

# 4 Biochemical and Molecular Identification

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## 4.1. Introduction

*Meloidogyne* identification has always presented challenges to the diagnostician. Conservative morphology, life stages in different habitats, wide host ranges, indistinct species boundaries or species complexes, sexual dimorphism, species with a potential hybrid origin, polyploidy, and over a century of human-aided dispersal are just some of the complicating features in the identification of *Meloidogyne* spp. Consider the infective stage: if *Meloidogyne* is present in a field, a soil nematode extraction typically recovers the small (< 0.6 mm) infective juvenile stage. Trained nematologists using a dissecting microscope can readily recognize members of the genus based on the fine stylet, the characteristically tapering tail, body movement or body shape if the juvenile is not moving. Yet even the most seasoned diagnostician would hesitate to assign an individual juvenile to a species. Morphometrics of juveniles can provide a relatively reliable assessment for species assignment (Hirshmann, 1985; Jepson, 1987; Karssen, 2002), but species-level identification, in practice, is complicated by genetic, climatic and

anthropogenic factors associated with the dynamic nature and global scope of present-day agricultural production. In other words, there is no guarantee that an agricultural field contains only a single species of *Meloidogyne* or that the diagnostic descriptions currently available cover all of the diversity in the genus and will permit reliable identification. Are seed potatoes, for example, which are routinely shipped across international borders, responsible for the widespread distribution of *Meloidogyne chitwoodi*? The planting of infected seed potatoes may occur in a field already infested with another *Meloidogyne* species, as it is now recognized that soils containing multiple *Meloidogyne* species are fairly common. Furthermore, *Meloidogyne enterolobii* (= *Meloidogyne mayaguensis*) was recognized as a problem in Florida when galls appeared on root-knot-resistant tomatoes, grown in response to persistent populations of *Meloidogyne incognita* (Brito *et al.*, 2004), and *Meloidogyne floridensis* was recognized as a distinct species following its discovery on root-knot-resistant peach rootstock (Carneiro *et al.*, 2000; Handoo *et al.*, 2004). *Meloidogyne paranaensis*, *Meloidogyne izalcoensis* and *M. mayaguensis* (now

*M. enterolobii*; see Hunt and Handoo, Chapter 3, this volume) were probably misdiagnosed on coffee for over 20 years due to over-reliance on perineal patterns and differential host tests (Carneiro *et al.*, 1996a,b, 2004a, 2005a).

Agricultural fields with multiple *Meloidogyne* species are not the only diagnostic challenge. A more basic concern is the actual genetic nature of our diagnostic target. As techniques have increased our ability to resolve more finely *Meloidogyne* genetics, it has become clear that many of our 'species' are collections of lineages that may or may not share a recent common ancestry. Common ancestry and descent provide the framework for the species concepts and the recognition of species boundaries. For *Meloidogyne*, this framework is still in the early stages of development (see Adams *et al.*, Chapter 5, this volume). In this chapter we briefly review the historical development of biochemical and molecular-based identification methods for root-knot nematodes. The application of these methods needs to be considered in terms of the cost and accuracy that they provide and will vary depending on the application, such as for routine quarantine or ecological studies, or for functional and evolutionary studies. Our lack of knowledge of the biogeography and evolutionary history of these organisms, and their genetics, is overlaid by complications that have been introduced through dispersal with agriculture, and this must also be remembered when using identification methods.

De Waele and Elsen (2007) noted that by 2006 about half (47) of the 92 nominal species (now 97, see Hunt and Handoo, Chapter 3, this volume) of *Meloidogyne* that they listed were described in the last 20 years; 29 of these were from Central and South America, Africa or Asia, with 14 of the new species from China. Thus, the possibility is high of encountering a new *Meloidogyne* species, particularly in tropical regions, where species diversity is rich. Where species identification is critical for intercepting and deploying appropriate quarantine steps, for the detection of emerging nematode threats and for appropriate nematode management, accurate identification is fundamental. Whilst new species continue to be described and identification methods improve, it is also important to recognize that often the expertise and facilities are lacking in parts of the world where *Meloidogyne* spp. are most prevalent and problematic. Basic education,

training and appropriate infrastructure and funding are required for *Meloidogyne* spp. diagnostics to be utilized where they are most needed and for the benefit of the international community.

'Emerging threats' have been highlighted through extensive surveys and the use of a range of diagnostic tools to aid species identification. Distributions and host ranges, morphological and molecular descriptions, in addition to revealing these new threats, also aid in defining the most stable diagnostic features and those that can be most practically utilized. For example, *M. mayaguensis*, first described in 1988 by Rammah and Hirschmann, is now recorded from West Africa (Senegal, Ivory Coast, Burkina Faso), South Africa, Malawi, the Caribbean (Puerto Rico, Cuba, Dominican Republic, Guadeloupe, Martinique, Trinidad), Brazil, Florida, and a glasshouse in France (De Waele and Elsen, 2007). It is now considered to be conspecific with *M. enterolobii*, described by Yang and Eisenback (1983) in China (Xu *et al.*, 2004), based on identical sequence of a mitochondrial DNA region obtained for both species (see Eisenback and Hunt, Chapter 2, this volume). The elevation in status of *M. enterolobii* as one of the most economically important root-knot nematode species has arisen through surveys that have established its wide geographic distribution and which have utilized biochemical and molecular diagnostics for identification (Fargette and Braaksma, 1990; Fargette *et al.*, 1996; Blok *et al.*, 2002). The recognition of its wide host range, combined with its virulence characteristics, makes it a major threat. The overlap of its morphometric characters with those of the most common tropical species (Brito *et al.*, 2004) has probably led to this species being misidentified in the past. Another species, *M. paranaensis*, which parasitizes coffee, has recently been shown to be widely distributed in coffee-growing regions in Central and South America and was probably confused with *M. incognita* until isozyme esterase phenotyping and RAPDs were used in its identification (Carneiro *et al.*, 1996a,b, 2004b; Hervé *et al.*, 2005). More species are likely to emerge as threats as further surveys are conducted and combined with more reliable diagnostic methods.

Before introducing the biochemical and molecular diagnostics, it is worth considering the sample types that may be used; the various life stages (egg, juvenile, female, male), root tissue or

soil may preclude or limit the suitability of a particular diagnostic method and influence the level of specificity and sensitivity that can be achieved. Bioassays involving nematocide testing or germplasm screening in which defined inoculums are used will have different requirements than field surveys and diversity studies. Assays that distinguish between biotypes require another level of discrimination. Traditional methods that require laborious extraction techniques and microscope observation combined with manual enumeration are still used frequently and may be the most efficient and cost-effective method available for some applications. Biochemical or molecular methods that combine identification with quantification with the potential for automation are still under development and beyond the resources of many organizations. However, examples are provided that illustrate both the practical benefits that biochemical and molecular diagnostics are bringing and how they are improving our understanding of *Meloidogyne* species.

## 4.2. Biochemical Methods

### 4.2.1. Isozymes

One of the earliest examples of the use of isozyme phenotypes to distinguish *Meloidogyne* spp. was published by Esbenshade and Triantaphyllou (1985), who reported esterase patterns from 16 *Meloidogyne* species, with the most common phenotypes being A2 and A3 (*Meloidogyne arenaria*), H1 (*Meloidogyne hapla*), I1 (*M. incognita*) and J3 (*Meloidogyne javanica*). In 1990, Esbenshade and Triantaphyllou used isozymes in their landmark survey involving approximately 300 populations originating from 65 countries and various continents. In later surveys, Carneiro *et al.* (2000) found 18 esterase phenotypes among 111 populations of *Meloidogyne* species in Brazil and other South American countries and Zu *et al.* (2004) examined 46 populations from 14 provinces in China and found five esterase phenotypes. Isozymes continue to be widely used for studies of *Meloidogyne* despite some of their limitations, and isozyme phenotypes for a large number of species have been published (Table 4.1). Schematic diagrams of isozyme patterns based on surveys, including those conducted in the International

*Meloidogyne* project, have been published (Bergé and Dalmaso, 1975; Dalmaso and Bergé, 1978; Fargette, 1978; Janati *et al.*, 1982; Esbenshade and Triantaphyllou, 1985, 1990; Carneiro *et al.*, 2000; Hernandez *et al.*, 2004) and provide important references.

Several isozyme systems have been used, with carboxylesterase/esterase EST (EC 3.1.1.1) proving to be most useful for discriminating *Meloidogyne* species, with others such as malate dehydrogenase MDH (1.1.1.37), superoxide dismutase SOD (1.15.1.1) and glutamate-oxaloacetate transaminase GOT (EC 2.6.1.1) also often included to confirm species identifications (Esbenshade and Triantaphyllou, 1985). Enzyme phenotypes are designated, indicating the *Meloidogyne* species that it specifies and the number of bands detected. Phenotypes with the same number of bands are differentiated by small letters (Esbenshade and Triantaphyllou, 1985, 1990). Enzyme patterns are usually compared with a known standard, frequently from *M. javanica*, which is included in the electrophoresis to determine migration distances. Isozymes are used primarily with the female egg-laying stage using single individuals (Dalmaso and Bergé, 1978), although the use of galled root tissue has also been reported. Miniaturization and automation of the electrophoresis systems and the use of precast polyacrylamide gels (i.e. PhastSystem, Pharmacia Ltd, Uppsala, Sweden) has made isozyme phenotyping a widely used technique (Esbenshade and Triantaphyllou, 1985; Karssen *et al.*, 1995; Chen *et al.*, 1998; Molinari, 2001). These systems are not technically sophisticated and more than one enzyme system can be stained on the same gel. Aside from the initial expense of equipment, the consumables required are relatively inexpensive and so isozymes are often used for field surveys and have been used for routine screening of glasshouse cultures to assure their species stability.

The relative stability of the isozyme phenotypes within *Meloidogyne* species (De Waele and Elsen, 2007) makes them an attractive system, although there are some complications. The occurrence of intraspecific variants and the difficulty in resolving size variants between species (e.g. esterase of *M. incognita* and *M. hapla*) has necessitated the use of more than one enzyme system to confirm the identity of some isolates. Malate dehydrogenase separates *M. hapla* from

**Table 4.1.** Esterase and malate dehydrogenase isozyme phenotypes of *Meloidogyne* spp. including atypical esterase patterns.

