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GENETIC DIVERSITY OF THE ROOT-LESION NEMATODE *PRATYLENCHUS PENETRANS*

Dissertação no âmbito do Mestrado em Biodiversidade e Biotecnologia Vegetal, orientada pela Doutora Ivânia Sofia Grasina Esteves e coorientada pela Doutora Joana Moura e Sá Cardoso, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia

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Genetic diversity of the root-lesion nematode *Pratylenchus penetrans*

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Abstract

Root-lesion nematodes (RLN), *Pratylenchus* spp., are a highly polyphagous group of plant-parasitic nematodes causing great economic losses worldwide. *Pratylenchus penetrans* is an important RLN species from an economic point of view, since it affects a wide range of cultivated plants. In Portugal, this species is found frequently in potato but despite the previous detection in this crop, little information is available on the morphometric and molecular characteristics of Portuguese isolates. The objective of this study was to assess the intra and inter-isolate variation of *P. penetrans* from Portugal, as well as their molecular relationships with isolates identified in other countries and hosts. In the first part of this study, the morphometric characters of five Portuguese *P. penetrans* isolates, propagated on *in vitro* carrot disc cultures, were compared in detail. Morphometrical variability within and between isolates were observed. However, differences fall within the range of the variability described previously in *P. penetrans* isolates from other parts of the world. Of the 15 morphometrical characters studied, the position of the vulva (V ratio) in the females and the stylet length in both females and males had the lowest coefficient of intra and inter-isolate variability. Their stability confirms that these characters are important in the diagnostics of this species. In the second part of the work, the cytochrome oxidase I (COI) gene and internal transcribed spacers (ITS) genomic region of the five isolates were selected for sequencing, in order to evaluate the intraspecific genetic diversity of this species. ITS region revealed higher genetic diversity than the COI, with 15 and 6 different haplotypes from the 15 ITS and 14 COI sequences, respectively. Intra and inter-isolate genetic diversity was found in all isolates with exception for one isolate in COI region. Results also showed that the differentiation of isolates from Portugal was not related with their geographical origin. In spite of the high intraspecific diversity found, phylogenetic analyses revealed that both COI and ITS regions allow the separation of *P. penetrans* species from other related species, such as *P. pinguicaudatus*, *P. fallax* and *P. thornei*. Additionally, no grouping of *P. penetrans* belonging to the same country or originated from the same host was found. The information obtained in this study contributes to increase understanding about the variability of *P. penetrans* occurring in Portuguese potato crops.

Keywords: COI; ITS; morphometry; Portugal; variability.

Resumo

Os nemátodes das lesões radiculares (NLR), *Pratylenchus* spp., são um grupo de nemátodes parasitas de plantas altamente polífagos, que têm um grande impacto económico a nível mundial. *Pratylenchus penetrans* é uma espécie importante de NLR do ponto de vista económico, pois afeta uma vasta gama de plantas cultivadas. Em Portugal, esta espécie é encontrada frequentemente em batateira, mas apesar da deteção prévia nesta cultura, existe pouca informação sobre as características morfométricas e moleculares de isolados portugueses. O objetivo deste estudo foi avaliar a variação inter e intra-isolados de *P. penetrans* em Portugal, bem como as relações moleculares com *P. penetrans* identificados noutros países e em outros hospedeiros. Na primeira parte deste estudo, os caracteres morfométricos de cinco isolados portugueses de *P. penetrans*, multiplicados *in vitro* em culturas de discos de cenoura, foram comparados, tendo sido encontrada variabilidade morfométrica dentro e entre os isolados. No entanto, as diferenças de variabilidade são semelhantes às descritas anteriormente para outros *P. penetrans* de outras partes do mundo. Dos 15 caracteres morfométricos estudados, a posição da vulva (razão de V) nas fêmeas e o comprimento do estilete, tanto nas fêmeas como nos machos, apresentaram o menor coeficiente de variabilidade intra e inter-isolado. A sua estabilidade confirma que estes caracteres são importantes no diagnóstico desta espécie. Na segunda parte do trabalho, foi avaliada a diversidade genética dos cinco isolados nas regiões genómicas COI e ITS. Os isolados apresentaram maior diversidade genética na região ITS do que na região COI, com 15 e 6 haplótipos diferentes, das 15 sequências ITS e 14 sequências COI, respetivamente. Foi encontrada diversidade genética intra isolado em todos os isolados analisados, com exceção de um isolado na região COI. Os resultados também mostraram que a diferenciação dos isolados de Portugal não estava relacionada com a sua origem geográfica. Apesar da elevada diversidade intraespecífica encontrada, as análises filogenéticas revelaram que ambas as regiões, COI e ITS, permitem separar as espécies de *P. penetrans* de outras espécies próximas, como *P. pinguicaudatus*, *P. fallax* e *P. thornei*. No entanto, não foi possível agrupar *P. penetrans* pertencentes ao mesmo país ou provenientes de hospedeiros semelhantes. A informação obtida neste estudo contribui para aumentar o conhecimento sobre a variabilidade de *P. penetrans* associados à cultura de batateira em Portugal.

Palavras Chave: COI; ITS; morfometria; Portugal; variabilidade.

GENERAL INTRODUCTION

NEMATODES

Nematodes, phylum Nematoda, are one of the most abundant groups of living animals and are arguably the most plentiful metazoans in both terrestrial and aquatic environments (Grewal *et al*., 2005; Begum *et al*., 2017). Around $4.4 \pm 0.64 \times 10^{20}$ nematodes inhabit the upper layer of soils across the globe with higher affluence in sub-Arctic regions than in temperate or tropical regions (Van Den Hoogen *et al*., 2019). Despite being very simple morphologically, they take advantage of a vast variety of habitats, essentially due to their great adaptability to adverse conditions and to their action as polyphagous species (Abad *et al*., 2010). Once identified, nematodes can be grouped according to their feeding habits, such as bacteriophages, fungivores, predators, omnivores and parasites of animals or plants (Van Den Hoogen *et al*., 2019). Nematodes play important roles in the ecological processes of the soil, for example, in the decomposition of organic matter and recycling of nutrients. In addition, they can be used as biological indicators of soil health (they enter the trophic networks) and the abundance of certain nematode groups can reflect the quality of the habitat (Bongers *et al*., 1999). Nematodes can also act as rhizosphere regulators through the dispersion of bacteria, viruses and fungi, and some can be used as biological control agents (Phillips *et al*., 2003). Nonetheless, a great majority are free-living species that feed on bacteria, fungi, protozoans while others are parasites of animals, including vertebrates, invertebrates and plants.

There are over 4,100 species of plant-parasitic nematodes (PPN) described to date out of 25,000 nematode species that have been described to date (Jones *et al*., 2013; Begum *et al*., 2017). PPN cause great yield losses in several crops, which are estimated to a total over 173 billion dollars per year (Nicol *et al*., 2011). In developing countries, about 14.6% of crop yield losses are caused by PPN and 8.8% in developed countries (Begum *et al*., 2017). PPN present an ample range of interactions with their plant hosts (Sijmons *et al*., 1994). Some nematodes never enter the host, just migrate through the soil, using roots as a fleeting food source as they encounter them, called migratory ectoparasites (*e.g. Trichodorus* spp., *Tylenchorhynchus* spp., *Xiphinema* spp.). Others are endoparasites, when they enter the host tissues to feed and to reproduce, causing considerable damage to the host (Jones *et al*., 2013). Some endoparasitic nematodes are sedentary, being embedded in the root during their development (*e.g. Globodera* spp., *Heterodera*, spp., *Meloidogyne* spp.), while others are migratory and spend much of their life time moving through soil and root tissues and destructively feeding on plant cells (*e.g. Ditylenchus* spp., *Hirschmanniella* spp., *Pratylenchus* spp., *Radopholus* spp.). Semi-endoparasites such as *Rotylenchus* spp. or some species of *Tylenchulus* spp. can partially penetrate the plant, with one part of the nematode inside the plant and the rest outside. These nematodes swell and do not move once they have entered

into the endoparasitic phase of their life cycle. Distinct plant tissues are infected by PPN, such as bulbs/tubers, flowers, fruits, leaves, roots, and stems, impairing the absorption and transport of water and nutrients (Castillo and Vovlas, 2007). After nematode infection, attacked plants become less tolerant to physical stresses and are more susceptible to attack by other pathogens. After plant decay, PPN can remain viable in the soil or may be dispersed in plant materials such as roots, seeds, and bulbs, being easily disseminated to areas where they might not exist. An accurate detection and identification of PPN are thus important for quarantine purposes and to define effective and sustainable integrated pest management programs.

Over the years, management of crop damage by PPN has been attained with the utilization of plant resistance, crop rotation and alternative cultural practices, chemical nematicides and biological control (Chitwood, 2002). None of these control methods has been shown to be totally effective and the most sustainable approach to nematode control is a combination of several strategies and tools (Dong and Zhang, 2006). For example, chemical control is generally used for PPN management, but due to environmental risks, high costs, limited availability in many developing countries or their diminished effectiveness following repeated applications, the use of chemical nematicides are being reassessed (Dong and Zhang, 2006). Genetic resistance is a good way for sustainable parasite control because of its low cost providing an effective solution compelling no extra resources and no additional costs (Waller *et al*., 2004). However, adoption of this strategy is uncertain, depending on the species and population densities of the nematodes.

ROOT-LESION NEMATODES, *PRATYLENCHUS* SPP.

Although the great majority of crop damage is caused by the sedentary endoparasitic root-knot nematodes (RKN), *Meloidogyne* spp., and cyst nematodes (CN), *Globodera* spp. and *Heterodera* spp., the root-lesion nematodes (RLN), *Pratylenchus* spp., are serious pests considered to be the third most important group of PPN after RKN and CN (Jones *et al*., 2013). RLN are obligate biotrophic, polyphagous and economically important migratory endoparasitic pests of agricultural, horticultural and industrial crops (Tan *et al*., 2013). RLN are mainly parasites of roots, despite they invade plant tubers, rhizomes, pods and occasionally some above-ground structures (Castillo and Vovlas, 2007). The extensive host range of RLN limits the use of crop rotation to manage these nematodes and once introduced in soil they are very difficult to manage. In addition, their damage can be related to pathogenicity and population densities (Orlando *et al*., 2020). RLN cause plant tissue necrosis due to the nematode migration and feeding. When RLN feed from the plant, they consume the plant cell cytoplasm

using their stylet, killing the plant cell, and moving ahead of the lesion. Due to damage of tissues, water and nutrient uptake by the roots is diminished, resulting in poor plant growth and yield (Begum *et al*., 2017). Infection can occur along the entire length of the root (Jones *et al*., 2013) and favour the entrance of other plant pathogens, such as fungi and bacteria (Castillo and Vovlas, 2007). From the 101 species of the genus *Pratylenchus* that have been identified, the most important species, in terms of economic damage they cause, are *P. brachyurus*, *P. coffeae*, *P. goodeyi*, *P. loosi*, *P. neglectus*, *P. penetrans*, *P. pratensis*, *P. scribneri*, *P. thornei*, *P. vulnus* and *P. zeae* (Castillo and Vovlas, 2007; Jones *et al*., 2014). Reliable identification methods are important for the successful management of these species. Unfortunately, it is difficult to separate RLN species based exclusively on their morphology because of the high intraspecific variability and the small number of diagnostic features present in the genus *Pratylenchus* (Waeyenberge *et al*., 2009). Management strategies for RLN control are thus often focused on limiting nematode reproduction before planting crops and include the application of nematicides, and cultural practices such as crop rotation, cover crops, biofumigation, and biocontrol agents (Orlando *et al*., 2020).

LIFE CYCLE OF ROOT-LESION NEMATODES

RLN diverge from other important root parasites as they enter and leave roots during their life cycle, move actively through soil, and penetrate the root for feeding and reproduction (Esteves *et al*., 2015). The life cycle of *Pratylenchus* lasts from 3-8 weeks, depending on the plant host and environmental conditions such as adequate temperature and moisture (Castillo and Vovlas, 2007). After embryonic development the first-stage juvenile (J1) within the egg moults to the second-stage juvenile (J2), remain inside the egg until hatch, commonly one week after egg deposition (Orlando *et al*., 2020). All subsequent juvenile (J3 and J4) and adult life stages of *Pratylenchus* are vermiform, mobile and can infect and feed on host plants (Figure 1). Females lay their eggs in clusters inside roots, or in adjacent soil near to the root. Males are common in some species (*e.g. P. coffeae* and *P. penetrans*) yet rare or absent in others (*e.g. P. crenatus*, *P. neglectus*, and *P. thornei*) and it is believed that *Pratylenchus* usually reproduce by parthenogenesis (Castillo and Vovlas, 2007; Orlando *et al*., 2020). After root penetration, invaded tissues turn brown due to the nematode feeding and migration within the root cortex. Affected cortical tissues collapse, resulting in plant cell death. After root decay, RLN leave lesions and may return to soil or attack other root portions. The temperature settles the duration of the life cycle and vary between species. Tropical species, such as *P. brachyurus* and *P. zeae*, can complete their life cycle in about 3-4 weeks at 30ºC. Species that prefer cooler climates, such as *P. penetrans*, may complete their life cycle in about 6-7 weeks at 20ºC on *Cryptomeria* seedlings (De Waele *et al*., 2002). Under adverse conditions,

longer term survival can occur at the egg stage. Further, RLN can survive in the soil for more than a year through anhydrobiosis (Jones *et al*., 2013).

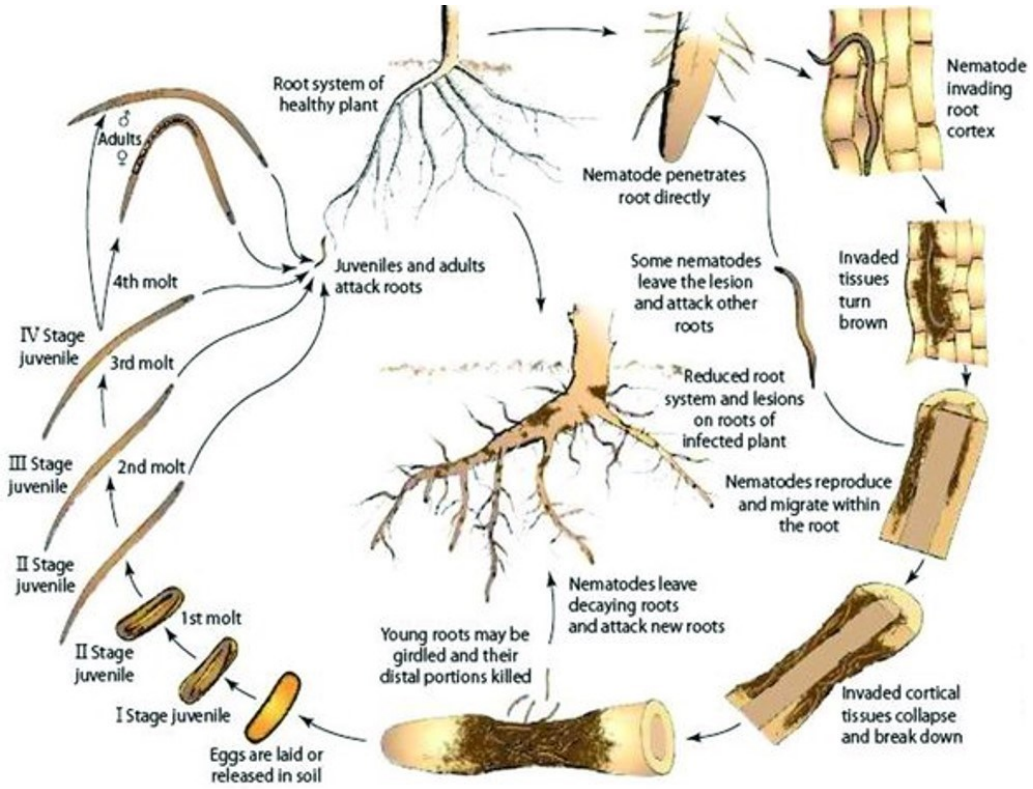

Figure 1 - Life cycle of root-lesion nematodes, *Pratylenchus* spp. (retrieved from Agrios, 2005).

MORPHOLOGICAL, MORPHOMETRIC AND MOLECULAR IDENTIFICATION OF *PRATYLENCHUS* SPP.

