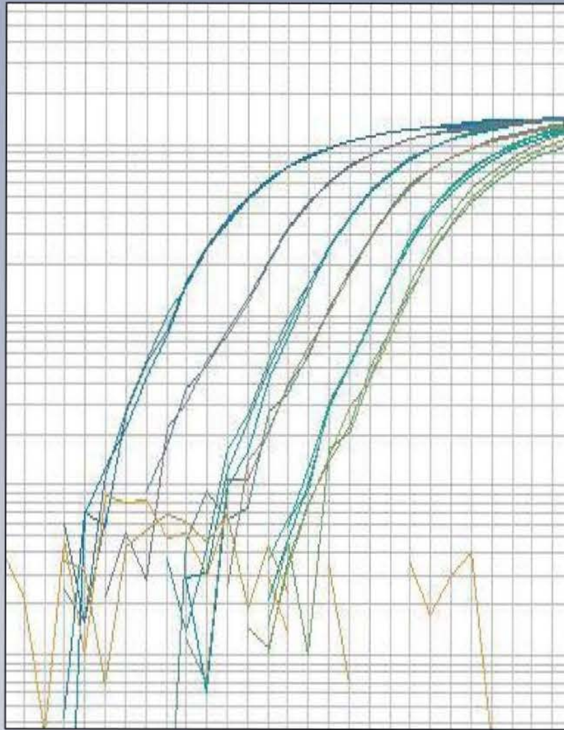

Cereal Cyst Nematodes: Molecular Identification and Quantification, and Screening for Resistance in Wheat

Fateh TOUMI

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Cereal Cyst Nematodes: Molecular Identification and Quantification, and Screening for Resistance in Wheat

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Cereal cyst nematodes: molecular identification and quantification, and screening for resistance in wheat

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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:

Graancystenematoden: moleculaire identificatie en kwantificatie, en screening voor resistentie in tarwe.

Illustration on the front cover: from up left to right, enlarged white females of *Heterodera latipons* attached to roots; field screening for resistance to *H. avenae*, symptoms of *H. filipjevi* damage in the field, and second-stage juvenile of *H. filipjevi*.

Illustration on the back cover: qPCR from dilution series of DNA extracted from 120 J2 to quantify *H. avenae.*

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The promoters:

Prof. dr. ir. Maurice Moens   Prof. dr. ir. Nicole Viaene   Prof. dr. ir. Luc Tirry

The author: ir. Fateh Toumi

Sonia, I am

Very weak to write about your strength!!!

Very strong to be a friend of you!!!

Very calm to write about your patience!!!

Very jealous of your success!!!

Very classic to write about your beauty!!!

You are the light of our life, my guide, my love.

I am coming back very soon.

Soso, I love you!!!

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List of abbreviations

AFLP: Amplified Fragment Length Polymorphism

BGRI: Borlaug Global Rust Initiative

CCN: Cereal cyst nematodes

CIMMYT: International Maize and Wheat Improvement Centre

COI: Cytochrome Oxidase subunit 1

Cre: Resistance gene to Cereal root eelworm

Ct: Threshold cycle

ddH_2O: Double distilled water

FAWWON: Facultative and Winter Wheat Observation Nursery

GBS: Genotyping By Sequencing

HS: Highly susceptible

Hsp90: Heat Shock Protein 90

ICARDA: International Centre for Agricultural Research in the Dry Areas

ICRISAT: International Crops Research Institute for the Semi-Arid Tropics

ILVO: Institute for Agricultural and Fisheries Research

ITS1: Internal transcribed spacer 1

ITS2: Internal transcribed spacer 2

IWWIP: The International Winter Wheat Improvement Programme

J1: First-stage juvenile

J2: Second-stage juvenile

J3: Third-stage juvenile

LD: Linkage disequilibrium

MBBISP: Monsanto's Beachell-Borlaug International Scholars Programme

MR: Moderately resistant

MS: Moderately susceptible

NGS: Next-Generation Sequencing

NTC: No-template control

PCA: Principal component analysis

PCR: Polymerase Chain Reaction

PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

Pf: final population density

Pi: Initial population density

PIC: polymorphic information content

QBOL: Quarantine Organisms Barcoding Of Life

qPCR: Quantitative Polymerase Chain Reaction

QTL: Quantitative trait loci

R: Resistant

rDNA: Ribosomal DNA

RLN: Root lesion nematode

S: Susceptible

SCN: Soybean cyst nematode

SHW: Synthetic hexaploid wheat

SNPs: Single-Nucleotide Polymorphisms

Ta: Annealing temperature

USSR: Union of Soviet Socialist Republics

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Summary

Cereal cyst nematodes (CCN), *Heterodera avenae, H. latipons*, and *H. filipjevi*, are considered major species on cereals. They have been reported frequently from several countries in Asia, Africa, USA, Europe. However, information on the nature and distribution of CCN species in Syria are rather limited. Therefore, I conducted a survey in north-eastern Syria to assess the distribution of CCN in the main wheat and barley growing areas. A total of 167 composite soil samples were collected from 167 wheat and barley fields. Cysts were extracted from soil, quantified and identified up to species level by using both morphological and molecular methods. The study revealed that 62% of the fields were infested with the three *Heterodera* species: *H. latipons, H. avenae,* and *H. filipjevi*. The most prevalent species was *H. latipons*, which was presented in 76% of the infested samples. *Heterodera avenae* was detected singly in 20% of the samples, while 11% consisted of mixed populations of other species. *Heterodera filipjevi* was never detected alone; it was found mixed with *H. avenae* and/or *H. latipons* in 9% of the samples. The high number of extracted cysts (up to 116 cysts 200 g^{-1} of soil) is most likely related to the monoculture crop producing system practised in that region and the fact those growers are unaware of the existence of resistant varieties.

Collected samples from the survey were identified molecularly *via* sequencing the ITS-regions of the rDNA. However, sequencing is relatively expensive and time-consuming compared to the use of species-specific PCR. Hence, I screened different DNA regions to check the possibility to use them efficiently to develop species-specific PCR assays to discriminate the three major CCN species accurately. The actin 1 gene was successfully used for establishing the assay to detect *H. latipons*. The actin 1 gene of eight *Heterodera* species was partially sequenced. A small fragment of the gene was suitable for the construction of a potentially useful species-specific primer for *H. latipons*. The optimised PCR was subsequently tested with several populations of 14 *Heterodera* species and 1 population of *Punctodera punctata*. 16 populations originating from six different countries represented *H. latipons*. The primer set (HLAT-ACT) was very specific. To further test its sensitivity, the PCR was conducted on DNA extracted from five second-stage juveniles (J2) of *H. latipons* mixed with 5 or 100 J2 belonging to *H. avenae*. The PCR was able to detect up to 1/10 dilution of the DNA obtained from 5 J2. The results showed that a specific and sensitive *H. latipons* species-specific PCR was constructed.

However, the same DNA part of the actin 1 gene was not useful for developing species-specific primers for *H. avenae* and *H. filipjevi.* Another investigated gene, the mitochondrial cytochrome oxidase subunit 1 (COI) gene, was successfully used to develop species-specific primers that could be used for the identification of *H. avenae* (AVEN-COI) and *H. filipjevi* (FILI-COI). The COI gene of 9 *Heterodera* spp. and *P. punctata* was partially sequenced, and the alignment showed variability between *H. avenae, H. filipjevi,* and other *Heterodera* species. Two sets of species-specific primers were developed for the identification of both species and the conditions for their use in PCR were optimised. The specificity of the designed primers was checked by comparison with 1 population of *P. punctata*, populations of 14 other *Heterodera* species, and 9 populations of *H. avenae* and 10 populations of *H. filipjevi* originating from different countries. To test the sensitivity, the PCR was run with DNA extracted from 5 J2 of *H. avenae* or 5 J2 of *H. filipjevi* mixed with DNA extracted from varying numbers of J2 of *H. latipons*. It was possible to detect as few as 5 J2 of *H. avenae* or *H. filipjevi* among 100 J2 of *H. latipons*. The two primers sets allow the detection of *H. avenae* and *H. filipjevi* where they occur in mixed populations with other *Heterodera* spp.

Precise identification and quantification of these three species are necessary to develop effective integrated pest control. Quantification *via* counting juveniles and eggs is extremely time-consuming. Hence, I checked the possibility to use the mitochondrial cytochrome oxidase subunit 1 (COI) gene to develop qPCR assays that could be used for the identification and quantification of *H. avenae* and *H. latipons*. Two qPCR primer sets for the identification and quantification of *H. avenae* and *H. latipons*, each set comprising two primers and a probe, were designed. After optimisation, the qPCR assays using DNA from a single J2 only were able to identify and quantify *H. avenae* and *H. latipons*. Their specificity was confirmed by the lack of amplification of J2 of 14 other *Heterodera* species. A qPCR using DNA extracted from 120 J2 + eggs of *H. avenae* and *H. latipons* resulted in steady Ct-values (Ct = 22.33 ± 0.1 and Ct= 21.83 ± 0.12, respectively). Dilution series of DNA extracted from 120 J2 + eggs of the two species were made. The assays for both species resulted in a standard curve showing a highly significant linearity between the Ct-values and the dilution rates (R^2 = 0.99; slope = -3.03 and R^2 = 0.99; slope = -3.28 for *H. avenae* and *H. latipons*, respectively). The two qPCR

assays provide a sensitive and valid tool for rapid detection and quantification of the two species whether they occur alone or in mixtures with other species. The COI gene was not useful to develop a qPCR assay to detect *H. filipjevi*.

Phenotyping and genotyping wheat lines against CCN are very important to provide the wheat-breeding programme with precise information about the lines to be used or to be eliminated out of a breeding programme. Hence, I screened (phenotyped) a total of 217 synthetic winter wheat lines representing three groups (group 1, group 2, and group 3), for resistance to *H. filipjevi* in the growth chamber. These lines were also genotyped using the Amplified Fragment Length Polymorphism (AFLP) technique. Genomic DNA extracted from the young leaves of all lines was digested with 3 restriction enzymes used in two combinations (*Eco*RI + *Mse*I, and *Pst*I + *Mse*I). AFLP was performed using two sets of primers for each enzyme combination. The average cyst number on 217 breeding lines ranged between 0 and 40 cysts per plant. Phenotyping the lines showed that in each group some lines are promising because of a low number of the newly developed cysts; those lines can be considered resistant (R) and moderate resistant (MR). Additionally, compared to the other two groups, lines belonging to group 3 showed the most frequent presence of R and MR phenotypes. The statistical analysis resulted in a significant positive correlation between the number of developed cysts and the root length, root surface and root volume in group 1 ($r = 0.11$, 0.14 and 0.15, respectively), the number of cysts and the number of root tips in group 2 ($r = 0.21$); and between the number of cysts and the root length and root volume in group 3 ($r = 0.25$ and 0.19, respectively). Interestingly, the biplot of the principal coordinate analysis of the AFLP DNA markers based on 2 factors clearly evidenced the genetic diversity of the resistant lines in all three groups. Additionally, the established dendrogram using the genetic distance index between all lines showed that all lines were regrouped into four well-separated clusters. However, lines were not grouped according to the resistance level (R, MR, MS, S, and HS).

In conclusion, this research showed that CCN are widely distributed in Syria. Cereal cysts nematodes can be identified and quantified molecularly precisely and rapidly

in one day including DNA extraction. Resistance genes are present in wheat lines, and promising cultivars can be used in breeding against cereal cysts nematodes.

Samenvatting

De graancysteaaltjes, *Heterodera avenae, H. latipons*, en *H. filipjevi*, worden beschouwd als belangrijke soorten voor granen. Hun aanwezigheid wordt frequent gemeld in verschillende landen van Azië, Afrika, USA, Europa. Informatie over hun levenswijze en verspreiding in Syrië is echter beperkt. Daarom heb ik een survey uitgevoerd in het noordoosten van Syrië om de verspreiding van graancysteaaltjes in de belangrijkste tarwe- en gerstproducerende gebieden te onderzoeken. Een totaal van 167 grondstalen werden verzameld uit evenveel velden met tarwe of gerst. De cysten werden uit de grond geëxtraheerd, geteld en geïdentificeerd tot op soortniveau met zowel morfologische als moleculaire methoden. Deze studie toonde aan dat 62% van de velden besmet waren met drie *Heterodera* soorten: *H. latipons, H. avenae* en *H. filipjevi*. De meest dominante soort was *H. latipons,* aanwezig in 76% van de besmette velden. *Heterodera avenae* kwam in 20% van de velden voor als enige soort, terwijl in 11% samen met een andere soort. *Heterodera filipjevi* werd nooit als enige soort gedetecteerd en werd steeds met *H. avenae* en/of *H. latipons* gevonden in 9% van de stalen. Het groot aantal verzamelde cysten (tot 116 cysten per 200 g grond) in deze gebieden kan hoogstwaarschijnlijk in verband worden gebracht met het telen van tarwe en gerst in een monocultuursysteem en het feit dat de telers geen kennis hebben over het bestaan van resistente variëteiten van graangewassen.

Cysten afkomstig uit grondstalen van de survey werden moleculair geïdentificeerd door het sequeneren van het ITS-gebied van het rDNA. Echter, sequeneren is een relatief dure en tijdverslindende methode in vergelijking met het uitvoeren van een soort-specifieke PCR. Daarom werden verschillende DNA-gebieden gescreend om uit te maken of het mogelijk was deze gebieden te gebruiken om soort-specifieke PCRs te ontwikkelen die de drie belangrijke soorten graancysteaaltjes accuraat kunnen identificeren. Het actine-gen 1 werd goed bevonden om een soort-specifieke PCR te ontwikkelen voor het detecteren van *H. latipons*. Hiervoor werd een deel van het gen van acht *Heterodera* soorten gesequeneerd en een klein fragment van het gen bleek uniek en bruikbaar om een soort-specifieke primer set voor de detectie van *H. latipons* te ontwikkelen. De geoptimaliseerde PCR werd vervolgens getest met verschillende populaties van 14 *Heterodera* soorten en één populatie van *Punctodera punctata*. Zestien populaties van *H. latipons* afkomstig uit zes verschillende landen werden eveneens

getest. De primer set (HLAT-ACT) bleek heel specifiek te werken. Om zijn gevoeligheid te testen werd de soort-specifieke PCR uitgevoerd met DNA geëxtraheerd uit 5 tweede stadium juvenielen (J2) van *H. latipons* gemengd met DNA afkomstig van 5 tot 100 J2 van *H. avenae*. De PCR kon het aaltje nog detecteren bij een 1/10 verdunning van het DNA uit 5 J2. Deze resultaten tonen aan dat een specifieke en gevoelige soort-specifieke PCR voor *H. latipons* was ontwikkeld.

