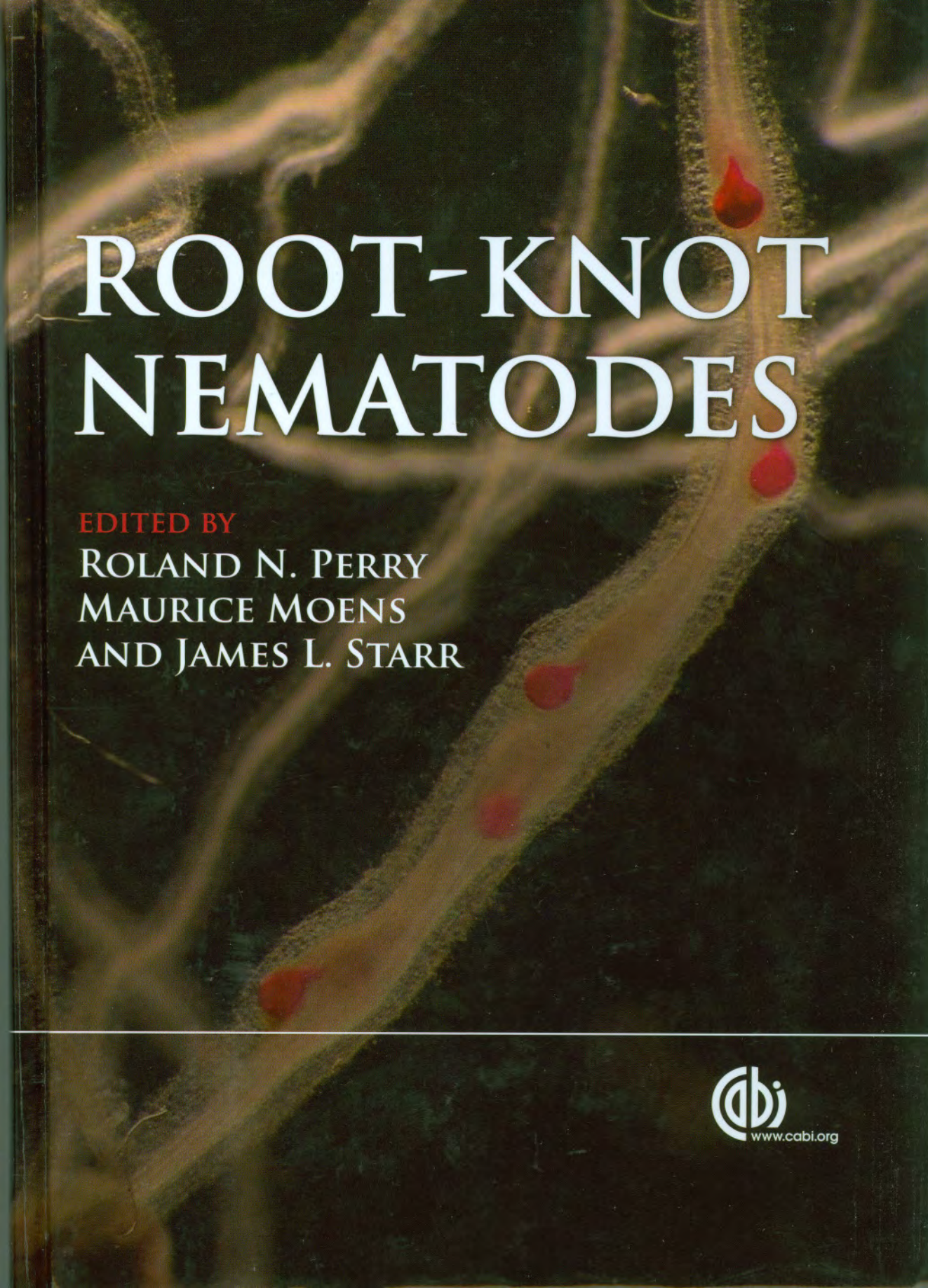
ROOT-KNOT
NEMATODES
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1 *Meloidogyne* Species – a Diverse Group of Novel and Important Plant Parasites

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1.1 Introduction

Root-knot nematodes are members of the genus *Meloidogyne* (Göldi, 1892), *Meloidogyne* is of Greek origin and means 'apple-shaped female'. They are an economically important polyphagous group of highly adapted obligate plant parasites, are distributed worldwide and parasitize nearly every species of higher plant. Typically they reproduce and feed on modified living plant cells within plant roots, where they induce small to large galls or root-knots, hence their vernacular name. The above-ground symptoms are not readily apparent and may be similar to those produced on any plants having a damaged and malfunctioning root system. Hosts may be heavily infected without showing external symptoms on the harvested products, e.g. symptomless potato tubers. The rapid rate of development and reproduction on good hosts results, in the majority of species, in several generations during one cropping season, leading to severe crop damage. Damage may consist of various degrees of stunting, lack of vigour, and wilting under moisture stress. Secondary infection by other pathogens often results in extensive decay of nematode-infected tissues. The common explanation for these above-ground symptoms is that *Meloidogyne* infection affects water and nutrient uptake and upward translocation by the root system. By disrupting the host plant physiology, root-knot nematodes may not only reduce crop yield but also product quality

 Root-knot Nematodes (eds R.N. Perry, M. Moens and J.L. Starr)

(e.g. of potatoes and carrots) and therefore are of great economic and social importance (see Greco and Di Vito, Chapter 11, this volume).

1.2 Impact

Damage and yield losses caused by plant pathogens, including plant-parasitic nematodes, are, on average, greater in tropical than in temperate regions because of greater pathogen diversity, more favourable environmental conditions for pathogen colonization, development, reproduction and dispersal, and lack of human, technical and financial resources to combat infections (De Waele and Elsen, 2007). Severity of damage caused by *Meloidogyne* can be species-specific and also vary by host, crop rotation, season and soil type (Greco *et al.*, 1992; Potter and Olthof, 1993). Similarly, economic thresholds vary, primarily depending on these same factors. Damage thresholds have been established for several crops, where the average is approximately 0.5–2 juveniles/g of soil (or from the lower limits of detection, over 1000 individuals/500 cm^3 of soil; see Greco and Di Vito, Chapter 11, this volume). In addition to direct costs, root-knot nematodes cause indirect costs because of the quarantine status of some species of *Meloidogyne* in several countries or regions. For example, *M. chitwoodi* is increasingly regulated because it is a serious pest of potato and other economically important crops such as carrot, and the known geographical distribution is still relatively limited. It is on the list of prohibited pests of many countries (Canada, the EU, Mexico and other countries in Latin America, and the Far East) (Hockland *et al.*, 2006). *Meloidogyne fallax*, another pest of potato, is recognized as an important pest by fewer countries (e.g. the EU), although it poses a similar economic risk. In the future, additional species of root-knot nematodes that might be added to the list of quarantine species include recently described species such as *M. minor* in Europe and *M. citri* in the USA.

1.3 History of the Genus

A more detailed account of the background literature, progress in descriptions and classification, and authorities for the species of the genus is given in Hunt and Handoo, Chapter 3, this volume; a brief summary is included here as part of the introduction to the genus and its basic biology. The first illustrated report of root-knot disease appeared during the middle of the 19th century when the clergyman Miles Joseph Berkeley (1855) first attributed galls detected on glasshouse cucumber roots to nematodes. The first description of a root-knot nematode was made by Cornu (1879); it was based on nematodes found in root galls that were detected on sainfoin (*Onobrychis sativus* Lam.) in the Loire valley, France. In 1887, Göldi briefly described and illustrated a root-knot nematode from coffee plants in Brazil and named it *M. exigua*. Although the 1887 publication was an advance copy or preprint of the full article subsequently published by Göldi in 1892, the 1887 article meets the requirements to establish the actual publication date for the genus and type species as 1887 (see Hunt and Handoo, Chapter 3, this volume for a full account of this decision).

The name *Heterodera marioni* was widely used for root-knot nematodes until 1949, when Chitwood removed the root-knot nematodes from the genus *Heterodera* because they differed from cyst nematodes. Since the oldest name for the genus was Göldi's *Meloidogyne*, that name had precedence. Chitwood redescribed *M. arenaria*, *M. exigua*, *M. incognita* and *M. javanica*, and described *M. hapla* and a variety of *M. incognita* he termed *M. incognita* var. *acrita*. The species were separated from each other on the basis of perineal pattern morphology, stylet knob shape, and length of stylet and dorsal gland orifice. Since Chitwood's publication, many more *Meloidogyne* species have been described (see Hunt and Handoo, Chapter 3, this volume, for a full list). Species descriptions gradually included increasing numbers of features (observed by light microscopy and/or electron microscopy) of females, males and second-stage juveniles (J2) (see Jepson, 1987, for a review, and Eisenback and Hunt, Chapter 2, this volume).

1.4 Current Trends in Species Identification

Research on cytogenetics, isozymes and the genome of root-knot nematodes, mostly since the

1970s, provided further evidence for the large diversity of species within the genus. Importantly, several new technologies have provided tools to assist in species identification. One of the most important has been the use of isozyme phenotypes, using PAGE of crude protein extracts and histochemical stains for non-specific esterases, superoxide dismutase, malate dehydrogenase, and glutamate oxaloacetate transaminase (Esbenshade and Triantaphyllou, 1985), with the esterases and malate dehydrogenase being the most useful for discriminating the four common *Meloidogyne* species: *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. These enzymes have also been used successfully with less common species such as *M. enterolobii* (= *M. mayaguensis*) (Brito *et al.*, 2004), *M. partityla* (Starr *et al.*, 1996), and *M. trifoliophila* (Mercer *et al.*, 1997). With the appropriate equipment it is possible to make the species identification on individual females. Ibrahim and Perry (1992) showed that PAGE combined with staining for esterases could be used with *Meloidogyne* females in galled roots, thus obviating the need to separate nematodes from the host tissue. It remains to be determined if these procedures will be able to discriminate all of the currently recognized species of this genus.

More recently, much effort has been given to the development of species-specific markers using variation in the genomes of the different species coupled with PCR techniques (see Blok and Powers, Chapter 4, this volume). There are now many reports that describe protocols for distinguishing numerous species using species-specific primers derived from the nuclear genome (Adam *et al.*, 2007) and the mitochondrial genome (Powers and Harris, 1993). A major benefit of these systems is that they can be used on an individual J2, the most common stage of the nematode found in the soil, thus eliminating the need to use mature females from roots of field samples (which is not possible if the host of interest is not actually growing at the time of sample collection) or to establish a culture in the glasshouse and wait for the development of the females. At present, there are no primer pairs available for many species, and multiple assays with different primer pairs are often needed to complete the identification. For some species the PCR amplification product must be digested with a restriction enzyme to complete the analysis (Powers and Harris, 1993). Finally, this approach requires some expensive equipment (the PCR thermocycler) and supplies, and a clean laboratory environment. None the less, these approaches are likely to play an ever-increasing role in species identification (especially by those not well trained or experienced in classical morphometrics) and will be used extensively by regulatory agencies.

1.5 Life Cycle

The root-knot nematode life cycle is summarized in Fig. 1.1 and Plate 36. Females lay eggs into gelatinous masses composed of a glycoprotein matrix, which is produced by rectal glands in the female, keeps the eggs together and protects them against environmental extremes and predation. The egg masses are usually found on the surface of galled roots, although they may also be embedded within the gall tissue. The egg mass is initially soft, sticky and hyaline but becomes firmer and dark brown with age. Surprisingly, there has been only limited analysis of the glycoproteins (Sharon and Spiegel, 1993) or other components of the gelatinous matrix, despite its obvious importance. In addition to providing some protection to the eggs from environmental extremes, it has been demonstrated that the matrix has antimicrobial properties (Orion and Kritzman, 1991).

Within the egg, embryogenesis proceeds to the first-stage juvenile, which moults to the infective J2. Hatch of the J2 is primarily dependent on temperature and sufficient moisture, although other factors, including root diffusate and generation, modify the hatching response (see Curtis *et al.*, Chapter 6, this volume) so that the J2 hatch when conditions are favourable for movement and host location. The ability of *Meloidogyne* to survive is enhanced by several physiological and biochemical adaptations, including delayed embryogenesis, quiescence and diapause, and lipid reserves that prolong viability until the J2 reaches and invades a host; these aspects are discussed in detail by Evans and Perry, Chapter 9, this volume. In the soil, the J2 is vulnerable and needs to locate a host as rapidly as possible. J2 are attracted to roots, and there is evidence that when both resistant and susceptible plant roots are present the susceptible ones are

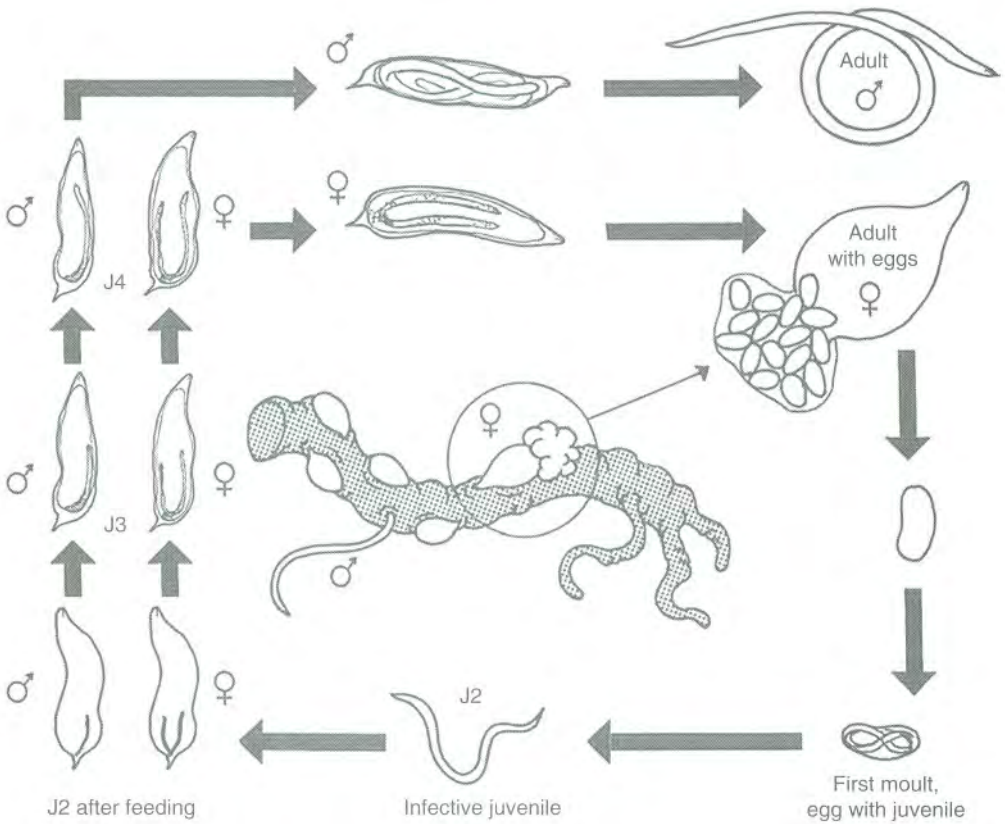

Fig. 1.1. Diagram of the life cycle of the root-knot nematode, *Meloidogyne*. J2: second-stage juvenile; J3: third-stage juvenile; J4: fourth-stage juvenile. (Adapted from Karssen and Moens, 2006.)

more attractive (see Curtis *et al.*, Chapter 6, this volume).

The invasive J2 commences feeding after it has invaded the root, usually behind the root tip, and moved through the root to initiate and develop a permanent feeding site. The feeding of the J2 on protoxylem and protophloem cells induces these cells to differentiate into specialized nurse cells, which are called giant cells. Once a giant cell is initiated, the nematode becomes sedentary and enlarges greatly to assume a 'sausage' shape. Under favourable conditions, the J2 stage moults to the third-stage juvenile (J3) after *c.*14 days, then to the fourth-stage juvenile (J4), and finally to the adult stage. The combined time for the J3 and J4 stages is much shorter than for the J2 or the adult, typically 4–6 days. J3 and J4 lack a functional stylet and do not feed. Males, when present, are vermiform and there is no evidence that they feed. Males may be found in parthenogenetic species when conditions are unfavourable for female development, such as when population densities are very high and presumably there is a limitation of food supply.

The initiation, development and maintenance of the giant cell is the subject of continuing investigation, facilitated by molecular techniques with the impetus of developing novel control strategies based on preventing giant cell formation or, more likely, development. These specialized feeding sites are remarkable for their complexity. They are greatly enlarged from typical phloem and xylem parenchyma, or cortical cells, with final cell volumes nearly 100-fold greater than normal root cells. The giant cells are functionally similar to syncytia induced by other plant-parasitic nematodes that have sedentary adult females, but are distinct in their development. Like syncytia, they are functional transfer cells, based on morphology (Jones and

Northcote, 1972) and because photosynthates pass through the giant cells before being ingested by the nematodes (Bird and Loveys, 1975). Unlike syncytia, each giant cell develops from a single initial cell rather than by coalescence of several adjacent cells. The giant cells are not only multinucleate, containing as many as 80 nuclei each, but individual nuclei within each giant cell are polyploid, some with *c.* eight-fold increase in chromosome number (Huang and Maggenti, 1969; Wiggers *et al.*, 1990). Thus, each giant cell may have up to a 600-fold increase in copy number of each plant gene. Several studies have documented the effects of nematode infection on gene expression, with a variety of genes being upregulated (Gheysen and Jones, 2006; Schaff *et al.*, 2007) and probably a greater number downregulated (Schaff *et al.*, 2007). A few studies on gene expression in the giant cells have reported that the mRNA for some genes can be present in giant cells at levels that are many fold greater than in non-infected root cells (Ramsey *et al.*, 2004; He *et al.*, 2005). These data demonstrate that the giant cells induced by all *Meloidogyne* spp. are unique examples of how parasites can affect normal host development. Most recently (Huang *et al.*, 2006), some progress has been made in the characterization of parasitism genes in the nematode that enable it fundamentally to alter plant growth and development for the benefit of the parasite (see Abad *et al.*, Chapter 7, this volume).

On the bases of cytogenetic studies on about 600 populations (representing 24 species) and in collaboration with the International *Meloidogyne* Project, Triantaphyllou (1985) was able to demonstrate that root-knot nematodes have undergone extensive cytogenetic diversification, probably unparalleled by that of any other animal group. Triantaphyllou concluded that characteristic features are the establishment of meiotic and mitotic parthenogenesis in association with various degrees of polyploidy and aneuploidy. Obligatory cross-fertilization also occurs in some diploid and polyploid forms (e.g. *M. kikuyensis* and *M. megatyla*), whereas facultative meiotic (automixis) (e.g. *M. exigua*, *M. chitwoodi* and *M. graminicola*) and obligatory mitotic parthenogenesis (apomixis) (e.g. *M. incognita*, *M. enterolobii* and *M. oryzae*) prevail in most polyploid and aneuploid forms (see Chitwood and Perry, Chapter 8, this volume).

The trend from amphimictic reproduction to apomixis is generally associated with shorter life cycles, higher reproductive rates and increasing importance as crop pathogens. Only a small number of species reproduce by amphimixis, i.e. with the obligatory fusion of a male and female gamete (e.g. *M. carolinensis*, *M. microtyla*, *M. pini*). These species are considered as minor root-knot nematode species because of their very restricted distribution, host range and economic impact (Jepson, 1987). Automictic root-knot nematode species are facultatively parthenogenetic; apomictic species are obligatory parthenogenetic. The apomictic mode of reproduction is found in the most important species in terms of geographic distribution and agronomic impact. There are two possible explanations for the worldwide distribution of the apomictic root-knot nematodes. Either they are indigenous in much or all of their current range, and therefore are very ancient species, or they are recently evolved and have been widely spread by agriculture (Trudgill and Blok, 2001). The former is widely viewed as unlikely, partly because parthenogenetic species are considered an evolutionary dead end. Most of the amphimictic and automictic species are diploid with a haploid chromosome number of 18. The majority of the apomictic species are polyploid or aneuploid and usually show a wide variation in chromosome number ($2n$ = 30–55 chromosomes) (Karssen and Moens, 2006; see Chitwood and Perry, Chapter 8, this volume). Root-knot nematode species further differ in their male-to-female ratio. Cross-fertilizing species such as *M. carolinensis* and *M. spartinae* usually have a 1:1 ratio. Species that reproduce by facultative or obligatory parthenogenesis such as *M. hapla* and *M. incognita* have variable sex ratios.

The root galling upon which the nematode's common name is based is quite variable among the different species of the genus and plant hosts (Plates 1–12). Some differences in galling among the different species of this genus are well known. *Meloidogyne hapla* is particularly known for the high incidence of adventitious roots that develop from root galls (Sasser, 1954; Plate 4). *Meloidogyne trifoliophila* on clover produces galls that are distinctly elongated, and the egg masses are more typically embedded within the galls than found erupting from the gall surface (Mercer *et al.*, 1997; Plates 6, 7). Other species have a tendency to produce galls at the root terminus. Root galls

can be quite small or indistinct on many hosts, which often results in failure to recognize that the plant is being parasitized. Graminaceous hosts rarely form galls. Most plants with fibrous or woody roots will have small or indistinct galls, especially early in a growing season or when nematode population densities are low. Infection sites of *M. partityla* on pecan that contain a single nematode do not form galls; rather, both the mature female and the egg mass are exposed on the root surface (Plate 8). Cotton and groundnut are examples of two highly susceptible crops in which root galls can be difficult to detect early in the growing season but massive galls can be evident at crop maturity. Plants with succulent roots, especially the cucurbits and tomato, develop the readily detectable galls for which the species is named, even with low infection incidence (Plates 2, 3, 9). Under extreme conditions, a plant's root system may be entirely gall tissue with no remaining fibrous roots.

1.5.1 Incompatible host reactions

The induction and maintenance of giant cells by *Meloidogyne* spp. and the associated physiological and molecular changes in a compatible host–parasite interaction are discussed by Bleve-Zacheo and Melillo (1997) and Abad *et al.* (Chapter 7, this volume). Resistance to *Meloidogyne* spp. is a much-researched topic and Veech (1981) provides an excellent summary of the older literature. Williamson and Roberts (Chapter 13, this volume) discuss resistance genes and the genetics of plant resistance to root-knot nematodes. The analysis of histochemical changes and signal transduction pathways using the resistant tomato plant–*Meloidogyne* model system has provided useful information on the changes associated with the incompatible response (Bleve-Zacheo *et al.*, 2007). Many plant defences against pathogens are regulated by signalling pathways in which jasmonic acid, for example, plays a key role. Soriano *et al.* (2004) showed that application of exogenous methyl jasmonate to roots of spinach and oats induced nematode resistance. Jasmonates induce *de novo* ecdysteroid synthesis in roots but Soriano *et al.* (2004) found that the invasion of spinach by *M. javanica* was impaired by the induction of ecdysteroid, indicating movement of ecdysteroid into the rhizosphere and sensitivity of the nematode to its presence; expression of resistance prior to invasion is uncommon. Cooper *et al.* (2005) demonstrated that jasmonic acid induced a systemic defence response that reduced the reproduction of avirulent *Meloidogyne* on susceptible tomato plants. Another resistant response associated with signal transduction during the hypersensitive reaction to pathogen invasion is the stress-induced oxidative burst. This response is complex and involves antioxidants, such as ascorbic acid. Application of ascorbic acid to susceptible tomato plants has been shown to inhibit invasion by *M. incognita*, and resistance has been associated with the ability to synthesize large amounts of ascorbic acid following J2 invasion (Arrigoni *et al.*, 1979). The oxidative burst occurs rapidly after nematode invasion, with a second burst associated with the hypersensitive reaction only detectable in the incompatible tomato–nematode interaction (Melillo *et al.*, 2006).

The responses associated with the resistance genes *Me1* and *Me3* in pepper differed (Bleve-Zacheo *et al.*, 1998). Many fewer J2 of *M. incognita* were able to invade the pepper line HDA149, carrying the *Me3* gene, compared with the line HDA330, carrying the *Me1* gene. The line HDA149 exhibited the typical early hypersensitive response to nematode invasion, while the resistance mechanism in HDA330 involved a delayed plant response after the J2 had set up several imperfect giant cells.

Antioxidant enzymes secreted by root-knot nematodes may be important to overcome the hypersensitive response of resistant roots and the associated generation of reactive oxygen species (Molinari *et al.*, 2008; Molinari, 2009). For example, a selected virulent isolate of *M. incognita* had greater activity of antioxidant enzymes, including catalase, superoxide dismutase and peroxidase, when compared with a near isogenic avirulent isolate and an avirulent field population (Molinari, 2009). However, it is not clear whether enhanced antioxidant activities contribute to the virulent phenotype, or whether they are a side effect.

1.6 Diversity in Biology

Species of root-knot nematodes demonstrate a large diversity in various aspects of their life cycles. With respect to their temperature requirements,

root-knot nematodes can be divided into two distinct groups of species, thermophils and cryophils, which can be separated by their ability to survive lipid-phase transitions that occur at 10 °C (Lyons *et al.*, 1975; see Evans and Perry, Chapter 9, this volume). *Meloidogyne chitwoodi*, *M. hapla* and probably *M. naasi* are cryophils and able to survive soil temperatures below 10 °C; *M. arenaria*, *M. javanica* and *M. exigua* are thermophils and do not have extended survival at temperatures below 10 °C. Like survival, hatching is primarily controlled by temperature (see Curtis *et al.*, Chapter 6, this volume). Thermotypes exist within species (e.g. Daulton and Nusbaum, 1961). Root-knot nematode species also differ in the number of generations they can produce per year; this number varies according to species and food availability. Usually there are many generations per year, but in some species (e.g. *M. naasi*) there is only one (Rivoal and Cook, 1993).

Root-knot nematodes demonstrate various degrees of specialization with respect to their host preference. Crops are usually better hosts than weeds (e.g. Mandulu and Trudgill, 1993; Hillocks *et al.*, 1995). Either man, when selecting crop plants, has inadvertently selected for increased susceptibility to root-knot nematode species, or the root-knot nematodes have been selected by repeated exposure to crop plants (Trudgill and Blok, 2001). Most amphimictic species have host ranges confined to a single subclass of plants, on either woody or perennial herbaceous hosts. *Meloidogyne spartinae* seems to be restricted to cordgrass (*Spartina* spp.), both *M. pini* and *M. megatyla* to *Pinus* spp. and *M. subartica* is confined to the Commelinidae (Jepson, 1987). Apart from *M. hapla*, which has a wide host range that does not include graminaceous species, the automictic species tend to have a narrow host range, while the mitotic species have a potential host range containing the majority of the higher plants (Trudgill and Blok, 2001). However, there are exceptions to these generalizations; for example, the apomictic species *M. quericiana* and *M. enterolobii* have restricted host ranges. The majority of apomictic species of root-knot nematodes appear to have a survival strategy based on a wide host range, which enables them to persist whatever the vegetation. They lack specific triggers for hatching, and hatch occurs as soon as the J2 has developed. On good hosts, where generation times are short and fecundity is high, several generations and rapid population increase occur. Consequently, as the growing season progresses, small populations of apomictic root-knot nematodes can become large and very damaging (Trudgill and Blok, 2001).

For those *Meloidogyne* spp. that have host ranges that are large (total number of hosts) and broad (large number of plant families with species susceptible to the nematode), it is somewhat amazing that there are quite distinct differences in their overlapping host ranges. Among the traditional four major species (which were four of the five original species described by Chitwood, 1949) there are some distinct and now classic differences. These four species (*M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*) have overlapping host ranges as they all infect many common vegetable crops, including tomato, which is often (but inappropriately) considered a universal host for *Meloidogyne* spp. *Meloidogyne hapla* reproduces only poorly or not at all on most grasses and grain crops. Of these four species, only some populations of *M. incognita* parasitize cotton. Groundnut is a good host for *M. hapla* and most *M. arenaria* populations but not for *M. incognita*. Most populations of *M. javanica* in the USA do not reproduce well on groundnut but populations of this species from India and northern Africa generally reproduce well on groundnut. Thus, even though they each have large, broad and overlapping host ranges, there are distinct differences in the host range of each of these species. Perhaps, despite the claims made above, some greater caution is warranted regarding statements of large host ranges. Given the continually increasing number of recognized species, how many of the previous reports on host ranges were based on incorrect species identification and/or having test populations that contained more than one species?

1.6.1 Concept of host races

Sasser (1952) was among the first to report the variation in host range within the original four major species. He later proposed the formal recognition of 'host races' within these species and proposed a standardized set of differential hosts for distinguishing these host races (Hartman and Sasser, 1985). Further, he proposed that the host

differential test could be used as an aid to species identification. Here it should be emphasized that Sasser never recommended the use of the host differentials as the sole basis for species identification. Since the 1970s, additional variation among the four major species has been recognized, especially among populations of *M. javanica*, which is now recognized as variable with respect to reproduction on pepper and groundnut, two important differentials for the host race test. This greatly complicates the use of the host race test as an aid to species identification. Perhaps more importantly, there has been a substantial number of new species described since the 1970s. Many of these recently described species have been found in mixed populations with one or more of the traditional major species and attack some of the same important crops. The North Carolina Host Race test does not clearly distinguish most of the newly described species from the original four. Similar variation in host range is reported for other species, with this variation being unrelated to the differential hosts used in the North Carolina Host Differential test.

Although the recognition of variation in host ranges is important, we suggest the formal recognition of the host races (often referred to simply as race) be discontinued. The host race concept has never been universally accepted, in part because it measured only a small portion of the potential variation in parasitic ability. Given the large number of hosts for many species, it is unlikely that the full extent of possible variation will ever be adequately characterized. Assigning each variant population a distinct number is likely to be very unwieldy with time. It had some value in the south-eastern USA because each of the differential hosts was an important and widely grown crop, but this is not always the case. Unfortunately, knowledge of host race rarely allowed one to predict accurately other behavioural characteristics of a population. Race 1 of *M. arenaria* is clearly not as aggressive on soybean as race 2 of that species (Ibrahim and Lewis, 1993) but this correlation of one aspect of behaviour with the host race status is the exception rather than the rule. Perhaps more importantly, the term race, when used in the context of plant disease, generally refers to populations of a pathogen that differ in virulence on host species that carry specific genes for resistance to other populations of that pathogenic species. This terminology is especially prevalent in the USA. The system also mixed variation in host range with variation in virulence. For example, the tobacco cv. NC95 carries specific resistance to *M. incognita*. Thus, variation in reproduction and galling among populations of *M. incognita* on tobacco actually indicates differences in virulence (the ability of some pathogen genotypes to have a compatible interaction with hosts carrying a specific gene for resistance to that pathogen species). Virulence within *M. arenaria*, *M. incognita* and *M. javanica* in relation to the *Mi* resistance gene in tomato is well documented (Semblat *et al.*, 2000). Similarly, variation among *M. incognita* populations with respect to different resistant cotton genotypes has been reported (Zhou *et al.*, 2000). Yet these examples of variation in virulence are unrelated to host race status. Ability to convey clearly information on the behaviour of a population via a proper and correct name is essential. It will be more useful when dealing with a population that has a variant host range to confirm that the population has been adequately identified and then to simply acknowledge the variant host range.

1.7 Major and Emerging Species

Discussion of *Meloidogyne* spp. frequently focuses on the major four species: the three tropical species, *M. incognita*, *M. arenaria* and *M. javanica*, and the temperate species, *M. hapla*. The recognition of these four species as being the 'major species' began with them being four of the five original species (Chitwood, 1949). That each has an extensive host range and that they are each globally distributed further contribute to their recognized importance. Finally, the often-cited publication by Taylor *et al.* (1982), that these four species comprised more than 99% of all species identified from a collection of 662 isolates from 65 countries, further strengthened their status as the major species. However, that survey had a couple of distinct biases that skewed the data. First, most samples were collected from warm temperate or tropical climates, with only 39 of the 662 samples from a climate where the coldest temperature was ≤5 °C. Additionally, although the samples were

collected from 121 different crop species, 33% of the samples were from just four crops (tomato, aubergine (= eggplant), tobacco and okra). Probably not surprisingly, tomato alone accounted for nearly 17% of all samples. None the less, the survey greatly strengthened the status of the four major species. Their widespread recognition has probably led to many cases of misidentification, e.g. once *M. hapla* was identified a few times parasitizing a given crop species in a particular region, most further finds of a root-knot nematode attacking that crop would probably be attributed to *M. hapla* without substantial scrutiny. The advent of isozyme phenotyping and species-specific DNA protocols has contributed greatly to our ability to determine rapidly, and with less equivocation than is possible with morphometric analysis, whether a given isolate belongs to one of the well-known species or is possibly a new species.

