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Root-lesion and cyst nematodes in cereal fields in Morocco: species identification, population diversity and crop resistance

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Root-lesion and cyst nematodes in cereal fields in Morocco: species identification, population diversity and crop resistance

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Root-lesion and cyst nematodes in cereal fields in Morocco: species identification, population diversity and crop resistance

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Thesis submitted in fulfillment of the requirements for
the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Wortellesie- en cystenematoden in de graanteelt in Marokko: soortbepaling, populatiediversiteit en gewasresistentie

Illustration on the front cover: from left to right, a perineal pattern and cyst of *Heterodera* species; Vermiform stage of *Pratylenchus thornei*; Plants showing poor growth and serious reduction in tillering caused by *Pratylenchus* spp.

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List of abbreviations

CCN : Cereal cyst nematodes

CDA: Canonical discriminant analysis

Ct : Cycle threshold

DGO: Dorsal esophageal gland orifice

DNA: Deoxyribonucleic Acid

TAF: Triethanolamine formalin solution

ITS: Internal transcribed spacer

J2: Second-stage juvenile

J3: Third-stage juvenile

J4: Fourth-stage juvenile

qPCR: Real-time PCR

QTL: quantitative trait locus

L: Line of wheat

LSU: 5.8S and 28S large subunit

NTC: Negative control

PCR : Polymerase chain reaction

Pi: Initial number of nematodes

Pf: Final number of nematodes

RLN : Root-lesion nematodes

Rf: Reproduction factor

SSU: Coding region of the 18S small subunit

WAI: Weeks after inoculation

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Summary

Cereal cyst nematodes (CCN, *Heterodera* spp.) and root-lesion nematodes (RLN, *Pratylenchus* spp.) are important plant-parasitic nematodes of wheat and exist in most of the cereal growing regions of the world. As there was limited information on the distribution of CCN and RLN species in wheat fields in Morocco, a survey was organized in 2011. A total of 75 soil and root samples were collected from fields in Gharb, Saiss, Zaers and Chaouia before the wheat and barley harvest (May to June). Cysts were extracted from soil by flotation and decanting through 200-µm sieves. Vermiform stages were extracted from roots and soil with an automated zonal centrifuge. They were identified up to species level using morphological and molecular methods. The survey revealed that 69% of the samples were infested with four species of *Pratylenchus*, *viz. P. thornei*, *P. penetrans*, *P. pseudocoffeae* and *P. pinguicaudatus*. The most prevalent species was *P. penetrans*, present in the four regions. Cereal cyst nematodes were found in 16% of the soil samples and were represented by two species, *viz*. *H. avenae* and *H. latipons*. *Heterodera avenae* was the most prevalent, occurring in 13% of the fields and associated with wheat in the 3 regions where it was found. *Heterodera latipons* was detected only in one sample, originating from Ain Jmaa (Saiss).

The morphological and molecular characteristics of 11 populations of CCN collected from different wheat growing regions of Morocco were studied. Morphometrics of cysts and second-stage juveniles (J2) were generally within the expected ranges for *H. avenae*; only the isolate from Aïn Jmaa showed morphometrics conforming those of *H. latipons.* When using species-specific primers for *H. avenae* and *H. latipons,* the specific bands of 109 bp and 204 bp, respectively, confirmed the morphological identification. In addition, the internal transcribed spacer (ITS) regions were sequenced to study the diversity of the 11 populations. These sequences were compared with those of *Heterodera* species available in the GenBank database (www.ncbi.nlm.nih.gov) and confirmed again the identity of the species. Ten sequences of the ITS-rDNA were similar (99-100%) to the sequences of *H. avenae* published in GenBank and three sequences, corresponding with one population, were similar (97-99%) to *H. latipons.*

During the survey of the wheat-growing area of Morocco, 17 populations of RLN were collected. They were identified on the basis of their morphological and morphometric characters, and by molecular methods. Microscopic observations of females and males demonstrated the occurrence of *P. penetrans* in 13 of the 17 samples; *P. thornei* and *P. pseudocoffeae* were detected in four samples from Zaers and a single sample from Settat,

respectively. A duplex PCR primer set was used to confirm the presence of *P. penetrans* while the species-specific forward primer PTHO and the common reverse primer D3B were used for *P. thornei*. For the remaining populations, the D2-D3 expansion segments of the 28S rRNA gene were amplified and the obtained sequences were compared with those of *Pratylenchus* species in the GenBank database. This comparison confirmed the morphological identifications and revealed a population of *P. pinguicaudatus*. The study of the phylogenetic relationship of the Moroccan *Pratylenchus* populations showed a high similarity (99-100%) between all *P. penetrans* populations. The population dynamics of six *Pratylenchus* populations from Morocco were evaluated on carrot-disk cultures at 4, 8 and 12 weeks after inoculation, and at 10, 15, 20 and 25°C. The optimum temperature for reproduction of all populations was 20°C. After 8 weeks at this temperature, nematode numbers increased up to 458-fold, 310-fold and 252-fold for the four populations of P. *penetrans*, the *P. thornei* and the *P. pseudocoffeae* populations, respectively.

A qPCR assay was developed to detect and quantify *P. penetrans* in a quick and accurate manner. A primer set, including two primers and a TaqMan probe, was designed based on the sequence of the β-1,4-endoglucanase gene. The assay was optimized by using the primers in a qPCR assay with SYBR green I dye and setting the qPCR program to different annealing temperatures ranging from 60°C to 64°C. Based on the Ct-values, the program with an annealing temperature of 63°C was retained. The assay with the probe was very sensitive as it was able to detect a single individual of *P. penetrans*, even when mixed with up to 80 individuals of *P. thornei*. The specificity of the reaction was confirmed by the lack of amplification of DNA from 28 populations of 18 other *Pratylenchus* species and from plant-parasitic nematodes from nine other genera. DNA from 21 different isolates of *P. penetrans* was amplified. DNA extraction from 80 individuals and quantification by qPCR was repeated four times; Ct-values showed consistent results (Ct=24.4±0.4). A dilution series from DNA of *P. penetrans* resulted in a standard curve showing a highly significant linearity between the Ct-values and the dilution rates (R^2=0.99; slope=−3.23; E=104 %). The tests showed a high correlation between the real numbers of nematodes and the numbers detected by the qPCR. The developed qPCR assay does not require expertise in nematode taxonomy and morphology, and can be used as a rapid diagnostic tool in research, as well as in diagnostic labs and extension services.

A real-time quantitative PCR assay was developed for the accurate detection and

quantification of another root-lesion nematode, *P. thornei*. A qPCR primer set, including two primers and a probe, was designed based on the sequence of the β-1,4-endoglucanase gene. The assay was optimised by using the primers with SYBR green I dye and setting the qPCR program to different annealing temperatures ranging from 62 to 69°C. Based on the Ct values, the program with an annealing temperature of 69°C was retained. The specificity of the qPCR assay including the probe was confirmed by the lack of amplification of DNA from 47 populations belonging to 15 other *Pratylenchus* species, while DNA from nine isolates from *P. thornei* was amplified. The assay was very sensitive as it was able to detect a single individual of *P. thornei*, even when mixed with up to 80 individuals of *P. penetrans*. DNA was extracted from exactly 80 *P. thornei* individuals. A dilution series from this DNA resulted in a standard curve showing a highly significant linearity between the Ct values and the dilution rates (R^2=0.98; slope=−3.38; E=97.6%). The qPCR assay proved to be specific and sensitive, thus providing a fast and accurate tool for detection and quantification of *P. thornei* during research, as well as for diagnostic labs.

Breeding for resistant varieties is one of the most effective methods to control nematodes. A collection of 14 spring wheat and of 11 winter wheat lines, developed at CIMMYT, for resistance to both nematode species. Individual plants were grown in sand in small tubes (15 × 20 × 120 mm) placed in a random design with ten replicates in the greenhouse. The resistance level was evaluated based on the numbers of nematodes extracted from both roots and soil of each line. Trials were terminated 9 weeks after nematode inoculation. The numbers of *P. penetrans* and *P. thornei* were determined using a microscope. Three lines (L9, L12 and L13) were found resistant to *P. thornei* and one of these (L9) was also resistant to *P. penetrans*. To investigate the stability of this resistance, J2 of *Heterodera avenae* were simultaneously inoculated. The reproduction of both lesion nematodes *P. penetrans* and *P. thornei*, was assessed both by counting and by using the developed qPCR assays. Our results showed that the wheat lines L9 and L9, L12, L13 remained resistant to *P. penetrans* and *P. thornei*, respectively. The outcome of this study is valuable to wheat breeding programmes in Morocco and the world. However, the resistant lines should be validated under natural field conditions. These findings are important to understand the background of the source(s) of resistance responsible for inhibition of nematode reproductions in promising wheat lines.

Samenvatting

Graancystenematoden (CCN) en wortellesienematoden (RLN, *Pratylenchus* spp.) zijn belangrijke plantparasitaire nematoden van tarwe en komen voor in de meeste regio's waar graan wordt geteeld in de wereld. Aangezien er weinig informatie bestaat over het voorkomen van CCN en RLN soorten in Marokko, werd er in 2011 een survey georganiseerd naar deze nematoden. In totaal werden 75 grond- en wortelmonsters genomen in velden uit de Gharb, Saiss, Zaers en Chaouia regio's, voorafgaand aan de oogst van tarwe en gerst (mei-juni). Cysten werden uit de grond geëxtraheerd met de drijf- en decanteertechniek, op zeven van 200-µm; de vermiforme stadia werden geëxtraheerd uit wortels en grond met een geautomatiseerde zonale centrifuge. Nematoden werden geïdentificeerd tot op soortniveau op basis van hun morfologie en met moleculaire methoden. Uit de survey bleek dat 69% van de monsters Pratylenchus-soorten bevatten: *P. thornei*, *P. penetrans*, *P. pseudocoffeae* en *P. pinguicaudatus*. De meest voorkomende soort was *P. penetrans*; deze kwam voor in de vier graanregio's. Graancysten werden gevonden in 16% van de grondmonsters en waren vertegenwoordigd door twee soorten, nl. *H. avenae* en *H. latipons*. *Heterodera avenae* was het prominenst aanwezig: in 13% van de tarwevelden en in 3 regio's. *Heterodera latipons* werd maar gedetecteerd in één monster, afkomstig van Aïn Jmaa (Saiss region).

De morfologische en moleculaire kenmerken van 11 populaties van CCN, afkomstig van verschillende tarwevelden in de 4 bestudeerde regio's van Marokko, werden onderzocht. De morfometrie van de cysten en de tweede-stadiumjuvenielen waren over het algemeen conform aan deze kenmerkend voor *H. avenae*; alleen het isolaat van Aïn Jmaa toonde een morfometrie dat voldeed aan de kenmerken van *H. latipons*. In een PCR test met soortspecifieke primers voor *H. avenae* en *H. latipons*, werden de karakteristieke banden van 109 bp en 204 bp, resp., verkregen, wat een bevestiging was van de morfologische identificatie. Daarnaast werden sequentiebepalingen van het ITS gebied uitgevoerd om de diversiteit van de 11 populaties te bestuderen. Deze sequenties werden vergeleken met deze van Heterodera-soorten in de GenBank databank (www.ncbi.nlm.nih.gov) en dit bevestigde opnieuw de identiteit van de soort. Tien sequenties van het ITS-rDNA waren vergelijkbaar (99-100%) met de sequenties van *H. avenae* gepubliceerd in GenBank en drie sequenties van eenzelfde populatie waren vergelijkbaar (97-99%) met deze van *H. latipons*.

Tijdens de survey van de graangebieden van Marokko werden 17 populaties RLN verzameld. Zewerden geïdentificeerd op basis van hun morfologische en morfometrische

kenmerken, alsook met moleculaire technieken. Microscopische waarnemingen van vrouwtjes en mannetjes toonden aan dat *P. penetrans* aanwezig was in 13 van de 17 monsters; *P. thornei* werd gedetecteerd in vier monsters van Zaers en *P. pseudocoffeae* in een enkel monster van Settat. Een duplex PCR werd gebruikt om de aanwezigheid van *P. penetrans* te bevestigen terwijl de species-specifieke voorwaartse primer PTHO en de gemeenschappelijke reverse primer D3B werden gebruikt voor *P. thornei*. Voor de resterende populaties werden de D2-D3 expansiesegmenten van het 28S rRNA-gen geamplificeerd en de verkregen sequenties werden vergeleken met die van Pratylenchus-soorten in de GenBank database. Deze vergelijking bevestigde de morfologische identificaties en bracht een populatie van *P. pinguicaudatus* aan het licht. De studie van de fylogenetische verwantschap van de Marokkaanse Pratylenchus populaties toonde een grote gelijkenis (99-100%) aan tussen alle *P. penetrans* populaties. Naast de identificatie werd ook de populatiedynamica van 6 Pratylenchus populaties geëvalueerd door ze te vermeerderen op wortelschijfjes. De evaluatie gebeurde 4, 8 en 12 weken na inoculatie, en dit na incubatie bij 10, 15, 20 en 25 ° C. De optimale temperatuur voor de vermeerdering van alle populaties was 20 ° C. Na 8 weken bij deze temperatuur was het aantal nematoden toegenomen met een factor 458, 310 en 252 voor *P. penetrans* (4 populaties), *P. thornei* en *P. pseudocoffeae*, respectievelijk.

Een qPCR test werd ontwikkeld zodat op een snelle en gerichte manier *P. penetrans* kan worden opgespoord en gekwantificeerd. Een qPCR primer set, waaronder twee primers en een TaqMan-probe, werd ontworpen gebaseerd op de sequentie van het β-1,4-endoglucanase gen. De test werd geoptimaliseerd door gebruik van de primers in een qPCR assay met SYBR green I kleurstof en het testen van het qPCR programma bij verschillende annealingstemperaturen (60°C tot 64°C). Gebaseerd op de Ct-waarden, werd het programma met een hybridisatietemperatuur van 63 ° C gehandhaafd. De ontwikkelde test is zeer gevoelig want het kan 1 individu van *P. penetrans* detecteren, zelfs indien gemengd met 80 individuen van *P. thornei*. De specificiteit van de reactie werd bevestigd door het ontbreken van enige amplificatie van het DNA van 28 populaties van 18 andere Pratylenchus-soorten en plantenparasiterende nematoden van 9 andere genera. DNA van 21 verschillende isolaten van *P. penetrans* werd wel geamplificeerd. DNA-extractie uit 80 individuen en kwantificering door qPCR werd vier keer herhaald; Ct-waarden toonden consistente resultaten (Ct = 24,4 ± 0,4). Met een verdunningsreeks van DNA van *P. penetrans* werd een standaardcurve opgemaakt met een zeer significante lineariteit tussen de Ct-waarden en de verdunning (R^2 = 0,99; hellingsgraad = -3,23; E = 104%). Er was een sterke correlatie tussen het werkelijke

aantal nematoden en het aantal gedetecteerd met behulp van de qPCR. De ontwikkelde qPCR assay laat toe dat personen zonder expertise in nematodetaxonomie een snelle identificatie kunnen uitvoeren, zowel in het kader van onderzoek, diagnostische laboratoria of advies aan telers.

Er werd ook een real-time kwantitatieve PCR assay ontwikkeld voor de nauwkeurige detectie en kwantificering van een andere wortellesienematode, nl. *P. thornei*. Een qPCR primer set, waaronder twee primers en een probe, werd ontworpen gebaseerd op de sequentie van het β-1,4-endoglucanase gen. De test werd geoptimaliseerd door gebruik van de primers met SYBR green I kleurstof en het qPCR programma werd getest met verschillende annealing temperaturen (62-69 °C). Gebaseerd op de Ct-waarden, werd het programma met een hybridisatietemperatuur van 69 ° C gehandhaafd. De specificiteit van de qPCR assay werd bevestigd door het ontbreken van enige amplificatie van DNA van 47 populaties die tot 15 andere Pratylenchus-soorten behoorden. DNA van negen isolaten van *P. thornei* werd wel geamplificeerd. De test was zeer gevoelig omdat het een enkel individu van *P. thornei* kan detecteren, zelfs indien gemengd met tot 80 individuen van *P. penetrans*. DNA werd geëxtraheerd uit exact 80 *P. thornei* individuen en een verdunningsreeks van dit DNA resulteerde in een standaardcurve met een zeer significante lineariteit tussen de Ct-waarden en de verdunning (R^2 = 0,98; hellingsgraad = -3,38; E = 97,6%). De qPCR assay bleek specifiek en gevoelig, waardoor een snel en nauwkeurig instrument voor detectie en kwantificering van *P. thornei* beschikbaar is voor onderzoek en diagnostische laboratoria.

Veredeling voor resistente rassen is een van de meest effectieve methoden om aaltjes te beheersen. Daarom werden 14 zomertarwe- en 11 wintertarwelijnen, ontwikkeld aan het CIMMYT, geëvalueerd voor resistentie tegen beide nematodensoorten. Individuele planten werden gezaaid in zand in buisjes (15 × 20 × 120 mm) die in een willekeurige orde werden opgesteld in de serre, elke tarwelijn in tien herhalingen. Het resistentieniveau werd geëvalueerd op basis van het aantal nematoden geëxtraheerd uit zowel wortels als grond van elke tarwelijn, 9 weken na de inoculatie met nematoden. Het aantal *P. penetrans* en *P. thornei* werden visueel bepaald met een microscoop. Drie lijnen (L9, L12 en L13) waren resistent tegen *P. thornei* en één daarvan (L9) was ook resistent tegen *P. penetrans*. Om de stabiliteit van deze resistentie te onderzoeken, werden tweede-stadium juvenielen van *H. avenae* -de meest voorkomende graancystenematode in Marokko- samen geïnoculeerd met telkens een van beide Pratyenchus-soorten. De reproductie van *P. penetrans* en *P. thornei* werd opnieuw

bepaald, zowel microscopisch als met de pas ontwikkelde qPCR methoden. De resultaten toonden dat de resistenties van tarwelijnen L9 en L9, L12, L13 tegen *P. penetrans* en *P. thornei*, respectievelijk, behouden bleven. Dit onderzoek is waardevol voor verdelingsprogramma's van tarwe, in Marokko en in de wereld. Toch moeten deze resistentiebronnen worden bevestigd onder veldomstandigheden. De bevindingen zijn interessant om de genetische achtergrond en het resistentiemechanisme in veelbelovende tarwelijnen te begrijpen.

CHAPTER 1

General introduction

Agricultural production is a major component of Moroccan economy with an annual production on 8.7 million hectares of which 2.9 million are devoted to wheat (Statagri, 2014). Obviously, wheat (*Triticum aestivum* and *T. durum*) is the most important crop in Morocco, ranked 15th among the wheat producing countries. In the period 2013-2014 the total production was estimated at 5.1 million tonnes (Statagri, 2014). However, this production does not satisfy Moroccan needs. Several factors are responsible for this low productivity (Jlibene & Nsarellah, 2011). Of these, availability of irrigation water, status of soil nutrients, as well as outbreaks of insect pests and diseases are major constraints to higher production (Shroyer *et al*., 1990; Barakat & Handoufe, 1998). Plant-parasitic nematodes are one of the main biotic causes of plant stress and yield loss of wheat worldwide (Nicol & Rivoal, 2008). Global annual wheat yield losses due to damage by plant-parasitic nematodes are estimated at 7%, representing annual monetary losses of 5.8 billon US $ (Sasser & Freckman, 1987). Actual losses may even be higher because no data are available from the many countries where nematological expertise is lacking. Moreover, yield losses due to nematodes are often neglected because of lack of conspicuous aboveground symptoms.

In cereals, plant-parasitic nematodes mostly belong to two groups, *viz.* cereal cyst nematodes (CCN, *Heterodera* spp.) and root-lesion nematodes (RLN, *Pratylenchus* spp.). These nematodes are distributed globally and implicated in significant economic yield loss. Considerable economic losses due to CCN have been reported from many regions of the world including North Africa (Sikora, 1988), West Asia (Al-Yahya *et al*., 1998; Tanha Maafi *et al*., 2007), Europe (Rivoal & Cook, 1993), Australia (Meagher & Brown, 1974) and the USA (Smiley *et al*., 2005a). The genus *Heterodera* comprises 12 species that affect roots of cereals and grasses (Subbotin *et al*., 2010). These species are designated as the *Heterodera avenae* group (Handoo, 2002). From this group, three species, *viz*. *H. avenae*, *H. filipjevi* and *H. latipons*, are the economically most important cyst nematode pests to cultivated cereals (McDonald & Nicol, 2005; Nicol & Rivoal, 2008). *Heterodera avenae* is widely distributed in temperate wheat-producing regions throughout the world (Rivoal & Cook, 1993; Nicol *et al*., 2003; Smiley & Nicol, 2009). The nematode has been detected in Australia (Ophel-Keller *et al*., 2008; Riley & Mc Kay, 2009), USA (Smiley *et al*., 2008), European countries (Nicol *et al*., 2011), India (Singh *et al*., 2009), China (Zheng *et al*., 1996; Peng *et al*., 2007) and several countries of Northern Africa, including Tunisia (Namouchi-Kachouri & B'Chir, 2008), Algeria (Haddadi & Mokabli, 2015) and Morocco (Mokrini *et al*., 2009). In that latter country, *H. avenae* is the most important nematode on wheat and can reduce yield of

intolerant wheat cultivars to 50% (Meskine *et al*., 1984; Rammah, 1994). The species was first detected in an irrigated wheat (*T. aestivum*) field in 1956 (Ritter, 1982).

Root lesion nematodes are widespread and are considered as one of the most important groups of plant-parasitic nematodes in the world (Smiley *et al*., 2005b; Castillo & Vovlas, 2007; Smiley & Nicol, 2009; Keil *et al*., 2009). Economic losses in cereals due to RLN have been reported from North Africa (Sikora, 1988), Australia (Taylor & McKay, 1993; Vanstone *et al*., 1995) and the USA (Smiley, 2010). Eight species of the genus *Pratylenchus* affect roots of cereals (Rivoal & Cook, 1993). Among them, *P. thornei*, *P. neglectus*, *P. penetrans* and *P. crenatus* are distributed worldwide and sometimes coexist (Nicol *et al*., 2003; Smiley & Nicol, 2009). In Morocco, *Pratylenchus* spp. constitute the most important group of plant-parasitic nematodes in different wheat growing areas (Meskine *et al*., 1984; Rammah, 1994). They cause extensive necrosis on the roots with consequent crop losses. Three species, *viz. P. penetrans*, *P. neglectus* and *P. thornei*, have been detected in different wheat growing areas (Ammati, 1987; Mokrini *et al*., 2009).

Rapid and reliable identification of nematodes are of paramount importance when developing an efficient strategy for monitoring or controlling potential pests. Morphological features are traditionally used to differentiate, identify and classify nematodes, and continue to play a basic role (Troccoli *et al*., 2008). However, nematode identification using morphological features needs a powerful microscope and trained and experienced people. Microscopic identification of *Heterodera* and *Pratylenchus* species is often difficult and time-consuming due to difficulties in distinguishing key diagnostic features for *Pratylenchus* (*e.g.*, morphology of the lips, tail and vulva) (Handoo & Golden, 1989) and great variability of taxonomic characteristics (*e.g.*, size and shape of cyst, shape of fenestrae, and presence or absence of underbridge) for *Heterodera* (Handoo, 2002). It is also problematic due to the high phenotypic plasticity among populations and the absence of clear diagnostic characteristics for cryptic species of *Pratylenchus* (Waeyenberge & Viaene, 2015).

For this reasons, biochemical and molecular characterisation are important complements to morphological identification. Several authors used isoenzymes to identify *Pratylenchus* species (Ibrahim *et al*., 1995; Andrés *et al*., 2001). However, this method needs a mass of nematodes from which total proteins are extracted. As a consequence isoenzymes are not suitable for *Pratylenchus* species identification (Yu *et al*., 2012). In addition, many DNA-based molecular diagnostics have been developed for detecting *Pratylenchus* spp. (Uehara *et al*., 1998a; Al-Banna *et al*., 2004; Waeyenberge *et al*., 2009) and *Heterodera* spp.

(*e.g.*, Toumi *et al*., 2013a,b); they are rapid and robust and can be used routinely. However, molecular methods require plenty of nematodes to extract DNA as well as appropriate equipment. Several quantitative PCR strategies have been developed which can be used to quantify different species in mixtures. Several specific and sensitive qPCR assays have enabled the detection and quantification of several species of *Pratylenchus* (Berry *et al*., 2008; Toyota *et al*., 2008; Yan & Smiley, 2013). Quantitative PCR (Gao *et al.,* 2006) can be used in multi-species experiments, which would have been prohibitive in the past using conventional morphological methods.

Moroccan CCN and RLN have only been studied with respect to their morphology (Meskine *et al*., 1984; Znasni, 2003; Mokrini *et al*., 2009); hence, there is no information available on their morphometrics and genetics, or on their potential pathogenicity.

Because of the importance of the damage caused by RLN, many attempts have been made to control these nematodes including cultural practices, chemical control, and use of resistant wheat cultivars (Smiley & Nicol, 2009; Dababat *et al*., 2011). The use of resistant cultivars, *i.e.* cultivars that have the capacity to prevent or reduce nematode multiplication (Cook & Evans, 1987; Nicol *et al*., 2001), is considered one of the most effective and economical methods for managing nematodes in different cropping systems including rainfed and an irrigated areas. A highly resistant cultivar allows no nematode reproduction. Partially or moderately resistant cultivars allow some reproduction, whereas moderately susceptible cultivars allow increase in nematode populations but at a slower rate (Trudgill, 1991). As nematode resistance generally targets one species, other nematode species present in the field might parasitize the resistant host (Bradley & Duffy, 1982). It has been demonstrated that the concurrent presence of different taxa of plant-parasitic nematodes in agricultural soils complicates nematode management (Niblack *et al.*, 1986; Stetina *et al*., 1997), especially when using resistant cultivars.

In view of these issues, the aim of this thesis was to study the diversity of CCN and RLN associated with wheat in Morocco, their reproduction and interaction on different wheat cultivars, and the development of molecular tools for the identification and quantification of prevailing *Pratylenchus* spp. To achieve this, the following objectives were defined:

- To determine the prevalence and distribution of CCN and RLN in different wheat-growing areas of Morocco (Chapter 3).
- To characterize Moroccan *Heterodera* populations associated with wheat morphologically, morphometrically and molecularly (Chapter 4).

- To characterize (morphologically, morphometrically and molecularly) the different *Pratylenchus* populations associated with wheat (*T. aestivum* and *T. durum*) in Morocco (Chapter 5).
- To study the effect of temperature on the *in vitro* reproductive fitness of five *Pratylenchus* populations from Morocco (Chapter 5).
- To develop a real-time protocol for the detection and quantification of *P. penetrans* and *P. thornei* (Chapter 6 and 7).
- To screen wheat germplasm for resistance against Moroccan *P. penetrans* and *P. thornei* (Chapter 8).
- To study the interaction between *Pratylenchus* spp. (*P. thornei* and *P. penetrans*) and *H. avenae* and the influence of concomitant infection on the resistance of wheat (Chapter 8).

CHAPTER 2

The importance, biology and management strategies of cereal cyst nematodes (*Heterodera* spp.) and root-lesion nematodes (*Pratylenchus* spp.)

A literature review

2.1 Introduction

Among the cereals, wheat (*Triticum aestivum* and *T. durum*) and barley (*Hordeum vulgare*) occupy the most prominent position in terms of production, acreage and source of nutrition, particularly in developing countries (Nicol *et al*., 2011). By 2030, the world production of cereals is expected to increase to 8 billion tons; that of *T. aestivum* is estimated to increase from 584 million tons (average 1995-1999) to 860 million tonnes (Hossain & Teixeira Da Silva, 2012). In Morocco, cereals occupy 75% of the cultivated area and account for 10–20% of the agricultural gross domestic product (Benabdelouahab *et al*., 2016). Cereal production of the season 2013-2014 was estimated at 6.8 million tonnes and includes 4.42 million tonnes of common wheat, 0.5 million tonnes of durum wheat and 0.4 million tonnes of barley, ranking 15th among the cereal producing countries (Anonymous, 2014). Productivity of soft wheat, durum wheat and barley is low, due to biotic and abiotic stresses. Consequently, Morocco is not self sufficient in wheat production most of the years and imports bread wheat for its domestic consumption (Balaghi *et al*., 2013). Cereal production occurs in most parts of the country but is mainly concentrated in 6 regions, each contributing differently to a certain type of cereal (Table 2.1)

Table 2.1. Contribution (%) of the main cereal producing regions of morocco in the national production of soft wheat, durum wheat and barley (Average of 1990-2011) (Balaghi *et al*., 2013).

Region	**Soft wheat**	**Durum wheat**	**Barley**
Tadla	11.4	5.1	4.5
Gharb	25.6	4.4	-
Chaouia	10.7	22.4	15.6
Saiss	14.6	16.4	4.4
Haouz	6.1	12.2	22.4
Oriental	-	-	11
Total	86.4	60.5	57.9

2.2 The cereal cyst nematodes (CCN)

Heterodera is a very important genus of the family Heteroderidae. Members of this genus are obligate parasites and different species attack different crops, often causing great economic damage. The genus is particular among nematode genera because of the ability of the female to transform into a tough brown cyst, which protects the eggs formed within their body.

2.2.1 Taxonomical position (Siddiqi, 2000)

Phylum Nematoda (Rudolphi, 1808)
Class Secernentea (von Linstow, 1905)
Order Tylenchida (Thornei, 1949)
Superfamily Tylenchoidea (Orley, 1888)
Family Heteroderidae (Filipjev and Schuurmans Stekhoven, 1941)
Subfamily Heteroderinae (Filipjev and Schuurmans Stekhoven, 1941)
Genus *Heterodera* (Schmidt, 1871)

2.2.2 Distribution and importance

Cereal cyst nematodes (CCN) form a group of several closely related species that have been documented as causing economic yield losses in wheat production systems in several parts of the world, including West Asia, North Africa, Europe, Australia and the United States of America (Rivoal & Cook, 1993; Nicol & Rivoal, 2008; Sahin *et al*., 2009; Yan & Smiley, 2009). Twelve species affect roots of cereals and grasses (Nicol & Rivoal, 2008; Subbotin *et al*., 2010), among which three species, *viz. H. avenae*, *H. latipons* and *H. filipjevi*, are considered the most economically important, and sometimes coexist (Rivoal & Cook, 1993; Abidou *et al*., 2005; Mc Donald & Nicol, 2005).

Out of these three species, *H. avenae* is the most widely distributed and damaging species in temperate wheat producing regions throughout the world (Rivoal & Cook, 1993). It is known as a major production constraint of cereals in Europe (Rivoal & Cook, 1993), Australia (Brown, 1984), India (Khan *et al*., 1990; Singh *et al*., 2009), North America (Miller, 1986), and in several countries of North Africa and West Asia (Sikora, 1988; Al-Yahya *et al*., 1998; Nicol *et al*., 2011). *Heterodera latipons* has been found in the Mediterranean region (Franklin, 1969; Sikora & Oostendorp, 1986; Greco, 1994), but has also been detected in the temperate continental climates of Southern Russia, Ukraine, Central Asian Republics (Subbotin *et al*., 1996), Iran (Talatschian *et al*., 1976), Europe (Stoyanov *et al*., 1982; Sabova *et al*., 1988), and Canada (Sewell, 1973). *Heterodera filipjevi* has been found in more continental climates such as Russia, Tadzhikistan (Madzhidow, 1981; Subbotin *et al*., 1999), but also in Pakistan, Turkey (Rumpenhorst *et al*., 1996), India (Rao *et al*., 2002), Norway (Holgado *et al*., 2004), and USA (Smiley *et al*., 2008). Several other species of *Heterodera* (*e.g.*, *H. hordicalis*; *H. zeae* and *H. bifenestra*) are reported on wheat but are not considered to be of major economic importance (Smiley & Nicol, 2009; Lambardo *et al*., 2009; Sharma *et al*., 2009).

In Morocco, *H. avenae* was reported for the first time in an irrigated wheat field in 1951 (Ritter, 1982). It has been increasingly detected over the last few years and is recognized as a damaging pathogen of wheat and barley in most cereal growing areas, especially in Zaers, Saiss, Chaioua and Doukkala (Meskine *et al*., 1984; Znasni, 2003; Mokrini *et al*., 2009). Recently, *H. latipons* was found for the first time in the wheat growing area of Saiss region (Ain Jmaa) (Chapter 3). However, *H. filipjevi* has never been reported in cereal fields in Moroccan.

Cereal cyst nematodes can cause considerable yield reduction, especially in temperate climates and semi-arid regions where they can aggravate drought stress (Rivoal & Cook, 1993). Yield losses caused by CCN can be up to 90% in severely infested fields (Rivoal & Cook, 1993; Riley *et al*., 2009). Several authors have reported that water stress is one of the key environmental conditions that can exacerbate damage caused by CCN (*e.g.,* Nicol *et al*., 2011). Yield losses due to *H. avenae* on wheat are reported to be 40-92% in Saudi Arabia (Ibrahimi *et al*., 1999), 10% in China (Peng *et al*., 2009), 40-50% in Morocco (Rammah, 1994), and 23-50% in Australia (Meagher, 1972). It has been calculated that *H. avenae* is responsible for annual yield losses of 72 million Australian dollars in Australia (Wallace, 1965; Brown, 1981). Several studies have shown the economic importance of *H. latipons* and *H. filipjevi* (Nicol *et al*., 2006; Hajihasani *et al*., 2010). Nicol *et al*. (2006) showed that *H. filipjevi* infestation can be highly destructive to *Triricum* spp. Hajihasani *et al*. (2010) performed a study on the effects of *H. filipjevi* on the yield and growth parameters of *T. aestivum* cv. Sardari (bread wheat). The authors concluded that a population density of 20 eggs or second-stage juveniles (J2) per g of soil reduced grain yield up to 55%, root dry weight up to 70%, aerial shoot dry weight up to 48%, spike height up to 36%, and plant height up to 32%. In addition to yield loss of wheat, Philis (1988) reported up to 50% yield loss of barley in fields infested with *H. latipons* in Cyprus. In Turkey, *H. filipjevi* was found in all wheat growing areas in the East Anatolia Region, the Central Anatolian Plateau (CAP) and transition zone (Sahin *et al*., 2009, Toktay *et al*., 2015) with an estimate of yield loss up to 50% in several rain-fed winter locations (Nicol *et al*., 2006).

2.2.3 Identification of *Heterodera* species

In the *H. avenae* group, as in each *Heterodera* group, only minor morphological and morphometrical differences distinguish species from each other. The structures of the cone top of the cyst, including fenestra, bullae and underbridge are used to separate the species.

However, the increasing number of species in this group makes reliable morphological identification more difficult and time consuming (Subbotin *et al*., 2003). Molecular identification could confirm traditional identification, especially for morphologically closely related species. Several molecular techniques can be used for the separation of species and populations of the *H. avenae* group, including species-specific primer sets for differentiating *H. avenae* and *H. filipjevi* (Toumi *et al*., 2013a; Yan *et al*., 2013; Waeyenberge & Viaene, 2015), sequences of ITS-rDNA (Ferris *et al*., 1994; Bekal *et al*., 1997; Subbotin *et al*., 2003) and restriction fragment length polymorphism (RFLP) (Subbotin *et al.*, 1999).

2.2.4 Cereal cyst nematode pathotypes

Numerous schemes have been developed to classify nematodes according to their parasitic capabilities (Triantaphyllou, 1987). The term "pathotype" refers to a biological entity (nematode population) that is distinguished by its inherent capacity (or inability) to multiply on a given host genotype with one or more genes for resistance (Trudgill, 1986). The pathotype scheme for CCN is based on their multiplication on host differentials of barley, oats and wheat cultivars in the *International Cereal Test Assortment* developed by Andersen & Andersen (1982). The test uses 12 barley (*Hordeum vulgare* L.), six oat (*Avena sativa* L.), and six wheat differential cultivars to define pathotypes of *H. avenae*. This scheme distinguishes three primary groups, based on host resistance reactions of barley cultivars carrying the resistance genes Rha1, Rha2, and Rha3. In Europe, North Africa, and Asia, most populations of *H. avenae* belong to groups 1 (Ha1) and 2 (Ha2) (Al-Hazmi *et al*., 2001; Cook & Noel, 2002; Mokabli *et al*., 2002; Mc Donald & Nicol, 2005; Znasni, 2003). Pathotypes of group 3 are mostly found in Australia, Europe, and North Africa (Rivoal & Cook, 1993; Mokabli *et al.*, 2002). In Morocco, Znasni (2003) reported the presence of two pathotype groups (Ha1 and Ha2). The characterization of the CCN species and pathotype is essential for developing resistance in breeding and and applying appropriate cultivars in nematode management programs.

2.2.5 Biology

The life cycle of members of the *H. avenae* group involves various stages, including the egg, four juvenile stages, and the adult nematode (Lambert & Bekal, 2002; Subbotin *et al*., 2010). The species completes one generation per growing season (Rivoal & Cook, 1993); to complete its life cycle, the nematode requires between three and four months under low soil

temperature (5-15°C) and high soil moisture (Griffin, 1998; Smiley & Nicol, 2009). Cyst nematodes are characterised by the developing female swelling and becoming a cyst, which contains several hundred eggs. Within the cyst, eggs may remain dormant in soil for several years. Each egg contains a single first-stage juvenile (J1), which moults inside the egg to become a second-stage juvenile (J2) (Figure 2.1). Emergence of J2 from eggs enclosed in brown cysts requires a period of dormancy (diapause) that differs among species and climatic region (Smiley & Nicol, 2009). The induction or suppression of dormancy by different temperatures regulates the hatching of juveniles. For *H. avenae*, two ecotypes appeared to differ in the induction or suppression of dormancy (diapause) by different thermal conditions (Rivoal, 1986). In Mediterranean climates, the diapause is acting when the climate is hot and dry; diapause is suppressed when the soil temperature falls and moisture rises (Rivoal & Cook, 1993).

Further research with North African populations (Algeria, Morocco and Tunisia), demonstrated hatching schemes relevant to the Mediterranean ecotype, with a higher optimum of hatching temperatures, which could express adaptation of populations to warmer climatic conditions (Rivoal & Nicol, 2009). Scholz & Sikora (2004) demonstrated that the hatch of *H. latipons* in Syria was similar to the Mediterranean ecotype of *H. avenae* from France and southwest Spain. By contrast, *H. filipjevi* originating from Turkey does not show any diapause as the juveniles hatch immediately at the beginning of the winter wheat growing period (Sahin *et al*., 2009).

Second-stage juveniles of CCN invade just behind the growing apex of the root tip (Seinhorst, 1986; Von Mende *et al*., 1998) and then pass through cells towards the stele where they initiate the development of a cluster of multinucleate feeding cells called a syncytium (Baldwin & Mundo-Ocampo, 1991). The J2 then go through a moult to the third-stage (J3). The syncytium provides food for the development and maturation of the juveniles to adult stage. The strong sexual dimorphism develops after the fourth-stage (J4). The adult males become vermiform and leave the root (Sijmons *et al*., 1994), whereas the females swell into a white lemon-shaped body that protrudes out of the surface of the root. After mating, the females produce eggs that are kept within their bodies. When all eggs have formed, the female dies and becomes a cyst, detached from the root (Smiley & Nicol 2009).

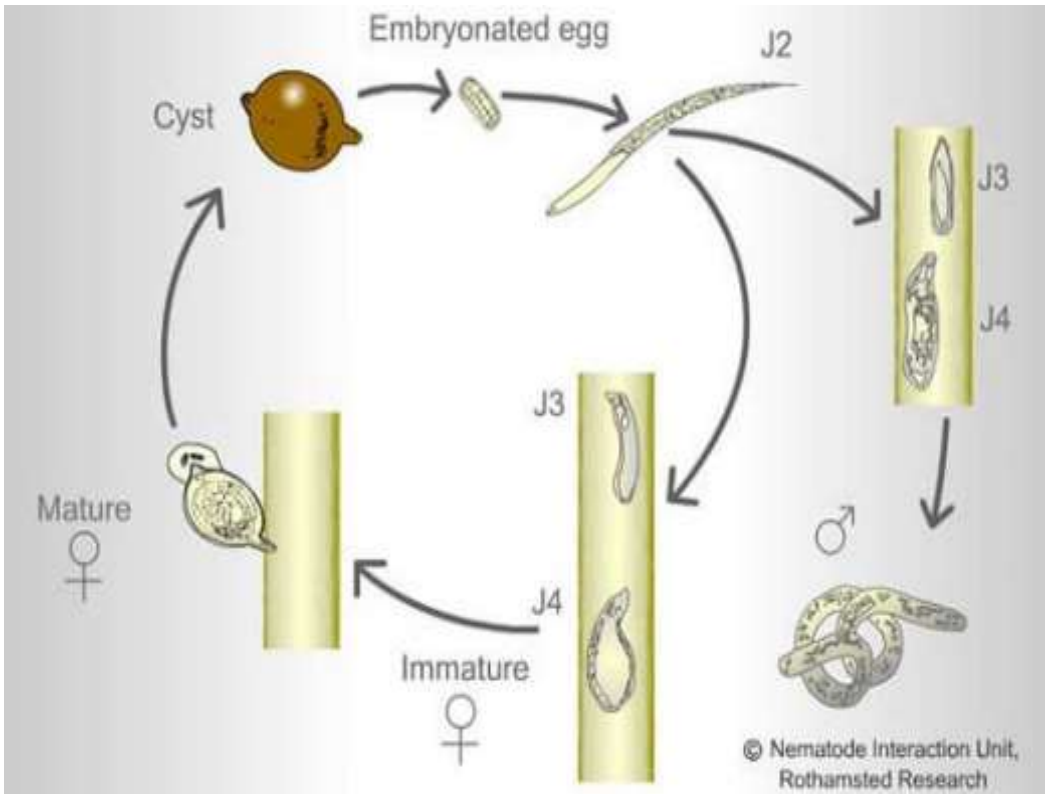

Figure 2.1. The life cycle of cereal cyst nematodes (*Heterodera* spp.) illustrating infective second-stage juveniles (J2) infecting roots and further stages of juveniles (J3 and J4) developing into adult females or males (Rothamsted Research) .

2.2.6 Symptoms

Aboveground symptoms caused by members of the *H. avenae* group include yellowing, poor tillering, stunting and patchy growth of the host plants. There may be a burning of the leaves, similar to that caused by drought conditions. This is due to the nematode interfering with the metabolic balance of the plant and inhibiting hydrostatic water pressure, which results in wilting (Griffin, 1988). The symptoms caused by members of the *H. avenae* group on the roots are different depending on the host. Wheat attacked by *H. avenae* shows increased root production such that the roots have a 'bushy knotted' appearance, usually with several females visible at each knot (Rivoal & Cook, 1993) (Figure 2.2). While *H. avenae* is far more common than *H. filipjevi*, these two species have similar host ranges and cause similar symptoms and economic losses (Smiley, 2009). Root symptoms often do not become recognizable until one to three months after planting, depending on climatic conditions and spring or winter wheat growth habits (Smiley & Nicol, 2009). Root systems of wheat and barley plants infested with *H. avenae* include elongation of the main root, bunched tips of rootlets and a knotted appearance due to cysts. Infected oat roots appear ‘ropery’ and

swollen (Smiley & Yan, 2010). Root symptoms of *H. latipons* are different from those seen with *H. avenae*, with no characteristic "knotting" caused by excessive production of lateral roots at the site of infection (Mor *et al*., 1992).

Figure 2.2. *Heterodera avenae* producing 'knotting' of wheat roots. (Photo by Vivien Vanstone, DAFWA).

2.2.7 Major methods of control

Reducing yield loss caused by cereal cyst nematodes requires control of CCN below the damage threshold or growing non-suceptible crops. This requires observations of population dynamics and yield losses on representative local cultivars under natural field conditions (Smiley & Nicol, 2009). Cultural practices based on rotations of non-hosts (non-cereals) and clean fallow can effectively control CCN (Dababat *et al*., 2015). Singh *et al*. (2009) showed that *H. avenae* population densities decreased by 70% after rotation with non-host crops like carrot (*Daucus carota*), fenugreek (*Trigonella foenum-graecum*), and onion (*Allium cepa*), or by fallow and summer ploughing. Nematicides can be applied, but are not preferred by the farmers because of the high cost per unit area in wheat (Dababat *et al*., 2015). However, when the nematode population in the soil is high, and other management approaches are inadequate, chemical control can bring the *H. avenae* population below damage threshold levels (Hague & Gowen, 1987).

Chemical control of nematodes is often considered economically and environmentally unacceptable (Mc Donald & Nicol, 2005; Viaene *et al*., 2013), so development of microbial antagonists for CCN might be one of the few remaining alternatives (Riley *et al*. 2010). A range of microorganisms has been investigated as potential biocontrol agents for CCN

including, *Pochonium chlamydosporium*, *Trichoderma longibrachiatum* and *Paecilomyces lilacinus* (Siddiqui & Mahmood, 1999; Kerry *et al*., 1984; Zhang *et al*., 2014). Likewise, some bacteria have been shown to offer potential as biocontrol agents. A bacteria similar to *Pasteuria* spp. was able to parasitise *H. avenae* and was shown to prevent 38 to 56 % of the juveniles from invading roots (Davies *et al*., 1990). Bansal *et al*. (1999) showed that *Azotobacter chroococcum* reduced cyst formation by 48%. However, little information has been gathered on biological control of CCN in recent years.

Soil solarisation offers an alternative management method to control nematodes (Viaene *et al*., 2013). Al-Rehiayani & Belal, (2009) showed that soil solarisation using polyethylene sheets during hot summer months in Al-Qassim (Saudi Arabia) was effective in reducing populations of *H. avenae* in wheat.

The potential use of biofuigation derived from plants or organisms is an alternative non-chemical for controlling many plant parasitic nematodes. For, cereal cyst nematodes, there is no information about their control by this approach. Haroon *et al*. (2009) used some medicinal plants (root extracts) for controlling *Heterodera zeae* and they concluded that *Calendula officinalis, Ambrosia maritime* and *Origanum vulgare* sigificantly reduced the hatching of eggs and mortality of *Heterodera zeae* compared to the controls. In addition, several studies have demonstrated the potential of brassicaveous crops to control the cyst nematode *Globodera pallida* in potato production (Lord *et al*., 2011; Ngala *et al*., 2015).

One of the most economic, environmental and promising methods of managing CCN is the use of resistant wheat germplasm (Dababat *et al*., 2015). Many sources of resistance in wheat germplasm have been reported (Nicol & Rivoal, 2008; Smiley & Nicol, 2009). Resistance sources around the world were obtained from wild wheat relatives through breeding programme (Ogbonnaya *et al*., 2001). At least nine single dominant resistance genes (*Cre* genes) have been found, many of which derive from wild relatives of wheat (Dababat *et al*., 2015). Six *Cre* genes (*Cre*2 to *Cr*7) were derived from *Aegilops* spp. (Jahier *et al*., 2001); other resistance genes were derived from *T. aestivum* (*Cr1* and *Cr8*) and rye (*Secale cereale*) lines (*CreR*) (Barloy *et al*., 1996). Sources of resistance to *H. avenae* have been collated and reviewed and, where possible, have had their genetic location and gene designation reported (Table 2.2) (Rivoal *et al*., 2001; Nicol *et al*., 2003; Mc Donald & Nicol, 2005; Nicol & Rivoal, 2008; Vanstone *et al*., 2008).

Some sources of resistance currently used to control *H. avenae* in wheat and barley in

Australia have been found to be effective against *H. latipons* (Moklabi *et al*., 2002). The Iraqi landrace AUS4930 is resistant to both *H. australis* (Australian pathotype Ha13) and the Turkish *H. filipjevi* (pathotype HF1) (Nicol & Rivoal, 2000). However, the use of resistance requires a sound knowledge of the virulence spectrum of the targeted species. Several studies showed that the wheat cultivars resistant to populations of *H. avenae* in one region were fully susceptible to populations of the same species in other regions (Bonfil *et al*., 2004; Smiley & Nicol, 2009).