Hetzelfde deel van het actine-gen 1 was echter niet bruikbaar voor de ontwikkeling van soort-specifieke primers voor de detectie van *H. avenae* en *H. filipjevi.* Een ander gen, het mitochondriaal cytochroom oxidase subunit 1 (COI) gen, kon wel met succes worden gebruikt om soort-specifieke primers voor de detectie van deze nematoden te ontwikkelen. Een deel van het COI-gen van 9 *Heterodera* soorten en *P. punctata* werden hiervoor gesequeneerd en vergeleken. Twee soort-specifieke primersets werden ontwikkeld voor de identificatie van *H. avenae* (AVEN-COI) en *H. filipjevi* (FILI-COI)*,* en de condities voor hun gebruik in een PCR werden geoptimaliseerd. De specificiteit van de ontwikkelde primers werd gecontroleerd met 1 populatie van *P. punctata,* 14 populaties van 14 andere *Heterodera* soorten, 9 populaties van *H. avenae* en 10 populaties van *H. filipjevi* afkomstig uit verschillende landen. Om de sensitiviteit van de soort-specifieke PCRs te bepalen, werden testen uitgevoerd met DNA geëxtraheerd uit 5 J2 van *H. avenae* of *H. filipjevi* gemengd met DNA geëxtraheerd uit een variërend aantal J2 van *H. latipons*. Het was mogelijk om slechts 5 J2 van *H. avenae* of *H. filipjevi* te detecteren tussen 100 J2 of *H. latipons*. De twee primersets lieten de detectie van *H. avenae* en *H. filipjevi* toe, ook indien ze gemengd voorkwamen met populaties van andere *Heterodera* soorten.

Een juiste identificatie en kwantificatie van de drie soorten graancysteaaltjes is noodzakelijk om effectieve geïntegreerde beheersingsmaatregelen te ontwikkelen. Kwantificeren door juvenielen en eieren van de cysten te tellen is zeer tijdrovend. Daarom werd de mogelijkheid onderzocht om het mitochondriaal cytochroom oxidase subunit 1 (COI) gen te gebruiken om een qPCR voor de identificatie en kwantificatie van *H. avenae* en *H. latipons* te ontwikkelen. Twee qPCR primersets, bestaande uit twee primers en een probe, werden ontwikkeld. Na optimalisatie waren de qPCR methoden in staat om *H.*

avenae en *H. latipons* te identificeren en te kwantificeren gebruik makend van DNA uit slechts 1 J2. Hun specificiteit werd bevestigd door het onvermogen om DNA van J2 uit 14 andere *Heterodera* soorten te amplificeren. Een qPCR-uitvoering met DNA geëxtraheerd uit 120 J2 en eieren van *H. avenae* en *H. latipons* resulteerde in stabiele Ct-waarden (Ct = 22,33 ± 0,1 en Ct= 21,83 ± 0,12, respectievelijk). Verdunningsreeksen van DNA geëxtraheerd uit 120 J2 en eieren van de twee soorten werden voorbereid. De qPCR van beide soorten resulteerde in een standaardcurve met een duidelijk significant lineair verband tussen de Ct-waarden en de verdunningsreeks (R^2 = 0,99; helling = 3,03 en R^2 = 0,99; helling = -3,28 voor *H. avenae* en *H. latipons*, respectievelijk). Beide qPCRs blijken gevoelige en valabele methoden te zijn om snel een van beide soorten te detecteren en te kwantificeren, of deze soorten nu afzonderlijk of gemengd met andere soorten voorkomen. Het COI-gen was echter onbruikbaar om een qPCR voor de detectie van *H. filipjevi* te ontwikkelen.

Genetisch materiaal van tarwe fenotypisch en genotypisch karakteriseren in relatie met graancysteaaltjes is heel belangrijk om veredelingsprogramma's van tarwe precieze informatie te verschaffen over welk genetisch materiaal te selecteren of juist te vermijden. In totaal werden 217 synthetisch wintertarwelijnen afkomstig van 3 verzamelingen gescreend in een groeikamer op resistentie tegen *H. filipjevi*. Hetzelfde genetisch materiaal werd ook genotypisch getypeerd door middel van 'Amplified Fragment Length Polymorphism' (AFLP). DNA geëxtraheerd uit blaadjes van opgekweekt genetisch materiaal werd behandeld met 2 combinaties van 3 verschillende restrictie-enzymen (*Eco*RI + *Mse*I, en *Pst*I + *Mse*I). AFLP werd uitgevoerd met 2 primersets voor elke combinatie van restrictie-enzymen. Het gemiddelde aantal cysten ontwikkeld op de 217 lijnen schommelde tussen 0 en 40 per plant. Het fenotypisch karakteriseren van de planten toonde aan dat in elke verzameling bepaalde lijnen veelbelovend zijn omwille van het klein aantal nieuwe cysten; dit genetisch materiaal werd als resistent (R) of gematigd resistent (MR) beschouwd. De derde verzameling bevatte, in vergelijking met de andere twee verzamelingen, meer R en MR materiaal.

De statistische analyse toonde een significante positieve correlatie tussen het aantal ontwikkelde cysten en de lengte van de wortels, de worteloppervlakte en het

wortelvolume in verzameling 1 (r = 0,11, 0,14 en 0,15 respectievelijk); tussen het aantal cysten en het aantal wortelpunten in groep 2 (r = 0,21); en tussen het aantal cysten, de lengte van de wortels en het wortelvolume in groep 3 (r =0,25 en 0,19 respectievelijk). Vermeldenswaardig is het resultaat van de ‘biplot principale coördinaten analyse’ van de AFLP DNA merkers gebaseerd op 2 factoren, dat duidelijk de genetische diversiteit bewees van de resistente lijnen in de drie verzamelingen. De dendrogram gebaseerd op de genetische afstandindex tussen alle lijnen, toonde aan dat alle lijnen gehergroepeerd werden in vier duidelijk onderscheiden clusters. De lijnen werden echter niet gegroepeerd op basis van hun graad van resistentie (R, MR, MS, S en HS).

Als besluit kan worden gesteld dat dit onderzoek heeft uitgewezen dat graancysteaaltjes wijd verspreid zijn in Syrië. Deze aaltjes kunnen nauwkeurig en vlug (in één dag) moleculair worden geïdentificeerd en gekwantificeerd. Resistentiegenen zijn aanwezig in nieuw genetisch materiaal van tarwe; daardoor kunnen veelbelovende tarwevariëteiten worden ontwikkeld tegen graancysteaaltjes.

Chapter 1

General introduction

The growth of the world population, unsustainable use of natural resources, as well as climate changes have a negative impact on food security in some regions of the world. Expanded investments in sustainable agriculture to increase productivity and to avoid losses, along with the reduction of wasted food and the promotion of healthier food diets, should avoid an increasing gap between food supply and demand (www.wheatinitiative.org/sites/default/files/attached_file/wheatinitiative_visiondocument.pdf). Globally, wheat is one of the three most important crops for humans and livestock (Shewry, 2009); it is the most important protein source and provides around 20% of the global calories (Breiman & Graur, 1995). In 2016, 742 million tonnes wheat were estimated to be produced on more than 223 million hectares (www.fao.org/worldfoodsituation/csdb/en/). However, in 2050, the world's population is expected to reach nine billion. It has been estimated that cereal production needs to increase by 50% by 2030 (Alexandratos & Bruinsma, 2012). Obviously, food security has become a critical global challenge for the 21st century.

Like all crops, cereals are exposed to biotic and abiotic stresses. Among the abiotic stresses, the temperature increase due to the global warming is becoming a serious problem, especially for wheat. Among the biotic stresses, plant-parasitic nematodes play an important role by decreasing the global crop yield by *ca.* 10% (Brown, 1985; Whitehead, 1998; Nicol & Rivoal, 2008). Nevertheless, they are frequently overlooked, as they are soil borne and microscopic. Moreover, their interaction with hosts generally results in fairly non pathogen-specific above-ground symptoms such as yellow lowered leaves, stunting and patches of poor growth. On wheat and barley, in addition to the root lesion nematodes (Riley & Kelly, 2002; Smiley *et al*., 2004), the cereal cysts nematodes (CCN) are economically the most important group, especially in non-irrigated wheat production systems (Sikora, 1988; Greco *et al*., 2002), Cereal cysts nematodes have been reported from West Asia, North Africa, Europe and the United States of America (Nicol & Rivoal, 2008; Riley *et al*., 2009). The cysts are easily spread to non-infested areas with soil carried on equipment, animals, shoes, roots, tubers, and by wind (in dust) and water (Smiley & Nicol, 2009).

According to the phylogenetic relationships, the Avenae group consists of the following closely related species of the genus *Heterodera*: *H. avenae*, *H. mani*, *H. hordecalis*, *H. latipons*, *H. australis*, *H. pratensis*, *H. arenaria*, *H. aucklandica*, *H. filipjevi*, *H. sturhani* and *H. ustinovi* (Subbotin *et al*., 2010a; Subbotin, 2015). However, in an older study based on the morphological characters, *H. bifinestra* (Cooper, 1955) was included in the Avenae group (Stone & Hill, 1982). Likewise, *H. spinicauda* (Wouts *et al*., 1995) was considered indicative of a new representative of Avenae group. The Avenae group is a complex because the group consists of closely related species. The differentiation for those species based on morphological and morphometric characters is difficult and sometimes impossible *i.e*., *H. avenae* and *H. australis* can be differentiated by molecular method only (Subbotin *et al*., 2010a). Also, *H. sturhani* species is morphologically similar to *H. pratensis* and *H. riparia* in many characteristics of cysts and second-stage juveniles. Therefore, for the correct species identification of some species, an integration of more than one method is needed (morphological, biological, molecular or biochemical) (Subbotin *et al*., 2010a).

Within the Avenae group, *H. avenae*, *H. filipjevi*, and *H. latipons* are the most common species (Sikora, 1988; Rivoal & Cook, 1993). However, the identification of these species is not always easy because considerable diversity is known to exist within the CCN complex with respect to morphological, molecular and pathological characteristics (Rivoal *et al*., 2003). Furthermore, it is not unusual to find the two or three species of CCN together in the same field (*e.g*., Öztürk *et al.,* 1998; Abidou *et al.,* 2005a; Hassan, 2008; Imren *et al.,* 2012; Yavuzaslanoglu *et al.,* 2012; Dababat *et al.,* 2014).

Accurate identification of the prevailing nematode species and knowledge of their population density are essential when designing effective control measures. As is common for other nematode species, CCN are traditionally identified on the basis of their morphology and morphometrics. Unfortunately, this is time-consuming and hardly applicable when species-mixtures need to be identified and quantified. However, it has been shown that DNA-based methods can be excellent tools complementing the traditional identification (Ferri *et al*., 2009). Species-specific PCR and qPCR overcome these difficulties, and might be used as a diagnostic tool allowing a high through-put. Next to the

frequently used ITS-rDNA, discriminating species-specific information can be found in several protein-coding genes such as actin, aldolase (Kovaleva *et al.*, 2005), β-tubulin (Sabo & Ferris, 2004), pectate lyase (Boer *et al.*, 2002), annexin (Patel *et al.*, 2010), chorismate mutase (Vanholme *et al.*, 2009), mitochondrial cytochrome oxidase subunit 1 (COI) (Derycke *et al.*, 2005), and heat shock protein 90 (hsp90) (Skantar & Carta, 2004). The successful use of any of these genes for developing species-specific PCR and/or qPCR assay will help to tackle the serious difficulties in CCN identification in Syria and Turkey due to the presence of species-mixtures (Abidou *et al.*, 2005a; Yavuzaslanoglu *et al.*, 2012).

Many strategies were developed to control CCN and include crop rotation with non-cereals, the use of chemical or biological control agents, the development of resistant wheat varieties, and agricultural practices such as quarantine, field sanitation, bare fallowing, weed control, organic and inorganic fertilisers (Smiley & Nicol, 2009; Dababat *et al.,* 2011; Dawabah *et al.,* 2015). Of these methods, incorporating resistance genes into wheat cultivars is considered to be the most environmentally friendly, and cost-effective control measure for reducing nematode populations below the economic damage threshold level (Gair *et al.,* 1969; Mitchinson *et al.*, 2009). However, the occurrence within and among populations of individuals that are highly variable in virulence (*i.e.* capacity to reproduce on resistant plants) and in reproductive capacity, is considered one of the major challenges to control CCN with resistant cultivars (Rivoal *et al.*, 2001; Mokabli *et al.*, 2002). Also, the existence of different pathotypes of *H. avenae* and *H. filipjevi* occur in different regions of the world and even within regions and individual fields. Pathotypes vary in their ability to reproduce on individual varieties and species of cereal crops (Smiley *et al.*, 2011).

The success and usefulness of resistance to CCNs depend on the effectiveness and durability of the sources of resistance. Therefore, different genera of the Poaceae have been screened in wheat-breeding programmes as potential sources of germplasm with resistance to CCN (Ogbonnaya *et al.,* 2001). So far, 9 resistance genes to *H. avenae* (*Cre*) (formerly called: Cereal root eelworm) were reported from different sources of *Triticum aestivum*, *T. tauschii, Aegilops ventricosa, Ae. triuncialis* and *Secale cereale.*

These genes confer total or partial resistance to different CCN pathotypes (Toktay *et al*., 2012; Dababat *et al*., 2014). New sources of resistance are being pyramided in high yielding varieties of known quality, and this should be further investigated for improving their resistance to CCN. Furthermore, a correlation between resistance to CCNs and grain yield at several key regional locations has not yet been established.

In view of these issues, the specific objectives of this research were:

1. Investigation of the distribution of CCN in wheat fields in north-eastern regions of Syria.
2. Screen different DNA regions to check their usefulness for the development of species-specific primers and probes for *H. latipons, H. avenae* and *H. filipjevi*.
3. Development of species-specific PCR and qPCR assays for the identification and the quantitative detection of the three above-mentioned species.
4. Investigate a selection of synthetic winter wheat lines with the aim of finding resistance against *H. filipjevi*:
    - Genotype the wheat lines using AFLP markers to assess their diversity.
    - Phenotype their host suitability to *H. filipjevi* and their root traits.
    - Identify superior lines with a high level of resistance to *H. filipjevi.*
    - Investigate the relation between plant response to nematodes and root parameters.

Chapter 2

Literature review

2.1 Importance of cereals

Small grain cereals include wheat, barley, oats, rye, triticale, rice and other species that constitute the world's most important source of food. They are critical components of local economies in developed and developing countries. About 70% of land devoted to producing food crops is planted to cereals. These crops supply 20% of calories and account for more than half of all harvested crop areas in the world (Breiman & Graur 1995; Dababat *et al.*, 2015). Production of small grain crops on a per unit area basis increased linearly from 1960 until about 2005 and then began to decelerate in the rate of annual gain. The rate of increase is projected to continue to decline through 2050 (Alexandratos & Bruinsma, 2012). Since most available prime land is already being used for crop production, land planted to wheat, the primary small grain crop, is not anticipated to increase appreciably; any increase will likely occur on land that is of only marginal to good productive capacity. Additionally, land suitable of being irrigated, and therefore capable of producing higher-yielding crops, is not anticipated to increase appreciably through 2050.

2.2 Global distribution of the Cereal Cyst Nematodes

The genus *Heterodera* is considered to be one of the oldest discovered genera of plant-parasitic nematodes after the two previously named genera *Anguina* (Scopoli, 1777) and *Tylenchus* (Bastian, 1865). In 1859, Schacht reported the first cyst-forming nematodes on roots of sugar beet. Later, Schmidt (1871) erected the genus *Heterodera* and described these nematodes as *H. schachtii*. When later, cyst nematodes were detected on other crops, it was thought that they belonged to *H. schachtii*. In 1874, Kühn reported cyst nematodes parasitising cereals in Germany. Later, Liebscher (1892) reported a species of the genus *Heterodera* affecting peas but differing from *H. schachtii* in that it did not affect oats, a recognised host of *H. schachtii* at that time; it was named *H. goettingiana*. The host specificity of the cyst nematode being recognised, more species could be identified. The potato cyst nematode, *H. rostochiensis*, was described from potatoes (Wollenweber, 1923) and the oat cyst nematode, *H. avenae*, from cereals (Wollenweber, 1924).

