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Review on Nematode Molecular Diagnostics: From Bands to Barcodes

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Abstract
Molecular methods of identification provide accurate, reliable diagnostic approaches for the identification of plant-parasitic nematodes. The promising and attractive results have generated increasing demands for applications in new fields and for better performing techniques. Initially, the techniques were used solely for taxonomic purposes, but increasingly became popular as a component of diagnostic information. Diagnostic procedures are now available to differentiate the plant-pathogenic species from related but non-pathogenic species. The microscopic size of plant parasitic nematodes poses problems and techniques have been developed to enrich samples to obtain qualitative and quantitative information on individual species. In addition, techniques are available to evaluate single nematodes, cysts or eggs of individual species in extracts from soil and plant tissue. DNA or RNA-based techniques are the most widely used approaches for identification, taxonomy and phylogenetic studies, although the development and use of other methods has been, and in some cases still is, important. DNA barcoding and the extraction of DNA from preserved specimens will aid considerably in diagnostic information. . In addition, further review is needed to identify all recovered nematode and evaluation of promising treatments for use in integrated disease management strategy to manage not only regulated species such as the potato cyst nematodes Globodera rostochiensis and Globodera pallida, and root-knot nematode Meloidogyne. chitwoodi, but also other related nematode diseases of plant.
Key Words: Barcoding, Diagnosis, DNA, Molecular, Nematode, RNA.

1. Introduction

The Phylum Nematoda is highly diverse in terms of species richness and one of the most abundant metazoan groups on earth. It is estimated that nematodes comprise nearly 90% of all multicellular organisms. Furthermore, Lambshead (1993) predicted the number of nematode species in marine habitats to be as high as one hundred million, although only 26,646 species have been described thus far from all habitats (Hugot *et al*., 2001). Nematodes are essentially aquatic organisms and most are microscopic in size (0.3-3.0 mm), living in a range of habitats, from oceans to the microscopic film of water surrounding soil particles. They occur as free living foragers and as obligate ecto and endoparasites. Based on their different feeding habits, terrestrial and marine nematodes can be divided into different functional (trophic) groups (Yeates *et al*., 1993). However, nematodes are considered one of the most difficult organisms to identify due their microscopic size, morphological similarity, limited number of distinguishable taxonomic characters and overlapping morphometric measurements. In a global context there is increasing demand for nematode taxonomists to assess community structure in relation to soil function (Wardle *et al*., 2004), to develop new tools for agronomic management and to address quarantine regulations (Powers, 2004). However, there are few trained nematode taxonomists remaining, primarily due to retirement without replacement and/or young scientists' lack of interest in classical taxonomy (Ferris, 1994; Coomans, 2002). While there is a continuing decline in classical taxonomic expertise of many taxa including Nematoda (Andre *et al*., 2001; Coomans,

2002), access to new technologies provides some new opportunities for nematode identification and there is an increasing reliance on developing molecular based diagnostic protocols to identify pests and pathogens. It is essential that the causative organism of plant disease is identified correctly in order to implement effective management strategies. Thus, to differentiate species using diagnostic techniques is a vital component of management of economically important pests, including plant-parasitic nematodes. Conventional methods for nematode identification rely on time-consuming morphological and morphometric analysis of several specimens of the target nematode. The accuracy and reliability of such identification depends largely on the experience and skill of the person making the diagnosis, and the number of such qualified and experienced nematode taxonomists is small and currently in decline. Molecular methods of nematode identification provide accurate, alternative diagnostic approaches (Punja *et al*., 2008).

Molecular diagnostics of nematodes will soon be commonplace. The field has moved beyond initial technical development to the present stage of data accumulation. PCR has enabled the rapid collection of vast amounts of genetic data, information that may provide the raw material for reliable nematode identification. Any investigator given a set of specific primers, a thermal cycler, a few stock reagents, and a means to evaluate the

amplified PCR product, can quickly generate enough data to require intensive bioinformatics analysis. One glance at a current nematology journal will confirm the abundance of papers comparing nematode nucleotide sequences or restriction enzyme patterns. In nematode identification the central question is no longer one of the relative importances of molecules versus morphology. A well-formulated approach to that question will create a solid framework for future studies of nematode biodiversity. Haste in the process of nematode characterization could lead to a molecular Tower of Babel. Nematode diagnostics has traditionally relied on careful measurement and comparison of morphological structures. This process has revealed significant variation in those structures. Often discrimination between species is based on average measurements from a population of individuals, a tricky business if populations consist of mixtures of closely related species. Nonetheless, over a century of descriptive biology has led to the recognition of approximately 25,000 nematode species and the widely held estimate that 10-40 times as many species remain to be described. Within the next decade each of the nominal species could conceivably be characterized by molecular methods. But the speed and ease of data acquisition may be lying the real difficulty of accurate nematode identification. The first step in the development of diagnostic protocols usually involves the selection of an “exemplar” or representative specimen of the taxon. The identification of the exemplar is critical. Paradoxically, this crucial first step is often the least critically examined step in diagnostic studies (Stevens JR, Schofield CJ., 2003). The construction of the rapidly growing molecular database must include a means to evaluate the quality of the data. Without adequate documentation or designation of voucher material we are left wondering what, exactly, does a DNA sequence represent. Ideally, each DNA sequence in Gen Bank or each published PCR-RFLP gel pattern should be accompanied by extensive information about that specimen. This information could include photographs, measurements, collection site data, and status of voucher material. The voucher material may consist of DNA, digitized images, video or a portion of the original specimen (De Ley P, Burt W., 2002). This has been the standard for traditional, morphological-based diagnostic research, and there should be no relaxing of those standards for molecular studies.

Moreover, diagnostic techniques that rely on the interaction between or separation of specific molecules include serological, biochemical and DNA or RNA-based approaches. Molecular diagnostics is a term used more specifically for the characterization of an organism based on information on its DNA or RNA structure. For diagnostic purposes, homologous genes or DNA or RNA fragments, whose sequence differs between species but is similar for all individuals of the same population or species are selected and compared. DNA- or RNA-based techniques are the most widely used approaches for identification, taxonomy and phylogenetic studies (Perry and Jones, 1998). However, the history of diagnostics of plant-parasitic nematodes shows that the development and use of other methods has been, and in some cases still is, important. Initially, the techniques were used solely for taxonomic purposes, but increasingly became popular as a component of diagnostic information for farmers, growers and advisors. Therefore, this review is necessitated to provide information on molecular diagnosis of nematode from bands to barcodes.

2. Molecular Diagnostics

Most methods of nematode diagnostics have some limitations. Species identification based on differences in morphological and morphometrical characters requires a lot of skill and is often inconclusive for individual nematodes. Isozyme or total protein analyses are relatively fast ways to identify root knot or cyst-forming nematode species. Differences in isozyme or protein patterns show significant consistency and are useful for species identification. However, reliable results can only be obtained with nematodes of specific developmental stage. DNA based diagnostics do not rely on the express products of the genome and are independent of environmental influence or developmental stage. Recent progress in nematode diagnostics has been achieved due to introducing the polymerase chain reaction (PCR), a powerful method with widespread application in many biological fields. A single nematode, egg or even a part of the nematode body could be identified using this technology. The majority of PCR-based techniques developed for nematode diagnostics indicate differences of the rRNA or mitochondrial DNA (mtDNA) gene sequences (Luc *et al*., 2005). The first report of DNA-based techniques to identify plant-parasitic nematodes was published over 20 years ago. Curran *et al*. (1985) used restriction fragment length polymorphisms (RFLPs) and demonstrated that this technique had greater discriminatory potential than serological and biochemical approaches. Several research groups used DNA probes for identification purposes, including Marshall and Crawford (1987) and Burrows and Perry (1988) who both used probes for PCN, and Palmer *et al*. (1992) who used probes to identify *Ditylenchus dipsaci*. Significant progress over the last decade in molecular diagnostics of nematodes has been due to the development and introduction of polymerase chain reaction (PCR). This method enables numerous copies to be obtained from a single or a few molecules of DNA extracted from an organism by chemical synthesis *in vitro*. It has been used extensively to identify species of plant-parasitic nematodes. Ibrahim *et al*. (1994) used PCR to amplify a fragment of the rDNA array from 12 species and populations of *Aphelenchoides*. RFLPs in the fragment were

used successfully to compare and differentiate species and populations. The PCR primers used to amplify the rDNA in this work were based on conserved sequences in the 18S and 26S ribosomal genes of *Caenorhabditis elegans* (Files and Hirsh, 1981) and were first used for work on plant-parasitic nematodes by Vrain *et al*. (1992), who examined intraspecific rDNA RFLPs in the *Xiphinema americanum* group. The wide application of PCR in diagnostics is a reflection of the advantages of the technique, which is very sensitive, rapid, easy to perform and inexpensive. PCR is used routinely for nematode diagnostics and has been comprehensively reviewed recently by Powers (2004), Blok (2005), Subbotin (2006) and Subbotin and Moens (2006).

Compared with biochemical approaches, molecular diagnostics has several advantages. It does not rely on expressed products and is not influenced by environmental conditions and development stage. Any development stage can be used for diagnosis. It is much more sensitive than any biochemical technique, and can be used with nanograms of DNA extracted from one nematode or even part of a nematode body. It can also be used with various types of samples, such as soil extracts, plant material or formalin- fixed samples. Ibrahim *et al*. (2001) conducted an elegant, comparative study estimating the efficiency of detection, identification and quantification of the two PCN species from field soil samples using IEF (Isoelectric Focusing), ELISA and PCR techniques with standard nematological methods. A greater number of positive results were obtained with PCR with specific primers than with any other method, indicating the greater sensitivity of this method. The results from ELISA did not agree with other methods because of partial crossreactivity of the two antibodies used. PCR and IEF results can be obtained in 1 day, whereas ELISA results are only available the next day. There were also differences in pricing for sample testing between these methods; the price for IEF testing was significantly higher than that for PCR or ELISA.