Pratylenchus species can be identified by means of morphological and morphometric characters (Carrasco-Ballesteros *et al*., 2007), but needs specialized expertise since most specific differences can only be observed using high magnifications. Although the genus *Pratylenchus* is easy recognisable from other PPN genera, the majority of morphological features of the species within this genus is very similar, while the variability of the morphometrical characters usually used by taxonomists to distinguish nematode species is huge (Castillo and Vovlas, 2007). Morphological features and molecular data from individual RLN adults are thus used in taxonomic studies. Depending on the species, adults of *Pratylenchus* are about 300-900 µm long and are relatively burly (Begum *et al*., 2017). The nematode anterior end can be identified by the lip area, wide, low, flattened anteriorly, not set off in the great majority of RLN species and by the presence of a short stylet moderately sclerotised and with rounded to oblong or anteriorly concave knobs (Luc, 1987). The average

size of the stylet in *Pratylenchus* is ca 16 µm, being shorter as 11.5 µm in some species (*e.g. Pratylenchus microstylus*) and larger as 23 µm in others (*e.g. P. macrostylus*) (Castillo and Vovlas, 2007). Below the stylet, in a relatively clear area after the pharynx procorpus, the oesophageal median bulb (metacorpus) can be seen. This structure is round, clearly distinct from procorpus, with valve prominent and is used for pumping food into the intestines. Just below of the median bulb, the excretory pore can be found, right before the esophageal glands. Esophageal glands are located at the posterior region of pharynx and are composed by one dorsal and two sub ventral unicellular glands. These glands overlap the nematode intestine on the ventral side of its body. The intestine can be recognized as a quite long dark region extending from the esophageal glands to the tail (Figure 2). After digestion, faeces are expelled through from the anus located in the posterior end of the nematode near the tail. The reproductive system of the adult female consists in a single, anteriorly directed, gonad and a post-vulval uterine sac. Spermatheca is large, oval to round, usually filled with sperm when males are present. The post-vulval uterine branch has a mean length of 23 µm although it is often difficult to determine the exact length of this structure (Castillo and Vovlas, 2007). The presence of the vulva is a defining characteristic of females and this organ is usually located at about 70-85% of the body length down from anterior end (Figure 3A). The female tail is 2 to 3 times the anal body diameter, with broad to narrowly rounded (rarely pointed) or truncated tip, which may be smooth or crenated (Luc, 1987). The shape and degree of annulation or crenation of the female tails are variable. Male RLN are usually smaller than females and are distinguished by the presence of a pair of spicules located near the tail (Figure 3B). The caudal alae, or bursa, found in males have crenate margins and enclose the tail tip. The spicules are simple, equal and arcuated. The identification of *Pratylenchus* spp. is usually based on female and male morphology, although females possess more diagnostic characters and are more common than males, which are unknown in a substantial number of species (Castillo and Vovlas, 2007).

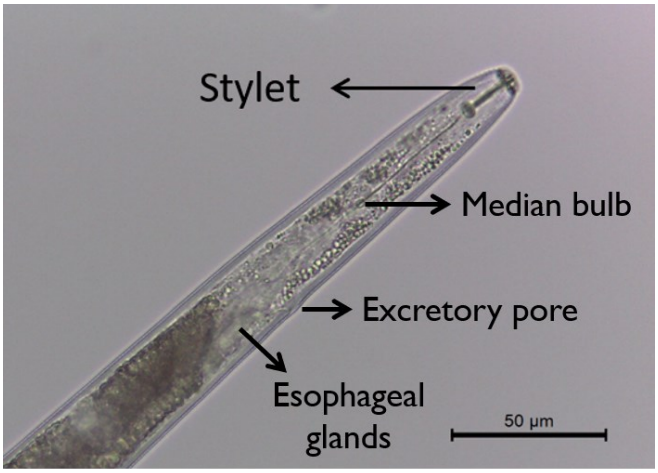

Figure 2 - Detail of the anterior end of a *Pratylenchus penetrans* showing the stylet, median bulb, excretory pore and esophageal glands.

Pratylenchus penetrans is characterised by labial region slightly offset, low, flat in front, with rounded outer margins, with three annuli, stylet with knobs varying from rounded to markedly cup-shaped anteriorly, pharynx overlapping the intestine ventrally in a lobe ca 1.5 body diameter long, excretory pore opposite to pharyngo-intestinal junction, spermatheca spherical, post-vulval uterine sac short undifferentiated, ca 1-1.5 vulval body diameter, ovary not extending to esophagous, tail generally rounded and smooth tip. Males are common and slightly smaller than females, but similar vermiform shape. Spicules slender and tail ca twice as long as cloacal body diameter, caudal alae irregularly crenate along margin, enveloping the tail tip (Roman and Hirschmann, 1969; Castillo and Vovlas, 2007). This species can be distinguished from closely related species by body and stylet length, number of lip annuli, labial framework, position of the vulva, shape of spermatheca and tail terminus (Castillo and Vovlas, 2007).

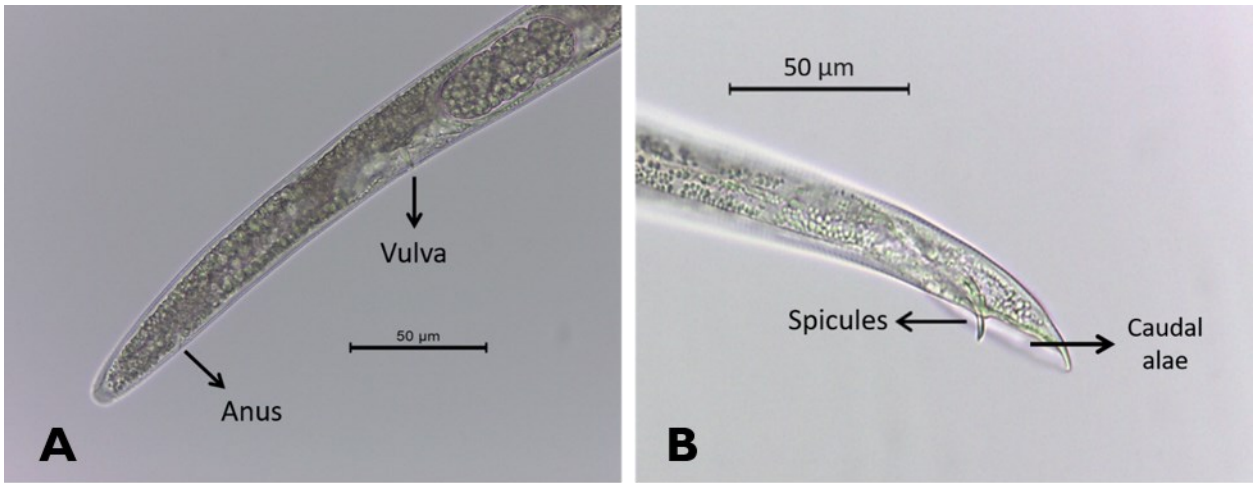

Figure 3 - Detail of the posterior end of a *Pratylenchus penetrans* female showing the vulva and anus (A) and male (B) with visible protruding pair of spicules and caudal alae enveloping the tail tip.

Considering the potential impact of RLN on agricultural crops, the correct identification and characterisation of *Pratylenchus* species is necessary, for example, to inform farmers on the application of suitable pest management strategies (Mirghasemi *et al*., 2019). Janssen *et al*. (2017) revealed an outstanding number of cryptic biodiversity within the genus *Pratylenchus*. Due to this fact, morphological identification of RLN should be complemented with molecular analysis for accurate diagnosis of this group of nematodes.

In the past, cytogenetic and biochemical methods based on isoenzyme analysis have been developed to identify RLN and to study intra-specific variation in isolates from different geographical locations (Roman and Triantaphyllou, 1969; Andrés *et al*., 2000), but these methods proved to be strenuous and time consuming in their application. Nowadays,

molecular techniques based on nucleic acid technology are used routinely in RLN diagnostics and molecular characterisation of *Pratylenchus* spp. The tandemly arranged ribosomal DNA (rDNA) 18S, 5.8S and 26S or 28S genes, separated by internal transcribed spacers (ITS) and bordered by intergenic regions (IGS), are present in multiple copies in the genome of eukaryotes. The coding regions are usually conserved even between distantly related species, while ITS spacers show considerable variability between different animal genomes. Non-coding but transcribed ITS sequences emerge briskly and can be used to compare more closely related RLN species and subspecies, however to compare more far-off taxa 18S, 5.8S and 28S coding sequences are used because they emerge slowly than the non-coding ITS regions (Jones *et al*., 2014). Molecular techniques based on restriction fragment length polymorphism (RFLP) analysis of the ribosomal ribonucleic acid (rRNA) genes (Waeyenberge *et al*., 2000) and sequencing of different fragments of the rDNA cluster, including ITS (De Luca *et al*., 2011; Wang *et al*., 2012), 18S (Subbotin *et al*., 2008; van den Elsen *et al*., 2009) and 26S (Al-Banna *et al*., 2004; Handoo *et al*., 2001; Subbotin *et al*., 2008) are currently used in diagnostics of RLN species (Janssen *et al*., 2017). In addition, sequencing of the mitochondrial DNA (mtDNA), cytochrome oxidase I (COI) gene (Palomares-Rius *et al*., 2014; Troccoli *et al*., 2016; Janssen *et al*., 2017; Singh *et al*., 2018; Divsalar *et al*., 2019; Mirghasemi *et al*., 2019) and the nuclear *hsp90* gene (De Luca *et al*., 2012; Palomares-Rius *et al*., 2014; Troccoli *et al*., 2016; Fanelli *et al*., 2018; Mirghasemi *et al*., 2019) have also been largely used in the molecular characterisation of RLN species. However, according to Janssen *et al*. (2017), despite the existence of a wide spectrum of identification methods, no specific approach has been widely accepted yet as most species identification methods have been tested for only a limited number of *Pratylenchus* taxa. Molecular techniques are thus important tools that aid in detection and characterisation of a RLN, alone or within mixtures of non-target nematodes. Such techniques are also useful to distinguish closely related and cryptic species, which are morphologically indistinguishable but yet genetically distinct (Powers, 2004). Nonetheless, misidentification is a significant problem for molecular analysis due to the use of sequences that have been incorrectly assigned to a species (Orlando *et al*., 2020).

PRATYLENCHUS PENETRANS

Pratylenchus penetrans is the most important species of RLN from an economic point of view (Vieira *et al*., 2015). This species presents a wide geographic distribution and is often reported as a limiting factor of several important agronomic crops, such as bean or potato, ornamental crops and fruit trees. In Europe, this species has been recently detected in several potato fields in Portugal, with population densities found above the crop economic thresholds in some regions (Esteves *et al*., 2015). In addition to the direct damage it causes to the plant,

P. penetrans increases the severity of diseases caused by fungi such as *Fusarium oxysporum* (Upadhaya *et al*., 2020), *Rhizoctonia solani* (Viketoft *et al*., 2020), *Verticillium dahliae* in potato (Rowe *et al*., 1987) and common scab, caused by the bacterium *Streptomyces scabies* (Holgado *et al*., 2009). *Pratylenchus penetrans* is widely distributed throughout temperate and tropical areas of the world (Thompson *et al*., 2010) and has been recorded on more than 400 hosts (Vieira *et al*., 2015).

Infected plants grow poorly, and crop growth is often uneven (Figure 4). Affected roots have distinct dark brown/reddish lesions and they can be extensive in size, especially when plants are grown in soils with high *P. penetrans* densities (Figure 5). Infected roots with *P. penetrans* usually are thinner and exhibit a reduced size, for example, carrots may have deferred maturity. *Pratylenchus penetrans* differs from other species in the genus *Pratylenchus* due to the hosts they can infect, even though it causes identical visual symptoms on plants. When invading the roots of strawberry, for instance, *P. penetrans* cause discoloration and necrosis of certain tissues and hyperplasia in others. Townshend *et al*. (1963) demonstrated a similar relationship in apple, celery, and peach. Surface of potato tubers infected with *P. penetrans* show symptoms like common scab, marked by cross-shaped lesions typical of the pathogen (Holgado *et al*., 2009).

Figure 4 - Field pea crop infested with *Pratylenchus penetrans* (retrieved from Collins, 2016).

Figure 5 - Maize roots infected with *Pratylenchus penetrans*. Different densities from left (0) to right (256) with individuals per gram of soil (right root with the higher nematode density) (retrieved from Moens and Perry, 2009).

According to Mizukubo and Adachi (1997), the life cycle of *P. penetrans* in *Trifolium repens* was estimated to be 46.4, 38.5, 28.1, 26.2, and 22.4 days at 17, 20, 25, 27, and 30°C, respectively. Soil temperature affects nematode activities, influences, or regulates parameters such as egg-laying rate, female maturity rate, mortality of each stage, and duration of oviposition. However, soil temperature it is often an uncontrollable variable which may be remarkably influential at the field (Ferris, 1970). The nematodes outlast in soil or roots, throughout their larval and adult stages, vermiform and motile, and all stages from J2 on, can infect plants by penetrating the root and invading the cortex (Mitreva *et al*., 2004). The regions of root hair development and the elongation zone are the major entry points. Variability between *P. penetrans* isolates in terms of reproduction and pathogenicity have been reported, which suggests the existence of intraspecific variability in the *Pratylenchus* genome (France and Brodie, 1995). The possibility that other related RLN species are conspecific with *P. penetrans* is also implied, due to the validity of many characters used in species identification. Although its extensive distribution and plentiful hosts, information about *P. penetrans* intraspecific variability is still limited and further studies on intraspecific and interspecific variation are thus needed.

The first record of *P. penetrans* in Portugal was associated with olive trees in Coimbra district, Centro region (Abrantes, 1980). This species was later found in roots and soil in several hosts, such as potato, tomato or maize (Abrantes *et al*., 1987). Despite its wide plant host range, until now, this RLN species has been essentially detected in Portugal in potato fields (Esteves *et al*., 2015). Recently, Rusinque *et al*. (2020) first reported *P. penetrans* parasitizing amaryllis (*Hippeastrum* x *hybridum*) on soil and root samples collected from potted plants produced in greenhouses in Montijo, Setúbal district.

OBJECTIVES

Pratylenchus penetrans is an important PPN worldwide because can cause significant damage to a wide variety of economical important crops (Jones *et al*., 2013). In Portugal, this species was found frequently infecting potato roots (Esteves *et al*., 2015) and isolates, previously collected from different regions of mainland Portugal, are being maintained at NEMATO-lab, CFE – Coimbra University. In spite of the previous detection of *P. penetrans* in potato crops, little information is still available on the morphometric and molecular characteristics of these isolates. Information about the intra and inter-specific variation of *P. penetrans* parasitizing potato will be useful to understand the genetic diversity of this species, and relationships with other *P. penetrans* identified in other countries and hosts. Knowledge obtained can be valuable in help defining effective strategies for RLN management in potato crops.

The main goal of this work was to assess the genetic diversity of *P. penetrans* from Portugal.

The specific objectives were:

- To obtain *P. penetrans* inoculum for morphometric and molecular studies (chapter 1);
- To evaluate the morphometric variability of isolates (chapter 1);
- To confirm the identification of isolates using molecular markers (chapter 2);
- To assess genetic diversity, geographical and host relations among the isolates (chapter 2).

CHAPTER 1

MORPHOMETRIC VARIABILITY OF *PRATYLENCHUS PENETRANS* ISOLATES

INTRODUCTION

Pratylenchus species can be morphologically differentiated from other genera in the family Pratylenchidae by the number of gonads forming the female genital system (i.e., one vs two), whether or not the caudal alae of males reach to the tip of the tail, sexual dimorphism, presence or absence of deirids and by the orientation of the esophageal gland lobe overlap (Luc, 1987). For discrimination of species within the genus *Pratylenchus*, the following diagnostic morphometric characters used are: overall body length, stylet length, maximum body width, body width at anus, vulva-anus distance, size of spicules and tail length. Numerous studies have demonstrated that some measurements, such as stylet length and V ratio, are less modified by biotic or abiotic factors and proved to be reliable diagnostic characters in the genus *Pratylenchus* (Castillo and Vovlas, 2007). Nonetheless, RLN species identification is difficult due to the overlapping of several morphological and morphometric characters.

Several RLN species such as *P. arlingtoni*, *P. brachyurus*, *P. convallariae*, *P. dunensis*, *P. fallax*, *P. oleae*, *P. pinguicaudatus* share many morphological characteristics with *P. penetrans* and for this reason the validity of several of these taxa has been questioned (Subbotin *et al*., 2008; Palomares-Rius *et al*., 2014). *Pratylenchus fallax* was originally described and differentiated from *P. penetrans* by Seinhorst (1968). Tarte and Mai (1976) afterwards considered both species to be conspecific, as morphological traits showed considerable intraspecific variability as a result of variable environmental conditions. Environmental factors may influence main morphologic characters in *P. penetrans*, especially the host plant. Such variability can also be found in isolates from different geographical locations (Tarte and Mai, 1976). Furthermore, intraspecific morphological variability has been demonstrated in *P. penetrans* (Tarte and Mai, 1976). As a result, the taxonomic value of some of the characters, such as the length of the body and tail, median bulb and vulva lips, a and b ratios, is dubious, making precise identification of this species difficult (Waeyenberge *et al*., 2009).