Later, many more cyst nematodes were detected on cereals. These cereal cyst nematodes (CCN) form a complex of several closely related species, which are distributed worldwide on Poaceae (Rivoal & Cook, 1993; Nicol, 2002; Nicol *et al.,* 2004; Nicol & Rivoal, 2008). Among the CCN species, *H. avenae* was the first to be reported (Kühn, 1874; Wollenweber, 1924), followed by the Mediterranean *H. latipons* (Franklin, 1969), the north European *H. hordecalis* (Andersson, 1974), the eastern European *H. filipjevi* (Madzhidov, 1981) and several others (Wouts *et al*., 1995). So far, 11 species of the CCN group have been described. However, *H. avenae*, *H. latipons,* and *H. filipjevi* are considered the most economically important species in cereals worldwide (Figure 2.1) (Rivoal & Cook, 1993; Nicol & Rivoal, 2008).

The absence of any fossil of cyst nematodes made the origin estimation difficult. However, biogeography of cyst nematodes, together with their host and molecular data, may provide some evidence on the approximate time and area of origin of cyst nematodes (Subbotin *et al*., 2010a), where the Mediterranean and the Middle Asia regions suggested to be the centre of origin for the genus *Heterodera* (Krall & Krall, 1978).

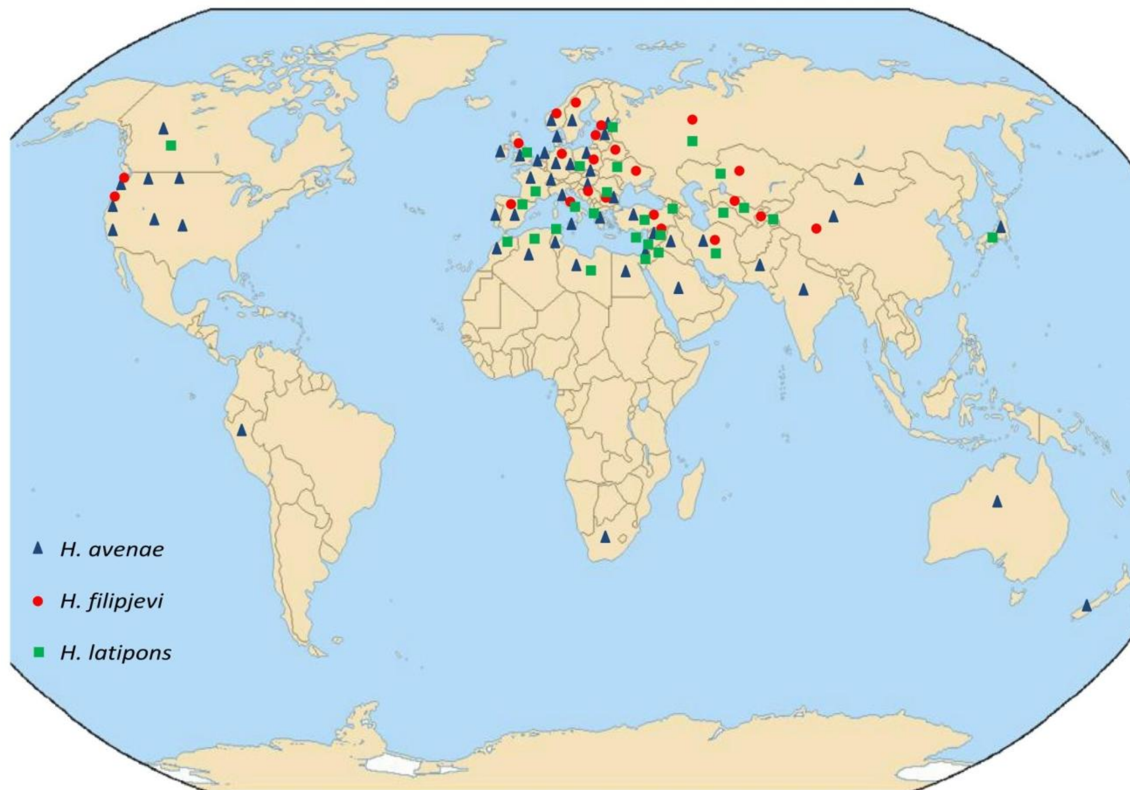

Figure 2.1. Distribution of the three-major species of cereal cyst nematodes.

Heterodera avenae (common name: cereal or oat cyst nematode) is the most widely distributed species of the CCN. After it was first reported in Germany, it was subsequently reported in most European countries: Italy (Mezetti, 1953), Portugal (Macara, 1963), Spain (Tobar, 1963), Greece (Hirschmann *et al*., 1966), former Yugoslavia (Grujicic, 1966), France (Sosa, 1966; Rivoal, 1977), Belgium (Coomans, 1989), UK (Gair *et al*., 1969), and from non-European countries such as Tunisia (Delanoue, 1953), India (Vasudeva, 1958), Peru (Krusberg & Hirschmann, 1958), Canada (Kort, 1972), New Zealand (Grandison, 1975), Australia (Davidson, 1930). Pakistan (Maqbool, 1988), Japan, South Africa, Syria, Iran, Egypt, Morocco and Libya (Sikora, 1988), China (Chen *et al*., 1989), Algeria (Mokabli *et al.,* 2002), Turkey (Rumpenhorst *et al.,* 1996) and Saudi Arabia (Youssif, 1987). In the USA, *H. avenae* was first reported in 1974 from Washington and western Oregon (Jensen *et al.,* 1975). It has subsequently been reported in seven western states: Idaho, Montana, California, Colorado, Oregon, Washington, and Utah (Hafez & Golden, 1984; 1985; Hafez *et al.,* 1992; Smiley *et al.,* 1994). *Heterodera avenae* in Australia was later described as *H. australis* (Subbotin *et*

al., 2002), whereas *H. avenae* in China was recently described as *H. sturhani* (Subbotin, 2015).

In the 1960s, another cyst nematode was detected in the Mediterranean region (Israel and Libya) on the roots of stunted wheat plants. It was described as a new species and named *H. latipons* based on morphological characteristics of the Israel population (Kort, 1960; Franklin, 1969). *Heterodera latipons* (Mediterranean cereal cyst nematode) has a wide distribution and is essentially distributed in the Mediterranean region, *viz.* Italy and Libya (Kort, 1972), Greece (Hirschmann *et al*., 1966), Cyprus (Philis, 1988), Spain (Romero, 1980), Morocco (Mokrini *et al*., 2012), and in the Middle-East where it was found in Syria (Sikora & Oostendorp, 1986), Jordan (Yousef & Jacob, 1994), Lebanon (Greco *et al.,* 2002) and Turkey (Rumpenhorst *et al*., 1996). It was also detected in more or less temperate continental climates of several republics of the former USSR (Mulvey & Golden, 1983; Subbotin *et al.,* 1996), in Iran (Talatchian *et al.,* 1976; Tanha Maafi *et al.,* 2007), Japan (Momota, 1979), and Europe including Bulgaria (Stoyanov, 1982), Czech Republic (Sabova *et al*., 1988) and UK (Anon, 2005), Canada (Sewell, 1973).

Filipjev and Schuurmans Stekhoven (1941) reported populations of CCN in the Sverdlovsk region (Russia) and identified these as *H. avenae.* Later, similar populations were found in Ukraine and other republics of the former USSR (Kirjanova, 1969). In the 1970s, heavy infestations were reported from wheat fields near the Dashtak, Kuljab region, and Tajikistan. Several differences in morphometrics and the presence of a light underbridge in the vulval cone of the cysts supported the description of this nematode as a new species, *viz*. *H. filipjevi* (Madzhidov 1981; Subbotin *et al.,* 2010b). *Heterodera filipjevi* is the third major CCN species (the rye cyst nematode). Previously in Sweden, populations of this nematode were named as the Gotland strain of *H. avenae* (Andersson, 1973; Cook & Noel, 2002). Later, *H. filipjevi* was found in Iran (Sturhan & Rumpenhorst, 1996; Tanha Maafi *et al.,* 2003) where it is the most dominant and widespread species of CCN (Damadzahed & Ansaripour, 2001; Tanha Maafi *et al.,* 2007). It was further reported from Poland, Spain and Sweden (Stelter, 1984), Belarus and Bulgaria (Bossis & Rivoal, 1996), former USSR (Balakhnina, 1989), Turkey (Rumpenhorst *et al.,* 1996), India (Bishnoi & Bajaj, 2002), Norway (Holgado *et al.,* 2004a), Italy (Madani *et al*., 2004),

Germany (Grosse & Kohlmüller, 2004), UK (Mitchinson, 2009), Syria (Abidou *et al.,* 2005a), China, India, Kazakhstan (Subbotin *et al*., 2010b), and the USA (Smiley *et al.,* 2008; Smiley, 2009a)

2.3 Yield loss and economic importance

Cereal cyst nematodes can cause considerable yield losses, especially in semi-arid regions, temperate climates, and where monoculture systems exist (Rivoal & Cook, 1993). The use of nematicides is considered the only option in evaluating the yield loss due to CCN damage in the field (Brown *et al*., 1970; Simon, 1980; Rivoal & Sarr, 1983). Multiplication of the majority of plant-parasitic nematodes, including CCN, is relatively slow. Root nematodes only cause yield reduction when harmful densities are already present in the soil at the time of planting of the crop (Pi) (Schomaker & Been, 2013). As a consequence, the relation between the Pi of CCN and growth and yield of wheat is very important to estimate the putative economic loss (Ibrahim *et al.,* 1999).

Yield loss caused by *Heterodera* spp. on wheat and barley was reported from different regions. In Australia, the loss caused by *H. avenae* (currently called *H. australis*) was estimated to reach up to 20% in barley and up to 50% in wheat (Meagher, 1972). Ibrahim *et al.* (1999) reported losses of 40-92% in wheat and 17-77% in barley in Saudi Arabia. Ten to forty percent loss in wheat was reported in China (currently called *H. sturhani*) (Peng *et al*., 2007) and 40-50% in India (Mathur *et al.,* 1980). In a study in Rajasthan conducted by Handa and Yadav (1991), applying nematicides reduced yield loss of up to 44% in barley and 35% in wheat. Namouchi-Kachouri *et al.* (2007) reported yield loss of 19-86% in barley and 26-96% in wheat in Tunisia. Yield losses of 15 to 20% in wheat in Pakistan (Maqbool, 1988) and 50% in wheat in Israel (Mor *et al*., 1992) were documented. Holgado *et al.* (2003) reported losses in wheat yield caused by *H. avenae* and *H. filipjevi* of 50% in Norway. In Syria, Hassan *et al.* (2010) investigated the effect of *H. avenae* on the plant growth, yield, and nematode reproduction in durum and bread wheat cultivars under field conditions, where a reduction in yield was 57% and 50% in grain and 50% and 45% in straw in durum and bread wheat, respectively. In the USA,

Smiley *et al.* (1994) reported that *H. avenae* reduced wheat yield by 50% in heavily infested irrigated fields.

Heterodera filipjevi also decreases cereal yield. Several reports confirmed the economic yield loss in many countries under different climatic conditions. The economic importance of *H. filipjevi* on winter wheat was determined under rain-fed conditions in Turkey, showing an average yield loss between 42 and 50% (Nicol *et al.,* 2006). In Iran, the yield loss due to *H. filipjevi* was investigated on winter wheat in monoculture. The grain yield loss was estimated at 48% with a Pi density of 20 eggs and J2 (g soil)$^{-1}$ while the aerial shoot yield loss was 40% (Hajihasani *et al.,* 2010a).

Compared with the above-mentioned species, yield losses in wheat caused by *H. latipons* are not well documented. However, it was reported to be an important nematode on barley and durum wheat production in temperate and semi-arid regions (Sikora, 1988; Ismail *et al.,* 2001; Scholz, 2001; Scholz & Sikora, 2004). *Heterodera latipons* is believed to cause less damage to cereals compared with *H. avenae* (Mor *et al*., 1992; 2008). However, in Cyprus, *H. latipons* was reported to decrease barley yield by 50%. The loss was greatest under severe drought conditions and monoculture systems (Philis, 1988; 1997). In Syria, grain and straw losses caused by *H. latipons* could be an important constraint on barley and durum wheat production in semiarid regions (Scholz, 2001). In Iran, *H. latipons* significantly reduced grain yield of winter wheat up to 55%, root dry weight with 70%, aerial shoot dry weight with 48%, spike height up to 36% and plant height up to 32% (Hajihasani *et al.,* 2010b).

2.4 Life cycle of the Cereal Cyst Nematodes

Like other cyst nematodes, the life cycle of CCN begins as an egg contained in a cyst. In and out of this egg develop four successive juvenile stages leading to the mature adult stages (male and female). Cereal cyst nematodes have one generation per year (Greco *et al*., 2002). The mature cysts are brown and an immobile. The first-stage juvenile (J1) moults to the second-stage juvenile (J2) inside the egg. The emergence of J2 out of the egg and the cyst is triggered by specific interactions among soil temperature and moisture and to some extent by exudates from host roots (Smiley & Nicol, 2009). As a

survival strategy, not all juveniles hatch at the same time. A proportion of J2 is retained within the cyst. Once the J2 of CCN are released into the soil, they begin to search for a suitable host, relying primarily on gradients of chemicals including CO_2, amino acids and sugars released by the roots of the host (Perry, 1997; Subbotin *et al.,* 2010a; Turner & Subbotin, 2013). Usually, the newly hatched J2 invade the host root directly behind the growing root tip in meristematic tissue and migrate intracellularly to the stelar region through disruptive stylet action and secretions. Then, J2 will select a competent root cell to inject secretions from its pharyngeal glands to form the syncytium (= a group of feeding cells) by day four after the invasion (Williams & Fisher, 1993; Davis *et al.,* 2000; Vanholme *et al.,* 2004). A syncytium develops from the dissolution of neighbouring cell walls forming an enlarged and metabolically active multinucleate cell that serves as the nutrient source (Seah *et al.,* 2000). Once the syncytium is formed, the J2 start feeding on it and moult to third-stage juveniles (J3). At the fourth moult (end of the J4 stage), males develop at a similar rate in the same root as the females. Males are non-feeding, leave the root and live for only a short time in the soil. Females attract males via sex pheromones. One female may mate with several males. After mating, the embryos develop within the egg until the formation of the J2 while still within the female's body (Turner & Subbotin, 2013). Female nematodes die, and their cuticle becomes a tough protective layer called the cyst. Cyst colour will change from white to mid/dark-brown after female death (Seah *et al.,* 2000). Cysts contain several hundreds of embryonated eggs and unhatched J2 and are considered as a dormant stage. Depending on the species and environmental conditions, the unhatched J2 can remain within the protective cyst for many years of dormancy as a survival strategy (Turner & Subbotin, 2013).