Notwithstanding the increasing number of new *Meloidogyne* species, the four major ones are undoubtedly of immense economic importance and quite possibly still deserving of their status. They have been the subject of a considerable amount of research, which is reflected in the following chapters. Most knowledge of *Meloidogyne* spp. is based on studies of one or more of these four major species. Further, most of the more fundamental studies on these four species have been based on research with either tomato or tobacco as the host species. In the era of molecular genetics, future studies will depend on model systems for which the genetics of the host are well characterized. *Medicago truncatula* (Dhandaydham *et al.*, 2008) is an example of such a model system and has the advantage of being a legume; many legume crop species are very susceptible to one or more root-knot nematode species. Descriptions and hosts of 12 species, including the four major species, of economic importance in different geographical areas are given by Hunt and Handoo, Chapter 3, this volume, where naming authorities for all species of *Meloidogyne* are provided. Here we describe some species that we consider to be of 'emerging' importance. It is to be expected that changing selection pressures due to evolving cropping and management systems, especially the use of host resistance, will result in a dynamic landscape with regard to important *Meloidogyne* spp.

1.7.1 *Meloidogyne enterolobii* (= *Meloidogyne mayaguensis*)

This nematode is currently considered as one of the most important root-knot nematode species because of its wide geographical distribution, its wide host range, and its ability to overcome the resistance of important crop plants, such as genotypes of tomato, pepper, and some agronomic crops that carry the *Mi-1* gene, which confers resistance to *M. javanica*, *M. arenaria* and *M. incognita* (Fargette, 1987; Fargette *et al.*, 1994). This nematode was originally described from a population collected from aubergine (*Solanum melongena*) in Puerto Rico (Rammah and Hirshmann, 1988). Subsequently, it has been detected in Africa (Fargette *et al.*, 1994; Duponnois *et al.*, 1995; Willers, 1997; Trudgill *et al.*, 2000), the USA (Brito *et al.*, 2004), South and Central America (Decker and Rodriguez-Fuentes, 1989; Carneiro *et al.*, 2000, 2001; Trudgill *et al.*, 2000) and Europe (Blok *et al.*, 2002).

Other recorded hosts include vegetables, and other crops, such as bell pepper (*Capsicum annuum*), soybean (*Glycine max*), sweet potato (*Ipomoea batatas*), tobacco (*Nicotiana tabacum*) and watermelon (*Citrullus lanatus*). A tropical fruit tree, guava (*Psidium guajava*), is also a good host of this nematode (Plate 20). Spanish needle (*Bidens pilosa*), a weed host, has also been identified. In Cuba, reproduction was observed on coffee (*Coffea arabica* cv. Caturra), bean (*Phaseolus vulgaris* cv. Icapijao), beet (*Beta vulgaris*), broccoli (*Brassica oleracea* var. Botrytis), celery (*Apium graveolens* cv. Utah), horsebean (*Cannavalia ensiformis*), parsley (*Petroselynum crispum* cv. Plain), potato (*Solanum tuberosum*) and pumpkin (*Cucurbita* sp.). In Florida, this nematode has been found in roots of angel trumpet (*Brugmansia* cv. Sunray), basil (*Ocimum* sp.), cape honeysuckle (*Tecomaria capensis*), glory bush (*Tibouchina* cv. Compacta and *Tibouchina elegans*), carpet bugleweed (*Ajuga reptans*) and Uganda glorybower (*Clerodendrum ugandense*).

1.7.2 *Meloidogyne paranaensis*

This species was detected on coffee in Paraná state, Brazil, from which it is named (Carneiro *et al.*, 1996). Coffee (*C. arabica*) is the primary host of this species. However, the nematode has also been detected on soybean (Dinnys Roese *et al.*,

2004). In host studies, tobacco, tomato and watermelon were reported as suitable hosts (Carneiro *et al.*, 1996). Decline and dieback of coffee trees, and yield suppression of up to 50%, are associated with nematode infection in Brazil, where the damage occurring on coffee in Paraná state was initially erroneously attributed to *M. incognita* (Carneiro *et al.*, 1996). Currently the nematode has been detected only in the USA (including Hawaii), Central America, the Caribbean, Guatemala and South America.

Specific damage caused by *M. paranaensis* on roots of coffee typically does not involve gall formation. Instead, *M. paranaensis* causes the taproot of coffee to crack and split, as well as damaging other root tissue. Necrosis also occurs where females are embedded and near the giant cells where feeding occurs. Above-ground symptoms generally range from chlorosis and reduced plant growth to death (Carneiro *et al.*, 1996).

Meloidogyne paranaensis may occur by itself or in mixed populations with other *Meloidogyne* spp. (Carneiro *et al.*, 1996). Inserra *et al.* (2003) suggested that this nematode may lower yield potentials by 50%. This estimate is based on information provided by Carneiro *et al.* (1996), but the original authors only suggested that this particular species may '[account] for approximately 52% of all root-knot nematode infestations in Paraná'. Carneiro *et al.* (1996) do not comment on the magnitude of damage when these infestations occur.

1.7.3 *Meloidogyne fallax* and *Meloidogyne chitwoodi*

Meloidogyne chitwoodi and *M. fallax* are closely related species parasitizing monocotyledons and dicotyledons, including several major crop plants such as potatoes, carrots and tomatoes (Santo *et al.*, 1980; O'Bannon *et al.*, 1982; Brinkman *et al.*, 1996; Karssen, 2002 (Plates 10, 11, 22, 25–27)).

Meloidogyne chitwoodi was first described from roots and tubers of potato in a field near Quincy, Washington state, USA. The species has been recorded from Argentina, Belgium, Germany, The Netherlands, Portugal, several states of the USA, Mexico and South Africa (EPPO, 2004). It is not clear whether this is its area of origin. In Europe, the nematode was first detected in the Netherlands, but a review of old illustrations and old specimens of *Meloidogyne* suggests that they may have occurred earlier (EPPO, 1991). The species can begin development when soil temperature rises above 5 °C and requires 600–800 degree-days to complete the first generation; subsequent generations require only 500–600 degree-days (Pinkerton *et al.*, 1991). Intraspecific variation in *M. chitwoodi* is manifest by the occurrence of three biotypes that can be distinguished based on reproduction on lucerne cv. Thor, carrot cv. Red Cored Chantenay, and *Solanum bulbocastanum* Dun. SB22 (Santo and Pinkerton, 1985; Mojtahedi *et al.*, 1988, 1994).

Symptoms caused by *M. chitwoodi* vary according to host, population density of the nematode and environmental conditions. Galls produced on potato tubers are often not easily detected. On carrots, galls appear mainly on the lenticels and reduce the commercial value of the crop (Wesemael and Moens, 2008).

Meloidogyne fallax was detected for the first time in 1992 in a field north of Baexem (The Netherlands) and initially identified as 'a deviating *M. chitwoodi* population' (Karssen, 1994). After this first report, it was recorded on potato at several locations in the southern and south-eastern part of the Netherlands and eventually described as a separate species (Karssen, 1996). Later it was also found in a plastic tunnel house in France (Daher *et al.*, 1996), and in Belgium (Waeyenberge and Moens, 2001) and Germany (Schmitz *et al.*, 1998). The species has been detected outside Europe, in New Zealand (Marshall *et al.*, 2001), Australia (Nobbs *et al.*, 2001) and South Africa (Fourie *et al.*, 2001). Above-ground symptoms of heavily infested plants include stunting and yellowing, while below-ground galling is typical. Variations in host ranges among different populations have not been described for *M. fallax*.

Successful hybridization was not obtained when *M. fallax* and *M. chitwoodi* were crossed; the F1 was viable, but the F2 second-stage juveniles were not viable and showed morphological distortions (van der Beek and Karssen, 1997).

The root galls produced by *M. chitwoodi* and *M. fallax* are comparable to those produced by several other root-knot species, relatively small galls in general, without secondary roots emerging from them (as found in *M. hapla*). On potato tubers, *M. chitwoodi* and *M. fallax* cause numerous small, pimple-like raised areas on the surface (with *M. hapla* these swellings are not evident).

There is a marked contrast in the hatching response of the two species (see Curtis *et al.*, Chapter 6, this volume). Hatching of J2 of *M. chitwoodi* produced on young plants does not require host root diffusate stimulus, whereas at the end of the plant growing season, egg masses contained a percentage of unhatched J2 that require host root diffusate to cause hatch. This form of obligate quiescence at the end of the host growing season was not found in *M. fallax*, which hatched well in water and did not require hatch stimulation from root diffusate, irrespective of the age of the plant on which the egg masses were produced (Wesemael *et al.*, 2006).

1.7.4 *Meloidogyne minor*

Karssen *et al.* (2004) described *M. minor*, which appeared to be the causal agent of yellow patch disease on several golf courses in the British Isles and root-knot symptoms in one potato field in The Netherlands in 2000 (Plates 12–15). A joint pest risk analysis (PRA) by nematologists in The Netherlands and the UK for the EU region established that *M. minor* was present mainly on coastal sand dunes, golf courses and sports grounds in the British Isles, and in The Netherlands *M. minor* was found on several golf courses, sports grounds and pasture fields. *Meloidogyne minor* has been reported on turfgrass in Belgium (Viaene *et al.*, 2007) but it is not known if it is indigenous to Europe and present in other EU countries. Additional surveys are required to determine its distribution and economic importance, but the PRA concluded that, with the current knowledge, *M. minor* was primarily a problem for golf courses, and it is not yet possible to determine whether quarantine measures are appropriate. It is presumed that its spread cannot readily be controlled since it can be carried on footwear and sports equipment. The PRA document will be available on the web sites of the Dutch and British NPPOs (National Plant Protection Organizations; www.minlnv.nl/pd and www.defra.gov.uk).

1.8 Interactions with Other Plant Pathogens

Meloidogyne spp. frequently play a role in disease interactions (Khan, 1993; see Manzanilla-López and Starr, Chapter 10, this volume), especially with other soil-borne pathogens. Plant pathologists often attribute these interactions to wounds made by the nematodes, which ignores the profound effects of parasitism by *Meloidogyne* spp. on plant physiology and gene expression. Regardless of the underlying mechanisms, the numerous interactions with other root and vascular pathogens only serve to exacerbate the ultimate damage to the crop and increase crop losses. Sometimes *Meloidogyne* spp. and ectoparasitic nematodes appear mutually antagonistic. However, interactions between these two groups may be beneficial for one or both of the species (Eisenback, 1993).

1.9 Management and Control

All methods for control of plant pathogens, including parasitic nematodes, can be categorized under one or more principles (Table 1.1). All of the various methods for control of nematodes fit within one of these principles. Management of nematodes (see Nyczepir and Thomas, Chapter 18, and Coyne *et al.*, Chapter 19, this volume) involves the manipulation of nematode densities to non-injurious or sub-economic threshold levels using several measures in relation to the whole production system, whereas control implies the use of a single measure to reduce or eliminate nematode pests, which in most cases is not possible (Thomason and Caswell, 1987). Maintenance of diversity is an objective of management but not of control, and of increasing importance is the additional need to take into consideration the impact of the pest management strategy on biodiversity and the ecological balance in the soil. Biological control (see Hallmann *et al.*, Chapter 17, this volume) is the management of plant diseases and pests with the aid of living organisms, including predators and parasites of organisms that kill or damage their hosts and also microbes that indirectly influence the establishment, function and survival of pathogens and pests. Plant resistance (see Williamson and Roberts, Chapter 13, and Starr and Mercer, Chapter 14, this volume) is biologically based but is considered a distinct approach to control and management.

As with most plant-parasitic nematodes, preventing the introduction and spread of species of

Table 1.1. Principles for control of plant pathogens.

Exclusion	Prohibiting, frequently by governmental regulations, the entry of the pathogen into a region or locale where it does not exist
Eradication	The complete or partial removal of the pathogen from a region, locale, or field (e.g. soil fumigation)
Avoidance	Avoiding environments and conditions that favour pathogen activity (e.g. altering planting date)
Protection	Usually the use of pesticides to inhibit pathogen activity (e.g. use of non-fumigant nematicides)
Resistance	Altering the genetic constitution of the host so it is able to inhibit pathogen activity (for nematodes, this is typically inhibition of reproduction)
Therapy	Action taken after infection has occurred to limit further development of the pathogen (e.g. hot water treatments of infected bulbs or corms)

Meloidogyne is a vital component of management strategies. *Meloidogyne* species may be spread on farm machinery and may be present in planting material, such as corms, bulbs or roots, but are not found in seeds. Cleaning machinery before use is recommended, and planting material can be discarded if infected, or treated with chemicals or hot water to reduce the numbers of *Meloidogyne*. Only seedlings produced in *Meloidogyne*-free seedbeds should be transplanted.

Listing species as quarantine organisms reduces the risks of spread through international trade. In general, root-knot nematodes are not regulated as a group because the major economically important species are already widely distributed (Hockland *et al.*, 2006). However, *M. chitwoodi* is of increasing importance, primarily because it is a serious pest of economically important crops such as potatoes and carrots, and is on the lists of prohibited pests of many countries (Canada, the EU, Mexico, and other countries in Latin America and the Far East). In the future, with the increase in the number of new species being described, initially with limited knowledge of distribution, more species are likely to be of regulatory concern.

Various cultural and physical control methods have been used with varying degrees of success, but often these methods are only of local or regional relevance. For example, soil solarization (Gaur and Perry, 1991) is only of use in regions where sufficient solar energy is available for long periods of time. Similarly, in some climates, ploughing at intervals of 2–4 weeks during the dry season exposes eggs and J2 to desiccation, killing many in the upper layers of soil. Population densities can be reduced by organic amendments, and flooding land to a depth of 10 cm or more for several months is also effective. The adverse effect of *Tagetes* species is highly variable, depending on the combination of *Tagetes* species and cultivar, and the species and population of *Meloidogyne*. It appears that reduction of *M. incognita* by marigold (*Tagetes patula*) is primarily due to an antagonistic or trap crop effect; J2 enter roots but there is neither giant cell formation nor a hypersensitive reaction.

The era of nematicides was the 1950s, 1960s, 1970s and 1980s. Their overall effectiveness is often cited as a reason why other alternative management systems did not receive greater attention for many years. Starting in the late 1970s, the use of some fumigants, initially 1,2-dibromo-3-chloropropane, was greatly restricted or forbidden entirely. That was followed by similar restrictions and eventual suspension (in some cases by corporate rather than governmental decision) of some of the granular nematicides. Currently, the list of available and effective nematicides is very short. Unfortunately, due in part to the small market share of nematicides relative to herbicides or insecticides, there is little prospect for new effective materials in the near future.

1.10 Conclusions and Future Directions

De Waele and Elsen (2007) attribute the general lack of awareness in tropical countries of even the

existence of plant-parasitic nematodes to the microscopic nature of nematodes, the lack of characteristic symptoms they cause, and the farmers' limited previous exposure to extension and community information. This is probably universally true. Knowledge of the pest involves identification of the species present and, clearly, the provision of better species descriptions, including all available information on morphology, morphometrics, genetics, phylogenetics, etc., is essential (see Eisenback and Hunt, Chapter 2, this volume). Future research in nematode systematics should comprise well-focused taxonomy based on a combination of classical and molecular methods (Coomans, 2002), and we consider that papers on *Meloidogyne* spp. should include a brief description of the method used to identify the population(s). A relevant example of the need for this was provided when a closer examination of *Meloidogyne* populations associated with coffee in Brazil, combining morphological observations with molecular diagnostics, led to the description of several new *Meloidogyne* species (Plate 17) and the suggestion that *Meloidogyne* spp. populations on coffee from Brazil and other Central and South American countries must frequently have been misidentified (Carneiro *et al.*, 2004). That many of the recently described species have been found in association with more common species suggests that such misidentications may have occurred elsewhere.

There needs to be a uniform method to assess and disseminate information on damage caused by the nematode species. Costs of produce fluctuate greatly and are often not comparable between countries. Thus, economic losses are relevant data only for a particular year. Yield loss data should be in a form that can be easily converted to costs if comparison between years is required. But this presupposes a uniform estimation of damage, which may not be yield loss per se but is likely to include the amount of harvested crop that is unmarketable because of damage by *Meloidogyne* spp.

In this chapter we have recommended abandoning the host race system proposed by Hartman and Sasser (1985) that has been used by many scientists. This is not to dismiss the importance in variation in parasitic abilities but is a recognition that the system is no longer adequate for the greatly expanded genus. The variation in host ranges and parasitic fitness of the more than 90 currently recognized species on individual hosts now appears to be too great to be categorized by a numerical host race designation. With the increasing use of resistant hosts for control of root-knot nematodes, eliminating the use of the host race also avoids potential confusion with races (or pathotypes) that vary in virulence on resistant host genotypes.

This introductory chapter has set the scene for the subsequent chapters, where aspects of nematode biology, host–plant interactions and control will be discussed in depth. Information on the genomes of *M. hapla* and *M. incognita* will enable features of obligate parasitism to be defined, and the genomic information may aid in the identification of novel control targets and the refining of environmentally acceptable management options. Management of *Meloidogyne* in developed and resource-poor regions will be central to the provision of sufficient food for the ever-increasing global population.

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2 General Morphology

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2.1 General Morphology

After their first discovery on the roots of cucumber in an English glasshouse (Berkeley, 1855), the root-knot nematodes were soon recognized as important pathogens on numerous host plants all around the world (Greeff, 1864; Licopoli, 1875; Cornu, 1879; Jobert, 1878; Örley, 1880; Bellati and Saccardo, 1881; Müller, 1884; Treub, 1885; Atkinson, 1889; Neal, 1889; Cobb, 1890; Palumbo, 1892; Viala, 1893; van Breda de Haan, 1899; Lavergne, 1901). Near the end of the 19th century, recognition of the economic importance of root-knot nematodes stimulated several detailed morphological studies of these nematodes (Müller, 1884; Göldi, 1887; Atkinson, 1889; Neal, 1889; Cobb, 1890). Although additional contributions on the morphology of all stages in the life cycle of root-knot nematodes were made by Nagakura (1930), the most significant study in this respect was by Chitwood (1949), who revealed that the root-knot nematodes comprised several different species, including *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949 and *M. hapla* Chitwood, 1949.

Histological studies (Elsea, 1951; Maggenti and Allen, 1960) added important details to our understanding of the morphology of the root-knot nematodes, and, shortly thereafter, the use of the transmission electron microscope (TEM) greatly enhanced our knowledge of the fine structure of these nematodes (Bird, 1958, 1959, 1979b; Bird and Rogers, 1965a,b; Bird and Saurer, 1967; Bird and Soeffky, 1972; Baldwin and Hirschmann, 1973, 1975, 1976; Dropkin and Acedo, 1974; Bird and McClure, 1976; Johnson and Graham, 1976; McClure and Bird, 1976; Wergin and Endo, 1976; Baldwin *et al.*, 1977; Endo and Wergin, 1977; Goldstein and Triantaphyllou, 1980; Shepherd and Clark, 1983), as well as the changes in morphology that occur during the onset of parasitism (Bird 1967, 1968a,b, 1969, 1971a).

Additional observations on the external morphology of the various life stages were illuminated by the use of the scanning electron micro-

scope (SEM). This instrument clarified our understanding of the perineal pattern (Yik and Birchfield, 1978), the morphology of the second-stage juvenile (J2) (Eisenback and Hirschmann, 1979a,b), male (Eisenback and Hirschmann, 1980, 1981) and female heads, and excised stylets (Eisenback *et al.*, 1980). SEM of excised stylets of the J2 revealed minute differences among the four best-known species (Eisenback, 1982).

The morphology of the root-knot nematodes has been reviewed by several authors (Whitehead, 1968; Bird, 1971b, 1979b; Franklin, 1971, 1978; Esser *et al.*, 1976; Taylor and Sasser, 1978; de Guiran and Ritter, 1979; Jepson, 1983a,b,c, 1987; Eisenback, 1985a,b; Hirschmann, 1985a,b; Eisenback and Triantaphyllou, 1991; Kleynhans, 1991; Karssen, 2002; Karssen and Moens, 2006).

The morphological details of root-knot nematodes are important for the identification of species (Chitwood, 1949; Esser *et al.*, 1976; Franklin, 1978; Taylor and Sasser, 1978; Eisenback and Hirschmann, 1981; Eisenback *et al.*, 1981; Jepson, 1983a,b,c, 1987; Hirschmann, 1985b; Kleynhans, 1991; Karssen, 2002; Karssen and Moens, 2006) and for identifying phylogenetic relationships (Franklin, 1971; Wouts, 1979; Hirschmann, 1985a). In addition, these morphological details are often used to determine physiological function (Elsea, 1951; Maggenti and Allen, 1960; Bird and Saurer, 1967; Bird, 1968a,b, 1969, 1979c; Baldwin and Hirschmann, 1973, 1975, 1976; Dropkin and Acedo, 1974; McClure and Bird, 1976; Wergin and Endo, 1976; Dropkin and Bird, 1978; Viglierchio, 1979; Shepherd and Clark, 1983).

Morphological observations are useful in interpreting the interaction of the environment with the nematode (Papadopoulou and Triantaphyllou, 1982), and also provide insight into the intricate host–parasite relationship (Bird, 1979a; de Guiran and Ritter, 1979); this interaction may have an influence on nematode morphology (Bird, 1967, 1968a,b, 1971a).

During the complex life cycle of the root-knot nematodes (Fig. 1.1), the morphology changes from a one-celled zygote to a small, vermiform, first-stage juvenile, which moults once in the egg to become the infective J2, the stage that subsequently hatches from the egg. The infective juveniles may move freely out from the egg mass into the surrounding soil and search for the root tip of a suitable host plant or, in the case of second and further generations of an established infection,

Fig. 2.1. Scanning electron micrograph of root-knot nematode male (left), female (centre) and 13 second-stage juveniles (right), showing general body shape and relative dimensions of the stages. After Eisenback and Triantaphyllou (1991), courtesy of Marcel Dekker, Inc.

may migrate entirely within the root tissue before establishing a new feeding site. The root cortex is penetrated near the zone of differentiation, the J2 migrating predominantly intercellularly toward the root tip and then making a 180° turn back towards the zone of differentiation (Wyss *et al.*, 1992). Once inside the vascular cylinder the J2 initiate a series of three to eight giant cells, where they feed for 3–8 weeks and greatly increase in size by swelling into a sausage-shaped juvenile with a characteristically spicate tail region. The swollen J2 rapidly moult into short-lived third- and fourth-stage juveniles. Fourth-stage juveniles that will develop into males become vermiform after the third moult, but juveniles destined to become females remain swollen. Both types of fourth-stage juveniles moult once more to become either a mature male or a female. The morphology of the J2 and the adult male and female (see Fig. 2.1 for comparison of the general appearance of these stages) will now be described according to body system.

2.1.1 Second-stage juvenile (Figs 2.1–2.9)

Depending upon certain environmental signals, some J2 may enter into a diapause and remain in the egg, where they overwinter (de Guiran and Ritter, 1979; see Evans and Perry, Chapter 9, this volume). Those that hatch from the egg are quite mobile and capable of moving long distances

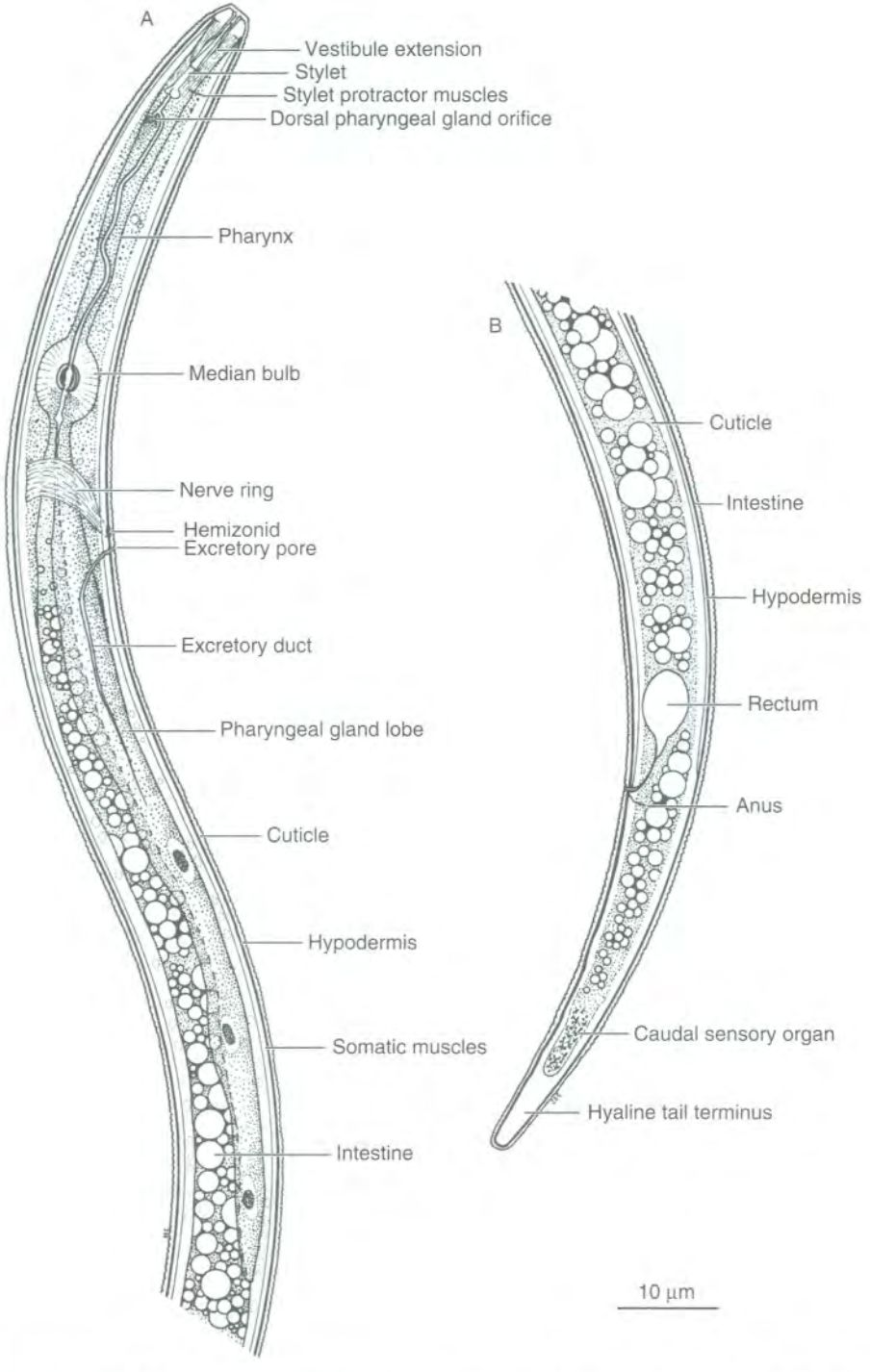

Fig. 2.2. Drawings of second-stage juvenile root-knot nematode. A: anterior region; B: posterior region. After Eisenback (1985a), courtesy of N.C. State University Graphics.

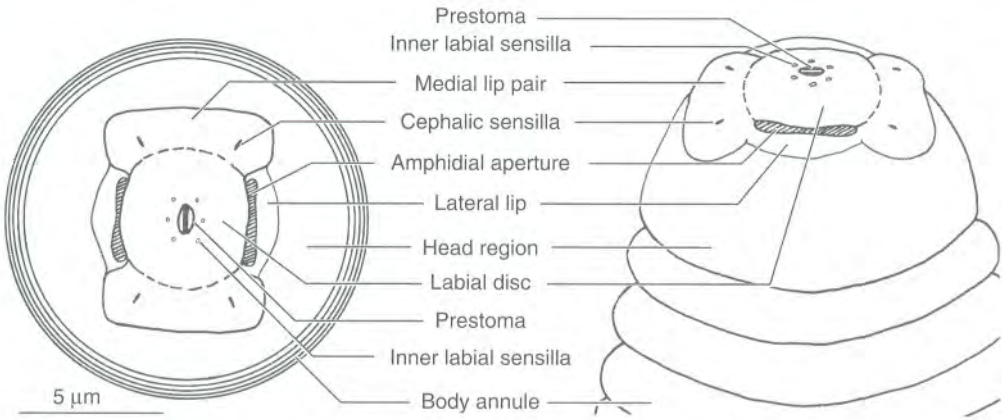

Fig. 2.3. Drawing of anterior end of second-stage juvenile of root-knot nematode, as revealed by scanning electron microscopy, in face and lateral views. After Eisenback and Hirschmann (1979b), courtesy of *Journal of Nematology*.

(40–100 cm) vertically within the soil profile when soil moisture levels are optimum. Mobility allows the J2 to find a suitable host root tip, to penetrate into the cortex and then move to the preferred feeding site. The body wall, together with its protective cuticle and somatic muscles, is controlled by the nervous system, which allows the nematode to respond to environmental cues (see Curtis *et al.*, Chapter 6, this volume) that enable it to move to a suitable site for establishing a host–parasite relationship. The digestive system (Fig. 2.2) initiates the formation of a feeding site, and the nutrients that the nematode absorbs from the plant are stored in the intestine, which becomes enlarged, resulting in a swollen juvenile that is no longer capable of movement. The energy stored in the intestine is eventually transferred to the small genital primordium, which subsequently develops into the adult reproductive systems (Triantaphyllou, 1960, 1962, 1979; Papadopoulou and Triantaphyllou, 1982).

2.1.2 Male (Figs 2.1, 2.9–2.16)

Males become vermiform after the third moult and remain vermiform during the fourth moult into the adult stage, the nematode being enclosed within the shed cuticles of the previous stages. They become mobile as adults and eventually emerge from the retained cuticles before leaving the gall and entering the soil phase. There they migrate and attempt to find a female for sexual reproduction. As with the J2, the body wall and nervous system allow the male nematode to respond to environmental cues and to move through the soil in its search for a mate. In species that reproduce by amphimixis males are quiet common, but in parthenogenetic species males may be very rare and are unnecessary for reproduction (Triantaphyllou, 1979). Unlike the J2 and female, the male does not feed, all of the energy required for the development of its reproductive system being obtained while it was a J2. As a consequence, the male pharyngeal glands are degenerate and probably not functional, while the intestine serves as a storage organ for the food reserves obtained as a J2 and supplies energy to the reproductive system for the production of sperm.

2.1.3 Female (Figs 2.1, 2.17–2.23)

Females are pear-shaped and sedentary (Figs 2.17, 2.18), although the neck region remains musculated and allows the nematode to change head position so that she can feed on one of several giant cells. The increase in body size and change in shape adds volume to the reproductive system, which is in close contact with the large amorphous intestine. While the digestive system is specialized for the maintenance of the giant cells and the withdrawal of nutrients from the plant, the intestine is less specialized and serves primarily as a storage organ for the nutrients

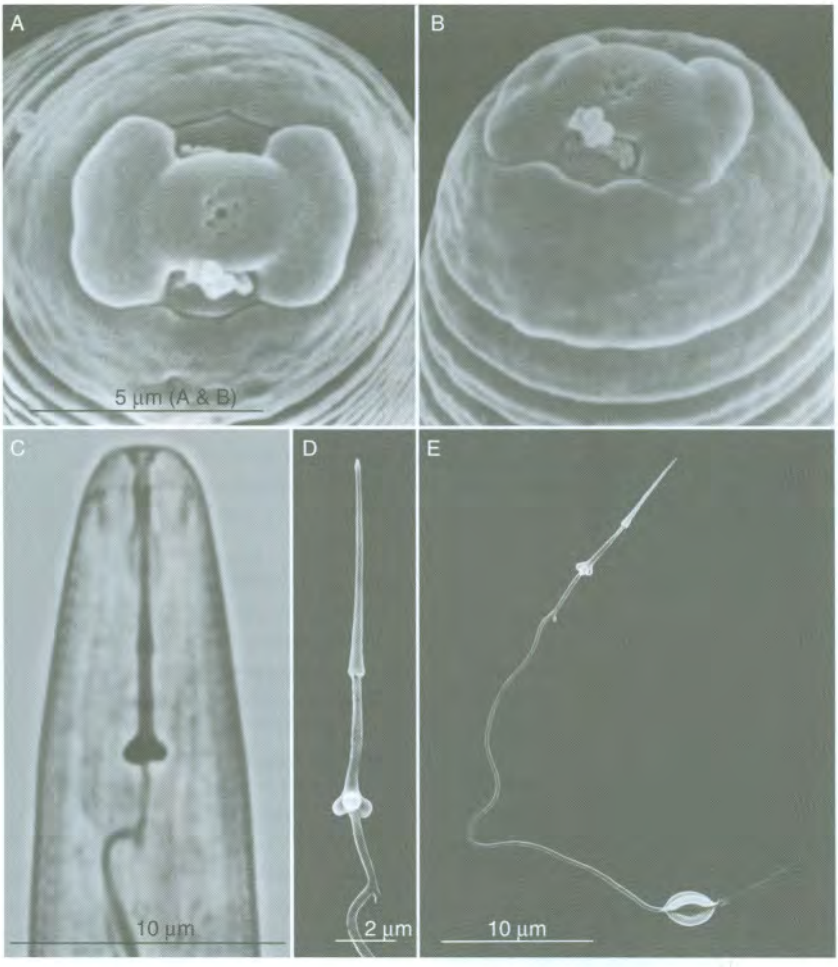

Fig. 2.4. Micrographs of second-stage juvenile root-knot nematode. A: scanning electron micrograph (SEM) of anterior end in face view; B: SEM of anterior end in lateral view; C: light micrograph of anterior, showing shape of head and stylet morphology; D: SEM of excised stylet; E: SEM of excised stylet with attached cuticular lumen lining of entire pharynx. After Eisenback (1982) and Eisenback and Hirschmann (1979a), courtesy of *Journal of Nematology* and *Scanning Electron Microscopy*.

ingested from the plant. Oddly, the intestine is not attached to the rectum and therefore has a blind ending. Instead, six large rectal gland cells empty their contents through the anus to form the gelatinous matrix that surrounds and protects the eggs as they are deposited through the vulva. The two ovaries of the female genital system are in direct contact with the intestine so that nutrients stored there simply diffuse across a few cell membranes to become available for growth of the oogonia and oocytes into mature eggs.

