9 Molecular Diagnostics of Plant-parasitic Nematodes

R.N. Perry, S.A. Subbotin and M. Moens

Abstract
Biochemical and molecular methods of identification provide accurate, reliable diagnostic approaches for the identification of plant-parasitic nematodes. Initially, the techniques were used solely for taxonomic purposes, but increasingly became popular as a component of diagnostic information for farmers, growers and advisors. 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. Background information on early, pioneering work is presented as a prelude to discussion of diagnostic approaches. These include the use of isoelectric focusing (IEF) and restriction fragment length polymorphisms (RFLPs), progressing to antibody approaches and current polymerase chain reaction (PCR)-based techniques. 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 bar coding and the extraction of DNA from preserved specimens will aid considerably in diagnostic information and these are discussed in the context of the future requirements of accurate and rapid diagnostic protocols.

Introduction

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

 Biotechnology and Plant Disease Management
(eds Z.K. Punja, S.H. De Boer and H. Sanfaçon)

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. Biochemical and molecular methods of nematode identification provide accurate, alternative diagnostic approaches.

For growers and extension workers, economically important species of plant-parasitic nematodes have to be detected from soil samples prior to planting. Frequently, closely related and morphologically similar species are present as mixtures in soil samples and it is vital that diagnostic procedures are available to differentiate the 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. To assess damage after plant growth, nematodes have to be extracted from plant tissue and techniques have been developed to overcome difficulties associated with this approach. This chapter will indicate the techniques used to evaluate individual nematodes, cysts or eggs of individual species in extracts from soil and plant tissue. The goal of growers and extension workers is on-site diagnostics, and this is now a realistic objective.

Plant health agencies also need molecular techniques to aid the identification of pathogenic nematodes. The implementation of phytosanitary measures is of major importance in reducing the adverse impact of plant-parasitic nematodes (Hockland *et al.*, 2006). Nematodes that may be of phytosanitary importance are intercepted by plant health inspectors at points of entry of goods into a country. Frequently, these nematodes are species that have the potential to become significant pests if allowed to enter and establish. Internationally standardized diagnostic protocols are needed to limit or avoid the spread of these species, and rapid and accurate molecular techniques are especially important where morphological identification is difficult or where only immature specimens have been intercepted.

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.

Background

The work of Webster and Hooper (1968) was among the first papers on differentiation of plant-parasitic nematodes which used serological techniques to examine differences between species of the genera *Heterodera* and *Ditylenchus*. Electrophoretic methods separate proteins based on their size or isoelectric point. Trudgill and Carpenter (1971) used polyacrylamide gel electrophoresis (PAGE) to differentiate pathotypes of potato cyst nematodes (PCN), which paved the way for the subsequent description of a new species, *Globodera pallida*, and its differentiation from *Globodera rostochiensis*. Isoelectric focusing (IEF) was used by Fleming and Marks (1982) to separate and quantify the two PCN species in mixed populations. Bakker and Gommers (1982) used two-dimensional electrophoresis combined with protein analysis as a further refinement for differentiation of PCN species. The two species of PCN are economically important pests, mainly in Europe. Globally, species of the root-knot nematode genus, *Meloidogyne*, are of primary economic importance. In the USA, differentiation of *Meloidogyne* spp. was achieved by Esbenshade and Triantaphyllou (1985) using PAGE combined with staining for esterases, a technique still in routine use. Ibrahim and Perry (1992) showed that this approach could be used with *Meloidogyne* females in galled roots, thus obviating the need to separate nematodes from the host tissue. This technique was used on other genera of plant-parasitic nematodes, such as *Aphelenchoides* (Ibrahim *et al.*, 1994). Monoclonal antibodies (MAbs) were used by Schots *et al.* (1986) as a more sensitive technique to differentiate between PCNs. Robinson (1989) used MAbs in a quantitative assay for *Meloidogyne*. The separation of *Meloidogyne incognita* has been achieved using MAbs raised to non-specific esterases, an approach that avoids the need to separate the esterases by electrophoresis (Ibrahim *et al.*, 1996). Antibodies are being used in a method to enhance detection of nematodes in soil samples (described later).

Isoelectric Focusing

Of the techniques mentioned above, IEF is currently being used extensively in many nematology diagnostics laboratories using the automated electrophoresis equipment, PhastSystem, developed by Pharmacia Corp. IEF is used routinely to differentiate the two PCN species, *G. rostochiensis* and *G. pallida*, based on the presence of species-specific bands (Karssen *et al.*, 1995); however, this technique has not yet been tested against other morphologically similar *Globodera* spp., such as *G. artemisiae*, *G. millefolii* and *G. tabacum*. The IEF method may also be useful to differentiate other cyst nematodes (Sturhan and Rumpenhorst, 1996).

For routine identification of *Meloidogyne* spp., perineal patterns (the cuticular lines and associated structures surrounding the vulval region) are often used as the main diagnostic feature. However, species such as

M. paranaensis, *M. konensis* and *M. mayaguensis* have perineal patterns similar to *M. incognita* or *M. arenaria* and could be misidentified (Carneiro *et al.*, 2000). Carneiro *et al.* (2004) concluded that the perineal pattern can be used only as a complementary tool; enzyme characterization is important for checking the morphological consistency of the identification. Extensive enzymatic studies have demonstrated that *Meloidogyne* spp. can be differentiated by enzyme phenotypes (Esbenshade and Triantaphyllou, 1985; Fargette, 1987). Isozyme phenotypes of adult females, especially esterase and malate dehydrogenase, are species-specific and are useful as reliable characters for *Meloidogyne* identification (Esbenshade and Triantaphyllou, 1990; Carneiro *et al.*, 2004). Progress in electrophoretic procedures has made possible nematode identification from the protein extract of a single female in less than 2 h from the time the females are collected from infected plant roots (Esbenshade and Triantaphyllou, 1990). Descriptions of new species of root-knot nematodes are now usually accompanied by phenotypic profiles for both these isozymes (Castillo *et al.*, 2003; Karssen *et al.*, 2004; Carneiro *et al.*, 2005). One of the main disadvantages of the IEF method is its sensitivity to nematode age. Only young adult females are used for such diagnostics.

Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analyses thermolabile, non-volatile organic compounds, especially those of high molecular mass, and is used successfully in biochemistry for the analysis of proteins, peptides, glycoproteins, oligosaccharides and oligonucleotides. Identification is based on the generation of diagnostic MALDI-TOFMS protein profiles of the test organisms. In this technique, positively charged ions are generated and analysed after laser irradiation of the organism in extracts after disruption of cells, or in intact whole cells of the organism in the presence of an ultraviolet (UV)-absorbing matrix. Recently, MALDI-TOFMS was successfully used for nematode diagnostics (Navas *et al.*, 2002; Perera *et al.*, 2005). Perera *et al.* (2005) used two methods, grinding up nematodes and direct analysis of intact nematodes. After standardization, these methods were applied to analyse the seed-gall nematodes, *Anguina tritici* and *Anguina funesta*, and the root-knot nematode, *Meloidogyne javanica*. Typical protein profiles and diagnostic peaks were identified for these nematode species and for mixtures of *Anguina* spp. (Fig. 9.1). Compared with other biochemical methods, MALDI-TOFMS is a promising technique that enables rapid and reliable identification of plant-parasitic nematodes because sample preparation is simple and analysis is rapid. It could be also automated in terms of sample plate loading, generation of spectra (up to 400-place sample plates), and automated peak calling, leading to automation of species identification (Perera *et al.*, 2005).