Table 2.2. Main sources of genes used in bread wheat (*Triticum aestivum*) for resistance to the cereal cyst nematode *Heterodera avenae* (after Smiley & Nicol, 2009).

Cereal species	Genotype	Resistance gene and location	Use in cultivars
T. aestivum	Loros, AUS10894	*Cre*1 (formerly Ccn1) on chromosome 2BL	NW Europe, Australia, NW USA
	Katyil	Con	Australia
	Festiguay	*Cre*8 (formerly CreF) on chromosome 7L or 6B	Australia
	AUS4930 = Iraq48	Possible identical genetic location as Cre1: also resistant to *P. thornei*	Under evaluation in Australia, France and CIMMYT
	Molineux	Chromosome 1B	Australia
	Raj MR1	One dominant gene	Released cv. in India
T. durum	Psathias 7654, 7655	Not known	Not known
Triticosecale	T701-4-6	*Cre*R on chromosome 6RL	Australia
	Drira	Not known	Australia
	Ningadhu		
	Tahara	Not known	Not known
	Salvo	Not known	UK
Secale cereale	R173 family	*Cre*R on chromosome 6RL	Australia
Aegilops tauschii	CPI 110813	*Cre*4 on chromosome 2DL	Australian synthetic hexaploid lines
	AUS 18913	*Cre*3 on chromosome 2DL	Australian advanced breeding lines
A. peregrine	1	*Cre* (3S) with Rkn2 on chromosome 3S, CRX not yet located	Not known
A. longissima	18	Not known	France
A. geniculate	79, MZ1, MZ61, MZ77, MZ124	Not known	France
A. triuancialis	TR-353	*Cre*7 (formerly *Cre*A et)	France
A. ventricosa	VPM 1	*Cre*5 (formerly *Cre*X) on chromosome 2AS *Cre*2 (formerly *Cre*X) on genome N	Spain

2.3 The root-lesion nematodes (RLN) *Pratylenchus* spp.

Root-lesion nematodes of the genus *Pratylenchus* are recognised worldwide as one of the major constraints of crops of primary economic importance, including cereals, coffee, corn, banana, legumes, potato and many fruits (Castillo & Vovlas, 2007). The genus *Pratylenchus* was established by Filipjev (1936) as a group of nematodes possessing a tylenchoid pharynx overlapping the anterior portion of the intestine and a uniovarial gonad in adult females. Species of *Pratylenchus* are very similar in gross morphology and most specific differences can only be detected using high magnifications. This means that, although the genus is easily recognisable, it is extremely difficult to construct satisfactory keys for species determination (Castillo & Vovlas, 2007). The following classification of the genus *Pratylenchus* spp. is based on Siddiqi (2000).

2.3.1 Taxonomical position

Phylum Nematoda (Rudolphi, 1808)
Class Secernentea (von Linstow, 1905)
Subclass Tylenchia (Inglis, 1983)
Order Tylenchida (Thorne, 1949)
Suborder Tylenchina (Thorne, 1949)
Superfamily Hoplolaimoidea (Filipjev, 1934)
Family Pratylenchidae (Thorne, 1949)
Subfamily Pratylenchinae (Thorne, 1949)
Genus *Pratylenchus* (Filipjev, 1936)

2.3.2 Life cycle

Pratylenchus spp. are migratory endoparasites (Moens & Perry, 2009). All mobile life stages of *Pratylenchus* species are parasitic as both adults and juveniles can penetrate, migrate and feed within roots (Bridge & Starr, 2007). As is typical of other plant-parasitic nematodes, *Pratylenchus* species develop within the egg to the J1 that moults to J2, which then hatches from the egg (Figure 2.3) (Davis & MacGuidwin, 2000). The nematodes further moult through stages three (J3) and four (J4) to become fully developed adults. Each subsequent moult results in an increase in size and sexual development (Luc *et al*., 2005). Mobile juveniles and adult stages can enter and leave roots (Figure 2.3). They may become entirely embedded within root tissue and migrate from cell to cell within that tissue and spend most of their life cycle in host plant roots, but can also be found at the root surface and in adjacent soil. Females can deposit eggs in the roots and in the soil (Pudasaini *et al*., 2008). Eggs are released into the soil during root degradation (Castillo & Vovlas, 2007). Lesion nematodes

appear to be attracted to host roots, especially to the region of root hair production and the root tip (Peng & Moens, 1999). There are differences in the sites and mechanisms of root penetration by different species of *Pratylenchus* in various hosts. *Pratylenchus penetrans* explores the root by robbing its lip region along the surface of epidermal cells and protracting their stylet enough to touch but not penetrate the walls (Zunke, 1990). In lucerne and clover, *P. penetrans* preferred to penetrate the main roots where lateral roots ruptured the cortex and migrated through the cortex of main roots into lateral roots (Townshend *et al*., 1989). In other studies *P. penetrans* aggregated and penetrated in the zone of root elongation of turf grasses (Troll & Rhode, 1966). Castillo *et al*. (1998) found that both females and juveniles of *P. thornei* penetrated the roots of chickpea without any preference of site.

Pratylenchus thornei and *P. neglectus* are parthenogenic (De Waele & Elsen, 2002), i.e. females produce fertile eggs without copulation with a male. By contrast, *P. penetrans* is an amphimictic species, i.e. male and female must mate before fertile eggs are produced. Populations of *P. penetrans* therefore include nearly equal proportions of males and females (Smiley, 2010). Long-term survival under adverse conditions can occur at the egg stage (Castillo & Vovlas, 2007).

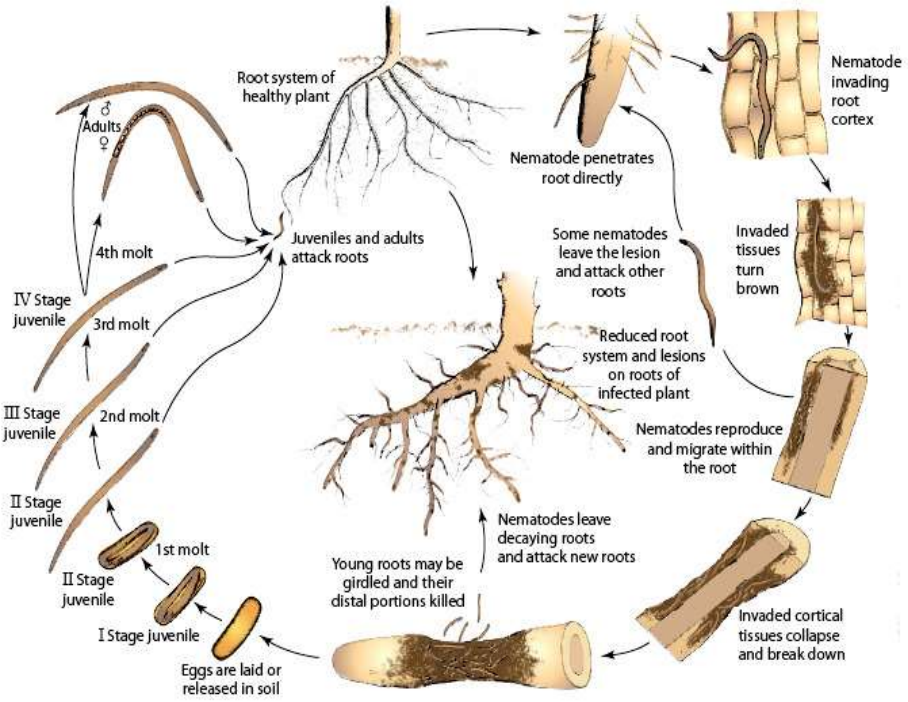

Figure 2.3. Life and disease cycle of root lesion nematodes (*Pratylenchus* spp.) (Agrios, 1997).

Nematodes of the genus *Pratylenchus* can complete their life cycle in 45 to 65 days depending on the species, the amount of available food sources, temperature, host species and moisture (Taylor *et al*., 2000). The optimum conditions for development vary with the

species. *Pratylenchus* spp. can complete three to six generations within the roots during one crop-growing season (Taylor *et al*., 2000). On the basis of laboratory observations, life cycle duration has been estimated for several nematode-host plant combinations. The time required to complete the life cycle varies considerably depending on temperature and moisture. In red clover, *P. penetrans* completed a generation in 54-65 days and produced 16-35 eggs per female at a rate of 1-2 eggs per day at 24°C (Turner & Chapman, 1972). The generation time of *P. penetrans in vitro* was estimated as 46, 38, 28, 26 and 23 days at 17, 20, 25, 27 and 30°C, respectively (Mizukubo & Adachi, 1997). On carrot callus, the complete life cycle of *P. coffeae* at 30°C was 27-28 days, that of *P. penetrans* at 24°C was 34-35 days and that of *P. loosi* at 20°C was 45-46 days (Wu *et al*., 2002). Similarly, the life cycle of *P. thornei* was completed in about 25-35 days on carrot discs at 20-25°C (Castillo *et al*., 1995).

2.3.3 Importance and geographical distribution of *Pratylenchus* species in wheat

The migratory endoparasitic genus *Pratylenchus* is the second group of nematodes considered economically important on wheat production systems (Smiley *et al*., 2005b; Castillo & Vovlas, 2007; Smiley & Nicol, 2009; Keil *et al*., 2009). At least eight species of the genus *Pratylenchus* affect roots of cereals (Rivoal & Cook, 1993). Among them, *P. thornei*, *P. neglectus*, *P. penetrans* and *P. crenatus* have a worldwide distribution, and sometimes coexist (Nicol *et al*., 2003; Smiley & Nicol, 2009).

The geographic distribution of *Pratylenchus* species depends mostly on both the prevalence of host plants supporting reproduction and abiotic factors (mainly temperature) (Castillo & Vovlas, 2007). In the Pacific Northwest of the USA (Oregon, Washington, Idaho), *Pratylenchus* species have been identified in more than 90% of dry land cropping wheat fields with predominance of *P. neglectus* and *P. thornei* (Smiley *et al*., 2004; Sheedy *et al*., 2008). In the northern grains region of Australia (Queensland and northern New South Wales), the presence of *P. thornei* and *P. neglectus* has been known since the 1960's and yield loss in wheat caused by *P. thornei* has been demonstrated since the late 1970's (Thompson *et al*., 2008). However, *P. thornei* is the predominant species of RLN in wheat and causes estimated annual losses of $33 million (Brennan & Murray, 1989). Several studies reported that the spring wheat yields elsewhere in the world have been reduced by as much as 32% by *P. neglectus* and 69% by *P. thornei* (Mc Donald & Nicol, 2005; Smiley & Nicol, 2009; Thompson *et al*., 2008). Similar yield losses from these species have been reported for spring wheat in Oregon (Smiley *et al*., 2005b). Armstrong *et al*. (1993) reported that the winter

wheat yields were reduced by 32% by *P. thornei* in Colorado. In the Isparta province of Turkey, three *Pratylenchus* species (*P. thornei*, *P. neglectus*, *P. scribneri)* have been identified in wheat (Sogut *et al*., 2008). Yield losses due to *P. thornei* are estimated at 20% on wheat in Turkey (Toktay, 2008). Pourjam *et al*. (1999) reported that *P. thornei* and *P. neglectus* are the most common species of *Pratylenchus* in Iran. *Pratylenchus penetrans* also parasitizes wheat and barley, and resulted in yield loss of 10-19% in Canada (Nicol & Rivoal, 2008). In Morocco, *Pratylenchus* spp. is the most important group of nematodes in different cereal growing areas followed by nematodes of the *H. avenae* group (Meskine *et al*., 1984; Rammah, 1994; Mokrini *et al*., 2009); RLN cause extensive necrosis on the roots with consequent crop losses. *Pratylenchus penetrans* is the most abundant and widespread RLN in Morocco (Chapter 3). The species was recovered from 70% of the soil samples, with population densities of 32-123 nematodes 100 ml^{-1} of soil and 67-102 nematodes g^{-1} of root of wheat.

2.3.4 Symptoms

Root lesions are the main symptoms on plants invaded by *Pratylenchus* species. RLN cause degradation of cells in the epidermis and cortex of underground plant organs. This reduces the amount of root branching and the ability of roots to absorb water and nutrients (Smiley, 2010). Wheat roots infested with RLN display sloughing of cortical and epidermal cells, degradation of lateral roots, and loss of root hairs (Vanstone *et al*., 1998). Generally, infected cereal roots are browning as lesions rapidly coalesce to produce extensive areas of discolorations. Aboveground symptoms are non-specific. Overall, affected plants appear stunted with premature yellowing of older leaves, reduced tillering, and lower weight (Fulton *et al*., 1960; Smiley, 2010; Castillo & Vovlas, 2007). These symptoms are often confused with nutrient deficiencies, drought, root disease, barley yellow dwarf (Taylor *et al*., 1999; Smiley, 2010) or associated with other pathogens (Evans & Haydock, 1993). For instance, fields with high populations of root-lesion nematodes often have plant canopies that are irregular in height and maturation, as also occurs in plants affected by *Rhizoctonia* root rot (Smiley, 2010). Penetration of root tissues by RLN results in lesions that favour greater colonization by root-rotting fungi and by saprophytic bacteria, fungi, and nonparasitic nematodes (Moens & Perry, 2009; Smiley, 2010). These secondary organisms cause more intense rotting and discoloration than that caused by the root-lesion nematode alone. Cortical degradation and reduced root branching often are not visible until plants are six or more weeks old, and these root symptoms are often confused with those caused by *Pythium* or

Rhizoctonia root rot (Smiley, 2010). Colonization of *Fusarium oxysporum* in chickpea has been shown to increase with increasing population density of *P. thornei* (Castillo *et al*., 1998). More root lesions can occur when *P. neglectus*-infested wheat plants are co-infected with various fungal pathogens, including *Pythium irregulare*, which causes damping off, and *Gaumannomyces graminis*, the agent responsible for take-all disease (Taheri *et al*., 1994).

Differences in temporal developments of symptoms of *P. penetrans* invasion were observed on different hosts. For example, lesions in the proximal parts of the seminal roots of wheat appeared only after 6 weeks after inoculation with *P. thornei* (Baxter & Blake, 1968). However, lesions on strawberry roots appeared 17 days after inoculation when inoculated with *P. penetrans* (Townshend, 1963).

2.3.5 Management of *Pratylenchus* species on wheat

The choice of management tactic to reduce damage caused by root-lesion nematodes depends upon many factors. All tactics require accurate diagnosis of the species and population levels of *Pratylenchus* as assessed from soil and root samples taken from any given field. Action thresholds vary among *Pratylenchus* species and crops depending upon geographic location, crop value, and the potential for disease complexes (Davis & McGuidwin, 2000; Castillo & Vovlas, 2007). The damage thresholds of some species of Pratylenchus associated with cereals were determined in several studies (Table 2.3). Assessment of potential crop damage caused by *Pratylenchus* is usually based on population densities in soil at the time of planting, but also on densities in roots during the growing season.

Table 2.3. Damage threshold densities of cereal-*Pratylenchus* combinations

Pratylenchus spp.—Cereals	Damage threshold (Nematodes/cm soil)	Reference
P. crenatus — oat	0.33	Barker & Olthof (1976)
P. neglectus— barley	1.5	Rivoal & Cook, (1993)
P. thornei— wheat	0.5-1	Rivoal & Cook, (1993)
P. thornei— wheat	0.42	Nicol & Ortiz-Monasterio, (2004)
P. thornei— wheat	2.5	Thompson, (1993)
P. thornei— wheat	3	Nicol *et al.,* (1999)

The main purpose of controlling RLN is to avoid having significant yield losses. There are many reports about the different methods to reduce population densities of RLN.

2.3.5.1 Cultural practices

Cultural methods offer some control, but are often of limited effectiveness. To be of significance they need to be integrated with other control measures. The use of crop rotation is a limited option for RLN, due to their polyphagous nature (Nicol & Rivoal, 2008). Successful use of rotation requires a thorough understanding of the effectiveness of a particular rotation. Little information is available about the role of crop rotation in controlling RLN in wheat, although some field and laboratory work has been undertaken to better understand the hosting ability of cereals and leguminous crops to *P. thornei* and *P. neglectus* as hosts (Vanstone *et al*., 1998; Lasserre *et al*., 1994; Nicol, 1996). Nevertheless, some effective rotations have been developed. For example, in Sonora (Mexico), populations of *P. thornei* in wheat fields were reduced by rotations that include corn, cotton or soybean for 2 consecutive years (Van Gundy *et al*., 1974). In Queensland (Australia) wheat is rotated with the barley cv. Clipper to reduce populations of *P. thornei* (O'Brien, 1983). Control of *P. zeae* has been reported on rice by crop rotation with non-host crops such as legumes, *e.g*., mung bean (*Vigna radiata*) and black gram (*Vigna mungo*) (Prasad & Rao, 1978). The principle for the use of crop rotation to reduce RLN densities is that monoculture of a host plant usually results in increased population density and consequent yield losses (Castillo & Vovlas, 2007). However, some long-term monoculture experiences indicate that monoculture may also reduce *Pratylenchus* populations (Castillo & Vovlas, 2007). Andersen (1975) showed that *P. crenatus* and *P. neglectus* reached highest numbers in the first 3 years of barley monoculture, after which population densities decreased gradually and stabilized at a lower level.

Di Vito *et al*. (1991) showed that the solarisation of soil with polyethylene film for 6-8 weeks in chickpea fields reduced *P. thornei* populations by 50 %. Tillage is often considered as an option for control of soil-borne nematodes.

Other management practices are less effective in managing *Pratylenchus* populations. Field sanitation during the fallow phase is as important as during the in-crop phase, because *Pratylenchus* species multiply on many weed species in the genera *Avena, Brassica, Bromus, Malva* and *Rumex* (Vanstone & Russ, 2001). Smiley *et al*. (2004) reported that the presence of susceptible weeds or crop species between planted crops allows *Pratylenchus* to increase population density over a greater interval of the cropping system. Irrigated wheat yield in *P. thornei*-infested fields was improved by delaying planting by one month, presumably because seedlings overwintered with lower populations than for early planting (Van Gundy *et al*., 1974).

Fertilisation with inorganic sources of nitrogen has also been observed to modify populations of *Pratylenchus* spp. Dmowska & Ilieva (1995) reported that *Pratylenchus* spp. were more abundant in plots of barley fertilized with nitrogen over 22 years than in non-fertilised plots.

2.3.5.2 Resistance and tolerance

The use of resistant and tolerant cultivars is considered the most economical and environmentally acceptable means for control of RLN (Castillo *et al*., 1998). *Pratylenchus* nematodes readily multiply on a susceptible wheat cultivar to high population densities, which decrease wheat growth and yield; on a resistant cultivar the reproduction is much reduced with less yield loss. By contrast, a tolerant cultivar still has the capacity to grow and yield well in the presence of high numbers of nematodes. However, as tolerant cultivars allow nematode reproduction they do not reduce the numbers of *Pratylenchus* in the soil (Thompson *et al.*, 1999). The first source of superior tolerance to *P. thornei* in wheat lines was identified through targeted screening of cultivars. Tolerant cultivars such as Pelsart (Brennan *et al.*, 1994), Sunvale (Ellison *et al.*, 1995) and Baxter (Thompson *et al.*, 1999) were used to minimise the effects of *Pratylenchus*. These tolerant lines offered a 30% yield increase compared to other commercial cultivars available at the time (Thompson *et al.*, 1995). Ideally the resistance should be combined with tolerance (plants which have the ability to yield despite the attack of the nematode). The most detailed research on breeding for tolerance and resistance to *Pratylenchus* spp. has been carried out in Australia, where it was shown that a tolerant wheat variety grown in nematode-free fields or after nematicide treatment is a good option for controlling RLN (Thompson *et al*., 2008). The soil-borne pathogen programme at CIMMYT Turkey annually screens about 1,000 accessions of wheat from the Turkey – CIMMYT - ICARDA International Winter Wheat Improvement Program (www.iwwip.org) under growth room, greenhouse, and field conditions at various locations in Turkey. Cultivars are also screened for multiple disease resistance, such as resistance to different species of root lesion nematodes (*e.g. Pratylenchus thornei* and *P. neglectus*) (Toktay *et al*., 2013).

Many sources of resistant wheat germplasm have been reported for RLN (Thompson & Haak, 1997; Taylor *et al*., 2000; Toktay *et al*., 2012). Resistance in wheat against *P. thornei* (Vanstone *et al*., 1998; Thompson *et al*., 1999; 2009) and *P. neglectus* (Thompson *et al*., 1989) has been identified. In Australia, the first significant source of resistance to *P. thornei* was the bread wheat line GS50a selected from a severely infested field of the variety Ghatcher

(Thompson & Clewett, 1986). Thompson *et al*. (1999) showed that this line reduced *Pratylenchus* reproduction by more than ten-fold. Sheedy & Thompson (2009) investigated 274 accessions of Iranian wheat landraces and identified 25 accessions that were more resistant than 'GS50a'. Thompson *et al*. (2009) found additional sources of *P. thornei* resistance from screening wheat accession collections from West Asian and North African regions.

Resistance to *P. neglectus* has been investigated less than resistance to *P. thornei* because the latter species is the most frequent RLN detected on cereals in the world. For *P. neglectus*, the resistant gene, (*Rlnn1*) located on chromosome 7AL and originating from Australien variety 'Excalibur', has been identified and validated (Williams *et al*., 2002). As both *Pratylenchus* species are often found in the same field in mixed populations (Thompson *et al*., 2010), the development of wheat cultivars with resistance to both species is desirable. However, it should be noted that wheat cultivars with resistance or tolerance to *P. thornei* are not necessarily resistant or tolerant to *P. neglectus* and vice-versa, since resistance and tolerance to each species is genetically independent (Smiley & Nicol, 2009); this probably also applies to other species that infest wheat.

CHAPTER 3

Distribution of the members of the *Heterodera avenae* group and of *Pratylenchus* spp. on wheat and barley in Morocco

3.1 Introduction

Cereals are exposed to biotic and abiotic stresses. Among the biotic stresses, plant-parasitic nematodes play an important role in decreasing crop yield (Nicol & Rivoal, 2008). Cereal cyst nematodes (CCN) and root lesion nematodes (RLN) are known to be a major constraint to wheat production in several parts of the world. Significant economic losses due to CCN and RLN have been reported (See Chapter 2). In Morocco, *Heterodera avenae* was reported for the first time in 1951 when a cyst of the species was detected in a soil sample taken in Gharb region (Meagher, 1977). Later, the species was detected several times in different wheat-growing areas of Morocco. For years, *H. avenae* was the only species of cyst nematode known as a parasite of cereals in Morocco (Meskine *et al*., 1984; Rammah, 1994; Mokrini *et al*., 2009). Damage such as reduced tillering, early yellowing, early maturation, and reduced yield were reported by many investigators (Rivoal & Sarr, 1987; Nicol, 2002; Smiley *et al*., 2005b). With respect to their prevalence in cereal growing areas of Morocco, CCN are second after RLN (Meskine *et al*., 1984; Rammah, 1994; Mokrini *et al*., 2009).

Znasni (2003) reported that 26% of surveyed wheat fields were infested with RLN in the Eastern region of the country. *Pratylenchus thornei*, *P. crenatus* and *P. neglectus* were reported in different cereal-growing areas of Morocco (Meskine *et al*., 1984). Mokrini *et al*. (2009) reported RLN in 67% of soil samples taken in different cereal growing areas (Gharb, Tadla, Zaers and Saiss); population densities were estimated between 14-43 nematodes (100 g soil) $^{-1}$ and 80-200 nematodes (10 g root)$^{-1}$. Two species, *P. penetrans* and *P. thornei*, were found associated with wheat, barley and oat. In all cited Moroccan reports, identification of the CCN and RLN was based on their morphology. In view of this paucity of information and in order to orientate further nematological research, the objective of this part of my study was to establish the occurrence and distribution of *Pratylenchus* spp. and *Heterodera* spp. in the major wheat-growing areas of Morocco (Table 2.1).

3.2 Agro-ecological regions of Morocco

Morocco is located in the northwest corner of Africa, bordered by the Mediterranean Sea and the Atlantic Ocean in the north and west, respectively, by Algeria in the east, and by Mauritania in the south. The total land area is 710,850 km and includes several zones, among which are agricultural plains and river valleys, plateaus, and mountain chains (Berkat & Tazi, 2004). Morocco has a Mediterranean climate characterised by a dry and hot summer (4-6 months) and a short and cold winter at higher altitudes. The Mediterranean climate prevails

over much of northern and central Morocco, and is moderated by the oceanic influence. Moving southward, the climate becomes increasingly hot and arid with important temperature differences.

Based mainly on intensity of wheat and barley cropping (see 2.1), a survey was conducted in four regions of Morocco (Figure 3.1).

3.2.1 Gharb

The Gharb region is the largest agricultural area in Morocco. It is well known for its industrial crops such as sugar beet, sugar cane, in addition to conventional crops such as cereals and vegetables. It is dominated by sandy and clayey soils. The climate is Mediterranean with annual precipitations ranging between 480 and 600 mm; the average air temperature is 27°C in summer and 13°C in winter (Marouane *et al*., 2014).

3.2.2 Saiss

The Saiss plateau is mostly covered with rainfed agriculture; the annual rainfall ranges between 451 and 500 mm (Berkat & Tazi, 2004). The average air temperature in this region is 29°C in summer and 10°C in winter. Major crops are cereals, olives, vineyards, pulses, forages and oil crops.

3.2.3 Chaouia

This region is characterized by an annual rainfall ranging from 300 to 400 mm, and by mild winters. However, the dry season is relatively long (May-October). The average air temperature in this region is 22°C in summer and 12°C in winter (Younsi *et al*., 2001; Berkat & Tazi, 2004). The cropping system consists of cereals (wheat, barley and maize cover about 80% of the area), fallow (13%), vegetables, forages (3%), pulses and orchards. Vegetable production, mainly tomato, represents an important activity geared partly towards export. This zone is quite exposed to drought, as most of the crops are rainfed.

3.2.4 Zaers

This region is located along the Atlantic Ocean. It has a mild climate, shifting from cool in winter to warm days in the summer months. The average air temperature is 25°C and 12°C in summer and winter, respectively. The annual rainfall ranges between 500 and 520 mm.

Figure 3.1. The four major wheat and barley growing areas of Morocco surveyed for cereal cyst nematodes (*Heterodera avenae* group) and root lesion nematodes (*Pratylenchus* spp.). Gh: Gharb, Sa: Saiss, Za: Zaers, Ch: Chaouia.

3.3 Material and methods

The survey was carried before the harvest of cereals in 2011. Soil and root samples were taken in 75 fields (Table 3.1) spread over the four major cereal growing regions of Morocco (Gharb, Chaouia, Zaers and Saiss; Figure 3.1). Sixty-nine samples were taken from wheat fields; the remaining 6 samples were obtained from barley fields. Samples were taken where wheat or barley plants showed chlorotic and yellowing leaves and poor growth. Each sample (soil and root) was composed of 15 subsamples randomly collected per field. The sample was thoroughly mixed before 2000 g soil were put in a plastic bag and taken to the laboratory.

Table 3.1. Localities, provinces and regions sampled during a survey (May-June 2011) of Moroccan cereal-growing regions for cereal cyst nematodes, *Heterodera avenae* group, and *Pratylenchus* spp.

Sampling date	Regions and provinces	Number of fields surveyed	
		Wheat	Barley
	Gharb		
10/05/11	Sidi Slimane	7	-
	Kenitra	8	-
	Saiss		
	Haj Kaddor	3	-
	Ain Jmaa	6	1
	Meknes	3	-
	Ait Malk	1	-
	Sebaa Ayoune	2	-
	Ain Taoujdate	2	-
12/05/11	Mhaya	2	-
	Chaouia		
	Berrechid	4	1
	Mediouna	6	-
18/05/11	Settat	9	-
	Zaers		
	Ain El Aouda	2	-
	Marchouch	6	1
	Krina	1	
	Ouled Said	1	1
20/05/11	Sidi Bettach	6	2
	Total	69	6

From each soil sample, 100 cm soil was used to extract the vermiform stages of *Pratylenchus* spp. and *Heterodera* spp. using an automated zonal centrifuge (Hendrickx, 1995). Cysts were extracted from another 100 cm soil using the sieving (200-µm sieves) and flotation method (Shepherd, 1986). Counting and separation of cysts from soil debris and other organic materials retained on the filter paper, were carried out using a stereoscopic microscope. Roots were washed, cut into pieces of 1 cm, mixed well and 10 g of root pieces were macerated in a blender for 20 s. All extracts were examined using a stereomicroscope. *Pratylenchus* species were identified based on the morphology and morphometric features of females and males, using relevant references of *Pratylenchus* spp. (Shen & Allen, 1953; Loof, 1960; Castillo & Vovlas, 2007). Cysts were identified on the basis of the vulval cone structures and measurements (Handoo, 2002). Second-stage juveniles were recovered from cysts kept in water in watch glasses and used for morphological identification.

3.4 Results

3.4.1 Cereal cyst nematodes

3.4.1.1 Field infestation and distribution

Details on the morphological identification of cysts are shown in Chapter 4. CCN were found in three out of four wheat-producing regions of Morocco at a frequency of 15% (Table 3.2). No CCN were detected in the 15 soil and root samples from the Gharb region. However, CCN were widely spread in the important cereal growing areas (Saiss, Chaouia and Zaers). The highest incidence was found in both Saiss and Chaouia (20% of fields); cysts were not found in some provinces i.e. Meknes, Sebaa Ayoune and Mhaya (Saiss region). *Heterodera avenae* was the most dominant species (14% of the surveyed fields); *H. latipons* was only found in wheat in Ain Jmaa (Saiss) (Chapter 4). The population densities of CCN in soil samples ranged from 6 to 155 cysts (100 g soil)$^{-1}$. The highest levels of infestation were found in Marchouch (155 cysts (100 g soil)$^{-1}$). Soil population density varied between 247 and 301 eggs and juveniles (100 g soil)$^{-1}$. *Heterodera avenae* was found only in wheat fields (Table 3.2).

3.4.2 Root-lesion nematodes *Pratylenchus* spp.

3.4.2.1 Field infestation

Details on identification of RLN are shown in chapter 5. Fifty-two out of 75 localities sampled (= 69%) were

positive for root-lesion nematodes. *Pratylenchus* spp. occurred in 41 wheat fields (60%) and all barley fields (100%). Infested fields showed a serious reduction in tillering, patches of stunted plants, leaf yellowing, and extensive necrosis of the roots (Figure 3.2). Four species were detected, *viz. P. penetrans*, *P. thornei*, *P. pseudocoffeae* and *P. pinguicaudatus*. *Pratylenchus penetrans* was the most dominant species. Densities of mobile stages of *P. penetrans* in wheat fields ranged from 32 to 123 (100 g soil)$^{-1}$ and from 76 to 102 (10 g root)$^{-1}$. In barley fields densities of mobile stages were similar and ranged from 6 to 112 (100 g soil)$^{-1}$ and 2 to 91 (10 g root)$^{-1}$ (Table 3.3). The highest density of *P. penetrans* was recorded in Sidi Slimane with 123 *P. penetrans* (all vermiform stages) (100 g soil)$^{-1}$. The ratio mobile stages of both species *P. pinguicaudatus* and *P. penetrans* in the same sample collected from Chaouia were 6 and 34 (100 g soil) $^{-1}$ respectively.

Table 3.2. Occurrence of cyst nematodes of the *Heterodera avenae* group in wheat and barley fields in Morocco (survey May-June 2011).

Regions and provinces	Number of fields surveyed		*Heterodera* species	Highest number of cysts (100 soil)$^{-1}$	
	Wheat	Barley		Wheat	Barley
Gharb					
Sidi Slimane	7 (0)*	-	-	-	-
Kenitra	8 (0)	-	-	-	-
Saiss					
Haj Kaddor	3 (1)	-	*H. avenae*	70	-
Ain Jmaa	6 (1)	1 (0)	*H. latipons*	65	-
Meknes	3 (0)	-	-	-	-
Ait Malk	1 (1)	-	*H. avenae*	8	-
Sebaa Ayoune	2 (0)	-	-	-	-
Ain Taoujdate	2 (1)	-	*H. avenae*	6	-
Mhaya	2 (0)	-	-		-
Chaouia					
Berrechid	4 (1)	1 (0)	*H. avenae*	50	-
Mediouna	6 (1)	-	*H. avenae*	70	-
Settat	9 (2)	-	*H. avenae*	25	-
Zaers					
Ain El Aouda	2 (0)	-	-	-	-
Marchouch	6 (3)	1 (0)	*H. avenae*	155	-
Krina	1 (0)		-	-	-
Ouled Said	1 (0)	1 (0)	-	-	-
Sidi Bettach	6 (0)	2 (0)	-	-	-
Total	69 (11)	4(0)			

*: The number between brackets indicates the number of infested fields.

Figure 3.2. Plants showing poor growth and serious reduction in tillering caused by *Pratylenchus* spp.

Table 3.3. Occurrence of root-lesion nematodes (*Pratylenchus* spp.) in wheat and barley fields in Morocco (survey May-June 2011).

Regions and provinces	Number of fields surveyed		*Pratylenchus* species	Density in 100 g soil		Density in 10 g roots	
	Wheat	Barley		Wheat	Barley	Wheat	Barley
Gharb							
Sidi Slimane	7 (7)*	-	*P. penetrans*	(32-123)**	-	(7 - 81)**	-
Kénitra	8 (3)	-	*P. penetrans*	(0 - 9)	-	(0 - 22)	-
Saiss							
Taso	4 (1)	-	*P. penetrans*	7	-	21	-
Ain Jmaa	6 (2)	1 (1)	*P. penetrans*	(0 – 32)	(0 - 6)	0	2
Meknes	3 (3)		*P. pen+P. th*	(11 - 37)	-	(9 - 61)	-
Ait Malk	1 (1)		*P. penetrans*	23	-	7	-
Sebaa Ayoune	1 (1)		*P. penetrans*	19	-	11	-
Ain Taoujdate	2 (2)		*P. penetrans*	(11 - 23)	-	(3 - 6)	-
Mhaya	2 (2)		*P. penetrans*	(45 - 52)	-	(8 - 71)	-
Chaouia							
Berrechid	4 (4)	1 (1)	*P. penetrans*	(0 - 28)	37	(2 - 45)	12
Mediouna	6 (5)		*P. pen+P. pse*	(0 - 46)	-	(2 - 23)	-
Settat	9 (6)		*P. pen+ P. pi*	(0 - 67)	-	(0 - 43)	-
Zaers							
Ain El Aouda	2 (2)		*P. pen+P. th*	(45 - 78)	-	(67 - 102)	-
Marchouch	5 (3)	1 (1)	*P. pen+P. th*	(0 - 41)	27	(0 - 17)	-
Krina	1 (0)		-		-	0	-
Ouled said	2 (1)	1 (1)	*P. penetrans*	5	25	57	91
Sidi Bettach	6 (3)	2 (2)	*P. pen+P. th*	(0 - 56)	(6 - 112)	(0 - 69)	87
Total	69 (46)	6 (6)					

*: The number between brackets indicates the number of infested fields. -: none
**(Range) numbers per 100g soil; (Range) numbers per 10g root; *P. pen*: *P. penetrans*; *P. th*: *P. thornei*; *P. pse*: *P. pseudocoffeae*; *P. pi*: *P. pinguicaudatus.*

3.5 Discussion

The results of the survey demonstrate that CCN occur in the three of the four major cereal-growing areas of Morocco, and RLN in all four regions. This is similar to earlier findings reported by Meskine *et al*. (1984), Znasni (2003) and Mokrini *et al*. (2009), with the exception of CCN being found in Gharb by these authors. The CCN are represented by two species, *viz*. *H. avenae* and *H. latipons*. *Heterodera avenae* was the most prevalent species occurring in 13% of the fields and was associated with wheat in the 3 regions where this nematode was found. *Heterodera latipons* was detected in one sample originating from Ain Jmaa (Saiss) and is herewith reported for the first time in Morocco (Chapter 4).

Although *H. avenae* is widely distributed in the three regions studied, with important variations in incidence between different regions. The lowest (15%) incidences are found in

Saiss and Zaers, whereas the highest (20%) frequencies are detected in Chaouia. These proportions of infestation are mostly greater than those observed in earlier surveys in the same regions (Himmich, 1987; Meskine *et al*, 1984). This variation can be attributed to environmental factors and agricultural practices. In both Chaouia and Zaers, which showed the highest level of infestation, soil textures are clay-marl and sandy loam. However, *H. avenae* was also common in the clay soils of Saiss. That means that *H. avenae* is found in a wide range of soil types.

An earlier survey (Znasni, 2003) reported *H. avenae* to be associated with oats and barley in morocco. A more recent survey showed *H. avenae* associated with bread wheat (*T. aestivum*) (Mokrini *et al*., 2009). In this study, *H. avenae* was only detected in wheat fields. The highest infestations were found in the provinces Marchouch, Mediona and Haj Kaddor, where the average number of cysts (100 g soil)$^{-1}$ reached 155, 70 and 70, respectively. As the distance between Marchouch and Mediona is only 80 km, one can easily imagine that the spread of the nematode is due to the contaminated movement of soil attached to farm machineries. The high number of extracted cysts (up to 155 cysts (100 g soil)$^{-1}$) and their distribution are probably influenced respectively by the monoculture practiced in the region and climatic differences. *Heterodera latipons* is adapted to Mediterranean climatic conditions, where as *H. avenae* develops in more temperate climates (Nicol *et al*., 2003). Both Zaers and Chaouia have a temperate climate with a minimum temperature ranging between 5-20°C and 5-10°C, respectively, which allows the juveniles to emerge from eggs in cysts and penetrate the root in great numbers (Meskine *et al*., 1984). *Heterodera avenae* was not detected in samples from Gharb. This observation may be attributed to the limited number of samples (15 samples) compared with other regions. Here, wheat is irregularly produced and mainly rotated with vegetables. This practice may reduce the multiplication and spread of the species.

Heterodera avenae, H. filipjevi and *H. latipons* are considered to be the most common species of the *H. avenae* group worldwide (Nicol *et al*., 2011). However, local differences in importance between CCN species do exist. The first species was reported from many countries with different climatic types throughout the world (Sturhan & Rumpenhorst, 1996). In Tunisia, Namouchi-Kachouri & B'Chir (2005) reported that *H. avenae* was the dominant species associated with wheat and barley. Ahmadi & Maafi (2014) reported that 38% of the surveyed cereal fields in Iran were infested with CCN of which *H. filipjevi* was the dominant species; the distribution of *H. avenae* and *H. latipons* was limited to West and Southwest Iran (Tanha Maafi *et al*., 2012). Rumpenhorst *et al*. (1996) reported species of the *H. avenae* group

to be present in 41% of the samples taken in the Central Anatolian Plateau (CAP) in Turkey. Recently Toktay *et al*., (2015) reported 56% incidence of CCN represented by *H. filipjevi* and *H. latipons* in the East Anatolia region of Turkey. The predominant species in the rain-fed winter wheat productions systems of the CAP was *H. filipjevi* (Sahin *et al*., 2009). Still regarding the CAP, Abidou *et al*. (2005) reported 85% incidence of CCN represented by *H. filipjevi* and *H. latipons*. In the Eastern Mediterranean of Turkey, Imrin *et al*., (2015) found *H. avenae*, *H. latipon*s and *H. filipjevi* either pure or mixed in populations.

Four *Pratylenchus* species, *viz. P. penetrans*, *P. thornei, P. pseudocoffeae* and *P. pinguicaudatus*, were detected in this survey. RLN were found in 69% of the samples. They were most prominent in Chaouia (80%) and least in both Saiss and Zaers (65%), which is less than the 84% reported for these regions by Meskine *et al*. (1984). In Gharb, the percentage of RLN infestation was 66%, i.e. more than 26% reported by Sbihi (2003). RLN were found in 100% and 66% of the barley and wheat fields, respectively. *Pratylenchus penetrans* was detected in the four regions, where as *P. thornei* was found only in Zaers and Chaouia. Both *P. penetrans* and *P. thornei* were previously reported in Gharb, Tadla, Saiss and Zaers (Meskine *et al*., 1984; Mokrini *et al*., 2009). These two species were now either found singly or in mixed populations. Of the two species, *P. penetrans* was the most frequently detected. It was found alone in 27 samples out of 75. In Gharb, this species was detected alone, but in other regions (Saiss and Zaers) it was present together with *P. thornei.* Observations of mixed populations of RLN were reported earlier in Morocco (Ammati, 1987; Meskine & Abbad Andaloussi, 1992; Mokrini *et al*., 2009). Meskine *et al*. (1984) reported the presence of *P. crenatus* and *P. neglectus* from Morocco. The fact that both species were not detected in the current survey, suggests that they are not widely distributed. Next to the above-mentioned *Pratylenchus* species, two other species, *viz. P. pinguicaudatus* and *P. pseudocoffeae*, were detected in the current survey. Corbett, (1974), found *P. pinguicaudatus* only in wheat at Rothamsted, England. In addition, this species was found associated with wheat and barley in Alhama, Southeastern Spain (Talvera & Tobar Jimenez, 1997). Troccoli and Di Vito, (2002) found *P. pinguicaudatus* associated faba bean in Tunisia. However, *Pratylenchus pseudocoffeae* has been recorded in Florida, USA on aster (Inserra *et al*., 1998). Mohammad Deimi *et al*. (2009) detected *P. pseudocoffeae* associated with chrysanthemum in Iran. Recently, Kim *et al*. (2016) detected the same species in soil and root samples from chrysanthemum field located in Chilgok and Geumsan counties in Korea.

Pratylenchus spp. are reported on wheat and other cereals in many countries with

different climates throughout the world (Castillo & Vovlas, 2007). At least eight species of RLN can parasitize cereals. *Pratylenchus thornei* is the most studied species on wheat. It is found in Syria (Greco *et al*., 1984; Saxena *et al*., 1988), Pakistan and India (Maqbool, 1988), Algeria (Troccoli *et al*., 1992) and Italy (Lamberti, 1981). In Turkey, Sahin *et al.* (2009) reported *P. thornei* and *P. neglectus*, occasionally found together. Abidou *et al*. (2005) found about 40% of the soil samples infected with *P. thornei* and/or *P. neglectus* in CAP in Turkey. In Australia, Israel, Oregon (USA) and Mexico, *P. thornei* reduced wheat yield by 85%, 70%, 50% and 37%, respectively (Smiley, 2010). *Pratylenchus neglectus* was also reported associated with wheat in Australia and NW America (Vanstone *et al*., 1998; Taylor *et al*., 1999). *Pratylenchus penetrans* parasitizes wheat and barley, and reduces yield by 10 to 19% in Canada (Nicol & Rivoal, 2008). There is no information on the economic importance of *Pratylenchus* species in Morocco.

It is clear from this study that *H. avenae*, *P. thornei* and *P. penetrans* are widespread in wheat growing areas of Morocco. In view of the estimates of nematode densities obtained in this survey, one can assume that these nematodes probably damage cereals in many cases. Field studies on the population dynamics and the damage function are necessary to estimate the economic impact of these nematodes on cereals. More surveys are needed in other regions in order to determine their importance. Since the nematode can cause considerable damage to wheat, one of the main cereals produced in Morocco, care should be taken to prevent the spread to other regions. Further investigations are necessary to identify the pathotype of Moroccan CCN populations. Sources of resistance to both nematode groups should be searched for and used in cereal breeding programmes.

CHAPTER 4

Identification

of cereal cyst nematodes, *Heterodera* spp., from Morocco

Mokrini, **F.**, Waeyenberge, L., Viaene, N. & Moens, M. (2012). First report of the cereal cyst nematode *Heterodera latipons* on wheat in Morocco. *Plant Disease* 96 (5), 774.

4.1 Introduction

Cereal cyst nematodes (CCN) form a group of several closely related species. Twelve out of 80 species belonging to the genus *Heterodera* (Subbotin *et al.*, 2010) affect roots of cereals and grasses (Yan & Smiley, 2009). Three species (*Heterodera avenae*, *H. filipjevi* and *H. latipons*) are among the economically most important cyst nematode pests of cultivated cereals (Rivoal & Cook, 1993; Nicol, 2002). *Heterodera avenae* is widely distributed in temperate wheat-producing regions throughout the world (Nicol, 2002; Smiley & Nicol, 2009). *Heterodera latipons* is found in the Mediterranean regions, eastern and northern Europe, the Middle and Near East, and North and South Africa, Asia and North America (Greco *et al*., 2002; Abidou *et al*., 2005; Smiley & Nicol, 2009), whilst *H. filipjevi* has been reported from eastern and northern Europe, Central and West Asia, the Middle East, the Indian subcontinent and North America (Rivoal *et al*., 2003; Rumpenhorst *et al*., 1996; Holgado *et al*., 2004; Smiley & Nicol, 2009). Earlier reports from Morocco mention only *H. avenae* as representative of the CCN. The nematode was detected for the first time in 1951 in an irrigated wheat (*T. aestivum*) field in the Gharb region (Ritter, 1982). More populations of *H. avenae* were found during later surveys (Meskine *et al*., 1984; Sbihi, 2003; Znasni, 2003; Mokrini *et al*., 2009). In all of these Moroccan studies, cysts were identified using morphological features only; morphometrical and molecular identification were not considered.

The taxonomy of the *H. avenae* group and its members has been the object of several review papers (Mulvey & Golden, 1983; Ferris *et al*., 1994; Subbotin *et al*., 1996; Handoo, 2002). Species belonging to this group form a complex, and invade and reproduce only in roots of cereals and grasses (Smiley & Yan, 2015). Within the *H. avenae* group, only minor morphological and morphometrical differences distinguish the species from each other (Subbotin *et al.,* 1999; Handoo, 2002). The increasing number of species in this group makes morphological and morphometrical identification more difficult, time consuming and requires appropriate skills (Subbotin *et al*., 2003). This is especially the case for samples of which the content is not predictable, e.g. not field samples of known species composition where nematode densities need to be determined, but samples of unknown origin or where other species are suspected. Nevertheless, accurate identification of members of the *H. avenae* group is needed as an initial step in designing effective control measures. This is especially important when searching for potential sources of host-plant resistance against *Heterodera* species (Dababat *et al*., 2015). In addition, rapid and accurate identification is highly

significant for quarantine purposes. For these reasons, the development of molecular methods to identify members of the *H. avenae* group has been the goal of numerous studies. The internal transcribed spacer regions of ribosomal genes (rDNA–ITS) were found to be useful to differentiate species within the *H. avenae* group (Bekal *et al*., 1997; Subbotin *et al*., 2000; Zheng *et al*., 2000; Rivoal *et al*., 2003). Additionally, the comparison of sequences of the ITS-rDNA region of unknown species with those published and deposited in GenBank facilitated fast identification of most species of cyst-forming nematode (Subbotin *et al*., 1999; 2000).

Because Moroccan CCN have mainly been identified on the basis of their morphology (see above) very little information is available on the diversity and variability of their morphometrics and genetics. Only Subbotin *et al*. (2003) published three sequences of *H. avenae* from Morocco. To fill these gaps, I conducted a survey in the major wheat growing areas in Morocco (chapter 3) with following main objectives: (a) to collect, identify and compare both cysts and J2s of populations of CCN using morphological, morphometrical and molecular approaches including species-specific PCR and sequencing of the ITS-rDNA expansion segments, and (b) to determine the phylogenetic relationships between these populations.