2.1.4 Egg

Eggs of root-knot nematodes vary in size and shape but are typically approximately 95 µm long and 40 µm in diameter (Saigusa, 1957; Bird and McClure, 1976; McClure and Bird, 1976). The eggshell consists of an outer vitelline layer approximately 30 nm thick, a middle chitinous layer about 400 nm thick and an inner glycolipid layer of varying thickness. The glycolipid layer makes the egg very resistant to harsh chemicals

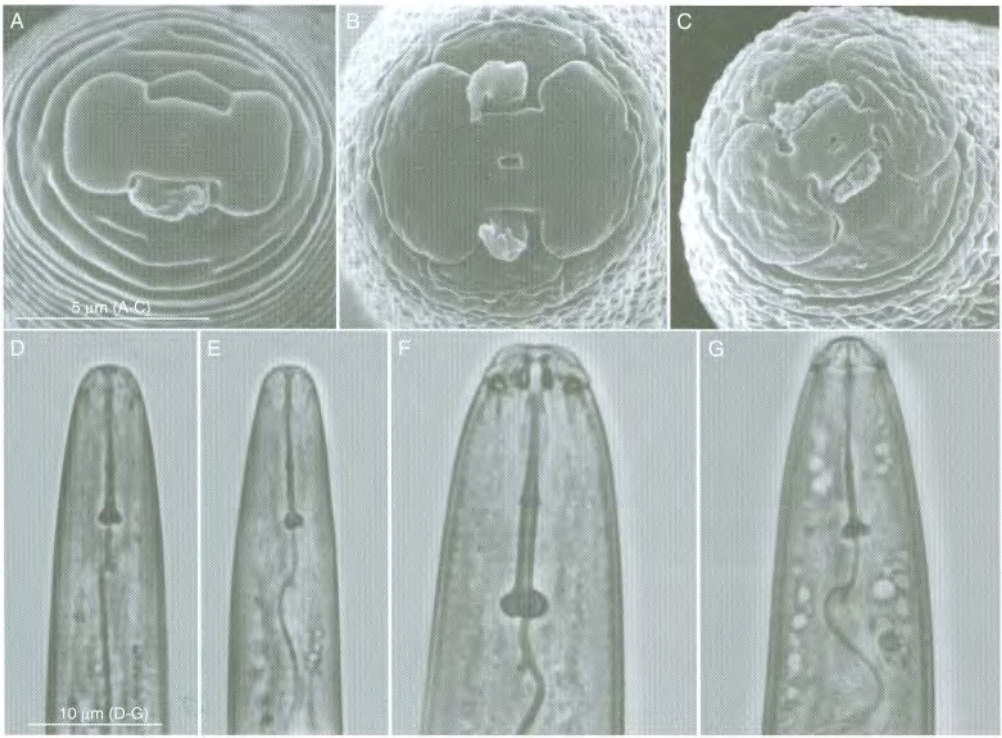

Fig. 2.5. Micrographs of anterior end of second-stage juvenile root-knot nematode. Scanning electron micrographs. A: *Meloidogyne incognita*; B: *M. brevicauda*; and C: *M. nataliae*. Light micrographs. D: *M. arenaria*; E: *M. hapla*; F: *M. brevicauda*; G: *M. nataliae*. After Eisenback (1988), courtesy of Plenum Press.

and, as a consequence, this stage is not sensitive to toxins such as common nematicides.

2.2 Body Wall

In the female, the body wall protects her from the outside environment, but in the J2 and male it also enables them to move through the soil. The body wall has three major layers: the cuticle, hypodermis and somatic muscles (Bird, 1979b). Much of the nervous system is contained within the hypodermis.

2.2.1 Cuticle

The non-cellular, elastic cuticle is secreted by the hypodermis and covers the entire body and all of the openings, including the lining of the pharynx, the amphidial canals, the secretory/excretory duct, the phasmidial ducts, the male cloaca, the J2 rectum and the female vulva (Bird, 1979b). It functions as an interface between the organism and the harsh environment found in the soil, and protects the mobile juvenile and male from numerous biological, chemical and physical hazards. The diffusion of liquids through the body wall is controlled by the structure of the cuticle. Furthermore, the cuticle restricts the body from increasing in diameter and, in concert with the high turgor pressure inside the pseudocoelom, acts as an exoskeleton that enables the nematode to move freely through the soil or plant tissue.

Like most nematodes, the cuticle of the J2 and male has three layers: cortical, medial and basal. The female, however, only has a cortical and basal layer (Bird, 1979b). These layers vary in thickness according to the life stage. In the J2 the cuticle is 0.3–0.4 µm thick, whereas it is 1.5 µm thick in the male and 4–6 µm in the female, where

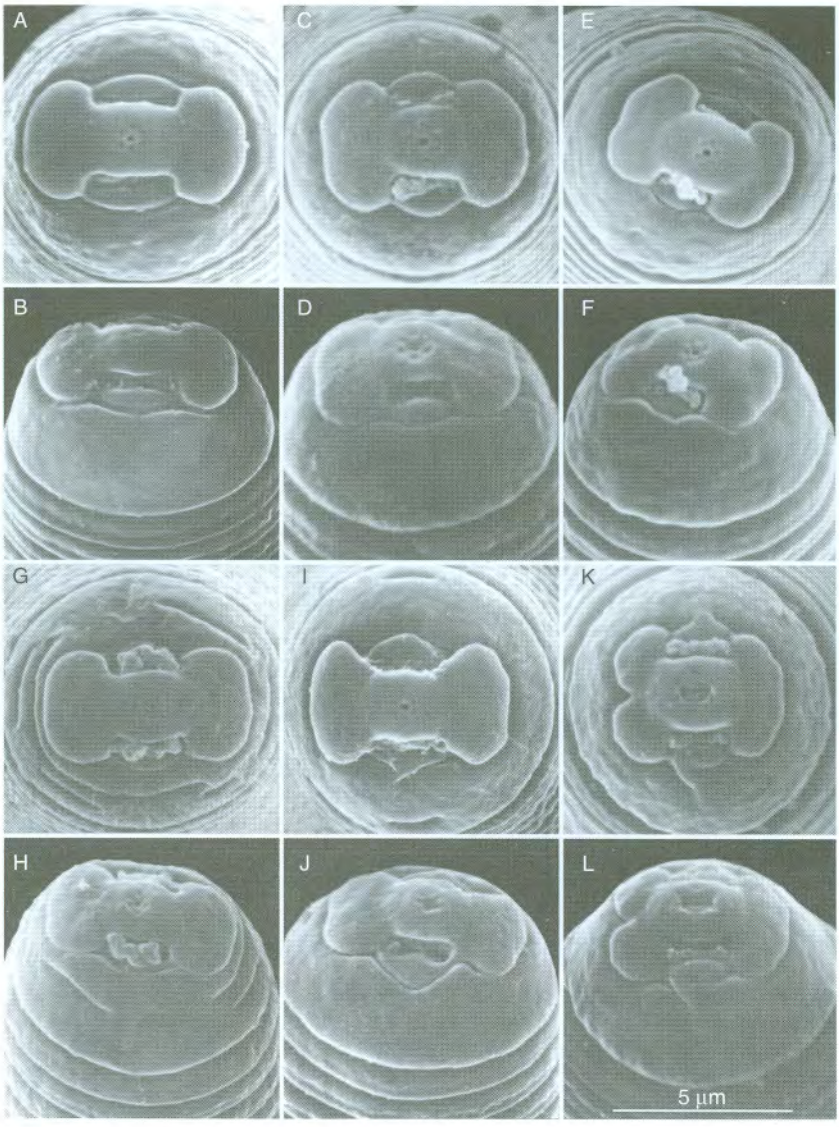

Fig. 2.6. Scanning electron micrographs of face and lateral views of anterior end of second-stage juveniles of several species of root-knot nematodes. A, B: *Meloidogyne arenaria*; C, D: *M. exigua*; E, F: *M. hapla*; G, H: *M. incognita*; I, J: *M. javanica*; K, L: *M. megatyla*. After Eisenback and Hirschmann (1979a), courtesy of *Scanning Electron Microscopy*.

it is thickest in the perineal region and thinnest near the anterior end (Bird, 1979b).

The cortical layer in all three stages is divided into a tri-part external cortical layer composed of two thin, dark-staining regions with a thin, lightly staining inner region, and a thicker internal cortical layer. In the J2, the external cortical layer varies from 35 to 40 nm thick, while in the male it is 100 nm thick and in the females it varies from 100 to 125 nm (Baldwin and Hirschmann, 1975; Johnson and Graham, 1976; Bird, 1979b). The internal cortical layer is much thicker than the external cortical layer, being 50–100 nm thick in the J2, 400–500 nm thick in the male and 700–900 nm thick in the female

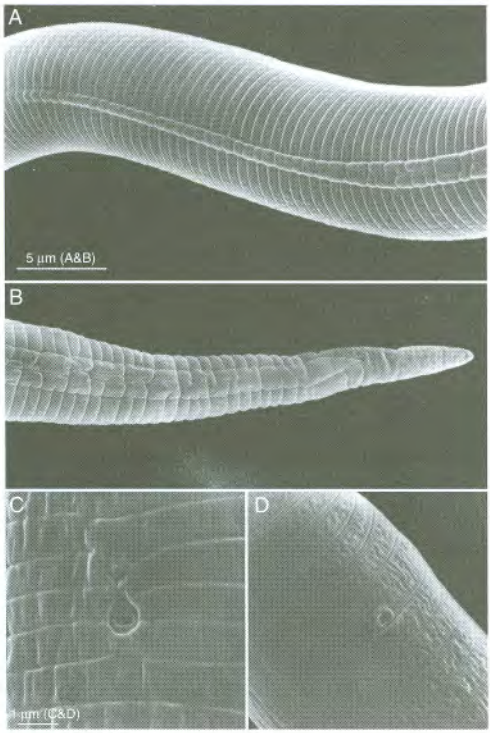

Fig. 2.7. Scanning electron micrographs of second-stage juvenile root-knot nematode. A: anterior portion of body, showing the regular body annulation and beginning of the lateral field; B: posterior end of body, showing end of lateral field and tail terminus; C: secretory/excretory pore; D: anal opening. After Eisenback and Hirschmann (1979b), courtesy of *Journal of Nematology*.

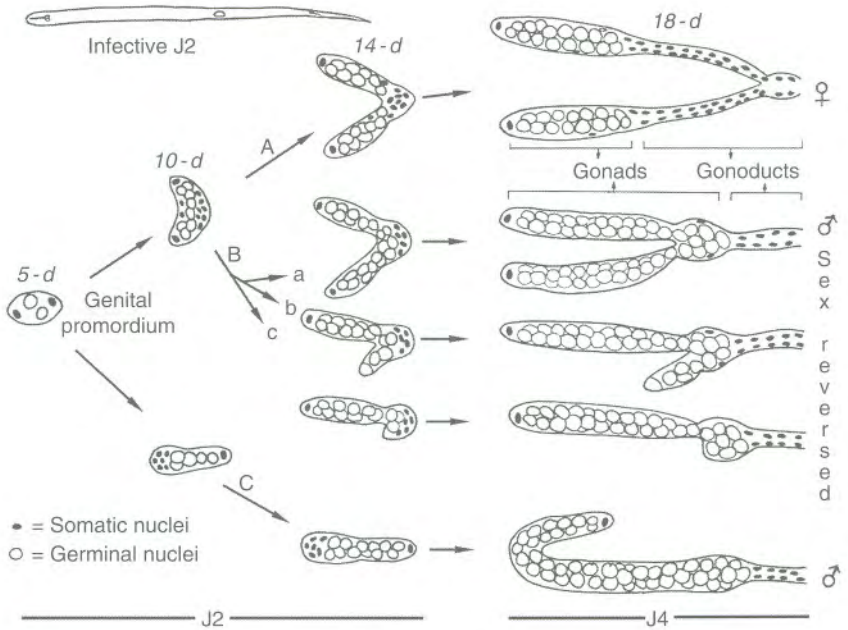

Fig. 2.8. Development of genital primordium into a normal female gonad with two ovaries, sex-reversed gonads of males with two testes and a normal male with only one testis. After Papadopoulou and Triantaphyllou (1982), courtesy of *Journal of Nematology*.

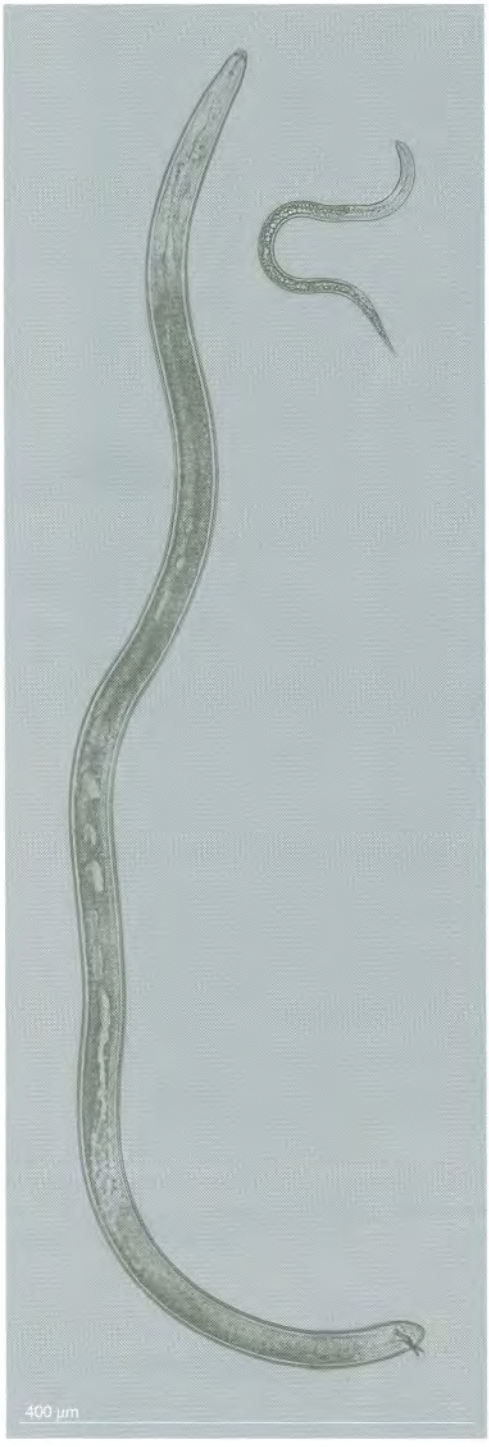

Fig. 2.9. Light micrograph of male and second-stage juvenile root-knot nematode (original).

(Baldwin and Hirschmann, 1975; Johnson and Graham, 1976; Bird, 1979b). Variation in the thickness of this layer is caused by the indentations of the body annulations, the layer being thinner beneath each annulation and thicker under each body annule; this layer is fibrillar. Unlike the other layers of the cuticle, which are broken down and recycled by the hypodermis, the external cortical layer must be cast off during the moulting process.

Although the medial layer is present in the vermiform, mobile J2 and male, it is lacking in the female. This layer may be continuous with the substrate of the internal cortical layer, but it contains electron-dense globules that may be filled with a liquid in the living nematodes. In the J2, the medial layer varies greatly in thickness where it occurs under the body annules (100 nm thick) but disappears completely under the annulations. In the male, this layer is present beneath the annulations and annules but varies in thickness from 300 to 400 nm where the globules are more dense between the annules, and in the lateral fields near the anterior end (Baldwin and Hirschmann, 1975; Johnson and Graham, 1976; Bird, 1979b).

The basal layer is the thickest of the three layers. It is 100–125 nm thick in the J2, moderately thick in the male (400–500 nm) and very thick in the female (3000–4000 nm). In both the male and pre-parasitic juvenile this layer is striated by two sets of laminae orientated at right angles to one another (Popham and Webster, 1978). However, in the female these striae are absent. The orientation of the parallel striae, which occur approximately 22 nm from each other, forms crystalline laminae, which may be either structurally important in protecting the nematode from the harsh external environment or a necessary structural component vital for movement through the soil. A few days after the J2 establishes a host–parasite relationship with the plant, the striae break down and disappear. They are not found in any of the other juvenile stages and reappear only in the cuticle of the mobile male (Bird, 1971b). The laminae appear to be modified beneath the lateral fields where the striae fork, and are replaced by obliquely orientated fibrillar layers at the edges of the lateral field. They are absent from the anterior end of the nematode beyond the normal body annules. Likewise, in the tail tip of the J2 the striae become very disorganized (Bird, 1979c).

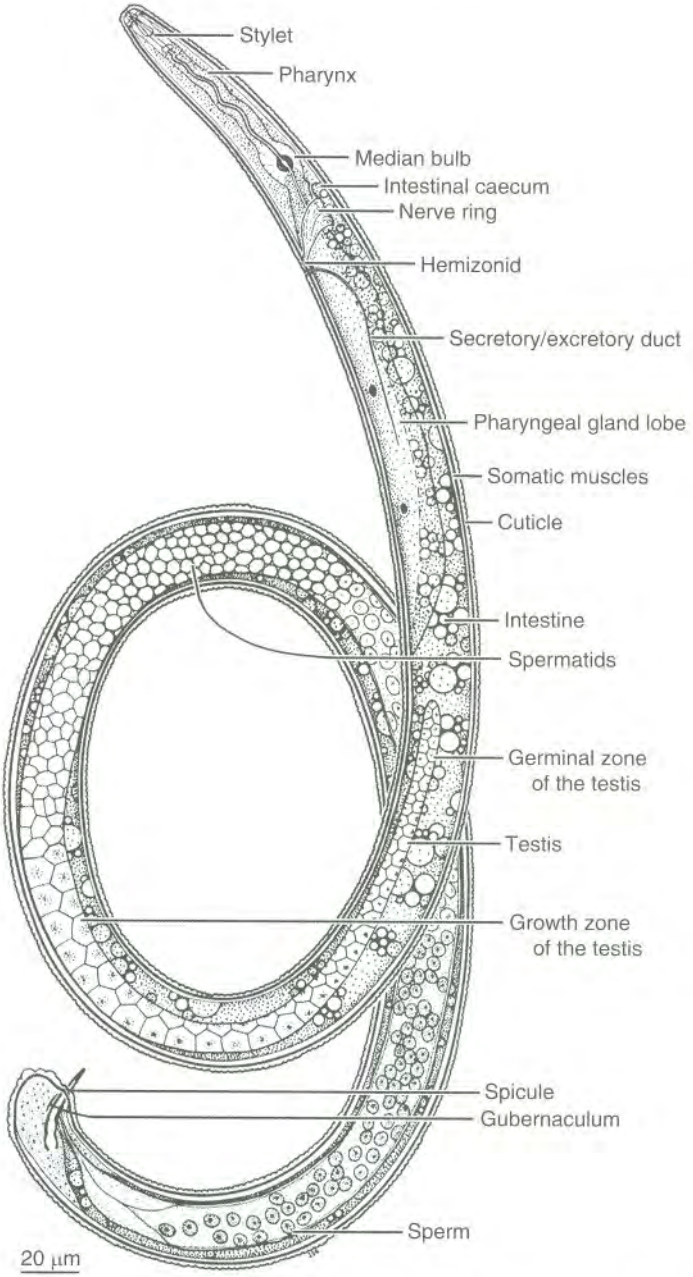

Fig. 2.10. Drawing of entire specimen of male root-knot nematode. After Eisenback (1985a), courtesy of N.C. State University Graphics.

The J2, male and female have similar external cuticular markings, but these differ in their expression (Eisenback and Hirschmann, 1979a,b, 1980, 1981; Eisenback *et al.*, 1980; Jepson, 1987; Kleynhans, 1991; Karssen and Moens, 2006). In all three life stages the slit-like stomatal opening is situated within an ovoid, prestomatal depression. The prestoma is surrounded by six small,

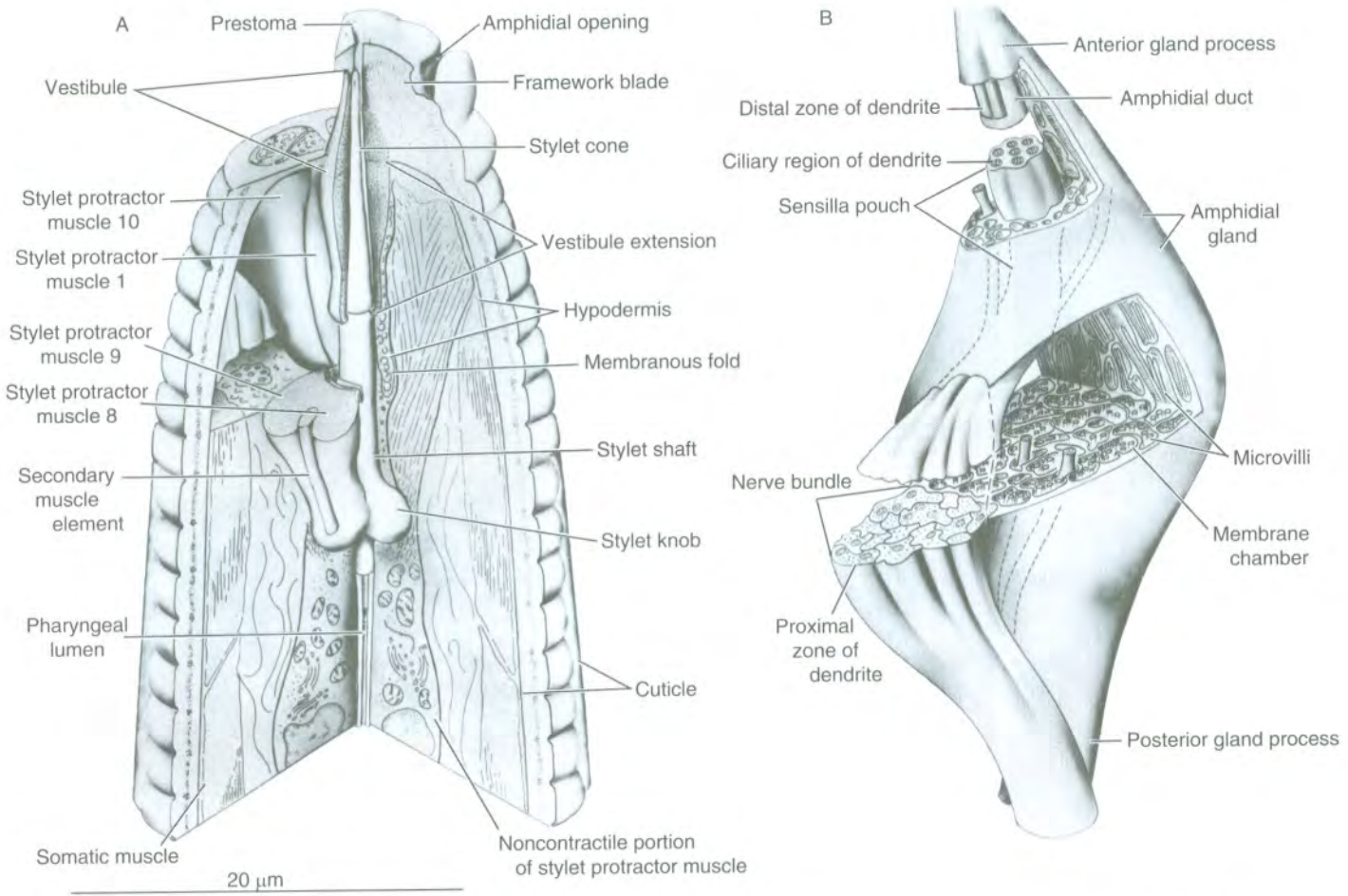

Fig. 2.11. 3-D reconstructions of male root-knot nematode as revealed by transmission electron microscopy. A: anterior end; B: amphid. After Baldwin and Hirschmann (1973, 1976), courtesy of *Journal of Nematology*.

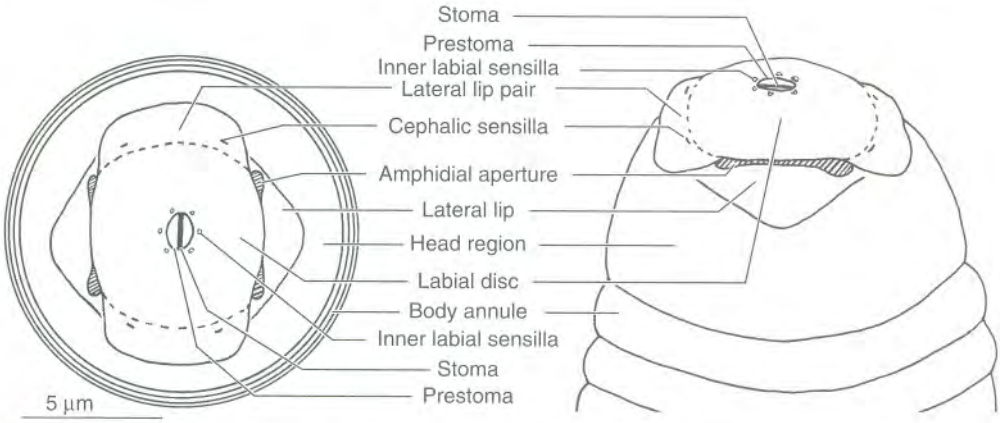

Fig. 2.12. Drawings of anterior end of male root-knot nematode in face and lateral views, as revealed by scanning electron microscope. After Eisenback and Hirschmann (1981), courtesy of *Journal of Nematology*.

pit-like openings of the inner labial sensilla (see Figs 2.6 and 2.13F, for example). In both male and female these sensilla sometimes occur on the edge of the prestoma, causing it to appear hexagonal. The prestoma, stoma and inner labial sensilla are located on the labial disc. In the J2 this disc is moderately sized (Fig. 2.3), but in the male it is relatively large (Fig. 2.12), while in the female it is relatively small (Fig. 2.19). This structure is generally fused with the medial lip pairs and slightly covers the amphidial apertures in face view, to form the head cap. Each medial lip pair contains the nerve endings of two cephalic sensilla, which are located just beneath the cuticle, their positions being sometimes visible as slight depressions. The lateral lips occur posterior to the amphidial apertures and can be either fused with the medial lip pairs or completely or partially fused with the head region. The lips are not a part of the head cap but instead form part of the head region.

Located between the labial disc and lateral lips, the amphidial apertures are elongated ovals in the J2 (Figs 2.3, 2.4A,B, 2.5A–C, 2.6) and female (Figs 2.19, 2.22A,B), but are more slit-like in the male (Figs 2.12, 2.13D–G, 2.14A–C). Depending on the speed of fixation, these openings are often plugged with secretions from the amphidial gland. The secretions are thought to be necessary for cleansing the nerve endings of the amphids.

A large head annule (= the head region) occurs posterior to the head cap and lateral lips (Figs 2.3, 2.12, 2.19). This region may be completely smooth or marked by one to seven complete or incomplete annulations (see Figs 2.13E, 2.14B, for example). Following the head region, regular, evenly spaced body annules continue along the entire length of the J2 and male. They are spaced 0.8–1.0 µm apart in the J2 (Fig. 2.7A) and 2.0–2.5 µm in the male (Fig. 2.15F,G). In the female, the head region is usually less distinct and may sometimes be difficult to distinguish from the regular body annulations (Fig. 2.22A,B). As the body annulations continue along the body of the female, they become less and less set-off, until they become more closely spaced and more distinct where they encircle the perineal area (Figs 2.22E,F, 2.23). The regular body annulations are interrupted near the base of the stylet by a wide, raised lateral field on each side of the body in the J2 and male. Furthermore, the body annulations are interrupted ventrally in all three life stages by the small, ovoid opening of the secretory/excretory pore and by the cloacal opening in the male and anus in the J2 and female (Eisenback and Hirschmann, 1979a,b; Eisenback and Triantaphyllou, 1991).

Commencing as a single ridge about 8–15 body annules from the anterior end (Fig. 2.7A), the lateral field runs the entire length of the nematode. Additional incisures appear along the length of the lateral field, forming three or four incisures in total. In some species the number of lateral lines may increase to more than four, but the number varies along the body and sometimes additional

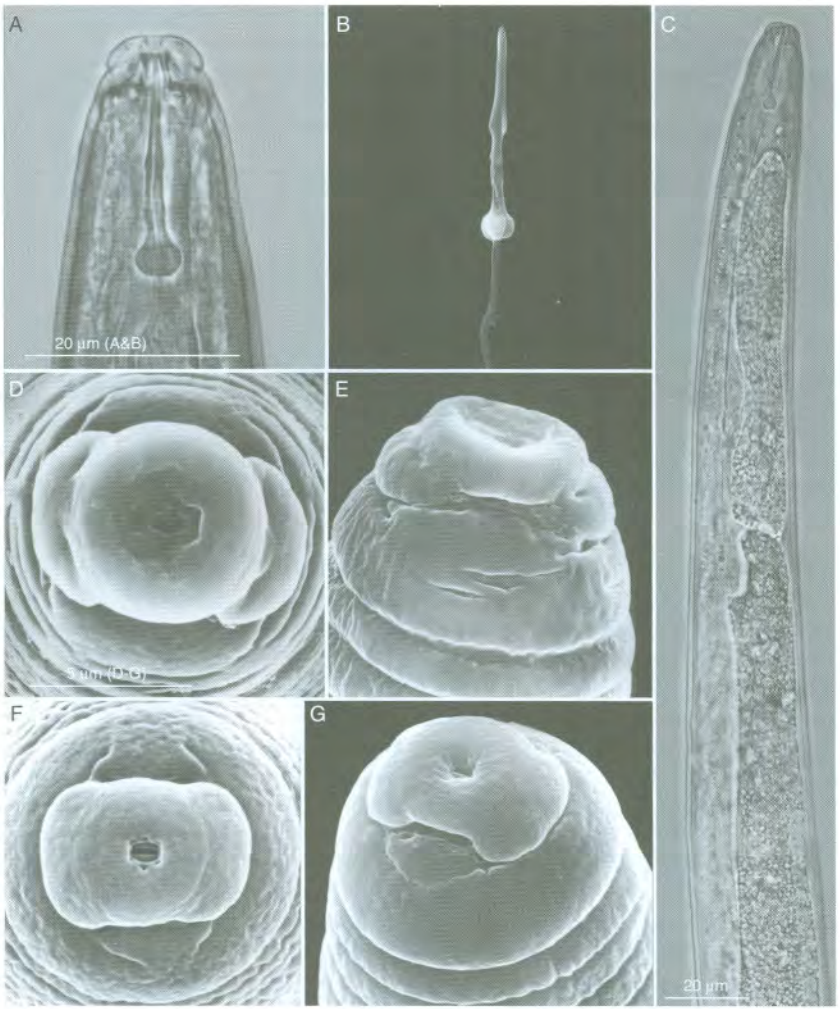

Fig. 2.13. Photomicrographs of male root-knot nematode. A: light micrograph of anterior end, showing head shape and stylet morphology; B: scanning electron micrograph (SEM) of excised male stylet with attached cuticular lumen lining of pharynx; C: light micrograph of anterior end, showing most of pharynx, including stylet, procorpus, metacorpus, basal gland lobe, intestinal caecum and intestine D: SEM of anterior end of *Meloidogyne incognita* in face view; E: SEM of anterior end of *M. incognita* in lateral view; F: SEM of anterior end of *M. hapla* in face view; G: SEM of anterior end of *M. hapla* in lateral view. After Eisenback and Hirschmann (1981), courtesy of *Journal of Nematology*.

folds can form between the lines, thereby making an exact count difficult. In the J2, the lateral fields narrow in the tail region where the two middle incisures merge, resulting in just three incisures. The middle incisure so formed then gradually disappears, the two outer incisures finally merging towards the end of the tail (Fig. 2.7B). In the male, however, the lateral fields appear either to encircle the tail tip or to become very wide and gradually merge with the tail terminus (Fig. 2.15D). The two inner incisures either disappear near the cloacal opening or occur almost all the way to the tail tip. The lateral field is areolated by transverse markings that correspond to the regular body annulation, although they are often incomplete and irregular (Fig. 2.7A,B).