Species	Esterase phenotype	Atypical esterase patterns	Mdh
<i>M. arabicida</i>	AR2 <sup>7</sup> , M1F1b <sup>22</sup>		N1 <sup>22</sup>
<i>M. ardenensis</i>			N1a <sup>23</sup>
<i>M. arenaria</i>	A1 <sup>18</sup> , A2 <sup>18</sup> , A3 <sup>18</sup>	S1-M1 <sup>18</sup> , S2-M1 <sup>18</sup> , M3-F1 <sup>18</sup>	N1 <sup>18</sup> , N3 <sup>18</sup>
<i>M. artiella</i>	M2-VF1 <sup>23</sup>		N1b <sup>23</sup>
<i>M. baetica</i>	Rm 0.31 <sup>12</sup>		
<i>M. carolinensis</i>	VS1-S1a <sup>18</sup>		H1 <sup>18</sup>
<i>M. chitwoodi</i>	S1 <sup>18</sup>		N1a <sup>18</sup>
<i>M. coffeicola</i>	C2 <sup>5</sup>		C1 <sup>5</sup>
<i>M. cruciani</i>	M3a <sup>18</sup>		N1 <sup>18</sup>
<i>M. duytisi</i>	VS1 <sup>23</sup>		N2 <sup>23</sup>
<i>M. dunensis</i>	VS1 <sup>28</sup>		N1c <sup>28</sup>
<i>M. enterolobii</i>	VS1-S1 <sup>18</sup>		N1a <sup>18</sup>
<i>M. ethiopica</i>	E3 <sup>9</sup>		N1 <sup>9</sup>
<i>M. exigua</i>	VS1 <sup>20</sup> , E1 (VF1) <sup>5</sup> , E1b (VF1) <sup>5</sup> , E2 <sup>11</sup> , E2a <sup>11</sup> , E3 <sup>11</sup>		N1 <sup>20</sup>
<i>M. fallax</i>	F3 <sup>33</sup>		N1b <sup>23</sup>
<i>M. floridensis</i>	P3 <sup>5, 21</sup>		N1 <sup>5</sup>
<i>M. graminicola</i>	VS1 <sup>18</sup>		N1a <sup>18</sup>
<i>M. graminis</i>	VS1 <sup>19</sup> G1 <sup>3</sup>		N4 <sup>19</sup> , N1a <sup>3</sup>
<i>M. hapla</i>	H1 <sup>18</sup>	A1 <sup>18</sup>	H1 <sup>18</sup>
<i>M. haplanaria</i>	Rm 0.61 <sup>17</sup>		Rm 0.44 <sup>17</sup>
<i>M. hispanica</i>	S2-M1 <sup>18</sup> , Hi3 <sup>6</sup>		N1 <sup>18</sup>
<i>M. incognita</i>	I1 <sup>18</sup> , I2 <sup>5</sup> , M1a <sup>22</sup>	S1 <sup>18</sup>	N1 <sup>18</sup>
<i>M. inornata</i>	I3 <sup>10</sup>		N1 <sup>5</sup>
<i>M. izalcoensis</i>	I4=S4 <sup>22, 8</sup>		N1 <sup>22</sup>
<i>M. javanica</i>	J3 <sup>18</sup> , J2a <sup>19</sup> , J2 <sup>32</sup>		N1 <sup>18, 15</sup>
<i>M. jianyangensis</i>	Rm 0.41, 0.45, 048 <sup>1</sup>		
<i>M. konaensis</i>	F1 <sup>16</sup> , K3 <sup>5</sup> , I1 <sup>31</sup> , F1-I1 <sup>31</sup>		N1 <sup>5</sup>
<i>M. kralli</i>			N1c <sup>23</sup>
<i>M. lusitanica</i>	P1 <sup>27</sup> , A1 <sup>23</sup>		P3 <sup>27</sup> , N1c <sup>23</sup>
<i>M. maritima</i>	VS1-S1 <sup>23</sup>		N1c <sup>23</sup>
<i>M. marylandi</i>	VS1 <sup>26</sup>		N1c <sup>26</sup>
<i>M. enterolobii</i>	VS1-S1 <sup>29</sup> , M2 <sup>5</sup>		N1a <sup>5</sup> , N3c <sup>29</sup>
<i>M. microcephala</i>	A1 <sup>18</sup>		N1 <sup>18</sup>
<i>M. microtyla</i>	M1 <sup>18</sup>		H1 <sup>18</sup>
<i>M. minor</i>	VS1 <sup>24</sup>		N1a <sup>24</sup>
<i>M. moroccensis</i>	A3 <sup>30</sup>		N1 <sup>30</sup>
<i>M. naasi</i>	VF1 <sup>18</sup>		N1a <sup>18</sup>
<i>M. oryzae</i>	VS1 <sup>18</sup>		N1a <sup>18</sup>
<i>M. panyuensis</i>	S1-F1 <sup>25</sup>		N1b <sup>25</sup>
<i>M. paranaensis</i>	P1 <sup>4, 5, 7</sup> , F1 <sup>5</sup> , P2 <sup>7</sup>		N1 <sup>5</sup>
<i>M. partityla</i>	Mp3 <sup>2</sup>		N1a <sup>2</sup>
<i>M. petuniae</i>	VS1-S1 <sup>14</sup>		N1 <sup>14</sup>
<i>M. plantani</i>	S1 <sup>18</sup>		N1a <sup>18</sup>
<i>M. querciana</i>	F1 <sup>18</sup>		N3a <sup>18</sup>

<sup>1</sup>Baojun *et al.* (1990); <sup>2</sup>Brito *et al.* (2008); <sup>3</sup>Brito, pers. comm.; <sup>4</sup>Carneiro *et al.* (1996b); <sup>5</sup>Carneiro *et al.* (2000); <sup>6</sup>Carneiro *et al.* (2004a); <sup>7</sup>Carneiro *et al.* (2004b); <sup>8</sup>Carneiro *et al.* (2005a); <sup>9</sup>Carneiro *et al.* (2007); <sup>10</sup>Carneiro *et al.* (2008); <sup>11</sup>Carneiro, pers. comm.; <sup>12</sup>Castillo *et al.* (2003); <sup>13</sup>Castro *et al.* (2003); <sup>14</sup>Charchar *et al.* (1999); <sup>15</sup>Cofcewick *et al.* (2005); <sup>16</sup>Eisenback *et al.* (1994); <sup>17</sup>Eisenback *et al.* (2003); <sup>18</sup>Esbenshade and Triantaphyllou (1985); <sup>19</sup>Esbenshade and Triantaphyllou (1987); <sup>20</sup>Esbenshade and Triantaphyllou (1990); <sup>21</sup>Handoo *et al.* (2004); <sup>22</sup>Hernandez *et al.* (2004); <sup>23</sup>Karssen and van Hoenselaar (1998); <sup>24</sup>Karssen *et al.* (2004); <sup>25</sup>Liao *et al.* (2005); <sup>26</sup>Oka *et al.* (2003); <sup>27</sup>Pais and Abrantes (1989); <sup>28</sup>Palomares Rius *et al.* (2007); <sup>29</sup>Rammah and Hirschmann (1988); <sup>30</sup>Rammah and Hirschmann (1990); <sup>31</sup>Sipes *et al.* (2005); <sup>32</sup>Tomaszewski *et al.* (1994); <sup>33</sup>van der Beek and Karssen (1997).

*M. incognita*, *M. arenaria* and *M. javanica*, whereas glutamate dehydrogenase separates *M. incognita* from *M. javanica*, *M. arenaria* and *M. hapla* (Esbenshade and Triantaphyllou, 1985). Poor signal intensity can also necessitate the use of several females (e.g. with *Meloidogyne exigua* (Carneiro *et al.*, 2000)).

In surveys concerning *Meloidogyne* biodiversity and nature conservancy, isozymes are a convenient first stage in species identification and have enabled species diversity and the frequency of particular species and their abundance to be determined. Females recovered after allowing multiplication of field samples on a generally susceptible host such as *Solanum lycopersicum* can be tested for their isozyme phenotype and the associated egg mass reserved for further characterization if necessary. Lima *et al.* (2005) used this approach in their study of the nematofauna of the Atlantic forest in Brazil, and Hernandez *et al.* (2004) in their survey of coffee-growing areas in Central America. Novel isozyme phenotypes have been frequently encountered in these surveys of biodiverse regions, adding to the understanding of the species ecology and biogeography of *Meloidogyne* spp.

The literature gives many examples of atypical isozyme phenotypes or those from undescribed species, some of which are resolved as new species in due course. Some examples of these are given here, but it remains one of the challenges of using isozymes where novel phenotypes are obtained, to relate these to previous examples in the literature. Esbenshade and Triantaphyllou (1985) listed F1, VS1, VS1, VS1-S1, VS1-M2, S1-M1, M3, A2 as undescribed phenotypes; Cenis *et al.* (1992) reported an atypical esterase pattern for *M. incognita* from Spain; Hernandez *et al.* (2004) found M1F1a (Rm 73.5, 78.0), M1F1b (Rm 73.5, 82.0) and Sa4 (Rm = 73.5, 78.0, 53.0, 59.0) esterase phenotypes with isolates from coffee in Central America; and Adam *et al.* (2005) described an S2 phenotype for an *M. incognita* isolate from Libya. Molinari *et al.* (2005) reported atypical EST patterns in their survey of populations from India, Venezuela, Cuba and Egypt. Lima *et al.* (2005) found an MC4 phenotype in their survey of montane forest in Brazil; Carneiro *et al.* (2005b) lists unknown populations, including esterase phenotype Br2, Rm 0.92, 1.02, in a survey of coffee in Brazil; Medina *et al.* (2007) found Est

S1, Est F2b and Est F2a in 20% of the samples from fig trees in Brazil; and Carneiro *et al.* (2007) found atypical esterase patterns L3 (Rm: 1.0, 1.1, 1.3) and V3/V4 with a minor band (Rm: 1.3) and three major bands (Rm: 0.9, 1.2, 1.3) in their survey of vineyards in Chile. Clearly many novel esterase patterns are still being discovered, and to determine whether these represent novel or aberrant patterns additional information from host range, geographic distributions and other biochemical, molecular or morphological features are needed. Intraspecific diversity or differences in the patterns obtained from different laboratories may also contribute to slight variations in phenotypes, as highlighted by Hernandez *et al.* (2004).

