Authors' Response

Anonymous Reviewer 1,

We are very grateful for your comments on our manuscript. Please find below my personal responses to your comments.

General comments: Overall this paper provides a decent review of the broad history of nematode taxonomy and the methods available for nematode classification. This paper is fitting for the SOIL journal and (with modifications) would provide a valuable contribution to the journal. I loved the idea of making nematode classification more accessible to a broader research audience. However, as it is written, the information is useful for a specific group of people, and needs clarification to apply to a broader audience. Currently, the paper reads as a list of facts/methods and barely hints at an overall aim and message. My specific comments follow.

Specific comments: -A statement of purpose/aims at the end of the introduction would be helpful - as it ends currently, it states that a number of reviews have been published, which left me wondering why should I be interested to read this one? -With a clear statement of purpose, the article can be reworked to support this -much time is spent discussing the particulars of some methods, but not others, perhaps a table of methods and pros/cons or uses would be helpful to clarify for the reader the different method options

Response: The concluding section of the introduction has been rewritten to have a purposeful statement. Here is the final part of the introduction:

'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 the possibility of increased use of nematode communities for evaluation of management strategies and assessments of ecosystem health'.

Tables summarizing the various techniques have also been included.

Technical comments: Please double check the formatting of scientific names (families, species) and be consistent throughout the paper. -Consider shortening or dividing up paragraphs, many paragraphs quite lengthy and could be logically divided -Please check the use of commas throughout p.1181, line 25 - to properly deal with the issue of what? p.1183, line 13 - incorrect use of "too" p.1183, line 19 - phasmids are sensory, not secretory p. 1188, line 7-8 - did you do this study? is this hypothetical?

Response: Scientific names have all been changed to the correct formats. Lengthy paragraphs have been appropriately divided.

Anonymous reviewer 2,

The authors are very grateful for your helpful comments and suggestions on this manuscript. Please see our responses to your comments and suggestions below.

On the general comments about the main message being unclear, the authors propose to review the introduction to ensure that the aim of the work is clearer.

Reviewer's comment: Abstract: "Some groups of nematodes are also known to cause significant losses to crop production" – apparently the authors refer to plant-parasitic nematodes

Response: Indeed, the reference is to plant-parasitic nematodes. We propose rephrasing that sentence to: "Some plant-parasitic species are also known to cause significant losses to plant production"

Reviewer's comment: " . . . knowledge of their diversity is still limited due to the difficulty in achieving species identification using morphological characters" Virtually all (if not all) species known so far are defined on morphological and/or histological autapomorphies. Hence, we can't determine whether our "knowledge of their diversity is still limited" due the above mentioned difficulty. I can relate 'diversity' to "species definition" or equivalent, but not to species identification. In short: a hard-to-understand statement

Response: Yes, it is true that almost all known species are defined on the basis of morphological/phenotypic characters. Please refer to the final response on abstract. This has been corrected

Reviewer's comment: "… useful means of circumventing the numerous limitations associated with classical morphology based identification" No, it is circumventing anything – it is just (enormously) the number of informative characters. There is no fundamental difference between morphological or DNA sequence-based characters.

Response: What is inferred here, as you mentioned, is that DNA provides more informative characters which in the case of morphology can be limited. As a consequence, this means that for some taxa this limited number of informative characters may not be enough to reach species identification. Under such situations, therefore, the DNA approach is helping to overcome this limitation by offering informative characters to base species identifications/delineation on. This has been changed in the revised version.

Reviewer's comments: "high throughput sequencing is facilitating advanced ecological and molecular studies". Rather, HGT allows for a shift in terms of time (and – therefore – resources) from data collection to data analysis. It gives researchers the opportunity to analyze numbers of samples (and sample size) that are required for proper statistical analyses

(and not dictated by "what can maximally be handled by a limited number of people"). Whether an ecological study is 'advanced' depends on other things.

Response: True, NGS offers the opportunity to analyse enormous and multiple samples simultaneously. The revised abstract is significantly different from the original version

'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 their species using phenotypic features. Molecular methodology provides a 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 of the current and almost-outdated high throughput sequencing platforms and their applications in both plant nematology and soil ecology'.

