Abstract

Nematodes represent a species-rich and morphologically diverse group of metazoans known to inhabit both aquatic and terrestrial environments. Their role as biological indicators and as key players in nutrient cycling has been well documented. Some plant-parasitic species are also known to cause significant losses to crop production. In spite of these, there still exists a huge gap in our knowledge of their diversity due to the enormity of time and expertise often involved in characterising species using phenotypic features. Molecular methodology provides useful means of complementing the limited number of reliable diagnostic characters available for morphology-based identification. We discuss herein some of the limitations of traditional taxonomy and how molecular methodologies, especially the use of high throughput sequencing, have assisted in carrying out large scale nematode community studies and characterisation of phytonematodes through rapid identification of multiple taxa. We also provide brief descriptions of some the current and almost-outdated high throughput sequencing platforms and their applications in both plant nematology and soil ecology.

Introduction

The phylum Nematoda is a species-rich taxonomic group that has been reported in abundant numbers across 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) reported 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 behaviour (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 highly fecund r-strategists, such as the bacterivorous rhabditidae to the large-bodied less fecund k-strategists, such as the omnivorous dorylaimida.

Previous studies have shown that prevailing physical conditions 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 a soil is stable or has undergone some recent perturbation, the nematode community is likely to differ from one place to another. The contribution of nematodes to nutrient cycling (Bardgett 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 the categorisation of nematodes into feeding groups, reproductive strategies and general responses to physical and organic disturbances (Bongers, 1990; Bongers and Ferris, 1999). Classifications into such functional groups are often means of simply lumping together individuals considered to have similar influence(s) on ecosystem functioning; and the validity of such grouping depends mainly on the underlying research objectives (Bongers and Bongers, 1998). Therefore, individuals within a group may not necessarily have any close phylogenetic connections. The family or genus level identification is often sufficiently informative enough for understanding nematodes' role in soil functioning, although species-level identification will certainly unravel more information pertaining to several key ecological concepts (Bongers and Bongers, 1998; Yeates, 2003). The drawback, however, is that their high abundance, minute size and conserved morphology (Decraemer and Hunt, 2006) preclude rapid and accurate identification of species. Consequently, this has severely limited the fraction of environmental samples analysed in nematode community studies, thus limiting the scale and resolution of many important ecological studies (Porazinska et al., 2010).

In terms of the need for accurate identification of nematodes to species level, research has largely focused on plant parasitic taxa, mainly due to the magnitude of direct economic losses they inflict on agriculture – an estimated USD118 billion in a single year (McCarter, 2009). Their management in field crops has for a long time been dependent on the use of nematicides (Hague and Gowen, 1987) which are being gradually phased out following the realisation of the impact that these nematicides pose to the environment (Akhtar and Malik, 2000). Some years ago the EU made some very important modifications to its policy on the use of pesticides to make it more sustainable and to reduce the risk it 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 some time now been sought to replace the loss of synthetic products (Kerry, 2000). Examples include crop rotation and host plant resistance. Effective implementation of such strategies often requires a good understanding of the taxonomy and biology of plant parasitic nematodes species being targeted. This is because most plant resistance genes are only effective against a narrow range of parasitic species or populations. Therefore, knowing the targeted parasitic species or population makes it easier to choose which plant genotype introduce into the field. And with respect to crop rotation, such knowledge will assist in choosing what plant to be used as a non-host in order to avoid further multiplication of the nematode pest.

The existence of character variation and physiological races within species are some of the problems associated with, but not limited to the taxonomy of plant parasitic nematodes (Allen and Sher, 1967). Such complications among other factors became the main catalysts for 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., 2005) have been used to complement the light microscopic approach for identification. Each of the above-mentioned techniques has certain constraints that limit its exclusive use as a 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, 2004; Blok, 2004, 2005). More recently, high throughput species identification using next generation sequencing (NGS) technology has also been applied for large scale nematode community studies to enhance better understanding of their diversity. This technique, known as metabarcoding has also been applied in the area of plant nematology as a means of analysing very large samples of important plant parasitic nematode groups for improved understanding of their distribution and diversities (Eves-Van Den Akker et al., 2016). This current review discusses some of the past and most current approaches to nematode identification and classification with some emphasis on the future use of high throughput species identification for large-scale nematode pest detection and on the possibility of increased use of nematode communities for evaluation of management strategies and assessments of ecosystem health.

Classical taxonomy

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 utilise phenotypic characters 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.

Prior to the introduction of molecular data, studies on phylogenetic relationships within nematology have been based on morphological characters. A notable challenge to the use of morphological characters for achieving a more natural classification is recognizing characters that are homologous from 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 when used alone may be 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 solely 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 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. The best option, therefore, remains to improve and optimize the process of collecting and analysing molecular data to make this tool exclusively powerful for species delineation.

Biochemical methods for nematode identification

Several biochemical and molecular approaches have been used for identification of nematodes. Genomic information at all levels has been utilized for identifying nematodes, from DNA sequence, the structure of molecules, genetic mutations to the presence versus absence of genes (Subbotin and Moens, 2007). 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 (Table 1).

The use of molecular data for identification of taxa has also 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 variations. The DNA 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 Castagnone-sereno et al., 1994), amplified fragment length polymorphism (AFLP) (Semblat et al., 1998; Marche et al., 2001), restriction 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) (Table 2). 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).

DNA barcoding

Molecular diagnostics of nematodes has over the years seen enormous progress. 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). 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 a substantial amount of differential characteristics in the form of sequence divergence (Blok, 2005).

Most molecular diagnostics have 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 (COI) 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 a 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 1 and 2 (ITS 1 and 2) positioned between 18S and 5.8S and between 5.8S and 28S respectively.

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 has been to build taxonomic reference libraries with sequences of standardized markers from different 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.

DNA barcoding has proven useful in our understanding of 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.

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

The rRNA genes (SSU and LSU) are preferred over the mitochondrial COI gene in most nematological studies due to the availability of sequences from more conserved regions for universal primer design. Moreover, the abundance of sequences of these two genes from described taxa in public databases makes 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 classically involves the analysis bulk DNA derived from environmental samples (Taberlet et al., 2012).