#### 4.2.2. Antibodies

Polyclonal and monoclonal antibodies have been produced for root-knot nematode identification purposes, as well as for investigations of the nematode surface and secretions, interactions with the host and other parasites, for localization studies, development of plantibodies and behavioural studies (Tastet *et al.*, 2001). Qualitative and quantitative features of immunoassays using poly- or monoclonal antibodies have determined their utility for diagnostic purposes. The sample type from which the antigen will be extracted and with which cross-reaction may occur, the life-stage and the antibody sensitivity and specificity all contribute to whether an immunoassay is appropriate for the particular application. In addition, the process of developing an antiserum requires a considerable investment and hence applications must justify these costs. Polyclonal antibodies tend to be highly sensitive; however, they may also be cross-reactive and lack the specificity required, and different batches may vary in their binding characteristics. Production of a polyclonal antibody to a diagnostic protein can overcome some of the problems with cross-reactivity but producing sufficient pure antigen can be challenging. Monoclonal antibodies (Mab) produced from cell lines can give high specificity and better reproducibility between batches but their production is expensive and cell lines can be unstable. Screening existing libraries for an antibody that has the required specificity is

another alternative but requires technical expertise. For routine testing, such as for nematicide or germplasm screening where a defined nematode species is involved, an enzyme-linked immunoabsorbent assay (ELISA) may be the most appropriate assay. However, when dealing with unknowns, such as in surveys or in quarantine situations, immunoassays are usually not the most appropriate technique to use.

The use of antibodies as diagnostic tools for *Meloidogyne* spp. is limited to a few examples, having mainly been superseded by DNA-based diagnostics, which generally have greater sensitivity and specificity. Davies *et al.* (1996) selected three Mabs that could distinguish females of *M. incognita*, *M. javanica* and *M. arenaria* by ELISA and dot blots; however, cross-reactivity was found when used in Western blots. Antisera raised to purified species-specific esterase bands did permit differentiation of *M. incognita* from *M. javanica* but the Mabs cross-reacted with other species of root-knot nematodes (Davies *et al.* 1996; Ibrahim *et al.* 1996). Ibrahim *et al.* (1996) raised a monoclonal antibody to purified esterase from *M. incognita* and were able to distinguish *M. incognita* from *M. javanica* in crude extracts of non-denatured protein. Tastet *et al.* (2001) used two-dimensional electrophoresis to identify a major protein of *M. chitwoodi* and *Meloidogyne fallax* that was not found in several other *Meloidogyne* species, and following internal amino-acid sequencing, a peptide was synthesized and used to raise antisera in rabbits. They were able to distinguish *M. chitwoodi* and *M. fallax* from eight other *Meloidogyne* species in a dot-blot hybridization with soluble proteins extracted from a single female.

Quantification of root-knot nematodes directly in soil using antibodies has not proved successful, and some level of nematode extraction has been required (Davies *et al.*, 1996). However, immunocapture to recover particular nematodes from mixtures has been achieved. Antiserum-coated magnetized beads (Dynabeads) were used to recover *M. arenaria* from mixtures with other species of nematodes (Chen *et al.*, 2001). Combining an enrichment approach with highly specific antibodies may provide a fruitful avenue for the future. Targets that are unique to particular species or races may be identified from the considerable sequence information that is being generated, and synthetic peptides that are based

on unique sequence regions could be used to raise antibodies and generate a new source of diagnostic antibodies.

### 4.3. DNA-based Methods

#### 4.3.1. DNA Extraction

Many methods have been reported for the extraction of DNA from bulk samples of second-stage juveniles (J2) as well as from single J2, females and males. Methods for the extraction of DNA from plant roots and galls infected with *Meloidogyne* and from soil samples are also available.

For single J2, DNA extraction methods include crushing the nematode on a glass slide with a pipette tip (<http://nematode.unl.edu/nemaid.pdf>), treatment of intact nematodes with NaOH (Stanton *et al.*, 1998), and proteinase K treatment following cutting in worm lysis buffer (Castagnone-Sereno *et al.*, 1995). A systematic diagnostic key for the identification of seven of the common and economically important *Meloidogyne* spp. by Adam *et al.* (2007) provides a logical process for molecular identification of individual nematodes in, at most, three steps. The extraction method used yields sufficient DNA for 15 PCR reactions and the key can be readily expanded to include more species. Multiple displacement amplification (MDA) of total genomic DNA from *Meloidogyne* spp. is also possible to increase the amount of template for molecular analyses from small samples (Skantar and Carta, 2005). For larger samples of juvenile nematodes or egg masses, extraction of DNA using phenol:chloroform (Blok *et al.*, 1997a) or DNA extraction kits such as those of Qiagen are suitable.

Nematodes extracted from soil using a Baermann funnel can be individually isolated for diagnostic analyses. Examples of the application of molecular diagnostics to DNA extracted from the total soil nematode communities are limited. Methods for the extraction of DNA directly from soil, including proteinase K digestion followed by phenol:chloroform extraction, sodium hydroxide extraction, and bead beating combined with a commercial kit for DNA recovery, have been compared by Donn *et al.* (2008), but use of these methods for detection of *Meloidogyne* spp. was not reported.

### 4.3.2. Restriction Fragment Length Polymorphisms (RFLPs)

Initially the use of restriction fragment length polymorphisms (RFLPs) to distinguish species and isolates of root-knot nematodes involved the extraction and purification of genomic DNA, restriction digestion and visualization of banding patterns following gel electrophoresis. An early example of the application of RFLPs to *Meloidogyne* spp. was reported by Curran *et al.* (1985, 1986). The DNA isolated from large numbers of eggs was digested and then subjected to electrophoresis in an agarose gel, followed by visualization of the DNA banding patterns with ethidium bromide. The patterns representing highly repeated regions of DNA allowed samples to be distinguished but required large amounts of DNA and, hence, prior culturing of the isolates. The patterns were often not clearly seen against the background smear of DNA. However, the advantage of the removal of dependency on a particular stage in the life cycle and the inclusion of the whole genome was apparent with this approach. Later, RFLPs were combined with DNA hybridization and the use of either probes labelled radioactively or a non-radioactive detection system using randomly selected clones from genomic DNA, mitochondrial DNA or satellite DNA sequences as probes (Curran and Webster, 1987; Castagnone-Sereno *et al.*, 1991; Gárate *et al.*, 1991; Cenis *et al.*, 1992; Piotte *et al.*, 1992, 1995; Xue *et al.*, 1992; Baum *et al.*, 1994; Hiatt *et al.*, 1995). Although interspecific discrimination was demonstrated in these experiments, the lack of sensitivity, i.e. the requirement for DNA from multiple individuals, the use of radioactivity and the relative complexity of the technique limited its application. The development of PCR has largely supplanted hybridization-based approaches to RFLP analysis for nematode species identification.

### 4.3.3. Satellite DNA Probes and PCR

Satellite DNAs (satDNAs) are highly repeated tandem arrays of short sequences (~70–2000 bp in length) that are associated with heterochromatin, centromeric and telomeric regions of chromosomes. The detection of satDNAs in nematode

tissue squashed on to a membrane and then hybridized with a satellite probe is an attractive diagnostic approach as it requires limited molecular equipment or expertise and can be used efficiently where there are large numbers of samples to screen, such as from field surveys. This method usually does not require DNA extraction or PCR amplification of the nematode DNA and, when used with the non-radioactive detection system DIG, is safe, stable and reusable (Castagnone-Sereno *et al.*, 1999). SatDNAs have different signature sequences and can differ in their copy number, length and polymorphic regions in *Meloidogyne* species (Meštrović *et al.*, 2006), and satDNA assays have been described for several species of *Meloidogyne*. The highly repetitive nature of satDNA aids in their ease of detection (satDNA comprises 2.5% of the genome of *M. incognita* (Piotte *et al.*, 1994) and 20% of *M. fallax* (Castagnone-Sereno *et al.*, 1998)), and the discovery that some satDNAs are divergent between different species has been exploited to develop various diagnostic probes for RFLPs, dot blots and for designing PCR assays. The distribution of these sequences in the genome and the mechanisms involved in their evolution are not well understood; however, with the determination of the genomic sequences of *M. incognita* and *M. hapla* (see Abad and Oppermann, Chapter 16, this volume), the number of different types and their location in the genome is being revealed and may help to understand how satDNA might be further exploited in the future for diagnostic purposes.

Examples of the use of satDNA as a diagnostic probe include repeat sequences from *M. hapla* that were radioactively labelled and had sufficient sensitivity to detect DNA from individual females of *M. hapla*, including those in root tissue (Piotte *et al.*, 1995; Dong *et al.*, 2001a); this probe detected *M. hapla* but not *M. chitwoodi* or *M. incognita*. Castagnone-Sereno *et al.* (2000) isolated a conserved Sau3A satDNA from *M. arenaria*, which was subsequently also described in *M. javanica* (Meštrović *et al.*, 2005). Randig *et al.* (2002a) cloned a BglII satellite from *M. exigua* and used it as a radioactively labelled probe to detect single individuals (J2, females, egg masses and galls squashed on to nylon membrane) and showed it to be specific to *M. exigua* when tested with eight other *Meloidogyne* spp. Similarly, single J2 of *M. chitwoodi* or *M. fallax* could be distinguished from *M. hapla* in a simple squash

blot using DIG-labelled probes from AluI satDNA pMcCo and pMfFd, and, conversely, *M. hapla* was detected and distinguished from *M. chitwoodi* or *M. fallax* with the pMhM satDNA probe isolated from *M. hapla* (Castagnone-Sereno *et al.*, 1998). Conversion of satellite DNA probes into a PCR-based detection system has provided an alternative approach for sensitive detection of *Meloidogyne* spp. This was demonstrated for *M. hapla* by Castagnone-Sereno *et al.* (1995).

