biodiversity knowledge of the world to any and all users, on the spot, in real time, now*.' However, it is not clear if Janzen's vision is achievable. Will a universal tool work only with DNA? Why not use image analysis to arrive at what Janzen wants?

On the other side of the spectrum, despite its demonstrable and tangible advantages, there is anxiety among biologists, especially taxonomists, that molecular barcoding will replace taxonomy as we know it and will reduce the biological complexity of an entire organism to a small fraction of the organism – a gene (Lipscomb et al., 2003; Will and Rubinoff, 2004). This general disquiet masks serious concerns with the scientific merit of specific barcoding proposals.

One such concern is associated with the nature of the use of DNA sequences for taxonomic purposes. Lee (2004) gave a detailed account of the limitations of using of DNA sequences for delimiting species boundaries. Lee (2004), however, failed to distinguish between the two fundamentally different approaches using DNA sequences to enhance inventory of biological diversity. The first approach aspires to find genes that hopefully will predict "species" as delimited by other non-molecular methods (Hebert et al., 2003a; Fre´zal and Leblois, 2008), but the second does not attempt to employ molecular data in line with what are traditionally considered "species" (Blaxter, 2003, 2004; Blaxter and Floyd, 2003; Floyd et al., 2002). This latter approach aspires to inventory biodiversity at the molecular level in an operational way (MOTUs) and clearly states that the inventoried elements of diversity will not necessarily reflect what normally are considered "species". Unfortunately, our understandings on the use and benefit of DNA sequences depend on which approach we are discussing.

The effort to harmonize DNA sequences with "species", however we define species, has the advantage of being biologically meaningful, thus extending its impact to not only inventory of biological diversity but also to the more practical and pressing issues of conservation. Such harmonization will link future, DNA-based works, with already accumulated information with regard to "species". However, reservations continue to linger even with the hope of harmonizing DNA sequences with "species": Ferguson (2002), for instance, argues that *"…genetic divergence is too crude a proxy for reproductive isolation and thus species boundaries*". Although currently there is a diverse array of species concepts, the fact that genetic exchange among individuals remains the driving force in maintaining a species in the form of reticulate populations lends credit to the weight biologists give to reproductive isolation. Limited studies demonstrated that a single gene may predict "species" boundaries in nematodes (Eyualem and Blaxter, 2003), but more recognize the limitations and use multiple genes that do the job in many animal groups (Fre´zal and Leblois, 2008).

Another concern, which seems to be the primary reason for the opposition to the idea of DNA barcoding, is associated with use of a single supposedly universal gene-the Cytochrome *c* Oxidase subunit I gene - as an ultimate DNA barcode in all animals (Hebert et al., 2003b). After extensive efforts for a number of years and criticism on theoretical and technical grounds (Blaxter, 2004; Kipling and Rubino, 2004; Lee, 2004; Moritz and Cicero, 2004; Powers, 2004; Bhadury et al., 2006), the initial "universal gene" idea gave way to a multi-gene approach (Fre´zal and Leblois, 2008).

Blaxter (2004), favoring the use of nuclear small sub unit ribosomal (SSU) and large subunit ribosomal (LSU) genes, discussed the relative utility of these DNA segments for barcoding. A key aspect missing from Blaxter's list of conditions for the selection of a DNA barcode was the congruence between the DNA barcode and a species-level resolution as defined by other methods of delimitation. MOTU, defined by DNA sequences, therefore, cannot be equated with "species" or any taxon category. While the utility of MOTU in the context of our current understanding of global species diversity may be limited, certainly it has an important role in evaluating genetic diversity within already defined taxa.

Does morphology matter?

Some biodiversity studies use molecular methods for taxa delimitation with the extreme aim of abandoning morphological data altogether (Edgcomb et al., 2002; Floyd et al.,

2002; López-Garcia et al., 2003). Such studies indisputably will contribute immensely to our understanding of genetic diversity, but their contribution to our understanding of the interaction of organisms with their environment will probably fall short of their intended goals because they lack morphological information which relates to function. Regardless, with the current level of information accrued, "*DNA sequences alone are not sufficient to characterize a species, but their unique reproducibility helps to guard against duplicate descriptions*" (Tautz et al., 2002). Despite its obvious advant-ages and the fact that it is possible to predict a certain level of gene expression from sequences, our current inability to predict ecological functionality of entire species from DNA sequences alone invalidates such a speedy jump to abandon morphology altogether. In short, molecular methods are best supplemental tools, as are other methods (Mallet and Willmott, 2003).

MOLECULAR METHODS USED FOR IDENTIFICATION OF NEMATODES WITH AGRICULTURAL IMPLICATIONS

For years, morphological identification was the only method widely used to identify nematodes. As our knowledge of nematodes of agronomical importance increased, it became clear that morphology alone did not reveal the complete picture of observed pathological differences between populations within morphologically delimited species. As a result, researchers have been looking for methods that can better predict observed pathological behaviors among populations within species.

To this end, numerous molecular techniques have been developed that are capable of identifying and quantifying nematodes at the species level and below. Techniques such as isoenzyme pattern analysis, restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, polymerase chain reaction (PCR), quantitative polymerase chain reaction (qPCR), and sequencing of diagnostic rDNA regions have all been used successfully to identify and quantify several agriculturally important plant-parasitic nematodes. These methods have their own advantages and limitations (Table 1). In the following sections we will briefly describe the different molecular methods used in nematode identification. Most of these methods have been widely used in the diagnostics of agriculturally important nematodes. DNA sequences of marker genes, Denaturing Gradient Gel Electrophoresis (DGGE) and the more recently developed method of pyrosequencing are the three methods employed in biodiversity studies of free-living forms.

Allozymes

Analysis of allozyme patterns was the first biochemical method to characterize and diagnose nematode species. The principle is based on the fact that differences in net charge, size, or conformation of proteins (isoelectric focusing) are due to differences in the number and composition of amino acids. These differences may be characteristic for a taxonomic group. However, there is no confirmation that proteins with similar electrophoretic patterns do indeed have similar amino acid sequences, i.e. similar patterns may not actually reflect identity on the genetic level.

