Morphological, biochemical and molecular approaches to the identification of *Meloidogyne incognita*, *Meloidogyne javanica* and *Meloidogyne arenaria* in Portugal

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Nowadays people know the price of
Everything and the value of nothing!
"Oscar Wilde"

Cuando la gratitud es tan absoluta
las palabras sobran.
"Alvaro Mutis"

Quem não sabe a arte,
Não a estima.
"Luís Vaz Camões"

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Love always
Leidy

Resumo

Os nemátodes-das-galhas-radiculares, *Meloidogyne* spp., causam anualmente prejuízos de milhões de euros, não só pela redução da quantidade mas também da qualidade dos produtos agrícolas. Assim, o principal objetivo deste trabalho consistiu em contribuir para o desenvolvimento de uma metodologia robusta de diagnóstico e enquadrada nos protocolos da EPPO para identificação das três principais espécies do nemátode das galhas (*M. incognita, M. arenaria* e *M. javanica*) nos Laboratorios de Genetica Molecular e Nematologia no INIAV Portugal. Para a verificação do material de referência, foram realizadas observações e respetivas medições das estruturas morfológicas dos juvenis de segundo estádio, assim como estudos moleculares. Não foi possível efetuar estudos bioquímicos com estas amostras, uma vez que não havia disponibilidade de fêmeas adultas. Os resultados obtidos nos estudos morfológicos e biométricos não foram conclusivos. Relativamente ao estudo molecular, também não foi possível identificar corretamente as três espécies.

Com o objetivo de identificar as espécies de *Meloidogyne* presentes em amostras da região Oeste de Portugal, foram efetuados estudos morfológicos, bioquímicos e moleculares. A morfologia do padrão perineal apontou a presença de duas espécies, *M. incognita* e *M. arenaria,* mas devido à variabilidade dos padrões perineais esta caracterização não foi considerada conclusiva. O mesmo aconteceu com as análises enzimáticas e moleculares. Os mesmos estudos foram conduzidos em material originário do Norte de Portugal. Os resultados morfológicos sugeriram a presença de *M. javanica,* resultado que foi confirmado bioquimicamente e por biologia molecular. Foram ainda realizadas amplificações por PCR usando *primers* universais para nematodes das amostras de referência e do Oeste de Portugal, com o objetivo de sequenciar os produtos PCR que apresentassem uma banda única do tamanho expectável. As sequências obtidas foram alinhadas e analisadas resultando uma homologia entre as três espécies em estudo e com outras que não pertencem ao género *Meloidogyne*. Embora não se tenha conseguido definir uma ferramenta de diagnóstico rápida e precisa para a identificação das três principais espécies do género *Meloidogyne*, a realização deste estudo permitiu determinar que a adequada identificação destes nemátodes só é possível mediante a conjugação dos três métodos de diagnóstico - morfológico, bioquímico e molecular.

Palavras-chave: Nemátodes-das-galhas-radiculares, *Meloidogyne*, padrões perineais, métodos bioquímicos, PCR.

Abstract

Plant parasitic nematodes are highly damaging pests in many crops of great economic importance. A substantial part of this damage is caused by infestations of root-knot nematodes (RKN), due to their wide geographical distribution as well as a vast range of host plants. The most important and widely distributed root-knot nematode species are *Meloidogyne incognita, M. arenaria* and *M. javanica* accounting for almost 95% of the occurrences, and so, an accurate and reliable identification it is primary to establish effective, sustainable and environmentally safe control measures.

The main goals of this study were to characterise morphologically, biochemically and molecularly Portuguese isolates and reference material from Netherlands Food and Consumer Product Safety Authority (NVWA), Wageningen, The Netherlands, in order to find the most suitable tools that can be used in the Laboratory of Molecular Genetics and Nematology at the INIAV in Portugal to assist in the identification of the three most common species of *Meloidogyne*. Morphological studies incorporated the examination of female's perineal pattern and measurements of second stage juveniles' features, which presented enough variation to confirm the unreliability and difficulty of morphological identification alone. Biochemical assays with isozymes such as esterases (EST) were performed on females and the PAGE enzymatic patterns obtained match those already described for *M. javanica*. Molecular analysis included PCRs using universal primers that target the 28S gene, ITS and IGS ribosomal DNA regions (rDNA) and the region between the cytochrome oxidase (COII) of the mitochondrial DNA (mtDNA) and 16S rDNA genes together with species-specific SCAR primers and sequencing and cloning. The sequences obtained from sequencing of PCR fragments and from cloned fragments were aligned and compared to those found in GenBank database through BLAST analysis with inconclusive results.

Although it was no possible to uncover an efficient tool for *Meloidogyne* identification this study pinpointed that the three approaches for root-knot nematode identification need to be used if we are to identify this species accurately and effectively. What's more this research can be used as platform for further studies regarding this species at the Nematology of INIAV Portugal.

Key words: Root-knot nematode, *Meloidogyne* spp., perineal patterns, esterase phenotype, PCR.

Resumo alargado

Os nemátodes parasitas de plantas são conhecidos pelos efeitos nocivos causados em muitas culturas economicamente importantes. Estes fitoparasitas podem ser ectoparasitas, vivendo fora do hospedeiro e alimentando-se a partir do exterior da raiz, ou endoparasitas, vivendo parte do seu ciclo de vida dentro de órgãos da planta hospedeira.

Os nemátodes-das-galhas-radiculares, *Meloidogyne* spp., são nemátodes endoparasitas sedentários de grande importância económica, parasitando praticamente as raízes de todas as plantas vasculares, e estando amplamente distribuídos tanto em regiões de clima quente e tropical como em regiões de clima temperado. Estes nemátodes causam anualmente prejuízos de milhões de euros, não só pela redução da quantidade mas também da qualidade dos produtos agrícolas.

Entre as espécies do género *Meloidogyne* as mais importantes são *Meloidogyne incognita, Meloidogyne arenaria* e *Meloidogyne javanica,* as quais se encontram amplamente distribuídas nas várias zonas geográficas, sobretudo nas regiões tropicais, representando 95% do total das ocorrências deste género. Por isso, a identificação correta destas espécies é essencial para a implementação de novas e efetivas estratégias de controlo.

Inicialmente, a identificação do género *Meloidogyne* era apenas baseada nas características morfológicas e biométricas. Contudo, além destas espécies serem morfologicamente semelhantes, apresentam uma grande variabilidade das características para diagnóstico dos juvenis de segundo estádio e dos padrões perineais das fêmeas, tornando a identificação morfológica difícil e laboriosa, mesmo para os especialistas. Por conseguinte, para ultrapassar estas as dificuldades, o padrão de atividade isoenzimática, nomeadamente a análise do polimorfismo das esterases de fêmeas, tem sido considerado um parâmetro bastante fiável. No entanto, esta metodologia só pode ser aplicada a fêmeas adultas. Havendo a necessidade de identificar as espécies de *Meloidogyne* em todas as fases do seu ciclo, têm sido desenvolvidas metodologias moleculares como alternativa.

Assim, o principal objetivo deste trabalho consistiu em contribuir para o desenvolvimento de uma metodologia robusta de diagnóstico e enquadrada nos protocolos da EPPO para identificação das três principais espécies do nemátode das galhas (*M. incognita, M. arenaria* e *M. javanica*) nos Laboratorios de Genetica Molecular e Nematologia no INIAV Portugal.

Visando a produção de material biológico para realização dos estudos morfológicos, biométricos, bioquímicos e moleculares, foram inoculadas plantas de tomate *Lycopersicum solanum* cv. Rio Grande com juvenis de segundo estádio e raízes infetadas provenientes do NVWA - Netherlands Food and Consumer Product Safety Authority, Wageningen. As plantas foram mantidas na estufa de quarentena durante quatro meses, após os quais as raízes e o solo foram avaliados, confirmando-se a ausência de galhas nas raízes e formas móveis no solo.

Para a verificação do material de referência proveniente de Wageningen, foram realizadas observações e respetivas medições das estruturas morfológicas dos juvenis de segundo estádio, assim como estudos moleculares. Não foi possível efetuar estudos bioquímicos com estas amostras, uma vez que não havia disponibilidade de fêmeas adultas. Os resultados obtidos nos estudos morfológicos e biométricos não foram conclusivos, dado que os valores das medições se sobrepõem e a similaridade das características morfológicas das três espécies é muito elevada. Relativamente ao estudo molecular, também não foi possível identificar corretamente as três espécies.

Com o objetivo de identificar as espécies de *Meloidogyne* causadoras de galhas nas raízes de tomateiros provenientes da região Oeste de Portugal, foram efetuados estudos morfológicos, bioquímicos e moleculares utilizando as fêmeas que foram retiradas das raízes de cinco plantas diferentes. A morfologia do padrão perineal apontou a presença de duas espécies, *M. incognita* e *M. arenaria,* mas devido à variabilidade dos padrões perineais esta caracterização não foi considerada conclusiva. O mesmo aconteceu com as análises enzimáticas e moleculares. Por isso, não foi possível identificar a ou as espécies presentes nestas amostras. Os mesmos estudos foram conduzidos em material originário do Norte de Portugal. Os resultados morfológicos sugeriram a presença de *M. javanica,* resultado que foi confirmado bioquimicamente através da análise dos padrões da isoenzima esterase e por biologia molecular através da PCR com *primers* específicos para *M. javanica*.

Foram ainda realizadas amplificações por PCR usando *primers* universais para nemátodes das amostras de referência e do Oeste de Portugal, com o objetivo de sequenciar os produtos PCR que apresentassem uma banda única do tamanho expectável. As sequências obtidas foram alinhadas e analisadas resultando uma homologia entre as três espécies em estudo e com outras que não pertencem ao género *Meloidogyne.*

Adicionalmente, os produtos PCR obtidos com os *primers* TW81/AB28 foram clonados e posteriormente sequenciados, tendo-se obtido resultados semelhantes aos anteriormente referidos.

Embora não se tenha conseguido definir uma ferramenta de diagnóstico rápida e precisa para a identificação das três principais espécies do género *Meloidogyne*, a realização deste estudo permitiu determinar que a adequada identificação destes nemátodes só é possível mediante a conjugação dos três métodos de diagnóstico - morfológico, bioquímico e molecular. De salientar a importância da manutenção das populações de *Meloidogyne* spp em estufa e a adequada colheita e preparação das amostras para as análises bioquímica e molecular. A experiência adquirida ao longo deste trabalho servirá como conhecimento de base para futuros estudos na Nematologia do INIAV envolvendo os nemátodes-das-galhas-radiculares.

Palavras-chave: Nemátodes-das-galhas-radiculares, *Meloidogyne*, padrões perineais, fenótipo de esterase, PCR.

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bp:	Base pairs
DNA:	Desoxyribonucleic Acid
rDNA	Ribosomal DNA
dNTP:	Deoxynucleotide triphosphate
EPPO:	European and Mediterranean Plant Protection Organization
INIAV:	Instituto Nacional de Investigação Agrária e Veterinária, I.P.
IPM:	Integrated pest management strategies
J2:	Second Stage Juvenile
NVWA:	Netherlands Food and Consumer Product Safety Authority, Wageningen
PAGE:	Polyacrylamide Gel Electrophoresis
PCR:	Polymerase Chain Reaction
RFLP:	Restriction Fragment Length Polymorphism
RNA:	Ribonucleic Acid
RKN:	Root-Knot Nematodes
SCAR:	Sequence Characterised Amplified Regions

General Introduction

The phylum Nematoda comprises a large number of described species, many of which are parasites of animals or plants as well as many free-living species (Cobb, 1915). Nematodes are a major eukaryotic group on earth, due to their ability to adapt to hostile and changing environmental conditions (Abad & Williamson, 2010).

The plant-parasitic nematodes feed on roots or parasitize the aerial portions of the plant. According to their mode of infection, the root parasites are classified as ectoparasites (feed from outside the host root) or as endoparasites (boring into and feeding from inside the host root) and, at the same time, they are classified as mobile (migrating while feeding) or sedentary (stationary while feeding). By far the most economically important group of plant parasitic nematodes is the sedentary endoparasites (Sasser & Freckman 1987; Koenning *et al*., 1999; Chitwood, 2003). This group is composed of two taxonomic groups, commonly referred as the cyst nematodes (*Heterodera* and *Globodera* spp.) and root-knot nematodes (*Meloidogyne* spp.*)*.

The root-knot nematodes (RKN) are members of the genus *Meloidogyne*, their name is of Greek origin and means 'apple-shaped female'. They are a group of highly adapted obligate plant parasites and their control presents a major global challenge (Karsen & Moens, 2006). Their presence was first noted on plants in the early 1800's when the Englishman M.J Berkeley in 1855 correlated the galls on cucumber roots with nematodes (Hunt & Handoo, 2009). Long after, in the early period of nematode taxonomy (1879-1948), RKN were mostly placed in the same genus as cyst nematodes, until 1949 when Chitwood separated RKN from cyst nematodes. He reassigned these species to the genus *Meloidogyne*, which was first named by Göeldi in a paper, published in 1887 and reprinted in 1892 (Hirschmann, 1985; Karssen, 2002).

Amongst the many genera of nematodes having some economic impact, *Meloidogyne* spp. are responsible for a large part of the annual multibillion losses attributed to nematode damage (Sasser *et al*., 1987). Economic losses due to *Meloidogyne* spp. are not only confined to yield reductions but also to an increase on production costs for farmers. Apart from these direct losses, many indirect losses such as waste of irrigation, water and fertilizers can occur. Moreover, during the last few years in Europe, chemical treatments have been restricted, limiting the management options or obliging farmers to apply more expensive control measures (Wesemael *et al*., 2011).

Aim of this study

The identification of *Meloidogyne* species has become increasingly important for the design of effective nematode management practices such as crop rotation and plant resistance which require precise species identification as well as for quarantine purposes (Hussey, 1990; Zijlstra 2000; Zijlstra & Van Hoof, 2006).

According to Karssen & Moens (2006), the best approach for a reliable identification is to integrate morphology, morphometrics, isozyme and DNA data, together with information on mode of reproduction, host plants and distribution. Therefore, the main purpose of this study was to develop efficient and robust tools in the Laboratory of Molecular Genetics and Nematology at INIAV (National Reference Laboratory of Plant Health) to assist in the identification of the most common and economically important species of *Meloidogyne* in Portugal. This development plays a crucial role since it helps to provide valid results to the requested analyses of samples of *Meloidogyne* species present in Portugal fields.

Based on the above, the overall objectives of the present work were:

- Morphological characterisation of second stage juveniles and females from reference material and North and West Portugal samples.
- Biochemical identification using analysis of the esterase phenotype of specimens from North and West Portugal as well as reference samples.
- Molecular identification of reference material and North and West Portugal samples using PCR with universal and species specific primers.

The Genus *Meloidogyne*

In an 1887 paper (reprinted in 1892) Göeldi described different diseases affecting coffee plants in the Rio de Janeiro province including the root-knot nematodes (RKN). He named the nematode *Meloidogyne exigua* and described and illustrated it briefly. His work is within the first findings on this nematode, highlighting its significance as causing serious problems in crops of high economic importance (Lordello & Lordello, 1983; Karssen 2002). From this description, Chitwood obtained the name we currently use for the RKN nematodes (Mitkowski, & Abawi, 2003). Root-knot nematodes are one of the oldest known parasitic nematodes of plants and have been of interest to nematologists worldwide probably due to their widespread distribution and success as parasites of economically important crops (Dong *et al*., 2001; Trudgill & Blok, 2001).

This genus comprises more than 80 species (Karssen, 2002) and on a worldwide basis includes the plant parasitic nematodes most economically damaging to crop protection. *Meloidogyne incognita, Meloidogyne javanica, Meloidogyne arenaria, Meloidogyne chitwoodi, Meloidogyne fallax* and *Meloidogyne hapla* account for more than 95% of the occurrences of this genus and are the most widely distributed species. Their wide host ranges enhance the impact of these species; the most common species are estimated to be able to infect more than 5500 plant species (Trudgill & Blok, 2001).

Meloidogyne incognita, M. javanica and M. arenaria are highly abundant in tropical climates but also in greenhouses of temperate regions; *M. chitwoodi*, *M. fallax* and *M. hapla* are major species in temperate climate. Furthermore, in respect of changing global trade pattern and crop production system, *M. minor* and *M. enterolobii* species are becoming emerging threats (Wesemael *et al*., 2011) for the temperate and tropical region respectively. As a result, the European and Mediterranean Plant Protection Organization (EPPO) has reported *Meloidogyne chitwoodi*, *M. fallax* and *M. enterolobii* as quarantine pests (EPPO, 2016).

In Portugal, nematology studies started in 1881 when Moraes observed for the first time the formation of galls in vine roots while working on a survey in Phylloxera (Reis, 1970). The description made by Moraes (1882) corresponds to the *Meloidogyne* genus and he also referred its occurrence to different parts of the country and to be always associated to *Vitis vinifera* L.

Many other references regarding this nematode appeared, until 1962 when Lima identified the first species in Portugal as being *Meloidogyne incognita* and *M. javanica* in peach

orchards. According to Lima (1962) the RKN were the most important plant parasitic nematodes due to its widespread distribution in the country.

Some other species of this genus have been found alone or in mixed populations in different regions of the centre and south, associated with several and important cultivated plants (Abrantes *et al.*, 2008; Maleita *et al*., 2011). Also, the presence of *M. chitwoodi* a species of quarantine was detected during a survey for potato cyst nematodes (PCN) *Globodera* spp. on two samples, one from Porto and one from the island of Madeira (Da conceição *et al*., 2009).

Life cycle and biology

Nematode growth and reproduction depend on the establishment of specialized feeding sites within the root. Consequently, these nematodes do not kill the host cells from which they feed. Instead, they induce a differentiation process that leads to the formation of giant cells. Each RKN triggers the development of five to seven giant cells (Christie, 1936; Jones, 1981; Abad *et al*., 2003; Jones & Goto, 2011).