In the female, regular body annules mark the neck region (Fig. 2.22D), where they become

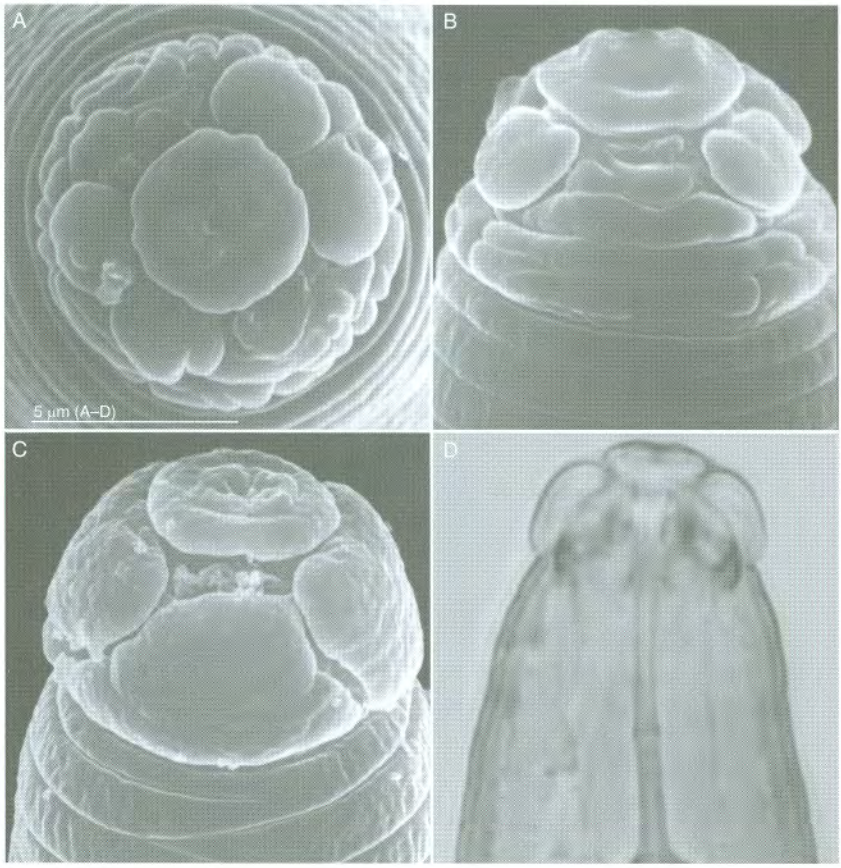

Fig. 2.14. Scanning electron micrographs (SEM) and light micrographs (LM) of male root-knot nematodes. A: SEM of *Meloidogyne nataliae* face view; B: SEM of *M. nataliae* in nearly lateral view; C: SEM of *M. brevicauda* in nearly lateral view. D: LM of *M. brevicauda*. After Eisenback (1988), courtesy of Plenum Press.

more shallow as they proceed down the body, until they nearly disappear altogether in the vicinity of the mid-body region (Eisenback *et al.*, 1980). Near the posterior end, these annulations reappear and become spaced closer together with deeper striae, thereby forming the characteristic, fingerprint-like perineal pattern, which encompasses the vulva, anus and tail tip (Figs 2.18, 2.22E,F, 2.23). The lateral fields are irregular and incomplete in the female, a feature that makes counting the number of incisures uncertain. These fields disappear in the middle of the body but may reappear posteriorly in a few species that have distinct lateral ridges in the perineal pattern (Eisenback *et al.*, 1981).

The phasmidial openings of the J2 and female may be obscured by other features within the lateral field, but in the male they appear as small slit-like openings within the inner incisures at the level of the cloacal opening (Fig. 2.15D) (Eisenback and Hirschmann, 1979a,b; Jepson, 1983c). Likewise, the secretory–excretory pore of the J2 (Fig. 2.7C), male (Fig. 2.15F,G) and female appears as a small, simple, rounded to ovoid ventral opening that may be located in a depression within the cuticle (Fig. 2.15G). Regular body annulations may be interrupted by the occurrence of this pore and these disruptions may be most severe in the female. The anus in the J2 is a small, rounded opening (Fig. 2.7D). In the female it is covered by a flap of cuticle and in the male the cloacal opening is transversely elongate and devoid of regular body annulations, both anteriorly and posteriorly (Fig. 2.15D).

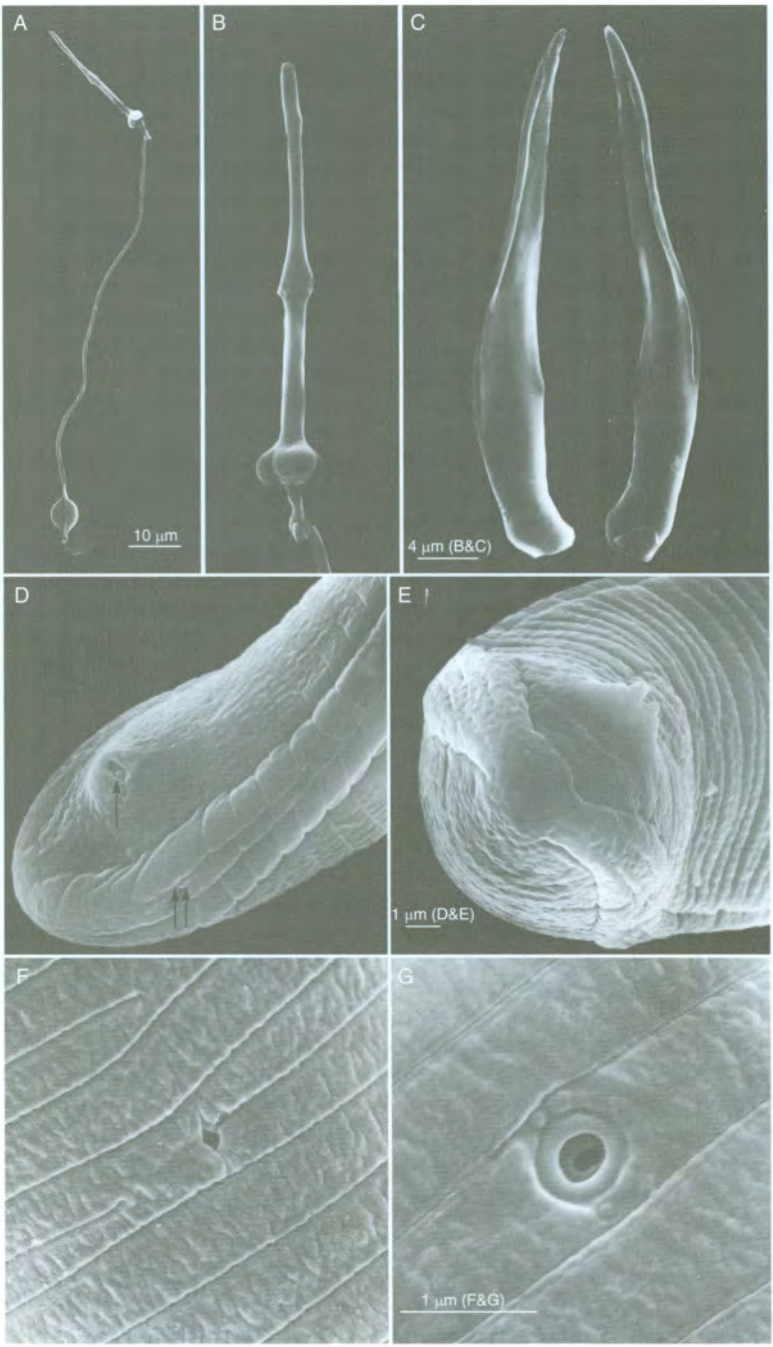

Fig. 2.15. Scanning electron micrographs (SEM) of male root-knot nematodes. A: excised stylet and cuticular lumen lining of pharynx; B: excised stylet; C: pair of spicules; D: posterior end, showing lateral field wrapping around tail, phasmidial opening (double arrows) and cloacal opening (single arrow) with tips of spicules protruding; E: posterior end of tail, showing lateral field and tips of spicules protruding through cloacal opening. F, G; secretory–excretory pore of a typical root-knot nematode and recessed opening of *Meloidogyne javanica*, respectively. After Eisenback (1985a), courtesy of N.C. State University Graphics.

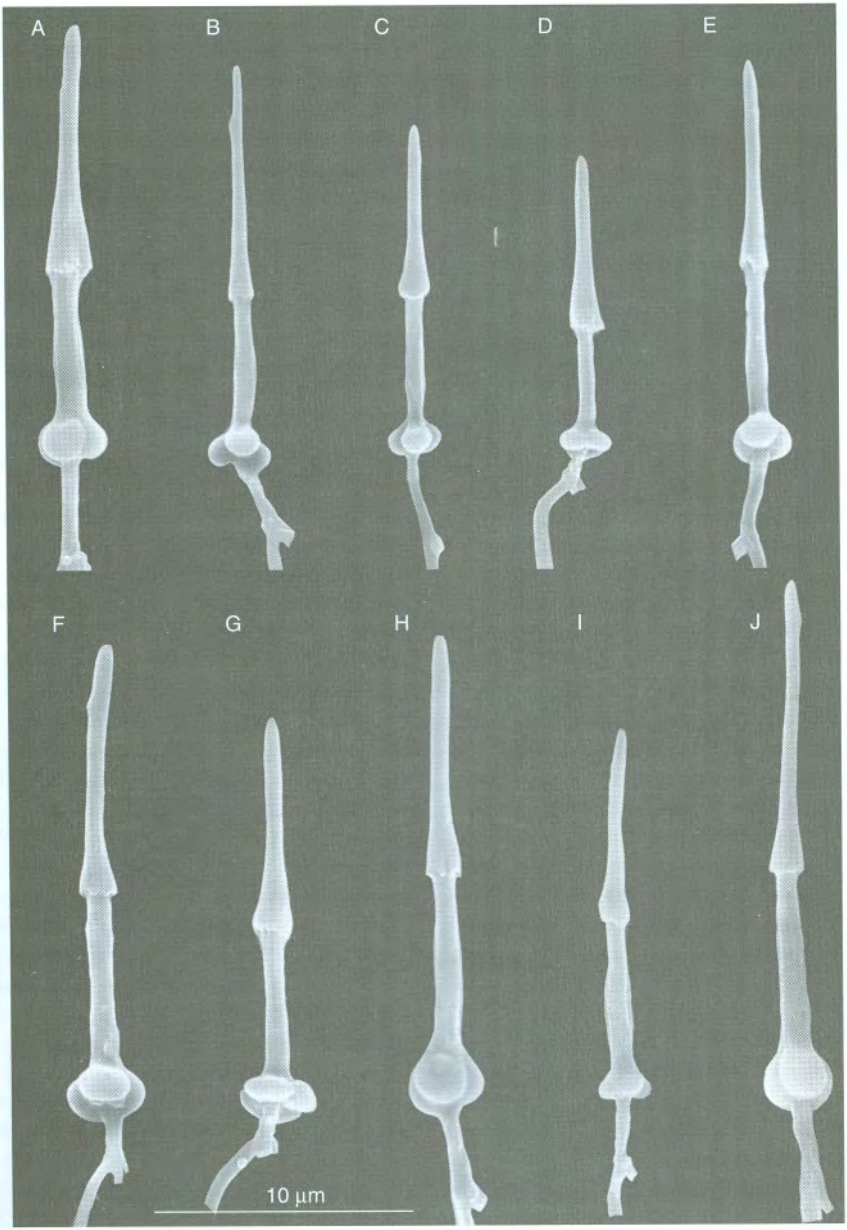

Fig. 2.16. Scanning electron micrographs of excised stylet of male root-knot nematodes. A: *Meloidogyne arenaria*; B: *M. carolinensis*; C: *M. exigua*; D: *M. graminicola*; E: *M. hapla*; F: *M. incognita*; G: *M. javanica*; H: *M. megatyla*; I: *M. naasi*; J: *M. nataliae*. After Eisenback and Hirschmann (1982), courtesy of *Scanning Electron Microscopy*.

2.2.2 Hypodermis

The hypodermis is a thin, living, plasma-membrane-bound syncytium between the cuticle and the somatic muscles. It secretes the cuticle and serves as the interface between the somatic muscles and the cuticle. The hypodermis lines the stomatal cavity, cephalic framework, sensory

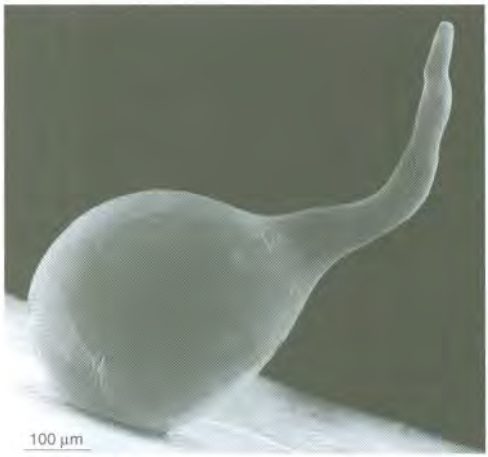

Fig. 2.17. Scanning electron micrograph of entire root-knot nematode female. After Eisenback and Triantaphyllou (1991), courtesy of Marcel Dekker, Inc.

structures and stylet shaft (Fig. 2.11A) (Elsea, 1951; Baldwin and Hirschmann, 1975; Johnson and Graham, 1976; Wergin and Endo, 1976). It extends between the stylet protractor muscle elements and expands between the somatic muscles to form four hypodermal chords: two lateral, one ventral and one dorsal. The lateral chords lie beneath the lateral fields and contain the secretory–excretory duct, longitudinal nerves, transverse nerves and many cell organelles, including mitochondria, ribosomes, Golgi bodies, smooth and rough endoplasmic reticulum, lipid globules and other organelles (Elsea; 1951; Dropkin and Acedo, 1974; Baldwin and Hirschmann, 1975; Johnson and Graham, 1976; Wergin and Endo, 1976). However, the interchordal hypodermis contains none of these organelles, having only nerve processes and numerous hemidesmosomes that serve as attachment points for the somatic muscles to the cuticle (Baldwin and Hirschmann, 1975; Bird, 1979a). The interchordal hypodermis is of varying thickness in the female and approximately 100 nm thick in the J2 and male (Elsea, 1951; Baldwin and Hirschmann, 1975; Bird, 1979a).

2.2.3 Somatic muscles

Somatic muscles are necessary for movement. In the J2, somatic muscles allow the nematode to move inside the egg and thereby facilitate hatch. They also enable it to move through the soil to find a suitable host (Bird, 1967). These muscles help the infective juvenile to penetrate the root and to move to a suitable site for establishment of the host–parasite relationship (Bird, 1967, 1971a). Likewise, somatic muscles in the males allow them to move out of the root tissues where they developed, and to move into the soil in the pursuit of an available mate. In the female, the somatic muscles occur only in the head region, where they allow the nematode to move her head in order to feed on one of several available giant cells (Elsea, 1951; Bird, 1979a,b). The rest of the body lacks these muscles as the female has abandoned her mobile lifestyle in exchange for a protected habitat inside the root system and an enlarged body that significantly increases her reproductive capacity.

Somatic muscle cells are spindle-shaped and grouped into four rows between the hypodermal chords in cross-section. In the J2 and male, longitudinally orientated muscles increase in number as they progress posteriorly down the body wall. There are two interchordal cells in the anterior end, three to four at the base of the stylet, four to five in the pharyngeal region and four to five for the remainder of the body wall (Baldwin and Hirschmann, 1975). The J2 has between 50 and 100 somatic muscle cells, whereas the male has more. In the J2 the somatic muscles end near the level of the anus, whereas they end near the level of the cloacal opening or at the base of the metacorpus in the male and female, respectively.

The contractile portion of a somatic muscle lies adjacent to the hypodermis and cuticle, whereas the non-contractile portion protrudes into the pseudocoelom. In cross-section, the contractile portion is obliquely striated with five to six cycles of I, A, H, A and I bands; however, the tips of the cells contain only fine filaments. Fine filaments only occur in the I bands; A bands contain thick filaments surrounded by a hexagonal pattern of 10–15 fine filaments, while H bands contain only thick filaments. The thin filaments of actin are 6 nm in diameter and the thick filaments of myosin are 22–24 nm in diameter. The muscles contract when the actin filaments slide past the myosin filaments, using the energy that is provided by the high-energy phosphate bonds of myosin.

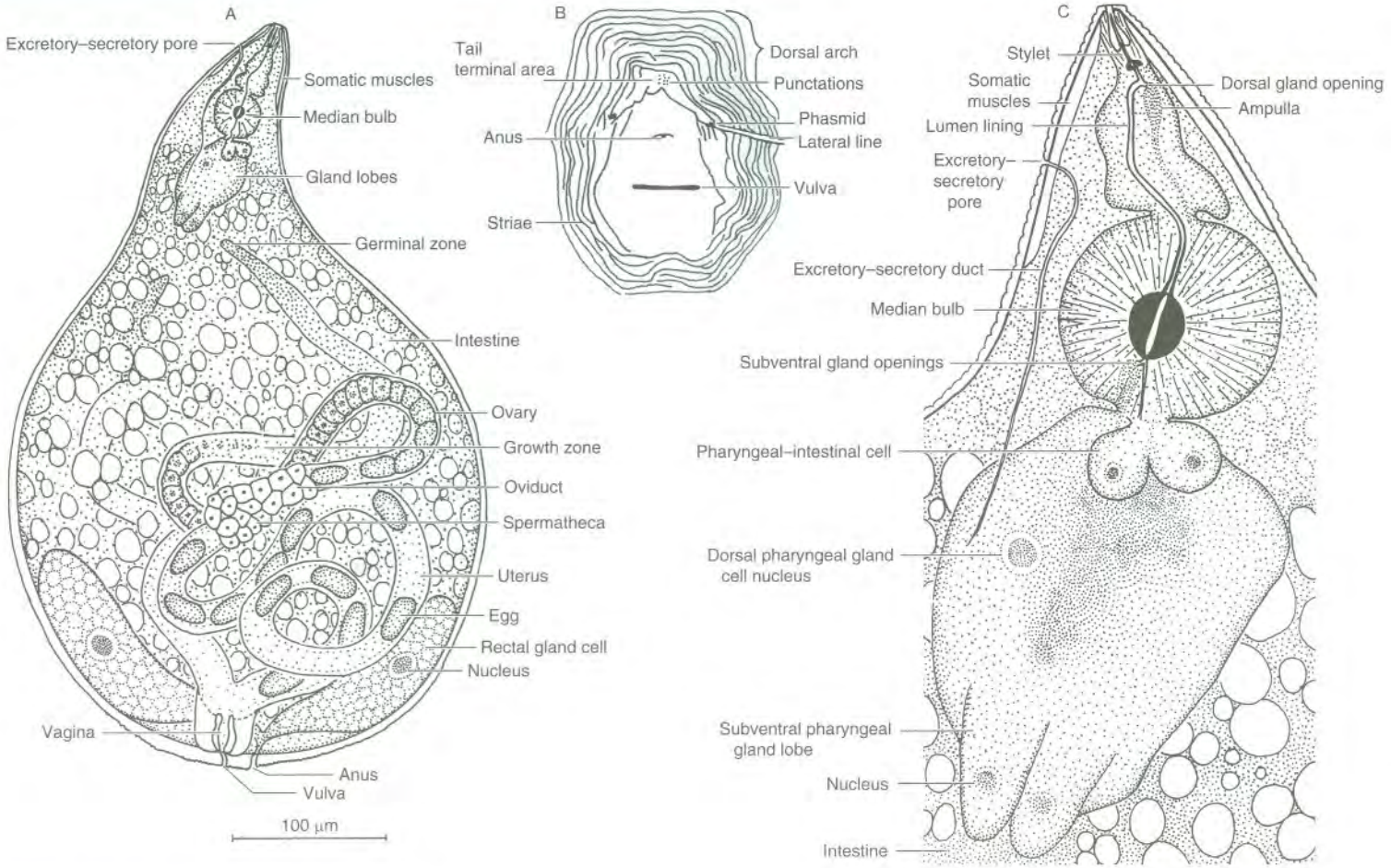

Fig. 2.18. Drawings of female root-knot nematode. A: entire nematode; B: typical perineal pattern; C: detailed view of anterior end. After Eisenback (1985a), courtesy of N.C. State University Graphics.

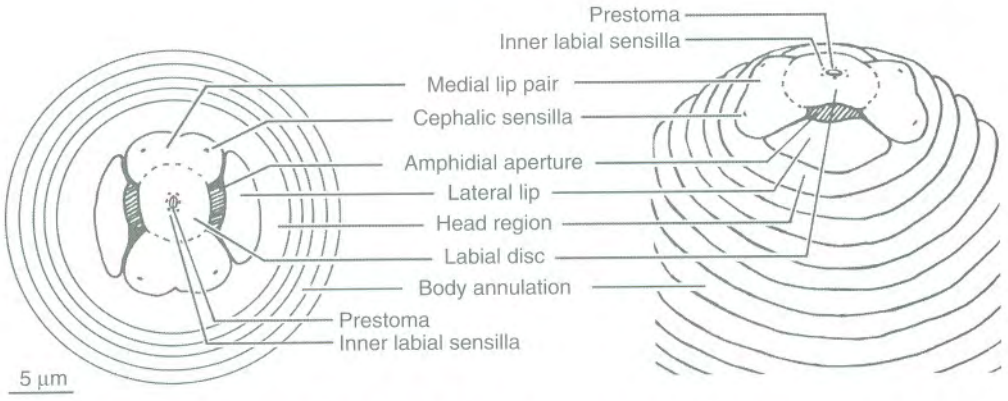

Fig. 2.19. Drawings of anterior end of female root-knot nematode in face and lateral views, as revealed by scanning electron microscope. After Eisenback *et al.* (1980), courtesy of *Journal of Nematology*.

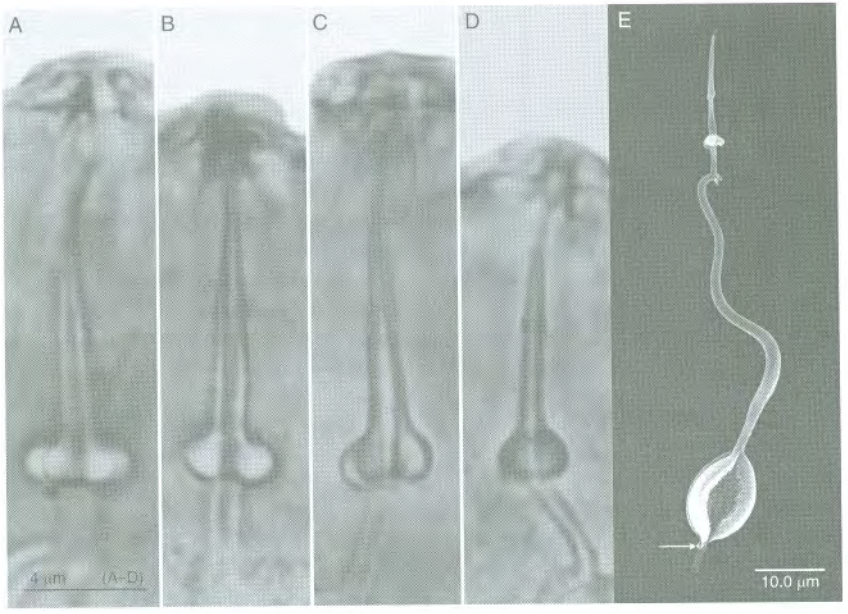

Fig. 2.20. Light micrographs of female stylet. A: *Meloidogyne incognita*; B: *M. javanica*; C: *M. arenaria*; D: *M. hapla*. E: scanning electron micrograph of stylet and cuticular lumen lining of entire pharynx (arrow marks the openings of the subventral pharyngeal glands). After Eisenback *et al.* (1980), courtesy of *Journal of Nematology*.

The non-contractile portion of the muscle cell is innervated by a muscle process that taps into one of the major bundles of longitudinal nerves (Baldwin and Hirschmann, 1973). Muscles in the two subventral quadrants are innervated by the ventral nerve, whereas those in the two subdorsal quadrants are innervated by the dorsal nerve chord. The cell nucleus, numerous mitochondria, lipid globules, glycogen and smooth endoplasmic reticulum occur in the non-contractile portion of the muscle, distinctly separating it from the contractile portion (Bird, 1971b; Baldwin and Hirschmann, 1975).

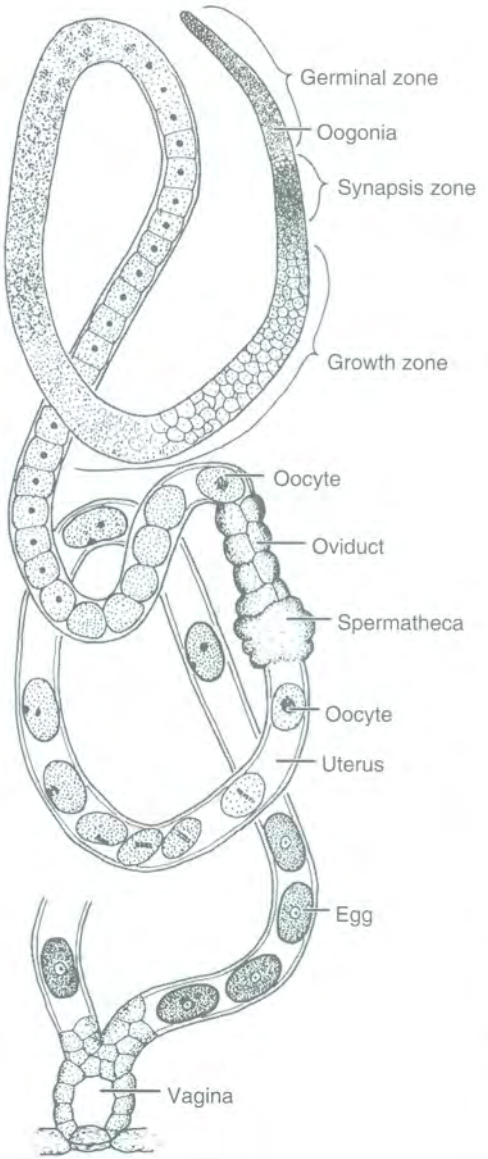

Fig. 2.21. Drawing of one of the two ovaries of a female root-knot nematode. After Triantaphyllou (1962), courtesy of *Nematologica*.

2.3 Nervous System

The nervous system provides the precise motor control that allows the nematode to move around in its environment and the sensory organs that enable the individual to respond to environmental cues. The J2 uses this system to move about inside the egg, to hatch from the egg and move through the soil, to sense the presence of a suitable host root, and to penetrate that host and migrate to the correct position for the development of the host–parasite relationship. In the male, the nervous system is used when migrating out of the root system and moving through the soil to find a suitable mate, while in the female it is utilized to move the head end from one giant cell to another both by coordinating movement of the head and by sensing the correct site for feeding to occur.

Little information is known about the nervous system of root-knot nematodes, the majority of observations relating to the anterior end of the J2 and male. Although the tail of the J2 has been studied, the nervous system in the male tail has not been investigated. Likewise, detailed studies of the female nervous system are not available.

The nerve ring, hemizonid, amphids and phasmids may be visible with the light microscope. The primitive coordinating centre of the nervous system, known as the nerve ring, occurs just posterior to the median bulb. It is formed from numerous nerve processes with their nucleated cell bodies lying anteriorly and posteriorly to the ring itself (Bird, 1971b). The hemizonid is probably a major lateroventral commissure of the nervous system and appears in lateral view as a transparent semicircle on the ventral surface of the body, usually near the secretory–excretory pore in the J2 (Fig. 2.2A) and male (Fig. 2.10).

Six longitudinal nerves extend from the nerve ring anteriorly and innervate the cephalic sensory structures, including the labial and cephalic sensilla, as well as the paired amphids. At least four nerves run posteriorly from the nerve ring, where they turn away from the pharynx and become embedded in the hypodermal chords: two lateral, one dorsal and one ventral. Transverse nerves interconnect all four of the longitudinal nerves. The somatic muscles in the subventral quadrants are innervated by the ventral chord and those in the subdorsal quadrants are innervated by the dorsal chord. The lateral nerves are responsible for innervating the sensory structures in the tail, including the phasmids and spicules in the male, and the phasmids and caudal sensory organ in the J2 (Wergin and Endo, 1976; Endo and Wergin, 1977).

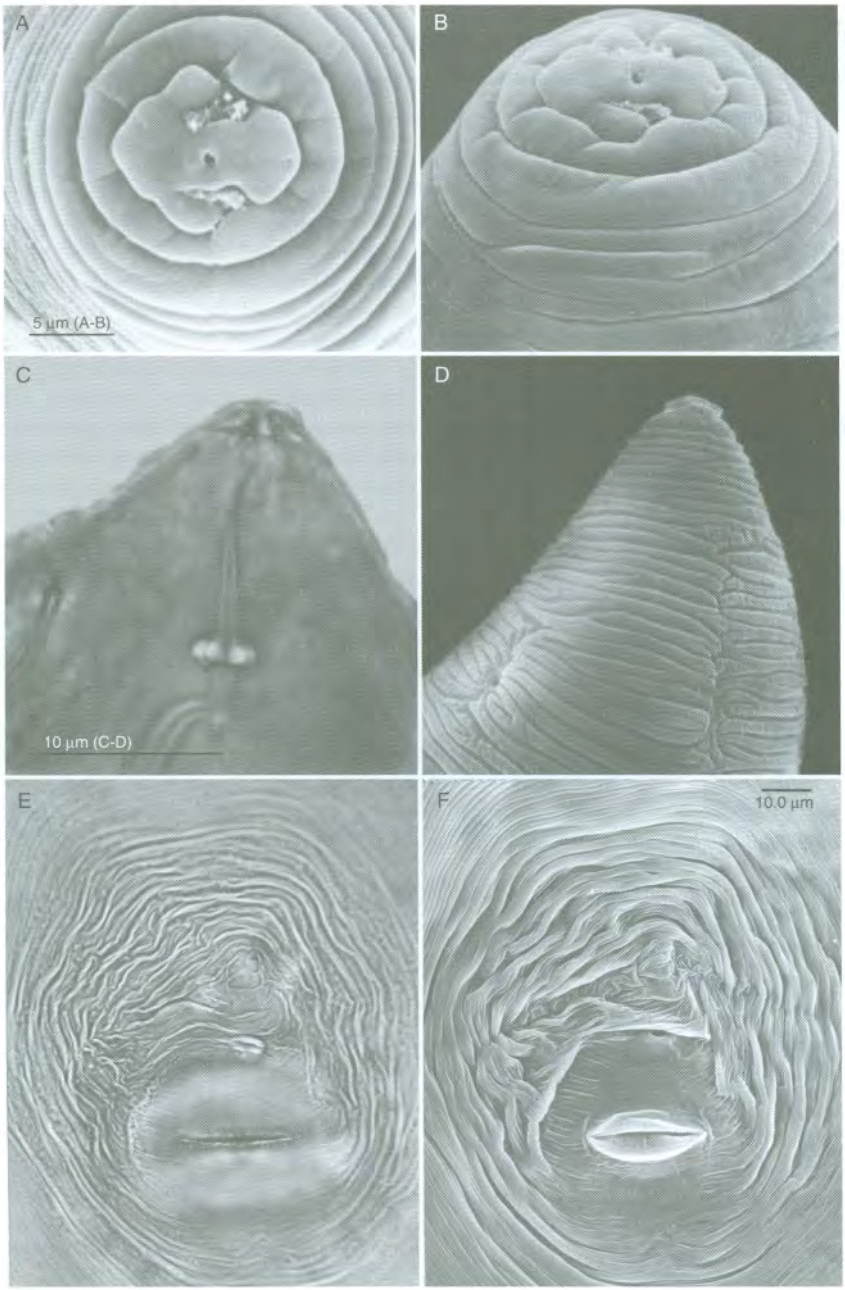

Fig. 2.22. Photomicrographs of a female root-knot nematode. A: scanning electron micrograph (SEM) of anterior end in face view; B: SEM of anterior end in lateral view; C: light micrograph (LM) of anterior end, showing stylet and secretory–excretory duct; D: SEM of anterior end, showing body annulations and secretory/excretory pore; E: LM of perineal pattern; F: SEM of perineal pattern. After Eisenback *et al.* (1980), courtesy of *Journal of Nematology*.