#### 4.3.4. Ribosomal DNA PCR

The ribosomal DNA (rDNA) repeating unit, including 18S, 28S, and 5.8S coding genes and the internal transcribed spacer (ITS), external transcribed spacer (ETS) and intergenic spacer (IGS) regions, has been used extensively for both phylogenetic studies and diagnostic purposes. The ITS regions are possibly the most widely used genetic markers among living organisms and the most common species-level marker used for plants, protists and fungi (Hajibabaei *et al.*, 2007). The multi-copy basis of rDNA provides ample target for PCR amplification, and sufficient variation and stability occurs within it for reliable discrimination of most species, although intraspecific variation has been found (Zijlstra *et al.*, 1995; Hugall *et al.*, 1999; Adam *et al.*, 2007) and there is evidence for intra-individual variation (Blok *et al.*, 1997b; Powers *et al.*, 1997; Zijlstra *et al.*, 1997; Hugall *et al.*, 1999). Differences in sequence variation occur between the regions of the rDNA cistron, with regions coding for structural RNAs (18S, 28S, 5.8S) showing greater conservation than the transcribed and non-transcribed intergenic regions (ITS, ETS, IGS). For diagnostic purposes, rDNA PCR amplification products that are polymorphic in size, with or without subsequent restriction enzyme digestion, have been used to identify many *Meloidogyne* spp. For example, PCR-RFLP of the ITS regions has been used to identify *M. arenaria*, *Meloidogyne camelliae*, *Meloidogyne mali*, *Meloidogyne marylandi*, *Meloidogyne suginamiensis* (Orui, 1999), *M. incognita*, *M. javanica*, *M. hapla*, *M. chitwoodi*, *M. fallax* (Zijlstra *et al.*, 1995) and *Meloidogyne naasi* (Schmitz *et al.*, 1998). Size polymorphisms of rDNA amplification products, where products are amplified from more than one species but the size is characteristic of a particular species, are used in the scheme of Adam *et al.* (2005).

Sequence analysis of rDNA is, however, increasingly being used for identification of *Meloidogyne* spp. (Powers, 2004), and this approach is useful when the resources are available and when supported with a sound phylogenetic basis for distinguishing species, which is validated with many isolates (see Adams *et al.*, Chapter 5, this volume). These analyses have also led to a published patent which describes primers based on sequence polymorphisms in rDNA for distinguishing *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *Meloidogyne microtyla*, *Meloidogyne ardenensis*, *Meloidogyne maritima*, *Meloidogyne duytsi*, *M. chitwoodi*, *M. fallax*, *Meloidogyne minor*, *M. naasi*, *Meloidogyne oryzae* and *Meloidogyne graminicola* (Helder *et al.*, 2008).

Various sets of primers are reported in the literature for amplifying different rDNA regions. The primers designed by Vrain *et al.* (1992) have been widely used to amplify the ITS region for *Meloidogyne* spp. and produce a product of ~800 bp, which can then be sequenced to produce species-specific primers or restriction enzyme digestions. For example, this approach was used by Zijlstra (1997) and Zijlstra *et al.* (2004), who sequenced rDNA ITS of *M. naasi*, *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. minor* and *M. incognita* and then designed specific primers for each species to produce products unique to each species. The ITS-RFLP approach, as well as producing characteristic digestion patterns, has been used to determine the composition of species in mixtures by comparing the intensity of bands produced for each species. This was demonstrated for mixtures of *M. hapla*, *M. chitwoodi*, *M. incognita* and *M. fallax* by Zijlstra *et al.* (1997).

Most reports have concluded that there is limited sequence polymorphism in the ITS sequences of the most common species – *M. incognita*, *M. javanica* and *M. arenaria* – to distinguish them, although Hugall *et al.* (1999), in their detailed sequence analyses, did reveal polymorphisms in the ITS region, which they suggested are indicative of gene lineages shared in these species and illustrate the potential for misidentification if ITS sequence is used exclusively for identification of these species. Because of the limited sequence polymorphism in ITS rDNA to distinguish *M. incognita*, *M. javanica* and *M. arenaria* reliably, specific sequenced characterized amplified region (SCAR) primers have been developed for these species. In Table 4.2, examples of species-specific primers are given, some of which are

based on rDNA sequences and others developed from RAPDs. Although these primers are described as 'species specific', they must be considered in relation to the species and isolates that have been used for comparison.

Other species combinations that have proved difficult to distinguish using morphological and biological features, such as *Meloidogyne hispanica* from *M. incognita* and *M. arenaria*, have been differentiated by comparing their sequences from ITS, 18S and D2-D3, a variable region within the 28S gene. However, *M. hispanica* has also been reported to have an identical ITS sequence to *Meloidogyne ethiopica*, suggesting caution is needed with this approach too, although these species can be differentiated by their D2-D3 sequences (Landa *et al.*, 2008). The IGS region has been found to contain repeated sequences and sequence polymorphisms that have been exploited to distinguish *M. chitwoodi* and *M. fallax* (Blok *et al.*, 1997a, 2002; Petersen *et al.*, 1997; Wishart *et al.*, 2002) from other species, including *M. enterolobii*, *M. hapla* and *M. incognita*/*M. javanica*/*M. arenaria*. Distinguishing species based on size polymorphisms of the amplification products has the additional advantage that the products act as positive controls, in contrast to species-specific primer sets, where a product is only obtained from the species that the primers are specific for and the negative results cannot be distinguished from failed reactions.

#### 4.3.5. Mitochondrial DNA

From the perspective of identification, the mitochondrial genome provides a rich source of genetic markers for identification (Rubinoff and Holland, 2005; Hu and Glasser, 2006). Multiple copies of the circular mitochondrial genome are contained within each cell, providing ample template for PCR assays. Uniparental inheritance and a low level of recombination facilitate the construction of phylogenies that can be used to address questions of species boundaries and variation among populations. Rates of evolution of the mitochondrial genome are generally higher than rates for corresponding nuclear genes, creating sufficient nucleotide variation for species-level analyses (Brown *et al.*, 1979). The conserved gene content among mitochondrial genomes of ani-

mals allows investigators to compare similar experimental approaches across widely divergent phyla. For example, postglacial recolonization patterns in Europe have been inferred by an examination of the mitochondrial cytochrome b gene of wood rats and their nematode parasites (Nieberding *et al.*, 2005). The Consortium for the Barcode of Life (<http://www.barcoding.si.edu/>) has exploited these mitochondrial features and proposed a worldwide initiative in which all known species are 'barcoded' by DNA sequence from the cytochrome oxidase subunit I (COI) gene. One objective of this initiative is to develop a rapid method to identify all known animal species. The initiative has generated considerable debate, particularly on theoretical and philosophical issues, with critics claiming proponents of the approach oversell the advantages and insights to be gained from a 'one-gene-fits-all' approach (Moritz and Cicero, 2004). Advocates point to a growing literature of empirical taxonomic studies that employ the COI barcode (Vogler and Monaghan, 2007). Several nematode taxa have been barcoded by COI, such as *Bursaphelenchus* (Ye *et al.*, 2007); however, studies on *Meloidogyne* are yet to be published.

A structural map of the mitochondrial genome of *Meloidogyne* was published by Okimoto *et al.* (1991), although not the full sequence. The map showed the location of 12 protein-coding genes, the large and small rRNA genes, and tRNA genes (Fig. 4.1). In gene content and overall structure, the *Meloidogyne* mitochondrial genome resembled other animal mtDNAs. It is a circular molecule with genes co-linearly arranged without intervening non-coding DNA sequences. However, several unique characteristics of the genome highlighted features that subsequently were incorporated into diagnostic assays. Gene order in *Meloidogyne* mitochondria differed from that of two other nematodes, *Ascaris suum* and *Caenorhabditis elegans* (Okimoto *et al.*, 1991). The differences in gene order allowed for the development of PCR-based diagnostic assays with reduced probability of false-positive amplifications. This could be accomplished by placing primer pairs in two genes that were not adjacent to each other in non-target mitochondrial genomes. A second feature, relatively rare in animal mitochondrial genomes, was the presence of non-coding, repeated sequences. Three sets of different-sized



repeats, 102, 63 and 8 nucleotides in length, are clustered apart from the protein-coding genes. Blok *et al.* (2002) discovered that amplification of the 63 bp repeating region by flanking primers produced a discrete 320 bp product with *M. enterolobii*, whereas other *Meloidogyne* species produced either a multi-banded pattern or no amplification product. Hyman and Whipple (1996) and Lunt *et al.* (2002) have explored the possibility of using the repeated region as a marker to examine population dynamics. The extreme variability of this region within and among offspring of the same parent makes this an intriguing target for genealogical studies, but amplification properties make it procedurally difficult to analyse (Lunt *et al.*, 2002). A second region of the *Meloidogyne* genome amenable to diagnostic development is the portion of the genome flanked by the COII gene and the large (16S) ribosomal gene. Between these two genes is the tRNA-His gene (53 bp) and, in the mitotically parthenogenetic species, non-coding sequences that include a stem and loop structure characteristic of the AT-rich region or control region of the mitochondrial molecule (Hugall *et al.*, 1994, 1997; Jeyaprakash *et al.*, 2006). This region was originally targeted as a potential means for differentiating the five common *Meloidogyne* species of different-sized amplified products generated by primers positioned in the 3' portion of COII and the 5' portion of 16S rRNA (Powers and Harris, 1993). Three size classes were recognized: (i) an approximately 530 bp amplification product was observed in *M. hapla*, which included the flanking portions of COII and 16S rDNA and the complete tRNA-His but no AT-rich region; (ii) a 1.1 kb amplification product found in *M. arenaria* included an approximately 570 bp AT-rich region; and (iii) *M. incognita* and *M. javanica* had the largest amplification products (~ 1.6 kb) due to an AT-rich region of approximately 1.0 kb. Today, more than 15 years later, many additional *Meloidogyne* species have been examined, resulting in numerous size classes (Fig. 4.1). These size classes result primarily from insertions and deletions in the AT-rich region. A large group of species fall into the smallest size class, those lacking an AT-rich region in the amplified product. Together with *M. hapla*, these include *M. chitwoodi*, *M. fallax*, *M. graminicola*, *Meloidogyne graminis*, *M. mali*, *M. marylandi*, *M.*

*microtyla*, *M. naasi*, *M. oryzae*, *M. suginamiensis* and *Meloidogyne trifolliophila*. Presumably this is the ancestral state for *Meloidogyne* since non-*Meloidogyne* species, such as *Nacobbus aberrans*, share this trait (Powers, unpublished observation). *M. mayaguensis* and *M. enterolobii* share a 167 bp AT-rich region, identical in size and sequence, a key feature which led to their synonymization (Blok *et al.*, 2002; Xu *et al.*, 2004). *M. arenaria* and *M. floridensis* share an intermediate-sized AT-rich region of 573 and 603 bp respectively, and *M. incognita*, *M. javanica* and other mitotically parthenogenetic species possess AT-rich regions that range from 963 to 1100 bp in size (Jeyaprakash *et al.*, 2006). Size classes of amplification will probably diminish in diagnostic value as more species are examined and distinctions among groups based on size alone are blurred. However, sequence polymorphism among species remains sufficient to construct diagnostic assays, keeping in mind that all diagnostic assays must be grounded in an understanding of species boundaries. The disparities among phylogenetic trees generated from 18S ribosomal DNA and mitochondrial DNA suggest a full understanding of *Meloidogyne* species boundaries is yet to be obtained (Tigano *et al.*, 2005).

