Diversity, phylogeny, characterization and diagnostics of root-knot and lesion nematodes

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Chapter 1

General introduction

1 Organisms under study: plant-parasitic nematodes

Nematodes (Nematoda), commonly referred to as round worms are a species-rich phylum within the Ecdysozoa, a monophyletic clade grouping all Metazoa with a exoskeleton which can be shed during a process called ecdysis (Telford *et al.*, 2008). The most well-known nematode is probably *Caenorhabditis elegans* which is a widely used model organism in molecular- and developmental biology (Eisenmann, 2005). Besides *C. elegans*, the phylum Nematoda contains 25,043 described species (Zhang, 2013), however, this number is known to be a serious underestimation as many nematode clades remain poorly studied. Yet, nematodes are known to be the most abundant multicellular animals on earth (Bongers & Ferris, 1999). The vast majority of nematodes commonly referred to as free-living nematodes feed on bacteria, fungi, protozoa, algae and/or other nematodes. These free-living nematodes are ubiquitous in one or more freshwater, marine or soil ecosystems. Besides free-living species the phylum contains an astonishing diversity of parasitic lifeforms. Various nematodes have evolved to parasitize an extremely wide spectrum of hosts, including a wide variety of animals (including humans) and virtually all species of vascular plants. Currently, around 4000 nematode species have been described as plant-parasites (Decraemer & Hunt, 2013). Interestingly, plant-parasitism is polyphyletic and has evolved independently in each of the three major clades of Nematoda (Fig. 1); i.e. Enoplia, Dorylaimia and Chromadoria (Blaxter *et al.*, 1998; Dorris *et al.*, 1999; Baldwin *et al.*, 2004). More specifically plant-parasitism is found within Trichodoridae, Longidoridae and Tylenchomorpha. According to recent studies several of these plant-parasite groups probably constitute several separate origins of parasitism (Quist *et al.*, 2015; Sánchez-Monge *et al.*, 2017). Interestingly, plant-parasitism appears to have evolved several times independently from fungivorous ancestors (Holterman *et al.*, 2006). The majority of plant-parasitic nematode species is found within the Tylenchoidea and within this group plant-parasites can be classified in three feeding types: (migratory) ectoparasites, migratory endoparasites and sedentary endoparasites. In this thesis we will focus on the genera *Pratylenchus* and *Meloidogyne*, the economically most important genera of migratory endoparasites and sedentary endoparasites, respectively. Interestingly, according to molecular phylogenetic data the sedentary endoparasitic nematodes of the genus *Meloidogyne* have evolved from migratory endoparasites, as the monophyletic genus *Meloidogyne* is embedded within the paraphyletic genus *Pratylenchus* (Bert *et al.*, 2008).

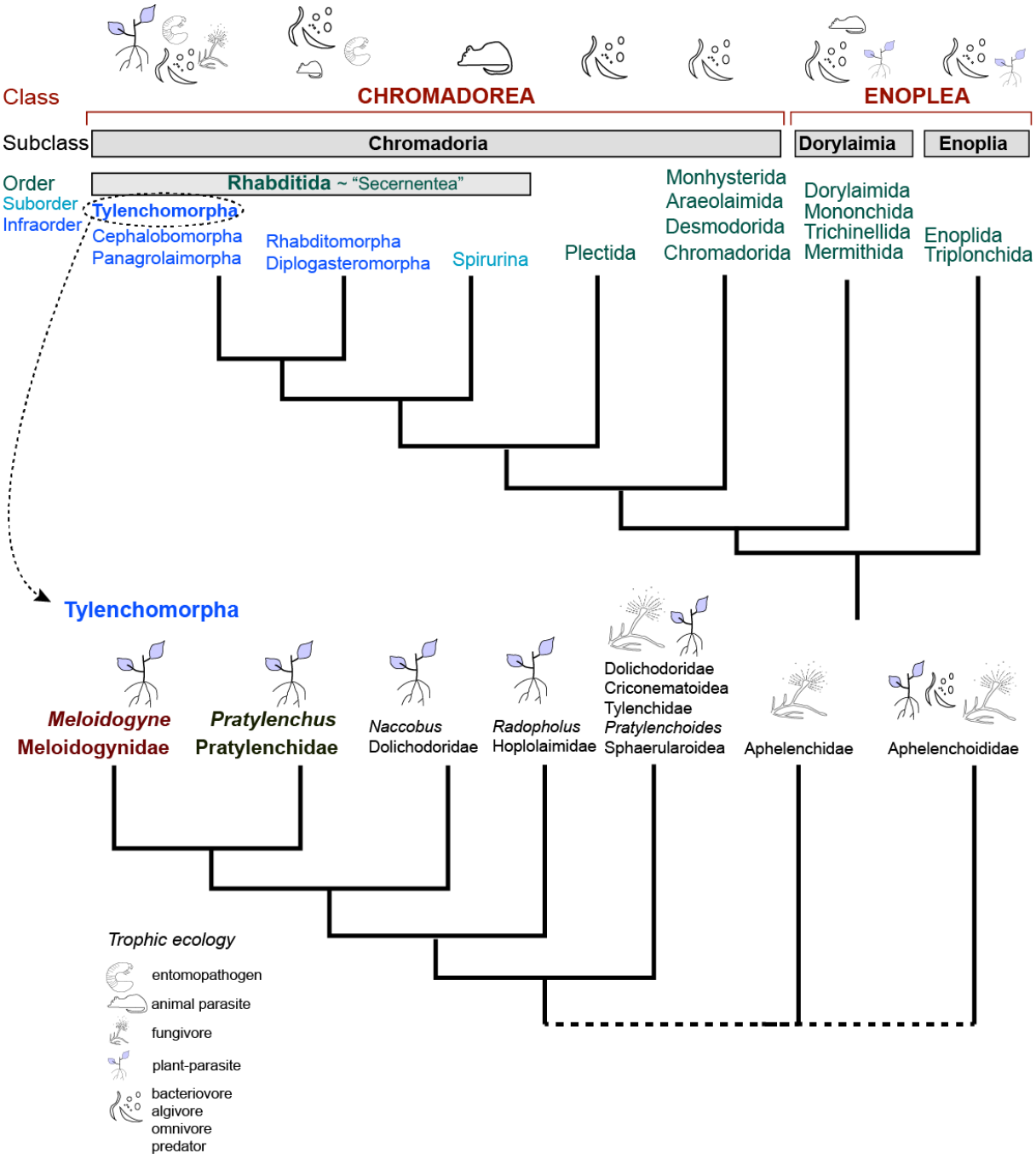


Figure 1. Above: overview of the phylogenetic relationships within the phylum Nematoda. Below: simplified phylogeny of the infraorder Tylenchomorpha, showing the phylogenetic position of the genera *Pratylenchus* and *Meloidogyne*. The different origins of parasitism are shown besides the branches of both trees. Figure modified from Bert *et al.* (2011).

1.1 Pratylenchus: root-lesion nematodes

The genus *Pratylenchus* was established by Filipjev (1936). Diagnostic characters include a pseudo-mono-prodelphic gonoduct, a tylenchoid pharynx with anterior intestine overlap and a typical head and stylet shape (Filipjev, 1936; Castillo & Vovlas, 2007; Geraert, 2013). The type species of the genus is *Pratylenchus pratensis*, which was first described as *Tylenchus pratensis* by de Man (1880). Ever since then the number of species has gradually increased, however, the exact number of species is dependent on the taxonomic interpretation followed (Ryss, 2002b; Ryss, 2002a; Castillo & Vovlas, 2007; Geraert, 2013). In 2013, 98 species were recognized by Geraert (2013) and after which, three additional species have been described: *Pratylenchus oleae* (Palomares-Rius et al., 2014), *Pratylenchus quasitereoides* (Hodda et al., 2014), and *Pratylenchus parazeae* (Wang et al., 2015), bringing the total species number to 101 morphologically-defined species. An overview of the general morphology of the genus *Pratylenchus* can be found in figure 2. A detailed account of *Pratylenchus* morphology can be found in Castillo & Vovlas (2007).

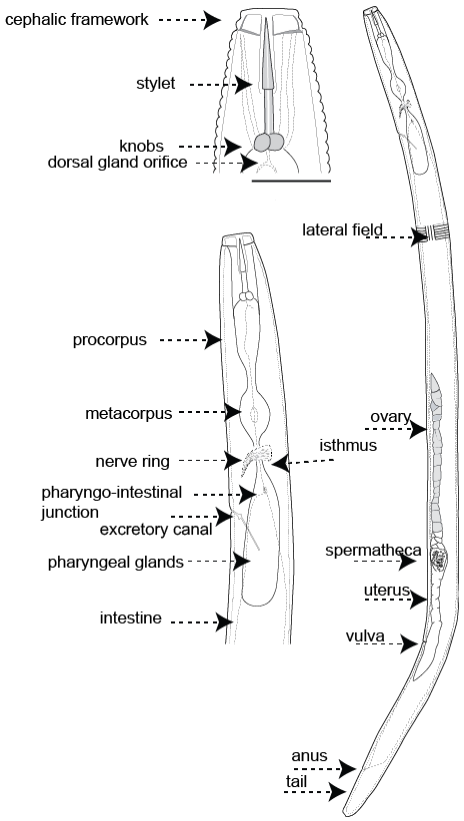


Figure 2. Schematic overview of the morphology of the genus *Pratylenchus* and Pratylenchidae in general. Figure modified from Bert & Borgonie (2006).

The typical live cycle includes four juvenile and an adult stage (Fig. 3). The first-stage juvenile (J1) molts into a second-stage juvenile (J2) inside the egg, subsequently this J2 hatches from the egg. The second-stage juveniles are like all other juvenile and adult stages motile and able to enter root tissue. Specimen move intracellular and are able to puncture plant cells and ingest cytoplasmic content using their hollow stylet (Zunke, 1990). During feeding the median bulb acts as a suction device while the pharyngeal lobe is thought to play a crucial role in the digestion of the cytoplasmic plant material (Zunke, 1990; Castillo & Vovlas, 2007; Jones & Fosu-Nyarko, 2014). Once the nematode reaches the adult-stage it becomes sexually active and will produce eggs as a result of sexual or parthenogenetic reproduction. The duration of a complete lifecycle is largely dependent on environmental conditions but ranges between 3 to 9 weeks (Jones & Fosu-Nyarko, 2014). Infected roots are characterized by brown lesions and necrotic areas, usually these lesions are caused by a combination of nematode feeding and secondary infections by bacteria or fungi, which benefit from the nematode induced damage in order to enter the plant tissue (Castillo & Vovlas, 2007; Jones & Fosu-Nyarko, 2014). According to molecular phylogenies the genus *Pratylenchus* is paraphyletic, sharing a monophyletic clade with the genera *Apratylenchus*, *Zygotylenchus* and *Meloidogyne* (Trinh et al., 2009; Palomares-Rius et al., 2010*)*. However, this paraphyletic relationship needs to be confirmed using phylogenomic data as relationships are generally poorly supported. Within the genus phylogenetic relationships have been elucidated mainly using ribosomal markers (Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010; De Luca *et al.*, 2011; Palomares-Rius *et al.*, 2014; Rybarczyk-Mydlowska *et al.*, 2014). One of the monophyletic groups embedded within the paraphyletic genus *Pratylenchus* is the genus *Meloidogyne*.

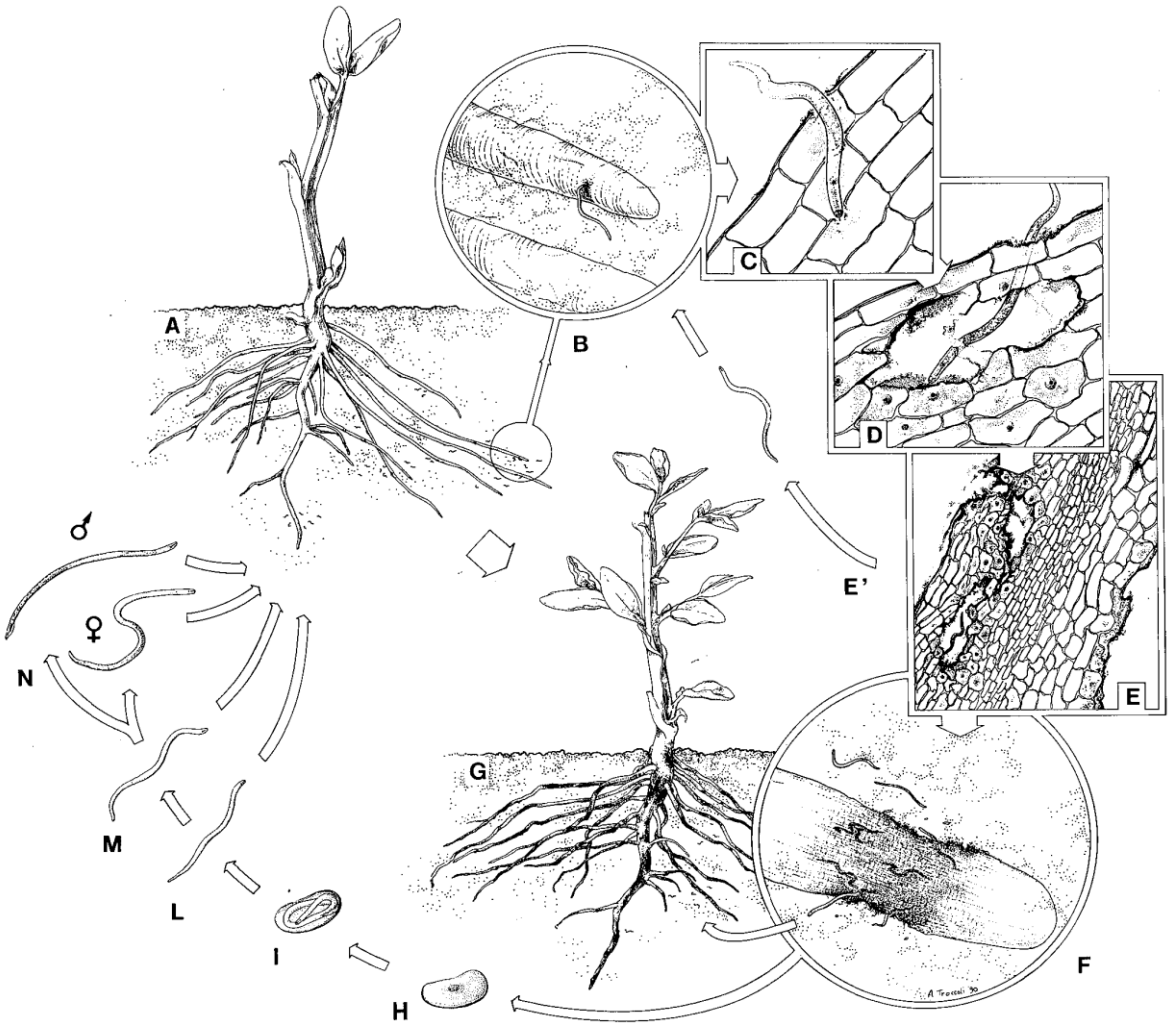


Figure 3. Schematic representation of the life cycle of *Pratylenchus penetrans* on faba bean. A: root system of healthy plant; B-D: Nematode penetration on roots; E, F: Cortical damage; G: Infected roots system necrotic lesions; H, I: Eggs; L, M: Juvenile specimens; N: adult specimens. After Vovlas & Troccoli (1990).

1.2 Meloidogyne: root-knot nematodes

Root-knot nematodes from the genus *Meloidogyne* are a cosmopolitan group of obligate plant parasites, able to parasitize virtually all species of higher plants. The genus was established by Göldi (1887), who described *M. exigua*, the type species of the genus. Formerly, root-knot nematodes and cyst-forming nematodes were placed within the Heteroderidae. However, based on morphological and later molecular data it became apparent that both groups of sedentary

endoparasites were the result of convergent evolution (Siddiqi, 1986; Jepson, 1987; De Ley & Blaxter, 2004). As a result the genus *Meloidogyne* was moved to a new subfamily, the Meloidogyninae Skarbilovich, 1959. The genus is now firmly established as a monophyletic clade in close relationship with the Pratylenchidae (Holterman *et al.*, 2009; van Megen *et al.*, 2009; Rybarczyk-Mydlowska *et al.*, 2014). Excellent reviews of the general morphology of species of *Meloidogyne* can be found in Whitehead (1968), Jepson 1987), Karssen (2002) and Eisenback & Hunt (2009). Currently, the genus *Meloidogyne* contains 101 described species, however, the species status of several species is debated (Karssen, 2002; Karssen *et al.*, 2012; Ahmed *et al.*, 2013). Traditionally, three major clades are recognized within the genus (Tigano *et al.*, 2005). Clade I contains *M. enterolobii* and the so-called *Meloidogyne incognita* group (MIG) (Pagan *et al.*, 2015), which among other species includes *M. incognita*, *M. javanica* and *M. arenaria*. This clade contains closely-related polyphagous mitotic parthenogenetic species (except for *M. floridensis*) which are geographically widespread. Clade II comprises *M. hapla* as most important representative, a species primarily known from temperate climates (Tandingan De Ley *et al.*, 2002). Clade III contains among other species; *M. fallax*, *M. chitwoodi*, *M. naasi*, *M. graminicola* and *M. minor* (Holterman *et al.*, 2009). Besides the three main clades the genus includes several early diverging lineages including, *M. mali*, *M. ichinohei*, *M. artiellia*, *M. baetica*, *M. coffeicola* and *M. camelliae* (Castillo *et al.*, 2003; Holterman *et al.*, 2009; Tomalova *et al.*, 2012; Ahmed *et al.*, 2013; Trisciuzzi *et al.*, 2014).

The life cycle of a root-knot nematode (Fig. 4) includes 4 juvenile stages and an adult life stage (Castagnone-Sereno *et al.*, 2013). After embryogenesis the first-stage juvenile develops and molts into a second-stage juvenile (J2) inside the egg. Under favorable environmental conditions the second-stage juvenile hatches from the egg and represents the infective life stage. The J2 stage is motile and will invade root tissue, inside the root tissue the J2 will establish a permanent feeding site (Castagnone-Sereno *et al.*, 2013). The feeding of the J2 will transform protoxylem and protophloem plant cells into specialized giant cells (Karssen *et al.*, 2013). These giant cells are multinucleate cells that act as a nutrient sink for the plant. As a result of giant cell feeding the J2 stage will gradually increase in size as it moults into a J3, J4 and finally the adult stage. Females will produce hundreds of eggs which are deposited in a gelatinous egg-mass. When present, males are motile and vermiform, however, they are not actively feeding on plant tissue (Karssen *et al.*, 2013).

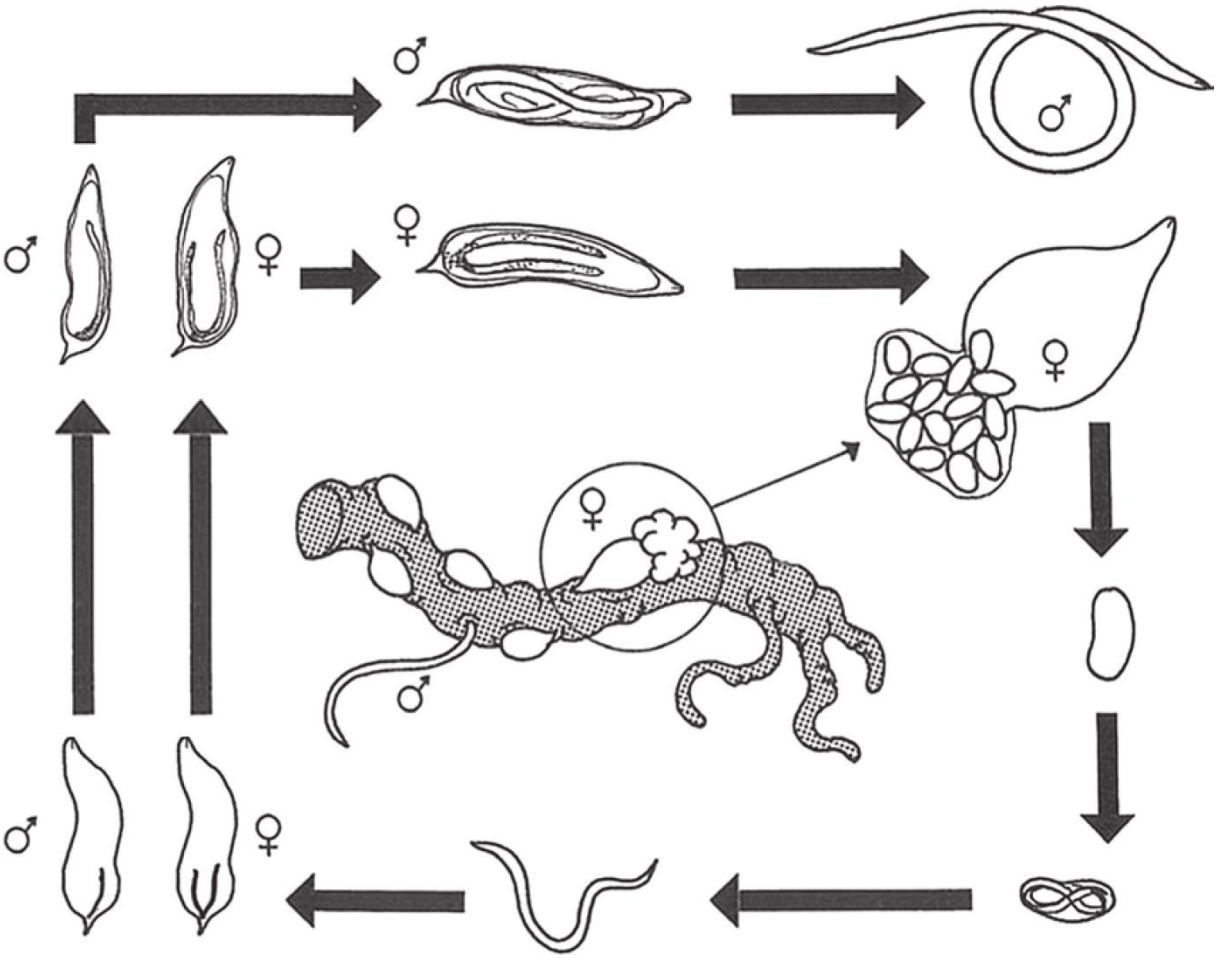


Figure 4. A schematic representation of the life cycle of *Meloidogyne*. Figure modified from Karssen & Moens (2006).

2 Economic importance

On the millennium summit of the United Nations, eight millennium development goals were established; the first goal was to eradicate extreme poverty and hunger by 2015. While many factors are involved, nematology can partially contribute to this issue (Ciancio, 2015). Indeed, plant-parasitic nematodes pose a serious threat to agriculture worldwide, moreover, plant-parasitic nematodes are considered to be an important limiting factor in vegetable protection, causing an elaborate use of pesticides in many areas (Sikora & Fernandez, 2005). While, data on the economic impact are scares and difficult to acquire, damage caused by plant-parasitic nematodes was estimated 77-80 billion dollars yearly (Sasser & Freckman, 1987; Nicol *et al.*, 2011). However, this number is generally believed to be a serious underestimation for several reasons (Sikora & Fernandez, 2005; Nicol *et al.*, 2011; Jones *et al.*, 2013; Ciancio, 2015): (i)

damage caused by plant-parasitic nematodes is in general less obvious in comparison to damage caused by other pathogens (for example fungi like *Fusarium* or damage caused by bacteria, viruses and insects can have very characteristic above ground symptoms) as it is mostly situated below ground while the above ground symptoms are nonspecific often resembling symptoms of nutrient deficiency; (ii) plant-parasitic nematodes can often act as a gateway for other pathogens such as bacteria, viruses and fungi; and (iii) in many developing countries there is a great unawareness of nematode problems among farmers. For this reason, nematode problems are especially significant in developing nations (Onkendi *et al.*, 2014), were the impact on food security can be severe (Ciancio, 2015).

In terms of economic loss the two genera dealt with in this thesis, root-knot nematodes and root-lesion nematodes rank as respectively first and third most important nematode species, cyst-nematodes are ranked second (Jones *et al.*, 2013). Plant-parasitic nematodes of the genus *Pratylenchus* are able to enter the root-system and migrate through the root and use their stylet to pierce plant cells and ingest the cytoplasmic content (Castillo & Vovlas, 2007). This causes the root to show typical root-lesions and eventually necrotic roots which are often found in combination with other soil-borne pathogens which can take advantage of the entrance in the root-system provided by the nematode (Jones & Fosu-Nyarko, 2014). In some cases, damage caused by root-lesion nematodes can cause a yield loss extending to 85% of the expected production (Nicol *et al.*, 2011). Several species of *Pratylenchus* such as *P. penetrans*, *P. brachyurus*, *P. coffeae* and *P. vulnus* have a wide geographical distribution and can parasitize a wide range of host plants, including a large variety of important economic crops (Castillo & Vovlas, 2007).

Endo-sedentary parasites of the genus *Meloidogyne* induce specific galling on plant roots. Root-knot-nematodes have a worldwide distribution and are able to parasitize almost all species of vascular plants (Trudgill & Blok, 2001). While most species are documented to be polyphagous, some early-diverging species are thought to be oligophagous (Holterman *et al.*, 2009). Damage caused by root-knot nematodes is partly dependent on environmental conditions and the considered hostplant. However, under favorable conditions many species have the potential of destroying complete harvests (Moens *et al.*, 2009; Nicol *et al.*, 2011; Seid *et al.*, 2015).

Overall, plant-parasitic nematode populations are difficult to control once established in a crop or field, both root-lesion and root-knot nematodes are known to parasitize a considerable range

of weed species, seriously complicating pest management (Belair *et al.*, 2007; Rich *et al.*, 2009; Kokalis-Burelle & Rosskopf, 2012). A wide variety of nematode control strategies is known. In the past nematode control was mainly achieved by using a variety of chemical nematicides in order to diminish nematode densities in the soil (Onkendi *et al.*, 2014). While chemical control is usually quit effective it greatly influences the complete soil ecosystem as it also kills beneficial nematodes and depending on the chemical other soil organisms, for this reason many chemical compounds used in the past have been taken out of production (Nyczepir & Thomas, 2009). However, the negative effects of chemical control can be limited when applied specifically (Cao, 2016). An interesting alternative for chemical control is genetic resistance breeding, this field was recently reviewed for plant-parasitic nematodes by Starr and Mercer (2009). However, while resistant varieties can be an efficient control strategy, resistance breaking is a profound problem often associated with root-knot nematodes (Onkendi *et al.*, 2014). In search for sustainable nematode control strategies, biological control using endoparasitic bacterial and fungal pathogens are being explored (Hallmann *et al.*, 2009). However, as infections of *Pratylenchus* and *Meloidogyne* are difficult to control once established in a field prophylactic measures remain of primary importance in plant management. In order to prevent infestation, several root-knot and root-lesion nematodes are quarantine species in many parts of the world. For example *Meloidogyne fallax*, *M. chitwoodi* and *M. enterolobii* are quarantine species in Europe according to the European Plant Protection Organization (EPPO), while *Pratylenchus goodeyi*, *P. convallariae* and *P. crenatus* are considered quarantine pathogens by the Florida Department of Agriculture and Consumers.

3 Identification of plant-parasitic nematodes

Correct pathogen diagnosis has proven to be of crucial importance in order to employ efficient control strategies (Onkendi *et al.*, 2014). Despite the importance of correct pathogen identification, both root-knot nematodes and root-lesion nematodes face similar identification problems. Traditionally, species from both genera were identified using morphological and morphometric characters, problematically, both genera are extremely conserved in morphology (Castillo & Vovlas, 2007; Hunt & Handoo, 2009). For the genus *Pratylenchus* frequently used morphological characters include head morphology, stylet shape, number of lip annuli, spermatheca shape, lateral field morphology and shape of the tail; a detailed account on the use of morphological characters in *Pratylenchus* can be found in Castillo and Vovlas (2007). Within

the genus *Meloidogyne* the perineal pattern (cuticle pattern around the vulva) is mostly used for species diagnosis, a comprehensive description on the morphological variation of perineal patterns can be found in Whitehead (1968), Jepson (1987) and Karssen (2002). Because of the limited amount of taxonomically useful characters, taxonomists have also explored Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy complemented with 3D reconstruction technologies in search for additional morphological features (Ragsdale & Baldwin, 2010). SEM visualization revealed that the lip pattern of *Pratylenchus* is a useful taxonomic feature in species identification (Subbotin *et al.*, 2008). SEM allows a more detailed study of the perennial pattern and head morphology in root-knot nematodes (Eisenback, 1991; Karssen, 2002). However, because of the limited amount of informative morphological characters, morphometrics have been widely used in nematode taxonomy. For *Pratylenchus* mainly morphometrics of females are used (Castillo & Vovlas, 2007; Geraert, 2013), while for *Meloidogyne* especially the morphometrics of second stage juveniles and males have proven to be useful for species identification (Whitehead, 1968; Jepson, 1987; Karssen, 2002).

Despite the tremendous efforts made by taxonomists in studying morphology, morphological identification remains greatly hampered by phenotypic plasticity and interspecific similarities (Roman & Hirschmann, 1969; Tarte & Mai, 1976; Castillo & Vovlas, 2007; Hunt & Handoo, 2009). These problems are clearly demonstrated by a recent study revealing the presence of different morphotypes within a single species, suggesting that several of the already-scarce morphological diagnostic features can be dependent on the reproductive strategy of a population (Troccoli *et al.*, 2016). Due to these limitations, morphological identification of root-knot and root-lesion nematodes is a time-consuming process that requires a great amount of expertise and high quality reference material. In order to complement morphology based identification, a wide variety of techniques have been proposed. Hartman and Sasser (1985) developed a technique based on differential host preferences in order to classify root-knot nematodes in different races. However, to date, no genetic, isozymatic, or cytogenetic basis has been established for these different host races, indicating that they do not, in fact, represent homological speciation events (Castagnone-Sereno *et al.*, 2013). Moreover, increasing the number of host plants has inevitably led to additional races (Robertson *et al.*, 2009). It has also been suggested that the virulence of *Meloidogyne* is mediated by epigenetic control (Perfus-Barbeoch *et al.*, 2014), thus rendering host specificity an inappropriate diagnostic technique.

In the 1970's Dickinson et al. (1971) and Dalmasso & Berge (1978) developed a biochemical-based diagnostic technique, reliant on isozyme profiles. Later Esbenshade and Triantaphyllou (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987) showed that variations in esterase and malate dehydrogenase isozyme profiles were extremely informative in differentiating most known species of *Meloidogyne* (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987; Carneiro *et al.*, 2000; Karssen, 2002). While the method is labor intensive and only applicable to young adult females it remains one of the most reliable and widely-used differentiation methods to date (Blok & Powers, 2009; Elling, 2013; Humphreys-Pereira *et al.*, 2014). Also for the genus *Pratylenchus* isozyme electrophoresis was explored as an identification strategy, however, it was never widely used because many specimens from the same species are necessary for an analysis (Ibrahim *et al.*, 1995; Andres *et al.*, 2000). Other biochemical techniques involving monoclonal or polyclonal antibodies appeared promising, yet additional research and validations are required before this method can be applied in practice (Ibrahim *et al.*, 1996). The first molecular diagnostic techniques were introduced by Curran *et al.* (1986), using restriction length polymorphisms (RFLP) of genomic DNA. Later a wide variety of molecular identification techniques have been developed, including satellite DNA (Castagnone-sereno *et al.*, 1995; Castagnone-Sereno *et al.*, 1999), restriction fragment analysis (Waeyenberge *et al.*, 2000; Pagan *et al.*, 2015), species specific primers in combination with gel electrophoresis (Zijlstra, 2000; Zijlstra *et al.*, 2000; Al-Banna *et al.*, 2004; Adam *et al.*, 2007), duplex PCR (Waeyenberge *et al.*, 2009) and qPCR (Zijlstra & Van Hoof, 2006; Mokrini *et al.*, 2013; Sapkota *et al.*, 2016). With the rapidly declining cost and improved availability of genetic sequencing and related technologies, theoretically, all the aforementioned methods could be replaced by DNA barcoding (Powers, 2004). In the context of phylogenetic analyses and identification the ribosomal gene cluster (18S, ITS, and 28S) has been widely used (Tandingan De Ley *et al.*, 2002; Tenente *et al.*, 2004; Tigano *et al.*, 2005; Subbotin *et al.*, 2008; Holterman *et al.*, 2009; Palomares-Rius *et al.*, 2010). Also mitochondrial genes including Cox1 and Cox2 (Kiewnick *et al.*, 2014) and the noncoding region between 16s and Cox2 were evaluated (Hugall *et al.*, 1994; Hugall *et al.*, 1997; Stanton *et al.*, 1997; Pagan *et al.*, 2015). In comparative phylogenetic analyses protein coding genes, such as RNA polymerase II, dystrophin, elongation factor 1-alpha and map1 have been used (Lunt, 2008; Tomalova *et al.*, 2012; Rybarczyk-Mydlowska *et al.*, 2014). These molecular identification

techniques and barcoding initiatives will be discussed in detail in the introduction of Chapter 2 for root-knot nematodes and in the introduction of Chapter 4 for root-lesion nematodes.

4 Variability in reproduction strategies and genome evolution

One of the most fascinating features of root-knot nematodes is their incredible cytogenetic variability. Most of the knowledge about nematode cytogenetics comes from the monumental work of Prof. Dr. Triantaphyllou during the second part of the twentieth century (Triantaphyllou, 1962; Triantaphyllou, 1963; Triantaphyllou, 1966; Triantaphyllou, 1973; Triantaphyllou & Hirschmann, 1980; Triantaphyllou, 1983; Triantaphyllou, 1984; Triantaphyllou, 1985b; Triantaphyllou, 1985a; Triantaphyllou, 1987; Triantaphyllou, 1990; Triantaphyllou & Hirschmann, 1997). From this research we know that root-knot nematodes exhibit a gigantic variation in chromosome number ranging from n=7 to 2n=56 chromosomes. The cytologically characterized species of root-knot nematodes and their chromosome complement were summarized by Chitwood and Perry (2009). This astonishing variability in chromosome numbers is associated with a wide variation in reproduction strategies ranging from amphimixis over meiotic parthenogenesis and mitotic parthenogenesis (Castagnone-Sereno, 2006; Castagnone-Sereno *et al.*, 2013). Interestingly, several species are able to reproduce by both sexual reproduction in the presence of males and by meiotic parthenogenesis in the absence of males (Triantaphyllou, 1985a; Van der Beek *et al.*, 1998). Triantaphyllou 1985 hypothesized that ancestral root-knot nematodes were amphimictic, from which parthenogenetic live forms evolved. High chromosome numbers were assumed to reflect different states of polyploidy. Interestingly, based on the cytological characterization the amphimictic *M. spartinae* and *M. kikuyensis*, where n=7 chromosomes was assumed to be the ancestral chromosome complement (Triantaphyllou, 1987; Triantaphyllou, 1990).

Within the genus *Meloidogyne* clade I is of particular interest as it contains a wide range of mitotic parthenogenetic species and a single meiotic parthenogenetic species, *M. floridensis*. The mitotic parthenogenetic species of this clade are currently thought to be the product of a recent hybridization event (Lunt, 2008; Fargette *et al.*, 2010). This was recently demonstrated by comparative genomics between *M. hapla* (Opperman et al., 2008), *M. incognita* (Abad *et al.*, 2008) and *M. floridensis* in which *M. floridensis* was demonstrated to be potentially one of the parental species in the hybrid origin of *M. incognita* (Lunt *et al.*, 2014). Interestingly, the

genome of *M. floridensis* also appeared to be of hybrid origin (Lunt *et al.*, 2014). Further genome sequencing and comparative genomic allowed confirmation of this hypothesis and additional evidence for polyploidy in tropical root-knot nematodes (Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017). These complex origins of species within the genus *Meloidogyne* species were already presumed based on the study of isozyme electrophoresis, as the complex isozyme patterns were thought to reflect the complex genomic composition of root-knot nematodes (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987). The concepts of cytological evolution of root-knot nematodes will be further introduced in the introduction of Chapter 3. In contrast to root-knot nematodes, there is little known about the cytogenetics of root-lesion nematodes. One of the few studies by Roman & Triantaphyllou (1969) indicated a comparable variability in chromosome numbers, ranging from n=5 to 2n=32. Also within root-lesion nematodes sexual, meiotic parthenogenetic and mitotic parthenogenetic reproduction strategies occur (Roman & Triantaphyllou, 1969). Interestingly, mitotic parthenogenetic species appear to be related with higher chromosome numbers and polyploidy (Roman & Triantaphyllou, 1969).

5 Aims

We have chosen the *Pratylenchus* and *Meloidogyne* as subject of this thesis because both genera are phylogenetically closely related and are economically important plant-parasites. Despite the economic significance of these pathogens routine identification practices are far from established. As a result the main aim of this thesis was to improve root-knot and root-lesion nematode characterization and identification. In order to improve identifications of these plant pathogens, several biological aspects of these species have to be studied, specifically:

a) As several groups of root-knot and root-lesion nematodes remain poorly studied, unknown biodiversity has to be characterized and taxonomic classifications have to be updated.

b) Species previously defined by morphological characters or isozyme pattern have to be linked to DNA sequences in order to allow molecular identification through DNA barcoding. These molecular barcodes also facilitate molecular species delimitation.

c) Cytology and evolution of reproductive strategies have to be comprehensively analyzed in order to improve our understanding of the evolutionary history of species. This will also contribute, in combination with DNA sequences, to define taxonomic entities.

The proposed strategy will generate comprehensive data and new insights that will allow to evaluate and revise current pathogen identification and improve pathogen identification in the future. This should significantly contribute to the systematics of plant-parasitic nematodes, biodiversity studies, biogeography and different aspects of nematode management.

6 Outline of this study

This thesis is divided into six chapters.

Chapter 1 contains a general introduction, illustrating the context in which this study is situated and the main aims of this work.

Chapter 2-5 contain the actual results of this study, each chapter includes a specific introduction, materials and methods, results and discussion section. All these chapters are modified from papers of SCI-indexed journals; Chapter 2 and 3 have been published, Chapter 3 and 4 are under review.

> **Chapter 2**, "Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution", presents a new DNA-barcode based identification strategy for tropical root-knot nematodes.
>
> **Chapter 3**, "Integrative taxonomy of root-knot nematodes reveals multiple independent origins of mitotic parthenogenesis", presents a taxonomic revision of African coffee root-knot nematodes and provides insight into the evolution of reproduction and oogenesis within the genus *Meloidogyne*.
>
> **Chapter 4**, "Molecular characterization and species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the *Penetrans* group (Nematoda: Pratylenchidae)", presents a taxonomic revision of the cryptic *Pratylenchus penetrans* species group using molecular species delimitations in order to allow DNA barcode-based diagnostics.
>
> **Chapter 5**, "The pitfalls of molecular species identification: a case study within the genus *Pratylenchus* (Nematoda: Pratylenchidae)", presents the problems faced in current molecular species identification practices.

Chapter 6 contains a general discussion, integrating the main findings of this study in a wider perspective.

Chapter 2

Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution

Modified from Janssen T., Karssen G., Verhaeven M., Coyne D., Bert W. 2016. Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. Scientific reports 6, 22591. http://www.nature.com/articles/srep22591

Cited by 11, IF 2014: 5.578, Q1 5/57.

1 Abstract

The polyphagous parthenogenetic root-knot nematodes of the genus *Meloidogyne* are considered to be the most significant nematode pest in sub-tropical and tropical agriculture. Despite the crucial need for correct diagnosis, identification of these pathogens remains problematic. The traditionally used diagnostic strategies, including morphometrics, host-range tests, biochemical and molecular techniques, now appear to be unreliable due to the recently-suggested hybrid origin of root-knot nematodes. In order to determine a suitable barcode region for these pathogens nine quickly-evolving mitochondrial coding genes were screened. Resulting haplotype networks revealed closely related lineages indicating a recent speciation, an anthropogenic-aided distribution through agricultural practices, and evidence for reticulate evolution within *M. arenaria.* Nonetheless, nucleotide polymorphisms harbor enough variation to distinguish these closely-related lineages. Furthermore, completeness of lineage sorting was verified by screening 80 populations from widespread geographical origins and variable hosts. Importantly, our results indicate that mitochondrial haplotypes are strongly linked and consistent with traditional esterase isozyme patterns, suggesting that different parthenogenetic lineages can be reliably identified using mitochondrial haplotypes. The study indicates that the barcode region Nad5 can reliably identify the major lineages of tropical root-knot nematodes.

2 Introduction

Root-knot nematodes of the genus *Meloidogyne* are considered to be the most significant nematode pest to crop production, causing multi-billion dollar annual losses worldwide (Agrios, 2005). Indeed, one species, *M. incognita,* is considered to be the world's most damaging crop pathogen (Trudgill & Blok, 2001). However, despite the crucial need for correct diagnosis of these pathogens, identification of root-knot nematodes continues to pose an obstacle, even for specialists, with reliable, routine identification methods far from established. Traditionally, researchers have relied on morphometrics and perennial patterns for species identification, which is now known to be greatly hampered by phenotypic plasticity and interspecific similarities (Hunt & Handoo, 2009). Hartman and Sasser (1985) also devised a technique based on differential host preferences, even though, to date, no genetic, isozymatic, or cytogenetic basis has been established for these different host races, indicating that they do not in fact represent homological speciation events (Castagnone-Sereno *et al.*, 2013). Moreover,

increasing the number of host plants has inevitably led to additional races (Robertson *et al.*, 2009). It has also been suggested that the virulence of *Meloidogyne* is mediated by epigenetic control (Perfus-Barbeoch *et al.*, 2014), thus rendering host specificity an inappropriate diagnostic technique.

In the mid-1980's Esbenshade and Triantaphyllou (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987) developed a biochemical-based diagnostic technique, reliant on isozyme profiles; variations in esterase and malate dehydrogenase isozyme profiles proved extremely informative in differentiating most known species of *Meloidogyne*. The main drawback to this method, however, is that the technique is only applicable to young adult females, with results often varying between laboratories, leading to suggestions that polymorphic enzyme profiles exist (Esbenshade & Triantaphyllou, 1985). Despite these shortcomings, isozyme electrophoresis remains one of the most reliable and widely-used differentiation methods (Blok & Powers, 2009; Elling, 2013; Humphreys-Pereira *et al.*, 2014). Further to this, biochemical techniques involving monoclonal or polyclonal antibodies appeared promising, yet additional research and validations are required before this method can be applied in practice (Ibrahim *et al.*, 1996).

The first molecular diagnostic techniques to differentiate species of *Meloidogyne* were introduced by Curran *et al.* (1986), using restriction length polymorphisms (RFLP) of genomic DNA. Satellite DNA, often in combination with probes, have also been explored for specific diagnosis (Castagnone-sereno *et al.*, 1995; Castagnone-Sereno *et al.*, 1999). Despite the fact that several of these techniques can distinguish between various species of *Meloidogyne*, none is used as frequently as the species-specific primers method. Species-specific primers have been developed to amplify sequence-characterized amplified regions (SCAR), which have been converted from diagnostic randomly amplified polymorphic DNA fragments (RAPDs) (Zijlstra, 2000; Zijlstra *et al.*, 2000; Adam *et al.*, 2007). This gel based technique is simple, life-stage independent, cost efficient and permits numerous samples to be run within a reasonable amount of time. In addition, the technique is regularly updated as new species-specific primers are developed (Correa *et al.*, 2014). However, some challenges remain associated with species-specific primers, such as ambiguous results, low sensitivity, occasionally poor band visibility, and lack of reproducibility between laboratories (Blok & Powers, 2009; Onkendi *et al.*, 2014).

With the rapidly declining cost and improved availability of genetic sequencing and related technologies, theoretically, all the aforementioned methods could be replaced by DNA barcoding (Powers, 2004). However, the search for an appropriate barcode region has so far proved notoriously difficult, especially for a clade of mostly mitotic parthenogenetic pathogens including *M. incognita*, *M. javanica*, and *M. arenaria*, commonly referred to as "tropical root-knot nematodes" or the *M. incognita* group (MIG) (Qiu *et al.*, 2006; Pagan *et al.*, 2015). These closely-related MIG lineages, together with the divergent *M. enterolobii*, form a phylogenetically, well-supported group, named clade I (Tandingan De Ley *et al.*, 2002; Tigano *et al.*, 2005).

The first barcode region to be evaluated was the ribosomal gene cluster but 18S and 28S rDNA appear to lack the resolution required to distinguish between these closely-related lineages (Landa *et al.*, 2008). Conversely, the quickly-evolving internal transcribed spacer (ITS) regions have been shown to contain multiple, highly divergent copies within a single individual (Hugall *et al.*, 1999). These divergent copies could well be linked to the hybrid origin of parthenogenetic root-knot nematodes, as suggested by Lunt *et al.* (2014), which would create difficulties for barcoding these hybrid lineages using nuclear markers (Lunt, 2008; Lunt *et al.*, 2014). Arguably, mitochondrial genes can partly circumvent these problems due to their uni-parental inheritance and high mutation rate (Gissi *et al.*, 2008; Pagan *et al.*, 2015). Therefore the intergenic region between 16s and Cox2 has become a focus for characterizing parthenogenetic *Meloidogyne* lineages. Based on this region a PCR-based detection method for root-knot nematodes was developed (Powers *et al.*, 1986; Powers & Harris, 1993) and using root-knot nematode species from Australian restriction fragment analysis of this region revealed a correspondence with isozyme phenotypes (Hugall *et al.*, 1994). Recently, progress towards a more reliable and durable technique was made by Pagan *et al.* (2015); as several MIG lineages could each be assigned unique mitochondrial haplotypes based on PCR fragment size and restriction cleavage patterns, which was assessed on a range of ethanol-preserved populations from Africa. In search of a suitable barcode region for the MIG, the traditionally-used cytochrome c oxidase 1 and 2 regions are reportedly insufficiently variable to reliably distinguish MIG lineages (Kiewnick *et al.*, 2014). Nonetheless, the development of a reliable barcode marker for these root-knot nematodes is of huge economic significance since correct

identification of these pathogens can be critical for making informed decisions for efficient and suitable management strategies.

A reliable barcode marker should preferably be a mitochondrial coding region, as intergenic regions have been shown to contain AT repeats that appear not to correlate with speciation events (Hugall *et al.*, 1997; Pagan *et al.*, 2015). The goal of the current study therefore, was to verify whether the coding genes of the mitochondrial genome of clade I root-knot nematodes harbor useful diagnostic barcoding regions. Primers for nine coding genes of the mitochondrial genome were developed. These were sequenced, screened for polymorphic nucleotide positions, and compared with traditional isozyme electrophoresis profiles. To ascertain if lineage sorting of polymorphic positions was complete, numerous populations from geographically widespread origins and variable host plants were screened. The ultimate aim was to provide a simple, efficient and reproducible barcoding assay for reliable identification of MIG pathogens.

3 Material and methods

3.1 Collection of populations, morphological identification and culturing

For this study 85 separate *Meloidogyne* populations were examined. Thirty-seven populations were obtained from pure cultures originating from the National Plant Protection Organization (Wageningen, the Netherlands), while the other populations were collected during four field surveys in three countries. Sampling of field-cultivated crops was undertaken in Tanzania, Pakistan, and Nigeria between 2012 and 2013, providing 48 populations. Comprehensive information on the geographical origin and the host plant species was collected for all populations (Table 1), which were all morphologically characterized based on second-stage juveniles (Jepson, 1987) and perennial patterns, when females were available, in order to ensure clade I species were included. Subsequently, each population was inoculated onto *Lycopersicon esculentum* cv. Moneymaker plants, individually, in pots containing sterile potting media, using a few egg masses or juveniles. Populations were maintained in the greenhouse at Wageningen at 23°C.

Table 1. Studied populations with their unique ID number together with esterase isozyme phenotype (Est), malate dehydrogenase isozyme phenotype (Mdh) and their respective host

plant. Additional information on sampling location and origin of the studied material. If samples were collected from the field, GPS coordinates are provided, if the studied material originated from a reference culture the unique identification code is provided (Plant Protection service, Wageningen).

Species	Specimen ID	Est	Mdh	Host plant	Location	GPS coordinates/ reference culture number
Meloidogyne enterolobii						
	T337	VS1-S1	N1a	*Solanum melongena*	Puerto rico, typematerial Meloidogyne mayaguensis received from Dr. V. Blok	E1834
	T382	VS1-S1	N1a	*Enterolobium contortisiliquum*	China, Hainan Island, type locality sample	E1470
	T424	VS1-S1	N1a	*Solanum lycopersicum*	USA, Florida, received from Dr. J. Brito	E7314-1 (no1-514-313 5/15/04)
	T441	VS1-S1	N1a	Cactaceae	Singapore, received from Dr. A. Castillo	E8336
	T463	VS1-S1	N1a	*Ulmus*	China	E4775-1
	T468	VS1-S1	N1a	*Capsicum annuum*	Mexico	E9491
	T536	VS1-S1	N1a	*Chlorophytum*	Fera, R. Lawson 3122513	E9578
Meloidogyne incognita						
	T384	I1	N1	*Daucus carota*	Morocco	E5942
	T161	I1	N1	*Ficus*	China	F0983
	T515	I1	N1	*Solanum tuberosum*	Italy	E1318
	T526	I1	N1	*Syngonium*	Togo	R91/2342
	T532	I1	N1	*Vitis*	Egypt, Monufia Governorate, El Sadat city	E9619-30a
	T540	I1	N1	*Philodendron selloum*	United States of America	F1763
	T552	I1	N1	*Ficus*	China	E2107-1
	Y29	I1	N1	*Dioscorea* (Yam)	Nigeria, Kogi, Idah	7°6’56”N,6°44’37”E
	Y57	I1	N1	*Celosia*	Nigeria, Oyo, Akobo	7°25’57”N,3°56’35”E
	C33	I1	N1	*Solanum aethiopicum*	Tanzania, Morogoro, Kipera	6°55’56.784”S, 37°32’3.408”E
	C41	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Kipera	6°56’13.452”S, 37°31’35.543”E
	C49	I1	N1	*Solanum aethiopicum*	Tanzania, Morogoro, Mlali	6°58’0.48”S, 37°31’18.12”E
	C53	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Mlali	6°58’0.192”S, 37°31’18.048”E
	C69	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Hembeti	6°17’27.096”S, 37°28’18156”E
	C81	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Msongozi	7°3’59.508”S, 37°20’41.279”E
	C87	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Msongozi	7°3’50.4”S, 37°20’34.044”E
	C95	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Msongozi	7°3’21.024”S, 37°19’53.867”E
	M4	I1	N1	*Capsicum annuum*	Tanzania, Morogoro, Mlali	6°57’4.248”S, 37°31’45.12”E
	M8	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Mlali	6°57’4.248”S, 37°31’45.12”E
	M15	I1	N1	*Capsicum annuum*	Tanzania, Morogoro, Mlumbilo-Mtibwa	6°11’42.72”S, 37°42’54.647”E
	M20	I1	N1	*Fabaceae* (Bean)	Tanzania, Morogoro, Muomero	6°17’52.836”S, 37°26’30.66”E

Species	Specimen ID	Est	Mdh	Host plant	Location	GPS coordinates/ reference culture number
	M21	I1	N1	*Solanum aethiopicum*	Tanzania, Morogoro, Muomero	6°17’52.836”S, 37°26’30.66”E
	M28	I1	N1	*Solanum lycopersicum*	Tanzania, Pwani, Bagamoyo-mtoni	6°27’10.62”S, 38°53’23.784”E
	M44	I1	N1	*Coffea*	Tanzania, Morogoro, Luale	7°7’57.108”S, 37°32’17.916”E
	M46	I1	N1	*Pisum sativum*	Tanzania, Morogoro, Luale	7°7’58.404”S, 37°32’6.792”E
	M49	I1	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Bunduki	7°1’53.184”S, 37°37’2.891”E
	A1	I1	N1	*Abelmoschus esculentus*	Pakistan, Faisalabad, Chak # 61 JB Dharoran	31°26'53.02"N, 72°58'14.00"E
	A3	I1	N1	*Abelmoschus esculentus*	Pakistan, Faisalabad, Chak # 146/RB II Khewa	31°36'6.34"N, 73°16'39.41"E
Meloidogyne javanica						
	T347	J3	N1	*Solanum lycopersicum*	Rwanda, Kayonza	1°57’16.6”S,30°31’16.9”E
	T417	J3	N1	*Carmona*	China	E9455
	T429	J3	N1	*Solanum lycopersicum*	Spain	F1836-3
	T485	J3	N1	*Ficus*	China	E1090-4
	T497	J3	N1	*Fabaceae* (Bean)	Morocco	E9492
	T509	J3	N1	*Solanum tuberosum*	Congo	E1387
	T520	J3	N1	Pistache	Iran	D4872
	Y60	J3	N1	*Dioscorea* (Yam)	Nigeria, Benue, Tsiabi	7°15’52”N,8°15’3”E
	C35	J3	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Kipera	6°55’56.784”S, 37°32’3.408”E
	C47	J3	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Mlali	6°57’3.708”S, 37°31’48.37”E
	C63	J3	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Dakawa	6°26’58.236”S, 37°31’53.184”E
	C89	J3	N1	*Solanum lycopersicum*	Tanzania, Morogoro, Msongozi	7°3’14.796”S, 37°22’39.971”E
	M14	J3	N1	*Brassica*	Tanzania, Morogoro, Mlumbilo-Mtibwa	6°11’40.884”S, 37°42’51.552”E
	M30	J3	N1	*Abelmoschus esculentus*	Tanzania, Pwani, Bagamoyo-mtoni	6°27’10.62”S, 38°53’23.784”E
	M39	J3	N1	*Solanum lycopersicum*	Tanzania, Dar-es-Salaam, Kisse	7°0’0”S, 39°0’0”E
	M40	J3	N1	*Brassica oleracea*	Tanzania, Morogoro, Msufini	6°17’15.432”S, 37°28’38.675”E
	M50	J3	N1	*Coffea*	Tanzania, Morogoro, Bunduki	7°1’54.12”S, 37°36’51.804”E
	A8	J3	N1	*Solanum melongena*	Plant Pathology Research Area (Culture), University of Agriculture, Faisalabad	/
	A21	J3	N1	*Abelmoschus esculentus*	Pakistan, Mandibahauddin, Phalia, Kadhar	32°25'48.27"N, 73°28'40.63"E
	A23	J3	N1	*Abelmoschus esculentus*	Pakistan, Mandibahauddin, Phalia, Chhohranwala	32°31'42.40"N, 73°44'4.20"E
	A24	J3	N1	*Abelmoschus esculentus*	Pakistan, Faisalabad, Chak # 225 RB Malkhanwala	31°21'48.23"N, 73° 7'5.87"E
	A25	J3	N1	*Cucurbita pepo*	Pakistan, Mandibahauddin, Phalia, Chhohranwala	32°31'53.60"N, 73°43'23.00"E

Species	Specimen ID	Est	Mdh	Host plant	Location	GPS coordinates/ reference culture number
	A29	J3	N1	*Solanum melongena*	Pakistan, Mandibahauddin, Phalia, Seeray	32°23'56.21"N, 73°32'35.66"E
	A30	J3	N1	*Cucurbita pepo*	Pakistan, Mandibahauddin, Phalia, Seeray	32°23'56.41"N, 73°32'33.48"E
	A31	J3	N1	*Abelmoschus esculentus*	Pakistan, Mandibahauddin, Phalia, Seeray	32°23'57.95"N, 73°32'35.54"E
	A32	J3	N1	*Cucurbita pepo*	Pakistan, Mandibahauddin, Phalia, Chhohranwala	32°31'39.89"N, 73°44'0.63"E
Meloidogyne arenaria						
	T311	A3	N1	unknown (extracted from soil)	Italie, Monsampolo del Tronto, Marché	F9497-6
	T332	A2	N1	Solanaceae	France	E9085
	T393	A2	N1	*Echiocactus grusonii*	Netherlands, greenhouse	E9279
	T411	A2	N1	*Calathea*	Costa Rica	F0428
	M41	A2	N1	*Allium cepa*	Tanzania, Morogoro, Msufini	6°17’15.432”S, 37°28’38.675”E
	T453	A2	N3	*Livistonia*	Sri Lanka	E9211
	T461	A2	N3	*Hosta*	USA	C8526
	Y19	A2	N3	*Dioscorea* (Yam)	Nigeria, Benue, Otukpo	7°11’31”N,8°7’59”E
	Y34	A2	N3	*Dioscorea* (Yam)	Nigeria, Niger, Tufakampani	9°14’29”N,6°54’59”E
***Meloidogyne* sp. 1**						
	T473	A2-S1-M1	N1	*Heliconia*	Tanzania	E8465
	T585	A2-S1-M1	N1	*Ficus*	China	D2055-1
***Meloidogyne* sp. 2**						
	T316	A1a-S1	N1	*Beta vulgaris*	Spain	C7720
	T576	A1a-S1	N1	*Solanum lycopersicum*	Guatemala	C7729
Meloidogyne luci						
	T326	L3	N1	*Solanum lycopersicum*	Dornberg, Slovenia	D9742
	T459	L3	N1	*Solanum lycopersicum*	Guatemala	F0034
	T693	L3	N1	*Daucus carota*	Iran	E4271
Meloidogyne inornata						
	T638	I3	N1	*Solanum lycopersicum*	Chili	F2484
	T695	I3	N1	*Solanum lycopersicum*	Chili	F2642
Meloidogyne ethiopica						
	T612	E3	N1	*Solanum lycopersicum*	Brazil, Charchar, received from R. Carneiro	E6089

3.2 Isozyme analysis

To confirm the morphological identification and purity of the cultures, esterase and malate dehydrogenase isozymes were analyzed according to Karssen *et al.* (1995). First, ten young females of each culture were isolated from roots in isotonic (0.9 %) NaCl solution. Individual females, after desalting in reagent-grade water on ice for 5 minutes, were loaded to sample wells containing 0.6 µl extraction buffer (20% sucrose, 2% triton X-100, 0.01% Bromophenol Blue), and subsequently macerated using a glass rod. This mixture was homogenised, and protein extractions were loaded onto a (8-25) polyacrylamide gradient gel and electrophoretically fractioned using a PhastSystem (Pharmacia Ltd, Uppsala, Sweden). In addition to the ten test samples, two *M. javanica* protein extractions were added to the centre of each gel to serve as a reference. After electrophoresis, gels were stained to examine for malate dehydrogenase (Mdh) and esterase (Est) activity for 5 and 45 minutes, respectively, rinsed with distilled water, and fixed using a 10% glycerol, 10% acetic acid, distilled water solution.

3.3 Mitochondrial DNA analysis

Genomic DNA of crushed individual females was extracted using worm lysis buffer and proteinase K (Bert *et al.*, 2008). Genomic DNA of individual second-stage juveniles or males was extracted using a quick alkaline lysis protocol adapted from Schneider *et al.* (2015); individual nematodes were transferred to 10 µl 0.05N NaOH, with 1 µl of 4.5% tween added. The mixture was heated to 95°C for 15 min, and after cooling to room temperature 40 µl of double-distilled water was added.

PCR primers were designed for 16S ribosomal RNA (16S), the Cytochrome c oxidase subunits 1, 2 and 3 (Cox1, Cox2, Cox3), Cytochrome b (Cytb), the NADH dehydrogenase subunits 1, 2, 3 and 5 (Nad1, Nad2, Nad3, Nad5) using PRIMER3(Untergasser *et al.*, 2012) implemented in GENEIOUS R6 (Biomatters; http://www.geneious.com). As a starting point for primer design a combination of recently published mitochondrial genomes: *M. incognita*, *M. chitwoodi* (Humphreys-Pereira & Elling, 2014), *M. graminicola* (Sun *et al.*, 2014) and contigs from the genomic next generation sequence data of *M. incognita* (Abad *et al.*, 2008), *M. hapla* (Opperman *et al.*, 2008) and *M. floridensis* (Lunt *et al.*, 2014) sequencing consortia were used. The resulting primer sequences are displayed in Table 2 together with the length, position and proportion of the amplified fragments.

Table 2. Sequences of newly developed primers and the position of the amplified fragment in relation to the total length of the mitochondrial coding sequence.