A typical metabarcoding approach proceeds as follows (i) extracting bulk DNA from the organisms or directly from the environment (ii) amplifying a selected DNA barcode 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 marker(s), 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 may also comprise 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). Sapkota et al. (2015) also tested and developed a new amplification approach to enable high throughput analysing of soil samples by directly extracting the DNA without a nematode extraction step. The authors reported very good coverage of the nematode diversity within the tested soils. However, detailed assessments of the efficiency of DNA recovery from the soil are generally lacking. Also, such a method will usually only allow for analysis of soil samples much smaller in volume than would otherwise be used if there would be an extraction step. 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). Nonetheless, with sufficient testing and validation, this approach can be immensely beneficial in the long run.

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 not unique to metabarcoding alone 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. The latter is continuously being improved to allow coverage of newly discovered taxa (Sapkota 2015). In contrast, it has been shown to have limited taxonomic resolution among certain taxa within the phylum Nematoda. Nonetheless, the SSU rRNA region is still the marker of choice for DNA metabarcoding of environmental samples where wider coverage remains essential, but species-level identification, not strictly important.

The COI gene, 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 suitable primer sets 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). A significant amount of errors has 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). While most of these errors have been attributed to technical factors such as PCR and sequencing errors, inappropriate protocols such as incorrect annealing temperatures and cycle numbers as well as human errors can contribute to the formation of sequence artefacts. 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 species-rich and genetically diverse (Fonseca et al., 2012).

According to Porazinska et al. (2012), up to 14% of raw sequence data can be made up of chimeras; and in clustered OTU datasets, they can constitute up to 40% of a dataset. Considering how rampant they may be in sequence datasets, there is always the risk of such hybrid sequences being classified as new taxa or unknown to science as is often the case in many metabarcoding studies. Stringent approaches to removing them from sequence data are, therefore, warranted. Several bioinformatic tools designed to identify and discard such hybrid sequences from the reads generated from high-throughput sequencing platforms are available (Beccuti et al., 2013). For biodiversity studies, the most commonly used ones are CHIMERA_CHECK, Pintail, Mallard, Bellerophon, ChimeraChecker, ChimeraSlayer, Perseus and UCHIME. Perseus 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 assumption is that chimeras are less abundant than their parents because they have undergone fewer cycles of amplification compared to their parents. Another method of chimera picking which is incorporated within the QIIME analysis pipeline is the blast fragments method which is based on the BLAST taxonomic-assignment (Altschul et al. 1990).

One other constraint to DNA barcoding 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 the accuracy of sequence information in the database. 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 efforts in widening the choices of potential barcode markers since that would mean channelling 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 the barcode (Porazinska et al., 2009, Creer et al., 2010, Bik et al., 2012).

Next generation sequencing technology

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 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 technologies. 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 that information on the abundance 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 operates including the chemistry and the instrumentations involved (Mardis, 2008; Metzker, 2005). 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). The main advantage to the use of 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. Recent reports indicate that Roche will soon withdraw support for this instrument marking an end to the 454 technology.

The 454 technology was soon followed by the Solexa/Illumina technology as the second NGS platform to be available commercially. 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 Roche 454 predecessor. One of its platforms, the MiSeq series currently can produce read lengths of up to 2x300 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 tends 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 on this platform (Ekblom and Galindo, 2011).

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 artefacts, 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 a 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 a 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., 2012).

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; Lallias, 2015). The relatively longer read lengths generated with this platform made it more suitable for metabarcoding analysis. Porazinska et al. (2009) carried out one of the early studies to

evaluate the suitability of NGS for nematode metabarcoding analysis while comparing two potential barcode regions from the SSU and LSU genomic regions. Using a combination of the two, up to 97% of the species in the tested community were detected in this study. Using either of these markers alone could not provide this high coverage of the diversity in the sample. The authors also found no correlation between the number of reads generated for each of the sampled taxa and their abundances. In fact, some of the less abundant taxa produced the highest number of reads. 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.

Using metabarcoding, Lallias et al. (2015) examined the variation in diversities of protists and microbial metazoans including nematodes across two distinct estuaries in the UK. They utilized the same small subunit nuclear rRNA gene marker as the one used by Fonseca et al. (2010) in a similar study on marine microbial eukaryotes. One of the key aspects of the outcome of this study was that patterns of the marine meiofauna diversity followed specific factors such as hydrodynamics, salinity range and granulometry depending on their life-history characteristics. In phytonematology, the metabarcoding approach targeting a region within the mitochondrial genome was used in a recent study to characterise populations of potato cyst nematodes from several Scottish soils (Eves-Van Den Akker et al., 2015). Besides this study describing the distribution of *Globodera pallida* mitotypes across Scotland, it also outlined how to carry out an accurate, high throughput and quantitative means of characterizing up to a thousand fields at the same time.

High throughput Next Generation Sequencing (NGS) methods have also been applied in sequencing complete mitochondrial genomes (Jex et al., 2008a, 2010). 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 amplifies the entire mitochondrial genome as two overlapping fragments of approximately 5 and 10 kb sizes (Hu et al., 2002) which then were subsequently bulked and 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). This exercise, if carried out for as many nematode species as possible, may enhance the utility of the complete mitochondrial genome for inferring phylogeny between related taxa. At the moment, this area remains to be properly explored. Although most widely adopted phylogenetic relationships derived from molecular data are based on the small subunit ribosomal RNA gene (Blaxter et al., 1998; Holterman et al., 2006; van Megen et al., 2009), information relating to phylogeny from the mitochondrial genome may increase greatly our understanding of relationships between nematodes.