4.2 Material and methods

4.2.1 Collection of populations of the *Heterodera avenae* group

Sampling was carried out during the wheat-growing season (May to June 2011) in four different regions representing the main wheat growing areas of Morocco (Chapter 3). Cysts were extracted from each soil sample using the modified Cobb decanting and sieving method (Cobb, 1918) (Chapter 3). After extraction, cysts were stored at 4°C.

4.2.2 Morphology and morphometrics of populations

Species identification was based on cyst vulval cone structures and measurements, as well as morphometric features of the J2s. The vulval cone of the cysts was cut and prepared for microscopic examination according to Hooper (1986). For each population, cones of ten mature cysts were mounted in glycerine jelly. The identification of the cysts was done on the basis of the structure of the underbridge, the shape of the semifenestra, and the development of the bullae (Handoo, 2002). For each population, juveniles were obtained from the same cysts, killed by gentle heat (warming up enough to kill the nematode but not too long not to

deform or destroy it), fixed in TAF, embedded in glycerol (De Grisse, 1969); permanent slides were made immediately. Ten J2s of the selected cyst populations were examined and measured using an Olympus BX51 compound microscope equipped with an Olympus image-capture system and software. Seven characters of juveniles (J2s) known to be important for taxonomic diagnosis in this group (Subbotin *et al*., 1999) were used for identfication. The obtained morphological and morphometric data were compared to each other and referenced to related published data (Handoo, 2002; Subbotin *et al*., 2003).

4.2.3 Morphometric data analysis

Morphometrical data were run through discriminant multivariante analysis to investigate the separate ability of 11 populations based on their morphometric characters. Canonical discriminant analysis (CDA) was performed using STATISTICA.

4.2.4 Molecular characterization

4.2.4.1 DNA Extraction

For each population, a single J2 isolated from a single cyst was transferred into an Eppendorf tube containing 25 µl double distilled water (ddH2O) and 25 µl nematode lysis buffer (final concentration: 200 mM NaCl, 200 mM Tris-HCl (pH8), 1% mercaptoethanol and 800 µg Proteinase K). The tubes were incubated at 65°C for 1.5 h and at 99°C for 5 min, consecutively (Holterman *et al*., 2006). The extracted DNA suspension was stored at −20 °C or used immediately for DNA amplification.

4.2.4.2 PCR with species-specific primers

The species-specific primers set AVEN-COI-forward (5'-GGG TTT TCG GTT ATT TGG-3' and AVEN-COI-reverse (5'-CGC CTA TCT AAA TCT ATA CCA-3') (Toumi *et al*., 2013a) together with the universal primers developed by Ferris *et al*. (1993), i.e. forward primer 5′-CGT AAC AAG GTA GCT GTA G-3′ and the reverse primer 5′-TCC TCC GCT AAA TGA TAT G-3′, were used to detect *H. avenae* in the DNA extracts of 11 populations. Extracts that were not identified as belonging to *H. avenae* were used in a PCR with the species-specific primers set Hla-acti-F (5'-ACT TCA TGA TCG AGT TGT AGG TGG ACT CG-3') and Hla-acti-F (5'-ACC TCA CTG ACT ACC GAT GAA GAT TC-3') (Toumi *et al*., 2013b) along with the universal reverse primers (Ferris *et al*., 1993) eventually to characterise *H. latipons*.

The PCR used to detect *H. avenae* was run as follows: 2 µl DNA extract (see above) were added to the PCR reaction mixture containing 21 µl ddH2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany) and 1 µM of each of the primers AVEN-COI (Toumi *et al*., 2013a) and Ferris *et al*. (1993). The thermal cycler programme consisted of 5 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C, followed by a final elongation step of 8 min at 72°C. For the detection of *H. latipons*, 2 µl of the DNA extract was added to the PCR reaction mixture containing 21 µl ddH2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany), and 1 µM of each of the primers Hla-acti (Toumi *et al*., 2013b) and Ferris *et al*. (1993). The programme of the thermal cycler consisted of 5 min at 95°C; 50 cycles of 30 s at 94°C, 45 s at 50°C and 45 s at 72°C, followed by a final elongation step of 8 min at 72°C.

Eventually, 5 µl of each PCR product was mixed with 1 µl of 6× loading buffer (Fermentas Life Sciences, Germany) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis (100 V, 40 min), the gel was stained with ethidium bromide (0.1 µg ml^{-1}) for 20 min, visualised and photographed under UV-light. The remaining PCR product was stored at –20°C.

4.2.4.3 PCR amplification and sequencing

The ITS-rDNA region was amplified using the primers 5′-CGT AAC AAG GTA GCT GTA G-3′ and 5′-TCC TCC GCT AAA TGA TAT G-3′ (Ferris *et al*., 1993). One µl of DNA was added to the PCR reaction mixture containing 22 µl ddH2O, 25 µl 2 × DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany) and 1 µM of both primers. The thermal cycler-program consisted of 5 min at 95°C; 35 cycles of 1 min at 94°C, 45 s at 49°C and 1 min at 72°C; followed by a final elongation step of 8 min at 72°C. After PCR, 5 µl of each PCR product was mixed with 1 µl of 6× loading buffer (Fermentas Life Sciences, Germany) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis (100 V for 40 min) the gel was stained with ethidium bromide (0.1 µg ml^{-1}) for 20 min, visualized and photographed under UV-light. The remaining PCR product was stored at -20°C. The amplification and electrophoresis process was repeated and both PCR products were pooled for purification. The purification process was done as described by the manufacturer's instructions (Wizard® SV Gel and PCR Clean-Up System Kit, Promega). DNA from each sample was sequenced (Macrogen, Seoul, South Korea) in both directions to obtain overlapping sequences of both DNA strands. The sequences were edited and analysed using software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit

7.0.4.1 (Hall, 1999). Finally, all sequences were blasted in GenBank (Sequin v. 9.00, http://www.ncbi.nlm.nih.gov/).

4.2.4.4 Phylogenetic analysis

Twenty-nine ITS sequences of *H. avenae* and *H. latipons* (13 new and 16 from GenBank; Figure 4.4) were aligned using Clustal W (Thompson *et al*., 1994) and visually checked. Differences between sequences were estimated using the DNA distance option provided by BioEdit sequence alignment editor (Hall, 1999). The alignment was imported into the software package Mega 5.0 (Tamura *et al*., 2011); after checking 24 different nucleotide substitution models, the model with the lowest BIC score (Bayesian Information Criterion) was retained for constructing a 60% consensus Neighbour-joining tree. To determine statistical consistency of the classification, bootstrap analysis using 1000 bootstrapped data sets was performed. As in Madani *et al*. (2004), *Meloidodera alni* (AF274419) was added as outgroup in the phylogenetic analysis. The analysis involved 23 nucleotide sequences with a total of 910 positions in the final dataset. The new sequences in this study were deposited in GenBank (accession numbers in Figure 4.4).

4.3 Results

4.3.1 Morphology and morphometrics

The survey yielded 11 *Heterodera* populations (Table 4.1). Ten populations were monospecific for *H. avenae* and 1 for *H. latipons.*

4.3.1.1 ***Heterodera avenae*** (Wollenweber, 1924)

Morphometrics

(See Tables 4.2 and 4.3)

Description

Cysts

Mostly lemon-shaped, with a protruding neck and vulvar cone. Cyst wall dark brown, bearing a zig-zag pattern. Vulval cone bifenestrate. No underbridge. Bullae in all populations. The cyst of populations H01, H03, H07 and H08 slightly bigger than other populations.

Second-stage juveniles

Body cylindrical, head slightly offset, tapering round tail tip. Stylet strong with shallow anteriorly concave basal knobs. Body length 503 to 640 µm; stylet length (22.3-27.9 µm); anteriorly concave basal knobs. Lateral field with four incisures.

Remarks

This species was detected in 10 populations (H01, H02, H03, H05, H06, H07, H08, H09, H10, H11). These populations were morphologically and morphometrically similar to populations described previously (Handoo, 2002; Subbotin *et al*., 2003).

4.3.1.2 ***Heterodera latipons*** (Franklin, 1969)

Morphometrics

(See Tables 4.4)

Description

Cysts

Lemon-shaped; cyst wall partially transparent, between light and dark brown; ridges in zigzag pattern. Fenestration with 2 distinct semifenestrae and strong underbridge, no bullae. Fenestra 64 µm (60-72 µm) long and 21 µm (18-25 µm) wide; underbridge 96 µm (85-115 µm) long, vulval slit length 8 µm (7-9 µm).

Second-stage juveniles

Cylindrical head slightly offset, round tail tip tapering. Compared with *H. avenae* populations body slightly shorter and short length of hyaline terminal tail. Body length 445 µm (412-472 µm), stylet length 24 µm (23-25 µm) and hyaline terminal tail 28 µm (24-31 µm). Lateral field with four incisures.

Remarks

Only one population of *H. latipons* was detected (Ain Jmaa, Saiss). The morphometrics and

morphological characters corresponded to those reported by Handoo (2002).

Table 4.1. Species and populations of *Heterodera* collected during a survey in wheat producing areas of Morocco.

Code	Location	Area	Host	Morphological identification	Identification using PCR		Result of sequencing
					H. avenae	*H. latipons*	
H01	Haj Kaddor	Saiss	DW	+	+	-	Ha
H02	Ait Malk	Saiss	W	+	+	-	Ha
H03	Ain Taoujdtae	Saiss	DW	+	+	-	Ha
H04	Ain Jmaa	Saiss	DW	+	-	+	Hl
H05	Marrakech	Zaers	W	+	+	-	Ha
H06	Marchouch	Zaers	DW	+	+	-	Ha
H07	Marchouch	Zaers	DW	+	+	-	Ha
H08	Mediona	Chaouia	W	+	+	-	Ha
H09	Berchd	Chaouia	W	+	+	-	Ha
H10	Settat	Chaouia	DW	+	+	-	Ha
H11	Settat	Chaouia	DW	+	+	-	Ha

+: Positive for the mentioned species; **–:** Negative for the mentioned species
Ha: *Heterodera avenae*; Hl: *Heterodera latipons*
DW : Durum Wheat ; W : Wheat

Table 4.2. Morphometric characters of cyst and vulval cone of *Heterodera avenae* populations from Morocco (n= 10). Measurements in µm and in form: mean ± standard deviation (range).

Population	Cyst				Vulval areas of cyst					
Code	Length without neck	Neck length	Width	L/W ratio	Semifenestra width	Fenestra length	Vulva slit length	Vulva bridge width	Underbridge	Bullae
H01*	823± 71 (683-911)	70±17 (40-98)	662±66 (570-742)	1.3±0.2	19±2.1 (18-25)	47±1.8 (44-49)	9±1.7 (6-13)	6±0.8 (5-7)	Absent	Present
H02	797±53 (708-886)	67±16 (40-95)	609±65 (490-680)	1.31±0.2	22±1.7 (20-25)	46±3.6 (38-49)	10±0.9 (9-11)	6±0.9 (5-8)	Absent	Present
H03	839±90 (683-961)	43±8 (31-55)	630±108 (458-742)	1.33±0.4	22±1.3 (20-24)	48±1.2 (46-50)	11±0.9 (10-12)	6±0.6 (6-8)	Absent	Present
H05	763±98 (587-867)	67±21 (45-120)	581±53 (470-625)	1.31±0.2	21±1.6 (18-23)	49±2 (45-51)	9±1.6 (8-11)	5±0.6 (5-7)	Absent	Present
H06	716±91 (596-842)	74±20 (41-111)	530±77 (470-712)	1.35±0.2	21±1 (19-22)	45±2.5 (41-48)	10±1 (8-11)	7±0.9 (6-8)	Absent	Present
H07	810±114 (613-940)	72±17 (56-107)	613±86 (495-710)	1.32±0.3	20±1.4 (18-22)	42±2.2 (40-47)	9±0.9 (8-10)	6±1.4 (5-8)	Absent	Present
H08	858±99 (581-911)	69±12 (50-91)	631±54 (521-693)	1.36±0.3	19±1.7 (18-23)	44±2.4 (41-47)	9±0.8 (8-10)	6±0.8 (5-7)	Absent	Present
H09	674±102 (598-875)	79±13 (50-96)	522±63 (445-667)	1.29±0.3	19±1.7 (18-22)	45±3.8 (40-51)	10±1 (8-11)	6±1.2 (5-8)	Absent	Present
H10	783±103 (590-913)	65±10 (59-94)	584±63 (410-640)	1.34±0.3	20±2.1 (18-23)	49±2.8 (42-52)	8±1.2 (7-11)	5±0.6 (5-7)	Absent	Present
H11	766±95 (602-877)	61±14 (49-95)	580±94 (409-711)	1.32±0.2	21±1.6 (18-23)	49±2.5 (42-52)	9±1.4 (8-11)	7±1.5 (5-8)	Absent	Present
Handoo (2002)	710 (580-975)	61±14 (49-95)	580±94 (409-711)	1.32±0.2	-	49±2.5 (42-52)	9±1.4 (8-11)	7±1.5 (5-8)	Absent	Present
Znasni (2003)	763 (575-937)	59 (37-91)	583 (458-972)	1.31	20 (17-24)	49 (43-56)	10 (8-11)		Absent	Present

***** Codes for populations: see Table 4.1.

Table 4.3. Morphometrics of second-stage juveniles of *Heterodera avenae* populations collected in Morocco (n= 10). Measurements in µm and in form: mean ± standard deviation (range).

Code	Body length	Body Width	Stylet length	Tail length	Lateral lines	Hyaline terminal length	DGO	Hyaline tail length/ Stylet length
H01*	590±25.1 (546-621)	22.5±0.5 (21.4-23.1)	24.2±1.4 (22.3-26.2)	67.4±5.8 (60.2-74.7)	4	43.2±4.7 (37.7-52)	5.7±0.2 (5.2-5.9)	1.7±0.1 (1.6-2)
H02	567±27.9 (520-597)	23±0.6 (21.8-23.8)	24.2±1.3 (23-27)	68.2±2.9 (60.5-78)	4	44±4.6 (38-50.4)	5.4±0.4 (4.9-6.1)	1.8±0.2 (1.6-2.2)
H03	578±30.8 (540-631)	22.7±0.8 (21.1-24.3)	25.5±0.9 (24.3-26.7)	61±4.6 (56-71.6)	4	43.5±4.2 (39-51.2)	5.3±0.3 (4.9-5.8)	1.7±0.1 (1.6-2)
H05	576±43.8 (503-639)	23.1±0.5 (22.1-24)	26.1±1.1 (24.7-27.9)	66±5.9 (59.1-77)	4	43±3.9 (38.3-51)	5.4±0.3 (5-5.9)	1.6±0.1 (1.5-1.8)
H06	581±40 (522-645)	22.4±0.9 (21-23.7)	25.2±0.8 (24.2-26.6)	64.2±5.2 (57.3-72.1)	4	43.4±3.7 (39-50)	5.6±0.3 (5.03-6.1)	1.8±0.1 (1.7-2.2)
H07	577±17.7 (557-611)	22.6±0.8 (20.9-23.6)	24.6±0.5 (24.1-25.8)	60.2±4.1 (57-68.6)	4	44±3.3 (39-50.5)	5.3±0.1 (5- 5.7)	1.8±0.1 (1.7-2.1)
H08	566±28.1 (521-614)	21.98±0.7 (21-23.2)	26.2±0.7 (25.4-27.7)	65.6±3 (59.2-69.5)	4	42.3±3.2 (38.2-49)	5.4±0.3 (5-5.7)	1.6±0.2 (1.5-2.1)
H09	592±16.2 (570-623)	22.4±0.9 (21.5-24.2)	26.2±0.7 (25.4-27.4)	63.2±5.1 (57.4-71.2)	4	44±3.8 (39-51)	5.3±0.3 (4.9-5.7)	1.7±0.1 (1.6-2.0)
H10	561±34.7 (526-623)	22±0.7 (21.7-23.8)	25.3±0.8 (24.7-27.5)	66.4±4.1 (60.5-73.3)	4	41±3.6 (35-48)	5.4±0.2 (5-5.8)	1.7±0.1 (1.6-2)
H11	573±30 (539-624)	23±0.5 (22.3-24)	25.8±1.1 (24.6-27.2)	69±4.9 (62.7-77.3)	4	44±3.9 (38.4-50)	5.1±0.4 (4.7-5.8)	1.8±0.2 (1.6-2.1)
Handoo (2002)	577 (520-620)	20-24	27 (24-28)	68 (58-70)	4	41 (35-45)		
Znasni (2003)	522 (492-633)	22 (21-23)	24 (23-26)	65 (62-68)	4	45 (39-54)	5.5 (5-6.1)	-

* Codes for populations: see Table 4.1

Table 4.4. Morphometrics of cysts and second-stage juveniles of the *Heterodera latipons* population collected in Morocco (n= 10). Measurements in µm and in form: mean ± standard deviation (range).

Population Characters	H04*	(Handoo, 2002)
Cyst		
Cyst length without neck (L)	590±23.3 (551-632)	525 (348-645)
Neck length	75±7.9 (65-90)	83 (58-103)
Cyst width (W)	393±47.7 (310-490)	414 (277-510)
Vulval cone		
Fenestra length	64±4.1 (60-72)	67 (58-76)
Semi fenestra width	21±2.1 (18-25)	-
Underbridge length	96±10.9 (85-115)	103 (80-125)
Vulval slit length	8±0.6 (7-9)	7 (6-9)
Vulval bridge width	27±3 (24-33)	33 (18-39)
Bullae	Absent	Absent
Second stage juveniles		
Body length	445±25 (412-472)	454 (401-478)
Body width	19±0.8 (18-21)	21 (19-22)
Stylet length (SL)	24±1 (23-25)	24 (23-25)
Tail length	50± 3 (46-54)	48 (42-54)
Lateral lines	4	4
Hyaline terminal length (HL)	28±3 (24-31)	27 (20-31)
HL/SL ratio	1.3±0.2 (1.1-1.5)	-
DGO	4.7±0.2 (4.5-5.1)	5 (4-5)

* Codes for populations: see Table 4.1.

4.3.2 Canonical discriminant analysis (CDA)

Morphometrics of cyst and J2s of the eleven populations identified morphologically were used in a canonical discriminant analysis (CDA). Using a combination of 8 morphometrical characters for the cysts and 6 morphometrical characters for the J2s (Table

4.5), the CDA clearly separated the two species from each other (Figure 4.1). Six morphometric traits of cysts and J2s (length without neck, fenestra length, fenestra width, vulva bridge width, hyaline terminal length and DGO) provided the most useful taxonomic characters for identification and discrimination (Table 4.5). It was not possible to separate the ten populations of *H. avenae* by CDA (Figure 4.2).

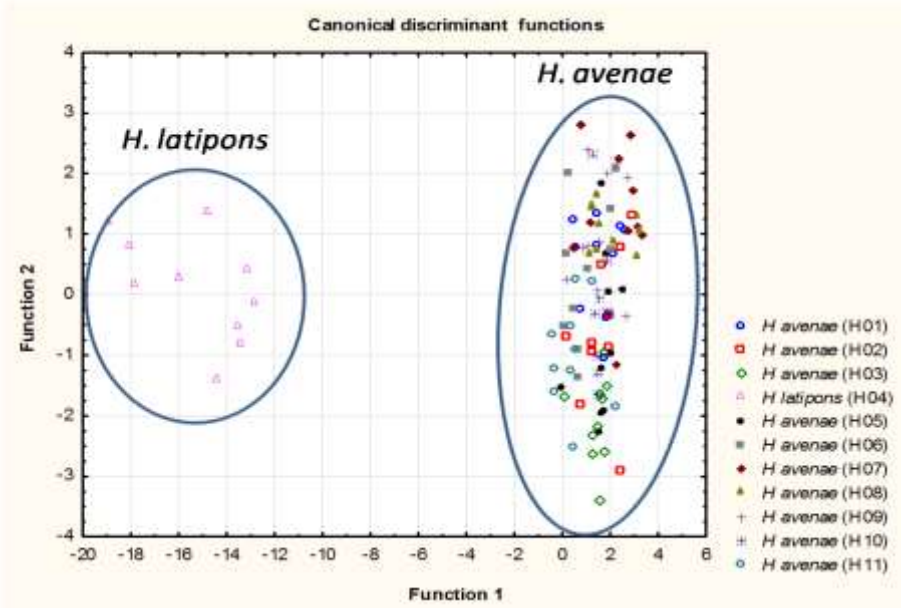

Figure 4.1. The two main canonical discriminant analysis of populations of the *Heterodera avenae* group collected from different cereal growing areas of Morocco, based on 14 morphological characters of cysts and J2s.

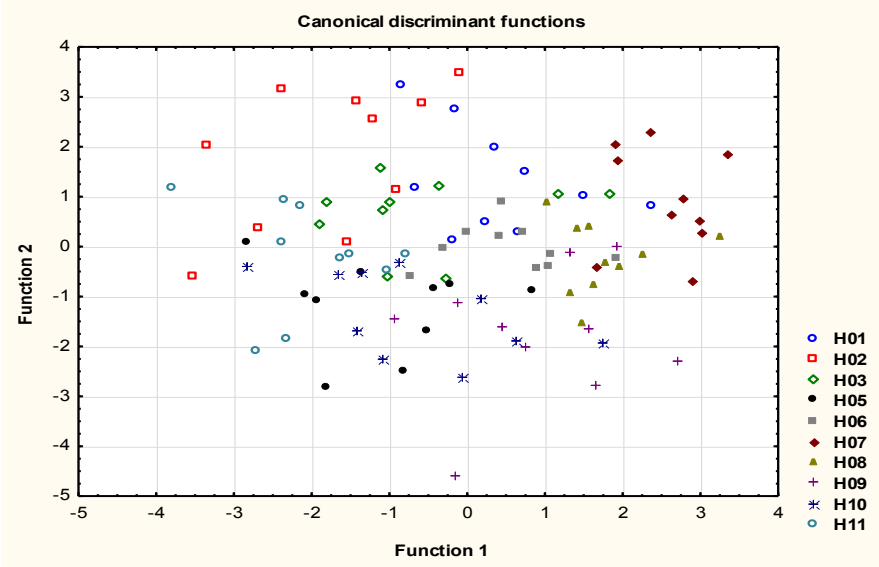

Figure 4.2. The two main canonical discriminant analysis of *Heterodera avenae* collected from different cereal growing areas of Morocco, based on 14 morphological characters.

Table 4.5. Standardized coefficients for canonical variants of cysts and second-stage juveniles of *Heterodera avenae* populations from Morocco.

Selected characters	Function 1	Function 2
Cyst		
Length without neck	0.03	-0.27
Neck length	-0.039	-0.24
Width	0.13	0.01
L/W ratio	0.19	-0.21
Fenestra		
Length	-0.58	0.52
Width	-0.02	0.65
Vulva slit length	0.13	0.18
Vulva bridge with	-0.75	-0.22
Second-stage juveniles		
Body length	0.18	-0.1
Body width	0.2	0.42
Stylet length	-0.008	-0.11
Tail length	0.04	0.67
Hyaline terminal length	0.42	-0.34
DGO	0.37	-0.11

4.3.3 Molecular characterisation

4.3.3.1 Species-specific PCR and sequencing

The *H. avenae*-specific primers PCR (AVEN-COI) amplified a band of 109 bp for 10 samples (H01, H02, H03, H05, H06, H07, H08, H09, H10 and H11) (Figure 4.3). This means that of out of 11 populations, 10 populations were molecularly identified as *H. avenae*. For the sample (1 population) not identified as *H. avenae*, the *H. latipons*-specific primers (Hlat-act) amplified a specific band of 204 bp.

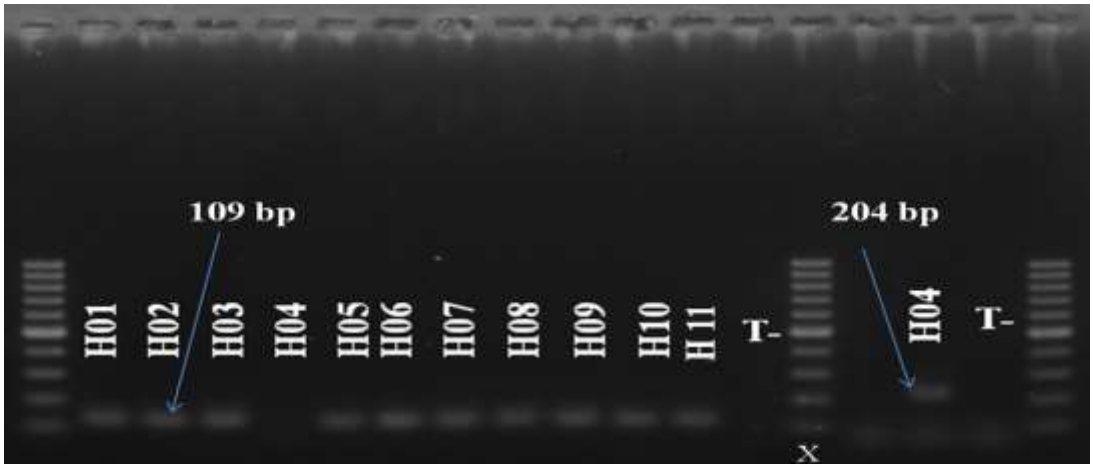

Figure 4.3. Results of specific PCR for *Heterodera avenae* (H01, H02, H03, H05, H06, H07, H08, H09, H10, H11) and *H. latipons* (H04**).** X = 100 bp DNA ladder (Promega Benelux), T-: negative control, codes see Table 4.1.

4.3.3.2 ITS sequence and analyses

The comparison of ITS-rDNA sequences of *H. avenae* and *H. latipons* populations among themselves and with sequences of *Heterodera* species available in GenBank is presented in Figure 4.4. The comparison confirmed the identification of the species using morphological features and species-specific PCR. Ten sequences of the ITS-rDNA were similar (99-100%) to the sequences of *H. avenae* published in GenBank (AY148363, AY148364, AY148360, AY148359, AY148361, AY148362, AY148354, AY148358, AY148367, AY148368, AY148369) and three sequences (JQ319035, JQ319036 and JQ319037) were similar (97-99%) to *H. latipons.* On the basis of the topology of the calculated majority rule, 60% consensus Maximum Likelihood tree for all the Moroccan populations collected in the survey and the 3 Moroccon populations in GenBank (AY148367, AY148368, AY148369, Subbotin et al., 2013 and 16 *Heterodera* spp. from GenBank, two major groups of *Heterodera* were revealed (Figure 4.4). In Group I (bootstrap value = 100%), two subgroups were identified. The first one comprised all *H. avenae* populations from Morocco together with eight other *H. avenae* populations available in GenBank. The second subgroup comprised only one population of *H. avenae* from China (AF264181). Group II held two sub-groups. The first one (bootstrap value = 97%) contained a *H. latipons* population from Iran (AF498382) and Syria (JX024182). The second subgroup (bootstrap value = 99%) contained the *H. latipons* population from Morocco together with a Jordanian population of the same species (HM560790). As this result consisted a first record of *H. latipons* from Morocco, the sequences of this population were deposited in GenBank under accessions numbers JQ319035 and JQ319036.

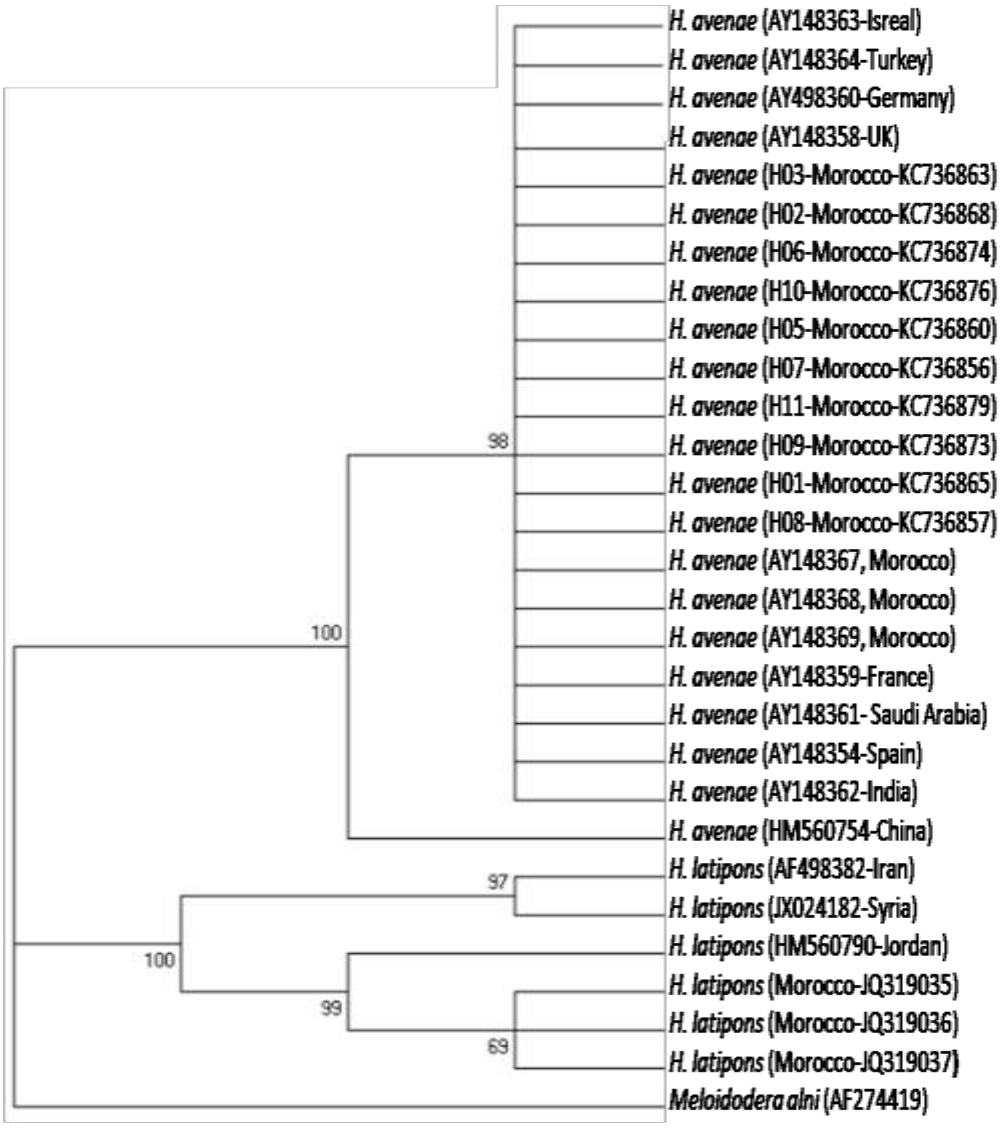

Figure 4.4. The topology of the majority rule 60 consensus Maximum Likelihood tree for all populations studied with addition of *Heterodera* populations obtained from GenBank based on the sequence alignment of the ITS-rDNA**.** For the list with the abbreviations of the population codes see Table 4.1.

4.4 Discussion

Two species, *viz*. *H. avenae* and *H. latipons* were detected during the survey of cyst nematodes in the major cereal-cultivating areas of Morocco. The latter species is detected for the first time in the country; it was found in a wheat field in Ain Jmaa (Saiss). Both species are economically very important as they cause serious losses in wheat production systems in several parts of the world (Nicol & Rivoal, 2008). Previous surveys in the area had revealed the presence of *H. avenae* only (Meskine *et al*., 1984; Ammati, 1987; Znasni, 2003; Mokrini *et al*., 2009). In this study, cysts of *H. avenae* were found only in wheat fields (Saiss, Chaouia

and Zaers regions of Morocco). However, the absence of cysts in barley fields is probably related to the rotation with vegetables or food legumes, practiced in these fields. Moreover, farmers in in the main cereal growing areas of Morocco prefer to grow wheat, so less fields of barley were sampled, hence reducing the chances for detecting infestations with cysts.

Both species were distinguishable easily on the basis of the cyst morphology. All cysts of *H. avenae* had prominent bullae, but no underbridge; *H. latipons*, however, had a strong underbridge and lacked distinct bullae in the vulval cone. Previous studies (Wouts & Sturhan, 1995; Rivoal *et al*., 2003; Subbotin *et al*., 2003) reported the same morphological characteristics that separated *H. avenae* from *H. latipons*. Abidou *et al.* (2005) showed specific differentiation between *H. latipons* and both *H. avenae* and *H. filipjevi*, based on a strong and deep underbridge without bullae. Recently, Baklawa *et al*. (2015) reported the discrimination of Egyptian *H. avenae* populations from German *H. filipjevi* and *H. avenae* populations based on the presence (or absence) of an underbridge and bullae. Compared to *H. latipons*, J2s of *H. avenae* have a longer tail, stylet and hyaline part of tail. More studies (Subbotin *et al*., 1999; Abidou *et al*., 2005) reported *H. latipons* populations to have a shorter body, stylet and hyaline tail length than *H. avenae*.

The CDA of morphometrical data of both cysts and J2s revealed the most important characteristics to discriminate *H. avenae* from *H. latipons*, i.e. length of cyst without neck, fenestra length, fenestra width, vulva bridge width, length hyaline tail terminal, and DGO. CDA has already successfully been used to analyse morphometric data of *Heterodera* spp. Studying Spanish and British populations of *H. avenae* and *H. filipjevi*, Valdeolivas & Romero (1990) showed that CDA clearly separated the two species. The authors reported five morphological traits of juveniles (*viz.* c, stylet length, hyaline part of tail, length of body and tail) and length as well as width of cyst provided the most useful taxonomic characters for discrimination of both species (*H. avenae* and *H. filipjevi*). Dawabah *et al*. (2012) reported some characters that can be utilized for the separation of different populations within *H. avenae*. These characters include J2s midbody width, J2s body width at the anus and tail length/ body width at the anus. Unlike the findings of Dawabah *et al*. (2012), the Moroccan populations of *H. avenae* did not segregate into groups, because of the low intraspecific variation of their morphometric characters. This lack of intraspecific differences based on morphological characters was confirmed by the lack of intraspecif polymorphism based on the study of ITS sequences.

Species-specific primers for PCR have been developed to complement the traditional species identification of *H. avenae* (Toumi *et al*., 2013a; Yan *et al*., 2013), *H. latipons* (Toumi

et al., 2013b) and *H. filipjevi* (Toumi *et al*., 2013a; Yan *et al*., 2013). Several genes were successfully used to discriminate between many species of *Heterdera*. Yan & Smiley (2013) developed species-specific primer sets to detect *H. avenae* and *H. filipjevi* on the basis of the ITS region of DNA and Peng *et al.* (2013) developed species-specific SCAR-PCR assay to detect *H. filipjevi*. When using the species-specific primers developed for both *H. avenae* and *H. latipons* (Toumi *et al*., 2013a, b) we obtained the characteristic bands of 109 bp and 204 bp respectively, confirming their morphological identification. This confirms the specificity of the primers sets.

In addition to the morphology, morphometric, species-specific primers, the sequence comparison of the ITS region clearly separates the Moroccan *H. avenae* from *H. latipons.* This rDNA region has been commonly used to separate nematodes at species level, including the genus *Heterodera* (Bekal *et al*., 1997; Subbotin *et al*., 2003; Ou *et al*., 2008; Fu *et al*., 2011; Hesar *et al*., 2012). The results reported here did not show any intraspecific polymorphism between Moroccan populations of *H. avenae* based on the ITS sequences. These results are in agreement with Baklawa *et al*. (2015) who found that *H. avenae* populations originating from different localities of Egypt clustered together in the same group and had high similarities to each other. Likewise, Abidou *et al*. (2005) reported the absence of intraspecific polymorphism among French and Syrian populations of *H. avenae*. However, polymorphism among different populations of both *H. avenae* and *H. latipons* was reported previously (Bekal *et al*., 1997; Subbotin *et al.,* 1999; Rivoal *et al*., 2003; Madani *et al*., 2004; Imren *et al*., 2015). Imren *et al*. (2015) showed intraspecific polymorphism among populations of *H. avenae* originating from Eastern Mediterranean region of Turkey based on the ITS sequences. Similarly, Subbotin *et al*. (2003) reported that *H. avenae* populations from Africa and Asia could be distinguished from *H. avenae* populations from Europe based on the ITS sequences. Hesar *et al*. (2012) found that the phylogenetic relationships (full ITS) within *H. avenae*, grouped an Iranian population of *H. avenae* with populations from India and Israel, while European populations of *H. avenae* from Germany, France and Spain formed another cluster. In the study reported here, based on data of the ITS region, the Moroccan populations of *H. avenae* clustered with *H. avenae* populations from Europe and Asia. The data also confirmed previous results in the phylogram presented by Madani *et al*. (2004), in which a Moroccan population of *H. avenae* clustered with populations from France, Turkey and Israel. Moreover, the three *H. latipons* sequences from Morocco obtained from the same population were identical to each other and also to a sequence in GenBank from a *H. latipons* population from Jordan (HM560790).

This is the first report on the presence of *H. latipons* in Morocco. This species has been identified in Mediterranean, eastern and northern European, West Asian and North African cereal-producing regions (Franklin, 1969; Stoyanov, 1982. Subbotin *et al*., 1996; Greco *et al*., 2002; Nicol & Rivoal, 2008). Probably, the species is more widely spread inside and outside Morocco than currently known. Therefore, it might be interesting to re-examine earlier findings of the cereal cyst nematode *H. avenae* in the country.

This is the first report providing the integrated morphometric, morphological and molecular characterization of cereal cyst nematode populations from Morocco. Further investigations are necessary to identify the pathotypes of the *H. avenae* and *H. latipons* populations of the Saiss, Gharb, Chaouia and Zaers regions of Morocco, as well as suitable resistance sources to be used in cereal breeding programmes.

CHAPTER 5

Diversity of root-lesion nematodes (*Pratylenchus* spp.) associated with wheat (*Triticum aestivum* and *T. durum*) in Morocco

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Mokrini, **F.**, Waeyenberge, L., Viaene, N., Abbad Andaloussi, F. & Moens, M. (2016). Diversity of root-lesion nematodes (*Pratylenchus* spp.) associated with wheat (*Triticum aestivum* and *T. durum*) in Morocco. *Nematology* 18 (7), 781-801.

5.1 Introduction

Cereals are exposed to biotic and abiotic stresses. Among the biotic stresses, plant-parasitic nematodes play an important role in decreasing crop yield (Nicol, 2002; Nicol & Rivoal, 2008). The damage they cause to cereals has been estimated at $US80 billion per year (Nicol *et al*., 2011). Root-lesion nematodes (RLN; *Pratylenchus* spp.) are widespread and one of the most important groups of plant-parasitic nematodes (Jones *et al*., 2013). Significant economic losses due to RLN have been reported from Europe (Lasserre *et al*., 1994), Australia (Thompson *et al*., 1993, 2008) and the USA (Smiley *et al*., 2004).

Eight *Pratylenchus* species affect roots of cereals (Rivoal & Cook, 1993). Among them, *P. thornei* Sher & Allen, *P. neglectus* (Rensch) Filipjev & Schuurmans Stekhoven, *P. penetrans* (Cobb) Filipjev & Schuurmans Stekhoven and *P. crenatus* Loof have a worldwide distribution, and sometimes coexist (Nicol *et al*., 2003). In cereal growing areas of Morocco, *Pratylenchus* spp. are the most important plant-parasitic nematodes (Meskine & Abbad Andaloussi, 1992; Mokrini *et al*., 2012). They cause extensive necrosis of the roots with consequent crop losses. *Pratylenchus penetrans* was recovered from 70% of the soil samples taken during a nematode survey of wheat growing areas, with population densities of 32-123 nematodes 100 ml^{-1} of soil and 67-102 nematodes g^{-1} of roots (Mokrini *et al*., 2012).

Traditionally, identification of *Pratylenchus* species relies on morphological characters of the adult stages (Loof, 1991; Handoo *et al*., 2001). However, diversity in morphology and morphometrics among and within the species has been reported frequently as the result of different environmental conditions, including host plant (*e.g.*, Román & Hirschmann, 1969; Doucet *et al*., 2001). This morphological diversity makes diagnosing of *Pratylenchus* species time consuming and difficult to achieve.

Diversity also exists at the genetic level between and within *Pratylenchus* species (Orui, 1996; Uehara *et al*., 1998b; Waeyenberge *et al*., 2000). It allows separation of species on the basis of different DNA fragments and makes molecular characterisation a useful complement to morphological identification (Orui, 1996; Waeyenberge *et al*., 2000; Mizukubo *et al*., 2003). Genetic variation further allows the study of the phylogeny of the pratylenchids (Subbotin *et al.*, 2008) by comparing sequences of the ITS rDNA (*e.g.*, Waeyenberge *et al*., 2000; Mizukubo *et al*., 2003; de la Peña *et al*., 2006) or the D2-D3 segment of 28S rDNA (*e.g.*, Al-Banna *et al*., 2004; De Luca *et al*., 2004; Subbotin *et al*., 2008). Intraspecific variation in the ITS rDNA has been observed in several *Pratylenchus*

species (Uehara *et al*., 1998b; Waeyenberge *et al*., 2000; Mizukubo *et al*., 2003).

Earlier research also revealed differences in pathogenicity between *Pratylenchus* populations; *e.g.*, *P. vulnus* Allen & Jensen on peach almond hybrids and apple rootstocks (Pinochet *et al.*, 1993), *P. coffeae* Goodey on sweet potato (Mizukubo & Sano, 1997) and *P. neglectus* on potato (Hafez *et al.*, 1999). Trinh *et al.* (2011) demonstrated the high pathogenicity of Vietnamese isolates of *P. coffeae* on Arabica coffee. The isolates differed in reproductive fitness, which was linked to their pathogenicity. However, the host is very important to study the virulence of these isolates and to confirm their reproduction fitness. Reproductive fitness is one of the major components of pathogenicity (Shaner *et al*., 1992); it is an important feature for the assessment and understanding of disease reactions of plants to pathogens. To compare the reproductive fitness of *Pratylenchus* populations, the use of *in vitro* monoxenic cultures, such as carrot disk cultures (Moody *et al*., 1973), offers a suitable approach since this technique provides homogenous environmental conditions including a constant temperature.

To our knowledge, Moroccan RLN have only been studied with respect to their morphology (Meskine & Abbad Andaloussi, 1992; Mokrini *et al*., 2009); hence, there is no information available on the diversity of their morphometrics and genetics, nor of their potential pathogenicity. For this reason, we conducted a survey of the major wheat growing areas in Morocco (see Chapter 3), with the following main objectives: *i*) to collect, identify and compare *Pratylenchus* species and populations using morphological, morphometric and molecular approaches including species-specific PCR and sequencing of the D2-D3 28S rDNA expansion segments; and *ii*) to assess the diversity in population dynamics and optimum temperature for reproduction between Moroccan *Pratylenchus* species and populations by examining their *in vitro* reproduction.

5.2 Material and Methods

5.2.1 *Pratylenchus* populations

A nematode survey was conducted in 2011 in four cereal-growing areas of Morocco, *i.e.*, Zaers, Chaouia, Gharb and Saiss (Table 5.1). For each region, 15 to 20 fields were sampled, yielding a total of 75 soil and root samples. Samples were taken where plants showed chlorotic leaves and poor growth. Each sample was composed of 15 subsamples randomly collected in the rhizosphere of several plants at a depth of 5-20 cm. The sample was

thoroughly mixed before *ca* 2000 g was taken to the laboratory. Nematodes were extracted from 100 g of soil and 10 g of roots (when present) using an automated zonal centrifuge (Hendrickx, 1995). The extracts were examined for the presence of the genus *Pratylenchus* using a compound microscope; a total of 17 *Pratylenchus* populations were obtained.

5.2.2 Morphological identification

From each isolate, adults were recovered from carrot disks, fixed (Netscher & Seinhorst, 1969) and transferred to anhydrous glycerin (Seinhorst, 1959). Ten females or males were transferred to a drop of glycerin and covered with a cover slip. The nematodes were identified using the morphometrics and the morphological features of ten females and ten males as suggested by Ryss (1988) and Castillo & Vovlas (2007). Measurements were taken with an Olympus BX51 compound microscope equipped with an Olympus image-capture system and software (Cell^D).

5.2.3 Molecular observations

5.2.3.1 DNA extraction

For each of the populations, DNA was extracted from 1 to 6 individuals separately, depending on the number of nematodes available. Nematodes were transferred to an Eppendorf tube containing 25 µl double distilled water and 25 µl nematode lysis buffer (final concentration: 200 mM NaCl, 200 mM Tris-HCl (pH = 8), 1% mercaptoethanol and 800 µg proteinase K). The tubes were incubated at 65°C for 1.5 h and 99°C for 5 min, consecutively (Holterman *et al*., 2006). A total of 52 DNA extracts were obtained from 17 populations (Table 5.1). They were stored at –20°C or used immediately for DNA amplification.

5.2.3.2 PCR with species-specific primers

The species-specific primers PpenA (5'-TGA CTA TAT GAC ACA TTT RAA CTT G-3') and AB28 (5'-ATA TGC TTA AGT TCA GCG GGA-3') (Waeyenberge *et al*., 2009) together with the universal primers (De Ley *et al*., 1999) D3A (5'-GAC CCG TCT TGA AAC ACG GA-3') and D3B (5'- TCG GAA GGA ACC AGC TAC TA-3') were used to detect *P. penetrans* in all 52 DNA extracts. The DNA extracts not identified as *P. penetrans* were used in a PCR with the species-specific forward primer PTHO (5'-TAG GGC AGT AGG TTG

TCG GC-3') along with the universal reverse primer D3B (Al-Banna *et al*., 2004) to detect *P. thornei*.

To detect *P. penetrans*, 2 µl of DNA extract (see above) were added to the PCR reaction mixture containing 21 µl ddH_2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany) and 1 µM of each of the primers Ppen, AB28, D3A and D3B. The thermal cycler program consisted of 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C, followed by a final elongation step of 8 min at 72°C. For the detection of *P. thornei*, 2 µl of the DNA extract was added to the PCR reaction mixture containing 22 µl ddH_2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany), and 1 µM of both primers PTHO and D3B. The program of the thermal cycler consisted of 8 min at 95°C; 35 cycles of 30 s at 94°C, 45 s at 60°C and 1 min at 72°C, followed by a final elongation step of 8 min at 72°C. Five µl of each PCR product was mixed then with 1 µl of 6× loading buffer (Fermentas Life Sciences, Germany) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis (100 V, 40 min), the gel was stained with ethidium bromide (0.1 µg ml^{-1}) for 20 min, visualised and photographed under UV-light. The remaining PCR product was stored at –20°C.

5.2.3.3 Sequencing of D2-D3 expansion segments

For DNA extracts where the species-specific PCR for both *P. penetrans* and *P. thornei* were negative, the D2-D3 region was amplified with the forward D2A (5'-ACA AGTACC GTG AGG GAA AGT TG-3') and reverse D3B (5'-TCG GAA GGA ACCAGC TAC TA-3') primers according to De Ley *et al*. (1999), and subsequently sequenced. Additionally, to confirm the positive results obtained by the species-specific PCR, we sequenced the PCR-product from one DNA extract of each population identified as *P. penetrans* or *P. thornei,* except for 2 populations of *P. thornei* of which extracts were depleted.

In each case, 2 µl DNA extract (see above) was added to the PCR reaction mixture containing 22 µl ddH_2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany) and 1 µM of both primers (D2A and D3B). The thermal cycler program consisted of 5 min at 95°C; 40 cycles of 30 s at 94°C, 45 s at 55°C and 1 min at 72°C; this was followed by a final elongation step of 7 min at 72°C. After PCR amplification, 5 µl of each PCR product was mixed with 1 µl of 6× loading buffer (Fermentas Life Sciences, Germany) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis (100 V, 40 min) the gel was stained with ethidium bromide (0.1 µg ml^{-1}) for 20 min, visualised and

photographed under UV-light. The remaining PCR product was stored at –20°C. The amplification and electrophoresis process were repeated and both PCR products were pooled for purification. The purification process was done as described by the manufacturer's instructions (Wizard® SV Gel and PCR Clean-Up System Kit, Promega). The purified PCR products were sequenced (Macrogen, The Netherlands) in both directions to obtain overlapping sequences of the forward and reverse DNA strands. The sequences were edited and analysed using the software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall, 1999). Finally, all sequences were blasted in GenBank (Sequin v. 9.00, http://www.ncbi.nlm.nih.gov/) to reveal their identity.