MATERIALS AND METHODS

PRATYLENCHUS PENETRANS ISOLATES

Five *P. penetrans* isolates, obtained previously from potato roots sampled in the North and Centre regions of mainland Portugal (Esteves *et al*., 2015), were used in this study. Isolates from a single gravid female were propagated on carrot discs. Two out of the five isolates shared the same sampling geographic origin (Table 1.1).

Table 1.1 - *Pratylenchus penetrans* isolates used in this study and respective geographical origin.

Isolate	GPS coordinates	Locality
A21L2	41°16' 18"N 8°41' 23" W	Aveleda, Maia
A24L1	41°15' 27"N 8° 40' 30" W	Vila Nova da Telha, Maia
A34L3	40°37' 28" N 8° 38' 19" W	Aveiro
A44L2	40°23'25.2"N 8°30'07.7"W	Coimbra
A44L4		

IN VITRO REARING

Pratylenchus penetrans isolates were multiplied on carrot discs as this method allows the rapid mass production of migratory endoparasites (Boisseau *et al*., 2008). For preparation of carrot discs, whole carrots with tops removed were washed with running tap water and were transferred to a vertical laminar flow chamber. For disinfection, carrots were rinsed with sterile tap water, followed by 70% alcohol (v/v) and were flamed for a few seconds. Then, with the aid of a sterile scalpel, epidermis was removed flaming the blade at each cut, and discs of approximately 5 mm thickness were cut (Figure 1.1A). Individual discs were then transferred to Petri plates (5 mm diameter) and were left exposed to ultraviolet light for 3 hours. Finally, plates were sealed with parafilm and stored at room temperature, until nematode inoculation.

Nematodes were inoculated into newly disinfected discs inside the laminar flow cabinet to avoid the development of bacterial/fungal contamination. Ten microliters aliquots containing a suspension of 20 to 50 nematodes in tap water, were placed in the centre of the disinfected carrot discs where an incision has been made (Figure 1.1B). About 50 discs were inoculated per isolate. Plates were sealed with parafilm and were incubated at room temperature for 2-3 months, until changes in the aspect of the carrot discs were observed (Figure 1.1C).

The extraction of nematodes was done in sterile glass bowls filled with autoclaved tap water with 0.1% streptomycin sulphate (Fisher Scientific, USA), using a sieve to support the carrot disc (Figure 1.1D). Nematodes were collected daily for 3 days, after migration into the water. Using a micropipette, nematodes that have deposited on the bottom of bowls were transferred into small excavated glass blocks (~3 mL). Water with antibiotic was progressively

removed and replaced with autoclaved tap water. Suspensions were distributed into 1.5 mL centrifuge tubes and the extracted nematodes were used for both morphometric and molecular analyses.

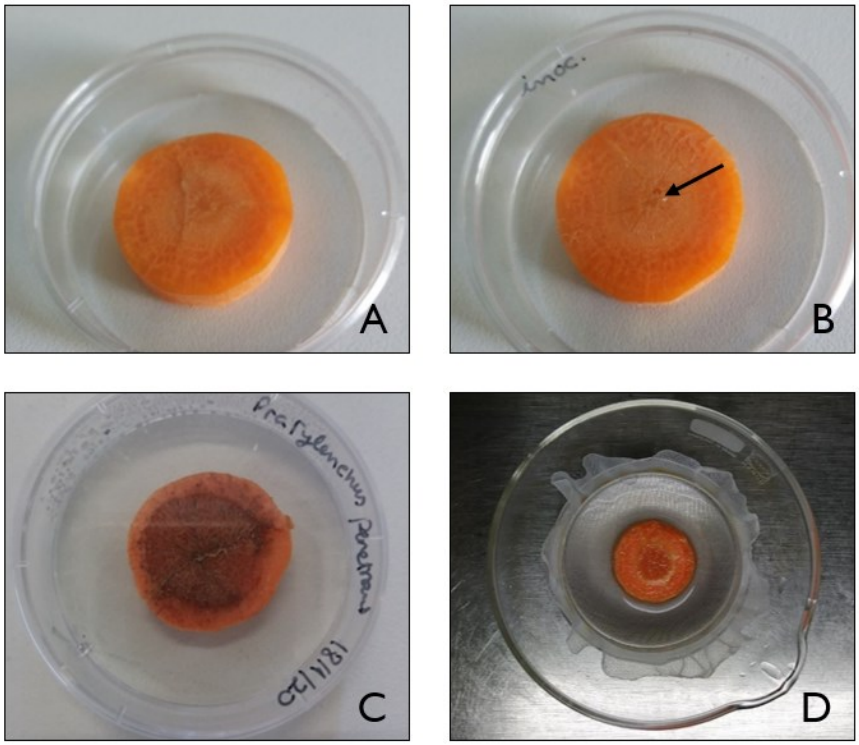

Figure 1.1 - Aspect of carrot discs: a) before inoculation; b) at inoculation (arrow showing area where *Pratylenchus penetrans* were inoculated); c) after nematode multiplication; d) glass bowl containing sieve to support the carrot disc at extraction.

MORPHOMETRIC ANALYSES

Twenty individual adults from each isolate (10 females and 10 males), picked at random from suspensions using a fine bristle glued to the end of a mounted needle, were transferred into a drop of water on a glass slide and were used for morphometric analyses. Before covering slides with the coverslips, nematodes were immobilized by gently heating the slide underneath, just enough to stop movement. Nematode measurements of characters considered useful for morphometric characterisation were made using a Leica DM2500 microscope equipped with a Leica ICC50HD digital camera and LAS 4.8.0 software (Leica). The following morphometric variables were assessed: overall body length (L); stylet length; distance from anterior end to centre of median bulb; distance from anterior end to excretory pore; distance from anterior end to esophageal glands; distance from anterior end to vulva; maximum body width; body width at anus; vulva-anus distance; tail length and the taxonomic ratios a, b', c, c' and V (Man, 1880). The a ratio corresponds to body length/maximum body width, b' = body length/distance from anterior end to esophageal glands, c = body length/tail

length, c' = tail length/tail diameter at anus and V = position of vulva from anterior end expressed as percentage of body length. All measurements were expressed in micrometres (µm). As example, measurement of the overall body length in a *P. penetrans* female is shown in Figure 1.2.

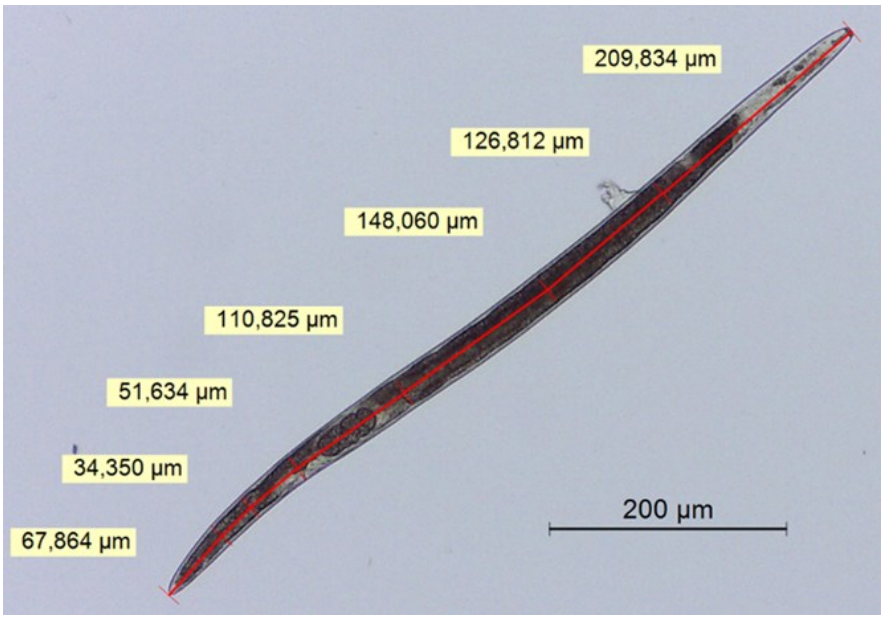

Figure 1.2 - Measurement of the overall body length (L) on a *Pratylenchus penetrans* female using LAS 4.8.0 software (Leica).

The morphometric values of isolates were compared with those previously published for other isolates of *P. penetrans* (Loof *et al*., 1960; Roman and Hirschmann, 1969; Tarte and Mai, 1976; Mokrini *et al*., 2016; Janssen *et al*., 2017). To assess the morphometric variation of the isolates, data was subjected to one-way analysis of variance (ANOVA) using Statsoft Statistica® V.7, after ensuring that the assumptions of normality and constant variance were met, as checked by using the Shapiro-Wilk and Levene's tests, respectively. Logarithmic and square root transformations were applied to data whenever needed. Following ANOVA, Fisher LSD test at 95% confidence level was done to test differences between isolates. The non-parametric Kruskal-Wallis test was applied to data that could not meet the ANOVA assumptions of normality and variance even after function transformations. The coefficient of variability (CV) was calculated by dividing the respective means with the standard deviations, to determine which characters were most stable and more variable among isolates. Possible correlations between different morphometric characters were assessed using the two-tailed Pearson correlation in Statsoft Statistica® V.7.

RESULTS AND DISCUSSION

All the five *P. penetrans* isolates used in this study were multiplied on carrot discs and sufficient nematode inoculum could be obtained. As the multiplication progressed, changes in the aspect of the carrot discs were observed, most discs acquired a brownish-gold colour as a result of nematode feeding. Although the average number of nematodes extracted from carrots was not rigorously quantified for each isolate, few nematodes of A21L2 and A34L3 isolates were recovered from the discs (~50 nematodes/disc), suggesting that these isolates had a poor reproduction rate. Conversely, several hundreds of nematodes (500 to 1000 nematodes/disc) could be extracted from isolates A24L1, A44L2 and A44L4. Thus, although the exact numbers of nematodes, that were extracted, were not rigorously quantified, fewer nematodes from A21L2 and A34L3 isolates were recovered from the carrot discs. The use of variable male/female ratios at inoculation may have affected the final nematode densities that were obtained. However, it is also possible that the reproductive ability may be variable among isolates. In the past, differences in the reproduction ability of two *P. penetrans* from New York (USA) on potato clones were reported (France and Brodie, 1995). More recently, intraspecific differences in *P. penetrans* reproductive fitness have been found by Mokrini *et al*. (2016) in four populations from Morocco. Since the reproductive fitness together with virulence are major components of pathogenicity (Shaner *et al*., 1992), further studies should be focused in clarifying possible differences in the reproduction of *P. penetrans* isolates from Portugal.

A range of morphometric characters useful in RLN diagnostics were measured from a total of 100 *P. penetrans* adults and over 500 images were recorded for analysis of the five different isolates. The morphometric measurements of *P. penetrans* isolates of Portugal were similar to other populations described by Loof (1960), except the variation of the c ratio in both A34L3 females and males, overall body length of A24L1 and A44L2 males and spicule length of A34L3 and A44L2 (Tables 1.2 & 1.3). Males of these two isolates showed the greatest spicule size of all males examined. Spicule size of males and overall body length of *P. penetrans* isolates from Portugal were also greater than those observed by Mokrini *et al.* (2016) in populations associated to maize in Morocco. Nevertheless, the size of most morphometric characters measured in this study matched the descriptions of isolates of *P. penetrans* from other countries (Loof *et al*., 1960; Roman and Hirschmann, 1969; Tarte and Mai, 1976; Castillo and Vovlas, 2007; Mokrini *et al.*, 2016; Janssen *et al*., 2017).

Morphometric comparisons using ANOVA revealed a high degree of intra and inter-isolate variability on most studied characters. Nine out of fifteen morphometric characters studied in *P. penetrans* females, varied significantly among isolates ($p < 0.05$) (Table 1.2).

Inter-isolate variability was high for the overall body length, anterior end to excretory pore, anterior end to vulva, body width at anus, vulva-anus distance, tail length and ratios b', c and c', whereas the distance of anterior end to median bulb, anterior end to pharyngeal gland lobe, maximum body width, stylet length, ratios a and V of females were not significantly different between isolates ($p > 0.05$). In males, significant inter-isolate variability was found in nine out of thirteen morphometric characters: overall body length, anterior end to median bulb, anterior end to excretory pore, spicule length, tail length and a, b', c and c' ratios ($p < 0.05$). The distance from anterior end to esophageal glands, body width at anus, maximum body width and stylet length were similar among isolates ($p > 0.05$) (Table 1.3).

The measurements of stylet length and V ratio of females, had the lowest CV intra and inter-isolates, and showed to be stable characters, as previously reported by Roman and Hirschmann (1969) and Tarte and Mai (1976). The highest values of CV in females were found in tail length, vulva-anus distance and c' ratio (Table 1.4). In males, c' ratio, tail and spicule length were the most variable characters between isolates, confirming the results given by the ANOVA (Table 1.3). High variability was found in the posterior region of nematodes.

Table 1.2 - Morphometric characters of *Pratylenchus penetrans* females from Portugal. All measurements are in µm. Data are means of 10 nematodes ± standard deviation (range). In each row means followed by the same letters do not differ significantly at p > 0.05, according to the Fisher LSD and Kruskal-Wallis test. *Variable was not significantly different at p > 0.05 according to the Kruskal-Wallis test.

Character	Isolate					Loof (1960)
	A21L2	A24L1	A34L3	A44L2	A44L4	
L	630.3 ± 59.4 ^{a,b} (527.5-720.0)	723.5 ± 93.9 ^{a,b} (603.3-858.8)	600.8 ± 86.3 ^{a,b} (522.1-812.1)	712.2 ± 61.0 ^{b,c,d} (615.5-802.5)	695.6 ± 82.4 ^{c,d} (605.3-869.5)	343-811
a	22.8 ± 3.5 ^{a} (17.4-28.3)	21.6 ± 3.0 ^{a} (18.5-26.9)	20.1 ± 2.3 ^{a} (16.1-23.8)	23.1 ± 3.1 ^{a} (20.1-29.3)	21.4 ± 2.4 ^{a} (16.5-25.0)	19-32
b' *	5.1 ± 0.6 ^{a,b,c} (4.3-6.4)	5.3 ± 0.5 ^{a,b} (4.8-6.3)	4.4 ± 0.4 ^{b,c} (3.8-5.2)	5.3 ± 0.5 ^{a,b} (4.6-6.0)	5.9 ± 2.0 ^{a,b} (4.3-10.5)	
c	21.9 ± 3.3 ^{a} (18.1-29.6)	22.9 ± 3.2 ^{b} (17.6-27.2)	25.6 ± 2.8 ^{b} (21.1-28.9)	21.2 ± 2.3 ^{b} (18.5-25.6)	21.3 ± 2.3 ^{b} (18.2-24.6)	15-24
c'	2.0 ± 0.3 ^{a} (1.4-2.5)	1.9 ± 0.4 ^{b} (1.5-2.7)	1.5 ± 0.3 ^{b} (1.2-1.9)	1.9 ± 0.4 ^{b} (1.4-2.4)	2.0 ± 0.3 ^{b} (1.5-2.4)	
V *	81.1 ± 2.2 ^{a} (75.7-83.7)	81.0 ± 1.7 ^{a} (77.7-83.9)	82.5 ± 2.2 ^{a} (78.3-85.9)	80.6 ± 2.0 ^{a} (77.0-83.5)	80.3 ± 4.0 ^{a} (69.8-83.4)	75-84
Stylet length *	16.9 ± 0.8 ^{a} (16.0-18.0)	16.2 ± 0.6 ^{a} (15.4-17.2)	16.9 ± 1.2 ^{a} (15.7-20.1)	16.2 ± 0.6 ^{a} (15.2-17.3)	16.5 ± 0.7 ^{a} (15.5-18.0)	15-17
Anterior end to centre of median bulb	57.2 ± 3.6 ^{a} (50.7-62.3)	63.5 ± 7.6 ^{a} (49.4-71.8)	61.8 ± 5.1 ^{a} (53.7-70.5)	61.9 ± 2.7 ^{a} (56.0-66.2)	60.9 ± 5.3 ^{a} (49.4-67.9)	
Anterior end to excretory pore	89.0 ± 9.1 ^{a,b} (78.4-105.6)	98.7 ± 9.3 ^{a,b,c} (81.6-113.3)	91.4 ± 9.7 ^{b,c,d} (83.9-116.8)	104.7 ± 4.3 ^{b,c,d} (96.0-111.7)	92.9 ± 15.3 ^{c,d} (71.7-114.5)	
Anterior end to esophageal glands *	125.4 ± 15.6 ^{a} (103.4-147.7)	136.2 ± 15.3 ^{a} (108.4-156.7)	137.3 ± 10.5 ^{a} (126.2-155.2)	134.0 ± 9.2 ^{a} (120.7-151.9)	124.7 ± 21.7 ^{a} (82.4-148.0)	
Anterior end to vulva	510.6 ± 45.5 ^{a} (423.7-575.9)	586.1 ± 77.5 ^{a} (485.6-695.8)	496.0 ± 77.8 ^{a} (427.0-697.0)	574.5 ± 53.9 ^{a} (474.2-637.5)	558.9 ± 77.6 ^{a} (475.9-724.8)	
Maximum body width	28.2 ± 5.1 ^{a} (21.9-39.2)	34.1 ± 6.7 ^{a} (23.5-43.8)	30.0 ± 3.7 ^{a} (23.6-34.2)	31.2 ± 4.5 ^{a} (23.2-36.4)	33.0 ± 5.9 ^{a} (24.2-43.4)	
Body width at anus	14.8 ± 1.7 ^{a,b} (11.6-17.3)	17.5 ± 2.5 ^{a,b,c} (13.2-21.0)	15.6 ± 1.8 ^{a,b,c} (13.5-19.2)	18.2 ± 2.8 ^{b,c,d} (13.2-24.0)	16.9 ± 2.3 ^{c,d} (13.9-20.2)	
Vulva-anus distance	87.6 ± 16.4 ^{a} (64.9-118.7)	103.5 ± 17.2 ^{a} (72.7-127.7)	79.6 ± 14.6 ^{a,b,c} (58.9-111.0)	104.6 ± 18.0 ^{b,c,d} (80.9-141.7)	94.1 ± 11.3 ^{c,d} (72.9-109.0)	
Tail length	29.4 ± 4.9 ^{a,b} (19.0-35.8)	31.8 ± 2.6 ^{a,b,c} (27.7-35.6)	23.7 ± 3.9 ^{a,b,c} (19.5-31.4)	34.0 ± 4.2 ^{b,c} (26.6-41.0)	32.8 ± 3.9 ^{d} (25.9-38.4)	

*variables were analysed using the non-parametric Kruskal-Wallis test because data could not meet ANOVA assumption of normality

Table 1.3 - Morphometric characters of *Pratylenchus penetrans* males from Portugal. All measurements are in µm. Data are means of 10 nematodes ± standard deviation (range). In each row means followed by the same letters do not differ significantly at p > 0.05, according to the Fisher LSD and Kruskal-Wallis test. *Variable was not significantly different at p > 0.05 according to the Kruskal-Wallis test.