#### 4.3.6. Sequence Characterized Amplified Regions (SCARs)

Specific primers have been developed to PCR-amplify diagnostic repetitive regions of sequence: sequence characterized amplified regions (SCARs). Typically, characteristic repetitive sequences have been identified following an analysis of a panel of isolates from several *Meloidogyne* species with short RAPD primers of eight to ten nucleotides; the differential bands are isolated, sequenced and long specific primers designed. Examples of 'species specific' primer sets based on RAPD product and rDNA sequences are shown in Table 4.2 for ten species. For several species there are choices of primer sets. The sensitivity and the specificity of these primer sets will vary and depend on the number of species and isolates that they have been tested with. There are also examples where several sets of SCAR primers have been used together in multiplex reactions, which allows several species

**Table 4.2.** 'Species specific' primers for *Meloidogyne* identification. See references for species and isolates used for validation of these primers.

Species	Primer set (5'-3')	Amplicon length	Reference
<i>M. arenaria</i>	TCGGCGATAGAGGTAAATGAC TCGGCGATAGACTACAAC	420 bp	Zijlstra <i>et al.</i> , 2000
	TCGAGGGCATCTAATAAAGG GGGCTGAATAATCAAAGGAA	950 bp	Dong <i>et al.</i> , 2001b
<i>M. chitwoodi</i>	CCAATGATAGAGATAGGAAC CTGGCTTCCTCTTGTCAAA	400 bp	Williamson <i>et al.</i> , 1997
	GATCTATGGCAGATGGTATGGA AGCCAAAACAGCGACCGTCTAC	900 bp	Petersen <i>et al.</i> , 1997
<i>M. exigua</i>	TGGAGAGCAGCAGGAGAAAGA GGTCTGAGTGAGGACAAGAGTA	800 bp	Zijlstra, 2000
	CATCCGTGCTGTAGCTGCGAG CTCCGTGGGAAGAAAGACTG	562 bp	Randig <i>et al.</i> , 2002a
<i>M. fallax</i>	TGGGTAGTGGTCCCCTCTG AGCCAAAACAGCGACCGTCTAC	1100 bp	Petersen <i>et al.</i> , 1997
	CCAAACTATCGTAATGCATTATT GGACACAGTAATTCATGAGCTAG	515 bp	Zijlstra, 2000
<i>M. hapla</i>	CAGGCCCTTCCAGCTAAAGA CTTCGTTGGGGAAGTGAAGA	960 bp	Williamson <i>et al.</i> , 1997
	TGACGGCGGTGAGTGCGA TGACGGCGGTACCTCATAG	610 bp	Zijlstra, 2000
<i>M. incognita</i>	GGCTGAGCATAGTAGATGATGTT ACCCATTAAGAGGAGTTTTGC	1500 bp	Dong <i>et al.</i> , 2001b
	GGATGGCGTGCTTTCAAC AAAAATCCCCTCGAAAAATCCACC	440 bp	Wishart <i>et al.</i> , 2002
<i>M. javanica</i>	CTCTGCCCAATGAGCTGTCC CTCTGCCCTCACATTAGG	1200 bp	Zijlstra <i>et al.</i> , 2000
	TAGGCAGTAGGTTGTCGGG CAGATATCTCTGCATTGGTGC	1350 bp	Dong <i>et al.</i> , 2001b
<i>M. mayaguensis</i>	GGGATGTGTAAATGCTCCTG CCCGCTACACCCTCAACTTC	399 bp	Randig <i>et al.</i> , 2002a
	GTGAGGATTCAGCTCCCCAG ACGAGGAACATACTTCTCCGTCC	955 bp	Meng <i>et al.</i> , 2004
<i>M. naasi</i>	CCTTAATGTCAACACTAGAGCC GGCCTTAACCGACAATTAGA	1650 bp	Dong <i>et al.</i> , 2001b
	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACATAC	670 bp	Zijlstra <i>et al.</i> , 2000
<i>M. paranaensis</i>	ACGCTAGAATTCGACCCTGG GGTACCAGAAGCAGCCATGC	517 bp	Meng <i>et al.</i> , 2004
	GAAATTGCTTTATTGTTACTAAG TAGCCACAGCAAATAGTTTTTC	322 bp	Blok <i>et al.</i> , 2002
<i>M. mayaguensis</i>	CTCTTTATGGAGAATAATCGT CCTCCGCTTACTGATATG	433 bp	Zijlstra <i>et al.</i> , 2004
	GCCCGACTCCATTTGACGGA CCGTCCAGATCCATCGAAGTC	208 bp	Randig <i>et al.</i> , 2002b

to be identified in a single reaction (Zijlstra, 2000; Randig *et al.*, 2002b). Interference between primers can be a problem in multiplexing so that specificity is compromised and usually multiplexing only works with a limited number of primers.

#### 4.3.7. Random Amplified Polymorphic DNA (RAPDs)

Random amplified polymorphic DNA (RAPDs) have been developed to examine intra- and

interspecific relationships of *Meloidogyne* spp. (Blok *et al.*, 1997b), from which SCAR primers for species identification have been developed (see Section 4.3.6), and they have been used directly to assist with species identification. Characteristic amplification patterns that are obtained with certain RAPD primers are used to distinguish individuals. Species-specific diagnostic primers are preferred for identification purposes as the relative high annealing temperatures that are used with species-specific primers enhance their specificity. However, occasionally ambiguous results are obtained with specific primers, possibly due to a polymorphism within the binding site of the primers or a deletion within the amplification region which leads to an atypical size of amplification product. In these instances RAPDs have been used, even with individual nematodes, to assist with identifications (Adam *et al.*, 2007). Orui (1999) used RAPD amplification with DNA extracted from single J2 or males to distinguish ten *Meloidogyne* spp., and Randig *et al.* (2001) observed stable RAPD profiles from single females and showed that they remained stable for three subsequent generations using DNA equivalent to a quarter of a female nematode in each reaction. Adam *et al.* (2007) also found consistent amplification patterns from individual J2, females and males of *M. javanica* using RAPDs. Obtaining reproducible amplification patterns with RAPDs requires rigorous application of procedures; however, they are useful in certain circumstances.

#### 4.3.8. Other PCR Targets

The potential for using RKN pathogenicity and avirulence factors for diagnostic purposes remains largely unexplored and may provide rational bases for deployment of resistance and cropping regimes in the future. For example, the pharyngeal gland protein SEC 1 sequence was used by Tesaová *et al.* (2003) to distinguish *M. incognita* from *M. javanica*, *M. arenaria*, *M. hapla*, *M. chitwoodi* and *M. fallax*, although the molecular bases for the differentiation was not explained.

#### 4.3.9. Real-time PCR

Few examples have been published using real-time PCR for identification and quantification of

root-knot nematodes. Increased sensitivity compared with conventional PCR, simultaneous detection of more than one species and the absence of post-PCR processing steps are advantages; however, real-time PCR does require specialized equipment and reagents. The use of probes can increase the specificity of real-time PCR assays, and minor sequence polymorphisms can be exploited with novel chemistries in the probes that maximize sequence discrimination, particularly when size differences in the products cannot be distinguished reliably or when heteroduplex formation may confound interpretations. Applications include ecological studies involving species mixtures or for examination of quarantine samples where closely related species may be present. Zijlstra and van Hoof (2006) reported a real-time multiplex test for *M. chitwoodi* and *M. fallax*, two species that are sympatric and of economic and quarantine importance in a number of countries. Ciancio *et al.* (2005) and Toyota *et al.* (2008) have reported real-time PCR primers for *M. incognita*, and Berry *et al.* (2008) have reported real-time PCR primers for *M. javanica*. Stirling *et al.* (2004) describe the use of real-time PCR to evaluate a risk assessment of *Meloidogyne* spp. damage to tomato using 400g soil samples; however, primer sequences are not provided.

#### 4.3.10. Microarrays

The potential of microarray technology for diagnosis of plant-parasitic nematodes in complex samples is a new approach being developed. The principle has already been demonstrated for the detection of human and plant pathogens with oligonucleotide spotted arrays. Microarrays can circumvent some of the limitations of multiplex PCR where several optimized primer sets are used in a reaction and interference/competition/loss in specificity in the amplification reactions, as well as problems in discriminating the products, can be problematic. An attraction of microarrays is the potential to monitor a large number of possible targets simultaneously, a feature that is important for plant protection organizations with responsibility for many different organisms as well as for those conducting ecological studies involving complex communities. The specificity of microarrays is dependent on unique signature sequences being available for each species.

However, the large number of sequences (probes for specific targets) that can be screened simultaneously allows for more than one capture probe to be used for each species, thus increasing the confidence in the results. Improvements in the sensitivity and specificity of arrays with shorter and more sequence-specific oligonucleotides have been made as well as in the chemistries of the probes. Major issues that remain are the amplification of unknowns from complex samples and the non-expected behaviour of some probes, in which sequences that have mismatches with the target hybridize better than the perfectly matched target (Frederique Pasquer, pers. comm.), as well as cost. Examples of published microarray results that include *Meloidogyne* species are still limited (Szemes *et al.*, 2005; François *et al.*, 2006; van Doorn *et al.*, 2007), but they illustrate the potential of the technology. To obtain the sensitivity that is required for the detection of nematodes, amplification of the target DNA of the nematode is necessary. Multiplex amplification strategies involve either amplification with generic primers or multiple primer sets that target a genomic region containing species-specific information (to be recognized on the microarray); both approaches face serious limitations. Targeting a conserved genome region limits the analysis to a taxonomically defined group, while combining several primer sets may present a significant technical challenge. François *et al.* (2006) generated PCR products from *M. chitwoodi* using specific primers labelled with Cyanine 3 or Cyanine 5 fluorescent dyes, and hybridized them overnight to the microarray. They were able to detect *M. chitwoodi* in pure and mixed samples (i.e. when *M. chitwoodi* DNA was mixed with DNA from a congeneric nematode species), and they found that simultaneous hybridization of the microarray with two amplified targets labelled with different dyes gave no significant competition between the targets. Padlock probes offer a means of combining pathogen-specific molecular recognition and universal amplification. In combination with a microarray, padlock probe technology has been shown to enable the sensitive simultaneous detection of ten different plant pathogens, among them *M. hapla* (Szemes *et al.*, 2005). More recently, a similar approach using OpenArrays enabled the quantitative multiplex detection of 13 plant pathogens, among them *M. hapla* (van Doorn *et al.*, 2007). Microarrays do offer the possibility of a uniform and standardized detection system for a

wide range of pathogens and further developments are expected in the future.