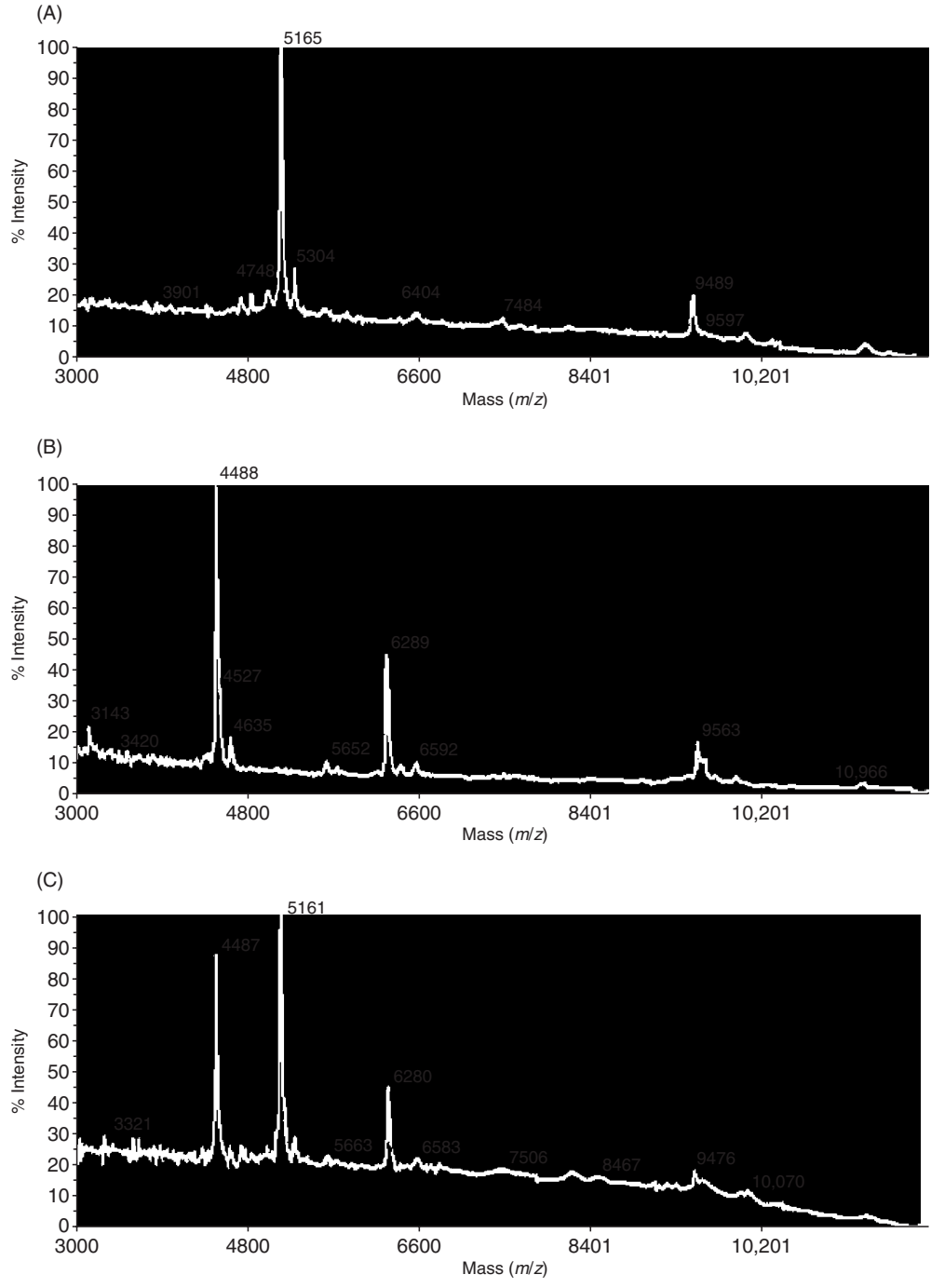

Fig. 9.1. MALDI-TOFMS protein profiles generated by direct analysis of intact nematodes: (A) *Anguina tritici*; (B) *Anguina funesta*; (C) *A. tritici* and *A. funesta* mixture (Perera *et al*., 2005).

Molecular Diagnostics

The first report of DNA-based techniques to identify plant-parasitic nematodes was published over 20 years ago. This seminal work by 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. For example, 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, 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 cross-reactivity 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 meth-

ods; the price for IEF testing was significantly higher than that for PCR or ELISA.

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.

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, 2003) using an antibody-based capture system (Fig. 9.2). 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).

DNA extraction

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).

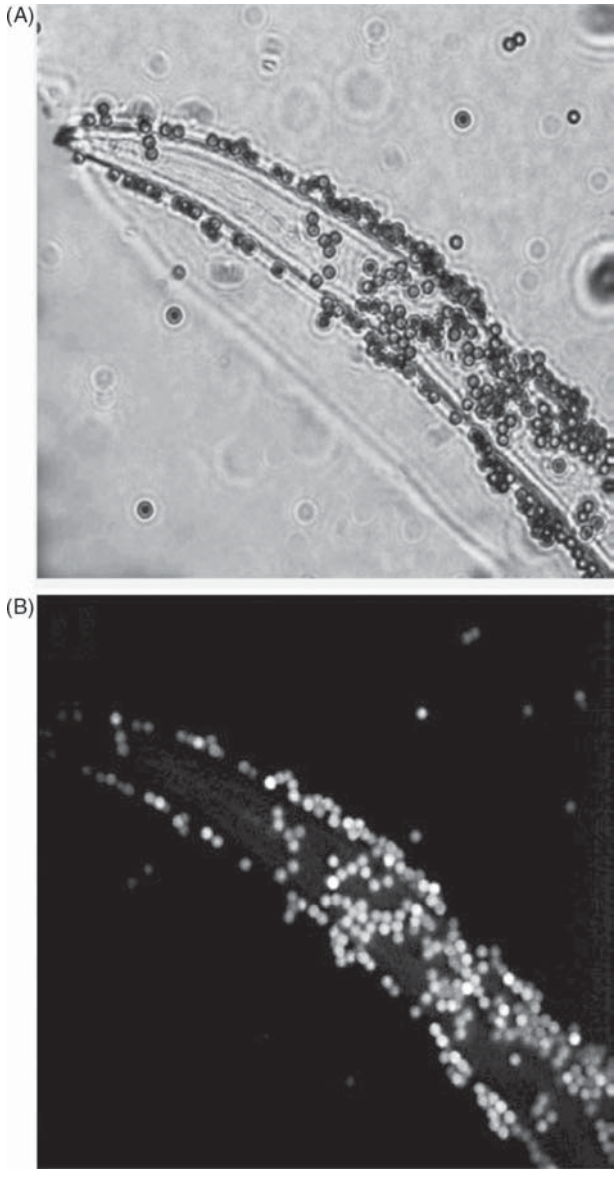

Fig. 9.2. *Meloidogyne arenaria* second-stage juveniles (J2) captured using immunomagnetic capture. J2 are coated with Dynabeads. (A) Bright field image; (B) fluorescence optics (Chen *et al.*, 2001).

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 formalin-fixed nematode specimens.

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 cyto-

chrome 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).

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 strand conformation polymorphism (SSCP) and real-time PCR.

PCR-RFLP

This method is used to diagnose monospecific samples and contains two steps: (i) amplification of gene marker region using universal primers (Table 9.1); and (ii) digestion of the resulting amplicon by restriction enzymes. PCR-based techniques have been used for diagnostics of many

Table 9.1. Universal primers useful for nematode diagnostics.

Code	Sequence (5'–3')	Target DNA region	References
C2F3 1108	GGT CAA TGT TCA GAA ATT TGT GG TAC CTT TGA CCA ATC ACG CT	Intergenic region between COII and 16S rRNA gene of mtDNA	Powers and Harris (1993)
18S rDNA1.58S	TTG ATT ACG TCC CTG CCC TTT GCC ACC TAG TGA GCC GCG CA	ITS1 of rRNA	Szalanski *et al.* (1997)
18S 26S	TTG ATT ACG TCC CTG CCC TTT TTT CAC TCG CCG TTA CTA AGG	ITS1-5.8S-ITS2 of rRNA	Vrain *et al.* (1992)
F194 F195	CGT AAC AAG GTA GCT GTA G TCC TCC GCT AAA TGA TAT G	ITS1-5.8S-ITS2 of rRNA	Ferris *et al.* (1993)
D2A D3B	ACA AGT ACC GTG AGG GAA AGT TG TCG GAA GGA ACC AGC TAC TA	D2-D3 region of 28S rRNA	De Ley *et al.* (1999)
TW81 AB28	GTT TCC GTA GGT GAA CCT GC ATA TGC TTA AGT TCA GCG GGT	ITS1-5.8S-ITS2 of rRNA	Joyce *et al.* (1994)
SSU18A SSU26R	AAAGATTAAGCCATGCATG CATTCTTGGCAAATGCTTTCG	18S rRNA	Blaxter *et al.* (1998)
Nem_18S_F Nem_18S_R	CGCGAATRGCTCATTACAACAGC GGGCGGTATCTGATCGCC	18S rRNA	Floyd *et al.* (2005)
18sl.2 18srb:	GGCGATCAGATACCGCCCTAGTT TACAAAGGGCAGGGACGTAAT	18S rRNA	Powers *et al.* (2005)

nematode groups (Table 9.2); here, we focus mainly on the applications for diagnosis of root-knot and cyst nematodes.

For diagnostics of root-knot nematodes, several gene markers, including intergenic region of mtDNA, nuclear ITS-rRNA, intergenic region of rRNA, 18S rRNA and D2–D3 expansion segment of 28S rRNA gene, have been developed and used. A detailed description of the discrimination of species of root-knot nematodes using PCR of the intergenic region located between the cytochrome oxidase subunit II (COII) and 16S rRNA (lRNA) of mtDNA has been provided by Powers and Harris (1993). It has been shown that the amplification length and RFLP of this fragment enable the separation of five major *Meloidogyne* spp. Subsequently, this region was studied intensively in several root-knot nematode species using PCR-RFLP and sequencing techniques (Hugall *et al.*, 1994, 1997; Williamson *et al.*, 1997; Orui, 1998; Stanton *et al.*, 1998; Blok *et al.*, 2002; Tigano *et al.*, 2005). Powers *et al.* (2005) described a modified, detailed procedure for PCR-RFLP identi-

Table 9.2. PCR-RFLP assays used for diagnosis of nematodes.

Nematode genera	Target DNA sequence	References
Heterodera	ITS of rRNA	Bekal *et al*. (1997); Szalanski *et al*. (1997); Orui (1997); Subbotin *et al*. (2000a); Tanha Maafi *et al*. (2003)
Globodera	ITS of rRNA	Fleming and Mowat (1993); Thiéry and Mugniéry (1996); Subbotin *et al*. (1999, 2000b)
Bursaphelenchus	ITS of rRNA	Hoyer *et al*. (1998); Iwahori *et al*. (1998); Braasch *et al*. (2001); Burgermeister *et al*. (2005)
Ditylenchus	ITS of rRNA	Wendt *et al*. (1993)
Aphelenchoides	ITS of rRNA	Ibrahim *et al*. (1994)
Pratylenchus	ITS of rRNA	Orui (1995); Orui and Mizukubo (1999); Waeyenberge *et al*. (2000)
Xiphinema	ITS of rRNA	Vrain *et al*. (1992)
Radopholus	ITS of rRNA	Elbadri *et al*. (2002)
Anguina	ITS of rRNA	Powers *et al*. (2001)
Meloidogyne	MtDNA	Powers and Harris (1993); Orui (1998); Blok *et al*. (2002); Xu *et al*. (2004); Powers *et al*. (2005)
Meloidogyne	ITS of rRNA	Zijlstra *et al*. (1995); Schmitz *et al*. (1998)
Nacobbus	ITS of rRNA	Reid *et al*. (2003)

fication of *Meloidogyne* spp. using two marker regions, a fragment of mitochondrial DNA located between the COII and 16S rRNA (Powers and Harris, 1993) and a partly amplified 18S ribosomal rRNA gene. The mitochondrial gene region provided greater species discrimination and revealed intraspecific variation among many isolates. The samples were amplified by C2F3/1108 primer set (Table 9.1), and the size of individual amplification products was determined in subsequent assays (Fig. 9.3).