Gene (Length of CDS in bp[a])	Primer name	Primer sequence 5' – 3'	Primer position[a]	Fragment length (bp)	Gene coverage (%)
Cytochrome c oxidase subunit 1 (1522)	COX1F	ATCCTCCTTTGATGATTGATGG	374	996	65
	COX1R	AACTCAATAAAGAACCAATAGAAG	1369		
Cytochrome c oxidase subunit 2 (693)	COX2F	TTGAATTTAAGTGTTGTTTATTAC	155	432	62
	COX2R	GATTAATACCACAAATCTCTGAAC	586		
Cytochrome c oxidase subunit 3 (762)	COX3F	TTTTGCTGAGGATTAATAGG	171	397	52
	COX3R	TAAACTTCCATAAATACCATCAC	567		
NADH dehydrogenase subunit 1 (850)	NAD1F2	ATTAGATTATTAACTTTACTGGAGCG	40	558	66
	NAD1R2	GGAAAGAGAAAGTGAATTAGTGAGA	597		
NADH dehydrogenase subunit 2 (802)	NAD2F	GTATTATTAATATTTTGTAGGAAT	103	610	76
	NAD2R	ATATTAACTGACTTATTATCCC	712		
NADH dehydrogenase subunit 3 (315)	NAD3F	AATGAAAAATTCTTATTTCGAAAG	75	219	70
	NAD3R	ATATATTTTCATTCCAAAACTAAA	293		
NADH dehydrogenase subunit 5 (1474)	NAD5F2	TATTTTTTGTTTGAGATATATTAG	257	610	41
	NAD5R1	CGTGAATCTTGATTTTCCATTTTT	866		
Cytochrome b (1015)	CYTBF	TGAGGTTAATAATGGTTGGTTAATTCG	165	801	79
	CYTBR	GGGAGCCAAGAACCAGTTTT	965		
16S ribosomal RNA (804)	16SF	GCTCATTGTTAAAGAAAAGC	339	399	50
	16SR	GTTGTGAAATAGAGTTGTT	737		

[a] Length of the coding sequence and primer position within the gene are given according to mitochondrial genome of *Meloidogyne incognita* (Abad *et al.*, 2008).

PCR amplification was carried out using the standard Taq DNA polymerase mixture (Qiagen, Germany), employing 2 µl genomic DNA extraction and 0.4 mM of each primer. The PCR amplification conditions were: initial desaturation for 2 min at 94 °C, followed by 40 cycles of 60 secs at 94 °C, 60 secs at 45 °C, 90 secs at 72 °C, and finally an extension for 10 min at 72 °C. For NADH dehydrogenase subunit 1, Cytochrome c oxidase and Cytochrome b the annealing temperature was increased to 55 °C. PCR products were electrophoretically fractioned on a 1% agarose gel and stained with ethidium bromide. Successful reactions were purified and sequenced commercially by Macrogen Inc. (Europe) in forward and reverse direction. Consensus sequences were assembled using GENEIOUS R6. All contigs were subjected to a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov) to check for

possible contaminations. Reliability and reproducibility of PCR amplification was tested by sequencing Nad1 twice using a different primer combination NAD1F1 (TCA AAT TCG TTT AGG ACC AAC) and Nad1R1 (CGA ATT GTT TAT CCT CGT TTT C) and by substituting Taq DNA polymerase by Phusion® High-Fidelity DNA Polymerase. To check heteroplasmy within a population and within a single individual, respectively multiple individuals of a single population were sequenced and four populations were cloned using pGEM®-T Easy Vector Systems Promega *i.e. M. javanica* T417 (3 sequenced clones), *M. javanica* T520 (9 sequenced clones), *M. incognita* T515 (3 sequenced clones) and *M. incognita* T540 (4 sequenced clones). All mitochondrial sequences were translated on the TranslatorX web server (Abascal *et al.*, 2010) using the invertebrate genetic code and aligned by its amino acid translation using MAFFT 7.157 (Katoh & Standley, 2013). Haplotype networks were calculated using the median joining algorithm as implemented in Network 4.6 (Bandelt *et al.*, 1999), http://www.fluxus-engineering.com/**)**, gaps were coded as unknown characters. Haplotype diagrams were redrawn in ADOBE® ILLUSTRATOR® CS3. Liberal P ID values, inter- and intra-lineage species variability were calculated with the species delineation plugin of GENEIOUS R6 (Masters *et al.*, 2011) using a UPGMA tree, and distances were calculated according to the Jukes-Cantor model. In all analyses the generated sequences in the current study were complemented with mitochondrial haplotypes extracted from the mitochondrial genome sequences of *M. incognita* (Humphreys-Pereira & Elling, 2014), *M. javanica*, *M. enterolobii* and *M. arenaria* (Humphreys-Pereira & Elling, 2015) and haplotypes extracted from whole genome sequences of *M. incognita* (Abad *et al.*, 2008) and *M. floridensis* (Lunt *et al.*, 2014).

4 Results

4.1 Sampling and isozyme electrophoresis

Among the 80 populations of root-knot nematodes examined 10 different esterase profiles and three different malate dehydrogenase patterns were identified (Table 1). These profiles represent 11 lineages of *Meloidogyne*, of which two appear new to science (see Fig. 1). In total seven populations of *M. enterolobii* were identified, including specimens originating from the type localities of *M. enterolobii* and its junior synonym *M. mayaguensis* (Karssen *et al.*, 2012). The most frequently-occurring lineages in the dataset were *M. incognita* (28 populations) and

M. javanica (26 populations), originating from a range of host plants and a wide geographical distribution (Table 1). *Meloidogyne incognita* was represented by both the I1 and I2 phenotype, although the I1 phenotype only occurred when esterase bands were weakly visible, indicating that the absence of the secondary esterase band represents an analysis artifact. For this reason all *M. incognita* esterase phenotypes in this study are defined as I1. *Meloidogyne arenaria* is represented by three isozyme profiles: the A2N1 type (4 populations), the A2N3 type (4 populations) and the A3N1 type (1 population). Three populations of *M. luci*, two populations of *M. inornata* and one population of *M. ethiopica* had an L3, I3 and E3 esterase phenotype, respectively. In addition to these known isozyme patterns two previously unrecorded esterase profiles were discovered (Fig. 1); one occurring in two populations, one originating from China from *Ficus* and one from Tanzania from *Heliconia*. The Mdh pattern of these populations was characterised as the N1 phenotype (Fig. 1b). The esterase phenotype displays three clear bands, of which the two fast migrating bands are positioned in the same location as for the *M. arenaria* A2 phenotype, while the slowest migrating band and its slightly faster migrating weak band occur in a similar position as the S1-M1 phenotype (Fig.1a). Both the A2 and S1-M1 phenotype have previously been associated with *M. arenaria* (Esbenshade & Triantaphyllou, 1985) indicating that our combined A2-S1-M1 pattern should be considered an atypical, possibly hybrid, *M. arenaria* pattern. A second novel pattern was associated with two *Meloidogyne* populations, one originating from Spain (Beet) and one from Guatemala (Tomato). The Mdh activity displayed a N1 phenotype (Fig. 1d) and the esterase phenotype consists of two bands of which the faster migrating band is more pronounced (Fig. 1c). This esterase phenotype is not directly related to any other described *Meloidogyne* esterase phenotype indicating a new, undescribed lineage. The slow migrating band is in the S1 position (Esbenshade & Triantaphyllou, 1985) while the fast migrating band is in a new position, herein named A1a. This new esterase phenotype is therefore referred to as A1a-S1.

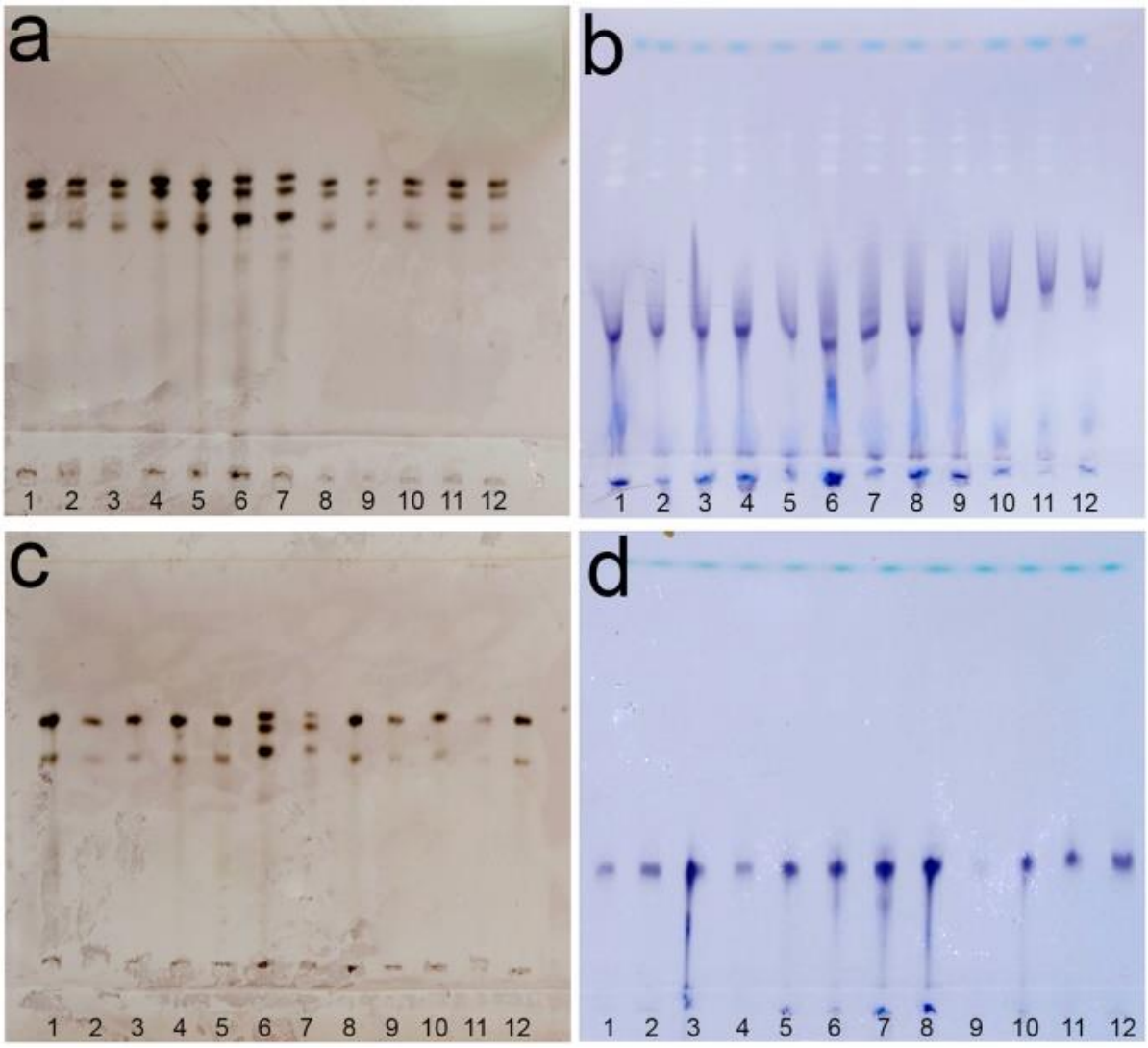


Figure 1. isozyme profiles of two undescribed MIG lineages. Lane 6 and 7 represent *Meloidogyne javanica* reference phenotypes, lane 1–5 and 8-12 represent undescribed MIG lineages. a) esterase A2-S1-M1 phenotype of *Meloidogyne* sp. 1, b) malate dehydrogenase N1 phenotype of *Meloidogyne* sp. 1, c) esterase A1a-S1 phenotype of *Meloidogyne* sp. 2, d) malate dehydrogenase N1 phenotype of *Meloidogyne* sp. 2.

4.2 Identification of polymorphic sites within the mitochondrial genome

From the 80 geographically widespread populations, 305 sequences and 22 mitochondrial haplotypes were generated (Table S3), corresponding to 11 isozyme based lineages of clade I root-knot nematodes. Identical results with different primer combinations and different DNA polymerases. Sequence results for multiple individuals within one population (tested for 11 populations, see Table S3). Cloning data revealed limited heteroplasmy within a single individual, but never associated with informative species specific nucleotide positions, *i.e.* 0.16% variation (1 nucleotide position) in one clone of T417, 0.16% variation in two clones of T520, 0.33% variation in one clone of T540, while no variation was detected in T515.

4.2.1 Meloidogyne enterolobii

The seven populations of *M. enterolobii* showed identical sequences for the eight analysed gene fragments (Table S3), except for the population originating from the type locality of the former *M. mayaguensis*, which displayed a single mutation in the Cox3 fragment. The *M. enterolobii* haplotype was clearly divergent from the MIG lineages, as our gene fragments were different in 29 nucleotide positions (7.7%) in 16S, 93 positions (11.1%) in Cox1, 30 positions (7.5%) in Cox2, 39 positions (10.8%) in Cox3, 81 positions (10.8%) in Cytb, 68 positions (15.4%) in Nad1, 22 positions (10.1%) in Nad3 and 79 positions (18%) in Nad5, placing *M. enterolobii* in a clearly phylogenetically distinct position within clade I.

4.2.2 MIG lineages

The 16S fragment revealed six polymorphic sites (1.5%), including two *M. javanica*-specific mutations, a *M. incognita* and a *Meloidogyne* sp. 2-specific site (Fig. S1). Additionally, in one population of *M. arenaria* and one population of *M. incognita* an additional single mutation within the 16S fragment was encountered.

The Cox1 fragment contained seven variable positions, but with only 0.7% variable sites and five different haplotypes, this gene is one of the most conserved regions sequenced in this study (Fig. S2). Nevertheless, it revealed five *Meloidogyne* sp. 2-specific sites, one *Meloidogyne* sp. 1-specific mutation and two sites displaying variability between different populations of *M. luci* and *M. inornata*.

Our Cox2 fragment revealed five haplotypes (Fig. S1). One is shared between *M. luci* and *M. inornata*, and one is shared among 10 populations of *M. incognita* and our only representative population of *M. ethiopica*. A third haplotype has two *Meloidogyne* sp. 2-specific sites. Cox2 was not able to differentiate between *M. arenaria*, *M. javanica*, *M. floridensis* and *Meloidogyne* sp. 1 H1, which are grouped in a fourth haplotype, while a fifth haplotype composes *Meloidogyne* sp. 1 H2.

The Cox3 gene fragment reveals four haplotypes (Fig. S1). One is characteristic for five *M. arenaria* populations including three isozyme types (A2N1, A3N1 and A2N3). A second haplotype has two lineage-specific sites for *Meloidogyne* sp. 2. A third is shared among *M. luci*, *M. ethiopica* and *M. inornata* and a fourth groups *M. javanica*, *M. incognita*, *Meloidogyne* sp. 1, two *M. arenaria* populations and *M. floridensis*.

The Cytb fragment contained a lineage-specific haplotype for *M. ethiopica*, *M. floridensis*, *Meloidogyne* sp. 2 and distinguished several populations of *M. arenaria*, but failed to separate the other lineages included in the current study (Fig. S2).

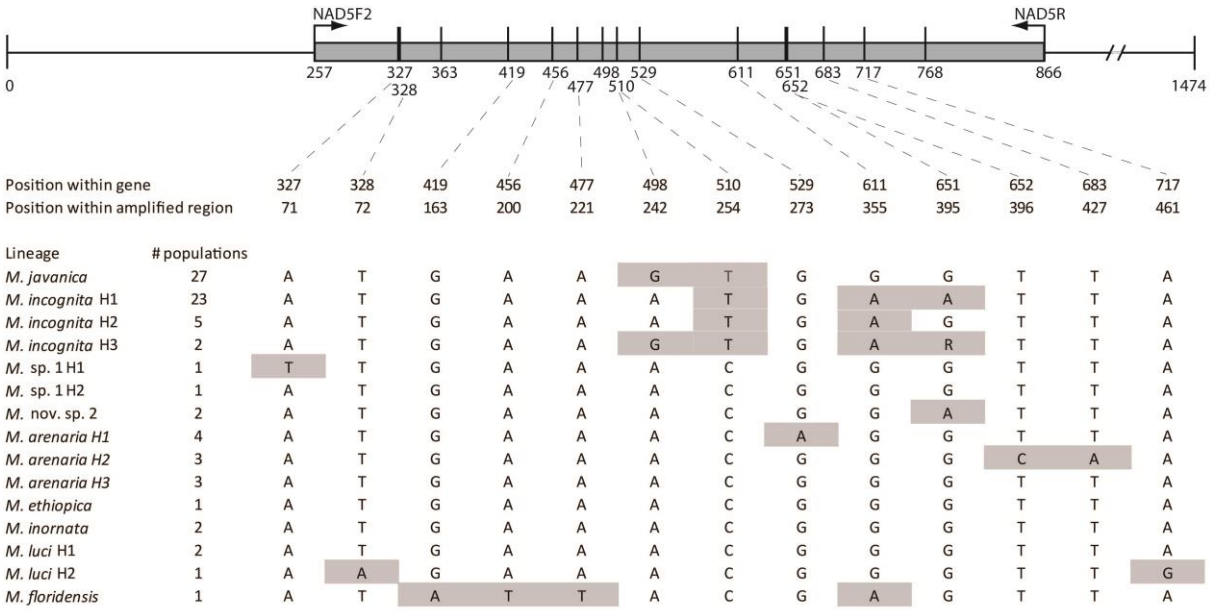
Our Nad1 gene reveals seven haplotypes (Fig. S2). These represent a *M. javanica*, *M. luci*, *M. floridensis*, *Meloidogyne* sp. 1 H1, *Meloidogyne* sp. 2. and an *M. incognita*-specific haplotype, each differing in one nucleotide position. Nad1 did not differentiate between *M. arenaria*, *Meloidogyne* sp. 1 H2, and *M. ethiopica*, although one nucleotide position showed variability between various *M. arenaria* populations.

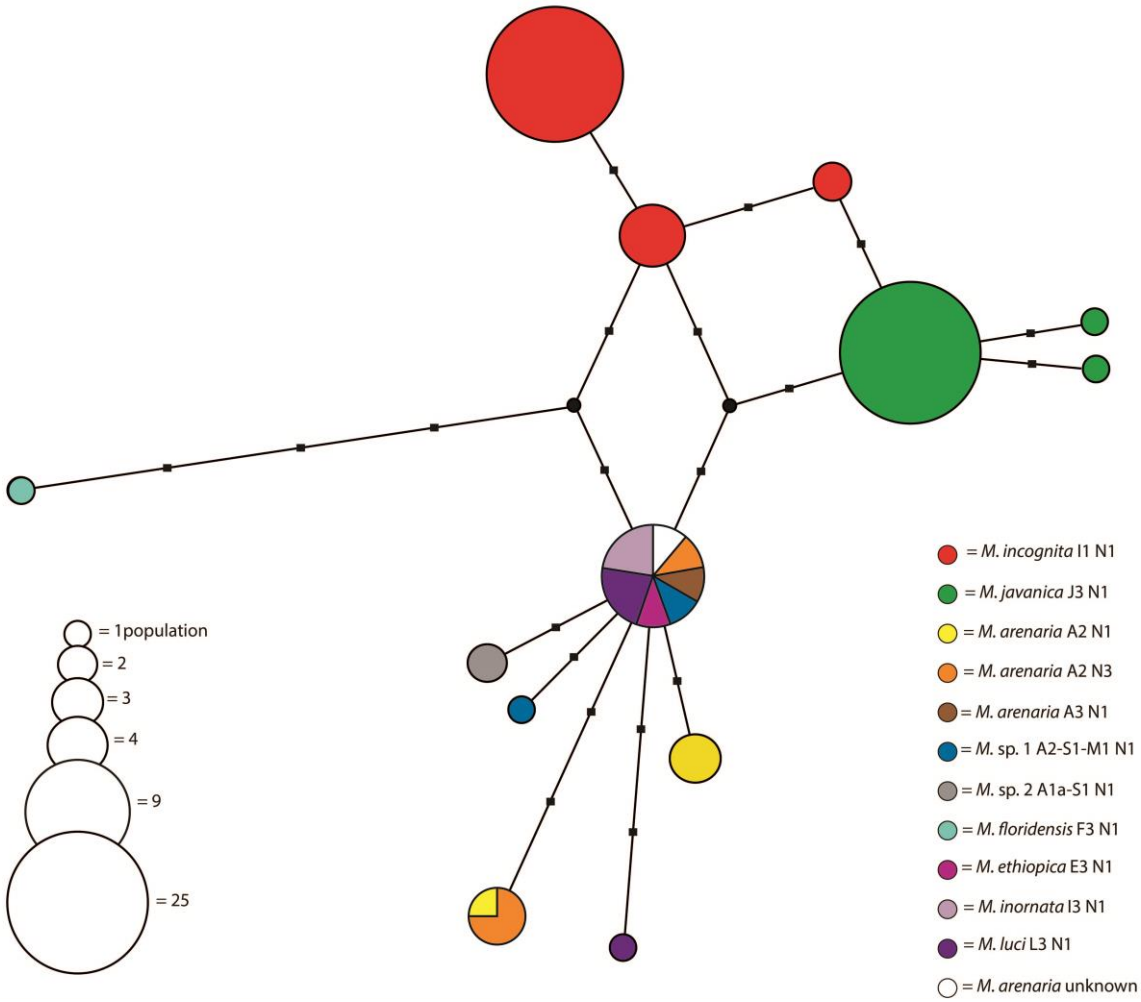
The Nad2 fragment contains eight polymorphic positions, revealing 10 haplotypes (Fig. S2). A first haplotype is *M. javanica*-specific differing in at least two mutations from the other MIG lineages. Also *Meloidogyne* sp. 2 and *M. inornata* each have a lineage-specific haplotype, while *M. incognita* is represented by four closely-related specific haplotypes. Another haplotype is shared by *M. luci*, *M. ethiopica* and *M. floridensis*. The final haplotype groups *M. arenaria*, *Meloidogyne* sp. 1 and one population of *M. arenaria* differing in a single nucleotide position.

The Nad3 fragment was identical for all 17 representative sequenced populations (Table S3). Conversely, with 15 polymorphic positions, the Nad5 fragment is the most variable with a variation of 2.46% and representing 13 haplotypes out of 78 sequenced populations (Fig. 2).

The *M. incognita* I2 esterase type is represented by three closely-related haplotypes differing in only one or two positions from each other. Of 27 *M. javanica* populations, 25 shared the same haplotype, while two populations had a closely-related haplotype differing in only one position. Another common haplotype was shared by *M. inornata*, *Meloidogyne* sp. 1, *M. arenaria* H3, *M. ethiopica* and two of the three *M. luci* populations. This haplotype most likely corresponds to the haplotype G, as defined and recovered by Pagan *et al.* (2015) based on restriction fragment analysis of the intergenic region between 16S and Cox2. The third *M. luci* population included in the current study had a slightly different haplotype, differing in two positions from the other two populations and also *Meloidogyne* sp. 1, which is associated with a second distinct haplotype. Additionally, two *M. arenaria* haplotypes were recovered, each differing in one or two nucleotide positions. However, no link was observed between isozyme phenotypes and mitochondrial haplotypes among the different populations of *M. arenaria*. *Meloidogyne floridensis* is associated with the most divergent haplotype, differing in at least four positions to its closest relatives. Finally for the Nad5 fragment, a lineage-specific haplotype was recorded for *Meloidogyne* sp. 2.

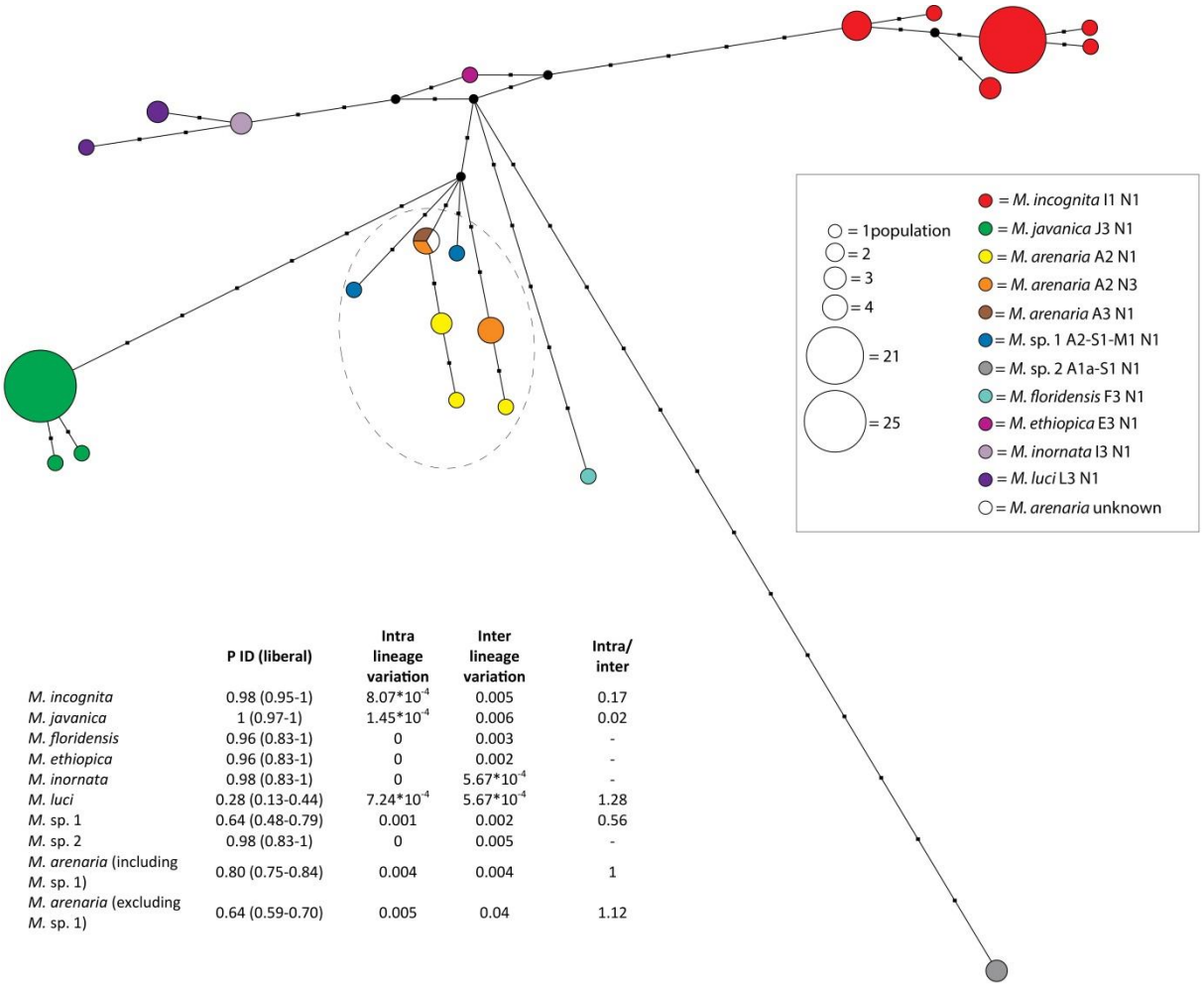
Figure 2. (Next page) Nad5 barcode gene sequence comparison between MIG lineages. A schematic overview of the gene shows the position and length of the amplified fragment, primer position and position of polymorphic nucleotide positions. Alongside the schematic overview an overview table shows the polymorphic nucleotide positions for comparison with barcode sequences as well as the number of populations studied. The haplotype network shows the relationships between different haplotypes, circle size is equivalent to the number of studied populations and branch length is equivalent to the number of mutations (shown as black squares). Different isozyme phenotypes are displayed by different colors, median vectors are shown as black circles. Within the Nad5 gene two *Meloidogyne javanica* populations (T347 and T417) each have an extra mutation which are not shown in the schematic overview. H1, H2 and H3 indicate different haplotypes of a certain lineage. *Meloidogyne incognita* H3 displays a heterozygous position at site 395 indicated with a degenerate base “R” in the table.


NAD5F2
NAD5R
0
257
327
328
363
419
456
477
498
510
529
611
651
652
683
717
768
866
1474
Position within gene
Position within amplified region
327 328 419 456 477 498 510 529 611 651 652 683 717
71 72 163 200 221 242 254 273 355 395 396 427 461
Lineage
populations
M. javanica 27 A T G A A G T G G G T T A
M. incognita H1 23 A T G A A A T G A A T T A
M. incognita H2 5 A T G A A A T G A G T T A
M. incognita H3 2 A T G A A G T G A R T T A
M. sp. 1 H1 1 T T G A A A C G G G T T A
M. sp. 1 H2 1 A T G A A A C G G G T T A
M. nov. sp. 2 2 A T G A A A C G G A T T A
M. arenaria H1 4 A T G A A A C A G G T T A
M. arenaria H2 3 A T G A A A C G G G C A A
M. arenaria H3 3 A T G A A A C G G G T T A
M. ethiopica 1 A T G A A A C G G G T T A
M. inornata 2 A T G A A A C G G G T T A
M. luci H1 2 A T G A A A C G G G T T A
M. luci H2 1 A A G A A A C G G G T T G
M. floridensis 1 A T A T T A C G A G T T A


= 1population
= 2
= 3
= 4
= 9
= 25
= M. incognita I1 N1
= M. javanica J3 N1
= M. arenaria A2 N1
= M. arenaria A2 N3
= M. arenaria A3 N1
= M. sp. 1 A2-S1-M1 N1
= M. sp. 2 A1a-S1 N1
= M. floridensis F3 N1
= M. ethiopica E3 N1
= M. inornata I3 N1
= M. luci L3 N1
= M. arenaria unknown

4.3 Multi-gene haplotype network

A haplotype network was constructed using a concatenated alignment of the Cox1, Cox2, Cox3, 16S, Nad2 and Nad5 gene fragments. This multi-gene haplotype network clearly separates the major lineages of root-knot nematodes (Fig. 3). Both *M. javanica*, *M. incognita*, *M. floridensis* and *Meloidogyne* sp. 2 each occur in a clearly-separated position, supported by several lineage-specific sites. This separated position is confirmed by the low intra-/inter-lineage variability ratio of these lineages (Fig. 3). This intra-/inter variability ratio is higher for *M. arenaria* and *M. luci*, suggesting that intra-lineage variability is lower relative to inter-lineage variability with the nearest neighboring lineage. High P ID (liberal) values (Masters *et al.*, 2011) indicate a high probability of correctly identifying these lineages using BLAST, DNA Barcoding or tree placement. Interestingly, however, both *M. incognita* and *M. javanica* show some intraspecific mitochondrial variability. Furthermore, the closely related isozyme phenotypes of *M. ethiopica*, *M. inornata* and *M. luci* occupy separate positions in accordance to their mitochondrial haplotypes. Two distinct haplotypes for *M. luci* were also observed, occurring in a paraphyletic position, one shared between two populations originating from Iran and Slovenia and another haplotype from Guatemala. All ten included *M. arenaria* populations form a largely unresolved cloud of closely related haplotypes (Fig. 3). Surprisingly, the A3 esterase phenotype shared a haplotype with an A2 phenotype population and the haplotype extracted from the mitochondrial genome of *M. arenaria* (Humphreys-Pereira & Elling, 2015) for which the isozyme profile is unknown. This indicates that different isozyme phenotypes are not necessarily associated with different mitochondrial haplotypes. The slightly different haplotypes of the two *Meloidogyne* sp. 1 populations are closely associated with the *M. arenaria* cloud, indicating that *Meloidogyne* sp. 1 should be considered an *M. arenaria* variant, as already indicated by its esterase isozyme phenotype. Overall, the concatenated mitochondrial haplotype network clearly separates different lineages of parthenogenetic root-knot nematodes, demonstrating a clear link with isozyme phenotypes.



	P ID (liberal)	Intra lineage variation	Inter lineage variation	Intra/ inter
M. incognita	0.98 (0.95-1)	$8.07*10^{-4}$	0.005	0.17
M. javanica	1 (0.97-1)	$1.45*10^{-4}$	0.006	0.02
M. floridensis	0.96 (0.83-1)	0	0.003	-
M. ethiopica	0.96 (0.83-1)	0	0.002	-
M. inornata	0.98 (0.83-1)	0	$5.67*10^{-4}$	-
M. luci	0.28 (0.13-0.44)	$7.24*10^{-4}$	$5.67*10^{-4}$	1.28
M. sp. 1	0.64 (0.48-0.79)	0.001	0.002	0.56
M. sp. 2	0.98 (0.83-1)	0	0.005	-
M. arenaria (including *M.* sp. 1)	0.80 (0.75-0.84)	0.004	0.004	1
M. arenaria (excluding *M.* sp. 1)	0.64 (0.59-0.70)	0.005	0.04	1.12

Figure 3. Multi-gene haplotype network of studied MIG lineages as calculated from 16S, Cox1, Cox2, Cox3, Cytb, Nad1, Nad2 and Nad5 gene fragments. The haplotype network shows the relationships between different haplotypes, circle size is equivalent to the number of studied populations and branch length is equivalent to the number of mutations (shown as black squares). Different isozyme phenotypes are displayed by different colors, median vectors are shown as black circles. The M*eloidogyne arenaria* group is highlighted by a dashed circle. The table shows P ID (liberal) values indicating the probability of correctly identifying these lineages using BLAST, DNA Barcoding or tree placement; intra-lineage variation; inter-lineage variation to closest neighboring lineage and a ratio of intra- and inter lineage specific variation indicating the degree of separation of the lineage.

5 Discussion

Although the appointment of lineage-specific barcodes for MIG root-knot nematodes is known to be problematic, since several nuclear and mitochondrial candidate genes were found to be unsuitable (Hugall *et al.*, 1999; Lunt, 2008; Kiewnick *et al.*, 2014), we were able to find consistent differences between 11 isozyme lineages of root-knot nematodes based on nucleotide polymorphisms originating from nine coding genes of the mitochondrial genome. While non-coding genes have been shown to contain insertions prone to heteroplasmy (Hugall *et al.*, 1997; Whipple *et al.*, 1998; Humphreys-Pereira & Elling, 2015; Pagan *et al.*, 2015), we found no evidence for variable insertions within coding genes, only a very limited amount of heteroplasmic positions within a single individual was recovered. However, this variation was not associated with species specific nucleotide positions, indicating that barcode accuracy is not influenced by heteroplasmy.

As previously highlighted in various studies (Lunt, 2008; Fargette *et al.*, 2010; Kiewnick *et al.*, 2014; Humphreys-Pereira & Elling, 2015) the only clearly divergent species in clade I is *M. enterolobii*, differing in all seven sequenced mitochondrial gene fragments (7.5% - 18% divergent). Consequently *M. enterolobii* can easily be identified using any of the sequenced mitochondrial coding gene fragments. Moreover, its haplotype is identical to the mitochondrial genome sequence of *M. enterolobii* (Humphreys-Pereira & Elling, 2015) with virtually no mitochondrial variation between the seven geographically widespread populations of *M. enterolobii* observed. Between the type locality of *M. mayaguensis* (Rammah & Hirschmann, 1988) and *M. enterolobii* (Yang & Eisenback, 1983), just one single mutation in Cox3 was observed. However, this single mutation is considered insufficient to re-erect *M. mayaguensis* as a separate taxon, and thus further supports the synonymisation between *M. mayaguensis* and *M. enterolobii* (Karssen *et al.*, 2012) based on host range, isozyme phenotype and morphological data.

Except for *M. enterolobii*, clade I comprises extremely closely-related parthenogenetic lineages, known as the MIG (Powers & Sandall, 1988; Hugall *et al.*, 1994; Lunt, 2008; Fargette *et al.*, 2010; Humphreys-Pereira & Elling, 2015). This close relationship is here confirmed, based on the mitochondrial coding genes, such as the Nad3 gene fragment, which is completely identical for all MIG lineages. Also, the widely used barcode gene Cox1 is too conserved

(Kiewnick *et al.*, 2014), as it can only reliably differentiate *Meloidogyne* sp. 2 from the other MIG lineages. The limited diversity of mitochondrial coding genes confirms the recent origin of these MIG root-knot nematodes (Lunt, 2008), yet our study reveals that most of the mitochondrial coding genes exhibit some degree of diversity, generally varying between 0 and 1.5%, resulting in informative mitochondrial haplotypes. Remarkably, these mitochondrial haplotypes correspond clearly with isozyme patterns. This reflects earlier restriction fragment analysis of the intergenic region between 16S and Cox2 (Hugall *et al.*, 1994), indicating that the low, but consistent, diversity between different haplotypes can be informative in lineage identification.

Pagan *et al.* (2015) described one *M. incognita*-specific and one *M. javanica*-specific site within the 16S gene, which were subsequently used as lineage-specific restriction sites, the specificity of which was confirmed using numerous root-knot nematode populations from Africa. In the current study, we further confirm these lineage-specific sites, and additionally identify four more *M. incognita*-specific sites and five *M. javanica*-specific sites, which are directly connected to I1 (I2) and J3 esterase phenotypes, respectively. The specificity of these sites was confirmed based on 30 *M. incognita* and 27 *M. javanica* populations of widespread geographic origin, indicating that lineage sorting is complete. Moreover, the most common *M. incognita* mitochondrial haplotype was identical to the haplotype from the mitochondrial genome sequence of *M. incognita* generated by Humphreys-Pereira & Elling (2014) and to the mitochondrial haplotype extracted from the complete genome sequence of *M. incognita* (Abad *et al.*, 2008). Also, the most common *M. javanica* haplotype corresponded with its recently published mitochondrial genome (Humphreys-Pereira & Elling, 2015). We also found that the unique three banded *M. floridensis* esterase phenotype is associated with a lineage-specific mitochondrial haplotype containing seven lineage-specific sites and a separate position in the haplotype network. This confers with its aberrant meiotic parthenogenetic mode of reproduction and its isolated position according to RAPD data (Handoo *et al.*, 2004).

Furthermore, our analysis indicates that even very closely-related esterase phenotypes can be reliably identified using mitochondrial haplotypes. For example, the E3, I3 and L3 phenotypes of *M. ethiopica*, *M. inornata* and *M. luci*, respectively, were for the first time connected to distinct but related haplotypes. Surprisingly though, we recovered two separate haplotypes for

M. luci, which were both more closely related to *M. inornata* than to the other haplotype of *M. luci*. Both haplotypes occurred in populations originating from separate geographical regions (e.g. Guatemala, Iran and Slovenia), indicating either that the L3 phenotype evolved convergently from the I3 pattern, or alternatively, that the I3 phenotype is the result of reticulate evolution, which was recently suggested to play an important role during the evolution of the MIG (Lunt *et al.*, 2014). This latter scenario seems plausible as the E3, I3 and L3 phenotypes are associated with striking variations in somatic chromosome numbers, varying from 2n= 36-38 over 2n= 42-46 to 3n= 54-58, respectively (Carneiro *et al.*, 2014), inferring that these haplotypes and associated isozyme profiles originate from different hybridization events. To clarify the precise origin of these closely-related parthenogenic lineages and their taxonomic status, additional assessment of a broader range of populations from across a wide geographic distribution, in combination with genomic analysis, is necessary.

The three isozyme patterns observed for *Meloidogyne arenaria* (A2N1, A3N1, A2N3) are represented by a largely-unresolved cloud of related haplotypes in our network, where some level of variability is observed. Specifically the A2N3 phenotype occurs in two separate positions in the network, some displaying an identical mitochondrial haplotype with an A3N1 phenotype population, while the A2N1 phenotype appears to be linked to different mitochondrial haplotypes, indicating that Mdh phenotypes are not lineage-specific. Interestingly, intraspecific variability of both H1 and H3 Mdh phenotypes, has already been reported for *M. mali* (Ahmed *et al.*, 2013). That different isozyme phenotypes can be associated with the same mitochondrial haplotype in *M. arenaria* is consistent with a wide variation in karyology, with chromosome numbers varying from 30-38 over 40-48 to 51-56 (Triantaphyllou, 1963; Esbenshade & Triantaphyllou, 1985), indicating several levels of polyploidy. Consequently, the available evidence combined indicates that different lineages of *M. arenaria* have been involved in recent hybridization events. This assumption is further supported by the fact that the A3 phenotype appears to be associated with higher (52-54) chromosome numbers (Esbenshade & Triantaphyllou, 1985), while sharing all its esterase alleles with the A1 phenotype (absent in our analysis) and the A2 phenotype. Moreover, the A2 S1-M1 phenotype of *Meloidogyne* sp. 1 (haplotypes are close to the *M. arenaria* cloud) appears to be a combination of two previously reported *M. arenaria* phenotypes (A2 and S1-M1)

(1985). This suggests that *M. arenaria* actually comprises a random combination of lineages with different isozyme phenotypes and mitochondrial haplotypes.

The newly described esterase isozyme phenotype (Fig. 1 c, d) of *Meloidogyne* sp. 2 shows a very distinct mitochondrial haplotype, displaying 16 lineage-specific mutations, establishing it as the most divergent lineage of the MIG to date (Fig. 3). Additional information on this deviating lineage, which appears to have a wide geographical distribution (Spain and Guatemala) is necessary, including its mode of reproduction, cytogenetic composition and host range, in order to understand its divergent phylogenetic position. The observation that the two newly determined isozyme patterns also relate to distinct mitochondrial haplotypes provides a strong indication of the high potential value of mitochondrial haplotypes for separating lineages and root-knot nematode diagnostics. With the exception of *M. arenaria* A3, it is further demonstrated that each esterase phenotype is associated with a specific mitochondrial haplotype and that our multi-gene haplotype network shows a high degree of resemblance with the phylogenetic tree of Esbenshade and Triantaphyllou (1987) as derived from the evaluation of isozymic data. This accordance between biochemical and molecular identification techniques provides a great opportunity to evaluate species concepts within the genus *Meloidogyne*, especially in combination with upcoming genomic information.

Due to the mostly parthenogenetic nature and suggested hybrid origin of *Meloidogyne* lineages in clade I (Lunt *et al.* 2014), it is difficult to link mitochondrial haplotypes with actual speciation events. Haplotype variation can occur among lineages with the same isozymic pattern (e.g. *Meloidogyne* sp. 1, *M. luci, M. incognita, M. javanica*), while in rare cases, reticulate evolution enables species to possess the same mitochondrial haplotype but different isozymatic patterns, indicating a different genomic composition (e.g. *M. arenaria* A3 and A2, see above). Haplotype variation may be a consequence of accumulated mutations following hybridization and can be considered intraspecific variability. Alternatively, these nucleotide polymorphisms could reflect the diversity generated by crosses within an ancestral gene pool, which were later fixed by hybridization and parthenogenesis (Fargette *et al.*, 2010). In the latter case, individual mitochondrial haplotypes can be considered separate hybrid lineages, possibly each with a distinct genomic composition. Arguably, both explanations may have played a role in shaping the presently-observed genetic diversity within root-knot nematode mitochondrial genomes. To

unravel the precise origin and diversity of clade I mitochondrial haplotypes, additional knowledge on the structure and origin of their genome is crucial towards revealing whether hybridization in this group is traceable to unique hybridization events or, alternatively, that hybridization is rampant and constantly leads to new lineages of parthenogenetic root-knot nematodes. Nevertheless, in both scenarios a mitochondrial haplotype-based identification is preferable over nuclear gene-based identification or morphological determination (Hugall *et al.*, 1994; Castagnone-Sereno *et al.*, 2013; Pagan *et al.*, 2015), especially since mitochondrial haplotypes are unequivocally linked with isozyme phenotypes, which continue to be considered a superior diagnostic strategy for root-knot nematodes (Blok & Powers, 2009; Elling, 2013). Furthermore, it is suggested that while the species conundrum within the MIG continues to be resolved, 'lineages' should be used as a preferred term over 'species'. Yet, for convenience, the term species remains useful for the well-established 'species', although in effect they represent a more or less random combination of lineages.

Interestingly, most root-knot nematode lineages identified in the current study have a global distribution (Table 1). The observation that identical multi-gene mitochondrial haplotypes can have a global distribution favors the hypothesis that this distribution was caused by humans through agricultural practices and does not pre-date human crop exchange and agricultural development (Castagnone-Sereno *et al.*, 2013). If worldwide distribution would pre-date agricultural development a much larger variation in mitochondrial haplotypes between lineages from distant locations could be expected, especially because parthenogenetic reproduction most likely implies that single nucleotide polymorphisms remain separated between different populations.

The current study demonstrates that root-knot nematodes from clade I can be reliably identified using mitochondrial haplotypes. The Nad5 gene fragment contains the largest number of variable positions and is therefore the preferred barcoding gene for clade I *Meloidogyne* spp.. Sequencing the Nad5 fragment allows a reliable identification of the most common MIG lineages, i.e. *M. incognita*, *M. javanica*, most populations of *M. arenaria* but also *M. floridensis and Meloidogyne* sp. 2. However, the relatively uncommon, closely-related lineages i.e. *M. ethiopica, M. inornata, M. luci, Meloidogyne* sp. 1 and some *M. arenaria* are clustered in one Nad5 haplotype. In comparison, most of these lineages were also grouped in the same haplotype

by Pagan *et al.* (2015) (therein described as haplotype G) based on restriction fragment analysis of the intergenic spacer between Cox2 and 16S. To separate these closely-related lineages requires sequencing of an additional gene (Cox2), preferably in combination with isozyme electrophoresis. In comparison with other diagnostic strategies the proposed DNA barcoding method has several distinct advantages: (i) it is not life stage dependent, which is vital in studying root-knot nematodes, as second-stage juveniles (and males) represent the only free-living stage (Adam *et al.*, 2007; Castagnone-Sereno *et al.*, 2013); (ii) a single individual is sufficient, which is important as species mixtures are common; (iii) barcoding does not provide a yes/no answer but does help to identify unforeseen plant threats or unknown lineages; (iv) the protocol can be performed in a relatively short time span, in combination with the suggested quick DNA extraction method, which omits a time-consuming proteinase K step, enabling sequence-based lineage identification within a single day; (v) the resulting sequences can be analysed in a comparative population genetic framework using haplotype networks; (vi) barcoding using coding genes does not suffer from heteroplasmy between or within single individuals; (vii) and possibly most importantly, barcoding can produce highly reproducible results between laboratories.

6 Supplementary information Chapter 2

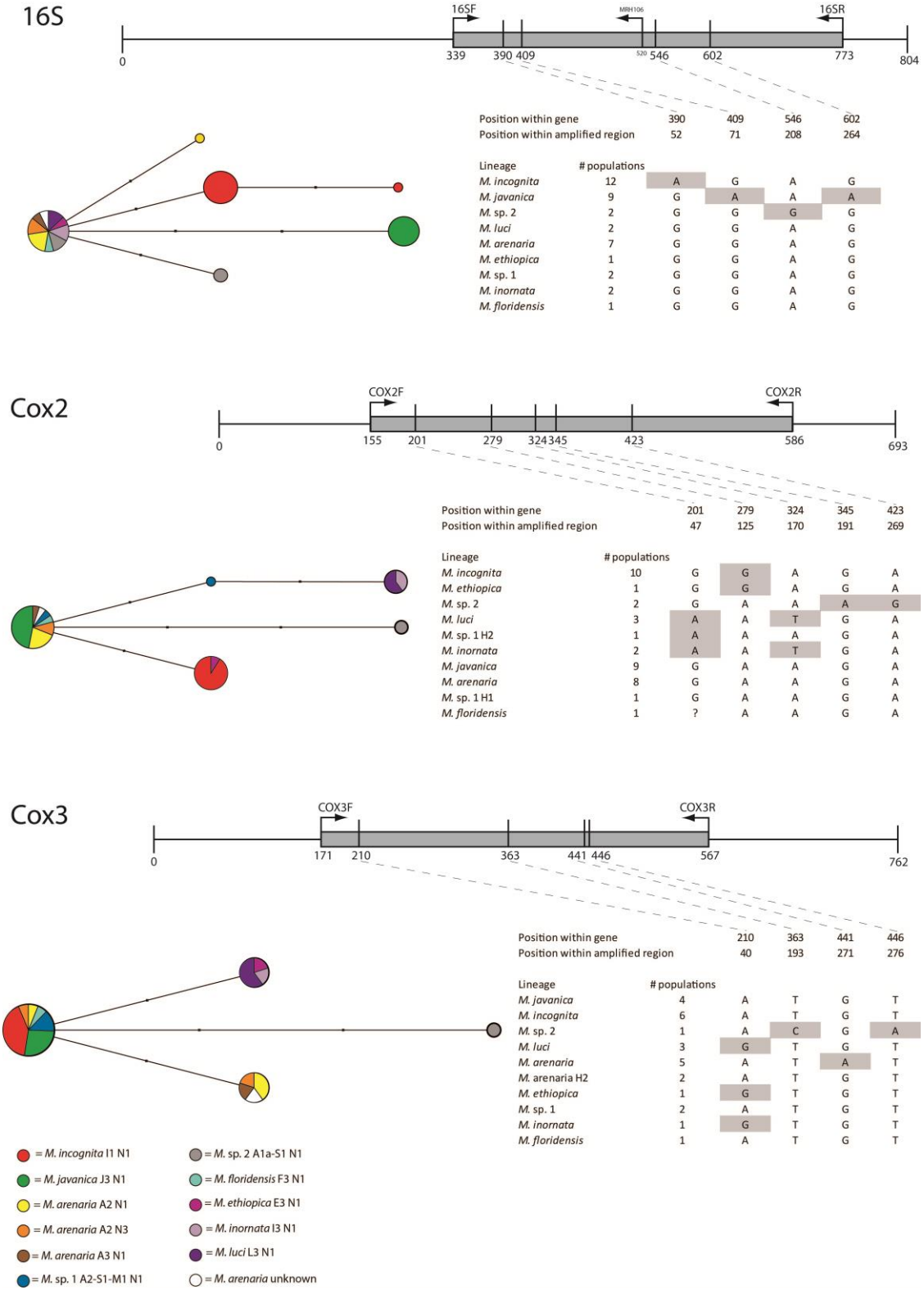
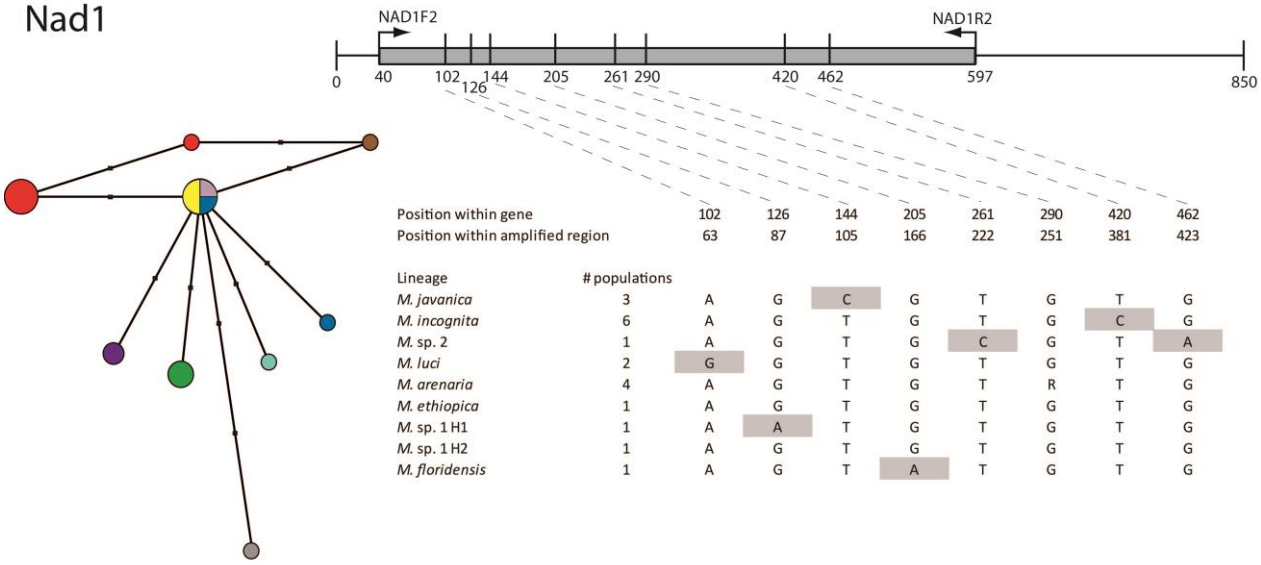


Figure S1. 16S, Cox2 and Cox3 gene sequence comparison between MIG lineages. For each gene fragment a schematic overview is provided, alongside an overview table and a haplotype network. The schematic overview shows the position and length of the amplified fragment, primer position and position of polymorphic nucleotide positions. The overview table shows the polymorphic nucleotide positions as well as the number of populations studied. The haplotype network shows the relationships between different haplotypes, circle size is equivalent to the number of studied populations and branch length is equivalent to the number of mutations (shown as black squares). Different isozyme phenotypes are displayed by different colors. Within the 16S gene two populations (*M. incognita* T532 and *M. arenaria* T332) have an extra mutation which are not shown in the schematic overview.

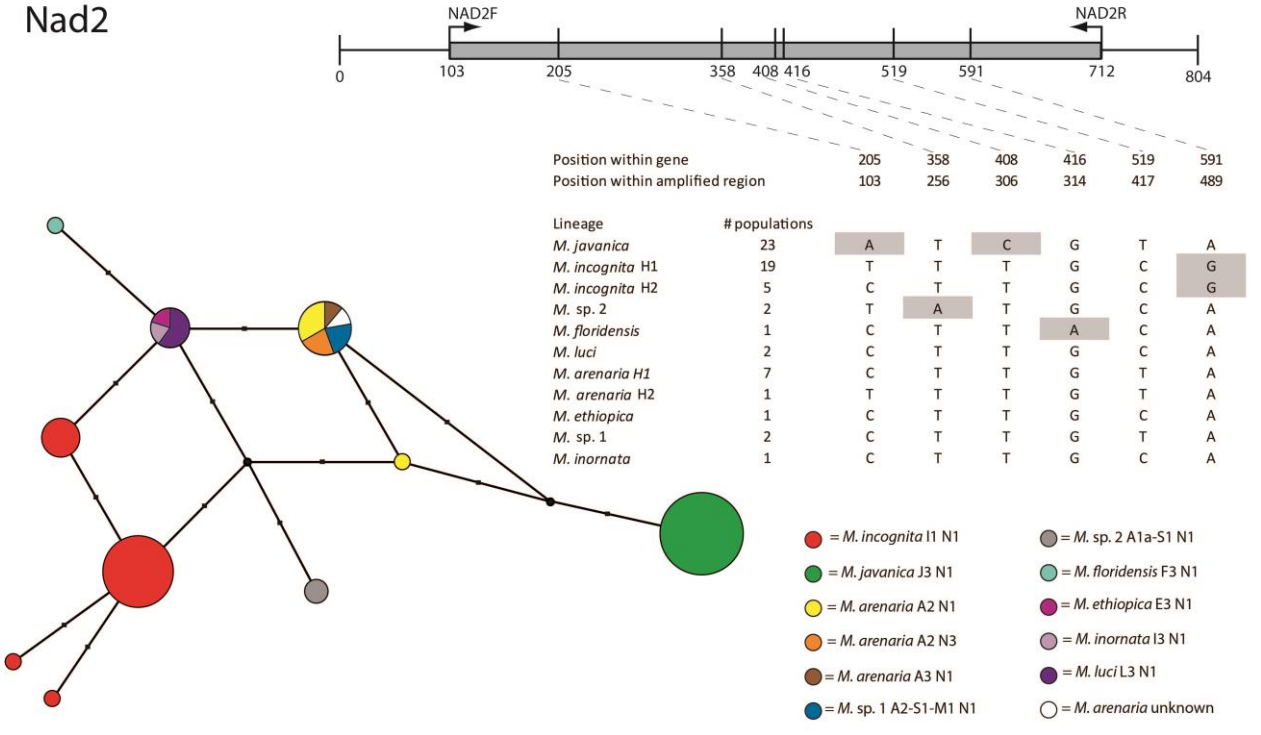
Figure S2. (Next page) Nad1 and Nad2 gene sequence comparison between MIG lineages. For each gene fragment a schematic overview is provided, alongside an overview table and a haplotype network. The schematic overview shows the position and length of the amplified fragment, primer position and position of polymorphic nucleotide positions. The overview table shows the polymorphic nucleotide positions as well as the number of populations studied. The haplotype network shows the relationships between different haplotypes, circle size is equivalent to the number of studied populations and branch length is equivalent to the number of mutations (shown as black squares). Different isozyme phenotypes are displayed by different colors, median vectors are shown as black circles. Within the Nad2 gene two *M. incognita* (C87 and M15) populations each have an extra mutation which are not shown in the schematic overview. For the Cox1 and Cytb fragment an overview table of the polymorphic nucleotide positions is provided.

Nad1



Position within gene		102	126	144	205	261	290	420	462
Position within amplified region		63	87	105	166	222	251	381	423
Lineage	# populations								
M. javanica	3	A	G	C	G	T	G	T	G
M. incognita	6	A	G	T	G	T	G	C	G
M. sp. 2	1	A	G	T	G	C	G	T	A
M. luci	2	G	G	T	G	T	G	T	G
M. arenaria	4	A	G	T	G	T	R	T	G
M. ethiopica	1	A	G	T	G	T	G	T	G
M. sp. 1 H1	1	A	A	T	G	T	G	T	G
M. sp. 1 H2	1	A	G	T	G	T	G	T	G
M. floridensis	1	A	G	T	A	T	G	T	G

Nad2



Position within gene		205	358	408	416	519	591
Position within amplified region		103	256	306	314	417	489
Lineage	# populations						
M. javanica	23	A	T	C	G	T	A
M. incognita H1	19	T	T	T	G	C	G
M. incognita H2	5	C	T	T	G	C	G
M. sp. 2	2	T	A	T	G	C	A
M. floridensis	1	C	T	T	A	C	A
M. luci	2	C	T	T	G	C	A
M. arenaria H1	7	C	T	T	G	T	A
M. arenaria H2	1	T	T	T	G	T	A
M. ethiopica	1	C	T	T	G	C	A
M. sp. 1	2	C	T	T	G	T	A
M. inornata	1	C	T	T	G	C	A

- = *M. incognita* I1 N1
- = *M. javanica* J3 N1
- = *M. arenaria* A2 N1
- = *M. arenaria* A2 N3
- = *M. arenaria* A3 N1
- = *M.* sp. 1 A2-S1-M1 N1
- = *M.* sp. 2 A1a-S1 N1
- = *M. floridensis* F3 N1
- = *M. ethiopica* E3 N1
- = *M. inornata* I3 N1
- = *M. luci* L3 N1
- = *M. arenaria* unknown

Cox1

Position within gene		558	603	846	969	1068	1119	1188
Position within amplified region		185	230	473	596	695	746	815
Lineage	# populations							
M. javanica	3	G	T	T	T	T	T	T
M. incognita	6	G	T	T	T	T	T	T
M. sp. 2	1	A	C	C	C	T	T	C
M. arenaria	4	G	T	T	T	T	T	T
M. ethiopica	1	G	T	T	T	T	T	T
M. sp. 1 H1	1	G	T	T	T	T	T	T
M. sp. 1 H2	1	G	T	T	T	T	C	T
M. inornata	2	G	T	T	T	T	T	T
M. floridensis	1	G	T	T	T	T	T	T
M. luci H1	2	G	T	T	T	C	T	T
M. luci H2	1	G	T	T	T	T	T	?