2.1. Keeping nematodes for molecular studies and DNA extraction

The efficiency of DNA extraction from a sample depends on how nematodes have been prepared and fixed. Various methods of fixation have been proposed and described, but the best approach is to use live nematodes for diagnostics. If the period between nematode extraction and molecular analyses is several days or weeks, nematodes may be kept at low temperatures before use. In some cases, quarantine regulations do not allow live nematodes to be kept and transported, so nematodes should be heated briefly to kill them but leave the DNA undamaged. Often, during long field sampling trips, it is not possible to keep nematodes at low temperatures, so fixation in 75–90% alcohol, glycerol or simply drying the nematodes in a plastic tube are alternative methods to save nematode DNA for further molecular study. For long-term storage, formalin has been used as a fixative in nematology for many years. It had been assumed that the effects of formalin fixation caused fixed specimens to be unsuitable for DNA analysis, but several methods of DNA extraction from formalin-fixed and glycerine-embedded nematodes stored for days or even years have been tested and have shown promising results (De Giorgi *et al*., 1994; Thomas *et al*., 1997; Bhadury *et al*., 2005; Rubtsova *et al*., 2005). However, although De Giorgi *et al*. (1994) amplified DNA fragments from fixed nematodes, they reported several artificial mutations in sequences recovered from formalin-fixed nematodes. Artefacts could be the consequence of formalin damaging or cross-linking cytosine nucleotides on either strand, so that DNA polymerase would not recognize them and instead of guanosine incorporate adenosine, thereby creating an artificial C–T or G–A mutation (Williams *et al*., 1999). In contrast, Thomas *et al*. (1997) and Bhadury *et al*. (2005) did not find ambiguities in sequences obtained from formalin-fixed nematodes after a few days of storage. Rubtsova *et al*. (2005) even reported successful sequencing without ambiguities of a short fragment of the D2 expansion segment of 28S rRNA amplified from *Longidorus* spp. kept in permanent slides for more than 10 years. The development of a successful protocol for DNA extraction and PCR from formalin-fixed and glycerine-embedded nematodes from permanent slides, presently kept in many taxonomic collections in different countries, will provide new opportunities to analyse rare species with limited distribution and, potentially, enable many diagnostic problems in nematology to be solved. A solution called DESS, which contains dimethyl sulphoxide, disodium ethylene diamine tetraacetic acid (EDTA) and saturated NaCl, has been shown to preserve nematode morphology equally as well as formalin fixation and allowed PCR to be performed on individual nematodes (Yoder *et al*., 2006). In the future, this may be the solution of choice for nematode preservation.

2.2. Antibody approach for sample enrichment

The quantity and quality of DNA is very important for successful diagnosis. In many cases, one specimen, either adult, juvenile or egg, or even part of a nematode might be enough for molecular identification. However, for greater reliability, the use of several specimens of the target nematode is always preferable. Detection of plant-parasitic nematodes in samples is a difficult task due to their microscopic size and uneven dispersal in the soil. A method to enrich nematode extracts from soil samples was proposed and developed by Chen *et al*. (2001) using an antibody-based capture system. In this method, an antibody which recognizes the surface of target nematodes is incubated with a nematode suspension extracted from a field sample. Then, magnetic beads coated with the

secondary antibody are added and a magnet is used to capture target nematodes while other nematodes are discarded (Chen *et al*., 2003). The immunomagnetic capture system has been shown to be effective for the enrichment of *Meloidogyne spp., X. americanum* and *G. rostochiensis* from total nematode extracts from soil, with up to 80% of the target nematode being recovered (Chen *et al*., 2001, 2003). The antibody-based capture system is an effective method of detecting specific nematodes in mixed soil samples and results in samples containing target nematodes in large numbers, which are suitable for further diagnostics techniques (Chen *et al*., 2003).

2.3. Whole genomic amplification

The problem of being able to obtain only limited DNA from nematode samples might also be solved using whole genomic amplification (WGA) approaches. Using these methods, it is possible to generate microgram quantities of DNA starting with as little as a few nanograms of genomic DNA from a single nematode specimen or even part of nematode body. Multiple-displacement amplification (MDA) is a relatively novel technique for WGA. It uses the highly processive Phi29 DNA polymerase and random exonuclease-resistant primers in an isothermal amplification reaction (Dean *et al*., 2001). Successful amplifications of MDA have been reported using nematode DNA from species of *Bursaphelenchus* and *Meloidogyne*. Application of this method to nematode samples significantly improved efficiency of amplification of ribosomal and protein coding genes (Metge and Burgermeister, 2005; Skantar and Carta, 2005). Skantar and Carta (2005) considered that the potential applications of MDA to nematode identification are far-reaching. Using MDA, it should be possible to archive genetic material from individual nematodes, thereby eliminating the need for more labour-intensive culture methods. MDA could facilitate the development and production of DNA 'type species' that may be shared among scientists, or enable large quantities of genetic material from rare specimens to be archived. Genome amplification is also tolerant of sample degradation and might usefully be applied to formal in fixed nematode specimens.

2.4. DNA sequence targets for diagnostics

There are two main approaches to select target DNA sequences for diagnostic purposes: (i) to use known conserved genes, common to all nematode species, and to explore the specific sequence variation in order to distinguish species; and (ii) to randomly screen the whole genome and find specific DNA fragments that could be used as markers for diagnostics. At present, the first approach is more widely used for nematode diagnostics. The main region targeted for this diagnostic development is nuclear ribosomal RNA genes, especially the internal transcribed spacers 1 (ITS1) and 2 (ITS2), which are situated between 18S and 5.8S, and 5.8S and 28S rRNA genes, respectively. The choice of these genes is partly historical, because they were the first to be characterized in nematodes, and partly due to advantages in methodology, because these genes are present in a cell in many copies and, thus, can be amplified relatively easily from a small sample. Ribosomal genes and their spacers have undergone different mutation rates, and this enables different regions to be used for diagnostics at a higher taxonomic level, such as family and genus, down to species, subspecies or even population levels. Modern diagnostics of nematodes from the genera *Heterodera*, *Globodera*, *Bursaphelenchus*, *Pratylenchus*, *Anguina*, *Ditylenchus*, *Nacobbus* and *Radopholus* are based on nucleotide polymorphisms in sequences of the ITSs. To distinguish most species of root-knot nematodes, the intergenetic spacer (IGS) of nuclear rRNA (Petersen and Vrain, 1996), which is between 28S and 18S rRNA genes, and the intergenic spacer of mitochondrial DNA (Powers and Harris, 1993; Powers *et al*., 2005), which is between the 5' portion of cytochrome oxidase subunit II and large ribosomal rRNA genes, are used in addition to the ITS-rRNA. Species of root-knot nematodes can be separated based on the length as well as on the nucleotide polymorphism of the amplified fragments, when amplified by PCR primers in the flanking genes (Powers and Harris, 1993; Petersen and Vrain, 1996). Sequence analyses of the 18S rRNA gene (Floyd *et al*., 2002) and the D2–D3 expansion segments of the 28S rRNA for many tylenchid nematodes (Subbotin *et al*., 2006) and longidorids (Rubtsova *et al*., 2005; He *et al*., 2005) have revealed that these genes are also reliable diagnostic targets at the species level. Other genes that are increasingly being used for diagnostic purposes include the major sperm protein (Setterquist *et al*., 1996), heat shock Hsp90 (Skantar and Carta, 2004) and actin (Kovaleva *et al*., 2005) genes. It is evident that recent progress in nematode genome sequencing and expressed sequence tag (EST) projects (Scholl and Bird, 2005) will give more promising and reliable gene candidates for diagnostic developments.

Targets for development of a diagnostic method also can be identified by screening random regions of the genome to find DNA fragment sequences that are unique for a particular taxon. This can be done using PCR-based techniques, such as random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP), which provide randomly generated fragments from the genome. These fragments are separated by gel electrophoresis and the patterns are compared for different taxa. Potential diagnostic and unique bands are extracted, cloned and sequenced and then used to design specific sequence-characterized amplified

region (SCAR) primers (Zijlstra, 2000). Satellite DNA is present in the genome of almost all eukaryotic organisms and is composed of highly repetitive sequences organized as long arrays of tandemly repeated elements. Satellite DNA sequences have been characterized from a number of plant-parasitic nematodes and have proven to be species-specific, thus constituting useful diagnostic tools for identification of species of agronomic interest (Grenier *et al*., 1997; Castagnone- Sereno *et al*., 1999; He *et al*., 2003).

2.5. DNA extraction

The first step in molecular diagnostic procedures is the preparation of the template DNA. Several protocols for the extraction of nucleic acids from nematodes are available (Curran *et al*., 1985; Caswell- Chen *et al*., 1992; Blok *et al*., 1997). Some of these allow the isolation of microgram quantities of pure genomic DNA. However, because only small quantities of starting DNA are required for PCR amplification, simplified and rapid procedures generally can be used (Harris *et al*., 1990; Subbotin *et al.*, 2000; Waeyenberge *et al*., 2000; Floyd *et al*., 2002). Using different extraction methods and commercial kits, nematode DNA can be obtained directly from soil samples (Nazar *et al*., 1995; Waite *et al.*, 2003). Furthermore, extraction of DNA from formalin- fixed materials or nematodes embedded in glycerine on slides provides a new opportunity for molecular examination of reference materials (Thomas *et al*., 1997). Moreover, using proteinase K is the most useful, cheap and rapid approach to extract DNA from nematodes (Waeyenberge *et al*., 2000). It consists of two steps: (i) mechanical destruction of nematode body and tissues in a tube using ultrasonic homogenizer or other tools, or repeatedly freezing samples in liquid nitrogen; and (ii) chemical lyses with proteinase K in a buffer for 1 h or several hours with subsequent brief inactivation of this enzyme at high temperature. Chelex resin protocols can be also successfully applied for DNA extraction from nematodes (Walsh *et al*., 1991). Various chemical treatments are also applied to remove cell components and purify the DNA. Phenol or phenol with chloroform extractions is often employed to remove proteins and ethanol is then used to precipitate and concentrate the DNA. Stanton *et al*. (1998) described an efficient method of DNA extraction from nematodes using chemical lyses in alkali solution without prior mechanical breaking of nematode bodies. Effective DNA extraction can be achieved by using commercial kits developed by different companies. These approaches rely on DNA binding to silica in the presence of a high concentration of chaotropic salt (Boom *et al*., 1990).