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 combination of markers. Several markers have been tested on different nematode groups and they have exhibited varying degrees of performances. However, there still seems to be no known marker that possesses all the key features of an ideal marker- very slow substitution rate within flanking regions for ease of amplification with a universal primer, sufficient mutations to allow for inter-specific delimitation and enough intra-specific similarity across the entire phylum. The choice of DNA region to target largely relies on the objectives of the particular study. One may target any of the mitochondrial DNA-based markers such the COI, Nad5, 16S, COI and Nad2 if the study demands species-level resolution or to the level of populations covering a narrow diversity such as a family or genus. In plant nematology, a number of closely related species within groups such as the cyst and root-knot nematodes have been successfully identified using DNA markers within the mitochondrial genome (Eves-Van Den Akker et al., 2015; Janssen et al., 2016). If, on the other hand, the study demands a wider coverage without a strict requirement for species-level identification, as in community level analysis where computation of diversity indices usually only require family or genus level identification (Bongers, 1990), any of the markers within rRNA genes can be suitable.

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 sequences of four distinct bases, thus providing an unambiguous reference for rapid identification (Bucklin et al., 2011). 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 recognising 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. Moreover, 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 analysing 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 much 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 and this may eventually facilitate the use of nematodes as bioindicators.

Acknowledgements

The authors wish to thank EUPHRESCO for the funding. We would like to thank Bex Lawson of Fera for providing some nematological articles used in preparing this manuscript. We also appreciate the inputs from Ian Adams and Giles Budge in the form of suggestions and comments.

References

Abebe, E., Mekete, T., and Thomas,W. K.: A critique of current methods in nematode taxonomy, Afr. J. Biotechnol., 10, 312–323, 2013.

Agatha, S. and Strüder-Kypke, M. C.: Phylogeny of the order Choreotrichida (Ciliophora, Spirotricha, Oligotrichea) as inferred from morphology, ultrastructure, ontogenesis, and SSr- RNA gene sequences, Eur. J. Protistol., 43, 37–63, 2007.

Akhtar, M. and Malik, A.: Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review, Bioresource Technol., 74, 35–47, 2000.

Allen, M. and Sher, S.: Taxonomic problems concerning the phytoparasitic nematodes, Annu. Rev. Phytopathol., 5, 247–262, 1967.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J.: Basic local alignment search tool., J. Mol. Biol., 215(3), 403–10, doi:10.1016/S0022-2836(05)80360-2, 1990.

Bardgett, R. D. and Chan, K. F.: Experimental evidence that soil fauna enhance nutrient mineralization and plant nutrient uptake in montane grassland ecosystems, Soil Biol. Biochem., 31(7), 1007–1014, doi:10.1016/S0038-0717(99)00014-0, 1999.

Beccuti, M., Carrara, M., Cordero, F., Donatelli, S., and Calogero, R. A.: The structure of state-of-art gene fusion-finder algorithms, Genome Bioinformatics, 1, 1–6, 2013.

Bergé, J.-B. and Dalmasso, A.: Caractéristiques biochimiques de quelques populations de Meloidogyne hapla et Melodogyne spp, Cah ORSTOM. Série Biologie: Nématologie, 10, 263–271, 1975

Bik, H. M., Porazinska, D. L., Creer, S., Caporaso, J. G., Knight, R., and Thomas, W. K.: Sequencing our way towards understanding global eukaryotic biodiversity, Trends Ecol. Evol., 27, 233–243, 2012.

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R., Mackey, L. Y., Dorris, M., and Frisse, L. M.: A molecular evolutionary framework for the phylum Nematoda, Nature, 392, 71–75, 1998.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., and Abebe, E.: Defining operational taxonomic units using DNA barcode data, Philos. T. R. Soc. B, 360, 1935–1943, 2005.

Blok, V. C. and Powers, T. O.: Biochemical and molecular identification, in: Root-knot

551 nematodes, edited by: Perry, R. N., Moens, M., and Starr, J. L., CABI Wallingford, UK, 98–
552 118, 2009.

553 Blok, V. C.: Molecular diagnostics for plant-parasitic nematodes, Proceedings of the Fourth
554 International Congress of Nematology, June 2002, Tenerife, Spain, Tenerife, Spain, 2004,
555 195–206, 2004.

556 Blok, V.: Achievements in and future prospects for molecular diagnostics of plant-parasitic
557 nematodes, Can. J. Plant. Pathol., 27, 176–185, 2005.

558 Boisselier-Dubayle, M. and Gofas, S.: Genetic relationships between marine and
559 marginalmarine populations of Cerithium species from the Mediterranean Sea, Mar. Biol.,
560 135, 671– 682, 1999.

561 Bongers, T. and Ferris, H.: Nematode community structure as a bioindicator in environmental
562 monitoring, Trends Ecol. Evol., 14, 224–228, 1999.

563 Bongers, T., and Bongers, M.: Functional diversity of nematodes. *Applied Soil Ecology*, 10(3),
564 pp.239–251, 1998.

565 Bongers, T.: The maturity index: an ecological measure of environmental disturbance based
566 on nematode species composition, Oecologia, 83, 14–19, 1990.

567 Bucklin, A., Steinke, D. and Blanco-Bercial, L.: DNA barcoding of marine metazoa, Ann. Rev.
568 Mar. Sci., 3, 471–508, 2011.

569 Bongers, T.: The maturity index: an ecological measure of environmental disturbance based
570 on nematode species composition, Oecologia, 83, 14–19, 1990.

571 Cantalapiedra-Navarrete, C., Navas-Cortés, J. A., Liébanas, G., Vovlas, N., Subbotin, S. A.,
572 Palomares-Rius, J. E., and Castillo, P.: Comparative molecular and morphological
573 characterisations in the nematode genus Rotylenchus: Rotylenchus paravitis n. sp., an
574 example of cryptic speciation, Zool. Anz.-A Journal of Comparative Zoology, 252, 246–268,
575 2013.

576 Carneiro, R. M., Almeida, M. R. A., and Quénéhervé, P.: Enzyme phenotypes of Meloidogyne
577 spp. populations, Nematology, 2, 645–654, 2000

578 Carpenter, A., Hiatt, E., Lewis, S., and Abbott, A.: Genomic RFLP analysis of Meloidogyne
579 arenaria race 2 populations, J. Nematol., 24, 23–28, 1992

580 Carrasco-Ballesteros, S., Castillo, P., Adams, B., and Pérez-Artés, E.: Identification of
581 Pratylenchus thornei, the cereal and legume root-lesion nematode, based on SCAR-PCR
582 and satellite DNA, Eur. J. Plant. Pathol., 118, 115–125, 2007.