Reviewer's comment: Introduction: "the criteria for allocating individuals into these groupings have often been questioned since even species within the same trophic group are known to sometimes vary in their source of food and response to disturbances" More fundamental point of criticism – the usefulness/validity of 'trophic groups' depends very much on the underlying research question. If this question is about carbon or nitrogen fluxes through a soil food web, this might be valid. For more detailed questions, it should be noticed that "trophic groups" are composed of phylogenetically fully unrelated taxa that only have one thing in common – they roughly (!) prefer the same kind of food.

Response: A sentence or two along the lines of the validity of trophic group classification has been included in the revised version.

'Classifications into such functional groups are often means of simply lumping together individuals considered to have similar influence 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 phylogenetic links. 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)'.

Reviewer's comment: "for species level identification is vital to accurate and precise computation of nematode indices as determiners of sediment quality" – At species level? For by far most free- living nematodes virtually no ecological information is available at species level. Hence, there is no reason to label this as being 'vital'.

Response: The reviewer is correct. Perhaps a more appropriate way to put this is "for identification to at least the genus level is important for more accurate and precise

computation of nematode indices".

Reviewer's comment: "as well as the existence of intraspecific variations and cryptic species (valid species species that morphologically indistinguishable)" – for the purpose indicated here ("computation of nematode indices as determiners of sediment quality" (what about soil?)), I would suggest not to put any effort in such subtleties (there are many, more basic hurdles to be overcome). Note "species species".
Response: Correction-"species"
By sediment, we meant any substrate inhabited by nematodes. Sediment is not the right terminology, since this only refers to aquatic habitats. Correction- "soil"

Reviewer's comment: "categorizing nematodes based on higher level classifications such as families and feeding guilds" – again, the taxonomic resolution required is variable will be defined by the underlying research question.
Response: This has been duly addressed in the modified manuscript

Reviewer's comments: "... recently made some very important modifications to its policy" "(Regulation 2009/1107/EC OL and Directive 2009/128/EC)" – Recently? This is 7 years ago.
Response: This has been corrected. We removed 'recently'.

Reviewer's comments: "These alternative approaches will undoubtedly rely" – why the two most important ones, crop rotation and host plant resistances, are not mentioned?
Response: We agree that they have to be mentioned since they are among the alternatives we were referring to here. They (both crop rotation and plant resistances) can only be effectively implemented if we have knowledge of the plant parasitic nematode (PPN) present in the field. In line with reviewer's comment, the text in the manuscript has been replaced with:

'Alternative non-chemical options have for sometime 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. Most plant resistance genes are effective only against a narrow range of parasitic species or populations. Therefore, knowing the targeted parasitic species or population makes easier the choice of which plant genotype introduce into the field'.

Reviewer's comments: "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" These techniques are used for very distinct (and non-comparable) reasons: host tests for pathotyping, SEM for the generation of additional morphological characters, and isozyme analysis for species identification (actually also life stage identification).
Response: It is true that these techniques have quite distinct applications. However, all three serve the same general purpose, which is to identify relevant differences between types/species and complement light microscopy.
"Each one of the above mentioned alternatives to light microscope-based approaches" We would then remove the underlined phrase above (in the sentence that follows) since it implies

that these approaches can substitute morphology-based identification which does not apply to all of them.

Reviewer's comments: molecular methods of plant parasitic nematode identification discussing in depth the different markers and DNA target regions used for discriminating species, their future prospects and limitations (Powers et al., 1997; Powers, 2004; Blok, 2004, 2005)". (. . . I am afraid with quite some overlap with the current manuscript.
Response: The intention was to make mention of some of the identification techniques used in the recent past and these were of course covered in more details in the cited papers above. This final revised version will have little overlap with the publications we cited above.

Reviewer's comment: The phylum Nematoda: "as Priapulida, the Kinorhyncha as well as their closest sister taxon the Nematomorpha". Non-relevant in this context, covered in more detail in various other recent papers.
Response: It is true that there is no reason to try covering the above-mentioned phyla. However, we only mentioned them only for the sake of comparison. The idea was to state how diverse they are compared to other close relatives. We agree that to most specialists, this may seem like an old story. Therefore, this section has been taken out.

Reviewer's comment: "This has however been disputed by De Ley (2000) and De Ley et al. (2005) who argued that this theory simply emanates from the failure of light microscopy to provide enough resolution, thus precluding" – skip, in 2016 this is a non-discussion / irrelevant.
Response: Skipped this as suggested by Reviewer.