Cyt b

Position within gene		219	292	405	521	676	820	841
Position within amplified		55	128	241	357	512	656	677
Lineage	# populations							
M. javanica	3	A	A	A	C	T	G	G
M. incognita	6	A	A	A	C	T	G	G
M. sp. 2	1	G	A	A	C	T	G	G
M. luci	2	A	A	A	C	T	G	G
M. arenaria H1	1	A	A	A	C	T	G	A
M. arenaria H2	2	A	A	C/A	C	T	G	A
M. arenaria H3	1	A	G	A	C	T	G	G
M. ethiopica	1	A	A	A	C	A	G	G
M. sp. 1 H1	2	A	A	A	C	T	A	G
M. sp. 1 H2	2	A	A	A	C	T	G	G
M. floridensis	1	A	A	A	T	T	G	G

Table S3: Overview of mitochondrial gene sequences used in this study with their respective Genbank accession numbers

Species	Specimen ID	16S	Cox1	Cox2	Cox3	Cytb	Nad1	Nad2	Nad3	Nad5
Meloidogyne enterolobii										
	T337	KU372433	KU372161	KU372188	KU372222	KU372243	KU372260		KU372335	KU372359
	T382			KU372186						
	T424									
	T441	KU372435		KU372187						
	T463	KU372431		KU372189						KU372358
	T468	KU372434		KU372184	KU372223	KU372242	KU372259		KU372336	
	T536	KU372432		KU372185						
	(1)	KP202351	KP202351	KP202351	KP202351	KP202351	KP202351	KP202351	KP202351	KP202351
Meloidogyne incognita										
	T384	KU372444	KU372165		KU372225			KU372284	KU372338	KU372373
	T161	KU372438		KU372198			KU372263	KU372288		KU372388
	T515	KU372442*		KU372195*				KU372289*		KU372372*
	T526	KU372445		KU372191				KU372299		KU372361
	T532	KU372437*		KU372193*				KU372292*		KU372386*
	T540	KU372443	KU372164	KU372196	KU372228	KU372246	KU372265	KU372287	KU372339	KU372366
	T552	KU372446		KU372197				KU372285		KU372381
	Y29									KU372362
	Y57									KU372374
	C33									KU372365
	C41	KU372440				KU372248		KU372293		KU372382
	C49							KU372282		KU372379
	C53									KU372364
	C69							KU372297		KU372368
	C81							KU372296		KU372369
	C87							KU372281		KU372376
	C95							KU372298		KU372370
	M4							KU372294		KU372363
	M8							KU372300		KU372380
	M15							KU372286		KU372371
	M20							KU372283		KU372383
	M21							KU372291		KU372384
	M28	KU372439	KU372163	KU372194	KU372226	KU372247	KU372264	KU372302	KU372340	KU372377
	M44							KU372301		KU372387
	M46									KU372375
	M49									KU372367
	A1	KU372441	KU372166	KU372192	KU372227	KU372245	KU372262	KU372290	KU372341	KU372378

Species	Specimen ID	16S	Cox1	Cox2	Cox3	Cytb	Nad1	Nad2	Nad3	Nad5
	A3							KU372295		KU372385
	(2)	KJ476151	KJ476151	KJ476151	KJ476151	KJ476151	KJ476151	KJ476151	KJ476151	KJ476151
	(3)	CABB00000000	CABB00000000	CABB00000000	CABB00000000	CABB00000000	CABB00000000	CABB00000000	CABB00000000	CABB00000000
Meloidogyne javanica										
	T347	KU372450	KU372169	KU372208	KU372231	KU372249	KU372266	KU372319	KU372343	KU372392
	T417	KU372451		KU372204				KU372318		KU372391
	T429	KU372453*		KU372206*				KU372321*		KU372401*
	T485	KU372454		KU372201				KU372311		KU372410
	T497	KU372455		KU372207				KU372320		KU372394
	T509	KU372448		KU372205						KU372398
	T520	KU372452		KU372203				KU372317		KU372396
	Y60									KU372416
	C35							KU372312		KU372415
	C47							KU372313		KU372413
	C63							KU372325		KU372407
	C89							KU372324		KU372412
	M14							KU372314		KU372397
	M30							KU372305		KU372408
	M39							KU372315		KU372409
	M40				KU372230			KU372310	KU372344	KU372405
	M50									KU372395
	A8							KU372308		KU372393
	A21							KU372316		KU372414
	A23							KU372306		KU372399
	A24							KU372322		KU372406
	A25							KU372304		KU372402
	A29							KU372309		KU372404
	A30							KU372323		KU372400
	A31									KU372411
	A32	KU372449	KU372170	KU372202	KU372232	KU372250	KU372267	KU372307	KU372345	KU372403
	(1)	KP202352	KP202352	KP202352	KP202352	KP202352	KP202352	KP202352	KP202352	KP202352
Meloidogyne arenaria										
	T311	KU372428	KU372159	KU372177	KU372217	KU372240	KU372256	KU372278	KU372333	KU372357
	T332	KU372426*	KU372160	KU372181*	KU372219	KU372239	KU372258	KU372277*	KU372334	KU372354*
	T393	KU372430*		KU372183*	KU372221			KU372279*		KU372350*
	T411	KU372425		KU372178				KU372274		KU372356
	M41	KU372424	KU372158	KU372179	KU372220	KU372241	KU372257	KU372273	KU372332	KU372349
	T453	KU372429		KU372180	KU372218			KU372276		KU372351
	T461	KU372427		KU372182	KU372216			KU372275		KU372352*
	Y19									KU372355
	Y34									KU372353

Species	Specimen ID	16S	Cox1	Cox2	Cox3	Cytb	Nad1	Nad2	Nad3	Nad5
	(1)	KP202350	KP202350	KP202350	KP202350	KP202350	KP202350	KP202350	KP202350	KP202350
***Meloidogyne* sp. 1**										
	T473	KU372458	KU372174	KU372212	KU372236	KU372253	KU372271	KU372329	KU372342	KU372420
	T585	KU372459	KU372175	KU372213	KU372237	KU372254	KU372270	KU372328	KU372347	KU372421 *
***Meloidogyne* sp. 2**										
	T316	KU372460	KU372176	KU372214	KU372238	KU372255	KU372272	KU372331	KU372348	KU372423 *
	T576	KU372461		KU372215				KU372330		KU372422
Meloidogyne luci										
	T326	KU372457	KU372173	KU372210	KU372233	KU372252	KU372268	KU372327	KU372346	KU372417 *
	T459	KU372456	KU372171	KU372211	KU372235	KU372251	KU372269	KU372326		KU372418
	T693		KU372172	KU372209	KU372234					KU372419 *
Meloidogyne inornata										
	T638	KU372447	KU372167	KU372200				KU372303		KU372390
	T695		KU372168	KU372199	KU372229					KU372389
Meloidogyne ethiopica										
	T612	KU372436	KU372162	KU372190	KU372224	KU372244	KU372261	KU372280	KU372337	KU372360 *
Meloidogyne floridensis										
	(4)	CCDZ00000000	CCDZ00000000	CCDZ00000000	CCDZ00000000	CCDZ00000000	CCDZ00000000	CCDZ00000000	CCDZ00000000	CCDZ00000000

(1) Mitochondrial coding genes extracted from complete mitochondrial genome sequence (Humphreys-Pereira & Elling, 2015)
(2) Mitochondrial coding genes extracted from complete mitochondrial genome sequence (Humphreys-Pereira & Elling, 2014)
(3) Mitochondrial coding genes extracted from next generation sequencing reads of complete genome (Abad *et al.*, 2008)
(4) Mitochondrial coding genes extracted from next generation sequencing reads of complete genome (Lunt *et al.*, 2014)
*This gene was sequenced for two different individuals from the same population, revealing the same haplotype

Chapter 3

Integrative taxonomy of root-knot nematodes reveals multiple independent origins of mitotic parthenogenesis

Modified from Janssen T., Karssen G., Topalović O., Coyne D., Bert W. 2017. Integrative taxonomy of root-knot nematodes reveals multiple independent origins of mitotic parthenogenesis. PLoS ONE 12(3). http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0172190

Cited by 2, IF 2015: 3.057, Q1 11/63.

1 Abstract

During sampling of several *Coffea arabica* plantations in Tanzania severe root galling, caused by a root-knot nematode was observed. From pure cultures, morphology and morphometrics of juveniles and females matched perfectly with *Meloidogyne africana,* whereas morphology of the males matched identically with those of *Meloidogyne decalineata*. Based on their Cox1 sequence, however, the recovered juveniles, females and males were confirmed to belong to the same species, creating a taxonomic conundrum. Adding further to this puzzle, re-examination of *M. oteifae* type material showed insufficient morphological evidence to maintain its status as a separate species. Consequently, *M. decalineata* and *M. oteifae* are synonymized with *M. africana,* which is herewith redescribed based on results of light and scanning electron microscopy, ribosomal and mitochondrial DNA sequences, isozyme electrophoresis, along with bionomic and cytogenetic features. Multi-gene phylogenetic analysis placed *M. africana* outside of the three major clades, together with *M. coffeicola, M. ichinohei* and *M. camelliae*. This phylogenetic position was confirmed by several morphological features, including cellular structure of the spermatheca, egg mass position, perineal pattern and head shape. Moreover, *M. africana* was found to be a polyphagous species, demonstrating that "early-branching" species of *Meloidogyne* are not as oligophagous as had previously been assumed. Cytogenetic information indicates *M. africana* (2n=21) and *M. ardenensis* (2n=51-54) to be a triploid mitotic parthenogenetic species, revealing at least four independent origins of mitotic parthenogenesis within the genus *Meloidogyne*. Furthermore, *M. mali* (n=12) was found to reproduce by amphimixis, indicating that amphimictic species with a limited number of chromosomes are widespread in the genus, potentially reflecting the ancestral state of the genus. The wide variation in chromosome numbers and associated changes in reproduction modes indicate that cytogenetic evolution played a crucial role in the speciation of root-knot nematodes and plant-parasitic nematodes in general.

2 Introduction

Coffee (*Coffea arabica* L.) is one of the most important cash crops worldwide and the second most important traded commodity after oil, with an estimated total export value of US$ 19.1 billion in 2012/2013 (ICO, 2014). An estimated 100 million people worldwide are dependent on growing coffee, most of them from developing tropical countries (Vega *et al.*, 2003). In Africa, coffee generates substantial income for rural communities and is a primary source of income for an estimated 10 million households in 25 countries (ICO, 2015). However, coffee production in Africa is declining, by approximately 17% since the 1970's (ICO, 2015), while in other regions coffee production has essentially doubled over the last 50 years. Meanwhile, global coffee consumption continues to rise at an accelerating rate (ICO, 2014). There are various reasons for the coffee production decline in Africa, among them losses due to pests and diseases, and the costs involved in dealing with them. Pesticides, for example, account for over 30% of coffee production costs (ICO, 2014; ICO, 2015). Of the various ailments that plague coffee production, plant-parasitic nematodes, in particular root-knot nematodes (*Meloidogyne* spp.), are an especially significant, yet relatively overlooked threat. In South and Central America, where most of the available information on nematode pests of coffee has been attained, nematodes are recognized as highly damaging pests, responsible for the complete destruction of coffee plantations, to the point of forcing a shift to other crops, such as sugar cane (Campos & Villain, 2005). Often, coffee can only be cultivated when grafted onto nematode-resistant root-stocks.

In South and Central America the root-knot nematodes that cause damage to coffee roots are *M. exigua* Göldi, 1887, *M. incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. coffeicola* Lordello & Zamith, 1960, *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996, *M. hapla* Chitwood, 1949, *M. arenaria* Chitwood, 1949, *M. inornata* Lordello, 1956, *M. arabicida* Lopez & Salazar, 1989, *M. konaensis* Eisenback, Bernard and Schmitt, 1994, *M. enterolobii* Rammah & Hirschmann 1988, *M. izalcoensis* Carneiro, Almeida, Gomes and Hernandez, 2005 and *M. lopezi* Humphreys-Pereira, Flores-Chaves, Gomez, Salazar, Gomez-Alpizar & Elling, 2014 (Carneiro & Cofcewicz, 2008; Humphreys-Pereira *et al.*, 2014). Most of these species can be identified using species-specific primers that have been developed to

amplify sequence-characterized amplified regions (SCAR), having themselves been converted from diagnostic randomly amplified polymorphic DNA fragments (RAPDs) (Randig *et al.*, 2002; Correa *et al.*, 2013).

Despite the importance of root-knot nematodes on coffee, there is virtually no information on nematodes of coffee in Africa (Carneiro & Cofcewicz, 2008). *Meloidogyne* spp. reported on coffee in Africa include the widely distributed *M. incognita*, *M. javanica*, (Campos & Villain, 2005) and recently *M. izalcoensis* and *M. hapla* (Jorge Junior *et al.*, 2016). There are also five species which have been almost exclusively reported from Africa, *M. africana* Whitehead, 1959, *M. kikuyensis* De Grisse, 1960, *M. oteifae* Elmiligy 1968, *M. megadora* Whitehead, 1968 and *M. decalineata* Whitehead, 1968. *Meloidogyne africana* has been reported on *C. arabica* in Kenya (Whitehead, 1959; Campos & Villain, 2005) and Tanzania (Bridge, 1984), on *C. canephora* L. in Democratic Republic of Congo (Campos *et al.*, 1990), on pepper (*Capsicum annuum* L.) in Sudan (Yassin & Zeidan, 1982) and once outside of Africa on maize (*Zea mays* L.) in India (Chitwood & Toung, 1960). *Meloidogyne kikuyensis* was originally described from *Pennisetum clandestinum* Hochst. in Kenya (De Grisse, 1960) but was also reported from coffee by Whitehead (1969). *Meloidogyne oteifae* was described from *Pueraria javanica* Benth. and *C. canephora* in Congo, but has not been reported since (Elmiligy, 1968). *Meloidogyne megadora* was originally described from *C. arabica* and *C. canephora* in the Republic of Angola (Whitehead, 1968) and later reported from Uganda and São Tomé and Príncipe (de Almeida & Santos, 2002; Campos & Villain, 2005). *Meloidogyne decalineata* was also reported in coffee nurseries on São Tomé Island (Lordello & Fazuoli, 1980).

Since the monumental taxonomical work of Whitehead (Whitehead, 1959; Whitehead, 1968; Whitehead, 1969), progress on the taxonomy of African root-knot nematodes has been limited. Problematically, most descriptions are only based on a limited number of morphological features (Carneiro & Cofcewicz, 2008), causing problems in species diagnostics, since morphological identification of root-knot nematodes and indeed of nematodes in general is known to be greatly hampered by phenotypic plasticity (Eyualem & Blaxter, 2003; Hunt & Handoo, 2009). As a result of this, and limited local expertise, root-knot nematode infections on coffee in Africa are rarely identified up to species level (Onkendi *et al.*, 2014).

In 2013, severe root-galling was observed in several coffee plantations in the Lushoto and Mbelei districts of Tanzania. Intriguingly, initial phylogenetic analyses revealed the root-knot nematode to be outside the three classically recognised clades within the genus (Tandingan De Ley *et al.*, 2002), such species are not frequently encountered in field surveys, and as a consequence, little morphological and isozymic information is available, while cytogenetic information is missing completely (Castagnone-Sereno *et al.*, 2013). Interestingly, *M. coffeicola*, also a coffee root-knot nematode species from Brazil, is also considered to be to be outside the three well-known clades (Tomalova *et al.*, 2012), while *M. ichinohei* Araki, 1992, a root-knot nematode from Japan parasitizing *Iris laevigata* Fisch. & Mey., 1839 (Holterman *et al.*, 2009), *M. camelliae* Golden, 1979 and an undescribed species of *Meloidogyne* from *Sansevieria* sp. are reported occupying a paraphyletic phylogenetic position in the genus, based on ribosomal rDNA (Holterman *et al.*, 2009; Trisciuzzi *et al.*, 2014). However, studying oogenesis of these uncommon species would most likely allow insight into the complex cytogenetic history of the genus *Meloidogyne* (Castagnone-Sereno *et al.*, 2013).

Formerly, amphimictic root-knot nematodes were hypothesized to be the ancestral state within the genus *Meloidogyne*, meiotic parthenogenetic species derived from them and mitotic parthenogenetic species evolved from meiotic parthenogenetic species (Triantaphyllou, 1985a; Triantaphyllou, 1990; Castagnone-Sereno, 2006). This hypothesis was primarily based on the low chromosome number of obligatory amphimictic species: *M. spartinae* (Rau & Fassuliotis, 1965) Whitehead, 1968 (Triantaphyllou, 1987) and *M. kikuyensis* (Triantaphyllou, 1990). Both species have n=7 chromosomes, while *M. carolinensis* Eisenback, 1982, *M. megatyla* Baldwin & Sasser, 1979 and *M. microtyla* Mulvey, Townshend & Potter, 1975 have n=18 chromosomes (Triantaphyllou, 1985a). However, molecular phylogenies demonstrated that *M. microtyla* (Tandingan De Ley *et al.*, 2002) and *M. spartinae* (Plantard *et al.*, 2007; Holterman *et al.*, 2009) did not occur at their assumed early diverging position, while the assumed meiotic parthenogenetic species *M. artiellia* Franklin, 1961 (cytology of this species was never formally studied) does take an early diverging position (De Giorgi *et al.*, 1996; De Giorgi *et al.*, 2002; Castagnone-Sereno *et al.*, 2013). Consequently, this provided support for the alternative hypothesis of Triantaphyllou (1985a), in which meiotic parthenogenetic species reflect the ancestral state in comparison to amphimictic and mitotic parthenogenetic species (Castagnone-Sereno *et al.*, 2013). Moreover, mitotic parthenogenetic species are reported to have several

origins, one in clade I in which all species are mitotic parthenogens, except *M. floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar & Higgins, 2004, and one in clade II in which *M. hapla* race B and *M. partityla* are described to be mitotic parthenogens (Marais & Kruger, 1991; Van der Beek *et al.*, 1998), while in in clade III *M. oryzae* Maas, Sanders & Dede, 1978 is the only apomictic species among facultative meiotic parthenogens (Tandingan De Ley *et al.*, 2002; Holterman *et al.*, 2009; Castagnone-Sereno *et al.*, 2013).

Recovery and culturing of *M. africana* facilitated the current study on the evolution of reproduction and oogenesis within the genus. However, to identify this species a taxonomic conundrum needed to be resolved, based on the limited available morphological and molecular information for African coffee root-knot nematodes. Therefore, the first objective of the current study was to perform an integrative taxonomic description based on LM, SEM, four loci (18S, ITS, 28S, Cox1) and isozyme phenotyping, which remains essential for accurate diagnosis of root-knot nematodes (Hunt & Handoo, 2009). The second objective was to gain insight into the bionomics and mode of reproduction by studying host symptoms and cytology of the parasite. The final objective was to place morphological, isozyme, bionomic and cytological findings in an evolutionary perspective using a multi-gene phylogeny of the genus *Meloidogyne* to reveal insight into the evolution of reproduction and oogenesis within the genus.

3 Material and methods

3.1 Collection of populations, culturing and host-range test

In September 2013, root samples of *C. arabica* showing a characteristic root-knot galling were collected from nine fields from two villages in the West Usambaras Lushoto Mountain Reserve (Tanzania): Mbelei (-4.83166,38.39883; -4.82946,38.39694; -4.82824,38.395778; -4.83183,38.39866; -4.83025,38.399120; -04.79879,038.30156) and Lushoto (-4.79879,38.30156; -4.80096,38.29975; -04.82946,038.39694). No specific permit was required for this sampling and this study did not involve endangered species. Nematodes were extracted from soil using a modified Baermann funnel (Hooper, 1986) and subsequently fixed in DESS solution (Yoder *et al.*, 2006). From each sample, infected roots were directly fixed in DESS solution. Second-stage juveniles were hand-picked from dissected galls of fresh roots using a

stereomicroscope and inoculated onto *C. arabica* seedlings in individual pots containing sterile potting media and maintained in a greenhouse of the National Plant Protection Organization (Wageningen, the Netherlands) at 23°C. Nematodes from this *C. arabica* culture were extracted using a variety of techniques including the modified Baermann funnel, Oostenbrink elutriator, centrifugal sugar flotation, mistifier and gall dissection (van Bezooijen, 2006). A host-range test was performed by inoculating 1500 juveniles on *Sanseveiria* sp. and *Solanum lycopersicum* L. (cv. Moneymaker).

3.2 Morphological characterization

Nematodes were studied in temporary preparations sealed with nail-polish using an Olympus BX50 DIC microscope (Olympus Optical), DESS fixed specimens were first transferred to water for 1 hour before mounting in temporary slides. Morphological vouchers were created using a combination of movies and photomicrographs with an Olympus C5060Wz camera, which are available online at http://www.nematodes.myspecies.info and on the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.9f63r). Vouchered nematodes were subsequently picked from temporary mounts and processed for DNA extraction. Nematodes from *C. arabica* cultures were fixed in TAF (Triethanolamine 2%, Formalin 7%, distilled water 91%) at 70°C and processed to anhydrous glycerine, following the method of Seinhorst (1962) modified by Sohlenius and Sandor (1987). These TAF fixed specimen were used for a profound morphometric analysis. Comparative morphological analysis of each live stage as presented in section 1 are based on a combination of field caught populations and the population in culture. The cellular architecture of the gonads of egg laying females was examined after dissection in temporary mounts according to the method of Bert et al. (2002); movies of spermatheca morphology are available online on Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.9f63r). For scanning electron microscopy (SEM) nematodes were fixed in 600 µl fresh 4% Paraformaldehyde fixative buffered with Phosphate Buffer Saline (PBS) and 1% glycerol and heated for 3 seconds in a 750W microwave. Subsequently specimens were dehydrated in a seven-step graded series of ethanol solutions and critical-point dried using liquid CO_2, mounted on stubs with carbon discs, coated with gold 25 nm. Specimens were studied and photographed with a JSM-840 EM (JEOL) electron microscope at 12 kV.

3.3 Isozyme analysis

Esterase and malate dehydrogenase isozymes were analysed according to Karssen et al. (1995). Ten young females were isolated from root cultures in isotonic (0.9%) NaCl solution. Individual females, after desalting in reagent-grade water on ice for 5 minutes, were loaded to sample wells containing 0.6 µl extraction buffer (20% sucrose, 2% triton X-100, 0.01% Bromophenol Blue), and subsequently macerated using a glass rod. This mixture was homogenized, and protein extractions were loaded onto a (8-25) polyacrylamide gradient gel and electrophoretically fractioned using a PhastSystem (Pharmacia Ltd., Uppsala, Sweden). In addition to the ten test samples, two *M. javanica* protein extractions were added to the centre of each gel to serve as a reference. After electrophoresis, gels were stained to examine for malate dehydrogenase (Mdh) and esterase (Est) activity for 5 and 45 minutes, respectively, rinsed with distilled water, and fixed using a 10% glycerol, 10% acetic acid, distilled water solution.

3.4 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from both live specimen and specimen fixed in DESS solution. Genomic DNA of individual crushed females was extracted using worm lysis buffer and proteinase K (Bert *et al.*, 2008) while genomic DNA of juveniles and males was extracted using the quick alkaline lysis protocol described by Janssen et al. (2016). Briefly, individual nematodes were transferred to a mixture of 10 µl 0.05N NaOH and 1 µl of 4.5% Tween. The mixture was heated to 95°C for 15 min, and after cooling to room temperature 40 µl of double-distilled water was added. PCR amplification was performed using toptaq DNA polymerase (QIAGEN, Germany), in a volume of 25 µl using a Bio-Rad T100^{TM} thermocycler. PCR mixtures were prepared according to the manufacturer's protocol with 0.4 µM of each primer and 2 µl of single nematode DNA extraction. The 28S rDNA fragment D2A (ACA AGT ACC GTG AGG GAA AGT TG) and D3B (TCG GAA GGA ACC AGC TAC TA) primers were used according to De Ley et al. (1999). The 18S rDNA gene was amplified using G18S4 (GCT TGT CTC AAA GAT TAA GCC) and 18P (TGA TCC WKC YGC AGG TTC AC) with internal sequencing primers 4F (CAA GGA CGA WAG TTW GAG G) and 4R (GTA TCT GAT CGC CKT CGA WC) according to Bert et al. (2008). The internal transcribed rDNA spacer (ITS) was amplified using VRAIN2F (CTT TGT ACA CAC CGC CCG TCG CT) and VRAIN2R (TTT CAC TCG CCG TTA CTA AGG GAA TC) subsequently cloned using pGEM ® -T easy vector systems (Promega) and sequenced using universal M13F and M13R primers.

The Cytochrome c oxidase subunit 1 (Cox1) gene fragment was amplified using JB3 (TTT TTT GGG CAT CCT GAG GTT TAT) and JB4.5 (TAA AGA AAG AAC ATA ATG AAA ATG) (Bowles *et al.*, 1992; Derycke *et al.*, 2010b). Sanger sequencing of purified PCR fragments was carried out in forward and reverse direction by Macrogen (Europe). Contigs were assembled using GENEIOUS R6.1.8 (Biomatters; http://www.Geneious.com). All contigs were subjected to BLAST searches to check for possible contaminations on http://www.ncbi.nlm.nih.gov.

3.5 Sequence analysis

Multiple sequence alignments of single ribosomal genes (18S, 28S and ITS) were made using the Q-INS-i algorithm in MAFFT 7.271 (http://mafft.cbrc.jp/alignment/server/index.html), which accounts for the secondary structure of rRNA (Katoh & Standley, 2013). Cox1 sequences were translated using the TranslatorX webserver (http://translatorx.co.uk/) (Abascal *et al.*, 2010), using the invertebrate genetic code, and the nucleotides aligned according to an amino acid alignment constructed using MAFFT. Multiple sequence alignments are available on Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.9f63r). Post alignment trimming was conducted using the parametric profiling method of ALISCORE2.2 (Misof & Misof, 2009). Gaps were treated as a 5th character and the default sliding window was used. The best fitting substitution model was estimated for each gene using the Akaike Information Criterion in jModelTest 2.1.2 (Darriba *et al.*, 2012). Single gene alignments were concatenated with GENEIOUS R6.1.8 (S1 Table). Phylogenetic analyses of single genes were conducted by Bayesian methods, while the phylogeny of the concatenated alignment was conducted using both Bayesian and maximum likelihood methods. Maximum likelihood analyses were conducted using RaxML 8.0 (Stamatakis, 2014) with 5000 bootstrap replicates under the GTR + I + G model treating every gene as a separate partition. Bayesian phylogenetic analyses were conducted using MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003). Two separated analysis were performed using the default priors and the GTR + I + G model using three heated (temp=0.2) and one cold chain per analysis. Gaps were treated as missing data and in the multi-gene analysis each gene and different Cox1 codon positions were treated as a different partitions. Analyses were run for 20 million generations, sampling trees every 500th generation. Run convergence was assessed using standard deviation of split frequencies and Potential Scale Reduction Factors (PSRF). Of the results 25% were discarded as burnin and burnin size was evaluated using a generation/Log-likelihood scatterplot. Reproduction modes were traced along

the Bayesian majority rule consensus tree phylogeny using maximum parsimony and maximum likelihood methods implemented in Mesquite 3.10 (Maddison & Maddison, 2016).

3.6 Cytological staining

Egg laying females were dissected from freshly collected coffee roots, smeared on microscope slides and stained according to Triantaphyllou (1985b). Smears were hydrolysed by submerging the slide for 6 minutes in 1N HCl, fixed for 30-60 minutes in freshly prepared fixative consisting of 75% absolute ethanol and 25% acetic acid, stained for 30 minutes in propionic orcein and washed using 45% propionic acid. Preparations were sealed using nail polish and oogenesis was studied using an Olympus BX51 DIC microscope (Olympus Optical). Chromosome numbers were estimated from late-prophase or early-metaphase chromosomal planes, because in these stages the chromosomes are discrete and can be counted accurately (Triantaphyllou, 1985a). Multifocal movies were made from chromosome planes and subsequently analysed and counted frame-by-frame using ImageJ software(Schneider *et al.*, 2012). Movies are available online at http://www.nematodes.myspecies.info and on the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.9f63r).

4 Results and discussion

4.1 Morphological identification of coffee root-knot nematodes from Tanzania

From all nine sampled locations a *Meloidogyne* species was isolated. These specimen were morphologically similar and, based on their Cox1 sequence (see section 3.3.1), were confirmed to belong to the same species. As currently the descriptions of African coffee root-knot nematodes are limited to morphological features, the morphology of this species is compared with original type material of *M. africana, M. oteifae, M. megadora* and *M. decalineata*. For morphological comparison both a cultured population on *Coffea arabica* and field caught populations were used.

4.1.1 Females

A morphological and morphometric comparison of females and perineal patterns between *M. africana, M. oteifae, M. megadora* and *M. decalineata* revealed that these species are less different than previously considered (Table 1). Whitehead (1968) described the female body of *M. decalineata* without or with a very slight protuberance, however, a clear protuberance was

observed in paratype specimens. Perineal patterns of *M. decalineata* and *M. oteifae* were differentiated from *M. africana* by exhibiting a narrow versus a wide lateral field (Elmiligy, 1968; Whitehead, 1968), however, examination of type material revealed no significant morphological differences. *Meloidogyne oteifae* was originally further differentiated from *M. africana* by the circles of striae, which are crossed by other striae radiating from the vulva (Elmiligy, 1968), however, the striation pattern around the vulva observed in paratype specimens of *M. africana* and *M. decalineata* was identical. Also, the morphology of the female head is very similar for all considered species (Table 1, Fig 1 and 2). Thus, no female morphological or morphometric characters separate our populations, *M. africana*, *M. oteifae*, *M. megadora* and *M. decalineata.*

Table 1: Comparison of female morphological and morphometric characters between species of *Meloidogyne* parasitizing coffee in Africa.

	M. africana Whitehead 1959[5]	*M. oteifae* Elmiligy 1968	*M. decalineata* Whitehead 1968	*M. megadora* Whitehead 1968	*Meloidogyne sp.*
n	17	10	20	12	22
L	760±73 (660-910)	600 (520-680)	819±133 (649-1041)	683±87 (554-845)	615±95 (400.0-770.0)
Stylet	15	13.5 (13-14)	14 (12-17)	15 (13-17)	14.3±0.8 (13.0-15.5)
Stylet knobs width	/	/	/	3 (3-5)	3.1±0.3 (3.0-4.0)
DGO	4-9	3.5 (3-4)	4	6 (4-8)	5.7±0.8 (4.5-7.0)
a	1.6±0.16 (1.4-1.9)	/	1.6±0.20 (1.2-2.1)	1.45±0.218 (1.1-1.8)	1.6±0.2 (1.3-2.4)
Body shape	pyroid	pyroid	pyroid	pyroid	pyroid
Protuberance	present	present[1]	present[2]	mostly present	present
ES porus	16-30 annules	12-15 annules	20-50 annules	8-30 annules	14-35 annules
Phasmids	close to tail terminus	close to tail terminus	close to tail terminus	close to tail terminus	close to tail terminus
Elevated perenium	present	present[3]	present	sometimes present	present
Start lateral field	faint	faint	rudimentary lateral field in some patterns	not generally visible, posterior part with striae	faint
Dorsal arch	low	low	low	low	low
Stria	very fine	very fine	very fine	very fine	very fine
Stria surrounding vulva	sometimes striae surrounding vulva	striae surrounding vulva[4]	sometimes striae between tail and vulva	/	sometimes striae surrounding vulva
Stylet knobs	rounding or tending to flatten anterior	rounded	rounded or backward sloping	back-sloped	rounded
Head annules	1 or 2 annules behind headcap	difficult to distinguish	2 annules behind headcap	3 annules behind headcap	1 or 2 annules behind headcap

[1] Not mentioned in the original description of Elmiligy (1968) but clearly observed in paratype slides.
[2] Whitehead (1968) mentions posterior end without or with very slight protuberance, however, we observe a clear protuberance in all paratype specimen.
[3] Elmigly (1968) mentiones the perineal pattern not raised on a knob, however, we observe a clearly elevated perenium in several paratype perineal patterns.
[4] Elmigly (1968) differentiated *M. oteifae* from *M. africana* by the circles of striae which are crossed by other striae radiating from the vulva and by the absence of the wide relatively clear area in the lateral field. However, we observe exactly the same striation around the vulva in several paratype specimen of *M. africana* and we observed no difference in morphology of the lateral field between paratype material of *M. oteifae* and *M. africana*.
[5] Standard deviations are wrongly calculated in Whitehead (1959), standard deviations in this table are taken from Whitehead (1968)

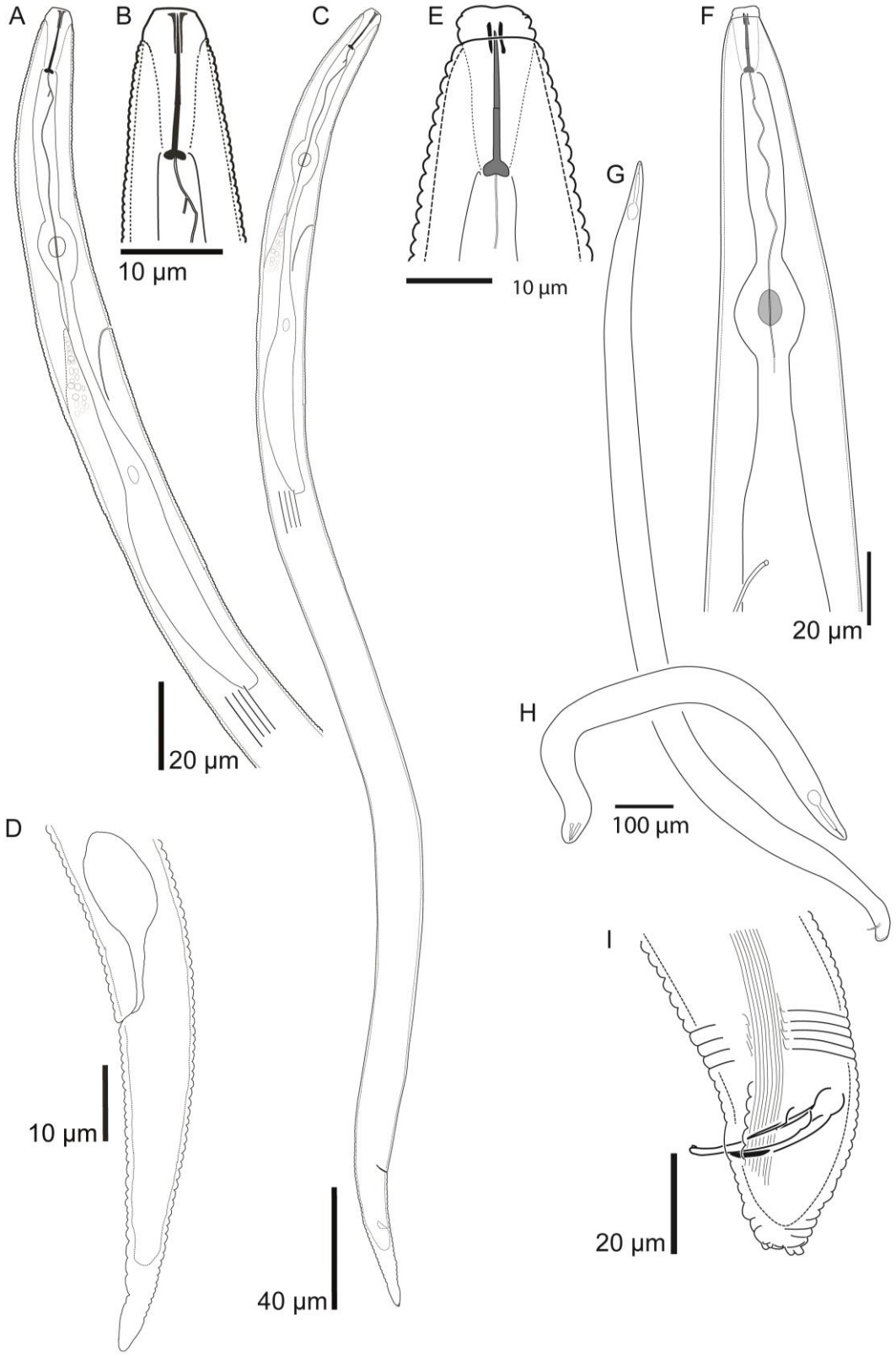


Figure 1. Camera lucida drawings of *Meloidogyne africana* from Tanzania. (A) second-stage juvenile anterior body; (B) second-stage juvenile head; (C) second-stage juvenile habitus; (D)

second-stage juvenile tail; (E) male head; (F) male anterior body; (G, H) variable male habitus during development as sex-reversed females; (I) male tail.

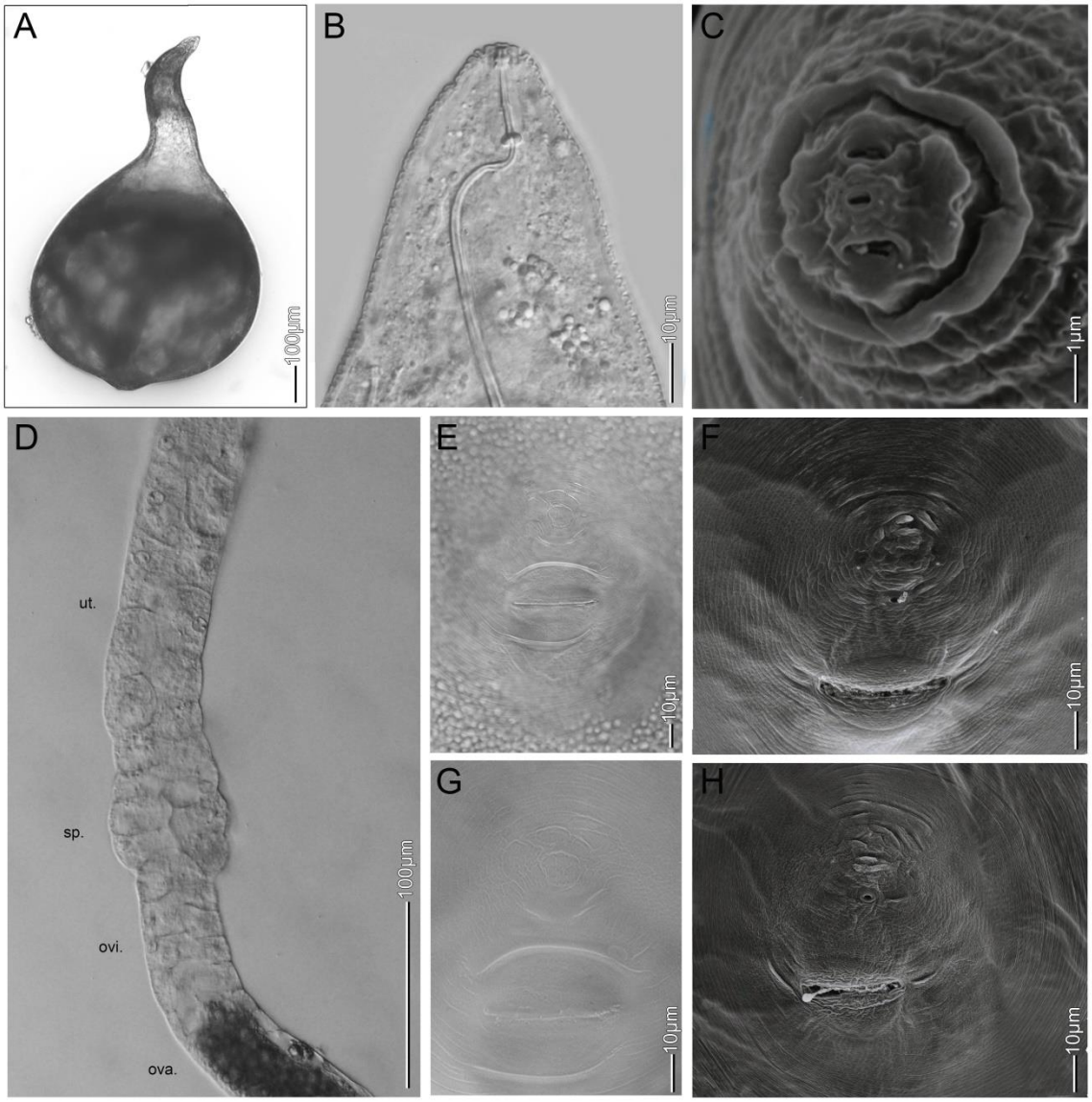


Figure 2. Light microscopy and SEM of *Meloidogyne africana* females. (A) general habitus with characteristic protuberance; (B) head, lateral view; (C) cephalic region (en face view); (D) gonad morphology of uterus (ut.), spermatheca (sp.), oviduct (ovi.) and ovarium (ova.); (E, G) photomicrographs of perineal pattern; (F,H) SEM photograph of perineal pattern.

4.1.2 Second-stage juveniles

The morphological comparison between second-stage juveniles reveals strong similarities between our population, and both *M. africana* and *M. oteifae* (Table 2). By contrast, the juvenile characteristics of *M. decalineata* match well with *M. javanica*. The total length, tail length, hyaline tail terminus of *M. decalineata* is markedly longer compared to *M. africana, M. oteifae* and our population, and the tail terminus has a subacute pointed terminus (vs rounded tail terminus). These differences are unexpected as *M. decalineata* was reported from the same site and host in the Lushoto district as three of our populations (Whitehead, 1969). Furthermore, a long juvenile tail usually implies that phasmids are positioned far apart in the perineal pattern, due to swelling of the juvenile body during transition to the female live stage. In *M. decalineata* the phasmids are positioned close to each other in the perineal pattern (Table 1). These observations indicate that *M. decalineata* was most likely described from a species mixture and that the described juveniles of *M. decalineata* belong to *M. javanica*. This error is possible as *M. javanica* has been reported from coffee and weeds from coffee plantations in East Africa and is a commonly occurring root-knot nematode in the region ((Whitehead, 1969); personal observations TJ). Additionally, Whitehead (1969) reports *M. decalineata* to be a very common species in the village of Lushoto, however, after profound sampling only *M. africana*-like juveniles were recovered during the current sampling. In conclusion, the differences between juveniles of our population (Fig 1 and 3), *M. africana* and *M. oteifae* are insignificant and the juveniles that have been described for *M. decalineata* (Whitehead, 1968) are here considered to belong to *M. javanica*. Juveniles of *M. megadora* have a longer tail with a shorter hyaline terminus in comparison to our population, *M. africana* and *M. oteifae.*

Table 2. Comparison of second-stage juvenile morphological and morphometric characters between species of *Meloidogyne* parasitizing coffee in Africa.

	M. africana Whitehead 1959[7]	*M. oteifae* Elmiligy 1968	*M. decalineata* Whitehead 1968	*M. javanica* Eisenback and Triantaphyllou 1981	*M. megadora* Whitehead 1968	*Meloidogyne sp.*
n	25	30	25	90	26	30
L	420±5 (380-470)	370 (320-400)	543±24 (471-573)	488 (402-560)	451±27 (413-548)	422.0±39 (352.0-535.5)
Stylet	14.8±1.5 (12-18)	12 (11-13)	12.4±0.68 (10.7-13.7)	10-12	12.0±0.61 (10.7-13.2)	11.5±0.5 (10.5±12.5)
DGO	3	3	3.5 (3.0-4.0)[2]	3.5	3.9±1.14 (2.3-4.8)	4.0±0.6 (3.0-5.5)
Tail	40±1.7 (30-48)	around 48[3]	48±2 (44-52)	56 (51-63)	53±3 (47-58)	42.1±1.9 (39.0-46.0)
Hyaline tail terminus	7.2±1.4 (Jepson 1987)	Around 10 (Jepson 1987)	15.5±3.1 (Jepson 1987)	13.7 (9-18)	6.3±0.6 (Jepson 1987)	10.5±1.3 (8.0-13.0)
a	24.4±1.75 (22-28)	26.5 (22-29)	36.3±1.94 (32.8-40.0)	/	27.8±2.31 (23.1-32.9)	25.5±3.1 (19.5-31.1)
c	10.8±1.82 (7.3-14.3)	8 (7.5-9.2)	11.2±0.51 (10.3-12.2)	/	8.4±0.64 (7.6-11.0)	10.1±1.0 (7.8-12.7)
Tail shape	blunt rounded terminus	tail tapering to a rounded terminus	tail tapering smoothly or irregularly to subacute terminus	tail tapering smoothly or irregularly to subacute terminus[4]	tail tapering irregularly ending in a subacute variously end	blunt rounded terminus
Head annules	2	difficult to distinguish	3-4	3-4	3	2
Lateral field lines	4[1]	4	4	4	4(2 clear + 2 fainter)	4
Areolation lateral field	outer bands areolated[4]	/	outer bands areolated	outer bands areolated[4]	/	outer bands areolated
Hemizonid	/	/	above excretory pore	above excretory pore[4]	above excretory pore	above excretory pore
Phasmids	close to tail-tip	halfway or two thirds of tail	/	/	/	close to tail-tip
Stylet knobs	rounded	rounded	ovoid	ovoid[4]	rounded, back-sloped	rounded
Rectum	inflated[4]	inflated[5]	inflated[6]	inflated[4]	not inflated	inflated

[1] Whitehead (1959, 1968) did not observed the lateral field of second-stage juveniles. We observed 4 lines in the lateral field of paratype specimen. [2] Whitehead (1968) did not report the DGO length of *M. decalineata*, from paratype material DGO length was observed to be 3.5 (3.0-4.0), n=6. [3] Estimated from body length/tail length ratio.[4] Own observation from paratype specimen.[5] Elmigly (1968) reported the rectum of *M. oteifae* to be not inflated, however an inflated rectum was observed in paratype specimen.[6] Whitehead (1968) reported the

rectum of *M. decalineata* to be not inflated, however an inflated rectum was observed in 2 paratype specimen. [7]Standard deviations are wrongly calculated in Whitehead (1959), standard deviations in this table are taken from Whitehead (1968).

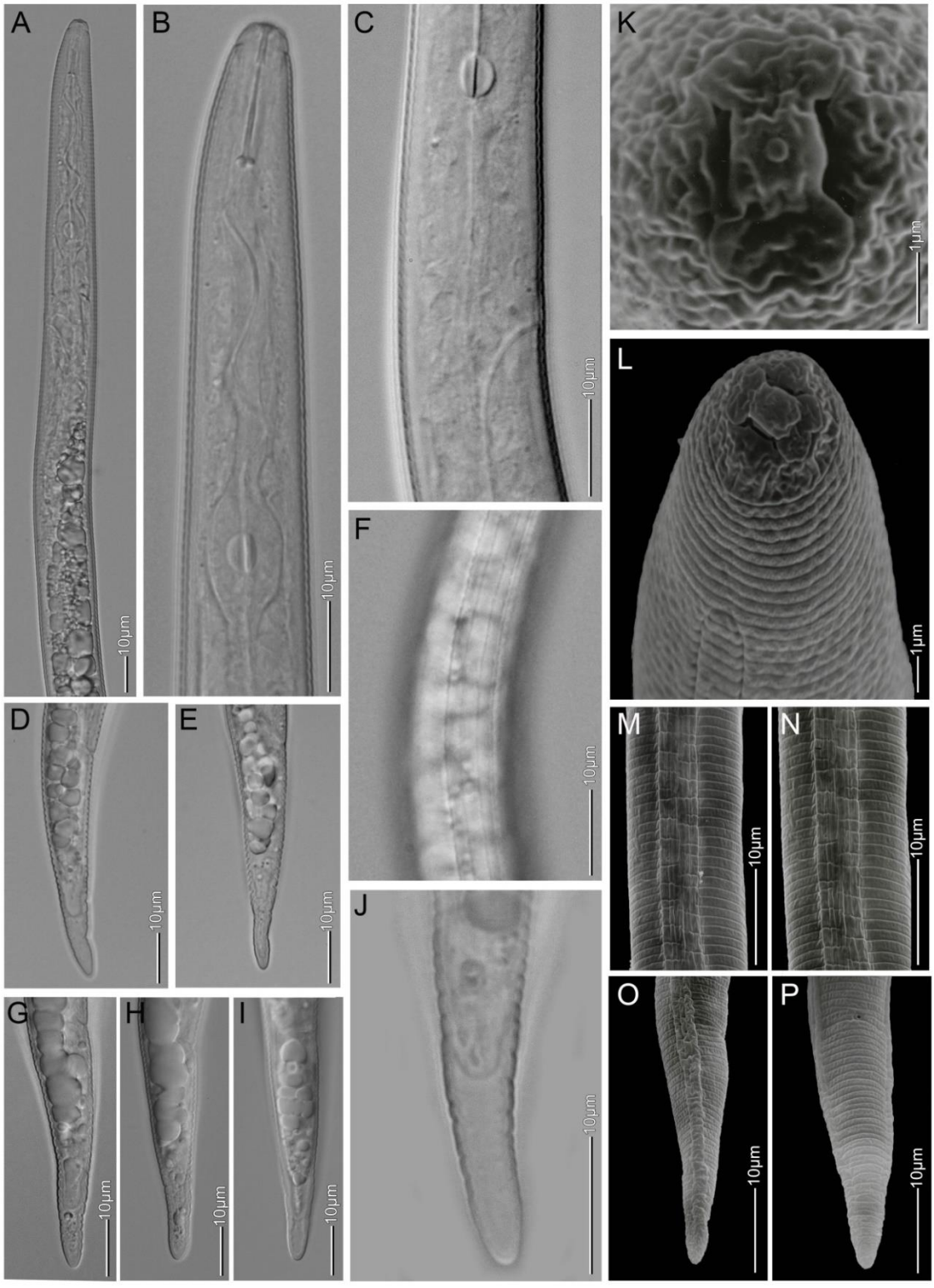


Figure 3. Light microscopy and SEM of *Meloidogyne africana* second-stage juveniles. (A,B) anterior body; (C) meta- and post-corpus region; (D, E, G, H, I) tail variation; (F) mid-body lateral field; (J)

hyaline tail region; (K) cephalic region, en face view; (L) cephalic region, lateral view; (M, N) SEM photographs of mid-body lateral field; (O) tail, lateral view; (P) tail, ventral view.

4.1.3 Males

In both cultured and field recovered populations the males show extreme variations in shape and length, ranging from unusual partly swollen dwarf males to typical long and slender males. Their length and maximum body width varies from 816-1750 µm and 36-66 µm, respectively, resulting in a highly variable 'a' ratio (see Table 3, Fig 1 and 4). Several males had a second atrophied, partly developed, testis instead of a single testis. Notably, males were recovered only from dissected galls of either culture or field populations and not from soil or roots using a mistifier, Oostenbrink elutriator or modified Baermann. This would indicate that males of this species do not exit the galls and therefore are not free-living. This is further supported by empty female spermatheca, which indicates that males play no role in the reproduction of this species (see also section 3.5.2). The variable body shape, sexual inactivity and occurrence of intersexual features indicates a distorted development of males, which most-likely corresponds to sex-reversed females in varying stages as described for *M. incognita* by Papadopoulou and Triantaphyllou (1982), who assumed that sex-reversal is mediated by hormonal balance and therefore greatly dependent on environmental conditions. However, we observed no differences in male development of cultured or field populations. Interestingly, similar to males from our populations, dwarf males were also reported alongside normally developed males of *M. megadora* by Whitehead (1968), which had a reduced stylet with rounded knobs, depending on the developmental stage. This implies that the morphology of the male stylet and the body shape are of limited taxonomical use for this species since they are developmental-dependant. Surprisingly, only three male specimens are reported for *M. decalineata* (Whitehead, 1968), indicating that males are uncommon, possibly pointing to a similar distorted development of males.

In agreement with other similarities, all our populations exhibit a characteristic lateral field, as described for *M. decalineata*, i.e. narrow, occupying one-fifth of the body width, with 10 to 13 lateral lines, while the lateral fields of *M. oteifae* and *M. megadora* exhibit a variable number of lateral lines. In contrast, the lateral field of *M. africana* exhibits 4 lateral lines only; careful examination of the type material of *M. africana* furthermore reveals a remarkable similarity with males of *M. hapla* (Table 3). Among other characters, the DGO, stylet length, stylet knob

shape, lip shape, a single short rarely reflexed testis and lateral field including typical striation observed in male type material match perfectly with *M. hapla*. This would indicate that the males of *M. africana* and *M. hapla* were quite conceivably mixed up by Whitehead (1959), which is possible given that Whitehead (1969) reported *M. africana* from *Pyrethrum*, a crop known to be a particularly good host of *M. hapla* (Whitehead, 1969; Parlevliet, 1971). Furthermore, we also recovered *M. hapla* from *C. arabica* in Tanzania (Mufindi, Iringa) and from *Achyranthes aspera* (Mbelei, Tanga), a commonly occurring weed in coffee plantations. Indeed, *M. hapla* frequently occurs in the tropics at cooler higher elevations, a typical habitat for coffee cultivation in Africa, where mixed populations of *M. africana* and *M. hapla* may consequently occur. Although Whitehead (1959) did not detail the extraction methods used, the fact that males of our *M. africana* could not be obtained using traditional mistifier, Oostenbrink elutriator or modified Baermann extraction methods supports the argument that the original males belong to a different species. For *M. oteifae* just one single male paratype specimen was deposited by Elmiligy (1968), which does not permit for a definitive diagnosis, but which shows a remarkable similarity with *M. javanica* males. In conclusion, our observations indicate that males of *M. africana* (Whitehead, 1959) belong to *M. hapla*, while males from our population correspond with *M. decalineata* males. This further complicates the African coffee root-knot nematode taxonomic conundrum.

Table 3. Comparison of male morphological and morphometric characters between species of *Meloidogyne* parasitizing coffee in Africa.

	M. africana Whitehead 1968	*M. hapla* Whitehead 1968	*M. oteifae* Elmiligy 1968	*M. decalineata* Whitehead 1968	*M. megadora* Whitehead 1968	*Meloidogyne sp.*
Male						
n	18	25	10	2	25	21
L	1470±197 (1200-1850)	1139±166 (791-1432)	1160 (980-1270)	1630, 1700	1906±330 (905-2277)	1285±245 (816.0-1750.0)
Stylet	20.7±1.08 (19-22)	20.0±1.28 (17.3-22.7)	22 (19-23)	20, 19	20.4±1.13 (18.3-21.9)	15.7±1.1 (14.0-18.0)
Stylet knobs width	/	3.5±0.52 (2.5-5.0)	/	/	5.1±0.64 (3.6-6.1)	3.5±0.4 (3.0-4.0)
DGO	(4-6)	2.9±0.23 (2.5-3.2)[1]	3.5 (3-4.5)	4	6.5±1.18 (4.0-8.3)	5±0.4 (4.0-6.0)
Spicules	(26-35)	25.7±2.42 (21.6-28.1)	33 (29-37)	33, 34, 36, 37	32.6±2.96 (25.2-36.0)	26.5±2.3 (24.0-31.0)
Gubernaculum	(7-9)	8.2±0.82 (7.2-9.4)	11 (10-12)	7	10.6±0.86 (9.4-11.9)	7.6±1.8 (6.0-10.0)
a	38.9±4.72 (31-50)	41.7±3.66 (33.3-47.0)	26 (25-28)	29.6, 42.5	52.8±7.06 (36.9-62.8)	26.0±4.0 (19.2-34.3)
Lateral field lines	4	4	4, 5 or more present in few specimen	10	mostly 4, 5 or 6 for short distance	10-13
Lateral field width	1/4 of body width	/	1/5 of body width	1/7 of body width	/	1/4 of body width
Testes	single, rarely reflexed	single, rarely reflexed	single, mostly reflexed	single	single, in 2 exceptional cases double	single, mostly reflexed

[1] Jepson reported the DGO of *M. hapla* to be 4.1±1.0 (2.7-5.4).

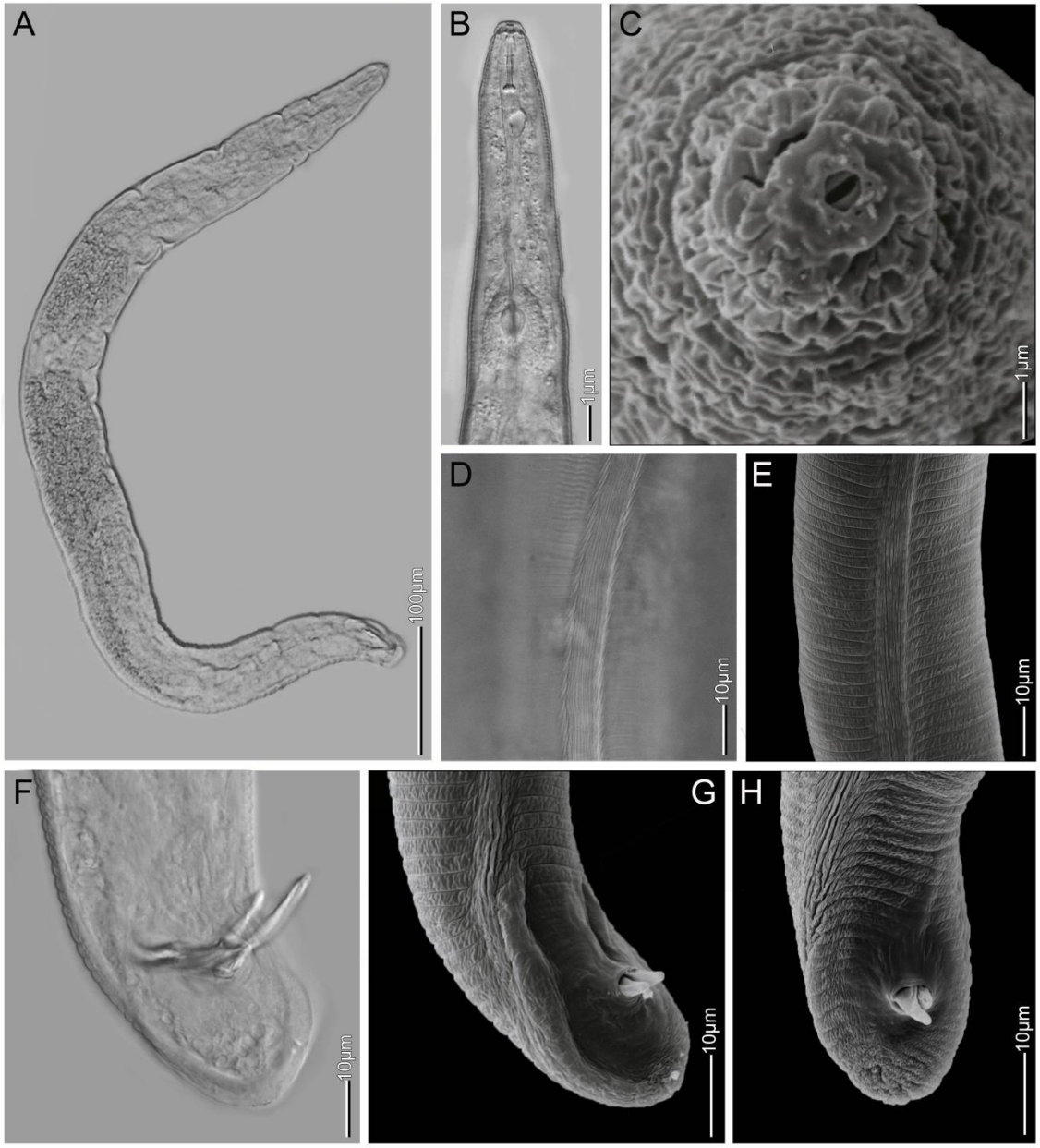


Figure 4. Light microscopy and SEM of *Meloidogyne africana* males. (A) habitus of dwarf sex-reversed female; (B) anterior body in lateral view; (C) cephalic region, en face view; (D, E) mid-body lateral field; (F, G, H) tail.

4.1.4 Conclusion of morphological identification

In the current study, in order to clarify the taxonomic status of African coffee root-knot nematodes, the features of females and juveniles need to be prioritised, as they are considered the superior *Meloidogyne* diagnostic characteristics (Jepson, 1987). The males of most of the investigated populations have a distorted development as sex-reversed females, and the originally described males of *M. africana* and *M. oteifae* are likely based on other species. From all of our current populations females and juveniles unequivocally match with *M. africana*. However, Whitehead (1959) assigned males of *M. hapla* as the holotype of *M. africana* and as such the existing type is not in taxonomic accord with *M. africana*. For the more recently described *M. decalineata*, the holotype has been correctly assigned but the juveniles have been described based on a different species. Retaining the original name *M. africana* and not *M. decalineata* will result in a greater taxonomic stability; irrespective of the current manuscript, other populations of this species will most likely be identified as *M. africana*, since males are rare. Hence, *M. africana* is not only the oldest available name but also in accord with the prevailing usage of the original name and therefore the name *M. africana* should be conserved. As a consequence *M. decalineata* is considered a junior synonym of *M. africana.* Reference material has been deposited in the collections of Wageningen and the Nematology Research Unit University Ghent (slides UGnem147-149).