5.2.3.4 Phylogenetic analysis

The obtained sequences were compiled with sequences of *Pratylenchus* species available in GenBank. The sequences were aligned with Clustal W (Thompson *et al.*, 1994) and visually checked. Differences between sequences were estimated using the DNA distance option provided by BioEdit sequence alignment editor (Hall, 1999). The alignment was imported into the software package Mega 5.0 (Tamura *et al*., 2011); after checking 24 different nucleotide substitution models, the model with the lowest BIC score was retained for constructing a 60% consensus Maximum Likelihood tree. *Radopholus similis* (Cobb) Thorne (KF234235) was added as outgroup. Bootstrap analysis with 100 replicates was performed to assess the degree of support for each clade on the tree.

5.2.4 Reproductive fitness

5.2.4.1 Nematode cultures

Starting from single females, four populations of *P. penetrans* (PZ3, PC2, PG18 and PS20), one population of *P. thornei* (PZ2) and one of *P. pseudocoffeae* Mizukubo (PC1) were multiplied *in vitro*. Each population was established on carrot disks (Moody *et al*., 1973), which were inoculated with one gravid female per disk and kept in an incubator at 21-22°C. When needed, the nematodes were extracted from the carrot disks on a Baermann funnel in a mistifier chamber (1950). Vigorous nematode inoculum was available 2 months later.

To assess population dynamics at different times and temperatures, batches of 120 carrot disks of each of the six selected populations were prepared. Twenty gravid females were inoculated per carrot disk and kept in a Petri dish sealed with Parafilm. Disks were

incubated in the dark at 10, 15, 20 or 25°C for 4, 8 or 12 weeks; they were completely randomised in the incubators. After every incubation time, ten carrot disks were used for evaluation of the nematode reproduction. The experiment was repeated to confirm the results.

5.2.4.2 Assessment of nematode reproduction

Nematodes were extracted from the carrot disks at the end of the incubation periods. Living mobile stages were collected after 48 h on a Baermann funnel in a mistifier chamber (OEPP/EPPO, 2013). Then, the same carrot disks were macerated in a blender for 1 min before nematode eggs were extracted using an automated centrifuge (Hendrickx, 1995) to collect the remaining immobile nematodes. The sum of the number of eggs, juveniles, females and males was the final population density (*Pf*) that was used to compute the reproduction factor (*Rf*) = *Pf*/*Pi* (*Pi*: initial nematode population density = inoculum density = 20 females).

5.2.5 Statistical analysis

The morphometric data of males and females were analysed in a canonical discriminant analysis (CDA) to investigate the distinctness of the populations. Data from both experiments on reproductive fitness were not significantly different and were therefore pooled for analysis, resulting in 20 replicates per combination (population × time × temperature). Data were analysed using SPSS 21 software. Numbers of female, male, eggs, juveniles and final total population density were subjected to a three-way ANOVA. Subsequently, the means were separated using Tukey's Honestly Significant Difference test ($P < 0.05$).

5.3 Results

The survey yielded 17 *Pratylenchus* populations (Table 5.1). Ten populations were monospecific for *P. penetrans*, one for *P. thornei*, one for *P. pseudocoffeae*, and three populations contained a mixture of two species (*P. penetrans* and *P. thornei*). Two populations, PZ7 and PZ8, from the Zaers region yielded only eight and five females, respectively. These limited numbers failed to multiply on carrot-disks; hence, morphometrics are not available for these populations. They were molecularly characterised as *P. pinguicaudatus* Corbett.

Table 5.1. Species and populations of *Pratylenchus* collected during a survey in wheat producing areas of Morocco (2011).

Code	Location	Area	**Region**	Morphological identification (**)	Identification using PCR with specific primer		Result of sequencing (**)
					P. penetrans	***P. thornei***	
PZ1 (6)	Ain El Aouda	Rabat	Zaers	Pp, Pth **	+	+ *	Pp, Pth **
PZ2 (6)	Ain El Aouda	Rabat	Zaers	Pth	-	+	Pth
PZ3 (6)	Merchouch	Rabat	Zaers	Pp, Pth	+	+	Pp, Pth
PZ4 (5)	Merchouch	Rabat	Zaers	Pp, Pth	+	+	Pp, Pth
PZ7 (5)	INRA Settat	Settat	Chaouia	/	-	–	Pping
PZ8 (4)	Sid Laaydi	Settat	Chaouia	/	-	–	Pping
PZ12 (1)	Oulad Said	Rabat	Zaers	Pp	+	/	Pp
PZ18 (1)	Oulad Said	Rabat	Zaers	Pp	+	/	Pp
PZ19 (2)	Oulad Said	Rabat	Zaers	Pp	+	–	Pp
PC1 (4)	INRA Settat	Settat	Chauoia	Ppseu	-	–	Ppseu
PC2 (2)	INRA Settat	Settat	Chaouia	Pp	+	–	Pp
PC3 (2)	Berrechid	Berrechid	Chaouia	Pp	+	–	Pp
PC20 (2)	Mediouna	Casablanca	Chaouia	Pp	+	–	Pp
PG18 (2)	Sidi Slimane	Sidi Slimane	Gharb	Pp	+	–	Pp
PS12 (1)	Ain Jmaa	Meknes	Saiss	Pp	+	/	Pp
PS14 (2)	Mhaya	Fes	Saiss	Pp	+	–	Pp
PS20 (1)	Ain Taoujdate	Fes	Saiss	Pp	+	/	Pp

Values in parentheses are the number of nematode specimen used for DNA extraction**. +:** Positive for the mentioned species; **–:** Negative for the mentioned species; /: not checked; ** Pp: *Pratylenchus penetrans*; Pth: *P. thornei*; Pping: *P. pinguicaudatus*; Ppseu: *P. pseudocoffeae*.

5.3.1 Morphology and morphometrics

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

Morphometrics

See Tables 5.2, 5.3.

Description

Female

Body slender and vermiform, straight to slightly curved ventrally when killed. Lip region set off, flat anteriorly with rounded outer margins, bearing three annules. Lateral field marked by four incisures. Stylet robust with rounded knobs, sometimes flattened anteriorly. Excretory pore at 79.6 (74-81.5) µm from anterior extremity (averages of 13 populations), located 2-3 annules posterior to hemizonid. Spermatheca rounded, filled with sperm Post-uterine sac longer than body diam. Vulva located at 78.1 (75-80.4)% of body length (averages of 13 populations). Tail generally cylindrical, 28.3 (26-30.5) µm long. Tail smooth, with hemispherical end, comprising 20-28 annules on ventral side.

Male

Morphologically similar to female, but smaller for all non-sexual characters. Stylet knobs variable in shape. Lateral field with four lines ending on bursa, spicules slender, gubernaculum ventrally curved. Bursa enclosing tail.

Remarks

This species (13 populations) was found in the four cereal growing areas of the country. The morphological features and the morphometrics of these populations were similar to other populations described by Loof (1960), Román & Hirschmann (1969) and Townshend (1991), except for variation of the a-ratio of populations PZ12 and PZ18. Females of these two populations showed the greatest a-ratio of all the females examined. The populations

showed different degrees of variability in morphology within the population. The most variable character was the tail terminus of females of *P. penetrans*; within population PC2, this feature ranged from smooth to distinctly crenate. In three out of 17 populations *P. penetrans* was found mixed with *P. thornei.*

5.3.1.2 *Pratylenchus thornei* Sher & Allen, 1953

Morphometrics

See Table 5.4.

Description

Female

Lip region relatively high, composed of three annules, not offset from body. Body contracted ventrally posterior to vulva. Lateral field marked by four incisures. Stylet moderately stout, with rounded to anteriorly flattened basal knobs. Tail terminus bluntly rounded to truncate. Vulva at 76.7(74-78) % of body length (average of four populations). Spermatheca difficult to observe, without spermatozoa when visible. Excretory pore 83 (80-85) µm (average of four populations) posterior to head, immediately posterior to hemizonid. Tail 18-25 annules, bluntly rounded, without striations around terminus.

Male

Absent in each of the four populations.

Remarks

This species was detected in four populations (PZ1, PZ2, PZ3 and PZ4). The populations were morphologically and morphometrically similar to populations described previously (Sher & Allen, 1953; Handoo & Golden, 1989; Pourjam *et al*., 1997). The morphometrics of females of these populations matched the original description with the

exception of PZ1, PZ2 and PZ4, for which we observed a c-ratio different from the original description. A similar observation was made for the b-ratio of females of PZ2.

5.3.1.3 *Pratylenchus pseudocoffeae* Mizukubo, 1992

Morphometrics

See Table 5.5

Description

Female

Body straight with posterior half slightly curved ventrally when heat-relaxed. Lip region with three annuli, relatively low, flattened, 2-3 µm high, 6-9 µm wide. Stylet knobs massive, shape variable but mostly broadly rounded or flattened anteriorly. Excretory pore at 87 (86-90) µm, located slightly anterior to level of cardia. Hemizonid flat, 2-3 annuli long, at level of excretory pore. Vulva located at 81.8 (78-83.4)% of body length. Spermatheca oblong, packed with sperm. Post-vulval uterine sac less than two vulval body diam. long. Tail subhemispherical or bluntly pointed with smooth terminus.

Male

Body generally straight when heat-relaxed, similar to female except for sexual dimorphism. Stylet length slightly shorter than in female (Table 5.5). Stylet knob shape mostly broadly rounded. Labial region with three annuli. Spicules arcuate, slightly longer than stylet. Gubernaculum simple.

Remarks

Only one population of *P. pseudocoffeae* was detected (Settat, region Chaouia). The morphometrics and morphological characters corresponded to those reported by Mizukubo (1992).

Table 5.2. . Morphometric characters of females of *Pratylenchus penetrans* populations from different localities in Morocco (n = 10). All measurements are in µm and in the form: mean ± s.d. (range).

	Populations													
Characters	PZ1	PZ4	PZ18	PZ19	PZ3	PZ12	PC2	PC3	PC20	PG18	PS12	PS14	PS20	(Loof, 1960)
L	605 ± 33.7 (567-678)	592 ± 22.5 (550-622)	612 ± 53 (530-693)	585 ± 50 (512-646)	605 ± 26.4 (570-655)	601 ± 66 (517-710)	556 ± 43 (490-612)	630 ± 37 (586-710)	556 ± 30 (590-685)	601 ± 49 (512-690)	578 ± 52 (461-643)	569 ± 41 (490-621)	586 ± 30 (522.3-621)	343-811
A	32.2 ± 2.1 (29.3-36)	31.1 ± 2.7 (26.7-35.1)	33.1 ± 2.1 (30.1-36.8)	31.8 ± 2.3 (27.8-33.9)	33.1 ± 1.5 (30-35)	32.8 ± 3.2 (28.4-37.9)	29.2 ± 1.6 (26.7-31.8)	30.6 ± 2.1 (27.4-34)	30.9 ± 2.2 (27.3-34.9)	31.3 ± 2.8 (27.1-35.2)	29.7 ± 4.1 (24-34.8)	29.9 ± 2.3 (27-33.9)	30.8 ± 3.1 (26.3-35.8)	19-32
B	6.6 ± 0.4 (6-7.6)	6.6 ± 0.2 (6.3-6.8)	7.2 ± 0.8 (6-8.7)	7 ± 0.6 (5.9-7.8)	6.9 ± 0.4 (6.4-7.9)	7.1 ± 0.7 (5.9.-8.5)	6.6 ± 0.7 (5.4-7.5)	7.7 ± 0.6 (7.1-8.7)	7.7 ± 0.6 (6.8-8.9)	7.2 ± 0.6 (6.2-8.5)	6.8 ± 0.6 (5.4-7.6)	6.9 ± 0.6 (6.1-7.6)	7.3 ± 0.5 (6.6-8)	5.3-7.9
b’	4.9 ± 0.2 (4.5-5.3)	4.4 ± 0.1 (4.3-4.6)	4.7 ± 0.4 (4.1-5.4)	4.6 ± 0.4 (3.9-5.1)	4.9 ± 0.3 (4.5-5.3)	4.9 ± 0.3 (4-5.8)	4.4 ± 0.6 (4-5)	5 ± 0.3 (4-5.9)	4.4 ± 0.3 (4-5)	4.7 ± 0.4 (4-5.8)	4.5 ± 0.4 (4-5)	4.5 ± 0.5 (4-5)	4.8 ± 0.4 (4-5.6)	
C	21.5 ± 1.5 (18.7-23.6)	21.7 ± 1.8 (19.1-25.6)	22.9 ± 3.3 (17.5-27.4)	20.7 ± 3.1 (17.3-28.3)	21.9 ± 1.7 (19.7-24.3)	21.8 ± 3 (18.6-26.9)	20 ± 1.5 (17.5-23)	20.6 ± 1.4 (17.3-22.1)	24.9 ± 5.8 (20-36.2)	19.7 ± 1.2 (17.9-21.4)	19 ± 1.1 (16.9-20.7)	19.6 ± 1.7 (15.7-22)	21.2 ± 1.6 (18.5-23.4)	15-24
c'	2.2 ± 0.2 (1.8-2.6)	2.1 ± 0.2 (1.7-2.5)	2.2 ± 0.2 (1.9-2.6)	2.3 ± 0.2 (2.1-2.9)	2.3 ± 0.2 (1.8-2.5)	2.2 ± 0.2 (1.8-2.4)	2 ± 0.2 (1.8-2.3)	2.3 ± 0.3 (1.7-2.8)	2.2 ± 0.3 (1.8-2.9)	2.1 ± 0.3 (1.5-2.7)	2.2 ± 0.2 (1.8-2.7)	1.9 ± 0.1 (1.7-2.1)	2 ± 0.2 (1.7-2.3)	
V	80.4 ± 1.1 (78-82)	79.2 ± 0.7 (77-80)	80.1 ± 0.7 (78-82)	78.7 ± 1.3 (76-80)	79.9 ± 0.5 (77-81)	78.7 ± 1.1 (76-82)	75 ± 1.3 (74-79)	77.8 ± 0.9 (76-80)	78.5 ± 1.7 (77-81)	78 ± 1.2 (76-80)	76.2 ± 0.3 (75-78)	76.2 ± 1.6 (75-78.9)	77 ± 2.1 (75-80)	75-84
Stylet length	15.6 ± 0.7 (14.6-17.2)	15.7 ± 0.5 (15-16.2)	16.3 ± 0.6 (15.8-17.4)	15.9 ± 0.5 (14.9-16.8)	16.3 ± 0.5 (15.6-17.4)	16 ± 0.4 (15.2-16.7)	15.6 ± 0.5 (14.8-16.3)	16.1 ± 0.9 (14.8-17.5)	16 ± 0.8 (14.8-17.2)	16.2 ± 0.7 (15.1-17.4)	16.1 ± 0.5 (15.2-17.2)	15.6 ± 0.5 (15-16.8)	16.3 ± 0.5 (15.6-17.2)	15-17
DGO	2.5 ± 0.3 (2.2-3.1)	2.9 ± 0.3 (2.4-3.3)	2.6 ± 0.2 (2.1-2.9)	2.7 ± 0.3 (2.1-3.2)	2.7 ± 0.2 (2.3-3.1)	2.6 ± 0.2 (2.1-2.9)	2.7 ± 0.4 (2.1-3.4)	2.5 ± 0.4 (2.1-3.3)	2.3 ± 0.1 (2.1-2.6)	2.8 ± 0.3 (2.3-3.4)	2.6 ± 0.3 (2.1-3.1)	2.8 ± 0.3 (2.5-3.2)	2.7 ± 0.3 (2.2-3.1)	
Max. body diam.	19 ± 0.8 (17.6-20.4)	19.1 ± 1.7 (17.6-21)	18.4 ± 0.8 (17.5-19.9)	18.3 ± 0.5 (17.3-19.2)	18.2 ± 0.6 (17.5-19.2)	18.3 ± 0.6 (17.5-19.7)	19 ± 0.7 (17.8-20.3)	20.6 ± 1.6 (17.3-22.7)	20.2 ± 1 (18.9-22.5)	19.2 ± 1.5 (16.7-21.5)	19.6 ± 2.2 (17-22.7)	19.1 ± 1.8 (16.7-22)	19.1 ± 1.5 (17.4-21.9)	
Excr. pore	81.2 ± 1.7 (78.8-84.2)	80.9 ± 1.4 (79.3-84.2)	81.2 ± 0.8 (79.4-82.3)	81.1 ± 1.1 (79.5-83.1)	81.5 ± 0.5 (80.6-82.4)	81.3 ± 1.2 (79.2-83)	75.7 ± 2.1 (72-78)	74 ± 2.5 (70-79)	75.3 ± 1.4 (73-78)	81.4 ± 1.2 (79.7-83)	81.2 ± 1.5 (78.2-83)	80.7 ± 1.6 (78.3-83)	80.3 ± 2.7 (76.3-85)	
L/excr. pore	7.4 ± 0.4 (7-8.3)	7.3 ± 0.2 (6.8-7.6)	7.5 ± 0.7 (6.5-8.7)	7.2 ± 0.6 (6.2-8)	7.4 ± 0.3 (6.9-7.9)	7.4 ± 0.8 (6.3-8.8)	7.3 ± 0.5 (6.3-7.9)	8.5 ± 0.6 (7.7-9.7)	8.3 ± 0.4 (7.6-8.9)	7.4 ± 0.6 (6.1-8.5)	7.1 ± 0.7 (5.6-8.1)	7.1 ± 0.5 (6-7.8)	7.3 ± 0.3 (6.6-7.8)	
Pharynx length	82.7 ± 5.7 (75.4-92.4)	88.9 ± 3.5 (84.2-94.3)	85.1 ± 4.2 (77.2-90.1)	83 ± 3.4 (79-89.3)	86.6 ± 3.8 (78.4-93.1)	84.2 ± 4.1 (78.4-88.4)	83.9 ± 3.8 (76.3-90.3)	81 ± 4.6 (74.3-87)	81.2 ± 4.4 (74.1-87)	82.8 ± 3.5 (76-87.1)	84.3 ± 3.1 (79.3-89.1)	82.5 ± 3.2 (78.2-87)	80.1 ± 3 (76.1-86.7)	
Pharyngeal overlap	39.3 ± 5.7 (28.5-46.2)	42.7 ± 2.9 (36.4-47)	43.2 ± 2.4 (39-47.2)	44.4 ± 2.2 (39.5-47.3)	34.7 ± 4.7 (28.4-44)	38.3 ± 3.8 (32.5-43.3)	42.6 ± 3.2 (38.2-47.1)	45 ± 2.1 (41.2-48.2)	44.6 ± 1.9 (42.2-47.6)	45.4 ± 1.7 (42-47.2)	42.5 ± 2.1 (39-46.3)	44.3 ± 1.7 (41.8-47)	42.7 ± 2.6 (39.3-47.2)	
Ovary	209.6 ± 16.6 (190-240)	200 ± 6.6 (192-211)	216 ± 7.6 (204-226)	232 ± 4.1 (227-241)	233 ± 5.6 (220-240.3)	229 ± 5.8 (223-239)	196.9 ± 3.5 (192-203.2)	193 ± 1.9 (190-197)	193.9 ± 2.5 (192-199)	204 ± 6.8 (192-213)	234 ± 10.2 (225-253)	234 ± 5.2 (223-241)	226 ± 9.6 (213-241)	
Post-uterine sac	28 ± 2.4 (25.1-32.3)	28.1 ± 1.9 (25.5-31.4)	26.5 ± 0.9 (24.7-27.5)	29.8 ± 0.7 (28.9-31.4)	30.9 ± 1.6 (28.8-34.4)	30.5 ± 1.3 (28.6-32.6)	29.2 ± 2.2 (25.5-32.3)	26.4 ± 1.8 (23.5-28.5)	26.7 ± 2.5 (22.1-29.8)	26.9 ± 2.9 (23.4-31.4)	27.3 ± 1.8 (23.4-29.4)	26.9 ± 1.7 (23.5-29)	26.2 ± 1.7 (22.1-28.4)	
Tail	28.3 ± 2.7 (24.5-31.5)	27.5 ± 2.8 (23.3-32.4)	27.1 ± 3.1 (23.4-33.2)	28.8 ± 4.6 (18.2-33.8)	27.7 ± 2.7 (23.8-32.8)	27.7 ± 1.8 (24.3-29.8)	28 ± 2.3 (23.9-31.5)	29.6 ± 2.9 (25.9-34.9)	26 ± 5.2 (18.4-32.5)	30.5 ± 2.9 (27.4-35.2)	30.4 ± 2.4 (27.3-35.4)	29.1 ± 1.9 (26.3-32)	27.7 ± 1.1 (25.8-29.8)	

Vulva to anus distance	89.8 ± 5.1 (82.9-101)	98.3 ± 6.1 (89.2-105)	93.2 ± 7.2 (83.2-103)	95 ± 6 (87.3-104)	93.5 ± 4.6 (85.2-97.8)	99.1 ± 6.3 (86.9-109)	110.6 ± 6.5 (98.2-119)	110.2 ± 6.1 (102-119)	108.8 ± 4.6 (101-116)	107.3 ± 8.3 (93.2-117)	105.8 ± 8.9 (92.4-117)	105 ± 7.2 (93-113)	102.4 ± 9.8 (91.3-117)
Lip diam.	7.6 ± 0.4 (7-8.2)	7.2 ± 0.4 (6.5-8.1)	6.6 ± 0.5 (6.1-7.7)	7.7 ± 0.4 (6.8-8.1)	7.1 ± 0.6 (6.1-8.1)	6.9 ± 0.4 (6.5-8.1)	7.2 ± 0.2 (6.8-7.7)	7 ± 0.6 (6.2-8.1)	7.2 ± 0.5 (6.5-8.4)	6.9 ± 0.5 (6.2-8.1)	7.6 ± 0.3 (7.2-8.3)	7.5 ± 0.6 (6.6-8.3)	7.3 ± 0.2 (6.8-7.5)
Lip height	2.4 ± 0.3 (2.1-3.1)	2.6 ± 0.2 (2.1-3.1)	2.4 ± 0.3 (2.1-3.1)	2.1 ± 0.2 (1.8-2.4)	2.4 ± 0.3 (2.1-2.9)	2.3 ± 0.3 (1.9-2.9)	2.4 ± 0.2 (2.1-2.8)	2.3 ± 0.2 (1.9-2.9)	2.6 ± 0.4 (2.1-3.2)	2.9 ± 0.2 (2.5-3.2)	2.4 ± 0.3 (1.9-2.8)	2.2 ± 0.2 (1.9-2.6)	2.4 ± 0.3 (1.9-2.8)

For codes for populations, see Table 5.1.

Table 5.3. Morphometric characters of males of *Pratylenchus penetrans* populations from different localities in Morocco (n = 10). All measurements are in µm and in the form: mean ± s.d. (range).

	Populations													
Characters	PZ1	PZ4	PZ18	PZ19	PZ3	PZ12	PC2	PC3	PC20	PG18	PS12	PS14	PS20	(Loof , 1960)
L	495 ± 22.6 (455-522)	502 ± 11.7 (481-521)	501 ± 7.4 (490-511)	516 ± 18.3 (494-560)	501 ± 5.2 (493-513)	501 ± 4.5 (495-508)	509 ± 8.2 (498-523)	499 ± 13.8 (481-531)	502 ± 8.5 (490-512)	499 ± 7.6 (485-509)	498 ± 15.5 (467-509)	509 ± 6.1 (498-519)	502 ± 6 (490-511)	305-574
A	27.5 ± 1.3 (25-29)	27.4 ± 1.1 (25.8-29)	27.7 ± 0.8 (26.5-29)	28.7 ± 1.6 (26-32)	27.9 ± 1.2 (26-29)	27.9 ± 0.6 (26.8-29.2)	28.4 ± 1.2 (27-30.2)	28.7 ± 1.3 (26.5-30.1)	28.2 ± 1.2 (26-29.4)	27.4 ± 0.7 (26-28.2)	27.3 ± 1.2 (25.4-29.4)	28.4 ± 0.8 (26.9-29.9)	27.8 ± 0.6 (26-29)	23-34
B	7.3 ± 0.3 (6.8-7.8)	6.8 ± 0.3 (6.4-7.5)	6.8 ± 0.3 (6.3-7.2)	7.7 ± 0.4 (7-8.6)	7.4 ± 0.2 (7-7.7)	7.6 ± 0.2 (7.3-8)	7.6 ± 0.2 (7.3-8)	7.3 ± 0.3 (6.9-8.1)	7.4 ± 0.3 (7-7.9)	7.3 ± 0.2 (7-7.6)	7.4 ± 0.3 (6.9-7.7)	7.5 ± 0.3 (7.1-8)	7.3 ± 0.2 (7-7.6)	5-8
b'	4.4 ± 0.2 (4-4.7)	4.4 ± 0.1 (4.2-4.7)	4.3 ± 0.1 (4.2-4.5)	4.6 ± 0.2 (4.4-4.9)	4.4 ± 0.1 (4-4.7)	4.5 ± 0.1 (4.4-4.7)	4.6 ± 0.1 (4.4-4.8)	4.5 ± 0.2 (4-4.9)	4.5 ± 0.1 (4.4-4.7)	4.5 ± 0.1 (4.3-4.7)	4.5 ± 0.2 (4.2-4.7)	4.5 ± 0.1 (4.2-4.7)	4.4 ± 0.1 (4-4.7)	
c	19.6 ± 1.3 (17-21.5)	20.3 ± 1.2 (18.3-22)	19.7 ± 1.1 (18.2-21.6)	20.2 ± 1.3 (17.8-22.9)	19.7 ± 0.6 (18-20.4)	20 ± 0.8 (18.8-21.5)	20.3 ± 0.7 (19-22)	19.9 ± 0.9 (18-22)	19.9 ± 0.8 (18.5-21)	20.1 ± 0.4 (19-21)	19.5 ± 0.9 (18-21.1)	20.1 ± 0.4 (19.6-21)	19.3 ± 0.8 (18-21)	16-22
c'	1.9 ± 0.1 (1.8-2.2)	2.1 ± 0.2 (1.9-2.4)	2.2 ± 0.2 (1.8-2.5)	2 ± 0.1 (1.8-2.4)	2 ± 0.1 (1-2.2)	2 ± 0.1 (1.8-2.2)	2 ± 0.1 (1.8-2.3)	2.1 ± 0.1 (1.9-2.2)	2.1 ± 0.1 (1.9-2.3)	2 ± 0.2 (1.8-2.4)	2,1 ± 0.2 (1.9-2.4)	1,9 ± 0.1 (1.8-2.2)	2 ± 0.1 (1-2.2)	
Stylet length	15.6 ± 0.3 (14.9-16)	15.5 ± 0.6 (14.9-16.3)	15.8 ± 0.5 (14.8-16.5)	16 ± 0.5 (14.9-17)	16 ± 0.4 (15-16.8)	16 ± 0.6 (15.3-17.1)	15.5 ± 0.5 (14.8-16.2)	15.2 ± 0.6 (14.3-16.1)	15.9 ± 0.5 (15-16.9)	15.4 ± 0.4 (14.9-16)	15.5 ± 0.5 (14.9-16.2)	15.1 ± 0.5 (14.3-16.1)	15.6 ± 0.4 (14.2-16.4)	
DGO	1.9 ± 0.2 (1.7-2.2)	1.8 ± 0.1 (1.6-2.1)	1.9 ± 0.1 (1.8-2.2)	2 ± 0.1 (1.8-2.2)	1.9 ± 0.1 (1-2)	1.9 ± 0.1 (1.7-2.1)	1.9 ± 0.1 (1.7-2.1)	1.9 ± 0.1 (1.7-2.1)	2 ± 0.1 (1.8-2.1)	1.9 ± 0.1 (1.7-2.1)	1.8 ± 0.1 (1.6-2.1)	1.9 ± 0.2 (1.7-2.2)	1.9 ± 0.1 (1-2)	
Max. body diam.	18.2 ± 0.5 (17.4-19)	18.3 ± 0.5 (17.8-19.5)	18 ± 0.5 (17.2-19.3)	18 ± 0.4 (17.2-18.8)	17.9 ± 0.7 (16-19)	17.9 ± 0.4 (17.4-18.7)	17.9 ± 0.6 (17-18.6)	17.4 ± 0.6 (16.7-18.4)	17.8 ± 0.7 (16.8-19)	18.2 ± 0.3 (17-18.9)	18.2 ± 0.4 (17-18.8)	17.9 ± 0.5 (17.4-19)	18 ± 0.4 (17-18.5)	
Excr. pore	77.3 ± 2.5 (74-81)	75.5 ± 3.6 (69.4-80.4)	75.2 ± 2.7 (71-78.4)	76.6 ± 3.4 (70-81)	76.1 ± 3 (71-80)	73.7 ± 2.5 (69.4-77.4)	72.8 ± 1.8 (70.4-75.3)	73.4 ± 1.9 (70-76)	76.4 ± 4.9 (70.6-87)	73.2 ± 2.5 (69.5-77)	72.8 ± 2.7 (69-77)	72.2 ± 1.9 (69.7-74.6)	72.9 ± 2.8 (69-77)	
L/excr. pore	6.4 ± 0.4 (5.7-6.9)	6.6 ± 0.4 (6.1-7.5)	6.7 ± 0.2 (6.3-7.1)	6.7 ± 0.4 (6.3-7.4)	6.6 ± 0.3 (6.2-7.2)	6.8 ± 0.2 (6.5-7.2)	7 ± 0.2 (6.7-7.3)	6.8 ± 0.2 (6.5-7.4)	6.6 ± 0.4 (5.6-7.1)	6.8 ± 0.3 (6.4-7.2)	6.8 ± 0.4 (6-7)	7 ± 0.2 (6.6-7.3)	6.9 ± 0.2 (6.5-7.4)	
Pharynx length	67.6 ± 3.3 (62-72.6)	73.8 ± 2.8 (69.8-78.2)	73.7 ± 4.1 (68.3-80.2)	67.3 ± 2.8 (60-70)	67.2 ± 1.2 (63-70)	66.3 ± 1.5 (62.8-67.8)	67.3 ± 2.2 (63.8-70.1)	68.1 ± 2.3 (63.6-71.6)	68.2 ± 2.3 (63-70.8)	68.2 ± 2.2 (65-71)	67.7 ± 2.8 (63-71)	67.7 ± 3 (62.7-71)	68.4 ± 1.2 (66-70)	
Pharyngeal overlap	43.7 ± 2.2 (41-45.6)	40.2 ± 2.6 (34.4-44.2)	41.4 ± 2.7 (37.1-45.6)	44.8 ± 2.4 (39-47)	45.4 ± 2.1 (42-49)	44.1 ± 1.2 (41.8-45.8)	43.5 ± 3.2 (38.7-47)	42.9 ± 1.7 (39.8-45.8)	43.3 ± 2.3 (38-45.7)	42.7 ± 2.1 (39-45.6)	43.3 ± 2.1 (39-45)	43.9 ± 1.1 (42.7-45.9)	44.4 ± 2.4 (39-47)	
T	41.9 ± 2.8 (38-46)	41.5 ± 1.3 (40.2-44.1)	40.7 ± 0.5 (40-41.5)	40 ± 1.5 (37-42)	41.8 ± 1.3 (40-44)	41.6 ± 1.2 (39.9-43.5)	41.4 ± 0.9 (39-42.5)	41.6 ± 1.4 (39-43)	40.9 ± 1 (39-42)	41.2 ± 1.1 (39-42.6)	41.9 ± 1.2 (40.1-44.4)	41.2 ± 0.8 (40-42.9)	41.6 ± 1.2 (39.1-42.8)	
Testis	207 ± 5.7 (199-215)	208 ± 4 (201-216)	203 ± 3.6 (199-211)	206 ± 4.8 (199-212)	209.5 ± 6.1 (201-220)	208 ± 5.6 (201-216)	211 ± 2.7 (208-217)	207 ± 5.3 (201-218)	205.7 ± 3.3 (201-210)	205 ± 5.1 (196-211)	208 ± 4.7 (199-213)	209 ± 4.3 (203-219)	208 ± 4.7 (199-213)	
Tail length	25.3 ± 1 (23.9-27)	24.7 ± 1.5 (22.2-27.1)	25.2 ± 1.5 (23-27)	25.6 ± 1.2 (23.9-27.8)	25.5 ± 0.9 (24-27)	25 ± 1 (23.7-26.7)	25.1 ± 0.7 (23.8-26.4)	25.1 ± 0.8 (23.9-26)	25.2 ± 1 (23-27.6)	24.8 ± 0.5 (23-25.8)	25.5 ± 1.4 (23.5-27.5)	25.3 ± 0.5 (24.6-26)	25.9 ± 1.1 (23-27)	
Lip diam.	7.1 ± 0.1 (6.9-7.4)	7.1 ± 0.2 (6.8-7.4)	7.1 ± 0.3 (6.5-7.6)	7.1 ± 0.1 (6.8-7.3)	7 ± 0.2 (6.7-7.2)	7 ± 0.1 (6.8-7.2)	6.9 ± 0.2 (6.6-7.3)	7.1 ± 0.2 (6.8-7.5)	7.1 ± 0.2 (6.8-7.5)	7.1 ± 0.2 (6.9-7.4)	7 ± 0.3 (6-7.5)	7.1 ± 0.1 (6.9-7.4)	7 ± 0.2 (6-7.6)	
Lip height	1.9 ± 0.3 (1.6-2.6)	2.4 ± 0.4 (1.8-2.9)	2.1 ± 0.2 (1.8-2.5)	2.1 ± 0.2 (1.8-2.4)	2.3 ± 0.2 (1.9-2.5)	2.1 ± 0.2 (1.8-2.4)	2.2 ± 0.2 (1.9-2.5)	2.3 ± 0.2 (1.8-2.5)	2.2 ± 0.2 (1.8-2.6)	2.2 ± 0.2 (1.9-2.5)	2.2 ± 0.2 (2-2.5)	2.2 ± 0.2 (1.9-2.5)	2.2 ± 0.2 (1.7-2.5)	

Spicule	14.7 ± 1.1 (13-16.3)	14.7 ± 1.1 (12.9-16.4)	14.9 ± 0.7 (14-16.6)	14.9 ± 0.6 (13.6-15.9)	14.8 ± 0.6 (13.4-15)	14.3 ± 0.7 (13-16.4)	14.5 ± 0.6 (13.8-15.3)	14.3 ± 0.6 (13.4-15.1)	14.6 ± 0.4 (13-15.3)	14.9 ± 0.6 (13-15.8)	15.2 ± 0.6 (14.5-16.3)	15.1 ± 0.6 (13.5-15.7)	15.5 ± 0.7 (14.2-16.4)	14-17
Gubernaculum	3.9 ± 0.2 (3.6-4.3)	4.8 ± 0.1 (4.6-5)	4.3 ± 0.4 (3.8-5.1)	4.3 ± 0.3 (3.9-4.7)	4.3 ± 0.3 (4-5)	4.1 ± 0.3 (3.8-4.6)	4.3 ± 0.2 (3.8-4.6)	4.1 ± 0.2 (3.8-4.6)	4.2 ± 0.3 (3.7-4.6)	4 ± 0.2 (3.8-4.4)	4.1 ± 0.1 (3.9-4.3)	4.2 ± 0.2 (3.9-4.7)	4.5 ± 0.2 (3.8-4.9)	3.9-4.2

For codes for populations, see Table 5.1.

Table 5.4. Morphometrics of females of four *Pratylenchus thornei* populations collected in Morocco (n = 10). All measurements are in µm and in the form: mean ± s.d. (range).

	Populations				
Characters	PZ1	PZ2	PZ3	PZ4	Sher & Allen (1953)
L	522 ± 55 (429-595)	603 ± 17.9 (571-626)	554 ± 38.2 (468-601)	550 ± 30.3 (489-608)	450-770
A	30 ± 5.7 (21-39)	32.8 ± 2.2 (30.1-34.6)	31.5 ± 2.6 (28.6-36.7)	31.4 ± 2.8 (27-36)	26-36
B	7.4 ± 0.6 (6.5-8.1)	8.8 ± 0.3 (8.3-9.2)	7.8 ± 0.7 (6.4-8.7)	7.5 ± 0.5 (6.8-8.5)	5.5-8
b'	4.8 ± 0.7 (3.7-5.8)	4.8 ± 0.3 (4.3-5.2)	4.7 ± 0.5 (3.9-5.5)	5.1 ± 0.4 (4.7-5.8)	
C	16.7 ± 1.8 (14.5-20.2)	20.1 ± 1.6 (17.8-22.9)	17.8 ± 1.5 (15.9-20.5)	17.7 ± 1.6 (15.9-20.4)	18-22
c'	2 ± 0.2 (1.7-2.4)	1.9 ± 0.2 (1.7-2.3)	1.9 ± 0.2 (1.6-2.3)	2.2 ± 0.2 (1.9-2.5)	
V	74.4 ± 2.6 (70.4-79.1)	78.5 ± 1.5 (75.5-80.4)	77.4 ± 1.7 (73.6-79.8)	76.8 ± 1.9 (73.4-79.7)	73-80
Stylet length	17.2 ± 1.1 (15.5-18.5)	16.1 ± 1.1 (15-18.3)	16.2 ± 0.4 (15.7-16.8)	17.5 ± 0.9 (15.5-18.5)	17-19
DGO	2 ± 0.1 (1.9-2.2)	2.1 ± 0.1 (1.9-2.4)	2.1 ± 0.1 (1.9-2.3)	2.1 ± 0.1 (1.8-2.3)	
Max. body diam.	17.7 ± 2.2 (13-19.8)	18.4 ± 0.9 (16.9-19.8)	17.6 ± 1.3 (15.7-19.7)	17.6 ± 0.9 (16.3-19.3)	
Excr. pore	80.8 ± 3.1 (75.4-86.3)	84.2 ± 2.6 (79.3-87.2)	84.9 ± 3 (79.3-88.2)	82 ± 2.4 (78.4-86.7)	
L/excr. pore	6.5 ± 0.7 (5.2-7.5)	7.1 ± 0.3 (6.6-7.7)	6.5 ± 0.5 (5.4-7.6)	6.7 ± 0.5 (5.6-7.6)	
Pharynx length	70.6 ± 3.3 (63.7-74.4)	68.4 ± 2.3 (64.3-71.5)	70.7 ± 2.4 (66.8-74.6)	73.5 ± 2.8 (69.8-78.3)	

Pharyngeal overlap	40.7 ± 3 (34-44.1)	40.1 ± 2.2 (36.4-43.5)	41.2 ± 1.8 (37.8-44.2)	43.4 ± 2.6 (39.8-46.7)
Post-uterine sac	26.2 ± 0.5 (25.3-27)	26.3 ± 0.5 (25.4-27.4)	26.4 ± 0.5 (25.7-27.1)	26.3 ± 0.5 (25.7-27.3)
Tail	31.1 ± 2.2 (28.5-35)	30.2 ± 2.5 (26.3-34)	31.1 ± 2.2 (27.9-34.2)	31.2 ± 2.4 (28.7-34.5)
Vulva to anus distance	101 ± 8.1 (88-111)	99 ± 6.6 (91-110)	93 ± 4.9 (87-103)	95 ± 6.6 (86-107)
Lip diam.	7.7 ± 0.2 (7.4-8.1)	7.7 ± 0.2 (7.4-8)	7.6 ± 0.2 (7.4-7.9)	7.7 ± 0.1 (7.5-7.9)
Lip height	2.7 ± 0.1 (2.6-3)	2.8 ± 0.1 (2.5-3)	2.7 ± 0.1 (2.6-2.9)	2.7 ± 0.2 (2.5-3)

For codes for populations, see Table 5.1.

Table 5.5. Morphometric characters of female and male *Pratylenchus pseudocoffeae* population PC1 collected in Morocco. All measurements are in µm and in the form: mean ± s.d. (range).

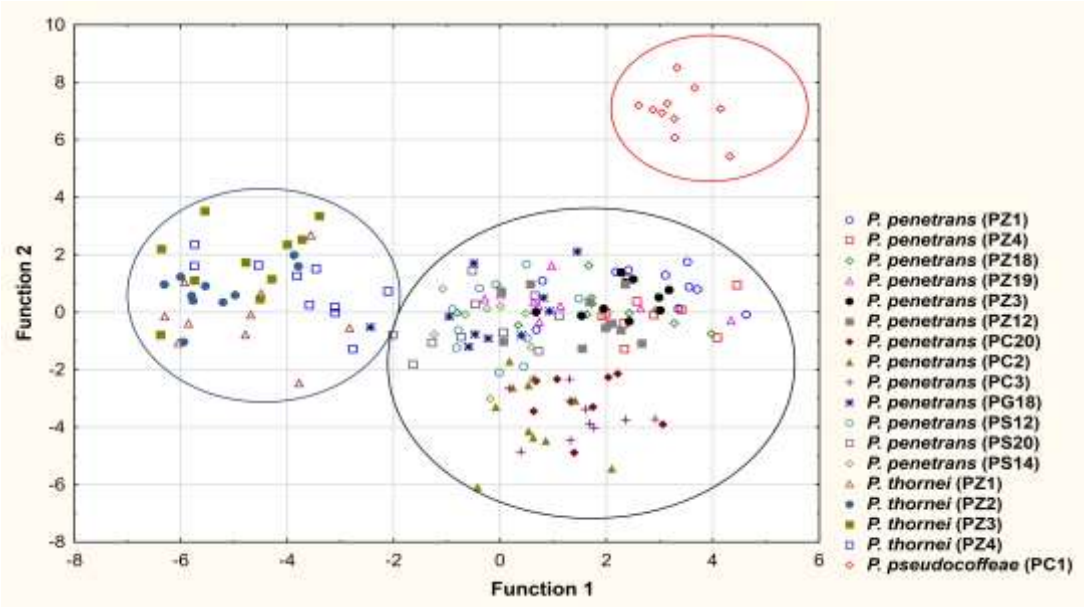
	Males		Females	
Characters	(n = 10)	Mizukubo (1992) (n = 11)	PC1 (n = 10)	Mizukubo (1992) (n = 50)
L	480 ± 16 (451-502)	490 (390-540)	508 ± 14.9 (480-523)	510 (410-620)
a	30.8 ± 1.3 (28-32)	30.6 (25.6-37)	27.8 ± 2.3 (25-30.7)	27.5 (22.6-32.1)
b	5.9 ± 0.2 (5.6-6.2)	6.1 (4.6-6.9)	5.9 ± 0.2 (5.3-6.2)	5.8 (4.7-7)
b'	3.3 ± 0.1 (3.2-3.4)	3.3 (2.9-3.8)	3.7 ± 0.1 (3.5-3.9)	3.1 (2.6-3.4)
c	19.9 ± 2 (17.4-24.5)	20.3 (18.1-23.9)	20.1 ± 0.8 (18.2-20.8)	19.3 (18-20)
c'	1.9 ± 0.1 (1.7-2.2)	2.4 (1.8-2.9)	2.4 ± 0.1 (2.2-2.7)	2.4 (1.8-2.5)
V	-	-	81.8 ± 0.4 (78-83.4)	81 (79-82)
Stylet length	14.8 ± 0.4 (14-15.5)	15 (14-15.5)	16 ± 0.4 (15.4-16.6)	16 (15-17)
DGO	2.7 ± 0.3 (2.2-3.2)	-	2.5 ± 0.2 (2.1-2.8)	2.5 (2.1-2.8)
Max. body diam.	15.5 ± 0.4 (15-16.1)	-	17.6 ± 1.6 (15.9-20.8)	-
Excr. pore	83.4 ± 0.2 (82.4-86.1)	-	87.8 ± 1.2 (86.2-90.1)	-
L/excr. pore	5.6 ± 0.2 (5.2-5.9)	-	5.7 ± 0.1 (5.4-6.1)	-
Pharynx length	81.6 ± 2.9 (76.4-85)	-	85.7 ± 1.2 (81.4-90.1)	-
Vulva to anus distance	-	-	66.5 ± 3.6 (61.3-72.3)	-
Tail	24.3 ± 2 (20-27.4)	24	25.2 ± 0.8 (24-26.4)	-
Spicule	17.1 ± 0.4 (16.2-17.8)	16.5		-
Gubernaculum	4.4 ± 0.2 (4.1-4.8)	5		-

5.3.2 Canonical discriminant analysis (CDA)

Using a combination of 14 morphometric characters for the females and 13 morphometric characters for the males (Table 5.6), the CDA clearly separated the three species from each other (Figure 5.1). Seven morphometric traits of females (L, a, b, c, body

diam., excretory pore and tail length) provided the most useful taxonomic characters for identification and discrimination (Table 5.6). The CDA for male morphometric characters equally allowed the separation of the two species of *Pratylenchus*; in this case four characters (a, b, body diam. and excretory pore) were the most valuable characters for species separation.

A)



B)

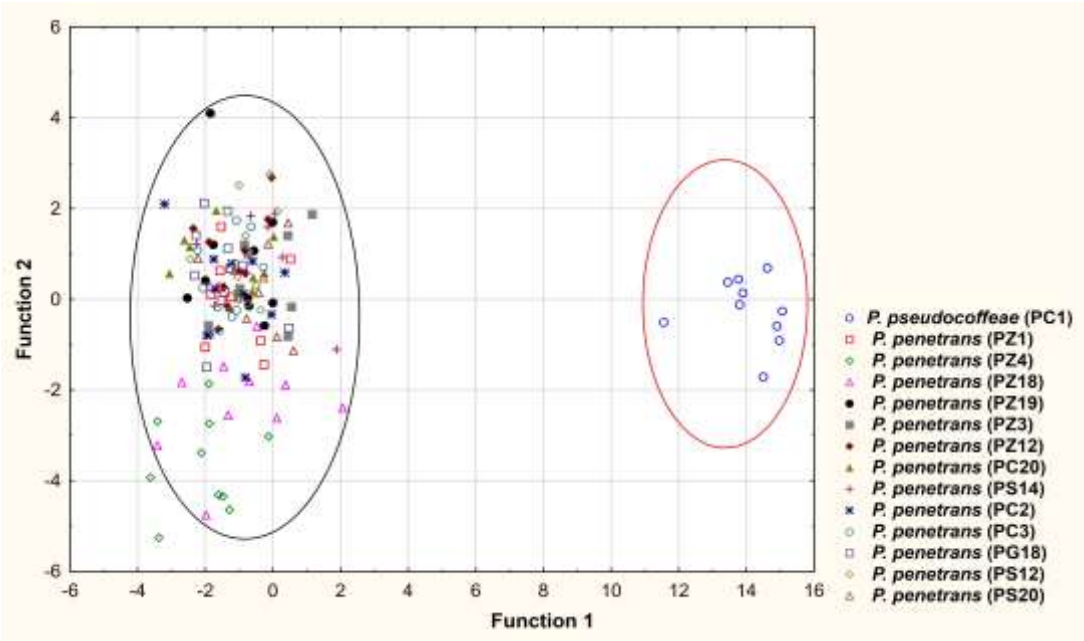


Figure 5.1. The two main canonical discriminant functions between 18 *Pratylenchus* populations from Morocco for females (A) and males (B), based on 14 morphological characters (see Table 5.6).

Table 5.6. Standardised coefficients for canonical variants of *Pratylenchus* spp. for females and males.