Reviewer's comment: "which is a relatively small fraction of the predicted number of species of ca. 1 million (Hugot et al., 2001)" – speculation about number of extant nematode species should be discussed in full detail or left out. In the context of this MS, I tend to opt for the latter.
Response: We skipped this part.

Reviewer's comment: "To properly deal with the issue of, De Ley (2000) suggested that reassessment of priorities is the best way to progress. He cited a number of steps to achieve this: . . ." Skip, irrelevant for this MS

Response: Took this part out.

Reviewer's comment: Predicted species diversity leaves so much more to do / Classical taxonomy and the vast taxonomic deficit Skip whole sections: speculation of number of species is not useful. Complaining in the same section about the decline of the number of taxonomists is quite "preaching to your own choir"-like. This is not the forum to do this.
Response: Left this part out.

Reviewer's comment: Changes within the classification systems: Too much overlap with (for instance) Systematic Position and Phylogeny by Paul de Ley and Mark Blaxter (De Ley P, Blaxter ML. 2002. Systematic position and phylogeny. In: Lee DL, editor. The biology of

nematodes. London: Taylor & Francis. p 1–30).
Response: Removed.

Reviewer's comment 6 Biochemical methods for nematode identification Skip all the historical overview-like elements 6.1 Protein based approach. For systematics and identification this outdated (key reason: protein expression depends on life stage / environmental conditions etc. – hence, unstable as marker) 6.2 DNA based approach p. 1189, lines 9-10: "The two ITS regions have been used in the past both as phylogenetic and diagnostic markers Right, ITS regions are very problematic as diagnostic marker. Two quotes from recent articles: "ITS sequences were studied to develop species-specific primers used in simple PCR reactions, e.g. , for detection of H. glycines (Subbotin et al. , 2001) and H. schachtii (Amiri et al. , 2002). However, polymorphism between rDNA repeats within a species like H. latipons makes designing a species-specific primer very difficult (Rivoal et al. , 2003)" (from Toumi et al. in Nematology 15 (2013) 709-717) "Moreover, polymorphism between ribosomal DNA (rDNA) repeats can occur within one species, e.g. H. avenae (Bekal et al. 1997; Zhao et al. 2011) and H. filipjevi (Subbotin et al. 2000; Subbotin et al. 2003). This polymorphism makes the design of a species- specific primer based on ITS-sequences very difficult" (from Toumi et al. in Eur J Plant Pathol (2013) 136:613–624) - suggestion: skip the section on ITS based identification (p. 1189. Line 3 – p. 1190, line 2.
Response
On the section of protein-based methods we summarized the application aspects significantly and only wrote on some few of their limitations. ITS discussion has been removed.

Reviewer's comment: p. 1193 (lines 19-23). "It should, however, not be confused with metagenomics, a term often used to refer to the genomic analysis of organisms from environmental samples (Handelsman, 2004; Tringe et al., 2005; Hugenholtz and Tyson, 2008). Another form of environmental DNA analysis that is just as common as, and often albeit wrongly used as synonym of, metagenomics is metagenetics" None of the authors are authorities in this field – hence skip & refrain from making strong statements on this topic
Response
Removed from the manuscript.

Reviewer's comment: 7 Limitations of high throughput DNA barcoding. p. 1195, lines 5-6.
"It has however, been shown to have limited taxonomic resolution among certain taxa within the phylum Nematoda". Note there is no "one-for-all" – so far SSU rDNA is the only one with reasonable phylum-wide coverage
Response: Yes, we agree there is currently no "one-for-all" marker. Included a sentence stating this in the conclusion.

Reviewer's comment: p. 1195, lines 14-15. "Another issue with DNA metabarcoding is its reliance on PCR (Taberlet et al., 2012). Significant amount of errors has been shown to accrue during amplification". Worthwhile mentioning: most of the time it is just improper use (!).
Response: Yes, we agree it will be worth mentioning some of the factors that can lead to such artefacts forming such as incorrect annealing temperature and cycle number.

Reviewer's comment: 8 Next generation sequencing technology p. 1196, lines 16-25. Skip, do the scientific community a favor, and don't explain Sanger sequencing here (!) – Note that

454 sequencing is almost phased out. In short: skip the historical overviews, and focus on current and near future approaches.

Response: Skipped the Sanger sequencing.

Reviewed 454 technology only by its advantages disadvantages and applications in nematology.