Zijlstra *et al*. (1995) were the first to use the amplified ITS region of nuclear rRNA for discrimination of *M. hapla*, *M. chitwoodi* and *M. fallax* from each other and from *M. incognita* and *M. javanica* by using several restriction enzymes. Using another set of primers, Schmitz *et al*. (1998) proposed the simultaneous use of two restriction enzymes, *Hinf*I/*Rsa*I or *Dra*I/*Rsa*I, for identification of several species of root-knot nematodes. However, studies based on these techniques did not reveal diagnostic enzymes that facilitated the separation of *M. incognita* and *M. javanica* (Xue *et al*., 1993; Zijlstra *et al*., 1995; Schmitz *et al*., 1998). Subsequent sequencing of the ITS-rRNA regions of *M. incognita*, *M. javanica* and *M. arenaria* revealed that these sequences were almost identical (Hugall *et al*., 1999). Similar sequences were found in several other closely related species, including *M. hispanica*, *M. morocciensis* (De Ley *et al*., 1999) and *M. mayaguensis* (Brito *et al*., 2004). Thus, unlike mtDNA, using the ITS-rRNA does not enable

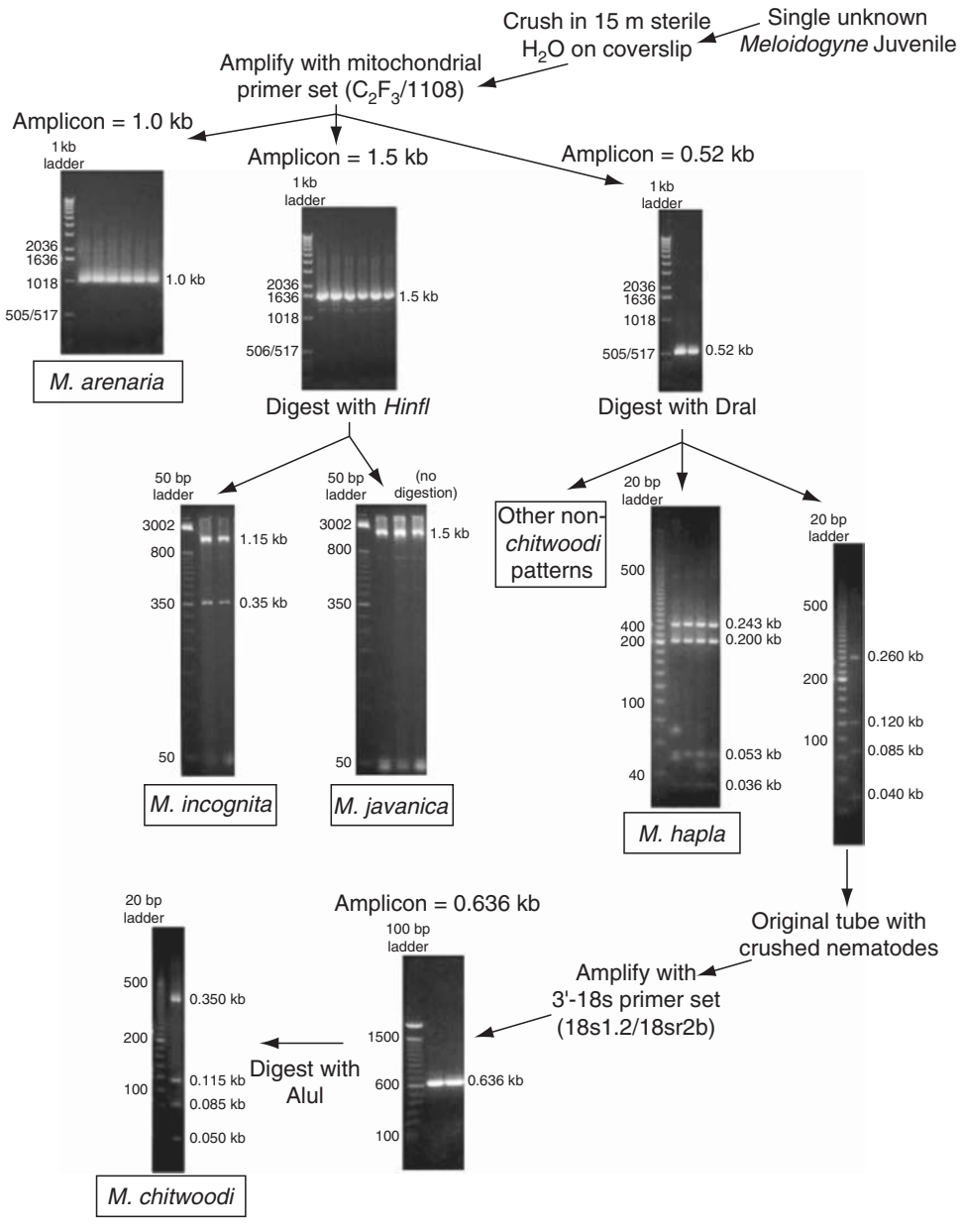

Fig. 9.3. Flow chart depicting the PCR-RFLP steps to identify *Meloidogyne* spp. (Powers *et al.*, 2005).

several agricultural important species of root-knot nematodes to be distinguished.

After sequencing nuclear intergenic rRNA spacer in *M. arenaria*, Vahidi and Honda (1991) found that it contained 5S-rRNA gene dividing the intergenic spacer into two spacers, one of them having nucleotide repeats. In one

species, the numbers of such repeats was variable, even within the genome of a single individual, whereas in some other species, the length of intergenic rRNA spacers was constant and could be used for species discrimination. Consequently, Petersen and Vrain (1996) proposed that the length of this spacer could be used to diagnose *M. chitwoodi*, *M. hapla* and *M. fallax*. Diagnostics of cyst nematodes is based on interspecific variation of the ITS-rRNA gene. After sequencing the rRNA gene from several nematodes, Ferris *et al.* (1993, 1994) suggested that the ITS region was conserved for populations of the same species and was sufficiently variable between species to distinguish among species of cyst nematodes. Subsequently, Szalanski *et al.* (1997) successfully applied PCR-RFLP of the ITS1 to differentiate *H. cruciferae*, *H. glycines*, *H. trifolii*, *H. schachtii*, *H. goettingiana* and *H. zeae*. This approach was also used to discriminate species from the Avenae group (Bekal *et al.*, 1997) and other cyst nematodes (Orui, 1997; Fleming *et al.*, 1998).

A comprehensive analysis of PCR-RFLP of the ITS-rRNA of cyst nematodes has been conducted by Subbotin and co-authors and published in a series of articles (Subbotin *et al.*, 1997, 1999, 2000a, 2003; Eroshenko *et al.*, 2001; Amiri *et al.*, 2002; Tanha Maafi *et al.*, 2003; Madani *et al.*, 2004). In these studies, universal TW81 and AB28 primers were used, which generated an amplicon of 1060 bp length, although this could vary in different species. Application of the RFLP technique to separate 26 species of cyst nematodes revealed several restriction enzymes, *Cfo*I, *Alu*I, *Bsu*RI, *Bsh*1236I and *ScrF*I, with greater discriminatory powers than other enzymes and which enabled more species of cyst nematodes to be characterized (Fig. 9.4).

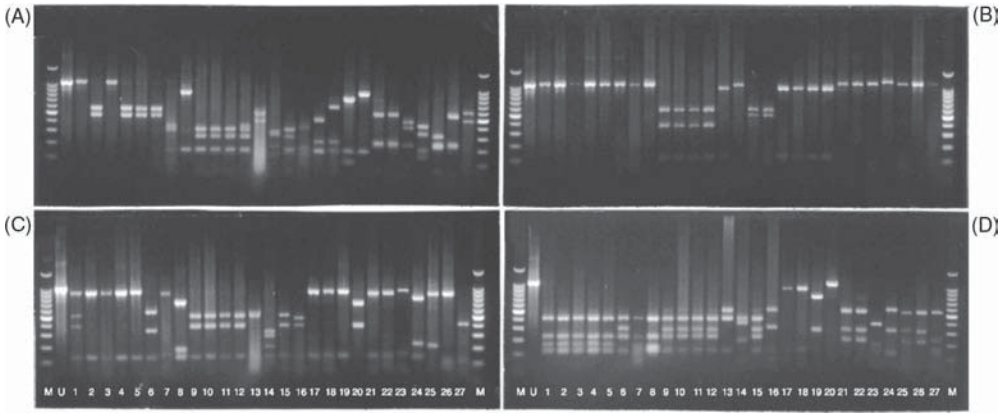

Fig. 9.4. Restriction fragments of amplified ITS regions of cyst-forming nematodes using TW81 and AB28 primers. Restriction enzymes: (A) *Alu*I; (B) *Ava*I; (C) *Bsh*1236I; (D) *Bsi*ZI. Lines: M – 100 bp DNA ladder (Promega, USA); U – unrestricted PCR product; 1 and 2 – *Heterodera avenae*, 3 – *H. arenaria*, 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 – *H. cardiolata*, 24 – *H. cyperi*, 25 – *H. goettingiana*, 26 – *H. urticae*, 27 – *Meloidodera alni* (Subbotin *et al.*, 2000a).