In addition, the morphology of *M. oteifae* females and juveniles correspond perfectly with *M. africana*, while males of *M. oteifae* show affinity with *M. javanica*. Moreover, *M. oteifae* (accepted for publication on 24 July 1968) does not appear to have been adequately compared with *M. decalineata* or *M. megadora* published in august 1968 (accepted for publication on 10 October 1967), which were published almost simultaneously. Therefore, *M. oteifae* is also considered to be a junior synonym of *M. africana*. For *M. megadora*, the females and males also show remarkable similarities to our populations of *M. africana*, including the presence of "dwarf" males. However, *M. megadora* is considered to be a valid species because the juveniles have a longer tail with a shorter hyaline terminus, and the esterase isozyme profile migrates faster in comparison to *M. africana* (Maleita *et al.*, 2012). Moreover, a recent molecular characterisation confirmed *M. megadora* to be a separate entity (Maleita *et al.*, 2016).

4.2 Redescription of *Meloidogyne africana* (Table 4, Fig 1, 2, 3 and 4)

Subfamily Meloidogyninae Skarbilovich, 1959

Genus *Meloidogyne* Göldi, 1887

Meloidogyne africana, Whitehead 1960
new syn. *Meloidogyne decalineata*, Whitehead 1968
new syn. *Meloidogyne oteifae*, Elmiligy 1968

Material examined. Meloidogyne africana: holotype and 3 paratype slides from Kamaara Coffee nursery, Meru district, Kenya (Rothamsted Nematode Collection 77/17/1-77/17/4); *Meloidogyne decalineata*: holotype and 3 paratype slides from Mawingo estate, Kilimanjaro, Tanganyika (Rothamsted Nematode Collection 77/10/1-77/10/4); *Meloidogyne megadora*: holotype and 3 paratype slides from Coffee research station, Amboim, Republic of Angola (Rothamsted Nematode Collection 77/13/1-77/13/4); *Meloidogyne oteifae*: holotype and 10 paratype slides from Congo (Ghent University Museum, Zoology Collection). Three *M. africana* populations from Lushoto, Tanzania and six populations from Mbelei, Tanzania.

Females. As described for *Meloidogyne africana* by Whitehead (Whitehead, 1959). Additionally, SEM of the perineal pattern supplements the light microscopic observations and drawings of Whitehead (1959) (Fig 1 and 2) and illustrates the typical characteristics of the perineal pattern. Faint striae forming a low dorsal arch, phasmids positioned adjacent to tail terminus, start of the lateral field of variable width and composed of irregular striae, perineal pattern on a raised perineum as a consequence of a clear protuberance, and vulva surrounded by circles of striae, which are sometimes crossed by other striae radiating from the vulva.

Juveniles. As described for *Meloidogyne africana* by Whitehead (1959). Body length has a wider range and the stylet is slightly shorter compared to the original description (Table 4). The lateral field, which was not described in the original description, is prominent with 4 lines (Fig 1 and 3), which may be areolated in the mid-body region. SEM observations indicate that more than 4 lines can be present in the mid-body region (Fig 3), providing an indication of the origin of the multiple lined lateral field of males. Second-stage juveniles have a characteristic rounded tail terminus with a long hyaline terminus (Fig 1 and 3) (Whitehead, 1959).

Males. As described for *Meloidogyne decalineata* by Whitehead (1968), although the morphometric values (Table 4) show a wider range compared to Whitehead (1968), which were based on three specimens only (Table 3).

Diagnosis. Meloidogyne africana is characterised by a distinct elevated perineal pattern with smooth striae, phasmids positioned close together and a variable lateral field. Males are variable in size with a narrow lateral field consisting of 10-13 lateral lines. Second-stage juveniles have a rounded tail, with a characteristic hyaline region. *Meloidogyne africana* is further characterised by a unique esterase isozyme phenotype. Based on ribosomal (18S and 28S rDNA) and mitochondrial (Cox1) sequences *M. africana* is differentiated from all members of clade I, II and III, and *M. coffeicola*, *M. cammeliae*, *M. ichinohei*, *M. mali*, *M. artiellia*, *M. beatica* (see section 3.3.1). *Meloidogyne africana* is differentiated from *M. megadora* by a shorter juvenile tail, a differential hyaline tail terminus, a different male lateral field and a more slowly migrating esterase isozyme profile (Maleita *et al.*, 2012). *Meloidogyne africana* is morphologically close to *M. acronea* Coetzee 1956, but is differentiated by the lateral lines, stylet of the male and the different juvenile tail.

Table 4. Morphometrics of *Meloidogyne africana* cultured on *Coffea arabica.* Mean ± SD (range), all measurements in µm.

Character	Females	Males	Second-stage juveniles
N	22	21	30
L	615±95 (400-770)	1285±245 (816-1750)	422±39 (352-536)
Greatest body diam.	375±59 (300-540)	50.0±7.6 (36.0-66.0)	16.8±2.3 (14.0-22.0)
Body diam. at anus	_	_	10.2±0.7 (9.0-12.0)
Head region height	_	3.3±0.5 (2.5-4.0)	2.7±0.5 (2.0-4.0)
Head region diam.	_	8.3±0.4 (7.5-9.0)	5.5±0.3 (5.0-6.0)
Neck length	217±48.1 (120-300)	_	_
Stylet	14.3±0.8 (13.0-15.5)	15.7±1.1 (14.0-18.0)	11.5±0.5 (10.5±12.5)
Stylet knobs width	3.1±0.3 (3.0-4.0)	3.5±0.4 (3.0-4.0)	1.9±0.2 (1.5±2.0)
DGO	5.7±0.8 (4.5-7.0)	5±0.4 (4.0-6.0)	4.0±0.6 (3.0-5.5)
Ant. end to metacorpus	_	64.0±8.2 (54.0-78.0)	46.0±5.6 (38.0-61.0)
Excretory pore-ant.end	_	150±24.9 (110-197)	75.5±6.9 (55.0-84.0)
Tail	_	_	42.1±1.9 (39.0-46.0)
Hyaline tail terminus	_	_	10.5±1.3 (8.0-13.0)
Spicules	_	26.5±2.3 (24.0-31.0)	_
Gubernaculum	_	7.6±1.8 (6.0-10.0)	_
a	1.6±0.2 (1.3-2.4)	26.0±4.0 (19.2-34.3)	25.5±3.1 (19.5-31.1)
c	_	_	10.1±1.0 (7.8-12.7)
c’	_	_	4.1±0.3 (3.5-4.7)
Body L/neck L	2.9±0.4 (2.3-3.8)	_	_
(Excretory pore/L)x100	_	11.7±2.0 (8.7-15.7)	17.8±1.2 (14.0-20.4)

4.3 Phylogenetic analysis, molecular diagnosis and evolutionary morphology

4.3.1 Phylogenetic analyses

The multi-gene sequence alignment of three genes (18S, 28S rDNA and Cox1), was 2847 base pairs in length, and the resulting phylogeny is in accordance with single gene phylogenies of 18S and 28S rDNA (Tandingan De Ley *et al.*, 2002; Holterman *et al.*, 2009; Ahmed *et al.*, 2013). The phylogenetic analysis revealed *M. africana*, *Meloidogyne* sp. and *M. coffeicola* to be outside of the three major clades (clade I, II and III) (Fig 5) based on the Bayesian analysis (posterior probability = 99) (Tandingan De Ley *et al.*, 2002). According to the maximum likelihood analysis, *M. ichinohei*, *M. camellieae* are also positioned outside these three major clades. Overall relationships between *M. africana, M. coffeicola, M. ichinohei* and *M. camelliae* are poorly supported and will need a phylogenomic approach in order to be resolved. The multi-gene phylogenetic analysis confirmed *M. africana* to be the sister taxon of an undescribed species of *Meloidogyne* from *Sansevieria* sp. (Holterman *et al.*, 2009), differing in 11 base pairs (0.7%) in 18S and 35 base pairs (7.5%) in the Cox1 fragment. In comparison to other root-knot nematodes the observed 7.5% Cox1 and 0.7% 18S rDNA divergence clearly constitute a different species (Kiewnick *et al.*, 2014; Janssen *et al.*, 2016), especially as 18S rDNA usually does not show sequence divergence for closely related species (Tang *et al.*, 2012; Kiewnick *et al.*, 2014).

The Cox1 multiple sequence alignment included 37 Cox1 sequences and was 432 base pairs in length. This alignment revealed only a single haplotype of *M. africana* based on the 16 sequenced specimens (8 females, 4 males and 4 juveniles) (Fig 6) and confirms that males, females and juveniles of different populations belong to the same species, *M. africana*. From five localities a single female was sequenced, from three locations a juvenile, a male and a juvenile were sequenced and from one location a male and a juvenile were sequenced. The presence of only one *M. africana* Cox1 haplotype in nine farms from Mbelei and Lusotho (Tanzania) indicates that intraspecific variability is low and that this species can be reliably identified using Cox1 DNA barcoding. However, a more extensive geographic sampling should be conducted in order to assess interspecific variability of *M. africana*, although recent studies show that most *Meloidogyne* spp. display little within-species genetic variability in Cox1 (Kiewnick *et al.*, 2014; Janssen *et al.*, 2016).

In order to differentiate *M. africana* from a broader range of species of *Meloidogyne* an 18S rDNA single gene phylogeny was constructed (Fig 7), based on a multiple sequence alignment of 1819 base pairs in length. The phylogenetic tree is in accordance with previous 18s rDNA based phylogenies (Tandingan De Ley *et al.*, 2002; Holterman *et al.*, 2009; Ahmed *et al.*, 2013) and confirms the species identity of *M. africana* and its position outside the 3 major clades of the genus.

Also, three ITS1-5.8S-ITS2 rDNA sequences were deposited on GenBank (accession numbers: KY433428-KY433430) because this remains a frequently used region for identification of plant-parasitic nematodes (Ye *et al.*, 2015; Maleita *et al.*, 2016). However, ITS has been described to exhibit multiple highly divergent copies in a single *Meloidogyne* individual due to the suggested hybrid origin of these species (Hugall *et al.*, 1999; Maleita *et al.*, 2016). Since *M. africana* is also suggested to be a triploid species (see section 3.5.2), ITS is considered to be unreliable for identification purposes. Furthermore, ITS sequences are extremely variable among root-knot nematodes (Hugall *et al.*, 1999; Blok & Powers, 2009) and multiple sequence alignments are unlikely to generate homologous nucleotide positions needed for a reliable phylogenetic analyses.

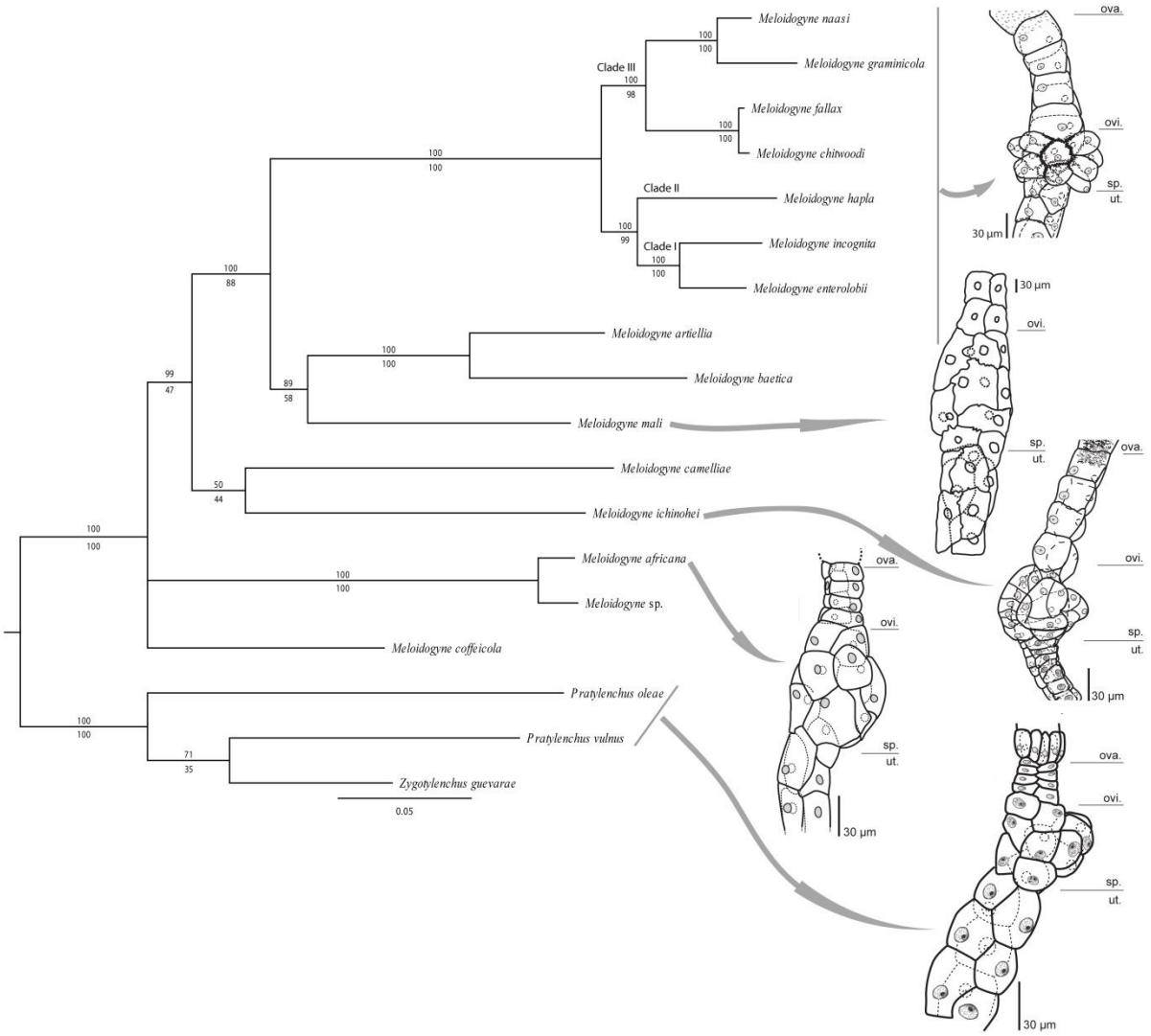


Figure 5. Molecular multi-gene phylogeny of the genus *Meloidogyne*. Consensus tree based on the combined nuclear 18S and 28S rDNA sequences and the mitochondrial Cox1 gene sequences. Values above branches are Bayesian posterior probabilities, values below branches are Maximum Likelihood bootstrap values. Overview of used sequences in S1 Table, for details on phylogenetic reconstruction see Materials and Methods. Evolutionary morphology of the female gonoduct is illustrated by drawings of the oviduct-spermatheca region from *Meloidogyne africana*, *Meloidogyne mali*, *Meloidogyne ichinohei*, *Meloidogyne hispanica* and *Pratylenchus thornei*; (ova.) ovaria; (ovi.) oviduct; (sp.) spermatheca; (ut.) uterus. Morphological drawing *M. ichinohei* and *M. hispanica* modified from Bert et al. (2002). *M. hispanica* is used as an example of typical gonoduct morphology in Clade I, II and III, only mophological exception is *M. microtyla* (Bert et al. 2002). Typical *Pratylenchus* gonoduct morphology is illustrated by a drawing of *Pratylenchus thornei*, modified from Bert et al. (2003).

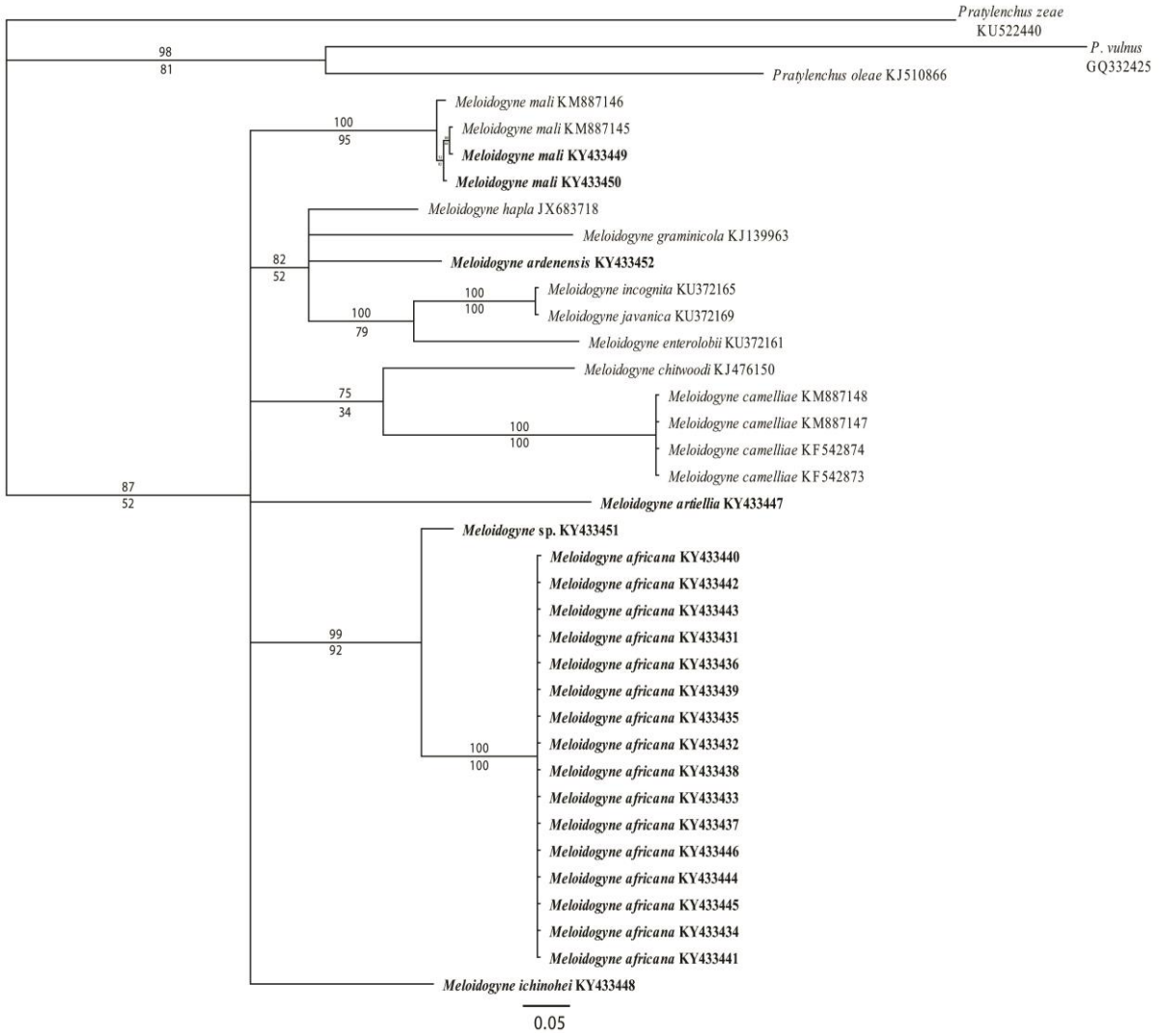


Figure 6. Majority rule consensus tree based on mitochondrial Cox1 sequences. Values above branches are Bayesian posterior probabilities, values below branches are Maximum Likelihood bootstrap values. For details on phylogenetic reconstruction see Materials and Methods. Genbank accession numbers are displayed behind the species name. Newly generated sequences are highlighted in bold.

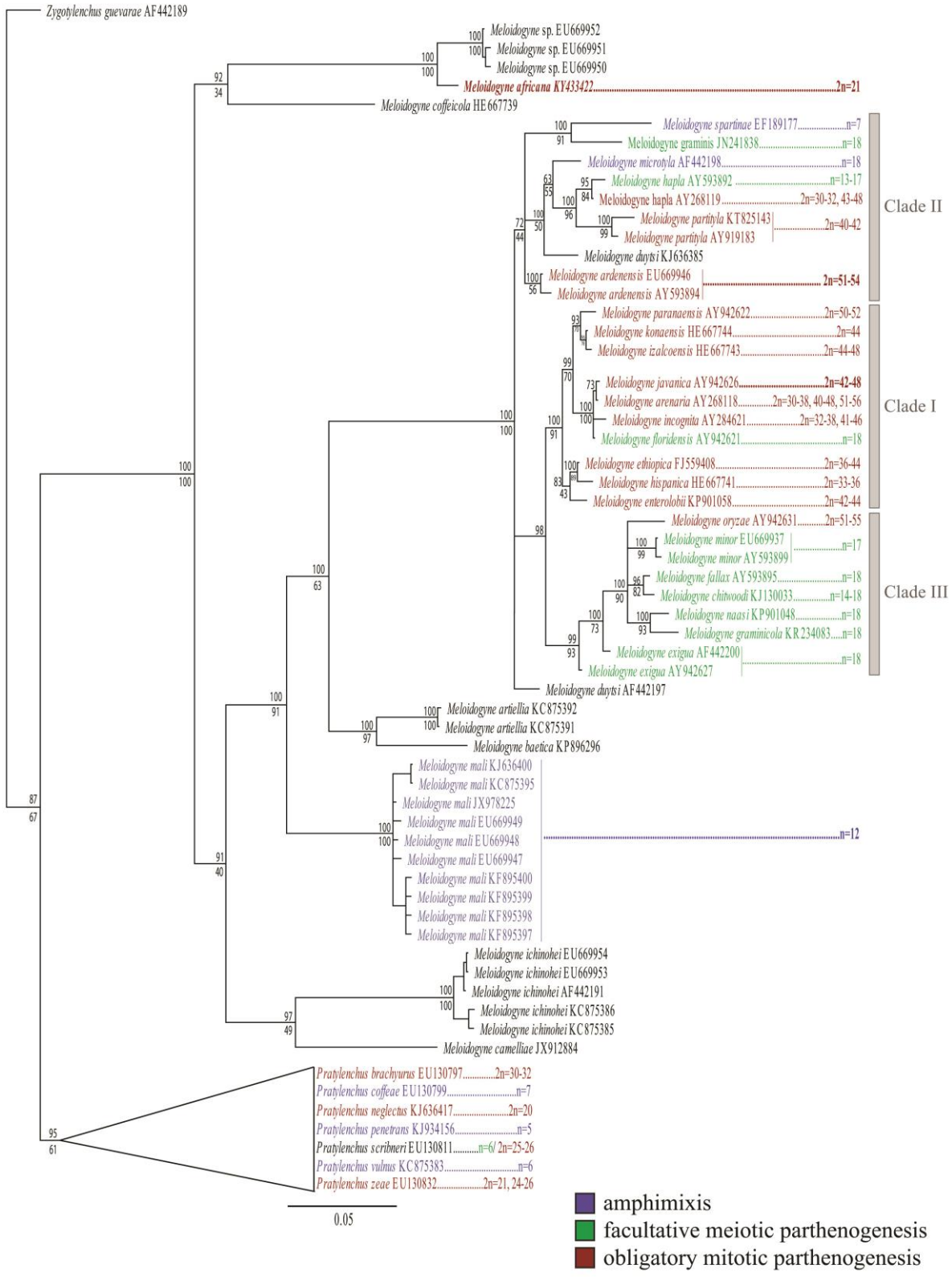


Figure 7. Majority rule consensus tree based on 18S ribosomal rDNA sequences with karyology. Values above branches are Bayesian posterior probabilities. Known karyotypes are displayed, reviewed by Chitwood and Perry (2009), newly generated karyotypes are highlighted in bold. Note there is no direct link between nucleotide sequences from NCBI and karyotyped populations. Amphimictic species highlighted in purple, facultative meiotic parthenogenetic species highlighted in green, mitotic parthenogenetic species highlighted in red.

4.3.2 Evolutionary morphology of the perineal pattern and protuberance

The perineal region of *Meloidogyne africana* appeared to show an ancestral pattern because it resembles a pre-adult of *M. javanica* (Whitehead, 1959) and this proposed ontogenetic pattern appears to correspond with the obtained phylogeny. In this regard, Karssen (2002) already noted that perineal patterns of all *Meloidogyne* pre-adults significantly differ from those of mature females because the pre-adult remains enclosed in the juvenile cuticle. Given that perineal patterns of pre-adult *M. javanica* (and other *Meloidogyne* spp.) are hemispherical-shaped, with a fine striation, they resemble the perineal pattern of *M. africana* adults. However, the hemispherical shape of the perineal pattern is directly linked to the presence of a protuberance in species of *Meloidogyne*, which is mostly associated with a finely striated perineal pattern (exceptions are *M. graminis* (Sledge & Golden, 1964) Whitehead, 1968, *M. marylandi* Jepson & Golden *in* Jepson, 1987 and *M. sasseri* Handoo, Heuttel & Golden, 1994, which show a protuberance in combination with a coarsely striated perineal pattern) (Karssen, 2002). This finely striated, hemispherical perineal pattern is most likely the result of less body expansion during the transition towards adult, which has evolved several times independently from each other, as has the presence of a protuberance. Thus, it is unclear if the finely striated perineal pattern and a protuberance of *M. africana*, *M. ichinohei and M. coffeicola* represents the ancestral state of the genus given that *M. camelliae*, *M. mali*, *M. beatica* Castillo, Vovlas, Subbotin & Trocolli, 2003 and *M. artiellia* show a coarsely striated perineal pattern without protuberance. Yet, incompletely swollen species with a protuberance might be considered as an intermediate step between vermiform nematodes of the genus *Pratylenchus* and completely globular nematodes of the genus *Meloidogyne*.

4.3.3 Evolutionary morphology of the female gonoduct

As in all Tylenchina, the two gonoduct branches of *M. africana* and *M. mali* each consist of a uterus, a spermatheca, an oviduct and an ovary (Fig 2 and 5). The uterus of *M. africana* and *M. mali* is a very long tricolumella with multiple cells as in all other *Meloidogyne* spp. (Bert *et al.*, 2002) but distinct from the short uterus of most Pratylenchidae (Bert *et al.*, 2003). This extended uterus convergently evolved in endoparasitic nematodes, including entomopathogenic steinernematids, false root-knot nematodes (*Naccobus* spp.), cyst- and root-knot nematodes (Bert *et al.*, 2008). The spermatheca of *M. africana* is not offset, consisting of 12-14 cells, without lobe-like cells and without interlaced cell boundaries (Fig 2 and 5). Also the

spermatheca of *M. mali* is not offset, without lobe-like cells, consisting of 13-16 cells (Fig 5), and cell boundaries are slightly interlaced but less interlaced than in *M. microtyla* (Bert *et al.*, 2002). Among root-knot nematodes the spermatheca morphology of both *M. africana* and *M. mali* is similar to *M. microtyla* and *M. ichinohei* in lacking lobe-like cells and clearly inter-laced cell boundaries, while in most other *Meloidogyne* spp. the spermatheca is composed of 16-18 lobe-like cells with interlaced boundaries (Bert *et al.*, 2002). Based on the obtained phylogeny, a spermatheca comprised of a limited number of cells, with not-interlaced and not-lobe like cells, could be an ancestral feature within the genus *Meloidogyne* because it is remarkably similar to spermatheca morphology of *Pratylenchus* (Bert *et al.*, 2003). In both *M. africana* and *M. mali* the oviduct consists of two rows of four cells, characteristic for all Pratylenchidae and most Tylenchomorpha (Geraert, 1983; Bert *et al.*, 2002; Bert *et al.*, 2003; Bert *et al.*, 2008).

4.4 Isozyme electrophoresis

Electrophoretic isozyme analysis of single young egg-laying females of *M. africana* revealed a unique esterase phenotype, consisting of two fast migrating esterase bands, designated as AF2 (Fig 8). The first is in approximately the same position as the fastest migrating *M. javanica* reference band (61.7%) and the second at 65.7% migrating speed. This phenotype resembles the slightly slower migrating M2-VF1 phenotype of *M. artiellia* (Karssen, 2002) and the faster migrating C2 and Me3 phenotypes of respectively *M. coffeicola* (Carneiro *et al.*, 2000) and *M. megadora* (Maleita *et al.*, 2012). The malate dehydrogenase isozyme analysis revealed a single broad band (migrating speed 45.3%) in a similar position as the H1 phenotype of *M. hapla.*

In order to obtain isozyme information for more species of *Meloidogyne,* the isozyme phenotypes of *Meloidogyne ichinohei* were studied, revealing another unique esterase phenotype. This phenotype consists of two clear slow migrating bands (relative migrating speed 53.0 and 56.1%), herein designated IC2 (Fig 8). The malate dehydrogenase isozyme analysis revealed a single broad band corresponding to the commonly occurring N1 phenotype. It has been observed that both the malate dehydrogenase isozyme analysis of *M. africana* and *M. ichinohei* are slightly smeared and have a broader appearance than the H1 and N1 phenotype respectively. This smeared appearance was not caused by an analysis artefact as it was verified in several separate analyses using different populations. Interestingly, both the *M. coffeicola* C1 phenotype (Carneiro *et al.*, 2000) and the *M. artiellia* N1b (Karssen, 2002) phenotype also have

a slightly smeared appearance implying that these differences could be informative and characteristic for *Meloidogyne* spp. outside the three major clades. These results indicate that *M. africana* and *M. ichinohei* can be reliably identified using isozyme electrophoresis, confirming that isozyme electrophoresis remains a highly reliable, if not labor intensive, diagnostic tool for root-knot nematodes (Blok & Powers, 2009; Elling, 2013; Janssen *et al.*, 2016).

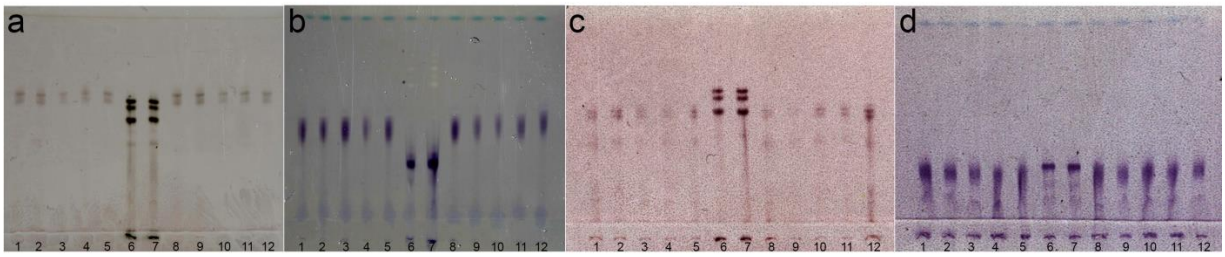


Figure 8. Isozyme profiles of *Meloidogyne africana* and *Meloidogyne ichinohei*. Lane 6 and 7 represent *Meloidogyne javanica* reference phenotypes, lane 1–5 and 8–12 represent phenotypes of *Meloidogyne africana* and *Meloidogyne ichinohei*. (a) esterase AF2 phenotype of *Meloidogyne africana*; (b) malate dehydrogenase H1 phenotype of *Meloidogyne africana*; (c) esterase IC2 phenotype of *Meloidogyne ichinohei*; (d) malate dehydrogenase N1 phenotype of *Meloidogyne ichinohei*.

4.5 Evolution of reproduction and oogenesis

4.5.1 Meloidogyne javanica

To re-establish the propionic orcein staining protocol of Triantaphyllou (1985), *M. javanica* reference material originating from Spain, F1836-3 (Janssen *et al.*, 2016), collected and maintained on *S. lycopersicum* was stained and compared to the *M. javanica* observations of Triantaphyllou (1962). Based on 8 late prophase to early metaphase chromosomal planes, our *M. javanica* population appeared to have 2n=44 chromosomes, identical to two *M. javanica* populations karyotyped by Triantaphyllou (1962) originating from England and Australia. Oocytes approaching the consistently empty spermatheca were observed to exhibit 44 univalent chromosomes, indicating that chromosome pairing did not take place during the zygotene stage, and therefore indicating that reproduction occurred by mitotic parthenogenesis.

4.5.2 *Meloidogyne africana*

Several mitotic divisions of the oogonia took place in the apical germinal zone. From 20 favourable late-prophase or early-metaphase chromosomal planes of these mitotic divisions, the chromosome number of *M. africana* was determined to be 2n=21. Similar to other *Meloidogyne* spp., the chromatin of young oocytes in the synapsis zone was found to be strongly orcein-stained (Triantaphyllou, 1985a). In the maturation zone, oocytes progressively were seen to increase in size, while chromatin in this region was only weakly stained. When matured oocytes approached the spermatheca, 21 univalent chromosomes were observed in four chromosomal planes at prophase, indicating that chromosome pairing did not take place during the zygotene stage. This observation, together with the spermatheca being consistently empty and the fact that the males were sexually inactive (see above), indicates that reproduction takes place by mitotic parthenogenesis. This is the first report of a species of *Meloidogyne* with a chromosome complement of 21. All other obligatory mitotic parthenogenetic *Meloidogyne* spp. are known to have at least 2n=30 chromosomes (Chitwood & Perry, 2009). Thus, *M. africana* constitutes the root-knot nematode with the lowest known number of chromosomes to reproduce by mitotic parthenogenesis. Interestingly, this mitotic parthenogenetic mode of reproduction correlates with the distorted development of sex-reversed females and the sexual inactivity of *M. africana* males, since this behaviour has so far been found only in mitotic parthenogenetic species (Papadopoulou & Triantaphyllou, 1982). However, these dysfunctional males are nevertheless thought to play an important role in ecological adaptation, as they reduce the population density in successive generations of parthenogenetic root-knot nematodes (Triantaphyllou, 1973; Castagnone-Sereno, 2006).

4.5.3 Meloidogyne ardenensis

A *M. ardenensis* population was collected from the roots of *Ligustrum* sp. (Wageningen, The Netherlands; GPS coordinates: 51.975688, 5.675987) in early spring, 2016, when young egg-laying females were present. The population was identified using both the morphology of juveniles and 28S rDNA and mitochondrial COX1 sequences. Similar to other *Meloidogyne* spp., several mitotic divisions were observed in the germinal zone. From 10 favourable late-prophase or early-metaphase planes of these mitotic divisions, the chromosome number of *M. ardenensis* was determined to be 2n=51-54, with the variation in chromosome number most likely due to the difficulty in the counting process, since chromosomes of *M. ardenensis* are

small and often positioned extremely close to one another. The mature oocytes approaching the spermatheca were found to comprise approximately 54 univalent chromosomes, with no oocytes observed with a haploid chromosome number, indicating only mitotic divisions and reproduction by mitotic parthenogenesis. This is in agreement with the high chromosome number of *M. ardenensis,* given that all polyploid species with more than 40 chromosomes reproduce by mitotic parthenogenesis (Triantaphyllou, 1962; Triantaphyllou, 1963; Triantaphyllou, 1984; Triantaphyllou, 1985a; Van der Beek *et al.*, 1998; Chitwood & Perry, 2009). Despite being parthenogenetic, males of *M. ardenensis* appear to be sexually active, as spermatheca in all specimens studied were filled. However, it is not uncommon that the spermatheca of mitotic parthenogenetic species are filled with sperm within the genus *Meloidogyne* (Triantaphyllou, 1962; Triantaphyllou, 1985a; Van der Beek *et al.*, 1998). In the case of *M. javanica* and *M. hapla* race B, a spermatozoon is even able to enter the oocyte, but the spermatozoon degenerates within the oocyte and fertilization does not occur (Triantaphyllou, 1962; Van der Beek *et al.*, 1998).

4.5.4 Meloidogyne mali

A *M. mali* population was collected from the roots of *Ulmus* sp. originating from the field trial "Mierenbos" (Wageningen, The Netherlands; GPS coordinates: 51.979623, 5.706362), a location previously sampled by Ahmed *et al.*(2013). The population was identified as *M. mali* based on juvenile and female morphology and on Cox1 DNA sequences. Young egg-laying females were collected from the field and directly used for cytogenetic staining. From 2 favourable late-prophase chromosome planes studied during mitotic divisions in the apical part of the germinal zone, the diploid chromosome number of *M. mali* was determined as 2n=24. After the oocytes increased in size in the maturation zone, 6 oocytes were observed with 12 bivalent chromosomes, showing that meiosis was taking place. In all of the specimens studied, the spermatheca was clearly filled with sperm, and in several eggs the inclusion of a sperm nucleus was observed. In one egg, the fusion between the small sperm nucleus and the larger egg nucleus was observed. These observations reveal that *M. mali* reproduces by amphimixis in the presence of males. However, no females with empty spermatheca have been isolated, and it remains to be confirmed whether *M. mali* is also capable of meiotic parthenogenesis. *Meloidogyne mali* is the first *Meloidogyne* spp. to be identified with a haploid chromosome

complement of n=12, demonstrating that chromosome numbers are even more variable than previously anticipated (Chitwood & Perry, 2009).

4.5.5 Evolution of reproduction and oogenesis in root-knot nematodes

In order to elucidate the evolution of reproduction and oogenesis within the genus *Meloidogyne,* chromosome number and reproduction mode of *Meloidogyne* spp. were plotted on the 18S rDNA phylogenetic tree (Fig 7). The results provide a completely new understanding on the evolution of reproduction within the genus *Meloidogyne*. To begin with, mitotic parthenogenesis has evolved independently on five occasions according to the maximum parsimony ancestral state reconstruction (Fig 9), being present in all three major clades and in *M. africana*. This evolutionary pattern is less resolved in the maximum likelihood ancestral state reconstruction as most of the ancestral nodes remain unresolved. In either case, our results contradict the traditional hypothesis that mitotic parthenogenetic species are restricted to one clade (Castagnonesereno *et al.*, 1993; Castagnone-Sereno, 2006). The presence of mitotic parthenogenesis in *Meloidogyne africana* was unexpected, as *Meloidogyne* spp. in this part of the tree were previously understood to reproduce by meiotic parthenogenesis (Castagnone-Sereno *et al.*, 2013). The presence of mitotic parthenogenesis within clade II, as observed for *M. ardenensis,* is in line with the observation of mitotic parthenogenesis in *M. hapla* race B (Triantaphyllou, 1966; Triantaphyllou, 1985a; Van der Beek *et al.*, 1998) and *M. partityla* (2n=40-42) (Marais & Kruger, 1991; Brito *et al.*, 2016). Curiously, the mitotic parthenogenetic species *M. oryzae* (2n= 51-55) and *M. ardenensis* (2n=51-54) both have a triploid genomic composition, in contrast to their closest phylogenetic relatives, which are facultative meiotic parthenogenetic, and have three times less chromosomes, namely n=18 (Fig 7). Similarly, in Clade I *M. inornata* Lordello, 1956 (2n=54-58) and some populations of *M. arenaria* (2n=51-56) appear to have a triploid genomic composition, in comparison with *M. floridensis* (n=18), the only meiotic parthenogenetic species within Clade I. However, most species in Clade I do not have an exact chromosome complement of 18, probably as a consequence of aneuploidy and polysomy, as well as structural chromosome rearrangements (Castagnone-Sereno *et al.*, 2013). These drastic changes in chromosome number are to be expected since root-knot nematodes, and indeed most other nematodes, have holocentric chromosomes with a diffuse centromere activity (Triantaphyllou, 1983; Melters *et al.*, 2012). Furthermore, it is certain that not all mitotic parthenogenetic *Meloidogyne* spp. have triploid genomes, with some populations

of *M. microcephala* and *M. hapla* reported to be tetraploid (Triantaphyllou, 1984; Triantaphyllou & Hirschmann, 1997).

These observations suggest that a triploid genomic composition is associated with mitotic parthenogenetic reproduction, as has already been observed in several Heteroderidae. In their case, the basic chromosome number of amphimictic species is assumed to be n=9, with *Heterodera trifolii* Goffart, 1932 (3n=24-28), *H. lespedezae* Golden & Cobb, 1963 (3n=27) and *H. sacchari* Luc & Merny, 1963 (3n=27) representing triploid mitotic parthenogenetic species (Triantaphyllou & Hirschmann, 1980). This has also been reported for a wide range of animals and plants (Comai, 2005). If mitotic parthenogenetic species have indeed evolved several times independently triggered by a triploid genome, it is also likely that *M. africana* has a triploid genomic composition. This would imply that *M. africana* evolved from an ancestral species with n=7 chromosomes. Interestingly, *M. spartinae* (Clade II) and *M. kikuyensis* have been both characterized as having n=7 chromosomes (Triantaphyllou, 1987; Triantaphyllou, 1990). It should also be noted that the chromosome number of *M. kikuyensis* and *M. spartinae* was mistakenly reported as n=9 in Castagnone-Sereno et al. (2013). Interestingly, *M. kikuyensis* is probably also positioned outside the three major clades as the spermatheca has a less pronounced lobe-like shape (Triantaphyllou, 1990) and because of the deviating male head and juvenile tail morphology (De Grisse, 1960; Eisenback & Spaull, 1988; Eisenback & Hunt, 2009). This phylogenetic placement was confirmed by preliminary molecular data (Eisenback J.D. personal communication).

The phylogenetic positions of the mitotic parthenogenetic *M. africana* with 2n=21 chromosomes (Fig 7) and the amphimictic *M. kikuyensis* with n=7 chromosomes favour the hypothesis of Triantaphyllou (Triantaphyllou, 1985a; Triantaphyllou, 1987) that sexual *Meloidogyne* spp. could lie close to the ancestry of the genus, and that mitotic parthenogenetic species evolved from them. Indeed the maximum parsimony ancestral state reconstruction shows meiosis to be present on this node, while the maximum likelihood ancestral state reconstruction is resolved equivocal. Yet, this hypothesis is consistent with the restricted chromosome numbers of the amphimictic *Pratylenchus penetrans* Cobb, 1917 (n=5), *P. vulnus* Allen & Jensen, 1951 (n=6) and *P. coffeae* (Zimmerman, 1898) Filipjev & Schuurmans Stekhoven, 1942 (n=7) (Roman & Triantaphyllou, 1969). Furthermore, in the most closely-related genus *Pratylenchus*, mitotic parthenogenetic reproduction also appears to have evolved

independently on several occasions, based on the chromosome numbers of *P. zeae* Graham, 1951 (2n=24-26), *P. scribner*i Steiner, 1943 (2n=25-26), *P. neglectus* (Rensch, 1924) Filipjev and Schuurmans Stekhoven, 1941 (2n=20) and *P. brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941 (2n=32) (Roman & Triantaphyllou, 1969) that are in an unrelated phylogenetic position (Subbotin *et al.*, 2008). This indicates that the basic haploid chromosome number of the genus *Meloidogyne* could possibly be n=7, as in *P. coffeae*, from which mitotic parthenogenetic species could have evolved with a triploid genome (3n=21). These triploid genomes are most likely the product of reticulate evolution through genome duplication, introgression or hybridization events.

However, based on this hypothesis it remains unclear how chromosome numbers evolved from n=7 to n=18 within the genus *Meloidogyne* (Triantaphyllou, 1985a). We propose two hypotheses: either this higher chromosome number was established by polyploidy, or alternatively it was established through fragmentation of chromosomes. While these hypotheses are not mutually exclusive, the latter idea appears the more likely, as chromosomes of *M. kikuyensis* are significantly larger than chromosomes of other *Meloidogyne* spp. (Triantaphyllou, 1990). This is a particularly attractive possibility, given that *M. kikuyensis* was occasionally observed to have an extra small chromosome that could itself have fragmented from another chromosome (Triantaphyllou, 1990). Moreover, the absence of centromere activity in *Meloidogyne* spp. indicates that fragmentation of chromosomes may occur more often than in other organisms with centralized centromere activity (Triantaphyllou, 1983; Melters *et al.*, 2012). Fragmentation also appears to have an evolutionary advantage over losing chromosomes, since it ensures that genetic material continues to be passed on to the next generation. Also, the fact that some *Meloidogyne* spp. have a large variation in chromosome number between n=7 and n=18 (*M. hapla* race A n=13-17, *M. chitwoodi* n=14-18 and *M. minor* n=17), as well as the phylogenetic position of *M. mali* (n=12) with an intermediate chromosome complement between n=7 and n=18 serve to add support to this hypothesis (Chitwood & Perry, 2009). Chromosome fragmentation or aneuploidy is already known for the nematode genus *Cactodera* (Heteroderidae), with a basic chromosome number of amphimictic species of n=9 having evolved to n=12-13 in the meiotic parthenogenetic *Cactodera betulae* through chromosome fragmentation or aneuploidy (Triantaphyllou & Hirschmann, 1980). Similar

processes have been documented in several insect species, such as Lepidoptera and Hemiptera (Schrader & Hughes-Schrader, 1955; Lukhtanov *et al.*, 2005).

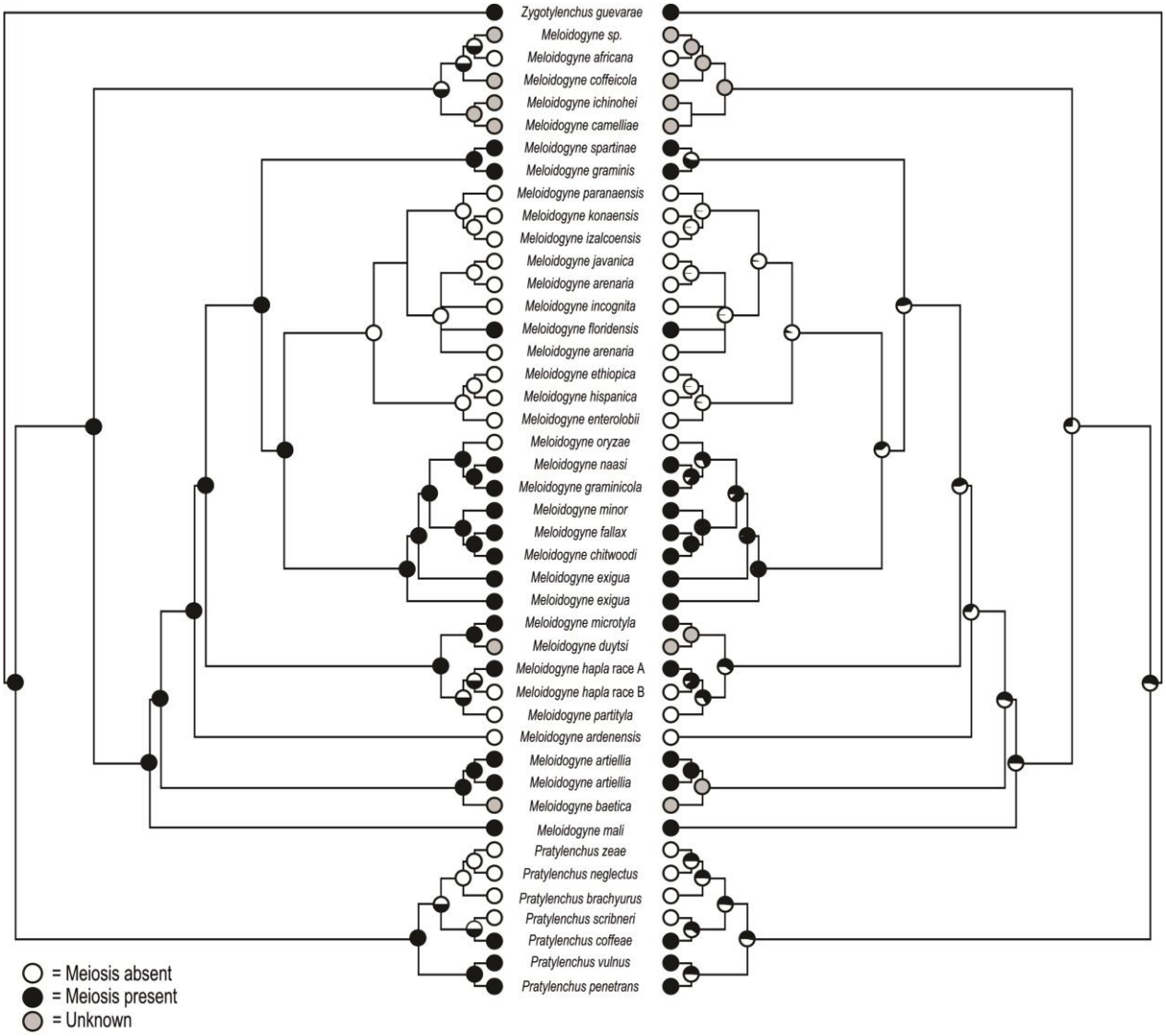


Figure 9. Maximum parsimony (left) and maximum likelihood (right) ancestral state reconstructions of the evolution of mitotic parthenogenesis and the associated loss of meiosis. Pie charts on internal nodes indicate the likelihoods of the different character states at each node and grey nodes indicate unknown character states. Presence and absence of meiosis are visualised in black and white, respectively.

4.6 Bionomics

Host tests revealed that *M. africana* was not only able to parasitize *C. arabica* but also *S. lycopersicum* (cv. Moneymaker) and *Sansevieria* sp. The above-ground symptoms include stunting, chlorosis and necrosis of leaves. In coffee roots, galls are usually positioned on the apical tip of the root, resulting in the impediment of root extension (Fig 10). Young galls are rounded, while older galls tend to be oval, 1-3 mm in size, and contain more than one female with egg sacs always embedded within the gall. This internal egg sac resembles *M. ichinohei* (Araki, 1992) Older roots often display cracking, sometimes causing the eggs to be expelled from the galls, an infection symptom also described for *M. coffeicola* (Lordello & Zamith, 1960), another species known to infect *Coffea* spp. in South America. On tomato roots, the symptoms caused by *M. africana* vary slightly: galls are much smaller and are more evenly distributed throughout the root system, with root tip galls less frequent. In general, the infection was less aggressive compared to coffee plants, indicating that tomato is less suitable as a host of *M. africana*. Interestingly, the galls of *M. exigua* have also been described as occupying a terminal position on coffee roots (Eisenback & Triantaphyllou, 1991), suggesting that this symptom might be host-dependent. Appearance and position of galls has been used as a taxonomic feature in many *Meloidogyne* spp. descriptions (Hunt & Handoo, 2009), although our results for *M. africana* indicate that many of these features (size, position) are host-dependent. Interestingly, no functional males were observed for *M. africana* on all three hostplants (*C. arabica*, *S. lycopersicum* and *Sansevieria* sp.). Other reported hosts of *M. africana* include *Z. mays* in India (Chitwood & Toung, 1960), *C. annuum* in Sudan (Yassin & Zeidan, 1982) and *Chrysanthemum cinerariaefolium* L., *Z. mays, Vigna catjang* (L.) Walp., *Syzygium aromaticum* (L.) Merrill & Perry, *C. arabica* and *Solanum tuberosum* L. in East Africa (Whitehead, 1969). Although these reports, based on morphological diagnostics, need to be confirmed, together with the results of our host-range test, they show that *M. africana* is a polyphagous species. Since *M. mali* and *M. camelliae*, are also confirmed polyphagous species (Golden, 1979; Ahmed *et al.*, 2013; Trisciuzzi *et al.*, 2014), this contradicts the hypothesis that *Meloidogyne* spp. show a tendency towards polyphagy, from the basal towards the more distally-positioned (Holterman *et al.*, 2009). This hypothesis, however, stems from limited phylogenetic-diverse sampling of root-knot nematodes, together with limited host range tests. Recent sampling and sequencing efforts (Tomalova *et al.*, 2012; Trisciuzzi *et al.*, 2014) have revealed additional species outside the three major clades, signifying that a much larger

phylogenetic biodiversity in root-knot nematodes can be expected, which is the key to fully understanding the evolution of the genus.

Figure 10. Symptoms caused by *Meloidogyne africana* on *Coffea arabica* and *Solanum lycopersicum* (cv. Moneymaker). (A) overview of galling on *Coffea arabica*; (B) detail of typical root-tip galls on *Coffea arabica*; (C) overview of galling on *Solanum lycopersicum;* (D) detail of galls on *Solanum lycopersicum.*

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6 Supplementary information Chapter 3

S1 table. Genbank accession numbers from sequences used to construct the concatenated phylogenetic analysis. Newly generated sequences in bold.

Species name	18S	28S	Cox1
Zygotylenchus guevarae	AF442189	JX261956	/
Pratylenchus oleae	KJ510864	KJ510856	KJ510866
P. vulnus	KC875383	KP161616	GQ332425
Meloidogyne ichinohei	AF442191	**KY433427**	**KY433448**
M. africana	**KY433422**	**KY433423**	**KY433434**
M. artiellia	KC875392	**KY433426**	**KY433447**
M. mali	KJ636400	JX978227	**KY433449**
M. sp.	EU669950	/	**KY433451**
M. baetica	KP896296	AY150369	/
M. camelliae	JX912884	KF542870	KM887148
M. coffeicola	HE667739	/	/
M. hapla	AY593892	KF430798	JX683718
M. incognita	AY284621	KP901085	KU372164
M. fallax	AY593895	KC241974	/
M. graminicola	KR234083	KR234084	KJ139963
M. chitwoodi	KJ130033	KC287193	KJ476150
M. enterolobii	KP901058	KP901079	JX683716
M. naasi	KP901048	KP901069	KM491211

Chapter 4

Molecular characterization and species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the *Penetrans* group (Nematoda: Pratylenchidae)

Modified from Janssen T., Karssen G., Orlando V., Subbotin S.A., Bert W. 2017. Molecular characterization and species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the *Penetrans* group (Nematoda: Pratylenchidae). Accepted with minor revisions in Molecular Phylogenetics and Evolution. IF 2016: 4.419, Q1: 32/166.

1 Abstract

Root-lesion nematodes of the genus *Pratylenchus* are an important pest parasitizing a wide range of vascular plants including several economically important crops. However, morphological diagnosis of the more than 100 species is problematic due to the low number of diagnostic features, high morphological plasticity and incomplete taxonomic descriptions. In order to employ barcoding based diagnostics, a link between morphology and species specific sequences has to be established. In this study, we reconstructed a multi-gene phylogeny of the *Pratylenchus penetrans* group using nuclear ribosomal and mitochondrial gene sequences. A combination of this phylogenetic framework with molecular species delineation analysis, population genetics, morphometric information and sequences from type location material allowed us to establish the species boundaries within the *Penetrans* group and as such clarify long-standing controversies about the taxonomic status of *P. penetrans*, *P. fallax* and *P. convallariae*. Our study also reveals a remarkable amount of cryptic biodiversity within the genus *Pratylenchus* confirming that identification on morphology alone can be inconclusive in this taxonomically confusing genus.

2 Introduction

Root-lesion nematodes of the genus *Pratylenchus* Filipjev 1936 are migratory endoparasites belonging to the family Pratylenchidae (Nematoda, Tylenchida). Root-lesion nematodes are ranked as the third most important group of plant parasitic nematodes in terms of economic loss in agriculture and horticulture (Castillo & Vovlas, 2007). In some cases, yield loss can extend up to 85% of the expected production (Nicol *et al.*, 2011). Moreover, *Pratylenchus* spp. are reported to act synergistically with some soil-borne pathogens, leading to even higher yield losses (Jones & Fosu-Nyarko, 2014). Several species of *Pratylenchus* such as *P. penetrans*, *P. brachyurus*, *P. coffeae* and *P. vulnus* have a wide geographical distribution and can parasitize a wide range of host plants, including a large variety of important economic crops (for a detailed list see Castillo & Vovlas, 2007). Problematically, several species of *Pratylenchus* are also known to parasitize a considerable range of weed species, seriously complicating pest management (Belair *et al.*, 2007). Overall, root-lesion nematodes are difficult to control once established in a field, forcing plant management towards prophylactic measures, while other

management strategies rely on crop rotation with an unsuitable host or host tolerance and genetic resistance (Castillo & Vovlas, 2007; Jones *et al.*, 2013; Jones & Fosu-Nyarko, 2014).

Within the genus *Pratylenchus*, 98 species were recognized by Geraert (2013) after which three additional species have been described: *Pratylenchus oleae* (Palomares-Rius *et al.*, 2014), *Pratylenchus quasitereoides* (Hodda *et al.*, 2014), and *Pratylenchus parazeae* (Wang *et al.*, 2015), bringing the total species number to 101. However, morphological diagnosis of root-lesion nematodes is problematic due to the low number of diagnostic features and high intraspecific variability (Roman & Hirschmann, 1969; Tarte & Mai, 1976; Castillo & Vovlas, 2007). These problems are clearly demonstrated by a recent study revealing the presence of different morphotypes within a single species, suggesting that several of the already scarce morphological diagnostic features might be dependent on the reproductive strategy of a population (Troccoli *et al.*, 2016). In order to properly diagnose *Pratylenchus* spp., a wide variety of biochemical and molecular methods have been proposed, including isozyme electrophoresis (Ibrahim *et al.*, 1995; Andres *et al.*, 2000), restriction fragment analysis of the internal transcribed spacer (ITS) of ribosomal RNA gene (Waeyenberge *et al.*, 2000), and sequencing of different fragments of the ribosomal gene cluster, including ITS (De Luca *et al.*, 2011; Wang *et al.*, 2012), 18S (Subbotin *et al.*, 2008; van Megen *et al.*, 2009) and 28S (Handoo *et al.*, 2001; Al-Banna *et al.*, 2004; Subbotin *et al.*, 2008). Species-specific primers have been developed for a variety of different species and genes (Handoo *et al.*, 2001; Al-Banna *et al.*, 2004; Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2014; Troccoli *et al.*, 2016) as well as in combination with duplex PCR (Waeyenberge *et al.*, 2009; De Luca *et al.*, 2012) and quantitative PCR methods (Mokrini *et al.*, 2013). Despite the existence of a wide spectrum of identification methods, no specific approach has been widely accepted yet and in fact most species identification methods have been tested for only a limited number of *Pratylenchus* taxa (Al-Banna *et al.*, 2004; Mokrini *et al.*, 2013; Palomares-Rius *et al.*, 2014; Troccoli *et al.*, 2016).

Most importantly, in order to employ molecular identification, traditional morphospecies would ideally be linked to molecular barcodes. In recent years, several species of *Pratylenchus* have been matched to molecular sequences, revealing the existence of cryptic species complexes (De Luca *et al.*, 2011; De Luca *et al.*, 2012). Collecting topotype material has often proved to be the only way to confidently connect DNA sequences to formerly described morphospecies

(Inserra *et al.*, 2007; De Luca *et al.*, 2010; Troccoli *et al.*, 2016). However, despite these efforts the vast majority of morphospecies remains unlinked to DNA sequences (Geraert, 2013).

Another problem is that the taxonomic status of several morphospecies is currently contested. This is especially true for the *Pratylenchus penetrans* species group (clade IV) (Subbotin *et al.*, 2008), in which species appear to share many morphological characteristics (Subbotin *et al.*, 2008). Along *P. penetrans* this clade IV contains *P. fallax, P. convallariae, P. pinguicaudatus*, *P. arlingtoni*, *P. dunensis*, *P. oleae* and *P. brachyurus* (Palomares-Rius *et al.*, 2014); however, the validity of several of these taxa has been questioned (Subbotin *et al.*, 2008). The taxonomic debate to clarify the validity of *P. penetrans* and *P. fallax* is an excellent example to illustrate the difficulties faced. *P. fallax* was originally described and differentiated from *P. penetrans* by Seinhorst (1968). Tarte and Mai (1976) subsequently considered both species to be conspecific, as morphological traits showed considerable intraspecific variability as a result of variable environmental conditions. Later *P. fallax* was re-erected as a separate species as breeding experiments yielded infertile offspring in interspecific breeding experiments (Perry *et al.*, 1980). *P. penetrans* and *P. fallax* were also differentiated using isozyme electrophoresis (Ibrahim *et al.*, 1995) and PCR-ITS-RFLP analysis (Waeyenberge *et al.*, 2000). Subsequently, sequence analysis of the D3 region of the 28S rRNA gene revealed that *P. fallax* and *P. convallariae* are 99% identical, both species were shown to be closely related to *P. penetrans* (96-97% similarity) (Carta *et al.*, 2001; Handoo *et al.*, 2001). Additional molecular characterization by Subbotin *et al*. (2008) further brought into question the validity of several species within the *Penetrans* group. Surprisingly, ITS rRNA gene phylogenies strongly suggested that *P. fallax* was also very closely related to *P. lentis* (Palomares-Rius *et al.*, 2010; Palomares-Rius *et al.*, 2014). At the same time, Holterman *et al*. (2006) and Jones *et al*. (2013) argued *P. convallariae*, *P. penetrans* and *P. fallax* to be conspecific. Despite this taxonomic confusion *P. fallax*, is currently recognized as a quarantine species in the USA. This regulation is affecting the trade of plants and plant products, adding even more importance to clarification of the taxonomic status of this species (Handoo *et al.*, 2001).