	Females		Males	
Selected characters	Root 1	Root 2	Root 1	Root 2
L	0.5	–1.89	–0.47	–0.44
A	0.18	1.53	0.33	–0.19
B	–1.2	-0.1	0.2	0.6
b'	–0.005	–0.023	–0.28	1.02
C	0.34	–0.22	0.23	–0.16
c'	0.35	0.008	–0.12	–0.13
V	0.4	0.94	-	-
Stylet length	–0.16	0.02	–0.21	–0.15
DGO	0.29	0.045	-	-
Max. body diam.	0.23	1.108	–0.007	–0.45
Excr. pore	–0.31	0.72	–0.008	–1.11
Pharynx overlap	0.13	0.04	0.15	0.1
Post-uterine sac	0.13	0.0004	-	-
Tail	–0.36	0.16	0.61	0.85
Spicule	-	-	0.24	0.18
Gubernaculum	-	-	0.21	0.1

5.3.3 Molecular characterisation

5.3.3.1 Species-specific PCR and sequences

The duplex PCR with the PpenA/AB28 and D3A/D3B primer pairs amplified a single band of 340 bp for all 52 DNA extracts. A second specific band of 660 bp was present only for PZ1, PZ3, PZ4, PZ12, PZ18, PZ19, PC2, PC20, PG18, PS12, PS14 and PS20 (Table 5.1, Figure. 5.2). This means that out of 52 DNA extracts (17 populations), 23 extracts (12 populations) were identified as *P. penetrans*. Two DNA extracts of PC3 were not detected by the species-specific PCR for *P. penetrans*; earlier they were morphologically identified as *P. penetrans*. For the remaining 29 extracts (nine populations) not identified as *P. penetrans*, the *P. thornei*-specific primer pair PHTO/D3B amplified a specific band of 288 bp for four DNA extracts originating from four populations (PZ1, PZ2, PZ3 and PZ4) (Figure. 5.3). Populations PZ1, PZ3 and PZ4 were composed of two species (*P. penetrans* and *P. thornei*) (Figures 5.2 and 5.3; Table 5.1).

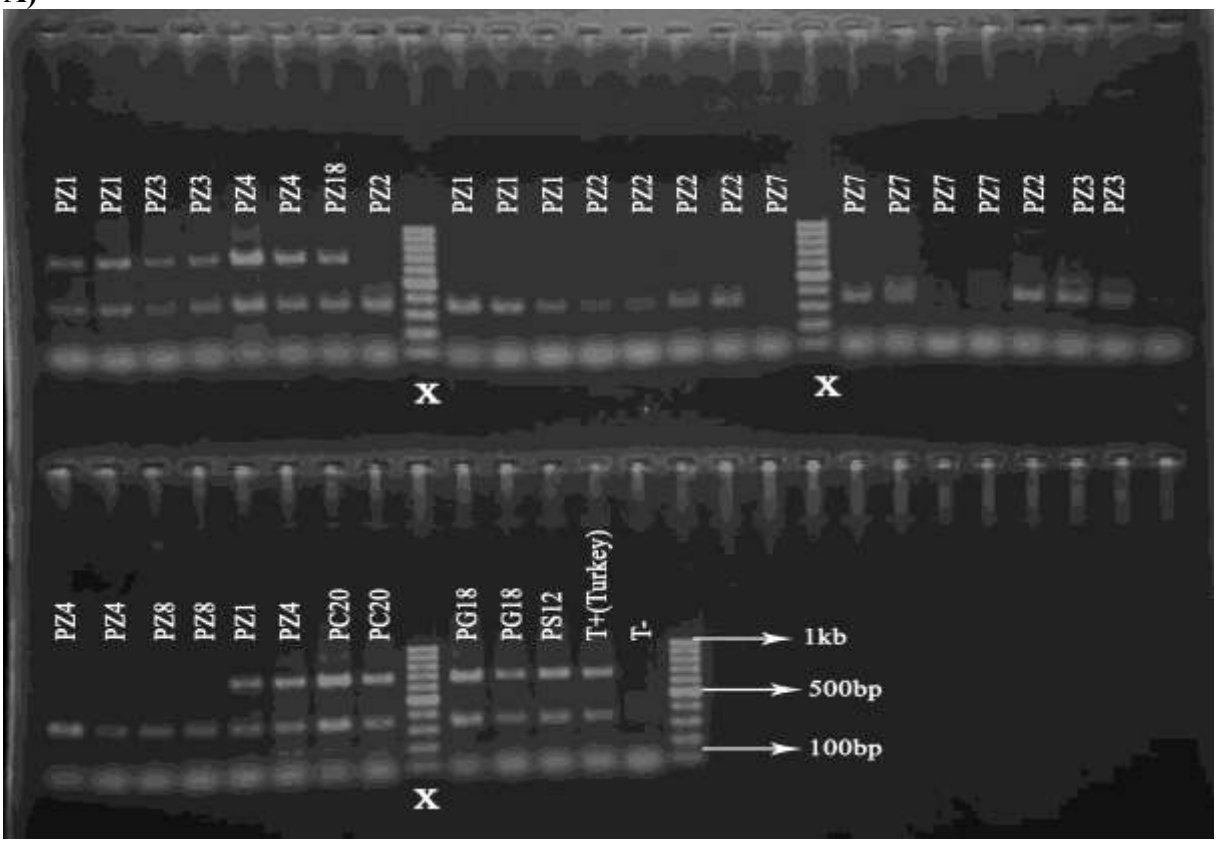
The comparison of the sequences of the D2-D3 28S rDNA expansion segments of the *P. penetrans* and *P. thornei* populations and three unidentified populations of *Pratylenchus* spp. (PZ7, PZ8 and PC1) from Morocco among themselves and with sequences of other

Pratylenchus species available in GenBank is presented in Figure 5.4. This comparison confirmed the identification of *P. penetrans* and *P. thornei* using both morphological features and species-specific PCR. In addition, the sequences revealed the two DNA extracts of PC3 to be *P. penetrans*; earlier they were morphologically identified as *P. penetrans* but not detected by the species-specific PCR.

Thirteen sequences of the D2-D3 28S rDNA expansion segments were similar (99-100%) to the sequences of *P. penetrans* published in GenBank, two sequences (PZ1 and PZ3) were similar (99%) to *P. thornei*, one sequence (PZ7) was similar (100%) to *P. pinguicaudatus*, three sequences (three individuals of PZ8) were similar (94%) to *P. pinguicaudatus*, and one sequence (PC1) was similar (99%) to *P. pseudocoffeae* and (97%) to the sequences of *P. scribneri*, *P. agilis* and *P. hexincisus* published in GenBank. The comparison of the D2-D3 28S rDNA expansion segments at intra-population level showed no divergence between PC3, PS12 and PS14; very low sequence divergence (0-0.3%) was observed for the other *P. penetrans* populations (Table 5.7). The D2-D3 sequence of the Moroccan population of *P. pinguicaudatus* (PZ7) had 100 % similarity with a sequence of this species deposited in GenBank (AJ545014) originating from a population from Tunisia. The sequence comparison of the two *P. thornei* populations from Morocco with the sequence of four *P. thornei* populations available in GenBank revealed the Moroccan sequences to be identical to the *P. thornei* sequence from Spain (EU130873). The relationships with other *Pratylenchus* species were measured through Bayesian Inference (BI) analysis. The obtained alignment presented 604 characters of which 187 were parsimony informative. On the basis of the topology of the calculated majority rule, 60% consensus Maximum Likelihood tree for all populations studied with addition of 13 *Pratylenchus* spp. from GenBank, two major groups of pratylenchids were revealed (Figure 5.4). In Group I (bootstrap value = 70%), two subgroups were found. The first group (Ia) comprised all *P. penetrans* populations from Morocco together with four other *P. penetrans* populations available in GenBank (bootstrap value = 99%). The second subgroup (Ib) comprised, *P. fallax* (AF264181), *P. convallariae* (AF196351) and *P. arligtoni* (AF307328). Group II (bootstrap value = 99%) comprised *P. pinguicudatus* PZ7 (KP289344) and *P. pinguicaudatus* from Tunisia (AJ545014). Group III (bootstrap value = 88%) contained the *P. pinguicaudatus* from Morocco (1PZ8, 2PZ8, 3PZ8). Group V held two subgroups. Subgroup Va (bootstrap value = 97%) comprised, *P. argilis* (EU130841), *P. hexincisus* (DQ498832) and *P. scribneri* (KT873859). Subgroup Vb (bootstrap value = 95%) contained the *P. pseudocoffeae* population from Morocco together with a population from USA of the same species (AF170444). Finally, group VI (bootstrap

value = 98%) contained all *P. thornei* (Moroccan and other) populations. The *P. thornei* populations form Morocco (PZ1, PZ3) clustered with a Spanish population of the same species (EU130873).

A)



B)

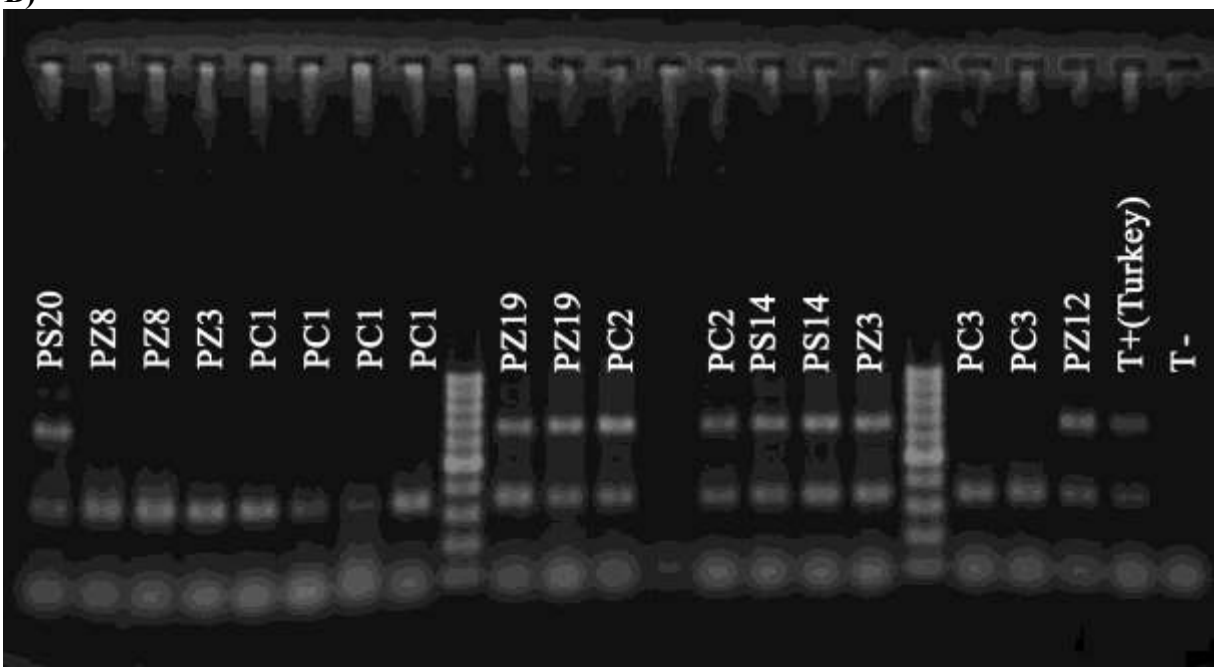


Figure 5.2. (A and B) Result of *Pratylenchus penetrans* species-specific duplex PCR (Waeyenberge *et al*., 2009) using all DNA extracts mentioned in Table 1. X = 100 bp DNA ladder (Promega Benelux), T–: negative control, T+: positive control.

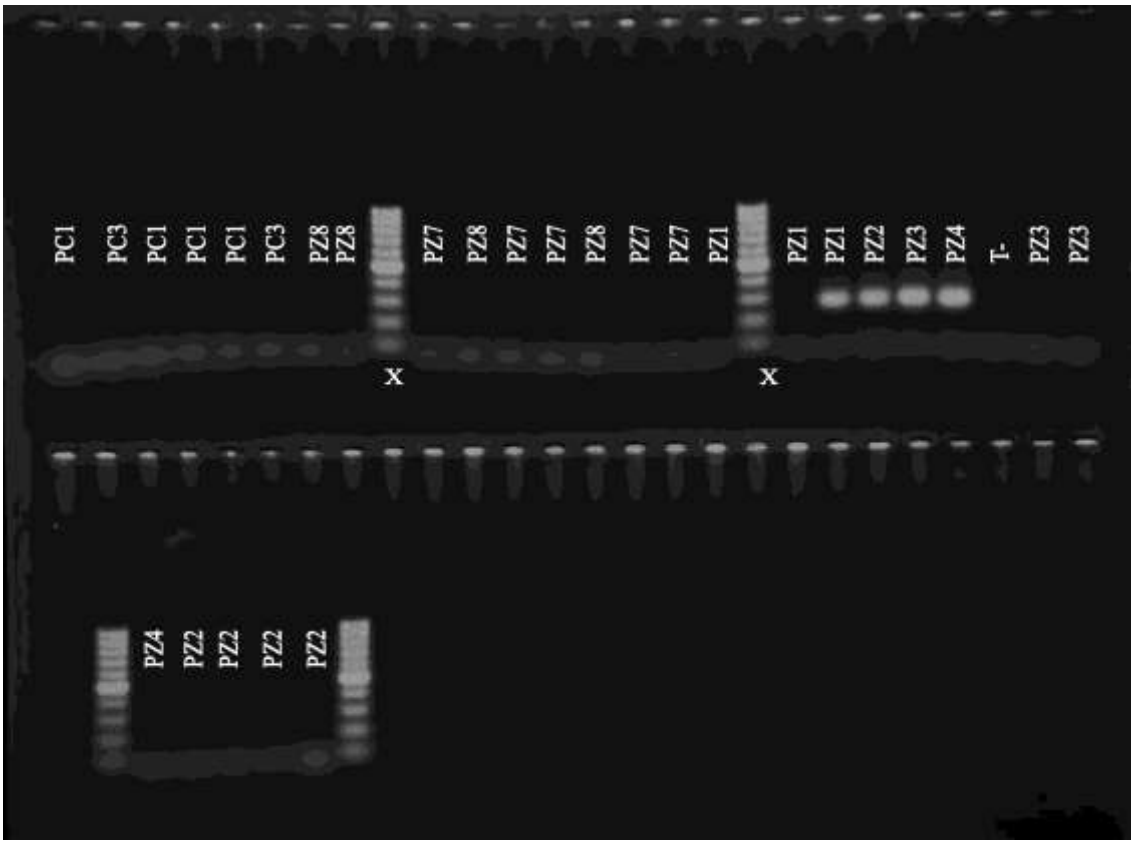


Figure 5.3. *Pratylenchus thornei* species-specific primers PTHO/D3B (Al-Banna *et al*., 2004) using DNA from 29 DNA extracts. X = 100 bp DNA ladder; T-: negative control

Table 5.7. Similarity in % between 13 *Pratylenchus penetrans* populations from Morocco, based on D2-D3 28S rDNA expansion segments sequences.

		% Similarity												
Populations	1	2	3	4	5	6	7	8	9	10	11	12	13
PZ1	1	100	99.8	100	100	99.8	100	100	99.8	100	99.8	100	99.8
PZ4	2		99.8	100	100	99.8	100	100	99.8	100	99.8	100	99.8
PZ18	3			99.8	99.8	99.7	99.8	99.8	99.8	99.8	99.8	99.8	99.8
PZ19	4				100	99.8	100	100	99.8	100	99.8	100	99.8
PZ3	5					99.8	100	100	99.8	100	99.8	100	99.8
PZ12	6						99.8	99.8	99.7	99.8	99.7	99.8	99.7
PC20	7							100	99.8	100	99.8	100	99.8
PC2	8								99.8	100	99.8	100	99.8
PC3	9									99.8	100	99.8	100
PG8	10										99.8	100	99.8
PS12	11											99.8	100
PS20	12												99.8
PS14	13												

For codes see Table 5.1.

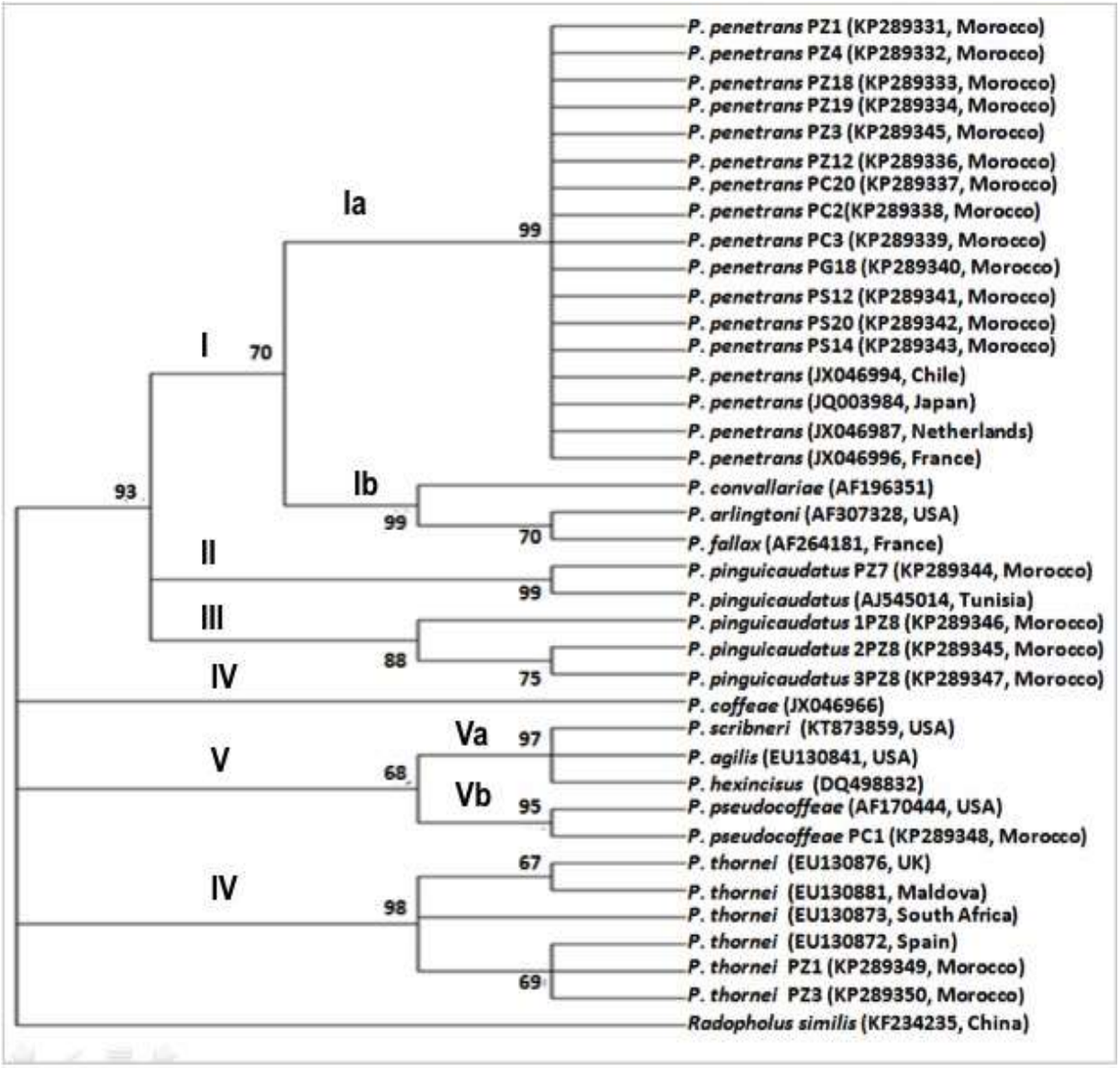


Figure 5.4. Maximum likelihood tree (60% majority rule) obtained from the analysis of the alignment of 20 sequences of *Pratylenchus penetrans*, *P. thornei*, *P. pseudocoffeae* and *P. pinguicaudatus* with addition of *Pratylenchus* sequences obtained from GenBank

5.3.4 Reproductive fitness

Significant differences in reproduction were observed between populations ($F = 19.1$; $df = 5$; $P < 0.0001$), temperature ($F = 5022$; $df = 3$; $P < 0.0001$) and time ($F = 4888.8$; $df = 2$; $P < 0.0001$). Also the interactions populations × temperature ($F = 76.4$; $df = 15$; $P < 0.0001$), population × time ($F = 22.9$; $df = 10$; $P < 0.0001$) and temperature × time ($F = 4888.8$; $df = 6$; $P < 0.0001$) were significant. Additionally, there was a significant 3-way interaction between populations, temperature and time ($F = 50.7$; $df = 30$; $P < 0.0001$).

At 10°C, all populations had increased 8 and 12 weeks after inoculation (WAI), but

not at 4 WAI (Table 5.8). The highest reproduction factor (*Rf*) at this temperature was found 12 WAI for *P. penetrans* PZ3 but was merely 3.7. The fewest nematodes (*Rf* = 0.4) were retrieved 4 WAI for *P. thornei* PZ2. At this temperature, all final populations consisted of females, juveniles, males and eggs, except for *P. thornei* PZ2 in which no males were found. In all populations, the fraction of eggs was the highest at each of the three observation times and varied between 61 and 92% (Figure 5.5).

At 15°C, more nematodes were extracted than inoculated for all populations, even after only 4 WAI (Table 5.8). The greatest reproduction was found 12 WAI for *P. penetrans* PC2 (*Rf* = 51.3) followed by *P. penetrans* PZ3 (*Rf* = 45.5); *P. pseudocoffeae* PC1 and *P. thornei* PZ2 had the lowest final *Rf* (21.7 and 26.9**,** respectively). At this temperature, all populations consisted of eggs, juveniles, females and males at all time points, except for *P. thornei* PZ2 for which no males were found. At 4 WAI, the proportion of eggs in the total population varied between 29% (*P. penetrans* PC2) and 39% (*P. penetrans* PS20). This percentage had increased by 8 WAI and ranged between 70% (*P. pseudocoffeae* PC1) and 82% (*P. penetrans* PC2 and PZ3). At 12 WAI, the percentage of eggs had decreased again to about the same numbers as at 4WAI (Figure 5.5). The remainder of the nematodes consisted of a mixture of juveniles, males and females in variable proportions, depending on the population.

At 20°C and 25°C, the *Rf* of all populations differed significantly between incubation times ($P < 0.05$) (Table 5.8). At 8 WAI, the *Rf* was greatest for all populations at both temperatures. The highest *Rf* was found 8 WAI at 20°C for *P. penetrans* PG18 (*Rf* = 458.6). At 25°C, all populations had multiplied already by a factor between 5.8 (PZ2) and 10.9 (PG18) 4 WAI (Table 8). At this temperature, at 8 WAI, *P. pseudocoffeae* PC1 had increased most (*Rf* = 217) while the *Rf* of the other populations was significantly less and varied between 65 (PS20) and 81 (PC2). The final *Rf* at 25°C (12 WAI) ranged from 22 (*P. penetrans* PS20) to 111 (*P. pseudocoffeae* PC1). The final populations at 20°C and 25°C consisted of females, juveniles, males (except *P. thornei*) and eggs; there was a greater percentage of females at 20 and 25°C than at the lower temperatures (Figure 5.5).

Table 5.8. Effect of time and temperature on the *in vitro* reproduction (*Rf*) on carrot disks of six *Pratylenchus* populations belonging to different species from Morocco (n = 20).

	Rf (4 weeks)					
T (°C)	PZ3	PC2	PG18	PS20	PZ2	PC1
10	0.5a*	0.8a	0.5b	0.5b	0.4b	0.6b
15	3.7a	3.4a	3.9a	4.1a	3.6a	3.8a
20	6.5ab	5.7c	6.8a	6.1bc	3.6e	4.7d
25	10.4a	8.7b	10.9a	9b	5.8c	8b
	Rf (8 weeks)					
T (°C)	PZ3	PC2	PG18	PS20	PZ2	PC1
10	1.3b	1.4b	1.6b	1b	1.2b	3.2a
15	41.8a	41.1a	44.7a	39.9a	39.8a	27.1b
20	350cd	413.5ab	458.6a	394.4bc	310.2d	252.3e
25	68.6bc	80.8b	77.9b	64.9c	70.7bc	217.3a
	Rf (12 weeks)					
T (°C)	PZ3	PC2	PG18	PS20	PZ2	PC1
10	3.7a	3.1ab	2.8b	2.7b	3b	2.9b
15	45.5b	51.3a	39.2c	35c	26.9d	21.7d
20	121b	112.1bc	115.2b	118.5b	103.7c	146.5a
25	25.8c	38.3c	23.8c	21.8c	68b	111a

Codes see Table 5.1.
* Means in rows at 4, 8 and 12 weeks after inoculation followed by the same letter do not differ significantly according to Tukey's test ($P < 0.05$).

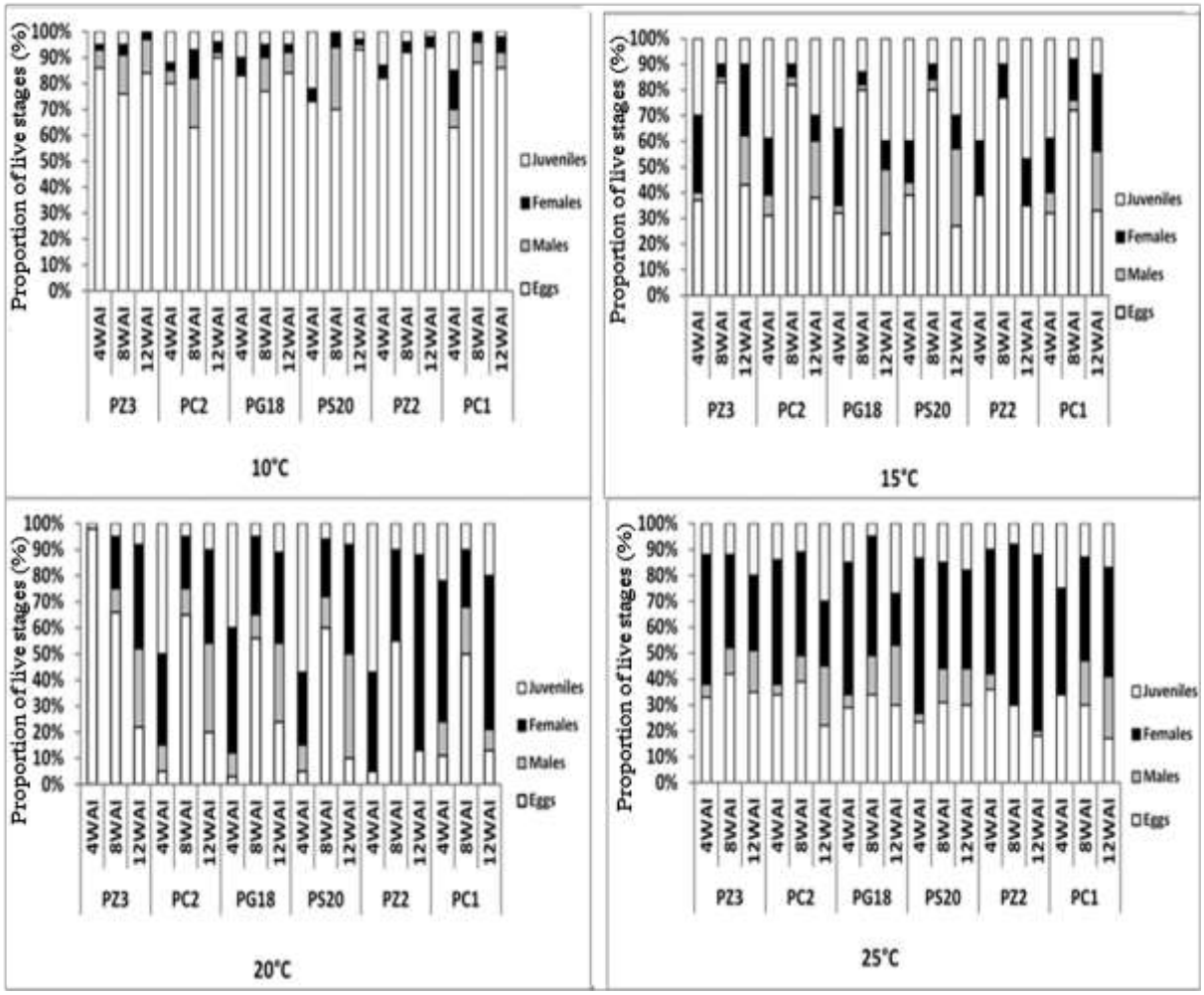


Figure 5.5. Effect of time and temperature on the *in vitro* population composition on carrot disks of six *Pratylenchus* populations belonging to different species (n = 20). Carrot disks inoculated with 20 females and incubated at 10, 15, 20 or 25°C. Observations made 4, 8 or 12 weeks after inoculation (WAI). For codes, see table 5.1

5.4 Discussion

Our survey of *Pratylenchus* in wheat-growing areas of Morocco yielded four species, *viz*., *P. penetrans*, *P. thornei*, *P. pinguicaudatus* and *P. pseudocoffeae*. The presence of these nematodes indicates potential damage to wheat and the need for appropriate management strategies. Previous surveys in the area had only revealed the presence of *P. penetrans* and *P. thornei* (Ammati, 1987; Meskine & Abbad Andaloussi, 1984; Mokrini *et al*., 2009). That means that the presence of *P. pinguicaudatus* and *P. pseudocoffeae* in Morocco is reported herein for the first time. Both, *P. penetrans* and *P. thornei* are economically important and considered serious pests of many hosts, including cereals (Castillo & Vovlas, 2007). However, nothing is known about the importance of *P. pinguicaudatus* and *P. pseudocoffeae* in cereals.

Traditionally, identification of *Pratylenchus* species relies on the morphology and morphometrics of females and males (when present). Variability in morphology is frequently observed among species of the genus *Pratylenchus* (*e.g*., Doucet *et al*., 2001; de la Peña *et al*., 2007; Troccoli *et al*., 2008). However, individual species subjected to varying environmental conditions and different host plants also differ in many morphometric relationships (Román & Hirschmann, 1969).

The CDA of morphometric data revealed the most important characteristics to discriminate *P. penetrans*, *P. thornei* and *P. pseudocoffeae*. Seven female characters allowed discrimination of these three species, whereas four male characters separated *P. penetrans* from *P. pseudocoffeae* (no males for *P. thornei*). Body diam. and distance from anterior end to excretory pore were the most important discriminating characters for the females and males. CDA has been used successfully to analyse morphometric data of *Pratylenchus* spp. Tuyet *et al*. (2013) reported that CDA enabled separation of ten populations of *P. coffeae* from Vietnam into three groups. The body length, b-ratio and distance from anterior end to the excretory pore were the most important characters of males to distinguish the populations in these groups. Unlike the findings of Tuyet *et al*. (2013), the Moroccan populations of *P. penetrans* did not segregate into groups.

Species-specific primers for PCR have been developed to complement the traditional species identification of *P. penetrans* (Uehara *et al*., 1998a; Waeyenberge *et al*., 2009), *P. thornei* (Al-Banna *et al*., 2004; Troccoli *et al*., 2008) and *P. neglectus* (Yan *et al*., 2008). Waeyenberge *et al*. (2009) demonstrated that the primers were able to detect a very large number of isolates of *P. penetrans* originating from all over the world. With the exception of two DNA extracts from Chaouia region of Morocco (PC3), the PCR confirmed the morphological identification of 12 of our populations as *P. penetrans*. The non-identification of the two individuals of PC3 might have been caused by a polymorphism at the binding site of the primers. Waeyenberge *et al.* (2009) commented on problems associated with species-specific primers, such as variation between sequences of isolates from different geographical origins. Sogut & Devran (2011) showed that the duplex PCR (PpenA, AB28/D3A, D3B) developed by Waeyenberge *et al*. (2009) did not always yield the two expected amplification products yet they demonstrated that the single primer pair (PpenA/AB28) successfully identified *P. penetrans*. When using the species-specific primers developed for *P. thornei* (Al-Banna *et al.*, 2004), we obtained the characteristic band of 288 bp for four populations, confirming their morphological identification. The same primer set was successfully used in other research (Sogut & Devran, 2011; Fayazi *et al*., 2012) and seems to be universal.

During the last two decades, sequences of the D2-D3 region have been commonly used to separate nematodes at species level, including pratylenchids (*e.g*., Duncan *et al*., 1999; Carta *et al*., 2001; Inserra *et al*., 2007; De Luca *et al*., 2004; de la Peña *et al*., 2007; Subbotin *et al*., 2008). Previous studies (Duncan *et al*., 1999; Al-Banna *et al*., 2004; Waeyenberge *et al*. 2009) demonstrated that this region is stable in length (*ca* 800 bp) within *Pratylenchus* and is therefore a suitable region for studying the phylogeny of the genus. The number of clades and their composition vary with both the gene(s) and the number of *Pratylenchus* spp. studied (Carta *et al*., 2001; De Luca *et al*., 2004; de la Peña *et al*., 2007; Subbotin *et al.*, 2008). The D2-D3 sequences of the Moroccan pratylenchids showed a high interspecific difference. *Pratylenchus penetrans* and *P. pinguicaudatus* were the closest species with 9.1% nucleotide divergence for the compared sequences. However, *P. penetrans* and *P. pseudocoffeae* showed a divergence of 25.4%. In the phylogenetic tree inferred from the D2-D3 region, the Moroccan populations of *P. penetrans* are localised in a large clade with *P. fallax* and *P. pinguicaudatus*, while *P. pseudocoffeae* is grouped with *P. thornei.* The topology of the consensus tree obtained in this study is relatively similar to the one obtained by Subbotin *et al*. (2008). Previous research using the D2-D3 region or the 18S rDNA gene as molecular marker (Subbotin *et al*., 2008; De Luca *et al*., 2011), always grouped *P. penetrans* with *P. pinguicaudatus* and *P. fallax*. However, Carta *et al*. (2001) when using the ITS region as marker, showed *P. fallax* to be close to *P. penetrans*, thereby confirming that *P. fallax* and *P. penetrans* are two different species, as suggested by previous studies (Perry *et al*., 1980; Ibrahim *et al*., 1994, Waeyenberge *et al*., 2000). A similar grouping was observed by De Luca *et al*. (2004) using D3 sequences. The clustering of *P. penetrans* with *P. pinguicaudatus* indicates a close phylogenetic relationship. The D2-D3 sequences obtained for the two populations of *P. thornei* (PZ3 and PZ4) were identical to each other and also to a sequence in GenBank from a *P. thornei* population from Spain (EU130873). Similar results were obtained when Subbotin *et al*. (2008) compared five *P. thornei* populations from different Mediterranean areas (Italy, Morocco, Tunisia and Spain).

At intraspecific level the D2-D3 sequences showed very low diversity (*P. penetrans*: 0-0.3%, *P. thornei*: 0-0.9%, *P. pinguicaudatus*: 0-4.3%*, P. pseudocoffeae*: 0.3%). Earlier, de la Peña *et al*. (2007) reported similar low divergences of D2-D3 sequences of *P. pratensis* (0-1.4%) and *P. dunensis* de la Peña, Moens, van Aelst & Karssen (1.7%). The relatively greater differences obtained for *P. pinguicaudatus* are difficult to explain. However, the absence of different geographical isolates of this species and of additional sequences in the database, does not allow any conclusion on these two sequences.

The multiplication of a selected number of six populations (*P. penetrans*: PZ3, PC2, PG18 and PS20, *P. thornei*: PZ2, and *P. pseudocoffeae*: PC1) was clearly influenced by the population, the temperature and the incubation time. The significant interaction between these three parameters indicated that the populations did not increase in a similar way with temperature and time. When kept at 10°C for 4 weeks, none of the populations was able to increase in number. Obviously, 10°C is close to the lower limit of the temperature range allowing multiplication of Moroccan *Pratylenchus* spp. Umesh & Ferris (1992) showed that *P. neglectus* reproduced at 10°C on excised barley roots in Petri dishes, but data on multiplication of *Pratylenchus* populations in carrot disks at this temperature are not available. Next to the availability of suitable host plants, the geographic distribution of pratylenchids is related to their temperature requirements (Castillo & Vovlas, 2007). In Morocco, the minimum soil temperature throughout the cereal production cycle (November to June) varies in the four regions surveyed. In Chaouia, the minimum temperatures range from 5°C (January) to 20°C (at the end of winter) (Balaghi *et al*., 2013). This range is favourable for the development of RLN as we observed an increase in *Pratylenchus* populations on carrot disks 8 WAI at 10°C and even earlier at 15°C and 20°C. Penetration of nematodes into the root system, which can seriously affect plant growth, is expected at tillering, which occurs from February onwards when temperatures are above 10°C. (Meskine & Abbad Andaloussi, 1992). In the Zaers and Gharb regions minimum temperatures are above 10°C during the elongation stage which allows nematode penetration during this period. However, in Saiss region, minimum temperatures are close to 0°C, but temperatures exceed 10°C by the end of March, corresponding to the ear emergence stage, thereby promoting nematode penetration of plant roots. Consequently, wheat production is more prone to RLN damage in Chaouia.

Amongst the temperatures studied, 20°C is clearly the optimum. In general, this value is in agreement with studies on the effects of temperature on the reproduction of several *Pratylenchus* species reported by several authors (Ascota & Malek, 1979; Castillo *et al*., 1995; 1996a, b; Thompson *et al*., 2015). As several studies have showed a relationship between reproductive fitness and pathogenicity (Sarah *et al*., 1993; Fallas *et al*., 1995; Trinh *et al*., 2011), we can assume that, at temperatures where high reproduction on carrot disks was noted, damage to cereal crops can be expected. In Morocco, temperatures around 20°C coincide with the elongation and earring stages of the wheat crop (Balaghi *et al*. 2013). Lesion nematode attacks during this period affect both the vegetative growth and the grain filling and can result in a significant reduction in grain production quantity and quality.

In our study, all three species of *Pratylenchus* multiplied very well on carrot disks at

20°C. However, the total numbers recovered per carrot disk varied with the species and population. Intraspecific differences in reproductive fitness of the four populations of *P. penetrans* were noticed at different temperatures. This difference among *P. penetrans* populations may be due to adaptation of the population to its environment (*e.g.*, climate, temperature, soil type) (Dao, 1970; Moens & Perry, 2009).

Fewer nematodes were retrieved 12 WAI than 8 WAI at 20 and 25°C in all six populations. This decrease in nematodes is probably caused by exhaustion of the food source. Such a decline has been observed in other carrot disk experiments and is influenced by the initial nematode population density, temperature and duration of the experiment (*e.g.*, Stoffelen *et al*., 1999; Tuyet *et al*., 2013). At 20°C and 25°C we noticed some deterioration of the carrot tissues 12 WAI, as well as nematodes migrating away from the carrot disks. This suggests that the high nematode densities resulted in the breakdown of carrot tissue as many plant cells were punctured by nematodes. The effect of temperature on the proportion of the different developmental stages was remarkable. At 10°C the nematode community on the carrot disks was mainly composed of eggs, even at 12 WAI. As the temperature increased, the prominent presence of eggs was much reduced in favour of juveniles, females and males (but not for PZ2 as the taxon has no males). Based on the high number of females in all populations at higher temperatures, the life cycle of most individuals was probably not completed at 10°C. However, at 20°C and 25°C the life cycle was already completed within 4 WAI, whilst egg laying had resumed. A similar life cycle duration at 20-25°C has been reported for *P. thornei* from Spain by Castillo *et al*. (1995). No males were observed for *P. thornei* (PZ2). Absence of males in populations of *P. thornei* has been reported previously (Verdejo-Lucas & Pinochet, 1992; Castillo *et al*., 1995, 1998).

The results presented here are the first providing the integrated morphometric, morphological and molecular characterisation of RLN populations from Morocco. Observations of nematode reproduction in carrot disks further showed clear differences between species and populations. These results provide basic information to develop a research programme that aims at establishing a control strategy against lesion nematodes. Additional experiments on nematode multiplication on wheat are necessary to confirm the observations in the laboratory on carrot disks and the pathogenicity of *P. penetrans* and *P. thornei* on wheat needs to be examined.

CHAPTER 6

Quantitative detection of the root-lesion nematode, *Pratylenchus penetrans*, using qPCR

Mokrini, F., Waeyenberge, L., Viaene, N , Abbad Andaloussi, F. & Moens. M (2013). Quantitative detection of the root-lesion nematode *Pratylenchus penetrans* using real-time PCR. The *European Journal of Plant Pathology* 137 (2), 403-413.

6.1 Introduction

The root-lesion nematode, *Pratylenchus penetrans*, is one of the most economically damaging plant-parasitic nematodes. It is widely distributed and is found on a wide variety of crops (Castillo & Vovlas 2007). In Europe, *P. penetrans* causes reductions in yield and/or quality of potato, vegetables, such as carrot, pea, bean, and ornamental plants, including rose, tulip and lily (Green & Verdejo 1985; Talavera *et al*., 2001; Pudasaini *et al*., 2007). The nematode also reduces productivity of potato in many production areas (Olthof & Potter, 1973; Bernard & Laughlin 1976; Olthof 1986, 1989; Ball-Coelho *et al*. 2003; Bélair *et al*., 2005; Holgado *et al*., 2009). In Québec, *P. penetrans* was reported as the dominant species in potato fields, and population densities above the damage threshold of 1,000 nematodes/kg soil were common (Olthof, 1987). This nematode was also recorded in Algeria and Tunisia on several crops (Troccoli *et al*. 1992). In Morocco, *P. penetrans* is the most common pratylenchid in different wheat producing areas (Meskine and Abbad Andaloussi, 1992; Mokrini *et al*. 2009, 2012).

It has been frequently demonstrated for diverse combinations of plants and nematodes, including *P. penetrans*, that a significant relationship exists between the pre-planting nematode density and the damage caused by the nematode on the host (Seinhorst, 1998; Sato *et al*., 2009). Hence, a correct identification and quantification of *P. penetrans* is of major importance in nematode control strategies. However, identification of *Pratylenchus* spp. based on morphology and morphometric traits of adults is time-consuming, requires ample skill and training from the observer, and is frequently inconclusive because of the small number of diagnostically valid characters (Luc 1987; Loof 1991). Moreover, *Pratylenchus* spp. are frequently present in mixed populations, which makes their identification and quantification even more difficult. Waeyenberge *et al*. (2000), De Luca *et al*. (2004) and Subbotin *et al*. (2006) demonstrated that DNA-based methods provide efficient tools for a precise and rapid identification of *Pratylenchus* species. PCR using species-specific primers constitute a major step forward in the development of diagnostic technology, which has successfully been used for sensitive detection of *Pratylenchus* species. Species-specific primers to detect *P. penetrans* in a conventional PCR have been developed (Uehara *et al*. 1998b; Al-Banna *et al*. 2004; Waeyenberge *et al*. 2009). However, these primers are not suitable to quantify the species. Recently, quantitative PCR (qPCR) strategies have been developed for *P. zeae* (Berry *et al*., 2008) and *P. thornei* (Yan *et al*., 2012). qPCR allows continuous monitoring of the sample during PCR using hybridization probes. The log-linear region can be easily identified

as the fluorescence data appear on the computer screen. Within this region, the number of cycles needed to obtain fluorescence above the background (Ct) is compared between samples and standards with known quantities of DNA. These data then can be used for quantification of the samples (Kingsnorth *et al*., 2003). The aim of the present study was to develop a rapid and precise method for the detection and quantification of *P. penetrans* in a nematode suspension using qPCR and to evaluate its efficacy.

6.2 Material and Methods

6.2.1 Nematodes populations and DNA extraction

Forty-nine isolates of 20 *Pratylenchus* species originating from several countries and hosts (Table 6.1) were used in this study. For several species, especially *P. penetrans*, more than one isolate was investigated to verify the specificity of the developed qPCR assay. From many of the isolates, DNA had already been extracted (all stages combined) and used in another study to develop a species-specific PCR for the detection of *P. penetrans* (Waeyenberge *et al*., 2009). This DNA was also used in our study. Other isolates were obtained during a survey conducted in different wheat growing areas in Morocco (Mokrini *et al*., 2012). The 21 Moroccan populations, comprising *P. penetrans*, *P. thornei* and *P. pseudocoffeae*, were identified on the basis of their morphology, morphometrics and D2-D3 28S rRNA gene sequences. From these latter populations, DNA was extracted as described by Holterman *et al*. (2006). For this purpose, one or five individuals (all stages combined) were hand-picked and transferred to an Eppendorf tube containing 25 µl double distilled water (ddH2O) and 25 µl nematode lysis buffer (final concentration: 200 mM NaCl, 200 mM Tris-HCl (pH8), 1% mercaptoethanol and 800 µg of Proteinase K). The tubes were incubated at 65°C for 1.5 h and 99°C for 5 min, consecutively. This DNA was used for testing the specificity of the selected primers and probe. For the sensitivity tests, the same DNA-extraction method was applied to obtain DNA from 1 to100 individuals (see below).