For example, *Cfo*I produced 16 polymorphic profiles enabling differentiation of 12 out of 26 species. Some enzymes produced species-specific profiles; for example, *Mva*I produced a specific profile for *H. schachtii*. Restriction of PCR product by seven enzymes usually enabled the most important species of cyst nematodes of the genus *Heterodera* to be distinguished from each other and from their sibling species (Fig. 9.4) (Subbotin *et al.*, 2000a). RFLP of the ITS region has been used to differentiate species of the genus *Globodera* parasitizing solanaceous plants (Fleming and Mowat, 1993; Szalanski *et al.*, 1997; Thiéry and Mugniéry, 1996; Fleming and Powers, 1998). For example, the two species of PCN could be distinguished by at least eight restriction enzymes (Subbotin *et al.*, 2000b).

PCR with specific primers

This type of PCR constitutes a new step in the development of DNA diagnostics and enables the detection of one or several species in a nematode mixture by a single PCR test, thus decreasing diagnostic time and costs. Diagnostics using PCR with specific primers has been developed for a wide range of plant-parasitic nematodes (Table 9.3). In multiplex PCR, several primer sets are utilized for amplification of several target genes. Such an approach has been developed for diagnostics of *H. glycines* (Fig. 9.5) (Subbotin *et al.*, 2001), *H. schachtii* (Amiri *et al.*, 2002) and *Bursaphelenchus xylophilus* (Jiang *et al.*, 2005). Multiplex PCR for detection of these species

Table 9.3. PCR with specific primer assays used for diagnosis of nematodes.

Nematode species and genera	References
Heterodera glycines	Subbotin *et al.* (2001)
Heterodera schachtii	Amiri *et al.* (2002)
Globodera rostochiensis and *G. pallida*	Mulholland *et al.* (1996); Bulman and Marshall (1997); Fullaondo *et al.* (1999)
Bursaphelenchus xylophilus	Liao *et al.* (2001); Matsunaga and Togashi (2004); Kang *et al.* (2004); Jiang *et al.* (2005); Leal *et al.* (2005); Castagnone *et al.* (2005)
Ditylenchus dipsaci	Esquibet *et al.* (2003); Subbotin *et al.* (2005)
Pratylenchus spp.	Uehara *et al.* (1998); Al-Banna *et al.* (2004)
Xiphinema spp.	Hübschen *et al.* (2004a)
Longidorus spp.	Hübschen *et al.* (2004b)
Paratrichodorus and *Trichodorus*	Boutsika *et al.* (2004)
Meloidogyne incognita	Zijlstra (2000); Dong *et al.* (2001); Randing *et al.* (2002)
Meloidogyne hapla	Williamson *et al.* (1997); Zijlstra (1997, 2000); Dong *et al.* (2001)
Meloidogyne chitwoodi	Williamson *et al.* (1997); Petersen *et al.* (1997); Zijlstra (2000)
Meloidogyne javanica	Zijlstra *et al.* (2000); Dong *et al.* (2001)
Meloidogyne arenaria	Zijlstra *et al.* (2000); Dong *et al.* (2001)
Meloidogyne naasi	Zijlstra *et al.* (2004)
Nacobbus spp.	Atkins *et al.* (2005)

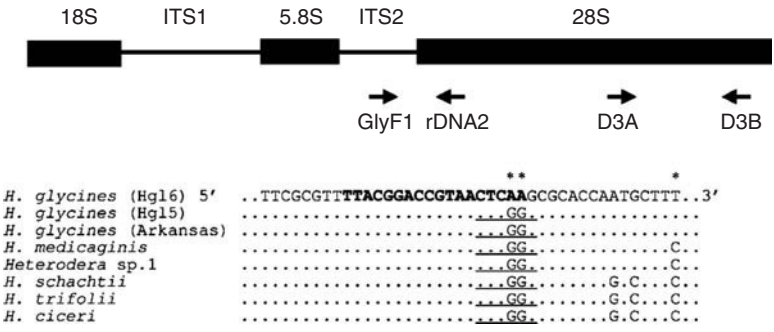

Fig. 9.5. Schematic representation showing positions of four primers in the rRNA genes used in the duplex PCR for identification of *Heterodera glycines* and ITS2 fragment alignment used for development of the species-specific primer (GlyF1), which is indicated in bold (Subbotin *et al.*, 2001).

includes two sets of primers: (i) the first is to amplify an internal control (e.g. universal primers for the expansion regions of 28S rRNA gene) confirming the presence of DNA in the sample and the success of PCR; and (ii) the second, including species-specific primer, is targeted to nematode DNA sequence of interest (Fig. 9.6). Nested PCR in a multiplex assay with specific SCAR primers has been developed for the diagnosis of several species of root-knot nematodes (Zijlstra, 2000).

PCR-SSCP

The PCR-SSCP technique can detect single nucleotide changes in the DNA fragments being studied due to the altered conformation mobility of the single strands of DNA during electrophoresis with a non-denaturing gel. Clapp *et al.* (2000) tested the PCR-SSCP method at two coastal dune locations in the Netherlands to determine the population structure of cyst nematodes. The ITS2 region was sufficiently variable within the taxa investigated to allow species to be separated on the basis of minor sequence variation. The PCR primers used in this study were effective for several species of cyst and root-knot nematodes.

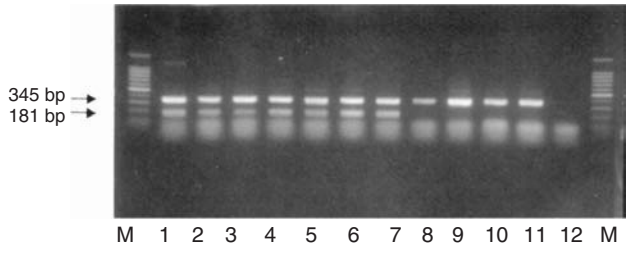

Fig. 9.6. Gel with PCR products (181 and 345 bp) generated by two sets of primers (GluyF1 + rDNA2 and D3A + D3B), respectively. Line: M – 100 bp DNA ladder (Promega, USA); 1–7: *Heterodera glycines*, 8: *H. schachtii*; 9: *H. ciceri*; 10: *H. medicaginis*; 11: *H. cajani*; 12: sample without nematode DNA (Subbotin *et al.*, 2001).

Real-time PCR

The PCR technique also permits the estimation of the number of nematodes in a sample. This has been successful only for nematodes of the same stage, e.g. second-stage juveniles of cyst nematodes and fourth-stage juveniles of *D. dipsaci*. It may be more difficult when trying to quantify nematodes of different stages. This technique indirectly measures the nematode number by assuming that the number of target DNA copies in the sample is proportional to the number of targeted nematodes. The real-time technique allows continuous monitoring of the sample during PCR using hybridization probes (TaqMan, molecular beacons) or dyes such as SYBR Green I. SYBR Green binds only to double-stranded DNA and becomes fluorescent only when bound. This dye has the virtue of being easy to use because it has no sequence specificity and it can be used to detect any PCR product. However, this virtue has a drawback, as the dye binds also to any non-specific product, including primer dimers. To overcome this problem, the melting curve analysis can be employed. The products of PCR reaction are melted by increasing the temperature of the sample. The non-specific product tends to melt at a much lower temperature than the longer specific product. Both the shape and position of the DNA melting curve area are a function of the GC/AT ratio. The length of amplicon can be used to differentiate amplification products separated by less than 2°C in T_m (the melting temperature) (Fig. 9.7).

Bates *et al.* (2002) were the first to use real-time PCR with SYBR Green I with plant-parasitic nematodes, when they used this technique to detect PCN. These authors found relatively large (4°C) differences in T_m between specific PCR products of *G. rostochiensis* and *G. pallida* amplified using the species-specific primers of Bulman and Marshall (1997). These differences ensure that melting peaks of these two products can be clearly distinguished in a multiplex reaction. By calculating the ratio of the melting peak height at T_m of each product and comparing it to the standard run under the same conditions, it was even possible to estimate the proportion of each product in the nematode mixture and finally to determine the ratio of juveniles of these two species in the sample. However, this method has not been applicable for absolute quantification of these nematodes. After testing, Madani *et al.* (2005) improved this approach and described methods for quantification of *G. pallida* and *H. schachtii* juveniles in a sample using the set with universal and species-specific primers. Validation tests showed a high correlation between real numbers of second-stage juveniles in a sample and the expected numbers detected by real-time PCR. A method for quantification using real-time PCR with SYBR Green I was also developed for estimation of the numbers of juveniles of the stem nematode, *D. dipsaci*, in soil samples (Subbotin *et al.*, 2005). All these methods showed a high level of specificity and sensitivity and enabled the detection of a single nematode in a sample. The background of other soil-inhabiting nematodes present in tested samples did not significantly compromise the accuracy of these assays. However, in most cases, accuracy of the quantification decreased with decreasing nematode numbers in the samples.