Therefor the goals of this study were therefore to: (i) explore the biodiversity of the *Penetrans* group species based on a combination of morphological and molecular data; (ii) elucidate the molecular phylogeny of the *Penetrans* group; (iii) use this phylogenetic framework to delimitate

species and clarify the taxonomic status of *P. arlingtoni*, *P. convallariae*, *P. dunensis*, *P. fallax*, *P. penetrans*, *P. pinguicaudatus* and four newly discovered undescribed species based on a link between sequence data and morphology; (iv) evaluate the correctness of species identification for sequences available in public databases; (v) assess the potential of using morphometric characters to distinguish species within the *Penetrans* group.

3 Material and methods

3.1 Nematode populations

Nematodes were extracted from soil and root material using a modified Baermann funnel and a mistifier (van Bezooijen, 2006), respectively (Hooper, 1986). Populations were sampled globally, however, the majority of samples were taken in the Netherlands focusing on fruit trees (*Malus* sp., *Prunus* spp., *Pyrus* sp.), grapes (*Vitis* spp.) and grasslands. The species: *P. dunensis*, *P. fallax* and *P. pinguicaudatus* were collected from the type localities, additional information on studied nematode species is given in Table 1.

3.2 Morphological study

Nematodes were studied in temporary slides sealed with nail-polish. Specimens were studied using an Olympus BX5 DIC microscope (Olympus Optical) and morphological vouchers were made using a combination of movies and photomicrographs made by an Olympus C5060Wz camera. To secure a link of morphometrics and sequences, measurements of these vouchers were made at the microscope or using ImageJ (Schneider *et al.*, 2012). Vouchered nematodes were subsequently picked from temporary mounts and processed for DNA extraction. The resulting digital specimen vouchers are available online at http://nematodes.myspecies.info/taxonomy/term/10645/specimens.

Remaining unvouchered nematodes were fixed in TAF at 70 °C and processed to anhydrous glycerin, following the method of Seinhorst (1962) modified by Sohlenius and Sandor (1987) and measured using an Olympus BX5 DIC microscope (Table 2). Only measurements (L, V, a, b, c, c', stylet length, body width, post-uterine sac length, tail length, and position of metacorpus, cardia, pharyngeal lobe, excretion pore, vulva in respect to anterior end) from vouchers with a direct link with the molecular data were analyzed using a Cluster, a Principal

Component Analysis (PCA) and a Canonical Discriminant Analysis (CDA). The Cluster analysis was done based on the default settings using Primer 6 (Clarke, 1993), after standardization and calculating an Euclidean distance matrix. The CDA and PCA analyses were only performed for those species with sufficient sequence-linked representatives (>3 individuals, i.e. *P. penetrans*, *P. fallax* and *Pratylenchus* sp. 4) using the CANDISC procedure in SAS® 9.4 to find the sets of variables that discriminate most between the populations, based on the pooled within variance-covariance matrix.

For Scanning Electron Microscopy (SEM) nematodes were fixed in 600 µl fresh 4% Paraformaldehyde fixative buffered with Phosphate Buffer Saline (PBS) and 1% glycerol and heated for 3 seconds in a 750W microwave. Subsequently, specimen were dehydrated in a seven-step graded series of ethanol solutions and critical-point dried using liquid CO_2, mounted on stubs with carbon discs, coated with gold (25nm). Specimen were studied and photographed with a JSM-840 EM (JEOL) electron microscope at 12 kV.

3.3 DNA extraction, PCR amplification and sequencing

Genomic DNA of individual nematodes was extracted using the quick alkaline lysis protocol described by Janssen *et al*. (2016). Briefly, individual nematodes were transferred to a mixture of 10 µl 0.05N NaOH and 1 µl of 4.5% tween. The mixture was heated to 95°C for 15 min, and after cooling to room temperature 40 µl of double-distilled water was added. PCR amplification was performed using TopTaq DNA polymerase (QIAGEN, Germany), in a volume of 25 µl using a Bio-Rad T100^{TM} thermocycler. PCR mixtures were prepared according to the manufacturer's protocol with 0.4 µM of each primer and 2 µl of single nematode DNA extraction. The 28S rDNA fragment D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG -3') and D3B (5'- TCG GAA GGA ACC AGC TAC TA -3') primers were used according to the protocol of De Ley *et al*. (1999). The internal transcribed rRNA gene spacer (ITS) was amplified using VRAIN2F (5'- CTT TGT ACA CAC CGC CCG TCG CT -3') and VRAIN2R (5'- TTT CAC TCG CCG TTA CTA AGG GAA TC -3') (Vrain *et al.*, 1992), subsequently cloned using pGEM ® -T easy vector systems (Promega) and sequenced using universal M13F and M13R primers. The cytochrome c oxidase subunit 1 (COI) gene fragment was amplified using JB3 (5'- TTT TTT GGG CAT CCT GAG GTT TAT -3') and JB4.5 (5'- TAA AGA AAG AAC ATA ATG AAA ATG -3') according to the protocol of Derycke *et al*. (2010b). Sanger

sequencing of purified PCR fragments was carried out in forward and reverse direction by Macrogen (Europe). Contigs were assembled using GENEIOUS R6.1.8 (Biomatters; http://www.geneious.com). All contigs were subjected to BLAST searches to check for possible contaminations on http://www.ncbi.nlm.nih.gov. The newly obtained sequences were submitted to the GenBank database, accession numbers are displayed in Table 1 and in the phylogenetic trees.

Table 1. Species and populations of *Pratylenchus* used in the study. * Subbotin et al. (2008), [1] No morphological voucher available, [2] Topotype material

Species	Locality	Specimen or sample code	COI haplotype	Host	GenBank accession number		
					D2-D3 of 28S rRNA	ITS of rRNA	*COI*
P. brachyurus	China, F0983	T162	Br1	*Ficus* sp.	-	KY828251-KY828252	KY817010
P. crenatus	The Netherlands, Baale-Nassau F0683-2	T133	Cr1	Grasses	KY828371	-	KY817014
P. crenatus	The Netherlands, Arkel F1145	T183	Cr1	*Pyrus* sp.	-	-	KY817006
P. crenatus	The Netherlands, Smilde F0712	T275	Cr1	Grasses	-	-	KY816997
P. crenatus	The Netherlands, Hei en Boeicop F1247	T297	Cr1	*Pyrus* sp.	-	-	KY816989
P. crenatus	The Netherlands, Heersch F2468	T702	Cr1	*Vitis* sp.	-	-	KY816969
P. crenatus	UK, England, Rothemstadt, broadbalk	T761	Cr1	Grasses	KY828370	-	KY816943
P. convallariae	Belgium, Wetteren	T267	Co1	Grasses	-	KY828256	KY817000
P. convallariae	Belgium, Wetteren	T268	Co1	Grasses	KY828373	-	KY816999
P. convallariae	Belgium, Wetteren	T269	-	Grasses	-	-	-
P. convallariae	USA, Ohio, Tipp City	CD1813[1]	Co2	Unknown plant	KY828372	-	KY817025
P. dunensis	The Netherlands, Groote Keeten	T67	Du1	*Ammophila arenaria*	KY828369	KY828244-KY828245	KY817019
P. dunensis	The Netherlands, Groote Keeten	T68	-	*Ammophila arenaria*	KY828368	-	-
P. dunensis	The Netherlands, Groote Keeten	T69	-	*Ammophila arenaria*	-	-	-
P. dunensis	The Netherlands, Groote Keeten	T70	-	*Ammophila arenaria*	-	-	-
P. fallax	The Netherlands, Middelharnis	T85	Fa1	Grasses	KY828367	-	KY817017
P. fallax	The Netherlands, Middelharnis	T87	-	Grasses	KY828366	-	-
P. fallax	The Netherlands, Smilde F0712	T272	Fa1	Grasses	KY828365	-	KY816998
P. fallax	The Netherlands, Baale-Nassau F0683-3	T283	Fa1	Grasses	KY828364	-	KY816996
P. fallax	The Netherlands, Baale-Nassau F0683-3	T284	Fa1	Grasses	-	-	KY816995

Species	Locality	Specimen or sample code	COI haplotype	Host	GenBank accession number		
					D2-D3 of 28S rRNA	ITS of rRNA	*COI*
P. fallax	The Netherlands, Uddel F0689	T286	Fa1	Grasses	-	-	KY816994
P. fallax	The Netherlands, Uddel F0689	T290	Fa1	Grasses	-	-	KY816993
P. fallax	The Netherlands, Doornenburg[2]	T353	Fa1	*Malus pumila*	KY828363	KY828258	KY816988
P. fallax	The Netherlands, Doornenburg[2]	T354	Fa1	*Malus pumila*	-	-	KY816987
P. fallax	The Netherlands, Heersch F2468	T700	Fa1	*Vitis vinifera*	-	-	KY816971
P. fallax	The Netherlands, Andelst F2420	T706	Fa1	*Prunus domestica*	-	-	KY816967
P. fallax	The Netherlands, Andelst F2420	T707	Fa1	*Prunus domestica*	-	-	KY816966
P. fallax	The Netherlands, Andelst F2420	T708	Fa1	*Prunus domestica*	-	-	KY816965
P. fallax	The Netherlands, Winssen F2399	T709	Fa1	*Prunus domestica*	-	-	KY816964
P. fallax	The Netherlands, Ochten F2421	T710	Fa1	*Prunus avium*	-	-	KY816963
P. fallax	The Netherlands, Ochten F2421	T712	Fa1	*Prunus avium*	-	-	KY816962
P. fallax	The Netherlands, Terwolde F2432	T717	Fa1	*Vitis vinifera*	-	-	KY816957
P. fallax	The Netherlands, Terwolde F2432	T718	Fa1	*Vitis vinifera*	-	-	KY816956
P. fallax	The Netherlands, Terwolde F2432	T719	Fa1	*Vitis vinifera*	-	-	KY816955
P. fallax	The Netherlands, Papendrecht F2473	T727	Fa1	*Prunus domestica*	-	-	KY816947
P. fallax	The Netherlands, Papendrecht F2473	T728	Fa1	*Prunus domestica*	-	-	KY816946
P. fallax	The Netherlands, Papendrecht F2473	T729	Fa1	*Prunus domestica*	-	-	KY816945
P. fallax	The Netherlands, Ysbrechtum F2455	V4C	Fa1	*Vitis vinifera*	KY828362	KY828272-KY828273	KY816938
P. fallax	The Netherlands, Ysbrechtum F2455	V5C	Fa1	*Vitis vinifera*	KY828361	-	KY816937
P. fallax	The Netherlands, Ysbrechtum F2455	V8C	Fa1	*Vitis vinifera*	KY828360	-	KY816935
P. fallax	The Netherlands, Doornenburg	T376	Fa2	*Malus pumila*	-	-	KY816986
P. fallax	The Netherlands, Doornenburg	T682	Fa2	*Pyrus* sp.	-	-	KY816978

Species	Locality	Specimen or sample code	COI haplotype	Host	GenBank accession number		
					D2-D3 of 28S rRNA	ITS of rRNA	*COI*
P. fallax	The Netherlands, Heersch F2468	T701	Fa2	*Vitis vinifera*	-	-	KY816970
P. fallax	The Netherlands, Winssen F2399	T705	Fa2	*Prunus domestica*	-	-	KY816968
P. fallax	The Netherlands, St.Oedenrode F2470	T685	Fa3	*Vitis vinifera*	-	-	KY816977
P. penetrans	The Netherlands, Kloetinge F2466	T720	Pe1	*Prunus avium*	-	-	KY816954
P. penetrans	The Netherlands, Vredepeel F770	T44	Pe2	*Daucus carota*	KY828359	-	KY817020
P. penetrans	Ethiopia, Shashamane	T225[1]	Pe2	*Zea mays*	KY828354	-	KY817001
P. penetrans	The Netherlands, Hei en Boeicop F1247	T296	Pe2	*Pyrus* sp.	-	-	KY816990
P. penetrans	The Netherlands, Schimmert F2433	T697	Pe2	*Prunus domestica*	-	-	KY816974
P. penetrans	The Netherlands, St.Oedenrode F2470	T730	Pe2	*Vitis vinifera*	-	-	KY816944
P. penetrans	France, Britany	CA192[1]	Pe2	*Malus pumila*	EU130859*	KY828242-KY828243	KY817022
P. penetrans	The Netherlands, Baarlo F2584	V3A	Pe3	*Malus pumila*	KY828347	KY828268-KY828269	KY816941
P. penetrans	The Netherlands, Stramproy F2425	T714	Pe4	*Prunus avium*	-	-	KY816960
P. penetrans	USA, California, Stanislaus County	CA91[1]	Pe5	*Vigna unguiculata*	EU130862*	-	KY817023
P. penetrans	The Netherlands, Zoetermeer F0716-1	T132	Pe6	Grasses	KY828358	-	KY817015
P. penetrans	The Netherlands, Kloetinge F2466	T722	Pe6	*Prunus avium*	-	-	KY816952
P. penetrans	Rwanda, Nyakiriba	T143	Pe7	*Allium cepa*	KY828357	KY828249-KY828250	KY817013
P. penetrans	Rwanda, Nyakiriba	T144	Pe7	*Allium cepa*	-	-	KY817012
P. penetrans	Rwanda, Nyakiriba	T145	Pe7	*Allium cepa*	-	-	KY817011
P. penetrans	The Netherlands, Arkel F1145	T184	Pe7	*Pyrus* sp.	-	-	KY817005

Species	Locality	Specimen or sample code	COI haplotype	Host	GenBank accession number		
					D2-D3 of 28S rRNA	ITS of rRNA	*COI*
P. penetrans	Rwanda, Bushoki	T200[1]	Pe7	*Solanum tuberosum*	KY828355	-	KY817004
P. penetrans	The Netherlands, Hei en Boeicop F1247	T295	Pe7	*Pyrus* sp.	KY828352	-	KY816991
P. penetrans	The Netherlands, Schimmert F2433	T698	Pe7	*Prunus domestica*	-	-	KY816973
P. penetrans	The Netherlands, Kloetinge F2466	T721	Pe7	*Prunus avium*	-	-	KY816953
P. penetrans	The Netherlands, Wemeldinge F2472	T724	Pe7	*Prunus avium*	-	-	KY816950
P. penetrans	The Netherlands, Wemeldinge F2472	T726	Pe8	*Prunus avium*	-	-	KY816948
P. penetrans	The Netherlands, Stramproy F2425	T713	Pe9	*Prunus avium*	-	-	KY816961
P. penetrans	The Netherlands, Kloetinge F2466	T723	Pe10	*Prunus avium*	-	-	KY816951
P. penetrans	The Netherlands, Zoetermeer F0716-1	T181	Pe11	Grasses	-	-	KY817007
P. penetrans	Colombia, San Vicente	T666	Pe11	*Physalis peruviana*	KY828351	-	KY816982
P. penetrans	Colombia, San Vicente	T677	Pe11	*Physalis peruviana*	KY828350	-	KY816981
P. penetrans	Colombia, San Vicente	T678	Pe11	*Physalis peruviana*	KY828349	-	KY816980
P. penetrans	Colombia, San Vicente	T679	Pe11	*Physalis peruviana*	-	-	KY816979
P. penetrans	The Netherlands, Wemeldinge F2472	T725	Pe11	*Prunus avium*	-	-	KY816949
P. penetrans	Japan, Aichi	CA85[1]	Pe11	*Brassica oleraceae*	EU130860*	-	KY817021
P. penetrans	The Netherlands, Baarlo F2584	V8A	Pe11	*Malus pumila*	KY828342	KY828274-KY828275	KY816936
P. penetrans	The Netherlands, Meijel F2537	V4B	Pe11	*Malus pumila*	KY828345	-	KY816939
P. penetrans	The Netherlands, Nagele F2612	V3F	Pe11	*Malus pumila*	KY828346	KY828270-KY828271	KY816940
P. penetrans	Japan, Chiba	CA193[1]	Pe12	*Daucus carota*	EU130857*	-	KY817024
P. penetrans	The Netherlands, Arkel F1145	T172	Pe13	*Pyrus* sp.	KY828356	-	KY817009
P. penetrans	The Netherlands, Apeldoorn F1179	T293	Pe13	*Pyrus* sp.	KY828353	KY828257	KY816992
P. penetrans	The Netherlands, Sambeek F2583	T686	Pe13	*Malus pumila*	-	-	KY816976

Species	Locality	Specimen or sample code	COI haplotype	Host	GenBank accession number		
					D2-D3 of 28S rRNA	ITS of rRNA	*COI*
P. penetrans	The Netherlands, Sambeek F2583	T687	Pe13	*Malus pumila*	-	-	KY816975
P. penetrans	The Netherlands, Schimmert F2433	T699	Pe13	*Prunus domestica*	-	-	KY816972
P. penetrans	The Netherlands, Meijel F2537	V1B	Pe14	*Malus pumila*	KY828348	KY828266-KY828267	KY816942
P. penetrans	The Netherlands, Stramproy F2425	T715	Pe15	*Prunus avium*	-	-	KY816959
P. penetrans	The Netherlands, Stramproy F2425	T716	Pe16	*Prunus avium*	-	-	KY816958
P. penetrans	The Netherlands, Meijel F2537	V6B	-	*Malus pumila*	KY828343	-	-
P. penetrans	The Netherlands, Baarlo F2584	V5A	-	*Malus pumila*	KY828344	-	-
P. penetrans	The Netherlands, Nagele F2612	V12F	-	*Malus pumila*	KY828341	-	-
P. penetrans	The Netherlands, Nagele F2612	V13F	-	*Malus pumila*	KY828340	-	-
P. penetrans	The Netherlands, Nagele F2612	V15F	-	*Malus pumila*	KY828339	-	-
P. pinguicaudatus	UK, England, Rothemstadt, broadbalk[2]	T572[1]	Pi1	*Triticum* sp.	-	KY828261-KY828263	KY816984
P. pinguicaudatus	UK, England, Rothemstadt, broadbalk[2]	CA191, CD2361[1]	-	*Triticum* sp.	KY828338	KY828239-KY828241	-
Pratylenchus sp. 1	Italy, Sicily, Villalba	T617	Sp1	*Lens culinaris*	KY828337	KY828264-KY828265	KY816983
Pratylenchus sp. 1	Italy, Sicily, Villalba	V13L	Sp1	*Lens culinaris*	KY828336	-	KY816934
Pratylenchus sp. 1	Italy, Sicily, Villalba	V15L	Sp1	*Lens culinaris*	KY828335	-	KY816933
Pratylenchus sp. 1	Italy, Sicily, Villalba	V29L	-	*Lens culinaris*	KY828334	-	-
Pratylenchus sp. 2	Nigeria, Ibadan, IITA trail field	T175	Sp2	Unknown	KY828333	-	KY817008
Pratylenchus sp. 2	Nigeria, Ibadan, IITA trail field	T194	-	Unknown	KY828332	-	-
Pratylenchus sp. 2	Nigeria, Ibadan, IITA trail field	T569	Sp2	Unknown	-	KY828259-KY828260	KY816985

Species	Locality	Specimen or sample code	COI haplotype	Host	GenBank accession number		
					D2-D3 of 28S rRNA	ITS of rRNA	*COI*
Pratylenchus sp. 3	Rwanda, Nyamata	T201	Sp3	*Zea mays*	KY828331	KY828253-KY828255	KY817003
Pratylenchus sp. 3	Rwanda, Nyamata	T202	Sp3	*Zea mays*	KY828330	-	KY817002
Pratylenchus sp. 3	Rwanda, Nyamata	T278	-	*Zea mays*	-	-	-
Pratylenchus sp. 4	Tunisia	T71a	-	*Phoenix dactylifera*	KY828329	KY828246-KY828248	-
Pratylenchus sp. 4	Tunisia	T72a	Sp4	*Phoenix dactylifera*	–	-	KY817018
Pratylenchus sp. 4	Tunisia	T97[1]	-	*Phoenix dactylifera*	KY828328	-	-
Pratylenchus sp. 4	Tunisia	T103a	-	*Phoenix dactylifera*	KY828327	-	-
Pratylenchus sp. 4	Tunisia	T104a	Sp4	*Phoenix dactylifera*	KY828326	-	KY817016
Pratylenchus sp. 4	Tunisia	T106a[1]	-	*Phoenix dactylifera*	KY828325	-	-

3.4 Sequence and phylogenetic analyses

The newly obtained and published nematode sequences for each gene (Handoo *et al.*, 2001; De Luca *et al.*, 2004; Subbotin *et al.*, 2008; Troccoli *et al.*, 2008; De Luca *et al.*, 2011; Mekete *et al.*, 2011; Wang *et al.*, 2012; Majd Taheri *et al.*, 2013; Palomares-Rius *et al.*, 2014; Kushida & Kondo, 2015; Mokrini *et al.*, 2016; Troccoli *et al.*, 2016) were aligned using ClustalX using default parameters. The best fit models of DNA evolution were obtained for each dataset using the program jModeltest 0.1.1 (Posada, 2008) under the Akaike information criterion. Bayesian phylogenetic analysis (BI) was carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis for each gene was initiated with a random starting tree and was run with four chains for 3.0×10^6 generations for COI and ITS datasets and 6.0×10^6 generations for the D2-D3 of 28S dataset. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples (20%), a 50% majority rule consensus tree was generated. Posterior Probabilities (PP) are given on appropriate clades. Pairwise divergences between the taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data using PAUP* 4.0b 10 (Swofford, 2002).

3.5 Species delimitation

Molecular species delimitation analyses were performed using the Generalized Mixed Yule Coalescent (GMYC) method (Pons *et al.*, 2006) and the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al.*, 2012). For the GMYC species delimitation method an ultrametric tree was constructed under an uncorrelated lognormal relaxed clock using BEAST v1.7.5 (Drummond *et al.*, 2012). Different codon positions were treated as different partitions. Duplicated haplotypes were removed from the dataset using COLLAPSE 1.2 (Posada, 2004) and outgroup group sequences were removed from the alignment. A constant size coalescent prior was used because the GMYC method uses a coalescent null model to explain tree branching patterns (Pons *et al.*, 2006). Default prior distributions were used except for the standard ucld.mean parameter which was changed into a uniform prior with initial value of 1, a lower bound of 0 and an upper bound of 100 for all three codon partitions. The analysis ran for 50000000 generations sampling every 5000th generation, 50% of the results were discarded as 'burnin'. From the resulting 20000 trees the maximum clade credibility tree was calculated with TreeAnnotator v1.7.5. On the resulting ultrametric tree both a single and a multiple-

treshold GMYC model was optimized (Fujisawa & Barraclough, 2013) using the SPLITS package (Ezard *et al.,* 2009) available for R. For the ABGD species delimitation method the alignment without outgroups and unique haplotypes was used. ABGD analyses were performed using the online version of the program (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html**)**, using the default program settings. Distances were calculated using the Jukes-Cantor (JC69) substitution model and the Kimura (K80) substitution model.

4 Results and taxonomic notes

4.1 Dataset and taxonomic notes

Using a combination of morphological taxonomic characters and molecular criteria, seven valid species of *Pratylenchus* were distinguished within the studied samples: *P. brachyurus, P. crenatus, P. convallariae, P. dunensis, P. fallax, P. penetrans, P. pinguicaudatus* and four unidentified and putative new species. The taxonomic status of several of these species is highly contested (Handoo *et al.*, 2001; Castillo & Vovlas, 2007; Subbotin *et al.*, 2008) and as a result some long lasting misconceptions exist**.** In the following section an attempt is made to clarify the taxonomic status of each species. In order to establish a clear link between DNA sequences and morphology, each sequenced specimen is linked to a morphological voucher available on http://www.nematodes.myspecies.info/ (Table 1). The link between morphology and sequences is crucial for the identification of formerly described morphospecies and is essential for the reproducibility of the generated hypotheses (Pleijel *et al.*, 2008). However, as species within the *Penetrans* group are morphologically very closely related, topotype material is often the only way to confidently link traditional morphospecies to DNA sequences (Inserra *et al.*, 2007; De Luca *et al.*, 2010; De Luca *et al.*, 2012; Troccoli *et al.*, 2016; Zamora-Araya *et al.*, 2016).

Brief morphological descriptions with illustrations (Figs 1-4), morphometric values (Table 2) and phylogenetic positions in the trees (Figs 5-7) are given for several species of *Pratylenchus* below.

4.1.1 *Pratylenchus arlingtoni* Handoo, Carta & Skantar, 2001

Pratylenchus arlingtoni was described to be parasitizing grasses at the Arlington National Cemetery (VA, USA) (Handoo *et al.*, 2001). While *P. arlingtoni* is morphologically closely related to *P. crenatus*, the D3 of 28S rRNA gene sequences of *P. arlingtoni* show that this species belongs to the *P. penetrans* complex (Fig. 5). This phylogenetic position is highly surprising given the conserved morphology of the other species within this species complex. However, our species delineation analysis reveals that the sequence of *P. arlingtoni* (AF307328) differs only 4 base pairs from our *P. convallariae* population (Fig. 5), indicating that Handoo *et al*. (2001) accidentally sequenced a *P. convallariae* specimen while describing the morphology of *P. crenatus*. This is not unlikely given that *P. crenatus* and *P. convallariae* share the same habitat and can be expected to co-exist in many grass habitats. Furthermore, *P. arlingtoni* was described to have a filled spermatheca on rare occasions (Handoo *et al.*, 2001), although, no males were reported for *P. arlingtoni*, and in the light of the current hypothesis this could point to the presence of *P. convallariae* in the sample. Additionally, re-examination of *P. arlingtoni* type material revealed a very close relationship with *P. crenatus*. Specifically, the same secretory-excretory duct swelling was observed in the type material as was described to be species specific for *P. crenatus* (Karssen & Bolk, 2000). A morphometric study of *P. crenatus* (Kumari, 2015) revealed that several morphometric values are more variable than originally assumed by Loof (1960), suggesting that pharynx length and b value are not useful in separating *P. arlingtoni* from *P. crenatus* as proposed by Handoo *et al*. (2001). Also it is questionable if pharyngeal gland length, c' value and post-uterine sac length relative to vulval-anal distance are useful to separate both species as they appear to be greatly variable and overlapping in range. Additionally, a SEM study of a *P. crenatus* population (Baarle-Nassau, the Netherlands, supplementary fig. 1) matches exactly with the SEM characteristics described for *P. arlingtoni*. Finally, in this current study *P. crenatus* was found in five different locations in the Netherlands and one in Harpenden, Rothamsted, UK and all populations matched with the morphology of *P. arlingtoni*. Therefore we consider *P. arlingtoni* to be a *species inquirendae* until its morphology and molecular identity have been reassessed.

4.1.2 Pratylenchus brachyurus (Godfrey 1929) Filipjev & Schuurmans Stekhoven, 1941

Pratylenchus brachyurus is a widely distributed species in tropical and sub-tropical regions. A *P. brachyurus* population was recovered from a *Ficus* sp. sample from China, its COI sequence represents the first mitochondrial gene sequence of the species (Fig. 6). The population displayed all characteristic morphological features of *P. brachyurus*: a labial region with two annuli, stylet with broadly rounded knobs, typical tail point, empty spermatheca and posteriorly-positioned vulva. Originally *P. brachyurus* was considered to be part of clade III (Subbotin *et al.*, 2008), later it was recovered as the earliest branching taxon of clade IV, albeit with poor support (Palomares-Rius *et al.*, 2014). This phylogenetic position was confirmed by our D2-D3 of 28S rRNA gene phylogenetic tree (posterior probability= 99%, Fig. 5).

4.1.3 *Pratylenchus convallariae* Seinhorst, 1959

(Fig. 1, Table 2)

Pratylenchus convallariae was originally described from Wassenaar, the Netherlands parasitizing *Convallaria majalis* L. Despite extensive sampling efforts in Wassenaar, *P. convallariae* was not recovered from the area. However, *P. convallariae* was recovered from grass in a sandy habitat (Wetteren, Belgium, GPS coordinates: 51.006643, 3.904379). The ITS sequences of this population are 92%-95% identical with sequences deposited by Waeyenberge *et al*. (FJ712907- FJ712911, Fig. 7), originating from a *C. majalis* grower in Lisse, The Netherlands, which is situated approximately 20 km from the type locality in Wassenaar. Moreover, these sequences are also closely related to another *P. convallariae* ITS sequence from China (HM469448, 91.1% - 94.6% similar, Fig. 7). The morphology of our population matches the morphology of the original description very well including the characteristic head and tail point morphology (Fig. 1). Morphometrics also agree with the original description including the vulva position, although specimens of our population were smaller, 378-438µm vs. 580-610µm (Table 2).

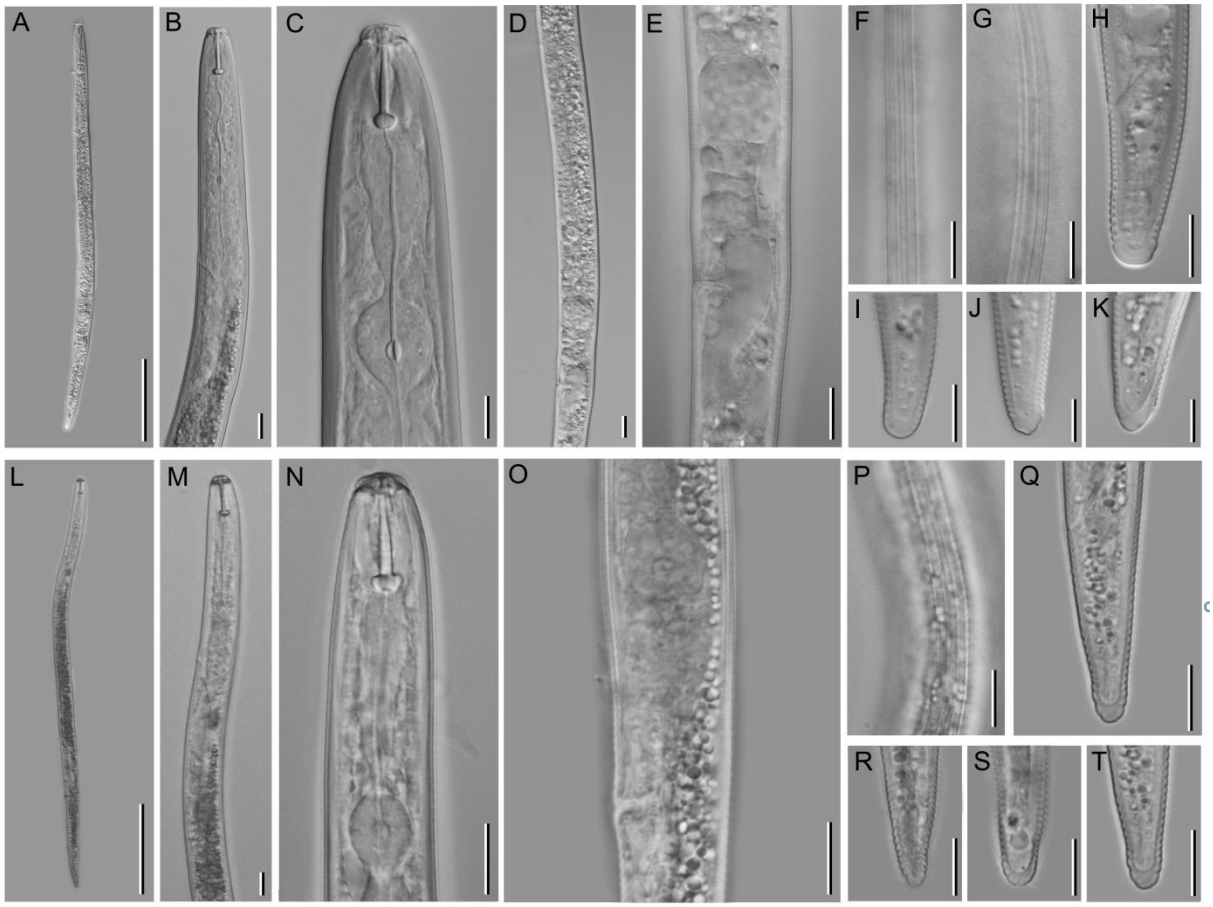

Figure 1. Photomicrographs of specimens of *Pratylenchus penetrans* (A-K) and *P. convallariae* (L-T). A: Entire female body; B, C: Female anterior region; D, E: Vulval region; F, G: Lateral field; H-K: Tail region. L: Entire female body; M, N: Female anterior region; O: Vulval region; P: Lateral field; Q-T: Tail region. Scale bars A, L: 100 µm; B-K, M-T: 10 µm.

Table 2. Morphometric measurements of females of *P. penetrans, P. fallax, P. convallariae* and *Pratylenchus* sp. 3 females. All measurements were made on glycerin fixed specimen, measurements are in µm and given as mean ± standard deviation (range).

	Baarlo (F2584)	Nagele (F2612)	Meijel (F2537)	Ysbrechtum (F2455)	Uddel (F0689)	Doornenburg Type locality	Wetteren	Rwanda
Species	*P. penetrans*	*P. penetrans*	*P. penetrans*	*P. fallax*	*P. fallax*	*P. fallax*	*P. convallariae*	*Pratylenchus* sp. 3
Character								
n	6	12	6	8	10	7	10	14
L	659 ± 54 (580-721)	684 ± 71 (555-792)	593 ± 82 (464-703)	527 ± 32 (487-594)	447 ± 46 (386-554)	471 ± 40 (412-524)	411 ± 20 (378-438)	531 ± 44 (469-600)
Stylet length	16 ± 0.7 (15-17)	16 ± 0.6 (15-17)	15 ± 1.2 (14-17)	16± 1 (14-19)	15 ± 0.5 (14-16)	16 ± 0.6 (15-17)	15 ± 1 (14-17)	13.6 ± 0.5 (13-14.6)
Anterior end to center of metacorpus	61 ± 4 (55-66)	60 ± 4 (53-66)	55 ± 8 (46-65)	57 ± 5 (46-63)	45 ± 4 (41-53)	46 ± 3 (40-50)	44 ± 2 (41-47)	54.7 ± 2.7 (50.9-59.6)
Anterior end to cardia	76 ± 5 (70-83)	76 ± 6 (67-87)	70 ± 8 (59-79)	70 ± 5 (61-78)	53 ± 4 (49 -62)	56 ± 5 (47-62)	51 ± 2 (48-55)	91.5 ± 6.6 (82.5-107.4)
Anterior end to pharyngeal gland lobe	146 ± 15 (120-161)	147 ± 15 (115-173)	93 ± 18 (70-110)	127 ± 9 (108-137)	78 ± 12 (64-103)	79 ± 9 (72-98)	106 ± 13 (84-119)	111 ± 15.3 (80-132)
Anterior end to EP	97 ± 11 (85-112)	99 ± 7 (88-110)	120 ± 23 (85-137)	87 ± 8.3 (78-96)	91 ± 11 (71-104)	108 ± 14 (78-117)	63 ± 6 (51-71)	76 ± 12.2 (49.8-90.5)
Anterior end to vulva	513 ± 66 (421-584)	540 ± 52 (431-631)	484 ± 76 (365-594)	413 ± 23 (384-460)	360 ± 34 (309 -427)	369 ± 25 (336-409)	323 ± 153 (308-353)	409 ± 40 (338-464)
Maximum body width	28 ± 3 (24-33)	27 ± 4 (20-36)	21 ± 1.2 (20-23)	21 ± 2 (20-25)	16 ± 1 (15-18)	16 ± 0.7 (15-17)	16 ± 1 (14-19)	21.6 ±1.8 (19-24.8)
Anal body width	13 ± 0.8 (12-14)	15 ± 2 (13-19)	12 ± 1 (11-13)	11 ± 0.7 (10-12)	9.6 ± 1 (8-11)	10 ± 0.5 (9-11)	10 ± 1 (8-12)	13.6 ± 1.7 (10.9-18)
Vulva-anus distance	106 ± 11 (84-115)	97 ± 9 (87-117)	80 ± 9 (67-90)	87 ± 12 (74-109)	63 ± 12 (52-91)	78 ± 16 (55-101)	63 ± 6 (49-70)	79.7 ± 11.4 (64-101.4)
Tail length	31 ± 3 (25-35)	32 ± 4 (27-41)	29 ± 2 (25-31)	26 ± 2 (23-31)	21 ± 2 (17-24)	24 ± 2 (22-27)	24 ± 3 (18-27)	31.7 ± 2.9 (26.2-36.6)
V	77 ± 6 (66 – 82)	79 ± 5 (67-84)	81 ± 2 (78-84)	78 ± 1 (77-80)	80 ± 2 (77-83)	78 ± 2 (74-81)	78 ± 2 (75-83)	77.6 ± 1.4 (75.3-79.7)
a	24 ± 2 (20 - 26)	25 ± 5 (18-35)	27 ± 3 (23-32)	25 ± 3 (19-29)	28 ± 2 (25-32)	29 ± 3 (24-33)	25 ± 2 (19-28)	24.5 ± 3.1 (19.8-29.6)
b	4.5 ± 0.1 (4.3 – 4.8)	4.6 ± 0.6 (3.7-5.5)	6.4 ± 0.7 (5.6-7.7)	4.1 ± 0.4 (3.8-4.8)	4.9 ± 0.7 (3.9-6)	6 ± 0.4 (5.3-6.6)	4 ± 0.5 (3.3-5)	5.7 ± 0.4 (5.2-6.8)
c	25 ± 9 (17-41)	22 ± 3 (15-27)	20 ± 1 (18-23)	20 ± 2 (16-23)	21 ± 3 (17-25)	20 ± 2 (17-22)	17 ± 2 (14-22)	16.3 ± 1.9 (12.8-20.1)
c’	2.1 ± 0.4 (1.3-2.5)	2.1 ± 0.3 (1.7-2.6)	2.5 ± 0.2 (2.2-2.72)	2.3 ± 0.2 (1.9-2.5)	1.3 ± 0.2 (1-1.6)	2.5 ± 0.2 (2.1-2.7)	2.5 ± 0.5 (1.5-3)	2.4 ± 0.3 (1.8-2.7)

4.1.4 *Pratylenchus dunensis* de la Pena, Moens, van Aelst & Karssen, 2006

(Fig. 2)

Pratylenchus dunensis material in this study was sampled from the type host *Ammophila arenaria* and from the type locality Groote Keeten, the Netherlands (GPS coordinates: 52.865208, 4.703626). The 28S sequences from our population match (99.1-99.5% similarity) with previously deposited sequences of the species description (de la Peña *et al.*, 2006).

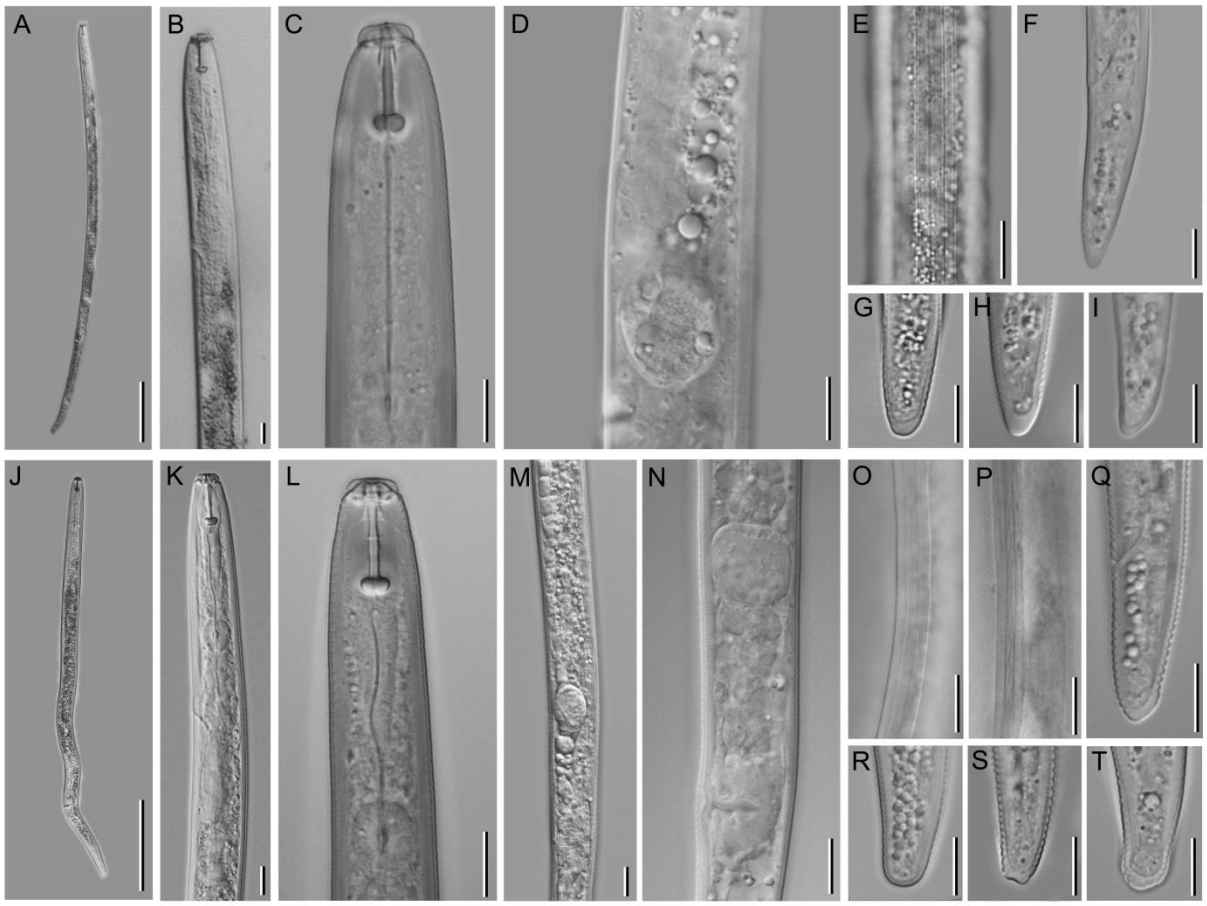

Figure 2. Photomicrographs of specimens of *Pratylenchus dunensis* (A-I) and *P. fallax* (J-T). A: Entire female body; B, C: Female anterior region; D: Vulval region; E: Lateral field; F-I: Tail region. J: Entire female body; K, L: Female anterior region; M, N: Vulval region; O, P: Lateral field; Q-T: Tail region. Scale bars A, J: 100 µm; B-I, K-T: 10 µm.

4.1.5 *Pratylenchus fallax* Seinhorst 1968

(Fig. 2, Table 2)

Pratylenchus fallax was originally described from an apple orchard near Doornenburg, the Netherlands (Seinhorst, 1968). Thanks to the WNE type material collection (Nematode Collection Europe, http://www.nce.nu/), we were able to determine the exact type locality, this orchard being situated near the Castle of Doornenburg (GPS coordinates: 51.894251, 6.000240). Within the orchard *P. fallax* was recovered from the roots of two apple trees. Our *P. fallax* population matched very closely in morphology (Fig. 2) and morphometrics (Table 2) with the population described by Seinhorst (1968). On the type locality, several other species of *Pratylenchus* were found: *P. crenatus* and *P. thornei* associated with grass and *P. pratensis* associated with *Prunus avium* (L.). Beside the type location, *P. fallax* was found associated with *Malus pumila*, *Prunus avium*, *Prunus domestica*, *Vitis vinifera*, *Pyrus* sp. and grasses in the Netherlands, representing in total 11 separate populations (Table 1). Based on the results of our study, we can conclude that the 28S and ITS sequences of *P. fallax* sequences that are currently available on GenBank have been misidentified: the D3 28S sequence of *P. fallax* (AF264181) obtained from *Convallaria majalis* L. and originated from France (Handoo *et al.*, 2001) does not match with our *P. fallax* topotype sequences. Instead this sequence is 99.5% similar to the *P. convallariae* population recovered in this study. Furthermore, five *P. fallax* ITS sequences (FJ712917- FJ712921) originating from Belgium do not match with our *P. fallax* topotype sequences but belongs to *P. lentis* (Troccoli *et al.*, 2008; Palomares-Rius *et al.*, 2010), *P. lentis* was recently considered a junior synonym of *P. pratensis* (Janssen *et al.*, 2017a).

4.1.6 *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941

(Fig. 1, Table 2)

Pratylenchus penetrans was originally described as *Tylenchus penetrans* by Cobb (1917), and later transferred to the genus *Pratylenchus* (Filipjev & Schuurmans Stekhoven, 1941). Since then *P. penetrans* has been recorded from over 350 host plants and appears to have a worldwide distribution in temperate regions (Castillo & Vovlas, 2007). In this study *P. penetrans* was recovered from *Malus pumila*, *Prunus avium*, *Prunus domestica*, *Pyrus* sp., *Zea mays*, *Vitis*

vinifera, Vigna unguiculata, Allium cepa, Solanum tuberosum, Physalis peruviana, Brassica oleraceae, Daucus carota and various grasses representing 20 separate populations of geographical widespread origin (Table 1). Morphometrics of the studied populations agree well with the original and other descriptions of this species (Castillo & Vovlas, 2007) (Table 2). One ITS sequence (FJ799117) has been uploaded as a *P. penetrans* sequence on GenBank but actually represents a *P. vulnus* sequence, suggesting that *P. vulnus* instead of *P. penetrans* (Chen *et al.*, 2009) is present on Strawberry in Taiwan.

4.1.7 *Pratylenchus pinguicaudatus* Corbett, 1969

Pratylenchus pinguicaudatus was descried from the Broadbalk trail field, Harpenden, Rothamsted, UK associated with wheat (Corbett, 1969). Subbotin *et al*. (2008) was the first who published 18S rRNA gene sequence of the topotype materials for *P. pinguicaudatus*. In this study we obtained the ITS, D2-D3 and *COI* gene sequences from this topotype material. The D2-D3 sequence did not match with those (AJ545014, KP289345-KP289347) of the root-lesion nematodes identified as *P. pinguicaudatus* from Tunisia and Morocco. Because these sequences clustered with those of *Pratylenchus* sp. 1, we consider these nematodes from North Africa as belonging to one or even two unidentified species.

4.1.8 *Pratylenchus* sp. 1

(Fig. 3)

In the summer of 2014 the type locality of *Pratylenchus lentis* was sampled in Villalba, Sicily, Italy (Troccoli *et al.*, 2008). This sampling revealed *P. lentis* to be a junior synonym of *P. pratensis* (Janssen *et al.*, 2017a). Besides *P. pratenis*, an unknown species of *Pratylenchus* was recovered from this location. Molecular species delimitation analyses indicate that this population represents a putative new species of the *Penetrans* group. *Pratylenchus* sp.1 is a bisexual species and morphologically similar to *P. penetrans,* sharing three lip annuli, rounded filled spermatheca, four lateral lines, a rounded tail tip and the same stylet morphology. Matrix

code for the tabular key proposed by Castillo & Vovlas (2007): A2, B2, C3, D2, E3, F4, G2, H1, I4, J1, K1.

4.1.9 *Pratylenchus* sp. 2

(Fig. 3)

Pratylenchus sp. 2 was collected from an unknown host in a trial field of IITA, Ibadan, Nigeria. Although only a limited number of specimens were recovered, molecular analysis clearly separate this species from any other molecularly characterized *Pratylenchus* (Fig. 5-7). Morphologically this species is similar with *Pratylenchus* sp. 3, *P. oleae*, *P. pinguicaudatus* and *P. elamini* in sharing three lip annuli, four lateral lines and a similar stylet morphology. Moreover, no males were found for *Pratylenchus* sp. 2 and the spermatheca was always empty despite egg development in the gonads, indicating an asexual mode of reproduction which is also shared with the three aforementioned species. Matrix code for the tabular key proposed by Castillo & Vovlas (2007): A2, B1, C2, D1, E2, F1, G2, H1, I3, J1, K1.

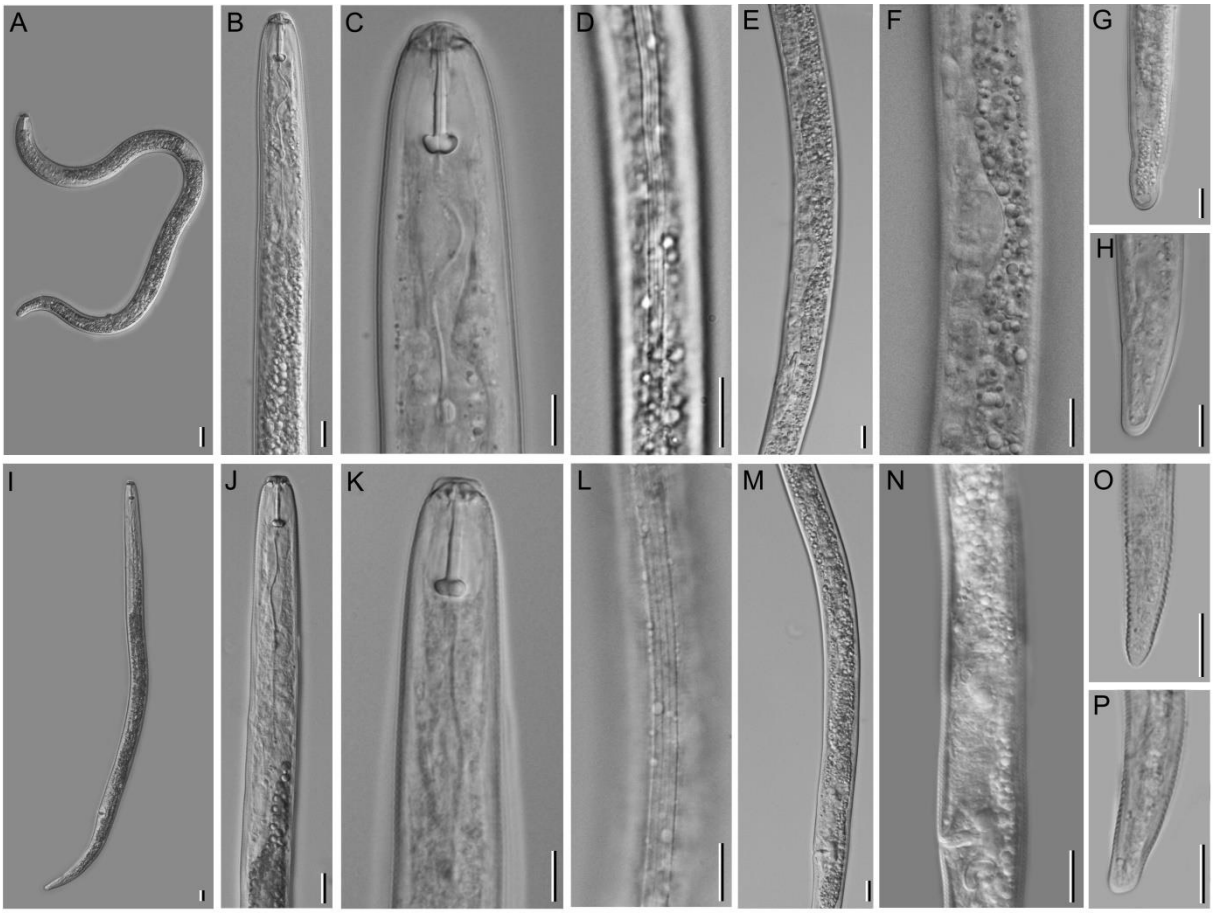

Figure 3. Photomicrographs of specimens of *Pratylenchus* sp. 1 parasitizing *Lens culinaris* in

Sicily (Italy (A-H) and *Pratylenchus* sp. 2 parasitizing an unknown host in Nigeria (I-P). A: Entire female body; B, C: Female anterior region; D: Lateral field; E, F: Vulval region; G, H: Tail region. I: Entire female body; J, K: Female anterior region; L: Lateral field; M, N: Vulval region; O, P: Tail region. Scale bars: 10 µm.

4.1.10 *Pratylenchus* sp. 3

(Fig. 4, Table 2)

Pratylenchus sp. 3 was recovered from Rwanda, Nyamata sector, Bugesera District, associated with *Zea mays*. Species delimitation analyses indicate this population to be a separate taxonomic entity. Morphologically this species is similar with *Pratylenchus* sp. 3, *P. oleae*, *P. pinguicaudatus* and *P. elamini*. With these species it shares the asexual mode of reproduction, empty reduced spermatheca, three lip annuli, and four lateral lines at times with oblique lines running in-between, and stylet morphology. Despite these similarities it differs by having a more variable and sometimes heavily crenate tail tip. Matrix code for the tabular key proposed by Castillo & Vovlas (2007): A2, B1, C2, D1, E2, F3(4), G3(2), H1, I1(2), J1, K2

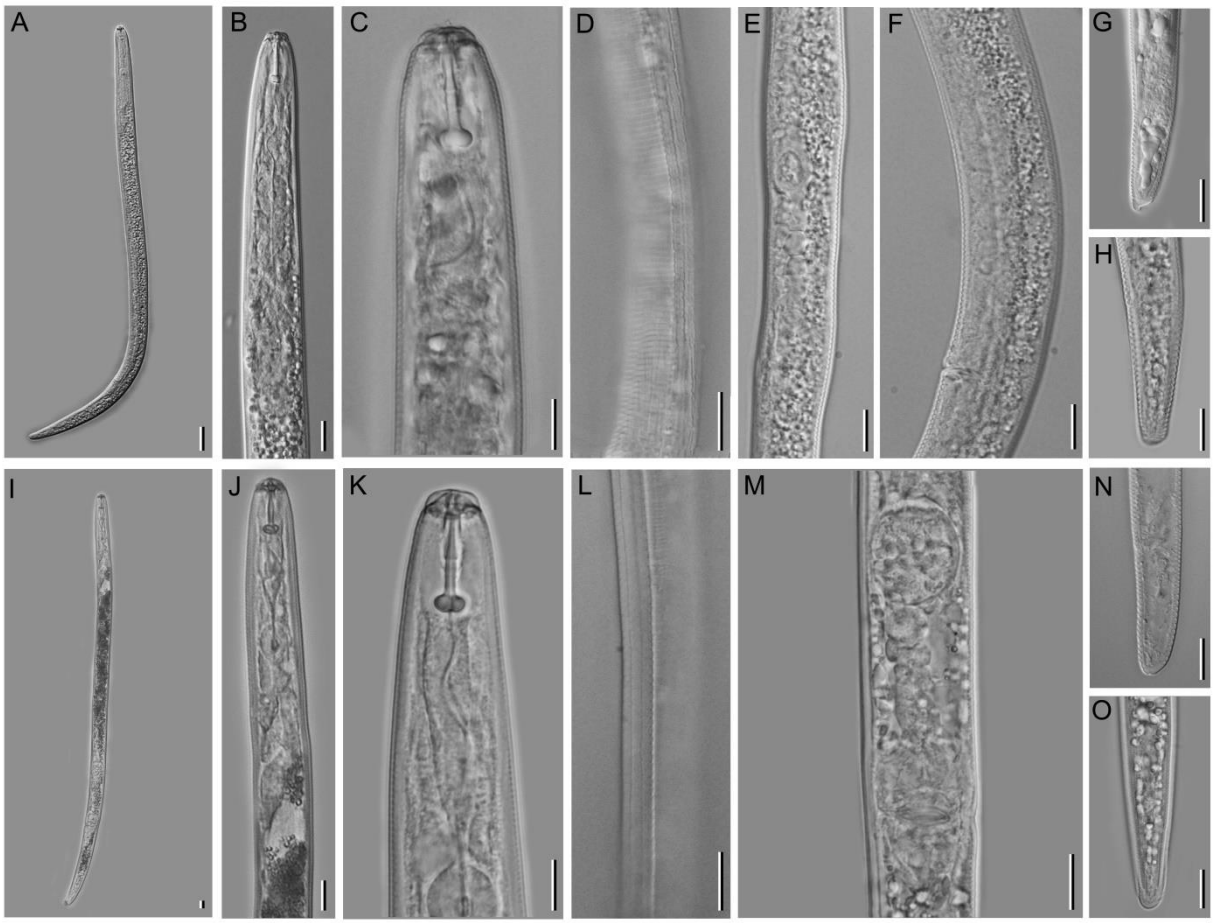

Figure 4. Photomicrographs of specimens of *Pratylenchus* sp. 3 parasitizing *Zea mays* in Rwanda (A-H) and *Pratylenchus* sp. 4 parasitizing *Phoenix dactylifera* in Tunisia (I-O). A: Entire female body; B, C: Female anterior region; D: Lateral field; E, F: Vulval region; G, H: Tail region. I: Entire female body; J, K: Female anterior region; L: Lateral field; M: Vulval region; N, O: Tail region. Scale bars: 10 µm.

4.1.11 *Pratylenchus* sp. 4

(Fig. 4)

Pratylenchus sp. 4 was found from *Phoenix dactylifera* in Tunisia. Morphologically this species is very similar with *Pratylenchus fallax* and *P. penetrans*, with which it shares 3 lip annuli, a filled rounded spermatheca, four lateral lines sometimes with additional oblique striation, stylet morphology and a sexual mode of reproduction. It is also similar in having a variable tail tip, which is in general rounded but can also be crenate (Fig. 4). As *Pratylenchus* sp. 4 had a widespread geographic occurrence in Tunisia on *Phoenix dactylifera* (unpublished data), it is highly likely that this species was previously identified as *P. penetrans* by Troccoli *et al*. (1992) from Algeria and by Edongali (1996) from Libya, especially given that its morphology and morphometrics are very similar to *P. penetrans* and our population from Tunisia. Matrix code for the tabular key proposed by Castillo & Vovlas (2007): A2, B1, C3, D2, E2, F4, G2, H1(2), I3, J1, K1.

4.1.12 Other unidentified species of *Pratylenchus* from the *Penetrans* group

Our sequence and phylogenetic analysis allowed to correct previous identification of two species of the group. The Iranian *Pratylenchus penetrans* D2-D3 of 28S rRNA gene sequence (JX261961) which was considered within the intraspecific range of *P. penetrans* by Majd Taheri *et al*. (2013) appeared in a fact to fall outside *P. penetrans* according to our data (Fig. 5). This species seems to be more closely related to *Pratylenchus* sp. 4 and *P. fallax* and is considered as an unidentified *Pratylenchus* in our tree. The D2-D3 sequences of Tunisian (AJ545014) and Moroccan samples (KP289344-KP289347) identified as *P. pinguicaudatus* by De Luca *et al*. (2004) and Mokrini *et al*. (2016), respectively, do not belong to this species, and, perhaps, belongs to *Pratylenchus* sp. 1 or an unidentified species. Additional morphological and molecular analyses must be carried out in order to diagnose these populations.

4.2 Phylogenetic relationships and evolution of parthenogenesis

In this study we analyzed the phylogenetic relationships within the *Penetrans* group using three gene fragments: the D2-D3 of 28S rRNA gene with the alignment of 163 *Pratylenchus* sequences, including 49 new ones (Fig. 5), the ITS rRNA gene with the alignment of 125 *Pratylenchus* sequences, including 37 new sequences (Fig. 6) and the *COI* gene with the alignment of 126 *Pratylenchus* sequences, including 91 new sequences (Fig. 7). The tree topologies obtained from these genes were generally congruent, except for the positions of several weakly supported clades. According to our analysis the *Penetrans* group contains following six valid species: *P. convallariae*, *P. dunensis*, *P. fallax*, *P. penetrans*, *P. oleae*, *P. pinguicaudatus* and several unidentified species. In all trees the *Penetrans* group was highly supported and according to the 28S rRNA gene, *P. brachyurus* forms the sister clade of this taxon. *Pratylenchus penetrans*, *P. fallax*, *P. convallariae*, *P. pinguicaudatus* and *Pratylenchus* sp. 4 form a monophyletic group in all trees and *Pratylenchus fallax* is the sister taxon of *Pratylenchus* sp. 4 or an unidentified *Pratylenchus* sp. from Iran. The phylogenetic relationships between *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 have not yet been resolved in our analysis, however, both species have been shown to be the earliest branching species within the *Penetrans* group. Interestingly, asexual reproducing species *P. pinguicaudatus*, *P. oleae*, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 do not form a monophyletic grouping in our phylogenetic analyses. As a consequence, parthenogenetic lineages appear to have evolved several times independently from sexually reproducing taxa, indicating independent origins of parthenogenesis through loss of meiosis.