Authors' general comment: Due to the major changes made to the current manuscript, we would like to propose a more appropriate title to replace the previous one "Technological advancements and their importance for nematode identification". We believe this is more befitting of the current state of the manuscript.

List of Relevant Changes

1. Abstract section has been mostly revised to clarify subjects the manuscript covers
2. A section has been included at the end of the introduction to statement the purpose of the review.
3. Sections covering the history of nematode classification have all been removed
4. The identification techniques covered in the original manuscript have now been summarized into a table with the short description, advantages, disadvantages and the applications of each.
5. A few recent publications in the area of metabarcoding in nematology have been included under the next generation sequencing section.
6. The paragraph on Sanger sequencing has been removed
7. Description of how 454 pyrosequencing platform works has been removed.

Marked-up Manuscript version. Changes have been underlined

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 their species using phenotypic features. Molecular methodology provides a 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 soil is stable or has undergone some recent perturbation, the soil nematode community is likely to differ from one place to another. The contribution of nematodes to nutrient cycling (Bardgett et al., 1999; 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 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 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, due mainly 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). The EU some years ago made some very important modifications to its policy on the use of pesticides to make it more sustainable and to reduce the risk this poses to human health and the environment. This has led to the re-evaluation (Regulation 2009/1107/EC OL and Directive 2009/128/EC) of various synthetic pesticides leaving only a few nematicides available for use by growers (Ntalli and Menkissoglu-Spiroudi, 2011). Alternative non-chemical options have for sometime now been sought to replace the loss of synthetic products (Kerry, 2000). 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 effective only against a narrow range of parasitic species or populations. Therefore, knowing the targeted parasitic species or population makes easier the choice of which plant genotype introduce into the field.

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 light microscopic approach for identification. Each of the above mentioned techniques have certain constraints that limit their use as quick, accurate and simple tool for nematode identification across the phylum. However, the use of molecular methods has continued to gain recognition for being fast, reliable and an easy diagnostic approach across many taxa within the phylum Nematoda (Floyd et al., 2002; De Ley et al., 2005).

It is important to mention that most of the pioneering works on molecular-based nematode

detection were developed on plant parasitic nematodes. As evidence of the importance of molecular data in taxonomy, it has become a common practice in recent times that most taxonomic descriptions comprise both morphology and morphometric studies as well as molecular analysis of the taxon's relatedness to other species (Handoo et al., 2004; Vovlas et al., 2011; Cantalapiedra-Navarrete et al., 2013). Over the past two decades there have been a number of published reviews on molecular methods of plant parasitic nematode identification discussing in depth the different markers and DNA target regions used for discriminating species, their future prospects and limitations (Powers, 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 utilize phenotypic characters and use this to effectively make a decision about the identity of an organism (Abebe et al., 2013). The continuous decline in the number of taxonomists has serious repercussions to our understanding of life's diversity. According to Coomans (2002) this waning number of specialists is also detrimental even to the quality of taxonomic researches that get published, since less qualified referees have to review such manuscripts.

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 singly delimit species, since all of the approaches can possibly fail at some point when used in isolation. This integrative approach has been successfully applied in some studies for examining species 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 them singly 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 have been utilized for identifying nematodes, from DNA sequence, the structure of molecules, genetic mutations to the presence versus absence of genes (Subbotin and Moens, 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 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 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

519 and 28S respectively.

520 Like all DNA based identification methods, DNA barcoding was designed for situations where
521 the morphology-based approach proved problematic. It is defined as the use of standardized
522 DNA regions as markers for rapid and accurate species identification (Hebert et al., 2005;
523 Blaxter, 2005). The key distinguishing feature between DNA barcoding and other molecular
524 diagnostic methods is the use of standardized markers in the former. Therefore, one of the
525 aims of the barcoding consortium is to build taxonomic reference libraries with sequences of
526 standardized markers from different organisms (Taberlet et al., 2012). Thus, by comparing
527 the sequences of such markers from unidentified organisms with these reference sequences,
528 their identities can be determined.

529 DNA barcoding has proven useful in our understanding of the degree of variation there is
530 between certain species and how these variations can obscure identification. For example,
531 the concept of cryptic species shows how morphology alone cannot be relied on for
532 discriminating phenotypically identical but valid species. Studies have shown that there are
533 several examples of cryptic species (e.g. *Tobrilus gracilis* (Ristau et al., 2013)) within the
534 phylum Nematoda that were previously considered to be the same species (Chilton et al.,
535 1995; Derycke et al., 2005; Fonseca et al., 2008). Barcoding also provides a means of
536 identifying rare species or specimens with limited availability.