2.6. PCR - based methods

PCR is the most important technique in diagnostics. Currently, there are several PCR-based methods used for nematode diagnostics: PCR-RFLP, PCR with specific primers, PCR-single and strand conformation polymorphism (SSCP). This enzymatic reaction allows *in vitro* amplification of target DNA fragments by up to a billion fold from complex DNA samples within a test tube. Any nucleic acid sequence can be detected by PCR amplification. The method requires a DNA template containing the region to be amplified, two oligonucleotide primers flanking this target region (Table 1), DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) mixed in buffer containing magnesium ions (MgCl2). The PCR is performed in tubes with final volumes of 20–100L. The PCR procedure consists of a succession of three steps which are determined by temperature condition: template denaturation at 95°C for 3-4 min, primer annealing at 55-60°C for 1-2 min and extension at 72°C for 1-2 min. The PCR is carried out for 30-40 cycles in a thermocycler with programmed heating and cooling. Finally, PCR products are separated electrophoretically according to their size on agarose or polyacrylamide gels and visualized by ethidium bromide under ultraviolet (UV) light or after silver staining. Once identified, nematode target DNA generated by PCR amplification can be characterized further by various analyses: restriction fragment length polymorphism (RFLP), single strand conformation polymorphism (SSCP) or sequencing. In general, utilization of PCR-based assays measuring intraspecific, interspecific, and intergeneric genetic variability has revealed numerous polymorphic nucleotide sites specific to a nematode genus or species.

Table 1. Universal primers frequently used for nematode diagnostics.

Code	Primer (5′–3′)	Amplified region	Reference
C2F3	GGT CAA TGT TCA GAA ATT TGT GG	3′ of COII to 16S	Powers and Harris (1993)
1108	TAC CTT TGA CCA ATC ACG CT	mitochondrial genes	
18S	TTG ATT ACG TCC CTG CCC TTT	ITS1 region of rDNA	Szalanski *et al.* (1997)
rDNA1.58S	GCC ACC TAG TGA GCC GCG CA		
18S	TTG ATT ACG TCC CTG CCC TTT	ITS1–5.8S–ITS2	Vrain *et al.* (1992)
26S	TTT CAC TCG CCG TTA CTA AGG	region of rDNA	
F194	CGT AAC AAG GTA GCT GTA G	ITS1–5.8S–ITS2	Ferris *et al.* (1993)
F195	TCC TCC GCT AAA TGA TAT G	region of rDNA	
SSU18A	AAA GAT TAA GCC ATG CAT G	18S gene of rDNA	Blaxter *et al.* (1998)
SSU26R	CAT TCT TGG CAA ATG CTT TCG		
D2A	ACA AGT ACC GTG AGG GAA AGT TG	D2–D3 expansion	De Ley *et al.* (1999)
D3B	TCG GAA GGA ACC AGC TAC TA	segments of	
TW81	GTT TCC GTA GGT GAA CCT GC	28S gene of rDNA	Joyce *et al.* (1994)
AB28	ATA TGC TTA AGT TCA GCG GGT	ITS1–5.8S–ITS2	
		region of rDNA	

2.6.1. PCR-RFLP (Restriction Fragment Length Polymorphism)

Variation in sequences in PCR products can be revealed by restriction endonuclease digestion. The PCR product obtained from different species or populations can be digested by a restriction enzyme and the resulting fragment is separated by electrophoresis. If there is some difference in sequences situated within the restriction site of the enzyme, the digestion of the PCR products will lead to different electrophoretic profiles. It has been shown that comparison of restriction patterns derived from amplified ITS regions is a very useful approach to distinguish species and populations of *Aphelenchoides* (Ibrahim *et al*., 1994), *Bursaphelenchus* (Hoyer *et al*., 1998), cyst-forming nematodes (Thiery and Mugniery, 1996; Bekal *et al*., 1997; Orui, 1997; Szalanski *et al*., 1997; Subbotin *et al*., 2000) (Fig. 1.), *Ditylenchus* (Wendt *et al*., 1993; Ibrahim *et al*., 1994), *Nacobbus* (Reid *et al.*, 2003), *Pratylenchus* (Orui, 1996; Waeyenberge *et al.*, 2000), *Radopholus* (Fallas *et al*., 1996), root knot nematodes (Zijlstra *et al.*, 1995; Schmitz *et al.*, 1998) and *Xiphinema* (Vrain *et al*., 1992). Comparison of RFLP profiles from newly obtained samples with those from known species provide a quick tool for nematode identification. PCR-RFLPs are especially suited to identify nematodes of monospecific probes; this strategy does not allow mixed species populations to be identified.

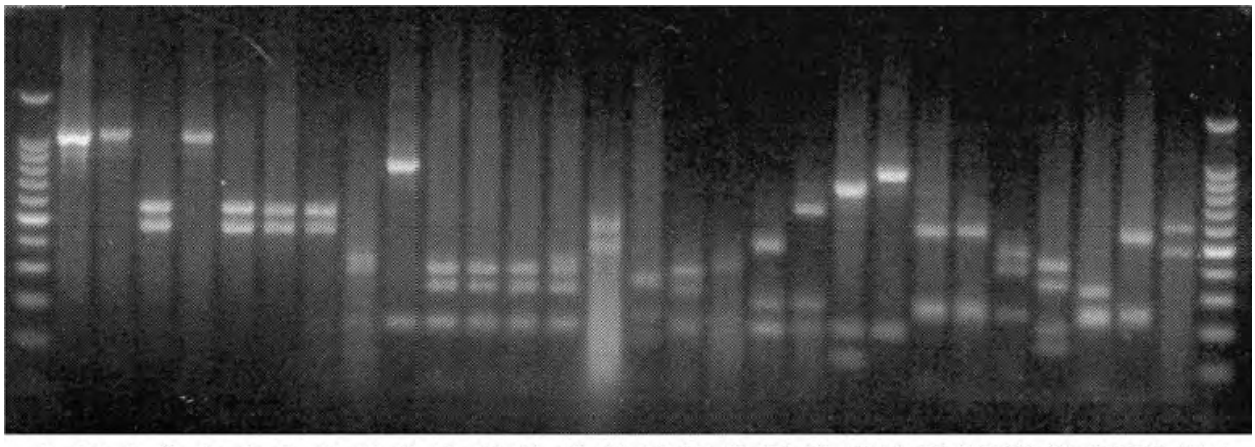

Fig.1. RFLP patterns obtained after *Alu*I digestion of the amplified PCR product of the ITS-rDNA for cystforming nematodes. L, 100 bp DNA ladder; U, unrestricted PCR product; 1, 2, *H. avenae*; 3, *H. arenaria*; 4, *H. filipjevi*; 5, *H. aucklandica*; 6, *H. ustinovi*; 7, *H. latipons*; 8, *H. hordecalis*; 9, *H. schachtii*; 10, *H. trifolii*; 11, *H. medicaginis*; 12, *H. ciceri*; 13, *H. salixophila*; 14, *H. oryzicola*; 15, *H. glycines*; 16, *H. cajani*; 17, *H. humuli*; 18, *H. ripae*; 19, *H. fici*; 20, *H. litoralis*; 21, *H. carotae*; 22, *H. cruciferae*; 23, *Heterodera* sp.; 24, *H. cyperi*; 25, *H. goettingiana*; 26, *H. urticae*; 27, *Meloidodera alni.* (Source: Subbotin *et al*., 2000).

2.6.2. PCR-SSCP (Single Strand Conformation Polymorphism)

This technique has been applied successfully for rapid identification of cyst-forming nematodes and root knot nematodes from cultures and field samples (Clapp *et al*., 2000). The distinguishing patterns obtained with PCR-SSCP are sequence dependent and utilize minor nucleotide differences across several hundred bases of sequences. It is a simple procedure where denatured, single-stranded PCR amplicons are separated electrophoretically in a non denaturing polyacrylamide gel. The length, position and extent of self-complementary base pairs affect the conformation taken up by the molecules and thus their electrophoretic mobility. This effect is enhanced by minor length polymorphisms and increasing amounts of sequence variation. SSCP patterns are highly reproducible between gels and generate two markers from each DNA sequence present. The band patterns are compared with those obtained from controls or from pattern databases.

2.6.3. Sequencing

Direct sequencing of PCR products or sequencing of cloned PCR fragments provides full characterization of amplified target DNA. One of the first applications of PCR in plant nematology was presented by Ferris *et al.* (1993), who used the ITS rDNA sequences to establish the taxonomic and phylogenetic relationships of cyst-forming nematodes. The sequences of the ITS regions, fragments of 18S and 28S of rRNA genes, have been examined for a wide range of plant parasitic nematodes (Subbotin *et al*., 2001b; Floyd *et al*., 2002; Reid *et al*., 2003). The comparison of newly obtained sequences from samples with those published or deposited in the GenBank is a most reliable approach for molecular identification. Increasing numbers of deposited nematode rDNA sequences as well as decreasing costs for sequence analyses will allow wider application of this still rather expensive procedure for routine nematode diagnostics in the future.

2.6.4. PCR with species-specific primers (Multiplex PCR)

PCR with specific primer combinations or multiplex PCR constitute a major development in DNA diagnostics and allow the detection of one or several species in a nematode mixture by a single PCR test, thus decreasing diagnostic time and costs. Species-specific primers are designed based on the broad knowledge of sequence divergence of the target DNA region in many populations of the same species and in closely related species. This knowledge allows the detection of populations with small differences in sequences, and avoids the amplification of an identical specific fragment in other species. The principle of this method is the alignment of the sequences from target and non-target organisms and the selection of primer mismatches to non-target organisms, but it shows sufficient homology for efficient priming and amplification of the target organism. In nematology, this diagnostic tool has been developed for identification of cyst-forming and root knot nematodes (Table 2) and *Pratylenchus* (Uehara *et al*., 1998), *Xiphinema* (Wang *et al*., 2003) and *Ditylenchus* (Esquibet *et al*., 2003). The multiplex PCR with specific primers for identification of several nematode targets in one assay is limited by the number of primer pairs that can be used in a single reaction and the number of bands that can be clearly identified without giving false-positive results. This technique requires precise optimization of the reaction conditions for the primer sets used simultaneously in the test.