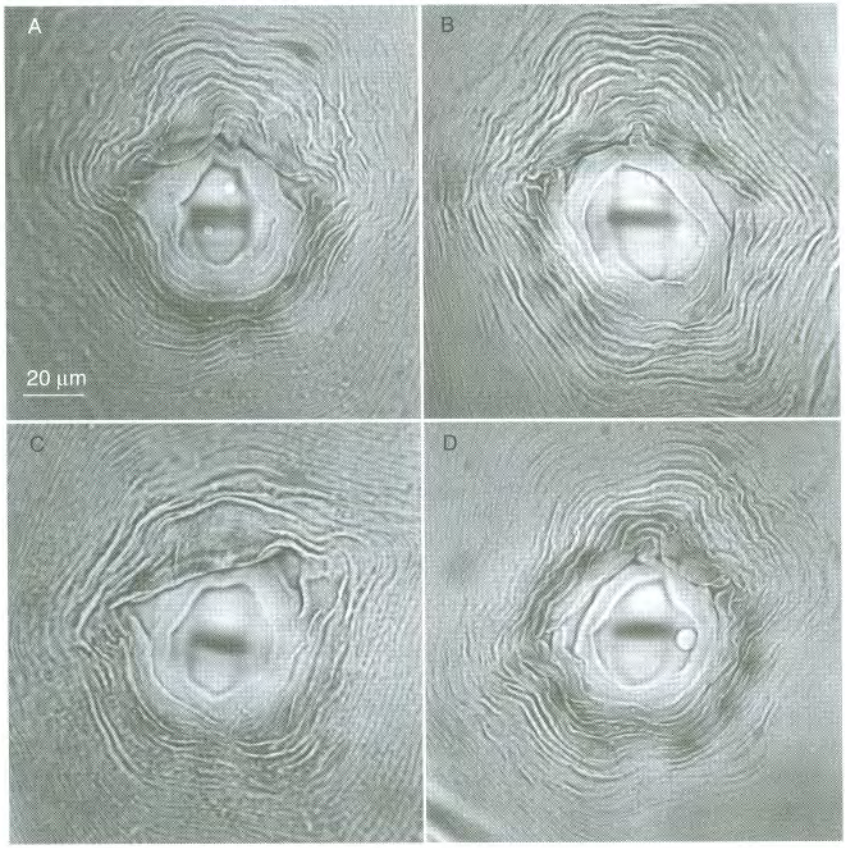

Fig. 2.23. Light micrographs of perineal patterns of female *Meloidogyne pini*. After Eisenback *et al.* (1985), courtesy of *Journal of Nematology*.

2.3.1 Cephalic sensory structures

Sensory structures in the anterior end of the J2, male and female are innervated by nerves extending anteriorly from the nerve ring. These structures include inner labial sensilla, cephalic sensilla, accessory sensory structures and the amphids (Figs 2.3, 2.12, 2.19). In all three life stages, the six inner labial sensilla open around the ovoid prestoma, where they function as chemoreceptors and probably assist during processes such as stylet insertion, feeding and stylet withdrawal. The pore-like openings of the inner labial sensilla are lined with cuticle secreted by the surrounding hypodermal tissue. Each pore contains the endings of two receptor cilia of different lengths, which probably function as chemoreceptors. The four cephalic sensilla are sometimes visible as slight depressions in the cuticle on the medial lip pairs. They are structurally similar to the inner labial sensilla but have just one ciliary receptor. Because they do not open to the external environment, these sensilla probably function as mechanoreceptors.

In the J2, several sensilla are contained in the anterior region of the nematode and may be analogous to the outer labial sensilla (Wergin and Endo, 1976; Endo and Wergin, 1977). These cilia end beneath the cuticle, are not surrounded by cuticle and may function as tactoreceptors. These accessory sensilla occur in the two subdorsal and two subventral sectors of the head region, and are associated with the inner labial sensilla and amphidial canals. Similar sensory structures are present in both male and female, but details of their morphology have not been presented.

Amphidial openings occur on the J2, male and female, and are located between the labial

disc and lateral lips. With SEM, these openings are often observed to be plugged by an exudate secreted by the amphidial gland (Figs 2.4A, B, 2.5A–C, 2.6). The amphids are made up of specialized support cells, nerve cells and secretory gland cells (Fig. 2.11B). The amphidial canals are lined with cuticle and surrounded by a support cell (the socket cell), which extends from the amphidial gland cell to the anterior portion of the canal. Seven modified cilia innervate the amphid and lie in the canal, ending near the aperture. The large, irregularly shaped amphidial gland cell (sheath cell) contains numerous mitochondria, microvilli, and intercellular spaces filled with ducts and granules (Wergin and Endo, 1976; Endo and Wergin, 1977). The amphids probably function as chemoreceptors and are essential for the nematode to interface with its environment.

2.3.2 Caudal sensory structures

In the J2, the phasmids are located between the anal opening and the tail tip, whereas in the male they occur near the level of the cloacal opening and in the female are located anterior to the tail terminus and within the perineal pattern. As revealed by TEM studies of the J2, the phasmids are characterized by a cuticle-lined duct, a nerve process and a gland cell (Bird, 1979c). Nerve tissue fills the hypodermis that surrounds the phasmidial gland cell. A nerve process extends from this nerve tissue into the basal layer of the cuticle and a single cilium innervates the phasmidial duct. A gland cell secretes an exudate into the phasmidial canal. Details of the phasmid structure of the male and female stages are probably similar to that of the J2, but they have not been studied in detail.

A caudal sensory organ (Fig. 2.2B) has been described occurring in the J2 tail directly anterior to the hyaline tail terminus (Bird, 1979c). It is 5–10 µm long and is made up of numerous branched nerve processes that originate from the median caudal nerve. This sensory structure is probably a tactoreceptor since it is completely contained within the hypodermal tissues.

In the male, the spicules are innervated by nerves contained within each hollow, cuticularized, organ (Eisenback and Hirschmann, 1980). These nerves open via two small pores at the tip of each spicule. Additional details of these nerves are lacking, but they clearly play a role in finding a mate and/or in copulation. They open to the environment, so these sensory structures probably function as chemoreceptors.

2.4 Digestive System

The digestive system is responsible for obtaining nutrients from a food source in order to support the life of the nematode, including normal metabolic activities, growth and development, movement and fuelling the reproductive system. In the J2 the digestive system is necessary to help the nematode hatch from the egg, penetrate a host root and establish and maintain a host–parasite relationship (Bird, 1967, 1968a,b, 1969; Bird and Saurer, 1967). All of the energy necessary for the three additional moults and development into an adult is obtained by the J2. Additional feeding occurs in the mature female to aid growth of the reproductive system, but the vermiform male does not feed at all (Triantaphyllou and Hirschmann, 1960).

The digestive system is composed of a stoma, pharynx, intestine and rectum (Figs 2.2, 2.10). The stoma is armed with a hollow, protrusible, hypodermic-needle-like stylet that serves as an interface between the nematode and the plant. The pharynx contains three specialized gland cells responsible for several functions in the host–parasite relationship, and a metacorpus that pumps substances from the gland cells into the plant and from the plant into the intestine. Because the food taken in by the nematode (at the expense of the plant) is so highly refined, digestion and absorption are unnecessary. The intestine serves simply as a storage organ and, in the female, is not even connected to the anal opening. Instead, six large rectal gland cells open through this orifice, where they secrete a voluminous gelatinous matrix that serves to protect the eggs as they are deposited to form the egg sac.

2.4.1 Stoma and pharynx

The ovoid prestoma is located on the labial disc where the slit-like stomatal opening guides the stylet as it is protruded into plant tissues. The

hypodermic-needle-like stylet lies within a cuticle-lined stomatal cavity surrounded by the hexaradiate cephalic framework (Baldwin and Hirschmann, 1973; Wergin and Endo, 1976; Endo and Wergin, 1977). This framework includes a basal ring, six blades and the vestibule containing the anterior end of the stylet (Fig. 2.11A). The vestibule continues posteriorly as the vestibule extension and connects to the stylet at the junction of the cone and shaft. The external layer of cuticle forming the body wall is continuous with the vestibule and its extension. The blades of the cephalic framework comprise one dorsal, one ventral, two subdorsal and two subventral blades. The lateral sectors are slightly larger than the others. These blades add structural support to the anterior end of the nematode, where they extend from the vestibule to the body wall. The basal ring of the cephalic framework is continuous with the basal layer of cuticle that makes up the body wall (Baldwin and Hirschmann, 1973; Wergin and Endo, 1976; Endo and Wergin, 1977).

Three groups of stylet protractor muscles attach to the body wall and cephalic framework anteriorly and to the stylet knobs posteriorly (Fig. 2.11A). The vestibule extension holds the stylet in place where it attaches at the junction of the shaft and cone. The stylet opens on the ventral side of the cone near the tip in the J2 and female, and approximately 2–3 μm from the tip in the male, at a point that is often marked by a slight protuberance (Fig. 2.16B,F,J) (Eisenback, 1982). The stylet is made up of three distinct parts: cone, shaft and three knobs, one dorsal and two subventral (Figs 2.4C–E, 2.5D–G, 2.13A,B, 2.15B, 2.16, 2.20). The cone is more stable and less soluble than the shaft and knobs, as attested by the fact that these two latter structures often disappear quite rapidly in permanently prepared specimens (Baldwin and Hirschmann, 1973; Wergin and Endo, 1976; Endo and Wergin, 1977).

The stylet protractor muscles force the anterior end of the stylet out through the stoma and into the cell wall of the host plant, where it is used to inject saliva into the cell. This saliva modifies the cell by changing it into a metabolic sink for the nematode, from which the nematode receives nutrients and energy (see Abad *et al.*, Chapter 7, this volume). The vestibule folds backward, and the flexible hypodermal tissues surrounding the stylet allow it to move back and forth. Since stylet retractor muscles are lacking, these same tissues enable the stylet to return to its normal position when the stylet protractor muscles relax, the process being facilitated by the release of tension in the anterior extended portion of the alimentary tract (Baldwin and Hirschmann, 1973; Wergin and Endo, 1976; Endo and Wergin, 1977).

The non-contractile portion of the stylet protractor muscles is contained within the anterior end of the procorpus (Baldwin and Hirschmann, 1973; Wergin and Endo, 1976; Endo and Wergin, 1977). Except in the metacorpus, the lumen lining of the pharynx is circular in cross-section. A short distance from the base of the stylet, at approximately 2–8 μm, the opening of the dorsal pharyngeal gland branches into several channels. The enlarged, muscular metacorpus serves as a pumping mechanism. Along with two strategically placed sphincter muscles, the metacorpus can pump substances either out through the stylet or back into the nematode. The lumen lining of the metacorpus is triradiate in cross-section and heavily sclerotized. The outer edges of this lumen lining are not very flexible but the inner sections are thin and flexible. When the muscles of the metacorpus contract, they pull the thin inner section apart and produce a very strong pumping action. The branched openings of the subventral pharyngeal glands are located in the metacorpus just posterior to the triradiate lumen lining (Baldwin *et al.*, 1977; Eisenback *et al.*, 1980; Eisenback and Hirschmann, 1981; Eisenback, 1982).

In the male, the pharynx is somewhat degenerate and probably not functional. The triradiate lumen lining of the metacorpus is thin and the muscles are poorly developed, sparse in number and disorganized. The lumen ends as a series of folded membranes that are surrounded by two to three pharyngeal–intestinal cells. Three digestive glands overlap the intestine ventrally. The dorsal gland cell is uninucleate and lies anterior to the two subventral gland cells. The dorsal gland cells form an ampulla at the base of the stylet, and the subventral gland cells form an ampulla at the base of the metacorpus (Baldwin *et al.*, 1977).

The three pharyngeal gland cells overlap each other and wrap around the intestine in the J2 (Fig. 2.2A). In the female, these gland cells form one large dorsal lobe and two smaller subventral lobes that are quite variable in morphology (Fig. 2.18B), but in the male they are often reduced

and degenerate. Two pharyngo-intestinal cells are present at the junction of the pharynx and intestine (Garcia-Martinez, 1982). They are small, rounded and uninucleate. In the male, however, these two cells are completely contained within the isthmus (Baldwin *et al.*, 1977).

2.4.2 Intestine

Few details of the intestine of root-knot nematodes have been resolved because its morphology is not very distinct. Numerous lipid globules fill the intestine of the J2 (Fig. 2.2), male (Fig. 2.10) and female (Fig. 2.18A). In the J2, a clearly defined lumen with microvilli is absent, but a partially defined lumen may be present in both male and female (Elsea, 1951; Bird, 1979a,b). In the male, an intestinal caecum commonly extends from the base of the isthmus to the base of the metacorpus (Baldwin *et al.*, 1977). In both male and female the intestine is syncytial. In the female, the connection to the rectum has been lost and the intestine functions primarily as a storage organ.

2.4.3 Rectum

The J2 anal opening is a small, round pore, usually contained within one body annule (Fig. 2.7D) (Eisenback and Hirschmann, 1979b). The lumen of the rectum is lined with cuticle that becomes thinner anteriorly until it merges with the plasma membrane. The diameter of the tail is nearly 8 µm, whereas the rectum is 6 µm in diameter (Bird, 1979c). The rectum, in its dilated state, contains a matrix resembling that extruded from the adult female rectal gland cells (Bird, 1979c).

In the J2 and female, the rectal lumen is surrounded by rectal gland cells. The intestine in the female is not connected to the rectum, and the same situation may be true for the J2. As soon as the J2 begins to feed, the rectal gland cells enlarge and become visible as six distinct cells (Fig. 2.18A).

In the male, the intestine and testis share a common duct, the cloaca, which opens about 6–8 µm from the tail tip. Additional details about the morphology of the male tail are lacking.

The anal opening of the female is located between the tail tip and the vulva. As the female matures, the rectal lumen decreases in size. Two subdorsal, two subventral and two lateral rectal glands are connected to the rectum. The uterus separates the two subventral glands, but in general the six glands are equally spaced around the posterior end of the body, where they are closely adpressed to the hypodermis. The rectal gland cells increase in size until they are nearly contiguous (Bird, 1968a,b, 1979b).

The rectal gland cells (Fig. 2.18A) are very large, approximately 100 µm long by 25 µm in diameter, and contain a very large nucleus, approximately 25 µm long by 15 µm in diameter (Maggenti and Allen, 1960; Bird and Rogers, 1965b). These are the largest nuclei in the female. The rectal gland cells contain dense cytoplasm filled with many Golgi bodies, mitochondria, anastomosing endoplasmic reticulum with many ribosomes, and multivesicular lamellae with a repeat periodicity of 75 Å. These cells with giant nuclei and numerous cell organelles are vital for the secretion of the gelatinous matrix. Visible sinus canals are present in each gland cell where they merge to form a single long duct that opens into the anterior end of the rectal lumen. The gelatinous matrix is secreted via the anal opening, which is opened by the anal depressor muscles attached to the rectum and body wall.

When the adult female is mature, copious quantities of gelatinous matrix are secreted, the combined volume of which may be larger than the female herself. The matrix contains proteins, a mucopolysaccharide and various enzymes (see Evans and Perry, Chapter 9, this volume). It forms an irregular, mesh-like structure that physically prevents the J2 from hatching during extreme periods of drought (Maggenti and Allen, 1960; Bird and Rogers, 1965b). It also contains antimicrobial compounds that protect the eggs from attack by soil-borne organisms. The numerous resources that the female uses to produce the gelatinous matrix demonstrate the value that it plays in the long-term survival of this highly evolved sedentary parasite (Bird, 1971a; Bird and Soeffky, 1972; Dropkin and Bird, 1978).

2.5 Secretory–Excretory System

The secretory–excretory system is marked on the body by a small, rounded opening located ventrally in the cuticle where the body annulations

are interrupted or slightly deviated. In the J2 (Fig. 2.7C) and male, the pore is located near the metacorpus and 2–8 µm from the hemizonid. However, in the female it is located anterior to the median bulb, often near the stylet base (Fig. 2.18A). The pore is approximately 0.2 µm in diameter in the J2 and 0.6 µm in the female. The duct of the secretory–excretory system extends posteriorly through the hypodermis and becomes a canal that is not lined with cuticle. In the female, the anterior end of this duct is surrounded by numerous vesicles (Bird, 1979b). In the J2, male and female stages, the posterior end of the secretory–excretory canal is closely related to a large sinus gland cell. Although the function of this system is not completely understood, it probably has an excretory function for removing toxic wastes and perhaps a secretory function as well (Bird, 1971b, 1979b).

2.6 Reproductive System

The reproductive system is closely associated with the digestive system, from which it receives all of its nutritional requirements. This system ensures that new individuals will be produced in the struggle for survival of the species. The genital primordium is very small in the pre-parasitic juveniles, but rapidly increases in size as soon as feeding commences (Fig. 2.8) (Papadopoulou and Triantaphyllou, 1982). In the pyriform female, it develops into two very long and convoluted ovaries (Figs 2.18A, 2.21). The shape of the female (Fig. 2.17) allows for this increase in the length of the ovaries, a feature that greatly enhances reproductive capacity. However, such a huge increase in fecundity comes at the expense of mobility. The male, in contrast, remains vermiform and mobile, although the reproductive system is of normal size (Elsea, 1951).

2.6.1 Second-stage juvenile

The genital primordium of the pre-parasitic J2 is formed from four cells, i.e. two small, flattened somatic cells surrounding two large, spherical germinal cells. The primordium lies parallel with the body wall at about 65% of the body length from the anterior end. As soon as feeding begins, it starts to increase in size and develops into either the ovaries or a testis (Fig. 2.8) (Papadopoulou and Triantaphyllou, 1982).

2.6.2 Male

Usually the male has just one testis, but when the environment affects sex expression some individuals may develop two testes. This change in sex, which is caused by various environmental effects, is called sex reversal (Fig. 2.8) (Triantaphyllou, 1979). It is thought to be advantageous to the survival of these obligate parasites because the mechanism reduces the population-induced stresses on the host plant. Males require less energy to produce; they do not feed as adults; they do not add progeny to the burden of the host; and, when present, they may increase genetic variation in the population as a result of sexual reproduction (Triantaphyllou, 1979; Papadopoulou and Triantaphyllou, 1982).

The male gonad is formed from the testis proper (which constitutes approximately one half of the length of the entire tube) and the vas deferens. A cap cell at the distal end of the testis is present in the undifferentiated genital primordium in the J2 (Triantaphyllou, 1979). All of the spermatogonia are derived from this cell in the germinal zone of the testis. The spermatogonia increase in size in the remaining growth zone of the testis proper. Following the growth zone, the glandular vas deferens empties ventrally into the cloaca. The entire gonad is covered by a single layer of epithelial cells (Shepherd and Clark, 1983).

Paired, cuticularized spicules are located within a pouch in the cloaca (Figs 2.10, 2.15C). As these structures are protruded through the cloacal opening they form a tube that facilitates an efficient transfer of sperm into the female vagina. The spicules have a head and shaft that consists of a hollow cytoplasmic core surrounding one or more nerves that open to the exterior via two small pores at the tip of each spicule. A gubernaculum is located dorsally to the spicules and serves to guide them out through the cloacal opening during protrusion and retraction. Spicule protractor and retractor muscles are attached to the spicule head and body wall.

Spermatozoa often pack the vas deferens. They are approximately 6 µm in diameter and 12 µm long. Spermatozoa are divided into two parts: one part contains the nucleus and is surrounded by mitochondria and fibrillar bodies; the other contains most of the cytoplasm, which is used to form several large pseudopodia necessary for crawling through the uterus to the spermatheca. Numerous filopodia are sometimes formed in the region of the spermatozoon that contains the nucleus (Goldstein and Triantaphyllou, 1980; Shepherd and Clark, 1983).

2.6.3 Female

The saccate female body (Fig. 2.17) is filled with two highly convoluted gonads that are in very close proximity to the digestive system, particularly to the nutrient-laden intestine. Approximately 60% of the female gonad is made up of the ovaries proper (Fig. 2.21). The anterior end of each ovary contains a cap cell, which initiates the germinal zone of the organ and produces all of the oogonia. The oogonia are arranged radially around a rachis, to which they are attached by a cytoplasmic bridge. The growth zone of the ovary follows as a region where the oogonia increase in size. The boundaries between the oocytes become more distinct in this zone and the rachis gradually disappears. The oocytes accumulate glycogen, refringent bodies and lipid globules as they pass in single file through the growth zone into the oviduct (Elsea, 1951; Triantaphyllou and Hirschmann, 1960; Triantaphyllou, 1962, 1979; McClure and Bird, 1976).

Each of the two staggered rows of cells in the oviduct (Fig. 2.21) contains four tightly packed cells with large cytoplasmic invaginations and large, irregularly shaped nuclei. The oocytes stretch and flatten these cells as they pass through the narrow valve in the lumen of the oviduct, although they become ovoid afterwards (Elsea, 1951; Triantaphyllou and Hirschmann, 1960; Triantaphyllou, 1962; 1979; McClure and Bird, 1976).

Immediately posterior to the oviduct, the spermatheca (Fig. 2.21) consists of 14–20 rounded, lobe-like cells that have a deeply invaginated margin of plasmalemma. Densely vesiculate bodies located between the lobes of the spermathecal wall have numerous microtubules that project into the lumen and assist in the formation of the protein membrane of the eggshell (McClure and Bird, 1976).

The uterus (Fig. 2.21) occurs posterior to the spermatheca and can be divided into distinct regions: (i) cells at the ovarial end, possessing large intracytoplasmic spaces lined with endoplasmic reticulum and producing the chitin layer; (ii) cells in the middle of the uterus, forming the glycolipid layer; and (iii) cells in the posterior end, consisting of dense cytoplasm with large areas of compact endoplasmic reticulum (Elsea, 1951; Triantaphyllou and Hirschmann, 1960; Triantaphyllou, 1962; 1979; McClure and Bird, 1976).

Near the posterior end of the gonad the two uteri join together to form a common duct (Fig. 2.21). Numerous muscles are attached to the vagina and radiate outward to attach to the body wall. These muscles contract to dilate the vagina during egg laying. The vagina itself is lined with thick cuticle. The vulva is located transversely on the posterior end of the body wall and is surrounded by two, slightly elevated vulval lips (Elsea, 1951).

2.7 Morphological Methods

Preparation of nematodes for SEM and TEM has been reviewed previously by Wergin (1981), Eisenback (1985c, 1991) and Carter (1991). Making perineal patterns for species identification has been outlined by Hartman and Sasser (1985), and additional methods for collecting and preparing nematodes for optical microscopy have been evaluated by Fortuner (1991).

2.8 Minimum Standards for Describing a New Species

With the increasing number of described species in the genus *Meloidogyne*, it is important that species descriptions conform to a general standard in order to facilitate accurate comparisons and differential diagnoses. Because of the relatively conserved morphology in the group, there is an increasing emphasis on techniques such as isozyme phenotyping and molecular sequences. No

new species description should be published without at least one of these techniques, a consideration that may require collaboration with workers from other laboratories where specialist methodologies such as molecular characterization are more readily available. New descriptions and other taxonomic work should be submitted to a journal of suitable standing in order to ensure appropriate peer scrutiny – there is little to be gained scientifically by 'hiding' such papers in either obscure journals or those that do not regularly publish manuscripts of a taxonomic nature.

Goodey (1959) provided an excellent 'master-class' on the data to be presented when describing new species. Although not specific to root-knot nematodes, his paper is still recommended reading for anyone embarking on such a task. Authors should also be careful not to cite a new species name in a paper that may be published before the 'official' description – a truly embarrassing and unnecessary calamity, culminating in the creation of a *nomen nudum*.

The description format presented here is a blend of traditional and modern methodologies. The intention is to provide an up-to-date guide to achieve a robust standard of description – and one that should be aimed at by all authors. Journal editors should also find the protocol useful when deciding whether descriptions submitted for publication are adequately supported by appropriate information.

2.8.1 The text

The account below is based, purely as an example, on the style of *Nematology*. It may easily be adapted to that required by alternative journals.

***Meloidogyne ???* n. sp.**

(Figs ?–?)

2.8.1.1 Measurements

See Table 2.1 for a list of characters that should be measured. At least 20 specimens each of the J2, male and mature female stages should be measured. State whether the material is fresh or has been fixed and processed to glycerine when

Table 2.1. Morphometrics of *Meloidogyne* ??? n. sp. All measurements in µm and in the form: mean ± standard deviation (range).

	Female		Male	J2
Character/stage	Holotype	Paratypes	Paratypes	Paratypes
n	–	20	20	20
L	√	√	√	√
a	√	√	√	√
c	–	–	√	√
T	–	–	√	–
Max. body diameter	√	√	√	√
Neck length	√	√	–	–
Stylet length	√	√	√	√
Stylet knob height	√	√	√	√
Stylet knob width	√	√	√	√
DGO	√	√	√	√
Excretory pore to anterior end	√	√	√	√
Interphasmidial distance	–	√	–	–
Vulva length	–	√	–	–
Vulva–anus distance	–	√	–	–
Tail length	–	–	√	√
Spicule length (median line)	–	–	√	–
Gubernaculum length	–	–	√	–
Testis length	–	–	√	–
Hyaline tail terminus (h)	–	–	–	√
h% (h/tail length × 100)	–	–	–	√

measured. Cite data as: mean ± standard deviation (range). The coefficient of variation may also be given. Measurements are usually expressed in µm, although body length of the male and mature female may be in mm. The majority of measurements are best rounded to the nearest micrometer. Spicules should be measured along the curved median line rather than the chord. When measuring the stylet take particular care that the conus tip is accurately determined (i.e. do not assume that the stylet ends at the anterior extremity of the labial region). Measurements should be made at an appropriate magnification. For smaller structures (e.g. stylet, spicules, J2 hyaline region, perineal pattern) this usually equates to using a ×100 oil immersion objective, although body length of mature females and males may be recorded using a ×10 objective. Ensure that the optical combination used during the measurement process has been properly calibrated. Be aware that adding components such as a drawing tube or NIC (Nomarski Interference Contrast) prism will alter the focal length of the system and hence the magnification factor, thereby necessitating recalibration. Measurements should ideally be taken using either a drawing tube or computer equipped with measurement software. For reasons of convenience and accuracy, the use of a calibrated eyepiece graticule is best restricted to acquiring the length of short, straight structures, such as the stylet.

2.8.1.2 Description

FEMALE. Body shape, presence or absence of terminal cone, form of head region, including annulation, general appearance of stylet and shape of knobs, DGO (dorsal gland orifice) position, position of excretory pore, perineal pattern (type of striae – smooth, wavy, etc., overall shape and proportion of parts dorsal and ventral to vulva, presence or absence of dorsal arch, presence of wings, lateral field development, other pertinent peculiarities, such as punctations, etc.).

MALE. Body form, head shape and form of annules, presence of labial disc, general appearance of stylet and shape of knobs, overlap of pharyngeal glands, spicule shape, lateral field development.

J2. Body form, head shape and form of annules, general appearance of stylet and shape of knobs, excretory pore position, hemizonid position in relation to excretory pore, overlap of pharyngeal glands, form of rectum (inflated or not), tail shape and form of tip, development of hyaline region.

EGG. Length and diameter and the ratio between the two are the main criteria.

2.8.1.3 Type host and locality

The original host of the species should be recorded, together with detailed data concerning the type locality. Altitude and/or GPS (global positioning system) coordinates should be cited if at all possible. If the type material stems from a culture on a host other than the type, then this should also be indicated and appropriate details supplied.

2.8.1.4 Type material

A mature female should be designated as the holotype, the other material examined by the author(s)being paratypes (note that the term 'allotype' is unregulated by the International Code of Zoological Nomenclature (ICZN) and has no meaning under the Code; it is best avoided). Type material should be processed to, and mounted in, glycerine as permanent mounts (J2, males, mature females and perineal patterns plus corresponding neck regions). Ensure that the cover slip is supported appropriately (glass rods or beads of slightly larger diameter than specimen, wax ring, etc.) to avoid squashing or pressing the nematodes. Labels must record all pertinent data (stage, paratype/holotype, host, locality, collection number, etc.). Deposition of fixed, but unmounted, material is also desirable, plus material preserved in a manner that enables subsequent molecular studies. Material should be deposited in at least two internationally recognized collections and preferably not restricted to author or laboratory collections. Ideally the number of paratypes, their life cycle stage and collection numbers designated by the depository should be recorded.

2.8.1.5 Diagnosis and relationships

The diagnostic characters of the proposed species should be listed stage by stage. This forms the *diagnosis* of the species and may be repeated in the *Summary* or *Abstract*. The relationships of the new species should then be established by means of

detailed comparison with similar species, using comparative data so that the former can be easily distinguished from its congeners. Try not to use expressions that may be ambiguous or subjective, e.g. avoid the use of unsupported phrases such as 'larger than' or 'smaller than' – much better to cite and compare the actual measurements, preferably by providing for each species the mean size and range of the structure in question so that the reader may additionally judge the utility of the comparison.

2.8.1.6 Etymology

The derivation of the specific epithet should be given so that is clear as to how the name was formed and what was the intention of the author (e.g. in case the name is wrongly formed and needs to be emended subsequently). Formation of names and endings must conform to the requirements of the Code. Note: if naming a species in honour of a man, the ending of the patronym must be *-i*; if after a woman, *-ae*.

2.8.1.7 Isozyme phenotype

Isozyme profiles such as esterase and malate dehydrogenase are necessary. A photograph of a good-quality gel with clear bands should be provided. A profile of a known species, such as *M. javanica* or *M. incognita*, should also be run and included next to that of the new species. The number of strong and weak bands should be recorded, together with the migration value (Rm).

2.8.1.8 Molecular characterization

Current molecular data include RAPD (random amplified polymorphic DNA) profiles and sequence data for the ITS (internal transcribed spacer) regions and D2/D3 (dopamine receptor) region. Sequences should be as complete as possible and must be deposited in GenBank or an equivalent open-access database. Accession number(s) must be published with the description. It is recognized that not all laboratories will have access to molecular techniques, but this lack should not prevent the retention of suitably preserved material that can be used for this purpose by other scientists, either as collaborators or subsequent to any published description. If molecular studies cannot be performed at the time of description it is advisable to preserve material to facilitate subsequent DNA analysis. Yoder *et al.* (2006) recommend placing live nematodes directly into DESS solution. Alternative methods include placing live nematodes into saturated NaCl solution, preferably stored at −20 °C, or placing several J2 into a small tube with minimal water and allowing the worms to desiccate before sealing and storing at −20 °C (S. Subbotin, California, 2008, personal communication).

2.8.1.9 Cytogenetics and karyology

Method of reproduction (e.g. meiotic parthenogenesis) and chromosome number should be stated, if known.

2.8.1.10 Bionomics

Host range, differential hosts and gall form are all useful items to record.

2.8.1.11 Remarks

Any other relevant information supporting the description.

2.8.2 The figures

It is imperative that the description of any new species is supported by appropriate line drawings – light micrographs on their own are not sufficient. Line drawings should ideally be supplemented by good-quality light micrographs and scanning electron micrographs.