#### 4.4. Conclusions and Future Directions

Biochemical and molecular methods for identification of *Meloidogyne* species are now widely used and, in some cases, essential for species diagnosis. They cannot, however, be used with confidence to identify all *Meloidogyne* species. A clear understanding of species boundaries and adequate sampling of known species across their geographic range are lacking (Adams *et al.*, Chapter 5, this volume). Particularly noteworthy are the recent conclusions of Lunt (2008), which strongly suggest that the tropical apomictic *Meloidogyne* species result from interspecific hybridizations, and this is also indicated in the genome sequence of *M. incognita* (Abad *et al.*, 2008). Depending on the nature of the interspecific hybridization and the parental species involved, these hybrids pose special difficulties for diagnostics based on single genetic loci. Several species, such as *M. chitwoodi* and *M. enterolobii*, are well characterized by multiple genetic markers and have been sampled across much of their known range. Other species, such as *M. floridensis* and *M. fallax*, have been characterized molecularly but are currently known from a relatively limited geographic region. The so-called major species – *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* – have been extensively studied biochemically and molecularly, resulting in an increasingly large set of 'atypical' diagnostic characters (see above). The unexpectedly high levels of intraspecific variation within the clonal, mitotically parthenogenetic species mirror their observed cytological and physiological variation (Castagnone-Sereno, 2006). *M. hapla* is readily differentiated morphologically and molecularly from the other three species; none the less, documentation of nuclear and mitochondrial variation in *M. hapla* has steadily accumulated (Peloquin *et al.*, 1993; Pottie *et al.*, 1995; Hugall *et al.*, 1997; Handoo *et al.*, 2005; Powers *et al.*, 2005). In addition to the continued evaluation of intraspecific variation, there is a pressing need to incorporate newly discovered tropical and Asian species of *Meloidogyne* into current identification protocols (De Waele and Elsen, 2007). Validation and adaptation of these

methods for different geographic regions and different working conditions is a challenging goal. To date, most studies employing molecular diagnostic methods have been conducted at academic or national institutions and few large-scale surveys, such as that conducted by Powers *et al.* (2005), have employed molecular diagnostics and taken the theory into practice. If routine use of molecular identification to meet regulatory demands or to enhance management decisions is a goal of diagnostics, then it will be necessary to emphasize methods that are robust, reliable and inexpensive. Given the current concerns in relation to climate change, food security and the glo-

bal transport of agricultural commodities, the use of diagnostics for *Meloidogyne* spp. is highly relevant.

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#### 4.6. References

- Abad, P., Gouzy, J., Aury, J.-M. *et al.* (2008) Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology* 26, 909–915.
- Adam, M.A.M., Phillips, M.S. and Blok, V.C. (2005) Identification of *Meloidogyne* spp. from north east Libya and comparison of their inter- and intro-specific genetic variation using RAPDs. *Nematology* 7, 599–609.
- Adam, M.A.M., Phillips, M.S. and Blok, V.C. (2007) Molecular diagnostic key for identification for single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology* 56, 190–197.
- Baojun, Y., Kaiji, H., Hui, C. and Weisheng, Z. (1990). A new species of root-knot nematode *Meloidogyne jianyangensis* n. sp. parasitizing mandarin orange. *Acta Phytopathologica Sinica* 20, 259–264.
- Baum, T.J., Lewis, S.A. and Dean, R.A. (1994) Isolation, characterization and application of DNA probes specific to *Meloidogyne arenaria*. *Phytopathology* 84, 489–494.
- Bergé, J.-B. and Dalmasso, A. (1975) Biochemical characteristics of several populations of *Meloidogyne hapla* and *Meloidogyne* spp. *Cahiers O.R.S.T.O.M. Serie Biologie, Nematologie* 10, 263–271.
- Berry, S.D., Fargette, M., Spaul, V.W., Morand, S. and Cadet, P. (2008) Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zaei*) and adagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes* 22, 168–176.
- Blok, V.C., Phillips, M.S. and Fargette, M. (1997a) Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root knot nematodes. *Journal of Nematology* 29, 16–22.
- Blok, V.C., Phillips, M.S., McNicol, J.W. and Fargette, M. (1997b) Genetic variation in tropical *Meloidogyne* spp. as shown by RAPDs. *Fundamental and Applied Nematology* 20, 127–133.
- Blok, V.C., Wishart, J., Fargette, M., Berthier, K. and Phillips, M.S. (2002) Mitochondrial differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology* 4, 773–781.
- Brito, J., Powers, T.O., Mullin, P.G., Inserra, R.N. and Dickson, D.W. (2004) Morphological and molecular characterisation of *Meloidogyne mayaguensis* isolates from Florida. *Journal of Nematology* 36, 232–240.
- Brito, J.A., Kaur, R., Cetintas, R., Stanley, J.D., Mendes, M.L., McAvoy, E.J., Powers, T.O. and Dickson, D.W. (2008) Identification and isozyme characterization of *Meloidogyne* spp. infecting horticultural and agronomic crops, and weed plants in Florida. *Nematology* 10, 757–766.
- Brown, W.M., George, M. Jr and Wilson, A.C. (1979) Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA* 76, 1967–1971.
- Carneiro, R.M.D.G., Almeida, M.R.A. and Carneiro, R.G. (1996a) Enzyme phenotypes of Brazilian populations of *Meloidogyne* spp. *Fundamental and Applied Nematology* 19, 555–560.

- Carneiro, R.M.D.G., Carneiro, R.G., Abrantes, I.M.O., Santos, M.S.N.A. and Almeida, M.R.A. (1996b) *Meloidogyne paranaensis* n. sp. (Nemata: Meloidogynidae), a root-knot nematode parasitizing coffee in Brazil. *Journal of Nematology* 28, 177–189.
- Carneiro, R.M.D.G., Almeida, M.R.A. and Quénéhervé, P. (2000) Enzyme phenotypes of *Meloidogyne* spp. populations. *Nematology* 2, 645–654.
- Carneiro, R.M.D.G., Tigano, M.S., Randig, O., Almeida, M.R.A. and Sarah, J.L. (2004a) 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., Ritta, M., Almeida, A. and Gomes, A.C.M.M. (2004b) First record of *Meloidogyne hispanica* Hirschmann, 1986 on squash in state of Bahia, Brazil. *Nematologia Brasileira* 28, 215–218.
- Carneiro, R.M.D.G., Almeida, M.R.A., Gomes, A.C.M.M. and Hernandez, A. (2005a) *Meloidogyne izardensis* n. sp. (Nematoda: Meloidogynidae), a root-knot nematode parasitizing coffee in El Salvador. *Nematology* 7, 819–832.
- Carneiro, R.M.D.G., Randig, O., Almeida, M.R.A. and Gonçalves, W. (2005b) Identification and characterization of *Meloidogyne* species on coffee from São Paulo and Minas Gerais states of Brazil using esterase phenotypes and SCAR-PCR multiplex. *Nematologia Brasileira* 29, 233–241.
- Carneiro, R.M.D.G., Almeida, M.R.A., Cofcewicz, E.T., Magunacelaya, J.C. and Aballay, E. (2007) *Meloidogyne ethiopica*, a major root-knot nematode parasitising *Vitis vinifera* and other crops in Chile. *Nematology* 9, 635–641.
- Carneiro, R.M.D.G., Mendes, M. De L., Almeida, M.R.A., dos Santos, M.F.A., Gomes, A.C.M.M. and Karssen, G. (2008) Additional information on *Meloidogyne inornata* Lordello, 1056 (Tylenchida: Meloidogynidae) and its characterisation as a valid species. *Nematology* 10, 123–136.
- Castagnone-Sereno, P. (2006) Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. *Heredity* 96, 282–289.
- Castagnone-Sereno, P., Piotte, C., Abad, P., Bongiovanni, M. and Dalmasso, A. (1991) Isolation of a repeated DNA probe showing polymorphism among *Meloidogyne incognita* populations. *Journal of Nematology* 23, 316–320.
- Castagnone-Sereno, P., Esparrago, G., Abad, P., Leroy, F. and Bongiovanni, M. (1995) Satellite DNA as a target for PCR-specific detection of the plant-parasitic nematode *Meloidogyne hapla*. *Current Genetics* 28, 566–570.
- Castagnone-Sereno, P., Semblat, J.P., Leroy, F. and Abad, P. (1998) A new *AluI* satellite DNA family in the root-knot nematode *Meloidogyne fallax*: relationships with satellites from the sympatric species *M. hapla* and *M. chitwoodi*. *Molecular Biology and Evolution* 15, 1115–1122.
- 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.
- Castagnone-Sereno, P., Leroy, F. and Abad, P. (2000) Cloning and characterization of an extremely conserved satellite DNA family from the root-knot nematode *Meloidogyne arenaria*. *Genome* 43, 346–353.
- 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.
- Castro, J.M.D.C.E., De Lima, R.D. and Carneiro, R.M.D.G. (2003) Isoenzymatic variability in Brazilian populations of *Meloidogyne* spp. from soybean. *Nematologia Brasileira* 27, 1–12.
- Cenis, J.L., Opperman, C.H. and Triantaphyllou, A.C. (1992) Cytogenetic, enzymatic and restriction fragment length polymorphism variation of *Meloidogyne* spp. from Spain. *Phytopathology* 82, 527–531.
- Charchar, J.M., Eisenback, J.D. and Hirschmann, H. (1999) *Meloidogyne petuniae* n. sp. (Nemata: Meloidogynidae), a root-knot nematode parasitic on petunia in Brazil. *Journal of Nematology* 31, 81–91.
- 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 magnetised beads. *Nematology* 3, 593–601.
- Chen, Y.F., Wu, J.Y., Hu, X.O. and Yu, S.F. (1998) Using PhastSystem for rapid identification of root-knot nematodes. *Acta Phytopathology Sinica* 28, 73–77.
- Ciancio, A., Loffredo, A., Paradies, F., Turturo, C. and Sialer, M.F. (2005) Detection of *Meloidogyne incognita* and *Pochonia chlamydosporia* by fluorogenic molecular probes. *EPPO Bulletin* 35, 157–164.
- Cofcewick, E.T., Carneiro, R.M.D., Randig, O., Chabrier, C. and Quénéhervé, P. (2005) Diversity of *Meloidogyne* spp. on *Musa* in Martinique, Guadeloup, and French Guiana. *Journal of Nematology* 37, 313–322.