Restriction fragment length polymorphism (RFLP)

RFLP is a technique in which species or populations are differentiated by the patterns of enzyme cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarities or differences in the band patterns generated are used to differentiate species (and even strains) from one another (Oliveira et al., 2006; Barsi and De Luca, 2008; Troccoli et al., 2008).

Amplified fragment length polymorphism (AFLP)

AFLP is a DNA fingerprinting technique, which detects DNA restriction fragments by means of PCR amplification. Similar to RFLP, this method requires the use of a restriction enzyme. The DNA is digested with two restriction enzymes, one that cuts frequently and one that cuts less frequently. However, unlike RFLP it involves a selective PCR amplification of the target fragments. The actual number of amplified fragments will be smaller than the number of total fragments digested during the restriction step. Gel electrophoresis analysis reveals a unique pattern (fingerprint) of fragments.

Random amplified polymorphic DNA (RAPD)

RAPD is employed to sequence an unknown DNA segment by using primers of random sequences. Similar to AFLP, this method is PCR-based, but it does not involve a restriction step. RAPD amplifies segments of DNA that are essentially unknown to the researcher. It has been used to assess genetic variation among isolates of the same species (Dong et al., 2001; Cofcewicz et al., 2005; Vieira et al., 2007; Devran et al., 2009) and between species (Cofcewicz et al., 2005). Bandi et al. (1995) compared patterns generated using RAPD with those of allozymes. They found that both methods basically revealed the polyspecific structure of the genus *Trichinella* although the RAPD method was potentially useful for detecting cryptic species. They also demonstrated

Table 1. Advantages, limitations, and applications of molecular methods in nematode identifications.

Method	Use	Limitations	Application example
Allozymes	- First biochemical method in nematode taxonomy - Provides comparable data between geographical isolates	- Lack of confirmed genetic basis - Cannot be used for single nematode studies - Life Stage specific - Lacks data on specific characters - Requires non-denatured proteins	- Nematode genera identified using this method includes: *Meloidogyne, Heterodera, Pratylenchus, Ditylenchus and Aphelenchus.* (Esbenshade & Triantaphyllou 1990; Navas et al., 2001).
Restriction fragment length polymorphism (RFLP)	- Suited to differentiate between closely related taxa based on presence/ absence of restriction fragment bands	- Lacks homology of characters - Requires large amounts of PCR products to use for different restriction enzymes.	-Detection of species or populations within species (Curran et al. 1985, 1986; Powers et al. 1986; Castagnone-Sereno et al. 1991, 1993; Garate et al. 1991; Cenis et al. 1992; Piotte et al. . 1992; Xue et al. 1992; Fargette et al. 1996).
Amplified fragment length polymorphism (AFLP)	- Suited to assess variation among individuals of the same species	- Lengthy procedure	- Detection of species in *Heterodera avenae* group (Subbotin et al., 1999), molecular characterization of *Pratylenchus* species (Waeyenberge et al. 2000) and the study of intraspecific variation in *Radopholus similes* (Elbadri et al., 2002).
Random amplified polymorphic DNA (RAPD)	- Unlike AFLP this method doesn't require a restriction step - Simple and rapid	- Not suitable to all organisms and lacks reproducibility - Sensitive to variations in primer and DNA concentration	- Detection of *Globodera rostochiensis, G. pallida, Meloidogyne incognita, M. javanica,* and *M. arenaria*. (Fullaondo et al., 1999; Zijlstra et al. 2000)
Real-time PCR	- Qualitative and quantitative detection of species - Rapid and precise	- Requires species-specific primers - Multiple species detection requires lengthy optimization	- Detection of potato cyst nematode (*G. pallida*) and beet cyst nematode (*Heterodera schachtii*), quantification of *Meloidogyne* spp. from tomatoes, *Pratylenchus thornei* and *Pratylenchus neglectus,* detection of *Bursaphelenchus xylophilus* (Madani et al., 2005, Stirling et al., 2004, Hollaway et al., 2004).

that not all random primers provided similar results in RAPD analysis.

Denaturing gradient gel electrophoresis (DGGE)

DGGE is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products. The principle is that DNA fragments of the same length but with different sequences can be separated. Separation is based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel (Muyzer et al., 1998). DGGE has been shown to underestimate nematode biodiversity (Foucher et al., 2004; Cook et al., 2005). But, even with this limitation, it can be useful to evaluate patterns of nematode diversity over time (Wang et al., 2008).

Multiplex PCR for the identification of multiple species in a single reaction

Detection methods based on single PCR offer a practical alternative to the traditional methods, but each amplification reaction can detect only a single species in a given time. Plant-parasitic nematode species found in natural

Table 1. Continued.

Method	Use	Limitations	Application example
Multiplex PCR	- Detects more than one species at a time - Cost effective - Rapid	- Involves optimization of reagent concentrations, variation in primer annealing temperatures, primer interactions, and amplification of non-specific products - Requires species-specific primers	- Identification of *Globodera pallida* and *G. rostochiensis* (Fullaondo et al., 1999), *Pratylenchus penetrans* and *P. scribneri* (Setterquist et al., 1996), *Meloidogyne chitwoodi* and *M. fallax* (Petersen et al., 1997), and *P. coffeae* and *P. loosi* (Uehara et al., 1998).
DNA sequencing	- Preferred from the above mentioned methods as factors that affect patterns in these methods (e.g. variation in primer and DNA concentration, DNA template quality, gel electrophoresis, and the type of DNA polymerase) can be controlled and the sequencing step can be optimized. - Fast and accurate	- Difficult in finding an ideal gene for taxonomic identification as well as phylogenetic inference , that works in all nematode groups - Choice of marker is still an open issue	Widely used in recent years; in various groups including family *Hoplolaimidae*, (Subbotin et al., 2007), order *Tylenchida* (Subbotin et al., 2006), suborder *Criconematina* (Subbotin et al., 2005), suborder *Cephalobina* (Nadler et al., 2006) , *Pratylenchus* (Al-Banna et al., 1997), *Acrobeloides* (De Ley et al., 1999), *Steinernema* (Stock et al., 2001), *Meloidogyne* (Castillo et al., 2003), *Longidorus* (Rubtsova et al., 2001; Mekete et al., 2009).
Denaturing gradient gel electrophoresis (DGGE)	- Easy and straightforward approach for nematode community analysis - Used to differentiate multiple species in a sample	- DNA extraction for DGGE problematic - Not useful for quantification of species richness in marine communities (Cook et al., 2005)	– Community analysis in different ecosystems (Foucher et al., 2004; Griffiths et al., 2006; Shi-Bin et al., 2008; Waite et al. 2003; Foucher et al., 2004)