1. Line drawings: although there is an increasing trend towards molecular-based characterization, it is still vital that good-quality line drawings of the J2, male and mature female are provided. Important features must be drawn at an appropriate magnification to ensure clarity. The primary function of the line drawings is to convey information in as unambiguous a manner as possible. They need not be 'artistic' (some computer-generated toning can be a positive nuisance when reproduced, for example) but should clearly indicate the salient features of the new species and also cover major variation in, for example, perineal pattern and stylet form. A good example of a clearly drawn figure is shown in Fig. 2.24. One could quibble about the fact that only a single perineal pattern is drawn, but in general all the features are defily presented with

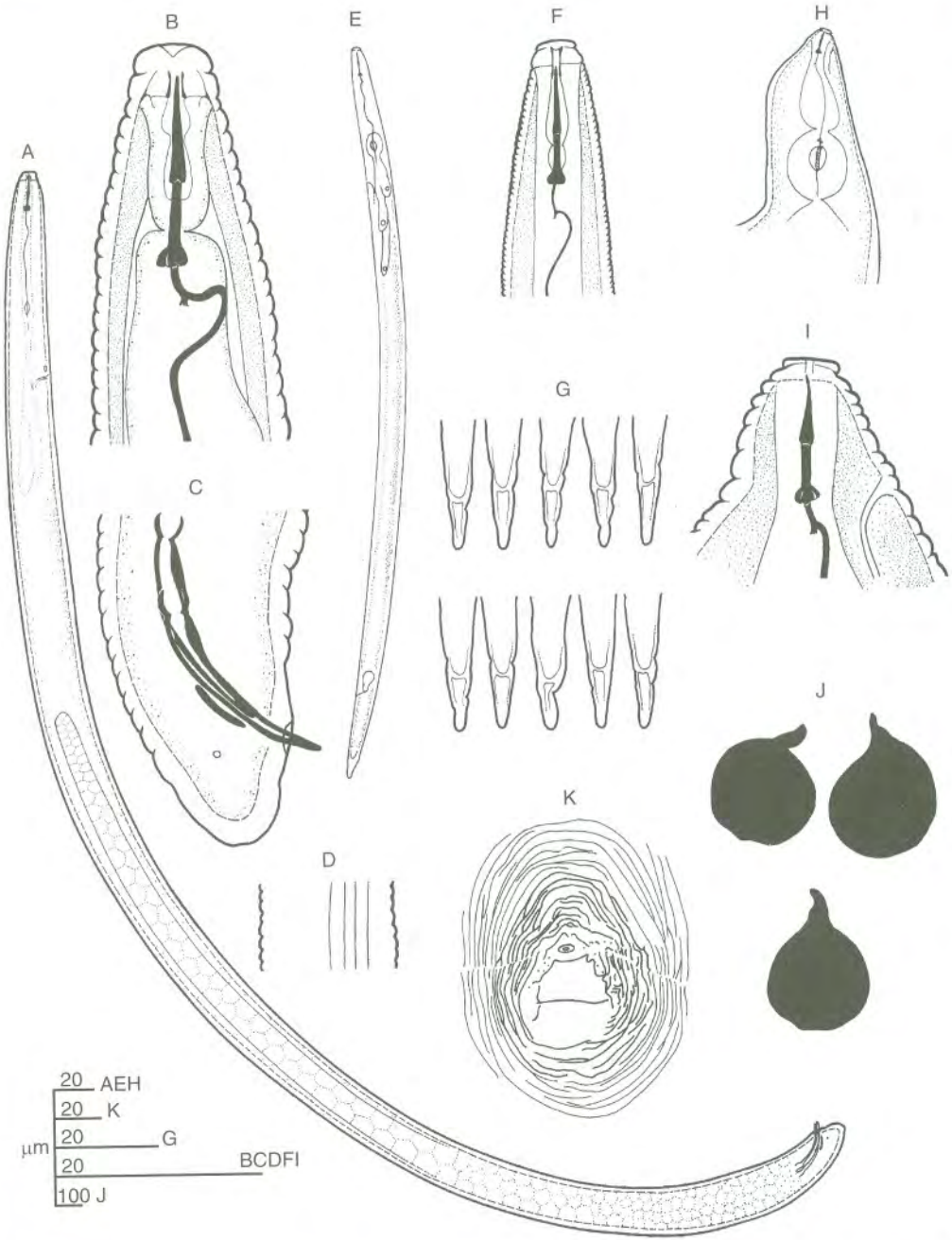

Fig. 2.24. Example of line drawings (of *Meloidogyne chitwoodi*) appropriate to illustrate the description of a new species. A: entire male; B: male anterior region; C: male tail region; D: male lateral field: E: entire J2; F: J2 anterior region; G: J2 tail regions; H, I: female anterior region, lateral view; J: entire females; K: perineal pattern.After Jepson (1985), courtesy of CAB International.

admirable simplicity. Relevant (and accurately calibrated) scales are, of course, an essential addition to any figure.

Suggested features to be reported upon are as follows, although others must be depicted if deemed by the author(s) to be diagnostic:

- J2: anterior region detail, stylet form and basal knob shape, pharyngeal region, including gland overlap, tail region and hyaline area.
- Male: anterior region detail, including that of the annules forming the labial region, stylet form and basal knob shape, pharyngeal region, including gland overlap, tail region, shape of spicules and gubernaculum, lateral field.
- Mature female: variation in general body form (in outline), anterior region detail, stylet form and basal knob shape, pharyngeal region, excretory pore position, perineal pattern form plus major variations encountered.

2. Light micrographs: provide a useful supplement to line drawings, particularly for perineal patterns. Good-quality photographs of the female anterior region, stylet and perineal pattern variation are useful, as are photographs of the male labial region, stylet and knobs and J2 labial and tail region.

3. Scanning electron micrographs: particularly useful for depicting the labial region (*en face* and lateral views needed) of male, female and J2, excised stylets of male and female and perineal pattern of mature female (see Fig. 2.25 for an example of suitable images).

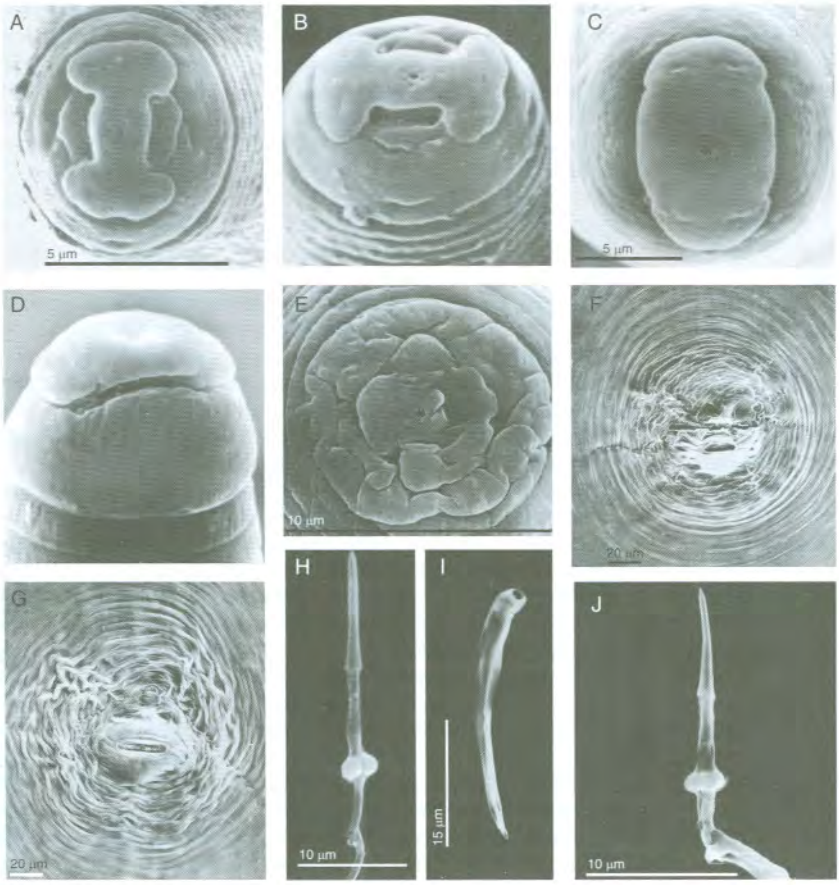

Fig. 2.25. Example of scanning electron micrographs appropriate to support the description of a new species. A, B: cephalic region of second-stage juvenile; C, D: cephalic region of male; E: cephalic region of female; F, G: female perineal pattern; H: male stylet; I: male spicule; J: female stylet. After Charchar *et al.* (2008), courtesy of *Nematology*.

2.8.2.1 Digital files

Very few original figures are now submitted to journals, the illustrations being scanned to digital files by the authors or their associates. This effectively bypasses much of the skill of the publisher's lithographer in delivering a high-quality print. When submitting digital files of figures it is important, therefore, that the correct format and resolution are employed. Black and white line figures are best scanned using the <sharp black and white line drawing> setting (or equivalent) of a professional-quality scanner. This will produce a very-high-quality bitmap file, which at 600 dpi resolution will only occupy about 500 kb when resized to page size and saved as a TIF with the LZW compression algorithm engaged. The 'common or garden' scanners, remarkable beasts that they are, seem to lack this scan option and the best that can be achieved is a greyscale image. Unfortunately, greyscale scans of black and white line drawings tend to generate abundant noise in the form of grey-hued pixels, which are either scattered over the white background of the figure or cluster around the lines, softening and degrading the image as a result. If a greyscale setting has to be employed then an acceptable compromise is to scan at a reasonably high resolution and then simultaneously reduce the file to page size and a resolution of 600 dpi. This file can be saved as a TIF with LZW compression or as a high-quality JPEG. Such images can be processed in a professional image package such as Photoshop® to reduce the effect of noise and thereby arrive at an acceptable result. Greyscale images and SEM micrographs should have a resolution of at least 300 dpi and be saved as high-quality JPEG. Be aware that many 'budget' scanners will automatically scan greyscale images (and also line drawings) as a colour RGB file – be sure to dump this redundant information before submitting the image as otherwise file size will be needlessly bloated by superfluous coding.

2.9 References

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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 assignation (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 *M. 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, *M. enterolobii* (= *M. mayaguensis*) was recognized as a problem in Florida when galls appeared on root-knot-resistant tomatoes, grown in response to persistent populations of *M. incognita* (Brito *et al.*, 2004), and *M. 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 parenaensis*, *M. 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 the 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. While 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, and 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, the USA (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 the 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 (RKN) 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 (random amplified polymorphic DNAs) 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 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 nematicide testing or germplasm screening in which defined inocula are used will have different requirements from 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* spp.

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 (*M. arenaria*), H1 (*M. hapla*), I1 (*M. incognita*) and J3 (*M. 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* spp. 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 Dalmasso, 1975; Dalmasso 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 each 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 (Dalmasso and Bergé, 1978), although the use of galled root tissue has also been reported (Ibrahim and Perry, 1992). Miniaturization and automation of the electrophoresis systems and the use of precast polyacrylamide gels (i.e. PhastSytem, 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. the esterases 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 (Mdh) isozyme phenotypes of *Meloidogyne* spp., including atypical esterase patterns.

Species	Esterase phenotype	Atypical esterase patterns	Mdh
M. arabicida	AR2[7], M1F1b[22]		N1[22]
M. ardenensis			N1a[23]
M. arenaria	A1[18], A2[18], A3[18]	S1-M1[18], S2-M1[18], M3-F1[18]	N1[18], N3[18]
M. artiella	M2-VF1[23]		N1b[23]
M. baetica	Rm 0.31[12]		
M. carolinensis	VS1-S1a[18]		H1[18]
M. chitwoodi	S1[18]		N1a[18]
M. coffeicola	C2[5]		C1[5]
M. cruciani	M3a[18]		N1[18]
M. duytsi	VS1[23]		N2[23]
M. dunensis	VS1[28]		N1c[28]
M. enterolobii	VS1-S1[18]		N1a[18]
M. ethiopica	E3[9]		N1[9]
M. exigua	VS1[20], E1 (VF1)[5], E1b (VF1)[5], E2[11], E2a[11], E3[11]		N1[20]
M. fallax	F3[33]		N1b[23]
M. floridensis	P3[5, 21]		N1[5]
M. graminicola	VS1[18]		N1a[18]
M. graminis	VS1[19] G1[3]		N4[19], N1a[3]
M. hapla	H1[18]	A1[18]	H1[18]
M. haplanaria	Rm 0.61[17]		Rm 0.44[17]
M. hispanica	S2-M1[16], Hi3[6]		N1[18]
M. incognita	I1[18], I2[5], M1a[22]	S1[18]	N1[18]
M. inornata	I3[10]		N1[5]
M. izalcoensis	I4=S4[8, 22]		N1[22]
M. javanica	J3[18], J2a[13], J2[32]		N1[18, 15]
M. jianyangensis	Rm 0.41, 0.45, 0.48[1]		
M. konaensis	F1[16], K3[5], I1[31], F1-I1[31]		N1[5]
M. kralli			N1c[23]
M. lusitanica	P1[27], A1[23]		P3[27], N1c[23]
M maritima	VS1-S1[23]		N1c[23]
M. marylandi	VS1[26]		N1c[26]
M. enterolobii	VS1-S1[29], M2[5]		N1a[5], N3c[29]
M. microcephala	A1[18]		N1[18]
M. microtyla	M1[18]		H1[18]
M. minor	VS1[24]		N1a[24]
M. morocciensis	A3[30]		N1[30]
M. naasi	VF1[18]		N1a[18]
M. oryzae	VS1[18]		N1a[18]
M. panyuensis	S1-F1[25]		N1b[25]
M. paranaensis	P1[4, 5, 7], F1[5], P2[7]		N1[5]
M. partityla	Mp3[2]		N1a[2]
M. petuniae	VS1-S1[14]		N1[14]
M. plantani	S1[18]		N1a[18]
M. querciana	F1[18]		N3a[18]

[1]Baojun *et al.* (1990); [2]Brito *et al.* (2008); [3]Brito, pers. comm.; [4]Carneiro *et al.* (1996b); [5]Carneiro *et al.* (2000); [6]Carneiro *et al.* (2004a); [7]Carneiro *et al.* (2004b); [8]Carneiro *et al.* (2005a); [9]Carneiro *et al.* (2007); [10]Carneiro *et al.* (2008); [11]Carneiro, pers. comm.; [12]Castillo *et al.* (2003); [13]Castro *et al.* (2003); [14]Charchar *et al.* (1999); [15]Cofcewick *et al.* (2005); [16]Eisenback *et al.* (1994); [17]Eisenback *et al.* (2003); [18]Esbenshade and Triantaphylllou (1985); [19]Esbenshade and Triantaphyllou (1987); [20]Esbenshade and Triantaphyllou (1990); [21]Handoo *et al.* (2004); [22]Hernandez *et al.* (2004); [23]Karssen and van Hoenselaar (1998); [24]Karssen *et al.* (2004); [25]Liao *et al.* (2005), [26]Oka *et al.* (2003); [27]Pais and Abrantes (1989); [28]Palomares Rius *et al.* (2007); [29]Rammah and Hirschmann (1988); [30]Rammah and Hirschmann (1990); [31]Sipes *et al.* (2005); [32]Tomaszewski *et al.* (1994); [33]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 *M. 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 Triantaphylllou (1985) listed F1, 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 whether 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 (Mabs) 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 alterative, but requires technical expertise. For routine testing, such as for nematicide or germplasm screening where a defined nematode species is involved, an 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 Mab 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 *M. 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 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 a 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, NaOH 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 (which uses digoxigenin-labelled DNA), 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* spp. (Meštrović *et al.*, 2006), and satDNAs assays have been described for several species of *Meloidogyne*. The highly repetitive nature of satDNAs 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 us 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 one of *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*, *M. camelliae*, *M. mali*, *M. marylandi*, *M. suginamiensis* (Orui, 1999), *M. incognita*, *M. javanica*, *M. hapla*, *M. chitwoodi*, *M. fallax* (Zijlstra *et al.*, 1995) and *M. 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*, *M. microtyla*, *M. ardenensis*, *M. maritima*, *M. duytsi*, *M. chitwoodi*, *M. fallax*, *M. minor*, *M. naasi*, *M. oryzae* and *M. 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 genetic 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

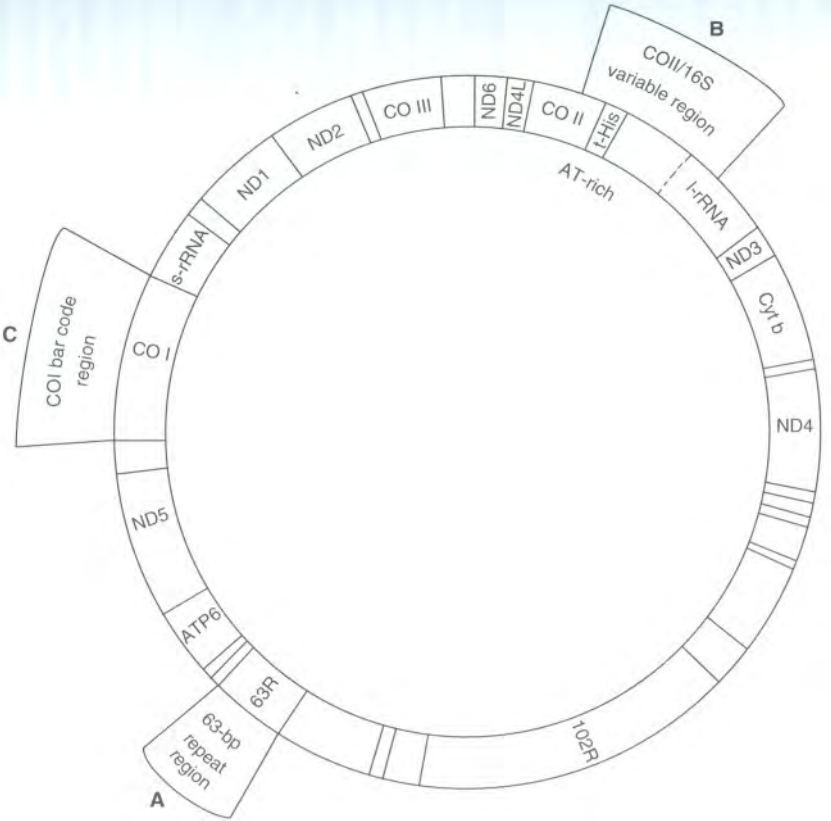
based on rDNA sequences and others developed from RAPDs. Although these primers are described as 'species specific', they must be considered in relation to the species and isolates that have been used for comparison.

Other species combinations that have proved difficult to distinguish using morphological and biological features, such as *M. 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 (mitochondrial DNA, mtDNA) 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 animals 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 'bar coded' 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 that 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 bar code (Vogler and Monaghan, 2007). Several nematode taxa have been bar coded 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 (transfer RNA) 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 colinearly 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



Approximate amplification product sizes

A 63-bp repeat region:
322 bp – *Meloidogyne enterolobii* (*M. mayaguensis*)

B COII/16S variable region:
Using primer set C_2F_3/1108
(C_2F_3 5'-GGTCAATGTTCAGAAATTTGTGG-3')
(1108 5'-TACCTTTGACCAATCACGCT-3')
1.5–1.6 kb – *M. arabicida, M. arenaria*, M. ethiopica, M. incognita, M. javanica*
1.2 kb – *M. paranaensis*
1.1 kb – *M. arenaria*, M. floridensis, M. morocciensis, M. thailandica*
750 bp – *M. enterolobii* (*M. mayaguensis*)
520–540 bp – *M. chitwoodi, M. fallax, M. graminicola, M. graminis, M. hapla, M. haplanaria, M. mali, M. marylandi, M. microtyla, M. naasi, M. oryzae, M. partityla, M. suginamiensis, M. trifoliophila*

C COI bar code region

*Hugall *et al.*, 1994

Fig. 4.1. *Meloidogyne* mitochondrial genome structure, showing regions used for diagnostics. (After Okimoto *et al.*, 1991 and sequences from NCBI (US National Center for Biotechnology Information).)

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 was found in *M. arenaria*, which 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 *M. trifoliophila*. Presumably this is the ancestral state for *Meloidogyne* since non-*Meloidogyne* spp., 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 synomyzation (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
M. arenaria	TCGGCGATAGAGGTAAATGAC TCGGCGATAGACACTACAACT	420 bp	Zijlstra *et al.*, 2000
	TCGAGGGCATCTAATAAAGG GGGCTGAATAATCAAAGGAA	950 bp	Dong *et al.*, 2001b
M. chitwoodi	CCAATGATAGAGATAGGAAC CTGGCTTCCTCTTGTCCAAA	400 bp	Williamson *et al.*, 1997
	GATCTATGGCAGATGGTATGGA AGCCAAAACAGCGACCGTCTAC	900 bp	Petersen *et al.*, 1997
	TGGAGAGCAGCAGGAGAAAGA GGTCTGAGTGAGGACAAGAGTA	800 bp	Zijlstra, 2000
M. exigua	CATCCGTGCTGTAGCTGCGAG CTCCGTGGGAAGAAAGACTG	562 bp	Randig *et al.*, 2002a
M. fallax	TGGGTAGTGGTCCCACTCTG AGCCAAAACAGCGACCGTCTAC	1100 bp	Petersen *et al.*, 1997
	CCAAACTATCGTAATGCATTATT GGACACAGTAATTCATGAGCTAG	515 bp	Zijlstra, 2000
M. hapla	CAGGCCCTTCCAGCTAAAGA CTTCGTTGGGGAACTGAAGA	960 bp	Williamson *et al.*, 1997
	TGACGGCGGTGAGTGCGA TGACGGCGGTACCTCATAG	610 bp	Zijlstra, 2000
	GGCTGAGCATAGTAGATGATGTT ACCCATTAAAGAGGAGTTTTGC	1500 bp	Dong *et al.*, 2001b
	GGATGGCGTGCTTTCAAC AAAAATCCCCTCGAAAAATCCACC	440 bp	Wishart *et al.*, 2002
M. incognita	CTCTGCCCAATGAGCTGTCC CTCTGCCCTCACATTAGG	1200 bp	Zijlstra *et al.*, 2000
	TAGGCAGTAGGTTGTCGGG CAGATATCTCTGCATTGGTGC	1350 bp	Dong *et al.*, 2001b
	GGGATGTGTAAATGCTCCTG CCCGCTACACCCTCAACTTC	399 bp	Randig *et al.*, 2002a
	GTGAGGATTCAGCTCCCCAG ACGAGGAACATACTTCTCCGTCC	955 bp	Meng *et al.*, 2004
M. javanica	CCTTAATGTCAACACTAGAGCC GGCCTTAACCGACAATTAGA	1650 bp	Dong *et al.*, 2001b
	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACTATAC	670 bp	Zijlstra *et al.*, 2000
	ACGCTAGAATTCGACCCTGG GGTACCAGAAGCAGCCATGC	517 bp	Meng *et al.*, 2004
M. enterolobii	GAAATTGCTTTATTGTTACTAAG TAGCCACAGCAAAATAGTTTTC	322 bp	Blok *et al.*, 2002
M. naasi	CTCTTTATGGAGAATAATCGT CCTCCGCTTACTGATATG	433 bp	Zijlstra *et al.*, 2004
M. paranaensis	GCCCGACTCCATTTGACGGA CCGTCCAGATCCATCGAAGTC	208 bp	Randig *et al.*, 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 (RAPD)

Random amplified polymorphic DNAs (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 relatively 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 Tesařová *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 the 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 400 g 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 have been made in the sensitivity and specificity of arrays with shorter and more sequence-specific oligonucleotides, 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, personal communication), as well as cost. Examples of published microarray results that include *Meloidogyne* spp. 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* spp. are now widely used and, in some cases, essential for species diagnosis. They cannot, however, be used with confidence to identify all *Meloidogyne* spp. A clear understanding of species boundaries and adequate sampling of known species across their geographic range are lacking (see 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; this is also indicated in the genome sequence of *M. incognita* (Abad *et al.*, 2008). Depending on the nature of the interspecific hybidization 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). *Meloidogyne 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; Piotte *et al.*, 1995; Hugall *et al.*, 1997; Handoo *et al.*, 2005; Powers *et al.*, 2005). In addition to the continued evaluation of intraspecfic 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 global transport of agricultural commodities, the use of diagnostics for *Meloidogyne* spp. is highly relevant.

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12 Sampling Root-knot Nematodes

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12.1 Introduction

Nematode population levels are estimated from samples of soil or plant tissue and the results are used for various purposes. They may be used in an advisory capacity to predict the need to impose management tactics to suppress populations to desired levels, or in research to study the responses of nematode populations to management tactics and other anthropogenic factors, or to derive relationships between population size and environmental factors. For most of these purposes an ideal nematode sample would reveal the spatial pattern of population densities within the sampled area. Such information would permit growers to select planting material best suited for various locations or to adopt variable rate methods for application of nematicides at levels needed to suppress local populations to non-damaging levels (Ferris, 1978; Wheeler and Bronson, 2004; Monfort *et al.*, 2007). It would facilitate research by permitting comparisons between spatial patterns of nematodes and those of the myriad physical and biotic forces to which they respond (Perry, 1998; Spiridinov *et al.*, 2007). However, nematode samples are expensive to collect and process: to estimate the population density of *Pratylenchus penetrans* in an area of 0.01 ha within 20% of the true mean with 95% confidence required approximately 7 h of labour (Proctor and Marks, 1974). Therefore, rather than estimating population densities at many discrete locations in a field, soil sampled at those locations (referred to hereafter as **cores**, after the method commonly used to collect them) is combined in a composite or bulk **sample**, which is mixed and then usually subsampled. Currently, growers can only approximate precision agriculture methods by delineating a sampling area into smaller units based on affordability and factors such as soil

 Root-knot Nematodes (eds R.N. Perry, M. Moens and J.L. Starr)

texture, cropping history or past crop performance, which are known to affect nematode population size (Barker and Campbell, 1981; Monfort *et al.*, 2007). Often as few as one to several bulk soil samples are taken in each delineated unit in a field. After mixing the soil, nematodes are commonly extracted from an additional subsample(s), which may comprise only 50–500 cm^3 of soil taken from areas as large as 2–5 ha (Barker and Imbriani, 1984). Population estimates from such small samples are clearly subject to substantial error. Thus, sampling intensity is usually dictated by the time and money growers and researchers are willing to invest rather than by what is required to achieve a specified level of accuracy (Been and Schomaker, 2000). The need for mechanized sampling devices has long been recognized as a major impediment to improving sampling effectiveness (Barker and Imbriani, 1984; Ferris *et al.*, 1990; Duncan, 1991; Been and Schomaker, 2000). Nevertheless, to make rational management decisions based on information from soil or plant samples requires some understanding of their reliability – the probability of achieving a specified degree of accuracy. Sample methods are devised and characterized in a variety of ways, most of which require knowledge of nematode spatial patterns in nature and how those patterns affect the frequency distributions of the sample data.

12.2 Nematode Spatial Patterns

The spatial patterns of nematodes in nature are patchy or aggregated at almost any resolution at which they have been studied (Goodell and Ferris, 1980, 1981; Duncan *et al.*, 1994a, 1995; Been and Schomaker, 1998). Samples from discrete locations within field plots as small as 1 m^2 or as large as a field will almost always result in counts with the variance larger than the mean (Wheeler *et al.*, 1992; Siddiqui and Shaukat, 2002). As populations grow they become relatively less aggregated (Taylor *et al.*, 1979; Noe and Barker, 1985). The degree of patchiness also depends on the spatial patterns of edaphic factors that affect nematodes (and their competitors and natural enemies), cultural practices that move soil and dictate the location and species of plant roots, and the biology of the nematode species itself. For example, reproduction of *Meloidogyne incognita* on cotton was much greater in coarse-textured soil than in fine-textured soil, whereas that for *Rotylenchulus reniformis* was highest in soils with moderate levels of clay and silt (Koenning *et al.*, 1996). The generally wide host range of most *Meloidogyne* species means that populations have greater opportunity to survive on weed hosts when non-host crops are grown, providing them with more and larger foci to initiate population growth on a subsequent host crop compared with species with very restricted host range, such as *Globodera pallida* (Been and Schomaker, 2006). The migratory behaviour, longevity, fecundity and oviposition behaviours of nematodes are characteristics that affect their spatial patterns in a field. Most species of *Meloidogyne* migrate readily as second-stage juveniles (J2), become sedentary after infection of the host, develop in 3–4 weeks, produce several hundreds of eggs in egg masses on roots, under optimum conditions, populations grow rapidly (Prot and Netscher, 1978; Prot and Van Gundy, 1981; Anwar and McKenry 2007). Even though distributions of eggs of *Meloidogyne* spp. are extremely patchy on a small scale compared with those of migratory nematodes, the combined traits of relatively many foci, ability to migrate readily and rapid population growth suggest that populations of most of the widely occurring species of *Meloidogyne* tend to be less aggregated at the field level than some of the other economically important nematode species (Goodell and Ferris, 1980; Noe and Campbell, 1985; Been and Schomaker, 2006; Herve *et al.*, 2005). By comparison, nematodes, such as *Tylenchulus semipenetrans*, that also oviposit large numbers of eggs in masses on roots, but which migrate less readily, may show greater aggregation across a field (Davis, 1984; Duncan *et al.*, 1995) than do most species of root-knot nematodes.

Nematode spatial patterns dictate the sampling patterns most likely to achieve accurate population estimates. Nematodes are rarely randomly distributed across a field, so random sampling is less likely than systematic sampling to detect and to describe accurately the average population density (Goodell and Ferris, 1981; Duncan *et al.*, 1994a,b; Been and Schomaker, 2000, 2006). Random sampling could easily fail to encounter a small focus of nematodes in a field that is otherwise non-infested, whereas systematic

sampling on a grid (including a regular zigzag pattern) with dimensions smaller than the focus should encounter nematodes and will also give appropriate weight to the larger, non-infested area. Similarly, in orchards, single core samples from more trees were shown to provide more accurate population estimates than multiple core samples taken from fewer trees (McSorley and Parrado, 1982a). For the same reason, collecting more small cores provides a more accurate mean estimate than an equivalent amount of soil collected as fewer large cores (Goodell and Ferris, 1981; McSorley and Parrado, 1982a, 1983; Been and Schomaker, 2006). Prot and Ferris (1992) demonstrated that for large-scale surveys a ten-core sample reliably detected most nematode species in two Californian fields as large as 7 ha, and that detection probability increased with distance between cores because microhabitats that influence the nematode spatial patterns were sampled more adequately. Grid dimensions can also be optimized with respect to spatial patterns. In row crops, nematodes are moved in the direction of the row by cultivation and they migrate along adjacent root systems, so densities are more highly concentrated within than across rows (Noe and Cambell, 1985; Been and Schomaker, 1998). Therefore, samples from grids with more points across rows than within rows are likely to estimate an average population density more accurately (Been and Schomaker, 2000). Similarly, the variability of population densities between plots tends to be lower in experimental plots with dimensions longer across than within rows (Noe and Campbell, 1985). The same principle applies when separate samples are collected from different strata in fields that comprise different soil conditions or previous or present host material. Stratification is intended to reduce the overall variability of the sampled populations by separately sampling areas likely to have lower or higher nematode numbers (Goodell and Ferris, 1981).

The depth to which samples are taken is another form of stratification, because nematodes have vertical spatial patterns that usually extend below the depth of a soil core. In a Californian vineyard, numerous species of *Meloidogyne* occurred at 120 cm and were detected at 330 cm depth (Ferris and McKenry, 1974). Therefore, the depth of sampling will affect the density of nematodes recovered per sample or per volume of soil. Nematode patterns are closely related to the vertical pattern of host root abundance (Ingham *et al.*, 1985; Duncan, 1986; Rodriguez-Kabana and Robertson, 1987; Verschoor *et al.*, 2001). Host root density is greatest in the surface soil horizons, so sampling to depths of 30 cm or less is usually adequate to reduce sample variability by recovering nematodes in the zone of their greatest density. However, in deep-rooted crops such as grape, samples to a depth of 60 cm were required to obtain the highest density and least variable estimates of *Meloidogyne* spp. (Ferris and McKenry, 1974). The highest density of *M. incognita* on banana was at 0–10 cm depth in the first-year crop but at 0–20 cm in the ratoon crop (Jonathan and Rajendran, 2003). The density of nematodes near the soil surface can provide a reasonable estimate of the total population to greater depths. Numbers of *T. semipenetrans* to a depth of 30 cm explained 73% of the variability in samples from 0 to 60 cm (Duncan 1986). *Meloidogyne hapla* on carrot, onion and weed fallow had similar patterns of abundance to a depth of 40 cm, with two-thirds of the population at 0–20 cm depth (Bélair 1998). Half of the numbers of *M. chitwoodi* to a depth of 70 cm were recovered in samples to either 20 or 34 cm in two fields with different cropping regimes (Wesemael and Moens, 2008).

Crop damage functions and nematode population models (see Greco and Di Vito, Chapter 11, this volume) that are functions of initial nematode density are non-linear because root damage and yield loss per nematode decreases with increasing population density (Seinhorst, 1965, 1998). These functions are usually developed in glasshouse, microplot or small-plot field trials using artificially uniform populations over a range of densities. As nematodes are not distributed uniformly within fields, the accuracy with which the mean nematode density in a field is predictive of yield loss and population change depends on the population spatial pattern. With increased aggregation, predictions from a mean tend to overestimate crop loss due to the increasing frequency of locations with no or fewer than the mean number of nematodes, and because damage per nematode is inflated for the fewer locations contributing high counts to the mean (Seinhorst, 1973; Perry, 1983; Noe and Barker, 1985). Damage functions can also underestimate damage that occurs in a few clusters in a field for which the mean density of the entire field is below

a damaging level. Approaches to resolving the problem of prediction from advisory samples have been addressed theoretically, but are not widely applied (Seinhorst, 1973; Perry, 1983; Noe and Barker, 1985; Hughes, 1990, 1999). Noe and Barker (1985) fitted the negative binomial model to frequency distributions of the counts of *M. incognita* from many quadrats in each of ten tobacco fields, and showed that the mean nematode density for the field was directly related to the aggregation parameter (k) of the negative binomial distribution. Using a published damage function, yield loss predictions from field means were compared with those from summing the effects of densities at discreet areas, as estimated from the associated probabilities from the negative binomial, to show that overestimation of yield loss in a field was inversely related to nematode mean density. Noe and Barker (1985) suggested deriving these relationships for specific crop–nematode systems in order to adjust estimates of yield loss from the field mean density. Others have suggested approximating this approach empirically by stratifying fields to estimate yields in discreet areas, although this approach is often cost-prohibitive using current sampling and processing methods (Perry, 1983).