Most *Meloidogyne* species have a similar life cycle (Figure 1) and their reproduction mode vary considerably, with a few *Meloidogyne* species producing cross-fertilised eggs after copulation (amphimictic), others reproducing by cross-fertilisation or meiotic parthenogenesis (automictic) and the last and most successful using obligatory mitotic parthenogenesis (apomictic) which is the case of the species under study (Triantaphyllou, 1985; Castagnone-Sereno, 2006; Jones & Goto, 2011). In this last form of reproduction there is neither reduction nor fusion of nuclei, and the egg directly develops into an embryo. When males are present, they can inseminate females, but the sperm nucleus degenerates and does not participate in fertilization (Triantaphyllou, 1962, 1963, 1981). Apomictic RKN species are diploid, triploid and sometimes tetraploid exhibiting a high response capacity to environmental selection (Castagnone-Sereno, 2002).

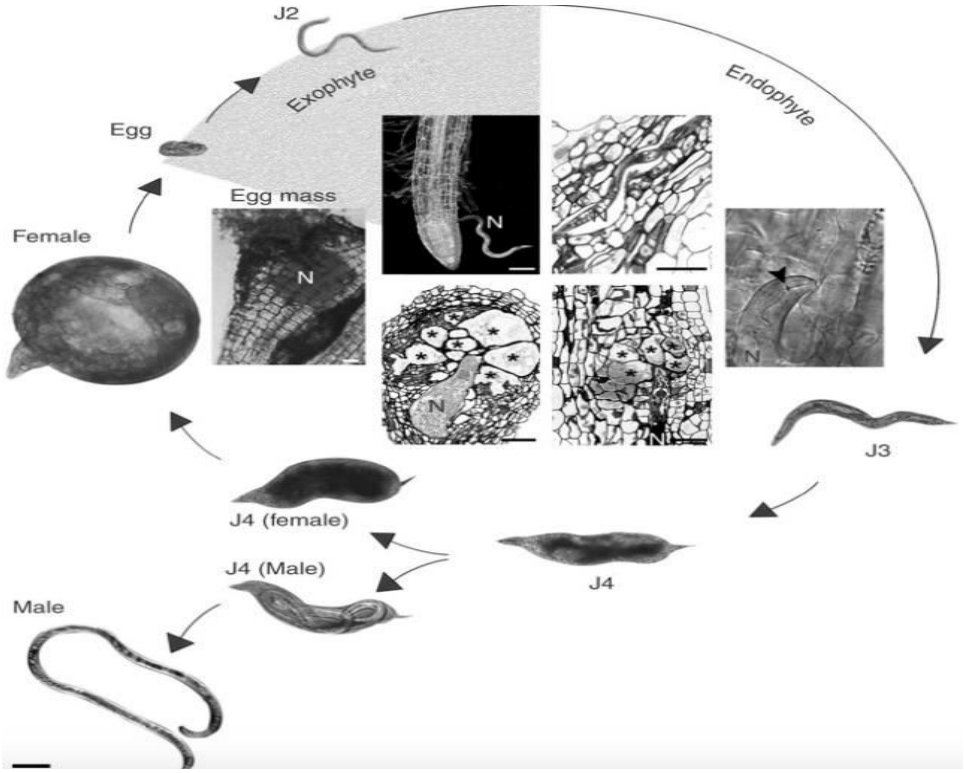

Figure 1 - Life cycle of root-knot nematodes, *Meloidogyne* spp.. N: Giant cells. (Adapted from Abad *et al.,* 2008).

Their life cycle begins when they hatch from eggs that are deposited in gelatinous masses on the soil surrounding a plant root. The mobile second-stage (J2s) hatches from the eggs searching for a host plant (Jones & Payne, 1978; Hussey, 1985; Jones & Goto, 2011; Saucet *et al*, 2016). Generally, they enter into the host tissue near the root tip by physical means (stylet) as well as cell wall degrading enzymes (Wyss *et al*., 1992; Wieczorek *et al*., 2014). They move intercellularly in the cell wall compartment towards root tip and turn around when they reach the apical meristem cells and move further to reach the differentiating vascular zone (Bird, 1961; Wyss *et al*., 1992; Goto *et al*., 2013; Saucet *et al*., 2016). There, they look for cells to induce them as multinucleate giant cells (Bird, 1961; Jones & Payne, 1978; Jones, 1981; Jones & Goto, 2011; Bartlem *et al*., 2014). For their growth and development, they take nutrients from these cells and follow three successive moults to be adults, the third and fourth stages do not feed and are short in duration. Females remain sedentary and are pear to globose in shape and occasionally vermiform males develop and migrate out of the roots (Abad *et al*., 2008).

Temperature is known to be influential on life cycle and biology of these nematode species'; activities such growth and development, mobility, infection capability and hatching are affected by its surrounding temperature. For instance, sex is determined by environmental conditions, with the frequency of males increasing in conditions of crowding or poor nutrition (Triantaphyllou, 1985; Tzortzakakis & Trudgill, 2005).

Symptoms

The primary symptom of RKN infection is the formation of typical galls on the roots of susceptible host plants (Figure 2). Nutrient and water uptake are substantially reduced because of the damaged root system, resulting in weak and poor-yielding plants (Abad *et al*., 2003).

Figure 2 - Root galls formed by *Meloidogyne*.

During parasitism, RKN establish and maintain an intimate relationship with their host. Severe infections result in reduced yields on numerous crops and can also affect consumer acceptance of many plants, including vegetables. The degree of root galling generally depends on three factors: nematode population density, *Meloidogyne* species and "races," and host plant species and even cultivar (Abad *et al*., 2003).

While most of root-knot nematode damages occur below ground, numerous symptoms can also be observed above ground. Severely affected plants will often wilt readily and may exhibit nutrient deficiency symptoms because galled roots have only limited ability to absorb and transport water and nutrients to the rest of the plant. Stunting is frequently observed as well as yellowing on host crops grown in root-knot nematode-infested fields, and crop yields are reduced (Starr *et al*., 2002).

Meloidogyne incognita

Meloidogyne incognita has a wide host range encompassing several hundreds of wild and cultivated plants; its pathogenicity mechanisms are believed to be conserved across plant genera, and even between dicotyledons and monocotyledons (Cabi, 2016).

This species is commonly found in hot regions and restricted to greenhouses in temperate climates (Karssen & Moens, 2006). According to the differentiating hosts from North Carolina there are four physiological races and although considered a species with little variability from the molecular point of view, there are four isozymatic variant phenotypes (Hartman & Sasser, 1985).

Meloidogyne incognita is morphologically similar to other species of *Meloidogyne* so confusion within this group is common, since the characters used are likely to be variable.

Meloidogyne arenaria

Meloidogyne arenaria is considered one of the most important species of this genus due to the economic impact it has worldwide. It is commonly found in hot regions and rarely in cold places (Carneiro *et al*., 2008). This species presents the highest morphological, isozymatic and molecular variation within this genus (Eisenback & Triantaphyllou, 1991). The host range of *M. arenaria* is extremely large and includes members from many plant families including monocotyledons, dicotyledons, and herbaceous and woody plants.

Two host races of *M. arenaria* have been recognized: race 1 infects and reproduces on groundnut, whereas populations of race 2 do not; the most common populations belong to race 1 and are triploid (3n = 51-56), race 2 populations are less common and are diploid (2n = 34-37) (Hartman & Sasser, 1985).

Meloidogyne javanica

Meloidogyne javanica is widely distributed in warm and tropical climates. The geographic range includes Africa, Australia, South America, Asia, the USA and greenhouses in Europe. According to differential host tests in North Carolina four races of *M. javanica* were found, where race 1 parasites tobacco, watermelon and tomato; race 2 parasites pepper besides the ones mentioned and race 3 parasites additionally to all the named hosts groundnut (Rammah & Hirschmann, 1990), and finally race 4 infects tobacco, watermelon, tomato, pepper and groundnut being cotton the only one immune (Rammah & Hirschmann, 1990; Carneiro *et al*., 2003).

Management and control

The eradication of nematodes from soil is a very challenging task. Management strategies have as main objective to increase crop yield by reducing the nematode population on soil and, consequently, limiting the damage to a level economically acceptable (Coyne *et al*., 2009).

Over the past century, to minimize crop losses caused by RKN, nematicides were widely used but, due to the adverse impacts on the environment and human health their use have been reduced resulting on the elimination of methyl bromide and others compounds from the market (Maleita, 2011).

Nowadays, the most successful approach to nematode control relies on integrated pest management strategies (IPM). IPM combines management options to maintain nematode densities below economic threshold levels. IPM techniques can still be difficult to implement against pathogens as aggressive and resilient as root-knot nematodes. Nevertheless, a combination of management tactics/tools, including cultural practices (rotations with non-host crops and cover crops that favour the build-up of nematode antagonists), resistant cultivars, and chemical soil treatments such as emamectin, dazomet, fenamiphos, metam-sodium and oxamyl, if necessary, generally provide acceptable control of root-knot nematodes. The extent of this success, however, is dependent upon having accurate *Meloidogyne* spp. identification, definition of damage threshold densities and available and readily acceptable resistant cultivar.

CHAPTER ONE:

MORPHOLOGYCAL STUDIES

1. INTRODUCTION

After their first discovery on the roots of cucumber in an English glasshouse, the RKN were soon recognized as important pathogens on numerous host plants all around the world. Many researches worked to contribute to the morphology of *Meloidogyne* on its different stages of life but it was Chitwood who found out that the RKN comprised several different species (Eisenback & Hunt, 2009).

Chitwood (1949) used morphological characterization as a first method for identification of RKN species. He studied the morphology of different kinds of nematodes and mentioned that the genus *Meloidogyne* was extremely adaptable and that their morphological characters showed considerable variations. The species concept introduced by Chitwood was based on female's perineal pattern morphology added with stylet knob shape and dorsal oesophageal gland orifice (DGO) length differences.

For many years, morphological identification based on the form of the perineal pattern of the mature female and various morphometric and morphological features of the J2 were relied upon in species determination since they are rapid and practical. Nevertheless, the presence of some variability between individuals (Eisenback, 1985; Hirschmann, 1985, Hussey, 1985; Karssen & van Aelst, 2001) and the increase in the number of species (Karssen, 2002) has limited the reliability of this type of identification. For instance, when using morphological characterization, mixed populations are not easily detected, as large numbers of specimens need to be examined for an accurate identification making necessary the development of new techniques for identification of these species (Hunt & Handoo, 2009).

Due to the morphological and morphometric similarity between species of *Meloidogyne*, the best approach to an optimal identification is to consider a combination of differential characteristics of females, males and second stage juveniles (Carneiro & Cofcewicz, 2008).

1.1. Characteristics for diagnosis

1.1.1 **Male characteristics.** Males are vermiform, annulated non-sedentary, 700-2000 µm long (Hunt & Handoo, 2009). Unlike the J2 and female, the male does not feed, all of the energy required for the development of its reproductive system being obtained while it was a J2. In parthenogenetic species males are very rare and unnecessary for reproduction (Triantaphyllou, 1979).

- **Head region**: The differences in head morphology with respect to shape and size of the head cap, presence or absence of annulation in the head region, and the way the head region fuses with the body are the main characters to be used to distinguish species and populations within species (Eisenback & Hirschmann, 1981).

- **Stylet:** Differences occur in size and shape of the cone, shaft and knobs. In the male the opening of the stylet lumen is located one-fourth the length of the cone from the stylet tip and the oesophageal lumen is smaller in diameter (Eisenback & Hirschmann, 1981).

- **Dorsal pharyngeal gland orifice (DGO):** This is the point at which the dorsal gland empties into the lumen of the oesophagus. This measurement presents variation in general; although some species can be distinguished on the basis of DGO distance (Eisenback & Hunt, 2009)

- **Excretory pore:** Oval shape opening located ventrally use to remove toxic waste from the nematode's body. The position of the excretory pore in males' shows large intraspecific variation so, its value as a differential characteristic is limited (Eisenback, 1985).

- **Hemizonoid:** A lens-like structure situated between the cuticle and hypodermal layer on the ventral side of the body just anterior to the excretory pore; generally believed to be associated with the nervous system (Hirschmann, 1985).

- **Tail:** The tail is small, rounded and without bursa, with the phasmids (sensory structures) located near the level of the cloacal opening (Eisenback, 1985).

- **Spicules:** paired, sclerotized structures which are the male copulatory organs. There seem to be slight differences in structure of the spicules between some of the species but no detailed comparative data is available (Hirschmann, 1985). The gubernaculum is the organ that guides the spicule during copulation.

1.1.2 **Female characteristics.** Round to pear-shaped with short projecting neck, white and sedentary. Vulva and anus located close together, terminal perineum with a fingerprint-like cuticular pattern, usually flattened, rarely elevated (Eisenback & Hunt, 2009). Stylet is slender, generally 12–15 µm long, with small basal knobs. Six large rectal glands secreting gelatinous material in which eggs are deposited. Eggs are not retained in the body (Eisenback, 1985).

- **Stylet:** The conical part of the stylet is curved dorsally, and the stylet lumen opens out ventrally near the stylet tip. The irregular margin of the cone overlaps the cylindrical shaft posteriorly, and the three basal knobs of the stylet may gradually fuse with the shaft or they may be distinctly set off (Eisenback *et al.* 1980)

- **Dorsal pharyngeal gland orifice (DGO):** The dorsal pharyngeal gland orifice is located behind the stylet knobs (Karssen & Moens, 2006). The range of the distance between the stylet knob base and dorsal pharyngeal gland orifice (DGO) is broad and seems variable within populations (Jepson, 1987).

- **Excretory pore:** The position of the excretory pore may be helpful in differentiating some species, although this character is quite variable within populations and species (Hirschmann, 1985).

- **Reproductive system:** In the pyriform female, it develops into two very long and convoluted ovaries. The shape of the female allows for this increase in the length of the ovaries, a feature that greatly enhances reproductive capacity (Hirschmann, 1985).

- **Perineal pattern:** The morphology of perineal patterns still remains the most important morphological character used for tentative species identification (Karssen, 2002). However, the presence of some variability in the perineal patterns between individuals of the same species (Eisenback, 1985; Hirschmann, 1985; Karssen & van Aelst, 2001) and the varied expertise of the people describing perineal patterns (Karssen, 2002) limit the accuracy of species identification based only on perineal patterns. Furthermore, species identifications based on morphological and morphometric characters requires a lot of skill and is time consuming (Hooper *et al*., 2005).

The perineal pattern is a unique and complex area located at the female posterior body region, comprises the vulva-anus zone (perineum), tail terminus, phasmids, lateral lines and surrounding cuticular striae (Figure 3); the striae are typical for each species (Eisenback, 1985).

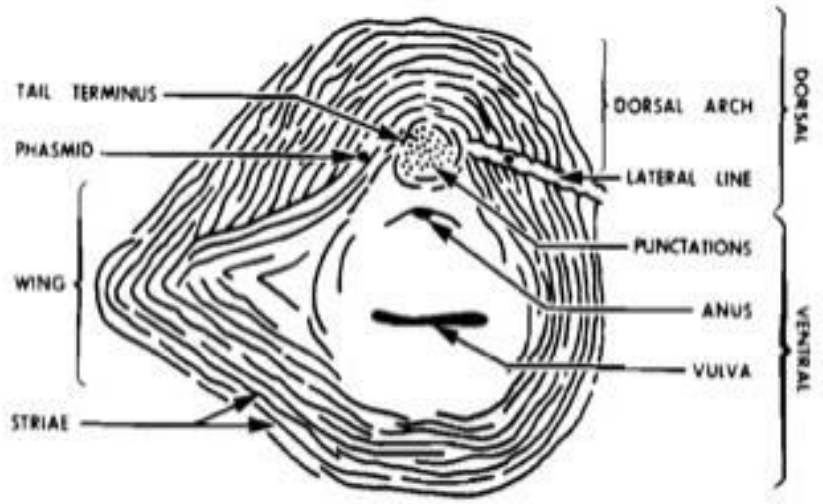

Figure 3 - Diagram of perineal pattern of a *Meloidogyne* female (Einsenback, 1985).

- ***Meloidogyne arenaria:*** Perineal pattern variable, rounded to ovoid with fine to coarse striae (Eisenback, 1985). Dorsal arch low, flattened with striae smooth or slightly wavy, continuous or broken, slightly bent towards tail tip at lateral line; generally forming shoulders on lateral portion of arch. Dorsal and ventral striae often meeting at an angle at lateral lines (Figure 4). (Hunt & Handoo, 2009).

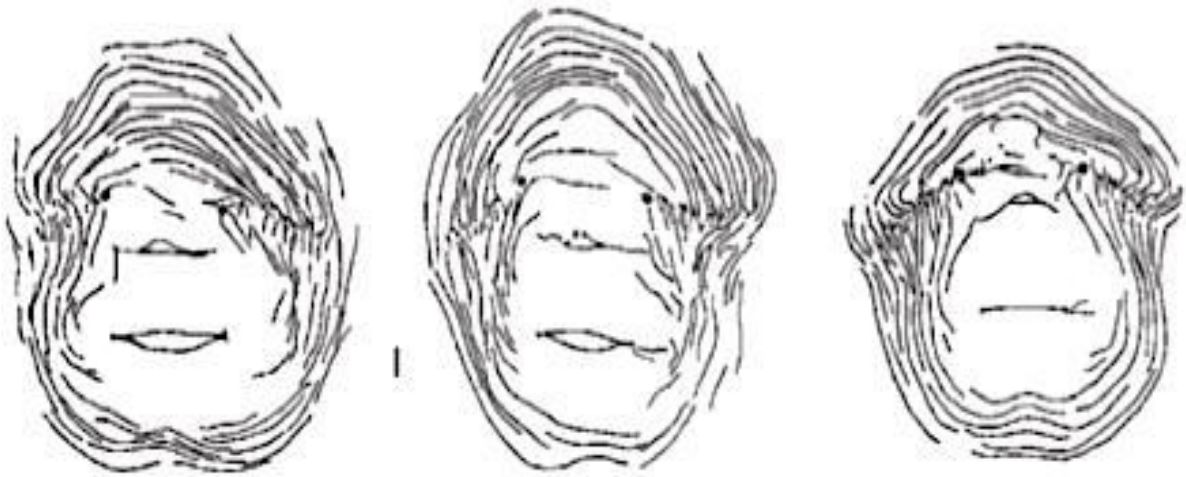

Figure 4 - Diagrams of variability of perineal patterns of *Meloidogyne arenaria* (after Chitwood, 1949; Orton Williams, 1975).