conditions are rarely, if ever, encountered singly. A more practical approach is the identification of multiple species in a single PCR reaction. Procedures that allow simultaneous detection and/or identification of different plant-parasitic nematodes have paramount importance because they require less time and cost. Multiplex PCR is a variant of PCR that enables simultaneous amplification of many target regions in one reaction by using more than one pair of primers. Since its first description in 1988 by Chamberlain et al., (1988), this method has been applied in many areas of DNA testing, including species identification, analyses of deletions, mutations, polymorphism, quantitative assays, and reverse transcription PCR (Elnifro et al., 2000; Subbotin and Moens, 2006).

DNA sequencing

Amplification and sequencing of diagnostic regions of nematode DNA have become the major source of new information for advancing our understanding of evolutionary and genetic relationships (Hajibabaei et al., 2007; Meldal et al., 2007). This has been possible for several years and has resulted in public DNA sequence databases that are available for BLAST-match searching (Porazinska et al., 2009). However, finding an ideal gene for each of the different purposes, taxonomic identification as well as phylogenetic inference and that work in all nematode groups and at all traditional taxonomic levels may be similarly difficult. Furthermore, the choice of a DNA locus that provides a species-specific designation is still an open issue (Porazinska et al., 2009).

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 for identification and molecular phylogenetic analysis of several groups of nematodes. These genes are multicopy, which makes them relatively easy to amplify. These regions have different modes and functions in evolution and are suited for use differentiating phylogenetically across the phylum and closely related populations and taxa (Al-Banna et al., 1997; Blaxter et al., 1998; Courtright et al., 2002; De Ley and Bert, 2002; Floyd et al., 2002).

Currently, the D2 and D3 expansion segments of the 28S rRNA and the partial 18S rRNA are being used extensively as the standard molecular marker for most plant-parasitic nematodes. These regions are highly effective in identifying different plant-parasitic groups because of the availability of several conserved primers

that amplify DNA from many taxa and because of the presence of phylogenetically informative sites (Blaxter et al., 1998; Subbotin et al., 2007). Sequence comparison of these genes from unknown species with those published in GenBank facilitates fast identification of most species of plant-parasitic nematodes (Ferris et al., 1999; Thiery and Mugniery, 1996; Orui 1997; Szalanski et al.,1997; Subbotin et al., 1999, 2000, 2001; Eroshenko et al., 2001; He et al., 2005).

With regard to free living nematodes, DNA sequencing has been used for rapid and accurate taxonomic identification and understanding population genetic structure in marine nematodes (De Ley et al., 2005; Derycke et al., 2007, 2008; Bhadury et al., 2006). Bhadury et al. (2006) studied nuclear and mitochondrial genomic regions and evaluated their potential use for marine nematode identification. Their results showed that 18S ribosomal RNA gene amplified most reliably from a range of taxa and can be useful for rapid molecular assignment of unknown nematode species. As is true for all taxa, this is practical only in cases where most members are sequenced and data is openly accessible for comparison. Groups with few sequenced members still pose a challenge to identification of hitherto unknown taxa. In a recent study Bhadury et al. (2008) studied the distribution of a single species *Terschellingia longicaudata* from a range of localities and habitats and reported that DNA sequences revealed the presence of cryptic species which based on morphology and morphometry were identified as conspecific. Consequently, in some marine groups, traditional morphological methods may underestimate biodiversity compared to DNA sequence data due to limited ability to detect species complexes.

Derycke et al. (2008), using DNA sequences, also revealed the insufficiency of morphology to detect marine nematode taxa; these authors revealed 10 sympatrically distributed cryptic species in the marine genus *Rhabditis* (*Pellioditis*). Derycke et al. (2007) studied the population genetic structure of populations identified morphologically as belonging to *Geomonhystera disjuncta* using two molecular markers (COI and ITS) and reported the presence of five cryptic taxa. These studies confirmed a wide and global distribution of some nematode species but also showed the possibility of a higher diversity being masked by inadequate species delimitation methods employed.

Bhadury et al. (2008), in agreement with Derycke et al. (2007), emphasized the need to use multiple markers for a better understanding of biodiversity in marine nematodes. Although morphology, as a method of delimiting taxa, is reported to be insufficient, often, what is overlooked is the researchers' level of taxonomic expertise. Taxonomic expertise and experience is difficult to measure but does vary widely. A researcher's inability to delimit a species morphologically is, therefore, not necessarily because morphology is inadequate as a method. Instead, in some instances, the taxonomist's lack of experience could be a determining factor. A recent study that addressed all these issues and can serve as an example of integration of methods in taxonomy was that of Sudhaus and Kiontke (2007). They studied the genus *Caenorhabditis* extensively and proposed the nominal species *Caenorhabditis brenneri* primarily based on "cross-breeding experiments, biogeography and DNA sequences".

Pyrosequencing of metagenomic samples in nematode biodiversity studies

Recently, pyrosequencing has emerged as a new sequencing technology (Creer et al., 2010). This technique is a widely applicable alternative technology for the detailed characterization of nucleic acids. Fundamentally different from DNA sequencing, pyrosequencing occurs by a DNA polymerase driven generation of inorganic pyrophosphate, with the formation of ATP, and the ATP-dependent conversion of luciferin to oxyluciferin. The generation of oxyluciferin causes the emission of light pulses, and the amplitude of each signal is directly related to the presence of one or more nucleotides. It is a non-electrophoretic real-time DNA sequencing method in which enzymatic reactions yield detectable light, proportional to the number of the incorporated nucleotides (Diggle and Clarke, 2004; Edwards et al., 2006).