A difference in the life cycle through the infection process was observed between *H. avenae* and *H. latipons* (Mor *et al.,* 1992; 2008). Second-stage juveniles of *H. avenae* attacked the root tip region inducing typical branching and swelling of roots. However, J2 of *H. latipons* penetrated at sites along roots more distant from the root tip. Hence, *H. latipons* did not produce clearly visible root symptoms in the early infection period or the seedling stage. Additionally, differences were observed in the infection process and in the feeding cell structures, where the growth inhibition caused by *H. avenae* was more severe than *H. latipons.*

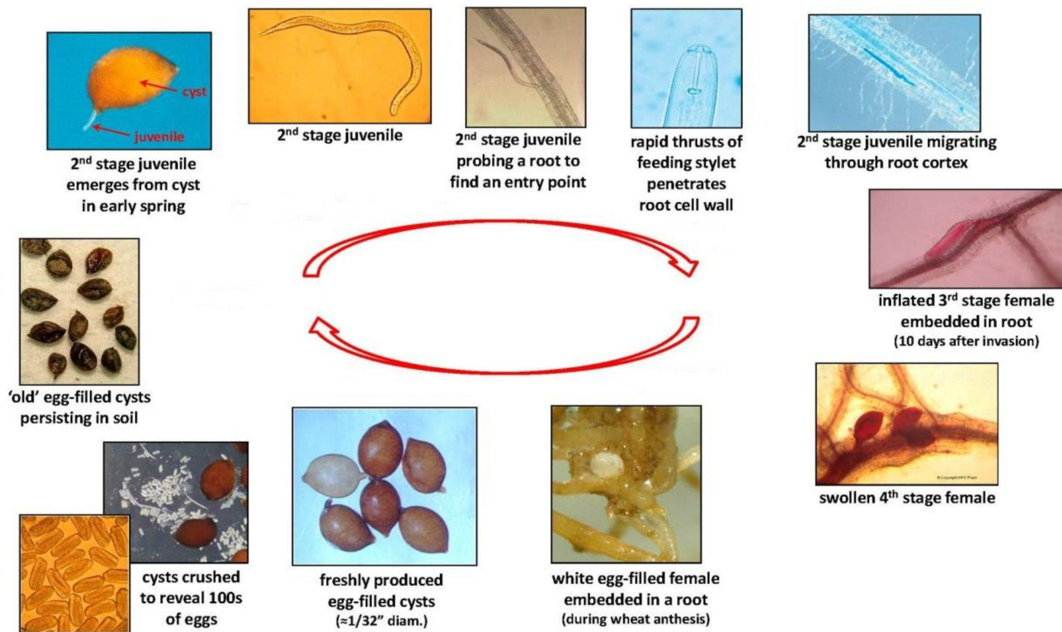

Figure 2.2. Life cycle of cereal cyst nematodes (Smiley, 2016).

2.5 Hatching

Quite a number of papers have dealt with the hatching process of the three-major species of CCN, using populations from different origins (Banyer & Fisher, 1971; Meagher, 1977; Rivoal, 1986; Nitao *et al.*, 1999; Ismail *et al.*, 2000; Mokabli *et al.*, 2001b; Scholz & Sikora, 2004; Al-Abed *et al.*, 2009; Sahin *et al.*, 2010). According to their origin, each species can hatch over a wide range of temperatures. Hatching of CCN is controlled by diapause, i.e. a state of arrested development whereby development does not continue until specific requirements have been satisfied, even if favourable conditions return. A range of environmental stresses on the female of the *H. avenae* initiates the diapause (Wright & Perry, 2006). *Heterodera latipons* hatches well at 10°C in Syria (Scholz & Sikora, 2004) and Jordan (Al-Abed *et al.,* 2009), with a maximum hatching of not more than 33% and one hatching peak at the end of January and beginning of February. The obtained hatching percentage for *H. latipons* was relatively low compared to *H. avenae* and *H. filipjevi* (Scholz & Sikora, 2004). Sahin *et al.* (2010) found the optimal hatch for Turkish populations of *H. filipjevi* in the range between 10 and 15°C; under *in*

vitro and field conditions, 94% of the J2 hatched from the eggs with two peaks recorded early October and the period between the end of January and early March.

In West Australia, *H. avenae* hatches optimally at a temperature between 10 and 15°C, with the hatching peak of 80% under the field conditions in late May (Banyer & Fisher, 1971; Meagher, 1977; Stanton & Eyres, 1994). Similar results were reported from France for *H. avenae* (Rivoal, 1986). Also, a relatively long incubation (six months) at 2°C was needed to enhance the hatch (70-80%) of *H. avenae* Swedish population (Ireholm, 1996). In Algeria, Mokabli *et al.* (2001b) kept two local populations at a wide range of constant temperatures (3, 7, 15, 20 and 25°C). After 11 months, cysts of the two populations were transferred for 2 months to either a high (25°C) or low (3°C) temperature and finally returned to the initial temperature. Both populations hatched over a wide range of constant temperatures (3-25°C) but differed in the times at which J2 emerged from the cysts.

Comparison of hatching of *H. avenae* kept at different constant temperatures or previously exposed during one or two months at low (5°C) or high (20°C) temperature, enabled the explanation of the diapause for the northern and southern ecotypes occurring in France (Rivoal, 1983). In the south of France, an obligatory diapause acts during the summer and autumn and is disrupted by low temperatures, which explains the winter hatching of this ecotype. By contrast, for the northern ecotype, a facultative diapause acts during winter and is broken by an increase in temperature, which leads to hatching in spring (Rivoal, 1983).

Hatching of *H. filipjevi* increased significantly when exposed to exudates of the susceptible wheat variety ‘Bezostaya’ compared to exudates of the resistant wheat variety ‘Sönmez’ (Sahin, 2010). Similarly, root exudates of the susceptible barley varieties ‘Tadmor’ and ‘Arta’ (Scholz, 2001) and the barley variety ‘Rum’ (Al-Abed *et al*., 2009) stimulated the hatching of *H. latipons* J2. However, hatching under the influence of exudates of susceptible cvs. was not significantly different from hatching in the control (water); the hatching in root exudates of the variety ‘Tadmor’ was lower than the hatching in water (Scholz, 2001). Nevertheless, after incubation at 5˚C, hatching of *H. latipons*

exposed to root exudates of both varieties was significantly greater than hatching in the control (water). This suggests that cultivars play a role in the production of signals that induce hatching of *H. latipons*, especially at low temperature. An experiment comparing root exudates of barley with those of sugar beet showed the mobility of J2 was greater towards the host barley compared to the non-host sugar beet, which demonstrated the high response specificity of *H. latipons* to cereals.

Plant age plays an important role in hatching through root exudates production (Ibrahim *et al.*, 1993). Root exudates of plant stage '2 unfolded leaves' are considered to be the best exudates for *H. latipons* hatching (Scholz & Sikora, 2004). Chemicals like zinc chloride ($ZnCl_2$) increase the *H. filipjevi* hatching rate by 30% more than sterilised water (Sahin, 2010). A similar result of increasing the hatch of *H. zeae* was obtained after immersion of cysts in 4 mM $ZnCl_2$ solution; it stimulated 10% extra emergence of J2 than occurred in tap water controls during 28 days (Hashmi & Krusberg, 1995). Adding fertilisers to corn plants resulted in more corn roots. Hence, more nematode infection sites were available which resulted in a doubling of the numbers of cysts of *H. zeae* and 2-3 times more J2 than in unfertilised cultures (Hashmi & Krusberg, 1995).

2.6 Resistance and tolerance of cereals to Cereal Cyst Nematodes

Development of cultivars with genetic resistance plus genetic tolerance has been achieved internationally. The use of resistant cultivars is the only durable method of control available for CCN; it is considered cost-efficient, environmentally safe and user-friendly (Dababat *et al.,* 2014). The effectiveness and usefulness of resistance to CCN depend on the efficacy, robustness and stability of the source of resistance, the interaction of the resistant accession and the nematode species, as well as on the correct identification of the nematode species and Pi (Dababat *et al.,* 2014). Genetic tolerance may be less specific than resistance, and tolerance offers acceptable alternatives where resistance is lacking. Tolerant cultivars may work against several nematode species simultaneously. However, breeding for tolerance is difficult because the criteria for selection are complex and expensive to define. Identification of greater tolerance by screening released cultivars may be more rewarding (Cook & Starr, 2006).

2.6.1 Resistance of cereals to Cereal Cyst Nematodes

Cultivars that greatly suppress or prevent the reproduction of nematodes are called resistant; those that allow moderate to high rates of reproduction are susceptible (Cook & Evans, 1987). The first resistant cultivar was bred by Nilsson-Ehle (1908). Later, resistant cultivars have been developed in Europe and India but have been most successful in Australia. In 1977, the rust-resistant wheat cv. Festiguay was introduced in Australia, CCN were unexpectedly controlled because the cultivar was also resistant to CCN (O'Brien & Fisher, 1977). One year later, a project was started to screen wheat lines for CCN resistance and tolerance; and in 1981 a project started to screen barley lines (Lewis *et al.*, 2009).

1.6.1.1 Screening for resistance

When screening cereals for resistance to *H. avenae*, Gill and Swarup (1971) used 100 freshly hatched J2 to inoculate one-week old seedlings grown in 10-cm diameter pots filled with sandy loam soil and kept in the greenhouse for two months. They considered plants with more than 5 cysts as good hosts, plants with1-5 cysts as poor hosts, and plants without cysts as non-hosts. However, the first generally accepted scoring criteria for *H. avenae*, were proposed by Andersen and Andersen (1982). They used two rates: *viz.* resistant plants (R), i.e. plants without cysts or with only a few cysts (1-2 cysts plant^{-1}), and susceptible plants (S), i.e. plants with many cysts. Later, Kaur *et al*. (2008) and Sharma *et al*. (2013) scored resistance to *H. avenae* using a rating scale with four classes: resistant (R), moderately resistant (MR), susceptible (S) and highly susceptible (HS). Another used strategy for resistance evaluation to CCN was the reproduction index (*Ri*). Many researchers reported the use of reproduction index which can be calculated by dividing the *Pf* (final population), on the *Pi* (initial population) and the *Ri* can be used in comparison with cultivars used as control and it is previous known response (Scholz, 2001; Hajihasani *et al*., 2010; Al-Abed *et al*., 2013). Depending on the *Ri*, a rating scale was developed to evaluate the resistance to *H. latipons* (Scholz, 2001), a variety was considered resistant when *Ri* = 0.5-0.9; moderately resistant when *Ri* = 1.0-1.2; and highly susceptible when *Ri* = 1.3-3.7.

During the five International Cereal Nematode Initiative Workshops (2009-2015), it became clear that research groups in different countries and institutes use their different methods for screening against CCN. Differences exist in inoculum (preparation, nematode stage and density), humidity, temperature, containers and soil. The lack of a common and standard screening method, including scales for resistance, makes the comparison of the results difficult. In 2009, Nicol *et al.* presented the first protocol and methodology for screening and evaluating resistance for both *H. filipjevi* and *H. avenae.* This scoring system (Nicol *et al.*, 2009) was used by Cui *et al.* (2016), and, with slight modifications, by Dababat *et al.* (2014) and Pariyar *et al.* (2016a). In each screening protocol, susceptible varieties and moderately resistant and/or resistant varieties should be included as references for classification (Nicol *et al.*, 2009; Dababat *et al.*, 2014).

1.6.1.2 Genes for resistance

In the last four decades, the low level of resistance for CCN in the genus *Triticum* led to the use of genes from alien cultivated or wild species (Bekal *et al.,* 1998). So, various species of *Triticum*, *Aegilops* and *Secale* have been screened through the wheat-breeding programmes as potential sources of germplasm with resistance to CCN (Ogbonnaya *et al.,* 2001). Nine resistance genes (*Cre*) to *H. avenae* (formerly called: Cereal root eelworm) were reported from different sources. The *Cre1* gene was characterised in the wheat line cv. Aus10894/Loros (Slootmaker *et al.,* 1974). The *Cre2*, *Cre5* (*CreX*) and *Cre6* genes were transferred from the wild *Aegilops ventricosa* (Delibes *et al.,* 1993; Jahier *et al.,* 1996; Ogbonnaya *et al.,* 2001), *Cre3* and *Cre4* from *T. tauschii* (synonym *Ae*. *squarrosa*, *Ae. tauschii*) (Eastwood *et al.,* 1991), *Cre7* (*CreAet*) from *Ae. triuncialis* (Romero *et al.,* 1998), *Cre8* (*CreF*) from the bread wheat cv. Festiguay (Paull *et al.,* 1998; Ogbonnaya *et al.,* 2001) and the *CreR* gene from *Secale cereale* (Asiedu *et al.,* 1990). All of these genes confer complete or partial resistance (*i.e.* resistance that is less than 100% inhibition of nematode reproduction in comparison to a chosen standard) to different CCN pathotypes.

Cre1 conferred resistance to the Australian *H. avenae* pathotype (*Ha13*) and several European pathotypes (*Ha11* and *Ha12*). Therefore, resistance genes, which

include *Cre1*, located on chromosome 2B of wheat (Slootmaker *et al.,* 1974), have been used in Europe and Australia. *Cre2* has exhibited a high level of resistance to pathotypes of *H. avenae Ha71* (Spanish), *Ha11* (British) and *Ha12–Ha41* (French) but was ineffective against *HgI–HgIII* (Swedish) and the Australian *Ha13* (Delibes *et al.,* 1993; Ogbonnaya *et al.,* 2001). *Cre3* confers resistance to Australian pathotypes but is susceptible to both the European *Ha11* and *Ha12* pathotypes (Eastwood *et al.,* 1991; Rivoal *et al.,* 2001). Partial resistance and tolerance against *Ha13* were exhibited in *Cre4* (Eastwood *et al.,* 1991; Nicol *et al*., 2001; Mulki *et al*., 2013). The *Cre5* gene showed partial resistance to French (*Ha12–Ha41*) and Australian (*Ha13*) pathotypes of *H. avenae* (Dosba *et al.,* 1978; Rivoal *et al.,* 1986; 1993; Jahier *et al.,* 2001; Ogbonnaya *et al.,* 2001). *Cre6* confers resistance for *Ha13* (Mulki *et al*., 2013).

To date, no complete resistance at once was reported by any of the *Cre* genes to the three species of CCN. Imren *et al.* (2012) showed that *Cre1*, *Cre3*, and *Cre7* provided resistance to both *H. avenae* and *H. latipons*, whereas *Cre8* and *CreR* showed resistance to *H. filipjevi* only. Toktay *et al.* (2012) screened lines with *Cre1* genes against *H. filipjevi* and the root lesion nematode (RLN) *Pratylenchus thornei*. Some lines showed resistance to both nematodes, whereas some lines were susceptible to either both or one of these nematodes. Therefore, there is no association among *H. filipjevi* Turkish population, *P. thornei* and the *Cre1* gene. no complete resistance and no relationship between *H. filipjevi* Turkish population and *P. thornei* resistance.