537 DNA barcoding may also be the only option available for identifying an organism when the
538 required life stage or specific sex for morphological identification is lacking or the morphology
539 of the specimen being studied is badly distorted. And finally on the control of pest movement
540 within trade where speed and accuracy of species identification is critical, barcoding offers a
541 quick and reliable means of detecting quarantine nematode species (Powers, 2004).

542 Hebert et al. (2003), in their heavily cited study on biological identifications through DNA
543 barcoding, proposed the use of COI of the mitochondrial DNA as a molecular marker for DNA
544 barcoding. As a result, COI has been widely used as standard barcode marker for metazoans
545 (Ferri et al., 2009). Different markers have been proposed for other groups of cellular
546 organisms. Markmann and Tautz, (2005) used the nuclear rRNA gene to study the diversity of
547 meiobenthos (small meiofauna that live in marine and freshwater sediments). Applying the
548 environmental metabarcoding approach, Fonseca et al. (2010) used the nuclear SSU gene of
549 the rRNA to study marine metazoan biodiversity. In plants, on the other hand, the preferred
550 barcode markers are ones found within the chloroplast genome, and identification often
551 entails the use of combination of two or more regions of this genome (Lahaye et al., 2008;
552 Hollingsworth et al., 2009) or with other nuclear genes (Tripathi et al., 2013). The nuclear
553 small subunit ribosomal RNA gene has also been successfully used as marker for studies
554 involving nematodes (Floyd et al., 2002; Porazinska et al., 2010).

555 The rRNA genes (SSU and LSU) are preferred over the mitochondrial COI gene in most
556 nematological studies due to the availability of more conserved regions for universal primer
557 design. Moreover, the abundance of sequences of these two genes from described taxa in

558 public databases make matching sequences for identification an easier job than when using
559 COI. In terms of resolution, however, COI is capable of discriminating between species more
560 than either of the rRNA genes. But a combination of the SSU and LSU genes has been shown
561 to be able to significantly improve the resolution, thereby achieving better detection levels
562 (Porazinska et al., 2009). With current advancements in sequencing technology resulting in
563 increasingly wide usage of next generation sequencing, a form of barcoding which has
564 recently gained much popularity is DNA metabarcoding. Taberlet et al. (2012) defined
565 metabarcoding as the automated identification of several species from a single bulk sample
566 containing multiples of different taxa. Using this approach, it is possible to carry out high
567 throughput identification of several species in a parallel fashion. DNA metabarcoding
568 classically involves the analysis bulk DNA derived from environmental samples (Taberlet et
569 al., 2012).

570 A typical metabarcoding approach proceeds as follows (i) extracting bulk DNA from the
571 organisms or directly from the environment (ii) amplifying a selected DNA barcode marker
572 region using universal primers (iii) sequencing all the amplified regions in parallel via a next
573 generation sequencing platform (iv) clustering of sequences into molecular operational
574 taxonomic units (MOTU) and (v) matching each MOTU against sequences of identified
575 organisms in a reference database (Valentini et al., 2009). Metabarcoding like standard
576 barcoding is based on the assumption that with appropriate barcode marker(s), each
577 molecular operational taxonomic unit can be assigned to a described species through its DNA
578 sequence (Orgiazzi et al., 2015) or identified as unknown if not yet described to assist with
579 the discovery of unknown biodiversity.