- Curran, J. and Webster, J.M. (1987) Identification of nematodes using restriction fragment length differences and species-specific DNA probes. *Canadian Journal of Plant Pathology* 9, 162–166.
- 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.
- Curran, J., McClure, M.A. and Webster, J.M. (1986) Genotypic differentiation of *Meloidogyne* populations by detection of restriction fragment length difference in total DNA. *Journal of Nematology* 18, 83–86.
- Dalmasso, A. and Bergé, J.-B. (1978) Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* spp.: application to the taxonomy of *Meloidogyne*. *Journal of Nematology* 10, 323–332.
- Davies, K.G., Curtis, R.H. and Evans, K. (1996) Serologically based diagnostic and quantification tests for nematodes. *Pesticide Science* 47, 81–87.
- De Waele, D. and Elsen, A. (2007) Challenges in tropical plant nematology. *Annual Review of Phytopathology* 45, 457–485.
- Dong, K., Dean, R.A., Fortnum, B.A. and Lewis, S.A. (2001a) A species-specific DNA probe for the identification of *Meloidogyne hapla*. *Nematropica* 31, 17–23.
- Dong, K., Dean, R.A., Fortnum, B.A. and Lewis, S.A. (2001b) Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. *Nematropica* 31, 271–280.
- Donn, S., Griffiths, B.S., Neilson, R. and Daniell, T.J. (2008) DNA extraction from soil nematodes for multi-sample community studies. *Applied Soil Ecology* 38, 20–26.
- Eisenbeck, J.D., Bernard, E.C. and Schmitt, D.P. (1994) Description of the Kona coffee root-knot nematode, *Meloidogyne konaensis* n. sp. *Journal of Nematology* 26, 363–374.
- Eisenback, J.D., Bernard, E.C., Starr, J.L., Lee, T.A. Jr and Tomaszewski, E.K. (2003) *Meloidogyne haplanaria* n. sp. (Nematoda: Meloidogynidae), a root-knot nematode parasitizing peanut in Texas. *Journal of Nematology* 35, 395–403.
- Esbenshade, P.R. and Triantaphyllou, A.C. (1985) Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* 17, 6–20.
- Esbenshade, P.R. and Triantaphyllou, A.C. (1987) Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). *Journal of Nematology* 19, 8–18.
- Esbenshade, P.R. and Triantaphyllou, A.C. (1990) Isozyme phenotypes for the identification of *Meloidogyne* species. *Journal of Nematology* 22, 10–15.
- Fargette, M. (1978) Use of the esterase phenotype in the taxonomy of the genus *Meloidogyne*. 2. Esterase phenotypes observed in West African populations and their characterisation. *Revue de Nématologie* 10, 45–56.
- Fargette, M. and Braaksma, R. (1990) Use of the esterase phenotype in taxonomy of the genus *Meloidogyne*. 3. A study of some 'B' race lines and their taxonomic position. *Revue de Nématologie* 13, 375–386.
- Fargette, M., Phillips, M.S., Blok, V.C., Waugh, R. and Trudgill, D.L. (1996) An RFLP study of relationships between species, populations and resistance-breaking lines of tropical species of *Meloidogyne*. *Fundamental and Applied Nematology* 19, 193–200.
- 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 the quarantine species *Meloidogyne chitwoodi*. *Molecular and Cellular Probes* 20, 64–69.
- Gárate, T., Robinson, M.P., Chacón, M.R. and Parkhouse, R.M.E. (1991) Characterization of species and races of the genus *Meloidogyne* by DNA restriction enzyme analysis. *Journal of Nematology* 23, 414–420.
- Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. and Hickey, D.A. (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics* 23, 167–172.
- Handoo, Z.A., Nyczepir, A.P., Esmenjaud, D., van der Beek, J.G., Castagnone-Sereno, P., Carta, L.K., Skantar, A.M. and Higgins, J.A. (2004) Morphological, molecular, and differential-host characterization of *Meloidogyne floridensis* n. sp. (Nematoda: Meloidogynidae), a root-knot nematode parasitizing peach in Florida. *Journal of Nematology* 36, 20–35.
- Handoo, Z.A., Skantar, A.M., Carta, L.K. and Schmitt, D.P. (2005) Morphological and molecular evaluation of a *Meloidogyne hapla* population damaging coffee (*Coffea arabica* L.) in Maui, Hawaii. *Journal of Nematology* 37, 136–145.
- Helder, J., Karssen, G., Van Den, E.S.J.J., Holterman, M.H.M., Veenhuizen, P.T.M., Landeweert, R., Hekman, H. and Bakker, J. (2008) Methods of detecting root knot nematodes. [www.freepatentsonline.com/EP1878803.html](http://www.freepatentsonline.com/EP1878803.html) (1/16/08).

- Hernandez, A., Fargette, M. and Sarah, J.L. (2004) Characterisation of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) from coffee plantations in Central America and Brazil. *Nematology* 6, 193–204.
- Hervé, G., Bertrand, B., Villain, L., Licardie, D. and Cilas, C. (2005) Distribution analyses of *Meloidogyne* spp. and *Pratylenchus coffeae sensu lato* in coffee plots in Costa Rica and Guatemala. *Plant Pathology* 54, 471–475.
- Hiatt, E.E., Georgi, L., Huston, S., Harshman, D.C., Lewis, S.A. and Abbott, A.G. (1995) Intra- and inter-population genome variation in *Meloidogyne arenaria*. *Journal of Nematology* 27, 143–152.
- Hirschmann, H. (1985) The genus *Meloidogyne* and morphological characters differentiating its species. In: Sasser, J.N. and Carter, C.C. (eds) *An Advanced Treatise on Meloidogyne. Volume 1, Biology and Control*. North Carolina State University Graphics, Raleigh, North Carolina, pp. 79–93.
- Hu, M. and Gasser, R.B. (2006) Mitochondrial genomes of parasitic nematodes – progress and perspectives. *Trends in Parasitology* 22, 78–84.
- Hugall, A., Moritz, C., Stanton, J. and Wolstenholme, D.R. (1994) Low, but strongly structured mitochondrial DNA diversity in root-knot nematodes (*Meloidogyne*). *Genetics* 136, 903–912.
- Hugall, A., Stanton, J. and Moritz, C. (1997) Evolution of the AT-rich mitochondrial DNA of the root knot nematode, *Meloidogyne hapla*. *Molecular and Biological 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 and Biological Evolution* 16, 157–164.
- Hyman, B.C. and Whipple, L.E. (1996) Application of mitochondrial DNA polymorphism to *Meloidogyne* molecular population biology. *Journal of Nematology* 20, 268–276.
- 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.
- Janati A., Bergé, J.-B., Triantaphyllou, A.C. and Dalmasso, A. (1982) Nouvelles données sur l'utilisation des isoestérases pour l'identification des *Meloidogyne*. *Revue de Nématologie* 5, 147–154.
- Jepson, S.B. (1987) *Identification of Root-knot Nematodes (Meloidogyne species)*. CABI Publishing, Wallingford, UK.
- Jeyaprakash, A., Tigano, M.S., Brito, J., Carneiro, R.M.D.G. and Dickson, D.W. (2006) Differentiation of *Meloidogyne floridensis* from *M. arenaria* using high-fidelity PCR amplified mitochondrial AT-rich sequences. *Nematropica* 36, 1–12.
- Karssen, G. (2002) *The Plant-parasitic Nematode Genus Meloidogyne Göldi, 1892 (Tylenchida) in Europe*. Koninklijke Brill NV, Leiden, The Netherlands.
- Karssen, G. and van Hoenselaar, T. (1998) Revision of the genus *Meloidogyne* Göldi, 1892 (Nematoda: Heteroderidae) in Europe. *Nematologica* 44, 713–788.
- 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.
- Landa, B.B., Palomares Rius, J.E., Vovlas, N., Carneiro, R.M.D.G., Maleita, C.M.N. and Abrantes, I.M. de O. (2008) Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease* 92, 1104–1110.
- Liao, J., Yang, W., Feng, Z. and Karssen, G. (2005). Description of *Meloidogyne panyuensis* sp. n. (Nematoda: Meloidogynidae), parasitic on peanut (*Arachis hypogea* L.) in China. *Russian Journal of Nematology* 13, 107–114.
- Lima, I.M., Souza, R.M., Silva, C.P. and Carneiro, R.M.D.G. (2005) *Meloidogyne* spp. from preserved areas of Atlantic forest in the state of Rio de Janeiro, Brazil. *Nematologia Brasileira* 29, 31–38.
- Lunt, D.H. (2008) Genetic tests of ancient asexuality in root knot nematodes reveal recent hybrid origins. *BMC Evolutionary Biology* 8, 194.
- Lunt, D.H., Whipple, L.E. and Hyman, B.C. (2002) Mitochondrial DNA variable number tandem repeats (VNTRs): utility and problems in molecular ecology. *Molecular Ecology* 7, 1441–1455.
- Medina, I.L., Gomes, C.B., Rossi, C. and Carneiro, R.M.D.G. (2007) Characterization and identification of root-knot nematodes (*Meloidogyne* spp.) from fig (*Ficus carica* L.) trees in Rio Grande do Sul and São Paulo states of Brazil. *Nematologia Brasileira* 30, 179–187.
- Meng, Q.-P., Long, H. and Xu, J.H. (2004) PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. *Acta Phytopathologica Sinica* 34, 204–210.