Gene pyramiding is a broad-spectrum technique for developing robust stress resistance in crops, and aimed at assembling multiple desirable genes into a single genotype to avoid breakdown of resistance mediated by the virulence genes of the pathogen (Ogbonnaya *et al*., 2001). Currently, gene pyramiding is a commonly used breeding strategy in self-pollinating crops like wheat and barley. Both genes, *Cre1* and *Cre8*, were pyramided successfully in Australia (Ogbonnaya *et al*., 2001; Barloy *et al*., 2007). Also, pyramiding into wheat of two genes from *Ae. variabilis* (*CreX* and *CreY*) showed that the level of resistance of the pyramided line was significantly higher than that of *CreX* and *CreY* single introgression lines. In addition, the pyramided genotype conferred resistance to the root-knot nematode *M. naasi* (Barloy *et al*., 2007*)*, probably

due to the *CreY* gene which may be the same as the gene conferring resistance to *M. naasi* (*Rkn-mn1*) described by Jahier *et al*. (1998).

2.6.2 Tolerance of cereals to Cereal Cyst Nematodes

Tolerance to plant-parasitic nematodes refers to the relative ability of a plant to sustain growth and yield well when parasitised by a nematode in comparison with non-invaded plants. The combination of resistance and tolerance in a plant provides the ideal management for plant-parasitic nematode (Cook & Starr, 2006; Rivoal & Nicol, 2009). While, the combination of resistance and intolerance in a plant is the second-best option (Smiley *et al*., 2013). Both resistance and tolerance are genetically independent, and cultivars resistant or tolerant to one species or pathotype are not necessarily resistant or tolerant to another species or pathotype (Smiley & Nicol, 2009; Toktay *et al*., 2012; Dababat *et al*., 2014). Plants that are sensitive and exhibit a significantly suppressed yield when invaded, are qualified as intolerant (Cook & Evans, 1987). The combination of intolerant and resistant plant can control the nematode population effectively but liable to damage when nematodes are numerous (Cook & Starr, 2006).

Tolerance is more difficult to assess and increases with plant age (Fisher, 1982a). Usually, tolerance is estimated in the field by comparing the yield of a specific plant cultivar in a naturally infested soil that is either left untreated or is treated with nematicides to reduce the impact of the existing nematode population (Smiley, 2009b). Very few experiments on tolerance have been reported. In France, wheat production was compared in lightly, moderately and highly *H. avenae* infested soils, in both field or controlled conditions (plastic pots) and soil resulting from long-term cultures with either susceptible oat cultivars (cv. Peniarth) or resistant oat (cv. Panema). *Heterodera avenae* affected the crop development, with a marked effect on the yield. The mechanisms and genetic determinism of the tolerance remain to be determined.

2.6.3 Pathotypes

A ‘pathotype’ can be defined as a group of nematodes that are morphologically identical, but can be distinguished from others of the same species by their pathogenicity

on specific hosts (host differentials). Nematodes of a certain pathotype have the common gene(s) for (a)virulence which differ(s) from gene or gene combinations found in other groups (pathotypes). The relative ability of a nematode taxon or population to damage a given plant can be defined by 'pathogenicity'. Virulence is a measure of the ability of a nematode to reproduce on a plant, particularly, when comparing populations of the same species for their ability to reproduce on resistant plants (Cook & Starr, 2006).

The *International Cereal Test Assortment for defining cereal cyst nematode pathotypes* (Andersen & Andersen, 1982) was developed for differentiating some populations of *H. avenae*, and later *H. filipjevi*, that differed in their ability to circumvent several resistance genes (virulence) included into cultivars called differential hosts (Cook & Noel, 2002; McDonald & Nicol, 2005). The test consisted of 12 barley (*Hordeum vulgare*), 6 oat (*Avena sativa* and *A. sterilis*) and 6 wheat (*Triticum aestivum* and *T. durum*) differential cultivars to characterise selected pathotypes of *H. avenae*. Later the set was reduced to 23 differential hosts (Cook & Rivoal, 1998; Turner & Rowe, 2006). Based on cyst multiplication on barley with known resistance genes (*Rha1*, *Rha2, Rha3*) (Andersen, 1961), three primary groups of pathotypes were distinguished (*Ha1, Ha2, Ha3*). The first two are widely distributed in Europe, North Africa, and Asia (Al-Hazmi *et al*., 2001; Cook & Noel, 2002; Mokabli *et al*., 2002; McDonald & Nicol 2005), whereas group *Ha3*, is mostly found in Australia, Europe and North Africa (Rivoal & Cook, 1993; Mokabli *et al*., 2002). Each pathotype group is further subdivided by their reactions on other differentials. The pathotype concept was established to differentiate northern European populations of *H. avenae*. Consequently, it was unable to define the pathotypes in other regions such as China, from where three undescribed pathotypes were reported (Nicol & Rivoal, 2008; Peng *et al*., 2007) before two more pathotypes *Ha43* (Yuan *et al*., 2010) and *Ha91* (Cui *et al*., 2015) were identified. The *International Cereal Test Assortment* did not define North American populations either (Smiley & Nicol, 2009; Smiley *et al*., 2011). Populations initially designated as pathotypes *Ha23* and *Ha33* (Andersen & Andersen, 1982) are now considered to be pathotypes of the closely related species *H. filipjevi* (Subbotin *et al*., 2003; Ozarslandan *et al*., 2010; Smiley *et al*., 2011; Toktay *et al*., 2013). Pathotyping of *H. latipons* has never been done. Due to gaining prevalence and economic importance of the three major CCN species on cereals, more

research has investigated and identified new sources of resistance that have the potential to control local populations of CCN species. As a result, it is necessary to expand and update the *International Cereal Test Assortment* by including local cereal cultivars/varieties (Cook & Noel, 2002; Smiley & Nicol, 2009; Smiley *et al*., 2011; Cui *et al*., 2015). Unfortunately, until now, molecular technology failed to distinguish pathotypes of CCN and markers for virulence traits.

2.7 Identification of Cereal Cyst Nematodes

The genus *Heterodera* contains 84 species (Subbotin *et al*., 2010b; Subbotin, 2015). Traditionally, the identification of *Heterodera* species is based on the morphology and morphometrics of the cysts and juveniles, and sometimes males (Rivoal *et al*., 2003). This identification is time-consuming and requires specialised skills and training by the observer, especially in the case of species-mixtures (Yan & Smiley, 2009). However, reliable and rapid identification of nematodes is considered of major importance in control strategies, monitoring, and controlling the movement or introduction of potential pests. Application of control measures, especially when considering resistant crops, requires accurate identification of the cyst nematode at species and sub-species level (*e.g.* pathotypes).

2.7.1 Biochemical techniques

In the 1990s, new techniques with great potential for differentiation between species appeared; they were based on the variability of biochemical traits of proteins (Bossis & Rivoal, 1996; Romero *et al*., 1996; Rumpenhorst *et al*., 1996; Mokabli *et al*., 2001a). The application of the enzyme-staining technique for characterisation of a single protein or small subset of proteins on gels provides a diagnostic method for CCN (Rumpenhorst, 1985; Subbotin *et al*., 1996; Subbotin *et al*., 2002; Holgado *et al*., 2004b). However, the high degree of polymorphisms within populations of the CCN species, can cause problems for diagnostics (Esbenshade & Triantaphyllou, 1988; Radice *et al*., 1988; Andres *et al*., 2001). Moreover, the influence of sample preparation, sample storage and the developmental stages of the nematodes sample on the banding patterns, caused that the technique was replaced by more sensitive DNA-based methods.

2.7.2 Molecular markers

Molecular markers (RAPD and PCR-RFLP of rDNA-ITS) have enabled various CCN species to be identified (Rivoal *et al.,* 2003; Subbotin *et al.,* 2003; Ophel-Keller *et al.*, 2008; Waeyenberge *et al.,* 2009). The analysis of coding genes (18S, 5.8S and 28S) of ribosomal DNA (rDNA), and the in-between two non-coding regions, called the internal transcribed spacer 1 and 2 (ITS1 and ITS2), have become a favourite way for nematode identification (Vrain *et al.,* 1992; Wendt *et al.,* 1993; Ferris *et al.,* 1993, 1994; Zijlstra *et al.,* 1995). Both spacers ITS1 and ITS2 are known to evolve faster and are therefore more variable than the coding genes. Consequently, they were useful for nematode identification and phylogenetic studies at the species level. Ferris *et al.* (1993; 1994) were the first to sequence ITS1 and ITS2 from several isolates of cyst nematodes belonging to the genus *Heterodera,* and to compare the sequences with those published from *Caenorhabditis elegans*. Shortly after, the use of molecular technologies started to increase exponentially (Rivoal *et al.,* 2003; Waeyenberge *et al.,* 2009). The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based on ITS-regions of the rDNA repeat units of *Heterodera* spp. has frequently been used for the identification of cyst nematodes (Bekal *et al.,* 1997; Subbotin *et al.,* 1999; 2000; Rivoal *et al.,* 2003; Madani *et al.,* 2004; Abidou *et al.,* 2005b; Smiley *et al.,* 2009; Yan & Smiley, 2009). It has been reported that restriction of the ITS amplicons with one or a combination of seven restriction enzymes (*Alu*I, *Ava*I, *Bsh*1236I, *Bsu*RI, *Cfo*I, *Mva*I and *Rsa*I) enables discrimination of agriculturally important cyst nematode species, both from one another and from their sibling species (Subbotin *et al.,* 2000). Species of *Heterodera* from the Avenae group can be differentiated from one another using the enzymes *Alu*I, *Cfo*I, *Hinf*I, *Ita*I, *Pst*I, *Rsa*I, *Taq*I and *Tru*9I, with the exception of *H. avenae* (type A) from *H. arenaria* because of a lack of differences between the restriction patterns obtained (Subbotin *et al.,* 2003). Five restriction enzymes (*Alu*I, *Rsa*I, *Bsu*RI, *Bsh1236*I and *Hin6*I) generate patterns that make it possible to distinguish between *H. latipons* and other *Heterodera* spp. However, PCR-RFLP has some limitations: (1) some enzymes are rare and thus expensive, (2) recognition sites are sometimes partly digested, (3) sometimes lack of specificity, and (4) insufficient resolution of small fragments visualised on agarose gels can interfere with a clear interpretation of the results (Waeyenberge *et al.,* 2009). In

addition, there is the phenomenon of heterogeneity or polymorphism as reported in *H. avenae* populations (Bekal *et al.,* 1997). Subbotin *et al.* (2000, 2003), reported intraspecific variations within the *Heterodera* species *H. betae*, *H. carotae*, *H. ciceri*, *H. cruciferae*, *H. filipjevi*, *H. glycines*, *H. pratensis*, *H. schachtii, H. trifolii, H. urticae* and *H. zeae*. Madani *et al.* (2004) and Rivoal *et al.* (2003) reported a relatively high level of sequence divergence between populations of *H. hordecalis* and suggested that two species may be grouped under this taxon.

2.7.3 DNA Sequencing

Recent progress in DNA sequencing nematode have been made and offered opportunities in the taxonomic analysis of nematodes (Waeyenberge *et al*., 2009). DNA sequencing costs have decreased more than 100-fold over the past decade due to the significant improvement of equipment, technology and process development for sequencing. However, *in silico* studies have also revealed that, in some cases, identical ITS-sequences can be found in morphologically clearly distinct *Heterodera* species such as *H. avenae* and *H. arenaria, H. carotae* and *H. cruciferae* (Subbotin *et al*., 2000). Furthermore, nematode identification via sequencing is still relatively expensive, laborious and time-consuming (Waeyenberge *et al.,* 2009).

2.7.4 Species-specific PCR

At the beginning of 2000, another approach to identifying *Heterodera* species was developed. In 2001, Amiri *et al.* designed a primer, using the available ITS-rDNA sequence information that was specific for species from the Schachtii group. One year later, Amiri *et al.* (2002) supplemented their research with a species-specific primer to detect only *H. schachtii*. In 2001, Subbotin *et al.* described a method to rapidly identify juveniles and cysts of the soybean cyst nematode (*H. glycines*), based on PCR with species-specific primers. A PCR with species-specific primers for *H. glycines* was developed by Ou *et al.* (2008). However, polymorphism between rDNA repeats within a species like *H. latipons* (Rivoal *et al.,* 2003), *H. avenae* (Bekal *et al.,* 1997; Zhao *et al.,* 2011) and *H. filipjevi* (Subbotin *et al.,* 2000; 2003) can cause the primer not to find its target. On the other hand, the analysis of the ITS-sequences revealed limited variations

for discriminating *H. avenae* and closely related species (Fu *et al.,* 2011), making designing a species-specific primer very difficult. Most recently, it was demonstrated that a species-specific PCR assay provides an efficient tool for an accurate, rapid and sensitive detection of the three species of CCN (Yan & Smiley, 2009; Qi *et al.,* 2012; Peng *et al.,* 2013; Yan *et al.,* 2013; chapter 4). However, the polymorphism within populations belonging to the same species can limit the species detection.

2.7.5 Quantitative PCR

Quantitative PCR (qPCR) is a simple and elegant technique useful in breeding programmes and extension activities. QPCR measures the nematode number indirectly by assuming that the number of target DNA copies in the sample is proportional to the number of targeted nematodes (Madani *et al*., 2005). The quantitative information in a qPCR comes from those few cycles (out of 40) where the amount of DNA grows exponentially above the threshold (Rasmussen, 1998). QPCR allows continuous monitoring of the amplification during PCR using hybridisation probes (TaqMan) or double-stranded dyes such as SYBR green I. With this technique, the increase of fluorescence signal appears on the computer screen and can be compared to a standard with known quantities of DNA. These data can be used to quantify the samples (Kingsnorth *et al.,* 2003). Quantitative PCR strategies have been developed for *H. schachtii* (Madani *et al.,* 2005) and *H. glycines* (Goto *et al.,* 2009; chapter 4). Since the qPCR equipment is relatively expensive, the technique is still limited especially in countries where labour is cheap.

2.8 Cereal Cyst Nematodes Control

In many countries where CCN occur, wheat is often one of the major food staples, and the control of the nematode is of considerable importance to improve both quantity and quality of the production. Furthermore, much of west Asia and north Africa is characterised by wheat monoculture systems, where rainfall or irrigation is limited. Such a cropping system frequently suffers drought stress; in these environments, the effects of the nematode damage can be augmented. Therefore, control of CCN is important.

However, CCN present a unique problem in their management due to the eggs protected inside the cyst (Riggs & Schuster, 1998). Eggs are protected against invasion by potential parasites, rapid desiccation, enhancing their ability to remain dormant for many years. Therefore, management strategies must be effective for more than one year. CCN have a relatively narrow host range, making appropriate crop rotation a possible option. Next to rotation, clean fallows, quarantine, cleaning machinery, field sanitation, weed control, organic and/or inorganic fertilisers, selecting sowing dates to escape hatching peak, and trap cropping (Rivoal & Nicol, 2009; Smiley & Nicol, 2009; Dababat *et al.*, 2011; Dawabah *et al.*, 2015). The mentioned agricultural practices could represent an efficient strategy to decrease nematode densities and reducing the effect of nematodes on wheat yields. Unfortunately, sometimes their use is limited by financial constraints. Similarly, if the speed of decline of a cyst nematode population in the soil is slow, control by crop rotation may entail a long and unacceptable interval between host crops (Whitehead, 1998).