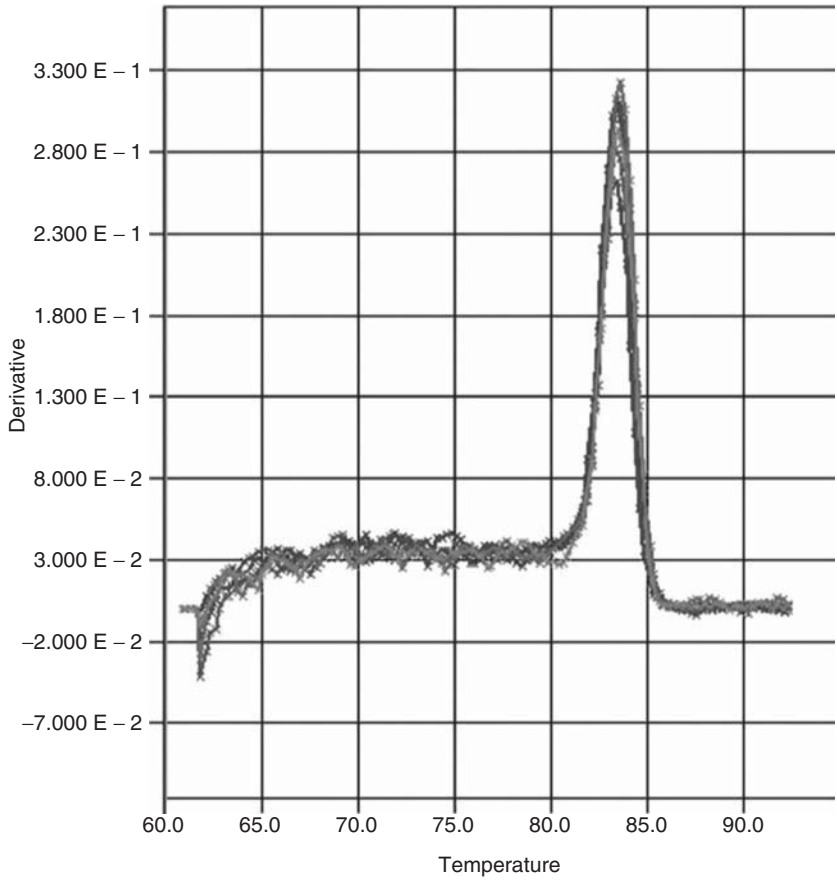

Fig. 9.7. Melting curve (fluorescence vs temperature) of specific amplicon for *Globodera pallida* (Madani *et al.,* 2005).

The disadvantage of using a fluorescent dye is that it binds to any double-stranded DNA and then it cannot be used for quantification of several targets in a multiplex real-time PCR, because it cannot distinguish between different sequences. In this case, sequence-specific fluorescent probes, such as TaqMan probes, are needed. Cao *et al.* (2005) developed a method for detecting the pinewood nematode, *B. xylophilus*, using TaqMan probes. The PCR assay detected DNA template concentrations as low as 0.01 ng. The Ct values were correlated with the DNA template concentration (R^2 = 0.996), indicating the validity of the assay and its potential for quantification of target DNA. The real-time PCR assay also detected DNA from single specimens of *B. xylophilus*. Recently, Holeva *et al.* (2006) described a novel diagnostic method for two virus–vector nematodes, *Paratrichodorus pachydermus* and *Trichodorus similis*, and associated tobacco rattle virus based on TaqMan probes. They demonstrated the potential of this assay for rapid, accurate and sensitive detection of both

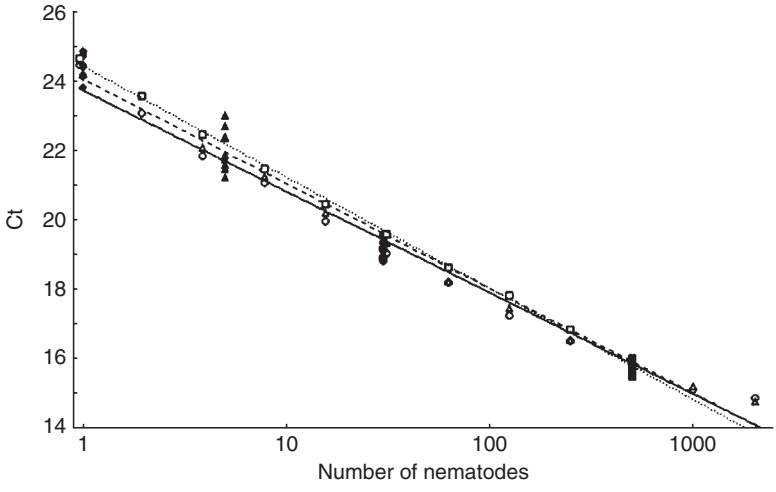

Fig. 9.8. Sensitivity, detection range and specificity of the real-time PCR assay for *Globodera pallida*. The reproducibility of the assay was determined by testing a dilution series of three independent DNA extractions. Black symbols represent the results of the validation test with known numbers of second-stage juveniles (1, 5, 30 or 500 juveniles) (Madani *et al.*, 2005).

trichodorid species and virus from field samples. The real-time PCR method is straightforward, sensitive and reproducible and, compared with conventional PCR methods, it has several advantages (Fig. 9.8). The technique allows a simultaneous faster detection and quantification of target DNA and the automated system overcomes the laborious process of estimating the quality of PCR product after electrophoresis.

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.

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.

DNA barcoding

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 18S-rRNA 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).

Future Developments and Directions

It is clear from the work over the last 20 years that molecular techniques are powerful tools for nematode diagnosis. By using them, scientists have solved a number of issues; however, an even larger number still remain unsolved. The promising and attractive results have generated increasing demands for applications in new fields and for better performing techniques. Diagnostic results are now expected more rapidly and samples are preferably examined on the spot. Direct diagnosis using soil samples without prior nematode extraction would further reduce the period of time required for diagnosis. Mobile molecular equipment may be particularly useful in quarantine applications, where the detection of single individuals is of paramount importance. Techniques must be able to distinguish between dead and living individuals.

Plant-parasitic nematodes live in communities comprised of variable numbers of species. The expectation of extension services goes beyond the molecular diagnosis of a single species, even if it is the major pathogen of a crop. Knowledge about the composition of the nematode community will help in designing a crop rotation sequence. For the same reason, the molecular quantification of plant-parasitic nematodes is extremely important. Molecular quantification has proven possible for a number of cyst nematodes and for *D. dipsaci*. However, for both of these groups, specimens of the same stage were used (second-stage and fourth-stage juveniles, respectively). Quantification of different stages will be necessary if molecular diagnostics is to be used by extension services. When planning a crop rotation, the identification of pathotypes (e.g. PCN) and races (e.g. *Meloidogyne*) is very important; currently, this identification is time-consuming and may not always be possible if nematode development is interrupted. This issue has been addressed molecularly, but still requires more research before a method can be used routinely.

The future of molecular nematode diagnostics is in the development of nanodiagnostics, which is still primarily in the research stage. Nanotechnology

extends the limits of molecular diagnostics to the nanoscale (one billionth of a metre). This scale of sensitivity as applied to diagnostics would include the detection of molecular interaction (Jain, 2003) and it is anticipated that many of the specific nanotechnologies will eventually be applied to the diagnostics of nematodes.

The small dimensions that are detectable using this technology have led to the use of nanochips, which employs the power of an electronic current to separate DNA probes to specific sites on the array, based on charge and size. Each test site on nanochips can be controlled electronically from the system's onboard computer. The use of the electronic-mediated hybridization reduces the time for detection of target DNA sequences to minutes instead of hours required with conventional approaches.

Another application of nanotechnology is the use of magnetic nanoparticles, bound to a suitable antibody, to label specific molecules, structures or microorganisms. Magnetic immunoassay techniques have been developed in which the magnetic field generated by the magnetically labelled targets is detected directly with a sensitive magnetometer (Jain, 2003).

DNA sequencing costs have fallen more than 100-fold over the past decade, fuelled in large part by tools, technologies and process improvements developed as part of the successful effort to sequence the human genome. New technologies open the door to the next generation of sequencing methods, which include, for example, pyrosequencing, sequencing-by-sequencing approach and sequencing using nanopores. There are many opportunities to reduce the cost and increase the throughput of DNA sequencing, which are likely to lead to very different and novel approaches to diagnostics.

References

Al-Banna, L., Ploeg, A.T., Williamson, V.M. and Kaloshian, I. (2004) Discrimination of six *Pratylenchus* species using PCR and species-specific primers. *Journal of Nematology* 36, 142–146.

Amiri, S., Subbotin, S.A. and Moens, M. (2002) Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* 108, 497–506.

Atkins, S.D., Manzanilla-Lopez, R.H., Franco, J., Peteira, B. and Kerry, B.R. (2005) A molecular diagnostic method for detecting *Nacobbus* in soil and in potato tubers. *Nematology* 7, 193–202.

Bakker, J. and Gommers, F.J. (1982) Differentiation of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, and of two *G. rostochiensis* pathotypes by means of two dimensional electrophoresis. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, Series C* 85, 309–314.

Bates, J.A., Taylor, E.J.A., Gans, P.T. and Thomas J.E. (2002) Determination of relative proportion of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. *Molecular Plant Pathology* 3, 153–161.

Bekal, S., Gauthier, J.P. and Rivoal, R. (1997) Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. *Genome* 40, 479–486.

Bhadury, P., Austen, C.M., Bilton, D.T., Lambshead, J.D., Rogers, A.D. and Smerdon, G.R. (2005) Combined morphological and molecular analysis of individual nematodes through short-term preservation in formalin. *Molecular Ecology Notes* 5, 965–968.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T. and Thomas, W.K. (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71–75.

Blok, V.C. (2005) Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Canadian Journal of Plant Pathology* 27, 176–185.