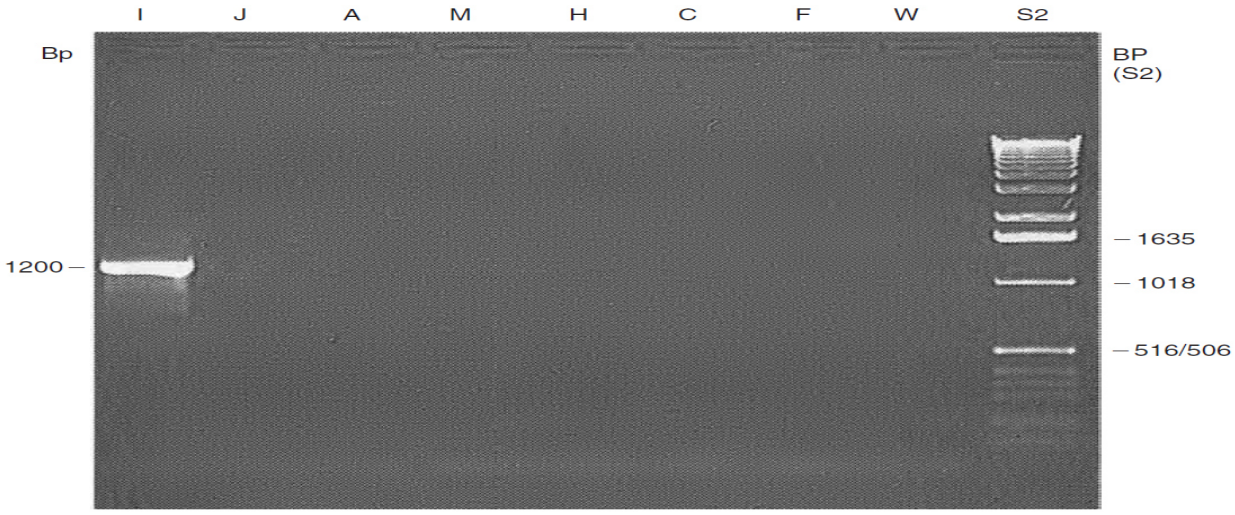

Fig.2. Amplification product of PCR with species-specific primer Finc/Rinc for *Meloidogyne incognita*. I, *Meloidogyne incognita*; J, *M. javanica*; A, *M. arenaria*; M, *M. mayaguensis*; H, *M. hapla*; C, *M. chitwoodi*; F, *M. fallax*; W, no template DNA control; S, size marker (Source: Zijlstra *et al*., 2000).

Table 2. Species-specific primers developed for identification of cyst-forming and root knot nematodes.

Species	Primer set (5′–3′)	Amplicon length	Reference
Globodera pallida	PITSp4 ACA ACA GCA ATC GTC GAG ITS5 GGA AGT AAA AGT CGT AAC AAG G	265 bp	Bulman and Marshall (1997)
Globodera pallida	TGT CCA TTC CTC TCC ACC AG CCG CTT CCC CAT TGC TTT CG	768 bp	Fullaondo *et al.* (1999)
Globodera pallida	GGT GAC TCG ACG ATT GCT GT GCA GTT GGC TAG CGA TCT TC	238 bp	Mulholland *et al.* (1996)
Globodera rostochiensis	PITSr3 AGC GCA GAC ATG CCG CAA ITS5 GGA AGT AAA AGT CGT AAC AAG G	434 bp	Bulman and Marshall (1997)
Globodera rostochiensis	GCA AGC CCA GCG TCA GCA AC GAA CAT CAA CCT CCT ATC GG	315 bp	Fullaondo *et al.* (1999)
Globodera rostochiensis	GGT GAC TCG ACG ATT GCT GT GCA GTT GGC TAG CGA TCT TC	391 bp	Mulholland *et al.* (1996)
Heterodera glycines	GlyF1 TTA CGG ACC GTA ACT CAA rDNA2 TTT CAC TCG CCG TTA CTA AGG	181 bp	Subbotin *et al.* (2001a)
Heterodera schachtii	SHF6 GTT CTT ACG TTA CTT CCA TW81 GTT TCC GTA GGT GAA CCT GC	200 bp	Amiri *et al.* (2002)
Meloidogyne arenaria	TCG AGG GCA TCT AAT AAA GG GGG CTG AAT AAT CAA AGG AA	950 bp	Dong *et al.* (2001)
Meloidogyne arenaria	Far TCG GCG ATA GAG GTA AAT GAC Rar TCG GCG ATA GAC ACT ACA ACT	420 bp	Zijlstra *et al.* (2000)
Meloidogyne chitwoodi	MC3F CCA ATG ATA GAG ATA GGA AC MC1R CTG GCT TCC TCT TGT CCA AA	400 bp	Williamson *et al.* (1997)
Meloidogyne chitwoodi	C64 GAT CTA TGG CAG ATG GTA TGG A 1839 AGC CAA AAC AGC GAC CGT CTA C	900 bp	Petersen *et al.* (1997)
Meloidogyne chitwoodi	Fc TGG AGA GCA GCA GGA GAA AGA Rc GGT CTG AGT GAG GAC AAG AGT A	800 bp	Zijlstra (2000)
Meloidogyne exigua	Ex-D15-F CAT CCG TGC TGT AGC TGC GAG Ex-D15-R CTC CGT GGG AAG AAA GAC TG	562 bp	Randing *et al.* (2002)
Meloidogyne fallax	F64 TGG GTA GTG GTC CCA CTC TG 1839 AGC CAA AAC AGC GAC CGT CTA C	1100 bp	Petersen *et al.* (1997)
Meloidogyne fallax	Ff CCA AAC TAT CGT AAT GCA TTA TT Rf GGA CAC AGT AAT TCA TGA GCT AG	515 bp	Zijlstra (2000)
Meloidogyne hapla	GGC TGA GCA TAG TAG ATG ATG TT ACC CAT TAA AGA GGA GTT TTG C	1500 bp	Dong *et al.* (2001)
Meloidogyne hapla	MH0F CAG GCC CTT CCA GCT AAA GA MH1R CTT CGT TGG GGA ACT GAA GA	960 bp	Williamson *et al.* (1997)
Meloidogyne hapla	Fh TGA CGG CGG TGA GTG CGA Rh TGA CGG CGG TAC CTC ATA G	610 bp	Zijlstra (2000)
Meloidogyne incognita	TAG GCA GTA GGT TGT CGG G CAG ATA TCT CTG CAT TGG TGC	1350 bp	Dong *et al.* (2001)
Meloidogyne incognita	Inc-K14-F GGG ATG TGT AAA TGC TCC TG Inc-K14-R CCC GCT ACA CCC TCA ACT TC	399 bp	Randing *et al.* (2002)
Meloidogyne incognita	Finc CTC TGC CCA ATG AGC TGT CC Rinc CTC TGC CCT CAC ATT AGG	1200 bp	Zijlstra *et al.* (2000)
Meloidogyne javanica	CCT TAA TGT CAA CAC TAG AGC C GGC CTT AAC CGA CAA TTA GA	1650 bp	Dong *et al.* (2001)
Meloidogyne javanica	Fjav GGT GCG CGA TTG AAC TGA GC Rjav CAG GCC CTT CAG TGG AAC TAT AC	670 bp	Zijlstra *et al.* (2000)
Meloidogyne paranaensis	Par-C09-F GCC CGA CTC CAT TTG ACG GA Par-C09-R CCG TCC AGA TCC ATC GAA GTC	208 bp	Randing *et al.* (2002)

2.6.5. Reverse dot-blot hybridization

This technique involves the use of PCR for simultaneous amplification and labelling of target DNA to generate digoxigenin-dUTP-labelled amplicons which are hybridized to specific immobilized oligonucleotide probes on a membrane. This approach can be used for simultaneous identification of many different nematodes from a single sample. Uehara *et al.* (1999) have demonstrated that this technology can be used for the identification of

Pratylenchus species (Fig. 3).

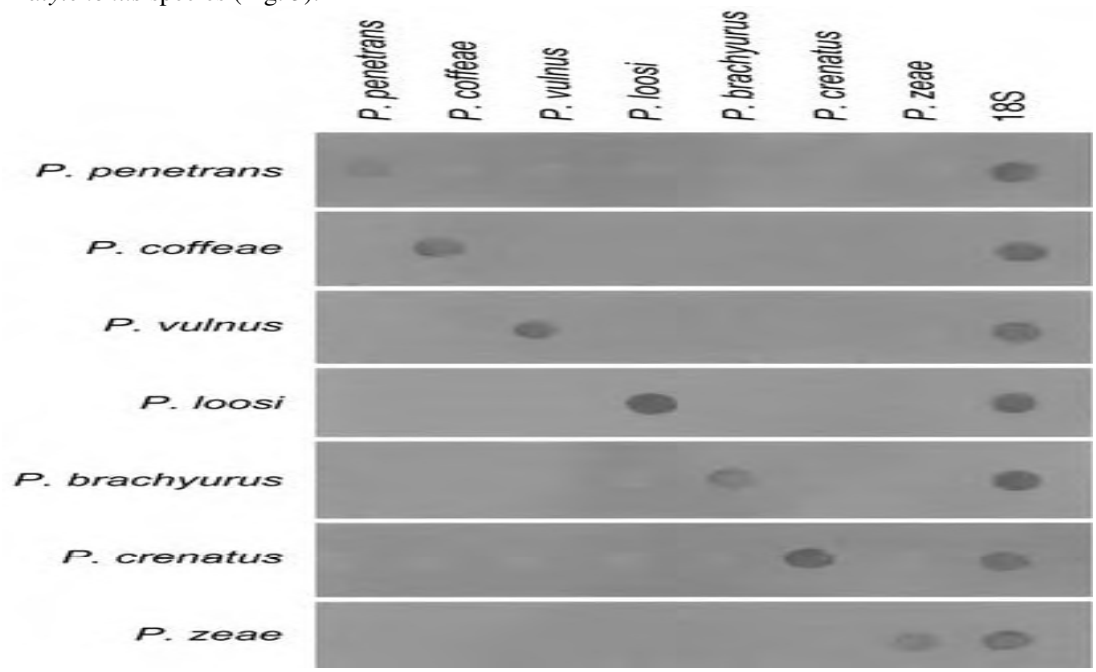

Fig.3. Reverse dot-blot hybridization with immobilized specific oligonucleotides. The *Pratylenchus* species listed on the left were used for each hybridization (Source: Uehara *et al.*, 1999).

2.7.6. RAPD-PCR (Random Amplified Polymorphic DNA- PCR)

In contrast to the above-mentioned classical PCR method, the random amplified polymorphic DNA PCR (RAPD-PCR) or PCR with arbitrary primer (AP-PCR) does not require any information on the primer design. This PCR technology uses a single random primer about ten nucleotides long, approximately 50% GC rich and lacking any internal inverted repeats. By lowering the annealing temperature during the amplification cycle, the primer anneals at random in the genome, allowing the synthesis of highly polymorphic amplification products. RAPD-PCR distinguishes nematode species, subspecies and races and is used for root knot nematodes (Cenis, 1993; Blok *et al.*, 1997) and cyst-forming nematodes (Caswell-Chen *et al.*, 1992; Thiery *et al.*, 1997) (Fig. 4). However, the reproducibility of the results is the most critical point for application of this technique for diagnostic purposes. Specific sequences for certain species or races, called SCARs (sequence characterized amplified regions), can be derived from RAPD fragments and further used to design species specific primers.