- ***Meloidogyne incognita:*** Perineal pattern typically oval to rounded with high, squared, dorsal arch; striae usually wavy, lateral field absent or weakly demarcated by forked striae (Figure 5) (Hunt & Handoo, 2009).

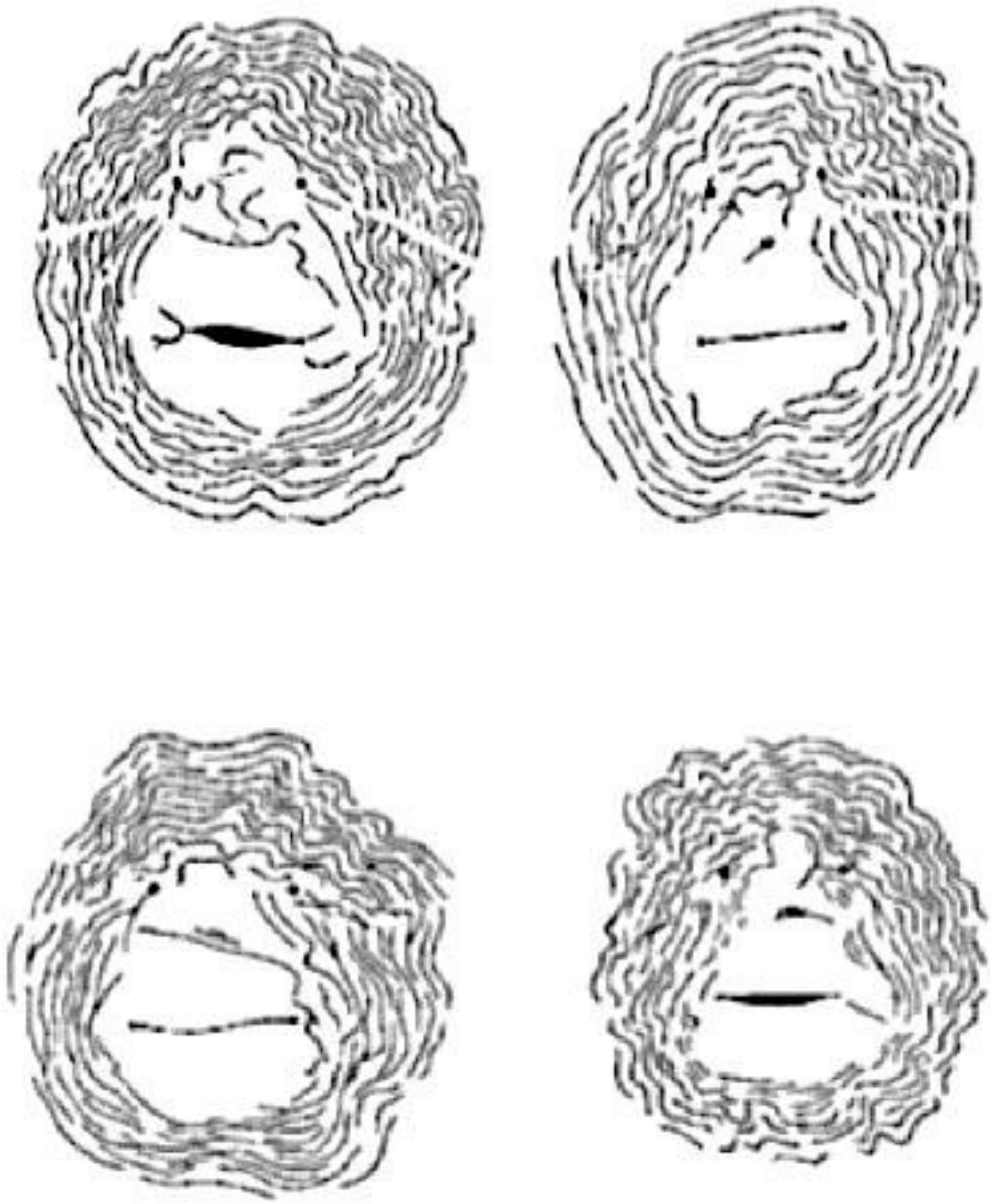

Figure 5 - Diagrams of variability of perineal patterns of *Meloidogyne incognita* (after Chitwood, 1949; Orton Williams, 1973).

- ***Meloidogyne javanica:*** The perineal patterns of M. *javanica* are unique because they contain lateral ridges that divide the dorsal and ventral striae (Eisenback, 1985). Generally, the ridges run the entire width of the pattern, but gradually disappear near the tail terminus. The dorsal arch is low and rounded to high and squarish and often contains a whorl in the tail terminal area. The striae are smooth to slightly wavy, and some striae may bend toward the vulval edges (Figure 6) (Hunt & Handoo, 2009).

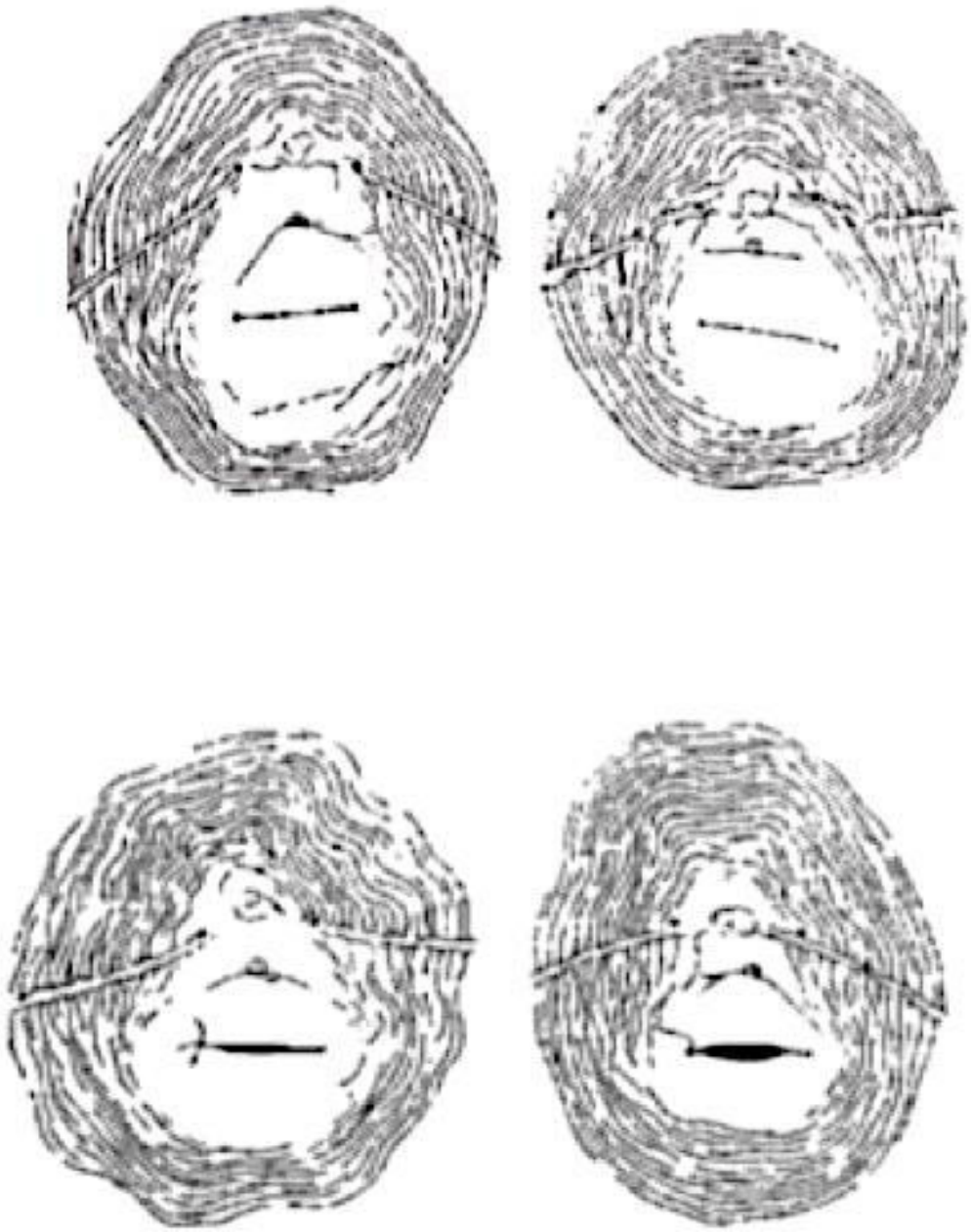

Figure 6 - Diagrams of variability of perineal patterns of *Meloidogyne javanica* (after Chitwood, 1949; Orton Williams, 1972).

1.1.3 **Juvenile characteristics.** The first stage has a blunt tail tip and moults within egg, the second and third moults occurring within cuticle of second stage. Second stage vermiform, migratory, ranging in size between 250 and 600 µm, (Karssen & Moens, 2006). Due to the small size of J2 it is difficult to identify all the cephalic structures. Second-stage juveniles have similar characteristics to males that can only be observed with an electron microscopy (Hunt & Handoo, 2009).

- **Body length:** There is considerable variation among species with respect to body length. The range of the genus is narrow and the measurements obviously overlap in many species, although the extreme values can be different, and differences in body length may occur also between populations of the same species (Hirschmann, 1985).

- **Stylet:** Stylet morphology is quite distinctive. Differences between species and populations occur especially in size and shape of the stylet knobs and in the way the knobs join the stylet shaft (Eisenback & Triantaphyllou, 1991). Juveniles have a delicate stylet with a size in media of 8 to18 µm long (Eisenback, 1985).

- **Dorsal pharyngeal gland orifice (DGO):** The distance of the DGO to the stylet base is an important distinguishing character, and groups of species may be differentiated by this measurement (Karssen & Moens, 2006). The distance from DGO to the stylet base is situated between 2 to 8 µm (Hunt & Handoo, 2009).

- **Reproductive system:** The genital primordium is very small in the pre-parasitic juveniles, but rapidly increases in size as soon as feeding commences (Hirschmann, 1985).

- **Tail and hyaline tail terminus:** Tail length is one of the most useful features because it varies considerably among species and has little intraspecific variation. Some species are clearly distinct from each other in overall range (Karssen & Moens, 2006). Length of the hyaline tail terminus is often very variable, although in some species it is clearly short and in others always long (Eisenback, 1985).

The hemizonid location in regard to the excretory pore is another character that is also helpful in identification of *Meloidogyne* (Hunt & Handoo, 2009).

Table 1 shows morphometric values of *Meloidogyne arenaria, Meloidogyne incognita* and *Meloidogyne javanica* reported by different authors. These values were used for comparison with the ones obtained in this study.

Table 1 - Reported morphometric values (in µm) for *Meloidogyne incognita, Meloidogyne arenaria* and *Meloidogyne javanica*. (Eisenback et al. 1981; Eisenback, 1985: Karssen & Moens, 2006; EPPO, 2016;)

Characteristic	*M. incognita*	*M. arenaria*	*M. javanica*
Body Length	360-400 (380)	400-490 (470)	400-560 (417)
Tail Length	43-65 (55)	45-70 (57.5)	51-63 (57)
Hyaline Terminus	6-11 (9)	6-15 (10.5)	10-19 (14.5)
DGO	2-3 (2.5)	3-4 (3.5)	3-4 (7.5)
Stylet Length	14-16 (15)	14-16 (15)	14-18 (16)

()- Numbers in brackets represent the mean value

2. MATERIALS AND METHODS

2.1. Nematode Isolates

Isolates of *M. incognita*, *M. arenaria and M. javanica* (Figure 7) extracted from soil samples received as reference material from the Netherlands Food and Consumer Product Safety Authority, Wageningen (NVWA) and Portuguese isolates extracted from infected roots and soil samples collected from North and West Portugal, provided by the Laboratory of Nematology (INIAV) were used in this study (Table 2).

Figure 7 - Reference material of *Meloidogyne arenaria, Meloidogyne incognita* and *Meloidogyne javanica* provided by Netherlands Food and Consumer Product Safety Authority, Wageningen (NL) (NVWA).

Table 2 - *Meloidogyne* isolates, host, geographic origin and studies performed in this work

Species	***Host plant***	***Geographic origin***	***Studies***
***M. incognita* Dutch NPPO (E2107-1)**	*Solanum lycopersicum*	The Netherlands	MM, ML
***M. arenaria* Dutch NPPO (E9279)**	*Solanum lycopersicum*	The Netherlands	MM, ML
***M. javanica* Dutch NPPO (E1387)**	*Solanum lycopersicum*	The Netherlands	MM, ML
***Meloidogyne* spp**	*Solanum lycopersicum*	West Portugal	MM,B,ML
***Meloidogyne* spp**	*Solanum lycopersicum*	North Portugal	MM,B,ML

MM: Morphometric, B: Biochemical, ML: Molecular

2.2 Inoculation of plant material

The nematode isolates from NVWA were propagated on tomato plants *(Solanum lycopersicum cv.* Rio Grande, considered susceptible to species *of Meloidogyne)* in a greenhouse in order to increase populations and to obtain inoculum to carry out the different studies proposed in this research. Fifteen pots were filled with a sterilised mixture of soil, sand and substrate (1:1:1). Seeds of tomato were previously germinated on moist filter paper at 23°C in Petri dishes (Figure 9). After three days, germinated seeds (those with emerging radicles) were transferred to a tray and two weeks later the seedlings were transplanted into the pots (Figure 8). Five pots with two-week-old tomato seedlings were artificially inoculated placing juveniles (J2) obtained from each of the species under study (*M. incognita, M. arenaria, M. javanica)* as well as chopped infected roots inside the pots.

The plants were transferred and maintained under greenhouse conditions at 18-27 ºC for 16 weeks.

Figure 8 - Germinated tomato seeds (*Solanum lycopersicum* cv. Rio Grande) and transplanted tomato seedlings.

Four months after inoculation, the roots and soil were analysed for the presence or absence of galls and juveniles respectively.

2.3 Morphological and morphometric studies

Morphological and morphometric studies were conducted on second-stage juveniles (J2) obtained from soil extraction and handpicked females from infected roots.

- **Juvenile preparation**

Juveniles were recovered from soil using flotation and sieving together with the centrifugal sugar flotation method (Laboratory of Nematology, INIAV Oeiras) which consist on: mixing 300 gr of soil in approximately 1 Lt of water and let the soil particles settle before pouring the water into the sieves (180 µm, 90 µm and 44 µm); the objective behind using three different sizes of sieves is to retain as much unwanted debris as possible. Once all the water has been poured, the residues caught in the finest sieve are washed into a centrifuge tube to be centrifuged for 4 minutes at 1750 rpm. After this centrifugation nematodes and any other heavy materials are forced to the bottom of the tube so the water is carefully discarded. Next, sugar is added and one more centrifugation takes place for 10 s at 1750 rpm; the nematodes will float in the sugar solution and the heaviest particles will go down to the bottom. At last, the sugar solution containing the nematodes is poured into the finest sieve and rinsed thoroughly with water to avoid damage and to be poured into the beakers.

The recovered J2 were individually transferred to a slide with a drop of water, to be gently heat killed using an alcohol burner and to be later observed at a light microscope.

The measurements made were achieved through drawing lines crossing approximately the middle of the specimen's body. The study took into account some of the most relevant measurements of second stage juveniles according to EPPO, 2016 as follows:

- Body length
- Stylet length
- Tail length
- Hyalines terminus length
- DGO

It is important to highlight that usually the influence of the morphological characteristics is higher than the morphometric on the diagnosis since as mentioned by Hunt & Handoo, (2009) the environment may have an effect on the nematode's morphometrics making them unreliable.

- **Female preparation**

Tomato roots from Portuguese samples were washed to remove soil and any other particles; immediately after, female extraction from vegetal tissues took place with the help of a disposable ophthalmic knife and a dissecting microscope. The females were then placed in glass blocks with lactic acid 45% for two days. Later, one by one were transferred to a slide with a drop of lactic acid 45% and cut with the ophthalmic knife to get the perineal patterns (Figure 9) for its identification at a light microscope.

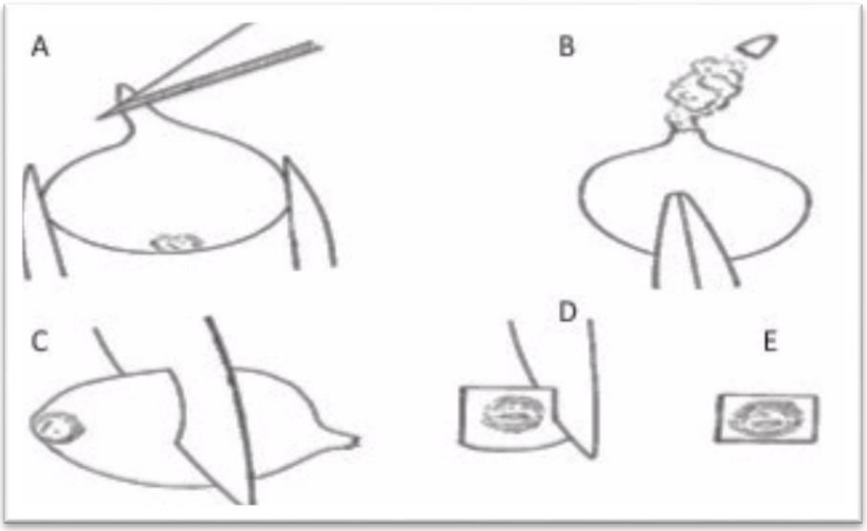

Figure 9 - Perineal pattern area cuts. A - Hold female's neck and body; B - excised female with neck region removed and body contents gently expelled; C - posterior body with perineal pattern removed; D - trimming surplus cuticle around perineal pattern; E - trimmed perineal pattern ready for mounting (Hartman & Sasser, 1985).