Blok, V.C., Wishart, J., Fargette, M., Berthier, K. and Phillips, M.S. (2002) Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology* 4, 773–781.

Blouin, M.S. (2002) Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology* 32, 527–531.

Blouin, M.S., Yowell, C.A., Courtney, C.H. and Dame, J.B. (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Molecular Biology and Evolution* 15, 1719–1727.

Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M. and van der Noordaa, J. (1990) Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 28, 495–503.

Boutsika, K., Phillips, M.S., MacFarlane, S.A., Brown, D.J.F., Holeva, R.C. and Blok, V.C. (2004) Molecular diagnostics of trichodorid nematodes and their associated *Tobacco rattle virus* (TBV). *Plant Pathology* 53, 110–116.

Braasch, H., Tomiczek, Ch., Metge, K., Hoyer, U., Burgermeister, W., Wulfert, I. and Schönfeld, U. (2001) Records of *Bursaphelenchus* spp. (Nematoda, Parasitaphelenchidae) in coniferous timber imported from the Asian part of Russia. *Forest Pathology* 31, 129–140.

Brito, J., Powers, T.O., Mullin, P.G., Inserra, R.N. and Dickson, D.W. (2004) Morphological and molecular characterization of *Meloidogyne mayaguensis* isolates from Florida. *Journal of Nematology* 36, 232–240.

Bulman, S.R. and Marshall, J.W. (1997) Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25, 123–129.

Burgermeister, W., Metge, K., Braasch, H. and Buchbach, E. (2005) ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. *Russian Journal of Nematology* 13, 29–42.

Burrows, P.R. and Perry, R.N. (1988) Two cloned DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis*. *Revue de Nématologie* 11, 453–457.

Cao, A.X., Liu, X.Z., Zhu, S.F. and Lu, B.S. (2005) Detection of the pinewood nematode, *Bursaphelenchus xylophilis*, using a real-time polymerase chain reaction assay. *Phytopathology* 95, 566–571.

Carneiro, R.M.D.G., Almeida, M.R.A. and Quénéherve, P. (2000) Enzyme phenotypes of *Meloidogyne* spp. populations. *Nematology* 2, 645–654.

Carneiro, R.M.D.G., Tigano, M.S., Randing, O., Almeida, M.R. and Sarah, J.-L. (2004) Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) on coffee from Brazil, Central America and Hawaii. *Nematology* 6, 287–298.

Carneiro, R.M.D.G., Almeida, M.R.A., Gomes, A.C.M.M. and Hernandez, A. (2005) *Meloidogyne izalcoensis* n. sp. (Nematode: Meloidogynidae) a root-knot nematode parasitising coffee in El Salvador. *Nematology* 7, 819–832.

Castagnone, C., Abad, P. and Castagnone-Sereno, P. (2005) Satellite DNA-based species-specific identification of single individuals of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *European Journal of Plant Pathology* 112, 191–193.

Castagnone-Sereno, P., Leroy, F., Bongiovanni, M., Zijlstra, C. and Abad, P. (1999) Specific diagnosis of two root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, with satellite DNA probes. *Phytopathology* 89, 380–384.

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

Chen, Q., Robertson, L., Jones, J.T., Blok, V.C., Phillips, M.S. and Brown, D.J.F. (2001) Capture of nematodes using antiserum and lectin-coated magnetized beads. *Nematology* 3, 593–601.

Chen, Q., Brown, D.J.F., Curtis, R.H. and Jones, J.T. (2003) Development of a magnetic capture system for recovery of *Xiphinema americanum*. *Annals of Applied Biology* 143, 283–289.

Clapp, J.P., Stoel, C.D. van der and Putten, W.H. van der (2000) Rapid identification of cyst (*Heterodera* spp., *Globodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformational polymorphism (PCR-SSCP) in cultures and field samples. *Molecular Ecology* 9, 1223–1232.

Curran, J., Baillie, D.L. and Webster, J.M. (1985) Use of genomic DNA restriction fragment length differences to identify nematode species. *Parasitology* 90, 137–144.

Dean, F.B., Nelson, J.R., Giesler, T.L. and Lasken, R.S. (2001) Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Research* 11, 1095–1099.

De Giorgi, C., Sialer, M.F. and Lamberti, F. (1994) Formalin-induced infidelity in PCR-amplified DNA fragments. *Molecular and Cellular Probes* 8, 459–462.

De Ley, I.T., Karssen, G., De Ley, P., Vierstraete, A., Waeyenberge, L., Moens, M. and Vanfleteren, J. (1999) Phylogenetic analyses of internal transcribed spacer region sequences within *Meloidogyne*. *Journal of Nematology* 31, 530–531.

De Ley, P., Tandingan De Ley, I., Morris, K., Abebe, E., Mundo, M., Yoder, M., Heras, J., Waumann, D., Rocha-Olivares, A., Burr, J., Baldwin, J.G. and Thomas, W.K. (2005) An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. *Philosophical Transactions of the Royal Society of London B* 272, 1945–1958.

Dong, K., Dean, R.A., Fortnum, B.A. and Lewis, S.A. (2001) Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne aranaria, M. hapla, M. incognita* and *M. javanica*. *Nematropica* 31, 273–282.

Elbadri, G.A.A., De Ley, P., Wayenberge, L., Vierstraete, A., Moens, M. and Vanfleteren, J. (2002) Interspecific variation in *Radopholus similis* isolates assessed with restriction fragment length polymorphism and DNA sequencing of the internal transcribed spacer region of the ribosomal RNA cistron. *International Journal for Parasitology* 32, 199–205.

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

Esbenshade, P.R. and Triantaphyllou, A.C. (1985) Use of enzyme phenotypes for identification of *Meloidogyne* species (Nematoda: Tylenchida). *Journal of Nematology* 17, 6–20.

Esbenshade, P.R. and Triantaphyllou, A.C. (1990) Isozyme phenotypes for the identification of *Meloidogyne* species. *Journal of Nematology* 22, 10–15.

Esquibet, M., Grenier, E., Plantard, O., Andaloussi, A. and Caubel, G. (2003) DNA polymorphism in the stem nematode *Ditylenchus dipsaci*: development of diagnostic markers for normal and giant races. *Genome* 46, 1077–1083.

Fargette, M. (1987) Use of the esterase phenotype in the taxonomy of the genus *Meloidogyne*. 2. Esterase phenotypes observed in West African populations and their characterization. *Revue de Nématologie* 10, 45–56.

Ferris, V.R., Ferris, J.M. and Faghihi, J. (1993) Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16, 177–184.

Ferris, V.R., Ferris, J.M., Faghihi, J. and Ireholm, A. (1994) Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *Journal of Nematology* 26, 144–151.

Files, J.G. and Hirsh, D. (1981) Ribosomal DNA of *Caenorhaditis elegans*. *Journal of Molecular Biology* 149, 223–240.

Fleming, C.C. and Marks, R.J. (1982) A method for the quantitative estimation of *Globodera rostochiensis* and *Globodera pallida* in mixed species samples. *Ministry of Agriculture, Northern Ireland Record of Agricultural Research* 30, 67–70.

Fleming, C.C. and Mowat, D.J. (1993) Potato cyst nematode diagnostics using the polymerase chain reaction. In: Ebbels, D. (ed.) *Proceedings of Plant Health and the European Single Market*. British Crop Protection Council, Farnham, UK, pp. 349–354.

Fleming, C.C. and Powers, T.O. (1998) Potato cyst nematode diagnostics; morphology, different hosts and biochemical technique. In: Marks, R.J. and Brodie, B.B. (eds) *Potato Cyst Nematode – Biology, Distribution and Control*. CAB International, Wallingford, UK, pp. 91–114.

Fleming, C.C., Turner, S.J., Powers, T.O. and Szalansky, A.L. (1998) Diagnostics of cyst nematodes: use of the polymerase chain reaction to determine species and estimate population level. *Aspect of Applied Biology* 52, 375–382.

Floyd, R., Eyualem, A., Papert, A. and Blaxter, M. (2002) Molecular barcodes for soil nematode identification. *Molecular Ecology* 11, 839–850.

Floyd, R.M., Rogers, A.D., Lambshead, J.D. and Smith, C.R. (2005) Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Molecular Ecology Note* 5, 611–612.

François, C., Kebdani, N., Barker, I., Tomlinson, J., Boonham, N. and Castagnone-Sereno, P. (2006) Towards specific diagnosis of plant-parasitic nematodes using DNA oligonucleotide microarray technology: a case study with quarantine species *Meloidogyne chitwoodi*. *Molecular and Cellular Probes* 20, 64–69.

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

Grenier, E., Castagnone-Sereno, P. and Abad, P. (1997) Satellite DNA sequences as taxonomic markers in nematodes of agronomic interest. *Parasitology Today* 13, 398–401.

He, Y., Li, H., Brown, D.J.F., Lamberti, F. and Moens, M. (2003) Isolation and characterisation of microsatellites for *Xiphinema index* using degenerate oligonucleotide primed PCR. *Nematology* 5, 809–819.

He, Y., Subbotin, S.A., Rubtsova, T.V., Lamberti, F., Brown, D.J.F. and Moens, M. (2005) A molecular phylogenetic approach to Longodoridae (Nematoda: Dorylaimida). *Nematology* 7, 111–124.

Hockland, S., Inserra, R.N., Millar, L. and Lehman, P.S. (2006) International plant health – putting legislation into practice. In: Perry, R.N. and Moens, M. (eds) *Plant Nematology*. CAB International, Wallingford, UK, pp. 327–345.