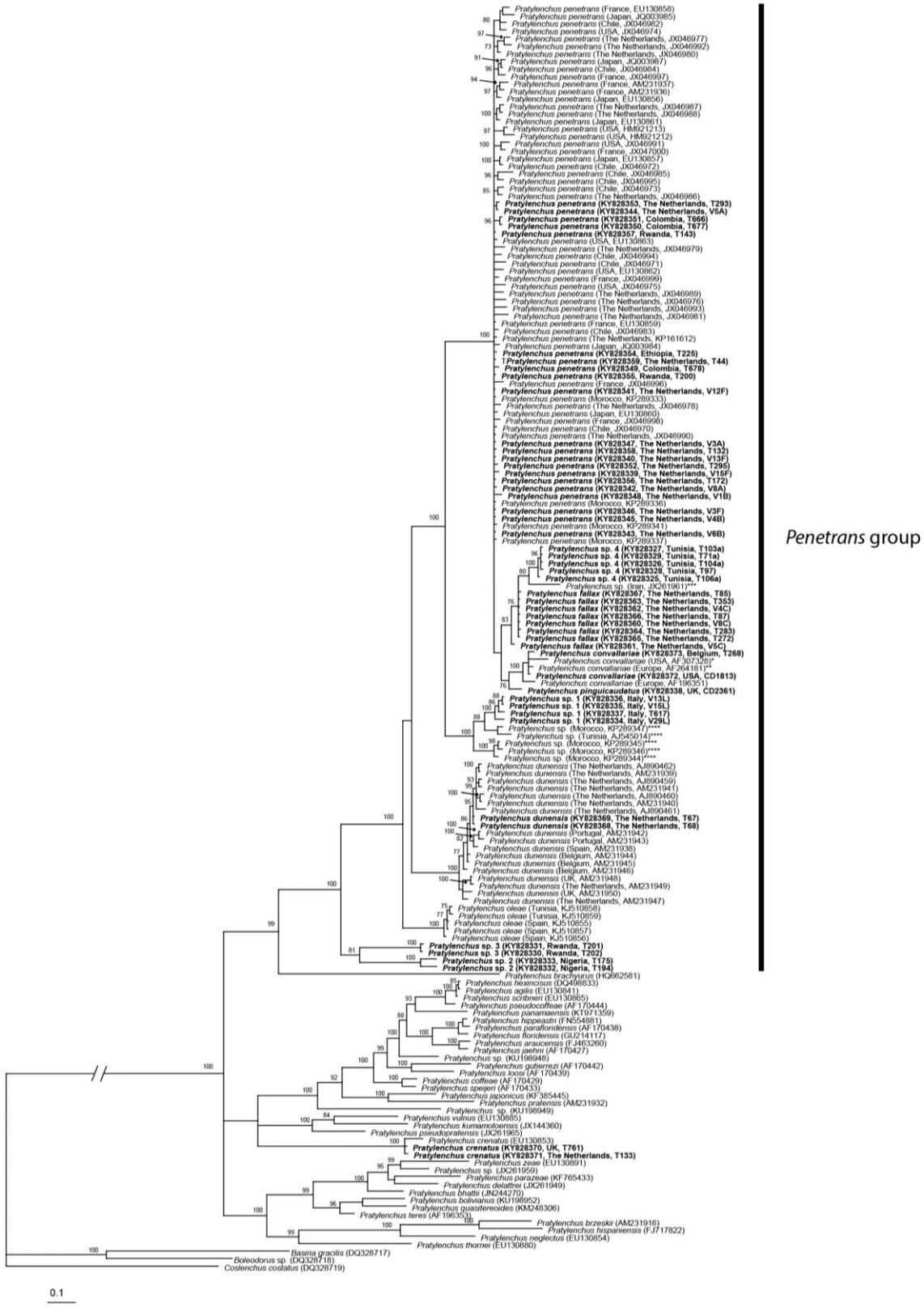

Figure 5. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the D2-D3 of the 28S rRNA gene sequences using the GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold. * - identified as *P. arlingtoni* by Handoo *et al.* (2001); ** - identified as *P. fallax et al.* (2001); *** - identified as *P. penetrans* by Majd Taheri *et al.* (2013); **** - identified as *P. pinguicaudatus* by De Luca *et al.* (2004) and Mokrini *et al.* (2016).

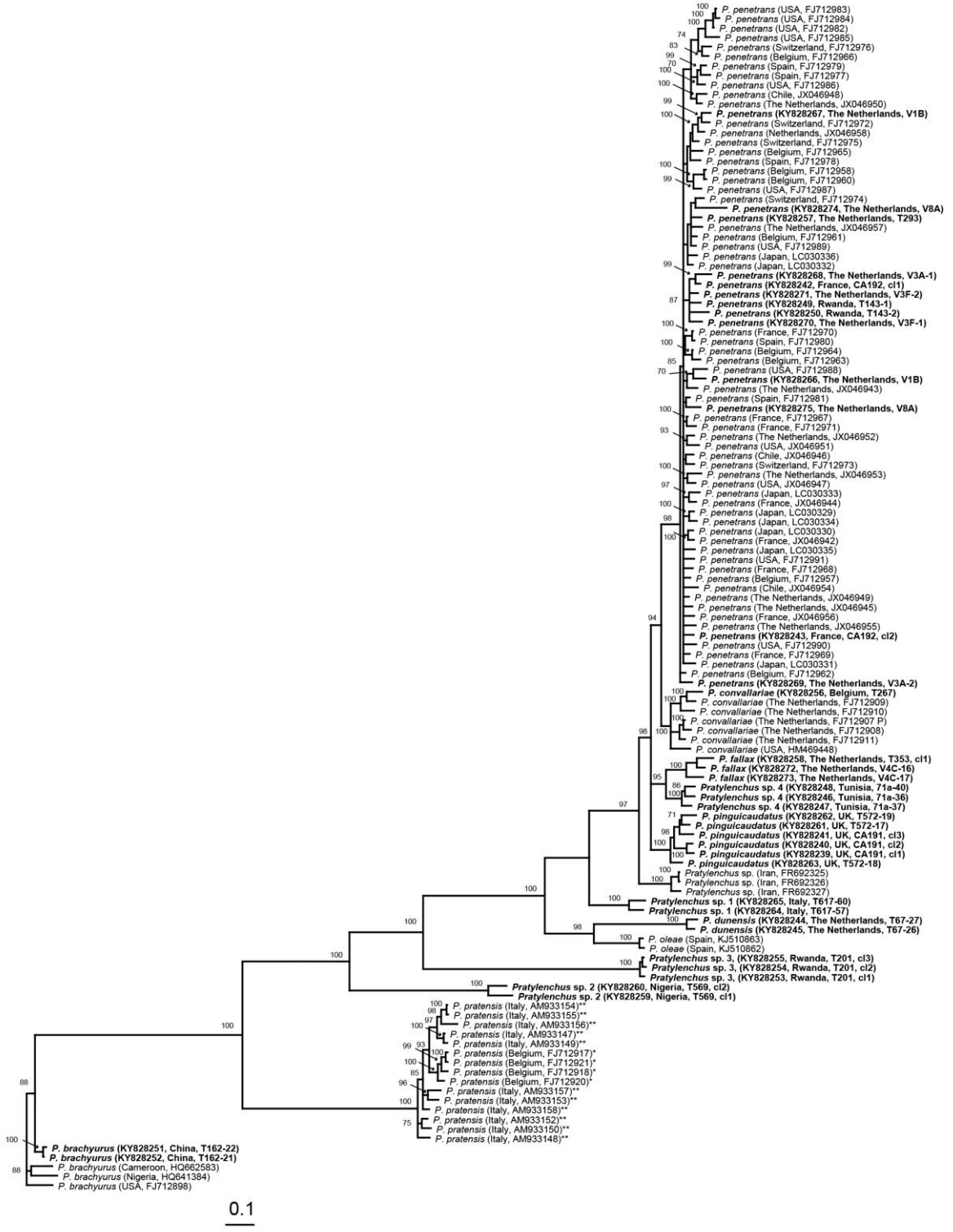

Figure 6. Phylogenetic relationships within *Penetrans* group of the genus *Pratylenchus* as inferred from Bayesian analysis of the ITS of RNA gene sequences using the GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are displayed in bold. * - identified as *P. fallax* in the GenBank.

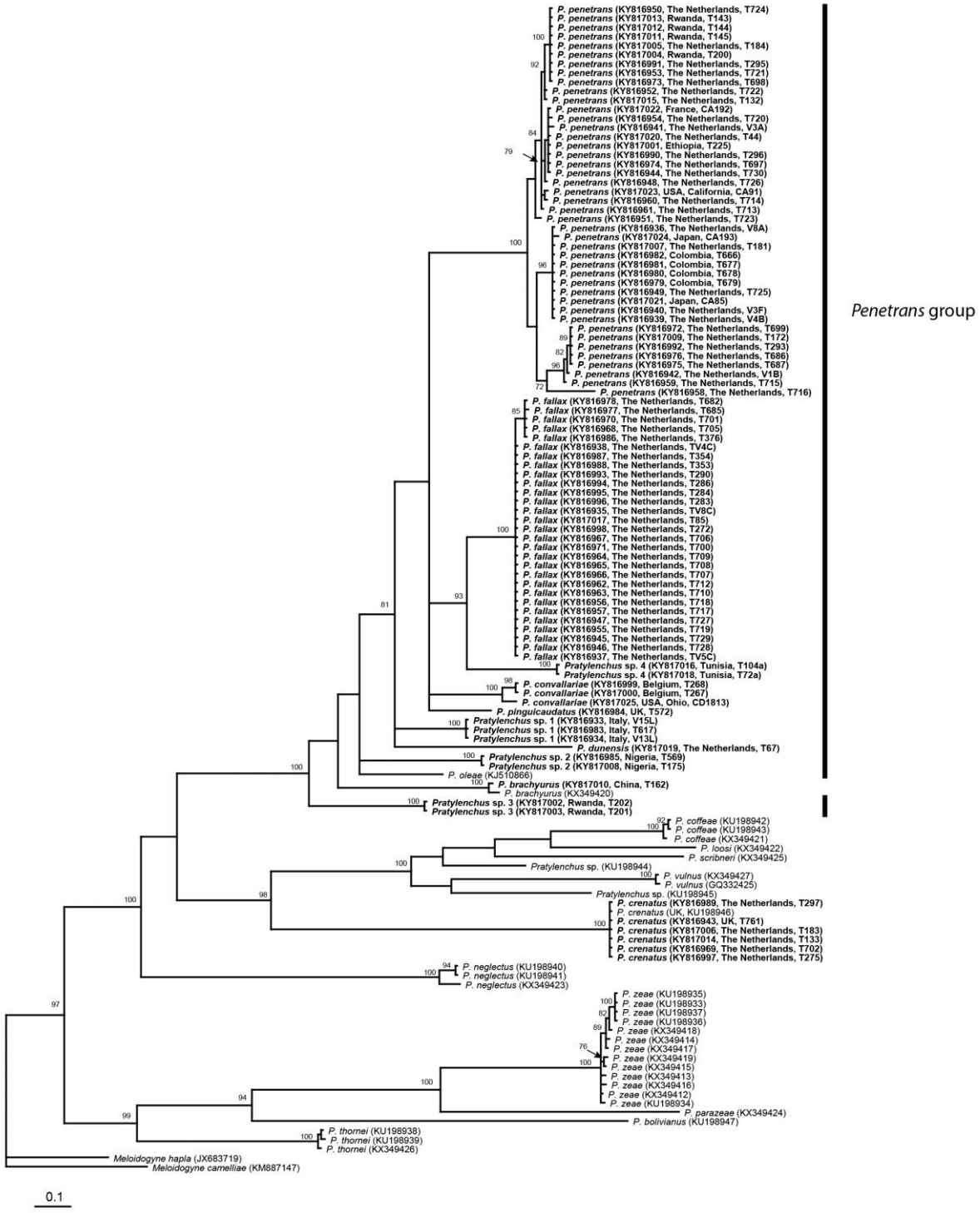

Figure 7. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the *COI* gene sequences using the GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are displayed in bold.

4.3 Molecular species delimitation

The *COI* alignment contained 95 sequences of the *Penetrans* group and was 405 base pairs long, revealing 30 different haplotypes. Molecular species delimitation using ABGD revealed 10 to 14 Operational Taxonomic Units (OTU's) (Table 4), differential operational taxonomic units were estimated within *P. penetrans* and *P. convallariae* according to different models and variable prior intraspecific divergence (Fig. 8, Table 4). The single threshold GMYC model predicted 11 operational taxonomic units, while the multiple threshold GMYC predicted two additional operational taxonomic units within *Pratylenchus penetrans*, yielding 13 operational taxonomic units (Fig. 8, Table 5). This variable number of estimated OTU's is not surprising given that molecular species delimitation analyses are known to generate a variety of different species hypotheses (Prevot *et al.*, 2013; Kekkonen & Hebert, 2014). In this study we opted to follow the most stringent hypothesis, where only the OTU's that are supported by all ABGD and GYMC species delineation methods were retained. The minimal number of OTU's as estimated by the ABGD analysis yielding 10 OTU's assuming a prior intraspecific divergence of 0.035938. Even following the most conservative estimate, *P. penetrans, P. fallax, P. convallariae, P. pinguicaudatus, P. oleae, P. dunensis*, and four new species are all delimitated as separate taxonomic entities, confirming signatures of independent evolution as predicted by population genetic theory (Fujisawa & Barraclough, 2013). Consequently, our species delimitation analyses confirmed the species hypotheses made by previous taxonomic studies (Cobb, 1917; Seinhorst, 1959; Seinhorst, 1968; Corbett, 1969; de la Peña *et al.*, 2006; Palomares-Rius *et al.*, 2014). Importantly, all these OTU's are confirmed as being monophyletic groupings in the *COI*, ITS and 28S phylogenies (Fig. 5-7, Table 5). Additionally, all OTU's represented by more than a single *COI* sequence had a significant Rodrigo's P(RD) value, again suggesting that the observed degree of distinctiveness of these OTU's is not the product of random coalescent processes (Table 5) (Rodrigo *et al.*, 2008). Also for the 28S rDNA region, all Rodrigo's P(RD) values were significant except those for *P. fallax* and *Pratylenchus* sp. 2 (Table 5). Furthermore, the Rosenberg's P(AB) probabilities were significant, rejecting the null hypothesis of random coalescence (Rosenberg, 2007), except for the *COI P. convallariae* and *Pratylenchus* sp.3 OTU's and the 28S rDNA *Pratylenchus* sp. 4 and *P. fallax* OTU's. All estimated *COI* OTU's are also associated with differential amino acid sequences. Amino acid variation between OTU's ranged from a minimum of 6 different amino acids (5%

difference) between *Pratylenchus* sp. 4 and *P. fallax* to a maximum of 27 amino acid differences (19.7% difference) between *Pratylenchus* sp. 3 and *P. dunensis* (Supplementary Table 1).

Interestingly, the amount of intraspecific variability was found to be highly variable, for example, *P. fallax* populations form the Netherlands showed only low intraspecific variability (Table 5), while *P. penetrans* from the Netherlands were found to contain very diverse haplotypes (Fig. 9). For *P. penetrans* the *COI* haplotypes were separated into four distinct groups (A, B, C, D in Fig. 9), representing up to 8.5% nucleotide divergence. These haplotype groups were even recognized as different OTU's in some of the GMYC and ABGD species delimitation analyses (Fig. 8), providing some evidence for different divergence events within *P. penetrans*. However, these different haplotype groups appear to occur sympatrically in several locations, in Stamproy (the Netherlands) Pe4, Pe9, Pe15 and Pe16 were found in the same locality, and also in Arkel, Zoetermeer, Schimmert, Wemmeldinge, representatives from two different haplotype groups were found in a single population (Table 1). Interestingly, the variation in *P. penetrans* nucleotide haplotypes represents virtually no amino acid sequence variability (only Pe16, Pe3 and haplotype group C had a single amino acid replacement (Supplementary Table 1), indicating that nucleotide variation is mainly situated in the third codon position (codon 1 and codon 2 were 99.1% and 100% identical while codon3 was only 91.4% identical) and that the haplotype groups A, B and C belong to respectively the same species. Our analyses indicate *COI* to be a good barcode gene as all OTU's do not only form monophyletic groupings in the *COI* phylogeny (Fig. 7), but are also associated with high P(ID) values (Table 5). Remarkably, some *P. penetrans* haplotypes were found to occupy a wide geographical distribution; the Pe7 haplotype is widely distributed in the Netherlands but also occurs in Ethiopia and Rwanda, and the Pe11 haplotype occurs in the Netherlands, Japan and Colombia (Fig. 9).

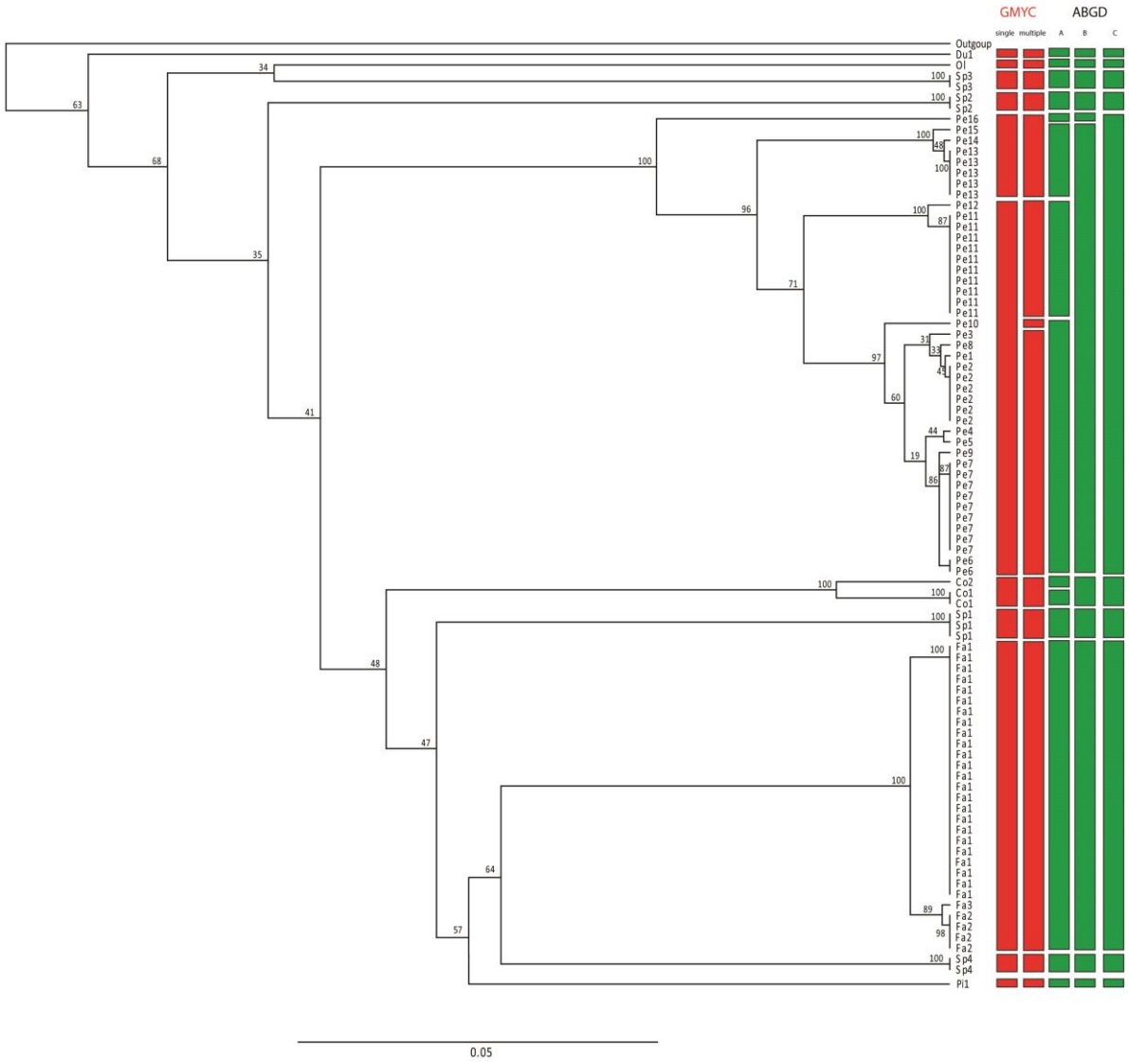

Figure 8. Molecular species delimitation analysis. Automated Barcode Gap Discovery (ABGD) and Generalized Mixed Yule Coalescent model (GMYC) are visualised on an ultrametric Bayesian tree of the *COI* gene. Different species are visualised in different colours. The right panel visualises the distribution of pairwise distances, showing a clear barcode gap between intraspecific and interspecific distances.

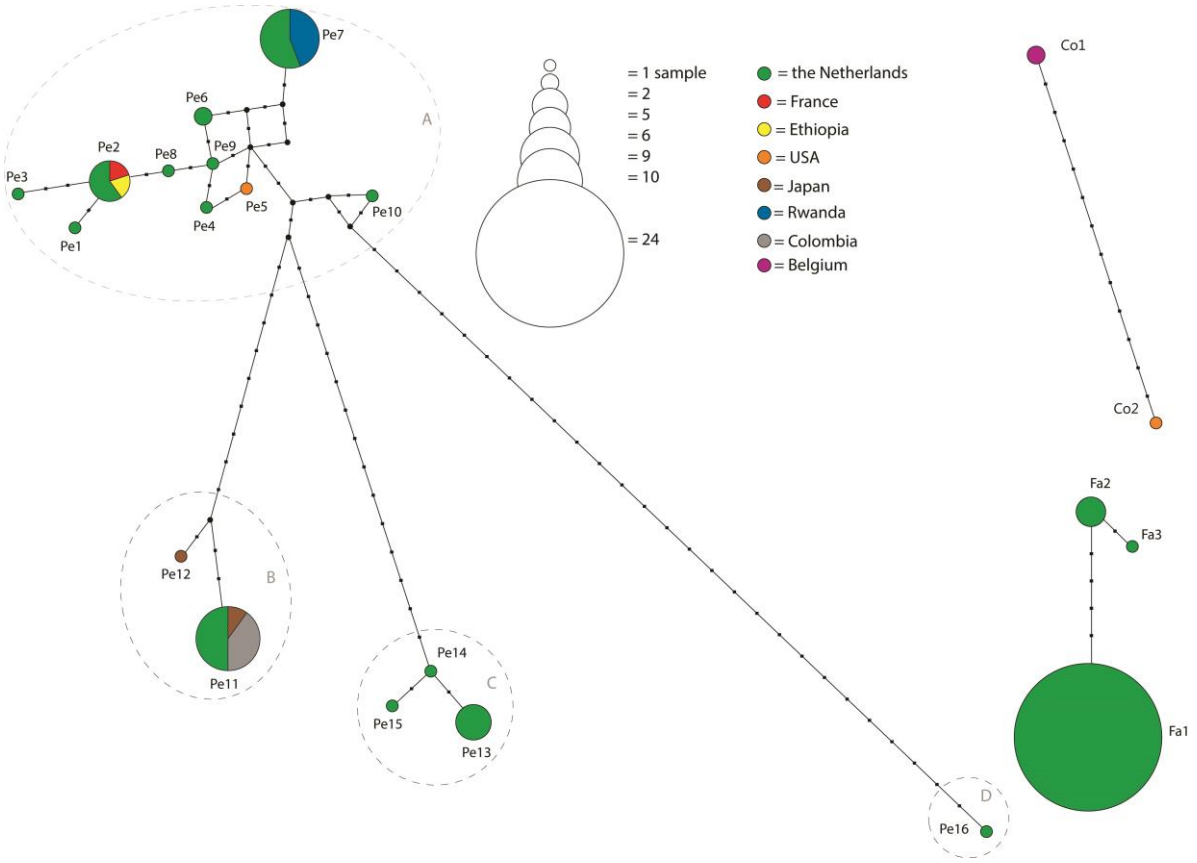

Figure 9. *COI* haplotype network of *Pratylenchus penetrans* (left), *Pratylenchus convallariae* (upper right) and *Pratylenchus fallax* (lower right). The haplotype network shows the relationships between different haplotypes, circle size is equivalent to the number of studied specimen and branch length is equivalent to the number of mutations (shown as black squares). Different geographic origins are displayed by different colors, median vectors are shown as black circles. Within *Pratylenchus penetrans*, different haplotype groups are highlighted by a dashed circle.

Table 3. Summary of ABGD species delimitation analysis results, according to different models and differential prior intraspecific divergence (P).

			Prior intraspecific divergence (P)							
model	X	Partition	0.001	0.00167	0.00279	0.00464	0.00774	0.01292	0.02154	0.03594
JC69	1.5	Initial	14	14	14	14	14	10	10	10
	1.5	recursive	14	14	14	14	14	11	11	10
K80	1.5	Initial	14	14	14	14	14	10	10	10
	1.5	recursive	14	14	14	14	14	11	11	10

Table 4. Summary of GMYC species delimitation analysis results according to single and multiple threshold models.

GMYC	Clusters	Entities	Likelihood null model	Likelihood GMYC model	Likelihood ratio	Likelihood ratio test	Treshold
Single	4	11	107.7124	114.6923	13.95977	0.0009304112***	-0.034947
Multiple	5	13	107.7124	114.9672	14.50958	0.000706782***	-0.034947
							-0.012446

Table 5. Results of GENEIOUS species delimitation plugin (Masters *et al.*, 2011). Posterior probability of each clade, intra specific variability showing the genetic variability among members of a putative species, inter specific variability showing the variability between a putative species and its closest relative. Ratio between intraspecific and interspecific variability provides a measure of genetic differentiation between the focal species and it's nearest neighboring species. PID strict refers to the mean probability with the 95% confidence interval of correctly identifying an unknown specimen of the focal species using placement on a tree with the criterion that it must fall within, but not sister to, the species clade. Rosenbergs P(AB) is the probability of reciprocal monophyly under a random coalescent model (Rosenberg, 2007). Rodrigo's P (RD) is the value estimating the probability that a clade has the observed degree of distinctiveness due to random coalescent processes (Rodrigo *et al.*, 2008).

COI								
OTU	Closest species	Clade support	Intra dist	Inter dist	Intra/inter	PID strict (95% confidence interval)	Rodrigo's P(RD)	Rosenberg s P(AB)
P. penetrans	Co	100	0.167	1.407	0.12	0.95 (0.90,1.0)	<0.05	2.4E-7
P. fallax	Sp4	100	0.030	0.905	0.03	0.98 (0.93,1.0)	<0.05	1.4E-4
P. convallariae	Pe	100	0.107	1.407	0.08	0.74 (0.57, 0.92)	<0.05	0.17
P. oleae	Sp1	-	0	1.301	-	-	-	-
P. dunensis	Co	-	0	1.614	-	-	-	-
P. pinguicaudatus	Co	-	0	0.172	-	-	-	-
P. sp. 1	Fa	100	0.014	1.253	0.01	0.78 (0.61,0.96)	<0.05	2.9E-7
P. sp. 2	Ol	100	0.013	1.438	0.01	0.59(0.44,0.74)	<0.05	6.4E-6
P. sp. 3	Ol	100	0.015	1.362	0.01	0.59(0.44,0.73)	<0.05	0.33
P. sp. 4	Fa	100	0.015	0.905	0.02	0.58(043,073)	<0.05	1.4E-4

28S rRNA								
OTU	Closest species	Clade support	Intra dist	Inter dist	Intra/inter	PID strict (95% confidence interval)	Rodrigo's P(RD)	Rosenberg s P(AB)
P. penetrans	Fa	98	0.246	0.622	0.40	0.87 (0.82,0.93)	<0.05	1.3E-9
P. fallax	Sp4	/	0.052	0.367	0.14	0.88 (0.78,0.99)	-	-
P. convallariae	Fa	100	0.197	0.621	0.32	0.72 (0.59,0.85)	<0.05	1.3E-9
P. oleae	Du	100	0.069	1.700	0.04	0.91 (0.78,1.0)	<0.05	2.6E-10
P. dunensis	Ol	100	0.249	1.700	0.15	0.94 (0.89,0.99)	<0.05	3.4E-24
P. sp. 1	Fa	100	0.188	1.434	0.13	0.78 (0.64,0.92)	<0.05	6.6E-9
P. sp. 2	Sp3	100	0.418	2.101	0.20	0.49 (0.34,0.64)	0.43	2.1E-6
P. sp. 3	Sp2	100	0.051	2.101	0.02	0.58 (0.43,0.73)	<0.05	2.2E-6
P. sp. 4	Fa	100	0.072	0.367	0.20	0.80 (0.68,0.93)	<0.05	0.07

4.4 Morphometrics and Morphology

A morphometric comparison of TAF fixed specimens of pure populations of *P. penetrans* (3), *P. fallax* (3), *P. convallariae* and *Pratylenchus* sp. 3 revealed both largely overlapping ranges between species and high intraspecific variability of morphometric characters (Table 2). The PCA analysis (Fig. 10) of pure populations *P. penetrans* (4), *P. fallax* (4), *P. convallariae*, *Pratylenchus* sp. 3 and *Pratylenchus* sp. 4 confirmed that morphometrics fail to separate different species within the *Pratylenchus penetrans* species complex. The CDA plot did not show a clear pattern related to species and all analyzed species are not significantly different

(Wilks' λ, P > 0.01). Based on the pooled within canonical structure, the morphometric characters that cause the relatively largest separation between the species, although insufficient, are b' V (Can1: -0.33) and the position of the excretory pore (Can1: 0.25). Also the cluster analysis failed to group specimen of different species in a monophyletic cluster, again indicating that intraspecific morphometric variability is larger than interspecific morphometric variability (results not shown).

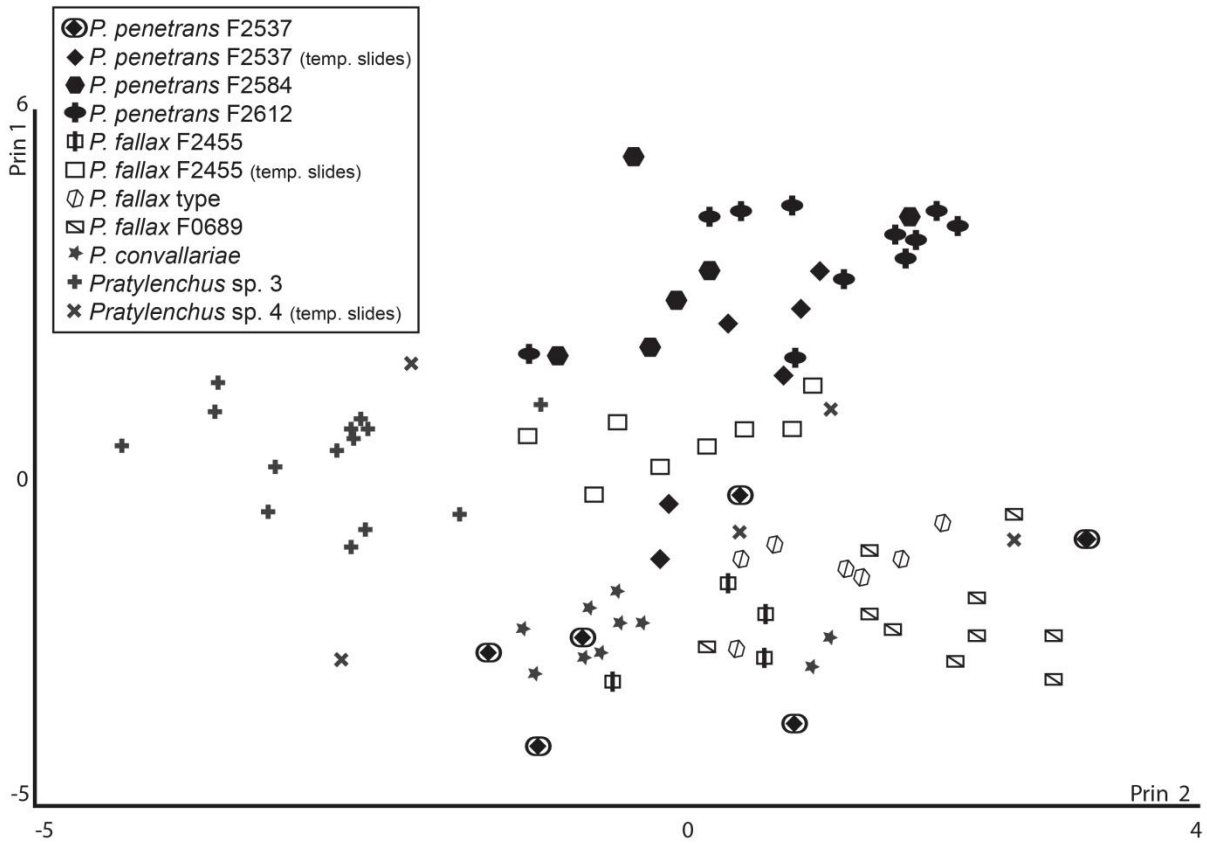

Figure 10. PCA plot of *Pratylenchus penetrans* complex species based on the female morphometric values, including L, V, a, b, c, c', stylet length, body width, post-uterine sac length, tail length, and position of metacorpus, cardia, pharyngeal lobe, excretion pore, vulva in respect to anterior end.

5 Discussion

Despite numerous claims in the past (Tarte & Mai, 1976; Handoo *et al.*, 2001; Holterman *et al.*, 2009) and a recent proposal by Helder in Jones *et al*. (2013) to synonymize *Pratylenchus fallax* and *P. convallariae* with *P. penetrans*, our study has clearly established these species as separate taxonomic entities with clear genetic boundaries according to ABGD and GMYC species delimitation. These different species were confirmed as being the result of speciation events rather than being the product of random coalescent processes through the use of Rosenberg's and Rodrigo's coefficients (Rosenberg, 2007; Rodrigo *et al.*, 2008) and phylogenetic analyses of three different genes. The hypothesis that these OTU's are separate logical entities holds at least true for *P. penetrans* and *P. fallax* which are reproducibly isolated (Perry *et al.*, 1980). Moreover, Holterman *et al*. (2009) and Helder in Jones et al. (2013) concluded *P. penetrans, P. fallax* and *P. convallariae* to be conspecific based on a limited sequencing of 18S rDNA, a marker which is known to greatly underestimate biodiversity (Tang *et al.*, 2012).

Besides *P. penetrans, P. fallax* and *P. convallariae* our species delimitation methods also indicate *P. pinguicaudatus, P. dunesis, P. oleae* and four new species to be separate taxonomic entities. As *P. arlingtoni* has been shown to be a *species inquirenda*, this brings the total of valid species within the *Penetrans* group to 10, while the taxonomic status of some unidentified species from Iran, Morocco and Tunisia (Fig. 5) remain to be investigated. *P. brachyurus* was identified as the early-branching sister taxon of the *Penetrans* group, confirming the phylogenetic position found by Palomares-Rius *et al*. (2014). However, this phylogenetic position needs to be confirmed using multi-locus sequencing data in order to acquire satisfactory phylogenetic support values.

Alongside *P. brachyurus, P. oleae*, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 were recovered as early-branching asexual lineages of the *Penetrans* group (clade IV). Surprisingly, these parthenogenetic lineages together with *P. pinguicaudatus* do not form a monophyletic clade. Indicating that these parthenogenetic lineages evolved several times independently from sexually reproducing taxa. Mitotic parthenogenetic *Pratylenchus* lineages were previously shown to be associated with higher chromosome numbers in comparison to sexually reproducing species (Roman & Triantaphyllou, 1969): i.e. *P. scribneri* (2n=25-26), *P. zeae*

(2n=21-26), *P. neglectus* (2n=20) and *P. brachyurus* (2n=30-32) as opposed to the sexually reproducing *P. penetrans* (n=5), *P. vulnus* (n=6) and *P. coffeae* (n=7). As the parthenogenetic *P. brachyurus* (2n=30-32) is positioned as the early-branching sister taxon of the *Penetrans* group (Palomares-Rius *et al.*, 2014), it is likely that *Pratylenchus* sp. 2, *Pratylenchus* sp. 3, *P. oleae* and *P. pinguicaudatus* are also associated with higher chromosome numbers given their asexual mode of reproduction. This indicates that a parthenogenetic reproduction mode might be triggered by a change in ploidy level through a genome duplication or hybridization and a consequently loss of meiosis as was previously suggested to be the case for root-knot nematodes (Lunt *et al.*, 2014; Janssen *et al.*, 2016; Janssen *et al.*, 2017c), and is also the case in many different metazoan taxa (Comai, 2005). In this study we tried to confirm this by karyotyping these Pratylenchus species, however, staining with propionic orcein and fluorescent dyes consistently failed. As a consequence this hypothesis remains to be confirmed, probably genome sequencing in combination with gene copy number analysis could provide a solution as already shown for root-knot nematodes (Lunt *et al.*, 2014). Interestingly, *Pratylenchus bolivianus* was recently shown to have both amphimictic and parthenogenetic populations (Troccoli *et al.*, 2016), also there the biological reason for change in reproductive strategy remains to be investigated.

Our phylogenetic analyses indicate that the *Penetrans* group might originate from Africa. The two earliest branching species of the *Penetrans* group, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 are recovered from Africa, and the other early-branching asexual species *P. oleae* was found in North Africa and at the Mediterranean Sea. Also, *P. brachyurus*, early-branching sister taxon of the *Penetrans* group, albeit with poor branch support, is widely distributed in Africa (Castillo & Vovlas, 2007; Palomares-Rius *et al.*, 2014). In this study *P. fallax* was found to be widely distributed in The Netherlands, parasitizing grasses, *Malus pumila*, *Prunus domestica*, *Prunus avium*, *Pyrus* sp. and *Vitis vinifera*, which confirms the previously suggested distribution pattern (Seinhorst, 1968; Seinhorst, 1977). However, based on morphological identifications, *P. fallax* has been reported to be widely distributed in Europe, and present in Russia, India, Japan and the USA (Castillo & Vovlas, 2007), associated with a variety of crops. Ideally these morphological identifications should be confirmed using molecular data. While intraspecific variability was low for *P. fallax* within the Netherlands, Dutch *P. penetrans* populations showed high intraspecific variability (Fig. 9). Interestingly, this intraspecific variability was mainly

confined to the third codon position of the *COI* gene. As a consequence this nucleotide variation was not associated with variations in amino acid sequences, indicating that this variability is biologically insignificant and confirming that different *P. penetrans* haplotypes are most likely conspecific. Recently, a similar variability pattern was discovered in *Longidorus orientalis*, displaying 15.5% intraspecific *COI* variability, which translated in only 1% intraspecific amino acid variation (Subbotin *et al.*, 2015).

Despite the large intraspecific variability recovered in *P. penetrans*, identical *P. penetrans* haplotypes were found to be geographically widespread. This wide distribution of haplotypes indicates that *P. penetrans* populations are not reproductively isolated, indicating that *P. penetrans* could have been spread anthropogenically through agricultural development and crop exchange. Anthropogenic distribution was previously suggested to explain the distribution patterns of other plant-parasitic nematodes, namely in root-knot nematodes (Castagnone-Sereno *et al.*, 2013; Janssen *et al.*, 2016) and cyst nematodes (Plantard *et al.*, 2008; Boucher *et al.*, 2013).

It has been demonstrated that morphometric characteristics are not useful for species diagnosis because of the large intraspecific morphological variability. In contrast to morphometrics, morphology can be used in some cases to identify species within the *Penetrans* group. However, morphological diagnostic characteristics are scarce and phenotypic plasticity is rampant as previously shown by Tarte and Mai (1976). For example, tail morphology is useful in some cases but cannot be used to differentiate all species of the *Penetrans* group, *Pratylenchus penetrans* can be differentiated from *P. fallax* based on a larger hyaline tail tip region, which is proportionally less crenate in a given population (Seinhorst, 1968). However, based on tail morphology *P. penetrans* is hard to differentiate from *Pratylenchus* sp. 1 and *Pratylenchus* sp. 4, while *P. fallax* is difficult to differentiate from *P. convallariae* (Castillo & Vovlas, 2007). The proportional spacing of lines in the lateral field was proposed as a way to differentiate *P. dunensis* from *P. penetrans* by de la Pena *et al*. (2006) and this is confirmed in our study. Nevertheless, other morphological characters associated with the lateral field are highly variable. Phenotypic plasticity within a single species includes areolation, striations and oblique lines within the lateral field and a large number of specimens must be examined in order to accurately diagnose the species of the *Pratylenchus penetrans* species complex. Especially

difficult to distinguish are the asexually reproducing species, *Pratylenchus* sp. 2, *Pratylenchus* sp. 3, *Pratylenchus oleae* and *Pratylenchus pinguicaudatus*, which share most of their morphological characteristics and are almost indistinguishable using morphology. Although *Pratylenchus* sp. 3 appears to have a more variable tail tip, also *Pratylenchus* sp. 2 and *Pratylenchus oleae* have a variable crenated tail tip (Palomares-Rius *et al.*, 2014), while *P. pinguicaudatus* was described as having a rounded tail tip (Corbett, 1969).

A further complication to morphological identification is the presence of sympatric species. For example, in this study, one sample form St. Oedenrode (the Netherlands) contained a mix of *P. penetrans* and *P. fallax*. Moreover, recently it has been noted that sexual and asexual populations of *Pratylenchus bolivianus* can be associated with differential morphotypes (Troccoli *et al.*, 2016). These findings need to be accounted for as reproduction modes are highly variable within the *Penetrans* group, and diagnostic characters related to the reproductive system may vary depending on the reproductive state of the population. As demonstrated by the numerous morphologically misidentified species on GenBank, morphological identification of species within the *Penetrans* group can only be reliably done by specialists with years of experience and only then in combination with high quality reference material. Furthermore, traditional morphological identification should be supplemented with molecular analysis in order to robustly diagnose populations.

Augmenting the complexity of the species diagnosis problem is the presence of several morphospecies that are morphologically similar to species in the *Pratylenchus penetrans* complex but which remain uncharacterized by molecular data. For example, *P. pseudofallax* and *P. subpenetrans* were differentiated form *P. fallax* and *P. penetrans* by morphometric characteristics (body length, length of the post uterine sac, stylet length, position of the vulva), the presence of males, the shape of the spermatheca and the shape of tail terminus (Taylor & Jenkins, 1957; Café-Filho & Huang, 1989). However, in reality differentiating *P. pseudofallax* and *P. subpenetrans* is difficult for the following main reasons: i) morphometric and morphological differentiating characters fall within the range of species within the *P. penetrans* complex as displayed by the ranges of morphometric variables in our study; ii) the presence of males and shape of the spermatheca might dependent on the reproductive phase of the population (Troccoli *et al.*, 2016); iii) the tail shape can be highly variable within species of the

P. penetrans complex; and iv) the reported hosts of *P. pseudofallax* (*Malus silvestris*) and *P. subpenetrans* (C*hrysanthemum* and ginseng) are also shown to be good hosts for *P. penetrans* (Castillo & Vovlas, 2007). Consequently, confirming the taxonomic status of *P. pseudofallax* and *P. subpenetrans* will require sequencing of topotype material as collecting topotype material has often proved to be the only way to confidently connect DNA sequences to formerly described morphospecies (Inserra *et al.*, 2007; De Luca *et al.*, 2010; Troccoli *et al.*, 2016).

DNA barcoding has been proposed as a successful diagnostic strategy in many organisms (Hebert *et al.*, 2003). DNA barcoding is especially useful in taxa that lack diagnostic morphological features (Hebert *et al.*, 2003). In this regard, DNA barcoding was explored for many nematode taxa including marine nematodes (Derycke *et al.*, 2010b), root-knot nematodes (Janssen *et al.*, 2016), Aphelenchoididae (Sánchez-Monge *et al.*, 2017). In the current study, the *COI* gene of the mitochondrial genome was explored as a barcode marker for *Pratylenchus*. Our results indicate *COI* to be a reliable barcode marker as indicated by strict P(ID) values, indicating that the mean probability of making a correct identification of an unknown specimen based on its placement in a tree is high when using *COI* as a barcode marker (Masters *et al.*, 2011). Although DNA barcoding was shown to be a reliable diagnostic strategy it is highly important to safeguard the link between DNA barcodes, morphology and sequences from topotype locations, as exemplified by the high number of misidentified species present on GenBank.

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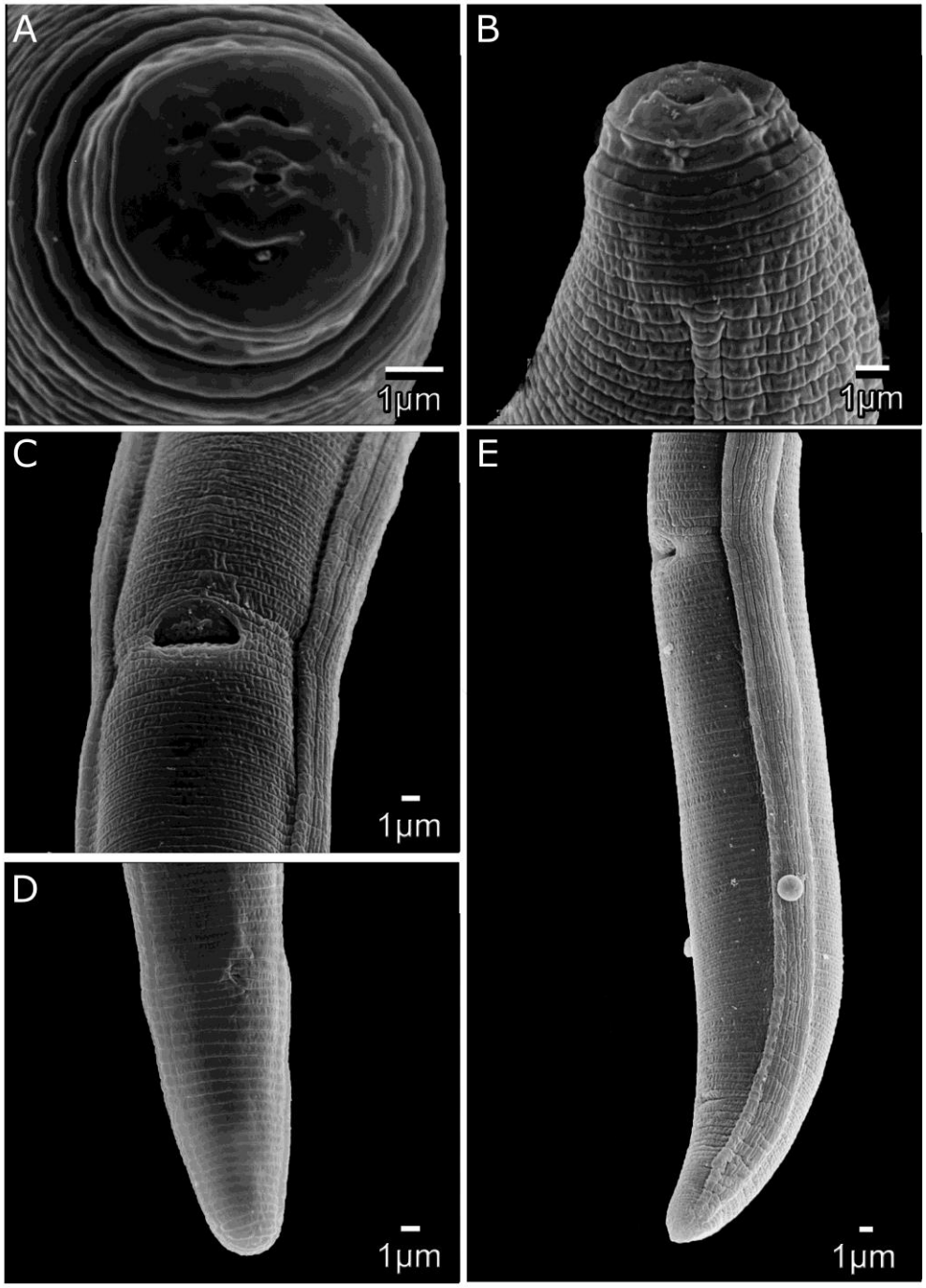
7 Supplementary info Chapter 4



Supplementary figure 1. Scanning Electron Microscopy of *Pratylenchus crenatus*, population was collected from Baale-Nassau, the Netherlands (F0683-2). A: lip region enface view; B: anterior region lateral view; C: vulval region ventral view; D: tail region ventral view; E: posterior region lateral view.

Supplementary table 1. COI fragment amino acid substitutions between different haplotypes of the *Penetrans* group.

	Sp3	Du1	Sp2	Ol	Pe12	Pe15	Pe14	Pe13	Pe16	Pe3	Pe11	Pe10	Pe6	Pe7	Pe4	Pe5	Pe9	Pe8	Pe1	Pe2	Sp4	Fa3	Fa2	Fa1	Sp1	Pi	Co2	Co1
Sp3		27	20	19	22	25	25	25	23	25	24	24	24	24	24	24	24	24	24	24	21	22	22	22	20	23	25	23
Du1	27		19	20	16	20	20	20	19	20	19	19	19	19	19	19	19	19	19	19	20	20	20	20	15	19	19	19
Sp2	20	19		13	17	21	21	21	20	21	20	20	20	20	20	20	20	20	20	20	15	15	15	15	12	16	17	15
Ol	19	20	13		15	20	20	20	19	20	19	19	19	19	19	19	19	19	19	19	13	13	13	13	10	14	14	13
Pe12	22	16	17	15		1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	12	9	9	9	9	7	7	8
Pe15	25	20	21	20	1		0	0	2	2	1	1	1	1	1	1	1	1	1	1	15	12	12	12	12	10	12	12
Pe14	25	20	21	20	1	0		0	2	2	1	1	1	1	1	1	1	1	1	1	15	12	12	12	12	10	12	12
Pe13	25	20	21	20	1	0	0		2	2	1	1	1	1	1	1	1	1	1	1	15	12	12	12	12	10	12	12
Pe16	23	19	20	19	1	2	2	2		2	1	1	1	1	1	1	1	1	1	1	14	11	11	11	11	9	11	11
Pe3	25	20	21	20	1	2	2	2	2		1	1	1	1	1	1	1	1	1	1	15	12	12	12	12	10	12	12
Pe11	24	19	20	19	0	1	1	1	1	1		0	0	0	0	0	0	0	0	0	14	11	11	11	11	9	11	11
Pe10	24	19	20	19	0	1	1	1	1	1	0		0	0	0	0	0	0	0	0	14	11	11	11	11	9	11	11
Pe6	24	19	20	19	0	1	1	1	1	1	0	0		0	0	0	0	0	0	0	14	11	11	11	11	9	11	11
Pe7	24	19	20	19	0	1	1	1	1	1	0	0	0		0	0	0	0	0	0	14	11	11	11	11	9	11	11
Pe4	24	19	20	19	0	1	1	1	1	1	0	0	0	0		0	0	0	0	0	14	11	11	11	11	9	11	11
Pe5	24	19	20	19	0	1	1	1	1	1	0	0	0	0	0		0	0	0	0	14	11	11	11	11	9	10	11
Pe9	24	19	20	19	0	1	1	1	1	1	0	0	0	0	0	0		0	0	0	14	11	11	11	11	9	11	11
Pe8	24	19	20	19	0	1	1	1	1	1	0	0	0	0	0	0	0		0	0	14	11	11	11	11	9	11	11
Pe1	24	19	20	19	0	1	1	1	1	1	0	0	0	0	0	0	0	0		0	14	11	11	11	11	9	11	11
Pe2	24	19	20	19	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0		14	11	11	11	11	9	11	11
Sp4	21	20	15	13	12	15	15	15	14	15	14	14	14	14	14	14	14	14	14	14		6	6	6	8	8	14	11
Fa3	22	20	15	13	9	12	12	12	11	12	11	11	11	11	11	11	11	11	11	11	6		0	0	8	7	12	9
Fa2	22	20	15	13	9	12	12	12	11	12	11	11	11	11	11	11	11	11	11	11	6	0		0	8	7	12	9
Fa1	22	20	15	13	9	12	12	12	11	12	11	11	11	11	11	11	11	11	11	11	6	0	0		8	7	12	9
Sp1	20	15	12	10	9	12	12	12	11	12	11	11	11	11	11	11	11	11	11	11	8	8	8	8		7	9	6
Pi	23	19	16	14	7	10	10	10	9	10	9	9	9	9	9	9	9	9	9	9	8	7	7	7	7		7	6
Co2	25	19	17	14	7	12	12	12	11	12	11	11	11	11	11	10	11	11	11	11	14	12	12	12	9	7		3
Co1	23	19	15	13	8	12	12	12	11	12	11	11	11	11	11	11	11	11	11	11	11	9	9	9	6	6	3	

Chapter 5

The pitfalls of molecular species identification: a case study within the genus *Pratylenchus* (Nematoda: Pratylenchidae)

Modified from Janssen T., Karssen G., Couvreur M., Waeyenberge L., Bert W. 2017. The pitfalls of molecular species identification: a case study within the genus *Pratylenchus* (Nematoda: Pratylenchidae). Accepted with minor revisions in Nematology.

1 Abstract

Comprehensive morphological and molecular analyses revealed that published ITS sequences of the economically important plant-parasitic nematode *Pratylenchus goodeyi* are actually sequences from distantly free-living bacterivorous 'cephalobs'. We demonstrated that this incorrect labeling resulted in a cascade of erroneous interpretations, as shown by the reports of "*Pratylenchus goodeyi"* on banana in China and on cotton in India. This clearly illustrates the risk of mislabeled sequences on public databases. Other mislabeled *Pratylenchus* cases are discussed to illustrate that this is not an isolated case. Hereby, *P. lentis* is considered a junior synonym of *P. pratensis* while *P. flakkensis* was for the first time linked to DNA sequences using topotype material. As taxonomic expertise is decreasing and sequence-based identification is growing rapidly the highlighted problem may even increase in the future. A strong link between morphology and DNA sequences will be of crucial importance in order to prevent sequence-based misidentifications.

2 Introduction

Molecular taxonomy and DNA barcoding provide a powerful tool for the identification of organisms and for studying biodiversity (Hebert *et al.*, 2003; Savolainen *et al.*, 2005; Kress & Erickson, 2008). Molecular identification is especially important for organisms for which morphological diagnostic characteristics are scarce. Due to the decreasing price and increased availability of sequence instruments, the amount of sequence data available on public DNA databases has exponentially grown over the last 10 years (Muir *et al.*, 2016). However, a substantial part of this sequence data appears to be incorrect, with faults ranging from sequence errors over mis-assemblies to mislabeled, unlabeled and misidentified sequences. In microbiology where morphological information is almost absent and identification is often purely based on 16s rDNA sequences the problem is well known (Vilgalys, 2003; Nilsson *et al.*, 2006; Lal & Lal, 2011). Nilsson *et al*. (2006) estimate that up to 20% of fungal sequences are actually misidentified. For eukaryotes, erroneous linking of sequence and organism was thought to be relatively uncommon due to the availability of morphological characters for their identification (Nilsson *et al.*, 2006). However, this problem appears not to be restricted to microbiology, as demonstrated here for Nematoda, using three case studies within the genus *Pratylenchus* (Pratylenchidae). Many species of *Pratylenchus* are very difficult to identify

morphologically, due to the lack of diagnostic characters, morphological interspecific plasticity and incomplete taxonomic descriptions (Castillo & Vovlas, 2007; Subbotin *et al.*, 2008; Troccoli *et al.*, 2016). As a result, DNA-based identification strategies are becoming more important (Waeyenberge *et al.*, 2000; Al-Banna *et al.*, 2004; Powers, 2004; Waeyenberge *et al.*, 2009; Mokrini *et al.*, 2013).

2.1 Case study 1: *Pratylenchus goodeyi* Sher and Allen 1953

Pratylenchus goodeyi is considered a major pest of banana and plantain (Castillo & Vovlas, 2007). It was originally described by Sher and Allen (1953) at Kew gardens in London from the roots of banana trees. Since then it has been reported from many banana producing regions; especially from Africa and southern European countries: the Canary Islands (Spain), Crete (Greece) and Madeira (Portugal) (Castillo & Vovlas, 2007). In Africa the parasite is often associated with higher altitudes and cooler temperatures (Price & Bridge, 1995). Surprisingly it has not been recorded in North or South America. *P. goodeyi* is differentiated from other root-lesion nematodes by the presence of 4 lip annuli, a large oblong spermatheca and a smooth dorsally sinuated tail tip (Sher & Allen, 1953; Loof, 1991).

In 2007, *P. goodeyi* was labeled as a cryptic species complex using sequence-based taxonomy (Waeyenberge, 2007) and in this study, five ITS sequences originating from Tenerife (Canary Islands, Spain) were made publicly available (FJ712922- FJ712926). In 2011, another *P. goodeyi* population (FR692324) was reported from the Canary islands by De Luca *et al.* (2011), *P. goodeyi* was also reported by Gokte-Narkhedkar *et al.* (2013) from cotton in India at three different locations and six *P. goodeyi* sequences were deposited on Genbank (KF275665, KF700243, KF840454, KF840455, KF840456, KF856291). In 2015, *P. goodeyi* was reported for the first time on banana in China (Zhang *et al.*, 2015). Both Waeyenberge (2007) and De Luca *et al.* (2011) reported a large discrepancy between *P. goodeyi* sequences from different geographical locations. This could indicate either the presence of a species complex as suggested by Waeyenberge (2007) or alternatively it could point to incorrectly labeled sequences as suggested by De Luca *et al.* (2011).

2.2 Case study 2: *Pratylenchus pratensis* (de Man, 1880) Filipjev, 1936

Pratylenchus pratensis was originally described as *Tylenchus pratensis* by de Man (1880). Later, the species was transferred to the genus *Anguillulina* (Goffart, 1929) and subsequently appointed as the type species of the newly erected genus *Pratylenchus* by Filipjev (1936). Later, *P. helophilus* Seinhorst, 1959 and *P. irregularis* Loof, 1960 were synonymized with *P. pratensis* (Loof, 1974; Castillo & Vovlas, 2007). *P. pratensis* has been recorded in Africa (Algeria, Libya, South Africa), Asia (India, Pakistan, China, Azerbaijan, Uzbekistan) and North America (Canada, USA, Mexico) but it was mainly reported from Europe being present in the Belgium, Bulgaria, Finland, Germany, Italy, Moldavia, Netherlands, Poland, Russia, Slovakia, Slovenia and Spain on a wide variety of crops (Castillo & Vovlas, 2007). Despite the numerous morphological reports of *P. pratensis*, there is relatively little sequence information available for this species. Nevertheless, seven 28S rDNA sequences were generated from five coastal areas in Belgium, the United Kingdom, Spain and Portugal by de la Peña *et al.* (2007). In addition five beta-1,4-endoglucanase gene sequences, five 18S rDNA sequences and two RNA polymerase II gene sequences were generated (Rybarczyk-Mydlowska *et al.*, 2012; Rybarczyk-Mydlowska *et al.*, 2014). However, morphological vouchers of these populations were not provided. In 2008, *P. lentis* Troccoli, De Luca, Handoo & Di Vito, 2008, morphologically very similar to *P. pratensis*, was described parasitizing roots of lentil (*Lens culinaris* Medik.) in Sicily (Italy) (Troccoli *et al.*, 2008) and this description was associated with twelve ITS sequences. Later, *P. lentis* appeared to be embedded within a *P. fallax* clade (Palomares-Rius *et al.*, 2010). However, this relationship has recently been shown to be the result of a *P. fallax* misidentification (Janssen *et al.*, 2017b).

2.3 Case study 3: *Pratylenchus flakkensis* Seinhorst, 1968

Pratylenchus flakkensis was originally described by Seinhorst (1968) from Middelharnis (the Netherlands) from a heavy loam soil under grass. Seinhorst (1968) also reported it from Ouddorp (Netherlands) and from Beckenham (United Kingdom). Later, *P. flakkensis* was reported by Ryss from Estonia and Russia (Ryss, 1986; Ryss, 1988; Ryss, 1992). *P. flakkensis* appears to be geographically widespread as it was also recorded from Pakistan, Peru, South Africa and Guadeloupe (Castillo & Vovlas, 2007). Despite the numerous morphological records of this species, no molecular data are currently available for this species.