580 Almost all DNA metabarcoding applications in nematology have mainly been based on the
581 analysis of bulk samples of entire organisms already isolated from the containing substrates
582 such as soil, water, plant material etc. (Porazinska et al., 2009; Porazinska et al., 2010; Creer
583 et al., 2010; Bik et al., 2012). Beyond multispecies identification from bulk samples of entire
584 extracted organisms, metabarcoding also may comprise the use of total and typically
585 degraded DNA extracted directly from environmental samples without prior isolation of
586 organisms (Taberlet et al., 2012). This approach, if successfully applied in nematology, can
587 help overcome the inconsistencies and poor recovery rates associated with various nematode
588 extraction methods (see, den Nijs and van den Berg, 2013). This method was applied for
589 community profiling of nematodes from European soils using the 18S rDNA (Waite et al.,
590 2003). Sapkota et al. (2015) also tested and developed new amplification approach to enable
591 high throughput analysing of soil samples by directly extracting the DNA without a nematode
592 extraction step. The authors reported very good coverage of the nematode diversity within
593 the tested soils. However, detailed assessment of the efficiency of DNA recovery from the soil
594 is generally lacking. Also, such a method will usually only allow for analysis of soil samples
595 much smaller in volume than would otherwise be used if there would be an extraction step.
596 Moreover, since most meiofaunal organisms are often found in substrates with volumes
597 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 only metabarcoding but is shared by the single species standard barcoding as well. As mentioned in earlier paragraphs, the SSU rRNA gene has been the most commonly used marker in nematode barcoding due to the availability of extensive database resources and the possibility of using conserved regions for designing versatile primers. The latter are continuously 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 SSU rRNA region is still the marker of choice for DNA metabarcoding of environmental samples where wider coverage remains essential and 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). 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 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 dataset. Considering how rampant they may be in sequence dataset, there is always the risk 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, thus, 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. Persues and UCHIME, operate on the assumption that chimeric sequences should be less frequent than the parental sequences (Edgar et al., 2011; Bik et al., 2012). In other words, the 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 fragment 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 many efforts to widen 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 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 single molecule

sequencer technology. Both the Polonator and the HeliScope are single molecule (shotgun) sequencing platforms; hence no amplification step is needed. These have the advantage of eliminating biodiversity inflation or artifacts often associated with PCR-based sequencing methods. The absence of PCR in their sequencing pipelines also means abundant information of taxa in samples, which are often obscured by amplification, can be revealed (Zhou et al., 2013). There have been several review articles that have covered in detail how each of these platforms operate including the chemistry and the instrumentations involved (Mardis, 2008; Metzker, 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 using this platform is the relatively long read lengths of the sequences, thus making assembly of contigs easier even in the absence of reference genomes. On the other hand, it has shallow sequencing coverage due to the few reads it generates per run (1 million sequences). It also has higher errors rates, especially when it encounters homopolymer repeats within the sequence (Ekblom and Galindo, 2011). These characteristics are some of the reasons why the technology has since been superseded by other approaches described below. 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 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 in 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 substantial amount of bias with coverage for only about 70% of the genome. On its ability to detect variants, it slightly outperformed the MiSeq, but in doing so recorded significant amount of false positives as well.

The SMRT sequencing technology by Pacific Biosciences is based on the natural process of DNA replication by DNA polymerase for real time sequencing of individual DNA molecules (Eid et al., 2009). Each dNTP has a specific fluorescence label attached to its terminal phosphate, which upon incorporation of a nucleotide gets detected immediately before it is cleaved off (www.pacificbiosciences.com/products/smrt-technology/). Features such as high speed, long read lengths, high fidelity and low cost per experiment have made this technology a desirable investment (Glenn, 2011; https://genohub.com/ngs-instrument-guide/). However, in comparison with the Ion Torrent and MiSeq sequencers, higher depth of coverage is required for calling of variants (Quail et al., 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 metagenetic analysis. Porazinska et al. (2009) carried out one of the early studies to evaluate the suitability of NGS for nematode metagenetic 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 only 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 low 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 metagenetics, Lallias et al. (2015) examined the variation in diversities of protists and microbial metazoans including nematodes across two distinct estuaries in 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 aspect 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, metagenetic 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 of 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 basically 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 exploited. 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 a combination of markers. Several markers have been tested on different nematode groups and these have exhibited varying degrees of performances. However, there still seem to be no known marker that can demonstrate all the key qualities required of an ideal marker- to contain a region of very low substitution rate for ease of amplification with a universal primer, to have regions of sufficient mutations to allow for inter-species delimitation while still maintaining sufficient within species similarity across the entire phylum. The choice of DNA region to target largely relies on the objectives of the 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 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 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 sequence 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 recognition of new species. It can facilitate the recognition and discrimination of cryptic species. This is especially useful when distinguishing invasive species from closely resembling but harmless species. 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 wide diversity of the phylum as possible, for now, at least in nematology, both the classical and molecular fields are needed for a better understanding of the biology and diversity of nematodes. With the speed and higher output that the molecular approaches introduce, nematode community analysis will be less laborious and this may eventually facilitate the use of nematodes as bioindicators.

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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).

1201

1202 Table 2 Summary of some of the DNA-based techniques for distinguishing between species/population of
1203 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 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)