Character	Isolate					Loof (1960)
	A21L2	A24L1	A34L3	A44L2	A44L4	
L	535.0 ± 30.7 [a] (483.3-582.4)	582.6 ± 35.0 [a] (529.0-629.4)	522.0 ± 32.2 [b] (470.5-580.5)	602.6 ± 36.0 [b] (531.5-670.1)	536.0 ± 17.9 [b] (508.2-570.0)	305-574
a *	24.6 ± 2.5 [a,b,c] (19.8-29.4)	28.2 ± 2.6 [a,b,c,d] (25.3-34.4)	24.6 ± 2.4 [b,c,d,e] (19.9-27.1)	28.5 ± 1.6 [b,c,d,e] (26.8-30.5)	29.3 ± 11.9 [c,d,e] (22.8-62.6)	23-34
b'	4.4 ± 0.5 [a] (3.4-4.9)	4.8 ± 0.4 [a] (4.2-5.5)	4.3 ± 0.2 [b] (4.0-4.6)	4.7 ± 0.2 [b] (4.4-5.1)	4.2 ± 0.4 [b] (3.7-4.8)	
c	21.1 ± 2.5 [a] (16.4-24.3)	20.4 ± 2.5 [a] (15.6-24.4)	24.2 ± 3.4 [b] (20.7-31.4)	21.6 ± 1.9 [a] (18.6-24.1)	19.7 ± 1.9 [a] (16.9-22.8)	16-22
c'	1.8 ± 0.2 [a,b] (1.6-2.2)	2.2 ± 0.4 [a,b,c] (1.5-2.7)	1.6 ± 0.2 [a,b,c] (1.2-2.0)	2.0 ± 0.3 [b,c,d] (1.5-2.4)	2.0 ± 0.3 [c,d] (1.7-2.5)	
Stylet length	15.8 ± 0.4 [a,b] (15.3-16.7)	15.0 ± 0.4 [a,b] (14.5-15.7)	15.7 ± 0.6 [a,b] (15.0-16.5)	15.6 ± 0.6 [a,b,c] (14.8-16.7)	15.9 ± 1.1 [b,c] (14.4-17.5)	
Anterior end to centre of median bulb	57.5 ± 3.1 [a] (52.7-62.9)	53.6 ± 4.6 [b,c] (45.7-59.2)	59.5 ± 3.4 [b,c] (54.6-63.7)	63.7 ± 4.4 [b,c,d] (54.9-71.5)	56.0 ± 3.9 [c,d] (49.5-60-5)	
Anterior end to excretory pore	85.0 ± 5.0 [a] (79.0-92.6)	83.2 ± 6.9 [a] (71.7-94.2)	84.5 ± 7.7 [a] (72.3-97.6)	94.9 ± 6.0 [b] (81.7-104.1)	87.1 ± 5.7 (77.0-95.9) [a]	
Anterior end to esophageal glands	124.0 ± 12.9 [a] (105.2-149.2)	122.3 ± 6.6 [a] (110.9-131.2)	122.5 ± 8.0 [a] (110.7-137.9)	127.7 ± 10.2 [a] (108.5-144.0)	127.9 ± 9.5 [a] (112.0-146.2)	
Maximum body width *	21.9 ± 2.4 [a] (18.2-27.1)	20.8 ± 1.7 [a] (17.7-23.5)	21.3 ± 1.8 [a] (19.1-25.0)	21.2 ± 1.7 [a] (19.2-24.4)	19.8 ± 4.3 [a] (8.6-23.2)	
Body width at anus	14.0 ± 1.4 [a] (12.0-15.7)	13.3 ± 0.7 [a] (12.4-14.6)	13.3 ± 0.7 [a] (12.2-14.9)	14.3 ± 1.2 [a] (12.3-15.8)	13.8 ± 0.7 [a] (12.9-14.8)	
Spicule Length	16.2 ± 0.8 [a] (15.2-17.5)	15.8 ± 1.5 [a] (13.8-18.0)	19.1 ± 1.6 [b] (16.6-21.6)	18.6 ± 1.7 [b] (15.9-21.9)	16.7 ± 1.4 [b] (14.4-18.5)	14-17
Tail length	25.6 ± 2.9 [a,b] (22.4-32.7)	29.0 ± 4.1 [a,b,c] (21.7-35.4)	21.9 ± 2.6 [a,b,c] (16.1-24.9)	28.2 ± 3.8 [b,c] (22.5-36.0)	27.4 ± 2.8 [d] (23.6-31.8)	

*variables were analysed using the non-parametric Kruskal-Wallis test because data could not meet ANOVA assumption of normality

Table 1.4 - Intra and inter-isolate Coefficient of Variability (%) of *Pratylenchus penetrans* females from Portugal

Character	Isolate					Inter-isolate Coefficient of Variability (%)
	A21L2	A24L1	A34L3	A44L2	A44L4	
L	9.4	13.0	14.4	8.6	11.8	8.0
a	15.3	13.8	11.3	13.2	11.4	5.5
b'	12.7	8.5	9.1	9.6	33.5	10.4
c	14.9	13.9	10.8	10.9	10.6	8.1
c'	16.2	19.2	17.1	19.2	14.3	11.1
V	2.8	2.0	2.7	2.5	5.0	1.0
Stylet length	4.6	3.9	7.2	3.9	4.5	2.1
Anterior end to centre of median bulb	6.4	11.9	8.2	4.4	8.7	3.9
Anterior end to excretory pore	10.3	9.5	10.6	4.1	16.5	6.6
Anterior end to esophageal glands	12.5	11.2	7.6	6.9	17.4	4.6
Anterior end to vulva	8.9	13.2	15.7	9.4	13.9	7.3
Maximum body width	18.2	19.8	12.4	14.4	17.9	7.5
Body width at anus	11.2	14.5	11.3	15.3	13.4	8.4
Vulva-anus distance	18.7	16.6	18.3	17.2	12.1	11.3
Tail length	16.6	8.3	16.5	12.4	12.0	13.4

Table 1.5 - Intra and inter-isolate Coefficient of Variability (%) of *Pratylenchus penetrans* males from Portugal.

Character	Isolate					Inter-isolate Coefficient of Variability (%)
	A21L2	A24L1	A34L3	A44L2	A44L4	
L	5.7	6.0	6.2	6.0	3.3	6.3
a	10.3	9.2	9.6	5.6	40.7	8.4
b'	10.7	8.1	4.5	4.4	8.7	5.8
c	11.6	12.2	13.9	8.9	9.6	8.0
c'	10.2	16.4	13.3	13.6	12.5	11.9
Stylet length	2.7	2.4	3.6	3.9	6.7	2.3
Anterior end to centre of median bulb	5.4	8.5	5.7	6.9	7.0	6.6
Anterior end to excretory pore	5.8	8.3	9.1	6.3	6.5	5.4
Anterior end to esophageal glands	10.4	5.4	6.5	8.0	7.5	2.2
Maximum body width	11.0	8.1	8.3	8.1	21.7	3.7
Body width at anus	9.9	5.6	5.3	8.4	5.0	3.2
Spicule Length	4.9	9.3	8.3	8.9	8.4	8.6
Tail length	11.3	14.2	12.0	13.4	10.2	10.7

The results given by two-tailed Pearson correlation showed significant correlations of some morphometric characters obtained from 50 females and 50 males of *P. penetrans* from Portugal (Tables 1.6 & 1.7). The overall body length of females revealed a high significant correlation with distance from anterior end to vulva ($r = 0.97$, $p \leq 0.05$) and vulva-anus distance ($r = 0.71$, $p \leq 0.05$). Positive correlations were also found between anterior end to vulva and maximum body width ($r = 0.77$, $p \leq 0.05$), maximum body width and body width at anus ($r = 0.71$, $p \leq 0.05$). The tail length of females and c ratio were negatively correlated ($r = - 0.71$, $p \leq 0.05$), whereas the c' ratio was correlated with ratios a ($r = 0.73$, $p \leq 0.05$) and c ($r = - 0.78$, $p \leq 0.05$). Stylet length, anterior end to median bulb, anterior end to excretory pore, excretory pore, anterior end to esophaegal glands, vulva-anus, V and b' ratio did not display highly relationships with other morphometric characters ($r < 0.70$) (Table 1.6). In males, overall body length, stylet length, anterior end to median bulb, anterior end to excretory pore, anterior end to esophageal glands, body width at anus, spicule length and ratio b' did not strongly correlate with each other or with any other morphometric data and the results did not show the same correlations found in females (Table 1.7). The a ratio and maximum body width were negatively correlated ($r = - 0.85$, $p \leq 0.05$), whereas the tail length showed a negative significant correlation with c ratio ($r = - 0.85$, $p \leq 0.05$) and positive correlation with c' ratio ($r = 0.89$, $p \leq 0.05$). The c ratio had a significant correlation with c' ratio ($r = 0.78$, $p \leq 0.05$) (Table 1.7).

Table 1.6 - Two-tailed Pearson correlation analysis for fifteen morphometric characters of *Pratylenchus penetrans* females from Portugal. *Correlation values greater than 0.7 or less than -0.7, significant at the 0.05 level.

	L	Stylet length	Median bulb	Excretory pore	Esophageal glands	Anterior end to vulva	Maximum body width	Body width at anus	Vulva-anus distance	Tail length	%V	a	b´	c	c'
L	1														
Stylet length	0.10	1													
Anterior end to centre of median bulb	0.55	0.31	1												
Anterior end to excretory pore	0.39	0.20	0.68	1											
Anterior end to esophageal glands	0.13	0.32	0.67	0.68	1										
Anterior end to vulva	0.97*	0.14	0.57	0.40	0.15	1									
Maximum body width	0.68	-0.04	0.46	0.20	0.03	0.71*	1								
Body width at anus	0.65	-0.20	0.33	0.22	-0.05	0.67	0.77*	1							
Vulva-anus distance	0.71*	0.01	0.48	0.37	0.18	0.58	0.35	0.33	1						
Tail length	0.66	-0.05	0.15	0.22	-0.08	0.54	0.20	0.37	0.65	1					
V	-0.04	0.15	0.12	0.05	0.12	0.20	0.17	0.15	-0.52	-0.45	1				
a	0.08	0.13	-0.07	0.15	0.08	0.01	-0.66	-0.40	0.26	0.41	-0.31	1			
b´	0.65	-0.15	-0.06	-0.26	-0.64	0.61	0.48	0.50	0.38	0.52	-0.10	0.00	1		
c	0.04	0.15	0.28	0.05	0.22	0.16	0.32	0.09	-0.18	-0.71*	0.52	-0.43	-0.10	1	
c'	0.10	0.12	-0.12	0.06	-0.05	-0.02	-0.44	-0.43	0.34	0.67	-0.55	0.73*	0.10	-0.78*	1

*correlation values greater than 0.7 or less than -0.7, significant at the 0.05 level

Table 1.7 - Two-tailed Pearson correlation analysis for thirteen morphometric characters of *Pratylenchus penetrans* males from Portugal. *Correlation values greater than 0.7 or less than -0.7, significant at the 0.05 level.

	L	Stylet length	Median bulb	Excretory pore	Esophageal glands	Maximum body width	Body width at anus	Spicule length	Tail length	a	b´	c	c'
L	1												
Stylet length	-0.22	1											
Anterior end to centre of median bulb	0.22	0.22	1										
Anterior end to excretory pore	0.52	0.09	0.49	1									
Anterior end to esophageal glands	0.27	-0.02	0.38	0.44	1								
Maximum body width	0.13	-0.24	0.15	0.09	0.28	1							
Body width at anus	0.19	-0.08	0.41	0.19	0.39	0.45	1						
Spicule length	0.11	-0.01	0.36	0.23	0.13	0.00	-0.04	1					
Tail length	0.57	-0.19	-0.13	0.25	0.24	0.22	0.16	-0.23	1				
a	0.24	0.23	-0.07	0.11	-0.15	-0.85*	-0.20	-0.06	0.03	1			
b´	0.61	-0.16	-0.14	0.05	-0.59	-0.12	-0.15	-0.04	0.28	0.33	1		
c	-0.10	0.07	0.26	0.03	-0.11	-0.15	-0.11	0.37	-0.85*	0.07	-0.01	1	
c'	0.46	-0.14	-0.32	0.14	0.05	0.00	-0.31	-0.23	0.89*	0.13	0.35	-0.78*	1

*correlation values greater than 0.7 or less than -0.7, significant at the 0.05 level

The morphometric analyses showed that measurements of main diagnostic characters of Portuguese isolates were similar to those found in *P. penetrans* by other authors in previous studies (Loof, 1960, Roman and Hirchmann, 1969, Castillo and Vovlas, 2007). The body size of the isolates from Portugal appears to be larger than that described in previous studies, however, these differences may be due to the fact that the measurements were made on nematodes which were propagated *in vitro* and not in field specimens, generally smaller. Another difference may be related to the fact that microscopic observations were made in nematodes without using a fixation method, such as the method of Seinhorst (1959) which includes hot fixation and gradual transfer of nematodes to glycerol for later measurements. The measurement software that was used in our study allows the capture of the image and simultaneous measurement of nematodes, without the need of preservation methods. Variations in morphometric characters can be caused from differences in fixation methods or changes in environmental conditions (Machado *et al*., 2015). Townshend (1991) reported that morphometric variations existed between populations of *P. penetrans* associated with strawberry and those associated with celery in Ontario, Canada. Furthermore, variations in size were also found between populations recovered from strawberry collected at different geographical areas (Townshend,1991).

In the five *P. penetrans* isolates from Portugal examined, the presence of substantial variability in morphometry within and between the isolates was observed. Intra and inter-isolate variability was found in most of the morphometric characters that were analysed in *P. penetrans* males and females. However, the results obtained with ANOVA and the analysis of the CV allowed to verify that the characters V and stylet size proved to be stable among isolates and between replicates within the same isolate. The stability of these characters confirms their usefulness for discriminating this species, as previously noted by Roman and Hirschmann (1969) and Tarte and Mai (1976). All other morphometrical characters, including those commonly used in nematode taxonomy, have shown relatively high coefficients of variation, such as overall body length, body width, anterior end to esophageal glands and a, b', c and c' ratios. Once again goes in line with other research studies, which referred similar results (Tarte and Mai, 1976; Roman and Hirschmann, 1969). This is confirmed by the Pearson correlation since the V ratio and stylet length did not correlate with any other morphometric character, supporting the ANOVA and the CV results.