Biological control is an environmentally friendly method that has the potential to reduce nematode multiplication and subsequent damage to crop plants (Kerry, 2000; Viaene *et al.*, 2006; Ashoub & Amara, 2010). Eggs of cyst nematodes are contained inside the female's body or cyst. Hence, cyst nematodes would appear to be the perfect target for the use of biological agents in their management by fungi or bacteria in the rhizosphere. Many studies reported the use of nematophagous fungi, *i.e. Nematophthora gynophila, Trichoderma longibrachiatum* and *Pochonia chlamydosporium,* for management and control both *H. avenae* and *H. filipjevi* populations densities (Kerry *et al.*, 1982a; 1982b; Holgado & Crump, 2003; Zhang *et al.*, 2014), and for *H. latipons* (Ismail *et al.*, 2001). Also, bacteria *i.e. Streptomyces* spp., *Bacillus* spp. and *Pasteuria* spp. was used and included in the management of CCN (Gokte & Swarup, 1988; Sayer *et al.*, 1991; Bansal *et al.*, 1999; Li *et al.*, 2011; Zhang *et al.*, 2016a,b). However, a test to evaluate the rhizosphere competence and long-term survival of the biocontrol agents in soil is needed. Additionally, the ultimate use of biological agents relies greatly on the agroecology of the cropping systems for persistence and effectiveness, which may be appropriate in more optimal cropping systems. Moreover, biological control may not be an appropriate management technique for in cereals regarding the relatively high cost.

The use of resistant cultivars requires a good knowledge and accurate identification of cyst nematodes species and their pathotype, next to the information on the Pi. With respect to the Pi, the threshold level for *H. avenae* infection was first estimated in UK at 5 eggs (g soil)$^{-1}$ (Gair *et al.*, 1969). In Australia, due to higher temperatures and more water-limited crop production than in temperate regions, different thresholds for *H. avenae* have been applied; initially 2 eggs (g soil)$^{-1}$ (Meagher & Brown, 1974) and later 1 egg (g soil)$^{-1}$ (Simon, 1980; King *et al.* 1982; Simon & Rovira, 1982). In 2008, Ophel-Keller *et al.* reported in Australia a low to moderate risk threshold at 5 eggs (g soil)$^{-1}$, and for moderate to high risk at 10 eggs (g soil)$^{-1}$.

The damage threshold density is a function of both genotypic and environmental factors (Smiley & Nicol, 2009). CCN are not strongly restricted by soil type but the damage is often greatest in light-textured and sandy soils (Smiley & Nicol, 2009). The damage threshold varies with soil type, climate, cultivar, nematode species, virulence, and ecotype. These variable influences on plant damage make it difficult to directly relate initial population density with a reduction in grain yield (Bonfil *et al.*, 2004). Generally, the threshold is increased by partial or full resistance reactions by a given cultivar. Also, the threshold usually decreases when plant growth is stressed by impediments to root penetration, drought, poor soil nutrition, or unfavourable temperature (Romero *et al.*, 1998; Smiley & Nicol, 2009). However, the use of resistant cultivars may lead to the selection of virulent pathotypes, or may entail a yield or quality penalty (Turner *et al.*, 1983; Whitehead, 1998).

Nematicides have been used widely and very effectively to control CCN (Whitehead, 1998). However, nematicides are expensive and usually toxic, require careful handling and appropriate use, may not give complete control when used alone, and the effectiveness of nematicides is reduced by their biological degradation by soil organisms. Therefore, several of the most effective have now been withdrawn from the market. Nevertheless, nematicides are likely to be needed for the foreseeable future as one of the elements in the integrated management of cyst nematodes.

The above-mentioned methods of agricultural practice, biological control and the use of resistant cultivars or by nematicides to control CCN, none of these methods is effective by itself in all situations and control is best when measures are combined (Whitehead, 1998).

Chapter 3

Distribution of the cereal cyst nematodes (*Heterodera* spp.) in wheat and barley fields in north-eastern regions of Syria[1]

[1]Redrafted after: **Fateh Toumi**, Ghassan Hassan, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie Nicol, Francis Ogbonnaya, Khaled Al-Assas, Taissir Abou Al-Fadil & Maurice Moens (2015). Distribution of the cereal cyst nematodes (*Heterodera* spp.) in wheat and barley fields in north-eastern regions of Syria. *Journal of Plant Diseases and Protection* 122, 255-263.

3.1 Introduction

Heterodera avenae, *H. filipjevi* and *H. latipons*, the three major species of the Avenae group are common in Turkey, a country adjacent to Syria. However, the dominant species varies with the region. *Heterodera avenae* and *H. latipons* were found in the south-eastern and eastern Mediterranean region, whereas *H. filipjevi* was prevalent in the Central Anatolian Plateau region (Öztürk *et al.,* 1998; Abidou *et al.,* 2005a; Yavuzaslanoglu *et al.,* 2012; Imren *et al.,* 2012; Dababat *et al.,* 2014). The three species were also found in cereal fields in Syria (Rivoal *et al.,* 2003; Abidou *et al.,* 2005a; Hassan *et al.,* 2010); they are widely distributed, with *H. latipons* being dominant (Scholz, 2001; Abidou *et al.,* 2005a).

Wheat and barley growing areas are distributed all over Syria. The annual wheat yield ranges between 3 and 4.5 million tonnes (Anonymous, 2010). Yield losses caused by *H. latipons* were estimated up to 20 and 30% in barley and durum wheat, respectively (Scholz, 2001). On wheat, losses caused by *H. avenae* can be as high as 57% (Hassan *et al.,* 2010). Referring to the economic importance of wheat, increasing concerns about the severity of the damage caused by CCN, and the little known information about the distribution of the three species of CCN in Syria, the objectives of this study were: (1) to determine the current prevalence, incidence and geographical distribution of CCN in the north-eastern region of Syria, and (2) to evaluate their infestation levels and to compare the obtained data with those from previous surveys carried out in the country (Scholz, 2001; Abidou *et al.,* 2005a; Hassan, 2008). The acquired knowledge should guide the development of the nematological research programme for cereals.

3.2 Material and Methods

3.2.1 Sampling

The survey was performed immediately after the harvest of cereals in 2009. Soil samples were taken in 167 fields (Table 3.1) spread over five of the major cereal growing provinces (Aleppo, Idlib, El-Raqqa, Deir Ez Zur and El-Hassake; Figure 3.1). Ninety-five samples were taken from rain-fed (40 samples) and irrigated wheat fields (55 samples);

the remaining 72 samples were obtained from barley fields (54 rain-fed and 18 irrigated). In each field, one composite sample (1-2 kg) was collected per hectare. All samples were taken at a soil depth of 5-20 cm after removing the top 5 cm layer. Each sample was composed of 15-25 subsamples; a distance of 30-50 m separated the subsamples. Subsamples were taken in a zigzag pattern across the field.

Figure 3.1. Map of Syria showing sampling areas for cereal cyst nematodes (*Heterodera* spp.).

3.2.2 Cyst extraction

Each soil sample was mixed thoroughly and slowly dried. A 1-mm sieve was used to eliminate all debris and pebbles. For each sample, cysts were extracted (Fenwick, 1940) from three 200 g subsamples. Extracted cysts were retained on a 250-µm sieve.

Shrunken and empty cysts were not considered. Eventually, the average was calculated for those three subsamples and expressed per 200 g of soil.

3.2.3 Nematode identification

For each sample, the vulval cone of 2-5 mature cysts was mounted in glycerine jelly. Identification of *Heterodera* species was based on the shape of the cyst, vulval slit, underbridge structure, and presence or absence of bullae (Handoo, 2002). Samples with fewer cysts were identified molecularly only.

Per sample, two cysts were transferred to separate 1.5 ml Eppendorf tubes containing 150 µl double distilled water (ddH_2O) (one cyst per tube). Cysts were crushed with a wooden tooth stick and 150 µl worm lysis buffer (final concentration 200 mM NaCl, 200 mM Tris-HCl (pH 8), 1% β-mercaptoethanol and 800 µg ml^{-1} Proteinase K) was added (Holterman *et al.,* 2006). Subsequently, all samples were incubated for 2 h at 60°C followed by 10 min at 99°C in a thermomixer with a rotation speed of 300 rpm. The extracted DNA was stored at -20°C for future use. From each DNA extract, amplification of the rDNA-ITS region was performed by adding 1 µl template DNA to the PCR reaction mixture containing 23 µl ddH_2O, 25 µl Dream *Taq* PCR Master Mix (2×) (Fermentas Life Sciences, Germany), 1 µM forward primer 5'-CGT AAC AAG GTA GCT GTA G-3' and 1 µM reverse primer 5'-TCC TCC GCT AAA TGA TAT G-3' (Ferris *et al.,* 1993). After electrophoresis of 5 µl PCR product in a 1.5% TAE buffered agarose gel (1 h, 100 V), the gel was stained in an ethidium bromide bath (1 mg l^{-1}) for 30 min and photographed under UV light. In the case of a positive result, the remainder of the PCR product was purified following the instructions of the Wizard SV Gel and PCR Clean-Up System kit (Promega Benelux, Leiden). Finally, the sequences of the purified PCR products were edited and analysed using software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall, 1999), and compared with sequences in GenBank (BlastN option, www.ncbi.nlm.nih.gov/) to reveal the identity of the *Heterodera* sample. Molecular identification was restricted to about 50% of the samples from each province, because of financial restrictions.

3.3 Results

The morphological observations resulted in the identification of three species (Table 3.1). Lemon-shaped cysts with a short vulval slit, clear and crowded bullae, and no underbridge in the vulval cone were identified as *H. avenae*. Cysts of *H. filipjevi* were also lemon-shaped, had big bullae, and a distinct underbridge, thick in the middle and thin at the ends. *Heterodera latipons* cysts were lemon-shaped, showed a short vulval slit, no bullae and a very strong underbridge with a pronounced enlargement in the middle (Figure 3.2).

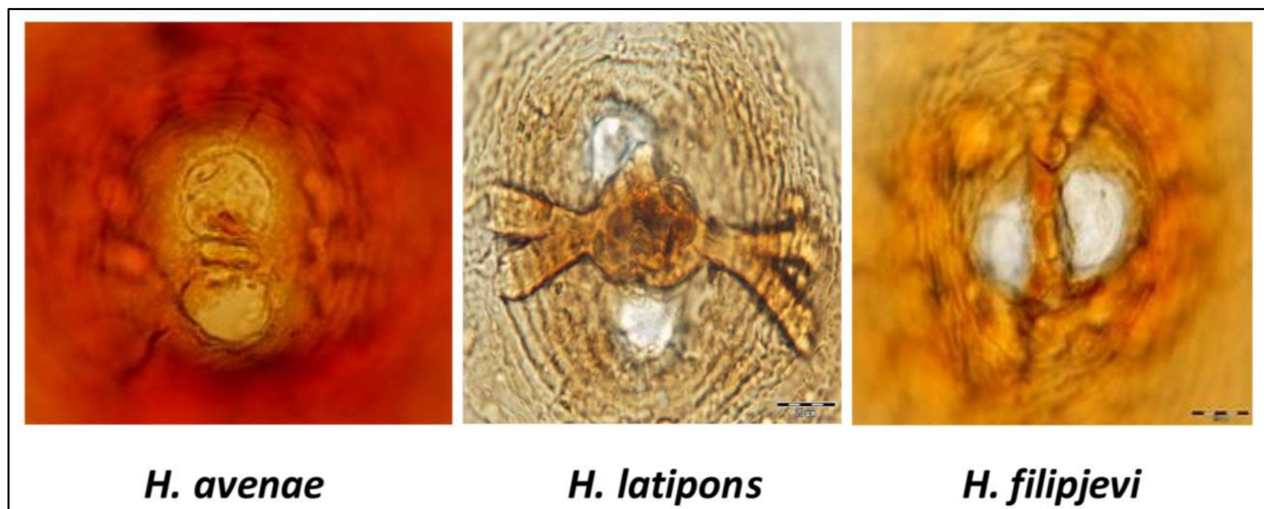

Figure 3.2. Vulval cone of the three species of cereal cyst nematodes detected during the survey.

Table 3.1. Results of the soil sampling for cereal cyst nematodes in fields of the five-major cereal growing provinces of the north-eastern region of Syria including the negative samples.

Code	Province	Village	Host	Irrigation	Number of cysts (200 g soil)$^{-1}$	Species
GH100	Aleppo	El-Shiokh Fokani	barley	rain-fed	1	*H. latipons* (Hl)
GH103	Aleppo	Mhajan	barley	rain-fed	8	Hl
GH108	Aleppo	Maran	barley	rain-fed	5	*H. avenae* (Ha)
GH110	Aleppo	Biaat El-Deniesh	barley	rain-fed	53	Ha
GH76	Aleppo	Thalthana	barley	rain-fed	11	Hl
GH77	Aleppo	Thalthana	barley	rain-fed	44	Hl
GH78	Aleppo	Aghtarien	barley	rain-fed	23	Hl
GH79	Aleppo	Aghtarien	barley	rain-fed	20	Hl
GH80	Aleppo	Hazwan	barley	rain-fed	1	Hl
GH81	Aleppo	Hazwan	barley	rain-fed	6	Hl
GH82	Aleppo	Hadadien	barley	rain-fed	24	Hl
GH84	Aleppo	Atien	barley	rain-fed	33	Hl
GH85	Aleppo	Atien	barley	rain-fed	55	Hl
GH86	Aleppo	El-Dana	barley	rain-fed	51	Hl
GH87	Aleppo	El-Dana	barley	rain-fed	9	Hl
GH88	Aleppo	El-Bab	barley	rain-fed	0	-*
GH90	Aleppo	El-Naziha	barley	rain-fed	40	Hl
GH91	Aleppo	El-Naziha	barley	rain-fed	19	Hl
GH92	Aleppo	Halab	barley	rain-fed	0	-
GH96	Aleppo	Brida	barley	rain-fed	1	Hl
GH97	Aleppo	Brida	barley	rain-fed	37	Hl
GH98	Aleppo	Moman	barley	rain-fed	1	Hl
GH101	Aleppo	El-Shiokh Fokani	wheat	rain-fed	1	Hl
GH102	Aleppo	Anadan	wheat	irrigated	4	Hl
GH104	Aleppo	El-Reshaf	wheat	rain-fed	7	Hl
GH105	Aleppo	Hwar el-Nahr	wheat	rain-fed	14	Hl
GH106	Aleppo	Der El-Jemal	wheat	irrigated	12	Hl
GH107	Aleppo	Marea	wheat	irrigated	24	Hl
GH109	Aleppo	Nobel	wheat	irrigated	21	Hl
GH83	Aleppo	Hadadien	wheat	irrigated	0	-
GH89	Aleppo	El-Bab	wheat	rain-fed	0	-