583 Castagnone-Sereno, P., Vanlerberghe-Masutti, F., and Leroy, F.: Genetic polymorphism
584 between and within Meloidogyne species detected with RAPD markers, Genome, 37, 904–
585 909, 1994.

586 Cenis, J.: Identification of Four Major Meloidogyne ssp. by Random Amplified Polymorphic

587 DNA (RAPD-PCR), Phytopathology, 83, 76–76, 1993.

588 Charchar, J. and Eisenback, J.: An improved technique to prepare perineal patterns of root-
589 knot nematodes for SEM, Nematol. Bras., 24, 245–247, 2000.

590 Chilton, N. B., Gasser, R. B., and Beveridge, I.: Di_ erences in a ribosomal DNA sequence of
591 morphologically indistinguishable species within the Hypodontus macropi complex
592 (Nematoda: Strongyloidea), Int. J. Parasitol., 25, 647–651, 1995.

593 Cobb, N. A.: Nematodes and their relationships, in: Yearbook of the United States Department
594 of Agriculture, 1914, US Government Printing Office, Washington DC, 457–490, 1915.

595 Coomans, A.: Present status and future of nematode systematics, Nematology, 4, 573–582,
596 2002.

597 Creer, S., Fonseca, V., Porazinska, D., Giblin-Davis, R., Sung, W., Power, D., Packer, M.,
598 Carvalho, G., Blaxter, M., and Lambshead, P.: Ultrasequencing of the meiofaunal
599 biosphere: practice, pitfalls and promises, Mol. Ecol., 19, 4–20, 2010.

600 Curran, J., McClure, M., and Webster, J.: Genotypic di_ erentiation of Meloidogyne
601 populations by detection of restriction fragment length di_ erence in total DNA, J.
602 Nematol., 18, 83–86, 1986.

603 Dayrat, B.: Towards integrative taxonomy, Biol. J. Linn. Soc., 85, 407–415, 2005

604 De Ley, P. and Blaxter, M.: Systematic position and phylogeny, in: The biology of nematodes,
605 edited by: Lee, D., Harwood Academic Publishers, Reading, 1–30, 2002.

606 De Ley, P., De Ley, I. T., Morris, K., Abebe, E., Mundo-Ocampo, M., Yoder, M., Heras, J.,
607 Waumann, D., Rocha-Olivares, A., and Burr, A. J.: An integrated approach to fast and
608 informative morphological vouchering of nematodes for applications in molecular
609 barcoding, Philos. T. R. Soc. B, 360, 1945–1958, 2005

610 De Ley, P.: A quick tour of nematode diversity and the backbone of nematode phylogeny, in:
611 WormBook, edited by: The Caenorhabditis elegans Research Community,
612 doi/10.1895/wormbook.1.7.1, available at: www.wormbook.org (last access: 10 August
613 2015), 2006.

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

616 Deagle, B. E., Jarman, 5 S. N., Coissac, E., Pompanon, F., and Taberlet, P.: DNA metabarcoding
617 and the cytochrome c oxidase subunit I marker: not a perfect match, Biology Lett., 10,
618 doi:10.1098/rsbl.2014.0562, 2014.

619 Decraemer, W. and Hunt, D.: Taxonomy and principal genera, in: Plant Nematology, edited
620 by: Perry, R. and Moens, M., CABI Publishing, Wallingford, UK, 3–32, 2006.

621 den Nijs, L. and van den Berg, W.: The added value of proficiency tests: choosing the proper
622 method for extracting Meloidogyne second-stage juveniles from soil, Nematology, 15,

623 143– 151, 2013.

624 Derycke, S., Remerie, T., Vierstraete, A., Backeljau, T., Vanfleteren, J., Vincx, M., and Moens,
625 T.: Mitochondrial DNA variation and cryptic speciation within the free-living marine
626 nematode Pellioditis marina, Mar. Ecol.-Prog. Ser., 300, 91–103, 2005.

627 Dorris, M., De Ley, P., and Blaxter, M.: Molecular analysis of nematode diversity and the
628 evolution of parasitism, Parasitol Today, 15, 188–193, 1999.

629 Drotz, M. K., Saura, A., and Nilsson, A. N.: The species delimitation problem applied to the
630 Agabus bipustulatus complex (Coleoptera, Dytiscidae) in north Scandinavia, Biol. J. Linn.
631 Soc., 73, 11–22, 2001.

632 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. and Knight, R.: UCHIME improves sensitivity
633 and speed of chimera detection, Bioinformatics, 27(16), 2194–2200, 2011.

634 Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P. and
635 Bettman, B.: Real-time DNA sequencing from single polymerase molecules, Science (80-.
636)., 323(5910), 133–138, 2009.

637 Eisenback, J. and Hirschmann, H.: Identification of Meloidogyne Species on the Basis of Head
638 Shape and, Stylet Morphology of the Male, J. Nematol., 13, 513–521, 1981.

639 Eisenback, J. D. and Hunt, D. J.: General Morphology, in: Root-knot Nematodes, edited by:
640 Perry R. N., Moens, M., and Starr J. L., CABI Wallingford, UK, 18–54, 2009.

641 Ekblom, R. and Galindo, J.: Applications of next generation sequencing in molecular ecology
642 of non-model organisms, Heredity, 107, 1–15, 2011.

643 Esbenshade, P. and Triantaphyllou, A.: Isozyme phenotypes for the identification of
644 Meloidogyne species, J. Nematol., 22, 10–15, 1990.

645 Esbenshade, P. and Triantaphyllou, A.: Use of enzyme phenotypes for identification of
646 Meloidogyne species, J. Nematol., 17, 6–20, 1985.

647 Eves-Van Den Akker, S., Lilley, C. J., Reid, A., Pickup, J., Anderson, E., Cock, P. J. A., Blaxter, M.,
648 Urwin, P. E., Jones, J. T. and Blok, V. C.: A metagenetic approach to determine the diversity
649 and distribution of cyst nematodes at the level of the country, the field and the individual,
650 Mol. Ecol., 24(23), 5842–5851, doi:10.1111/mec.13434, 2015.