One of the most important applications of this technology is the ability to identify large numbers of species from complex communities and recently it has been widely used to generate the genome sequences of complex environmental samples (Edwards et al., 2006; Joseph et al., 2009). Roesch et al. (2007) employed the method to investigate soil microbial diversity, and others have used it to study plant pathogenic fungal communities (Das et al., 2008; Buée et al., 2009). In nematology, it has been tested and has shown encouraging results for the analysis of nematode diversity from metagenomic samples (Creer et al., 2010). Porazinska et al. (2009) reported the use of high-throughput sequencing for metagenomic analysis of nematode diversity using coding sections of the small and large subunit of rRNA genes. The experiment was done in artificial metagenomic samples involving diverse reference nematodes in known abundances. The use of both SSU and LSU loci improved the detection at the species level from ~90% when either locus was used to 95% when both loci were used indicating that the use of multiple loci is a key factor in generating a more complete community profile. However, apart from presence/absence detection of taxa in the community, pyrosequencing of environmental samples failed to provide a complete picture of community structure; it failed to discriminate proportional abundance of each taxon in the artificial community (Porazinska et al., 2009). Nevertheless, the fact that a basic nematode community profile can be generated using this method is

by itself a significant leap from our current single nematode sequencing efforts and an important step towards the future, broad use of environmental DNA data to the study of nematode community structure.

Sequence databases

Our ability to infer relationships at all levels-among major groups, groups of closely related species, or geographically isolated populations of the same species-undoubtedly, provides an independent framework on which we can compare data from other studies such as morphological, morphometrical, embryological, and biological studies.

DNA sequencing and maintaining sequence databases are important activities for discovery of novel genetic properties, exploring phylogenetic affiliations, and in developing more specific primers and gene probes to address particular taxonomic questions. Gene sequences, once obtained, are to a large extent submitted to and maintained within various databases such as GenBank, the European Molecular Biology Laboratory (EMBL-Bank), the DNA Database of Japan (DDBJ), the NEMBASE, and the WORMBASE. These databases contain notably high-volume data sets from over 160,000 organisms, including bacteria, fungi, protozoa, nematodes and other fauna (http://www.ncbi.nlm.nih.gov/guide/taxonomy/). Comprehensive data on DNA sequences, morphology, taxonomy, phylogeny, ecology, accessible literature etc. would serve as crucial platform for training and research. The primary focus of DNA-based databases has hitherto been on plant parasitic and/or easily cultured nematodes (De Ley et al., 2005). Recent establishment of databases focused on general nematode morphology and taxonomic literature will strengthen research on free living forms: Nematol (http://nematol.unh.edu/index.php) that includes images, phylogenetic trees and sequences, and Nemys (http://nemys.ugent.be/) that hosts a rich collection of hard-to-find literature on taxonomy of nematodes.

CONCLUSION

Judging from current trends, nematode taxonomy in the future will inevitably become increasingly molecular. The practicality of molecular methods will work to the advantage of nematology, as the use of these methods will encourage the inclusion of this diverse animal group in biodiversity and environmental studies. Such an inclusion would be an important step towards a better understanding of the biology and ecology of nematodes. Therefore, molecular methods will help us realize our goals, i.e. to inventory and understand the biological diversity and ecological role of nematodes. Nonetheless, despite some difficulty associated with the use of morphology in the taxonomy of some groups of nematodes, the extensive amount of information accrued on various aspects of nematode biology is currently linked with morphology. Consequently, this makes the integration of morphology in taxonomic studies still relevant and essential.

ACKNOWLEDGMENTS

Eyualem Abebe was supported in part by the US Army Research Laboratory and US Army Research Office under Contract number W911NF-08-1-0402, and US National Science Foundation Award Number 0808632. We thank Paul De Ley, UC-Riverside, for commenting on an earlier version of the manuscript.

REFERENCES

Adams B (1998). Species concepts and the evolutionary paradigm in modern nematology. J. Nematol. 30: 1-21.

Adams B (2002). The species delimitation uncertainty principle. J. Nematol. 33: 153-160.

Agatha S, Strüder-Kypke MC (2007). Phylogeny of the order Choreotrichida (Ciliophora, Spirotricha, *Oligotrichea*) as inferred from morphology, ultrastructure, ontogenesis, and SSrRNA gene sequences. Eur. J. Parasitol. 43: 37-63.

Al-Banna L, Williamson V, Gardner SL (1997). Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. Mol. Phylogenet. Evol. 7: 94-102.

Bandi C, La Rosa G, Bardin M, Damiani G, Comncini S, Tasciotti L, Pozio E (1995). Random amplified polymorphic DNA fingerprints of the eight taxa of *Trichinella* and their comparison with allozyme analysis. J. Parasitol. 110: 401-407.

Barsi L, De Luca F (2008). Morphological and molecular characterization of two putative *Xiphinema americanum*-group species, *X. parasimile* and *X. simile* (Nematoda: Dorylaimida) from Serbia. Nematology, 10: 15-26.

Bhadury P, Austen M, Bilton D, Lambshead P, Rogers A, Smerdon J (2008). Evaluation of combined morphological and molecular techniques for marine nematode (*Terschellingia* spp.) identification. Mar. Biol.154: 509-518.

Bhadury P, Austen M, Bilton D, Lambshead P, Rogers A, Smerdon G (2006). Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. Aquat. Microb. Ecol. 44: 97-103

Blaxter M, Floyd R (2003). Molecular taxonomics for biodiversity surveys: already a reality. Trends Ecol. Evol. 18: 268-269.

Blaxter M (2003). Molecular systematics: counting angels with DNA. Nature 421: 122-124.

Blaxter M (2004). The promise of DNA taxonomy. Philos. Trans. R. Soc. B 359: 669-679.

Blaxter M, De Ley P, Garey J, Liu L, Scheldeman P, Vierstraete A, Vanfleteren J, Mackey L, Dorris M, Frisse L, Vida J, Thomas W (1998). Nature 392: 71-75.

Britten RJ (1986). Rate of DNA sequence evolution differ between taxonomic groups. Science, 231: 1393-1398.

Buée M, Reich M, Murat C, Morin E, Nilsson R, Uroz S, Martin F (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol.184: 449-456.

Castagnone-Sereno P, Piotte C, Abad P, Bongiovanni M, Dalmasso A. (1991). Isolation of a repeated DNA probe showing polymorphism among *Meloidogyne incognita* populations. J. Nematol. 23: 316-320.