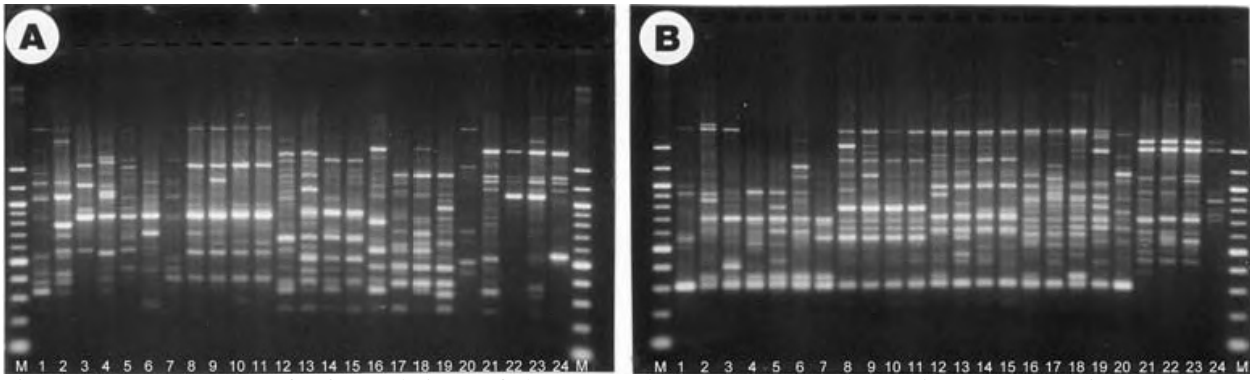

Fig. 4. RAPD patterns of 26 populations of the *Heterodera avenae* complex. Primers: A, A-16; B, A-18. Populations: 1, *H. avenae* (Taaken, Germany); 2, *H. avenae* (Santa Olalla, Spain); 3, *H. avenae* (Çukurova Ebene, Turkey); 4, *H. avenae* (Saudi Arabia); 5, *H. avenae* (Ha-hoola, Israel); 6, *H. avenae* (Israel); 7, *H. avenae* (near Delhi); 8, *H. australis* (South Australia, sample 3); 9, *H. australis* (Beulah, Australia); 10, *H. australis* (Victoria, Australia); 11, *H. australis* (Yorke Peninsular, Australia); 12, *H. mani* (Bayern, Germany); 13, *H. mani* (Heinsberg, Germany); 14, *H. mani* (Andernach, Germany); 15, *H. mani* (Germany); 16, *H. pratensis* (Missunde, Germany); 17, *H. pratensis* (Östergaard, Germany); 18, *H. pratensis* (Lindhöft, Germany); 19, *H. pratensis* (Lenggries, Germany); 20, *H. aucklandica* (One Tree Hill, New Zealand); 21, *H. filipjevi* (Saratov, Russia); 22, *H. filipjevi* (Akenham, England); 23, *H. filipjevi* (Torralba de Calatrava, Spain); 24, *H. filipjevi*

(Selçuklu, Turkey). M, 100 bp DNA ladder (Biolab). (Source: Subbotin *et al*., 2003.)

2.6.7. Amplified Fragment Length Polymorphism (AFLP)

The amplified fragment length polymorphism (AFLP) technique has been developed by Vos *et al.* (1995) and is based on the selective amplification of genomic restriction fragments. AFLP involves three steps: (i) digestion of DNA with two restriction enzymes and ligation of specific adapters to the restriction fragments; (ii) PCR amplification of a subset of the restriction/adapter fragments under stringent conditions; and (iii) gel electrophoresis analysis of the amplified restriction fragments. The AFLP technique has several advantages over RAPD in that it produces results that are very reproducible and it has higher resolutions generating many more amplified fragments. AFLP fingerprinting has been applied successfully for the evaluation of inter- and intraspecific genetic variation of cyst-forming nematodes (Folkertsma *et al*., 1996; Marche *et al*., 2001) and root knot nematodes (Semblat *et al*., 1998).

2.6.8. Problems with PCR-based techniques

Over many years, the application of various PCR methods with universal and specific primers for diagnostic purposes has revealed several problems. First, PCR amplifies DNA from live and dead specimens. This compromises the use of this method for the estimation of the efficiency of pesticide applications on nematode populations. Use of another reverse transcriptase (RT)-PCR technique can solve this problem. Using this approach, mRNA is extracted from live nematodes and then the RT converts RNA to cDNA, which is subsequently amplified and also could be quantified. The second limitation of this method is the probability of a *false-positive reaction*. Primer design is always based on existing knowledge of DNA sequences for target species and closely related nematodes. However, there is a possibility that similar fragments can be obtained for another previously non-investigated nematode as well as for the target species. The third limitation, which is the opposite of second one, is a probability of a *false-negative reaction*. Although a region with a conserved sequence should be used for primer design, the possibility cannot be excluded that some mutations occurred in this region in some specimens or populations of the target taxa. As a result, they might become non-detectable by the PCR test. The fourth limitation is a probability of sample contamination, which might occur during sample preparation and might give a false-positive reaction due to the great sensitivity of the PCR method. Following strict rules to prevent contamination during preparation of the PCR mixture is imperative for all diagnostics tests. These limitations indicate that, first, the PCR technique should be used intelligently and the researcher or advisor must be aware of the peculiarities of each method. Second, when there is any doubt, it is always necessary to confirm identification by several methods, including the use of traditional morphological features.

2.7. Hybridization DNA arrays

DNA arrays provide a powerful method for the next generation of diagnostics. The distinct advantage of this approach is that it combines DNA amplification with subsequent hybridization to oligonucleotide probes specific for multiple target sequences. DNA arrays can be used to detect many nematode species based on differences in ribosomal RNA gene. For example, nucleic acids can be extracted from a sample and then rRNA gene fragments amplified by PCR using sets of universal primers. The resulting PCR products can be hybridized to an array consisting of many oligonucleotide probes, which are designed to detect nematodes by genus or species and are based on discriminatory sequences. In general, arrays are described as macroarrays or microarrays, the difference being the size and density of the sample spots, the substrate of hybridization and the type of production. Although the potential of DNA array methods for nematological diagnostics has been recognized (Blok, 2005; Subbotin and Moens, 2006), little progress had been made in their use, and only few research papers have been published on this technique. A reverse dot-blot assay has been developed for identification of seven *Pratylenchus* spp. using oligonucleotides designed from the sequences of the ITS region of rRNA (Uehara *et al*., 1999). Recently, François *et al*. (2006) were successful in developing a DNA oligonucleotide microarray for identification of *M. chitwoodi* using two types of probes designed from SCAR and satellite DNA sequences.

2.8. DNA bar-coding

The bar-coding technique is based on the idea that a particular nucleotide sequence from a common gene can serve as a unique identifier for every species, and a single piece of DNA can identify all life forms on earth. Floyd *et al*. (2002) were the first to develop a 'molecular operation taxonomic unit' approach when they applied a molecular barcode, derived from single specimen PCR and sequencing of the 5' segment of the 18SrRNA gene, to estimate nematode diversity in Scottish grassland. The cytochrome c oxidase subunit I (COI) of mtDNA is emerging as the standard barcode for many animals. It is nearly 648 nucleotide pairs long in most groups. Mitochondrial DNA evolves much more quickly and contains more differences than the ribosomal gene or its spacer, making mtDNA more useful for distinguishing closely related species. The COI gene is not well characterized yet for plant-parasitic nematodes, except for a few genera; however, Blouin (2002) found that

mtDNA sequence variation among individuals of the same animal-parasitic nematode species averages from a fraction of a percent up to 2%, and the maximum difference observed between a pair of individuals that were clearly members of the same interbreeding population was 6%. MtDNA sequence difference between closely related species is typically in the range of 10–20%, so if two individuals differ by about 10% or more, one might question whether they really are conspecific (Blouin *et al*., 1998). A promising approach to standardize nematode identification using DNA bar cording is to characterize not one but two, or even more, gene regions, which must fit three following criteria: (i) show significant species-level genetic variability and divergence; (ii) be an appropriately short sequence length so as to facilitate DNA extraction and amplification; and (iii) contain conserved flanking sites for developing universal primers. Several DNA regions, such as 18S rRNA (Floyd *et al*., 2002), D2–D3 expansion segments of 28S rRNA (De Ley *et al*., 2005; Subbotin *et al*., 2006) or the ITS-RNA gene (De Ley *et al*., 2005), have been proposed for such a procedure. The ITS-rRNA region is more precisely characterized for many groups of plant-parasitic nematodes than any other gene fragment. The intraspecific variation for the ITS-rRNA sequence gene varies between nematode genera. For example, it typically does not exceed 1.3% for *D. dipsaci sensu stricto* (Subbotin *et al*., 2005), 1.4% for *Heterodera* spp. (Tanha Maafi *et al*., 2003; Madani *et al*., 2004) or 1.8% for *Globodera* spp. (Subbotin *et al*., 2000b). Observed differences greater than these values between a sample and a standard cast doubts about its co-specificity. Recent detailed sequence and phylogenetic studies indicated the presence of several sibling species that probably exist within currently defined species of plant-parasitic nematodes, such as *G. pallida* (Subbotin *et al*., 2000a,b), *H. avenae* (Subbotin *et al*., 2003), *D. dipsaci* (Subbotin *et al*., 2005), *B. xylophilus* and *B. mucronatus* (Zheng *et al*., 2003). However, in some cases, the ITS-rRNA does show differences between sequences and does not distinguish among some closely related and recently diverged species, such as *M. incognita*, *M. javanica* and *M. arenaria* (Hugall *et al*., 1999) or *H. avenae* and *H. arenaria* (Subbotin *et al*., 2003).