Patterns of *M. incognita, M. javanica* and *M. arenaria* were compared with diagrams published from previous studies (Chitwood, 1949; Orton Williams, 1972,1973,1975; Eisenback *et al*., 1981).

3. RESULTS

3.1 Inoculation

Tomato seedlings inoculated with reference material from NVWA Wageningen of the three species of *Meloidogyne* did not show any symptoms above ground that could indicate nematode infection. Moreover, once the plants were uprooted not *Meloidogyne* gall formation was observed even though a susceptible cultivar (Rio Grande) was used (Figure 10)

Figure 10 - Roots from inoculated tomato plants (cv. Rio Grande).

3.2 Reference material

As morphological characters of the species under study often vary considerably within populations, the identification must be based on a combination of morphological and morphometric characters. Morphometric measurements performed on second stage juveniles from reference material are reported in Table 3.

Table 3 - Morphometric values (in µm) of reference samples of *Meloidogyne incognita, Meloidogyne arenaria* and *Meloidogyne javanica*

Characteristic	*M. incognita* (n=5)	*M. arenaria* (n=5)	*M. javanica* (n=5)	***Reported values*** **Eisenback *et al.* 1981; Eisenback, 1985; Karsen & Moen, 2006; EPPO, 2016.**
Body length	371.2 – 394.4 ± 9.65 (377.6)	377.7 – 482.6 ± 44.38(421.3)	396.3 – 412.6 ± 5.39 (403.5)	*M. incognita*: 360-400 *M. arenaria*: 400-490 *M. javanica:* 400-560
Stylet length	16.2 – 18.2 ± 0.72 (17.2)	13.4 – 15.3 0.79 (14.5)	16.1 – 19.9 ± 070 (18.1)	*M. incognita*: 14-16 (15) *M. arenaria:* 14-16 (15) *M. javanica:* 14-18 (16)
DGO	2.5 - 3 ± 0.17 (2.7)	3 – 3.5 ± 0.20 (3.3)	2.4 – 2.7 ±0.17 (2.6)	*M. inc:ognita*: 2-3 (2.5) *M. arenaria*: 3-4 (3.5) *M. javanica*: 3-4 (3.5)
Tail length	47.2 – 55.5 ± 3.71 (50.1)	40.9 – 57.1 7.39 (48.1)	58.2 – 68.5 ± 3.67 (63.2)	*M. incognita*: 43-65 (54) *M. arenaria*: 45-70 (57.5) *M. javanica:* 51-63 (57)
Hyaline terminus	11.5 – 13.5 ± 0.52 (12.6)	12.8 – 13.8 0.36 (13.1)	10.2 – 13.5± 1.27 (12.3)	*M. incognita*: 6-11 (9) *M. arenaria*: 6-15 (10.5) *M. javanica*: 10-19 (14.5)

The mean values are given in brackets.

The observed juveniles were vermiform and moderately long, their head not offset from body. The stylet was slender with a sharp pointed stylet cone, cylindrical stylet shaft and rounded stylet knobs set off from the shaft (Figure 12). The tail region was narrow and the hyaline tail terminus was very distinct (Figure 13). Females were not available.

Second-stage juveniles of *M. incognita, M. arenaria* and *M. javanica* averaged 377.6 µm, 421.3 µm and 403.5 µm in length respectively and were between the typical ranges for *M.*

incognita, M. arenaria and *M. javanica*. The average stylet length of *M. incognita* and *M. javanica* and their range was greater in the populations under study. Tail morphometrics were not typical of *M. arenaria, M. javanica* or *M. incognita* (Table 3). Tail morphology was consistent with descriptions in the literature. *M. javanica's* tail tip was slightly curved which is a typical character of the species (Figure 13).The mean length of the hyaline tail terminus was 12.6 µm, 13.1 µm and 12.3 µm for each species respectively which is not in agreement with previous descriptions. Due to the scarcity of material, measurements were performed on 5 individuals, which may be one of the causes for the observed overlapping since for this kind of studies at least 10 specimens are required.

Morphological and morphometric data required a great deal of work. The data collected was inconclusive and the range of values for each of these characteristics overlapped just as has been observed by many other authors and illustrated in Figure 11. Whereby, these features were not sufficient to distinguish closely related *Meloidogyne* species.

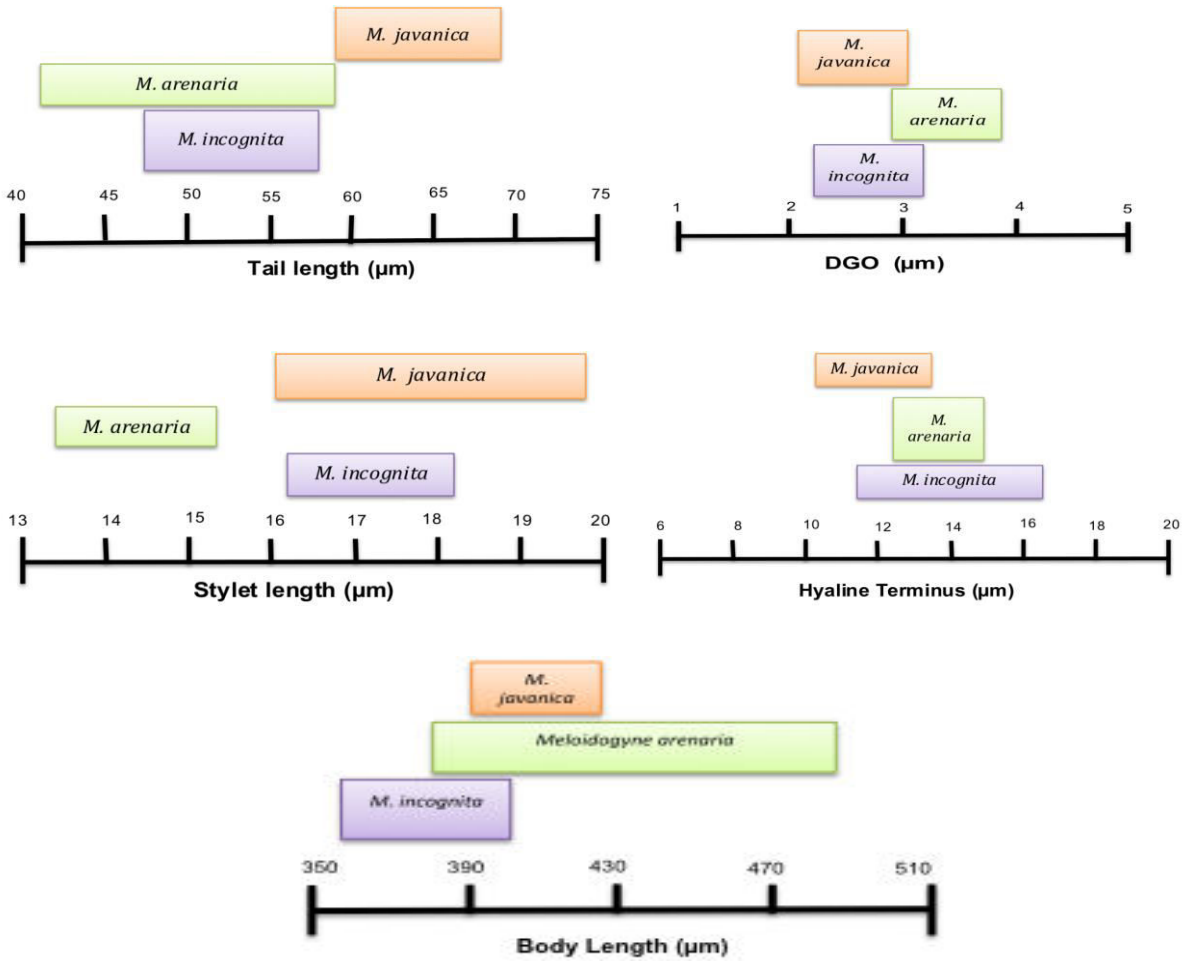

Figure 11 - Graphic representation of overlapped morphometric values of *Meloidogyne incognita, Meloidogyne arenaria* and *Meloidogyne javanica*.

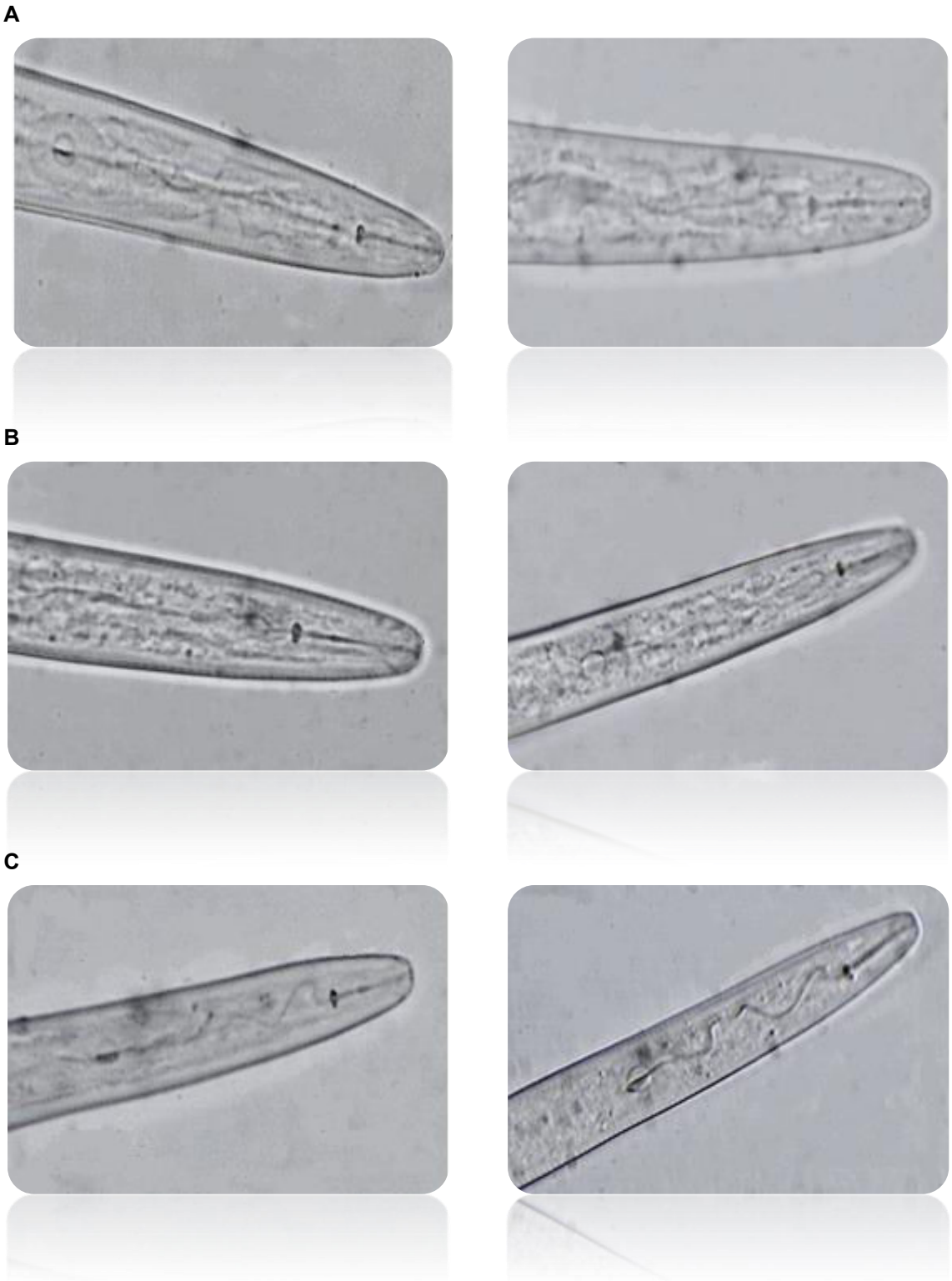

Figure 12 - Micrographs of second stage juveniles' anterior regions with stylets (400X). **A -** *Meloidogyne arenaria* **B -** *Meloidogyne incognita* **C -** *Meloidogyne javanica*.

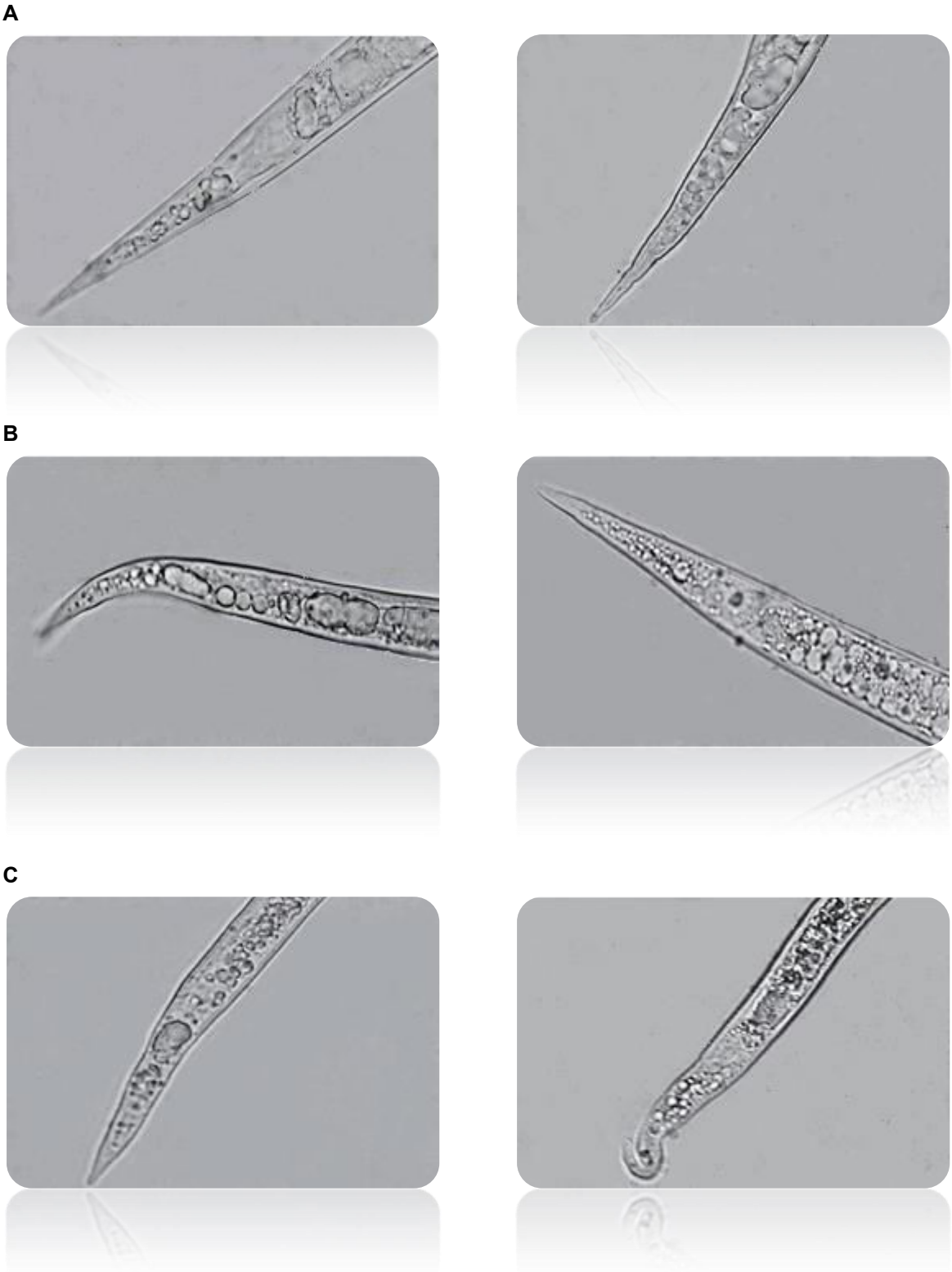

Figure 13 - Micrographs of second stage juveniles' tails (400X). **A -** *Meloidogyne arenaria* **B -** *Meloidogyne incognita* **C -** *Meloidogyne javanica.*

3.3 West Portugal samples

Infected roots from samples from West Portugal were analysed. J2 were not available so the identification of the species was achieved through the study of the female perineal pattern. This pattern is a valuable morphological feature. However, over the years there has been found significant variability, which undermined the value of this character for comparing *Meloidogyne* species.

Females obtained from infected roots were completely enclosed by galled tissue containing more than one female. The female's body was pearly white, pear shaped with anterior body portion commonly off-centre from a median plane and with almost terminal vulva. Their neck was short (Figure 14).