In order to provide an unequivocal link of DNA sequences with the above mentioned species of *Pratylenchus* and to clarify their taxonomic status, the goals of these case studies are to (i) characterize these species using a combination of morphological characteristics and molecular sequences from geographically different locations; (ii) determine their phylogenetic position within the genus; and (iii) enable reliable molecular diagnostics.

3 Materials and methods

3.1 Collection of nematode populations

Nematodes were extracted from soil and root material using a modified Baermann funnel (Hooper, 1986) or a mistifier (van Bezooijen, 2006). An overview of the collected populations can be found in Table 1.

3.2 Morphological characterization

Individual specimens were studied in temporary preparations sealed with nail-polish using an Olympus BX51 DIC microscope (Olympus Optical) and morphological vouchers were made using photomicrographs using a Nikon DS-Fi1. Measurements of morphometric characters were directly made using NIS-Elements D measuring software or on digital specimen vouchers using ImageJ (Schneider *et al.*, 2012). Vouchered nematodes were subsequently picked from these temporary mounts and processed for DNA extraction. The resulting digital specimen vouchers are available online at http://nematodes.myspecies.info/taxonomy/term/10645/specimens. Remaining unvouchered nematodes were fixed in TAF (2% triethanolamine, 8% formalin in distilled water) at 70 °C and processed to anhydrous glycerin, following the method of Seinhorst (1962) modified by Sohlenius and Sandor (1987) and measured as described above with Nikon measuring software. For Scanning Electron Microscopy (SEM) nematodes were fixed in 600 µl fresh 4% paraformaldehyde (PFA) fixative buffered with Phosphate Buffer Saline (PBS) and 1% glycerol and heated for 3 seconds in a 750W microwave. Subsequently, specimen where dehydrated in a seven-step graded series of ethanol solutions and critical-point dried using liquid CO_2, mounted on stubs with carbon discs, coated with gold (25 nm). Specimen were studied and photographed with a JSM-840 EM (JEOL) electron microscope at 12 kV.

3.3 DNA extraction, PCR amplification and sequencing

Genomic DNA of individual nematodes was extracted using the quick alkaline lysis protocol described by Janssen *et al.* (2016). Briefly, individual nematodes were transferred to a mixture of 10 µl 0.05N NaOH and 1 µl of 4.5% Tween 20. The mixture was heated to 95°C for 15 min, and after cooling to room temperature 40 µl of double-distilled water was added. PCR amplification was performed using Toptaq DNA polymerase (QIAGEN, Germany), in a volume of 25 µl using a Bio-Rad T100^{TM} thermocycler. PCR mixture was prepared according to the manufacturer's protocol with 0.4 µM of each primer and 2 µl of single nematode DNA extraction. The 28S rDNA fragment D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') primers were used according to the protocol of De Ley *et al*. (1999). The internal transcribed rDNA spacer (ITS) was amplified using VRAIN2F (5'-CTT TGT ACA CAC CGC CCG TCG CT-3') and VRAIN2R (5'-TTT CAC TCG CCG TTA CTA AGG GAA TC-3'), subsequently cloned using pGEM®-T Easy Vector systems (Promega) and sequenced using universal M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG A-3') primers. The Cytochrome c oxidase subunit 1 (COI) gene fragment was amplified using JB3 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and JB4.5 (5'-TAA AGA AAG AAC ATA ATG AAA ATG-3') according to the described protocol (Bowles *et al.*, 1992; Derycke *et al.*, 2010b). Sanger sequencing of purified PCR fragments was carried out in forward and reverse direction by Macrogen (Europe). Contigs were assembled using GENEIOUS R6.1.8 (Biomatters; http://www.Geneious.com). All contigs were subjected to BLAST searches to check for possible contaminations on http://www.ncbi.nlm.nih.gov.

3.4 Phylogenetic analysis

Ribosomal gene sequences were aligned with ClustalX (Larkin *et al.*, 2007) using default parameters, *COI* sequences were translated using the TranslatorX webserver (http://translatorx.co.uk/) (Abascal *et al.*, 2010), using the invertebrate genetic code, and the nucleotides aligned according to an amino acid alignment constructed using ClustalW. The best fit models of DNA evolution were obtained for each dataset using the program jModeltest 2 (Darriba *et al.*, 2012) under the Akaike information criterion. Bayesian phylogenetic analysis (BI) was carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis for each gene was initiated with a random starting tree and was run with four chains for 50 million

generations. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples (25%), a 50% majority rule consensus tree was generated. Posterior probabilities (PP) are given on appropriate clades.

Table 1. Species and populations of Pratylenchidae used in the study. Morphological vouchers are available on. http://nematodes.myspecies.info/taxonomy/term/10645/specimens. [1]No morphological voucher available

Species	Code	Country	Location	GPS coordinates	Host	28S rDNA	18S rDNA	Cox1	ITS
Pratylenchus goodeyi	T106,T107, T108, T165, T166, T167, T168, T169, T200[1]	LaPalma, Spain	San Juan	28.783772, -17.764273	Banana	KY828285-KY828286	KY828278	-	KY828309-KY828310
P. goodeyi	T206, T207, T208, T209	Belgium	Botanical garden, University Ghent	51.034947, 3.723358	Plantain	KY828287-KY828288	-	-	-
P. goodeyi	T222	Rwanda	Nyirangarama	-1.662589, 29.887836	Bean	KY828289	-	-	-
P. goodeyi	T226[1]	Ethiopia	East Shewa	7.288950, 38.701615	Maize	-	-	-	-
Pratylenchus flakkensis	T241, T242, T243	The Netherlands	Middelharnis	51.770211, 4.182428	Grass	KY828284	KY828277	KY828319	
P. flakkensis	T93, T96, T103, T104, T105	The Netherlands	Slootdorp	52.823096, 4.982603	Grass	KY828282	-	KY828313	-
P. flakkensis	T213, T214, T215, T216	Russia	Golitsino	55.616512, 36.995185	*Urtica dioica*	KY828283	-	-	-
Pratylenchus pratensis	T130, T131, T148, T149, T150, T155	The Netherlands	Noordwijkerhout	F0710	Grassland	KY828296-KY828297	KY828279	KY828314	-
P. pratensis	T683, T684	The Netherlands	Doornenburg	51.894631, 6.000663	*Pyrus* sp.	KY828299	-	-	-
P. pratensis	T704	The Netherlands	Winssen	-	*Prunus domestica*	-	-	-	-
P. pratensis	T616	Italy, Sicily	Villalba	-	*Lens culinaris*	KY828298	KY828280	-	KY828311
Pratylenchus vulnus	T73, T74	Tunisia	-	-	*Phoenix dactylifera*	KY828305-KY828306	-	KY828312	-
P. vulnus	T151	Wageningen	culture	-	*Rosa sp.*			KY828317	
Pratylenchus neglectus	T89	The Netherlands	Middelharnis	51.770211, 4.182428	Grass	KY828295	-	-	-

Species	Code	Country	Location	GPS coordinates	Host	28S rDNA	18S rDNA	Cox1	ITS
P. neglectus	T142	The Netherlands	Dronten	-	*Malus sp.*	-	-	KY828315	-
Pratylenchus hexincisus	T244, T245	Nigeria	Benue, Otukpo	8.13327, 7.19212	*Dioscorea alata*	KY828290	-	KY828320	-
P. hexincisus	T669, T671	Italy	Solanto	38.074542, 13.531327	*Lactuca sativa*	KY828293-KY828294	-	KY828322	-
P. hexincisus	T658, T659	China	Baishui, Shaanxi, China	-	*Malus sp.*	KY828291-KY828292	-	KY828321	-
Pratylenchus brzeskii	T174	Belgium	Het Zwin	51.366792, 3.357284	*Ammophila arenaria*	-	-	KY828318	-
Pratylenchus thornei	T33, T34, T37	The Netherlands	Slootdorp	52.859260, 4.977920	-	KY828300-KY828302	-	-	-
P. thornei	T146	The Netherlands	Phililipine	-	Grasses	-	-	KY828316	-
P. thornei	T219	Turkey	Mount Ararat		Grasses	KY828303	-	-	-
P. thornei	T763, T764	England	Rothemstadt, Broadbalk	/	Grasses	KY828304	-	KY828323-KY828324	-
Radopholus similis	T210	England	Londen, Kew Gardens	/	*Musa sp*.	KY828307	-	-	-
Acrobeloides cf. *nanus*	T254, T305, T306, T307, T308	Kenya	Kwale county	-4.186578, 39.380026	*Oryza sativa, Supa cultivar*	KY828281	KY828276	-	KY828308

4 Results

4.1 Case study 1: *Pratylenchus goodeyi*

To further assess *P. goodeyi* as a potential cryptic species complex (Waeyenberge, 2007) newly collected material of *P. goodeyi* from La Palma (Canary Islands, Spain) on banana, from plantain form the Ghent University botanical garden, from Rwanda on beans and from Ethiopia on maize (Table 1) was analyzed. Surprisingly, 28S rDNA sequences from these geographically distant and biologically divergent populations were 99.8-100% similar, showing only a single heterozygous position. Morphologically, our populations displayed the typical diagnostic characters of *P. goodeyi*: 4 lip annuli, large oblong spermatheca and the tail tapering to a narrow almost pointed smooth terminus (Fig. 1). Also morphometric characters matched well with the previously reported populations of *P. goodeyi* (Table 2) (Castillo & Vovlas, 2007). Furthermore, the enface lip pattern visualized by SEM was identical to the originally described lip pattern (Sher & Allen, 1953) (Fig. 1). Intriguingly, a remarkably different ITS sequence (only 54% similar) was observed between our populations and the populations described by Waeyenberge (2007) and Zhang *et al.* (2015). A detailed comparison of the previously generated "*P. goodeyi*" sequences revealed a close affinity with several Cephalobidae sequences (Fig. 2). After sequencing the ITS region for several Cephalobidae species, we found that *P. goodeyi* sequences from Waeyenberge (2007) and Zhang *et al.* (2015) are 98.6% similar to *Acrobeloides* cf. *nanus* (Fig. 2). The ITS sequence of this *Acrobeloides* cf. *nanus* is sister to the "*P. goodeyi*" sequences and they are together within a monophyletic Cephalobidae clade (Fig. 2). Additionally, six "*P. goodeyi*" sequences originating from three different localities in India were also found to group in this monophyletic Cephalobidae clade (Gokte-Narkhedkar *et al.*, 2013). Morphologically, the *Acrobeloides* cf. *nanus* population shows the same variation in lip region and tail morphology as previously reported for *Acrobeloides nanus* (Fig. 3) (Anderson, 1968; Boström & Gydemo, 1983). Phylogenetic analysis of 18S rDNA and 28S rDNA sequences confirm the phylogenetic position of *Acrobeloides* cf. *nanus* within the Cephalobidae and clearly visualize the genetic distance with our genuine *P. goodeyi* populations (supplementary figure A). According to 28S rDNA, *Acrobeloides* cf. *nanus* forms (as in Smythe and Nadler (2006)), a monophyletic clade with other *Acrobeloides* and *Cephalobus* sequences (data not shown). According to 28S rDNA and 18S rDNA our genuine *P. goodeyi* populations are positioned in clade VI, forming a monophyletic group together with

P. *zeae, P. parazeae, P. bhattii, P. delattrei* and *P. bolivianus* (Fig. 4 and 5). All data together confirm that the previously deposited sequences are cephalobid sequences that have been misidentified as *P. goodeyi*.

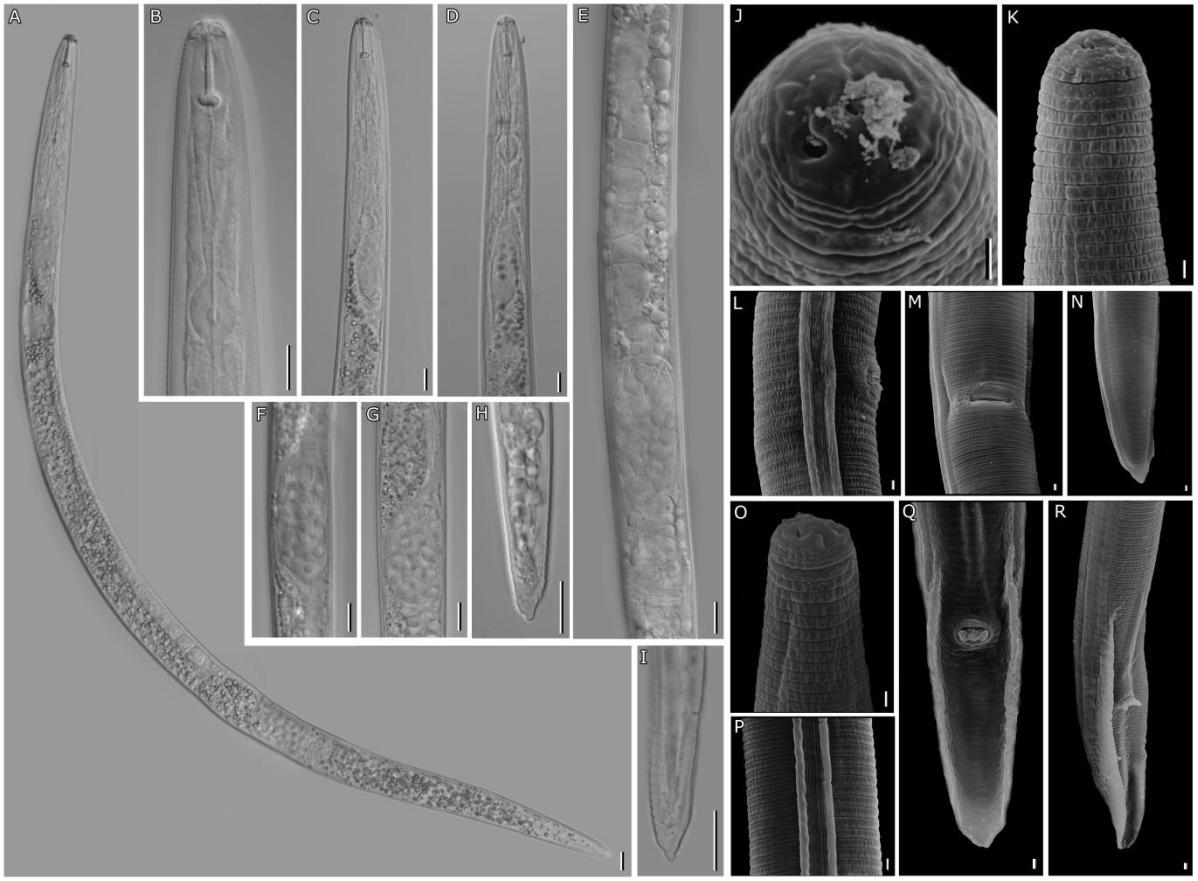


Figure 1. Light microscopy photomicrographs and SEM pictures of specimens of *Pratylenchus goodeyi*. A: entire female body; B-D: female anterior region; E: female gonoduct; F, G: spermatheca; H-I: tail region; J: female cephalic region, en face view; K: female cephalic region, lateral view; L: female lateral field at vulval region; M: female vulval region; N: female tail; O: male cephalic region, lateral view; P: male lateral field at mid-body; Q, R: male tail region. Light microscopy scale bars: 10 µm, SEM scale bars: 1 µm.

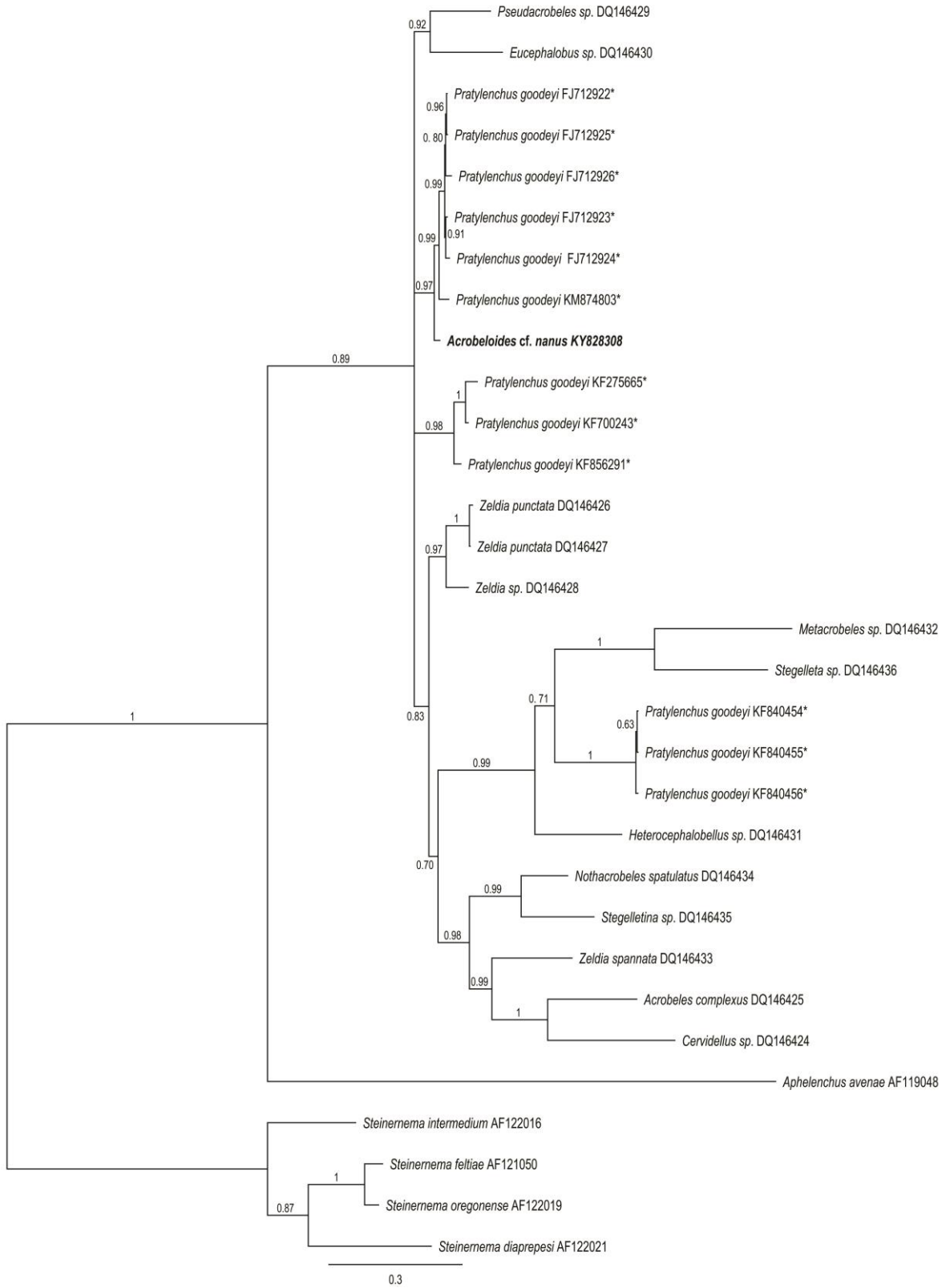


Figure 2. Phylogenetic position of misidentified *Pratylenchus goodeyi* sequences (FJ712922- FJ712926, KF275665, KF700243, KF840454, KF840455, KF840456, KF856291 and KM874803 indicated with a *) within the Cephalobidae as inferred from Bayesian analysis of the ITS of RNA gene sequences using GTR + I + G model. Posterior probabilities are given for appropriate clades. Newly obtained sequences are indicated in bold.

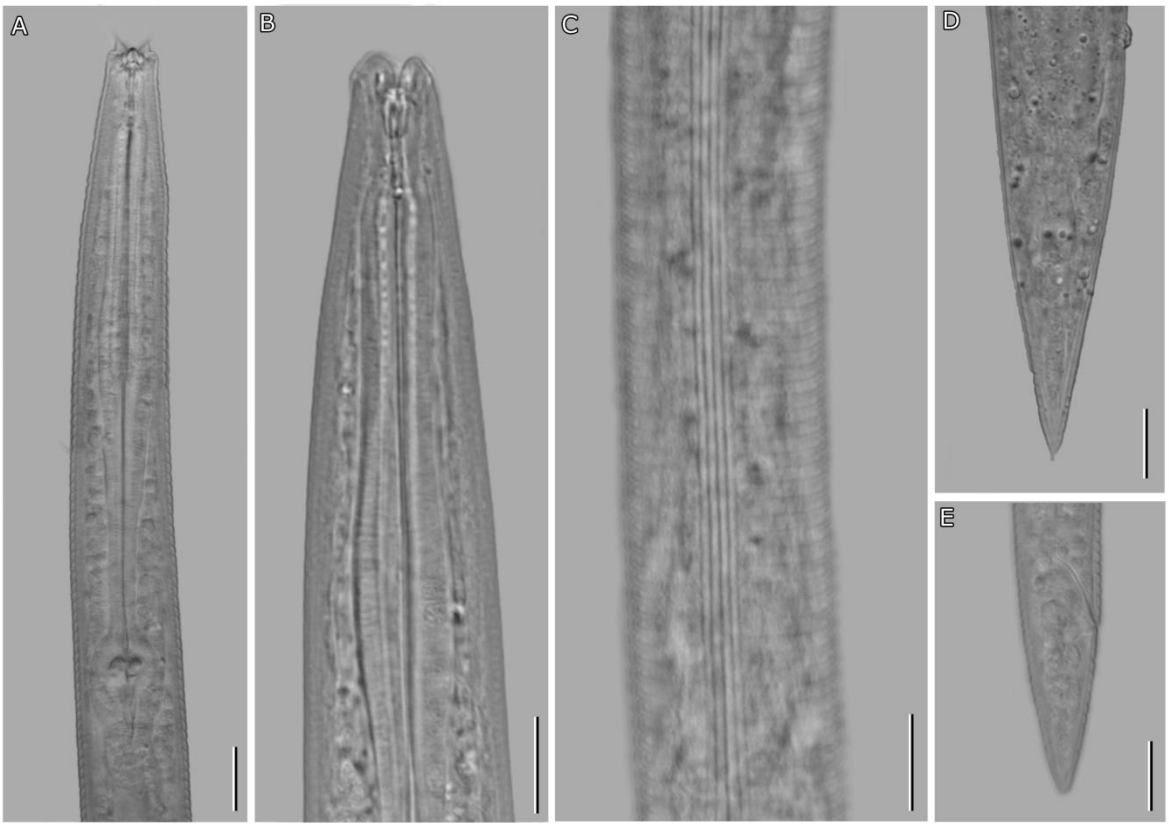


Figure 3. Light microscopy photomicrographs of specimens of *Acrobeloides* cf. *nanus.* A, B: female anterior region, visualizing morphological variation of labial probolae between different Specimens; C: lateral field at mid-body; D, E: female tail region, visualizing morphological variation of the tail tip between different specimens. Scale bars: 10 µm.

Figure 4. (Next page) Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the D2-D3 of the 28S rRNA gene sequences using GTR + I + G model. Posterior probabilities are given for appropriate clades. Newly obtained sequences are indicated in bold. *18S rRNA gene sequence obtained from topotype specimen of *Pratylenchus lentis. ITS* of RNA gene sequences from the same specimen matched with ITS sequences from Troccoli *et al.* (2008). **Previously misidentified as *Pratylenchus pratensis* (de la Peña *et al.*, 2007).


Apratylenchus binhi EF645137
Apratylenchus vietnamensis EF645138
Zygotylenchus guevarai JX261956
Pratylenchus brachyurus KF049925
Pratylenchus brachyurus KT948337
Pratylenchus brachyurus EU130842
Pratylenchus brachyurus KF712472
Pratylenchus brachyurus JN020922
Pratylenchus dunensis AJ890462
Pratylenchus dunensis AM231939
Pratylenchus dunensis AJ890459
Pratylenchus dunensis AM231941
Pratylenchus dunensis AJ890461
Pratylenchus dunensis AJ890460
Pratylenchus dunensis AM231940
T67 Pratylenchus dunensis KY828369
T68 Pratylenchus dunensis KY828368
Pratylenchus dunensis AM231942
Pratylenchus dunensis AM231943
Pratylenchus dunensis AM231938
Pratylenchus dunensis AM231944
Pratylenchus dunensis AM231945
Pratylenchus dunensis AM231946
Pratylenchus dunensis AM231947
Pratylenchus dunensis AM231950
Pratylenchus dunensis AM231948
Pratylenchus dunensis AM231949
T44 Pratylenchus penetrans KY828359
T200 Pratylenchus penetrans KY828355
T132 Pratylenchus penetrans KY828358
Pratylenchus penetrans JQ003984
T268 Pratylenchus convallariae KY828373
T283 Pratylenchus fallax KY828364
T353 Pratylenchus fallax KY828363
T71a Pratylenchus sp. 4 KY828329
T617 Pratylenchus sp. 1 KY828337
Pratylenchus oleae KJ510858
Pratylenchus oleae KJ510859 KJ510860
Pratylenchus oleae KJ510857
Pratylenchus oleae KJ510856
T202 Pratylenchus sp. 3 KY828330
T201 Pratylenchus sp. 3 KY828331
T175 Pratylenchus sp. 2 KY828333
Pratylenchus crenatus EU130852
Pratylenchus crenatus EU130853
Pratylenchus crenatus KM580543
Pratylenchus crenatus KM580545
Pratylenchus crenatus KM580544
Pratylenchus crenatus KJ493147 KJ493149
Pratylenchus crenatus KJ493148
Pratylenchus crenatus KJ493150
Pratylenchus kumamotoensis JX144360
Pratylenchus vulnus KP161615
Pratylenchus vulnus KP161616
Pratylenchus vulnus HM469437
Pratylenchus vulnus KF430799
Pratylenchus vulnus JQ892308
T73 Pratylenchus vulnus KY828305
Pratylenchus vulnus JQ003992
Pratylenchus vulnus JX261945
Pratylenchus vulnus JQ003993
Pratylenchus vulnus JQ003994
T74 Pratylenchus vulnus KY828306
T130 Pratylenchus pratensis KY828296
T684 Pratylenchus pratensis KY828299
T148 Pratylenchus pratensis KY828297
T616 Pratylenchus pratensis KY828298*
Pratylenchus pseudopratensis JX261965
Pratylenchus hexincisus DQ498832
Pratylenchus hexincisus DQ498832 DQ498833
T659 Pratylenchus hexincisus KY828291
T245 Pratylenchus hexincisus KY828290
T671 Pratylenchus hexincisus KY828293
Pratylenchus scribneri EU130864 JX047001 JX047004 KT873859
T669 Pratylenchus hexincisus KY828294
Pratylenchus scribneri JX047003
Pratylenchus scribneri JX047005
Pratylenchus scribneri EU130865
T658 Pratylenchus hexincisus KY828292
Pratylenchus scribneri JX047006
Pratylenchus pseudocoffeae AF170444
Pratylenchus pseudocoffeae KP289348
Pratylenchus pseudocoffeae KT175531
Pratylenchus pseudocoffeae KT175533
Pratylenchus pseudocoffeae KT971360
Pratylenchus pseudocoffeae KT175532
Pratylenchus scribneri DQ498830
Pratylenchus gutierrezi AF170441
Pratylenchus hippeastri GU214112
Pratylenchus parafloridensis AF170438
Pratylenchus floridensis GU214117
Pratylenchus araucensis FJ463261
Pratylenchus loosi EF446991
Pratylenchus coffeae JN809850
Pratylenchus coffeae JN809858
Pratylenchus coffeae JN809841
Pratylenchus sp. AM231929**
Pratylenchus sp. AM231930**
Pratylenchus sp. AM231932**
Pratylenchus sp. AM231931**
Pratylenchus sp. AM231934**
Pratylenchus sp. AM231935**
Pratylenchus sp. AM231933**
Pratylenchus japonicus KF385444
Pratylenchus brzeski AM231913 AM231914
Pratylenchus brzeski AM231915
Pratylenchus brzeski AJ890463
Pratylenchus brzeski AM231916 AM231918
Pratylenchus brzeski AM231917
Pratylenchus brzeski AM231925 AM231926 AM231927
Pratylenchus brzeski AM231922 AM231923 AM231924
Pratylenchus brzeski AM231928
Pratylenchus brzeski AM231912
Pratylenchus brzeski AM231912
Pratylenchus brzeski AM231911
Pratylenchus brzeski AM231919 AM231920 AM231921
T242 Pratylenchus flakkensis KY828284
T96 Pratylenchus flakkensis KY828282
T214 Pratylenchus flakkensis KY828283
Pratylenchus hispaniensis FJ717822
Pratylenchus neglectus KM200578
Pratylenchus neglectus KM200579
Pratylenchus neglectus EU130854
Pratylenchus neglectus JQ303333
Pratylenchus neglectus KM580547
Pratylenchus neglectus KM580550
Pratylenchus neglectus JX261947
Pratylenchus neglectus JX261951
Pratylenchus neglectus HM469438
Pratylenchus neglectus JX261946
T89 Pratylenchus neglectus KY828295
Pratylenchus neglectus KM580548
Pratylenchus neglectus KM580549
Pratylenchus neglectus KM580546
Pratylenchus thornei JX261960
Pratylenchus thornei KP289349 KP289350
T219 Pratylenchus thornei KY828303
T33 Pratylenchus thornei KY828300
T37 Pratylenchus thornei KY828302
Pratylenchus thornei KT213559
Pratylenchus thornei JX261954
Pratylenchus thornei EU130880
T763 Pratylenchus thornei KY828304
T34 Pratylenchus thornei KY828301
Pratylenchus thornei JX261955
Pratylenchus thornei JX261963
Pratylenchus delattrei JX261948
Pratylenchus delattrei JX261949
Pratylenchus zeae EU130890
Pratylenchus bhatlii JN244269
T165 Pratylenchus goodeyi KY828285
T207 Pratylenchus goodeyi KY828287
T166 Pratylenchus goodeyi KY828286
T209 Pratylenchus goodeyi KY828288
T222 Pratylenchus goodeyi KY828289
T664 Pratylenchus boliviarus
Radopholus similis EU555411
T210 Radopholus similis KY828307
Radopholus similis Q782258
Radopholus similis EU555410
Radopholus similis JQ782250
Radopholus similis KF234216
Belonolaimus longicaudatus DQ328710
Psilenchus sp. DQ328716
Coslenchus costatus DQ328719
Basiria gracilis DQ328717
Boleodorus sp. DQ328718
0.3

Figure 5. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the 18S rRNA gene sequences using GTR + I + G model. Posterior probabilities are given for appropriate clades. Newly obtained sequences are indicated in bold.

Table 2. Morphometric measurements of *P. goodeyi*, *P. flakkensis* and *P. pratensis* females. All measurements are in µm and given as mean ± standard deviation (range). *TAF fixed specimen mounted in glycerin, other populations are measured from temporary slides.

	La Palma	Botanical Garden Ghent	Rwanda*	Middelharnis*	Slootdorp*	Russia	Noordwijkerhout	Doornenburg*
♀	*Pratylenchus goodeyi*	*Pratylenchus goodeyi*	*Pratylenchus goodeyi*	*Pratylenchus flakkensis*	*Pratylenchus flakkensis*	*Pratylenchus flakkensis*	*Pratylenchus pratensis*	*Pratylenchus pratensis*
n	6	4	9	7	10	5	4	8
L	623 ± 49 (544-675)	595 ± 67.7 (496-640)	487 ± 48 (424-569)	433 ± 43.2 (373-503)	483 ± 39.8 (417-534)	508 ± 31.1 (479-548)	547 ± 71.6 (432-603)	541 ± 25.4 (505-578)
Stylet length	17.7 ± 0.7 (16.6-18.3)	17.2 ± 0.4 (16.7-17.7)	14.7 ± 0.6 (13.9-15.5)	16.9 ± 0.8 (15.7-18.0)	17.2 ± 0.6 (16.6-18.6)	17.5 ± 0.5 (16.8-18.2)	15.8 ± 0.5 (15.2-16.5)	16.2 ± 0.5 (15.6-17.2)
Anterior to center of metacorpus	60.7 ± 2.1 (57.6-63.1)	64.8 ± 2.1 (61.8-66.6)	53.6 ± 2.4 (49.6-58.1)	52.1 ± 6.7 (41.6-60.7)	48.3 ± 5.4 (40.9-57.4)	54.2 ± 1.4 (52.5-56.2)	54.5 ± 4.1 (48.7-58.3)	58.6 ± 3.0 (55.8-64.9)
Anterior end to cardia	82.5 ± 6.8 (74.2-88.8)	90.8 ± 4.9 (84.4-94.9)	93.7 ± 8.9 (86-108.4)	80.0 ± 11.6 (58.2-95.2)	75.1 ± 7.0 (59.2-86.5)	76.6 ± 6.2 (70.0-83.6)	84.2 ± 7.8 (76.3-93.4)	84.6 ± 8.7 (72.6-97.9)
Anterior to pharyngeal lobe	142.8 ± 8.6 (134-154)	154 ± 12.0 (137-164)	114 ± 12.4 (99-137.3)	136 ± 11.8 (122-156)	123 ± 11.9 (106-138)	124 ± 17.5 (98.6-143)	138 ± 14.0 (120-154)	140 ± 9.0 (126-155)
Anterior end to EP	102 ± 9,5 (78.3-102.4)	100.2 ± 6.4 (94.2-106)	78.5 ± 4.6 (71.1-85.6)	84.1 ± 132 (68.5-95.8)	75.9 ± 12.8 (55.1-99.4)	88.3 ± 4.5 (83.0-92.8)	92.0 ± 11.5- (73.5-102)	85.3 ± 6.8 (75,8-96.4)
Anterior end to vulva	471 ± 44,4 (397-507)	428 ± 47.1 (371-478)	357 ± 38 (304-430)	376 ± 132 (283-670)	370 ± 28.1 (316-415)	389 ± 23.4 (361-420)	420 ± 63.9 (331-473)	420 ± 15.0 (401-443)
Maximum body width	21.5 ± 1,6 (19.0-23.8)	23.0 ± 3.4 (10.1-26.9)	20.3 ± 2.3 (17.4-24.9)	19.7 ± 1.8 (17.9-22.6)	20.0 ± 1.7 (17.5-24.2)	18.9 ± 1.0 (17.9-20.4)	19.0 ± 3.2 (16.2-23.4)	19.4 ± 1.8 (16.7-22.5)
Anal body width	11,9 ± 1,5 (8.6-13.0)	11.8 ± 1.7 (10.1-14.1)	11.1 ± 0.9 (9.4-12.4)	13.0 ± 1.1 (11.3-14.0)	12.9 ± 1.1 (11.5-14.8)	12.6 ± 0.5 (12.2-13.5)	12.0 ± 1.7 (9.8-14.0)	12.1 ± 2.0 (10.0-16.4)
Post uterine sac length	30.2 ± 5,9 (23.8-39.4)	24.1 ± 1,8 (21.7-26.0)	15.6 ± 1.2 (13.3-17.1)	22.2 ± 1.6 (19.8-24.3)	22.4 ± 1.7 (19.4-24.2)	21.6 ± 1.7 (19.3-23.8)	28.5 ± 4.5 (24.6-33.8)	24.7 ± 3.8 (18.2-29.2)
Tail length	35.3 ± 5,2 (24.9-36.5)	34.4 ± 1.5 (32.8-36.0)	32.1 ± 3.2 (28.2-38)	30.8 ± 2.3 (27.2-33.9)	28.2 ± 2.5 (24.2-32.0)	29.4 ± 2.6 (26.7-33.5)	28.3 ± 2.6 (24.8-31.2)	27.6 ± 5.1 (18.5-34.7)
V	74.2 ± 1,7 (72.0-76.5)	72.0 ± 3.4 (67.9-74.8)	73 ± 1.5 (70.7-75.7)	85.5 ± 21.2 (75.6-133)	76.7 ± 3.1 (72.5-82.4)	76.6 ± 1.2 (75.4-78.3)	78.6 ± 1.1 (76.7-79.6)	77.6 ± 1.3 (75.8-79.3)
a	29.5 ± 1,4 (26.2-30.4)	26.0 ± 2.1 (23.7-28.7)	24.1 ± 2 (21.4-28.2)	22.0 ± 0.9 (20.3-23.5)	24.2 ± 1.5 (22.1-26.1)	26.8 ± 1.0 (25.3-27.9)	28.0 ± 3.1 (25.0-32.3)	28.1 ± 1.6 (25.7-30.3)
b	7.1 ± 0,8 (7.1-9.1)	6.5 ± 0.43 (5.9-6.8)	5.2 ± 0.6 (4.3-6.4)	5.5 ± 1.2 (4.2-8.0)	6.5 ± 0.5 (5.6-7.3)	6.7 ± 0.8 (5.8-7.8)	6.5 ± 0.6 (5.7-7.2)	6.4 ± 0.6 (5.5-7.2)
c	4.1 ± 0,4 (4.0-5.0)	3.8±0.2 (3.6-4.0)	2.9 ± 0.2 (2.6-3.3)	3.2 ± 0.4 (2.6-3.7)	4.0 ± 0.4 (3.4-4.5)	4.2 ± 0.8 (3.3-5.6)	3.9 ± 0.2 (3.6-4.1)	3.9 ± 0.2 (3.5-4.0)
c’	18,0 ± 4,5 (15.0-27.1)	17.3 ± 2.3 (14.1-19.5)	15.2 ± 0.7 (14-16.2)	14.1 ± 1.5 (11.9-15.0)	17.2 ± 1.1 (15.9-19.6)	17.3 ± 0.7 (16.3-17.9)	20.6 ± 4.4 (15.1-27.2)	20.2 ± 3.6 (16.7-27.3)

4.2 Case study 2: *Pratylenchus pratensis*

= *Tylenchus pratensis* de Man, 1880

= *Anguillulina pratensis* (de Man, 1880) Goffart, 1929

= *Pratylenchus helophilus* Seinhorst, 1959

= *Pratylenchus irregularis* Loof, 1960

= *Pratylenchus lentis* Troccoli, De Luca, Handoo and Di Vito, 2008

Pratylenchus pratensis was recovered from several places in the Netherlands, associated with grasses (Noordwijkerhout, 13 km away from the type locality at Leiden), *Pyrus sp.* (Doornenburg) and *Prunus domestica* (Winssen) (Table 1). All three populations showed the diagnostic morphological characters of *P. pratensis*, namely, labial region with three annuli, a finely annulated cuticle, four lateral lines, characteristic large elongated spermatheca shape, tail with 20-28 annuli and a mostly annulated tail terminus (Fig. 6) (Castillo & Vovlas, 2007). Morphometric characters were measured for populations from Doornenburg and Noordwijkerhout and matched well with the previously reported *P. pratensis* populations (Castillo & Vovlas, 2007) (Table 2). Moreover, our 18S rDNA sequence from Noordwijkerhout matches 99.4-100% with the *P. pratensis* sequences generated by Rybarczyk-Mydlowska *et al.* (2014). Generated 28S rDNA sequences were 99.1-99.6% similar between the two populations from Noordwijkerhout and Doornenburg, confirming that both populations belong to the same species. Surprisingly, these 28S rDNA sequences did not match with the *P. pratensis* sequences deposited by de la Peña *et al.* (2007) (16.2-16.8% divergence). While both our populations and the populations from de la Peña *et al.* (2007) are morphologically similar, our populations are more likely to represent the genuine *P. pratensis* because (i) morphologically *P. pratensis* was described to be closely related to *P. pseudopratensis* (Seinhorst, 1968) and this agrees with the position of our *P. pratensis* populations as they form a monophyletic clade with a *P. pseudopratensis* population from Majd Taheri *et al.* (2013), based on 28S rDNA (ii) the grass land with fruit trees in which our *P. pratensis* populations were found is very similar to the original type locality and the habitats previously reported for *P. pratensis* (Seinhorst, 1959; Loof, 1974; Castillo & Vovlas, 2007), while populations from de la Peña *et al.* (2007) were recovered from a highly different coastal dune habitat, and (iii) the 18S rDNA sequences from our *P. pratensis* population match with sequences generated by Rybarczyk-Mydlowska *et al.* (2014). As a result, the populations from de la Peña *et al.* (2007) are hypothesized to belong to

a separate species of *Pratylenchus*, sister to *P. japonicus* Ryss, 1988 according to our 28S rDNA phylogeny (Fig. 4) (Wang *et al.*, 2014).

In the summer of 2014 the type locality of *P. lentis* was sampled (Italy, Sicily, Villalba) (Troccoli *et al.*, 2008). On the type locality a species mixture of *Pratylenchus* sp. 1 (Janssen *et al.*, 2017b) and *P. lentis* was recovered. An ITS sequence of our *P. lentis* specimen was 96.3% similar to the ITS sequences of the original description, falling within the 7.1% intraspecific variability reported by Trocolli *et al.* (2008). However, an 18S rDNA sequence from the same *P. lentis* DNA extraction was identical to a *P. pratensis* sequence (KC875380) from the Netherlands (Rybarczyk-Mydlowska *et al.*, 2014) and 99.6% identical to a *P. pratensis* sequences from this study. The 28S rDNA sequences also from the same DNA isolate matched with two *P. pratensis* populations from Noordwijkerhout and Doornenburg (99.0-99.7% identical). Also mark that *P. pratensis* was already reported from *Trifolium* sp. in Italy (Puglia), and thus parasitizing the same plant family (Fabaceae) as *P. lentis* (Inserra *et al.*, 1979). Morphologically both *P. lentis* and *P. pratensis* were found to exhibit a highly variable tail tip. While Troccoli *et al*. (2008) reported *P. pratensis* to have a slightly shorter stylet and a more anterior vulva (V=75-78%) in comparison to *P. lentis*, these findings are contradicted by the morphometrics of our *P. pratensis* populations as V varied between 75.8% and 79.6% while stylet length varied between 15.2 µm and 17.2 µm (V= 76-80, Stylet length= 15.5-17.0 µm in *P. lentis*) (Troccoli *et al.*, 2008). Moreover, the spermatheca shape varied from roundish over oval to rectangular for *P. pratenis* populations and our *P. lentis* population. Additionally, no variation in cuticular annulation was observed between our *P. lentis* population and our *P. pratensis* populations. Based on all the above data *P. lentis* is conspecific with the morphologically similar *P. pratensis*. Thus, the excellent and comprehensibly described *P. lentis* should be considered a junior synonym of *P. pratensis* and the *P. pratenis* population of de la Peña *et al.* (2007) is considered a different species.

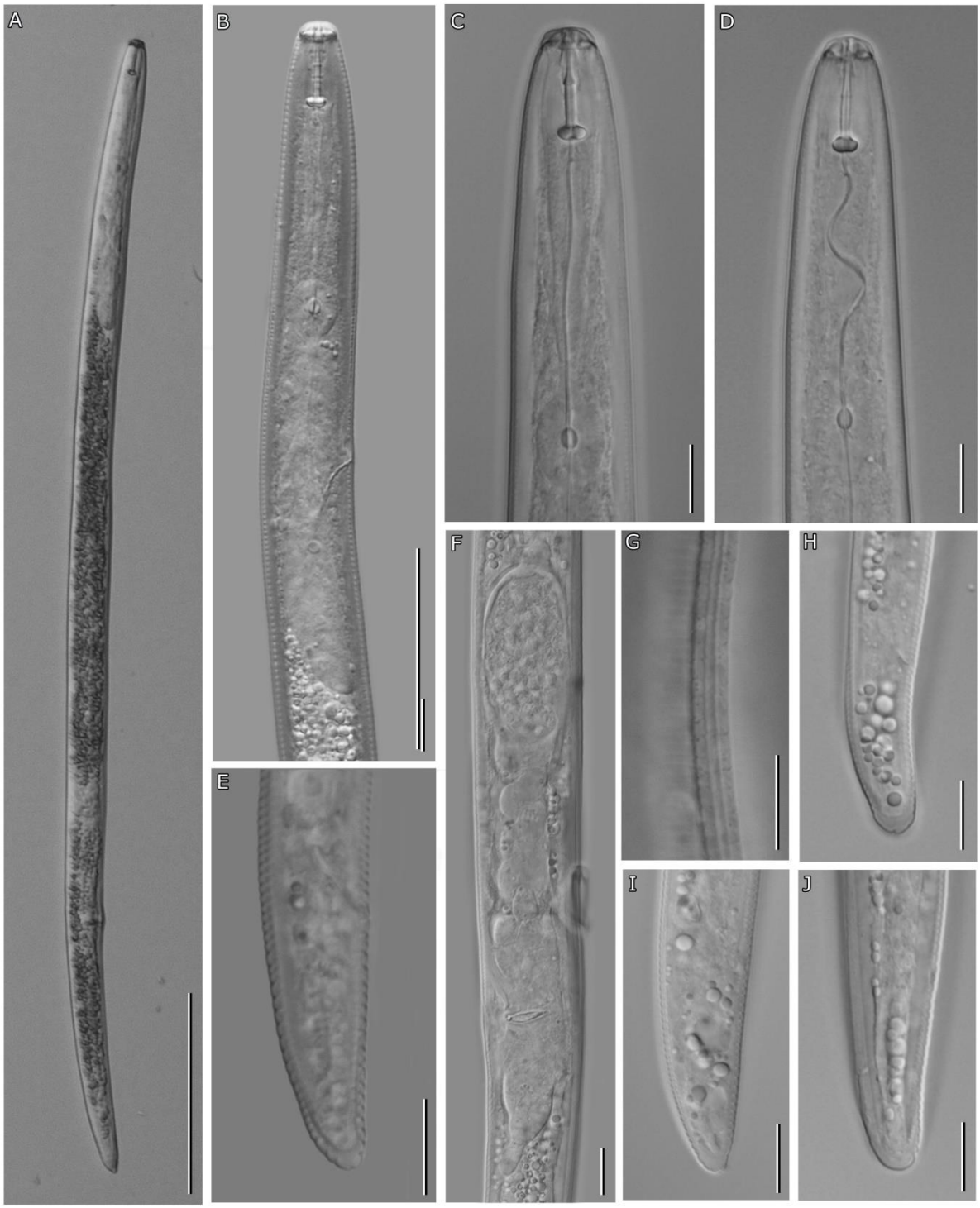


Figure 6. Light microscopy photomicrographs and SEM pictures of specimens of *Pratylenchus pratensis*. A: entire female body; B-D: female anterior region; F: posterior female gonoduct section; G: lateral field at mid-body; E, H-J: morphological variability in female tail region. Scale bar A: 100 µm, scale bars B-J: 10 µm.

4.3 Case study 3: *Pratylenchus flakkensis*

Pratylenchus flakkensis was recovered from its type locality, heavy loam soil under grass at Middelharnis (the Netherlands), associated with grasses (Slootdorp, the Netherlands), and with *Urtica dioica* (Golitsina, Russia). 28S rDNA sequences from these three populations were 99.4-99.8% similar, confirming that these populations belong to the same species. Also *COI* sequences of the *P. flakkensis* populations from Middelharnis and Slootdorp were found to be 96% similar (15 variable nucleotide positions). All populations showed the characteristic morphological features of *P. flakkensis*, *i.e.* a labial region with two lip annuli, a mostly annulated tail tip, a filled round to angular spermatheca, 4 lateral lines and a tail with 16-27 annuli (Fig. 7) (Seinhorst, 1968; Castillo & Vovlas, 2007). Also morphometric characters were found to be in line with previously reported populations of *P. flakkensis* (Table 2). The morphologically very similar *P. gibbicaudatus* Minagawa, 1982 was distinguished from *P. flakkensis* by a higher number of tail annuli (24-39 vs. 18-24) and this was confirmed by the range of annuli number found for *P. flakkensis* in this study (17-27) (Minagawa, 1982). Phylogenetic analysis of 18S rDNA and 28S rDNA sequences indicate *P. flakkensis* to be a member of clade V, together with *P. thornei*, *P. neglectus*, *P. hispaniensis* and *P. brzeskii* (Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010). Within clade V, *P. flakkensis* was found to be most closely related to *P. brzeskii* according to 28S rDNA sequences. Interestingly, the SEM enface lip pattern of *P. flakkensis* (Fig.7) was very similar to *P. thornei*, *P. neglectus* and *P. hispaniensis* in having an obtuse dumb-bell shaped lip pattern, indicating that this lip pattern could be an apomorphic feature of clade V (Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010).

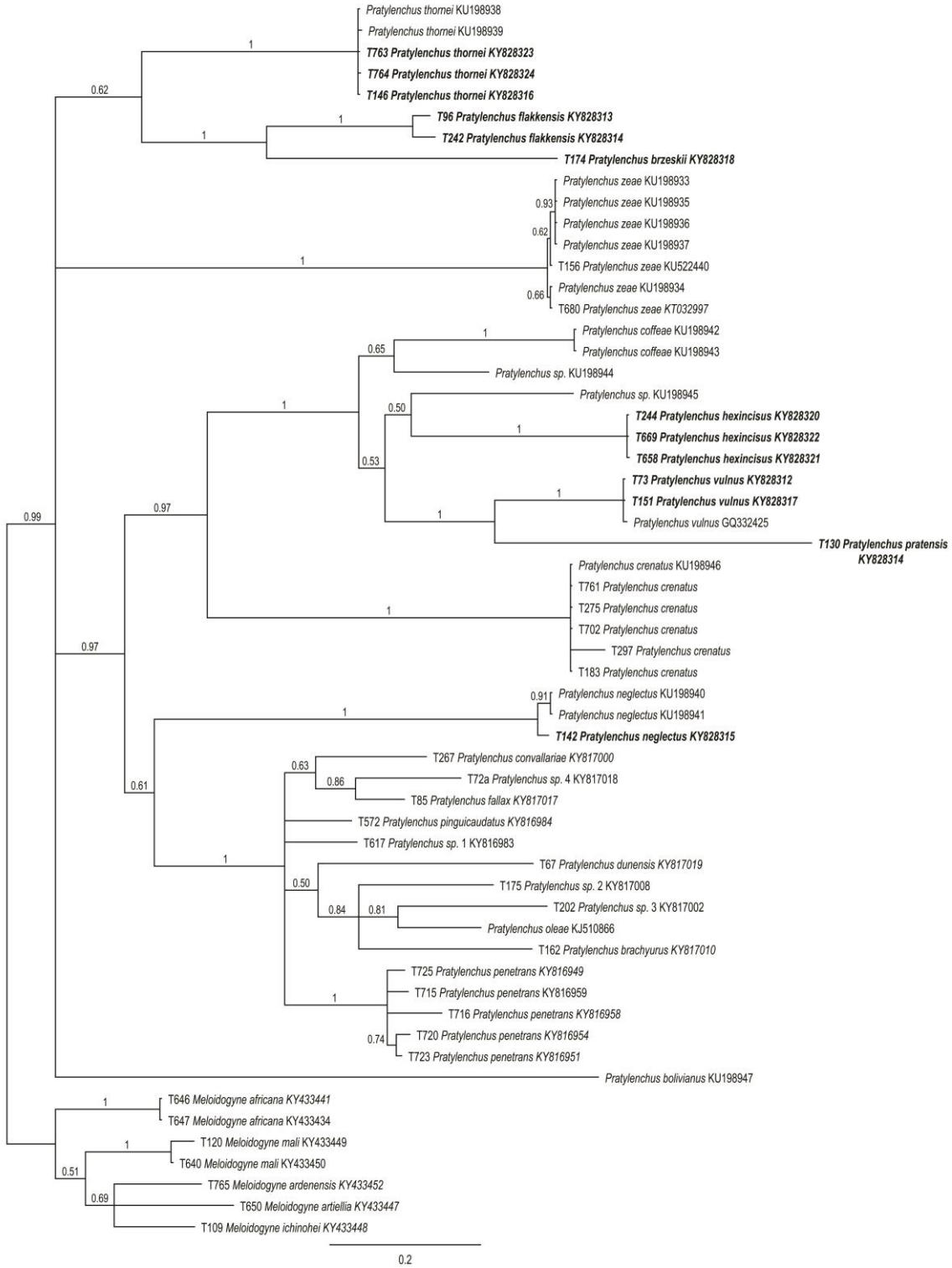


Figure 7. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the *COI* gene sequences using GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

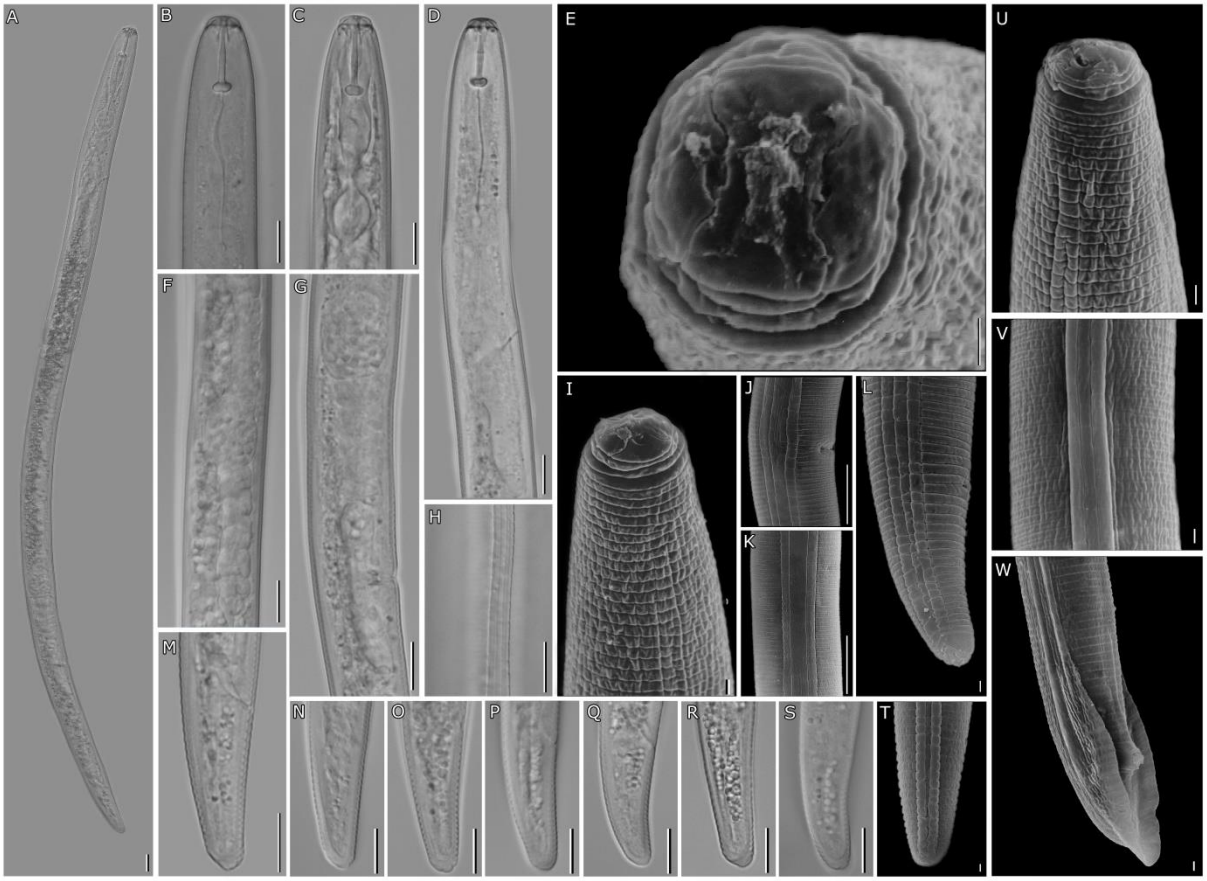


Figure 8. Light microscopy photomicrographs and SEM pictures of specimens of *Pratylenchus flakkensis*, A-T: female, U-W: male. A: entire female body; B-D: female anterior region; E: female cephalic region, en face view; F: spermathecal; G: posterior female gonoduct; H: lateral field at mid-body; I: female cephalic region, lateral view; J: lateral field at vulval region; K: lateral field at mid-body; L,M: tail region; N-T: morphological variation of the tail tip; U: male cephalic region, lateral view; V: male lateral field at mid-body; W: male tail region. Scale bars A-D,F-H, J,K, M-S: 10 µm; E, I, L, T-W: 1 µm.

5 Discussion

In this study it was clearly demonstrated that twelve published ITS sequences of the economically important plant-parasitic nematode *P. goodeyi* are actually sequences from distantly free-living bacterivorous cephalobs. Most likely, this original mislabeling was caused by a contamination of the DNA extraction by a free-living cephalob. Such contamination could happen when multiple individuals are used for a single DNA extraction. While the use of multiple specimen in a single DNA extraction leads to a number of problems it was and still is

a common practice in order to increase DNA concentration. Problematically, incorrect labeling of sequences can result in a cascade of erroneous interpretations. It also illustrates the danger of mislabeled sequences in public DNA databases, leading to misidentification, as shown by the reports of "*P. goodeyi"* on cotton in India (Gokte-Narkhedkar *et al.*, 2013) and on banana in China (Zhang *et al.*, 2015) based on cephalobid sequences. These misidentifications have a vast impact on pest management and international trade, as in this case *P. goodeyi* is listed as a quarantine species in some parts of the world (for example in Florida). Unfortunately, the associated 'domino effect' of misidentifications results in a large amount of further wrong sequence-based identifications. Furthermore, when two alternative sequences are available, one tends to have more trust in the alternative that is supported by most sequences (in this case the subdominant free-living nematode sequence). However, in these "identification" cases the data are not independent but are in fact all the result of one single mislabeling. Due to the apparently independent data the mislabeling mistake becomes more difficult to rectify. In this case the misidentification was almost rectified by De Luca *et al.* (2011) as the authors noted a large genetic discrepancy between the mislabeled *P. goodeyi* sequences, and a genuine *P. goodeyi* population. However, as ITS sequences failed to identify one of the populations as a population of *Acrobeloides*, the mislabeled sequences were not corrected. This indicates that ITS is not such a reliable barcoding region (De Luca *et al.*, 2011), as it suffers from substitution saturation on relatively low taxonomic level. If a more conserved barcode gene would have been selected the mislabeling would have been obvious.