DNA barcoding still causes a spirited reaction from many scientists. There are some potential limitations to barcoding, which relate to problems such as the presence of groups of organisms with little sequence diversity, a lack of resolution of recently diverged species, identification of hybrids and possible amplification of nuclear pseudogenes. Although all of these problems still exist, exploratory studies have shown that about 96% of eukaryotic species surveyed can be detected with barcoding, although most of these would also be resolvable with traditional means; however, the remaining 4% pose problems and can lead to error rates that are unacceptably high (up to 31% of false attributions) when relying on DNA barcoding alone. A further problem faced by the biologists who are trying to identify nematodes using barcoding is that currently there is insufficient information in databases for extensive nematode species identification based on DNA fragments. However, the increasing deposition of DNA sequences in public databases such as the GenBank and NemATOL will be beneficial for diagnostics (Powers, 2004).

2.8.1. DNA barcode as a diagnostic tool

The piece of DNA would be called a “DNA barcode” or a “barcode of life” (Besansky *et al*., 2003, Janzen DH., 2004, Pennisi E., 2003, Stoeckle M., 2003, Stoeckle *et al*., 2003). The DNA barcode is analogous to the Universal Product Codes commonly seen on retail products. Rather than a numeric code, the nucleotide sequence from a common gene would serve as a unique identifier for every species. Theoretically, a single 600-nucleotide fragment of protein-coding DNA could easily contain enough variation to identify the estimated 10 million species inhabiting the planet. Even a conserved gene fragment with evolutionary constraints on protein sequence variation could provide sufficient information for a universal barcode. In the example above, if the first two positions in the 200 codons of the 600-nucleotide fragment were invariant, and the third positions were constrained to two possible nucleotides by nucleotide bias, there would still be 2200 or 1060 possible sequences derived from this small fragment of DNA (Stoeckle M., 2003). Validation of the barcode concept, however, rests on more than theoretical information content of DNA. Based on experience of forensic sequencing at the World Trade Center, J. Craig Venter, from the Center for the Advancement of Genomics, estimated it would currently take 2 weeks to complete amplification and diagnostic single gene sequencing of 100,000 insect legs at a cost of $1 a sample. He speculated that within 5–10 years sequencing facilities will have the capacity to sequence entire mammalian genomes in seconds. Because of these technological advances and the information content of entire genomes, Venter advocates a strategy of whole genome sequencing as opposed to the development of a single gene barcode approach. Herbert *et al.* (2003) demonstrated that a barcode based on the mitochondrial gene cytochrome coxidase subunit 1 (COI) can provide accurate species identification across broad divisions of the animal kingdom. His proof-of-concept studies included a comparison of 13,000 congeneric species pairs representing 11 phyla. The estimated mean divergence of these species pairs was 11.3%, with 98% of the pairs exhibiting divergence at greater than 2%. The diversity of COI may be sufficient for barcoding animals, and used solely for the purpose of identification, the single gene approach appears promising. Given the rapid rate of species extinction and the accelerated threat of emerging diseases, many scientists believe the justification for

barcoding is compelling, and is a process that cannot be postponed without consequence. While argument exists about what a "barcode of life" might look like, research projects are underway to test the barcode concept. Regardless of the strategy ultimately adopted, it is clear that the process will affect the development of molecular diagnostics for nematodes. Nematodes are already among the first organisms used to test the barcode concept (Blaxter M., 2003, Floyd *et al*., 2002). Nematodes can rapidly be extracted, sorted, and counted from soil or other substrates. When phylogenetic breadth and diversity are important, nematodes extracted from a single 100 cc soil sample could provide representative species from many of the major taxonomic groups in the phylum, representing over 800 million years of phylogenetic divergence (Blaxter M., 1998). That same soil sample could include 50–100 different nematode species and thousands of individuals in all life stages (Bernard EC., 1992, Boag B, Yeates GW., 1998). The question of phylogenetic diversity and applicability is important in barcode development. A barcode that is readily incorporated into a phylogenetic framework takes advantage of the predictive power of evolutionary history.

Interpreting the signal from a 18S ribosomal DNA barcode, Floyd *et al*. (2003) were able to place unknown nematodes sampled from a Scottish upland grassland onto a phylogenetic tree. Currently, there is insufficient information in the nematode databases for extensive species identification based on the 18S barcode, but one glance at the tree permits an educated guess regarding the taxonomic affinities of their unknown nematode samples. The expanding 18S nematode tree (Blaxter *et al*., 1998, De Ley P, Blaxter M., 2002) will become a valuable resource through the collective efforts of contributors to GenBank and NemATOL, the NSF-funded nematode branch of the Tree of Life Project (*http://nematol.unh.edu/*). NemATOL will synergistically benefit diagnostic efforts by serving as a repository for sequence information and "collateral" biological data. Like Gen Bank with its Link Out connections, NemATOL is an open database containing an archived collection of nematode video images, morphological information, DNA sequences, alignments, and other reference material.

2.8.2. Genetic regions commonly used for diagnostics

Two genomic regions have been routinely characterized among nematode taxa: the ribosomal RNA array and the mitochondrial genome. The ribosomal RNA genes and their intervening sequences are the best-characterized genes or gene regions in Nematoda. The RNA genes themselves are highly conserved, but diagnostically useful variation is found both in discrete regions of the genes and in the length and sequence of the spacer regions. The ribosomal genes evolve slowly compared to their spacer regions, yet surprisingly, they retain considerable diagnostic value at the species level in Nematoda. The spacer regions have been widely applied as diagnostic markers at the species level, and their suitability to identify species is best evaluated on a case-by-case basis. Typically, the rRNA array consists of three ribosomal genes (rRNA), 18S, 5.8S, and 28S (26S). These genes, together with their spacer regions, are aligned in repeating units; a single unit in *C. briggsae* measures 7429–7431 bases (Stein *et al.,* 2003). *C. briggsae* has 55 tandemly aligned units, whereas *C. elegans* is believed to have 100–150 copies of the repeating unit located on chromosome I. Little is known of the chromosomal location of the rDNA array in other nematodes, although variation in the basic composition of the ribosomal array exists in the plant-parasitic nematode genera *Meloidogyne* (Vahidi *et al*., 1988). In that genus one copy of the 5S ribosomal gene is located in the intergenic spacer region between the 28S and 18S genes, an arrangement that has been exploited in several PCR-based diagnostic protocols (Blok *et al*., 1997). In *C. elegans* the 5S genes are distantly located in a tandem array of approximately 100 copies on chromosome V. The mitochondrial genome is a logical choice for species-specific diagnostic markers (Blouin MS., 2002, Hyman BC., 1988). The entire circular genome is present in high copy number and consists of rapidly evolving genes and conserved regions that allow broad phylogenetic application. Its uniparental inheritance, conserved structure, gene composition, and gene order, permit systematic and ecological genetic studies that span the animal kingdom. Interestingly, it is the unconventional structural elements of the nematode mitochondrial genome that have provided promising diagnostic opportunities. A similar gene order and lack of repetitive DNA sequences characterize most animal mitochondrial genomes (Brown WM., 1985). Nematodes, particularly those in the class Enoplea (Adenophorea), are characterized by a surprising variety in gene order (Lavrov DV, Brown WM., 2001). Both major nematode classes, Enoplea and Chromadorea (Secernentea), have mitochondrial genomes with unusual gene structure and repeated sequences. The repeated regions in *Meloidogyne* have been converted to species-specific markers and used to examine nematode population structure (Blok *et al*., 2002, Whipple *et al*., 1998).

2.8.2.1. rRNA and mtDNA genes

The rRNA genes are arranged as tandem repeats with several hundred copies per genome. Each repeat includes the small subunit (SSU) gene, or 18S gene, the 5.8S gene and the large subunit (LSU) gene, or 28S gene, the spacer region between the subunit and 5.8S gene, called the internal transcribed spacers (ITS1 and ITS2), and between the gene cluster, called the intergenic spacer (IGS). In the root knot nematodes, the 5S gene is found in

the IGS. The 18S gene evolves relatively slowly and is useful for comparison of distantly related groups, whereas ITS and IGS are considerably more variable and can be used to distinguish species or subspecies. Some regions of the 28S gene are also useful for species differentiation. MtDNA is a circular double-stranded closed small structure, which is present in large copy numbers in the cell. Rapid evolution rates of specific genes in the mtDNA, which evolve ten times faster and more than nuclear genes, resulted in accumulated sequence polymorphism. This allows this molecule to be used as a useful marker for differentiation of nematode populations and of closely related species.

3. Advantages of molecular diagnostics

3.1. Morphologically similar but genetically divergent

Nematodes are remarkably constrained morphologically. *Caenorhabditis elegans* and *C. briggsae* are two free-living nematodes whose discrimination would present a challenge to most trained nematode taxonomists. Yet in spite of their morphological similarity, a recent comparison of 338 orthologous genes (homologous genes derived from the same gene in the last common ancestor) resulted in an estimated date of divergence between the two species of 80–110 Mya (Stein *et al.,* 2003). This splitting event occurred 5-45 million years before the splitting of the mouse and human lineage (Waterston *et al.,* 2002). Whereas most of us feel comfortable in making the man mouse discrimination, this example sends a powerful message to those faced with identification of nematode species. Clearly, cryptic species must abound in the phylum Nematoda, and molecular techniques may be the only practical approach to recognize them.

3.2. Speed and Simplicity

Modern identification methods must provide accuracy, speed, reliability, affordability, and if possible, enable characterization of specimens new to science. Molecular approaches are becoming more widely applied to fulfill these new demands, in part because of the relative simplicity of their application in the laboratory. Most nematodes are approximately 1 mm in size and comprised of 1000 to 2000 cells. Conveniently, this size is suitable for a PCR reaction with a minimum effort required for sample preparation. There are no hard structural parts to a nematode that must be separated or circumvented to get at the DNA. In many cases, the nematode may simply be crushed in a droplet of water and a portion of the solution added directly to a PCR reaction (Powers To, Harris TS., 1993). Other preparation methods include a proteinase digestion and DNA purification step (Floyd *et al*., 2002, Stanton *et al*., 1998). Multiple amplifications (5-10) can routinely be achieved from a single nematode. The solution from a crushed nematode can be stored frozen indefinitely and thereby serve as a DNA voucher for future analysis.

3.3. Better targeting of problem Nematodes

With the pending elimination of general biocides, alternative strategies for managing nematodes by necessity must focus on control procedures for a specific nematode species, groups of species, or a sub specific taxa. Future diagnostic methods will require greater specificity across a broad range of nematode taxa.