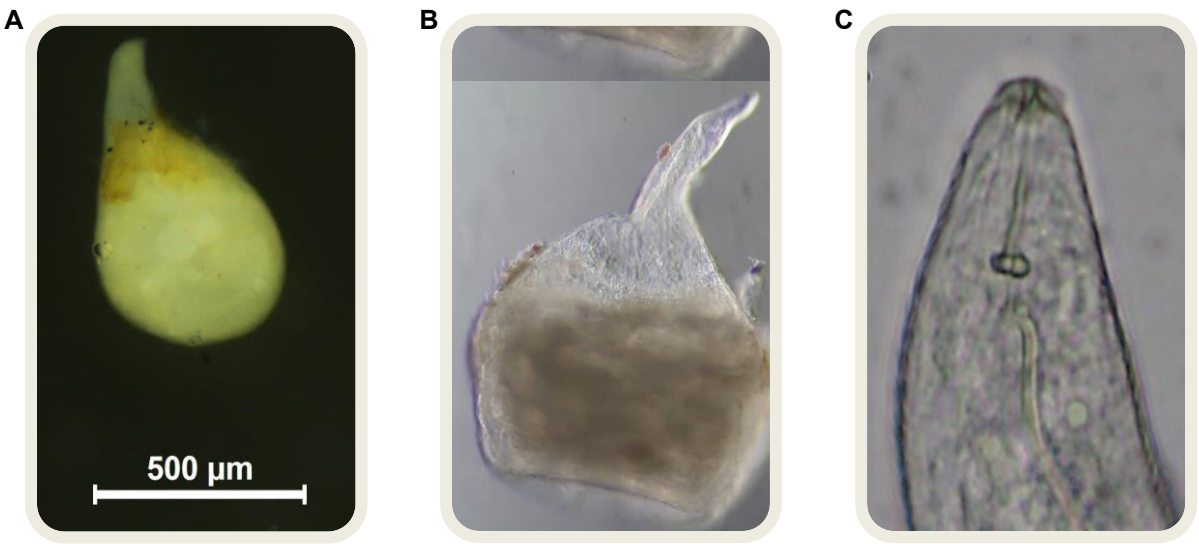

Figure 14 - Stereomicroscope and light microscope photographs of *Meloidogyne* females from West Portugal samples. **A -** whole specimen; **B -** whole specimen (1000X oil immersion) **C -** stylet (1000X oil immersion).

Microscopic examination of the perineal pattern morphology of adult females found in the infected roots indicates the presence of two different species, *M. incognita and M. arenaria*. However, the results are not conclusive since some characteristics are not completely specific and can be found in the three species under study.

Meloidogyne incognita perineal pattern was roughly oval, dorsal striae closely spaced, wavy to zigzag, forking to some extent at the lateral lines. The dorsal arch was trapezoid in shape, sometimes with a distinct tail whorl and numerous transverse striae at the side of the ventral part (Figure 15).

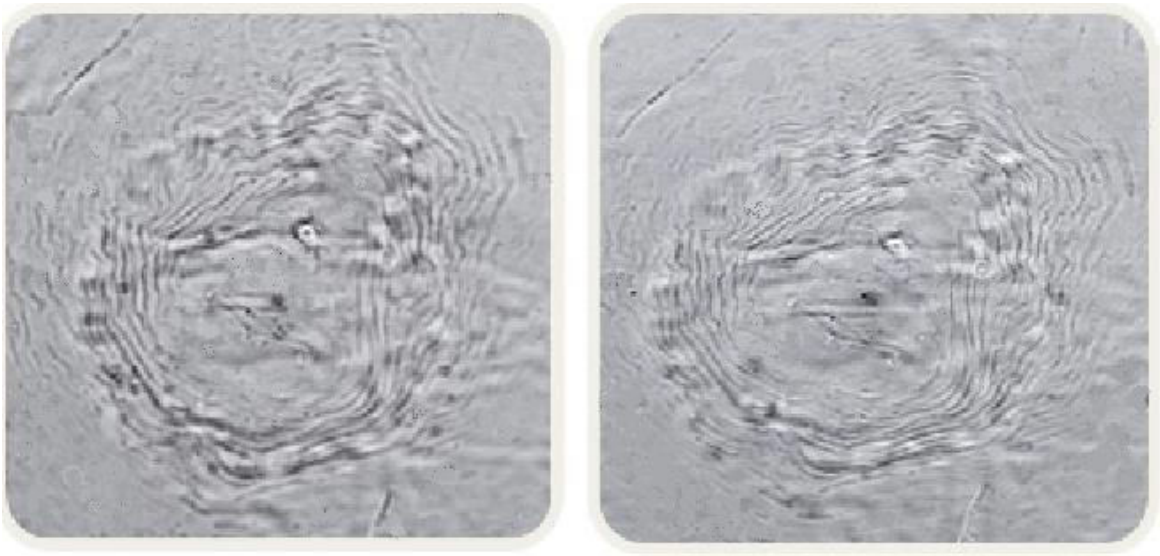

Figure 15 - Perineal pattern of *Meloidogyne incognita* (400X).

Meloidogyne arenaria's overall shape was rounded. Females showed low dorsal arch and the lines in the post-anal region are wavy and broken. Some striae bend towards the vulva. Phasmids were not visible and the lateral lines were weakly demarcated by forked striae (Figure 16).

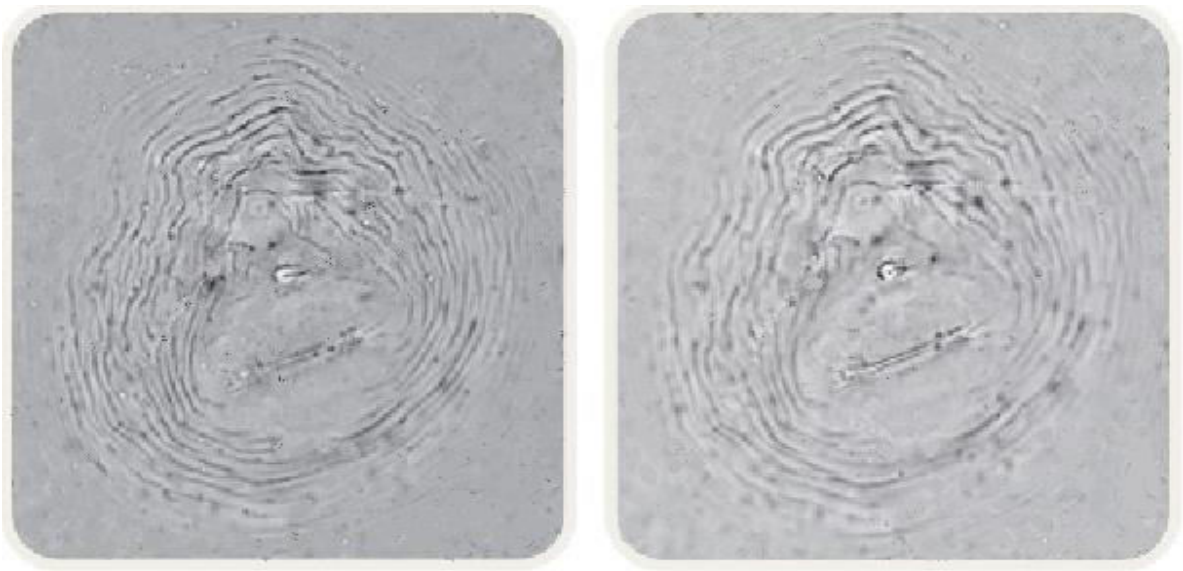

Figure 16 - Perineal pattern of *Meloidogyne arenaria* (400X).

3.4 North Portugal samples

Morphometrics performed on material from North Portugal are presented on Table 4. There were no major variations when compared to values previously reported in Table 3. However, those values overlap with other *Meloidogyne* species.

Table 4 - Morphometric values (in mm) performed on *Meloidogyne* spp. samples from North Portugal

Characteristic	*M. javanica* (n=5)	Reported values
Body length	390 - 540 (465)	400 - 560
Stylet length	14.5 - 18 (16.2)	14 -18 (16)
DGO	3 - 4 (3.5)	3 – 4 (3.5)
Tail length	49.9 - 60.8 (55.3)	51 - 63 (57)
Hyaline terminus	10 - 18 (14)	10 - 19 (14.5)

The mean values are given in brackets.

The perineal pattern was typical for *M. javanica* with a rounded pattern, striae interrupted laterally by a pair of conspicuous incisures extending on both sides of the tail terminus, low dorsal arch trapezoid shape and a tail whorl (Figure 17).

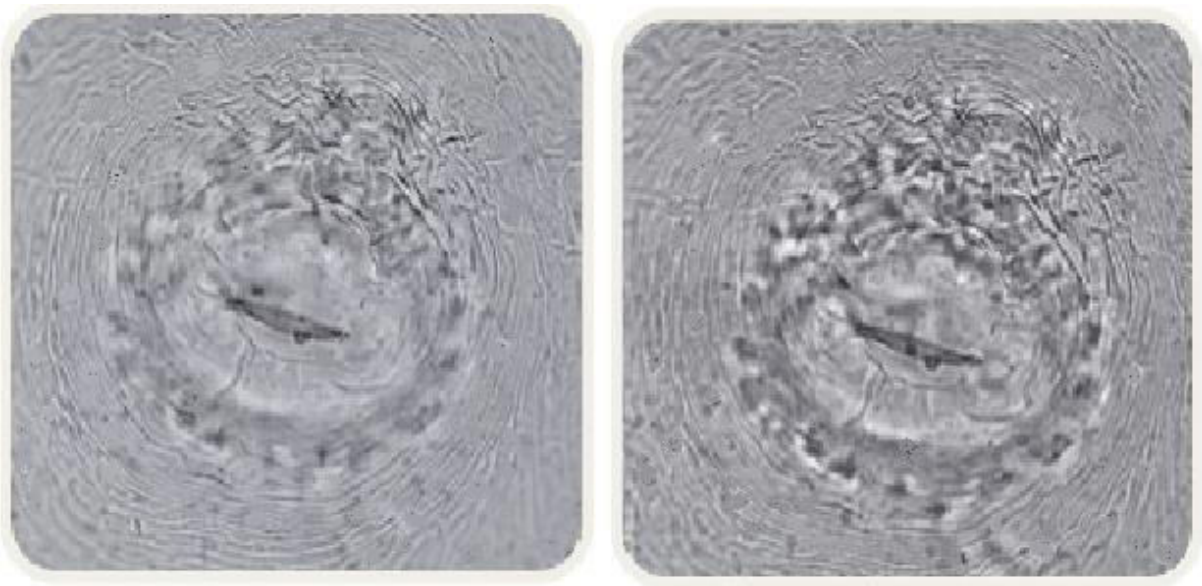

Figure 17 - Perineal patterns of *Meloidogyne javanica* (400X).

4. DISCUSSION

For the onset of this study, it was essential to have isolates of reference well identified and available for a wide range of methodologies to be used. For that reason, this work started with the experimental inoculation of seedlings of tomato (cv Rio Grande) with juveniles (J2) of *Meloidogyne* obtained from reference material from the Netherlands Food and Consumer Product Safety Authority, Wageningen (NVWA), to obtain and maintain the isolates collection. However, the expected infection of tomato seedlings did not happen probably because the greenhouse conditions, namely the temperature was cooler than expected for April and May and, as it is known these species prefer warmer climates. Also, it can be inferred that the material used may not have been biologically in conditions of producing infection, this due to a contamination as well as the inadequate storage during the delivery from the Netherlands with peaks of high and low temperature that could have affected the sample's quality.

Morphological features have been valuable tools for RKN identification due to its low cost and ease to learn the skills, with its accuracy depending on the number of characteristics to be evaluated and the number of specimens. On this study were examined the most relevant morphological and morphometric characteristics of *Meloidogyne* (females, and second-stage juveniles) according to Hirschman (1985) and EPPO (2016). The identification was made using a light microscope and proved to be a difficult task since sometimes it was not possible to see some of the characters or not great detail could be achieved so, for better image quality it is advisable to use differential interference contrast microscopy.

The measurements made on material from the Netherlands such as the juvenile body length, tail length, stylet length and lengths of hyaline tail terminus did not help on verifying the species identity. The obtained morphometric values for most of the characters overlapped and are within the expected range making them fall into more than one species description. For instance, *M. incognita* had range value between 371 µm to 394.4 µm but measures within this range can be easily misplaced into *M. arenaria* range value of 377.7 µm to 482.6 µm. For this reason, morphometrics on its own cannot be used to draw a conclusion on the identification of root-knot nematodes as already described by many authors.

Identification of *Meloidogyne* species was also attempted using morphology of perineal patterns since it is considered important in differentiating species of these nematodes. Nevertheless, on this study it was found that many of them presented a lot of similarities that can lead to misidentification of the species. To illustrate this, *M. javanica* commonly presents lateral lines that clearly divide the dorsal and ventral parts but those lateral lines were not

only spotted on *M. javanica* but also on *M. arenaria's* perineal pattern from West Portugal samples; this kind of incidents can cause confusion and lead to inadequate identifications of *Meloidogyne.* Same confusion has been already reported by Rammah & Hirschmann, (1990).

Additionally, for a reliable identification based on morphology, individuals from all stages should be examined which is not always possible. For instance, on this research individual samples did not have the three stages available; moreover, due to the parthenogenetic reproduction of the species under study males are not necessary, making them very rare specimens.

Finally, although morphological and morphometric data can be very helpful for tentative identification, it may not be enough to differentiate RKNs and their physiological/cytological races as they are closely related (Zijlstra, 2000).

CHAPTER 2: BIOCHEMICAL STUDIES

1. INTRODUCTION

The taxonomy of the genus *Meloidogyne* years ago was generally based on morphological and morphometric characters. However, variability of the perineal patterns and diagnostic morphological characters, life stages in different habitats, wide host ranges, indistinct species boundaries or species complexes, sexual dimorphism, species with a potential hybrid origin, polyploidy, and over a century of human-aided dispersal are up to today limitations that have led researchers to find another means of identification. Therefore, the integration of classical methods of identification with techniques such as the use of enzymatic and molecular markers is necessary to a more accurate and reliable species identification.

Extensive enzymatic studies have demonstrated that the major species of *Meloidogyne* can be differentiated by species-specific enzyme phenotypes, which can be revealed by polyacrylamide gel electrophoresis (PAGE) (Esbenshade & Triantaphyllou, 1990). Furthermore, recent progress in electrophoretic procedures have made possible, and also practical, the detection of the phenotype of one, two, and even more enzymes of a single *Meloidogyne* female (Carneiro *et al*., 2000).

Dickson verified that there were differences on the electrophoretic patterns of many enzymes developing the first demonstration that some enzymes may be species-specific and could be used in the identification of *Meloidogyne* species (Dickson *et al.,* 1971). Later on, in the mid-1980's Esbenshade and Triantaphyllou developed a biochemical-based diagnostic technique, reliant on isozyme profiles. They reported esterase patterns from 16 *Meloidogyne* species, with the most common phenotypes being A2 and A3 (*M. arenaria*), I1 and I2 (*M. incognita*) and J3 (*M. javanica*). Variations in esterase and malate dehydrogenase isozyme profiles proved to be extremely informative in differentiating most known *Meloidogyne* species (Esbenshade & Triantaphyllou, 1990). The main drawback to this method, however, is that the technique is only applicable to young adult females since they all are in a single developmental stage and are associated with the expression of a given gene product. Nonetheless, the adult stage is not readily isolated from the soil as it generally resides in the host. The ineffective second stage juveniles are usually in large numbers overshadowing the adult female stage.

Despite this shortcoming, isozyme electrophoresis remains one of the most reliable and widely used differentiation method (Janseen *et al.,* 2016). Since the female stage is often unavailable in soil samples, the isozyme method requires the time and space to establish and maintain populations in culture from single egg masses or single J2s in order to obtain this stage (Blok &Powers, 2009).

Several isozyme systems have been used, with carboxylesterase/esterase EST (EC 3.1.1.1) proving to be most useful for discriminating *Meloidogyne* species. Others such as malate dehydrogenase MDH (1.1.1.37), superoxide dismutase SOD (1.15.1.1) and glutamate-oxaloacetate transaminase GOT (EC 2.6.1.1) are also often included to confirm species identification (Esbenshade & Triantaphyllou, 1990). Enzyme patterns are usually compared with a known standard, frequently from *M. javanica*, which is included in the electrophoresis to determine migration distances (Esbenshade & Triantaphyllou, 1990).

The relative stability of the isozyme phenotypes within *Meloidogyne* species (De Waele & Elsen, 2007) makes them an attractive system, although there are some limitations. One is concerning the occurrence of intraspecific variants and the difficulty in resolving size variants between species which led to the need of using more than one enzyme system to confirm the identity of some isolates (Subbotin & Moens, 2006).

1.1 Isozymes

Since the 1960s a wide range of electrophoretic techniques have been developed to detect protein variation. The first of these techniques to be applied in nematology was the one-dimensional gel electrophoresis, in order to differentiate *Ditylenchus* from *Panagrellus* (Benton & Myers, 1966) and from four *Meloidogyne* species (Dickson *et al*., 1971). Since then these techniques have been improved and used for the identification of root-knot nematodes (RKN) species.

Isozymes are variants of a particular enzyme, which differ from one another in terms of their biochemical properties such as their amino acid sequence and substrate requirements. The change in amino acid sequence in isozymes contributes to a significant change in the electric charge thus, making it easy to identify them using gel electrophoresis. Some of these isozymes include: esterases (EST), malate dehydrogenase (MDH), superoxide dismutase (SOD), glutamate-oxaloacetate transaminase (GOT) and glutamate dehydrogenase (GDH).

Out of the five commonly studied enzyme patterns, esterases have been the most useful for differentiating the major Meloidogyne species (Esbenshade & Triantaphyllou, 1985; Cofcewicz *et al*., 2004).

1.1.1 Carboxylesterase/esterase

These are enzymes that hydrolyse carboxylate esters and are widespread in various organisms including animals, plants, and microorganisms. Carboxylesterases are defined as enzymes that catalyse the hydrolysis of acylglycerols with short chains (<10 carbon atoms). The standard substrate for carboxylesterase activity is tributyrine (Phythian, 1998).

Extensive enzymatic studies from several countries have demonstrated that species-specific enzyme phenotype could differentiate the major and most widely distributed species of *Meloidogyne* (Figure 18) (Esbenshade & Triantaphyllou, 1985). However, only phenotypes for 26 species are available (Esbenshade & Triantaphyllou, 1985; Pais & Abrantes, 1989; Carneiro *et al*., 1996).