In a second mislabeling example, it was shown that both the genuine and a wrongly-labeled *P. pratensis* population were linked to DNA sequences. However, as both populations were connected to a different marker (18S vs 28S) the problem was able to remain unnoticed for a long time. Furthermore, a putative new species was linked to a third marker, namely ITS. As such, the fact that *P. lentis* is conspecific with *P. pratensis* was also overlooked. Actually, mislabeled specimens are a symptom of a larger problem; 86% of the known eukaryotic species are still waiting for a description while the number of taxonomic experts is strongly decreasing (Mora *et al.*, 2011). Nowadays, many laboratories are shifting towards sequence-based identification but the majority of morphologically-described species remain unlinked to DNA sequences. In this study we have illustrated this by *P. flakkensis*, a commonly occurring species of *Pratylenchus* in Europe and Western-Asia that remained, until now, unlinked to DNA

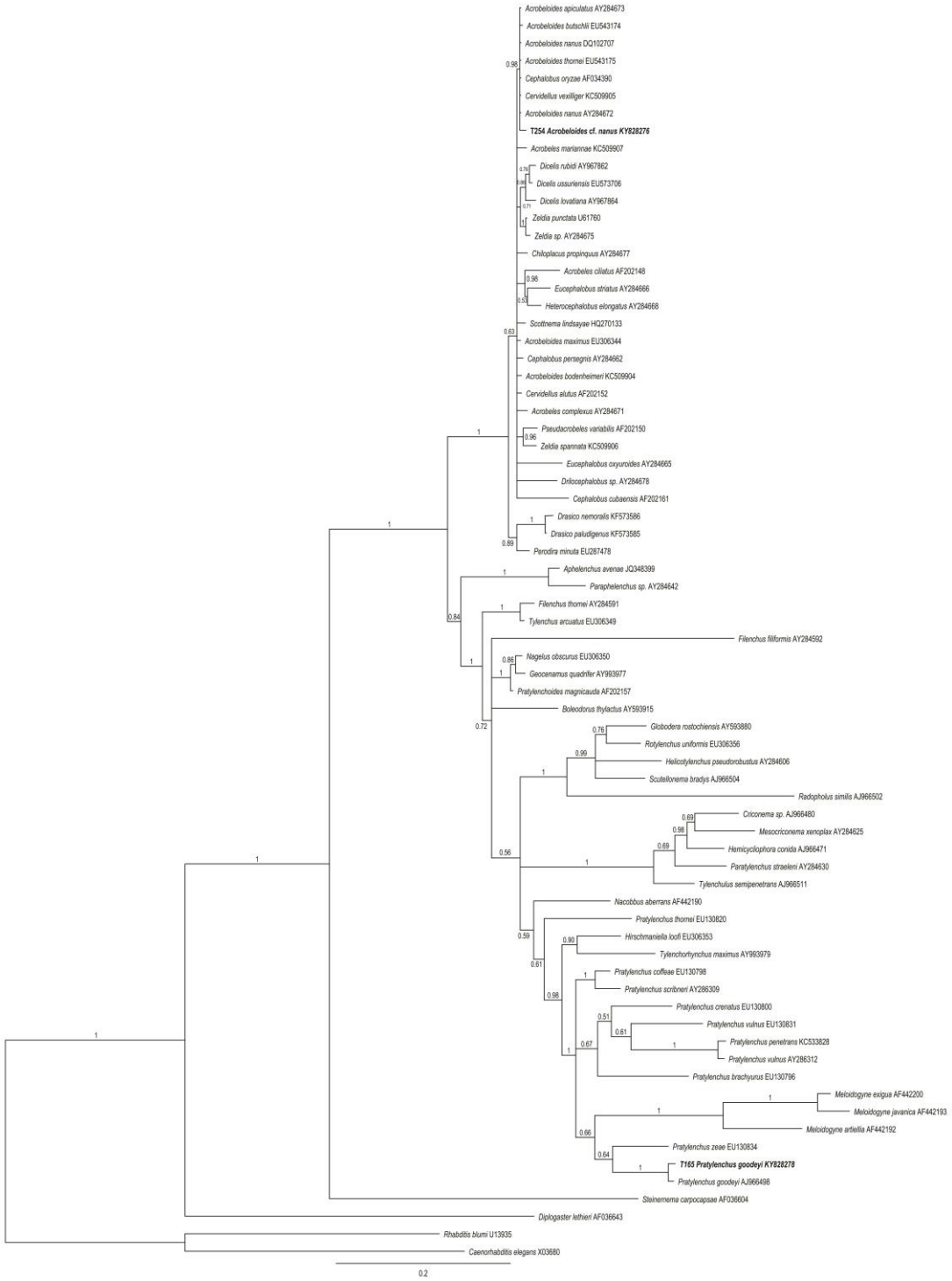
sequences. Within the genus *Pratylenchus* diagnostic, morphological characters are scarce, greatly hampering species identification (Castillo & Vovlas, 2007). Collecting topotype material is often the only option in order to link formerly described morphospecies to DNA sequences (De Luca *et al.*, 2012; Troccoli *et al.*, 2016; Zamora-Araya *et al.*, 2016; Janssen *et al.*, 2017b).

This study clearly illustrates the importance of a strong link between DNA sequences and morphological characters. Moreover, it shows that relying solely on either DNA sequences or on morphology for species identification is a flawed practice (Will *et al.*, 2005; Wheeler, 2008). Even a modest morphological description immediately provides possible connections to life history, ecology, behavior and taxonomic status while DNA sequences provide a wealth of characters for the study of molecular biology, phylogenetic relationships and population genetics. A connection between morphology and DNA sequences is therefore of crucial importance, again illustrating the value of depositing morphological vouchers with sequence material (De Ley *et al.*, 2005; Pleijel *et al.*, 2008). Mislabeling of sequences also poses a huge treat for pure sequence based identifications such as environmental sequencing, as these methods rely completely on the quality of the reference database (Dell'Anno *et al.*, 2015). Hence, safeguarding the link between DNA sequences and morphological characters is of crucial importance in order to prevent misidentifications and mislabeled sequences in public databases in the future.

6 Acknowledgements

We would like to thank Valeria Orlando to analyze the *P. lentis* population and Lisa Joos for processing of the photographical data and critical reading of this manuscript. Alliance Nyiragatare, Adem Esimo Beriso, Qing Xue, Yao Adjiguita Kolombia and Noura Chihani for collecting nematode populations. This work was supported by a special research fund UGent 01N02312.

7 Supplementary information Chapter 5



Supplementary info Fig. A. Phylogenetic relationships as inferred from Bayesian analysis of the 18S rRNA gene sequences using GTR + I + G model, visualizing the large genetic distance between genuine *Pratylenchus goodeyi* sequences and sequences of *Acrobeloides* cf. *nanus* and closely related misidentified *P. goodeyi* sequences. Posterior probabilities are given for appropriate clades. Newly obtained sequences are indicated in bold.

Chapter 6

General discussion

1 Diagnostic difficulties of plant-parasitic nematodes

Root-lesion and root-knot nematodes are morphologically extremely conserved genera (Castillo & Vovlas, 2007; Hunt & Handoo, 2009). Few morphological traits are taxonomically informative and, problematically, most are linked to reproductive or feeding mechanism and thus directly dependent on feeding habits and reproductive state of an individual or population. Moreover, nematode morphology is plagued by convergent evolution making it often difficult to determine homological traits (Ragsdale & Baldwin, 2010). As a result morphological identification of species from the genera *Meloidogyne* and *Pratylenchus* is a time consuming process, requiring a great amount of expertise and high quality reference material. In order to complement morphological diagnosis DNA barcoding presents a great opportunity (Powers, 2004). A large part of this thesis is devoted to facilitate DNA barcoding of *Meloidogyne* and *Pratylenchus*. In particular, we present a new barcode based identification method for tropical root-knot nematodes in this thesis (Chapter 2) and we have studied speciation within the *Pratylenchus penetrans* group in order to facilitate barcode based identifications in this morphologically conserved group of root-lesion nematodes (Chapter 4).

2 Spread of nematode species through agricultural practices

Our research revealed that several mitochondrial haplotypes from species of *Meloidogyne* and *Pratylenchus* are geographically remarkably widespread. Within the genus *Meloidogyne* several lineages, as defined by multi-gene mitochondrial haplotypes, such as *Meloidogyne* sp. 2, *M. enterolobii* and lineages of *M. incognita* and *M. javanica*, were found to have a global distribution (Chapter 2). Within the genus *Pratylenchus*, *P. penetrans, P. thornei* and *P. hexincisus* were shown to have widely distributed *COI* haplotypes. Similarly, *P. zeae* specimens with virtual identical *COI* haplotypes were found to be globally distributed (Troccoli *et al.*, 2016). The observation that identical mitochondrial haplotypes can have a global distribution favors the hypothesis that this distribution was caused by humans through agricultural practices and does not pre-date human crop exchange and agricultural development (Castagnone-Sereno *et al.*, 2013). If worldwide distribution would pre-date agricultural development a much larger variation in mitochondrial haplotypes between lineages from distant locations could be expected. This is especially true for lineages reproducing by mitotic parthenogenesis as this implies that single nucleotide polymorphisms remain separated between different populations.

In sexual reproducing species, such as *P. penetrans,* a wide distribution of haplotypes indicates that populations are not reproductively isolated (Chapter 4). Anthropogenic distribution was also previously indicated to explain the distribution patterns of cyst nematodes (Plantard *et al.*, 2008; Boucher *et al.*, 2013). Thus, it is highly likely that human aided dispersal through international crop exchange, agricultural development, field machinery and accidental dispersal has greatly contributed to the observed global distribution of many species of *Meloidogyne* and *Pratylenchus*. For example, recently it was shown that dispersal of root-lesion and root-knot nematodes occurred through grass sods trade (G. Karssen personal communication). Furthermore, the daily interception of samples infected with *Pratylenchus* or *Meloidogyne* in plant protection agencies around the world essentially prove that plant-parastitic nematodes are dispersed anthropogenically (G. Karssen personal communication). In order to inhibit the anthropogenic spread of plant-parasitic nematodes many countries have adopted strict constrains on the transportation of quarantine nematodes. These policies have definitely improved in some parts of the world through careful trade control, however, in developing nations this is not the case (Nicol *et al.*, 2011). While anthropogenic distribution of plant-parasitic nematodes might play an important role in dispersion of plant-parasitic nematodes, surprisingly, little is known about other means of dispersal. In 1994 Lehman reviewed the dispersal of phytoparasitic nematodes concluding that dispersal could take place by animals, plant tissue, water and wind (Lehman, 1994). Indeed, eggs of root-knot nematodes were found on faecal samples of humans and other endotherms, and from dust traps in Texas (Orr and Newton 1971) and India (Guar 1988). Moreover, root-lesion and root-knot nematodes were recovered from field runoff water and irrigation water (Lehman, 1994). These observations indicate that root-lesion and root-knot nematodes have relatively large natural dispersal capacity and are probably able to colonize large geographic areas with or without anthropogenic influence. Despite these preliminary results on dispersal capacity it is unlikely that *Pratylenchus* and *Meloidogyne* could fit into the "everything small is everywhere" hypothesis, in which small organisms are hypothesized to be ubiquitous with the environment determining the survival of a species on a certain location (Baas-Becking, 1934; Fontaneto, 2011). The main reason is that it is unlikely that plant-parasitic nematodes can colonize different continents without anthropogenic influence. Especially as our geographically widespread haplotypes require colonization events on a relatively regular basis in order to prevent geographic reproductive isolation and associated haplotype divergence. However, it is clear that more research is needed

to improve our understanding of natural dispersal capacities of plant-parasitic nematodes and to evaluate the usefulness of strict quarantine practises for controlling plant-parasitic nematode spread.

3 Need for careful selection of appropriate barcode genes

Originally ribosomal RNA genes were mainly used for phylogenetic purposes (Tandingan De Ley *et al.*, 2002; Subbotin *et al.*, 2008), however, it has been argued that ribosomal genes can be equally useful for identification and barcoding purposes as compared to mitochondrial genes (Vanhove *et al.*, 2013). Indeed, within the phylum Nematoda ribosomal genes have been widely used for molecular diagnostics, barcoding and environmental sequencing (Powers *et al.*, 1997; Powers, 2004; Powers *et al.*, 2009; Powers *et al.*, 2011). However, the selection of a reliable genetic marker for DNA barcoding is of crucial importance as has been illustrated in this study. For example the link of *P. goodeyi* with the wrong sequence by Waeyenberge (2007), Gokte-Narkhedkar et al. (2013) and Zhang et al. (2015) illustrates the danger of selecting a fast evolving barcode gene in combination with a poor reference dataset, while if a more slowly evolving marker had been selected the mislabeling would have been obvious. On the other hand, selecting a slowly-evolving barcode gene can lead to a serious underestimation of biodiversity. For example Helder in Jones *et al.* (2013) argued that *Pratylenchus penetrans, P. convallariae* and *P. fallax* are conspecific based on similar 18S rDNA sequences, a hypothesis clearly rejected by our species delimitation analysis (Chapter 4). In the current study we have used several genetic markers, each of which will be evaluated for its usefulness as a universal barcode gene for plant-parasitic nematodes in the following section.

3.1 18S rDNA

While 18S rDNA is an excellent marker for phylogenetic studies on a higher taxonomic level (Blaxter *et al.*, 1998; Tandingan De Ley *et al.*, 2002; Meldal *et al.*, 2007; van Megen *et al.*, 2009), several studies have shown that 18S rDNA lacks resolution at species level. This was found to be the case for Criconematina (Powers *et al.*, 2011), root-knot nematodes (Kiewnick *et al.*, 2014; Janssen *et al.*, 2017c), Rhabditidae (Fitch *et al.*, 1995), Heterorhabditidae (Liu *et al.*, 1997), Leptosomatidae (Derycke *et al.*, 2010a), several marine nematodes (Meldal *et al.*, 2007) and meiofauna in general (Tang *et al.*, 2012). Despite the fact that 18S rDNA will greatly underestimate species biodiversity, it is widely used as a barcode marker especially in

environmental sequencing (Powers *et al.*, 2009; Morise *et al.*, 2012; Dell'Anno *et al.*, 2015). One of the main advantages of 18S rDNA is the presence of highly conserved regions for which universal PCR primers can be designed. While it is often difficult to design universal applicable primers in faster evolving genes, this problem can be resolved by using primer cocktails for faster evolving genes (Ivanova *et al.*, 2007; Prosser *et al.*, 2013).

3.2 28S rDNA

The D2D3 region of 28S rDNA is a widely used marker in phylogenetic and taxonomical studies of several nematode taxa, including root-lesion (de la Peña *et al.*, 2007; Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010; De Luca *et al.*, 2012; Majd Taheri *et al.*, 2013; Palomares-Rius *et al.*, 2014; Troccoli *et al.*, 2016; Zamora-Araya *et al.*, 2016) and root-knot nematodes (Castillo *et al.*, 2003; Tenente *et al.*, 2004; Ahmed *et al.*, 2013; Ye *et al.*, 2015). Our study also demonstrated that 28S rDNA was able to resolve *COI* operational taxonomic units of the *Penetrans* group (Chapter 4). However, 28S rDNA is an evolutionary relatively conserved gene and lacks the resolution to separate several species of tropical root-knot nematodes (Tenente *et al.*, 2004; Seid *et al.*, 2017). Yet, the 28S rDNA has a higher mutation rate in comparison to the 18S rRNA gene, indicating it to be a superior marker for Nematoda biodiversity studies. However, careful evaluation of the region remains appropriate given the recent reports of high intra-genomic variability within 28S rDNA in the genus *Cephalenchus* (Pereira & Baldwin, 2016). Importantly, intra-genomic variability might be a problem associated with ribosomal genes in general, given the multicopy nature of the region in a genome (see also below).

3.3 ITS

ITS was used in this study to differentiate species of the *Penetrans* group (Chapter 4). ITS is widely used as a barcode marker for plant-parasitic nematodes as evidenced by the high number of ITS sequences present in public sequence databases. As shown in Chapter 4 the quickly evolving ITS region can be useful for separating closely-related species. However, in the current study, cloning of ITS fragments from a single specimen revealed considerable intra-individual variability within the ITS region (1.8%-42%), caused by single nucleotide mutations and large insertions and deletions (data not shown). Furthermore, several other *Pratylenchus penetrans* ITS sequences on GenBank contain large insertions e.g. (JX046955, JX046956). Thus, different copies of the ribosomal gene cluster in a single genome can have very different

ITS1 and ITS2 sequences. These insertions/deletions within the ITS region are unlikely to be the result of the cloning process or other technical aspects, since an identical protocol did not result in insertions/deletions in other genes (28S rDNA and *COI*). However, at this moment, exogenous induced variation cannot be completely excluded. Nevertheless, intra-individual genome variability is problematic for the recovery of homological nucleotide positions in a multiple sequence alignment, and might influence subsequent phylogenetic analyses (Pereira & Baldwin, 2016). The intraspecific variability within the ITS of *Pratylenchus* spp. was already relatively well known (Waeyenberge *et al.*, 2000; Troccoli *et al.*, 2008; De Luca *et al.*, 2011), yet intra-individual variability within the ITS region had hitherto only rarely been reported (Waeyenberge *et al.*, 2000; De Luca *et al.*, 2011). Our results indicate that large intra-individual variability in *Pratylenchus* is more common than previously anticipated. This is not surprising as intra-individual variability of ribosomal genes has already been found in several other nematode taxa, including Steinernematidae (Puza *et al.*, 2015), *Halicephalobus* (Yoshiga *et al.*, 2014), *Ditylenchus* (Subbotin *et al.*, 2011) and *Cephalenchus* (Pereira & Baldwin, 2016). This intra-individual variability can explain the fact that the *Pratylenchus* ITS region cannot be sequenced without cloning, since sequencing of variable-sized fragments will inevitably result in double peaks in the sequence chromatogram. Large intra-individual variability is also problematic in species delimitation as it violates the assumption of concerted evolution upon which these analyses are based (Pons *et al.*, 2006). Furthermore, intra-individual variability can obscure the boundaries between intraspecific and interspecific variation. Thus, if ITS is to be used for the diagnosis of *Pratylenchus*, it is advisable to sequence a representative amount of clones for each individual specimen (depending on the recovered variation), and this for multiple specimen per population of various populations in order to properly characterize intra-individual and intraspecific variability. Taken altogether, even though the ITS region is still widely used for the diagnosis of *Pratylenchus* spp. (Zhang *et al.*, 2015; Yan *et al.*, 2016), ITS appears to have serious limitations for species identification and taxonomy. However, ITS might still be a useful region to assess in combination with other more conserved markers like 18S, 28s and *COI*.

3.4 Mitochondrial genes

In this study mitochondrial coding genes proved to be reliable barcode genes. In Chapter 2 multi-gene mitochondrial barcodes were able to distinguish most tropical root-knot nematodes

and showed consistency with traditionally used isozyme profiles. In chapters 3, 4 and 5 cytochrome oxidase I proved to be a reliable gene for the identification of root-knot and root-lesion nematodes. This is not surprising as *COI* is probably the most used barcode gene for eukaryotes (Hebert *et al.*, 2003). Mitochondrial barcode genes have some distinct advantages including maternal inheritance, a direct link to amino acid sequences and a distinct genetic code. Moreover, most mitochondrial genes have a relatively high mutation rate, enough to reliably separate a wide range of species. Indeed, recent studies indicate *COI* to be a good barcode gene for several nematode clades (Derycke *et al.*, 2010b; van den Berg *et al.*, 2013; Kiewnick *et al.*, 2014; Troccoli *et al.*, 2016; Janssen *et al.*, 2017b; Sánchez-Monge *et al.*, 2017).

Despite the high potential of mitochondrial genes as barcode fragments, several potential drawbacks exist, including heteroplasmic nucleotide positions and nuclear mitochondrial pseudogenes (Numts), *i.e.* the transfer of genetic material from mitochondria to the nucleus and its integration into the nuclear genome. Numts have been demonstrated to be a problem for DNA barcoding studies in several eukaryotes (Bensasson *et al.*, 2001; Song *et al.*, 2008). While reports of Numts in nematode are limited they have been documented to be present in *Caenorhabditis elegans* (Bensasson et al., 2001). Especially as Numts have been widely reported among insects and other Ecdysozoa (Bensasson *et al.*, 2001; Martins *et al.*, 2007; Pamilo *et al.*, 2007; Song *et al.*, 2014) this topic and its potential problem for barcoding needs further investigation among Nematoda.

Another problem associated with mitochondrial genes is the difficulty to find conserved regions needed to design universally applicable primers. For root-lesion and root-knot nematodes this problem can be largely circumvented given the availability of genomes (Abad *et al.*, 2008; Opperman *et al.*, 2008; Sultana *et al.*, 2013; Humphreys-Pereira & Elling, 2014; Lunt *et al.*, 2014; Sun *et al.*, 2014; Burke *et al.*, 2015; Humphreys-Pereira & Elling, 2015). However, reliable primer design remains a problem in poorly studied nematode groups (De Ley *et al.*, 2005; Derycke *et al.*, 2010b). This problem of primer based sequencing will be most likely be solved in the future as genome sequencing becomes more affordable. However, careful selection and evaluation of barcode genes will continue to be important in the future in order to verify their homological origin (Derycke *et al.*, 2010b; Coissac *et al.*, 2016).

3.5 DNA barcoding-based diagnostics

For most nematodes one gene provides sufficient resolution, for root-lesion and especially root-knot nematodes multiple gene based identifications are helpful or even imperative. This can be done based on de currently available loci, but also the exploration of new loci will be important in the future. Multiple gene sequencing has already shown to be crucial for some tropical root-knot nematodes species in this study (Janssen *et al.*, 2016) and it will be important to develop a multiplex identification assay. Using the barcode based identification assay developed in Chapter 2 this can now be relatively easily achieved using a multiplex PCR assay of all coding genes in combination with next generation sequencing. A multiplex PCR assay is possible because the primers have been purposely designed to have the same annealing temperature. An interesting alternative would be the use of molecular inversion probes for sequencing of specific SNP's (Hardenbol *et al.*, 2005; Niedzicka *et al.*, 2016), the advantage of this method will be that molecular inversion probes allow an improved multiplexing, a higher specificity and a better reproducibility (Hardenbol *et al.*, 2005). Moreover, multiplexing different samples will allow identification of hundreds of individuals on a single flow cell. For root-knot nematodes this will be interesting as an infested field often contains a mixture of several different pathogens (Baidoo *et al.*, 2016).

4 Reference database quality determines quality of DNA barcoding studies

Due to the morphologically conserved nature of root-knot and root-lesion nematodes and probably plant-parasitic nematodes in general, linking described morphospecies with sequence data is problematic. This requires an intensive and time consuming morphological analysis, and in many cases collecting topotype material is the only way to confidently link morphospecies to DNA sequences (Troccoli *et al.*, 2016; Zamora-Araya *et al.*, 2016), which was done in several occasions in the framework of this work (Chapters 2, 4 and 5). The hidden diversity of *Pratylenchus* uncovered in this study augments the difficulty to link morphospecies with sequences. Sampling *Pratylenchus* spp. of the *Penetrans* group in a limited number of relatively well-studied agricultural habitats already resulted in the discovery of four new species (Chapter 4). A similar sampling effort in lesser known natural habitats most likely would result in an even larger number of undescribed species indicating that the currently 101 described species of *Pratylenchus* only represent a small fraction of the actual biodiversity in the genus. This

hypothesis may apply equally well to the genus *Meloidogyne*. As root-lesion and root-knot nematodes are arguably among the most-studied genera of plant-parasitic nematodes, one can only imagine the fraction of unknown biodiversity in plant-parasitic nematodes in general. As it will be close to impossible to formally describe all these species DNA barcoding provides, despite its disadvantages (Will *et al.*, 2005), an incredibly useful tool for the classification of this unknown biodiversity (Blaxter, 2016). However, when DNA barcoding is used for identification the quality of the database is of crucial importance (Ratnasingham & Hebert, 2007). In order to establish a falsifiable reference database, sequence names have to be accompanied with convincing evidence for species diagnosis (Pleijel *et al.*, 2008). Preferably this evidence includes integrative data such as morphometrics, morphological features, isozyme patterns, sample locality, etc. However, problematically, the opposite is often the current practice, few information is attached to DNA sequences leading to incorrect identifications with, especially for parasites, serious consequences as illustrated in Chapter 5 with the example of *P. goodeyi*. To secure the link of species with sequences, digital vouchering of specimens is extremely useful for documenting species as both morphological and morphometric information can be deduced from digital species vouchers (De Ley *et al.*, 2005). Moreover, digital species vouchering is more accessible as traditionally type specimen which often are poorly accessible in museum collections (Abebe *et al.*, 2011). However, for Nematoda the majority of biodiversity remains unlinked to sequences and a major effort is needed in order to link an adequate part of biodiversity to DNA sequences. The comprehensive work-flow associated with linking morphospecies to DNA sequences will undoubtedly have drastic taxonomical implications, such as , the recovery of new species complexes (for example the Penetrans group, see Chapter 4), synonymization of existing species (for example *P. lentis* was found to be a junior synonym of *P. pratensis*, see Chapter 5), and the recovery of undescribed species especially from geographically understudied regions and understudied natural habitats, illustrating the importance of fundamental research. Sufficient knowledge of the actual diversity is more than a merely scientific challenge, it is of crucial importance in the light of the development of next generation sequencing and metagenomics.

5 What constitutes a species in plant-parasitic nematodes?

In biodiversity studies often a predefined cut-off value for molecular variability is used to delimit species (Hebert *et al.*, 2003; Prevot *et al.*, 2013). We have demonstrated in our study

that these cut-off values can merely provide a rough species estimate as we have shown that the amount of molecular intraspecific variability is highly dependent on the species group under study. For example, in tropical root-knot nematodes we found that single nucleotide substitutions in mitochondrial genes might be associated with distinct species, while within *Pratylenchus penetrans* the intraspecific variability in *COI* can reach up to 8.5%. Thus, as thresholds for intraspecific variability are already highly variable for two closely-related genera, a uniform threshold has serious shortcomings to estimate the number of species in biodiversity studies. Moreover, it has been demonstrated that reproduction modes can have a large impact on genetic distances between species and interspecific variability within species. This was demonstrated in Rotifers were sexual reproducing taxa were associated with larger genetic gaps compared to asexual reproducing taxa (Tang *et al.*, 2014). Arguably asexual species could diversify faster in less discrete species as divergence does not depend on evolution of reproductive isolation (Tang *et al.*, 2014). As we have shown that the switch between sexual and asexual reproduction evolves multiple times in root-lesion and root-knot nematodes, this observation is of crucial importance for species delimitation in plant-parasitic nematodes. Species delimitation analyses of root-lesion and root-knot nematodes are further complicated by the presence of polyploidy. Polyploidy, established through hybridization or genome duplications, represents an important speciation strategy, effectively isolating lineages.

Given that intraspecific variability is highly variable, molecular species delimitation analyses can be useful in assessing genetic variability between different populations and to test species boundaries. In Chapter 4 we have used a variety of species delimitation methods to assess speciation within the *Penetrans* group. Remarkably, species boundaries were found to differ according to the used delimitation approaches and program settings. An observation which was already forwarded by several studies (Fujita *et al.*, 2012; Prevot *et al.*, 2013; Kekkonen & Hebert, 2014). Despite these differential outcomes, each method has a specific set of advantages and disadvantages. The ABGD method is straightforward, doesn't require a phylogenetic tree and automatically identifies the position of the barcode gap (Puillandre *et al.*, 2012), while the GMYC method relies on a combination of the coalescent theory and phylogenetic inference in order to delimitate species (Pons *et al.*, 2006). As the outcome is dependant of the method used, further tests of these species hypotheses are needed using a variety of methods. First, species delimitation analyses should be preferably tested using multiple molecular markers, in order to

exclude influence from specific gene trees (Fujita *et al.*, 2012). If available, morphological traits could help to support species hypotheses. Within the case of sexual reproducing species, congruence with biological species concept could be checked using interspecies breeding experiments in order to evaluate the presence of reproductive barriers (Perry *et al.*, 1980). Alternatively, other species concepts could be applied in order to further support species hypotheses.

In the past asexual lineages of root-knot nematodes of clade I have been largely separated according to differences in isozyme phenotypes (Chapter 2). Until recently every new isozyme phenotype was associated with a new species (Maleita *et al.*, 2012; Carneiro *et al.*, 2014; Humphreys-Pereira *et al.*, 2014). Moreover, isozyme variability has been demonstrated for a wide range of root-knot nematode species including *M. arenaria*, *M. paranaensis*, *M. mali* and *M. konaensis* (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987; Carneiro *et al.*, 1996; Carneiro *et al.*, 2000; Sipes *et al.*, 2005; Carneiro *et al.*, 2008; Ahmed *et al.*, 2013; Santos *et al.*, 2017). Also in *M. javanica* variable isozyme phenotypes have been recovered (Carneiro *et al.*, 1996), however, none of the recovered J2 esterase patterns has so far been sequenced to confirm that these isozyme phenotypes belong to *M. javanica*. In either case, in light of the recent discoveries about genome structure of root-knot nematodes and the mitochondrial haplotypes recovered in chapter 2 several species hypotheses have to be reevaluated. Especially as different isozyme profiles might be caused by the polymorphic and variable nature of root-knot nematode genomes (Blanc-Mathieu *et al.*, 2017). In order to delimit taxonomic entities, an integrative taxonomic aprouch will be necessary combining genomic, isozyme, and cytological data.

6 DNA barcoding and morphological diagnosis can be replaced by species-specific essays, but only for very well-known systems

In order to allow straightforward and cheap identification of pathogens many species-specific essays have been developed without the need for sequencing (Zijlstra, 2000; Zijlstra *et al.*, 2000; Randig *et al.*, 2002; Waeyenberge *et al.*, 2009; Niu *et al.*, 2011; Mokrini *et al.*, 2013; Correa *et al.*, 2014; Pagan *et al.*, 2015). Usually these identification strategies rely on species

specific primers or restriction fragment length analysis. The advantage of these methods is that they provide a fast yes-or-no answer to an identification attempt. However, in setting up such identification assays it is of crucial importance that the target species is properly characterized in advance, especially its intraspecific diversity. However, it appears that this often not the case. In this study we have tested species specific primers of Zijlstra *et al*. (2000) for *Meloidogyne javanica, M. incognita* and *M. arenaria*. For *M. javanica* and *M. incognita* we were unable to generate species-specific bands for several populations indicating that some isolates cannot be identified using these species specific primers (data not shown). We also confirmed the findings of Esbenshade & Triantaphyllou (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987) that *Meloidogyne arenaria* represents a species complex with a variety of isozyme phenotypes and most likely different ploidy levels (Chapter 2). Despite the fact that the *M. arenaria* is not a monophyletic group and cannot be molecularly delimited, species-specific primers for *M. arenaria* have been presented (Zijlstra et al., 2000). However, these species-specific primers were designed based on only four poorly-characterized populations of *M. arenaria* (Zijlstra et al., 2000). As demonstrated in this study, the complexity of the relationships among the *M. arenaria* lineages preclude the design of a reliable species specific primer combination and it is unclear which lineages of *M. arenaria* can be identified using currently known primer combinations. Indeed, several studies have already shown that *M. incognita, M. javanica* and *M. arenaria* primers are not truly species specific (Carneiro *et al.*, 2008; Devran & Sogut, 2009). Problematically, despite the clear limitations of species specific primers for root-knot nematodes, they are still widely used for the identification (Baidoo *et al.*, 2016; Neves *et al.*, 2017). Moreover, these primer combinations are used to develop new alternative identification strategies that rely on the same primer combination, for example in the application of loop-mediated isothermal amplification based identification for *Meloidogyne* (Niu *et al.*, 2011). Thus, the main problem is that several species-specific essays appear to be developed on a relatively limited number of populations (Correa *et al.*, 2013). Our results demonstrate that this is not a reliable methodology as species-specific essays can only be valid for well-known and clearly-characterized species, again highlighting the importance of fundamental research.

Regarding species identification in root-knot nematodes, especially *M. arenaria* remains difficult to define as it comprises a range of different lineages with variable mitochondrial

haplotypes in coding genes (Chapter 2) and noncoding genes (Pagan *et al.*, 2015). Moreover, *M. arenaria* contains lineages with different levels of polyploidy and a variety of isozyme phenotypes (Esbenshade & Triantaphyllou, 1985; Esbenshade & Triantaphyllou, 1987). A similar situation applies for the group of *Meloidogyne ethiopica*, *M. luci* and *M. inornata*, these species represent closely related mitochondrial haplotypes of lineages with highly variable chromosome numbers and variable isozyme patterns (Carneiro *et al.*, 2014). In order to elucidate whether all these lineages should be considered different species additional research is required, including a combination of genomic sequencing and conventional karyotype analysis. Especially as switches from sexual reproduction towards mitotic parthenogenesis appear to be linked with polyploidy and associated loss of meiosis (Chapter 3).

7 Genome evolution and loss of meiosis

One of the most striking feature of root-lesion and root-knot nematodes is their variability in genomic composition and associated variability in reproductive strategies (Roman & Triantaphyllou, 1969; Triantaphyllou, 1983; Triantaphyllou, 1985b; Triantaphyllou, 1985a; Castagnone-Sereno *et al.*, 2013). In this study, cytogenetic information in combination with a phylogenetic framework indicates two additional independent origins of polyploidy and associated mitotic parthenogenetic reproduction. Thus revealing at least five independent origins of mitotic parthenogenesis within root-knot nematodes (Chapter 3). Also evidence for reticulate evolution has been revealed for *M. arenaria* based on a combination of mitochondrial coding gene sequencing and isozyme electrophoresis (Chapter 2). As a result of whole genome sequencing of *Meloidogyne hapla* (Opperman *et al.*, 2008), *M. incognita* (Abad *et al.*, 2008) *M. floridensis* (Lunt *et al.*, 2014), *M. enterolobii* (Szitenberg *et al.*, 2017), *M. arenaria* (Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017) and *M. javanica* (Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017) we know now that the origin of mitotic parthenogenesis within clade I appears to be the result of a recent hybridization (Lunt *et al.*, 2014; Szitenberg *et al.*, 2017) which appears to compromise different ploidy levels (Blanc-Mathieu *et al.*, 2017). These different levels of polyploidy within clade I were already predicted based on genome size predictions (Lapp & Triantaphyllou, 1972; Pableo & Triantaphyllou, 1989) and cytological studies (Triantaphyllou, 1963; Triantaphyllou, 1966; Triantaphyllou & Hirschmann, 1980; Triantaphyllou, 1985a; Van der Beek *et al.*, 1998). Despite the polyploid nature of the nuclear

genome of several species of the MIG group, we found the mitochondrial genome to be very conserved (Chapter 2) and these findings have been confirmed by several other studies including genomic studies (Hugall *et al.*, 1994; Lunt, 2008; Fargette *et al.*, 2010; Pagan *et al.*, 2015; Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017). The conserved mitochondrial genome sequence within tropical root-knot nematodes, indicates that these putative hybrid species share a recent common maternal ancestor (Hugall *et al.*, 1994; Lunt, 2008; Fargette *et al.*, 2010; Pagan *et al.*, 2015; Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017).

In sharp contrast with the conserved nature of the mitochondrial genome stands the variability recovered in the nuclear genome as the large polyploid genomes were demonstrated to be composed of divergent homeologs (Lunt *et al.*, 2014; Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017). These recent studies also showed that species from the MIG group originated from a common hybridization event. This observation is in accordance with previous literature on comparative analysis of the ITS gene (Hugall *et al.*, 1999), and phylogenetic analysis of mitochondrial DNA and nuclear coding genes (Lunt, 2008; Fargette *et al.*, 2010). Interestingly, Szitenberg et al. (2017) found that *M. enterolobii* constitutes a separate origin of mitotic parthenogenesis in comparison to the other MIG species. Together with the newly discovered origins of mitotic parthenogenesis in Chapter 3 this brings the total number of independent origins of to at least six within the genus *Meloidogyne.*

In terms of genomic composition, Blanc-Mathieu et al. (2017) predicted *M. incognita* (triploid), *M. javanica* (tetraploid) and *M. arenaria* (tetra- to pentaploid) to have different ploidy levels. On the other hand, Szitenberg et al. (2017) predicted the genome of different MIG species to be hypotriploid. These contrasting results indicate that additional comparative genomic analysis are needed in order to clarify the exact genome structure of several species of the MIG. In either case, in this thesis we recovered cytological evidence for a triploid genome in several *Meloidogyne* species. Next to *M. africana* and *M. ardenensis* and some other species of clade I, also *M. oryzae* (Triantaphyllou, 1985b) was hypothesized to have a triploid genomic composition. The presence of triploid genomes is interesting as they could allow for polyploidization trough the triploid bridge pathway as described in plants (Blanc-Mathieu *et al.*, 2017). On the other hand triploid genomes have been described to show a tendency to slowly

evolve towards a diploid state, this would lead to a hypotriploid state as described by Szitenberg et al. (2017).

While a mitotic parthenogenetic way of reproduction is traditionally seen as an evolutionary dead end (Arrigo & Barker, 2012). In light of recent advances of genomic sequencing this view is slowly changing as it is recognized that under certain stress circumstances, polyploidy can have a distinct evolutionary advantage, for a review see Van de Peer et al. (2017). Moreover, accumulating evidence indicates that genome duplications played a crucial role in the evolution of plants (Adams & Wendel, 2005), animals (Simakov & Kawashima, 2016) and fungi (Kellis *et al.*, 2004). Also, among the tropical root-knot nematodes, despite the absence of meiosis, mitotic parthenogenetic species of the MIG were able to evolve in highly successful parasites, colonizing the majority of the planet (Trudgill & Blok, 2001). It was shown that the polyploid genomes of the MIG are highly dynamic and that variability between gene copies in a single genome can lead to different expression patterns (Blanc-Mathieu *et al.*, 2017), indicating that polyploid genomes have a lot of variability despite the loss of meiotic reproduction (Lunt *et al.*, 2014; Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017). Moreover, as shown by the numerous different origins of polyploidy within the genus it has been shown that genome duplication or hybridization plays an important role in the evolution of this genus. Most-likely genome variation in root-knot nematodes is not an unique phenomenon within Nematoda, as several other groups of nematodes show a high variability in chromosome numbers and reproductive strategies. Indeed, in this thesis we have shown that changes between asexual and sexual reproduction occur more frequently than previously anticipated in plant-parasitic nematodes. Within the *Penetrans* group it was shown that asexual lineages like *Pratylenchus pinguicaudatus*, *P. oleae*, *P. brachyurus*, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 did not form a monophyletic grouping, indicating independent evolution of asexual lineages (Chapter 4). Furthermore, *P. bolivianus* was found to exhibit two different morphotypes associated with sexual and asexual reproduction (Troccoli *et al.*, 2016). Ggiven that Roman & Triantaphyllou (1969) found that species of *Pratylenchus* also exhibit a large cytological variation in chromosome number, we can anticipate that polyploidy and associated changes in reproduction play an important role in the evolution of the genus *Pratylenchus*. Moreover, also in other groups of plant-parasitic nematodes like Heteroderidae similar polyploid genomic compositions

have been observed (Triantaphyllou & Hirschmann, 1980), indicating that chromosome evolution has played an important role in the evolution plant-parasitic nematodes in general.

While comparative genomic studies on root-knot nematodes of clade I have given insight in to the exciting evolutionary history of this clade (Lunt *et al.*, 2014; Blanc-Mathieu *et al.*, 2017; Szitenberg *et al.*, 2017). Several questions remain to be addressed. For example, within clade I, genomes of many species remain unknown. Of particular interest would be to include *M. ethiopica*, *M. luci*, *M. inornata* and more isolates of *M. arenaria* in a comparative genomic study. These species have a very interesting and closely related mitochondrial haplotypes, while their karyotype is highly variable (Chapter 2). Sequencing these lineages would allow more insight into the complex origins of the MIG and the evolution of closely related lineages. Also, the determination of the reproduction mode of *Meloidogyne* sp. 2 is of particular interest as it holds a distinct phylogenetic position based on mitochondrial gene sequences in comparison with the other MIG species (Chapter 2).

As initiated in Chapter 3, the chromosome composition of early diverging *Meloidogyne* spp. in comparison with its sister group *Pratylenchus* spp. remains to be further elucidates. Blanc-Mathieu *et al.* (2017) suggested the haploid chromosome number of n=18 as the ancestral haploid genome size for *Meloidogyne*. While the ancestral chromosome complement of clade I could well be n=18, the karyotypes of *M. mali* (n=12), *M. africana* (n=21) and *M. kikuyenis* (n=7) indicate the ancestral chromosome complement of the genus *Meloidogyne* to be smaller than n=18 (see Chapter 3 for a more extensive discussion). Most interestingly would be to combine the chromosome composition of early-branching *Meloidogyne* spp. with comparative genomics in order to provide insight into the evolutionary history of early branching root-knot nematodes.

8 General conclusion

- Chromosome evolution has played an important role in the evolution of root-knot and root-lesion nematodes, and probably plant-parasitic nematodes in general (Chapter 3).

- Mitotic parthenogenesis has multiple origins within *Meloidogyne* and *Pratylenchus*. Polyploidy is associated with mitotic parthenogenesis and is most likely established through genome duplications or hybridisation events, during this process meiosis is lost and lineages are reproductively isolated (Chapter 3 and 4).

- Mitochondrial coding gene sequencing provides a good strategy for species identification within tropical root-knot nematodes, as it is in agreement with isozyme electrophoresis based identifications (Chapter 2).

- Cytochrome oxidase 1 is a reliable barcode gene for the root-lesion nematodes of the *Penetrans* group. DNA barcoding is especially useful within the *Penetrans* group as morphometric characters fail to separate species (Chapter 4 and 5).

- Molecular species delimitation algorithms provide a useful approach for establishing and testing species hypothesis (Chapter 4).

- A strong link between DNA sequences and morphology is of crucial importance in order to safeguard the quality of sequence databases. Digital morphological vouchers present a great opportunity for linking sequences to morphology (Chapter 5).

- *Pratylenchus lentis* is a junior synonym of *P. pratenis* (Chapter 5).

- *Meloidogyne decalineata* and *M. oteifae* are a junior synonym of *Meloidogyne africana* (Chapter 3).

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Summary

The polyphagous plant-parasitic nematodes of the genera *Meloidogyne* and *Pratylenchus* are considered to among the most significant pests in agriculture worldwide. Despite the crucial need for correct diagnosis, identification of these pathogens remains problematic. The traditionally used diagnostic strategies for root-knot nematodes, including morphometrics, host-range tests, biochemical and molecular techniques, now appear to be unreliable due to the recently-suggested hybrid origin of root-knot nematodes. In Chapter 2, a new DNA barcode based identification essay was developed. In order to determine a suitable barcode region for these pathogens nine quickly-evolving mitochondrial coding genes were screened. Resulting haplotype networks revealed closely related lineages indicating a recent speciation, an anthropogenic-aided distribution through agricultural practices, and evidence for reticulate evolution within *M. arenaria.* Nonetheless, nucleotide polymorphisms harbor enough variation to distinguish these closely-related lineages. Furthermore, completeness of lineage sorting was verified by screening 80 populations from widespread geographical origins and variable hosts. Importantly, our results indicate that mitochondrial haplotypes are strongly linked and consistent with traditional esterase isozyme patterns, suggesting that different parthenogenetic lineages can be reliably identified using mitochondrial haplotypes. The study indicates that the barcode region Nad5 can reliably identify the major lineages of tropical root-knot nematodes.

In chapter 3, evolution of mitotic parthenogenesis is studied within the root-knot nematodes. During sampling of several *Coffea arabica* plantations in Tanzania severe root galling, caused by a root-knot nematode was observed. From pure cultures, morphology and morphometrics of juveniles and females matched perfectly with *Meloidogyne africana,* whereas morphology of the males matched identically with those of *Meloidogyne decalineata*. Based on their Cox1 sequence, however, the recovered juveniles, females and males were confirmed to belong to the same species, creating a taxonomic conundrum. Adding further to this puzzle, re-examination of *M. oteifae* type material showed insufficient morphological evidence to maintain its status as a separate species. Consequently, *M. decalineata* and *M. oteifae* are synonymized with *M. africana,* which is herewith redescribed based on results of light and scanning electron microscopy, ribosomal and mitochondrial DNA sequences, isozyme electrophoresis, along with bionomic and cytogenetic features. Multi-gene phylogenetic

analysis placed *M. africana* outside of the three major clades, together with *M. coffeicola*, *M. ichinohei* and *M. camelliae*. This phylogenetic position was confirmed by several morphological features, including cellular structure of the spermatheca, egg mass position, perineal pattern and head shape. Moreover, *M. africana* was found to be a polyphagous species, demonstrating that "early-branching" *Meloidogyne* spp. are not as oligophagous as had previously been assumed. Cytogenetic information indicates *M. africana* (2n=21) and *M. ardenensis* (2n=51-54) to be a triploid mitotic parthenogenetic species, revealing at least four independent origins of mitotic parthenogenesis within the genus *Meloidogyne*. Furthermore, *M. mali* (n=12) was found to reproduce by amphimixis, indicating that amphimictic species with a limited number of chromosomes are widespread in the genus, potentially reflecting the ancestral state of the genus. The wide variation in chromosome numbers and associated changes in reproduction modes indicate that cytogenetic evolution played a crucial role in the speciation of root-knot nematodes and plant-parasitic nematodes in general.

In Chapter 3 and 4, bacode based identification of root-lesion nematodes was studied. Morphological diagnosis of the more than 100 species of root-lesion nematodes is problematic due to the low number of diagnostic features, high morphological plasticity and incomplete taxonomic descriptions. In order to employ barcoding based diagnostics, a link between morphology and a species specific sequences has to be established. In chapter 4, we reconstructed a multi-gene phylogeny of the *Pratylenchus penetrans* group using nuclear ribosomal and mitochondrial gene sequences. A combination of this phylogenetic framework with molecular species delineation analysis, population genetics, morphometric information and sequences from type location material allowed us to establish the species boundaries within the *Penetrans* group and as such clarify long-standing controversies about the taxonomic status of *P. penetrans*, *P. fallax* and *P. convallariae*. Our study also reveals a remarkably amount of cryptic biodiversity within the genus *Pratylenchus* confirming that identification on morphology alone can be inconclusive in this taxonomically confusing genus.

In chaper 5, comprehensive morphological and molecular analyses revealed that published ITS sequences of the economically important plant-parasitic nematode *Pratylenchus goodeyi* are actually sequences from distantly free-living bacterivorous 'cephalobs'. We demonstrated that this incorrect labeling resulted in a cascade of erroneous interpretations, as shown by the reports of "*Pratylenchus goodeyi"* on banana in China and on cotton in India. This clearly illustrates the risk of mislabeled sequences on public databases. Other mislabeled *Pratylenchus* cases are discussed to illustrate that this is not an isolated case. Hereby, *P. lentis* is considered a junior synonym of *P. pratensis* while *P. flakkensis* was for the first time linked to DNA sequences using topotype material. The highlighted problem may even increase in the future as taxonomic expertise is decreasing and sequence-based identification is growing rapidly. A strong link between morphology and DNA sequences will be of crucial importance in order to prevent sequence-based misidentifications.

Samenvatting

Nematoden van de genera *Pratylenchus* en *Meloidogyne* worden wereldwijd beschouwd als één van de meest belangrijke pestsoorten in de landbouw. Ondanks het belang van correcte pest identificatie zijn er geen routine matige identificatie procedures voor handen. Traditioneel worden identificaties van wortelknobbel aaltjes gedaan met behulp van morfometrie, waardplant range testen, biochemische testen of moleculaire technieken. Recent is echter gebleken dat deze identificatie strategieën niet betrouwbaar zijn door de hybride oorsprong van veel tropische wortelknobbel nematoden. In hoofdstuk 2 van deze thesis werd een nieuwe identificatie methode ontwikkeld op basis van DNA barcoding van negen mitochondriale coderede genen. De resulterende haplotype netwerken tonen aan dat deze soorten zeer nauw verwant zijn, ook zijn deze soorten waarschijnlijk antropogeen verspreid zijn door middel van landbouw praktijken. Ook tonen deze haplotype netwerken aan dat *Meloidogyne arenaria* waarschijnlijk een hybride soort is. Toch is er voldoende nucleotide variatie aanwezig in de coderende genen van het mitochondriaal genoom om de verschillende soorten uit elkaar te houden. Ook waren DNA barcoding identificaties in overeenstemming met de traditioneel gebruikte isozym electroforese patronen. Van al de gescreende mitochondriale genen bleek Nad5 het meest bruikbaar voor barcode gebaseerde identificatie van tropische wortel knobbel aaltjes.

In hoofdstuk drie wordt de evolutie van mitotisch parthenogenetische voortplanting bestudeerd. Tijdens een staal name in Tanzania werden in verschillende zwaar aangetasten *Coffea arabica* plantages een ongekende wortelknobbel nematode terug gevonden. De morfologie van juvenielen en vrouwtjes was in overeenstemming met *Meloidogyne africana*, terwijl de mofologie van de mannetjes overeenkwam met *Meloidogyne decalineata*. Op basis van Cox1 sequenties werd echter bevestigd dat zowel juvenielen, vrouwtjes en mannetjes tot dezelfde soort behoorden. Als een gevolg hiervan werden *M. decalineata* en *M. oteifae gesynonimiseerd* met *M. africana.* Vervolgens werd een herbeschijving uitgevoerd op basis van verschillende genen, morfologie, mofometrie, scanning elektronen microscopie, isozym elektroforese en een cytogenetische studie. Fylogenetische analysen plaatse *M. africana* buiten de drie gekende clades van wortelknobbel aaltjes samen met *M. coffeicola, M. ichinohei* en *M. camelliae.* Deze basale positie werd bevestigd met behulp van morfologische kenmerken, cellulaire structuur

van de spermatheca, morfologie van de eimassas en mofologie van het periniale patroon. Daarnaast bleek dat *M. africana* in staat was om te parasiteren op verschillende gewassen. Met behulp van cytogenetische studies werd aangetoond dat *M. africana* (2n=21) en *M. ardenensis* (2n=51-54) triploide mitotische parthenogenetische soorten zijn terwijl *M. mali* (n=12) een seksuele soort bleek te zijn met een gelimiteerd aantal chromosomen. Aangezien deze seksuele soorten met een beperkt aantal chromosomen veel voorkomen binnen het genus, zou dit erop kunnen wijzen dat dit de ancestale situatie weergeeft. In deze studie werd ook aangetoond dat mitotisch parthenogenetische voortplanting minstens vier keer onafhankelijk is ontstaan binnen het genus *Meloidogyne*. Hieruit blijkt dat cytogenetische evolutie een cruciale rol heeft gespeeld tijdens de speciatie van het genus *Meloidogyne* en plant parasitaire nematoden in het algemeen.

Ook binnen het genus *Pratylenchus* is het bijzonder moeilijkheden om de meer dan 100 verschillende pest soorten te identificeren. Dit komt vooral door het gebrek aan morfologische kenmerken, morfologische plasticiteit en onvolledige morfologische beschrijvingen. Om DNA gebaseerde identificatie mogelijk te maken is het belangrijk om een link te maken tussen voormalig op basis van morfologie beschreven soorten te linken aan DNA sequenties. In hoofdtuk vier werd een fylogenie gemaakt op bis van verschillende genen van de *Pratylenchus penetrans* groep. Door de combinatie van mofologie, fylogenie, moleculaire soortsaflijning en populatie genetica konden verschillende taxonomische problemen worden opgelost. In deze studie werd een grote hoeveelheid cryptische biodiversiteit terug gevonden. Ook werd bewezen dat identificatie op basis van morfometrie niet mogelijk is. Daarnaast werd aangetoond dat DNA barcode gebaseerde identificatie met behulp van het Cox1 bijzonder betrouwbaar was.

In hoofdstuk vijf werd aangetoond dat gepubliceerde ITS sequenties van de economisch belangrijke plant parasiet *Pratylenchus goodeyi* eigenlijk behoren tot de vrij levende bacteriovore nematoden van de familie Cephalobidae. Deze foutieve identificatie zorgde voor een domino effect aan foutieve interpretaties en misidentificaties van *P. goodeyi* op banaan in China en op katoen in India. Deze misidentificaties maken duidelijk hoe gevaarlijk misgeidentificeerde sequenties zijn in publieke databanken. Dit blijkt ook nog uit enkele andere voorbeelden, zo wordt *P. lentis* gesynonimiseerd met *P. pratensis*, terwijl *P. flakkensis* voor de eerste maal gelinkt wordt met DNA sequenties. Het probleem van misgeidentificeerde sequenties in publieke databanken zal enkel toenemen in de toekomst omdat sequentie

gebaseerde identificaties snel aan populariteit wint terwijl morfologische expertise langzaam verdwijnt. Toch is een sterke link tussen morfologie en DNA sequenties bijzonder belangrijk voor het correct identificeren van pest soorten.

Curriculum vitae

Publications as first author:

1. **Janssen T.**, Vizoso D.B., Schulte G., Littlewood D.T.J., Waeschenbach A., Schärer L. 2015. The first multi-gene phylogeny of the Macrostomorpha sheds light on the evolution of sexual and asexual reproduction in basal Platyhelminthes. Molecular Phylogenetics and Evolution 92, 82-107. http://www.sciencedirect.com/science/article/pii/S1055790315001748 Cited by 5, IF 2014: 3.916, Q1 10/46.
2. **Janssen T.**, Karssen G., Verhaeven M., Coyne D., Bert W. 2016. Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. Scientific reports 6, 22591. http://www.nature.com/articles/srep22591 Cited by 11, IF 2014: 5.578, Q1 5/57.
3. **Janssen T.**, Karssen G., Topalović O., Coyne D., Bert W. 2017. Integrative taxonomy of root-knot nematodes reveals multiple independent origins of mitotic parthenogenesis. PLoS ONE 12(3). http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0172190 Cited by 2, IF 2015: 3.057, Q1 11/63.
4. **Janssen T.**, Karssen G., Orlando V., Subbotin S.A., Bert W. 2017. Molecular characterization and species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the *Penetrans* group (Nematoda: Pratylenchidae). Accepted with minor revisions in Molecular Phylogenetics and Evolution. IF 2016: 4.419, Q1: 32/166.
5. **Janssen T.**, Karssen G., Couvreur M., Waeyenberge L., Bert W. 2017. The pitfalls of molecular species identification: a case study within the genus *Pratylenchus* (Nematoda: Pratylenchidae). Accepted with minor revisions in Nematology. IF 2016: 1.162, Q2: 73/162.

Publications as co-author:

6. Zhanga S.L., Liu G.K., **Janssen T.,** Zhanga S.S., Xiaoa S., Lia S.T., Couvreur M. and Bert W. 2013. A new stem nematode associated with peanut pod rot in China morphological and molecular characterization of *Ditylenchus arachis* n. sp. (Nematoda Anguinidae). Plant Pathology 63, 1193-1206. Cited by 11, IF 2013: 2.969, Q1 7/79.

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8. Reygel P., Schockaert E., **Janssen T**. and Artois J.T. 2014. Two new species of *Carcharodorhynchus* Meixner, 1938 (Platyhelminthes: Rhabdocoela: Schizorhynchidae) from Brazil and Lanzarote. Marine biodiversity 44, 279-285. Cited by 4, IF2014: 1.104, Q3 25/44.
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12. Troccoli A., Subbotin S.A., Chitambar J.J., **Janssen T.**, Waeyenberge L., Stanley J.D., Duncan L.W., Agudelo P., Múnera Uribi G.E., Franco J. and Inserra R.N. 2016. Characterisation of amphimictic and parthenogenetic populations of *Pratylenchus bolivianus* Corbett, 1983 (Nematoda: Pratylenchidae) and their phylogenetic relationships with closely related species. Nematology, 00, 1-28. Cited by 3, IF 2014: 1.239, Q2 64/154.
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Symposia contributions

Oral symposia contributions as first author:

1. **Janssen, T.**, Vizoso D.B., Littlewood D.T.J., Waeschenbach A., Schulte G., Schärer L. 2012. The first comprehensive molecular phylogeny of the Macrostomorpha. 6th International Macrostomum meeting. Basel, Switserland. November 16 – November 18, 2012. Member of the organizing committee.

2. **Janssen, T.**, G. Karssen, M. Couvreur, and W. Bert. 2013. Phylogeny, taxonomy and evolution of reproduction within the genus *Pratylenchus*: a multi-gene approach combined with karyotyping. *10th International Nematology Symposium*. Moscow, Russia. June 30-July 5, 2013.
3. **Janssen, T.**, Karssen, G., Waeyenberge, L., Couvreur, M. & Bert, W. 2014. Providing a link between morphological descriptions and DNA barcoding to study speciation of the genus *Pratylenchus* in a robust phylogentic framework. *Journal of Nematology* 46, 180-180. *6th International Congress of Nematology*. Cape Town, South-Africa. May 4-May 9, 2014.
4. **Janssen, T.**, Vizoso D.B., Littlewood D.T.J., Waeschenbach A., Schulte G., Schärer L. 2014. The first multi-gene phylogeny of the Macrostomorpha sheds light on the evolution of sexual and asexual reproduction in basal Platyhelminthes. *8th International Macrostomum meeting*. Basel, Switserland. November 28-November 30, 2014.
5. **Janssen, T.**, Karssen, G. & Bert, W. 2015. Mitochondrial single nucleotide polymorphisms in mitochondrial coding genes are useful for identification of different parthenogenetic lineages of root-knot nematodes. *67th International Symposium on Crop Protection.* Ghent, Belgium. May 19, 2015.
6. **Janssen, T.**, Karssen, G., Orlando, V. & Bert, W. 2016. Multi-gene phylogenetics in combination with morphology allow species delineation and DNA barcode based diagnostics within a cryptic Pratylenchus species complex. *68th International Symposium on Crop Protection.* Ghent, Belgium. May 17, 2016.
7. **Janssen T.**, Karssen G., Verhaeven M., Coyne D., Bert W. 2016. Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *32nd European Nematology Society meeting*. Braga, Portugal. August 28-September 1, 2016.

Co-author symposia contributions:

8. Bert, W., **Janssen T.**, D. Slos, A. Y. Kolombia, M. Claeys, M. Couvreur, C. Mermans, P. Ensafi, J. S. Quisado, Q. Xue, G. Karssen, V. V. Yushin, and W. Decraemer. 2013. Nematode morphology, taxonomy and phylogeny at Ghent University: a diversity of integrated toolkits. *10th International Nematology Symposium*. Moscow, Russia. June 30-July 5, 2013.

9. Gheysen, G., Kyndt, T., De Carvalho, F. S., Hofte, M., Bert, W., **Janssen, T.**, Mibey, R. K. & Njira, P. N. 2014. Analysis of endophytic fungi and plant-parasitic nematodes from irregated and upland rice ecosystems in Kenia. *Journal of Nematology* 46, 167-168. Journal of Nematology. *6th International Congress of Nematology*. Cape Town, South-Africa. May 4-May 9, 2014.

10. Bert, W., Slos, D., **Janssen, T.**, Fonderie, P., Steel, H. & Decraemer, W. 2014. Recent methodological and theortical advances for species delimitation in contemporary nematode taxonomy. Journal of Nematology 46, 139-139. Cape Town, South-Africa. May 4-May 9, 2014.

11. Sánchez-Monge, A., **Janssen, T.** & Bert, W. 2015. Reverse taxonomy, phylogeny and DNA barcoding to illuminate the diversity of *Aphelenchoides*. *ONTA 47th Annual Meeting.* Varadero, Cuba. May 17-22, 2015.

Attended symposia:

12. 7th International Macrostomum meeting. Groningen, The Netherlands. November 29-December 1, 2013.
13. 65th International Symposium on Crop Protection. Ghent, Belgium. May 21, 2013
14. 66th International Symposium on Crop Protection. Ghent, Belgium. May 20, 2014
15. Symposium on next generation sequencing. Brussels, Belgium. October, 2013

Awards

1. A grant to attend the 6th International Congress of Nematology (Cape Town, South-Africa) was awarded by the International Society of Nematologists. May 4-May 9, 2014.
2. A grant to attend the 32nd European Nematology Society meeting (Braga, Portugal) was awarded by the European Society of Nematologists. August 28-September 1, 2016.

Peer Reviewer on invitation for the following scientific journals

1. Molecular Phylogenetics and Evolution
   Elsevier publishing
   IF 2014: 3.916, Q1 10/46, ISSN: 1055-7903
2. Nematology
   Brill publishing
   IF 2014: 1.239, Q2 64/154, ISSN: 1388-5545
3. Scientific Reports
   Nature publishing group
   IF 2014: 5.578, Q1 5/57, ISSN 2045-2322

Teaching

Practical assistant for the following courses at Ghent University:

Animal Cell Biology and Histology (J000305)

General Techniques in Nematology (C002805)

Quarantine Nematodes (C003679)

Functional evolution of invertebrates (C002766)

Mentor of the following thesis students:

1. Mermans, C. 2013. Biology meets development cooperation: characterization of plant-parasitic nematodes in Tanzania. Master Biology, UGent.
2. Pisman, M. 2013. Nematoden uit extreme habitats, de missing-link in nematoden fylogenie. Bachelor Biology, Ugent.
3. Vandewaerde, J. 2014. optimization of DNA extraction methods for mitochondrial genome sequencing of plant-parasitic nematodes. Master in Industrial Engineering KuLeuven.
4. Verhaeven, M. 2014. Biology meets development cooperation: Characterization of plant-parasitic nematodes in Africa. Master Biology, UGent.
5. Nyiragatare, A. 2014. Morphological and molecular characterization of plant parasitic nematodes in Rwandan crops. Master of Nematology, UGent.
6. Zia, A. 2014. Morphological and molecular characterization of plant parasitic nematodes of the genus *Meloidogyne* from Pakistan. European Master of Nematology, UGent.
7. Esimo Beriso, A. 2014. Morphological and molecular characterization of plant parasitic nematodes associated with maize field in southeastern of Ethiopia. Master of Nematology, UGent.
8. Maphane, T. 2014 Morphological and molecular characterization of plant parasitic nematodes from southern Botswana. Master of Nematology, UGent.
9. Orlando V. 2015. Morphological and Molecular characterization of plant parasitic nematodes from Sicily (Italy). Master of Nematology, UGent.
10. Topalovic, O. 2015. To unravel the status of a population of *Meloidogyne* sp. in Ireland. Master of Nematology, UGent.
11. Dung Pham, K. 2016. Plant parasitic nematodes from Vietnam. Master of Nematology, UGent.