- Meštrović, N., Randig, O., Abad, P., Plohl, M. and Castagnone-Sereno, P. (2005) Conserved and variable domains in satellite DNAs of mitotic parthenogenetic root-knot nematode species. *Gene* 362, 44–50.
- Meštrović, N., Castagnone-Sereno, P. and Plohl, M. (2006) High conservation of the differentially amplified MPA2 satellite DNA family in parthenogenetic root-knot nematodes. *Gene* 376, 260–267.
- Molinari, S. (2001) Polymorphism of esterase isozyme zymograms of *Meloidogyne* populations detected by PhastSystem. *Nematologica Mediterranea* 29, 63–66.
- Molinari, S., Lamberti, F., Crozzoli, R., Sharma, S.B. and Sánchez Portales, L. (2005) Isozyme patterns of exotic *Meloidogyne* spp. populations. *Nematologia Mediterranea* 33, 61–65.
- Moritz, C. and Cicero, C. (2004) DNA barcoding: promise and pitfalls. *PLoS Biology* 10, 1529–1531.
- Nieberding, C., Libois, R., Douady, C.J., Morand, S. and Michaux, J.R. (2005) Phylogeography of a nematode (*Heligmosomoides polygyrus*) in the Western Palearctic region: persistence of northern cryptic populations during ice ages? *Molecular Ecology* 14, 765–779.
- Oka, Y., Karssen, G. and Mor, M. (2003) Identification, host range and infection process of *Meloidogyne marylandi* from turf grass in Israel. *Nematology* 5, 727–734.
- Okimoto, R., Chamberlin, H.M., Macfarlane, J.L. and Wolstenholme, D.R. (1991) Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (*Meloidogyne*): nucleotide sequences genome location and potential for host-race identification. *Nucleic Acids Research* 19, 1619–1626.
- Orui, Y. (1999) Species identification of *Meloidogyne* spp. (Nematoda: Meloidogynidae) in Japan by random amplified polymorphic DNA (RAPD-PCR). *Japanese Journal of Nematology* 29, 7–15.
- Pais, C.S. and Abrantes, I.M. De O. (1989) Esterase and malate dehydrogenase phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* 21, 342–346.
- Palomares Rius, J.E., Volvas, N., Troccoli, A., Liébanas, G., Land, B.B. and Castillo, P. (2007) A new root-knot nematode parasitizing sea rocket from Spanish Mediterranean coastal dunes: *Meloidogyne dunensis* n. sp. (Nematoda: Meloidogynidae). *Journal of Nematology* 39, 190–202.
- Peloquin, J.J., Bird, M.D., Kaloshian, I. and Matthews, C. (1993) Isolates of *Meloidogyne hapla* with distinct mitochondrial genomes. *Journal of Nematology* 25, 239–243.
- Petersen, D.J., Zijlstra, C., Wishart, J., Blok, V. and Vrain, T.C. (1997) Specific probes efficiently distinguish root-knot nematode species using signature sequences in the ribosomal intergenic spacer. *Fundamental and Applied Nematology* 20, 619–626.
- Piotte, C., Castagnone-Sereno, P., Uijthof, J., Abad, P., Bongiovanni, M. and Dalmasso, A. (1992) Molecular characterization of species and populations of *Meloidogyne* from various geographic origins with repeated-DNA homologous probes. *Fundamental and Applied Nematology* 15, 271–276.
- Piotte, C., Castagnone-Sereno, P., Bongiovanni, M., Dalmasso, A. and Abad, P. (1994) Cloning and characterization of two satellite DNAs in the low-C-value genome of *Meloidogyne* spp. *Gene* 138, 175–180.
- Piotte, C., Castagnone-Sereno, P., Bongiovanni, M., Dalmasso, A. and Abad, P. (1995) Analysis of a satellite DNA from *Meloidogyne hapla* and its use as a diagnostic probe. *Phytopathology* 85, 458–462.
- 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 for the identification of five major *Meloidogyne* species. *Journal of Nematology* 25, 1–6.
- Powers, T.O., Todd, T.C., Burnell, A.M., Murray, P.C.B., Fleming, C.C., Szalanski, A.L., Adams, B.A. and Harris, T.S. (1997) The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. *Journal of Nematology* 29, 441–450.
- Powers, T.O., Mullin, P.G., Harris, T.S., Sutton, L.A. and Higgins, R.S. (2005) Incorporating molecular identification of *Meloidogyne* spp. into large-scale regional nematode survey. *Journal of Nematology* 37, 226–235.
- Rammah, A. and Hirschmann, H. (1988) *Meloidogyne mayaguensis* n. sp. (Meloidogynidae), a root-knot nematode from Puerto-Rico. *Journal of Nematology* 20, 65–68.
- Rammah, A. and Hirschmann, H. (1990) *Meloidogyne morocciensis* n.sp. (Meloidogyninae), a root-knot nematode from Morocco. *Journal of Nematology* 22, 279–292.
- Randig, O., Leroy, F., Bongiovanni, M. and Castagnone-Sereno, P. (2001) RAPD characterisation for single females of the root-knot nematodes, *Meloidogyne* spp. *European Journal of Plant Pathology* 107, 639–643.
- Randig, O., Bongiovanni, M., Carneiro, R.M.D.G., Sarah, J.-L. and Castagnone-Sereno, P. (2002a) A species-specific satellite DNA family in the genome of the coffee root-knot nematode *Meloidogyne exigua*: application to molecular diagnostics of the parasite. *Molecular Plant Pathology* 3, 431–437.

- Randig, O., Bongiovanni, M., Carneiro, R.M.D.G. and Castagnone-Sereno, P. (2002b) Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome* 45, 862–870.
- Rubinoff, D. and Holland, B.S. (2005) Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. *Systematic Biology* 54, 952–961.
- Schmitz, B., Burgermeister, W. and Braasch, H. (1998) Molecular genetic classification of Central European *Meloidogyne chitwoodi* and *M. fallax*. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 50, 310–317.
- Sipes, B.S., Schmitt, D.P., Xu, K. and Serracin, M. (2005) Esterase polymorphism in *Meloidogyne konaensis*. *Journal of Nematology* 37, 438–443.
- 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 the polymerase chain reaction. *Australian Plant Pathology* 27, 112–115.
- Stirling, G.R., Griffin, D., Ophel-Keller, K., McJay, A., Hartley, D., Curran, J., Stirling, A.M., Monsour, C., Winch, J. and Hardie, B. (2004) Combining an initial risk assessment process with DNA assays to improve prediction of soilborne diseases caused by root-knot nematode (*Meloidogyne* spp.) and *Fusarium oxysporum* f. sp. *lycopersici* in the Queensland tomato industry. *Australasian Plant Pathology* 33, 285–293.
- Szemes, M., Bonants, P., de Weerd, M., Baner, J., Landegren, U. and Schoen, C.D. (2005) Diagnostic application of padlock probes – multiplex detection of plant pathogens using universal microarrays. *Nucleic Acids Research* 33, e70.
- Tastet, C., Val, F., Lesage, M., Renault, L., Marché, L., Bossis, M. and Mugniéry, D. (2001) Application of a putative fatty-acid binding protein to discriminate serologically the two European quarantine root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, from other *Meloidogyne* species. *European Journal of Plant Pathology* 107, 821–832.
- Tesařová, B., Zouhar, M. and Ryšánek, P. (2003) Development of PCR for specific determination of root-knot nematode *Meloidogyne incognita*. *Plant Protection Sciences* 39, 23–28.
- 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.
- Tomaszewski, E.K., Khalil, M.A.M., El-Deeb, A.A., Powers, T.O. and Starr, J.L. (1994) *Meloidogyne javanica* parasitic on peanut. *Journal of Nematology* 26, 436–441.
- Toyota, K., Shirakashi, T., Sato, E. and Wada, S. (2008) Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Science and Plant Nutrition* 54, 72–76.
- van der Beek, J.G. and Karssen, G. (1997) Interspecific hybridisation of meiotic parthenogenetic *Meloidogyne chitwoodi* and *M. fallax*. *Phytopathology* 87, 1061–1066.
- van Doorn, R., Szemes, M., Bonants, P., Kowalchuk, G.A., Salles, J.F., Ortenberg, E. and Schoen, C.D. (2007) Quantitative multiplex detection of plant pathogens using a novel ligation probe-base system coupled with universal, high-throughput real-time PCR and OpenArrays. *BMC Genomics* 8, 276.
- Vogler, A.P. and Monaghan, M.T. (2007) Recent advances in DNA taxonomy. *Journal Zoological Systematics and Evolutionary Research* 45, 1–10.
- Vrain, T.C., Wakarchuk, D.A., Levesque, A.C. and Hamilton, R.L. (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563–573.
- 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.
- Wishart, J., Phillips, M.S. and Blok, V.C. (2002) Ribosomal intergenic spacer: a PCR diagnostic for *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. *Phytopathology* 92, 884–892.
- Xu, J., Liu, P., Meng, Q. 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., Beckenbach, K. and Webster, J.M. (1992) DNA hybridisation probes for studying the affinities of three *Meloidogyne* populations. *Fundamental and Applied Nematology* 15, 35–41.

- Yang, B. and Eisenback, J.D. (1983) *Meloidogyne enterolobii* n. sp. (Meloidogynidae), a root-knot nematode parasitizing pacara earpod tree in China. *Journal of Nematology* 15, 381–391.
- Ye, W., Giblin-Davis, R.M., Braasch, H., Morris, K. and Thomas, W.K. (2007) Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. *Molecular and Phylogenetic Evolution* 43, 1185–1197.
- 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. and van Hoof, R.A. (2006) A multiplex real-time polymerase chain reaction (TaqMan) assay for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax*. *Phytopathology* 96, 1255–1262.
- Zijlstra, C., Lever, A.E.M., Uenk, B.J. and van Sijfhout, C.H. (1995) Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* 85, 1231–1237.
- Zijlstra, C., Uenk, B.J. and van Sijfhout, C.H. (1997) A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. *Fundamental and Applied Nematology* 20, 59–63.
- Zijlstra, C., Donkers-Venne, D.T.H.M. and Fargette, M. (2000) Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised 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.
- Zu, J., Liu, P., Meng, Q. 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.