12.3 Characterizing Sample Accuracy and Reliability

Sample accuracy and reliability can be characterized using the mean–variance relationship for normally distributed data (Ferris *et al.*, 1990; Buntin, 2000). The confidence interval (CI) for a population of known mean (μ) and standard deviation (σ) is described by:

$$CI = \mu \pm z_{\alpha/2}\, \sigma / \sqrt{n} \qquad (12.1)$$

where z is the standard normal variate (1.96 at $P = 0.05$), α is the probability and n is the number of samples. Accuracy can be specified by equating the half-length of CI to a specified proportion (D) of the mean density

$$D\, \mu = z_{\alpha/2}\, \sigma / \sqrt{n} \qquad (12.2)$$

where b is an aggregation parameter (see Equation 12.7). Equation 12.2 can be rearranged to solve for z by estimating μ and σ from a preliminary sample mean ($\bar{x}$) and standard deviation (s)

$$z_{\alpha/2} = \sqrt{n}\, D\, \bar{x}/s. \qquad (12.3)$$

Equation 12.3 can be used to estimate the reliability of a population estimate obtained from a predetermined number of samples at a specified accuracy level (D $\bar{x}$). For example, if we choose to collect three samples from a population with mean and standard deviation equal to 1000 and 353, respectively, and we wish to know the frequency with which our population estimate will be no greater or smaller than 40% of the true mean, then $z = (\sqrt{3} \times 0.4 \times 1000) / 353 = 1.96$, and from a table of probabilities associated with standard normal variates, $P = 0.05$. We expect 95% of our population estimates from such a sample plan to be within 40% of the true mean.

Alternatively, rather than predefining n, we can select the value of z needed to achieve the desired precision and estimate an appropriate sample size by rearranging Equation 12.2 as:

$$n = z_{\alpha/2}^{\,2}\, s^2 / D^2\, \bar{x}^2 \qquad (12.4)$$

For a population at a given place and time, the parameter estimates used in Equations 12.3 and 12.4 can be derived several ways. Often, several bulk samples comprising a predetermined number of cores are collected, each across the entire sample area. Extraction of each bulk sample, or subsample(s) of each bulk sample, provides an estimate of the mean number of nematodes in the individual cores that comprise each sample. From the Central Limit Theorem, as the number of cores in a bulk sample increases, the distribution of sample estimates will approach normality, regardless of the spatial pattern in the field (Duncan and McSorley, 1987). Thus, Equations 12.3 and 12.4 are appropriate for characterizing data from bulk samples. However, samples comprising single cores or multiple cores taken at many discrete locations across the sample area (as in grid sampling) will yield population estimates that reflect the population spatial pattern, and the data will often be distributed according to the negative binomial model. The model can be fitted to such data to estimate the parameter k describing the degree of aggregation (McSorley and Parrado, 1982b). The relationship between the sample variance and k is approximated by:

$$s^2 \approx \bar{x} + \bar{x}^2 / k \qquad (12.5)$$

and has been used to estimate the variance or standard deviation in Equations 12.1–12.4 as in:

$$z_{\alpha/2} = D \sqrt{n} / \sqrt{(1 / \bar{x} + 1 / k)} \qquad (12.6)$$

Alternatively, algorithms can be written to sample from data sets of counts from single cores or samples from discrete areas within the sample area to develop simulated bulk samples with means that approach normality (Goodell and Ferris, 1981; McSorley and Parrado, 1983; Perry, 1983; Duncan *et al.*, 1993). Such an approach is especially useful to estimate optimum sampling as a function of numbers of cores per sample and samples per field, taking into account the time and cost to collect cores and to process samples (Goodell and Ferris, 1981).

As populations increase or decline, the variance changes as a function of the mean, a relationship that is well described by Taylor's Power Law (TPL) (Taylor, 1961)

$$\sigma^2 = a\,\mu^b \qquad (12.7)$$

where a is a scaling factor and b describes the degree of aggregation (b<1 approaches uniformity, b = 1 is random and b>1 is increasingly aggregated). Of the two parameters, b tends to be species-specific, whereas a is affected by the methodology – habitat, sample size, sample area size, collection and processing methodology (Ferris *et al.*, 1990; McSorley and Dickson, 1991; Duncan *et al.*, 1993, 2001). Taylor's law shows clearly that estimating the accuracy and reliability of different-sized samples based on sampling one field at one time applies only to populations of similar size. However, data from populations sampled in the same manner at different locations and/or times can be used to estimate parameters of TPL by regressing the log-transformed variances on the log-transformed means from each sampling event (i.e. $\log s^2 = \log a + b \log \bar{x}$) (Fig. 12.1). This approach permits the variance to be defined by the mean in order to optimize sampling across a range of population densities as in:

$$z_{\alpha/2} = \sqrt{n\,D^2 / a\,\bar{x}^{b-2}} \qquad (12.8)$$

Use of Equations 12.1–12.8 to investigate sample accuracy and reliability reveals some general principles of sampling. For a given sample size, accuracy and reliability increase dramatically with population density, especially at the lower density ranges (Fig. 12.2). Whereas σ^2 increases faster than μ when the spatial pattern is aggregated, σ increases more slowly than μ, resulting in decreasing coefficients of variation as populations grow. Therefore, the most accurate samples are obtained from locations and at times when population size is greatest (Singh and Gaur, 1997; Souza *et al.*, 2007). Similarly, for sampling methods that can accurately estimate population size at sub-damaging levels, the accuracy of predictions from damage functions will increase at

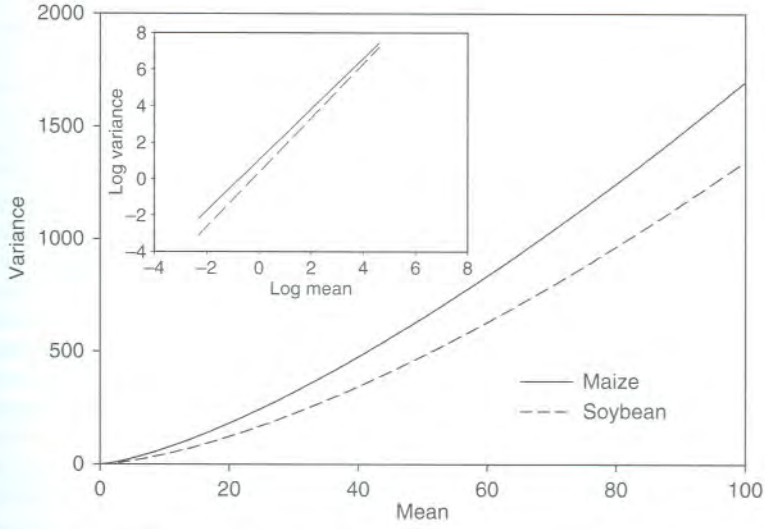

Fig. 12.1. The relationship between the variance and the mean population density for *Meloidogyne incognita* on maize and soybean as given by Taylor's Power Law ($\sigma^2 = a\,\mu^b$). Inset shows data transformed to permit parameter estimation through linear regression according to $\log s^2 = \log a + b \log \bar{x}$. Parameter values from McSorley and Dickson (1991).

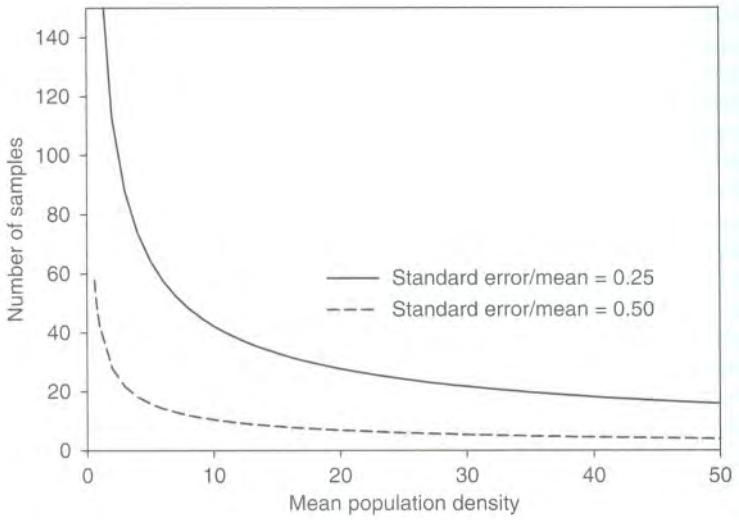

Fig. 12.2. Numbers of 12-core samples per 3 × 3 m area needed to estimate the population density of *Meloidogyne incognita* on soybean with estimated (from Equation 12.8, rearranged to solve for *n*) accuracy levels of 0.25 or 0.5 as a function of mean population density. Parameter values from McSorley and Dickson (1991).

higher, more-damaging densities, whereas less accurate estimates for smaller populations are of no economic consequence (Ferris *et al.*, 1990). The relationship between sample size and reliability (α) is not linear (Duncan *et al.*, 1996, 2001). It can be useful to characterize the reliability of samples of different size, rather than merely derive a sample size to achieve a specified accuracy and reliability (Neher and Campbell, 1996). For example, from Equations 12.3 and 12.4 it would require three bulk samples to estimate the mean with a 95% confidence interval half-length of 40% for a population with μ = 1000 and σ = 580. However, the likelihood of achieving the same accuracy with just two samples is almost the same (94%), but at a saving of one-third of the expense for sample acquisition and processing. Finally, whether for research or advisory purposes, nematode sampling plans rarely achieve confidence interval half-lengths of less than 40–50% with reasonable reliability (McSorley and Dickson, 1991). The utility for growers of population estimates with large error terms depends primarily on the size of the action threshold density. For species of *Meloidogyne* on various crops, typical tolerance limits range from just a few to more than 100 eggs and J2 per 100 cm^3 soil, with action thresholds being higher (Ehwaeti *et al.*, 1998; Asuaje *et al.*, 2004; Russo *et al.*, 2007). For other nematode species, such as *T. semipenetrans*, the action thresholds are as high as several thousand eggs and J2 per 100 cm^3 soil (Sorribas *et al.*, 2008). In Florida, most samples from citrus orchards reveal no or low numbers of *T. semipenetrans* (Duncan, 1999). Confidence interval half-lengths as high as 100% or more at low population density still result in small population estimates, which can be distinguished from the high action threshold for *T. semipenetrans*. Greater accuracy is required as the action threshold decreases, or as the frequency of damaging population levels increases, and the profitability of sampling becomes increasingly problematic.

Regulatory situations in which no nematodes can be tolerated represent the extreme case of high sampling costs associated with low threshold levels. Although applied sampling programmes do not include enough samples to allow mapping of nematode spatial patterns in a field, the most precise sampling programmes developed to date are based on such information (Been and Schomaker, 2000). The programmes are designed to detect the potato cyst nematode *G. pallida*, a regulated species for seed potato production. Fields with a wide range of different densities of potato cyst nematodes were sampled on grid pat-

terns to identify discrete patches of nematodes. These patches were further sampled at a finer grid resolution, which permitted accurate modelling of the dimensions of nematode patches as a function of the mean number of nematodes at the central point in the patch (Schomaker and Been, 1999). With this information, it is possible to optimize sampling programmes that address the challenging task of identifying fields with only a single patch of nematodes at densities below which symptoms will occur in the potato crop. A key aspect of the optimization is the ability to define the grid dimensions in the direction of rows and across rows to minimize both the labour needed to collect samples and the likelihood of not adequately sampling a patch due to an overly coarse grid. Sample plans derived by these methods were estimated to detect very small foci of cyst nematodes (two eggs/g soil) with 90% reliability, compared with just 12% using conventional sampling plans. Achieving such accuracy and reliability is expensive, requiring more than 300 cores and 6–7 bulk samples per ha. However, by substantially increasing the reliability of detecting small foci of cyst nematodes, potato growers in The Netherlands can confidently restrict soil fumigation to just infested parts of fields or grow less-profitable resistant varieties only when needed. The widespread adoption of the new sampling method resulted in an 80% reduction of soil fumigation between 1989 and 1994. A somewhat analogous situation is that in which plant propagation material must be certified as free of certain regulated nematodes. Assuming that infested material is properly identified if sampled, the probability of detection can be derived from the binomial distribution and depends on the size of the plant population, the percentage of plants infested, and the number of plants sampled. Tables have been published to determine, for example, that in a lot of 10,000 plants with either 1 or 5% infestation rates, sampling 500 or 100 plants, respectively, should detect the infestation 99% of the time (McSorley and Littell, 1993).

12.4 Sample Processing

Root-knot nematode infestation levels in fields can be estimated directly by enumerating some life stage of the nematode, or indirectly by measuring plant symptoms or damage.

When a soil/plant sample is taken, decisions have to be made about what is to be examined. It could be eggs and/or J2 in the soil, life stages within roots, root galls, females or number of egg masses. These are obtained variously, either directly from a field sample or from a bioassay of the soil sample. Different methods differ in the efficiency with which they separate nematodes from soil or plant tissue, and factors such as sample size and soil type have major effects on extraction efficiency (Campos and Campos, 2005; Qiu *et al.*, 2006). A commonly employed method (Baermann incubation) recovered only 30% as many plant-parasitic nematodes, but more than four times more omnivorous and predatory nematodes, than did centrifugal flotation (McSorley and Frederick 2004). The efficiency with which centrifugal flotation recovered *M. incognita* varied from 19 to 65%, depending on soil type (Hernandez and Lopez, 1989). Female and juvenile *M. incognita* were more efficiently separated from root tissue by a method that employed grinding rather than stirring, whereas the opposite occurred for eggs (Stetina *et al.*, 1997). Knowledge of extraction efficiency permits the comparison of results from different laboratories or methods, and protocols for efficiency estimation have been proposed (Ferris, 1987). Although detection thresholds and sample variability are inversely related to extraction efficiency, depending on the objectives, use of methods with lower efficiency may be adequate and justified for reasons of cost or convenience.

12.5 Extracting Nematodes from Soil

There are a number of methods for extracting eggs and juveniles. These are mainly some form of Baermann trays/funnels, decanting and sieving, elutriation (semi-automated) and centrifugal flotation. Baermann techniques are good for the recovery of clean, live samples; sieves are not needed and the necessary equipment is relatively inexpensive to construct. However, they rely on the nematodes being active and recovery percentages can be low. Recovery of *M. incognita* and other nematodes was accelerated by ten- to 100-fold by covering dishes to reduce evaporation and the resulting temperature gradient across the soil layer (Robinson and Heald, 1989).

Barker *et al.* (1969) found that Baermann methods worked very well for the extraction of *Meloidogyne* in the summer but not in the winter. Baermann trays or dishes are preferable to funnels. Sieving techniques or bucket sieving methods (Cobb, 1918) are relatively rudimentary but useful for the rapid extraction of active and inactive nematodes. Only two buckets and a set of sieves are required.

Elutriation techniques extract nematodes by using a vertical current of water, the rate of which is adapted to the particular size of the nematode. The efficiency of extraction is little different from that of the sieving techniques but does produce a cleaner sample. Various designs have been published, including those of Seinhorst (1956) and Oostenbrink (1960). Trudgill *et al.* (1973) and Winfield *et al.* (1987) produced fluidizing columns which are simple and adaptable. Semi-automated versions have also been produced (Byrd *et al.*, 1976).

Probably the most efficient extraction methods are flotation techniques. These rely on solutions of particular specific gravity such that the target organism(s) to be extracted float or are suspended in the solution. These methods can be useful for fast- or slow-moving nematodes as well as dead ones. The solutions may be made of sugars (sucrose), $MgSO_4$ or $ZnSO_4$, as well as of the rather more expensive manufactured compounds – Percol, Ficoll and Ludox (Viglierchio and Yamashita, 1983). Centrifugal flotation involves the mixing of water and soil (stones removed) followed by centrifugation, which pellets soil and nematodes. The supernatant with organic matter can then be removed. The pellet is resuspended in the suspending solution and re-centrifuged. The supernatant then contains the nematodes (Caveness and Jenson, 1955), which must be washed to minimize osmotic pressure and subsequent damage. This method was improved by Jenkins (1964) to handle larger volumes of soil up to 500 cm^3. Byrd *et al.* (1972) describe a method for the extraction of eggs by first extracting egg masses by elutriation. The egg masses are then dissolved in a 0.525% NaOCl solution to release the eggs, which are subsequently washed and stained by adding acid fuchsin solution and lactic acid. As well as extracting directly from soil, these techniques can be used to clean up samples which have been extracted by other means.

12.6 Extracting Nematodes from Plant Material

If it is felt that counting the number of egg masses is appropriate, the whole or parts of root systems can be sampled, washed free of soil and stained in a solution of Phloxine B (Hartman and Sasser, 1985) or an alternative dye (Thies *et al.*, 2002). The roots can be treated with a NaOCl solution in order to degrade the egg masses and release the eggs (Hussey and Barker, 1973). Thus, an egg count rather than an egg mass count can be obtained.

Various versions of the Baermann technique can be used to extract active nematodes from plant tissue, although it is worthwhile not to submerge the plant material or to let it dry out. The plant material will usually be chopped into small segments. The use of trays or dishes is preferable to funnels, as this maintains better oxygenation (Rodriguez-Kabana and Pope, 1981). Better still in this respect are mistifier techniques, whereby a mist is sprayed over the sample, usually intermittently.

Plant material can also be chopped up and placed in a domestic food blender. The time required to macerate the tissue depends on the type of plant material. The main aim is to blend for the minimum time needed to induce the nematodes to escape the tissue without harm to themselves. Comparing techniques, it has been observed that a brief exposure to 0.5% NaOCl (for 10 s) can enhance the efficiency of extraction (Stetina *et al.*, 1997). Enzymatic predigestion of tissue prior to mechanical maceration is seldom used but can increase extraction efficiency substantially (Kaplan and Davis, 1990; Julio *et al.*, 2003). Samples produced in these ways can be cleaned up using Baermann techniques if the nematodes are active, or by centrifugal flotation.

12.7 Root Gall Indices

Extraction of nematodes from soil samples does not always reveal their presence, especially at low densities. Olowe (2004) found in a survey of species of *Meloidogyne* in Nigeria that many negative samples proved positive when a bioassay was conducted using a susceptible host grown in the

apparently *Meloidogyne*-free soil. Barker (1985) also comments on bioassays as being the most sensitive in terms of low population densities. Typically the roots are recovered and the degree of galling assessed. This sampling of roots may be from a bioassay (Gugino *et al.*, 2008) or from hosts in the field directly (Bélair and Boivin, 1988). Various scales have been suggested, such as that of Bridge and Page (1980), which uses a 0–10 point scale, while others have proposed narrower scales. For example, Kinloch *et al.* (1987) used a 0–4 scale, where 0 = no galling, 0.2 = < 5%, 1 = 5–25%, 2 = 26–50%, 3 = 51–75%, and 4 = > 75% of the root surface galled. Barker (1985) summarizes these and suggests that a 0–6 scale is adequate in evaluating nematicide trials. Gall indices that are assessed directly in the field at harvest have the advantages of being relatively inexpensive and of measuring the effects of populations at their highest densities, which reduces sample variability. Bélair and Boivin (1988) related end-of-season gall indices of *M. hapla* to economic damage in the following carrot crop, thus providing a simple method by which growers can assess the need for nematode management in fields or even in just parts of fields. Mapping of maximum gall indices per ha on a large scale (350 ha) is being used to predict damage, monitor treatment efficacy and adjust management tactics in Morocco (Mateille *et al.*, 2005). A similar approach, using gall indices on lettuce seedlings to bioassay pre-plant soil samples for *M. hapla*, is currently used by New York vegetable growers (Gugino *et al.*, 2008). Although the assay requires 6 weeks for appropriate gall development, it does not require production of a susceptible host in the previous season, as do end-of-season field assays. Gall index bioassays of soil samples were an effective means of identifying edaphic factors associated with soils conducive to damage by *Meloidogyne* spp. in Mexico (Guzman-Plazola *et al.*, 2006).

12.8 Other Plant Symptoms

Other symptoms that can be observed above ground include stunting, chlorosis, wilting and leaf curling, but, being non-specific, they are not useful for quantification of nematode populations. However, above-ground symptoms can be used to prioritize sampling efforts and reduce the cost of an effective sampling programme. A two-step process was developed to predict crop loss in tomato in Australia, in which previous disease symptoms, combined with other risk factors such as soil texture and regional temperature, are used to estimate a numerical 'hazard index', which is used to determine whether to enumerate nematodes through soil sampling (Stirling *et al.*, 2004). Symptoms may occur on other plant parts, such as tubers. Coyne *et al.* (2006) were able to assess the distribution of *Meloidogyne* spp. on yam in West Africa by sampling yams from markets, and were able to determine in which countries and on which species of yam there were most infections by simple observation of the tubers. In Europe, where *M. chitwoodi* is a quarantine pest, more careful inspection is required to detect these nematodes and prevent their spread if found. Studies of the distribution of the nematodes within potato tubers and of the best way of extracting them revealed that zonal centrifugation yielded two to three times more eggs and juveniles of *M. chitwoodi* than elutriation and conventional centrifugation (Viaene *et al.*, 2007).

12.9 Research to Optimize Sampling Programmes for Root-knot Nematodes

Field sampling for agricultural prediction, survey sampling for ecological studies and agricultural resource allocation, and small-plot sampling for experimental purposes, have all been studied to optimize the cost of measuring population density of root-knot nematodes. Barker and Imbriani (1984) reviewed advisory sampling programmes, and noted that most recommended 20–50 cores collected systematically rather than randomly in areas of 2 ha or less in order to achieve population estimates of 30–50% of the mean in row crops, and slightly better in solidly planted perennials (Goodell and Ferris, 1981). Their overview remains largely current because sampling and extraction methods have changed little in the subsequent quarter-century. For example, the systematic collection of 50 cores in pineapple fields, recommended for management decisions, was shown to estimate *M. javanica* in two quarter-hectare fields with a standard error to mean ratio

of *c.* 30% (Stirling and Kopittke, 2000). McSorely and Parrado (1982b) reported similar levels of precision (standard error to mean ratio = 25%, or 95% confidence interval of *c.* 50%) for *M. incognita* estimated from 22 cores per quarter- to half-hectare areas of fallow vegetable fields. Gall index bioassays of *M. incognita* in vegetable fields (2–6 ha), required three to four 20-core composite samples to achieve 25% standard error to mean ratios, whereas use of 20 assays of individual cores resulted in ratios of 50% (i.e. confidence interval half-lengths = 100% of the mean) (McSorley and Parrado, 1983). Bélair and Boivin (1988) recommended a sequential sampling method with an error level of 0.05 to determine whether plant gall intensity on end-of-season carrots represented an economic threshold for the succeeding crop. The method requires comparing the cumulative gall index of a minimum of ten and maximum of 72 plants with a range of upper and lower index limits. At the point that the cumulative index exceeds (management required) or falls below (management unnecessary) the uncertainty range, sampling stops. Samples of 72 plants that remain within the uncertainty range are considered above the economic threshold.

The ability to identify and quantify DNA of soil organisms and the deregistration of increasing numbers of soil fumigants are two phenomena that may provide the impetus to develop more efficient methods of soil sampling for advisory purposes. DNA analyses have the advantage of potentially identifying any life stage of *Meloidogyne* to the species level (see Blok and Powers, Chapter 4, this volume). Tests can be designed to identify and quantify a suite of pest organisms from a single DNA sample. An Australian testing service processes up to 2500, 500-g soil samples annually, using real-time PCR to identify and quantify key lesion and cyst nematode pests of cereals, as well as key fungal disease organisms (Ophel-Keller *et al.*, 2008). Detection limits of these methods are lower than threshold levels, and population estimates for key nematodes have been validated against conventional methods and by using samples with augmented nematodes. Samples comprise 45 cores from 15 locations in fields previously stratified on the basis of historical yield maps, elevation, soil conductivity surveys, and even satellite reflectance bioimagery. The resulting information is currently the basis for nematode and disease management decisions on up to 100,000 ha of cereal. Similar tests have been developed and validated in a variety of soil types to identify and quantify species of *Meloidogyne* and other disease organisms in tomato, pineapple and other horticultural crops (Stirling *et al.*, 2004). With the decreasing availability and increased cost of soil fumigants and nematicides, soil sampling is likely to become increasingly important as a basis for more complex integrated pest management (IPM) approaches that involve greater use of rotations, resistance and biological control tactics (Stirling and Pattison, 2008). The use of partially mechanized DNA-based analyses that can accurately identify and quantify multiple pest and disease organisms will become increasingly more cost effective than current systems requiring greater time, labour and biological expertise (Stirling and Pattison, 2008).

A review of nematode survey data showed that reported species richness is highly correlated with sampling intensity, yet little work has been done to optimize sampling methods for regional nematode surveys (Boag and Yeates, 1998). Neher and Campbell (1996) investigated sample requirements necessary to distinguish regional differences in several ecological indices (maturity and diversity) used to describe nematode communities. Samples consisted of 20 cores (2.5 cm diameter × 20 cm depth) taken at equidistant intervals on a 90 m diagonal transect per field. They estimated that an optimum plan to achieve minimum correlations between samples in a field of 0.6, and a 90% probability of detecting a 10% difference in indices over time, requires two samples each from between 50 and 100 fields. The probability of detecting plant-parasitic nematodes in 10-core samples from lucerne fields between 2.5 and 7 ha was as high as 62–93% for species with relatively low average density (<30 per 100 g soil), and species such as *M. arenaria* with densities between 80 and 2000 per 100 g soil were always detected (Prot and Ferris, 1992). Sample pattern had little effect on species detection in these fields, although variability was lower in systematic than random samples.

Despite the widespread use of small-plot experiments in nematology, few studies report variability of counts among plots to optimize replication. For example, six to eight plots (2 rows × 2 m × 10–12 cores per plot) were needed to estimate most mean densities (10–100 J2 per 100 cm^3 soil) of *M. hapla* in potato fields with a coefficient of variation of 0.5 (Wheeler *et al.*, 1994, 2000).

12.10 Examples of Results from Sampling Programmes

12.10.1 Surveys

Sampling patterns, numbers, timing and the material sampled all depend on the objectives of the work. Survey work often only requires sampling at its most basic level, the objective usually being to investigate what nematodes are present and where. Sampling of soil and/or roots will often be carried out once. Such surveys may also be done in order to develop or verify molecular diagnostic tools. Hallmann *et al.* (2007), investigating the occurrence and importance of plant-parasitic nematodes (including *Meloidogyne*) in Germany, sampled 246 fields, taking 1 ha as a minimum area for a sample. Fields were selected mainly on the basis that they had known or suspected nematode problems, although some sites with no nematode history were used. To sample so many sites at a specific time of year was impractical, and samples were taken from each site over the course of a year. Each site was sampled in a zigzag manner, with 50 cores making up some 6 l of soil, which was mixed and subsampled (8% or 250 cm^3) for nematode extraction by centrifugation (Hooper *et al.*, 2005), followed by microscopic identification. However, there are difficulties in using this identification approach (see Blok and Powers, Chapter 4, this volume), even for experts, because of the high similarity between some species, as well as the fact that some *Meloidogyne* species have been poorly described (Eisenback, 1985). Olowe (2004) took a similar strategy in a survey of *Meloidogyne* spp. on cowpea in Nigeria but sampled both soil and roots from 248 farms (0.5–1.0 ha) with a spade to a depth of 10–15 cm. Again the samples were taken in a systematic zigzag path. The 'cores' from a farm were mixed and a 1.5 kg subsample taken. Rather than extract nematodes from the soil, galled roots were recovered by hand and females (a minimum of 30) recovered to be examined for perineal patterns. Samples without galled roots were bioassayed by planting a susceptible cowpea and looking for galling after 60 days. Using this approach, all fields sampled were found to harbour root-knot nematodes. The most commonly occurring species (*M. incognita*, *M. javanica*, *M. arenaria)* were identified and shown to vary with geographic location. The resulting information is being used to develop individual control strategies in different parts of Nigeria. These two examples illustrate similarities (sample pattern, sample area, composite sampling) and variability (core, sample and subsample sizes) in sampling methods commonly employed by different laboratories. They also illustrate the desirability of employing multiple detection methods, such as the use of a bioassay in conjunction with direct extraction of nematodes.

More intensive approaches can be taken, such as that of Fourie *et al.* (2001), who studied a range of nematode species (including *Meloidogyne* spp.) on soybean in South Africa. They sampled 17 locations containing replicated field trials of 30 different soybean genotypes each. Samples were taken by harvesting the roots and soil from 12 plants in each plot during flowering. A range of methods was then employed to examine the samples and for species identification and quantification. Nematodes from soil and roots were extracted by the sugar centrifugal–flotation method (Caveness and Jenson, 1955) or the maceration, sugar centrifugal–flotation method (Coolen and d'Herde, 1972), respectively. Nematode numbers were counted and some were fixed for species identification. A bioassay on tomato was also done to provide *Meloidogyne* specimens for species identity. Such a study was able to give more quantitative data about relative species abundance, rather than merely a qualitative assessment.

12.10.2 Field experimentation

When the objectives include determining the biology of the nematode/host interaction and ultimately investigating management strategies, sampling is required to evaluate experimental treatments (nematicide use, rotations, etc.). It is then most likely that sampling for nematodes will occur at least twice, pre- and post-planting or treatment, to estimate effectively the responses to treatments and sometimes to measure density-dependent population change. With root-knot nematodes, where there can often be more than one generation in a growing season, more frequent sampling may be undertaken.

In order to compare galling and yield on a number of soybean cultivars with a view to

developing management strategies, experiments were conducted in a field naturally infested with *M. arenaria* (Kinloch *et al.*, 1987). Nematode population density in the soil was determined immediately before planting and at harvest. Seven soil cores 2.5 cm diameter and 20 cm deep were taken from a 15-cm-wide band along the centre row of each plot. The cores were mixed and the nematodes extracted from a 100 cm^3 sample (15% of the total) by centrifugal flotation (Jenkins, 1964). Similar methods were used to study pre-planting and end-of season population levels of *M. incognita* during 8 years of various soybean–maize rotations (Kinloch *et al.*, 1985; Kinloch, 1986). These studies showed that in most situations there was a significant correlation between galling and yield, and between pre-plant densities, crop yield and final nematode densities.

A study of winter cover crops by McSorley and Gallaher (1992) compared nematode population growth during the summer on forage crops of corn, sorghum, soybean, cowpea and velvet bean at several sites in north Florida. They had seven experiments at seven different sites, which differed in the cover crops maintained on them during the winter of 1990/1 (wheat, rye, lupin, clover, crimson clover, hairy vetch and fallow (no crops or weeds)). Individual plots comprised four rows, 3.0 m long and 76 cm apart. Soil samples for nematode analysis were collected by removing and compositing six cores 2.5 cm deep × 20 cm long (*c.* 600 cm^3). Sampling took place at planting and post harvest. Nematodes were extracted from 100 cm^3 (17% of the total) subsamples with a modified sieving and centrifugation procedure. Of the nematodes observed in this study, *M. incognita* was the most suitable target for management by crop selection (velvet bean and resistant cowpea) and population growth on all crops was inversely density-dependent. McSorley and Gallaher (1993) extended this work, investigating maize and sorghum as cover crops using similar sampling methods They went on to pool their data from these field trials and were able to demonstrate that the relationship between $\log_n (P_f/P_i)$ and $\log_n (P_i)$, where P_f is the final population density and P_i is the initial population density, adequately estimated parameters in the population dynamics model of Seinhorst (1966).

Johnson and Campbell (1980) evaluated 31 crop-rotation systems during 5 years to determine the effects of cropping sequences, and of the nematicide fensulfothion, on nematode population densities of tomatoes seeded in the field, where the tomato plants were then harvested to be transplanted elsewhere. The soil was naturally infested with *M. incognita*, *M. javanica*, *Macroposthonia ornata*, *Paratrichodorus minor* and *Pratylenchus* spp. Soil samples (800 cm^3) were taken in February, June and October each year, beginning in June 1972 and continuing until June 1976. Soil samples consisted of a composite of 20 cores (2 cm diameter × 20 cm deep) collected randomly throughout the plot from the root zone of plants in each replicate. A 150 cm^3 (*c.* 19%) aliquot for each replicate was processed by a centrifugal–flotation method. Extracted nematodes were placed in calibrated dishes for identification and counting. In addition to the soil sampling, plants were selected randomly from each plot and examined for root galls. Roots were washed in tap water and indexed on a scale of 1–5. Immediately after the tomato plants were harvested, summer cover crop treatments were imposed on each plot. Sampling methods in this study were sufficient to demonstrate the inadequacy of the nematicide to control the nematodes, and demonstrated the need for extensive rotation between crops of transplant tomato to guarantee plants that meet certification requirements.