Table 6.1: Origin and codes of populations of *Pratylenchus* spp. and other nematode genera used in this study, together with the mean Ct value (0.05) and standard deviation obtained in a qPCR reaction.

code	Species	Host/soil	Origin	Ct
Pl1	*P. loosi*	native plants	USA	N/A
Pl2	*P. loosi*	Tea	Gilan, Iran	N/A
Pj	*P. jaehni*	Citrus	Sao Paulo, Brazil	N/A
Ph	*P. hippeastri*	Amaryllis	Florida, USA	N/A
Pgu1	*P. gutierrezi*	Maize	Kwazulu Natal, South Africa	N/A
Pgu2	*P. gutierrezi*	Coffee	Guatemala	N/A
Pg	*P. goodeyi*	Banana	Tenerife, Canary Islands	N/A
Pf1	*P. fallax*	Soil	Merelbeke, Belgium	N/A
Pf2	*P. fallax*	Soil	Redu, Belgium	N/A
Pcr1	*P. crenatus*	Soil	Gottem, Belgium	N/A
Pcr2	*P. crenatus*	Soil	Laukaa, Finland	N/A
Pcon	*P. convallariae*	*Convallaria*	Sassenheim, The Netherlands	N/A
Pcf	*P. coffeae*	Coffee	Vietnam	N/A
Pbr	*P. brachyurus*	Aster	Florida, USA	N/A
Pbo	*P. bolivianus*	*Alstroemeria*	West Sussex, UK	N/A
Pa	*P. agilis*	Maize	Maryland, USA	N/A
Pme	*P. mediterraneus*	Wheat	Saad, Isreal	N/A
Pne1	*P. neglectus*	Faba bean	Cerignola, Italy	N/A
Pne2	*P. neglectus*		Turkey	N/A
Ppi	*P. pinguicaudatus*	Faba bean	Beja, Tunisia	N/A
Pz	*P. zeae*	Grassland	Florida, USA	N/A
Pth1	*P. thorni*	Wheat	Ain Auda, Morocco	N/A
Pth2	*P. thorni*	Wheat	Marchouch, Morocco	N/A
Pth3	*P. thorni*	Wheat	Sidi Bettach, Morocco	N/A
Pth4	*P. thorni*	Wheat	Marchouch, Morocco	N/A
Pth5	*P. thorni*		Turkey	N/A
Pps1	*P. pseudocoffeae*	Wheat	Settat, Morocco	N/A
Pps2	*P. pseudocoffeae*		Iran	N/A
Ppe1	*P. penetrans*	Wheat	Turkey	28.9± 0.5
Ppe2	*P. penetrans*	Soil	Belgium	28.7± 0.1
Ppe3	*P. penetrans*	Soil	Kinrooi, Belgium	31.6± 0.3
Ppe4	*P. penetrans*	soil	The Netherlands	28.8± 1.3
Ppe5	*P. penetrans*	Soil	Kerkom, Belgium	29.3± 0.7
Ppe6	*P. penetrans*	Wheat	Ain auda, Morocco	28.5± 0.7
Ppe7	*P. penetrans*	Wheat	Marchouch, Morocco	28.4± 0.2
Ppe8	*P. penetrans*	Wheat	Ain auda, Morocco	28.2± 0.5
Ppe9	*P. penetrans*	Wheat	Ouled said, Morocco	29.9± 0.3
Ppe10	*P. penetrans*	Wheat	Sidi Bettach, Morocco	29.7± 0.5
Ppe11	*P. penetrans*	Wheat	Berchid, Morocco	27.2± 0.2
Ppe12	*P. penetrans*	Wheat	Mediona, Morocco	27.5± 0.4
Ppe13	*P. penetrans*	Wheat	Settat, Morocco	29.1± 0.7
Ppe14	*P. penetrans*	Wheat	Sidi slimane, Morocco	28.9± 0.1
Ppe15	*P. penetrans*	Wheat	Mhaya Morocco	29.4± 0.5

Ppe16	*P. penetrans*	Wheat	Ain Taoujdate, Morocco	27.1± 0.4
Ppe17	*P. penetrans*	Wheat	Sebaa ayoune, Morocco	29.4± 1.5
Ppe18	*P. penetrans*	Wheat	Meknes, Morocco	29± 0.1
Ppen19	*P. penetrans*	Wheat	Kenitra, Morocco	29.5± 0.3
Ppen20	*P. penetrans*	Wheat	Ait Malk, Morocco	28.7± 0.6
Ppen21	*P. penetrans*	Wheat	Taso, Morocco	29.3± 0.5
Glr	*Globodera rostochiensis*	Potato	Bioska, Serbia	N/A
Melh	*Meloidogyne hapla*	Culture	Belgium	N/A
Rad	*Radopholus duriophilus*	Coffee	Vietnam	N/A
Tys	*Tylenchulus semipenetrans*	Citrus	Gharb, Morocco	N/A
Xid	*Xiphinema diversicaudatum*	Citrus	Gharb, Morocco	N/A
Ha	*H. avenae*	Wheat	Zaers, Morocco	N/A
Hl	*H. latipons*	Wheat	Saiss, Morocco	N/A
Para	*Paratylenchus sp.*	Lettuce	Belgium	N/A
Scu	*Scutellonema sp.*	Yam	Ghana	N/A

N/A: not applicable

6.2.2 Development of primers and probe

We collected all gene sequences of *Pratylenchus* spp. available in the GenBank database in search for a DNA-region with potential for use as a diagnostic tool. However, we avoided the sequences based on ribosomal DNA as it is known that the LSU, SSU and D2D3 regions in this gene are not very suitable to distinguish *P. penetrans* from other closely related *Pratylenchus* spp. (Orui, 1996; Waeyenberge *et al*., 2000, 2009). Because most sequence information for several *Pratylenchus* species was found for the β-1,4-endoglucanase gene, we decided to retain this gene for further study. All retrieved β-1,4-endoglucanase gene sequences (Table 6.2; Figure 6.1) were aligned for the selection and design of primers and probes using the software package AlleleID 7.75. The sequences selected for the forward primer, reverse primer and probe were PpenMFor 3'-CCA ACC TCT GCT ACA CTA-5', PpenMRev 3'-CAG TGC CGT ATT CAG TGA-5' and PpMPb 3'-CAC TAT TAT GCC GC-5', respectively. The MGB-probe was labelled with 6-FAM (Life Technologies Europe).

Table 6.2. List with Genbank accession numbers based on β-1,4-endoglucanase used in this study for designing the primers and probe.

***Pratylenchus* species**	**Accession numbers**
P. penetrans	AB045781
P. penetrans	AB045780
P. penetrans	JN052038
P. penetrans	JN052037
P. penetrans	JN052036
P. penetrans	JN052035
P. vulnus	JN052050
P. vulnus	JN052051
P. thornei	JN052046
P. pratensis	JN052043
P. pratensis	JN052042
P. Pratensis	JN052044
P. neglectus	JN052029
P. neglectus	JN052030
P. neglectus	JN052031
P. neglectus	JN052032
P.neglectus	JN052033
P. neglectus	JN052034
P. convallariae	JN052028

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
PpAB045781      GCAATTTGTG CTACACCCTC CACTACTATG CCGCATCACA TAAACAGGAT
PpAB045780      CCAACCTCTG CTACACTATG CACTATTATG CCGCAACACA CAAACAATCG
PpJN052038      CCAACCTCTG CTACACTATG CACTATTATG CCGCAACACA CAAACAATCG
PpJN052037      ---------- ---------- ---------- ---------- ----------
PpJN052036      CCAACCTCTG CTACACTATG CACTATTATG CCGCAACGCA CAAACAATCG
PpJN052035      TCAACCTCTG CTACACTGTC CACTTTTATG CCGGTACACA CAAACAAGCG
PvJN052050      ---------- ---------- ---------- ---------- ----------
PvJN052051      CCAACATCAT GTACACCTTC CACTTCTATG CCGCCACCCA TGGCGCCACA
PthJN052046     CCAACTTGTG CTACACGCTG CACTTCTACG CGGCCTCCCA CAAACAGGAG
PprJN052043     ---------- ---------- ---------- ---------- ----------
PprJN052042     CCAACTTGTG CTACACCCTC CACTACTATG CCGCCTCCCA TAAGCAATCC
PprJN052044     CCAACTTGCG CTACACCCTC CACTACTATG CCGCCTCCCA TAAGCAATCG
PneJN052029     CCAACATCAT GTACACCTTC CACTTCTACG CGGCCGCCCA TGGCGCCTCC
PneJN052030     CCAACATCAT GTACACCTTC CACTTCTACG CGGCCACCCA TGGCCAGTCC
PneJN052031     CCAACATCAT GTACACCTTC CACTTCTACG CAGCCACCCA TGGCCAATCC
PneJN052032     CCAACCTGAT GTACACGCTG CACTACTACG CCGCCTCGCA CAAGCAGTCG
PneJN052034     CCAACTTGAT GTACACGCTG CACTACTACG CCGCCTCGCA CAAGCAGTCG
PcoJN052028     TCAATCTCTG CTACACTCTT CACTTTTATG CTGCATCACA TGGGCAATCA
PneJN052033     CCAACTTGAT GTACACGCTG CACTACTACG CCGCCTCGCA CAAGCAGTCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
PpAB045781      TTGCGCAACA AAGCGCAAGC GGCACTGAAC AA-------- ----------
PpAB045780      CTCCGCGACA AGACGCAAGC TGCATTGAAC AA-------- ----------
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PpJN052038   CTTCGCGACA AGACGCAAGC TGCATTGAAC AAGGTGAGGG AAGAA-----
PpJN052037   ---------- ---------- ---------- ---------- ----------
PpJN052036   CTTCGCGACA AGACGCAAGC TGCATTGAAC AAGGTGGGGG AAGAA-----
PpJN052035   TTTCGTGACA AAATGCAAGC TGCACTGAAC AAGGTGAAGA AAGAAGAATG
PvJN052050   ---------- ---------- ---------- ---------- ----------
PvJN052051   TACCGCACCA AAGTGCAAAC CGCTTATGAT AATGTATGGA TTTTTACATT
PthJN052046  CTGCGGAACA AGGCGCAGAC AGCGCTGAAC AATGTTTGTG TGCCAGACTT
PprJN052043  ---------- ---------- ---------- ---------- ----------
PprJN052042  CTCCGTGATA AGGCCACCGC CGCTTTGAAC AAGGTTGGCA ATGTGTGATG
PprJN052044  CTCCGTGATA AGGCCACCGC CGCTTTGAAC AAGGTTGGCA ATGTGTGAAC
PneJN052029  TACCGTACCA AAGTGCAGAC GGCCTACAAT AATGTGC--- ----------
PneJN052030  TATCGGGACA AAGTCACAAC GGCACGCAAC AATGTGGG-- ----------
PneJN052031  TATCGGGACA AAGTCACAAC GGCACACAAC AATGTGGG-- ----------
PneJN052032  CTGCGTGACA AGATCACCAC CGCCATCAAC AACG------ ----------
PneJN052034  CTGCGTGACA AGATCACCAC CGCCATCAAC AACG------ ----------
PcoJN052028  CTTCGGGACA AGACAACAGC TGCATTGAAC AAGGTGAGGG AAGGAATAGA
PneJN052033  CTGCGTGACA AGATCACCAC CGCCATCAAC AACG------ ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1060       1070       1080       1090       1100
PpAB045781   ---------- ---------- ---------- ---------- ----------
PpAB045780   ---------- ---------- ---------- ---------- ----------
PpJN052038   ---------- -GGAAGA--- ---------- -------AAA GAGAGTCTTC
PpJN052037   ---------- ---------- ---------- ---------- ----------
PpJN052036   ---------- -GACAAATTC ---------- -------AAC TAATGTTTTG
PpJN052035   GGAAGAGGAT AAATATAAAT ATCAGGAATG TGTCCCGTGC CAAAAACACG
PvJN052050   ---------- ---------- ---------- ---------- ----------
PvJN052051   TATTCCTTCT TGGCACTTTT TTCTACCATT TCTCTCAAAA AACAGACTTA
PthJN052046  ---------- -TCTCTGATT ---------- ---------- ---TGTATTG
PprJN052043  ---------- ---------- ---------- ---------- ----------
PprJN052042  ---------- -GATAGCCT- ---------- ---------- ---AGACTTA
PprJN052044  ---------- -GATGGTGT- ---------- ---------- ---AGGCTTA
PneJN052029  ---------- -----CTTCT TTCTAACGAT TT-------- -----ACCTA
PneJN052030  ---------- -----CCAAT TCTA--TGTC TTT------- -----AGGCA
PneJN052031  ---------- -----CCAGT TCTAAATGCC CTT------- -----AGGCA
PneJN052032  ---------- ---------- ---------- ---------- ----------
PneJN052034  ---------- ---------- ---------- ---------- ----------
PcoJN052028  ---------- -GAAAGGATT ACGAGGAGGA ATATGAGAAA AAGAGTCTTC
PneJN052033  ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1110       1120       1130       1140       1150
PpAB045781   ---------- ---------- ---------- --GAATGTCT GCATATTCGT
PpAB045780   ---------- ---------- ---------- --GGGTGTCT GCGTGTTCGT
PpJN052038   AATAAACATT TATG------ --------TT AAGGGTGTTT GCGTGTTTGT
PpJN052037   ---------- ---------- ---------- ---------- ----------
PpJN052036   TTTGTTTATT C--------- --------TC CAGGGTGTCT GCGTGTTCGT
PpJN052035   GAATAAAACG GGAAACGGGA CAGGAAATTT TTTTTTAAAA CAATTAATTC
PvJN052050   ---------- ---------- ---------- ---------- ----------
PvJN052051   AAAAGCCTTA TTATAAAATT AAAACAATTT GAGGGTCTTC CCGTGTTTGT
PthJN052046  AATAAGCAAT T--------- ---------G CAGGGCGCTT GCGTATTTGT
PprJN052043  ---------- ---------- ---------- ---------- ----------
PprJN052042  CAAGCCCCTC T-TCTCTCT- --------TG TAGAAAGTCT GCGTTTTCGT
PprJN052044  CAAGCCCCT- ----TATCC- --------TA TAGAAAGTCT GTGTTTTCGT
PneJN052029  AATGGCTTTC TTAT------ ---------T TAGGGAATTC CCATTTTCGT
PneJN052030  AATTCCCTCA AAAA------ ---------T CAGGGTCTGC CCATTTTCGT
PneJN052031  AATTCCCTCA AAAA------ ---------T AAGGGTCTTC CCATTTTCGT
PneJN052032  ---------- ---------- ---------- ----GCGCAG CCATCTTTGT
PneJN052034  ---------- ---------- ---------- ----GCGCAG CCATCTTTGT
```

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PcoJN052028  AATCAACATT TATTCATTT- --------TT CAGGGTGTTT GCATATTCGT
PneJN052033  ---------- ---------- ---------- ----GCGCAG CCATCTTTGT


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1160       1170       1180       1190       1200
PpAB045781   CACTGAATAC GGCACTGTCA ATGCAGATGG CAACGGCGGT ATGGACCAAG
PpAB045780   CACTGAATAC GGCACTGTCA GCGCTGATGG AAACGGCGGT ATGGACCAGG
PpJN052038   CACTGAATAC GGCACTGTCA GCGCTGATGG CAACGGCGGT ATGGACCAAG
PpJN052037   ---------- ---------- ---------- ---------- ----------
PpJN052036   CACTGAATAC GGCACTGTCA GCGCTGATGG CAACGGCGGT ATGGACCAAG
PpJN052035   ACCTAAAAAA CAAAAAATCA AGGAGAATAA AAAACGTACA ATTTAAGTTG
PvJN052050   ---------- ---------- ---------- ---------- ----------
PvJN052051   CACTGAATAT GGCACAACCG AGTCGAGCGG CGATGGCACT GTCGACACTT
PthJN052046  CACGGAGTAC GGCACGGTGG AAGCCAACGG CGGCGGCAAT CCGGACTCCG
PprJN052043  ---------- ---------- ---------- ---------- ----------
PprJN052042  ACCGAATAC GGTACCGTGA GTGCCGATGA CAATGGCGGC CTGGATGCCA
PprJN052044  CACTGAATAC GGTGTTGTGA GCGCCGATGG CAATGGCGGC GTTGATACGG
PneJN052029  CACCGAATAC GGCACCACAG AATCCAGTGG AGATGGCACT GTGGACACCT
PneJN052030  CACCAAATAC GGCACCACTG AATCGAGTGG AGACGGCACG GTGGACATCG
PneJN052031  CACCGAATAC GGCACCACAG AATCGAGCGG AGACGGCACG GTGGACATCT
PneJN052032  CACCGAGTTC GGCACCGTGG ACGCCAGCGG GGCCGGCAGT GTGGATGCCG
PneJN052034  CACCGAGTTC GGCACCGTGG ACGCCAGCGG GGCCGGCAGT GTGGATGCCG
PcoJN052028  GACTGAATAC GGCACTGTTA GCGCTGATGG CAACGGCGGT GTTGACCAAG
PneJN052033  CACCGAGTTC GGCACCGTGG ACGCCAGCGG GGCCGGCAGT GTGGATGCCG
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Figure 6.1. Alignment of a selection of our β-1,4-endoglucanase sequences (see table 6.2). Positions of the primers for *P. penetrans* are underlined and bold, the probe is bold, underlined and highlighted.

6.2.3 Real time PCR assay

All qPCR kits that were used (SensiFAST Probe Hi-ROX kit (2x) and SensiFAST SYBR Hi-ROX), were validated by the producer (Bioline Reagents Company, London, UK) on all commonly used real-time instruments and did not need further optimization regarding their composition. The SensiFAST SYBR Hi-ROX kit was only used to optimise the annealing temperature (by melting curve analysis). All other tests (specificity, sensitivity, construction of standard curve) were done with the SensiFAST Probe Hi-ROX (2x) kit. The finally retained *P. penetrans* species-specific qPCR assay is a TaqMan based assay.

6.2.3.1 Optimisation of the annealing temperature

We optimized the efficiency of the primers for different annealing temperatures with two Moroccan populations of *P. penetrans* (Ppe11, Ppe12) and one population of *P. thornei* (Pth2). The qPCR was performed for different annealing temperatures ranging from 60°C to 64°C in a final volume of 20 µl reaction mixture containing 10 µl of SensiFAST SYBR Hi-ROX (2×), 400 nM of each primer, and 3 µl of template DNA extracted from a single individual of Ppe11, Ppe12 or Pth2 (Table 6.3). Each sample was run in triplicate using an automated ABI PRISM 7900 HT sequence detection system (Applied Biosystems). The

amplification program consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C to 64° C for 30 s and 72°C for 1 min. The Sequence Detection Software (SDS) 2.4 was used to generate the amplification curves for each reaction. The threshold cycle number (Ct) was determined at a threshold set on 0.2. To differentiate species amplicons from non-specific products, a dissociation curve was generated after each reaction. Control samples without DNA template (NTC) were included in each experiment in duplicates.

6.2.3.2 Testing specificity of primers and probe

To determine whether the primers and probe were specific for amplification and detection of *P. penetrans*, DNA from 49 populations comprising 19 different *Pratylenchus* species was used, as well as DNA from plant-parasitic nematodes from 9 other genera (Table 6.1). Each sample was loaded in triplicate. A negative control sample was also prepared in two replicates using distilled water instead of a DNA template. All runs were done in a final volume of 20 µl containing 10 µl of a SensiFAST Probe Hi-ROX (2×), 400 nM of each primer, 200 nM of the probe and 3 µl of template DNA. The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 63°C for 30 s and 72°C for 1 min.

6.2.3.3 Testing sensitivity of primers and probe

To determine the sensitivity of the primers and probe for *P. penetrans*, two different experiments were run with SensiFAST Probe Hi-ROX (2×). The relationship between DNA concentration and Ct values was estimated in the first experiment as follows. DNA was extracted from three series of 1, 5, 10, 20, 40 and 80 individuals (all stages combined) of *P. penetrans* (Holterman *et al*., 2006). All DNA-extracts were run in triplicates, one for each series. A negative control was also prepared in two replications using distilled water instead of a DNA template. The second experiment examined the detection limit of one individual of *P. penetrans* in the presence of an increasing number of individuals (all stages combined) of *P. thornei*. Therefore, 1, 5, 10, 50 and 100 individuals (all stages combined) of *P. thornei* were hand-picked and transferred to an Eppendorf tube containing 25 µl water along with a single *P. penetrans*. DNA was extracted (Holterman *et al*., 2006) and two samples were taken from the extract. Three qPCR were run for each DNA extract. A negative control was also prepared in two replications using distilled water instead of a DNA template.

6.2.3.4 Construction of standard curve

DNA was extracted from four series of 80 individuals (all stages combined) of *P. penetrans* (Ppe14). A single qPCR was run for each DNA-extract and Ct-values were compared. Subsequently, all four tubes of DNA were mixed and a dilution series was prepared. The mixed DNA sample was serially diluted to 1/2, 1/4, 1/8, 1/16 and 1/80 of the original concentration (representing 80 individuals of *P. penetrans*). These concentrations were used as templates in a qPCR. Plotting logarithmic values of DNA concentration versus Ct-values generated a standard curve.

6.3 Results

6.3.1 Optimisation of the annealing temperature

Based on absence of non-specific product, the program with an annealing temperature of 63°C was selected (T). Increasing annealing temperatures resulted in higher Ct-values for *P. penetrans* (Table 6.3), whereas lower temperatures did not completely avoid the formation of non-specific products, as shown by the dissociation curves (Figures 6.2 and 6.3). At 62 °C, the dissociation curve of the qPCR test showed minor peaks for *P. thornei* (Figure 6.2) and a Ct value of 39 (Table 6.3) for this non-target species. At 63°C, the assay was able to detect a single individual of *P. penetrans* whereas no signals were observed in the NTC samples nor in the sample with *P. thornei* (Table 6.3). At this temperature, peaks for *P. thornei* were hardly noticeable in the dissociation curve while they were high for *P. penetrans* (Figure 6.3),

Table 6.3. Mean and standard deviation of Ct values obtained at different melting temperatures of DNA extracted from single individuals of two *P. penetrans* and one *P. thornei* population (n = 3)

	60°C		62°C		63°C		64°C	
Sample	**Mean Ct**	**SD**	**Mean Ct**	**SD**	**Mean Ct**	**SD**	**Mean Ct**	**SD**
P. penetrans (1)	26.2	0.1	30.4	0.4	31.3	0.4	34.5	0.4
P. penetrans (1)	27.5	0.2	30.1	0.7	31.5	1.1	35.2	1.6
P. thornei (1)	N/A	-	39.2	0.5	N/A	-	N/A	-
Negative control	39.5	0.4	N/A	-	N/A	-	N/A	-

N/A: not applicable

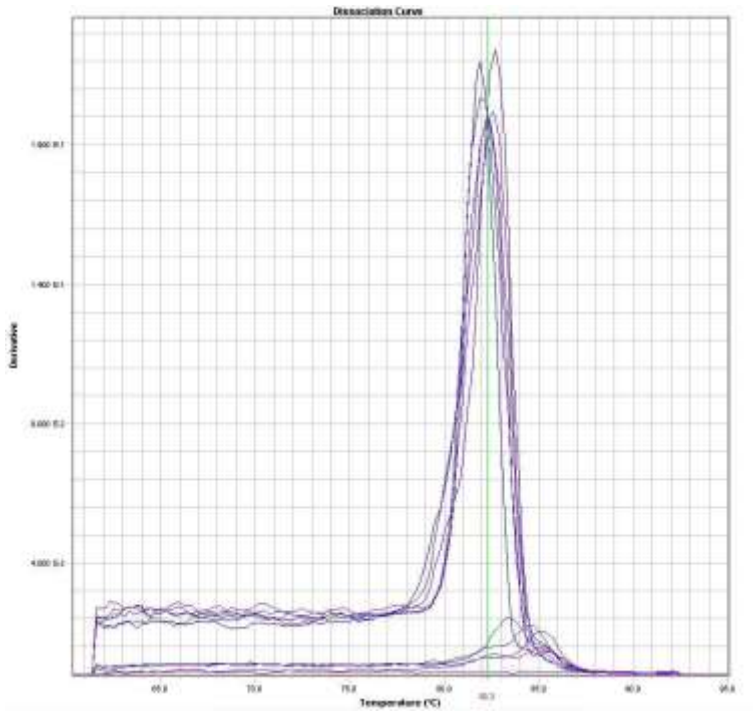

Figure 6.2. Dissociation curve of the qPCR test (SensiFAST SYBR Hi-ROX) with annealing temperature set at 62°C showing high peaks at ± 82.2°C of two *P. penetrans* populations (n=3) and minor peaks for a population of *P. thornei* (n=3) and NTC (n=2).

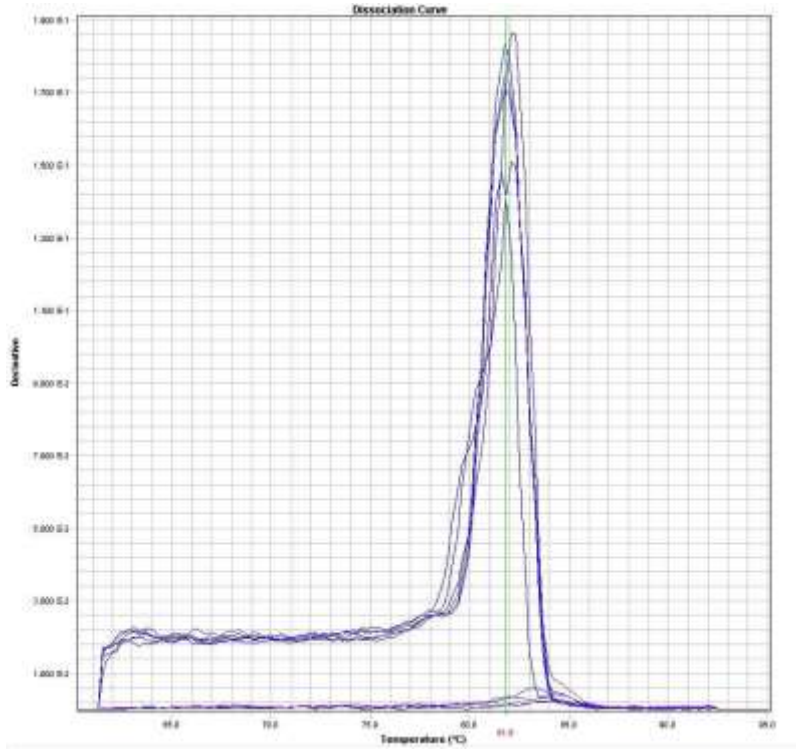

Figure 6.3. Dissociation curve of the qPCR test (SensiFAST SYBR Hi-ROX) with annealing temperature set at 63°C showing high peaks at ± 81.8°C of two *P. penetrans* populations (n=3) and very low peaks for a population of *P. thornei* (n =3) and NTC (n = 2).

6.3.2 Specificity of primer and probe set

The targeted fragment of all isolates of *P. penetrans* was amplified by utilizing the qPCR protocol with the primer pair PpenMFor/PpenMRev along with the probe PpMPb. The qPCR assay did not show any amplification of DNA from other *Pratylenchus* species, nor of DNA from species of other nematode genera (Table 6.1). In addition, DNA was not amplified or detected in any of the non-template controls that contained water instead of DNA. The Ct-values for DNA derived from 1-5 *P. penetrans* individuals from different populations from Morocco varied between 27.1 ± 0.4 and 29.9 ± 0.3 (Table 6.1).

6.3.3 Sensitivity of primers and probe

The qPCR assay (first experiment) successfully amplified DNA extracted from a nematode suspension containing 1, 5, 10, 20, 40 or 80 individuals of *P. penetrans*. The corresponding decreasing Ct-values were 32.5 ± 0.3, 30.1 ± 0.2, 29.9 ± 0.09, 28.6 ± 1.6, 27.1 ± 0.6, 26.5 ± 0.3 respectively. The Ct-value of the negative control was always undetermined.

The Ct-values obtained after qPCR, with DNA from a single *P. penetrans* in the presence of 1, 5, 10, 20, 40 and 80 individuals of *P. thornei* (second experiment) were almost the same: 32.3 ± 0.4, 32.1 ± 0.2, 31.9 ± 0.1, 32.2 ± 0.2, 31.9 ± 0.4 and 32.1 ± 0.3, respectively. The negative control was always undetermined. The Ct-values were significantly stable.

6.3.4 Construction of a standard curve

qPCR was run 4 times using DNA extracted from exactly 80 individuals of *P. penetrans*. Again, Ct-values showed a consistent result (Ct = 24.4 ± 0.4). The standard curve (Figure 6.4) generated from the data obtained with the qPCR of the serial dilution (Table 6.4) showed a highly significant relationship between the Ct-value and number of nematodes over the range studied ($R = 0.99$; slope = -3.23; E = 104%). Based on three sample replications, the ABI PRISM fluorescence detection system automatically calculated the starting number of *P. penetrans* by comparison of the Ct-values from the unknown samples with the values of the standard curve.

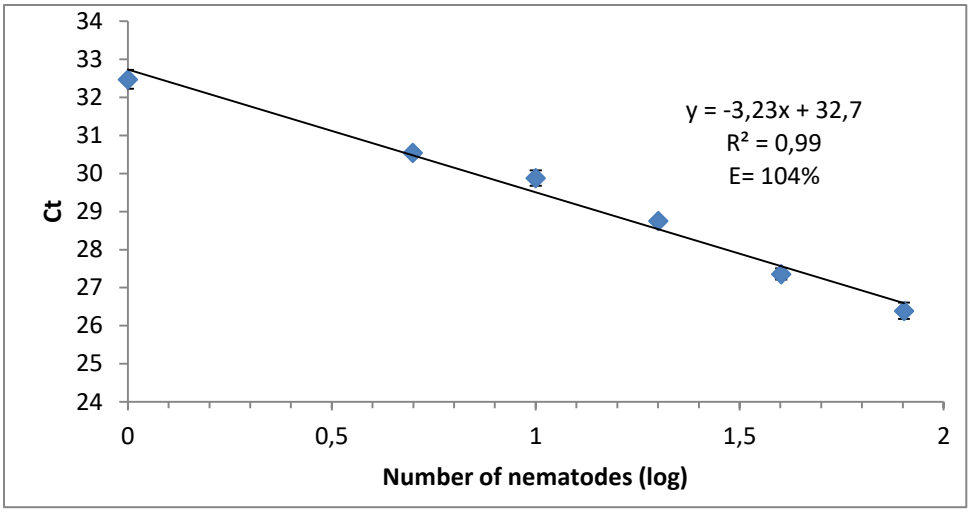

Figure 6.4. Standard curve of the qPCR assay (SensiFAST Probe Hi-ROX) for *P. penetrans*: threshold cycle number (Ct) plotted against the log of the number of individuals of *P. penetrans* (1, 5, 10, 20, 40, 80) (n = 3)

Table 6.4. Cycle threshold (Ct) values from a serial dilution of *Pratylenchus penetrans*

Serial dilution	Number of *P. penetrans*	Ct
1 :1	80	26,4 ± 0,21
1 :2	40	27,4 ± 0,14
1 :4	20	28,8 ± 0,06
1 :8	10	29,9 ± 0,2
1 :16	5	30,5 ± 0,08
1 :80	1	32,5 ± 0,25

6.4 Discussion

A rapid and reliable diagnostic test to quantify the presence of *P. penetrans* in samples is an essential step in the management of this economically very important plant-parasitic nematode. In this paper we report on the development of a qPCR assay for *P. penetrans* based on the β-1,4-endoglucanase gene. This gene may play a crucial role in plant cell wall-degradation during penetration and migration of nematodes in the host roots.

ITS-sequences of the ribosomal gene are frequently used for the development of molecular tools to identify plant-parasitic nematodes (Subbotin & Moens, 2006). However, Waeyenberge *et al*. (2000) and Uehara *et al*. (1999) demonstrated that ITS-sequences can vary in size between different *Pratylenchus* species, which makes sequence alignment to detect species-specific fragments problematic. In addition, ITS sequences show extensive

polymorphism within a species or an individual (Orui 1996; Waeyenberge *et al*. 2000). This drastically limits the number of potential DNA fragments suitable for the design of species-specific primers. Because of the limited availability of comparable sequences of *Pratylenchus* spp. in GenBank we decided to use the β-1,4-endoglucanase gene. To our knowledge, this gene has never been used to identify plant-parasitic nematodes.

The DNA-extraction method that we used appeared to be stable and capable of extracting DNA from up to 80 individuals. Five individuals, however, were suggested to be the maximum number of nematodes to be used in the DNA-extraction method described by Holterman *et al*. (2006). Our results showed the presence of an increasing amount of DNA extracted from a proportional increasing amount of nematodes.

The qPCR assay is very sensitive, reliably detecting the DNA of a single individual of *P. penetrans* when mixed with DNA from 80 individuals of *P. thornei*. This sensitivity compares well with findings reported for other nematode species. Madani *et al*. (2005) could detect a single second-stage juvenile of the cyst-forming nematodes *Globodera pallida* and *Heterodera schachtii*. Toyota *et al*. (2008) reported that real-time PCR sensitively detected a single second-stage of the cyst-forming nematodes *Globodera rostochiensis* from 1000 free-living nematodes.

Repeatability of the test was demonstrated by the similar Ct values (24.4 ± 0.4) obtained from 4 tests performed with 80 individuals of *P. penetrans,* and the almost equal Ct values of about 32 obtained in the 18 runs with 1 individual of *P. penetrans* mixed with variable numbers of *P. thornei*.

The assay has not only a high amplification efficiency, it is also was highly specific, showing a single amplicon in melting curve analyses and no specific amplification when using DNA from other species of *Pratylenchus*. The specificity was tested on several isolates of 19 different *Pratylenchus* species. Different populations of the morphologically and phylogenetically closely related species *P. fallax, P. convallariae* and *P. penetrans* (Subbotin *et al*., 2008) were also tested. The specific primers and probe did not produce any amplification for the 3 populations of *P. fallax* and *P. convallariae*, but were capable of detecting all 21 *P. penetrans* isolates tested, originating from all over the world. Neither was there amplification for the plant-parasitic species of nine other genera. Although the chosen target (the β-1,4-endoglucanase gene) to design de primers and probe are common for many nematode species, the developed primers were only compatible with *P. penetrans*.

The results of runs of the serial dilutions of DNA samples of *P. penetrans* as well as the runs conducted with DNA from increasing amounts of nematodes showed a highly

significant linearity ($R = 0.99$). The particular strength of the qPCR assay is that it is useful for quantifying *P. penetrans* in mixed populations of *Pratylenchus* spp., where visual identification of individual nematodes at species level is extremely difficult. Particularly, the juvenile stages are difficult to identify at species level because their species-specific features are not defined. Within the different developmental stages of pratylenchids, differences in size exist; second-stage juveniles of *Pratylenchus* spp. are small compared to adults of the same species. We believe that despite this difference, qPCR is able to quantify numbers of *P. penetrans* even when different developmental stages are mixed. The DNA signal can be converted into an accurate estimation of the number of individuals involved. Although the number of cells in an individual nematode increases during growth (Cunha *et al*., 1999), this given does not have a large influence on the estimation of the numbers of nematodes in a sample as the degree of accuracy required in agronomic and ecological studies will be much more determined by factors such as sampling and extraction efficiency.

CHAPTER7

The β-1,4-endoglucanase gene is suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei*

Mokrini, F., Waeyenberge, L., Viaene, N., Abbad Andaloussi, F. & Moens, M. (2014). The β-1,4-endoglucanase gene is suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei*, *Nematology* 16 (7), 789-796.

7.1 Introduction

The migratory endoparasitic nematode *Pratylenchus thornei* is an important pathogen of wheat in Europe, Africa, North America, Asia, the Middle East and Australia (Greco *et al.*, 1992; Troccoli, 1992; Smiley *et al.*, 2005a). In the Mediterranean basin, the nematode also causes severe yield decline of crops such as chickpea, faba bean (Glazer & Orion, 1983; Greco *et al.*, 1984) and pulse crops (Di Vito *et al.*, 1992).

Identification of *Pratylenchus* spp. based on morphology and morphometrics is time-consuming, requires ample skill from the observer, and is frequently inconclusive because of the small number of diagnostically valid characters (Luc, 1987; Loof, 1991). Therefore, traditional nematode identification is more and more supplemented with molecular observations. Waeyenberge *et al.* (2000), De Luca *et al.* (2004) and Subbotin *et al.* (2006) demonstrated that DNA-based methods could be used for identification of *Pratylenchus* species. Species-specific primers were developed for distinguishing species of *Pratylenchus*, including *P. thornei* (Al-Banna *et al.*, 2004; Carrasco-Ballesteros *et al.*, 2007; Yan *et al.*, 2008). However, these methods are not suitable to quantify the species.

In view of a high throughput detection and quantification of *P. thornei*, our objective was to develop a rapid and precise method for the detection and quantification of *P. thornei* in a nematode suspension using qPCR. A similar strategy has been used for the detection and quantification of *P. neglectus*, *P. vulnus*, *P. thornei* and *P. zeae* in DNA extracts of soil using primers designed from the ITS region of rDNA (Qiu *et al.*, 2007; Berry *et al.*, 2008; Toyota *et al.*, 2008; Yan & Smiley, 2013). Mokrini *et al.* (2013), however, developed a real-time PCR assay using species-specific primers and a probe based on the β-1,4-endoglucanase gene to detect and to quantify *P. penetrans* in a nematode suspension. Here we report on the use of the same gene to develop a qPCR protocol for the rapid, specific, sensitive and quantitative detection of *P. thornei*.

7.2 Material and methods

7.2.1 Nematode populations and DNA extraction

Forty-seven isolates of 15 *Pratylenchus* species originating from several countries and hosts (Table 7.1) were used in this study. For several species, especially *P. thornei*, more than one isolate was investigated to verify the specificity of the developed qPCR assay. DNA was extracted as described by Holterman *et al.* (2006). From many of the isolates, DNA was available (all vermiform stages) as it had been used in another study (Waeyenberge *et al.*,

2009). The Moroccan populations, *viz.*, *P. penetrans*, *P. thornei* and *P. pseudocoffeae*, were identified on the basis of their morphology, morphometrics and D2D3 28S rRNA gene sequences. The DNA was used for testing the specificity of the selected primers and probe. For the sensitivity tests, the same DNA-extraction method was used to obtain DNA from 1-80 individuals (see below).

Table 7.1. Origin and codes of populations of *Pratylenchus* spp. used in this study, together with the mean Ct value (0.03) and standard deviation, where determined, obtained in a qPCR reaction.

Code	Species	Host/Soil	Origin	Ct
Pa	*P. agilis*	Maize	USA	Und
Pbr2	*P. brachyurus*	Aster	Florida, USA	Und
Pcre	*P. crenatus*	Soil	Belgium	Und
Pc	*P. coffeae*	Coffee	Vietnam	Und
Pfa1	*P. fallax*	Soil	Merelbeke, Belgium	Und
Pfa2	*P. fallax*	Soil	Redu, Belgium	Und
Pg	*P. goodeyi*	Banana	Tenerife, Canary Islands	Und
Pgu	*P. gutierrezi*	Maize	South Africa	Und
Pme	*P. mediterraneus*	-	Spain	Und
Pneg	*P. neglectus*	-	Turkey	Und
Pping1	*P. pinguicaudatus*	-	Italy	Und
Pping2	*P. pinguicaudatus*	-	Morocco	Und
Pps1	*P. pseudocoffeae*	Wheat	Settat, Morocco	Und
Pps2	*P. pseudocoffeae*	-	Iran	Und
Pter	*P. teres*	-	South Africa	Und
Pz	*P. zeae*	-	Australia	Und
Ppe1	*P. penetrans*	Wheat	Turkey	Und
Ppe2	*P. penetrans*	Soil	Belgium	Und
Ppe3	*P. penetrans*	Soil	Kinrooi, Belgium	Und
Ppe4	*P. penetrans*	Soil	The Netherlands	Und
Ppe5	*P. penetrans*	Wheat	Ain Aouda, Morocco	Und
Ppe6	*P. penetrans*	Wheat	Marchouch, Morocco	Und
Ppe7	*P. penetrans*	Wheat	Mediona, Morocco	Und
Ppe8	*P. penetrans*	Wheat	Settat, Morocco	Und
Ppe9	*P. penetrans*	Wheat	Ain Taoujdate, Morocco	Und
Ppe10	*P. penetrans*	Wheat	Mhaya, Morocco	Und
Ppe11	*P. penetrans*	Wheat	SidiSlimane, Morocco	Und
Ppe12	*P. penetrans*	Wheat	Berchid, Morocco	Und
Pp	*P. penetrans*	-	Belgium	Und
PthN1	*P. thornei*	-	New Zealand	27.7 ± 0.6
PthN2	*P. thornei*	-	New Zealand	19.3 ± 0.6
PthMo	*P. thornei*	Almond	Souk El Gour, Morocco	22.6 ± 0.3
PthAus	*P. thornei*	-	Australia	29 ± 0.5
PthZ1	*P. thornei*	Wheat	Ain Aouda, Morocco	28.9 ± 0.4
PthZ2	*P. thornei*	Wheat	Ain Aouda, Morocco	28.5 ± 0.2
PthZ3	*P. thornei*	Wheat	Marchouch, Morocco	28.2 ± 0.5

PthZ4	*P. thornei*	Wheat	Marchouch, Morocco	30.1 ± 0.4
PthTu	*P. thornei*	Wheat	Turkey	27.9
Tys	*Tylenchulus semipenetrans*	Citrus	Gharb, Morocco	Und
Xid	*Xiphinema diversicaudatum*	Citrus	Gharb, Morocco	Und
Ha	*Heterodera avenae*	Wheat	Zaers, Morocco	Und
Hl	*Heterodera latipons*	Wheat	Saiss, Morocco	Und
Gro	*Globodera rostochiensis*	Potato	Bioska, Serbia	Und
Par	*Paratylenchus* sp.	Lettuce	Belgium	Und
Mh	*Meloidogyne hapla*	-	Serbia	Und
Rd	*Radopholus duriophilus*	Coffee	Vietnam	Und
Scu	*Scutellonema* sp.	Yam	Ghana	Und

Und = undetermined; – = unknown.

7.2.2 Development of primers and probe

We used the same sequence information of the β-1,4-endoglucanase gene of six *Pratylenchus* spp. available from GenBank as in Mokrini *et al.* (2013). The sequences were aligned using the software package AlleleID 7.75 (Figure 7.1). On the basis of this alignments we selected the following sequences for the forward primer, reverse primer and probe were PthMFor 3'-GGA TGC GGT CAT CAA GGC-5', PthMRev 3'-TTG GCT CTG GTG GTT CTG-5' and PthMPb 3'-CGA CTG GCA CGA CCA CAA CG-5', respectively. The MGB-probe was labelled with 6-VIC (Life Technologies Europe).

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
PpAB045781      C---AGCGGC TACCTGAGCA ACAAACAGAA CCAGAGGAAC ATGG---TCG
PpAB045780      C---AGCGGC TACTTAAGCA ATAAACAGGG CCAAATGAGC ATGG---TTG
PpJN052038      C---AGCGGC TACTTAAGCA ATAAACAGGG CCAAATGAGC ATGG---TTG
PpJN052037      TCCGAACACC CAATACAGCC GGCTTAAAAC CGTCATTGAT GCGGCCATAT
PpJN052036      C---AGCGGC TACTTAAGCA ACAAACAGGG CCAAATGAGC ATGG---TTG
PpJN052035      A---TTTATA CAACTATATT TTTTAAAAAA TTTAATAGTT TTTCT-TTGA
PvJN052050      C---AGCGGC TATTTGAGCA ATCAGGCCAC TCAAATGTCC CTGG---TCC
PvJN052051      C---AATTTT TGTTTGGGCT TATTTAGTGC CATCTTTTTT CTAGC-ATTT
PthJN052046     C---AACGGT TACCTGAGCA ATCCTTCCGG CCAGCAATCT CTCG---TGG
PprJN052043     C---ACCGGC TATTTGAGCA ATCCAAGCGG ACAGATGAGC TTGG---TCG
PprJN052042     ---------- ---------- ---------- ---------- ----------
PprJN052044     C---AACGGC TATTTGGCCA ACCAAGGCAC CCAAATGGCC TTGA---TCC
PneJN052029     ---------- ---------- ---------- ---------- ----------
PneJN052030     C---GGATAC TTGTCGGACC CCTCCGGGCA GATGGCCATG GTCG------
PneJN052031     C---GGCTAC TTGTCGGACC CCTCTGGACA GATGGCCATG GTGG------
PneJN052032     ---------- ---------- ---------- ---------- ----------
PneJN052034     C---AACAGC TATTTGAGCA ATCCTTCCGC CCAGATGGCA ATGG---TGG
PcoJN052028     T---AGCGGC TACATTAGTA ATAAACAAAA CACAATGAAC ATGA---TTG
PneJN052033     C---AACAGC TATTTGAGCA ATCCTTCCGC CCAGATGGCA ATGG---TGG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
PpAB045781      ACACGGTGAT AAAGGCGGCC ATTGCCCAGG GCAT-----T TACGTGATC-
PpAB045780      AGACGGTCAT AAAAGCAGCA ATTGCCGAAG GCAT-----T TATGTGCTC-
PpJN052038      AGACGGTCAT AAAAGCAGCA ATTGCCGAAG GCAT-----T TATGTGCTC-
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PpJN052037   CGTTGGGCAT TTATGTGATC GTGGATTGGC ATGTGTCCAC AACGTATCAA
PpJN052036   AGACGGTCAT AAAAGCAGCA ATTGCCGAAG GCAT-----T TATGTGCTC-
PpJN052035   AGATGGTAAA AAATAACCTT TTTTTAGAAA TTTTATTTTT CTTGCATCT-
PvJN052050   AGACCGTCAT CCAGGCCGCT ATCGACAATG GCAT-----T TATGTCATC-
PvJN052051   AGAGTCCCTA TCTAACTATG ATTTTACCAG CTTT-----T GATATTTTT-
PthJN052046  ATGCGGTCAT CAAGGCGGCC ATTGACCAGG GCAT-----C TACGTGATT-
PprJN052043  AGACGGTCAT AAATGCCGCC ATTGCCCAGG GCAT-----C TATGTCATC-
PprJN052042  ---------- ---------- ---------- ---------- ----------
PprJN052044  AGCTCATGAT CCAAGCGGCC ATTGACAACG GCAT-----C TACGTCATT-
PneJN052029  ---------- ---------- ---------- ---------- ----------
PneJN052030  AGACAGTCGT GCAGGCGGCC ATTGACCAGG GCAT-----T TACGTGATC-
PneJN052031  AAACGGTCGT GCAGGCGGCC ATTGACCAGG GCAT-----T TACGTGATC-
PneJN052032  ---------- ---------- ---------- ---------- ----------
PneJN052034  AGACGGTCAT CCAGGCGGCC ATCGCCGAGG GCAT-----C TACGTGATC-
PcoJN052028  AGACAGTCAT AAAAGCGGCA ATTGAGAATG GCAT-----T TATGTGCTT-
PneJN052033  AGACGGTCAT CCAGGCGGCC ATCGCCGAGG GCAT-----C TACGTGATC-

                  610        620        630        640        650
PpAB045781   ---------G TCGATTGGCA CGATCACAA- TG----CCCA GAACCATCTG
PpAB045780   ---------G TGGATTGGCA CGATCACAA- TG----CACA GAACCATCAA
PpJN052038   ---------G TGGATTGGCA CGATCACAA- TG----CACA GAACCATCAA
PpJN052037   TCACAGGCGG TAGGTGGAAA AATATATAAA TAAAACCACA TGATTTTATA
PpJN052036   ---------G TGGATTGGCA CGATCACAA- TG----CACA AAATCATCAG
PpJN052035   ---------T TTATTGAAAA AAATTGCAA- TTTTTTCTTC AAAACATGCA
PvJN052050   ---------G TCGACTGGCA TGACCACAA- TG----CTCA GAATCACAAG
PvJN052051   ---------T GAGATGGTAA CT-CCGCGC- TG----CCGA ----CTTTTC
PthJN052046  ---------G TCGACTGGCA CGACCACAA- CG----CACA GAACCACCAG
PprJN052043  ---------G TCGATTGGCA CGACCACAA- TG----CCCA GAATCATCAA
PprJN052042  ---------- ---------- CGACCATAA- TG----CCCA GAATCACAAG
PprJN052044  ---------G TCGACTGGCA CGACCATAA- TG----CCCA GGATCACAAG
PneJN052029  ---------- ---------- ----CGTGT- CG----GCCA ----CCTACC
PneJN052030  ---------A TCGACTGGCA ----CGTGT- CG----GCCA ----CCTACC
PneJN052031  ---------A TCGACTGGCA ----CGTGT- CG----GCCA ----CTTACC
PneJN052032  ---------- ---------- TGACCACAA- CG----CGCA GAACCACGTC
PneJN052034  ---------G TGGACTGGCA TGACCACAA- CG----CGCA GAACCACGTC
PcoJN052028  ---------G TGGATTGGCA CGACCACAA- TG----CACA GAATCATCAA
PneJN052033  ---------G TGGACTGGCA TGACCACAA- CG----CGCA GAACCACGTC

                  660        670        680        690        700
PpAB045781   AGTCAGG--- ---------- ---------- ---------- ----------
PpAB045780   AGCCAGG--- ---------- ---------- ---------- ----------
PpJN052038   AGCCAGGTGA AGGAGCTTGT TAGGTGACAA TGACAATAAT ATTATTGCTT
PpJN052037   CAGCAAAATT TATTTAAAAA TACGAATGGA TCATTCGTCA TTCATGAGTT
PpJN052036   AGCCAGGTAA AGAAGCTTAT TAGGTGACAA TGACAATAAT ATTATTTACT
PpJN052035   AAAGAATATA AATCCCAGCC TTGGTAATGA ATAACTTTAA AACCCGTTGC
PvJN052050   AGTCAAG--- ---------- ---------- ---------- ----------
PvJN052051   ACTCAAAACT C---AATCCT --ATTATTTA C----CTATA ATCTTCTTTC
PthJN052046  AGCCAAGCGG TAAGGCCGGC TTTTC-ATCA TGAG------ --AAAC----
PprJN052043  AGCCAGG--- ---------- ---------- ---------- ----------
PprJN052042  AGCCAGG--- ---------- ---------- ---------- ----------
PprJN052044  AGCCAGG--- ---------- ---------- ---------- ----------
PneJN052029  AGTCACAAGC TGTAAACCCC --CTCAGCCT T----GAATT CATTTCAGGC
PneJN052030  AGTCACAAGC GGTAGAGGGG -AATGGGCCG GA---TTATG GACTGGCCCA
PneJN052031  AGTCACAAGC GGTAGAGGGG GAATGGGCCG GA---TTATG GACTGGCCCA
PneJN052032  AGCCAGGCGG TCAGTTTTTC CTCCCCACTC CGAGC--TGT CCAAGCAGTA
PneJN052034  AGCCAGGCGG TCAGTTTTTC CTCCCCACTC CGAGC--TGT CCAAGCTGTA
PcoJN052028  AGCCAGGTGA AGAAGCCTAT TAGTTTATTA TGC---TTTT CTTATGGCTT
```

PneJN052033 AGCCAGGCGG TCAGTTTTTC CTCCCCACTC CGAGC--TGT CCAAGCTGTA

Figure 7.1. Alignment of a selection of β-1,4- endoglucanase sequences (see table 6.2). Positions of the primers for *P. thornei* are underlined and bold, the probe is bold, underlined and highlighted.