Code	Province	Village	Host	Irrigation	Number of cysts (200 g soil)$^{-1}$	Species
GH93	Aleppo	Halab	wheat	irrigated	0	-
GH94	Aleppo	Tel Refat	wheat	rain-fed	38	Hl
GH95	Aleppo	Tel Refat	wheat	rain-fed	5	Hl
GH99	Aleppo	Moman	wheat	irrigated	0	-
GH141	Deir Ez Zur	Hawie El-Maiadien	barley	irrigated	0	-
GH146	Deir Ez Zur	El-Tiba	barley	irrigated	3	Hl
GH147	Deir Ez Zur	El-Tiba	barley	irrigated	6	Hl
GH150	Deir Ez Zur	Bilaoom	barley	irrigated	10	Hl
GH151	Deir Ez Zur	Toob	barley	irrigated	0	-
GH154	Deir Ez Zur	Shakra	barley	rain-fed	5	Hl
GH156	Deir Ez Zur	Hsikia	barley	irrigated	7	Hl
GH160	Deir Ez Zur	Sealo El-Ankshi	barley	irrigated	0	-
GH161	Deir Ez Zur	Sealo El-Mawkiea	barley	irrigated	0	-
GH140	Deir Ez Zur	Hawie Mohsen	wheat	irrigated	4	Hl
GH142	Deir Ez Zur	El-Zebarie	wheat	irrigated	9	Ha
GH143	Deir Ez Zur	El-Zebarie	wheat	irrigated	6	Ha
GH144	Deir Ez Zur	El-Swieaiea	wheat	irrigated	0	-
GH145	Deir Ez Zur	El-Swieaiea	wheat	irrigated	5	Ha
GH148	Deir Ez Zur	Bolil	wheat	irrigated	0	-
GH149	Deir Ez Zur	Bolil	wheat	irrigated	0	-
GH152	Deir Ez Zur	Mohkan	wheat	irrigated	0	-
GH153	Deir Ez Zur	Morad	wheat	irrigated	8	Hl
GH155	Deir Ez Zur	Jeaiea	wheat	irrigated	0	-
GH157	Deir Ez Zur	Mohmadia	wheat	irrigated	0	-
GH158	Deir Ez Zur	Hatla	wheat	irrigated	0	-
GH159	Deir Ez Zur	Mriaia	wheat	irrigated	12	Ha
GH1	El-Hassake	Tel El-bazri	barley	rain-fed	1	Hl
GH10	El-Hassake	Rhaia	barley	irrigated	2	Hl
GH13	El-Hassake	Sibat	barley	rain-fed	24	Ha
GH14	El-Hassake	Sibat	barley	irrigated	14	Ha
GH17	El-Hassake	El-baghoz	barley	rain-fed	0	-
GH18	El-Hassake	El-baghoz	barley	rain-fed	0	-
GH2	El-Hassake	Tel El-bazri	barley	irrigated	2	Ha
GH20	El-Hassake	Khanamia	barley	rain-fed	10	Hl

Code	Province	Village	Host	Irrigation	Number of cysts (200 g soil)$^{-1}$	Species
GH27	El-Hassake	Boor saied	barley	rain-fed	0	-
GH3	El-Hassake	Khazna	barley	rain-fed	3	Hl
GH33	El-Hassake	Aloni	barley	rain-fed	0	-
GH34	El-Hassake	Aloni	barley	irrigated	0	-
GH35	El-Hassake	Twini	barley	irrigated	0	-
GH36	El-Hassake	Twini	barley	irrigated	2	Ha
GH37	El-Hassake	Tel Aswad	barley	rain-fed	41	Ha
GH38	El-Hassake	Tel Aswad	barley	rain-fed	48	Ha
GH39	El-Hassake	Tel Ailol	barley	rain-fed	35	Hl – Ha – *H. filipjevi* (Hf)
GH4	El-Hassake	Khazna	barley	rain-fed	17	Ha
GH40	El-Hassake	Tel Ailol	barley	rain-fed	27	Hl – Ha – Hf
GH41	El-Hassake	Tel Shaalan	barley	rain-fed	0	-
GH42	El-Hassake	Tel Shaalan	barley	rain-fed	0	-
GH5	El-Hassake	Kherbt El-khder	barley	rain-fed	1	Ha
GH57	El-Hassake	Tel Baidar	barley	rain-fed	0	-
GH6	El-Hassake	Kherbt El-khder	barley	rain-fed	0	-
GH65	El-Hassake	Am Kahfa	barley	rain-fed	60	Hl
GH66	El-Hassake	Am Roos	barley	irrigated	29	Ha
GH68	El-Hassake	Tel Tawiel	barley	irrigated	18	Ha
GH7	El-Hassake	Kherbt El-tamimi	barley	rain-fed	0	-
GH70	El-Hassake	El-Mazar	barley	rain-fed	36	Ha – Hl
GH71	El-Hassake	Zaher El-Arab	barley	irrigated	20	Hl
GH75	El-Hassake	El-bihera	barley	rain-fed	6	Hl
GH8	El-Hassake	Kherbt El-tamimi	barley	rain-fed	1	Hl
GH11	El-Hassake	Safia	wheat	irrigated	14	Hl
GH12	El-Hassake	Safia	wheat	rain-fed	0	-
GH15	El-Hassake	Khanka	wheat	irrigated	3	Ha – Hf
GH16	El-Hassake	Khanka	wheat	rain-fed	5	Hl
GH19	El-Hassake	Khanamia	wheat	rain-fed	27	Hl
GH21	El-Hassake	Salam Aliek	wheat	irrigated	14	Hl
GH22	El-Hassake	Salam Aliek	wheat	irrigated	3	Hl
GH23	El-Hassake	Tel-Skra	wheat	irrigated	0	-
GH24	El-Hassake	Tel-Skra	wheat	irrigated	0	-
GH25	El-Hassake	Jolbsan	wheat	rain-fed	5	Hl

Code	Province	Village	Host	Irrigation	Number of cysts (200 g soil)$^{-1}$	Species
GH26	El-Hassake	Jolbsan	wheat	irrigated	12	Ha – Hf
GH28	El-Hassake	Boor saied	wheat	rain-fed	0	-
GH29	El-Hassake	Kerkwi	wheat	rain-fed	16	Ha
GH30	El-Hassake	Kerkwi	wheat	rain-fed	18	Ha
GH31	El-Hassake	Jatal	wheat	irrigated	6	Hl – Ha
GH32	El-Hassake	Jatal	wheat	rain-fed	11	Hl – Ha
GH43	El-Hassake	Tel Khanzer	wheat	rain-fed	2	Hl
GH44	El-Hassake	Tel Khanzer	wheat	rain-fed	0	-
GH45	El-Hassake	Aamer	wheat	irrigated	11	Hl
GH46	El-Hassake	Aamer	wheat	rain-fed	0	-
GH47	El-Hassake	El-Hatimia	wheat	rain-fed	3	Hl
GH48	El-Hassake	El-Hatimia	wheat	rain-fed	0	-
GH49	El-Hassake	Bripha	wheat	rain-fed	12	Hl
GH50	El-Hassake	Bripha	wheat	rain-fed	3	Hl – Hf
GH51	El-Hassake	Ker Hsar	wheat	rain-fed	6	Hl
GH52	El-Hassake	Ker Hsar	wheat	irrigated	0	-
GH53	El-Hassake	Nes Tel	wheat	irrigated	1	Ha
GH54	El-Hassake	Nes Tel	wheat	rain-fed	0	-
GH55	El-Hassake	Damkhia El-Sakira	wheat	rain-fed	31	Hl
GH56	El-Hassake	Damkhia El-Sakira	wheat	irrigated	9	Ha
GH58	El-Hassake	Tel Baidar	wheat	rain-fed	20	Hl
GH59	El-Hassake	Jriba	wheat	irrigated	5	Ha – Hf
GH60	El-Hassake	Jriba	wheat	rain-fed	12	Ha – Hf
GH61	El-Hassake	Tel Khaled	wheat	irrigated	1	Hl
GH62	El-Hassake	Tel Khaled	wheat	rain-fed	17	Hl
GH63	El-Hassake	Rbieat	wheat	irrigated	3	Hl
GH64	El-Hassake	Tel Fares	wheat	rain-fed	8	Hl
GH67	El-Hassake	Himo	wheat	rain-fed	5	Hl
GH69	El-Hassake	Taalki	wheat	rain-fed	3	Ha
GH72	El-Hassake	Khass	wheat	irrigated	12	Hl – Ha – Hf
GH73	El-Hassake	Hawashia	wheat	rain-fed	22	Hl – Ha
GH74	El-Hassake	El-Karama	wheat	rain-fed	14	Hl – Hf
GH9	El-Hassake	Rhaia	wheat	irrigated	0	-
GH117	El-Raqqa	Moshahed	barley	rain-fed	1	Hl

Code	Province	Village	Host	Irrigation	Number of cysts (200 g soil)$^{-1}$	Species
GH118	El-Raqqa	Moshahed	barley	rain-fed	0	-
GH121	El-Raqqa	Bo Asii	barley	rain-fed	116	HI
GH125	El-Raqqa	Dkhailieb	barley	rain-fed	9	HI
GH129	El-Raqqa	Tina	barley	rain-fed	0	-
GH130	El-Raqqa	Tina	barley	rain-fed	0	-
GH137	El-Raqqa	Hafiat El-Wahab	barley	rain-fed	0	-
GH111	El-Raqqa	Kesrat El-shikh Jomaa	wheat	irrigated	0	-
GH112	El-Raqqa	Kesrat El-shikh Jomaa	wheat	irrigated	0	-
GH113	El-Raqqa	Tel El-Saman	wheat	irrigated	0	-
GH114	El-Raqqa	Tel El-Saman	wheat	irrigated	0	-
GH115	El-Raqqa	Tel El-Saman	wheat	irrigated	0	-
GH116	El-Raqqa	Tel El-Saman	wheat	irrigated	0	-
GH119	El-Raqqa	El-Wahda	wheat	irrigated	0	-
GH120	El-Raqqa	El-Wahda	wheat	irrigated	0	-
GH122	El-Raqqa	Bo Asii	wheat	rain-fed	19	HI
GH123	El-Raqqa	Hzima	wheat	irrigated	0	-
GH124	El-Raqqa	Hzima	wheat	irrigated	0	-
GH126	El-Raqqa	Dkhailieb	wheat	rain-fed	13	HI
GH127	El-Raqqa	Aien Isa	wheat	rain-fed	0	-
GH128	El-Raqqa	Aien Isa	wheat	rain-fed	5	HI
GH131	El-Raqqa	El-Raqqa	wheat	irrigated	0	-
GH132	El-Raqqa	El-Raqqa	wheat	irrigated	0	-
GH133	El-Raqqa	Baas	wheat	irrigated	0	-
GH134	El-Raqqa	Baas	wheat	irrigated	0	-
GH135	El-Raqqa	El-Yarmook	wheat	irrigated	0	-
GH136	El-Raqqa	El-Yarmook	wheat	irrigated	0	-
GH138	El-Raqqa	Mazrat Kirtaj	wheat	rain-fed	0	-
GH139	El-Raqqa	El-Asadia	wheat	irrigated	0	-
GH162	Idlib	Freka	barley	irrigated	9	HI
GH163	Idlib	Jeser Shokor	barley	rain-fed	0	-
GH164	Idlib	Jeser Shokor	wheat	irrigated	0	-
GH165	Idlib	Meles	wheat	rain-fed	11	HI
GH166	Idlib	Tel Khazal	wheat	rain-fed	4	HI

Code	Province	Village	Host	Irrigation	Number of cysts (200 g soil)$^{-1}$	Species
GH167	Idlib	Mart Mesrien	wheat	rain-fed	1	HI

***(-):** Negative samples.

Amplification of the rDNA-ITS region was successful for all selected samples; the PCR produced a single band with the expected size of 1100 bp for the three species (Figure 3.3) (Ferris *et al.*, 1993). No PCR products were obtained for the negative control without nematode DNA template. Sequencing of both DNA strands of the PCR products confirmed the morphological identification of corresponding samples.

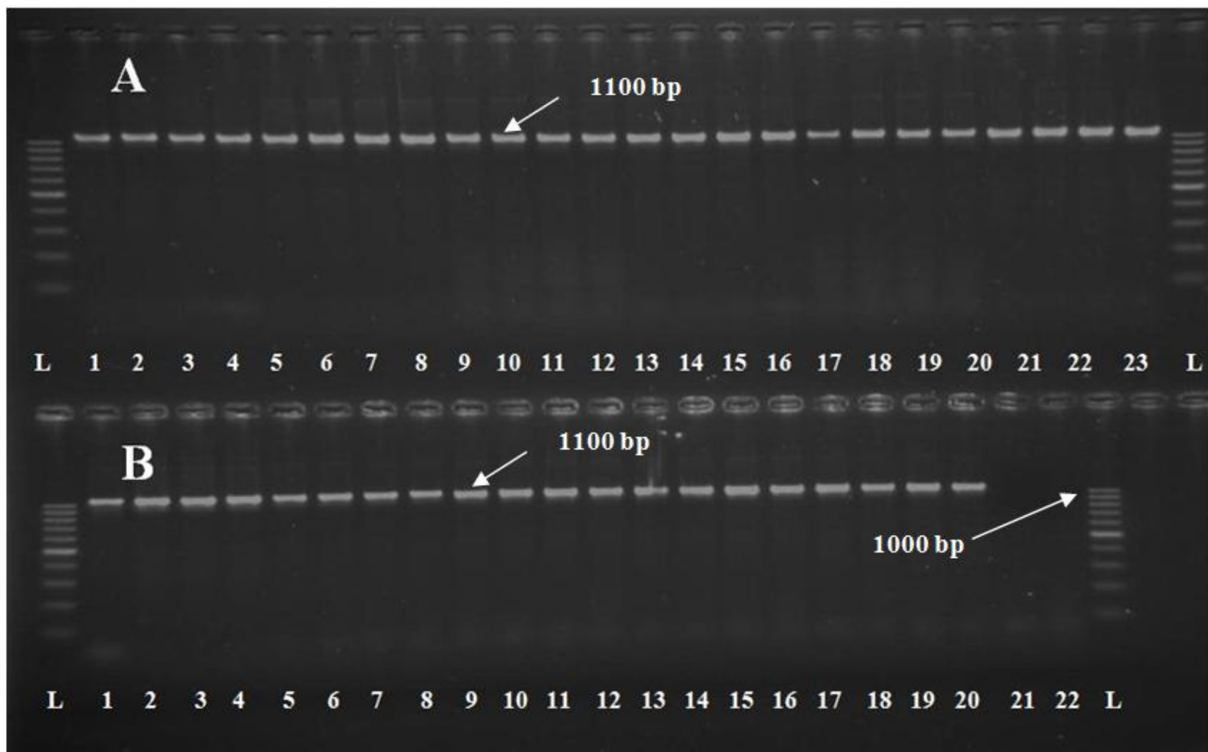

Figure 3.3. Selected results of the universal PCR of ITS-regions (rDNA) of *Heterodera* isolates taken in five Syrian provinces (Aleppo, Idlib, El-Raqqa, Deir Ez Zur and El-Hassake) (Table 3.1). A: 1-23 and B: 1-20: ITS of different *Heterodera* isolates (1100 bp) (Ferris *et al.,* 1993), 21-22: negative control, L: 100 bp DNA ladder (Fermentas Life Sciences).