A common approach to investigating the biology of the nematode/host interactions is to examine the relationship by fitting models, usually between yield and initial and/or final nematode population density (P_i and or P_f). In order to determine the economic threshold level for oriental melon production, Kim and Ferris (2002) examined the relationship of the yield of oriental melon (*Cucumis melo* L. cv. Geumssaragi-euncheon) grafted on to cv. Shintozoa (*Cucurbita maxima* × *Cucurbita moschata*) to population densities of *M. arenaria*. In order to obtain a range of P_i values of *M. arenaria*, plots were established by either no cultivation or continuous cultivation of oriental melon during the summer, and by addition of non-infested soil in autumn the year before the main trial. Pre-plant P_i levels were determined 4 months and 1 month before planting. Post-plant population densities were measured 2 and 4 months after planting. Composite samples consisted of 14 soil cores per plot (3 m of bed length). J2 of *M. arenaria* were extracted from 300 cm^3 (20%) of each sample by sugar-flotation–sieving (Southey, 1986). Due to

the low egg densities before planting, these were not assessed. Advisory threshold levels were estimated from the study because the resulting data were adequate to explain 42–45% of the variability of fruit yield either regressed against log-transformed initial numbers of nematodes in each plot, or fitted to Seinhorst's (1965) damage function as the model of the relationship between relative yield and the initial nematode population levels (Fig. 12.3). The data were also robust enough to reveal a strong relationship between population growth rate and initial densities (Fig. 12.4). By comparison, Ploeg and Phillips (2001) also examined the relationship between pre-planting densities of *M. incognita* and yield of melons (*Cucumis melo*) cv. Durango using both pot tests and a field trial. The field trial area was divided into four blocks of three 18.3-m-long beds. Markers were placed at 61 cm intervals in the centre of each bed and a composite soil sample consisting of three cores was collected from around each marker for nematode extraction. Nematodes were extracted from 100 g (10–20%) soil subsamples in a modified Baerman funnel technique (Rodriguez-Kabana and Pope, 1981). Melon growth in pots was well described by the Seinhorst (1965) damage function. However, despite a relatively intensive level of sampling in the field trial, just 26% of the variation was accounted for by the Seinhorst (1965) yield loss model (Fig. 12.5) and only 3% of the total variation in final population densities was accounted for when attempting to fit the Seinhorst (1967) population model to the data (Fig. 12.6).

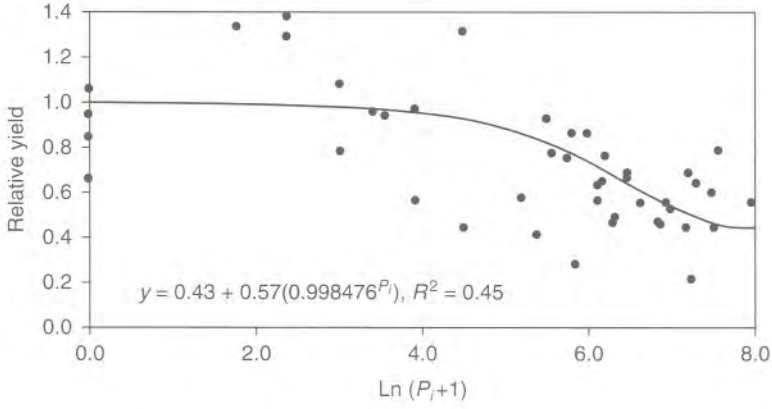

Fig. 12.3. The relationship between relative yield of oriental melon and initial nematode population density (P_i). (From Kim and Ferris, 2002.)

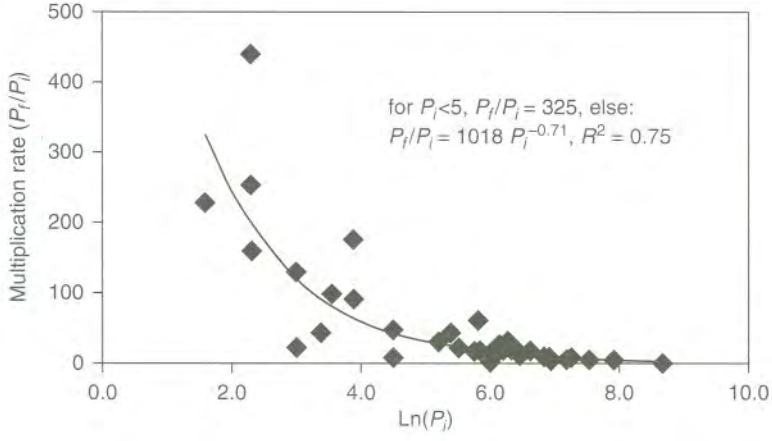

Fig. 12.4. The relationship between nematode multiplication rate and initial population density (P_i). (From Kim and Ferris, 2002.)

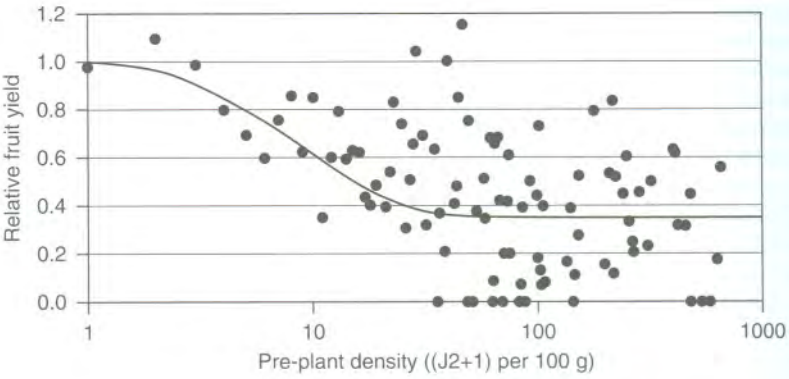

Fig. 12.5. The relationship between relative fruit yield of melon and initial (P_i) population density (second-stage juveniles/100 g soil). (From Ploeg and Phillips, 2001.)

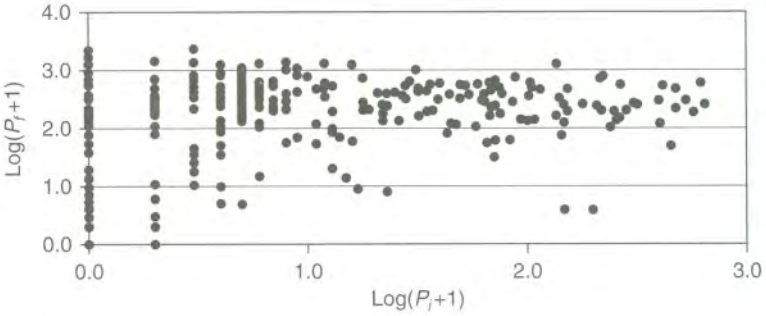

Fig. 12.6. The relationship between final (P_f) and initial (P_i) population density (second-stage juveniles/100 g soil). (From Ploeg and Phillips, 2001.)

Setting out to determine the effect of different crop rotations on *M. incognita* and *P. penetrans*, Kratochvil *et al.* (2004) conducted randomized trials in Maryland. To increase sample size and improve sampling technique to account for the spatial variability likely to be encountered, they split their plots into three subplots for sampling purposes, and took 20 soil cores from each. A composite sample of 500 cm^3 was then produced. Sampling of soil and roots amounted to a pre-planting, during-plant growth and post-harvest sample. Half of each sample was used for extraction of nematodes using a modified Baermann method. Just before harvest, root samples from five plants chosen at random from each subplot were assessed for root galling. Although the sampling was concentrated between the spring and autumn, it was possible to obtain some idea of the population density fluctuations under the different management treatments applied (Fig. 12.7).

Sampling more frequently can give a more complete picture of seasonal population change. Johnson *et al.* (1998) were interested in looking at rotations made up of combinations of cotton and soybean with triticale with and without nematicide treatment (fenamiphos). Field plots were established in an area naturally infested with *M. incognita* race 3, *P. brachyurus* and *Helicotylenchus dihystera*. The soil was sampled monthly, with 20 cores taken from the rows of each subplot. Nematodes were extracted from a 150 cm^3 subsample with centrifugal flotation (Fig. 12.8). Twenty plants were also dug from each plot and rated for root galling by *M. incognita* on a scale of 1 to 5 (Barker *et al.*, 1986). Similarly, Thomas and Clark (1983) conducted experiments in 1979 and 1980, sampling every 30 days during the growing

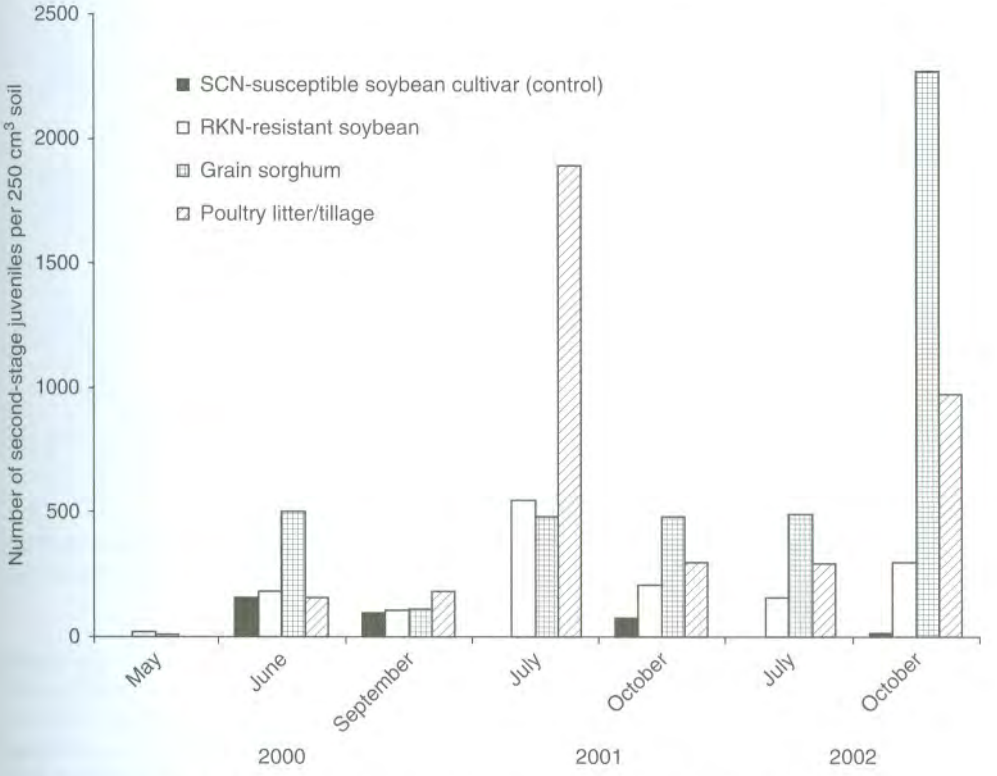

Fig. 12.7. Numbers of second-stage juveniles of root-knot nematodes per 250 cm^3 soil for seven sampling dates and four crop (or tillage) treatments. SCN = soybean cyst nematode; RKN = root knot nematode. (Data from Table 2 in Kratochvil *et al.*, 2004.)

season, to determine if an interaction exists between *R. reniformis* and *M. incognita* and, if so, the effects of this on yield and quality of sweet potatoes. Soil samples were taken approximately every 30 days using a 2 cm diameter soil probe, with five probes per plot to a depth of 15–20 cm, yielding approximately 250 cm^3. Unlike most other studies, the juveniles of *M. incognita* and the juveniles + young adults of *R. reniformis* were extracted from entire samples in a semi-automatic elutriator (Jenkins, 1964). In field plots with a high natural population density of *R. reniformis*, artificial infestations with high levels of *M. incognita* in both fumigated and non-fumigated treatments inhibited *R. reniformis*, while the final *M. incognita* juvenile population density was not affected. The results indicate that a competitive interaction exists, with each species capable of inhibiting the other and becoming the dominant population.

Even more intensive sampling over time was done by Pinkerton *et al.* (1991), who set out to determine the relationship between degree-day accumulation and population dynamics of *M. chitwoodi* in soil and potato tubers. Soil was sampled monthly during the overwintering period from harvest through to planting each year, and every 14 days during the growing season. Root samples from two plants selected from each plot were collected and examined for egg masses on each sampling date, until egg masses were detected (Dickson and Struble, 1965). J2 were extracted from 250 cm^3 soil samples (17% of the total) by wet sieving–centrifugation, and counted, thus allowing very detailed analysis of the relationship between temperature and seasonal population dynamics. This kind of study can have practical implications in terms of management, i.e. time of harvest, but could also be valuable in considering the effects of global warming on the potential distribution *M. chitwoodi* (Fig. 12.9). Samples of field soil were also collected at planting and harvest each season, and bioassayed with

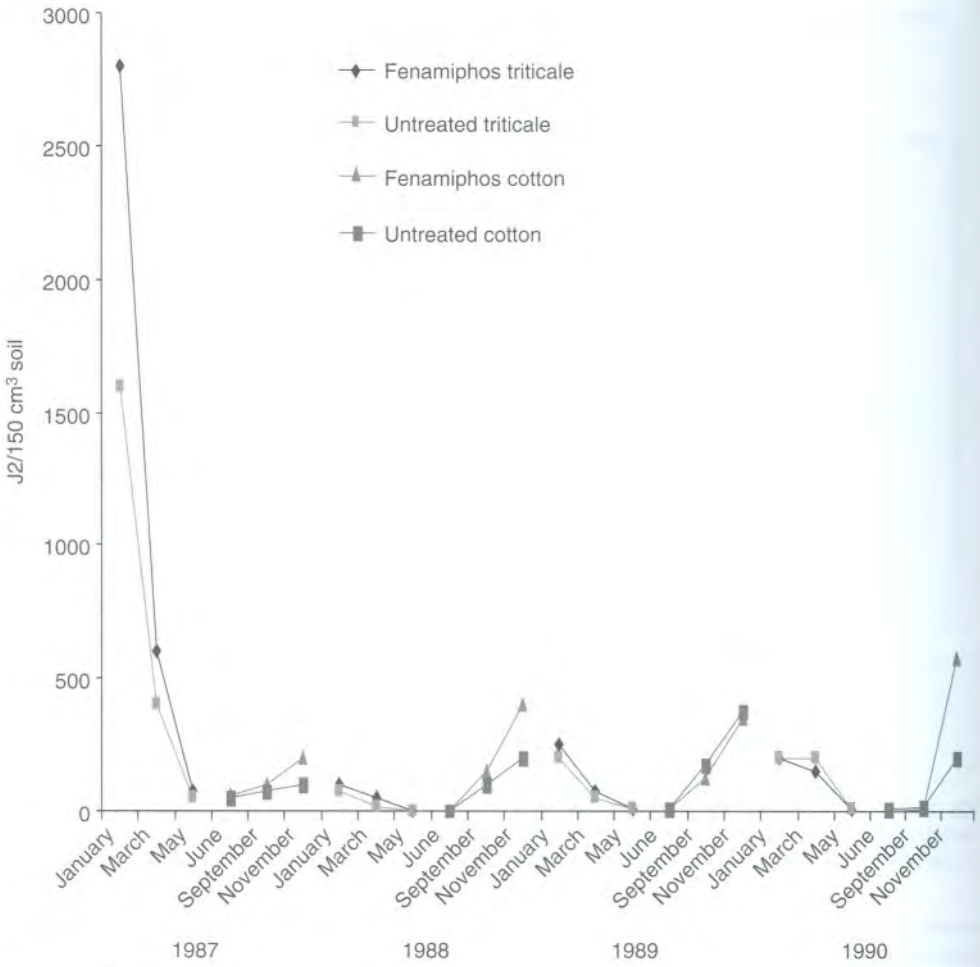

Fig. 12.8. The fluctuations of second-stage juveniles of root-knot nematodes per 150 cm^3 soil both temporally and with crop rotation, with or without nematicide. (Modified from Fig. 1 in Johnson *et al.*, 1998.)

a 3-week-old Columbia tomato plant to determine nematode infectivity. Plants were harvested after 21 days, the roots were stained (Byrd *et al.*, 1983) and the nematodes in the roots were counted. Five tubers from each plot on each sampling date were carefully scrubbed with a nylon scouring pad to remove the epidermis. A series of slices (0.75–1.5 mm thick) was cut tangentially from the tuber surface through the vascular ring. Approximately 5 g tissue per tuber was collected from four surfaces of each tuber. The slices were soaked in 1.5% NaOCl for 5 min, rinsed in tap water for at least 30 min, and stained. By combining the bioassay with the soil sampling, Pinkerton *et al.* (1991) were able to provide a detailed etiological record of the temporal development of all stages of nematode (J2, swollen J2 through to fourth-stage juveniles, females, and females with egg masses) in the plant parts and soil.

Sampling for nematodes in relation to biological control organisms, such as *Pasteuria penetrans* (see Hallmann *et al.*, Chapter 17, this volume), presents the same questions, as it is usual that the sampling is for the nematodes rather than the endospores. Chen *et al.* (1994) studied a mixed population of *M. incognita* and *M. javanica* in a tobacco field where the severity of root-knot in tobacco had decreased over time. *Pasteuria*

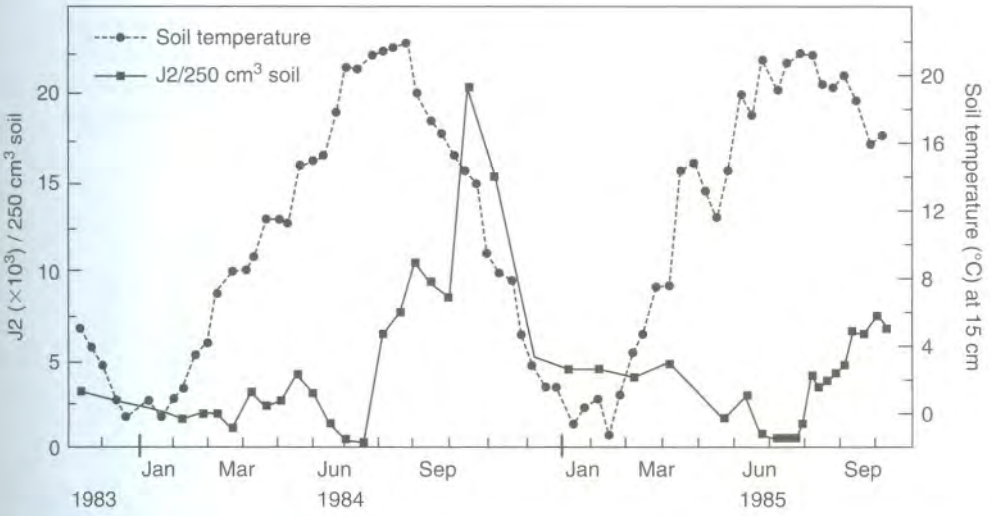

Fig. 12.9. The seasonal population dynamics of *Meloidogyne chitwoodi* and soil temperature 15 cm deep in an eastern Washington potato field. (From Fig.1 in Pinkerton *et al.*, 1991.)

penetrans was observed attached to J2 of *Meloidogyne* extracted from the soil. The site had been planted to tobacco continuously for 6 years prior to experimentation, with factorial design including different management strategies. Soil and root samples were taken with a bucket auger (10 cm diameter) from each plot in the root rhizosphere, to 25 cm depth. Soil was sampled four times through the growing season. The soil was mixed, and a 100 cm^3 (<2% of the total) subsample was taken from each sample to extract nematodes by a centrifugal-flotation technique. The numbers of J2 of *Meloidogyne* per 100 cm^3 soil were determined and the numbers of endospores attached to the cuticles of J2 were counted from 20 J2 per sample with an inverted light microscope.

Cetintas and Dickson (2005) also studied *P. penetrans*, but focused on its vertical distribution in field soil at a groundnut field in Florida that had been used to investigate the persistence and suppressiveness of *P. penetrans* to *M. arenaria*. Three different crop regimes were used. The soil was sampled annually for 4 years at five depths, down to 75 cm. Five cores per depth were taken using a bucket auger (10 cm diameter). One litre of soil at each depth was combined and mixed, and nematodes were extracted by centrifugal-flotation. The number of *P. penetrans* endospores on the first 20 J2 of *Meloidogyne* spp. observed was counted (Fig. 12.10).

Wesemael and Moens (2008) wanted to examine the importance of vertical distribution of *M. chitwoodi* in different rotations of field-grown vegetables. They chose two naturally infested fields and selected a 20 × 2 m plot in each. The plots were sampled every 3 or 4 weeks for 2 years. Fifteen soil cores 2.5 cm diameter × 70 cm were taken; each was divided into 10 cm segments and the corresponding segments were pooled. They found that the vertical distribution was consistent within each field irrespective of crop, suggesting that the main factors involved in any differences were soil type.

Investigating the vertical distribution of *M. arenaria* in a groundnut field, Rodriguez-Kabana and Robertson (1987) sampled soil over a 15-month period. They used a 5 cm diameter, hydraulically operated cylindrical probe to obtain cores 1 m long. They then cut this into 20 cm sections to produce a model of the depth distribution of the nematode (Fig. 12.11).

The results from soil sampling are usually obtained by extracting nematodes from the soil in some way and counting them, and sometimes by using the soil to conduct a bioassay. For example, a simple, visual soil bioassay was developed to assess *M. hapla* soil-infestation levels in order to facilitate the implementation of management practices (Gugino *et al.*, 2008). This involved the evaluation of the galling of lettuce as a bioassay plant in pots representing

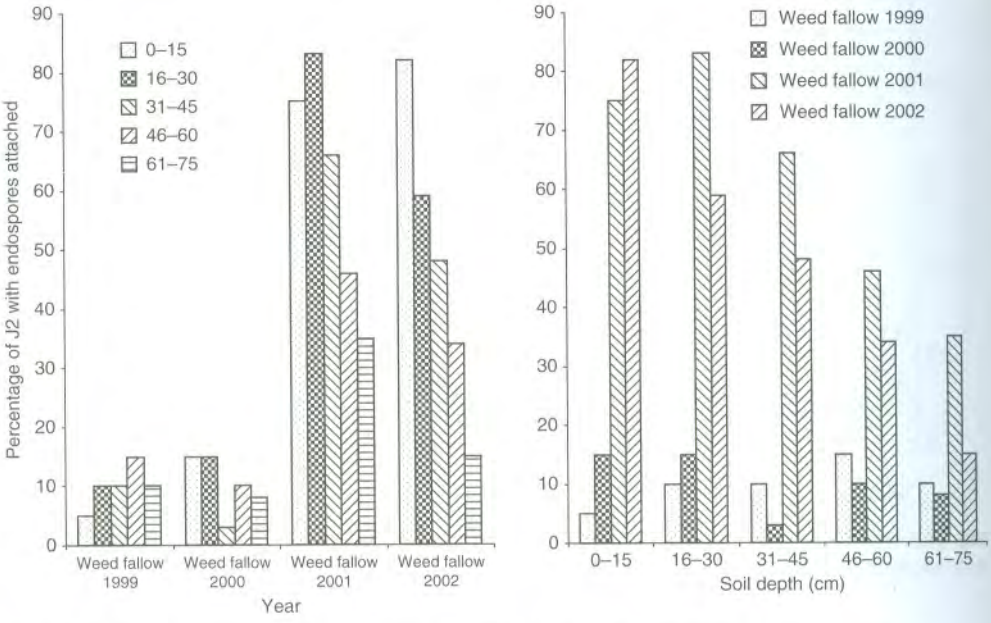

Fig. 12.10. The percentages of second-stage juveniles (J2) of *Meloidogyne* spp. with *Pasteuria penetrans* endospores attached and average numbers of endospores attached per J2 (as determined by soil extraction) from soil collected at five soil depths during groundnut harvest for the years 1999 to 2002 following 9 years of weed fallow. (Data from Table 2 in Cetintas and Dickson, 2005.)

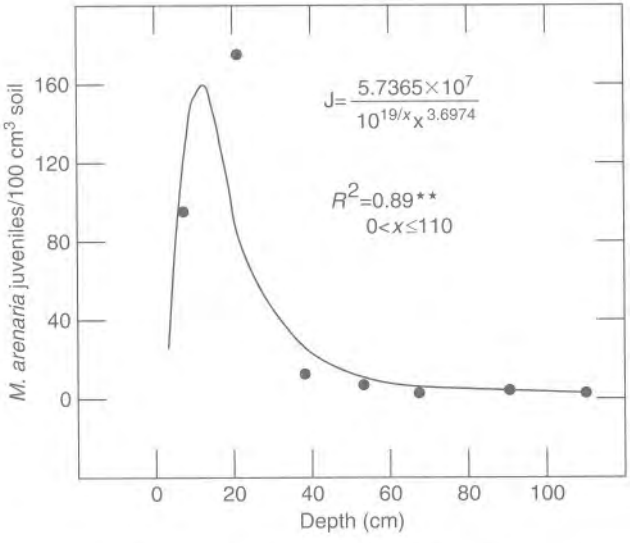

Fig. 12.11. The numbers of juveniles of *Meloidogyne arenaria* in a groundnut field as a function of soil depth. J = number of juveniles; x = soil depth. (From Fig. 2 in Rodriguez-Kabana and Robertson, 1987.)

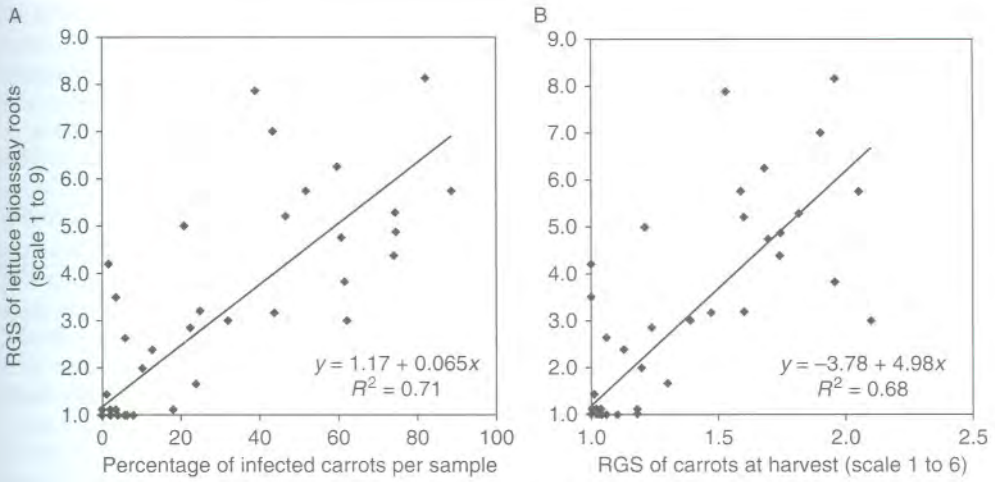

Fig. 12.12. The relationship between the percentage of infected carrot roots (A) and root-galling severity (RGS) (B) of mature carrots collected from 33 commercial fields over 3 years compared with the RGS ratings obtained from the glasshouse soil bioassay with lettuce conducted with corresponding composite soil samples. (From Fig. 2 in Gugino *et al.*, 2008.)

four composite soil samples per field. This proved an effective way of relating nematode infestation to disease levels on carrot (Fig. 12.12).

12.11 Conclusions and Future Directions

The familiar disease triangle concept – that crop loss occurs only when a host and pathogen coexist in an appropriate environment – reflects an aspect central to some current efforts to extend the utility of sampling nematode populations. When Australian pineapple growers lost access to ethylene dibromide, they relied increasingly on sampling programmes to make informed decisions on the need for and efficacy of new nematode management tactics. Stirling and Pattison (2008) point out that 'as with many monitoring programmes for crop pests', growers eventually submitted fewer samples each year as they began to recognize the edaphic conditions conducive to damage by nematodes. In some soils, the numbers of root-knot nematodes may be periodically above or below threshold densities, whereas in others they may never merit management. Elucidating soil factors that favour population growth by different nematode species should permit the adoption of site-specific management of nematodes using existing GPS (global positioning system)-based, variable-rate technologies that are currently uneconomic if nematode populations must be assessed directly (Wheeler *et al.*, 1999; Wrather *et al.*, 2002; Avendaño *et al.*, 2004). Indeed, technologies that integrate GPS to monitor variables, such as crop yield, plant height, canopy density, aerial reflectance and bulk soil electrical conductivity, continuously in real time are increasingly exploited to find relationships between soils, nematodes and crop damage (Ortiz *et al.*, 2007). These technologies have dramatically reduced the cost of collecting large data sets to develop soil and yield maps that can also be used to select experimental sites with the appropriate levels of variation in which to study nematode spatial patterns. For example, bulk soil electrical conductivity (EC_a) was inversely related to clay content and population density of *M. incognita* in Louisiana cotton fields (Wolcott *et al.*, 2004). In fields with multiple nematode pests, the spatial patterns of *M. incognita* that aggregate in coarse-textured soil could be distinguished from those of *R. reniformis*, found mainly in finer-textured soil, as estimated by EC_a (Overstreet *et al.*, 2009). These relationships permitted delineation of nematode management zones in fields based on the mean

EC_a value of the various zones (Fig. 12.13). Nematicide application was shown to increase yield more in management zones with low EC_a that contain large numbers of *M. incognita* than in zones with greater clay content and few root-knot nematodes (Overstreet *et al.*, 2005). Some growers are now attempting to adopt this technology to identify parts of fields, without the need for nematode sampling, in which management of root-knot nematodes is not profitable (C. Overstreet, 2009, personal communication). Engineering advances that improve the efficiency and precision of measuring edaphic and plant variables are increasingly likely to leverage research and extension efforts to estimate nematode spatial patterns in fields.

Grower impetus to quantify root-knot nematodes in conducive habitats will increase with advances in the variety and quality of information available from individual soil samples, and in response to the increased cost or reduced availability of chemical controls. The theoretical basis exists to relate sampling intensity to control costs and loss-prediction reliability, but the usefulness of such approaches is constrained by the high cost of sampling (Ferris, 1984; Ferris *et al.*, 1990). The ability of molecular methods, particularly real-time PCR, to identify and quantify root-knot nematodes in samples of nematodes extracted from soil with precision as good as or better than conventional methods is well established (Qiu *et al.*, 2006; Adam *et al.*, 2007; Berry *et al.*, 2008; Toyota *et al.*, 2008). This approach largely obviates the need for taxonomic expertise in diagnostic laboratories, and has tremendous potential for increased throughput via mechanization. Further cost reduction can be achieved by technical improvements in methodology, such as the design of lower-cost probes and the development of multiplex systems to quantify multiple nematode species in addition to other types of soil-borne pathogens (Ophel-Keller *et al.*, 2008). Given the promise shown by these new technologies to detect and infer nematode spatial patterns in order to predict the need for resistant planting material, or to guide the precision application of other control measures, it would seem likely that the development of mechanized sampling and extraction methods will receive renewed attention.

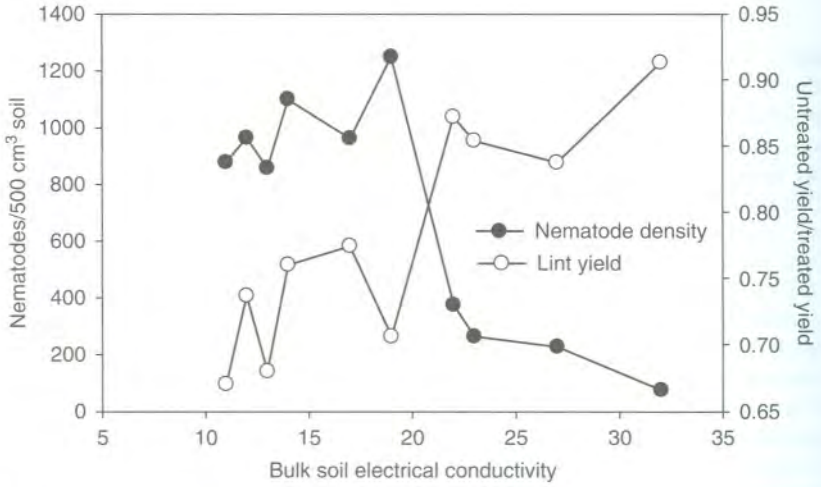

Fig. 12.13. The relationship between mean population density of *Meloidogyne incognita*, cotton yield response to treatment with Telone and mean bulk soil electrical conductivity in a 32-ha field in northeastern Louisiana infested with root-knot nematode. (Modified from Overstreet *et al.*, 2005.)

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