Holeva, R., Phillips, M.S., Neilson, R., Brown, D.J.F., Young, V., Boutsika, K. and Blok, V.C. (2006) Real-time PCR detection and quantification of vector trichodorid nematodes and *Tobacco rattle virus*. *Molecular and Cellular Probes* 20, 203–211.

Hoyer, U., Burgermeister, W. and Braasch, H. (1998) Identification of *Bursaphelenchus* species (Nematoda, Aphelenchoididae) on the basis of amplified ribosomal DNA (ITS-RFLP). *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 50, 273–277.

Hübschen, J., Kling, L., Ipach, U., Zinkernagel., V., Bosselut, N., Esmenjaud, D., Brown, D.J.F. and Neilson, R. (2004a) Validation of the specificity and sensitivity of species-specific primers that provide a reliable molecular diagnostic for *Xiphinema diversicaudatum*, *X. index* and *X. vuittenezi*. *European Journal of Plant Pathology* 110, 779–788.

Hübschen, J., Kling, L., Zinkernagel., V., Brown, D.J.F. and Neilson, R. (2004b) Development and validation of species-specific primers that provide a molecular diagnostic for virus–vector longodorid nematodes and related species in German viticulture. *European Journal of Plant Pathology* 110, 883–891.

Hugall, A., Moritz, C., Stanton, J. and Wolstenholme, D. (1994) Low, but strong structured mitochondrial DNA diversity in root-knot nematodes (*Meloidogyne*). *Genetics* 136, 903–912.

Hugall, A.F., Stanton, C. and Moritz, C. (1997) Evolution of the AT-rich mitochondrial DNA of the root knot nematode, *Meloidogyne hapla*. *Molecular Biology and Evolution* 14, 40–48.

Hugall, A., Stanton, J. and Moritz, C. (1999) Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution* 16, 157–164.

Ibrahim, S.K. and Perry, R.N. (1992) Use of esterase patterns of females and galled roots for the identification of species of *Meloidogyne*. *Fundamental and Applied Nematology* 16, 187–190.

Ibrahim, S.K., Perry, R.N., Burrows, P.R. and Hooper, D.J. (1994) Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. *Journal of Nematology* 26, 412–421.

Ibrahim, S.K., Davies, K.G. and Perry, R.N. (1996) Identification of the root-knot nematode, *Meloidogyne incognita*, using monoclonal antibodies raised to non-specific esterases. *Physiological and Molecular Plant Pathology* 49, 79–88.

Ibrahim, S.K., Minnis, S.T., Barker, A.D.P., Russell, M.D., Haydock, P.P.J., Evans, K., Grove, I.G., Woods, S.R. and Wilcox, A. (2001) Evaluation of PCR, IEF and ELISA techniques for the detection and identification of potato cyst nematodes from field soil samples in England and Wales. *Pest Management Science* 57, 1068–1074.

Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K. and Futai, K. (1998) PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundamental and Applied Nematology* 21, 655–666.

Jain, K.K. (2003) Nanodiagnostics: application of nanotechnology in molecular diagnostics. *Expert Review of Molecular Diagnostics* 3, 153–161.

Jiang, L.Q., Zheng, J.W., Waeyenberge, L., Subbotin, S.A. and Moens, M. (2005) Duplex PCR based identification of *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970. *Russian Journal of Nematology* 13, 115–121.

Joyce, S.A., Reid, A., Driver, F. and Curran, J. (1994) Application of polymerase chain reaction (PCR) methods to identification of entomopathogenic nematodes. In: Burnell, A.M., Ehlers, R.-U. and Masson, J.P. (eds) *COST 812 Biotechnology: Genetics of Entomopathogenic Nematode-Bacterium Complexes*. Proceedings of Symposium and Workshop, St. Patrick's College, Maynooth, Co. Kildare, Ireland. Luxembourg, European Commission, DG XII, pp. 178–187.

Kang, J.S., Choi, K.S., Shin, S.C., Moon, I.S., Lee, S.G. and Lee, S.H. (2004) Development of an efficient PCR-based diagnosis protocol for the identification of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *Nematology* 6, 279–285.

Karssen, G., van Hoenselaar, T., Verkerk-Bakker, B. and Janssen, R. (1995) Species identification of cyst and root-knot nematodes from potato by electrophoresis of individual females. *Electrophoresis* 16, 105–109.

Karssen, G., Bolk, R.J., van Aelst, A.C., van den Beld, I., Kox, L.F.F., Korthals, G., Molendijk, L., Zijlstra, C., van Hoof, R. and Cook, R. (2004) Description of *Meloidogyne minor* n. sp. (Nematoda: Meloidogynidae), a root-knot nematode associated with yellow patch disease in golf courses. *Nematology* 6, 59–72.

Kovaleva, E.S., Subbotin, S.A., Masler, E.P. and Chitwood, D.J. (2005) Molecular characterization of the actin gene from cyst nematodes in comparison to those from other nematodes. *Comparative Parasitology* 72, 39–49.

Leal, I., Green, M., Allen, E., Humble, L. and Rott, M. (2005) An effective PCR-based diagnostic method for the detection of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) in wood samples from lodgepole pine. *Nematology* 7, 833–842.

Liao, J.L., Zhang, L.H. and Feng, Z.X. (2001) Reliable identification of *Bursaphelenchus xylophilus* by rDNA amplification, *Nematologia Mediterranea* 19, 131–135.

Madani, M., Vovlas, N., Castillo, P., Subbotin, S.A. and Moens, M. (2004) Molecular characterization of cyst nematode species (*Heterodera* spp.) from the Mediterranean basin using RFLPs and sequences of ITS-rDNA. *Journal of Phytopathology* 152, 229–234.

Madani, M., Subbotin, S.A. and Moens, M. (2005) Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. *Molecular and Cellular Probes* 19, 81–86.

Marshall, J. and Crawford, A.M. (1987) A cloned DNA fragment that can be used as a sensitive probe to distinguish *Globodera pallida* from *Globodera rostochiensis* and other cyst forming nematodes. *Journal of Nematology* 19, 541 (abstract).

Matsunaga, K. and Togashi, K. (2004) A simple method for discriminating *Bursaphelenchus xylophilus* and *B. mucronatus* by species-specific polymerase chain reaction primer pairs. *Nematology* 6, 273–277.

Metge, K. and Burgermeister, W. (2005) Multiple displacement amplification of DNA for ITS-RFLP analysis of individual juveniles of *Bursaphelenchus*. *Nematology* 7, 253–257.

Mulholland, V., Carde, L., O'Donnell, K.L., Fleming, C.C. and Powers, T.O. (1996) Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. In: Marshall, C. (ed.) *Proceedings of Diagnostics in Crop Production Symposium*. British Crop Production Council, Farnham, UK, pp. 247–252.

Navas, A., López, J.A., Espárrago, G., Camafeita, E. and Albar, J.P. (2002) Protein viability in *Meloidogyne* spp. (Nematoda: Meloidogynidae) revealed by two-dimensional gel electrophoresis and mass spectrometry. *Journal of Proteome Research* 1, 421–427.

Orui, Y. (1995) Discrimination of the main *Pratylenchus* species (Nematoda: Pratylenchidae) in Japan by PCR-RFLP analysis. *Applied Entomology and Zoology* 31, 505–514.

Orui, Y. (1997) Discrimination of *Globodera rostochiensis* and four *Heterodera* species (Nematoda: Heteroderidae) by PCR-RFLP analysis. *Japanese Journal of Nematology* 27, 67–75.

Orui, Y. (1998) Identification of Japanese species of the genus *Meloidogyne* (Nematoda: Meloidogynidae) by PCR-RFLP analysis. *Applied Entomology and Zoology* 33, 43–51.

Orui, Y. and Mizukubo, T. (1999) Discrimination of seven *Pratylenchus* species (Nematoda: Pratylenchidae) in Japan by PCR-RFLP analysis. *Applied Entomology and Zoology* 34, 205–211.

Palmer, H.M., Atkinson, H.J. and Perry, R.N. (1992) Monoclonal antibodies specific to surface expressed antigens of *Ditylenchus dipsaci*. *Fundamental and Applied Nematology* 15, 511–515.

Perera, M.R., Vanstone, V.A. and Jones, M.G.K. (2005) A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 19, 1454–1460.

Perry, R.N. and Jones, J.T. (1998) The use of molecular biology techniques in Plant Nematology: past, present and future. *Russian Journal of Nematology* 6, 47–56.

Petersen, D.J. and Vrain, T.C. (1996) Rapid identification of *Meloidogyne chitwoodi, M. hapla*, and *M. fallax* using PCR primers to amplify their ribosomal intergenic spacer. *Fundamental and Applied Nematology* 19, 601–605.

Petersen, D.J., Zijlstra, C., Wishart, J., Blok, V. and Vrain, T.C. (1997) Specific probes efficiently distinguish root-knot nematode species using signature sequence in the ribosomal intergenetic spacer. *Fundamental and Applied Nematology* 20, 619–626.

Powers, T. (2004) Nematode molecular diagnostics: from bands to barcodes. *Annual Review of Phytopathology* 42, 367–383.

Powers, T.O. and Harris, T.S. (1993) A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* 25, 1–6.

Powers, T.O. Szalanski, A.I., Mullin, P.G., Harris, T.S. Bertozzi, T. and Griesbach, J.A. (2001) Identification of seed gall nematodes of agronomic and regular concern with PCR-RFLP of ITS1. *Journal of Nematology* 33, 191–194.

Powers, T.O., Mullin, P.G., Harris, T.S., Sutton, L.A. and Higgins, R.S. (2005) Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology* 37, 226–235.