7.2.3 Real time PCR assay

7.2.3.1 Optimisation of the annealing temperature

All qPCR kits (SensiFAST Probe Hi-ROX kit and SensiFAST SYBR Hi-ROX) were validated by the producer (Bioline Reagents Company, London, UK) on all commonly used real-time instruments and did not need further optimisation regarding their composition. qPCR using SYBR Green I dye was done under annealing temperatures ranging from 62-69°C in a final volume of 20 µl reaction mixture containing 10 µl of SensiFAST SYBR Hi-ROX (2×), 400 nM of each primer, and 3 µl of template DNA extracted from a single individual of *P. thornei* PthZ1 and five individuals of PthZ2 (Table 7.2). Each sample was run in duplicate using an automated ABI PRISM 7900 HT sequence detection system (Applied Biosystems). The amplification program consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62-69°C for 30 s and 72°C for 1 min. Sequence Detection Software (SDS) 2.4 was used to generate the amplification curves for each reaction. The threshold cycle number (Ct) was determined at a threshold set on 0.2. To differentiate species amplicons from non-specific products, a dissociation curve was generated after each reaction. Control samples without DNA template (NTC) were included in each experiment in duplicates.

7.2.3.2 Testing specificity of primers and probe

To determine whether the primers and probe were specific for amplification and detection of *P. thornei*, DNA from 47 populations comprising 15 different *Pratylenchus* species (Table 7.1) was used. Each sample was loaded in triplicate. A negative control sample was also prepared in two replicates using distilled water instead of a DNA template. All runs were done in a final volume of 20 µl containing 10 µl of a SensiFAST Probe Hi-ROX (2×), 400 nM of each primer, 200 nM of the probe and 3 µl of template DNA. The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 69°C for 30 s and 72°C for 1 min.

7.2.3.3 Testing sensitivity of primers and probe

The sensitivity of the primers and probe was determined in two different experiments run with SensiFAST Probe Hi-ROX (2×). The relationship between DNA concentration and Ct-values was estimated in a first experiment in which DNA was extracted from three series of 1, 5, 10, 20, 40 and 80 individuals (all vermiform stages) of *P. thornei* (Holterman *et al.*, 2006). All DNA-extracts were run in triplicate. A negative control was prepared in two replicates using distilled water instead of a DNA template. The second experiment examined the detection limit of one individual of *P. thornei* in the presence of an increasing number of individuals (all vermiform stages) of *P. penetrans*. One, 5, 10, 20, 40 and 80 individuals (all vermiform stages) of *P. penetrans* were hand-picked and transferred to an Eppendorf tube containing 25 µl water along with a single *P. thornei*. DNA was extracted (Holterman *et al.* 2006). Three qPCR were run for each DNA extract. A negative control was also prepared in two replications using distilled water instead of a DNA template.

7.2.3.4 Construction of standard curve

DNA was extracted (Holterman *et al.*, 2006) from three times 80 individuals (all juvenile stages without eggs) of *P. thornei* (PthZ1). A single qPCR was run for each DNA-extract and Ct-values were compared. Subsequently, all three tubes of DNA were mixed and a dilution series was prepared. The mixed DNA sample was serially diluted to 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 of the original concentration representing 80 individuals of *P. thornei*. These concentrations were used as templates in a qPCR. Plotting logarithmic values of DNA concentration versus Ct-values generated a standard curve.

7.2.3.5 Robustness of the method

To verify if the qPCR method is able to detect and quantify nematodes extracted from a soil sample, eight soil samples from a running pot test with cereals infected with *P. thornei* were analysed. Nematodes were extracted from soil and roots with zonal centrifugation (Hendrickx, 1995). The whole nematode suspension was first counted using a microscope and then used for DNA extraction, followed by the developed qPCR assay.

7.3 Results

7.3.1 Optimisation of the annealing temperature

Based on absence of non-specific product, 69°C was selected as annealing temperature. Increasing temperatures resulted in higher Ct-values. At 62°C, 63°C, 65°C and 68°C, the dissociation curve of the qPCR test showed multiple peaks (Figures 7.2, 7.3). At 69°C, the assay was able to detect a single individual of *P. thornei* whereas no signals were observed in the NTC samples (Table 7.2). Non-specific fluorescence due to amplification of primer-dimers or other non-specific amplification product was not observed.

Table 7.2. Mean and standard deviation of Ct values obtained at different melting temperatures of DNA extracted from single individuals (PthZ1) and five individuals (PthZ2) of *Pratylenchus thornei* (n = 2).

	62°C		63°C		65°C		68°C		69°C	
Sample	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
PthZ1 (1)	28.1	0.6	28.9	1.2	29	0.2	30.2	0.5	31.9	0.2
PthZ2 (5)	27.6	0.2	27.9	0.4	28.4	0.1	29.2	0.5	29.4	1
Negative control	Und	–	> 35	–	Und	–	Und	–	Und	–

Und = Undetermined

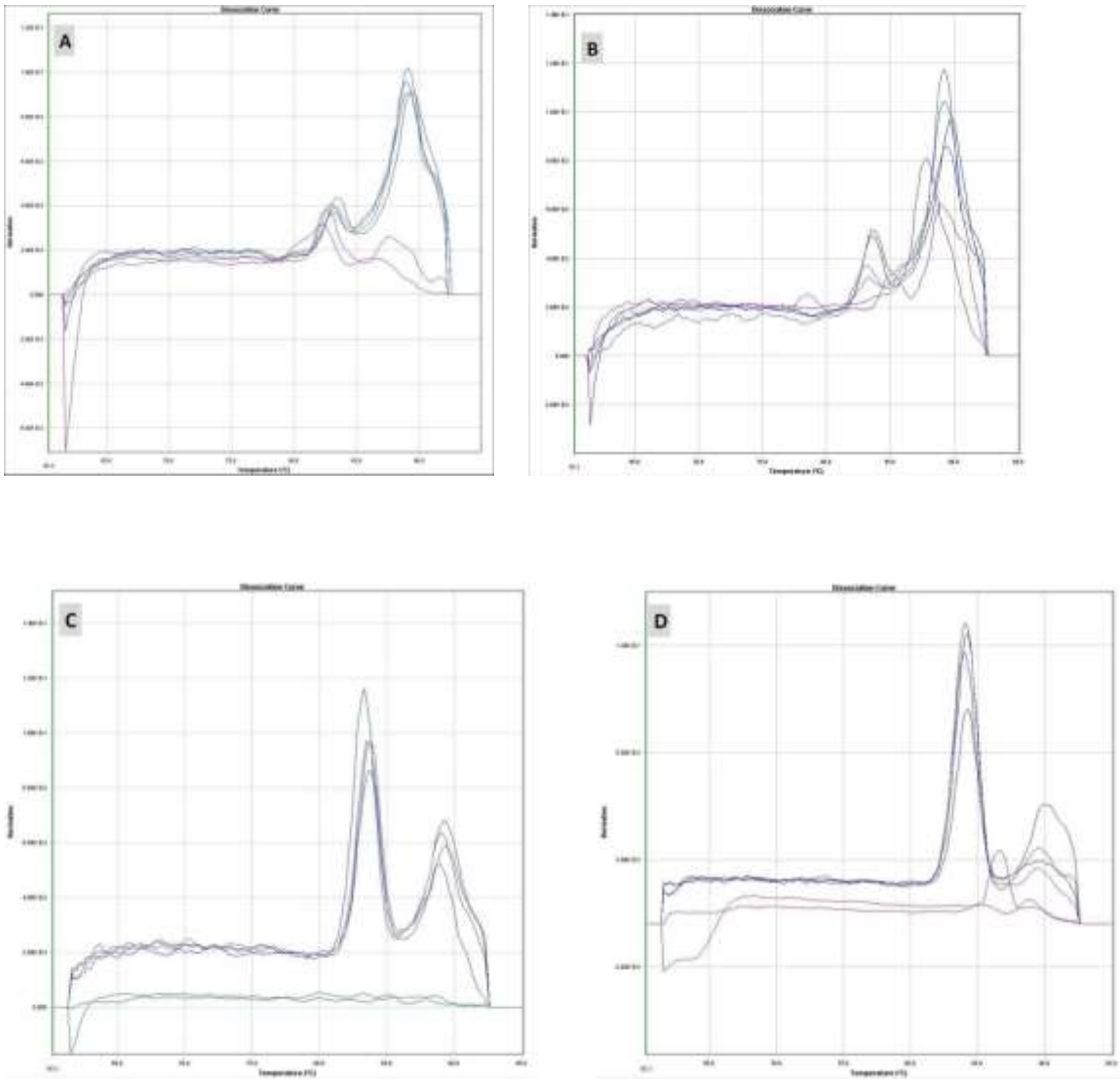

Figure 7.2. A, B: Dissociation curve of the qPCR test (SensiFast SYBR Hi-ROX) with annealing temperatures set at 62°C and 63°C showing multiple peaks of two *Pratylenchus thornei* populations (n = 3) and NTC (n = 2); C, D: Dissociation curve of the qPCR test (SensiFast SYBR Hi-ROX) with annealing temperatures set at 65°C and 68°C showing multiple peaks of two *P. thornei* populations (n = 3) and NTC (n = 2)

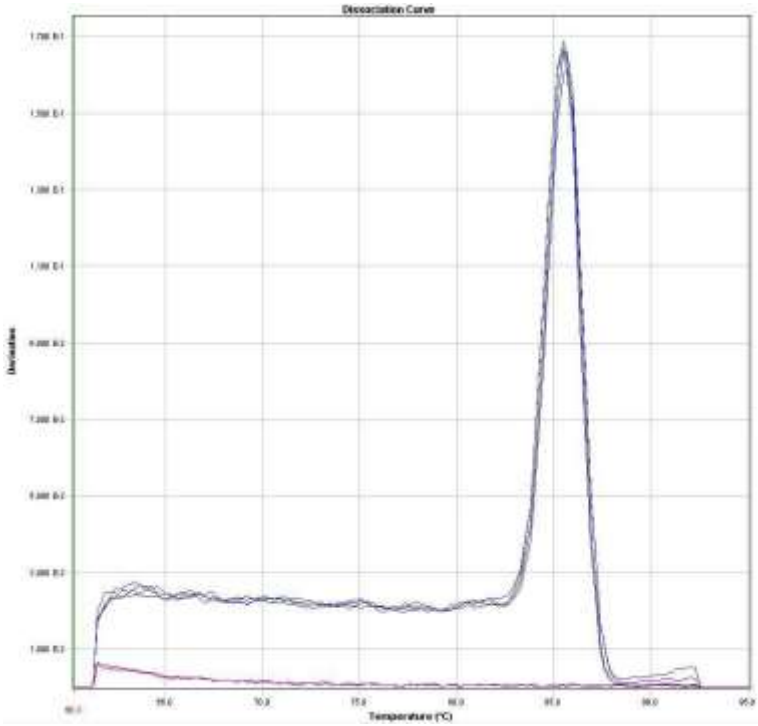

Figure 7.3. Dissociation curve of the qPCR test (SensiFast SYBR Hi-ROX) with annealing temperature set at 69°C showing high peaks at ± 85.5 of two *Pratylenchus thornei* populations (n = 3) and NTC (n = 2).

7.3.2 Specificity of primer and probe set

The target fragment (88 bp) of all *P. thornei* isolates was amplified running the qPCR protocol with the primer pair PthMFor and PthMRev, and the probe PthMPb. DNA from other *Pratylenchus* species, or from species of other nematode genera tested, was not amplified (Table 7.1). In addition, DNA was not amplified or detected in any of the controls that contained water instead of DNA. The Ct-values for DNA derived from 1-5 *P. thornei* individuals from different populations varied between 27.7 ± 0.6 and 30.1 ± 0.4 with the exception of two isolates. These isolates (PthN2; PthMo) were cultured, and pure genomic DNA from thousands of individuals was obtained using a DNA extraction kit about 7 years ago (High pure PCR template preparation kit, Roche diagnostics). The Ct-values of the latter were lower (19.38; 22.6) because of the more concentrated DNA (Table 7.1).

7.3.3 Sensitivity of primers and probe

The qPCR (first experiment) successfully amplified DNA extracted from all *P. thornei* quantities (1, 5, 10, 20, 40, or 80 individuals). The corresponding Ct-values were 28.7 ± 0.5, 26.9 ± 0.2, 26.1 ± 0.1, 24.8 ± 0.2, 23.9 ± 0.2, and 23.1 ± 0.2. The Ct-value of the negative control was always undetermined. The Ct-values obtained after qPCR with DNA from a

single *P. thornei* in the presence of increasing individuals of *P. penetrans* (second experiment) were almost constant: 28.8 ± 0.1, 28.6 ± 0.4, 28.7 ± 0.1, 28.9 ± 0.3, 28.8 ± 0.5 and 28.8 ± 1, respectively. The negative control was always undetermined. The Ct-values were very stable.

7.3.4 Construction of a standard curve

The primers PthMFor, PthMRev and PthMPb generated a PCR product. No fluorescent signal was recorded from negative control. The threshold was set on 0.03, measuring amplification during the logarithmic phase of the qPCR (Figure 7.4). qPCR was run three times using DNA extracted from 80 individuals of *P. thornei*. A standard curve for *P. thornei* was generated using a serial dilution of *P. thornei* DNA (Figure 7.4; Table 7.3). It showed a highly significant relationship between the Ct-value and number of nematodes over the range studied (R^2 = 0.98; slope = –3.38; E = 97.6%). Based on three sample replications, the ABI PRISM fluorescence detection system automatically calculated the starting number of *P. thornei* by comparison of the Ct-values from the unknown samples with the values of the standard curve.

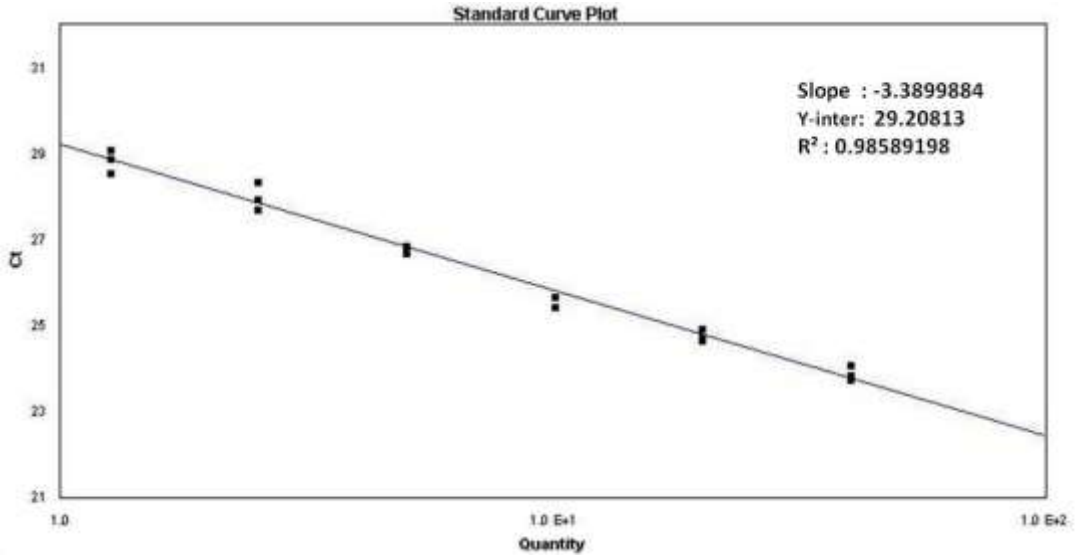

Figure 7.4. Standard curve of the qPCR assay for *Pratylenchus thornei* threshold cycle number (Ct) plotted against the log of the number of individuals of *P. thornei* (1, 5, 10, 20, 40) (n = 3).

Table 7.3. Cycle threshold (Ct) values from a serial dilution of *Pratylenchus thornei.*

Serial dilution	Number of *P. thornei*	Ct
1 : 2	40	23.9 ± 0.1
1 : 4	20	24.7 ± 0.7
1 : 8	10	25.6 ± 0.1
1 : 16	5	26.7 ± 0.1
1 : 32	2.5	28 ± 0.3
1 : 64	1.25	28.8 ± 0.3

7.3.5 Robustness of the method

The eight soil samples contained between 26 and 228 vermiform stages of *P. thornei* (no eggs) and some saprophytic nematodes. About the same, or very similar, numbers of *P. thornei* were counted using visual assessment as with the qPCR method (Table 7.4).

Table 7.4. Cycle threshold (Ct) values of nematodes from soil and root samples with *Pratylenchus thornei.* The total number of *P.thornei* in the nematode suspension was counted, then a subsample of the DNA-extract of the nematodes was used for qPCR. The calculated number of *P. thornei* is the number of individuals for the whole sample.

	Ct-	Number of *P. thornei*	
Sample	Value	Calculated	Counted
1	27.46	228.0	233
2	30.80	38.4	40
3	29.41	81.0	82
4	30.25	102.8	107
5	29.68	70.0	73
6	29. 95	60.4	65
7	32.79	26.0	29
8	26.39	103.2	106

7.4 Discussion

A qPCR assay aimed at the detection of the migratory endoparasitic nematode, *P. thornei*, was successfully developed using sequences of the β-1,4-endoglucanase gene of *Pratylenchus* spp. This gene plays a crucial role in plant cell wall-degradation during penetration and migration of nematodes in the host roots. Although demonstrated by other authors that the rDNA-ITS region can be used for qPCR species-specific primer and probe design for *Pratylenchus* species (Yan *et al*., 2012, 2013), the use of sequences of the β-1,4-endoglucanase gene overcomes potential difficulties when using ITS-rDNA. Several studies showed that ITS-sequences could vary in size between *Pratylenchus* species, making sequence alignment to detect species-specific fragments problematic (Uehara *et al.*, 1998b; De Luca *et al*., 2011). In addition, ITS sequences show extensive polymorphism within a species or an individual (Waeyenberge *et al.*, 2009; De Luca *et al*., 2011). The β-1,4-endoglucanase gene was used by Mokrini *et al.* (2013) to develop a qPCR assay for the identification of *P. penetrans*. We decided to continue using the β-1,4-endoglucanase gene for the development of a similar assay for *P. thornei* as we had the sequences for many relevant nematode species and populations needed in this study at our disposal.

The assay allowed accurate and consistent detection of the DNA of single individuals of *P. thornei* when mixed with DNA from 80 individuals of *P. penetrans*. This sensitivity compares well with findings reported for other nematode species, *viz*., *P. neglectus* (Yan & Smiley, 2013), *P. penetrans* (Mokrini *et al.*, 2013), *P. penetrans* (Sato *et al.*, 2007), *Globodera rostochiensis* (Toyota *et al.*, 2008), *G. pallida* and *Heterodera schachtii* (Madani *et al.*, 2005), and *Meloidogyne incognita* (Ciancio *et al.*, 2005). Yan *et al.* (2012) reported on an ITS-based primer for *P. thornei* with a sensitivity of a single juvenile or single female of *P. thornei* in a single gram of soil.

The assay has not only a high amplification efficiency, it is also highly specific, showing a single amplicon in melting curve analyses and no specific amplification when using DNA from other species of *Pratylenchus*, including the closely related *P. mediterraneus* (De Luca *et al.*, 2004).

Our runs of serial dilutions of DNA of *P. thornei*, as well as runs with DNA extracted from increasing amounts of nematodes showed a highly significant linear relationship between the Ct-value and number of *P. thornei*. These results are similar to those obtained with the qPCR test developed for *P. penetrans* by Mokrini *et al.* (2013). Yan *et al.* (2013) reported a significant positive relationship between the numbers of *P. neglectus* added to soil

and the numbers quantified using their soil standard curve and qPCR. They found much variation between the qPCR tests of replicate samples of *P. neglectus* added at 20 and 40 nematodes per g of soil. The authors mention this is probably due to the commercial kits used for extraction of DNA directly from soil causing variable number of nematodes to be disrupted and releasing their DNA during vortexing steps. Our tests were performed with nematode suspensions in water, requiring extraction of nematodes from soil prior to performing the qPCR test, but increasing accuracy in quantification. We demonstrated that the test also performs very well with nematodes extracted from soil using centrifugation, thus indicating its robustness.

This qPCR assay has the capacity of simultaneously detecting and quantifying *P. thornei* in mixed populations of *Pratylenchus* spp. where visual identification of individual nematodes at species level is extremely difficult. This technique does not require expertise in nematode taxonomy and morphology, and can be used as a rapid diagnostic tool in research, as well as in diagnostic laboratories to avoid the time-consuming steps of traditional nematode extraction, microscopic identification, and quantification.

CHAPTER 8

Screening for resistance to *Pratylenchus penetrans* and *P. thornei* in wheat lines, with or without co-inoculation with the *Heterodera avenae*, using qPCR as a technique for nematode quantification

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8.1 Introduction

Root-lesion nematodes (RLN) are considered the most important group of plant-parasitic nematodes attacking cereals on a worldwide basis (Smiley & Nicol, 2009). They comprise a group of closely related *Pratylenchus* species that have been documented to cause economic yield losses, especially in wheat production systems in north Africa, Australia, the United States, and parts of Europe (Nicol *et al*., 2003; Thompson *et al*., 2008; Vanstone *et al*., 2008; Smiley & Nicol, 2009). Eight species of RLN are known to be parasitic on small grain cereals. Of these, *Pratylenchus thornei* and *P. penetrans* are considered the most economically important (Rivoal & Cook, 1993; Mc Donald & Nicol, 2005). The value of Australian wheat production lost to *P. thornei* has been estimated at $AU 69 million/year in the northern region (Thompson *et al*., 2008) and $AU 190 million/year in the southern and western grain regions (Vanstone *et al*., 2008). RLN feed, migrate and reproduce inside the root cortex of their host, resulting in lesions and debilitated root systems that are inefficient at taking up nutrients and water from the soil. Evidence of the belowground pathogenic activity of the nematodes can be seen as aboveground symptoms of chlorosis and wilting of leaves, and reduced tillering (Moens & Perry, 2009). In Morocco, *Pratylenchus* spp. are the most prevalent group of nematodes in different cereal growing regions followed by cyst nematodes of the *Heterodera avenae* group (Meskine *et al*., 1984; Rammah, 1994; Chapters 3 and 4). The results of a survey of wheat producing regions in Morocco (Chapter 3) demonstrated *P. penetrans* to be the most abundant and widespread species that was recovered from 70% of soil samples. The population densities varied between 32 and 123 nematodes $(100\ g)^{-1}$ of soil and between 67 and 102 nematodes $(10\ g)^{-1}$ of root of wheat. The related species *P. thornei* is also common in Morocco, and both species can be found in a same field.

So far, many attempts have been made to control RLN in cereals around the world, including cultural practices, chemical control, and development of resistant varieties (Smiley & Nicol, 2009; Dababat *et al*., 2011). The use of resistant accessions is considered the most effective and economical method for managing nematodes as it is environmentally sustainable and requires no additional equipment or cost. Tolerant cultivars suffer little yield reduction even though their roots can be invaded by nematodes, whereas resistant cultivars reduce the rate of nematode multiplication in the roots (Roberts, 2002). Several resistant wheat accessions against *P. thornei* have been identified (Vanstone *et al*., 1998; Thompson *et al*., 1999; Toktay *et al*., 2012). For example, the Iraqi landrace AUS4930 was found to be resistant to the root-lesion nematode *P. thornei* as well as to the cyst nematode *H. avenae*

(Australian pathotype Ha13) (Toktay, 2008; Nicol & Rivoal, 2009). The soil-borne pathogen programme of CIMMYT-ICARDA screens annually about 1000 accessions from the International Winter Wheat Improvement Program (www.iwwip.org) under growth room, greenhouse, and field conditions at various locations. Cultivars are also screened for multiple disease resistance, including the root lesion nematodes *P. thornei* and *P. neglectus* (Dababat *et al*., 2015).

The presence of several taxa of plant-parasitic nematodes in agricultural soils is a complication in nematode management (Stetina *et al*., 1997). This is particularly challenging when using resistant cultivars because most nematode resistance is targeted at one species, which may be present with other species that could parasitize the resistant host (Bradley & Duffy, 1982). Competition between different soil-borne parasitic nematodes associated with economic damage has been reported for several crops (Yang, 1976; Lasserre *et al*., 1994; Brinkman *et al.*, 2004; Moens *et al*., 2006; Melakeberhan & Dey, 2003; Brinkman *et al.*, 2005). Gay and Bird (1973) found that the root-knot nematode *Meloidogyne incognita* was inhibited by the presence of *P. brachyurus* on cotton. Brinkman *et al*. (2005) reported that *P. penetrans* suppressed the abundance of *H. avenae* on dune grass *Ammophila arenaria*. Rivoal *et al*. (1995) recorded suppression of *P. neglectus* in the presence of *H. avenae* on oats. Lamberti *et al*. (2001) reported that *M. incognita* in olive suppressed reproduction of *P. vulnus*. Similarly, the infection rate of *H. glycines* on soybean decreased with increasing proportions of *P. penetrans* (Melakeberhan & Dey, 2003). Interspecific competition has been suggested between *P. coffeae* and *M. exigua* on coffee in Costa Rica (Bertrand *et al*., 1998) and Guatemala (Herve *et al*., 2005).

Surveys of cereal fields in the major wheat and barley cultivating areas of Turkey (Sahin *et al*., 2009), Iran (Abdollahi, 2010) and Morocco (Znasni, 2003; Mokrini *et al*., 2009; 2012) showed that both *P. penetrans* and *P. thornei* are often found together with *H. avenae*. Thus, it would be interesting to know when introducing or breeding for new cultivars if the infection by both a *Pratylenchus* species and *H. avenae* influences the resistance level.

The correct identification and quantification of nematode species is a fundamental step in control strategiesRecently, quantitative PCR strategies have been developed for *P. penetrans* (Chapter 6) and *P. thornei* (Chapter 7) as well as for *H. avenae* (Toumi *et al*., 2013a) and provide a rapid alternative to microscopic identification. Yan *et al*. (2008) reported differences between *P. neglectus* or *P. thornei* estimates obtained by microscopy and qPCR. The authors attributed this discrepancy to the difficulty in identifying and quantifying both *Pratylenchus* species using traditional microscopy-based methods and also to the uneven

distribution of nematodes in soil. In Chapter 7 it was concluded that there were no differences between the number of *P. thornei* counted using a microscope and numbers estimated by the qPCR assay from a suspension of cultured nematodes.

In view of these issues, the objectives of this study were (1) to identify resistant wheat lines against the root-lesion nematodes *P. thornei* and *P. penetrans* in pot experiments under greenhouse conditions, (2) to investigate the effects of co-inoculating *H. avenae* on the reproduction of the lesion nematodes *P. penetrans* and *P. thornei* on resistant wheat lines under controlled conditions, hence on the stability of the resistance when more than one nematode species are present, and (3) to compare the enumeration of vermiform stages of both *Pratylenchus* sp. using the traditional visual counting through microscopy or using qPCR (Chapters 6 and 7).

8.2 Material and Methods

8.2.1 Screening for resistance against the root lesion nematodes *P. thornei* and *P. penetrans*

8.2.1.1 Plants

Twenty-five lines of wheat provided by CYMMIT were screened for resistance against a population of *P. thornei* and of *P. penetrans*. The set of germplasm represented a collection of 14 lines of spring wheat (SW) and 11 lines of winter wheat (WW) (Tables 8.1 and 8.2). The durum wheat cultivar Ourgh, susceptible to both *P. thornei* and *P. penetrans,* was used as a standard. Two independent experiments were conducted to phenotype the set of wheat against *P. thornei* and *P. penetrans* in greenhouse conditions. About 15 surface sterilized seeds of each line were placed on moistened filter paper in sterilized Petri dishes. Seeds were germinated at 23 to 25°C for 3 to 4 days. Seeds of winter wheat cultivars had first been vernalized by keeping them at 5°C for 3 weeks. One seedling with 3 seminal roots was transplanted into a plastic folding tube (15 x 20 x 120 mm) filled with a mixture of sand, field soil and organic matter (70:29:1 V/V). The field soil and sand had been sieved and sterilized at 100°C before use. For each line of wheat, thirty screening tubes were devided over 3 pots (15 cm diameter) with 10 screening tubes in each pot. The spaces around the tubes were filled with sand to keep the tubes upright. Thirty replicates of each line were tested. The 75 pots (3 x 25 lines), were arranged in a completely randomized design in a greenhouse with temperatures between 22°C and 24°C. Plants were sprayed daily with water using an

atomizer. The same experimental protocol was used for *P. thornei* as for *P. penetrans*.

8.2.1.2 Nematode inoculum

Experiments were carried out using one populations of each species (*P. penetrans* and *P. thornei*), collected from Gharb and Zaers regions of Morocco, respectively. These two populations were maintained *in vitro* on carrot-disc cultures according to Moody *et al*. (1973). Nematodes were extracted by placing infected chopped carrot discs on Baermann funnels in a mist chamber (OEPP/EPPO, 2013) for 3 days. Nematode suspensions, containing all vermiform stages, were prepared using tap water. One week after planting, each seedling was inoculated with a nematode suspension containing 400 individuals of either *P. thornei* (experiment 1) or *P. penetrans* (experiment 2). This inoculum density was found to be well suited for nematode screening in cereals (Keil *et al*., 2009; Toktay *et al.*, 2012). Nematodes were applied with a pipette into 3 holes of 2 cm deep made at 0.5 cm distance from the stem base.

8.2.1.3 Assessment of resistance

Plants were harvested nine weeks after inoculation and aboveground plant parts were removed. To ensure easy removal of the plants from the soil, the sides of the plastic tubes were pressed to loosen the soil. The soil was then removed from the roots by gently shaking the plants. The roots were washed separately for every plant. Nematodes were released from the roots by cutting the root system in 2-cm pieces and macerating them in water for 1 min at high speed in a commercial blender (Waring). Nematodes were extracted from this mixture and also from the soil of each tube using an automated zonal centrifuge (Hendrickx, 1995). All vermiform stages of *P. thornei* or *P. penetrans* in the obtained nematode suspensions were counted using a stereomicroscope. When there were less than about 1000 individuals, nematodes were counted in four batches. For more than 1000 individuals per sample, a dilution was made and 1 ml of nematode suspension was counted in three replications. The number of extracted nematodes per plant was calculated. For the evaluation of the susceptibility of the wheat lines against *P. penetrans* and *P. thornei*, a reproduction factor *Rf* = Pf/Pi was calculated for each plant, where Pf = total number of nematodes from both soil and roots in each tube at harvest and Pi = initial number of nematode inoculated in the tube (i.e. 400). Wheat lines, which, based on their reproduction factor ($Rf < 1$), gave a resistant reaction against *P. thornei* or *P. penetrans* were re-phenotyped for data validation.

8.2.2 Dual inoculation of *H. avenae* and *P. penetrans* or *P. thornei*

8.2.2.1 Nematode inoculum

The populations of *P. thornei* and *P. penetrans* used in the screening described above were also used in these experiments. Cysts of *H. avenae* were obtained from soil samples collected from a field in Marchoch, Zaers region, Morocco. They were extracted from soil using a sieving and flotation method (Shepherd, 1986), then surface sterilized with 0.5% NaOCl for 10 min and finally rinsed several times in distilled water. Eventually, the cysts were kept for two months in a refrigerator at a temperature of 4°C before they were transferred into an incubator at a temperature of 10°C to enhance hatching (Dababat *et al*., 2014). The hatched second-stage juveniles (J2) were used as inoculum.

8.2.2.2 Plants and inoculation procedure

Three lines (L9, L12 and L13) and one line (L9) found resistant against *P. thornei* and *P. penetrans,* respectively, were tested once more under the same conditions as in the first experiments. Each seedling was placed in a conical screening tube (100 mm long x 15 mm diam) instead of a plastic folding tube. Two experiments (experiment 3 with *P. thornei*, experiment 4 with *P. penetrans*) were conducted in a growth chamber set at 16 h light, 21°C and 70% RH.

In experiment 3, the three wheat lines (L9, L12 and L13) were inoculated with (a) 400 *P. thornei* (all stages combined), (b) 400 J2 of *H. avenae,* or (c) 400 *P. thornei* (all stages) + 400 J2 of *H. avenae* (exp. 3). Similarly, in experiment 4, line (L9) was inoculated with (a) 400 *P. penetrans* (all stages combined), (b) 400 J2 of *H. avenae,* or (3) a mixture of 400 *P. penetrans* + 400 *H. avenae.* The susceptible durum wheat cv. Ourgh (control) was inoculated with 400 *P. thornei* (exp. 3) or 400 *P. penetrans* (exp. 4). Ten replicate tubes per inoculum treatment were arranged in a completely randomized design in tube racks placed above a shallow dish holding water. As the lower tips of tubes were about 2 cm inside the water, plants received water as needed. Both experiments (3 and 4) were conducted at the same time.

8.2.2.3 Evaluation of resistance

Nine weeks after inoculation, shoots were removed and cysts were extracted from soil on 200-µm sieves by the sieving and flotation method (Shepherd, 1986). The roots of each

wheat line were washed separately and the rinsing water was added to the beaker with the soil. Roots were visually checked for presence of cysts. Nematodes inside the roots were released by cutting the root system in 2-cm pieces followed by maceration in water (1 min at high speed in a commercial blender; Waring). Vermiform stages of *Pratylenchus* and *H. avenae* were extracted from both soil and the mixed roots using an automated zonal centrifuge (Hendrickx, 1995). The number of cysts (cysts on roots and in soil) and vermiform stages of *P. thornei*, *P. penetrans*, or *H. avenae* in the obtained nematode suspensions (about 35 ml) were counted using a stereomicroscope. The number of extracted vermiform stages of *P. penetrans*, *P. thornei* and *H. avenae* per plant was calculated. For the evaluation of the susceptibility of the wheat lines against *P. penetrans* and *P. thornei*, a reproduction factor *Rf* = Pf/Pi was calculated for each plant (see below).

To validate the qPCR methods for quantification of *P. penetrans* (Chapter 6) and *P. thornei* (Chapter, 7) on nematodes extracted from soil, all samples containing these species were also used in a species-specific qPCR. After counting the vermiform stages of *P. penetrans*, *P. thornei* or *H. avenae* extracted from soil and roots with zonal centrifugation, the obtained nematode suspension was transferred into a 40 ml conical tube where nematodes were allowed to settle down for 3 h. Then, 3 ml nematode suspension was pipetted from the bottom of each tube and distributed equally in two microtubes of 1.5 ml. After centrifugation at 12,000 rpm for 10 min, the supernatant was removed. DNA was extracted from nematodes from each of the two microtubes as described by Holterman *et al.* (2006). The microtubes were incubated at 65°C for 1.5 h and 99°C for 5 min, consecutively. The reaction mixture and PCR-program were as described in chapters 6 and 7. The standard curves as obtained in Chapters 6 and 7 were used.

8.2.2.4 Statistical analysis

Data of the screening tests were analysed with one-way analysis of variance (ANOVA) using SPSS software for Windows (SPSS Inc., Illinois, USA). Differences in reproduction of nematodes between wheat accessions (exp. 1 and 2) were checked with Tukey's test for comparison of means, when the F-value was significant at $P < 0.05$. The reproduction of *P. thornei* between three resistant wheat lines (exp. 3) counted using the microscope and by the qPCR method was subjected to a two-way-factorial ANOVA to determine the effects of the nematode treatment and wheat lines. The Student Newman-Keuls test ($P < 0.05$) was used for mean separation. Differences among the nematode treatments in

experiment 4 were assessed using a one-way analysis and the means were separated using Tukey's test ($P < 0.05$). The influence of the inoculation density of nematodes on the vegetative growth was analysed with two-way ANOVA using SPSS software for windows in (exp. 3) and with one-way ANOVA in (exp. 4). Correlation between the two estimates of nematode numbers; *viz*. the real-time PCR assay and visual counts (microscopy method) was determined.

8.3 Results

8.3.1 Screening of wheat lines for resistance to *P. penetrans* and *P. thornei*

Both, *P. penetrans* and *P. thornei* were able to survive and even increase on most lines. Nine weeks after inoculation, the number of vermiform stages of *P. penetrans* and *P. thornei* in the 25 lines of wheat ranged from 360 to 2128 and from 240 to 2040 per plant, respectively (Tables 8.1 and 8.2). On the susceptible line (Ourgh), the average final numbers per plant were 1285 and 1804, for *P. penetrans* and *P. thornei,* respectively. The lowest average number of nematodes per plant was found in line L9 (360 *P. penetrans* in soil and root), whereas the highest average number of nematodes per plant was found in line L24 (2128 *P. penetrans*) (Table 8.1). The corresponding reproduction factor (*Rf*) varied from 0.9 to 5.3, for lines L9 and L24, respectively. The total number of nematodes (roots and soil) on L9 was significantly lower than on other lines; L9 was the only line on which fewer specimens of *P. penetrans* were found after 9 weeks than the number that was inoculated (*Rf* = 0.9). There were no lines without nematode infection, i.e. none showed immunity to *P. penetrans*.

The *Rf* of *P. thornei* on the 25 lines varied from 0.6 (L9) to 5.1 (L8) (Table 8.2). The *Rf* of three lines (L9, L12 and L13) was less than 1 and the number of *P. thornei* in roots of L9 (84) as well as in soil (156) were the lowest of all lines. The sibling lines L12 and L13 had similar numbers of *P. thornei* in their roots, but more nematodes were found in soil of L13, resulting in *Rf*-values of 0.7 and 0.9 for L12 and L13, respectively. The highest number of nematodes in soil and roots was found in line L8 with 2040 *P. thornei* (vermiform stages) per plant (Table 8.2).

Because of the relatively low reproduction rate (*Rf* <1) on L9, L12 and L13, these lines were tested again, together with the control (Ourgh). Nine weeks after inoculation, the mean number of *P. penetrans* in roots and soil of L9 was 321 per plant. The final numbers of *P. thornei* on lines L9, L12, 13 were 288, 250 and 310, respectively. The corresponding *Rf*

varied from 0.6 to 0.8. These results confirm the resistance of a number of lines to *P. penetrans* (L9) and *P. thornei* (L9, L12 and L13).

Table 8.1. Average numbers of vermiform *Pratylenchus penetrans* per plant (n= 30) of different lines of wheat, 12 weeks after inoculation with 400 vermiform stages of *P. penetrans* (exp.1).

Code	Line	Accession* number	Type of wheat**	*P. penetrans*							Reaction***
				Root (Mean)	Root (Range)	Soil (Mean)	Soil (Range)	Total	Range	Rf (Pf/Pi)	
L1	6R (6D)	30883	SW	261 g	203-290	379 k	350-460	640 ± 33.7	521-801	1.6	S
L2	FRAME	20591	SW	198 i	165-231	522 j	385-632	720 ± 71.3	589-840	1.8	S
L3	SILVERSTAR		SW	523 b	480-571	997 c	796-1156	1520 ± 89.5	1287-1677	3.8	S
L4	VP5053	30903	SW	453 c	390-497	907 d	652-986	1360 ± 68.6	1200-1528	3.4	S
L5	T-2003	20628	SW	437 c	408-471	1243 b	1195-1289	1680 ± 35	1548-1796	4.2	S
L6	RAJ 1		SW	503 b	422-539	1497 a	1470-1534	2000 ± 32	1891-2126	5	S
L7	ID-2150	20626	SW	209 i	171-242	791 ef	741-825	1000 ± 33.4	893-1130	2.5	S
L8	MILAN	990659	SW	326 e	286-370	634 i	587-671	960 ± 30.2	885-1087	2.4	S
L9	AUS 4930.7/2 PASTOR	30857	SW	98 k	71-124	262 l	220-320	360 ± 22.3	269-390	0.9	R
L10	AUS GS50AT34/SUNCO	30798	SW	201 i	167-240	439 j	382-511	640 ± 30.5	571-721	1.6	S
L11	VL411R	30898	SW	232 h	183-270	608 i	421-657	840 ± 51.5	780-892	2.1	S
L12	CROC_1/AE.SQUARROSA (224)	20615	SW	102 k	77-134	458 j	381-555	560 ± 48.5	519-661	1.4	S
L13	CROC_1/AE.SQUARROSA (224)	20616	SW	283 f	241-317	357 k	311-432	640 ± 28	590-702	1.6	S
L14	VP1620	30901	SW	204 i	170-240	636 i	590-681	840 ± 116	791-961	2.1	S
L15	F130L1.12/ATTILA	980872	WW	387 d	351-420	773 g	723-812	1160 ± 26.8	1002-1326	2.9	S
L16	SONMEZ		WW	178 j	127-242	822 e	780-898	1000 ± 46.5	927-1056	2.5	S
L17	CPI133859		WW	104 c	16-137	416 m	378-467	520 ± 35. 3	481-601	1.3	S
L18	CPI133872		WW	204 i	169-137	636 i	580-678	840 ± 38.5	791-902	2.1	S
L19	KATE A-1	950590	WW	598 a	561-640	1202 b	1115-1245	1800 ± 37.7	1759-1902	4.5	S
L20	PRINS		WW	321 e	271-372	439 j	401-487	760 ± 29.4	744-802	1.9	S
L21	MIRZABEY2000		WW	309 e	266-365	691 h	641-735	1000 ± 33.8	956-1122	2.5	S
L22	AU/CO652337//2CA8-155/3/F474S1-1.1	50484	WW	595 a	570-629	805 f	780-841	1400 ± 25.8	1321-1522	3.5	S
L23	F372		WW	384 d	322-451	856 e	801-923	1240 ± 31.2	1181-1382	3.1	S
L24	TAIKONG		WW	627 a	566-702	1501 a	1458-1589	2128 ± 36.7	1998-2320	5.3	S
L25	ZHONGYU		WW	523 b	408-611	923 d	845-1123	1446 ± 41.7	1234-1600	3.6	S
Ls	OURGH			395 d	376-482	895 d	812-976	1285 ± 29.9	1190-1367	3.2	S

*Number assigned by CYMMIT, ** SW : Spring wheat, WW : Winter wheat, *** R : Resistant, S : Susceptible.
Means with the same letter in the same column are not significantly different at $P < 0.05$, according to Tukey's test.

Table 8.2. Numbers of vermiform *Pratylenchus thornei* per plant (n=30) of different lines of wheat, 12 weeks after nematode inoculation with 400 vermiform stages of *P. thornei* (exp.2).

Code	Line	Accession* Number	Type of wheat**	*P. thornei*							Reaction***
				Root (Mean)	Root (Range)	Soil (Mean)	Soil (Range)	Total	Range	Rf (Pf/Pi)	
L1	6R (6D)	30883	SW	140 k	91-183	460 g	406-503	600 ± 40	577-620	1.5	S
L2	FRAME	20591	SW	300 g	266-361	1220 b	922-1303	1520 ± 78	1479-1602	3.8	S
L3	SILVERSTAR		SW	390 ef	289-432	530 f	477-581	920 ± 30	878-1001	2.3	S
L4	VP5053	30903	SW	247 h	209-378	793 d	734-835	1040 ± 41	989-1191	2.6	S
L5	T-2003	20628	SW	366 f	267-476	634 e	564-693	1000 ± 76	986-1020	2.5	S
L6	RAJ 1		SW	746 a	680-893	534 f	449-595	1280 ± 56	1202-1425	3.2	S
L7	ID-2150	20626	SW	528 c	471-613	432 g	361-497	960 ± 54	891-1001	2.4	S
L8	MILAN	990659	SW	610 b	566-673	1430 a	1377-1498	2040 ± 44	1901-2199	5.1	S
L9	AUS 4930.7/2 PASTOR	30857	SW	84	56-140	156 k	83-223	240 ± 48	170-393	0.6	R
L10	AUS GS50AT34/SUNCO	30798	SW	147 jk	106-193	293 h	254-344	440 ± 37	370-563	1.1	S
L11	VL411R	30898	SW	319 g	270-372	641 e	578-699	960 ± 43	898-1042	2.4	S
L12	CROC_1/AE.SQUARROSA (224)		SW	173 j	134-237	107 l	70-143	280 ± 27	170-390	0.7	R
L13	CROC_1/AE.SQUARROSA (224)	20616	SW	156 jk	111-212	204 j	166-270	360 ± 36	277-524	0.9	R
L14	VP1620	30901	SW	211 i	170-256	789 d	731-882	1000 ± 37	941-1062	2.5	S
L15	F130L1.12/ATTILA	980872	WW	411 ed	371-455	429 g	355-479	840 ± 45	711-989	2.1	S
L16	SONMEZ		WW	433 d	386-478	327 h	291-377	760 ± 36	536-821	1.9	S
L17	CPI133859		WW	333g	277-392	507 f	416-562	840 ± 41	790-910	2.1	S
L18	CPI133872		WW	211 i	165-264	789 d	722-845	1000 ± 35	885-1051	2.5	S
L19	KATE A-1	950590	WW	283 g	222-345	237 i	173-290	520 ± 36	406-570	1.3	S
L20	PRINS		WW	174 j	143-241	306 h	243-352	480 ± 40	390-581	1.2	S
L21	MIRZABEY2000		WW	246 h	178-312	514 f	422-592	760 ± 48	663-820	1.9	S
L22	AU/CO652337//2CA8-155/3/F474S1-1.1	50484	WW	423 d	361-487	897 c	853-945	1320 ± 39	1245-1563	3.3	S
L23	F372		WW	321 g	241-389	439 g	390-489	760 ± 52	604-811	1.9	S
L24	TAIKONG		WW	227 ih	162-376	293 h	209-379	520 ± 65	488-587	1.3	S
L25	ZHONGYU		WW	233 h	134-321	760 d	612-835	1093 ± 49	937-1123	2.5	S
Ls	OURGH			314 g	269-375	1490 a	1381-1557	1804 ± 78	1756-1980	4.7	S

*Number assigned by CYMMIT, ** SW : Spring wheat, WW : Winter wheat, *** R : Resistant, S : Susceptible.
Means with the same letter in the same column are not significantly different at $P < 0.05$, according to Tukey's test.

8.3.2 Interaction between *P. thornei* and *H. avenae* and their influence on vegetative growth of wheat (nematodes counted under microscope)

Significant differences in reproduction of *P. thornei* were found between the nematode treatments and wheat lines. When *P. thornei* was inoculated alone, there were differences ($P = 0.04$) between the number of *P. thornei* extracted from the three lines of resistant wheat: 143 (L9), 160 (L12) and 303 (L13) (Table 8.3). This corresponded with reproduction factors for L9, L12 and L13 of 0.4, 0.4 and 0.8, respectively. When mixed with *H. avenae*, differences ($P < 0.000$) were still observed in the total number of *P. thornei* among the lines tested. When *P. thornei* and *H. avenae* cohabited the root, the number of *P. thornei* extracted from roots was decreased in lines L9 and L12, but not in L13, when compared with *P. thornei* in single inoculation. This interaction was significant between wheat lines and nematode treatmenets. In mixed inoculations, the total number of *P. thornei* per plant was reduced for lines L9, L12 and L13 to 45, 92 and 280, respectively (Figure 8.1, Table 8.3).