3.4. Application to all life stages

Surveys of nematode communities illustrate the difficulties in species identification. For accurate identification, most keys require critical decisions on adult characteristics. Nematode populations often are comprised of a large percentage of juveniles. In groups such as Dorylaimida successful navigation of keys requires both sexes, yet specimens are found in such low frequency that species determinations must be made on a single sex. Molecular markers can, and in some situations must, be used to identify all life stages. For example, only molecular methods have proved to be reliable in identifying infective juveniles of *M. mayaguensis* in the soil (Brito *et al*., 2004).

3.5. Mixed Nematode populations

Seldom in an agro ecosystem is an individual plant fed upon by a single species of plant-parasitic nematode. Multiple plant-parasitic species are typically recovered from the roots of a single plant, and in some situations, the community composition of plant-feeding nematodes in an agronomic soil reflects the specific sequence of cropping systems and rotation schemes over the course of several years. Monitoring the identity and abundance of all the nematodes in the soil community has been advocated as a key component in the modern vision of integrated pest management (Neher DA., 2001). Even when a subset of the nematode community is targeted for control, researchers are discovering that nematode diversity is greater than previously expected. For example, it appears that many agronomic soils contain mixtures of root-knot species (*Meloidogyne*), each species with its unique host ranges and life-history characteristics.

3.6. Emerging parasites

Improved molecular diagnostics will help to identify and address unforeseen threats to agricultural crops. A recent survey of nurseries and agronomic soils in southern Florida found nematode communities comprised of at least three nominal species of root-knot nematodes (Brito *et al*., 2004). One of the species recovered from Florida fields, *M. mayaguensis*, has the ability to reproduce on nematode-resistant rootstock, and had gone undetected due to its morphological similarity to *M. incognita* (and the fact that the common management practice, application of general biocides, did not require discrimination among nematode species).

3.7. Import and Export

The discrimination of nematodes at the species level becomes essential when agricultural commodities move across international borders. In 2000, the Nematology Laboratory in the California Department of Food and Agriculture made an average of 15 quarantine-related identifications each day (Luna and Chitambar, 2003). Quarantine regulations target dozens of species depending on the commodity. Many of these regulated species are members of extremely large genera containing numerous described species. In the case of regulated species such as the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*, and the Columbia root-knot nematode *M. chitwoodi*, the presence of a single individual can lead to the rejection of an entire shipment of potatoes intended for export. That one individual may exist among numerous nontarget, but closely related, species. In order to address quarantine and management problems, speed, sensitivity, accuracy, specificity, and broad applicability will be required features of any prospective nematode identification protocol.

4. *Meloidogyne*

Siddiqi (2000) lists 80 species of *Meloidogyne*. It is probably the most economically important group of nematodes, and the need to identify the infective juvenile stages places them among the most difficult plant-parasitic nematodes to identify. The genus includes highly damaging, cosmopolitan species as well as extremely localized species of unknown economic significance. *Meloidogyne* species are generally considered major problems of tropical crops, although several species are well adapted to colder climates. A relatively small subset of the described species has been examined by molecular methods. The genetic diversity within *Meloidogyne* becomes apparent when species outside the mitotic parthenogenetic "trio" of similar species, *M. arenaria*, *M. incognita*, and *M. javanica*, are compared. De Ley *et al.* (2002) included 14 *Meloidogyne* species in a phylogenetic comparison using nearly complete 18S rDNA sequence (1577–1597 bp). Within the genus there was up to 10% sequence divergence in pairwise comparisons among species, corresponding to considerable diversity for a relatively conserved gene. Positive from a diagnostic perspective are 1–89 autapomorphies, characters unique to a taxon, observed among this collection of species. These unique character states at polymorphic sites in the 18S gene can be exploited in the design of species-specific primer binding sites. Although the investigators only used a single isolate to represent each species in this study, validation through the comparison of additional isolates would support the 18S sequence as a barcode of sufficient variation to identify *Meloidogyne* species. Chen *et al*. (2003) focused on the D3 expansion region in the 28S ribosomal gene from the trio of mitotic parthenogenetic species plus seven mitotic parthenogenetic isolates of *M. hapla*. Sequence analysis of the 350-bp region showed a fundamental division between the isolates of *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla*. Species boundaries among the trio were obscured by the possession of five different D3 haplotypes among the three species, with one haplotype found in isolates of all three species. Four D3 haplotypes were found among isolates of *M. incognita*. A single D3 haplotype was recovered from *M. javanica*. This study sheds light on the origin of the mitotic parthenogenetic species and possible hybridogenetic speciation events, but suggests caution in applying the D3 region alone as a species diagnostic. The internal transcribed spacer region (ITS) and the intergenic spacer region (IGS) display considerably more variation among *Meloidogyne* species than does the D3 region. Petersen & Vrain (1996) demonstrated that PCR primers designed to amplify IGS could discriminate between the facultatively parthenogenetic species *M. chitwoodi* and the morphologically indistinguishable parasite of potato, *M. fallax*. A 100-bp size difference in the amplified IGS product separated the two species, and *M. hapla* IGS was approximately 2 kb smaller than both species. Four isolates of *M. hapla*, three of *M. chitwoodi*, and three of *M. fallax* were used in this study. Zijlstra *et al*. (1995) examined 25 isolates of *M. hapla*, *M. chitwoodi*, and *M. fallax* and determined that restriction digestion of the amplified ITS product could differentiate among the three species. Using these three species, mixed species populations were tested by digesting the amplified ITS product from DNA extracted from the species mixtures. Good correspondence was observed between the ratio of digestion fragments derived from the different species and the known species frequency in the mixtures (Zijlstra *et al*., 1995). ITS may perform well as a diagnostic for *Meloidogyne* species that reproduce sexually or by facultative parthenogenesis, but it is clear from the research of Hugall *et al*. (1999) that the use of the ITS region for identification of the mitotically parthenogenetic species could easily lead to misdiagnosis. Earlier, Hugall *et al*. (1994, 1997) demonstrated that mitochondrial haplotypes completely corresponded to groupings based on esterase isozyme phenotypes. This strict correlationwas recently

demonstrated in an examination of 46 *Meloidogyne* populations from 14 provinces in China (Xu *et al*., 2004). According to the designations of Hugall *et al*. (1997), mitochondrial haplotype B corresponded to the I1 esterase pattern of *M. incognita*, haplotype D to the J3 esterase pattern of *M. javanica*, and *M. arenaria* had two haplotypes, A, which corresponded the A1 esterase pattern, and C, which was associated with esterase pattern A2 and A3. The ITS region, however, displays remarkable patterns of variation, undoubtedly the echo of a hybrid ancestry. There are two divergent and distinct clusters of ITS variants found within these three species. The divergence between ITS clusters ranges from 14% to 18%. Variants from both clusters can be recovered from isolates containing each of the mitochondrial haplotypes. Hugall *et al*. (1999) examined one ITS cluster in depth by sequencing multiple clones from isolates representing the four mitochondrial haplotypes. In a total of 151 clones they recovered 89 different ITS variants with an average total nucleotide diversity of 7.2%. Typically, an individual of these three species possesses 9–13 ITS variants. As in the D3 region, patterns of ITS variation may help sort out potential parents in a hybrid speciation event, but they will be misleading as a species diagnostic.

The unusual location of the 5S ribosomal gene in the IGS region of *Meloidogyne* inspired Blok *et al.* (1997) to design primers in the 5S gene and the 5_ portion of the 18S gene to assess polymorphism. They discovered that the amplified product of the tropical root-knot nematode *M. mayaguensis* differed sufficiently from other species found in the tropics to sort species by product size on agarose gels. The mitotically parthenogenetic triowas nearly identical in sequence, with *M. incognita* exhibiting a single substitution and deletion for the 715-bp region when compared with *M. arenaria* and *M. javanica*. The latter two species displayed intraspecific variation among cloned IGS products. The mitochondrial genome shows extensive variation in *Meloidogyne*. Powers & Harris (1993) investigated a region of the mitochondrial genome between the 5_ portion of the cytochrome oxidase subunit II (COII) gene and the large (16S) ribosomal gene. Between these two genes lies the tRNA histidine and an intervening region characterized by insertions or deletions resulting in size polymorphism when amplified by PCR primers in the flanking genes. At least four major size classes have been identified (Blok *et al*., 2002, Orui Y., 1998, Powers TO, Harris TS., 1993, Stanton *et al*., 1997). The smallest class, in which only a few to 60 nucleotides comprise the intervening sequence, is found in sexually reproducing *Meloidogyne* species, most facultative parthenogenetic species, and mitotic parthenogenetic *M. hapla*. *M. mayaguensis* has two copies of the tRNA histidine gene, creating an amplification product approximately 160 bp larger than *M. hapla* (Blok *et al*., 2002). *M. arenaria* haplotype A (esterase AI) has an intervening sequence 370 bp larger than *M. mayaguensis*, but approximately 600 bp smaller than mitochondrial haplotypes B, C, and D, which correspond to the mitotically parthenogenetic species *M. incognita*, *M. arenaria*, and *M. javanica*. Stanton *et al*. (1997) outlined a PCR-RFLP diagnostic protocol based on restriction site polymorphism that could distinguish six closely related haplotypes found in *Meloidogyne* species. Okimoto *et al*. (1991) characterized a unique region of noncoding tandem repeats in the *Meloidogyne* mitochondrial genome and suggested that it might serve to identify species and host races. Due to the distribution of multiple variants within individuals, the series of 63-bp repeats has been more useful in population studies, where it has been used to partition variation at different hierarchical levels of the mitotically parthenogenetic species (Dautova *et al*., 2002, Lund HD, Whipple EL, Hyman CB., 1998, Whipple LE, Lunt DH, Hyman BC., 1998). This region recently was recognized for its diagnostic potential by its ability to discriminate *M. mayaguensis* based on the consistent presence of a single 322-bp size product when amplified with primers binding to the flanking tRNA serine and noncoding region (Blok *et al*., 2002). Using the same primer set, the mitotically parthenogenetic species produce multiple amplification products of varying intensity, or no product as in the case of *M. hapla*.