651 Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., Guerrero, R., Ferté, H., Bandi, C., Martin,
652 C., and Casiraghi, M.: Integrated taxonomy: traditional approach and DNA barcoding for
653 the identification of filarioid worms and related parasites (Nematoda), Front Zool., 6,
654 doi:10.1186/1742-9994-6-1, 2009.

655 Ferris, V., Ferris, J., Faghihi, J., and Ireholm, A.: Comparisons of isolates of Heterodera avenae
656 using 2-D PAGE protein patterns and ribosomal DNA, J. Nematol., 26, 144–151, 1994.

657 Floyd, R., Abebe, E., Papert, A., and Blaxter, M.: Molecular barcodes for soil nematode
658 identification, Mol. Ecol., 11, 839–850, 2002

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

661 Fonseca, V. G., Carvalho, G. R., Sung, W., Johnson, H. F., Power, D. M., Neill, S. P., Packer, M.,
662 Blaxter, M. L., Lambshead, P. J. D. and Thomas, W. K.: Second-generation environmental
663 sequencing unmasks marine metazoan biodiversity, Nat. Commun., 1, 98, 2010.

664 Fonseca, V. G., Nichols, B., Lallias, D., Quince, C., Carvalho, G. R., Power, D. M. and Creer, S.:
665 Sample richness and genetic diversity as drivers of chimera formation in nSSU metagenetic
666 analyses, Nucleic Acids Res., 40(9), e66–e66, 2012.

667 Fourie, H., Zijlstra, C. and McDonald, A.: Identification of root-knot nematode species
668 occurring in South Africa using the SCAR-PCR technique, Nematology, 3(7), 675–680,
669 doi:http://dx.doi.org/10.1163/156854101753536046, 2001.

670 Glenn, T. C.: Field guide to next generation DNA sequencers, Mol. Ecol. Resour., 11, 759–769,
671 2011.

672 Groombridge, B.: Global biodiversity: status of the Earth's living resources, Chapman & Hall,
673 London, UK, 1992.

674 Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., Ciulla, D.,
675 Tabbaa, D., Highlander, S. K., and Sodergren, E.: Chimeric 16S rRNA sequence formation
676 and detection in Sanger and 454-pyrosequenced PCR amplicons, Genome Res., 21, 494–
677 504, 2011.

678 Hague, N. and Gowen, S.: Chemical control of nematodes, Principles and practice of
679 nematode control in crops, 131–178, 1987.

680 Handoo, Z., Nyczepir, A., Esmenjaud, D., Van der Beek, J., Castagnone-Sereno, P., Carta, L.,
681 Skantar, A., and Higgins, J.: Morphological, molecular, and differential-host
682 characterization of Meloidogyne floridensis n. sp. (Nematoda: Meloidogynidae), a root-
683 knot nematode parasitizing peach in Florida, J. Nematol., 36, 20–35, 2004

684 Harris, T., Sandall, L., and Powers, T. O.: Identification of single Meloidogyne juveniles by
685 polymerase chain reaction amplification of mitochondrial DNA, J. Nematol., 22, 518–524,
686 1990

687 Hebert, P. D. and Gregory, T. R.: The promise of DNA barcoding for taxonomy, Syst. Biol., 54,
688 852–859, 2005.

689 Hebert, P. D., Cywinska, A., and Ball, S. L.: Biological identifications through DNA barcodes, P.
690 Roy. Soc. Lond. B Bio., 270, 313–321, 2003.

691 Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der
692 Bank, M., Chase, M. W., Cowan, R. S., Erickson, D. L., and Fazekas, A. J.: A DNA barcode for
693 land plants, Proc. Natl. Aca. Sci., 106, 12794–12797, 2009.

694 Holterman, M. H., Oggenfuss, M., Frey, J. E., and Kiewnick, S.: Evaluation of High resolution
695 Melting Curve Analysis as a New Tool for Root-knot Nematode Diagnostics, J. Phytopathol.,

160, 59–66, 2012.

Holterman, M., Karssen, G., Van Den Elsen, S., Van Megen, H., Bakker, J., and Helder, J.: Small subunit rDNA-based phylogeny of the Tylenchida sheds light on relationships among some high impact plant-parasitic nematodes and the evolution of plant feeding, Phytopathology, 99, 227–235, 2009.

Holterman, M., van der Wur_ , A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J., and Helder, J.: Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades, Mol. Biol. Evol., 23, 1792–1800, 2006.

Höss, S., Claus, E., Von der Ohe, P. C., Brinke, M., Güde, H., Heininger, P., and Traunspurger, Sochová, I., Hofman, J., and Holoubek, I.: Using nematodes in soil ecotoxicology, Environ. Int., 32, 374–383, 2006

Hu, M., Jex, A. R., Campbell, B. E., and Gasser, R. B.: Long PCR amplification of the entire mitochondrial genome from individual helminths for direct sequencing, Nat. Protoc., 2, 2339– 2344, 2007.

Huber, T., Faulkner, G., and Hugenholtz, P.: Bellerophon: a program to detect chimeric sequences in multiple sequence alignments, Bioinformatics, 20, 2317–2319, 2004.

Hyman, E. D.: A new method of sequencing DNA, Anal. Biochem., 174, 423–436, 1988.

Ibrahim, S., Davies, K., and Perry, R.: Identification of the root-knot nematode, Meloidogyne incognita, using monoclonal antibodies raised to non-specific esterases, Physiol. Mol. Plant. P., 49, 79–88, 1996.

Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. and Bert, W.: Mitochondrial coding genome analysis of tropical root-knot nematodes (Meloidogyne) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution, Sci. Rep., 6, 2016.

Jex, A. R., Hu, M., Littlewood, D. T. J., Waeschenbach, A., and Gasser, R. B.: Using 454 technology for long-PCR based sequencing of the complete mitochondrial genome from single Haemonchus contortus (Nematoda), BMC genomics, 9, 2008a.