Castagnone-Sereno P, Piotte C, Uijthof J, Abad P, Wajnberg E, Vanlerberghe-Masutti F, Bongiovanni M, Dalmasso A (1993). Phylogenetic relationships between amphimictic and parthenogenetic nematodes of the genus *Meloidogyne* as inferred from repetitive DNA analysis. Heredity 70: 195-204.

Castillo P, Vovlas N, Subbotin S, Troccoli A (2003). A new root-knot nematode: *Meloidogyne baetica* n. sp. (Nematoda: Heteroderidae) parasitizing wild olive in Southern Spain. Phytopathology, 93: 1093-1102.

Cenis J, Opperman C, Triantaphyllou A (1992). Cytogenetic, enzymatic and restriction fragment length polymorphism variation of *Meloidogyne* spp. from Spain. Phytopathology, 82: 527-531.

Chamberlain J, Gibbs R, Ranier J, Nguyen P, Thomas C (1988). Detection screening of the duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. 16: 11141-11156.

Chilton N, Gasser R, Beveridge I (1995). Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: *Strongyloidea*). Parasitol. Int. 25: 647-651.

Cofcewicz ET, Carneiro RM, Randig DG, Chabrier O, Quénéhervé C (2005). Diversity of *Meloidogyne* spp. on *Musa* in Martinique, Guadeloupe, and French Guiana. J. Nematol. 37: 313-322.

Cook A, Bhadury P, Debenham N, Meldal B, Blaxter M, Smerdon G, Austen M, Lambshead P, Rogers A (2005). Denaturing gradient gel electrophoresis (DGGE) as a tool for identification of marine nematodes. Mar. Ecol. Prog. Ser. 291: 103-113.

Courtright EM, Wall DH, Virginia RA, Frisse LM, Vida JT, Thomas WK (2000). Nuclear and mitochondrial DNA sequence diversity in the Antarctic nematode *Scottnema lindsayae*. J. Nematol. 32: 143-153.

Creer S, Fonseca VG, Porazinska DL, Giblin-Davis RM, Sung W, Power DM, Packer M, Carvalho GM, Blaxter M, Lambshead PJD, Thomas WK (2010). Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. Mol. Ecol. 19: 4-20.

Crompton DWT (1988). The prevalence of Ascariasis. Parasitol. Today, 4: 162-169.

Curran J, Baillie D, Webster J (1985). Use of genomic DNA restriction fragment length differences to identify nematode species. Parasitology, 90: 137-144.

Curran J, McClure M, Webster J (1986). Genotypic differentiation of *Meloidogyne* populations by detection of restriction fragment length difference in total DNA. J. Nematol. 18: 83-86.

Das M, Ehrlich K, Cotty P (2008). Use of pyrosequencing to quantify incidence of a specific *Aspergillus flavus* strain within complex fungal communities associated with commercial cotton crops. Phytopathology, 98: 282-288.

De Ley P (2000). Lost in worm space: phylogeny and morphology as road maps to nematode diversity. Nematology, 2: 9-16.

De Ley P (2006). A quick tour of nematode diversity and the backbone of nematode phylogeny. WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.41.1, http://www.wormbook.org. (April 29, 2010).

De Ley P, Bert W (2002). Video capture and editing as a tool for the storage, distribution, and illustration of morphological characters of nematodes. J. Nematol. 34: 296-302.

De Ley P, De Ley I, Morris K, Abebe E, Mundo M, Yoder M, Heras J, Waumann D, Rocha-Olivares A, Burr J, Baldwin J, Thomas W (2005) An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. Philos. Trans. R. Soc. B 360: 1945-1958

De Ley P, Felix M, Frisse L, Nadler S, Sternberg P, Thomas W (1999). Molecular and morphological characterization of two reproductively isolated species with mirror-image anatomy (Nematoda: *Cephalobidae*). Nematology, 1: 591-612.

Derycke S, Backeljau T, Vlaeminck C, Vierstraete A, Vanfleteren J, Vincx M, Moens T (2007). Spatiotemporal analysis of population genetic structure in *Geomonhystera disjuncta* (Nematoda, *Monhysteridae*) reveals high levels of molecular diversity. Mar. Biol. 151: 1799-1812.

Derycke S, Remerie T, Backeljau T, Vierstraete A, Vanfleteren J, Vincx M, Moens T (2008). Phylogeography of the *Rhabditis* (*Pellioditis*) *marina* species complex: evidence for long-distance dispersal, and for range expansions and restricted gene flow in the northeast Atlantic. Mol. Ecol. 17: 3306-3323.

Devran Z, Mutlu N, Özarslandan N, Elekcioğlu IH (2009). Identification and genetic diversity of *Meloidogyne chitwoodi* in potato production areas of Turkey. Nematropica, 39: 75-83.

Diggle M, Clarke S (2004). Pyrosequencing, sequence typing at the speed of light. Mol. Biotechnol. 28: 129-138.

Dong K, Dean RA, Fortnum BA, Lewis SA (2001). Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria, M. hapla , M. incognita* and *M. javanica.* Nematropica, 31: 271-280.

Edgcomb V, Kysela D, Teske A, De Vera G, Sogin M (2002). Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. Proc. Natl. Acad. Sci. USA, 99: 7658-7662.

Edwards R, Rodriguez-Brito B, Wegley L, Haynes M, Breitbart M, Peterson D, Saar M, Alexander S, Alexander E, Rohwer F (2006). Using pyrosequencing to shed light on deep mine microbial ecology. Genomics, 7: 1-12.

Elbadri G, De Ley P, Waeyenberge L, Verstraete A, Moens M, Vanfleteren J (2002). Intraspecific variation in *Radopholus similis* isolates assessed with restriction fragment length polymorphism and DNA sequencing of the internal transcribed spacer region of the ribosomal RNA cistron. Parasitol. Int. 32: 199-205.

Elnifro E, Ashshi A, Cooper R, Klapper P (2000). Multiplex PCR: optimization and application in diagnostic virology. Clin. Microbiol.13: 559-570.