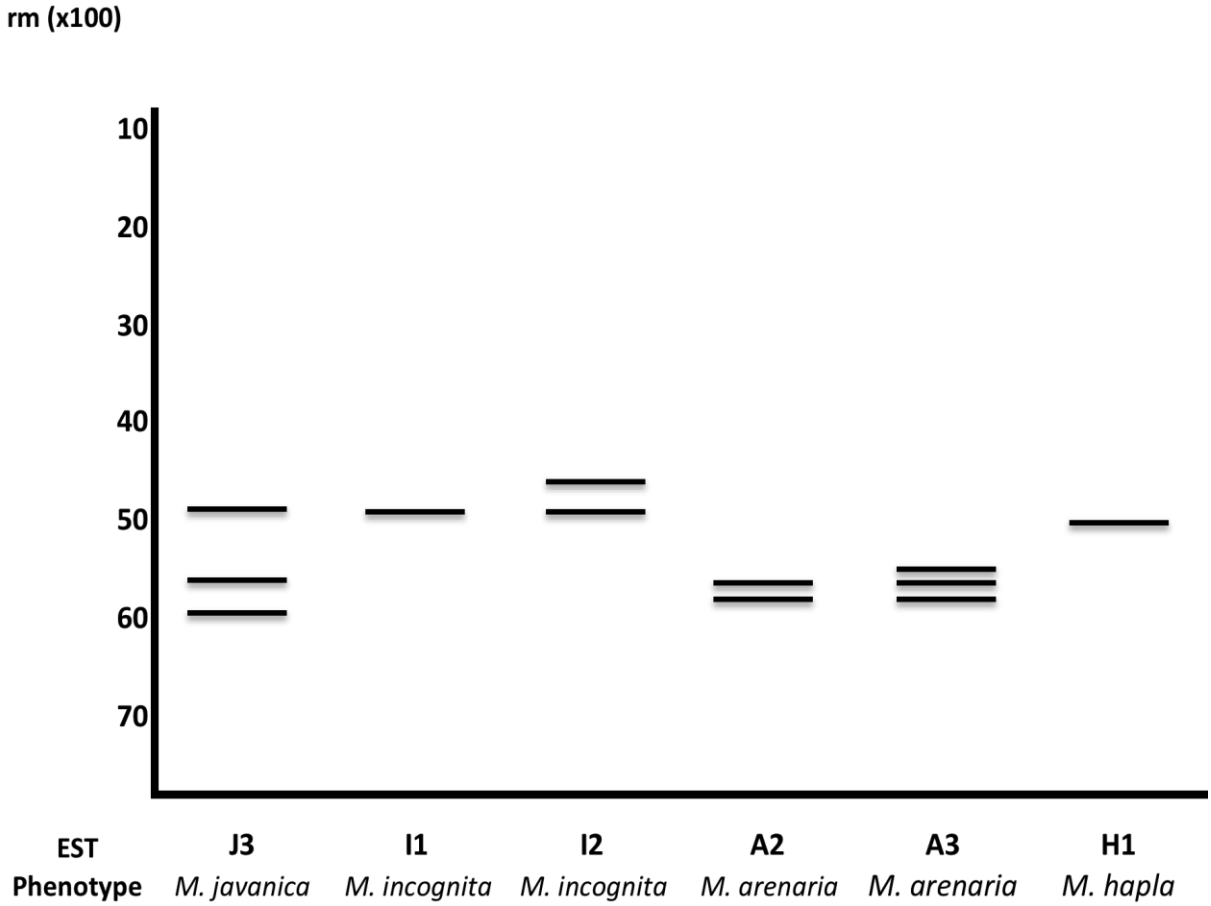

Figure 18 **-** Esterase (EST) phenotypes of the major species of *Meloidogyne.* Esterase types: J_3 = *Meloidogyne javanica*; I_1 = *Meloidogyne incognita* races 2 and 3); I_2 = *Meloidogyne incognita* races 1 and 4; A_3, A_2 = *Meloidogyne arenaria* race 2; H_1 = *Meloidogyne hapla*. (Dickson *et* al., 1971; Pais & Abrantes, 1989;).

2. MATERIALS AND METHODS

2.1 Sample preparation

Young egg-laying, plump, pearly white females from infected tomato roots from the North of Portugal were handpicked under a dissecting microscope and collected in isotonic 0,9% sodium chloride solution in order to avoid possible osmotic disruption in plain water. Following isolation, the females were transferred with the help of dissecting pins to micro-haematocrit capillary tubes containing 5 µl of extraction buffer (20% Sucrose and 1%Triton X-100) to avoid the loss of enzyme activity. The specimens were macerated with a pestle against the bottom of the tube, frozen and stored at -20 °C until electrophoresis (no longer than 3 months). Shortly, before electrophoresis the samples were thawed and centrifuged at 10000 g, -5°C for 15 minutes. After centrifugation there is a formation of three different layers, the upper layer formed by the lipid portion of the extract, the bottom layer formed by cuticle and other particles and the clear aqueous phase which was then used directly for electrophoresis.

2.2 Polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was carried out in vertical polyacrylamide gels, 1mm thick, in a Mini-Protean II (Bio-Rad Laboratories, Hercules, California, USA).

The separating gel was 7% polyacrylamide, pH 8,8, the stacking gel was 3% polyacrylamide, pH 6,8 and Tris-glycine solution pH 8,3 was used as running buffer (Appendix1). The electrophoresis was carried out at 6 mA/gel during the first 10 minutes and then at 20 mA/gel for about 50 minutes or until the bromophenol blue had migrated to the end of the gel.

Following electrophoresis, the gels were stained to examine for esterase activity with Fast Blue staining and α-naphthyl acetate substrate. The regions with enzymatic activity were shown as one or more dark bands.

3. RESULTS

Initial biochemical studies were performed using isolates from west Portugal. However, due to poor handling of females there was not esterase activity to be reported.

On later assays, isozyme analysis was also conducted using suitable young adult females extracted from samples collected from North Portugal. In these isolates, nine bands of esterase activity were observed (Figure 19), comprising three phenotypes. The three-esterase bands observed correspond to M. *javanica* when compared to esterase phenotypes already described by different authors as shown before in Figure 18.

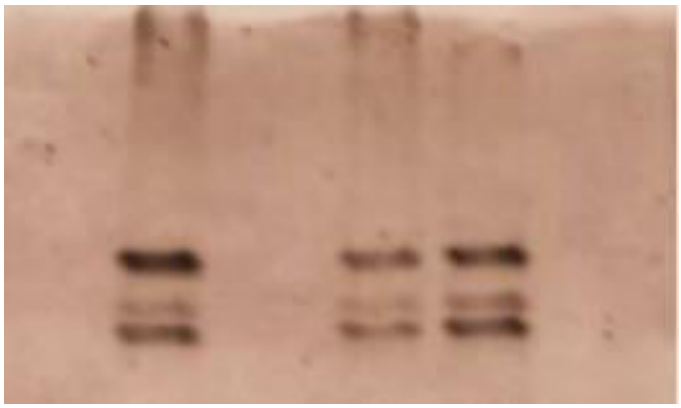

Figure 19 - Esterase phenotype of protein homogenates from three egg-laying females of *Meloidogyne* spp. from North Portugal.

4. DISCUSSION

Many studies have shown the usefulness of isozyme analysis, especially esterase phenotype, as the quicker, reliable and stable method to identify *Meloidogyne* spp. (Esbenshade & Triantaphyllou, 1985; Carneiro *et al*., 2000). And in fact, it should be the first step in any identification procedure for root-knot nematodes as it was observed during the study.

This technique uses primarily egg-laying females, which must be globose with a pearly white colour and in optimal conditions for extraction since any degradation on them can alter the final result. Usually the enzyme patterns are compared with a known standard, frequently from *M. javanica*, which is included in the electrophoresis. Unfortunately, in this study was not possible to get a *M. javanica* control sample for comparison so that, the profiles were compared to diagrams from previous reports.

The first attempt to obtain phenotype patterns of *Meloidogyne* was by using samples from West Portugal, but all times this procedure was tried the expected outcome was not achieved. This was probably because the females were not in ideal conditions for protein extraction.

The second attempt was carried out with samples from North Portugal. *Meloidogyne* females were retrieved more efficiently, probably because better skills had been acquired by then to deal with the specimens. The results show a clear profile that was identified as *M. javanica* when comparing with the diagrams available in the literature. This technique apart from identifying the root-nematode species it was also useful to show that not only the biochemical procedure can influence the results but also the preparation of the material can do it in greater proportions.

CHAPTER 3: MOLECULAR STUDIES

1. INTRODUCTION

Isozymes continue to be widely used for studies of *Meloidogyne* species despite being suitable for a specific developmental stage (mature females). However, since agricultural soils do not contain *Meloidogyne* adult females the identification of second stage juveniles will improve crop management decisions (Power & Harris, 1993).

In surveys targeting *Meloidogyne* species, isozymes can be used as a convenient preliminary stage in species identification. Remarkably, many useful esterase patterns are still being discovered, but to determine their specificity and sensitivity, other additional identification method such as molecular identification should be employed. That is why DNA-based diagnostics provide an attractive solution, since they do not rely on the expressed products of the genome, they are independent of environmental influence and of the stage of the nematode life cycle, and are potentially extremely discriminating.

Until now, a great variety of molecular approaches have been adopted to identify *Meloidogyne* spp. (Blok & Powers, 2009). These methods include the use of target regions such as: the mitochondrial DNA (mtDNA) (Tigano *et al*., 2005); intergenic spacer region (IGS) (Blok *et al*., 1997; Wishart *et al*., 2002; Adam *et al*., 2007); external transcribed spacer region (ETS) and internal transcribed spacer regions (ITS) (Palomares *et al*., 2007). Others include use of sequence characterized amplified region (SCAR) markers (Zijlstra, 2000; Randig *et al*., 2002; Tigano *et al.,* 2010).

Due to the evolutionary closeness lineage and polyploidy genomics between the root-knot species *M. arenaria*, *M. incognita* and *M. javanica,* the development of molecular techniques for identification is more difficult (Triantaphyllou, 1985; Al Banna *et al*., 1997). There is a wide availability of molecular identification reports for these species (Chacon *et al*., 1991; Powers & Harris, 1993; Baum *et al*., 1994; Zijlstra *et al*. 2000; Qiu *et al*., 2006; Adam *et al*. 2007). Techniques to distinguish between various *Meloidogyne* species have been developed3, but 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)(Janssen, 2016).

1.1 Ribosomal DNA

Comparative analysis of coding and noncoding regions of ribosomal DNA (rDNA) is a widely used tool for species and subspecies identification of many organisms (Zijlstra e*t al.,* 1995). Ribosomal DNA genes (18S, 28S and 5.8S) are arranged in tandem repeats that are kept apart by internal and external transcribed spacers (ITS and ETS) and adjacent to them are the intergenic spacer regions (IGS) (Figure 20).

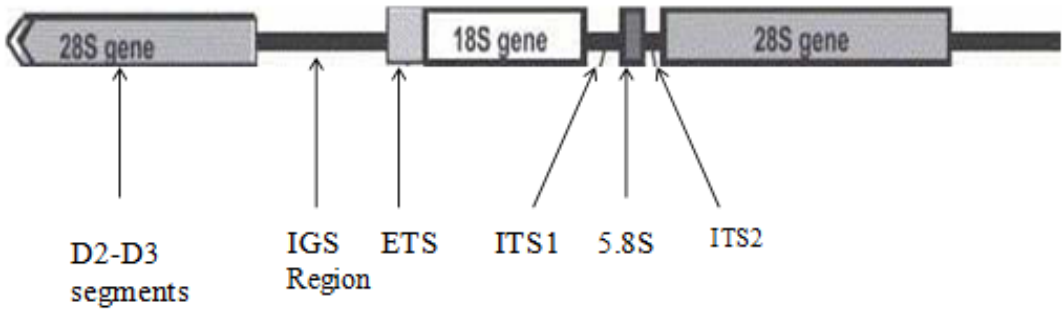

Figure 20 **-** Schematic diagram of the multigenic family of ribosomal DNA (rDNA) (Adapted from Douda et al., 2013).

The 18S, 28S and 5.8S coding genes are usually used in nematode diagnostics since they are greatly conserved compared to the transcribed and non-transcribed regions (ITS, ETS and IGS), that is the sequences of the rDNA genes are highly conserved whereas there is less conservation within the internal transcribed spacers (ITS) regions and little homology is found in the non-transcribed spacer regions (Albana *et al*., 1997). The more conserved sequences are the most useful for classification at higher taxonomic levels (genus to phylum) while the ITS sequences are useful at species and subspecies levels (Hyman & Powers, 1991).

1.2 Mitochondrial DNA

The mitochondrial genome of the majority of nematodes include 12 protein-coding genes, all components of the oxidative phosphorylation system including subunits of cytochrome c oxidase (COI – COII); 22 transfer RNA (tRNA) genes and rRNA genes encoding SSU and LSU rRNAS (Subbotin & Moens, 2006)

From the perspective of identification, the mitochondrial genome (mtDNA) (Figure 21) provides a rich source of genetic markers for identification (Rubinoff & Holland, 2005; Hu &

Glasser, 2006). Multiple copies of the circular mitochondrial genome are contained within each cell, providing ample template for PCR assays. mtDNA has been widely used not only for molecular phylogenetic relationship and evolutionary studies, but also for species identification and genetic investigations due to the abundance, small size, maternal inheritance, relatively rapid evolutionary rate and lack of genetic recombination of mtDNA (Liu *et al*., 2012).

An important region of the mitochondrial genome of *Meloidogyne* for diagnostic development is the portion of the genome flanked by the COII gene and the large (16S) ribosomal gene (Blok & Powers, 2009). In *Meloidogyne*, the mitochondrial DNA segment including 3′ end of *COII* and the 5′ portion of *16S* gene plays an increasingly important role in the differentiation of major *Meloidogyne* species (Powers & Harris, 1993).

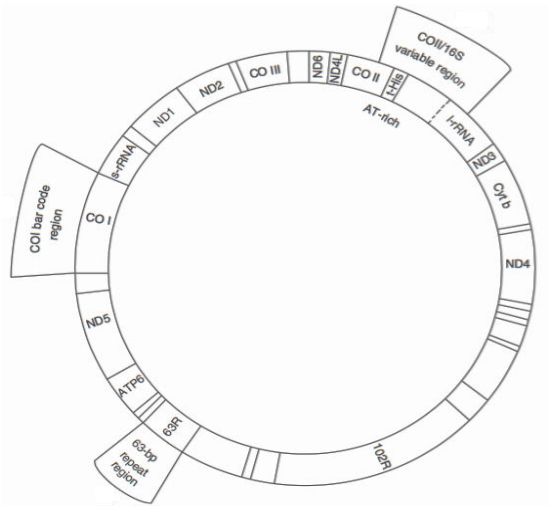

Figure 21 - *Meloidogyne* mitochondrial genome structure. (After Okimoto et al.,1991).

1.3 Sequence Characterized Amplified Regions (SCARs)

Specific primers have been developed to PCR-amplify diagnostic repetitive regions of sequence (SCARs). To develop this, the genetic diversity within certain species was assessed by RAPD (*Random Amplification of Polymorphic DNA)*, then RAPD-markers specific to these species were selected in order to convert them into specific characterized amplified region (SCAR) markers (Paran & Michelmore, 1993). SCAR-PCR methods have the potential to be used in routine diagnostic applications using DNA extracts from single juveniles, soil samples or even infected plant materials (Zijlistra, 2000).

2. MATERIALS AND METHODS

2.1 DNA Extraction

For molecular analysis, total genomic DNA was isolated from juveniles, females, egg masses and galls as listed in Table 5.

Table 5 - DNA extraction, dates and material extracted

Number	Date	Reference material	West Portugal	North Portugal
1	11 April 2016	1 juvenile/tube		
2	05 May 2016	5 juveniles/tube		
3	01 June 2016		1&7 juveniles/tube 1 egg mass/tube	
4	04 August 2016			1 female/tube

Juveniles, females, egg masses and galls were handpicked and transferred to Eppendorf tubes with 10 µl of sterile water, frozen in liquid nitrogen and then crushed with a micro pestle (Eppendorf, Hamburg, Germany). The homogenate was incubated in lysis buffer and 20 µl of Proteinase K (100µg ml-1) at 56 °C for approximately 3 hours. Following the incubation DNA extraction was carried out using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.2 Polymerase Chain Reaction

Several PCR with universal and species-specific primers were performed. Universal primers were used to amplify the rDNA internal transcribed spacer (ITS) and intergenic spacers (IGS) regions, the expansion segment D2/D3 of the 28S rDNA and the mtDNA region located between the 3' region of the COII and the 5' end region of the 16S rDNA (Figures 20 and 21). Species-specific primers that amplify SCAR markers were also used in this study. All primers were synthesised by STAB VIDA Facilities (Lisbon, Portugal).

Total DNA isolated from 5 individuals (J2), 1-individual (J2), females and egg masses were used to test the performance of the various combinations of primers.

All PCR reactions were performed in a 25 µl final volume and to optimise the amplification were assayed two different amplification kits: Dream Taq PCR Master Mix Kit (2X) (Thermo Scientific, Germany) and Promega Go Taq Flexi DNA Polymerase Kit (Promega, Madison) (Appendix 2).

PCR reactions were performed in a Biometra TGradient thermo cycler (Biometra, Göttingen, Germany) and several thermal cycling conditions were tested: Initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 1min, annealing temperature according to the primers (Appendix 3) and an extension at 72 °C for 1 min. A final extension at 72 °C for 10 min. The products were resolved by electrophoresis at 5 V.cm-1 in agarose gel (1,5%) containing 0,5 µg/ml ethidium bromide and 0,5x TBE running buffer. Data analysis was visualised by VersaDoc Imaging System (BioRad, USA). A great variety of assays were carried out for PCR optimisation.

2.3 Sequencing

To verify the identity of the reference material from NVWA, some bands corresponding to the PCR amplification products, obtained with universal primers, were isolated from gel and purified with the Gene JET™ PCR purification Kit (Fermentas, Germany) following the manufacturer's instructions.

The purified PCR products (Appendix 4) were sequenced in forward direction at STABVida Sequencing Laboratory (Lisbon, Portugal) on a DNA analyser ABI PRISM 3730xl (Applied Bio systems).