Jex, A. R., Littlewood, D. T. J., and Gasser, R. B.: Toward next-generation sequencing of mitochondrial genomes – focus on parasitic worms of animals and biotechnological implications, Biotechnol. Adv., 28, 151–159, 2010.

Jex, A. R., Waeschenbach, A., Littlewood, D. T. J., Hu, M., and Gasser, R. B.: The Mitochondrial Genome of Toxocara canis, Plos Neglect. Trop. D., 2, e273, doi:10.1371/journal.pntd.0000273, 2008b.

Jones, P., Ambler, D., and Robinson, M.: The application of monoclonal antibodies to the diagnosis of plant pathogens and pests, Proc. Brighton Crop., 1988, 767–776, 1988.

Karssen, G., Van Hoenselaar, T., Verkerk-Bakker, B., and Janssen, R.: Species identification of cyst and root-knot nematodes from potato by electrophoresis of individual females,

733 Electrophoresis, 16, 105–109, 1995.

734 Kerry, B. R.: Rhizosphere interactions and the exploitation of microbial agents for the
735 biological control of plant-parasitic nematodes, Annu. Rev. Phytopathol., 38, 423–441,
736 2000.

737 Kiewnick, S., Holterman, M., van den Elsen, S., van Megen, H., Frey, J. E., and Helder, J.:
738 Comparison of two short DNA barcoding loci (COI and COII) and two longer ribosomal DNA
739 genes (SSU & LSU rRNA) for specimen identification among quarantine root-knot
740 nematodes (Meloidogyne spp.) and their close relatives, Eur. J. Plant. Pathol., 140, 97–110,
741 2014.

742 Lahaye, R., Van der Bank, M., Bogarin, D.,Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit,
743 S., Barraclough, T. G., and Savolainen, V.: DNA barcoding the floras of biodiversity hotspots,
744 Proc. Natl. Aca. Sci., 105, 2923–2928, 2008.

745 Lallias, D., Hiddink, J. G., Fonseca, V. G., Gaspar, J. M., Sung, W., Neill, S. P., Barnes, N., Ferrero,
746 T., Hall, N. and Lambshead, P. J. D.: Environmental metabarcoding reveals heterogeneous
747 drivers of microbial eukaryote diversity in contrasting estuarine ecosystems, ISME J., 9(5),
748 1208–1221, 2015.

749 Lambshead, P.: Marine nematode diversity, in: Advances and Perspectives: Nematode
750 Morphology, Physiology and Ecology, edited by: Chen, W. Y., Chen, S. Y., and Dickson, S.
751 W., CABI Publishing, Wallingford, UK, 438–468, 2004.

752 Marché, L., Valette, S., Grenier, E., and Mugniéry, D.: Intra-species DNA polymorphism in the
753 tobacco cyst nematode complex (Globodera tabacum) using AFLP, Genome, 44, 941–946,
754 2001.

755 Marcussen, T.: Evolution, phylogeography, and taxonomy within the Viola alba complex
756 (Violaceae), Plant. Syst. Evo., 237, 51–74, 2003

757 Mardis, E. R.: Next-generation DNA sequencing methods, Annu. Rev. Genomics Hum. Genet.,
758 9, 387–402, 2008.

759 Markmann, M. and Tautz, D.: Reverse taxonomy: an approach towards determining the
760 diversity of meiobenthic organisms based on ribosomal RNA signature sequences, Philos.
761 T. R. Soc. B., 360, 1917–1924, 2005.

762 Mayr, E. and Ashlock, P. D.: The Science of Taxonomy, in Principles of Systematic Zoology, pp.
763 1–14., 1991.

764 McCarter, J. P.: Cell Biology of Plant Nematode Parasitism, edited by R. H. Berg and C. G.
765 Taylor, pp. 239 267, Springer Berlin Heidelberg, Berlin, Heidelberg., 2009.

766 Metzker, M. L.: Emerging technologies in DNA sequencing, Genome Res., 15, 1767–1776,
767 2005.

768 Nakamura, K., Oshima, T., Morimoto, T., Ikeda, S., Yoshikawa, H., Shiwa, Y., Ishikawa, S., Linak,
769 M. C., Hirai, A., and Takahashi, H.: Sequence-specific error profile of Illumina sequencers,

770 Nucleic Acids Res., 39, e90, doi:10.1093/nar/gkr344, 2011.

771 Neher, D. A.: Role of nematodes in soil health and their use as indicators, J. Nematol, 33, 161–
772 168, 2001

773 Ntalli, N. G. and Menkissoglu-Spiroudi, U.: Pesticides of botanical origin: a promising tool in
774 plant protection, in: Pesticides – Formulations, Effects, Fate, edited by: Stoytcheva, M., In-
775 Tech Europe, 3–24, 2011.

776 Orgiazzi, A., Dunbar, M. B., Panagos, P., de Groot, G. A., and Lemanceau, P.: Soil biodiversity
777 and DNA barcodes: opportunities and challenges, Soil. Biol. Biochem., 80, 244–250, 2015.

778 Payan, L., and Dickson, D.: Comparison of populations of Pratylenchus brachyurus based on
779 isozyme phenotypes, J. Nematol., 22, 538–545, 1990.

780 Perera, M. R., Taylor, S. P., Vanstone, V. A., and Jones, M. G.: Protein biomarkers to distinguish
781 oat and lucerne races of the stem nematode, Ditylenchus dipsaci, with quarantine
782 significance for Western Australia, Nematology, 11, 555–563, 2009.

783 Petersen, D. and Vrain, T.: Rapid identification of Meloidogyne chitwoodi, M. hapla, and M.
784 fallax using PCR primers to amplify their ribosomal intergenic spacer, Fund. Appl. Nematol.,
785 19, 601–605, 1996.

786 Porazinska, D. L., GIBLIN-DAVIS, R. M., Faller, L., Farmerie, W., Kanzaki, N., Morris, K., Powers,
787 T. O., Tucker, A. E., Sung, W., and Thomas, W. K.: Evaluating high-throughput sequencing
788 as a method for metagenomic analysis of nematode diversity, Mol. Ecol. Resour., 9, 1439–
789 1450, 2009.