CHAPTER 2

GENETIC DIVERSITY OF *PRATYLENCHUS PENETRANS*

INTRODUCTION

MOLECULAR DIAGNOSTICS OF RLN

Species-specific primers for different *Pratylenchus* spp. have been designed for conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR). PCR is a relatively simple and widely used molecular biology technique to amplify and detect DNA. PCR is highly sensitive and requires minimal DNA template for amplification and detection of target sequences. qPCR is used to detect, characterise and quantify nucleic acids for numerous applications and allows, not only the qualitative detection of target organisms, but also their quantification, proving a faster alternative to methods that rely on the identification and counting of nematodes by microscopy (Orlando *et al*., 2020). Several PCR-based diagnostic assays have been developed for identification and/or quantification of *P. penetrans* based on the ITS rDNA, D2-D3 expansion segment of 28S rDNA and β-1,4-endoglucanase gene. These species-specific primers have been developed for distinguishing RLN and the obtained PCR products correspond to a positive/negative result without the need for subsequent analysis of the amplicon (Table 2.1). The D2-D3 is found in a repeat, shows inter-specific variability in *Pratylenchus* spp., allowing the species discrimination in the genus *Pratylenchus*. It is a suitable region for diagnostic purposes and improves the specificity of the PCR and the sensitivity of detection (Al-Banna *et al*., 2004).

Table 2.1 - Species-specific primers of D2-D3 expansion segment of 28S rDNA, ITS rDNA and β-1,4-endoglucanase used in the DNA amplification of *Pratylenchus penetrans* (adapted from Orlando *et al*., 2020).

DNA region		Primer sequence (5'-3')	PCR product size (bp)	Molecular assay	Reference
D2-D3 expansion segment of 28S rDNA	F	TAAAGAATCCGCAAGGATAC	278	Conventional PCR	Al-Banna *et al*. (2004)
	R	TCGGAAGGAACCAGCTACTA			
	F	ACATGGTCGACACGGTGATA	520	Conventional PCR	Mekete *et al*. (2011)
	R	TGTTGCGCAAATCCTGTTTA			
	F	GGTTTTCGGGCTCATATGGGTTC	111	SYBR Green qPCR	Baidoo *et al*. (2017)
	R	TTTACGCCGAGAGTGGGATTGTG			
	F	GAGACTTTCGAGAAGGCGATATG	176	TaqMan qPCR	Dauphinais *et al*. (2018)
	R	AGGACCGAATTGGCAGAAG			
ITS rDNA	F	ATGATGGAAGTGTCCGCCT	462	Conventional PCR	Uehara *et al*. (1998)
	R	CCCAAACGACGGTCAAAAGG			
	F	ATTCCGTCCGTGGTTGCTATG	134	SYBR Green qPCR	Sato *et al*. (2007)
	R	GCCGAGTGATCCACCGATAAG			
	F	TGACTATATGACACATTTRAACTTG	660	Duplex PCR	Waeyenberge *et al*. (2009)
	R	ATATGCTTAAGTTCAGCGGGT			
	F	AATGTGTCTCGCCCTGAGG	80	TaqMan qPCR	Oliveira *et al*. (2017)
	R	GCAACCACGGACGGAATAC			
β-1,4-endoglucanase	F	CCAACCTCTGCTACACTA	-	TaqMan qPCR	Mokrini *et al*. (2013)
	R	CAGTGCCGTATTCAGTGA			
	F	GGCATTTATGTG(A/C)TCGTGGATTGGC	528	Conventional PCR	Peetz and Zasada (2016)
	R	GTTGCCATCAGCGCTGACAGTG			

GENETIC DIVERSITY IN RLN

Despite the fact that the genus *Pratylenchus* spp. has a great variety of species, a considerable number of RLN species share many morphological characters and, because of that, the taxonomic status of several species is currently contested. Identification of *Pratylenchus* is thus curbed by the resemblance among species in some cases and by the significant intraspecific variability of both morphological and morphometric diagnostic characters (Subbotin *et al*., 2008).

In respect to *P. penetrans*, high levels of polymorphism were found when analysing the morphological and morphometrical variation in populations from different geographical locations, and it was suggested that *P. convallariae* and *P. fallax* could be polymorphic variants of *P. penetrans* (Tarte and Mai, 1976). Later, Perry *et al*. (1980) detected infertile F1 progeny after interspecific crosses between *P. penetrans* and *P. fallax* in sterile culture and concluded that these species were valid. Several isoenzymes (esterase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase) discriminating *P. fallax* and *P. penetrans* and differences in the ITS rDNA length and in the ITS RFLPs between these two species were reported, respectively, by Ibrahim *et al*., (1995) and Waeyenberge *et al*., (2000). Nevertheless, a detailed study of *P. arlingtoni*, *P. convallariae*, *P. fallax* and *P. penetrans* revealed minor morphological and morphometrical differences (Handoo *et al*., 2001). Subsequently, *P. fallax* and *P. convallariae* were shown to be closely related to *P. penetrans* (96 - 97% similarity) after sequence analysis of the D2-D3 region of the 28S rRNA gene (Carta *et al*., 2001; Handoo *et al*., 2001). Phylogenetic analyses of sequences of D2-D3 of 28S rDNA or partial 18S rDNA conducted by Subbotin *et al*. (2008) exhibited a distinct clade that grouped *P. penetrans* with *P. convallariae*, *P. arlingtoni P. fallax, P. pinguicaudatus and P. dunensis* in clade IV. Later, Palomares-Rius *et al*. (2014) added *P. oleae* and *P. brachyurus* into this clade. Since *P. fallax* is a quarantine pest in many countries, the clarification of the taxonomic status of these species is essential.

Janssen *et al*. (2017) reconstructed a multi-gene phylogeny of the *Penetrans* group (clade IV) using the ITS, D2-D3 of the 28S rDNA regions from nuclear rDNA and the COI gene from mtDNA. Using a combination of phylogenetic data with molecular species delineation analysis, population genetics, morphometric information and sequences, the authors were able to confirm the taxonomic status of *P. penetrans*, *P. fallax* and *P. convallariae,* clarifying the boundaries within the *Penetrans* group. In the same study, *P. fallax* populations demonstrated low intraspecific variability whereas *P. penetrans* showed awfully diverse haplotypes, with extremely variable intraspecific variability. Nonetheless, identical *P.*

penetrans haplotypes were found to be geographically widespread, suggesting that *P. penetrans* could have spread anthropogenically through agricultural development and crop exchange (Janssen *et al*., 2017).

De Luca *et al*. (2011) also observed intraspecific variability in *P. penetrans* in the D3 region, as well as Fanelli *et al*. (2018), based on sequence analysis and phylogenetic reconstruction of the ITS and D2-D3 regions. Phylogenetic analysis, performed by De Luca *et al*. (2011), revealed high intraspecific variability in ITS sequences of several *Pratylenchus* species. Sequence analyses showed high sequence variability not only between populations or isolates but also within individuals. The nucleotide dissimilarities for each species varied, for example, up to 7% for *P. vulnus* and *P. neglectus*, up to 6% for *P. thornei*, up to 5% for *P. lentis* and up to 1% for *P. bolivianus*, *P. mediterraneus*, and *P. pseudocoffeae*. At species level, such great variability had not been noticed in other PPN (De Luca *et al*., 2011). The same study concluded that ITS sequences allow a clear separation of the *Pratylenchus* species in spite of the high intraspecific variability. Intraspecific ITS sequence variation, was found to be minor within species such as *P. goodyei* and *P. vulnus*, however in other species, for instance *P. coffeae*, the variation among populations are substantial.

MATERIALS AND METHODS

PCR, CLONING AND SEQUENCING

Total DNA was extracted from mixed developmental stages of *P. penetrans* using the commercial kit “DNeasy Blood & Tissue” (Qiagen, Germany).

To confirm the molecular identification of the five Portuguese isolates, DNA was amplified using the species-specific primer set (PPEN/D3B) designed by Al-Banna *et al*. (2004) to amplify the variable region within the D2-D3 expansion region of the 28S rDNA of *P. penetrans* (Figure 2.1). The PCR reaction mixture contained 2,5 µL 1× PCR reaction buffer, 1.25 mM $MgCl_2$, 200 µM dNTPs and 2.0 µM of each primer, 2 U of BioTaq DNA polymerase (Bioline, Meridien Bioscience, United Kingdom) 20-50 ng of extracted DNA and sterile water up to a volume of 25 µL. Reactions were carried out in a Thermal Cycler (Bio-Rad, Spain) with an initial denaturation step of 95ºC for 3 minutes followed by 35 reaction cycles of 95ºC for 1 minute, annealing at 62ºC for 1 minute, extension at 72ºC for 1 minute, and a final extension at 72ºC for 7 minutes. PCR products were separated by electrophoresis in 2 % Tris-Borate - EDTA (TBE) -buffered agarose stained with GreenSafe (Nzytech, Portugal) and visualized under UV light.

Two genomic regions were selected for *P. penetrans* intraspecific variability analysis, COI gene and ITS region were amplified by PCR. For COI gene, PCR amplification was performed using the forward primer JB3 (5' – TTTTTTGGGCATCCTGAGGTTTAT – 3') and the reverse primer JB4.5 (5' – TAAAGAAAGAACATAATGAAAATG – 3') (Derycke *et al*., 2005). The PCR reaction mixture contained 2.5 µL 1× PCR reaction buffer, 1.25 mM $MgCl_2$, 200 µM dNTPs and 2.0 µM of each primer, 2 U of BioTaq DNA polymerase, 20-50 ng of extracted DNA and sterile water up to a volume of 50 µL. For ITS region amplification, the forward primer PRATTW81 (5' – GTAGGTGAACCTGCTGCTG – 3') and reverse primer AB28 (5' – ATATGCTTAAGTTCAGCGGGT – 3') (Waeyenberge *et al*., 2009) (Figure 2.1) were used. The PCR reaction mixture contained 5 µL10× PCR reaction buffer, 1.25 mM MgCl2, 200 µM dNTPs, 0.5 µM of each primer, 2 U of BioTaq Polymerase, 20-50 ng of extracted DNA and sterile water up to a volume of 50 µL. Reactions were carried out in a Thermal Cycler (Bio-Rad, Spain) with an initial denaturation step of 95ºC for 3 minutes followed by 35 reaction cycles of 94ºC for 30 seconds, annealing for 30 seconds at 54ºC and 60ºC for COI region and ITS region, respectively, extension at 72ºC for 30 seconds, and a final extension at 72ºC for 7 minutes. After electrophoresis in a 1% TBE buffered agarose gel (1 h, 100 V) to check the amplification success, the PCR products were purified following the instructions included using the "NucleoSpin® Gel and PCR Clean-up" kit (Macherey-Nagel, Germany). Subsequently, the DNA concentrations of the purified PCR products were measured in a Nanodrop (ThermoFisher).

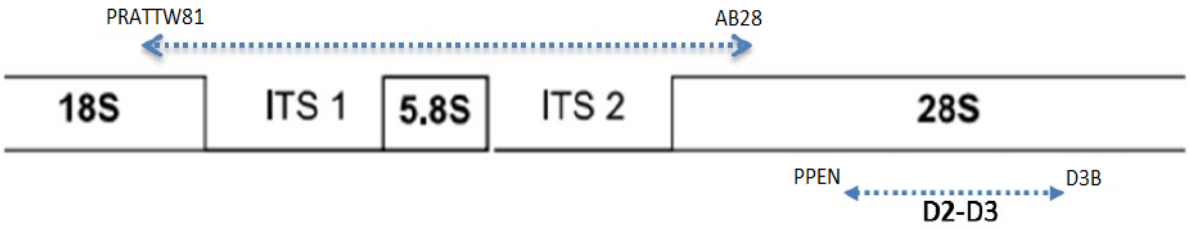

Figure 2.1 - Structure of the ribosomal DNA gene family in nematodes showing location of primers PPEN/D3B and PRATTW81/AB28 used in this study.

Purified PCR products were ligated into pGEM®-T Easy Vector (Promega, USA) using 50 ng vector in a 10 µL reaction with 3 U T4 DNA Ligase (Promega) and 22 ng of purified COI or 36 ng of ITS products in 1× Rapid Ligation Buffer (Promega). Ligation reactions were incubated for 1 h at room temperature. Then, 2 µL of the ligation product was used to transform *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) as described in the manufacturer's protocols. An agar Luria-Bertani (LB) medium with ampicillin, IPTG (Promega)

and X-GAL (Promega), was prepared for *E. coli* colonies to grow, according to standard protocols. From the obtained blue colonies (non-recombinant) and white colonies (recombinant), five white colonies per isolate were selected and further grown in 1 mL LB medium with ampicilin. Plasmidic DNA from the cultures were purified using Nzymini Prep (Nzytech, Portugal). Purified DNA was digested with *Eco*RI restriction enzyme (Bioron, Germany) to confirm the correct length of the cloned fragment. DNA from selected positive clones were sent for sequencing (Macrogen, Spain) in both directions using universal primers M13F and M13R, to obtain overlapping sequences of the forward and reverse DNA strand.

SEQUENCES ANALYSES

Sequences analyses and alignments were carried out using BioEdit (Hall, 1999). Homologous sequences in the databases were searched using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997). Sequence statistics, such as nucleotide diversity (Pi), haplotype diversity (Hd), average number of nucleotide differences (k) and mismatch distributions were estimated using DnaSP 6.12.03 software (Rozas *et al*., 2017). Intra-isolate sequence analyses were carried out from the alignments obtained with sequences of each isolate and overall sequence diversity with the alignment obtained with all sequences of the five isolates.

PHYLOGENETIC AND MOLECULAR EVOLUTIONARY ANALYSES

Phylogenetic and molecular evolutionary analyses were conducted in MEGA v10.1.8 software (Kumar *et al*., 2018). Phylogenetic trees were constructed by the Neighbor-Joining method (Saitou and Nei, 1987) with 1000 replications of bootstrap, with the genetic distances computed using the Maximum Composite Likelihood model (Tamura *et al*., 2004). Ambiguous positions were removed for each sequence pair (pairwise deletion option), using the COI and ITS nucleotide sequence alignments of isolates from Portugal and homologous sequences retrieved from GenBank database (Table 2.2). Genetic distance between sequences from the Portuguese isolates was also estimated by pairwise distance with pairwise deletion option and standard error estimated by a bootstrap procedure (1000 replicates), using the alignments of COI and ITS nucleotide sequences determined in this study. Additionally, Tajima's D neutrality tests (Tajima, 1989), which distinguish between a DNA sequence evolving randomly (or neutrally) and one evolving under a non-random process, were performed using the total number of mutations in DnaSP v6.12.03 software.

The correlation between genetic and geographic distance of *P. penetrans* isolates was also assessed by computing the determined pairwise distance versus the logarithmic function

of the distance (Km) between the sampling locations of each of the five isolates. Points with geographic distance of 0 Km were removed from analysis. The significance of this correlation was calculated using Pearson correlation in Statsoft Statistica® V.7.

Table 2.2 - Sequences used in this study.