Most of the diagnostic protocols mentioned above, based on mitochondrial DNA, will be limited in application as barcode technology since they exploit unique characteristics of the *Meloidogyne* mitochondrial genome. Differences in mitochondrial gene order limit approaches that use primer sets that cross gene boundaries, and the amplification of dynamically changing noncoding repeated sequences makes phylogenetic analysis extremely difficult. There has been no systematic survey of COI sequences among plant-parasitic nematodes, although the proof-of-concept papers of Herbert *et al*. (2003, 2003) and a recent analysis of the tylenchid insect associate *Howardula* suggest COI will provide good species resolution (Perlman *et al*., 2003). Anonymous loci in *Meloidogyne* have been evaluated through RAPD PCR and AFLP approaches (Blok VC, Phillips MS, Fargette M., 1997, Dautova *et al*., 2002, Fargette *et al*., 1996, Guirao P, Moya A, Cenis JL., 1995, Williamson *et al*., 1997). Notable among these studies is the conversion of species-specific amplified products to sequence-tagged sites for the identification of *M. chitwoodi* and *M. hapla* (Williamson *et al*., 1997). Their multiplex diagnostic assaywas tested with 7 North American isolates of *M. hapla* and 14 isolates of *M. chitwoodi* from the U.S. Pacific Northwest. PCR produced uniquely sized amplification products in reactions using single juvenile nematodes. Additional testing with other closely related species will further validate this assay.

5. *Heterodera* and *globodera*

Heterodera and *Globodera* are generally considered pests of temperate regions, although approximately one third of the 65 described species of *Heterodera* are known from the tropics (Siddiqi MR., 2000). The potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, are among the most heavily regulated nematodes in agricultural commerce. The resistant cyst stage contributes to their widespread dispersal. The cyst nematodes currently may be the best-characterized group of plant parasitic nematodes for application of DNA barcoding. Largely through the systematic survey work of Subbotin and coworkers, there exists an extensive database of heteroderid ITS sequences in GenBank (Amiri S, Subbotin SA, Moens M., 2002, Clapp *et al*., 2000, Subbotin *et al*., 2003, Zeng *et al*., 2000). Over one third of the described species have a representative ITS sequence deposited in GenBank, and phylogenetic analysis suggests a substantial number of new species may be recovered in molecular surveys (Subbotin *et al*., 2003). Descriptions of new species, however, may require a better understanding of intraspecific variation in *Heterodera*. Most of the recent studies have incorporated ITS-based PCR-RFLP approaches to species identification or used multiplex primer sets, one set as a positive control for amplification and a second set that includes a species-specific primer. Accordingly, these studies have assessed intraspecific variation by evaluating the presence or absence of key restriction sites in the case of PCR-RFLP or primer binding sites in the multiplex PCR assays. For example, Subbotin *et al*. (2001) tested 53 isolates of *H. glycines* using a multiplex assay that included a *H. glycines*-specific primer that bound to a region in ITS2. The primer-binding site in *H. glycines* differed from closely related *H. medicaginis* only in the final 3_ nucleotide of the primer. Amplification products were only produced in the 53 isolates of *H. glycines*. A similar multiplex assay was conducted with similar results for 37 isolates of *H. schachtii* (Amiri S, Subbotin SA, Moens M., 2002). These studies demonstrate that our delimitation of species boundaries for those cyst nematodes studied to date are supported by specific fixed nucleotides that can be exploited for species identification. Phylogenetic analyses further support the grouping of these species into familiar "species complexes" characterized, in part, by host associations (Subbotin *et al*., 2001, Tanha *et al*., 2003). Subbotin *et al*. (2003) conducted a remarkably thorough analysis of the *H. avenae* species complex, generating 70 ITS sequences from isolates providing worldwide representation of the group. Not surprisingly, extensive variation was observed among the nine species comprising the species complex, and isolates identified as *H. avenae* included several discretely different genotypes. PCR-RFLP and sequencing studies clearly demonstrate that intraspecific variation is common in the ITS region and likely to uncover new species. This variation has also been evidenced as bands of light intensity observed when restriction digestion products are separated on agarose gels or as variant sequences derived from cloned amplified PCR product (Subbotin *et al*., 2000, Tanha *et al*., 2003). In the heteroderid ITS region 0.5%–1.5% intraspecific sequence variation is not uncommon. How this variation is partitioned within individuals, among individuals, or among populations is not yet well understood. *Globodera rostochiensis* and *G. pallida* can be discriminated by several approaches. Subbotin *et al*. (2000) identified three restriction enzymes that differentially digest the ITS region producing patterns consistent with perceived species boundaries. These boundaries are supported by a phylogenetic analysis of 41 *Globodera* and related ITS nucleotide sequences, providing an evolutionary framework for species delimitation (Subbotin *et al*., 2000). Four distinct clades were identified for members of the genus parasitizing solanaceous plants, corresponding to *G. pallida*, *G. rostochiensis*, *G. tabacum*, and an unidentified species from South America. The D3 expansion region exhibited no nucleotide differences between the two potato cyst species. Mulholland *et al*. (Mulholland *et al*., 1996) designed a multiplex PCR assay for both species using species-specific primers located in ITS1. Bulman & Marshall (Bulman SR, Marshall JW., 1997) also designed a multiplex PCR reaction from ITS to distinguish the two potato cyst species. Stratford & Shields (Stratford *et al*., 1992) used primers specific for a *trans*-spliced leader gene and the 5S ribosomal gene to develop a PCR assay based on amplified product size differences in the two species. A remarkable discovery in *Globodera* is the unique structural organization of the mitochondrial genome of *G. pallida*. Armstrong *et al*. (2003) determined that unlike typical higher metazoa, which possess a single, circular mitochondrial genome, *G. pallida* possesses six or more small circular genomes ranging in size from 6.3–9.5 kb.

6. *Pratylenchus* and *nacobbus*

Pratylenchus species are distributed worldwide. Siddiqi (2000) lists 89 species in the genus. They are all identified by exceptionally subtle morphological characteristics, most often observed in the adult stages. Key diagnostic features such as number of lip annules, shape of the tail terminus, relative length of the esophageal gland overlap, length of the post uterine sac, and even lateral line number may be difficult to determine. Variability of these characters compounds the identification problem. Waeyenberge *et al*. (2000) characterized 18 species of *Pratylenchus* by PCR-RFLP of the ITS region. A minimum of two enzymes are necessary for discrimination of this collection of species. The conclusions in this study must be considered preliminary since 10 of the species were represented by a single isolate.Aremarkable ITS size difference exists within the genus; approximately 350 bp separate the smallest and largest amplified ITS region among the species. Intraspecific

ITS variation has been observed in *Pratylenchus*, particularly within isolates of *P. coffeae* (Orui Y., 1996, Uehara *et al*., 1998, Waeyenberge *et al*., 2000). Difficulties in the identification of *P. coffeae* were addressed by Duncan *et al*. (1999) and underscore the necessity of maintaining specimen vouchers that can be reexamined if necessary by additional molecular markers. Carta *et al*. (2001) used the D3 region of the 28S ribosomal gene in a phylogenetic analysis of 19 *Pratylenchus* species. Seventeen of the species had unique D3 sequences, although only one species was represented by more than a single isolate. Carta *et al.* (2001) also highlight some of the problems in selecting representative isolates of certain nominal species. This study, together with an earlier examination by Al-Banna *et al*. (1997) of 10 *Pratylenchus* species using D3 clearly illustrate difficulties associated with assessing species boundaries and relationships in a poorly characterized genus comprised of amphimictic, parthenogenetic, and polyploid species. *Nacobbus*, the false root-knot nematode, is a regulated species in which species determinations shape quarantine decisions. A turbulent taxonomic history and evidence of physiological variation led to the belief that *N. aberrans*, the primary pest species in the genus, is actually a species complex (Ibrahim *et al*., 1997, Jatala P., 1993, Jatala P, Golden M., 1977). Recent ITS sequencing from 12 isolates from South America and Mexico tends to confirm the existence of distinct subgroups (Reid A, Podrucka K., 2004). Missing from these analyses are crucial North American biotypes and representative of the type species, *N. dorsalis*.

7. *Xiphinema*

The dorylaim plant-parasites pose special challenges for molecular diagnostics. They are found in relatively low frequency in samples, multiple species are typically present in fields, and it is important to identify potential virus vectors. Wang *et al*. (2003) designed sets of primers from ITS sequences to identify four species of *Xiphinema* known to transmit virus. These primer sets worked well in multiplex combinations in amplifications from individual specimens. Hubschen *et al.* (2004) extended the testing of these primers to include dilution series of vector: nonvector *Xiphinema* DNA at levels of 1:800. The primers were further tested in experiments in which a single vector species was added to a community of nematodes extracted from the soil. Following bulk DNA extraction the vector-specific primers were able to detect the presence of the vector specimen. The recent studies of Hubschen *et al*. (2004) and others working with nematode virus vectors are notable in the effort spent in tests of validation for the diagnostic markers.

8. Summary

Molecular methods of identification provide accurate, reliable diagnostic approaches for the identification of plant-parasitic nematodes. The promising and attractive results have generated increasing demands for applications in new fields and for better performing techniques. Initially, the techniques were used solely for taxonomic purposes, but increasingly became popular as a component of diagnostic information. Diagnostic procedures are now available to differentiate the plant-pathogenic species from related but non-pathogenic species. The microscopic size of plant parasitic nematodes poses problems and techniques have been developed to enrich samples to obtain qualitative and quantitative information on individual species. In addition, techniques are available to evaluate single nematodes, cysts or eggs of individual species in extracts from soil and plant tissue. DNA or RNA-based techniques are the most widely used approaches for identification, taxonomy and phylogenetic studies, although the development and use of other methods has been, and in some cases still is, important. DNA barcoding and the extraction of DNA from preserved specimens will aid considerably in diagnostic information. Moreover, one message emerging from current diagnostic research is that the ITS region has sufficient information to diagnose most nematode species. There are, however, cases where ITS will misdiagnose species, such as the mitotically parthenogenetic species of *Meloidogyne* and other species of recent hybrid origin. For greater confidence in species delimitation and ultimately successful barcoding, additional unlinked genetic regions are necessary for better diagnostic coverage of the phylum. The mitochondrial gene COI is one good candidate that would complement ITS as a locus unlinked to the nuclear ribosomal gene region. Additionally, COI could help resolve questions of parentage in species of hybrid origin and link to ongoing barcoding efforts in other groups of animals. The 18S gene is surprisingly informative at the species level in Nematoda and has the advantage of direct incorporation into the Nematode Tree of Life. As a barcode of slightly coarser taxonomic resolution, 18S can readily be incorporated into current studies of nematode biodiversity. Above all, it must be recognized that species determinations have an essential practical application in nematology. Regulatory decisions depend on quick, accurate identification. Increasingly, economic management options require greater resolution in nematode identification.

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