Randing, O., Bongiovanni, M., Carneiro, R.M.D.G. and Castagnone-Sereno, P. (2002) Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome* 45, 862–870.

Reid, A., Manzanilla-Lopez, R.H. and Hunt, D.J. (2003) *Nacobbus aberrans* (Thorne, 1935) Thorne and Allen, 1944 (Nematode: Pratylenchidae); a nascent species complex revealed by RFLP analysis and sequencing of the ITS-rDNA region. *Nematology* 5, 441–451.

Robinson, M.P. (1989) Quantification of soil and plant populations of *Meloidogyne* using immunoassay techniques. *Journal of Nematology* 21, 583–584.

Rubtsova, T.V., Moens, M. and Subbotin, S.A. (2005) PCR amplification of a rRNA gene fragment from formalin-fixed and glycerine-embedded nematodes from permanent slides. *Russian Journal of Nematology* 13, 137–140.

Schmitz, V.B., Burgermeister, W. and Braasch, H. (1998) Molecular genetic classification of Central European *Meloidogyne chitwoodi* and *M. fallax* populations. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 50, 310–317.

Scholl, E.H. and Bird, D.M. (2005) Resolving tylenchid evolutionary relationships through multiple gene analysis derived from EST data. *Molecular Phylogenetics and Evolution* 36, 536–545.

Schots, A., Bakker, J., Egberts, E. and Gommers, F.J. (1986) Monoclonal antibodies against potato cyst nematodes. *Revue de Nématologie* 9, 309.

Setterquist, R.A., Smith, G.K., Jones, R. and Fox, G.E. (1996) Diagnostic probes targeting the major sperm protein gene that may be useful in the molecular identification of nematodes. *Journal of Nematology* 28, 414–421.

Skantar, A.M. and Carta, L.K. (2004) Molecular characterization and phylogenetic evaluation of the Hsp90 gene from selected nematodes. *Journal of Nematology* 36, 466–480.

Skantar, A.M. and Carta, L.K. (2005) Multiple displacement amplification (MDA) of total genomic DNA from *Meloidogyne* spp. and comparison to crude DNA extracts in PCR of ITS1, 28S D2–D3 rDNA and Hsp90. *Nematology* 7, 285–293.

Stanton, J.M., McNicol, C.D. and Steele, V. (1998) Non-manual lysis of second stage *Meloidogyne* juveniles for identification of pure and mixed samples based on polymerase chain reaction. *Australian Plant Pathology* 27, 112–115.

Sturhan, D. and Rumpenhorst, H.J. (1996) Untersuchungen über den *Heterodera avenae*-Komplex. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Fortwirtschaft, Berlin-Dahlem* 317, 75–91.

Subbotin, S.A. (2006) Molecular diagnostics of plant parasitic nematodes using the polymerase chain reaction. In: Zinovieva, S.V., Perevertin, K.A., Romanenko, N.D. *et al.* (eds) *Applied Nematology.* Nauka, Russia, pp. 228–252.

Subbotin, S.A. and Moens, M. (2006) Molecular taxonomy and phylogeny. In: Perry, R.N. and Moens, M. (eds) *Plant Nematology*. CAB International, Wallingford, UK, pp. 33–58.

Subbotin, S.A., Sturhan, D., Waeyenberge, L. and Moens, M. (1997) *Heterodera riparia* sp. n. (Tylenchida: Heteroderidae) from common nettle, *Urtica dioica* L., and rDNA-RFLP separation of species from the *H. humuli* group. *Russian Journal of Nematology* 5, 143–157.

Subbotin, S.A., Halford, P.D. and Perry, R.N. (1999) Identification of populations of potato cyst nematodes from Russia using protein electrophoresis, rDNA-RFLPs and RAPDs. *Russian Journal of Nematology* 7, 57–63.

Subbotin, S.A., Waeyenberge, L. and Moens, M. (2000a) Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLPs. *Nematology* 2, 153–164.

Subbotin, S.A., Halford, P.D., Warry, A. and Perry, R.N. (2000b) Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising Solanaceae. *Nematology* 2, 591–604.

Subbotin, S.A., Peng, D. and Moens, M. (2001) A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology* 3, 365–370.

Subbotin, S.A., Sturhan, D., Rumpenhorst, H.J. and Moens, M. (2003) Molecular and morphological characterisation of the *Heterodera avenae* complex species (Tylenchida: Heteroderidae) *Nematology* 5, 515–538.

Subbotin, S.A., Madani, M., Krall, E., Sturhan, D. and Moens, M. (2005) Molecular diagnostics, taxonomy and phylogeny of the stem nematode *Ditylenchus dipsaci* species complex based on the sequences of the ITS-rDNA. *Phytopathology* 95, 1308–1315.

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

Stanton, J.M., McNicol, C.D. and Steele, V. (1998) Non-manual lysis of second stage *Meloidogyne* juveniles for identification of pure and mixed samples based on polymerase chain reaction. *Australian Plant Pathology* 27, 112–115.

Szalanski, A.L., Sui, D.D., Harris, T.S. and Powers, T.O. (1997) Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology* 29, 255–267.

Tanha Maafi, Z., Subbotin, S.A. and Moens, M. (2003) Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on the ITS sequences of rDNA. *Nematology* 5, 99–111.

Thiéry, M. and Mugniéry, D. (1996) Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of Solanaceous plants. *Fundamental and Applied Nematology* 19, 471–479.

Thomas, W.K., Vida, J.T., Frisse, L.M., Mundo, M. and Baldwin, J.G. (1997) DNA sequences from formalin-fixed nematodes: integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29, 250–254.

Tigano, M.S., Carneiro, R.M.D.G., Jeyaprakash, A., Dickson, D.W. and Adams, B.J. (2005) Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. *Nematology* 7, 851–862.

Trudgill, D.L. and Carpenter, J.M. (1971) Disc electrophoresis of proteins of *Heterodera* species and pathotypes of *Heterodera rostochiensis*. *Annals of Applied Biology* 69, 34–41.

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

Uehara, T., Kushida, A. and Momota, Y. (1999) Rapid and sensitive identification of *Pratylenchus* spp. using reverse dot blot hybridization. *Nematology* 1, 549–555.

Vahidi, H. and Honda, B.M. (1991) Repeats and subrepeats in the intergenic spacer of rDNA from the nematode *Meloidogyne arenaria arenaria*. *Molecular and General Genetics* 227, 334–336.

Vrain, T.C., Wakarchuk, D.A., Levesque, A.C. and Hamilton, R.I. (1992) Intraspecific rDNA restriction fragment length polymorphisms in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563–574.

Waeyenberge, L., Ryss, A., Moens, M., Pinochet, J. and Vrain, T.C. (2000) Molecular characterization of 18 *Pratylenchus* species using rDNA restriction fragment length polymorphism. *Nematology* 2, 135–142.

Walsh, S.P., Metzger, D.A. and Higuchi, R. (1991) Chelex 100 as medium for simple extraction of DNA for PCR-based typing from forensic material. *Biological Techniques* 10, 506–513.

Webster, J.M. and Hooper, D.J. (1968) Serological and morphological studies on the inter- and intraspecific differences of the plant-parasitic nematodes *Heterodera* and *Ditylenchus*. *Parasitology* 58, 878–891.

Wendt, K.R., Vrain, T.C. and Webster, J.M. (1993) Separation of three species of *Ditylenchus* and some host races of *D. dipsaci* by restriction fragment length polymorphism. *Journal of Nematology* 25, 555–563.

Williams, C., Pohtén, F., Moberg, C., Söderkvist, P., Uhlén, M., Pontén, J., Sitbon, G. and Lundeberg, J. (1999) A high frequency of sequence alterations is due to formalin fixation of archival specimens. *American Journal of Pathology* 155, 1467–1471.

Williamson, V.M., Caswell-Chen, E.P., Westerdahl, B.B., Wu, F.F. and Caryl, G. (1997) A PCR assay to identify and distinguish single juveniles of *Meloidogyne hapla* and *M. chitwoodi*. *Journal of Nematology* 29, 9–15.

Xu, J.H., Liu, P.L., Meng, Q.P. and Long, H. (2004) Characterisation of *Meloidogyne* species from China using isozyme phenotypes and amplified mitochondrial DNA restriction fragment length polymorphism. *European Journal of Plant Pathology* 110, 309–315.

Xue, B., Baillie, D.L. and Webster, J.M. (1993) Amplified fragment length polymorphisms of *Meloidogyne* spp. using oligonucleotide primers. *Fundamental and Applied Nematology* 16, 481–487.

Yoder, M., Tandingan De Ley, I., King, I.W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Poiras, L. and De Ley, P. (2006) DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. *Nematology* 8, 367–376.

Zheng, J., Subbotin, S.A., He, S., Gu, J. and Moens, M. (2003) Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. *Russian Journal of Nematology* 11, 17–22.

Zijlstra, C. (1997) A fast PCR assay to identify *Meloidogyne hapla*, *M. chitwoodi*, and *M. fallax*, and to sensitively differentiate them from each other and from *M. incognita* in mixtures. *Fundamental and Applied Nematology* 20, 505–511.

Zijlstra, C. (2000) Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology* 106, 283–290.

Zijlstra, C., Lever, A.E.M., Uenk, B.J. and Van Silfhout, C.H. (1995) Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* 85, 1231–1237.

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

Zijlstra, C., van Hoof, R. and Donkers-Venne, D. (2004) A PCR test to detect the cereal root-knot nematode *Meloidogyne naasi*. *European Journal of Plant Pathology* 110, 855–860.