Species	Isolate	Region	Host	Accession Number	
				COI	ITS
Pratylenchus fallax	T353	The Netherlands, Doornenburg	*Malus pumila*	KY816988	KY828258
P. fallax	V4 C	The Netherlands, Ysbrechtum	*Vitis vinifera*	KY816938	KY828272 - KY828273
P. penetrans	T143	Rwanda, Nyakiriba	*Allium cepa*	KY817013	-
P. penetrans	T293	The Netherlands, Apeldoorn	*Pyrus* sp.	KY816992	-
P. penetrans	V3 A	The Netherlands, Baarlo	*M. pumila*	KY816941	KY828268 - KY828269
P. penetrans	V8 A	The Netherlands, Baarlo	*M. pumila*	KY816936	KY828274
P. penetrans	V1B	The Netherlands, Meijel	*M. pumila*	KY816942	KY828266
P. penetrans	V3 F	The Netherlands, Nagele	*M. pumila*	KY816940	KY828270 - KY828271
P. penetrans	N3678	USA, Minnesota	*Zea mays*	MK877982	-
P. penetrans	N6260	USA, Fairbanks County	*Paeonia* sp.	MK877984	-
P. penetrans	N7126	USA, Otoe County	*Malus* sp.	MK877987	-
P. penetrans	N7198	USA, Idaho	*Solanum tuberosum*	MK877988	-
P. penetrans	N7199	USA, Idaho	*S. tuberosum*	MK877989	-
P. penetrans	N7200	USA, Idaho	*S. tuberosum*	MK877990	-
P. penetrans	N7201	USA, Idaho	*S. tuberosum*	MK877991	-
P. penetrans	N7202	USA, Idaho	*S. tuberosum*	MK877992	-
P. penetrans	P147033	USA, Portage County	*S. tuberosum*	MK877995	-
P. penetrans	P147034	USA, Portage County	*S. tuberosum*	MK877996	-
P. penetrans	P147035	USA, Portage County	*S. tuberosum*	MK877997	-
P. penetrans	P148032	USA, Portage County	*S. tuberosum*	MK877998	-
P. penetrans	c12	Canada, Kentville	*Prunus* sp.	-	MK282740
P. penetrans	862	Chile	*Lillium* sp.	-	JX046946
P. penetrans	GY	France	*Prunus* sp.	-	JX046944
P. penetrans	JGM	France	*Sambucus* sp.	-	JX046942
P. penetrans	CA192	France, Britany	*M. pumila*	-	KY828242
P. penetrans	CA192	France, Britany	*M. pumila*	-	KY828243
P. penetrans	Pp18KL1	Long Island, USA	*S. tuberosum*	-	FJ712987
P. penetrans	Pp18KL2	Long Island, USA	*S. tuberosum*	-	FJ712988
P. penetrans	Pp18KL3	Long Island, USA	*S. tuberosum*	-	FJ712989
P. penetrans	Pp18KL4	Long Island, USA	*S. tuberosum*	-	FJ712990
P. penetrans	Pp18KL5	Long Island, USA	*S. tuberosum*	-	FJ712991
P. penetrans	F1	MN, USA	*S. tuberosum*	-	KX842607
P. penetrans	F2	MN, USA	*S. tuberosum*	-	KX842608
P. penetrans	F3	MN, USA	*S. tuberosum*	-	KX842609
P. penetrans	F4	MN, USA	*S. tuberosum*	-	KX842610
P. penetrans	F5	MN, USA	*S. tuberosum*	-	KX842611
P. penetrans	F6	MN, USA	*S. tuberosum*	-	KX842612
P. penetrans	F7	MN, USA	*S. tuberosum*	-	KX842613
P. penetrans	Pp17KL1	Monroe County, USA	*Prunus cerasus*	-	FJ712982
P. penetrans	Pp12KL1	Rennes, France	*Malus* sp.	-	FJ712967
P. penetrans	T143	Rwanda, Nyakiriba	*A. cepa*	-	KY828249
P. penetrans	T143	Rwanda, Nyakiriba	*A. cepa*	-	KY828250
P. penetrans	Pp14KL1	Spain	*Malus* sp.	-	FJ712977
P. penetrans	9827	The Netherlands	*Iris* sp.	-	JX046949
P. penetrans	5118	The Netherlands	*Lillium* sp.	-	JX046950
P. penetrans	T293	The Netherlands, Apeldoorn	*Pyrus* sp.	-	KY828257
P. penetrans	Pp1KL1	Tongeren, Belgium	*Rubus* sp.	-	FJ712957
P. penetrans	YIN	USA	*Acer x freemanii*	-	JX046947
P. penetrans	Pp2KL1	Zandhoven, Belgium	*Z. mays*	-	FJ712962
P. pinguicaudatus	T572	UK, England, Rothemstadt	*Triticum* sp.	KY816984	KY828261 - KY828262 - KY828263
P. thornei	N3786	California, USA	*V. vinifera*	MK878270	-
P. thornei	PthKL1	Santaella, Spain	*Cicer arietinum*	-	FJ713002

RESULTS AND DISCUSSION

SPECIES-SPECIFIC AMPLIFICATION OF D2-D3 REGION

The molecular identification of the five isolates was first confirmed by PCR, using the species-specific primer set PPEN/D3B, for amplification of the variable region within the D2-D3 expansion region of the 28S rDNA of *P. penetrans* (Al-Banna *et al*., 2004). The amplified product of approximately 280 bp was obtained for all the five isolates, confirming the identity of these isolates as *P. penetrans* (Figure 2.2).

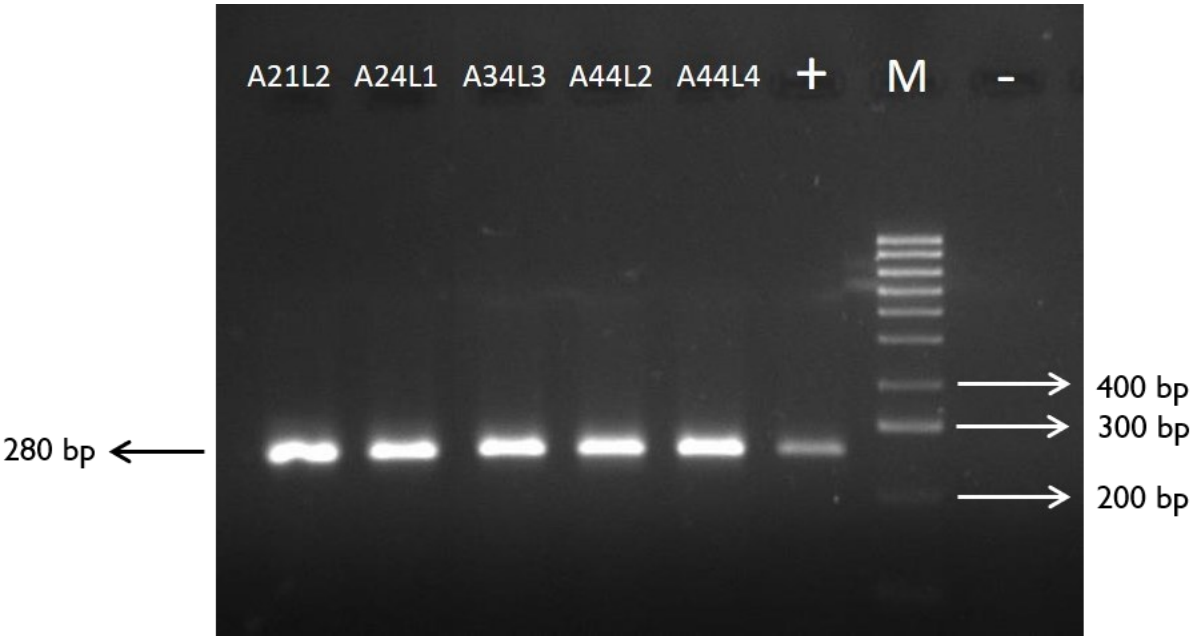

Figure 2.2 – D2-D3 expansion region of the 28S rDNA PCR products of five *Pratylenchus penetrans* isolates from Portugal, amplified using the species-specific primer set PPEN/D3B; (+): positive control; (-): negative control; M: Hyperladder IV (1000 bp), Bioline.

GENETIC DIVERSITY OF *PRATYLENCHUS PENETRANS* PORTUGUESE ISOLATES

Two genomic regions, COI and ITS, were selected for evaluation of *P. penetrans* isolates genetic diversity. For COI region, a fragment of ca. 440 bp was obtained by PCR for all five *P. penetrans* isolates from Portugal (Figure 2.3A).

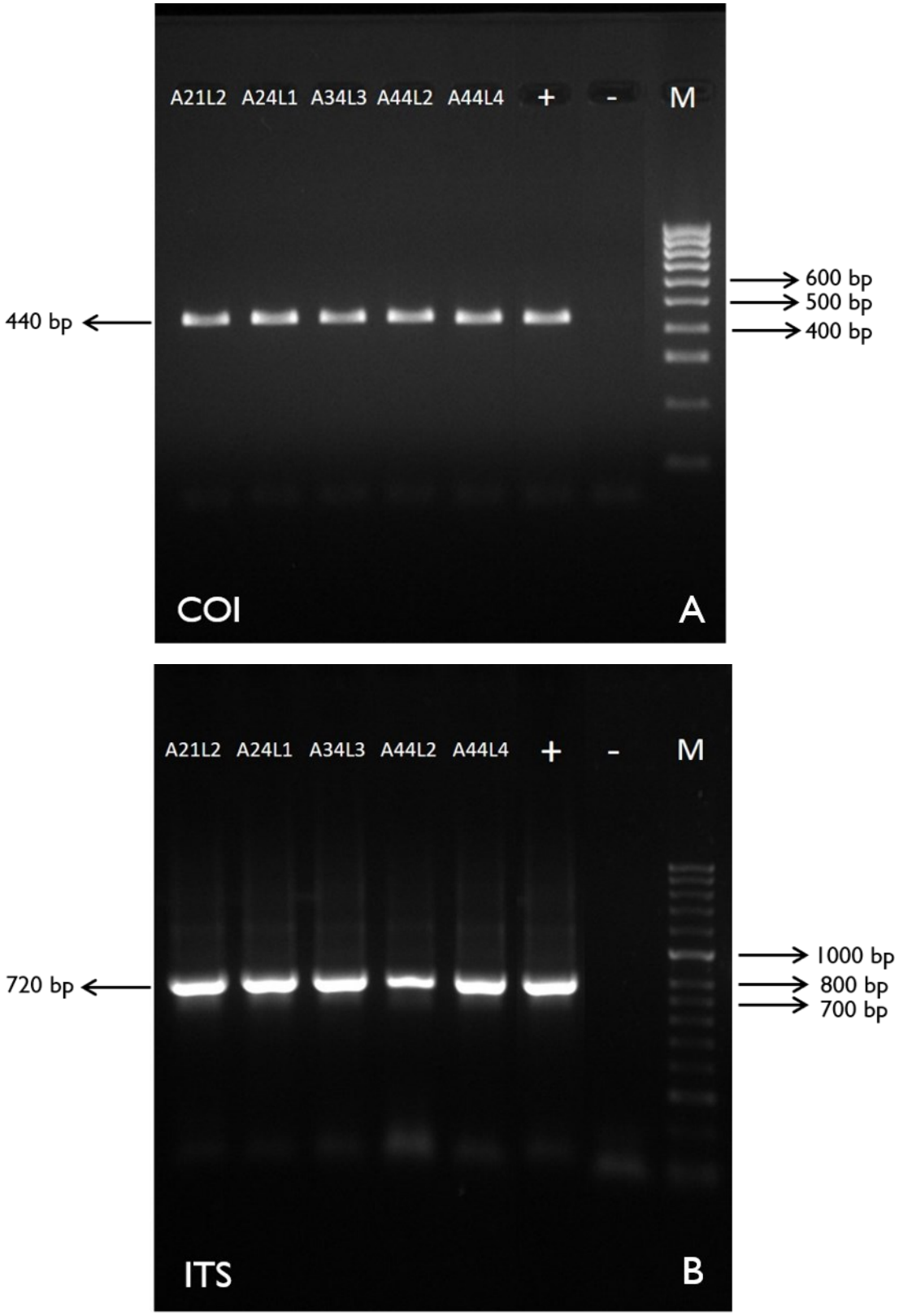

Figure 2.3 - COI (A) and ITS (B) PCR products of the five isolates of *Pratylenchus penetrans* from Portugal amplified with primers JB3/JB4.5 and PRATTW81/AB28, respectively; (+): positive control; (-): negative control; M: (COI) Hyperladder IV (1000 bp); (ITS) Hyperladder II (2000 bp), Bioline.

The PCR COI fragment of the five isolates were purified and cloned. Three positive clones of each isolate were sequenced, except for A21L2 isolate for which only two clones were successfully sequenced. All COI sequences presented 441 bp length, except one clone of A21L2 with 440 bp in length. For ITS region, a PCR fragment of approximately 720 bp was obtained for all the five isolates (Figure 2.3B). These products were purified from gel and cloned. Three positive clones for each isolate were successfully sequenced and presented lengths ranging from 711-723 bp.

A preliminary BLAST search against NCBI database of the determined COI and ITS sequences confirmed the species identity with sequences homologies ranging from 94.00% to 100.00% and 91.94% to 98.46% to other *P. penetrans* COI and ITS sequences, respectively, present in the database.

The length variation on all clones of ITS region and the sequence analysis revealed high variability, not only between isolates but also within isolates, with a high number of polymorphic (S) and mutation (Eta) sites then the one found for COI region. All 14 COI sequences and 15 ITS sequences corresponded, respectively, to 6 and 15 different haplotypes (Table 2.3). For COI region, a low number of polymorphic and mutation sites were found considering each isolate or even considering all isolates. The COI intra-isolate Pi was lower for A44L2 isolate (Pi = 0.00000), with all three clones being identical, and higher for A21L2 isolate (Pi = 0.00455). Intra-isolate Pi for ITS region was lower for A34L3 isolate (Pi = 0.00940) and higher for A44L2 isolate (Pi = 0.05768) (Table 2.3).

Considering all isolates, a higher Pi was found for the ITS region (Pi = 0.03185) than for the COI region (Pi = 0.00492). From neutrality tests, estimated Tajima's D values were 0.90207 ($p > 0.10$) and -1.51991 ($p > 0.10$) for COI and ITS regions respectively, indicating that the changes were not significant, and all sequences underwent neutral selection (Table 2.3).

Table 2.3 - Genetic diversity of cloned COI and ITS regions of five *Pratylenchus penetrans* isolates from Portugal.

Isolate	Genomic Region	Number of clones	Sequences length (bp)	Number of polymorphic sites (S)	Total number of mutations (Eta)	Number of Haplotypes	Haplotype diversity (Hd) (standard deviation)	Nucleotide diversity (Pi) (standard deviation)	Average number of nucleotide differences (k)	Tajima's D (statistical significance)
A21L2	COI	2	440; 441	2	2	2	1.000 (0.500)	0.00455 (0.00227)	2.000	-
	ITS	3	713; 717; 723	35	35	3	1.000 (0.272)	0.03291 (0.00909)	23.333	-
A24L1	COI	3	441; 441; 441	1	1	2	0.667 (0.314)	0.00151 (0.00071)	0.667	-
	ITS	3	711; 713; 716	38	42	3	1.000 (0.272)	0.03766 (0.01035)	26.667	-
A34L3	COI	3	441; 441; 441	1	1	2	0.667 (0.314)	0.00151 (0.00071)	0.667	-
	ITS	3	711; 711; 713	10	10	3	1.000 (0.272)	0.00940 (0.00401)	6.667	-
A44L2	COI	3	441; 441; 441	0	0	1	0.000 (0.000)	0.00000 (0.00000)	0.000	-
	ITS	3	713; 715; 718	60	62	3	1.000 (2.272)	0.05768 (0.01659)	40.667	-
A44L4	COI	3	441; 441; 441	2	2	2	0.667 (0.314)	0.00302 (0.00143)	1.333	-
	ITS	3	714; 714; 716	20	20	3	1.000 (0.272)	0.01878 (0.00722)	13.333	-
All 5 isolates	COI	14	-	9	9	6	0.681 (0.132)	0.00492 (0.00149)	2.165	p > 0.10 not significant
	ITS	15	-	99	109	15	1.000 (0.024)	0.03185 (0.00358)	21.914	p > 0.10 not significant

EVOLUTION AND PHYLOGENETIC RELATIONSHIPS

The estimate genetic distance between sequences of COI region revealed low nucleotide divergence with a minimum of 0.0000 base substitutions per site, considering both, intra and inter-isolate divergence. A maximum of 0.0162 base substitutions per base was found between isolates A44L4 clone 3 and A21L2 clone 1, isolates A44L4 clone 3 and A21L2 clone 2 and isolates A44L4 clone 3 and A34L3 clone 1 (Table 2.4 & Supplementary Table 1). On the other hand, ITS region revealed much higher nucleotide divergence with a maximum of 0.0763, between isolates A24L1 clone 2 and A44L2 clone 1, and that value decreases for 0.0157, between isolates A21L2 clone 3 and A34L3 clone 1. Within isolates the highest value of nucleotide divergence was detected on A44L2 with 0.0683, and the lowest value of base substitutions per site was on A34L3 with 0.0014 (Table 2.5 & Supplementary Table 2). Additionally, the mismatch distribution of both COI and ITS sequences revealed to be a multimodal distribution with several peaks of pairwise differences, excluding the possibility of abrupt selection events. However, the average number of k in COI region was lower than in ITS region (Figure 2.4).

Table 2.4 - Data on COI region genetic distance and geographic distance among the five *Pratylenchus penetrans* isolates from Portugal. Genetic distance estimated by number of nucleotide substitutions per site (inferior side) and geographical distance estimated using the logarithmic function of the distance (LOG Km) between the sampling locations of each of the five isolates (superior side).