Plants were smaller in mixed inoculations than in single inoculations with *P. thornei* (Table 8.4). In single inoculation, there was no significant difference in root weight between the lines L9, L12 and L13 due to *P. thornei*. In general, root weight in mixed inoculations was slightly less than root weight in single inoculations of either *P. thornei* or *H. avenae.*

8.3.3 Interaction between *P. penetrans* and *H. avenae* and their influence on vegetative growth of wheat (nematodes counted under microscope)

The numbers of *P. penetrans* extracted from soil and roots of resistant line L9 were reduced when *H. avenae* and *P. penetrans* were inoculated simultaneously compared to when *P. penetrans* was inoculated alone (Table 8.3, Figure 8.2). A total of 315 nematodes of *P. penetrans* were found in the soil and roots per plant in the single inoculation compared with 167 nematodes extracted from soil and roots in the concomitant inoculation with equal numbers of juveniles of *H. avenae*. Consequently, the reproduction factor of *P. penetrans* was lower in the presence of *H. avenae*: 0.4 instead of 0.8.

The plant height in mixed inoculations was 29.3 cm, which is lower than in single inoculation where plants reached 34.2 cm on average. Root weight, however, was not affected. In single inoculation, *H. avenae* caused a significant reduction in plant height of line 9 compared to *P. penetrans* (Table 8.4).

Table 8.3. Interaction of *Pratylenchus thornei* or *P. penetrans* with *Heterodera avenae* in three lines of resistant wheat to *P. thornei* (exp. 3) and one line resistant to *P. penetrans* (exp. 4).

Nematode	Wheat lines	Root lesion nematodes (all vermiform stages)					*H. avenae*	
		Root** (per plant)	Soil** (tube)	Total** Counted	Rf (Pf/Pi)	Total qPCR ***	Cysts (soil)	Vermiform stages (root and soil)
Pth *	L9	41 b	99 b	143 c ± 40	0.4	107 bc ± 40	-	-
	L12	25 a	135 c	160 d ± 49	0.4	140 c ± 48	-	-
	L13	24 a	279 d	303 f ± 52	0.8	278 d± 49	-	-
	Control	666	1151	1817 ± 64	4.6	1779 ± 75	-	-
Pth+Ha	L9	19 a	26 a	45 a± 11	0.1	26 a ± 7	10 ± 1.7	410 ± 39
	L12	17 a	75 b	92 b ± 19	0.2	72 b ± 15	13 ± 2.7	453 ± 40.4
	L13	95 c	185 c	280 e ± 86	0.7	249 d ± 68	7 ± 2.1	558 ± 47
Ha	L9	-	-	-	-	-	6 ± 1.6	438 ± 51,2
	L12	-	-	-	-	-	6 ± 1.9	504 ± 27.7
	L13	-	-	-	-	-	7 ± 2.2	391 ± 47
	Control	-	-	-	-	-	12 ± 2.8	821 ± 56
Ppen	L9	86 b	229 b	315 b ± 49	0.8	236 b ± 76	-	-
	Control	567	1373	1940 ± 31	4.9	1214	-	-
Ppen + Ha	L9	65 a	102 a	167 a ± 42	0.4	126 a ± 41	6 ± 1.4	504 ± 12
Ha	L9	-	-	-	-	-	9 ± 2.3	754± 32

*Pth: *P. thornei,* Ppen: *P. penetrans,* Ha: *H. avenae*

**: Means in column per nematode treatment followed by different letters are significantly different (n = 10) according to Student Newman-Keuls test. Means of totals are followed by standard error.

***: Soil and root. The calculated number of *P. thornei* or *P.penetrans* is the number of individuals for the whole sample. (as described in Chapter 7).

Table 8.4. Effects of the intraction of *Pratylenchus thornei* (exp. 3) or *P. penetrans* (exp. 4) with *Heterodera avenae* on plant growth.

Nematode	Wheat lines	Plant growth	
		Height (cm)**	Root weight (g)
Pth *	L9	32.2 a	1.19 ab
	L12	41.1 b	1.3 b
	L13	44.7 b	1.3 b
	Control	24.5	0.9
Pth+Ha	L9	30.3 a	1.15 a
	L12	33.3 a	1.27 ab
	L13	33.2 a	1.24 ab
Ha	L9	30.0	1.4
	L12	33.7	1.4
	L13	33.0	1.8
	Control	22.3	0.8
Ppen	L9	34.2 b	1.25 b
	Control	22.2	1.15
Ppen + Ha	L9	29.3 a	1.28 b
Ha	L9	30.0 a	1.1 a

*Pth: *P. thornei,* Ppen: *P. penetrans,* Ha: *H. avenae*

**: Means in column per nematode treatment followed by different letters are significantly different (n = 10) according to Student Newman-Keuls.

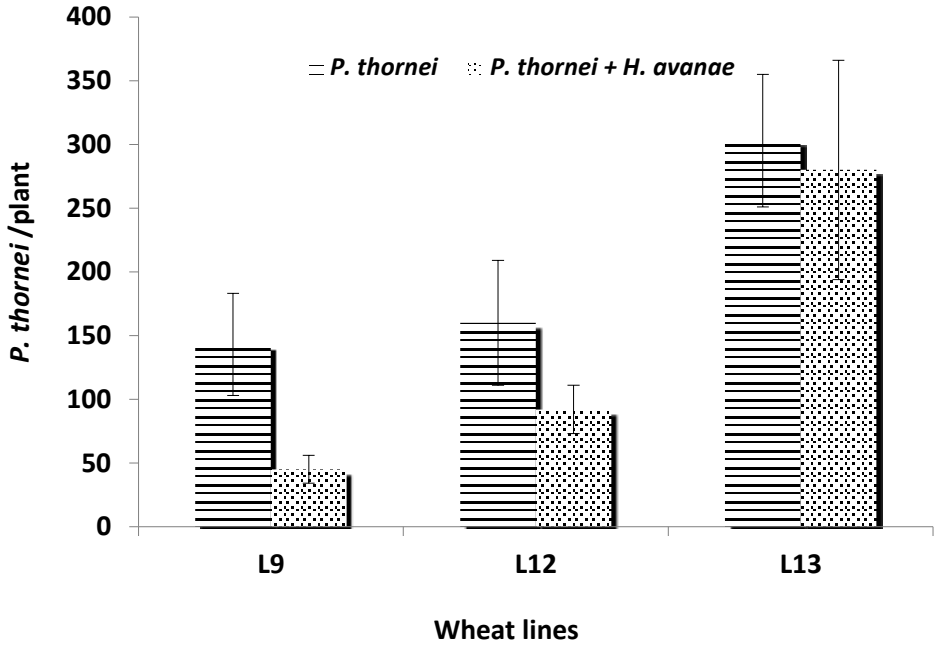

Figure 8.1. Effect of co-inoculation of *Heterodera avenae* and *Pratylenchus thornei* in three resistant wheat lines (L9, L12 and L13).

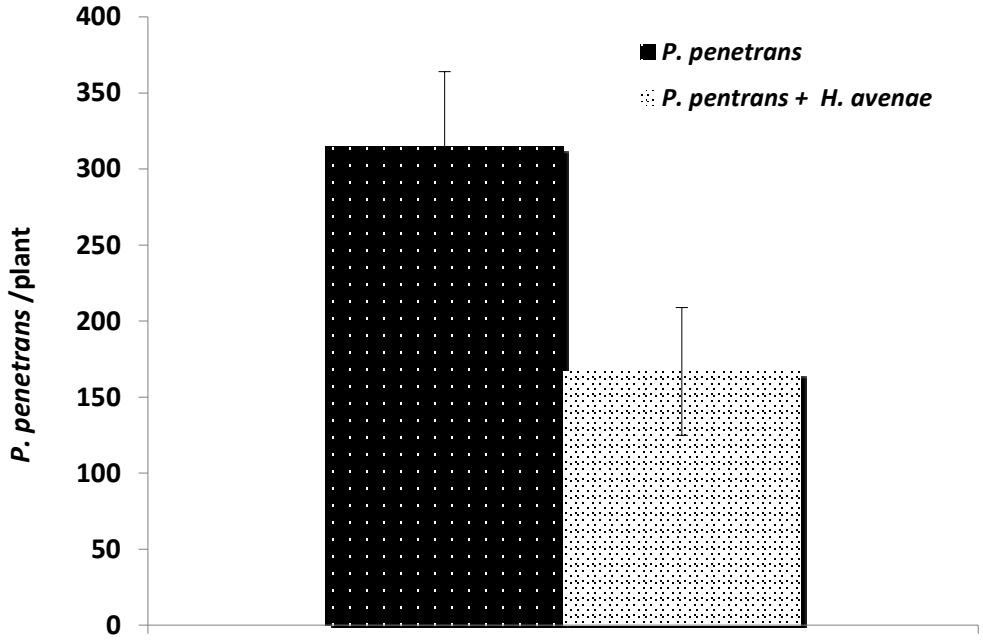

Figure 8.2. Effect of co-inoculation of *Heterodera avenae* and *Pratylenchus penetrans* in resistant wheat line (L9).

8.3.4 Relationship between numbers of nematodes detected by qPCR and microscope

The resistance (R, resistant or S, susceptible) of three lines of wheat was evaluated based on the numbers of nematodes extracted from roots and soil. These numbers were also determined with qPCR assays (Table 8.3). There was a positive correlation between the numbers of *Pratylenchus* spp. detected with the qPCR assay and the numbers counted under the microscope in single inoculations as well as in nematode suspensions where *H. avenae* was present (Figure 8.3). In single inoculations with *P. thornei* (Figure 8.3A) there was a strong ($R = 0.96$, $P < 0.001$, n= 10) positive correlation between the numbers based on the qPCR and those obtained by visual observation; counts based on qPCR underestimated the numbers of *P. thornei*. In mixed inoculations, there was also a positive relationship ($R = 0.88$; $P < 0.001$, n = 10) between the data generated by the two methods (Figure 8.3B), and the estimates of *P. thornei* were also higher using the microscopic method than with the qPCR. For quantification of *P. penetrans*, the estimates of the numbers of *P. penetrans* based on qPCR and those obtained by microscopy were not very well correlated ($R = 0.64$; $P < 0.05$, n = 10), but this relationship was better in mixed inoculation, the ($R = 0.80$; $P < 0.05$, n = 10) (Figure 8.3D). The estimates of *P. penetrans* in mixed inoculations were higher when using the microscopic method than with the qPCR, as was observed for *P. thornei*.

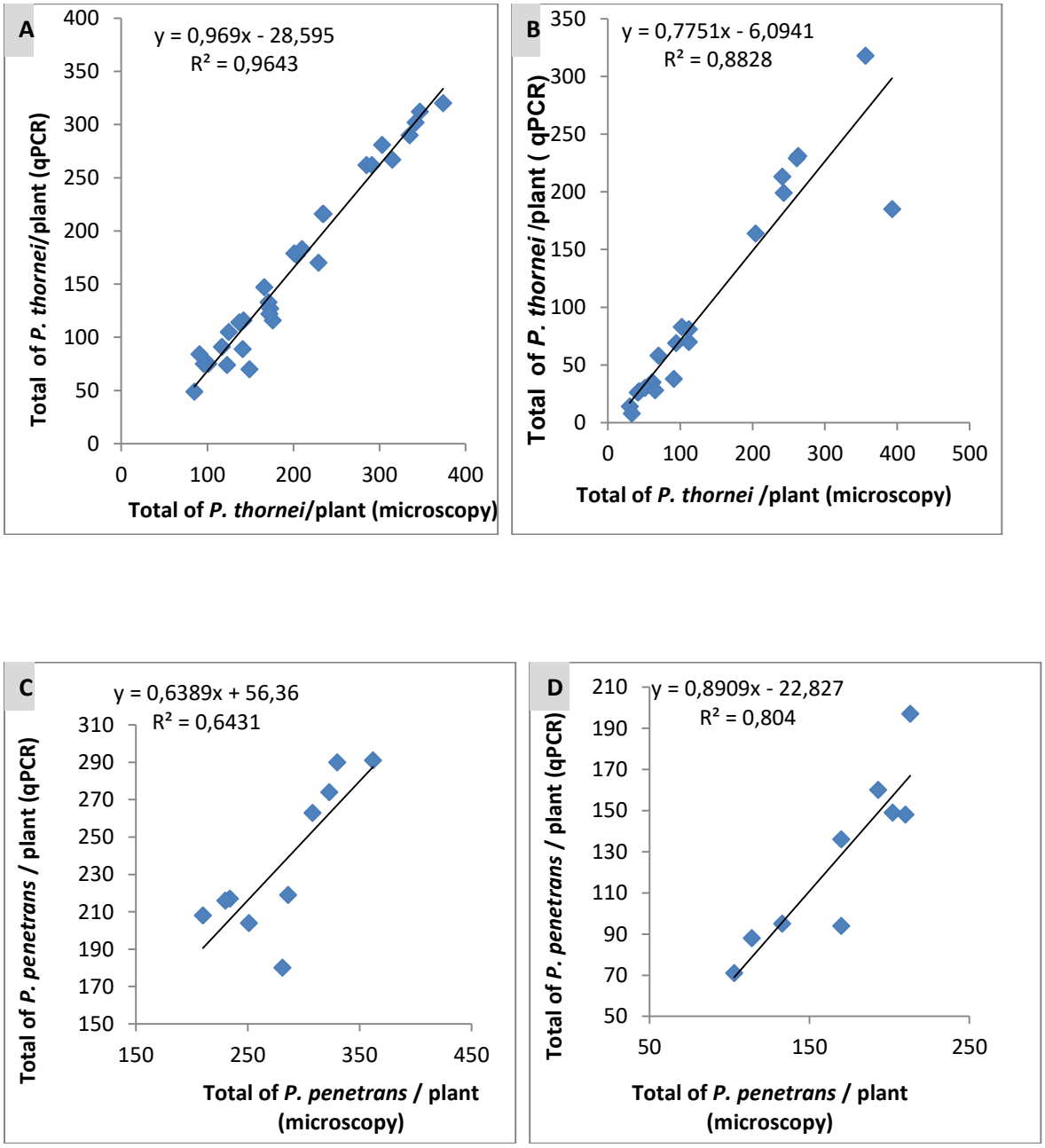

Figure 8.3. Comparison of number of nematodes obtained after counting with those obtained after qPCR. (A) Total number of *Pratylenchus thornei* per plant in three resistant lines of wheat after single inoculation; **(B)** Total number of *P. thornei* in three resistant lines of wheat after mixed inoculation with *Heterodera avenae.* **(C)** Total number of *P. penetrans* per plant in a single line after single inoculation. **D)** Total number of *P. penetrans* per plant in a single line after mixed inoculation with *H. avenae*

8.4 Discussion

Pratylenchus penetrans and *P. thornei* are the most important *Pratylenchus* species in different wheat-growing areas of Morocco (Chapter 5). These root-lesion nematodes are

migratory endoparasites of a wide range of crops (Williams *et al*., 2002). Their control by varietal resistance offers sustainable management of these important species. The term resistance is used to describe the ability of a plant to suppress development or multiplication of nematodes (Roberts, 2002; Smiley *et al*., 2005a). A highly resistant plant allows no nematode multiplication. Moderately resistant plants allow some intermediate amounts of multiplication. Use of resistance for the management of nematodes is expected to be a vital management component in the future (Roberts, 2002).

In view of this, *P. penetrans* and *P. thornei* were the objects of studies on varietal resistance in wheat. The inoculum was obtained from populations of both species multiplied on carrot disks (Chapter 5); one population of each species was eventually selected to evaluate the resistance of wheat lines provided by CYMMIT. The choice of the population was based on the rate of its multiplication on carrot disks. The total density of nematodes in soil and roots measured plant suitability to both species 9 weeks after inoculation. Nematode density can be determined either in the soil, or in the roots, or both. However, because *P. penetrans* and *P. thornei* are migratory endoparasites it was necessary to extract nematodes from both roots and soil. The 9-week period was selected on the basis of reports in the literature. Keil *et al*. (2009) reported that the best harvesting time to extract *P. thornei* on wheat was less than 12 weeks. Toktay *et al*. (2012) evaluated two different times for harvesting *P. thornei* during screening tests. Because harvesting after 9 weeks showed a lower number of nematodes in resistant germplasm than susceptible, the authors concluded that harvesting after 9 weeks is more appropriate with low standard error than 13 weeks for screening experiments and now it is used as a standard at CYMMIT for screening tests of wheat lines to *Pratylenchus* spp. (Toktay *et al*., 2015).

The wheat lines varied from poor to very good hosts for both *P. penetrans* and *P. thornei.* Three lines, *viz*. L9 (AUS 4930.7/2 PASTOR), L12 (CROC_1/AE.SQUARROSA (224); 20215) and L3 (CROC_1/AE.SQUARROSA (224); 20216) were resistant to *P. thornei*; L9 (AUS 4930.7/2 PASTOR) was also resistant to *P. penetrans*. Varying levels of resistance to root lesion nematodes of wheat lines were reported earlier (Zwart *et al*., 2005; Toktay, 2008; Toktay *et al*., 2012; Thompson *et al*., 2015). Linsell *et al*. (2014) concluded that the resistance to root-lesion nematodes wheat has never been associated with inability to penetrate roots. In addition, similar penetration rates were observed in susceptible and resistant bean cultivars when inoculated with *P. scribneri*. Accordingly, they showed that penetration rates of *P. thornei* did not differ between resistant and susceptible roots of wheat (*Triticum aestivum*), both on agar and in sand, even after 16 days of penetration (Linsell *et al*.,

2014). Similarly, Talavera & Vanstone (2001) observed *P. thornei* penetrating resistant wheat cultivars.

Thompson & Seymour (2011) reported that two wheat cultivars Morocco 426 and Iraq 43 were the best of the parents tested in a glasshouse for breeding for resistance to *P. thornei.* Kranti and Kanwar (2012) tested twenty wheat lines against *P. thornei* and reported that the lines AUS 15854, PBW 343, PBW 550, Raj MR 1, Raj 3765, CIMMYT line CROC_1/AE. SQUARROSA (224)//OPATA, WH 542, WH 896 and WHD 943 were resistant to an Indian population of *P. thornei*. My results on L12 and L13 (lines in common with the screening of Kranti & Kanwar, 2012) with a Moroccan population of *P. thornei* confirm this finding. Toktay *et al*. (2012) reported that wheat cv. Adana 99 is moderately resistant to root lesion nematode (*P. thornei*). In this study, we only used R or S, because we are only interested in completely resistant lines of wheat. Differences in nematode multiplication rates may be, influenced by genetic factor(s) in the host which results in susceptibility or resistance (Griffin, 1982). A major quantitative trait locus QTL was identified on Chromosome 6DS and was associated with resistance to both *P. thorne*i and *P. neglectus*. These two traits were designated as QRlnt.lrc-6D.1 and QRlnn.lrc-6D.1 respectively. QRlnt.lrc-2B and QTL QRlnt.lrc-6D.2, which located on chromosome 2BS and 6DL respectively, were also associated with resistance to *P. thornei* (Yu *et al.* 2012). Resistance to cereal cyst nematodes is controlled by a single gene, whilst resistance to root lesion nematodes is quantitative and controlled by a number of genes (Toktay *et al*., 2006; Nicol *et al*., 2009). In recent years, effective sources of resistance to *P. thornei* and *P. neglectus* have been identified and mapped in synthetic hexaploid wheat lines (Thompson *et al.,* 2009, Toktay *et al*., 2006). Resistance loci on chromosomes 1B, 2B and 6D were found with the line AUS4930 7.2 x Pastor population (Toktay *et al*., 2006). Similarly, in the CROC x PASTOR population, 2 resistance loci located on chromosomes 1B and 3B, were identified. In my screening test, L9 (AUS 4930.7/2 PASTOR) was resistant to both *P. thornei* and *P. penetrans*. Obviously, dual resistance to both *P. thornei* and *P. penetrans* is desirable when they commonly occur together as *P. thornei and P. neglectus* do in the northern grain region of Australian (Thompson *et al*., 2010).

Concomitant infestations of wheat fields with different species of plant-parasitic nematodes are common. In Morocco combinations of *P. penetrans* or *P. thornei* and *H. avenae* occur with high frequency in wheat fields (Znasni, 2003; Chapter 3). To unravel the interaction between nematode species with different feeding patterns, species of two genera were inoculated simultaneously on wheat lines, *viz*. *Pratylenchus* (migratory endoparasitic)

and *Heterodera* (sedentary endoparasitic). After single species inoculations the numbers of *P. thornei* and *P. penetrans* were greater than after concurrent inoculations with *H. avenae* in lines L9 and L12. The reduction of *P. thornei* in mixed inoculations was significant in the root of L9 and L12 and soil of three lines (L9, L12 and L13) of wheat. The mutual inhibitory effects appear to be caused by competition for root space. When observing penetration of rose roots by *P. penetrans*, Peng & Moens (1999) reported that the nematodes aggregated at different sites. This observation was made previously by Zunke (1990) who suggested that after the breakdown of an epidermal cell, this location becomes attractive to other *P. penetrans* leading to an accumulation of nematodes at this site. The same authors also observed that *P. penetrans* individuals could move out of the roots after their initial penetration and feeding. Competition in varying degrees between two or more nematodes species has been demonstrated by a number of other workers. Estores & Chen (1972) reported that *P. penetrans* and *M. incognita* depressed the population of each other in tomato. O'Bannon *et al*. (1976) found that mixed inoculation of *Radopholus similis* and *P. coffeae* on citrus resulted in lower populations of each species than in separate inoculation.

The main result in this study is that the three lines of wheat (L9, L12 and L13) resistant to *P. thornei* and one resistant line (L9) to *P. penetrans* kept their resistance even in an mixed inoculation with *H. avenae*. These three lines were as effective in limiting the reproduction of *P. penetrans* (L9) and *P. thornei* (L9, L12, L13) in both experiments (3 and 4), as previously observed in the experiments 1 and 2 of the screenings, and their resistant response was not altered by the presence of *H. avenae*. Niblack *et al*. (1986) showed that the soybean resistance to either *M. incognita* or *H. glycines* is unaffected also in concomitant infections with these nematodes. This study demonstrates that the sedentary endoparasite *H. avenae* is a competitor to both *P. thornei* and *P. penetrans* as it reduces the multiplication of the two *Pratylenchus* species. However, several studies have shown that *Pratylenchus* spp. inhibit *Heterodera* spp. and *Meloidogyne* spp. (Eisenback, 1993; Lasserre *et al*., 1994; Umesh *et al*., 1994). The effects of the species on each other are generally related to the nature of parasitism. Eisenback (1985) reported that there is an interspecific relationship between sedentary and migratory endoparasites, the close nematode-host relationship established by the sedentary species may make the host either more or less suitable for the latter. However, nematode interaction can be affected by timing of inoculations. Chitamber & Raski, (1984) reported that reproduction of *P. vulnus* was greatly inhibited after 125 days when *Meloidogyne incognita* was inoculated one month prior to *P. vulnus*, but in simultaneous inoculations the inhibition was delayed until 250 days. In this study, we inoculated with the

hatched J2 of *H. avenae* as described in several studies (Nicol *et al*., 2009; Toktay *et al*., 2012) because inoculation with cysts would have hatched gradually over time and could influence the competition with the juveniles of *Pratylenchus*. In addition, migratory endoparasites are less advanced parasites than sedentary endoparasites, which establish a complex relationship with the host and alter plant physiology. This change in physiology often affects the suitability of the host for the migratory endoparasites (Khan, 1993). Differences in results between earlier published experiments and experiments reported here, might be explained by the fact that in the latter resistant lines against *P. thornei* and *P. penetrans* were used, whilst in previous studies, the interaction between *H. avenae* and RLN was examined on susceptible wheat cvs.

Plants were smaller after mixed inoculations than after single inoculations (Table 8.3). The reductions of growth parameters after mixed inoculations may be attributed to root injury due to penetration and feeding by nematodes belonging to two genera leading to impairment of the efficiency of root systems to absorb water.

The real-time PCR assay was able to estimate the number of both *P. thornei* and *P. penetrans* in single and mixed inoculations with *H. avenae* from soil and root samples. In the experiments 3 and 4 there was a good and positive correlation between the numbers detected by real-time PCR and those obtained by counting under the microscope. However, the real-time PCR generally resulted in lower nematode counts than microscopic observations. Several researchers have reported under or overestimation of nematode numbers using real-time PCR. Min *et al*. (2012) reported a higher density of *P. penetrans* based on DNA extracted directly from soil and used in qPCR, compared with visual counts of nematodes obtained from the soils using the Baermann method. Ophel-Keller *et al.* (2008) reported that a DNA qPCR assay tended to overestimate population densities of *P. thornei* compared with microscopic counting. Yan *et al*. (2012), reported a significant positive correlation between the numbers of *P. thornei* based on DNA extracted from soil followed by real-time PCR and visual counts of nematodes extracted with the Whitehead tray. They showed that the counts determined by the real-time PCR were larger than the numbers derived from the visual counts, but that this overestimate was not significant. Berry *et al*. (2008), however, found that real-time PCR tended to underestimate the numbers of nematodes (*M. javanica*, *P. zeae* and *Xiphinema elongatum*). Previous data with real-time PCR (Chapters 6 and 7) indicate that life stages of *P. penetrans* and *P. thornei* in individual samples do not affect real-time PCR detection and quantification, therefore, are unlikely to be an explication for the under-estimation of nematodes calculated by PCR compared to the counts with the microscope. However, in the

case of *P. penetrans*, Sato *et al*. (2007) found that the Ct values of larger body sizes (male and female) were significantly lower than those from a small juvenile. Therefore, the population density of *P. penetrans* may change depending on the composition of the life stages (male, female and juveniles) that are present in a sample. However, the lower numbers of nematodes detected with the real-time PCR in the current study can probably be attributed to the dilution effect when preparing the samples for qPCR. For microscopy, nematodes were enumerated from the whole suspension, while for qPCR, the obtained nematode suspension was first transferred into a 40 ml tube where nematodes were allowed to settle down for three hours, then, 3 ml nematode suspension was pipetted from the bottom of the tube to extract DNA from. It is possible that some nematodes had not settled into the lower 3 ml, or that nematodes were lost during pipetting.

In any case, real-time PCR assay can offer an alternative assay to the time-consuming traditional method of morphological counting. This study demonstrated that the qPCR developed in previous studies (Chapters 6 and 7) can be used to detect and quantify *P. penetrans* and *P. thornei* in populations mixed with *H. avenae*. Among the 25 lines of wheat that were screened, L9 and (L9, L12, L13) were found to be resistant to *P. penetrans* and *P. thornei,* respectively, even in the presence of *H. avenae*. This chapter reports on the screening for resistance and interaction between two species of *Pratylenchus* and *H. avenae* from Morocco in wheat lines and shows promising results for enhanced wheat breeding. However, the field performance of these lines against root-lesion nematode attacks should be evaluated before they are released to the farmers.

CHAPTER 9

General discussion

Plant-parasitic nematodes, especially cereal cyst nematodes (CCNs; *Heterodera avenae* group) and root-lesion nematodes (RLN; *Pratylenchus* spp.), are major economic constraints in many wheat (*Triticum aestivum*) growing areas of the world (Nicol, 2002; Safari *et al*., 2005). This was also observed in earlier surveys in Morocco (Meskine *et al.,* 1984; Znasni, 2003; Mokrini *et al*., 2009) in which species from both genera were prevalent and present in high population densities in the different wheat-growing areas. In cereals, species of cyst nematodes, *viz. H. avenae, H. filipjevi* and *H. latipons* are considered the most economically important in global wheat production systems (Subbotin *et al*., 2003). Their unambiguous identification is essential for management practices to be successful. Their taxonomy is based traditionally on the morphology and morphometrics of cysts and second-stage juveniles. Several multivariate analysis methods are used to analyze the genetic variability and to investigate the differences between populations of *Heterodera* species. Most species of CCN can be differentiated from each other on the basis of morphological and morphometric features (Subbotin *et al.,* 1999; Handoo, 2002). However, morphological identifications are usually based upon minor differences among species, so correct identifications can be difficult to achieve, particularly if a quick diagnosis is needed and mixture of more than one species in the same samples (Subbotin *et al*., 2003).. The cyst characters, such as the presence (the weak to medium) or absence of bullae and underbridge, have already been mentioned in this study. These two characters could be verified easily and were found to be useful for distinguishing *H. avenae* from *H. latipons*. Recently, many DNA-based molecular diagnostics have been developed for detecting *Heterodera* spp. (*e.g.*, Toumi *et al*., 2013a,b); they are rapid and robust and can be used routinely.

Few Moroccan populations of *H. avenae* have been characterized morphologically, whilst no detailed descriptions of this species were published. Because of this lack of information, I identified and described more Moroccan populations of cereal cyst nematodes isolated from cereal fields in Morocco. Different approaches were used, *viz.* morphological and morphometrical observations using light microscopy, species-specific primers, molecular analyses of sequences and phylogenetic analyses.

Morphometric and/or morphological features allowed the separation of the collected populations into two species, *i.e. H. avenae* and *H. latipons*. The structures of the vulval cone of the cysts, i.e. fenestra, bullae, and underbridge, were useful features to separate the species (Chapters 3 and 4). Cereal cyst nematode species are differentiated on the basis of the vulval sections (type of fenestration, fenestral length, semi-fenestral width, bridge width of the vulva, presence of the underbridge and vulva slit length) as well as characters of J2 (body

length, midbody width, a-ratio, tail length, hyaline tail length, stylet length, and distance between dorsal gland duct opening to stylet base) (Subbotin *et al*. 1999, Handoo 2002). Valuable new information was collected on the distribution of *H. avenae* in the major wheat-growing regions of Morocco. Besides this, *H. latipons* (infecting wheat in Aïn Jemâa, Saiss) was detected for the first time in Morocco. The morphological and morphometrical observations were confirmed by PCR using the species-specific primers developed for *H. avenae* and *H. latipons* by Toumi *et al*. (2013a, b). The PCR method has potential to be used for routine diagnostic tests improving the control of these species. These results were further confirmed by comparison of rDNA-ITS region sequences of the Moroccan populations with sequences of *Heterodera* species available in GenBank (www.ncbi.nlm.nih.gov). Ten Moroccan sequences of the ITS-rDNA were similar (99-100%) to the sequences of *H. avenae* published in GenBank and three sequences were similar (97-99%) to *H. latipons*. Obviously, species-specific PCR is a rapid and a useful tool for identification of *Heterodera* spp. The survey showed that *H. avenae* is more wide spread in Morocco than previously thought (Meskine *et al*., 1984; Mokrini *et al*., 2009). *Heterodera avenae* and *H. latipons* are generally considered to be very important, causing economic yield losses in wheat production systems in several parts of the world (Nicol & Rivoal, 2008).

This is the first Moroccan survey using morphometric, morphological and molecular information to identify *Heterodera* species collected in different wheat producing areas. Their correct identification should aid effective management programmes. This study also highlights the importance of a holistic approach for *Heterodera* spp. diagnosis based primarily on morphology and morphometrics, but where possible integrated with molecular tools including species-specific primers and molecular analyses of DNA sequences in order to obtain appropriate and reliable identification. A similar strategy was also used in previous studies (Subbotin *et al*., 2003; Abidou *et al*., 2005; Dawabah *et al.*, 2012; Baklawa *et al*., 2015; Imren *et al*., 2015).

Next to cyst nematodes, *Pratylenchus* species play a prominent role on cereals. The genus comprises more than 70 described species and is distributed worldwide (Jones & Fosu-Nyarko, 2014). Eight species have been detected on cereals (Rivoal & Cook, 1993). Similar to *Heterodera* species, the accurate identification and understanding of the (genetic) diversity of *Pratylenchus* species is the first step in designing proper pest management programmes. This can only be achieved through comprehensive and accurate surveys. The occurrence of *Pratylenchus penetrans* and *P. thornei* alone or in mixed populations from samples collected

from 75 cereal fields, clearly demonstrated these species to be widespread in Morocco as they occurred in 41 of the sampled fields. In earlier reports (Meskine *et al*., 1984; Ammati, 1987), these species were considered the most common ones. With respect to geographical distribution, *P. thornei* was ranked first. However, I found *P. penetrans* more widely distributed than *P. thornei*. Because my survey considered a much larger area than the previous ones, the differences between the earlier surveys and the one reported here may be ascribed to differences in soil characteristics and climatic conditions of the studied areas. *Pratylenchus thornei* is the most studied species on wheat in different parts of the world (Greco *et al*., 1984; Nicol *et al*., 1999; Sahin *et al*., 2009; Smiley & Nicol, 2009; Abidou *et al*., 2005). Abidou *et al*. (2005) found about 40% of the soil samples infected with *P. thornei* and/or *P. neglectus* in CAP in Turkey. *Pratylenchus penetrans* parasitizes wheat and barley, and reduces yield by 10 to 19% in Canada (Nicol & Rivoal, 2008). The genus *Pratylenchus* was represented by two more species, *viz.* P. *pinguicaudatus* and *P. pseudocoffeae*, which were reported for the first time in Morocco. Differentiation of both *P. penetrans* and *P. thornei* from other *Pratylenchus* spp., mainly *P. pinguicaudatus*, can be very difficult using morphological traits. These species are quite similar and can be easily confused. Unfortunately, no information exists on the importance of *P. pinguicaudatus* and *P. pseudocoffeae* in cereals. Field studies on the population dynamics and the damage function are necessary to estimate the economic impact of these nematodes on cereals.

Similar to what was done for *Heterodera* spp., a combination of tools (morphology, morphometric, species-specific primers and DNA sequence data) was used to identify and characterize the *Pratylenchus* species collected in Morocco. The traditional identification of RLN based on morphology and morphometrics of females and males (when present) is a challenging task that demands considerable skills because of variability in morphology is frequently observed among and within species of the genus *Pratylenchus* (*e.g.*, Román & Hirschmann, 1969; Doucet *et al*., 2001). In my study (Chapter 5), the morphological and morphometrical characteristics of RLN, *viz*. *P. thornei*, *P. penetrans* and *P. pseudocoffeae* from different cereal producing areas of Morocco were consistent with the original reports of each species. Variability of some morphological characters of *P. penetrans* (PC2), such as the tail terminus of female, was observed. This variation could be attributed to the possibility to have mixing population. Variations in morphological and morphometric characters could be derived from changes in environmental conditions (Román & Hirschmann, 1969). In spite of the variability of the morphological character observed in this population, the CDA analysis was able to discriminate the three species of *Pratylenchus* from each other.

Molecular methods provide useful information for species separation and are essential in order to differentiate different species of *Pratylenchus* (Waeyenberge *et al*., 2000; De Luca *et al*., 2004; Subbotin *et al*., 2006). In addition to the morphology, PCR with species-specific primers and sequences of the ITS region of the r-DNA have been employed in several studies to resolve the identity of various *Pratylenchus* species (De Luca *et al*., 2004; de la Peña *et al*., 2007; Subbotin *et al*., 2008). The results obtained by species-specific PCR primers developed by Al-Banna *et al*. (2004) and Waeyenberge *et al*. (2009) agreed with the identification based on the morphological features. In addition, sequencing of the D2D3 region of the r-DNA allowed the differentiation of the Moroccan *Pratylenchus* species. The D2D3 sequences from most of *Pratylenchus* species identified were highly similar to the reference sequences deposited in GenBank, NCBI database. Moreover, the D2D3 sequences of the Moroccan pratylenchids showed a high interspecific difference. *Pratylenchus penetrans* and *P. pinguicaudatus* were the closest species with 9.1% nucleotide divergence for the compared sequences. However, *P. penetrans* and *P. pseudocoffeae* showed a divergence of 25.4%. In addition, the D2D3 sequences obtained for the *P. penetrans* populations from Morocco were highly similar. My results are the first providing the integrated morphometric, morphological and molecular characterisation of RLN populations from Morocco. However, analysis of morphological characters remains the standard method for routine identification of pratylenchids, as molecular analysis also needs to refer to morphological characters. Nevertheless, the combination of morphological and molecular observations for species description and diagnosis of species are strongly recommended.

Reproductive fitness is one of the major components of pathogenicity (Shaner *et al*., 1992); it is an important feature for the assessment and understanding of disease reactions of plants to pathogens. To compare the reproductive fitness of *Pratylenchus* populations, the use of *in vitro* monoxenic cultures, such as carrot disk cultures, offers a suitable approach since this technique provides homogenous environmental conditions, including a constant temperature, little space, and no maintenance. In chapter 5, I evaluated the *in vitro* reproductive fitness on carrot discs of six *Pratylenchus* populations collected from different wheat growing areas of Morocco: four *P. penetrans*, one *P. thornei* and one *P. pseudocoffeae* population. All three species of *Pratylenchus* multiplied very well on carrot disks. However, the total numbers recovered per carrot disk varied with the species and population. Intraspecific differences in reproductive fitness of the four populations of *P. penetrans* were noticed at different temperatures. As the populations originated from different regions, this

difference among *P. penetrans* populations may be due to adaptation of the population to its environment (*e.g.*, climate, temperature, soil type) (Dao, 1970; Moens & Perry, 2009). Comparing reproduction at different temperatures, I concluded that the optimum temperature for all populations was 20°C. After 8 weeks at this temperature, nematode numbers increased up to 458-fold, 310-fold and 252-fold for the four populations of *P. penetrans*, the *P. thornei* and the *P. pseudocoffeae* population, respectively. These results provide basic information to develop a research programme that aims at establishing a control strategy against RLN. Additional experiments on nematode multiplication on wheat are necessary to confirm the observations in the laboratory on carrot disks and the pathogenicity of *P. penetrans* and *P. thornei* on wheat needs to be examined.

The correct identification and quantification of *Pratylenchus* spp. is a fundamental step in nematode control strategies. However, the identification of *P. penetrans* and *P. thornei* based on morphology and morphometric traits is time consuming and requires specialized skills. Moreover, *Pratylenchus* spp. are frequently present in mixed populations (Chapter 5), which makes their identification and quantification even more difficult. Therefore, molecular tools are useful for the identification and discrimination between species, also their precise quantification. It was demonstrated that a species-specific PCR assay provides an efficient tool for an accurate, rapid and sensitive detection of *P. penetrans* or *P. thornei* (Uehara *et al*. 1998a; Al-Banna *et al*. 2004; Carrasco-Ballesteros *et al*., 2007; Yan *et al*., 2008; Waeyenberge *et al*. 2009). However, none of the species-specific primers were developed for quantification purposes, yet quantification is very essential in breeding programmes and extension activities. I developed two qPCR assays for the accurate detection and quantification of *P. penetrans* (Chapter 6) and *P. thornei* (Chapter 7), based on the sequence of the *β-1,4-endoglucanase* gene. The ITS-rDNA region is not always suitable for developing qPCR primers: ITS sequences vary in size between *Pratylenchus* species, making sequence alignment to detect species-specific fragments problematic (De Luca *et al.,* 2011). In addition, ITS sequences show extensive polymorphism within a species or an individual (Waeyenberge *et al*., 2009; De Luca *et al*., 2011). To avoid problems when developing primers for the qPCR detection of *P. thornei* and *P. penetrans*, I explored the possibilities with the *β-1,4-endoglucanase* gene, a gene that has never been used for quantitative detection (qPCR) of plant-parasitic nematodes. Both qPCR assays not only resulted in high amplification efficiency, they were also highly specific. No specific amplification was generated when using DNA from other species of the genus *Pratylenchus* or other plant-parasitic nematodes. Also, both assays were able to detect all populations of *P. penetrans* (Chapter 6) and *P.*

thornei (Chapter 7) that were used in this study. Importantly, the sensitivity of both qPCR assays allowed accurate and consistent detection of even 1 individual of *P. thornei* (Chapter 6) or 1 individual of *P. penetrans* (Chapter 7). The sensitivity of the *P. thornei* and *P. penetrans* assays, was comparable with that observed from other qPCR assays. Sato *et al*., (2007) could detect a single *P. penetrans* individual in a sample with an abundant number of free-living nematodes using SYBR Green I based qPCR method. Yan *et al*., (2012) developed a real-time PCR assay for *P. thornei* and detected one second-stage juvenile in one gram of soil. The developed qPCR assays provide a sensitive means for the rapid detection and reliable quantification of individuals of both *P. penetrans* and *P. thornei*. The real-time PCR assays that I developed were able to estimate the number of both *P. thornei* and *P. penetrans* in single and mixed inoculations with *H. avenae* from soil and root samples (Chapter 8). This method does not require expertise in nematode taxonomy and morphology, and can be used as a rapid diagnostic tool in research, as well as in diagnostic labs and extension services advising farmers for pest management.

The use of resistant cultivars is an effective and environmentally friendly method for the reduction of nematode populations below damaging levels. In Chapter 8, I evaluated a collection of 14 lines of spring wheat and 11 lines of winter wheat for their resistance to *P. penetrans* and *P. thornei* in the greenhouse. The resistance level was evaluated based on the numbers of nematodes extracted from roots and soil of each line, 9 weeks after infestation. My results indicated that three lines of wheat, *viz*. L9 Iraqi land race (AUS4920.7/2); L12 CYMMIT synthetic derivative (CROC_1/AE.SQUARROSA (224)//OPATA (20615); L13 CYMMIT synthetic derivative (CROC_1/AE.SQUARROSA (224)//OPATA (20616) were rated as resistant against *P. thornei* of which one line L9 (AUS4920.7/2), was also found resistant against *P. penetrans*. These results were in agreement with those of Toktay *et al*. (2006), Toktay (2008), Rivoal & Nicol (2009), Kranti & Kanwar (2011). Thompson *et al*., (2010) reported that the dual resistance to both *P. thornei* and *P. neglectus* is desirable as they commonly occur in fields together. Also in Morocco where 6% of the survey samples contained both species (Chapter 3), a wheat cultivar such as L9, with resistance to both species, would be useful. Generally, the investigated wheat sources of *Pratylenchus* resistance (AUS5205, AUS11984 and GS50a) do not contain resistance to both species at the same locus (Farsi *et al*., 1995; Thompson *et al*., 1999). Thompson & Seymour, (2011), reported that two cultivars Morocco 426 and Iraq 43 were the best of the parents tested for breeding for resistance to *P. thornei*. Kranti & Kanwar, (2012) reported that the wheat line

CROC_1/AE.SQUARROSA(224)//OPATA was found resistant to an Indian population of *P. thornei*, confirming my result about the resistance of the corresponding lines L12 and L13 against *P. thornei*

Pratylenchus penetrans and *P. thornei* are often found together with *H. avenae* (Sahin *et al.*, 2009; Mokrini *et al.,* 2009; Chapter 3). A breeding programme for nematode resistance, should therefore consider the most common and damaging nematode species. For this reason, I co-inoculated juveniles of *H. avenae*, on the cultivars that were found resistant and assessed the reproduction of both lesion nematodes *P. penetrans* and *P. thornei.* The results showed that, in the presence of *H. avenae*, the wheat lines L9, L12, and L3 remained resistant to *P. penetrans* and *P. thornei*. These findings show promising results for enhanced wheat breeding. However, the field performance of these lines should be evaluated.

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Curriculum Vitae

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2- Education

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1999-2005: **Agronomy engineer degree** at Hassan II Institute of Agronomy and Veterinary medicine, Rabat-Morocco, (Diploma of agronomy engineer), option: **Plant Protection**.

3- Current position

2006 - to date: Head of Nematology lab (Plant Nematology) (INRA-Agadir, Morocco).

4- Publications

1- **Mokrini, F**., Waeyenberge, L., Viaene, N., Abbad Andaloussi, F. & Moens, M. (2016). Diversity of root-lesion nematodes (*Pratylenchus* spp.) associated with wheat (*Triticum aestivum* and *T. durum*) in Morocco, *Nematology*. **http://booksandjournals.brillonline.com/content/journals/10.1163/15685411-00002993.**

2- Afechtal, M., Jamai, H., **Mokrini, F.,** Essarioui, A., Sbaghi, M., Dababat, A.A. (2016). First Report of Hop stunt viroid Infecting Citrus Trees in Morocco. *Plant Disease*, 100 **http://apsjournals.apsnet.org/doi/full/10.1094/PDIS-02-16-0156-PDN**

3- **Mokrini, F.,** Waeyenberge, L., Viaene, N., Abbad Andaloussi, F. & Moens, M. (2014). β-1,4-endoglucanase gene suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei, Nematology* 16 (7), pp 789-796.

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4- **Mokrini, F.,** Abbad Andaloussi, F., Waeyenberge, L., Viaene, N. & Moens, M. (2014). First report of the dagger nematode *Xiphinema diversicaudatum* (Nematoda: Longidoridae) on citrus in Morocco. *Plant Disease*, 98 (4), p575 http://apsjournals.apsnet.org/doi/abs/10.1094/PDIS-07-13-0764-PDN

5- **Mokrini, F.,** Waeyenberge, L., Viaene, N ., Abbad Andaloussi, F. & Moens. M (2013). Quantitative detection of the root-lesion nematode *Pratylenchus penetrans* using real-time PCR. *The European Journal of Plant Pathology*. 137 (2), pp 403-413. http://link.springer.com/article/10.1007%2Fs10658-013-0252-1

6- **Mokrini, F.,** Waeyenberge, L., Viaene, N. & Moens, M. (2012). First report of the cereal cyst nematode *Heterodera latipons* on wheat in Morocco. *Plant Disease*, 96 (5), 774 . http://apsjournals.apsnet.org/doi/abs/10.1094/PDIS-11-11-0999-PDN

5- Proceedings

1- **Mokrini, F.,** Waeyenberge, L., Viaene, N., Abbad Andaloussi, F and Moens, M. **(2015).** Resistance to the root-lesion nematodes (*Pratylenchus penetrans* and *P. thornei*) in wheat germplasm. *Proceedings of the 5th International Cereal Nematode Initiative Workshop*, 12-15 September, Ankara, Turkey, pp194-204.

2- **Mokrini, F., Waeyenberge**, L., Viaene, N., Abbad Andaloussi, F and Moens, M. (2014). β-1,4-endoglucanase gene suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei. Proceedings of the 66th International Symposium on Crop Protection*, Ghent University, Belgium.

3- **Mokrini, F.,** Waeyenberge, L., Viaene, N., Abbad Andaloussi, F and Moens, M. (2013)**.** Detection and quantification of the root-lesion nematode *Pratylenchus penetrans* using real-time PCR. *10th International Congress of Plant Pathology, Molecular Diagnostics of Plant Pathogens*, 25-30 August, China**.** Oral presentation.

4- **Mokrini, F.,** Waeyenberge, L., Viaene, N., Abbad Andaloussi, F. & Moens, M. (2013). Morphometrical and molecular characterization of root lesion nematodes (*Pratylenchus* spp.) on wheat in Morocco. *4th Workshop of the International Ceral Cyst Nematodes Initiative*. 22-24 August, China**.** Oral presentation.

5- **Mokrini, F.,** Waeyenberge, L., Viaene, N. & Moens, M. (2012). Occurrence of nematodes of the *Heterodera avenae* group and *Pratylenchus* spp. on wheat and barley in Morocco. *31st International Symposium of the European Society of Nematologists*, 23-27 September 2012, Adana, Turkey. Oral presentation.

6- **Mokrini, F.,** Waeyenberge, L., Viaene, N. & Moens, M. (2012). Morphological and molecular characterization of Moroccan cereal cyst nematode populations (*Heterodera* spp.). *3rd International Cereal Cyst Nematodes Initiative Workshop* 22-23 September 2012, Adana, Turkey. Oral presentation.

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