Nucleotide sequences were edited and analysed using BioEdit v7.2.0 (Hall, 2007) program and were compared with related sequences deposited in GenBank using BLAST engine search (http://www.ncbi.nlm.nih.gov/) for sequence similarity searching.

2.4 Cloning and sequencing

PCR products from ITS rDNA region obtained with primers TW81 and AB28 were chosen for cloning, using TA Cloning® kit Dual promoter with pCR®II (Invitrogen, Life Technologies) according to the manufacturer's instructions. Previously, an addition of A-overhangs was

carried out before the cloning process started, since PCR products were obtained with antecedence. Five positive colonies for each sample were screened by colony PCR with vector primers, in order to confirm the ligation of the insert. The clones containing the insert of expected size (See appendix 4) were sent for sequencing in both directions at STAB VIDA Facilities (Lisbon, Portugal) using a DNA analyser ABI PROSM 3730 xl (Applied Bio system).

To identify *Meloidogyne* species, the obtained raw sequences were checked and edited using sequence manipulation suite programme. Consensus sequences acquired were compared to those deposited in GenBank database through BLAST tool for sequence homology.

3. RESULTS

The ITS region from a wide taxonomic range of nematodes, including plant-parasitic species is considered as a taxonomic marker and it is referred by Powers *et al*. (1997) that there is not a single nematode species that has failed to provide an amplification product of the ITS region when amplified with universal PCR primer sets. This author also states that the size of the amplified product aids in the initial divergence among members of the group.

Based on that, the first approach to this study was to carry out an initial PCR amplification using the primers targeting the rDNA ITS region (ITS1/Vrain2) and the DNA isolated from second stage *Meloidogyne* juveniles from reference material as well as other nematode DNA, already available in the Laboratory of Genetics. Amplified products are shown in (Figure 22); *M. incognita, M. arenaria* and *M. javanica* yield a fragment size of 700 bp, which is in agreement to what previous studies have reported.

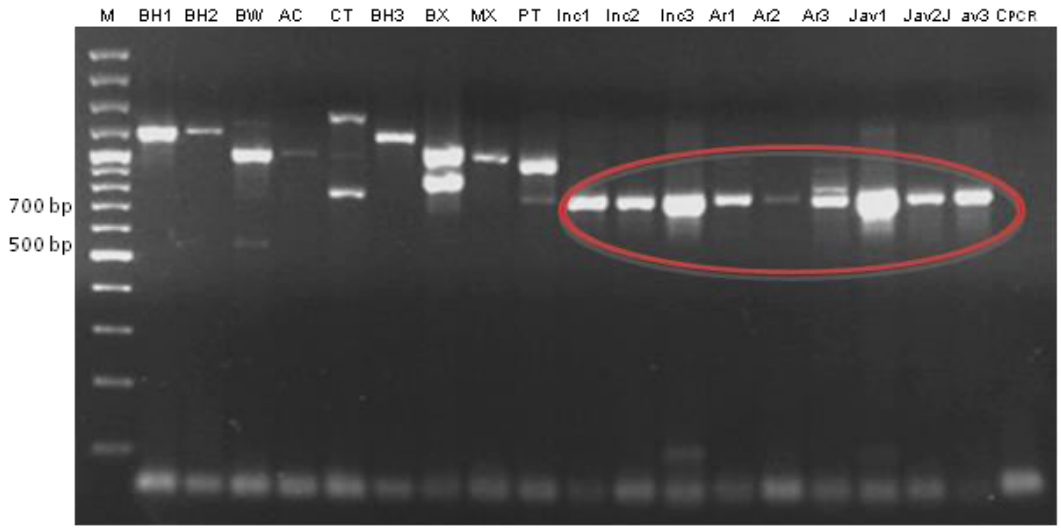

Figure 22 - Amplification products obtained from ten nematode isolates using ITS1 and Vrain2R primers. M: DNA marker (Gene Ruler 100 bp DNA, Thermo Scientific); BH1-BH3: *Bursaphelencus hofmanii*; BW: *Bursaphelencus willibaldi*; AC: *Acrobeloides*; BX: *Bursaphelencus xylophilus*; MX: *Mesocricoma xeno*plax; PT: *Pratylenchus*; Inc1-Inc3- *Meloidogyne incognita*: Ar1- Ar3: *Meloidogyne arenaria*; Jav1- Jav3: *Meloidogyne javanica*; C_{PCR}: PCR Control.

Considering that the results confirmed the presence of enough/viable DNA in the samples and that the DNA amplifications were successful, the next step was to carry out PCR amplifications using species-specific primers and DNA isolated from the same reference material received from the Netherlands and also soil samples collected from West Portugal.

Unfortunately, PCR products with SCAR species-specific primers for *M. incognita, M. arenaria* and *M. javanica* did not have any amplification results despite carrying out a great amount of assays to optimise the amplification conditions such as concentrations of magnesium chloride, DNA and primers, annealing temperatures and different amplification parameters. In order to overcome these difficulties, it was necessary to appeal to PCR products sequencing with universal primers.

PCR reactions employing universal primers (TW81/AB28, D3A/D3 and D2A/D3) targeting 28S rDNA gene for nematodes were performed. The fragment size obtained from amplifications with primers TW81/AB28 was around 700 bp (Figure 23), which was consistent with earlier studies.

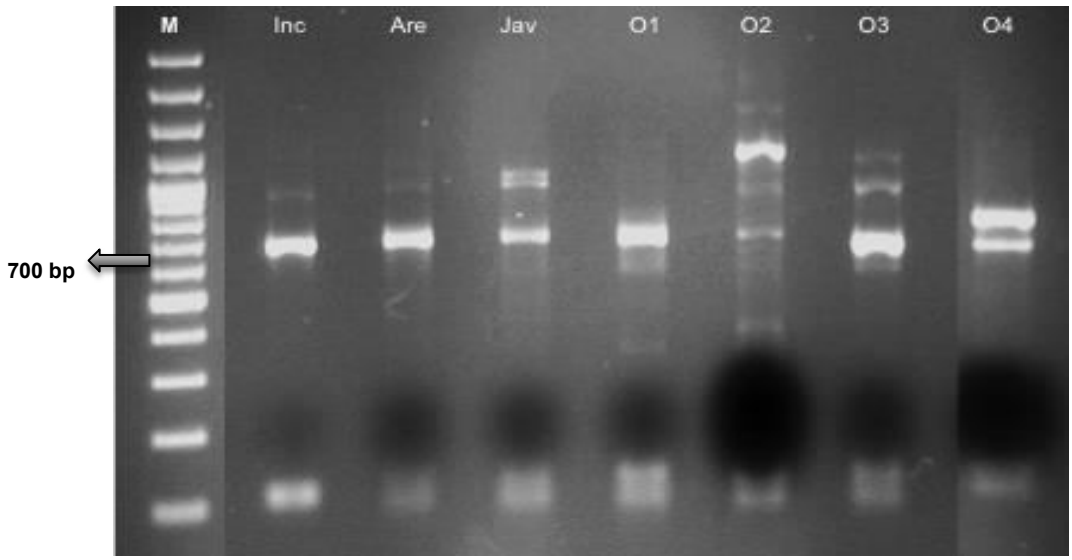

Figure 23 - DNA amplification products obtained from seven isolates of *Meloidogyne* spp. using universal primers TW81/AB28. M: DNA marker (GeneRuler 100 bp DNA Ladder, Thermo Scientific); Inc: *Meloidogyne incognita*; Are: *Meloidogyne arenaria*; Jav: *Meloidogyne javanica*; O1: 3294 West Portugal; O2: 3295 West Portugal; O3: 3296 West Portugal and O4: 3297 West Portugal.

A fragment size of 350 bp was achieved using primers D3A/D3B (Figure 24), which is in agreement with prior descriptions.

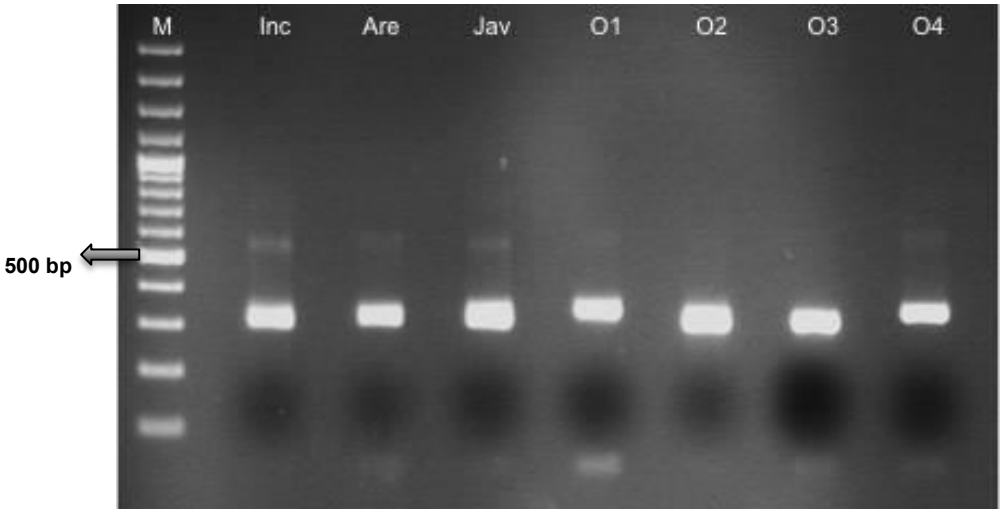

Figure 24 - DNA amplification products obtained from seven isolates of *Meloidogyne* spp. using universal primers D3A/D3B. M: DNA marker (GeneRuler 100 bp DNA Ladder, Thermo Scientific); Inc: *Meloidogyne incognita*; Are: *Meloidogyne arenaria*; Jav: *Meloidogyne javanica*; O1: 3294 West Portugal; O2: 3295 West Portugal; O3: 3296 West Portugal and O4: 3297 West Portugal.

PCR products using primers D2A/D3 showed an amplification fragment of 800 pb (Figure 25), which is in accordance with previous studies.

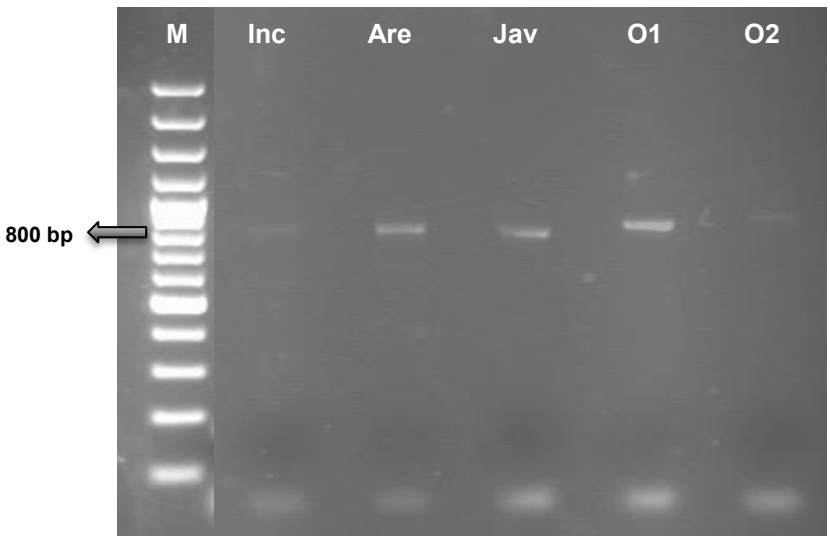

Figure 25 - DNA amplification products obtained from three isolates of *Meloidogyne* spp. using universal primers D2A/D3B. M: DNA marker (GeneRuler 100 bp DNA Ladder, Thermo Scientific); Inc: *Meloidogyne incog*nita; Are: *Meloidogyne arenaria*; Jav: *Meloidogyne javanica*; O1: 3294 West Portugal; O2: 3295 West Portugal.

PCR products were purified and a second amplification took place to confirm the existence of a single band. Selected samples showing single fragments were sent for sequencing at STAB Vida Sequencing Laboratory (Lisbon, Portugal).

Specimens from North Portugal were identified as *M. javanica* through morphology and biochemical methods. In order to confirm this result, the species-specific primers (Fjav/Rjav) were used, as well as universal primers (TW81/AB28 and PR81/AB28) in case sequencing was needed. As can be seen in Figure 26 the results are in complete accordance with what it was expected; a fragment size of 600 bp was achieved using species-specific primers and a fragment of 700 bp using universal primers confirming the identity of the samples as *M. javanica.*

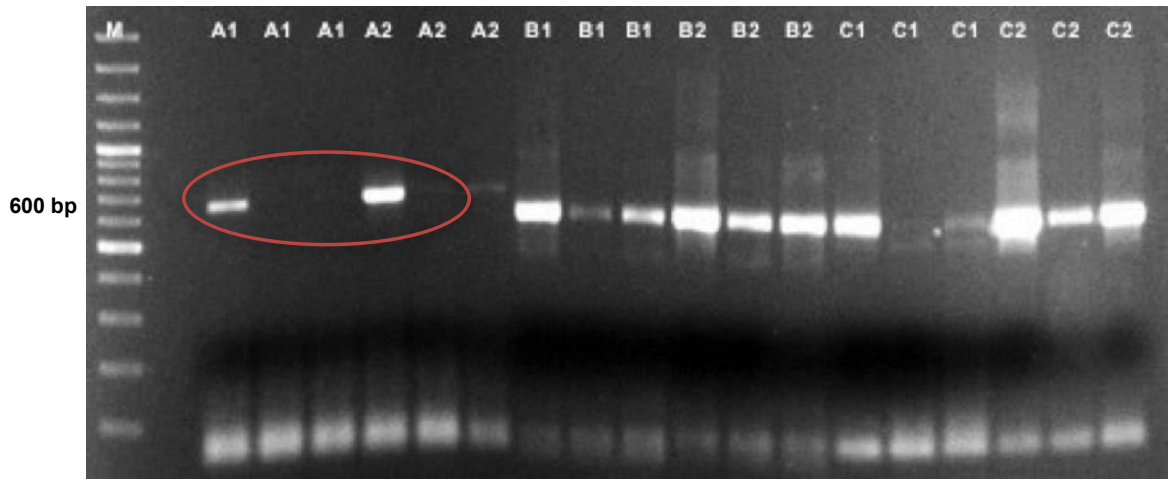

Figure 26 - DNA amplification products obtained from three isolates of *Meloidogyne* spp.. from North Portugal samples using species-specific primers (Fjav/Rjav) (A), universal primers TW81/AB28 (B) and PR81/AB28 (C); 1: 1µl DNA and 2: 5µl DNA. M: DNA marker (GeneRuler 100 bp DNA Ladder, Thermo Scientific).

Owing to the problems encountered in the verification and identification of the reference material from NVWA and West Portugal employing universal and specific primers, the next approach was to clone the samples comprising the specific primer regions. Colony PCR screening showed two different sizes of fragments, the first one corresponds to the vector on its own (240bp) and the second one that has the expected size (900bp) relates to the vector + insert of interest.

Table 6 shows the results from the sequence similarity search in the GenBank Database using the BLASTn program supported by the National Centre for Biotechnology Information (NCBI).

Table 6 - Alignment results from direct sequencing and cloning

Code sequence	Description	Query cover	E value	Ident	Accession No
JAV2B	**Meloidogyne javanica*	100%	0.0	100%	KX646188.1
	**Meloidogyne javanica*	100%	0.0	100%	KP901084.1
	**Meloidogyne javanica*	100%	0.0	100%	KP901083.1
	**Meloidogyne incognita*	100%	0.0	100%	KP901072.1
	**Meloidogyne incognita*	100%	0.0	100%	KP901070.1
ARE3B	**Meloidogyne javanica*	100%	0.0	99%	KX646188.1
	**Meloidogyne javanica*	100%	0.0	99%	KP901084.1
	**Meloidogyne javanica*	100%	0.0	99%	KP901083.1
	**Meloidogyne incognita*	100%	0.0	99%	KP901072.1
	**Meloidogyne incognita*	100%	0.0	99%	KP901070.1
INC1B	Sequence in poor conditions				
B1INC	*Fungal sp	100%	0.0	94%	KT996068.1
	*Fungal sp	100%	0.0	94%	KT996067.1
	*Fungal sp	100%	0.0	94%	KM265990.1
	*Fungal endophyte	100%	0.0	94%	KM265593.1
	*Plectosphaerella	100%	0.0	94%	KF435381.1
B2ARE	*Uncultured fungus clone 2168	98%	4e-71	95%	KP898063.1
	* Sarea resinae	88%	1e-70	98%	AY590792.1
	* Sarea resinae	88%	2e-68	97%	JF440615.1
	* Sarea resinae strain ASR H15	88%	2e-64	96%	JX421720.1
	* Uncultured fungus clone 2168	98%	4e-61	92%	KP897867.1
B3JAV	*Pyrenochaeta sp. MUT 5325	100%	0.0	98%	KU255067.1
	*Pyrenochaeta sp. MUT 5366	100%	0.0	98%	KU158171.1
	*Pleosporaceae sp.	100%	0.0	98%	KF428669.1
	*Pleosporaceae sp.	100%	0.0	98%	KF428350.1
	**Alternaria* spp.	100%	0.0	98%	GU934500.1

Code sequence	Description	Query cover	E value	Ident	Accession No
B63297B	Sequence in poor conditions				
3294D3	Sequence could not be obtained				
3294Far	Sequence could not be obtained				

Cloning					
I3	*Uncultured eukaryote genomic DNA	93%	0.0	98%	HE605278.1
	**Colpoda inflate*	78%	0.0	97%	KM222071.1
	*Uncultured eukaryote genes for ITS1	88%	0.0	92%	AB222616.1
	**Begonia fenicis*	84%	6e-140	84%	AJ491193.1
	**Colpoda cucullus*	40%	2e-135	96%	AB684383.1
AREB2	**Meloidogyne ethiopica*	98%	0.0	100%	KF482366.1
	**Meloidogyne* sp.	98%	0.0	100%	JX465590.1
	**Meloidogyne* sp.	98%	0.0	99%	JX465589.1
	**Meloidogyne* sp.	98%	0.0	99%	JX465587.1
	**Meloidogyne hispanica*	98%	0.0	99%	KU521802.1
JAVB5	*Uncultured Ascomycota clone	100%	0.0	99%	JX998693.1
	*Uncultured Ascomycota clone	100%	0.0	99%	JX998692.1
	**Alternaria* sp.	100%	0.0	98%	KU059938.1
	**Alternaria* sp.	100%	0.0	98%	KU059824.1
	**Pyrenochaeta gentianicola*	100%	0.0	98%	KU204576.1
96C	**Plectosphaerella oligotrophica*	100%	0.0	99%	KX446769.1
	**Plectosphaerella cucumerina*	100%	0.0	99%	KU059968.1
	**Plectosphaerella cucumerina*	100%	0.0	99%	KU059809.1
	*Uncultured *Plectosphaerella*	100%	0.0	99%	HG936781.1
	*Uncultured *Plectosphaerella*	100%	0.0	99%	HG936780.1
97B	*Cloning vector pamGFP5’	60%	3e-68	99%	JX156629.1
	*Cloning vector pamGFP	60%	3e-68	99%	JX156628.1
	*Cloning vector phis-5GFP5’	60%	1e-67	99%	JX156633.1
	*Cloning vector phis-5GFP	60%	1e-67	99%	JX156632.1
	*Cloning vector pPGKneo-II	60%	1e-67	99%	AF335420.3

For some sequences obtained either directly from PCR products or from cloned fragments, the *DNA* sequencing chromatogram quality was poor with a high background noise and odd peaks present underneath the main sequence peaks. So, it was not possible to use them for similarity search in the BLAST database.