	COI clones													
	A21L2_1	A21L2_2	A24L1_1	A24L1_2	A24L1_3	A34L3_1	A34L3_2	A34L3_3	A44L2_1	A44L2_2	A44L2_3	A44L4_1	A44L4_2	A44L4_3
A21L2_1		-	0.301	0.301	0.301	1.858	1.858	1.858	1.997	1.997	1.997	1.997	1.997	1.997
A21L2_2	0.0046		0.301	0.301	0.301	1.858	1.858	1.858	1.997	1.997	1.997	1.997	1.997	1.997
A24L1_1	0.0023	0.0023		-	-	1.848	1.848	1.848	1.989	1.989	1.989	1.989	1.989	1.989
A24L1_2	0.0023	0.0023	0.0000		-	1.848	1.848	1.848	1.989	1.989	1.989	1.989	1.989	1.989
A24L1_3	0.0046	0.0046	0.0023	0.0023		1.848	1.848	1.848	1.989	1.989	1.989	1.989	1.989	1.989
A34L3_1	0.0023	0.0046	0.0023	0.0023	0.0046		-	-	1.456	1.456	1.456	1.456	1.456	1.456
A34L3_2	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023		-	1.456	1.456	1.456	1.456	1.456	1.456
A34L3_3	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000		1.456	1.456	1.456	1.456	1.456	1.456
A44L2_1	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000	0.0000		-	-	-	-	-
A44L2_2	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000	0.0000	0.0000		-	-	-	-
A44L2_3	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000	0.0000	0.0000	0.0000		-	-	-
A44L4_1	0.0115	0.0115	0.0092	0.0092	0.0115	0.0115	0.0092	0.0092	0.0092	0.0092	0.0092		-	-
A44L4_2	0.0115	0.0115	0.0092	0.0092	0.0115	0.0115	0.0092	0.0092	0.0092	0.0092	0.0092	0.0000		-
A44L4_3	0.0162	0.0162	0.0138	0.0138	0.0161	0.0162	0.0138	0.0138	0.0138	0.0138	0.0138	0.0046	0.0046	

Table 2.5 - Data on ITS region genetic distance and geographic distance among the five *Pratylenchus penetrans* isolates from Portugal. Genetic distance, estimated by number of nucleotide substitutions per site (inferior side) and geographical distance estimated using the logarithmic function of the distance (LOG Km) between the sampling locations of each of the five isolates (superior side).

	ITS clones														
	A21L2_1	A21L2_2	A21L2_3	A24L1_1	A24L1_2	A24L1_3	A34L3_1	A34L3_2	A34L3_3	A44L2_1	A44L2_2	A44L2_3	A44L4_1	A44L4_2	A44L4_3
A21L2_1		-	-	0.301	0.301	0.301	1.858	1.858	1.858	1.997	1.997	1.997	1.997	1.997	1.997
A21L2_2	0.0390		-	0.301	0.301	0.301	1.858	1.858	1.858	1.997	1.997	1.997	1.997	1.997	1.997
A21L2_3	0.0362	0.0347		0.301	0.301	0.301	1.858	1.858	1.858	1.997	1.997	1.997	1.997	1.997	1.997
A24L1_1	0.0360	0.0317	0.0392		-	-	1.848	1.848	1.848	1.989	1.989	1.989	1.989	1.989	1.989
A24L1_2	0.0540	0.0482	0.0408	0.0448		-	1.848	1.848	1.848	1.989	1.989	1.989	1.989	1.989	1.989
A24L1_3	0.0423	0.0332	0.0172	0.0378	0.0424		1.848	1.848	1.848	1.989	1.989	1.989	1.989	1.989	1.989
A34L3_1	0.0333	0.0244	0.0157	0.0318	0.0333	0.0201		-	-	1.456	1.456	1.456	1.456	1.456	1.456
A34L3_2	0.0348	0.0259	0.0172	0.0332	0.0348	0.0216	0.0014		-	1.456	1.456	1.456	1.456	1.456	1.456
A34L3_3	0.0347	0.0259	0.0215	0.0302	0.0347	0.0245	0.0128	0.0143		1.456	1.456	1.456	1.456	1.456	1.456
A44L2_1	0.0623	0.0606	0.0486	0.0606	0.0763	0.0549	0.0533	0.0548	0.0501		-	-	-	-	-
A44L2_2	0.0571	0.0495	0.0392	0.0463	0.0271	0.0423	0.0347	0.0362	0.0376	0.0683		-	-	-	-
A44L2_3	0.0408	0.0388	0.0289	0.0377	0.0438	0.0303	0.0245	0.0250	0.0274	0.0655	0.0481		-	-	-
A44L4_1	0.0375	0.0360	0.0243	0.0359	0.0331	0.0376	0.0258	0.0272	0.0286	0.0604	0.0404	0.0376		-	-
A44L4_2	0.0345	0.0302	0.0304	0.0330	0.0406	0.0379	0.0290	0.0304	0.0304	0.0592	0.0391	0.0334	0.0273		-
A44L4_3	0.0374	0.0332	0.0333	0.0360	0.0436	0.0409	0.0319	0.0334	0.0333	0.0623	0.0420	0.0349	0.0302	0.0056	

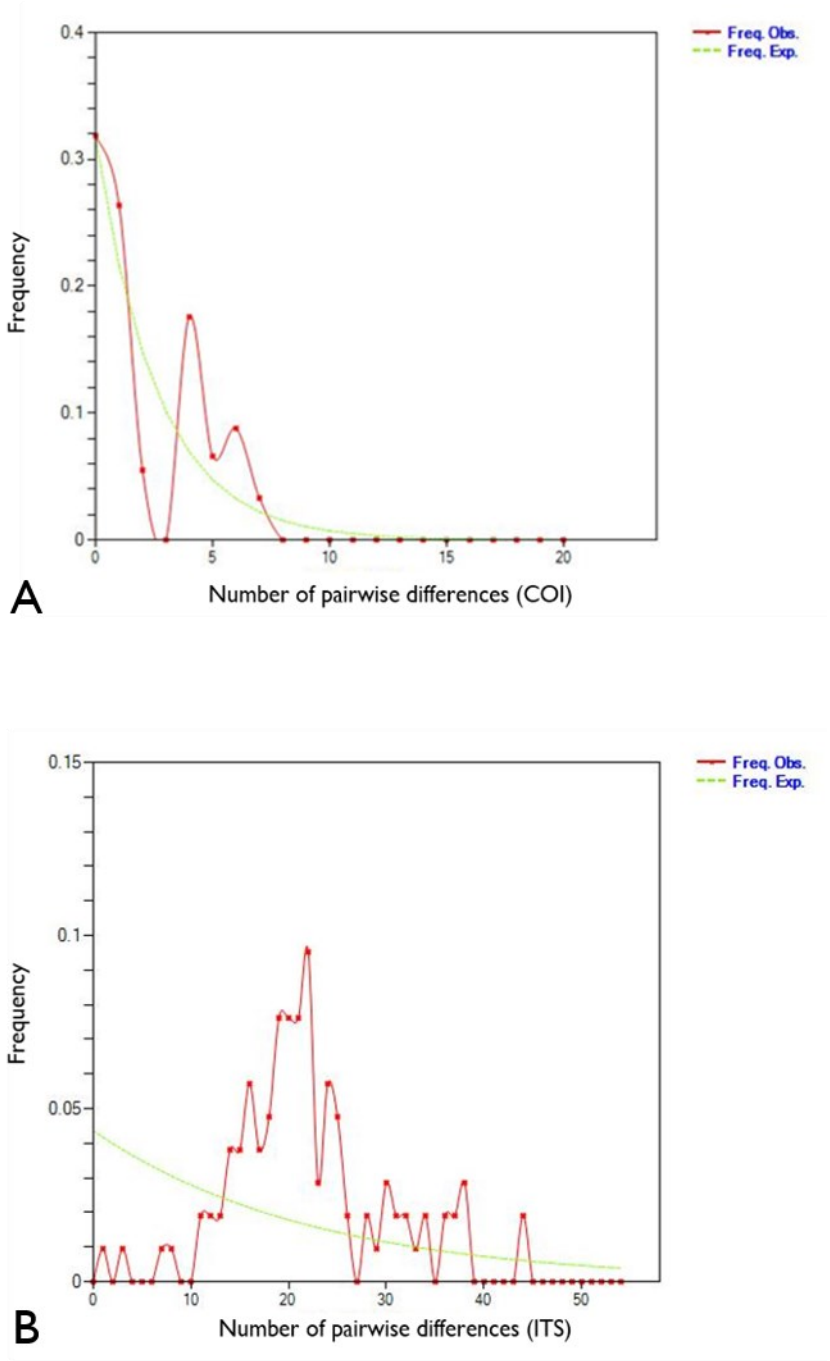

Figure 2.4 - Mismatch distribution of COI (A) and ITS (B) sequences of the five *Pratylenchus penetrans* isolates from Portugal.

The possible correlation between genetic distance and geographic distance of the five *P. penetrans* isolates were also investigated, considering both COI (Table 2.4) and ITS regions (Table 2.5), and revealed that there were no significant correlation between these two variables with a Pearson correlation of r = 0.11910 (p > 0.05) for COI region (Figure 2.5) and r = 0.07238 (p > 0.05) for ITS region (Figure 2.6).

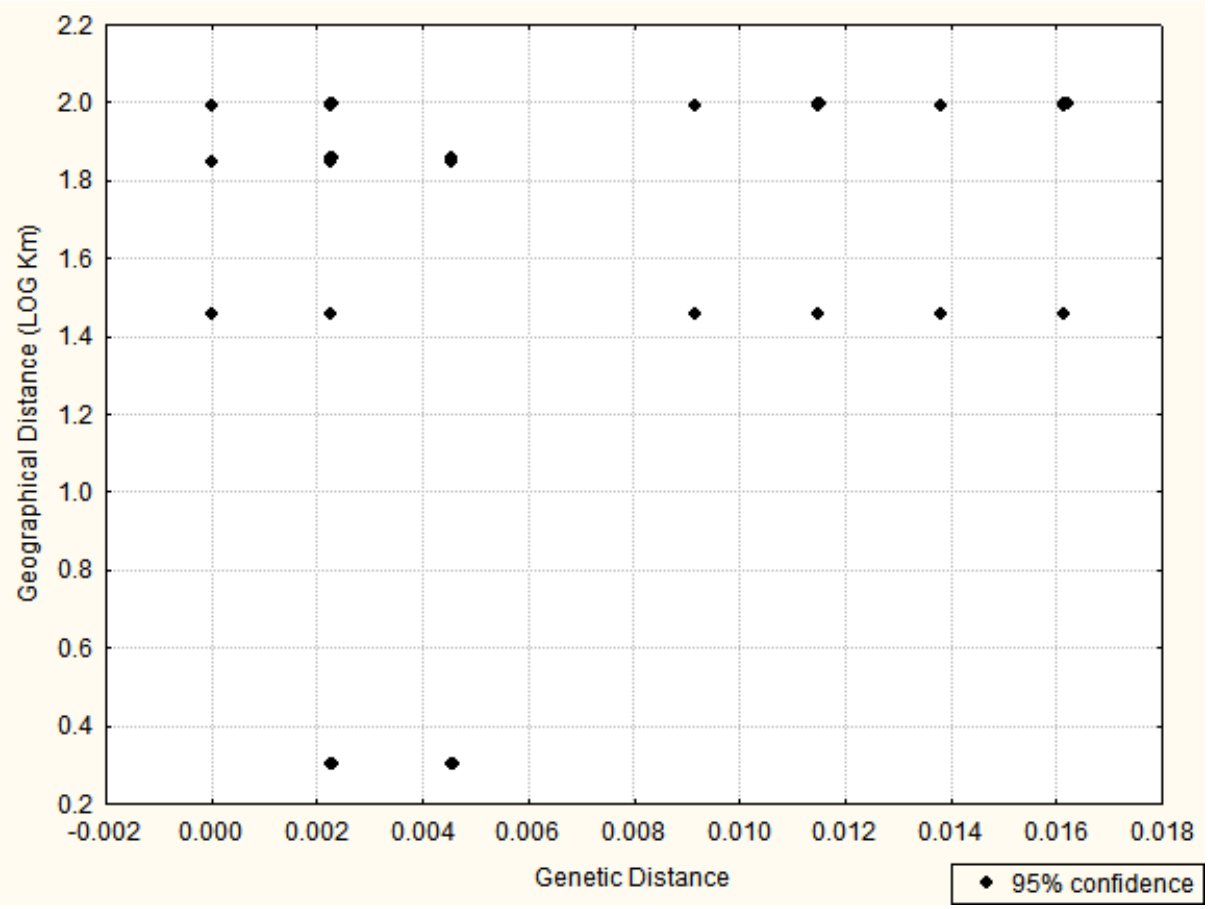

Figure 2.5 - Correlation between COI region genetic distance and geographic distance for the five *Pratylenchus penetrans* isolates from Portugal. Pearson correlation of r = 0.11910 (p > 0.05). Points with geographic distance = 0 were removed from analysis.

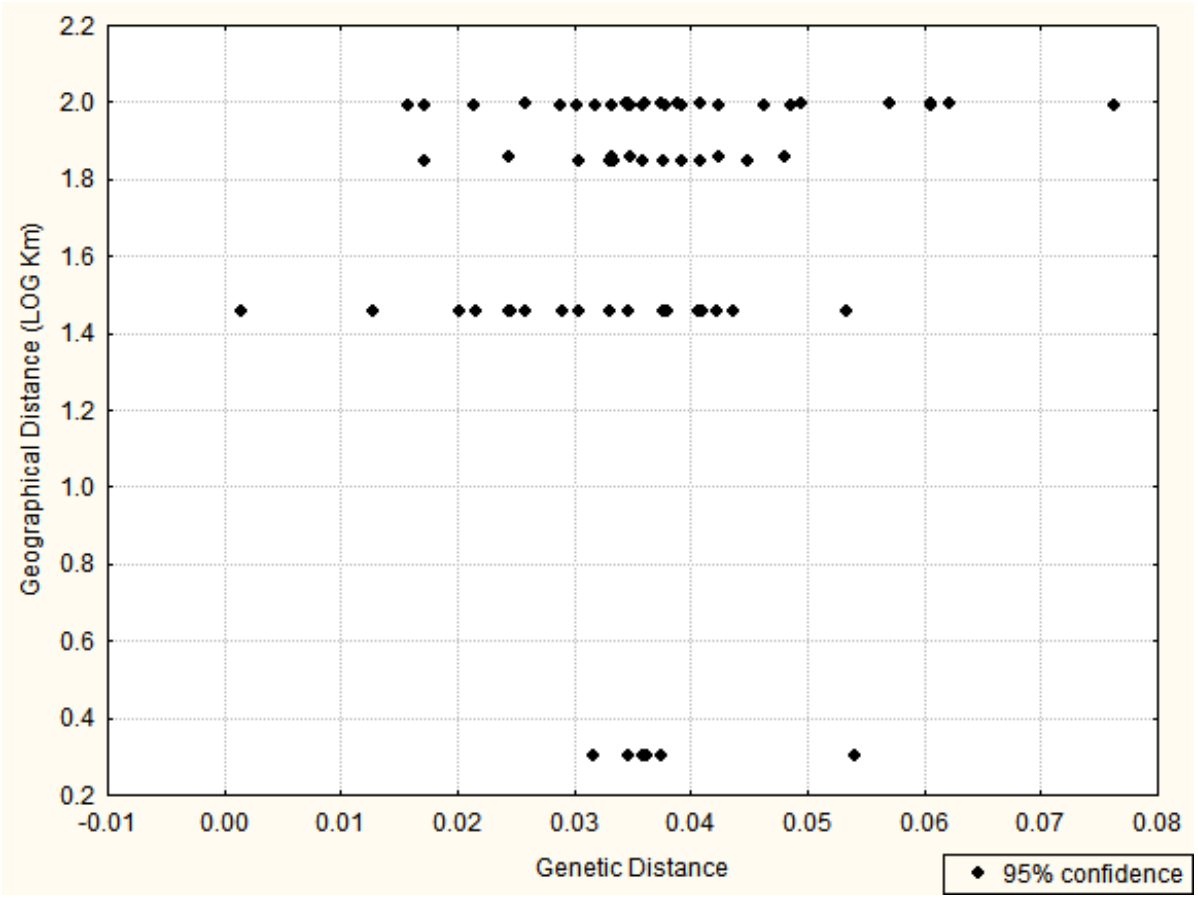

Figure 2.6 - Correlation between ITS region genetic distance and geographic distance for the five *Pratylenchus penetrans* isolates from Portugal. Pearson correlation of r = 0.07238 (p > 0.05). Points with geographic distance = 0 were removed from analysis.

In this study, the phylogenetic relationships within the *P. penetrans* species were analysed using the COI gene with the alignment of 35 *Pratylenchus* sequences, 32 of *P. penetrans*, including the 14 new sequences determined in this study, two of *P. fallax*, one of *P. pinguicaudatus* and one sequence from *P. thornei* used as outgroup (Figure 2.7). Phylogenetic analysis based on *P. penetrans* COI sequences revealed low divergence between sequences from the same isolate and also from different isolates. All COI sequences from Portuguese *P. penetrans* isolates group together and with other *P. penetrans* isolates, revealing a closer relationship with one Dutch isolate from apple (KY816941), one African isolate from onion (KY817013) and five American isolates from potato (MK877988; MK877989; MK877990; MK877991; MK877992).

Phylogenetic analysis based on *P. penetrans* ITS sequences was performed with the alignment of 56 *Pratylenchus* sequences, 50 of *P. penetrans*, including the 15 new sequences, three of *P. fallax*, three of *P. pinguicaudatus* and also one *P. thornei* sequence (Figure 2.8) and showed that our isolates clearly group up with other *P. penetrans* isolates available on GenBank but ITS sequences from the same isolates do not group together, reflecting the high intra and inter-isolate estimated ITS divergence. Additionally, no grouping of isolates belonging to the same country or originated from the same host was found. It is visible on both trees the differences between the four species included in the phylogenetic analysis, as they do not group together.