790 Porazinska, D. L., Giblin-Davis, R. M., Sung, W., and Thomas, W. K.: Linking operational
791 clustered taxonomic units (OCTUs) from parallel ultra sequencing (PUS) to nematode
792 species, Zootaxa, 2427, 55–63, 2010.

793 Porazinska, D. L., Giblin-Davis, R. M., Sung, W., and Thomas, W. K.: The nature and frequency
794 of chimeras in eukaryotic metagenetic samples, J. Nematol., 44, 18–25, 2012.

795 Powers, T. O. and Fleming, C. C.: Biochemical and molecular characterization, in: The
796 physiology and biochemistry of free-living and plant-parasitic nematodes, edited by: Perry,
797 R. and Wright, D., CABI Publishing, Wallingford, UK, 355–380, 1998

798 Powers, T. O., Mullin, P., Harris, T., Sutton, L., and Higgins, R.: Incorporating molecular
799 identification of Meloidogyne spp. into a large-scale regional nematode survey, J.
800 Nematol., 37, 226–235, 2005.

801 Powers, T.: Nematode molecular diagnostics: from bands to barcodes, Annu. Rev.
802 Phytopathol., 42, 367–383, 2004.

803 Quail, M. A., Smith, M., Coupland, P., Otto, T. D., Harris, S. R., Connor, T. R., Bertoni, A.,
804 Swerdlow, H. P. and Gu, Y.: A tale of three next generation sequencing platforms:
805 comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers, BMC
806 Genomics, 13(1), 1, 2012.

807 Ristau, K., Steinfartz, S., and Traunspurger, W.: First evidence of cryptic species diversity and
808 significant population structure in a widespread freshwater nematode morphospecies
809 (Tobrilus gracilis) , Mol. Ecol., 22, 4562–4575, 2013

810 Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., Leamon, J. H.,
811 Johnson, K., Milgrew, M. J., and Edwards, M.: An integrated semiconductor device enabling
812 non-optical genome sequencing, Nature, 475, 348–352, 2011.

813 Sapkota, R. and Nicolaisen, M.: High-throughput sequencing of nematode communities from
814 total soil DNA extractions, BMC Ecol., 15(1), 1–8, doi:10.1186/s12898-014-0034-4, 2015.

815 Sasser, J. and Carter, C. C.: Root-knot nematodes (Meloidogyne spp.): Identification,
816 morphological and physiological variation, host range, ecology, and control, Nematology
817 in the southern region of the United States, Southern Cooperative Series Bulletin, 276, 21–
818 32, 1982.

819 Sasser, J. N.: Identification and host-parasite relationships of certain root-knot nematodes
820 (Meloidogyne spp.), Technical Bulletin, Maryland Agricultural Experiment Station, A-77, 31
821 pp., 1954.

822 Schots, A., Gommers, F. J., Bakker, J., and Egberts, E.: Serological di_ erentiation of
823 plantparasitic nematode species with polyclonal and monoclonal antibodies, J. Nematol.,
824 22, 16– 23, 1990.

825 Schroder, J., Bailey, J., Conway, T., and Zobel, J.: Reference-free validation of short read data,
826 PLoS One, 5, e12681, doi:10.1371/journal.pone.0012681, 2010.

827 Semblat, J., Wajnberg, E., Dalmasso, A., Abad, P., and Castagnone-Sereno, P.: High-resolution
828 DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis, Mol.
829 Ecol., 7, 119–125, 1998.

830 Shaw, A. J. and Allen, B.: Phylogenetic relationships, morphological incongruence, and
831 geographic speciation in the Fontinalaceae (Bryophyta), Mol. Phylogenet. Evol., 16, 225–
832 237, 2000.

833 Subbotin, S. and Moens, M.: Molecular diagnostics of plant-parasitic nematodes, in: Plant
834 Nematology, edited by: Perry, R. and Moens, M., CABI Wallingford, UK, 33–58, 2007.

835 Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., and Willerslev, E.: Towards next
836 generation biodiversity assessment using DNA metabarcoding, Mol. Ecol., 21, 2045–2050,
837 2012.

838 Tastet, C., Val, F., Lesage, M., Renault, L., Marché, L., Bossis, M., and Mugniéry, D.: Application
839 of a putative fatty-acid binding protein to discriminate serologically the two European
840 quarantine root-knot nematodes, Meloidogyne chitwoodi and M. fallax, from other
841 Meloidogyne species, Eur. J. Plant Pathol., 107, 821–832, 2001

842 Tietjen, J. H.: Ecology of deep-sea nematodes from the Puerto Rico Trench area and Hatteras
843 Abyssal Plain, Deep Sea Res. Pt. A, 36, 1579–1594, 1989.

844 Tripathi, A. M., Tyagi, A., Kumar, A., Singh, A., Singh, S., Chaudhary, L. B., and Roy,S.: The
845 internal transcribed spacer (ITS) region and trnhH-psbA are suitable candidateloci for DNA
846 barcoding of tropical tree species of India, PloS one, 8,
847 e57934,doi:10.1371/journal.pone.0057934, 2013.

848 Valentini, A., Pompanon, F., and Taberlet, P.: DNA barcoding for ecologists, Trends Ecol. Evol.,
849 24, 110–117, 2009.

850 van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T.,
851 Holovachov, O., Bakker, J., and Helder, J.: A phylogenetic tree of nematodes based on
852 about 1200 full-length small subunit ribosomal DNA sequences, Nematology, 11, 927–950,
853 2009.

854 van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T.,
855 Holovachov, O., Bakker, J., and Helder, J.: A phylogenetic tree of nematodes based on
856 about 1200 full-length small subunit ribosomal DNA sequences, Nematology, 11, 927–950,
857 2009.

