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Technological advancements and their importance for nematode identification

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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 this, 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 We also provide brief descriptions of some the current and almost-outdated high-throughput sequencing platforms and their applications in both plant nematology and soil ecology.

1 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 et al., 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 metre 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). 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, and 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 and Chan, 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. Furthermore, as permanent community members (being unable to escape habitat disturbance), they serve as important biological indicators of sediment quality (Bongers and Ferris, 1999; Sochová et al., 2006; Wilson and Kakouli-Duarte, 2009; Höss et al., 2006).

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. Family- or genus-level identification is often sufficiently informative 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 USD 118 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 to 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 (Bergé 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., 2015). 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.

2 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 recognising 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 Jr. and Marshall (2003), in their review of 12 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 et al., 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 characterising 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 optimise the process of collecting and analysing molecular data to make this tool exclusively powerful for species delineation.

3 Biochemical methods for nematode identification

Several biochemical and molecular approaches have been used for identification of nematodes. Genomic information at all levels has been utilised for identifying nematodes, from DNA sequence, the structure of molecules, and 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 sulfate 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/ionisation time-of-flight mass spectrometry (MALDI-TOFMS) (Perera et al., 2009) are the methods that have been utilised 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; Marché et al., 2001), restriction fragment length polymorphism (RFLP) (Curran et al., 1986; Carpenter et al., 1992) and sequence characterised

Table 1. Summary of some of the protein-based techniques for distinguishing between 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).

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).

4 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, and applicable to all nematode life stages; provide highly specific means of identifying taxa (Powers, 2004); and, most of all, provide a substantial number 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 spacers 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 inter-

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

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 primers 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 Marché 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)

nal 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 standardised DNA regions as markers for rapid and accurate species identification (Hebert and Gregory, 2005; Blaxter et al., 2005). The key distinguishing feature between DNA barcoding and other molecular diagnostic methods is the use of standardised markers in the former. Therefore, one of the aims of the barcoding consortium has been to build taxonomic reference libraries with sequences of standardised 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. 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. Nonetheless, 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 multiple 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, and plant material (Porazinska et al., 2009, 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 and Nicolaisen (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.

5 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 and Nicolaisen, 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 is 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 number of errors have been shown to accrue during amplification (Haas et al., 2011; Porazinska et al., 2012). These errors often lead to misinterpretation of diversity within samples, mainly due to the formation of chimeras (Huber et al., 2004; Edgar et al., 2011). 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 characterised 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 characterised 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).

6 Next-generation sequencing technology

In spite of the immense improvements made to the capillary electrophoresis sequencing method, the cost of sequencing, time and labour needed were still too high for the growing demands for DNA sequence information (Metzker, 2005) – this remained the case until the introduction of the various 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 on 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 artefacts 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 to 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 Illumina (Solexa) technology as the second NGS platform to be available commercially. Illumina technology 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, can currently produce read lengths of up to 2×300 bp (http://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 increased 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 to 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^+ ions 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 it recorded a significant number 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 (http://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 se-

quencers, 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 et al., 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 11 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 utilised 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 characterising up to a thousand fields at the same time.

High-throughput 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., 2007), which 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 greatly increase our understanding of relationships between nematodes.

7 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 requires 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 a 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.

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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 SSrRNA 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, 403–410, 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, 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 Bioinform., 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., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., and Abebe, E.: Defining operational taxonomic units using DNA barcode data, Philos. T. Roy. Soc. B, 360, 1935–1943, 2005.

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.

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

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

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

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

Bongers, T. and Bongers, M.: Functional diversity of nematodes, Appl. Soil Ecol., 10, 239–251, 1998.

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

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

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

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

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

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

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

Cenis, J.: Identification of Four Major Meloidogyne ssp. by Random Amplified Polymorphic DNA (RAPD-PCR), Phytopathology, 83, 76, 1993.

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

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

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

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

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

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

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

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., and Taberlet, P.: DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match, Biol. Lett., 10, doi:10.1098/rsbl.20140562, 2014

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

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

De Ley, P.: A quick tour of nematode diversity and the backbone of nematode phylogeny, in: WormBook, edited by: The Caenorhabditis elegans Research Community, doi:10.1895/wormbook.1.7.1, 2006.

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

den Nijs, L. and van den Berg, W.: The added value of proficiency tests: choosing the proper method for extracting Meloidogyne second-stage juveniles from soil, Nematology, 15, 143– 151, 2013.

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

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

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

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

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

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

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

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

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

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

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

Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., Guerrero, R., Ferté, H., Bandi, C., Martin, C., and Casiraghi, M.: Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda), Front. Zool., 6, 1–12, doi:10.1186/1742-9994-6-1, 2009.

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

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

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

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

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

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

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

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

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

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

Handoo, Z., Nyczepir, A., Esmenjaud, D., Van der Beek, J., Castagnone-Sereno, P., Carta, L., Skantar, A., and Higgins, J.: Morphological, molecular, and differential-host characterization of Meloidogyne floridensis n. sp. (Nematoda: Meloidogynidae),

a root-knot nematode parasitizing peach in Florida, J. Nematol., 36, 20–35, 2004.

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

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

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

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

Holterman, M. H., van der Wurff, 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.

Holterman, M. H., 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. H., Oggenfuss, M., Frey, J. E., and Kiewnick, S.: Evaluation of High resolution Melting Curve Analysis as a New Tool for Root-knot Nematode Diagnostics, J. Phytopathol., 160, 59–66, 2012.

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, B. C.: Molecular diagnosis of Meloidogyne species, J. Nematol., 22, 24, 1990.

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, 22591, doi:10.1038/srep22591, 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, 1, doi:10.1186/1471-2164-9-11, 2008a.

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.

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.

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, Electrophoresis, 16, 105–109, 1995.

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

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

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

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

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

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

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

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

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

Mayr, E. and Ashlock, P. D.: The Science of Taxonomy, in: Principles of Systematic Zoology, McGraw-Hill, New York, 1–14, 1991.

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

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

Nakamura, K., Oshima, T., Morimoto, T., Ikeda, S., Yoshikawa, H., Shiwa, Y., Ishikawa, S., Linak, M. C., Hirai, A., and Takahashi, H.: Sequence-specific error profile of Illumina sequencers, Nucleic Acids Res., 39, e90, doi:10.1093/nar/gkr344, 2011.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sasser, J. N. and Carter, C. C.: Root-knot nematodes (Meloidogyne spp.): Identification, morphological and physiological variation, host range, ecology, and control, Nematology in the southern region of the United States, South. Cooperat. Ser. Bull., 276, 21–32, 1982.

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

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

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

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

Sites Jr., J. W. and Marshall, J. C.: Operational criteria for delimiting species, Annu. Rev. Ecol. Evol. Syst., 35, 199–227, 2004.

Sochová, I., Hofman, J. and Holoubek, I.: Using nematodes in soil ecotoxicology, Environ. Int., 32, 374–383, 2006.

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

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

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

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

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

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

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

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

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

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

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

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

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

Yeates, G. W.: Variation in soil nematode diversity under pasture with soil and year, Soil Biol. Biochem., 16, 95–102, 1984.

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

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

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

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

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