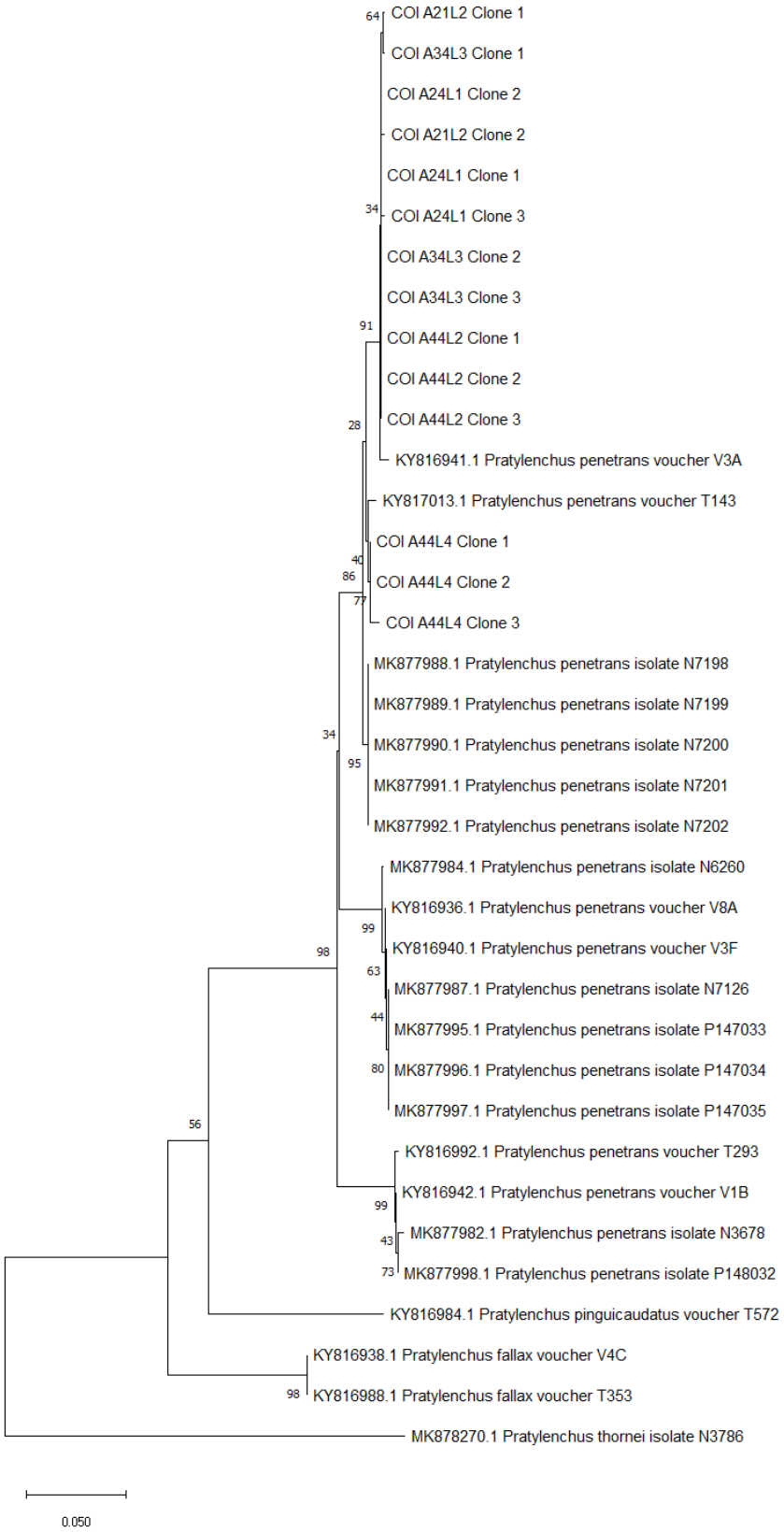

Figure 2.7 - Neighbor-Joining phylogenetic tree based on COI sequences of *Pratylenchus penetrans, P. pinguicaudatus* and *P. fallax*. COI sequence from *P. thornei* was used as outgroup. Bootstrap values are shown next to the branches and scale bar represents nucleotide substitutions per site.

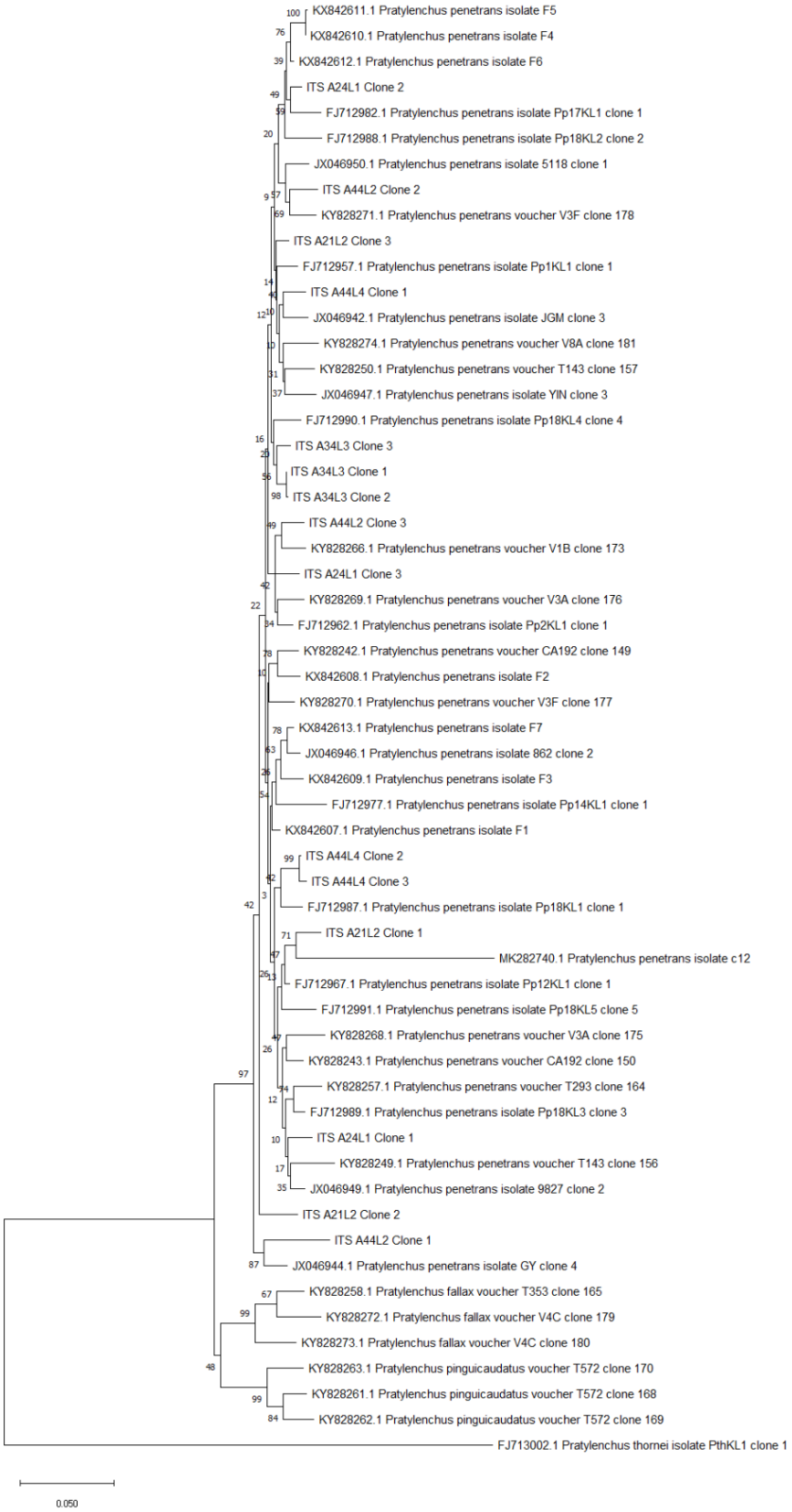

Figure 2.8 - Neighbor-Joining phylogenetic tree based on ITS sequences of *Pratylenchus penetrans, P. pinguicaudatus* and *P. fallax*. ITS sequence from *P. thornei* was used as outgroup. Bootstrap values are shown next to the branches and scale bar represents nucleotide substitutions per site.

The intraspecific variation and few species-specific diagnostic morphometric characteristics on *P. penetrans* species make the RLN identification difficult and only accessible for experienced taxonomists. To overcome those difficulties, molecular methods for *P. penetrans* identification were developed and the species-specific primers for PCR based on the D2-D3 region of 28S rDNA developed by All-Banna *et al*. (2004) were used in this study to successfully confirm the identity of all the five isolates as *P. penetrans*.

The COI and ITS genomic regions from these five *P. penetrans* isolates were selected for sequencing in order to evaluate the intraspecific genetic diversity of this species. From the two regions, the ITS region revealed higher genetic diversity than the COI region with 15 and 6 different haplotypes from the 15 ITS and 14 COI sequences, respectively. Besides, inter-isolate genetic diversity also intra-isolate genetic diversity was found in all isolates with exception for one isolate in COI region. In spite of this high intraspecific diversity found for *P. penetrans*, phylogenetic analyses revealed that both COI and ITS regions allow the separation of *P. penetrans* species from other related species, such as *P. pinguicaudatus*, *P. fallax* and *P. thornei* which is in accordance to that previously reported (Janssen *et al*., 2017; De Luca *et al*., 2011). Additionally, no grouping of isolates belonging to the same country or originated from the same host was found in phylogenetic analyses of both COI and ITS genomic regions. This is in accordance with the no correlation of genetic and geographic distance found among the five *P. penetrans* isolates, indicating that geographical distance is not the main factor leading to the differentiation of isolates. Janssen *et al*. (2017) referred that despite the large intraspecific variability recovered in *P. penetrans*, identical haplotypes were found to be geographically widespread.

CONCLUSIONS

In this study, *Pratylenchus penetrans* isolates from potato in different geographic locations of Portugal were characterised for the first time, using both morphometric and molecular analyses. Although the detailed comparative morphometrical study on five *P. penetrans* isolates from Portugal revealed the presence of substantial variability within and between these isolates, these differences fall within the range of the morphometrical variability described previously in *P. penetrans* from other parts of the world. High genetic diversity was found among isolates and this diversity is not only a result of the diversity found between isolates but also due to the diversity found within each isolate. As a future perspective, the information obtained contributed to increase understanding about this relevant PPN in potato crops, and may be used further in larger genetic studies, focusing this nematode species. Future research should also be conducted to evaluate if differences in pathogenicity between *P. penetrans* isolates are related to the observed morphometric and molecular variability.

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SUPPLEMENTARY DATA

Supplementary table 1 - Data on COI region genetic distance and respective standard deviation of the five *Pratylenchus penetrans* isolates from Portugal. Genetic distance estimated by number of nucleotide substitutions per site (inferior side) and respective standard deviation (superior side).

	COI clones													
	A21L2_1	A21L2_2	A24L1_1	A24L1_2	A24L1_3	A34L3_1	A34L3_2	A34L3_3	A44L2_1	A44L2_2	A44L2_3	A44L4_1	A44L4_2	A44L4_3
A21L2_1		0.0030	0.0021	0.0021	0.0030	0.0021	0.0021	0.0021	0.0021	0.0021	0.0021	0.0051	0.0051	0.0061
A21L2_2	0.0046		0.0022	0.0022	0.0031	0.0031	0.0022	0.0022	0.0022	0.0022	0.0022	0.0050	0.0050	0.0060
A24L1_1	0.0023	0.0023		0.0000	0.0022	0.0023	0.0000	0.0000	0.0000	0.0000	0.0000	0.0045	0.0045	0.0055
A24L1_2	0.0023	0.0023	0.0000		0.0022	0.0023	0.0000	0.0000	0.0000	0.0000	0.0000	0.0045	0.0045	0.0055
A24L1_3	0.0046	0.0046	0.0023	0.0023		0.0033	0.0022	0.0022	0.0022	0.0022	0.0022	0.0050	0.0050	0.0060
A34L3_1	0.0023	0.0046	0.0023	0.0023	0.0046		0.0023	0.0023	0.0023	0.0023	0.0023	0.0051	0.0051	0.0060
A34L3_2	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023		0.0000	0.0000	0.0000	0.0000	0.0045	0.0045	0.0055
A34L3_3	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000		0.0000	0.0000	0.0000	0.0045	0.0045	0.0055
A44L2_1	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000	0.0000		0.0000	0.0000	0.0045	0.0045	0.0055
A44L2_2	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000	0.0000	0.0000		0.0000	0.0045	0.0045	0.0055
A44L2_3	0.0023	0.0023	0.0000	0.0000	0.0023	0.0023	0.0000	0.0000	0.0000	0.0000		0.0045	0.0045	0.0055
A44L4_1	0.0115	0.0115	0.0092	0.0092	0.0115	0.0115	0.0092	0.0092	0.0092	0.0092	0.0092		0.0000	0.0032
A44L4_2	0.0115	0.0115	0.0092	0.0092	0.0115	0.0115	0.0092	0.0092	0.0092	0.0092	0.0092	0.0000		0.0032
A44L4_3	0.0162	0.0162	0.0138	0.0138	0.0161	0.0162	0.0138	0.0138	0.0138	0.0138	0.0138	0.0046	0.0046	

Supplementary table 2 - Data on ITS region genetic distance and respective standard deviation of the five *Pratylenchus penetrans* isolates from Portugal. Genetic distance estimated by number of nucleotide substitutions per site (inferior side) and respective standard deviation (superior side).

	ITS clones														
	A21L2_1	A21L2_2	A21L2_3	A24L1_1	A24L1_2	A24L1_3	A34L3_1	A34L3_2	A34L3_3	A44L2_1	A44L2_2	A44L2_3	A44L4_1	A44L4_2	A44L4_3
A21L2_1		0.0077	0.0075	0.008	0.009	0.0081	0.0072	0.0073	0.0072	0.0106	0.0098	0.0080	0.0078	0.0074	0.0078
A21L2_2	0.0390		0.0073	0.007	0.008	0.0071	0.0062	0.0064	0.0062	0.0104	0.0090	0.0077	0.0073	0.0065	0.0069
A21L2_3	0.0362	0.0347		0.008	0.008	0.0049	0.0046	0.0049	0.0055	0.0093	0.0077	0.0066	0.0061	0.0068	0.0071
A24L1_1	0.0360	0.0317	0.0392		0.009	0.0078	0.0072	0.0074	0.0069	0.0103	0.0088	0.0078	0.0074	0.0072	0.0076
A24L1_2	0.0540	0.0481	0.0408	0.045		0.0081	0.0071	0.0072	0.0071	0.0117	0.0063	0.0083	0.0068	0.0079	0.0083
A24L1_3	0.0423	0.0332	0.0172	0.038	0.042		0.0054	0.0056	0.0060	0.0097	0.0080	0.0067	0.0076	0.0074	0.0078
A34L3_1	0.0333	0.0244	0.0157	0.032	0.033	0.0201		0.0014	0.0043	0.0097	0.0075	0.0061	0.0061	0.0065	0.0068
A34L3_2	0.0348	0.0259	0.0172	0.033	0.035	0.0216	0.0014		0.0045	0.0099	0.0077	0.0062	0.0062	0.0067	0.0070
A34L3_3	0.0347	0.0259	0.0215	0.030	0.035	0.0245	0.0128	0.0143		0.0095	0.0077	0.0065	0.0062	0.0066	0.0070
A44L2_1	0.0623	0.0606	0.0486	0.061	0.076	0.0549	0.0533	0.0548	0.0501		0.0112	0.0110	0.0101	0.0104	0.0107
A44L2_2	0.0571	0.0495	0.0392	0.046	0.027	0.0423	0.0347	0.0362	0.0376	0.0683		0.0089	0.0079	0.0080	0.0084
A44L2_3	0.0408	0.0388	0.0289	0.038	0.044	0.0303	0.0245	0.0260	0.0274	0.0655	0.0481		0.0074	0.0069	0.0072
A44L4_1	0.0375	0.0360	0.0243	0.036	0.033	0.0376	0.0257	0.0272	0.0286	0.0604	0.0404	0.0376		0.0063	0.0067
A44L4_2	0.0345	0.0302	0.0304	0.033	0.041	0.0379	0.0290	0.0304	0.0304	0.0592	0.0391	0.0334	0.0273		0.0029
A44L4_3	0.0374	0.0332	0.0333	0.036	0.044	0.0409	0.0319	0.0334	0.0333	0.0623	0.0420	0.0349	0.0302	0.0056	