858 Vovlas, N., Troccoli, A., Palomares-Rius, J. E., De Luca, F., Liébanas, G., Landa, B. B., Subbotin,
859 S. A., and Castillo, P.: Ditylenchus gigas n. sp. parasitizing broad bean: a new stem
860 nematode singled out from the Ditylenchus dipsaci species complex using a polyphasic
861 approach with molecular phylogeny, Plant Pathol., 60, 762–775, 2011

862 W.: Nematode species at risk – a metric to assess pollution in soft sediments of freshwaters,
863 Environ. Int., 37, 940–949, 2011

864 Waite, I. S., O'Donnell, A. G., Harrison, A., Davies, J. T., Colvan, S. R., Ekschmitt, K., Dogan, H.,
865 Wolters, V., Bongers, T., and Bongers, M.: Design and evaluation of nematode 18S rDNA
866 primers for PCR and denaturing gradient gel electrophoresis (DGGE) of soil community
867 DNA, Soil Biol. Biochem., 35, 1165–1173, 2003.

868 Wardle, D., Yeates, G., Barker, G. and Bonner, K.: The influence of plant litter diversity on
869 decomposer abundance and diversity, Soil Biol. Biochem., 38(5), 1052–1062,
870 doi:10.1016/j.soilbio.2005.09.003, 2006.

871 Williams, S.: Species boundaries in the starfish genus Linckia, Mar. Biol., 136, 137–148, 2000.

872 Wilson, E. O.: A global biodiversity map, Science, 289, 2279–2279, 2000.

873 Wilson, M. J. and Khakouli-Duarte, T.: Nematodes as environmental indicators, CABI
874 Publishing, Wallingford, UK, 326 pp., 2009.

875 Yeates, G. W.: Nematodes as soil indicators: functional and biodiversity aspects, Biol. Fert.
876 Soils, 37, 199–210, 2003.

877 Yeates, G., Bongers, T., De Goede, R., Freckman, D., and Georgieva, S.: Feeding habits in soil
878 nematode families and genera – an outline for soil ecologists, J. Nematol., 25, 315–331,
879 1993.

880 Yeates, G.: Variation in soil nematode diversity under pasture with soil and year, Soil Biol.

881 Biochem., 16, 95–102, 1984.

882 Zhou, X., Li, Y., Liu, S., Yang, Q., Su, X., Zhou, L., Tang, M., Fu, R., Li, J., and Huang, Q.: Ultradeep
883 sequencing enables high-fidelity recovery of biodiversity for bulk arthropod samples
884 without PCR amplification, GigaScience, 2, 1–12, 2013.

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

888 Zijlstra, C.: Identification of Meloidogyne chitwoodi, M. fallax and M. hapla based on SCAR
889 PCR: a powerful way of enabling reliable identification of populations or individuals that
890 share common traits, Eur. J. Plant. Pathol., 106, 283–290, 2000.

891

892 Table 1 Summary of some of the protein-based techniques for distinguishing between
893 species/population of nematodes, their advantages, disadvantages and applications.

Approach	Principle	Advantages	Disadvantages	Applications
Isozyme analysis	Patterns of gel-separated isoenzyme bands used to identify species	1. Robust and easy to carry out. 2. To date, offers an excellent means of identifying tropical root-knot nematode species. 3. Extracts from a single sedentary female sufficient for reliable identification	1. Dependent on a particular life-stage of the nematode (young female). 2. Being protein-based subjects this method to influence of environmental conditions (e.g. type of host)	Widely used to separate species of cyst and root-knot nematodes (Esbenshade and Triantaphyllou, 1990: Karssen et al., 1995)
Two-dimensional polyacrylamide gel electrophoresis	Soluble proteins separated on the basis of their charges and masses on a gel	This method allows the separation of proteins with an even better resolution.	1. Subject to environmental variations.	Used to compare *Heterodera avenae* isolates (Ferris et al., 1994)
Antibody-based serological techniques	Antibodies are raised against species of nematodes and used to detect them	1. Can provide good specificity and sensitivity. 2. Can reliably distinguish between the two species of potato cyst nematodes.	Occasional cross-reactivity can affect specificity.	Monoclonal antibody used to test major *Meloidogyne* species (Ibrahim et al., 1996).

894

895

896

897 Table 2 Summary of some of the DNA-based techniques for distinguishing between species/population of
898 nematodes, their advantages, disadvantages and applications.

899

Markers	Principle	Advantages	Disadvantages	Applications
Restriction fragment length polymorphism (RFLP/PCR-RFLP)	Sequence polymorphism between species results in distinct cleaving sites for restriction enzymes, thus resulting in variable number of fragments with diverse sizes	1. The technique is fairly reproducible 2. Simple and inexpensive	Requires prior knowledge of the sequence of DNA region for design of primers or probes.	Using this technique, Carpenter et al. (1992) distinguished between three populations of a *Meloidogyne arenaria* race called race 2
Random amplification of polymorphic DNA (RAPD)	A short primer set is used which anneal to several sites on the DNA. If two of the annealed short primer happen to be close and opposite to each other, they will produce an amplicon. Difference in the gel fingerprints of amplicons separates species or populations.	1. Sequence information of DNA region not a prerequisite. 2. Simple and inexpensive	Technique may lack reproducibility.	Used to distinguish between species and populations of *Meloidogyne* from different origins. Castagnone-sereno et al. (1994)
Amplified fragment length polymorphism (AFLP)	This involves a series of PCR steps in which separate sets of primers are used to selectively amplify some subsets of products of each preceding PCR step. All selected fragments are run on a gel to product unique fingerprints.	1. Requires no prior knowledge of the sequence of the DNA region. 2. Highly reproducible.	1. Complex technique to carry out. 2. Expensive	Used to typify the genetic variability within the tobacco cyst nematode (TCN) complex Marche et al. (2001)
Sequence Characterised Amplified Region (SCAR)	A specific distinguishing marker from the fingerprint of a specific taxon or life stage of a species is isolated and amplified. This becomes a SCAR by which that taxon or life stage is identified.	1. Provides a rapid means of screening individuals. 2. Can be highly specific	May be labour-intensive.	Successfully used for identifying species of root-knot nematodes (Zijlstra et al., 2000; Fourie et al., 2001)

900