Eroshenko A, Subbotin S, Kazachenko I (2001). *Heterodera vallicola* sp. n. (Tylenchida: *Heteroderidae*) from elm trees, *Ulmus japonica* in the Primorsky territory, the Russian Far East, with rDNA identification of closely related species. Russ. J. Nematol. 9: 9-17.

Esbenshade P, Triantaphyllou A (1990). Isozyme phenotypes for the identification of *Meloidogyne* species. J. Nematol. 22: 10-15.

Eyualem A, Blaxter M (2003). Comparison of biological, molecular and morphological methods of species identification in a set of cultured *Panagrolaimus* isolates. J. Nematol. 35: 119-128.

Eyualem A, Baldwin J, Adams B, Hope D, Gardner S, Huettel R, Mullin P, Powers T, Sharma J, Ye W, Thomas W (2006). A position paper on the electronic publication of nematode taxonomic manuscripts. J. Nematol. 38: 305-311.

Eyualem A, Grizzle R, Hope D, Thomas W (2004). Nematode diversity in the Gulf of Maine, USA, and a web-accessible relational database. J. Mar. Biol. Assoc. U. K. 84: 1159-1167.

Fargette M, Phillips M, Blok V, Waugh R, Trudgill D (1996). An RFLP study of relationships between species, populations, and resistance-breaking lines of tropical species of *Meloidogyne*. Fundam. Appl. Nematol.19: 193-200.

Ferguson J (2002). On the use of genetic divergence for identifying species. Biol. J. Linn. Soc. 75: 509-516.

Ferris V (1983). Phylogeny, historical biogeography and the species concept in soil nematodes. In: Stone A, Platt H & Khalil L (Eds). Concepts in nematode systematics. London: Academic Press, pp. 143-161.

Ferris V, Krall E, Faghihi J, Ferris J (1999). Phylogenetic relationships of *Globodera millefolii*, *G. artemisiae*, and *Cactodera salina* based on ITS region of ribosomal DNA. J. Nematol. 31: 498-507.

Floyd R, Eyualem A, Papert A, Blaxter M (2002). Molecular barcodes for soil nematode identification. Mol. Ecol. 11: 839-850.

Fonseca G, Derycke S, Moens T (2008). Integrative taxonomy in two free-living nematode species complexes. Biol. J. Linn. Soc. 94: 737-753.

Foucher A, Bongers T, Noble L, Wilson M (2004). Assessment of nematode biodiversity using DGGE of 18S rDNA following extraction of nematodes from soil. Soil Biol. Biochem. 36: 2027-2032.

Fre´zal L, Leblois L (2008). Four years of DNA barcoding: Current advances and prospects. Infect. Genet. Evol. 8: 727-736

Fullaondo A, Barrena E, Viribay M, Barrena I, Salazar A, Ritter E (1999). Identification of potato cyst nematode species *Globodera rostochiensis* and *G. pallida* by PCR using species specific primer combinations. Nematology, 1: 157-163.

Garate T, Robinson M, Chacon M, Park-House R (1991). Characterization of species and races of the genus *Meloidogyne* by restriction enzyme analysis. J. Nematol. 23: 414-420.

Godfrey H 2002). Challenges for taxonomy. Nature, 417: 17-19.

Griffiths B, Donn S, Neilson R, Dniell T (2006). Molecular sequencing and morphological analysis of a nematode community. Appl. Soil Ecol. 32: 325-337.

Griffiths H, Sinclair W, Boudon-Padieu E, Daire X, Lee I, Sfalanga A, Bertaccini A (1999). Phytoplasma associated with elm yellows:

molecular variability and differentiation from related organisms. Plant Dis. 83: 1101-1104.
Hajibabaei M, Singer G, Hebert P, Hickey D (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 23: 167-172.
Hallan J (2007). Synopsis of the Described Nematoda of the World. http://insects.tamu.edu/research/collection/hallan/Nematoda/Family/0NematodaIndex0.htm (April 29, 2010).
He Y, Subbotin S, Rubtsova T, Lamberti F, Brown D, Moens M (2005). A molecular phylogenetic approach to Longidoridae (Nematoda: Dorylaimida). Nematology, 7: 111-112.
Hebert P, Ratnasingham S, DeWard J (2003a). Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc. R. Soc. London, Ser. B 270: S96-S99.
Hebert P, Cywinska A, Ball S, DeWaard J (2003b). Biological identifications through DNA barcodes. Proc. R. Soc. London, Ser. B 270: 313-321.
Hey J (2006). On the failure of modern species concepts. Curr. Trends Ecol. Evol. 21: 447-450.
Hoberg E, Monsen K, Kutz S, Blouin M (1999). Structure, biodiversity, and historical biogeography of nematode faunas in holarctic ruminants: morphological and molecular diagnoses for *Teladorsagia boreoarcticus* n. sp. (Nematoda: *Ostertagiinae*), a dimorphic cryptic species in muskoxen (*Ovibos moschatus*). J. Parasitol. 85: 910-934.
Hollaway G, Ophel-Keller K, Taylor, S, Burns R, McKay A (2004). Effect of soil water content, sampling method and sample storage on the quantification of root lesion nematode (*Pratylenchus* spp.) by different methods. Australas. Plant Pathol. 32: 73-79.
Hugot J (2002). Proposal for a network devoted to the study of nematology and helmithology. Nematology, 4: 563-565.
Hugot J, Baujard P, Morand S (2001). Biodiversity in helminths and nematodes as a field study: an overview. Nematology, 3: 199-208.
Hunt D (1997). Nematode species: Concepts and identification strategies exemplified by the Longidoridae, Steinernematidae, and Heterorhabditidae. In: Claridge M, Dawah H & Wilson M (Eds). Species: The units of biodiversity, The Systematic Association Special Volume Series 54. London: Chapman and Hall, pp. 221-245.
Janzen D (2004). Now is the time. Philos. Trans. R. Soc. B 359: 731-732.
Joseph FP, Highlander S, Luna AR, Gibbs RA, Versalovic J (2009). Metagenomic pyrosequencing and microbial identification. Clin. Chem. 55: 856-866.
Kipling W, Rubino D (2004). Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics, 20: 47-55.
Kotliar N (2000). Application of the new keystone-species concept to prairie dogs: how well does it work? Conserv. Biol. 14: 1715-1721.
Lambshead PJD (2004). Marine nematode biodiversity. In: Chen ZX, Chen SY & Dickson DW (eds.). Nematology, Advances and Perspectives ACSE-TUP Book Series, pp. 436-467.
Lazarova SS, Malloch G, Oliveira CMG, Hübschen J, Neilson R (2006). Ribosomal and Mitochondrial DNA Analyses of *Xiphinema americanum* Group Populations. J. Nematol. 38: 404-410.
Lee M (2004). The molecularisation of taxonomy. Invertebr. Syst. 18: 1-6.
Lipscomb D, Platnick N, Wheeler Q (2003). The intellectual content of taxonomy: a comment on DNA taxonomy. Trends Ecol. Evol. 18: 64-66.
López-Garcia P, Philippe H, Gail F, Moreira D (2003). Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. Proc. Natl. Acad. Sci. USA, 100: 697-702.
Madani M, Subbotin S, Moens M (2005). Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. Mol. Cell. Probes, 19: 81-86.
Mallatt J, Craig CW, Yoder MJ (2010). Nearly complete rRNA genes assembled from across the metazoan animals: Effects of more taxa, a structure-based alignment, and paired-sites evolutionary models on phylogeny reconstruction. Mol. Phylogenet. Evol. 55: 1-17
Mallet J, Willmott K (2003). Taxonomy: renaissance or Tower of Babel. Trends Ecol. Evol. 18: 57-59.
Mayden R (1997). A hierarchy of species concepts: The denouement in the saga of the species problem. In: Claridge M, Dawah H & Wilson M (Eds). Species: The units of biodiversity, The Systematic Association Special Volume Series 54. London: Chapman and Hall, pp. 381-424.
Mayr E, Ashlock P (1991). Principles of systematic zoology. 2nd ed. McGraw-Hill, New York, USA, p. 428.
McCarter J (2009). Molecular Approaches Toward Resistance to Plant-Parasitic Nematodes. Plant Cell Monograph, doi:10.1007/7089_2008_32. 12/24/09. (April 29, 2010).
Mekete T, Grey M, Niblack T (2009). Distribution, morphological description, and molecular characterization of *Xiphinema and Longidorous* spp. associated with plants (*Miscanthus* spp. and *Panicum virgatum*) used for biofuels. GCB Bioenergy 1: 257-266.
Meldal BHM, Debenham NJ, De Ley P, De Ley IT, Vanfleteren JR, Vierstraete AR, Bert W, Borgonie, G, Moens T, Tyler PA, Austen MA, Blaxter ML, Rogers AD, Lambshead PJD (2007). An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. Mol. Phylogenet. Evol. 42: 622-636.
Moritz C, Cicero C (2004). DNA barcoding: promise and pitfalls. PLoS Biology, 2: e354. doi:10.1371/journal.pbio.0020354. 12/24/09
Muyzer G, Smalla K (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Leeuwenhoek,* 73: 127-141.
Nadler S (2002). Species delimitation and nematode biodiversity: phylogenies rule. Nematology, 4: 615-625.
Nadler S, De Ley P, Mundo-Ocampo M, Smythe A, Stock S, Bumbarger D, Adams B, De Ley I, Holovachov O, Baldwin J (2006). Phylogeny of Cephalobina (Nematoda): Molecular evidence for recurrent evolution of problem and incongruence with traditional classifications. Mol. Phylogenet. Evol. 40: 696-711.
Navas A, Castagnone-Sereno P, Blazquez J, Esperrago G (2001). Genetic structure and diversity within local populations of *Meloidogyne* (Nematoda: *Meloidogynidae*). Nematology, 3: 243-253.
Oliveira C, Fenton B, Malloch G, Brown D, Neilson R (2005). Development of species-specific primers for the ectoparasitic nematode species *Xiphinema brevicolle, X. diffusum, X. elongatum, X. lfacolum, and X. longicaudatum* (nematoda: *Longidoridae*) based on ribosomal DNA sequences. Ann. Appl. Biol. 146: 281-288.
Oliveira CMG, Ferraz LCCB, Neilson R (2006). *Xiphinema krugi*, Species Complex or Complex of Cryptic Species? J. Nematol. 38: 418-428.
Orui Y (1997). Discrimination of *Globodera rostochiensis* and four *Heterodera* species (Nematoda: *Heteroderidae*) by PCR-RFLP analysis. Jpn. J. Nematol. 27: 67-75.
Perry N (2010). The ecological importance of species and the Noah's Ark problem. Ecol. Econ. 69: 478-485.
Petersen D, Zijlstra C, Wishart J, Blok V, Vrain T (1997). Specific probes efficiently distinguish root-knot nematode species using signature sequence in the ribosomal intergenetic spacer. Fundam. Appl. Nematol. 20: 619-626.
Piotte C, Castagnone-Sereno P, Uijthof J, Abad P, Bongiovanni M, Dalmasso A (1992). Molecular characterization of species and populations of *Meloidogyne* from various geographic origins with repeated-DNA homologous probes. Fundam. Appl. Nematol. 15: 271-276.
Porazinska D, Giblin-Davis R, Powers T, Farmerie W, Kanzaki N, Morris K, Sung W, Thomas W (2009). Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. Mol. Ecol. Resour. 9: 439-1450.
Powers T (2004). Nematode molecular diagnostics: From Bands to Barcodes. Annu. Rev. Phytopathol. 42: 367-383.
Powers T, Platzer E, Hyman B (1986). Species-specific restriction site polymorphism in root-knot nematode mitochondrial DNA. J. Nematol. 18: 288-293.
Powers T, Todd T, Burnell A, Murray P, Fleming C, Szalanski A, Adams B, Harris T (l997). The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. J. Nematol. 29: 441-450.
Roesch L, Fulthorpe R, Riva A, Casella G, Hadwin A, Kent A, Daroub S, Camargo F, Farmerieand W, Triplett E (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J. 1: 283-290.

Rubtsova T, Subbotin S, Brown D, Moens M (2001). Description of *Longidorus sturhani* sp. n. (Nematoda: Longidoridae) and molecular characterisation of several longidorid species from Western Europe. Russ. J. Nematol. 9: 127-136.