Sequences obtained from PCR products of samples B53297A, JAV2B, ARE3B showed almost 100% affinity with the three species of *Meloidogyne* under study. Similarly, the sequence obtained from the cloned sample AREB2 showed 98% analogy not only with *Meloidogyne arenaria* but also with *Meloidogyne hapla, Meloidogyne incognita, Meloidogyne enterelobii, Meloidogyne ethiopica* and *Meloidogyne hispanica.* Thus, confirming it was not possible to give an accurate identification of the species.

Many other sequences revealed high similarities with fungal species suggesting the material could have been contaminated.

4. DISCUSSION

Due to the need of a rapid and reliable identification of RKN, one of the approaches to this work was based on molecular methods in which PCR-based assays were performed using second stage juveniles DNA isolated from soil samples of reference material from NVWA, Wageningen and also from national samples collected in fields from West and North Portugal.

DNA amplifications were carried out using universal primers for nuclear ribosomal DNA regions namely the rDNA internal transcribed spacer (ITS) and intergenic spacers (IGS) regions for the expansion segment D2/D3 of the 28S rRNA and also for mitochondrial DNA regions located between 3' region of COII and the 5' end region of 16S rRNA. However, the banding profiles of PCR products were neither consistent nor reproducible even when all the conditions were kept constant. To illustrate this further, an initial amplification with primers ITS1/Vrain2 was achieved but in a second attempt there were not amplified DNA fragments.

Owing to the inconsistencies of the results, changes in all factors that could influence the end result such as annealing temperatures and PCR reaction components were made. Nevertheless, after many attempts, it was not possible to get amplification results. Some reasons referred as the possible cause for these results are related to DNA: its poor quality when used as template, the presence of inhibitors, or eventually its degradation due to a nuclease contamination, or the yield of DNA after isolation. Knowing the critical importance of the purity and quality of template DNA, for this study a commercial DNA extraction kit was used. Regarding the concentration of DNA for PCR reactions it has been described that for higher complexity templates (i.e. genomic DNA) 1 ng to 1 µg of DNA per 50 µl reaction is enough.

In the next step, SCAR species-specific primers were tested in the same samples and no DNA amplifications were obtained, even after many attempts. Nonetheless, an unusual outcome was seen on samples from West Portugal using specific primers (FAR/RAR) for *M. arenaria,* where the expected size fragment was 420 bp and the obtained fragment size was of 150 bp. Once again, these results suggest that the samples could have been contaminated. Equally important, species-specific primers SCAR helped to confirm the identity of the specimens found in samples from North Portugal as *Meloidogyne javanica*.

Primers targeting the 3' portion of COII and the 5' portion of 16S rRNA were assessed since previous studies have shown its effectiveness on the identification of *Meloidogyne* species. Under the present work various assays were performed using the primers (C2F3/1108-

MRH106) aiming to identify the species under study. However, contrary to what preceding studies showed there was not DNA amplification.

Many attempts to distinguish the three species of *Meloidogyne* with the universal primers specific for nematodes (TW81/AB28, D3A/D3B and D2A/D3) were tried with the purpose of sending the PCR products for sequencing. Primers (TW81/AB28) showed double bands with a main fragment of around 750-800 bp, the primers (D3A/D3B) had an amplified fragment of 350 bp and primers (D2A/D3) showed a band size of 800 bp agreeing with what it has been reported.

The obtained sequences validated the fact *M. incognita, M. arenaria* and *M. javanica* are almost identical and therefore confirming the similarity within the genus *Meloidogyne*. All this reaffirms the difficulty on root-knot nematode identification.

Sequences obtained from cloned fragments of *M. incognita* and *M. javanica* did not show any homology with *Meloidogyne* but instead with many species of fungus, contrary to this *M. arenaria* presented homology with more than five different species of *Meloidogyne*. Despite having carried out many assays it was not possible to prove the identity of the material from NVWA, Wageningen nor to identify the species found in the material from West Portugal.

To sum up, despite the difficulties with the DNA amplification, it should be noted that the usage of nuclear and mitochondrial markers has many advantages, for instance, the possibility of being applied on J2, females and males depending on what it is available, as well as the likelihood of RKN detection using a robust and accurate PCR assay. Nonetheless, due to the hybrid origin of parthenogenetic RKNs as suggested by Lunt *et al*., (2014), the results must be obtained by analysing multiple marker genes of the mtDNA (Janssen *et al*., 2016) since it is an important aspect of root-nematodes that needs to be taken into account to avoid misidentifications and to optimise management strategies.

General Discussion and Conclusion

One of the most widely distributed pests causing economically important damages in a large number of crops are the nematodes of the genus *Meloidogyne.* Four species in particular, *M. incognita, M. arenaria, M. javanica* and *M. hapla,* which due to their reproductive rate, short generation time and wide host range, are very difficult to control (Trudgill & Block, 2001).

This study was conducted in the Laboratories of Molecular Genetics and Nematology at the INIAV, that have an increasing need of accurately identify these nematodes. The objective was to develop an identification tool for the three species of *Meloidogyne* under study, but as could be seen along this work, the diagnosis of species and races of *Meloidogyne* is challenging because of poorly defined boundaries among species, intraspecific variability, potential hybrid origin, and polyploidy. The three studied species are so closely related that their identification was even more defiant than expected.

Based on the above, the three available approaches (morphological, biochemical and molecular) were treated as individual tools of verification and identification but as this study progressed it became evident the need of using a conjunction of methods based on morphometric and biochemical features together with molecular techniques. This fact confirms that one single technique cannot be relied upon, since *Meloidogyne'*s complexity require all available methodologies in order to get more accurate diagnostics that will lead to better management decisions and control measures.

Consequently, it was found that morphological and morphometric identification was arduous, measurements overlapped within the species and perineal patterns were useful but variable. For instance, lateral lines of perineal area could be seen in all patterns that inclined people to identify the species as *M. javanica*. Only when the patterns of the three species were compared it could be spotted that the lateral lines in *M. javanica* were much more profound and well defined, confirming the inaccuracy and difficulty of this type of identification.

Conversely, the effectiveness of isozyme analysis has been proven through previous researches and was performed three times throughout this dissertation with positive results on the last attempt. Despite having reliable results, isozyme analysis is a very restrictive technique since it only works with a specific developmental stage (mature females).

Regarding the molecular approach, DNA extraction was performed using a commercial kit. However, there are many other available methods that were not tested on this research. As a result, when using universal primers it could be confirmed the existence of viable DNA on the

different samples. Equally, many SCARs species-specific primers were assessed, resulting in the no amplification of the PCR products, which is in complete disagreement with earlier studies. Successful results were only obtained with samples from North Portugal; this gives some evidence to suggest that we could have been dealing with species outside the ones under study or that the samples were contaminated. In addition, sequencing and cloning of samples from The Netherlands and West Portugal could not provide conclusive results in the species identification.

As a matter of fact, a possible explanation for the above results lays on the fact that when isolating DNA from small organisms in natural environments such as soil or plants, it is often impossible to avoid co-extracting DNA from many other biological materials that may not be of interest such as microorganisms and fungal spores. The PCR can be used to isolate and amplify a gene of interest from a mixed sample of DNA. However, all ribosomal genes are universally present in eukaryotes and commonly used PCR primers bind to highly conserved regions of the gene, which will potentially amplify any that is homologue, regardless of its organism of origin.

Moreover, although the PCR process is fast, straightforward and able to determine the species identity irrespective of the developmental stage and from small amounts of tissues, its reliability is uncertain due to the intraspecific variability and closeness within the three species so, it is always necessary to confirm identification by analysing multiple marker genes.

In conclusion, an accurate identification poses a great challenge for planning root-knot nematode management strategies. The presence of *M. incognita, M. javanica* and *M. arenaria* in agricultural areas from Portugal is of concern and if not managed, root-knot nematodes can cause significant loss in the quality and quantity of common vegetable crop production in Portugal.

Additionally, the execution of these three approaches is not always possible as it is the case of laboratories with many samples to process so, isozyme analysis should be the first tool used towards the identification of *Meloidogyne* when females are available since it has been proven to be very reliable. Also, for future studies it is recommended the use of different DNA extraction techniques to compare their efficiency and be able to make the best choice.

Lastly, due to the difficulty on *Meloidogyne* species identification and to ensure all developmental stages are available, it is advisable not only to have soil samples but also root samples from current crops or any weeds present in the field, so that the laboratories are able to perform detail studies and deliver valid results.

Finally, this work brings some insight on morphology, morphometric, biochemical and molecular characteristics of Portuguese samples. Therefore these results are of great interest to the future diagnosis work that will be conducted at the INIAV.

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APPENDIX 1

PREPARATION OF POLYACRYLAMIDE GELS

The stock solutions used for polyacrylamide gel preparation are described on Table A. The separation gel is prepared first, using the stock solutions on the proportions indicated on table B and without the amomonium persulfate (APS); once all had been mixed properly it was added the APS. Immeadiately after, the solution was poured into the glass plates and overlay with propanol to flattened the surface. The gel was allowed to polymerize for about 15min, the propanol was poured off and the gel's surface rinsed with distilled water. While the separation gel was polymerizing the preparation of the stacking gel started following the same procedure as before. Once the gel had been poured between the glass plates a comb was placed just to mark the wells where the samples were going to be loaded

Table A. Stock Solutions for polyacrylamide gel preparation

Polyacrylamide Solution (36,5:1)	
Acrylamide	29.2g
Bisacrylamide	0.8g
	Water up to 100ml and filter

Tris-HCL 1.0M pH 8.8	
TRIS base	60.55g
	Water up to 500ml

Tris-HCL 0.5M pH 6.8	
TRIS base	30.275g
	Water up to 500ml

Ammonium Persulfate Solution (APS)	
Ammonium persulfate	0.1g
	Water up to 1ml

Electrophoresis Buffer pH 8.3	
TRIS Base	3.03g
Glycine	14.4g
	Water up to 1000ml

Sodium phosphate Buffer 0.2m pH 7.2	
Disodium phosphate 0.1M	144ml
Monisodium phosphate 0.1m	56ml
	Water up to 400ml

The separating and stacking gels were homogeneous 7% and 3% polyacrylamide Table B

Table B. Polyacrylamida Gel Preparation

Separation gel solution 7%	
Polyacrylamide	2.33ml
Tris-HCl 1.0M pH 8.8	3.75ml
TEMED (tetramethylethylenediamine)	50µl
	Water up to 10ml
Ammonium persulfate (APS)10%	50µl
Stacking Gel Solution (3%)	
Polyacrilamide	0.5ml
Tris HCl 0.5M pH 6.8	1.25ml
TEMED	25µl
	Water up to 5ml
APS 10%	25µl

Table C. Esterase's Staining Solution

STAINING SOLUTION (Add solution B to A while stirring)	
Solution A	
Sodium Phosphate buffer 0.2M pH7.2	100ml
Fast Blue RR	60mg
Solution B	
Acetone	2ml
α – naphthyl acetate	80mg

APPENDIX 2

Table A. PCR reaction's components

	Component	Volume (µl)	Final Concentration
Dream Taq PCR Master Mix Kit	DreamTaq Master mix (2x)	12,5	(1x)
	Primer Forward (10µM)	2,0	(0,8µM)
	Primer Reverse (10µM)	2,0	(0,8µM)
	Distilled and sterile water	7,5	
	DNA	1,0	
Promega Go taq Flexi DNA Polymerase Kit	Go taq Flexi Buffer (5x)	5,0	(1x)
	MgCl2 (25mM)	2,5	(2,5mM)
	dNTPS (20mM)	0,5	(0,2mM)
	Primer Forward (10µM)	1,0	(0,5µM)
	Primer Reverse (10µM)	1,0	(0,5µM)
	Go taq Polymerase (5U/µl)	0,25	(1.25U)
	Distilled and sterile water	13,75	
	DNA	1,0	

APPENDIX 3

Table A. Universal primers codes for molecular identification of *Meloidogyne incognita, Meloidogyne javanica* and *Meloidogyne arenaria*, their sequences and sources

Code	Region	PCR Product (bp)	Sequence 5'-3'	Ta (ºC)	Reference
S-ITS1/ Vrain2R	ITS1	750	TTGATTACGTCCCTGCCCTTT TTTCACTCGCCGTTACTAAGGGAATC	55°C	Vrain *et al*., 1992
D2A -D2* /D3B	28S rDNA	800	*ACAAGTACCGTGAGGGAAAGT ACAAGTACCGTGAGGGAAAGTTG TCGGAAGGAACCAGCTACTA	53°C	De ley *et al*., 1999
194/195	IGS	720	TTAACTTGCCAGATCGGACG TCTAATGAGCCGTACGC	50°C	Blok *et al*., 1997
***TW81-PR81/ AB28**	28S rDNA	700	*GTTTCCGTAGGTGAACCTGC GTAGGTGAACCTGCTGCTG ATATGCTTAAGTTCAGCGGGT	55°C	Joyce *et al*., 1994
D3A/D3B	28S rDNA	350	GACCCGTCTTGAAACACGGA TCGGAAGGAACCAGCTACTA	55°C	Chen *et al*., 2003
C2F3/ 1108	COII 16S	¥1700 ∂1100	GGTCAATGTTCAGAAATTTGTGG TACCTTTGACCAATCACGCT	50°C	Power & Harris, 1993
C2F3/ MRH106	COII 16S rRna	650	GGTCAATGTTCAGAAATTTGTGG AAT TTCTAAAGACTTTTCTTAGT	56°C	Staton *et al*., 1997

Ta –Temperature of annealing

∂-Fragment size belonging to *M. arenaria* ¥: Fragment size belonging to *M. incognita* and *M. javanica.*

Table B. Species-specific primers codes used for molecular identification of the three most important species of *Meloidogyne*, their sources and sequences

Code	Species	PCR Product (bp)	Sequence 5'-3'	Ta (ºC)	Reference
Finc/Rinc	*M. incognita*	1200	CTCTGCCCAATGAGCTGTCC CTCTGCCCTCACATTAGG-	54°C	Zijlstra *et al*.,2000
Far/Rar	*M. arenaria*	420	TCGGCGATAGAGGTAAATGAC TCGGCGATAGACACTACAACT	56°C	
Fjav/Rjav	*M. javanica*	670	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACTATAC	60°C	
DIF/DIR	*M. incognita*	1350-1370	TAGGCAGTAGGTTGTCGGG CAGATATCTCTGCATTGGTGC	62°C	*Dong *et al*.,2001
DJF/DJR	*M. javanica*	1650	CCTTAATGTCAACACTAGAGCC	52°C	
DAF/DAR	*M. arenaria*	950	TCGAGGGCATCTAATAAAGG GGGCTGAATATTCAAAGGAA	58°C	
MIF/MIR	*M. incognita*	999	GTGAGGATTCAGCTCCCCAG ACGAGGAACATACTTCTCCGTCC	60°C	Meng *et al*.,2004
incK14F/ incK14r	*M. incognita*	399	CCCGCTACACCCTCAACTTC GGGATGTGTAAATGCTCCTG	60°C	Randig *et al*.,2002
SEC1F/ SEC1R	*M. incognita*	502	GGGCAAGTAAGGATGCTCTG GCACCTCTTTCATAGCCACG	56°C	Teserova *et al*.,2003
MIGF/MIGR	*M. arenaria, M. javanica M. incognita*	500	ACACAGGGGAAAGTTTGCCA GAGTAAGGCGAAGCATATCC	58°C 1	Qiu *et al*., 2006

*Primers developed by Dong *et al*., 2001 have been indicated as DIF/DIR, DFJ/DFR and DAF/DAR in the present table since any specific acronym has no been given in their published article.

APPENDIX 4

PCR amplicons	Primer Forward	Molecular size (Kb)
JAV2B	D2A	0.8
ARE3B		
INC1B		
B1 INCOG	TW81	0.7
B2 ARE		
B3 JAV		
B5 3297A		
B6 3297B		
3294 D3	D3A	0.3
3294 ARE	Far	0.4

Table A. Sequenced PCR amplicons

Table B. Sequenced cloned amplicons

Cloned amplicons	Primer Forward	Molecular Size (Kb)
I3	T7	0.9
AREB2		
JAVB5		
96C		
97B		