Schwarz EM (2005). Genomic classification of protein-coding gene families (September 23, 2005), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.29.1, http://www.wormbook.org. (November 10, 2009).

Setterquist R, Smith G, Jones R, Fox G (1996). Diagnostic probes targeting the major sperm protein gene that may be useful in the molecular identification of nematodes. J. Nematol. 28: 414-421.

Shi-Bin W, Liang L, Ju W, Yong J, Si-Wei J (2008). PCR-DGGE analysis of nematode diversity in Cu-contaminated soil. Pedosphere 18: 621-627.

Stirling G, Griffin D, Ophel-Keller K, McKay A, Hartley D, Curran J, Stirling A, Monsour C, Winch J, Hardie B (2004). Combining an initial risk assessment process with DNA assays to improve prediction of soil borne diseases caused by root-knot nematode (*Meloidogyne* spp.) and *Fusarium oxysporum* f. sp. *lycopersici* in the Queensland tomato industry. Australas. Plant Pathol. 33: 285-293.

Stock S, Campbell J, Nadler S (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. J. Parasitol. 87: 877-889.

Subbotin S, Moens M (2006). Molecular Taxonomy and Phylogeny. In: Perry R & Moens M (Eds). Plant Nematol. CABI, UK, pp. 33-51.

Subbotin S, Halford P, Perry R (1999). Identification of populations of potato cyst nematodes from Russia using protein electrophoresis, rDNA-RFLP and RAPDS. Russ. J. Nematol. 7: 57-63.

Subbotin S, Halford P, Warry A, Perry R (2000). Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitizing solanaceous plants. Nematology, 2: 591-604.

Subbotin S, Sturhan D, Chizhov V, Vovlas N, Baldwin J (2006). Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. Nematology, 8: 455-474.

Subbotin S, Sturhan D, Vovlas N, Castillo P, Tanyi T, Moens M, Baldwin J (2007). Application of the secondary structure model of rRNA for phylogeny: D2-D3 expansion segments of the LSU gene of plant-parasitic nematodes from the family *Hoplolaimidae* Filipjev, 1934. Mol. Phylogenet. Evol. 43: 881-890.

Subbotin S, Vierstraete A, De Ley P, Rowe J, Waeyenberge L, Moens M, Vanfleteren JR (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, *Heteroderidae*) based on analysis of sequences from the ITS regions of ribosomal DNA. Mol. Phylogenet. Evol. 21: 1-16.

Subbotin S, Vovlas N, Crozzoli R, Sturhan D, Lamberti F, Moens M, Baldwin J (2005). Phylogeny of Criconematina Siddiqi, 1980 (Nematoda: Tylenchida) based on morphology and D2-D3 expansion segments of the 28S-rRNA gene sequences with application of a secondary structure model. Nematology, 7: 927-944.

Sudhaus W, Kiontke K (2007). Comparison of the cryptic nematode species *Caenorhabditis brenneri* sp. n. and *C. remanei* (Nematoda: *Rhabditidae*) with the stem species pattern of the Caenorhabditis Elegans group. Zootaxa, 1456: 45-62.

Szalanski A, Sui D, Harris T, Powers T (1997). Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. J. Nematol. 29: 255-267.

Tautz D, Arctander P, Minelli A, Thomas R, Vogler A (2002). DNA points the way ahead in taxonomy. Nature, 418: p. 479.

Thiery M, Mugniery D (1996). Interspecific rDNA restriction fragment length polymorphism in Globodera species, parasites of Solanaceous plants. Fundam. Appl. Nematol. 19: 471-480.

Troccoli A, De luca F, Handoo ZA, Di vito M (2008). Morphological and molecular characterization of *Pratylenchus lentis* n. sp. (Nematoda: *Pratylenchidae*) from Sicily. J. Nematol 40: 190-196.

Uehara T, Mizukubo T, Kushida A, Momota Y (1998). Identification of Pratylenchus coffeae and P. loosi using specific primers for PCR amplification of ribosomal DNA. Nematologica, 44: 357-368.

Van Regenmortel MHV (2010). Logical puzzles and scientific controversies: The nature of species, viruses and living organisms. Syst. Appl. Microbiol. 33: 1-6.

Velasko J (2008). The internodal species concept: a response to 'The tree, the network, and the species'. Biol. J. Linn. Soc. 93: 865-869.

Vieira P, Burgermeister W, Mota M, Metge K, Silva G (2007). Lack of Genetic Variation of *Bursaphelenchus xylophilus* in Portugal Revealed by RAPD-PCR Analyses. J. Nematol. 39: 118-126.

Waeyenberge L, Ryss A, Moens M, Pinochet J, Vrain T (2000). Molecular characterization of 18 Pratylenchus species using rDNA Restriction Fragment Length Polymorphism. Nematology, 2: 135-142.

Waite I, O'Donnell A, Harrison A, Davies J, Colvan S, Ekschmitt K, Dogan H, Wolters V, Bongers T, Bongers M, Bakonyi G, Nagy P, Papatheodorou E, Stamou G, Bostrom S (2003). Design and evaluation of nematode 18S rDNA primers for PCR and denaturing gradient gel electrophoresis (DGGE) of soil community DNA. Soil Biol. Biochem. 35: 165-173.

Wang J, Frasier T, Yang S, White B (2008). Detecting recent speciation events: the case of the finless porpoise (genus *Neophocaena*). Heredity, 101: 145-155.

Wang SB, Li Q, Liang WJ, Jiang Y, Jiang SW (2008). PCR-DGGE Analysis of Nematode Diversity in Cu-Contaminated Soil. Pedosphere, 18: 621-627.

Will K, Rubinoff D (2004). Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20: 47-55.

World Resources Institute. (2003). http://www.wri.org/wri/biodiv/brintro.html. (December 24, 2009)

Xue B, Baillie D, Beckenbach K, Webster J (1992). DNA hybridization probes for studying the affinities of three *Meloidogyne* populations. Fundam. Appl. Nematol. 15: 35-41.

Zijlstra C, Donkers-Venne D, Fargette M (2000). Identification of *Meloidogyne incognita, M, javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based PCR assays. Nematology, 2: 847-853.