According to the results of both morphological and molecular identifications, the three-major species of the Avenae group, *viz. H. latipons*, *H. avenae* and *H. filipjevi*, were present in 62% of the 104 fields surveyed in the region (Figure 3.4). *Heterodera latipons* was the most prevailing species in wheat and barley fields throughout the surveyed sites (79 fields*, i.e.* 76% of the infested fields). The highest population density was found in El-Raqqa (116 cysts (200 g)$^{-1}$ soil). This species was found singly in 70 fields (67% of the infested fields), and occurred mixed with *H. avenae* and/or *H. filipjevi* or both in nine fields

(9% of the infested fields) (Figure 3.5). All fields in El-Raqqa and Idlib were infested only with *H. latipons* (Figure 3.5). *Heterodera avenae* was found in 32 fields (31% of the infested fields). The highest incidence was in Aleppo (53 cysts (200 g)$^{-1}$). *Heterodera avenae* occurred singly in 21 fields (20%), and mixed with *H. latipons* or *H. filipjevi* in 11 fields (11%) (Figure 3.4). *Heterodera filipjevi* occurred only in El-Hassake in 9% of infested fields (9 fields) always in a mix with *H. latipons* and/or *H. avenae* (Figure 3.4).

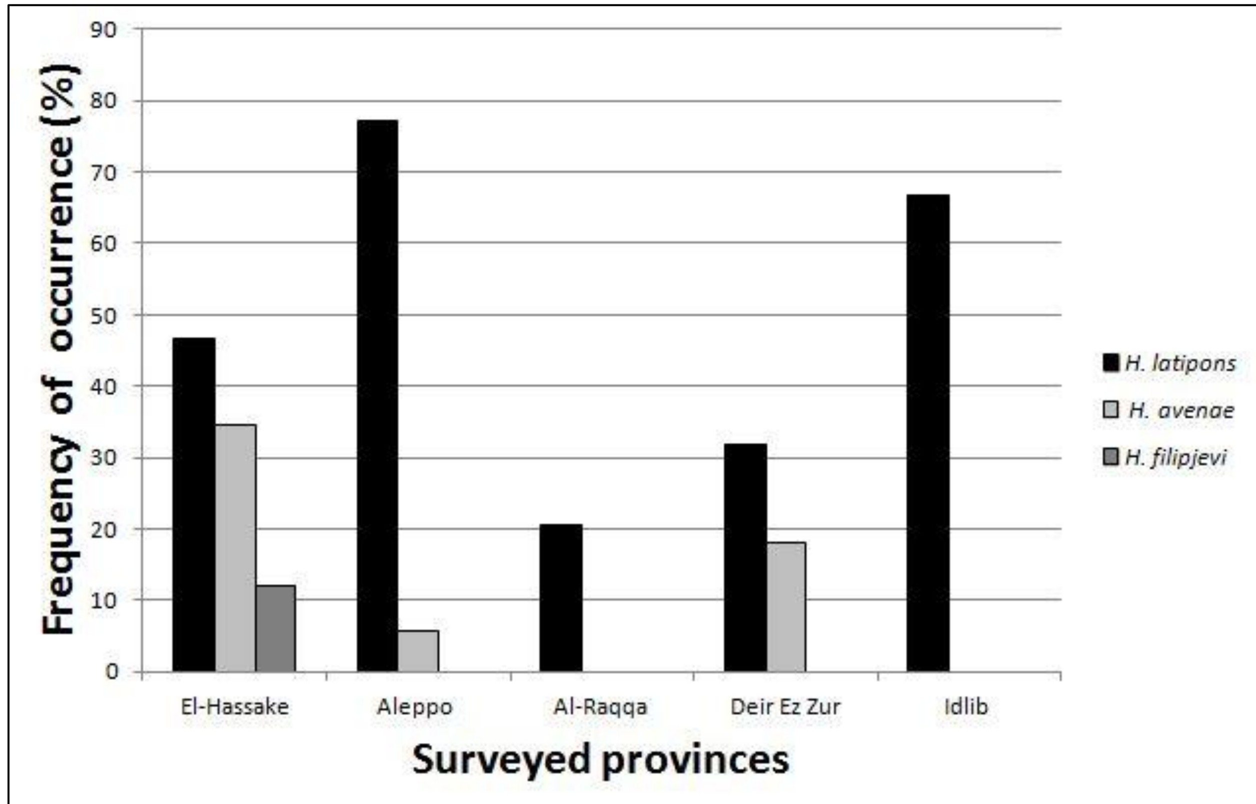

Figure 3.4. Occurrence of three species of cereal cyst nematodes in the five Syrian provinces surveyed.

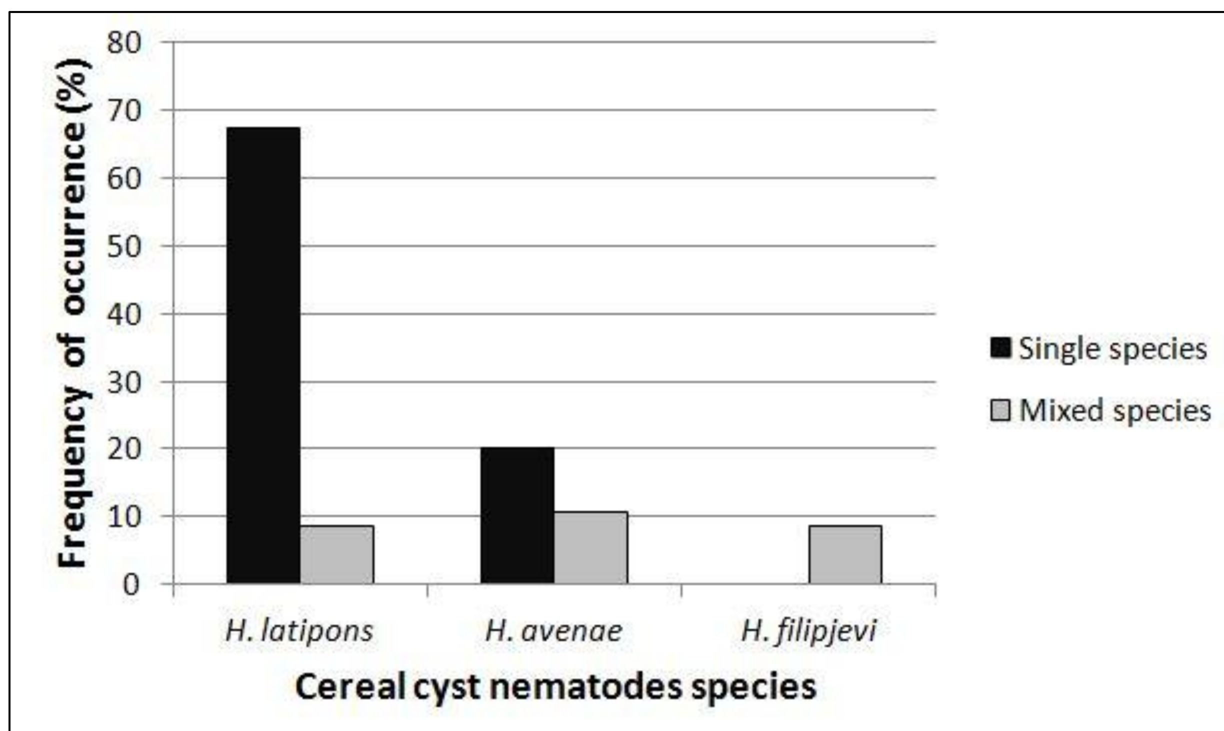

Figure 3.5. Occurrence of three species of cereal cyst nematodes, single or mixed with each other.

High infestation levels of nematodes were found in barley fields (69% of the fields). However, only in three provinces out of the five surveyed, the infestation levels in barley fields were higher than in wheat fields: Aleppo (91%), Deir Ez Zur (56%) and El-Raqqa (43%) (Figure 3.6). The highest numbers of cysts in each province per 200 g of soil reached up to 116, 60, 55, 10 and 9 in El-Raqqa, El-Hassake, Aleppo, Deir Ez Zur and Idlib provinces, respectively. Infestations in wheat fields generally were less abundant and occurred in 57% of the total wheat fields. However, in the provinces El-Hassake (77%) and Idlib (75%), the infestation levels in wheat fields were higher than in barley fields (Figure 3.6). The highest numbers of cysts per 200 g of soil in wheat fields were 38 in Aleppo and 31 in El-Hassake.

Twenty-five percent of the samples from the remaining sites in these five provinces had fewer than 7 cysts $(200\ g)^{-1}$ of soil, while 15% had fewer than 3 cysts per 200 g of soil. In about 38% of all the investigated fields, no cysts were detected.

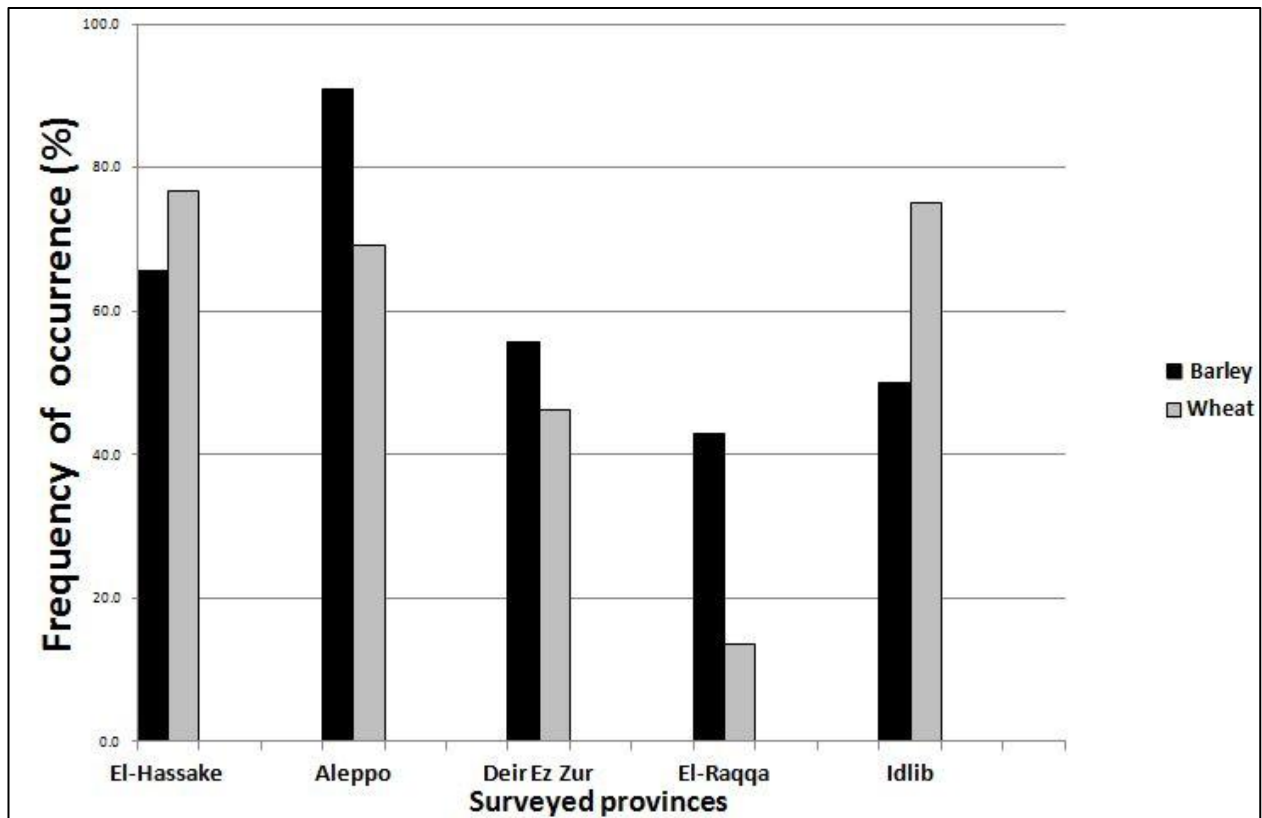

Figure 3.6. Occurrence of cereal cyst nematodes according to the host (barley - wheat) in five Syrian provinces.

3.4 Discussion

This survey demonstrates that the major cereal growing areas in Syria are commonly infested by three species belonging to the Avenae group. As was reported earlier (Scholz, 2001; Abidou *et al.,* 2005a; Hassan, 2008) *H. latipons* is the most widely distributed species and occurs in all regions. Heavy infestations of cyst nematodes, especially *H. latipons*, were found most commonly in barley fields in the dry areas of Syria (< 300 mm annual rainfall). In the last decade, because of the increase in the incidence of drought conditions, farmers prefer to grow barley instead of wheat because unlike wheat, barley can be grown with a limited rainfall. The monoculture of barley further aims at a higher yield to feed a larger number of animal stock (Scholz, 2001; Nicol *et al.,* 2004).

The survey also shows that *H. avenae* and *H. filipjevi* are more widespread than previously reported (Scholz, 2001; Abidou *et al.*, 2005a; Hassan, 2008). Abidou *et al.*

(2005a) described *H. filipjevi* from only one location, whereas in this survey, the species was detected at nine locations close to the Turkish border. This result suggests a further extension of the spread of *H. filipjevi* in Syria and Turkey (Imren *et al.*, 2012). *Heterodera filipjevi* is common in temperate climates in many European countries (Chapter 2); the East European region is considered to be the centre of distribution of the species (Rumpenhorst *et al.*, 1996). The areas I found infested with *H. filipjevi* are somehow similar in climate to the center of distribution of *H. filipjevi*, as they were situated at higher altitude with lower temperature and more rainfall than areas where *H. avenae* and *H. latipons* were detected abundantly. These findings confirm the differences in the ecoregional distribution of the three-major species of CCN and their link with the geographic and climate difference, in addition to the suggested early divergence between tropical and temperate heteroderid species (Subbotin *et al.*, 2001a). It would be worth to develop some phylogeography approaches to establishing the principles and processes determining the geographical distribution of these differentiation lineages. Fields in the other four provinces (Aleppo, Idlib, El-Raqqa and Deir Ez Zur) were not found infested with *H. filipjevi*. Additionally, all fields in El-Raqqa and Idlib were free of *H. avenae*. However, due to the relatively low number of collected samples compared with wide area surveyed, the strict outcome of this investigation regarding the negative samples (*e.g.* one or two species do not exist in a province) may not reflect the accurate occurrence of CCN. Therefore, an intensive survey of CCN distribution is still required in those provinces.

The morphological identification of the collected cysts was not straightforward because of the presence of species-mixtures. Moreover, in some samples, the number of cysts was low and not enough to allow morphological identification. Hence, identification via sequencing was used. However, sequencing is relatively expensive, time-consuming, and because of a large number of samples, approximately half the number of samples had to be sequenced. Subsequent species-specific PCR assays enabled fast and accurate identification (Chapter 4).

The wide distribution of CCN species in the surveyed regions and the high numbers of cysts in some samples is probably related to (1) the monoculture of cereals

practised in these regions, where damage caused by the nematodes is likely to be greater as well (Ibrahim *et al.*, 1999, Smiley & Nicol, 2009; Ali *et al.*, 2015), (2) the fact that farmers are unaware of the existence of resistant varieties, (3) the strong wind in the north-eastern regions in Syria responsible for the aerial spread of soil and cysts to other locations, (4) an irrigation system which is still not well developed but allows water to carry cysts to lower situated fields, and (5) non-use of agricultural practices such as clean fallow, weed control and cleaning machinery. Research by Kerry (1982b) showed that after many years of monoculture of cereals, *H. avenae* populations declined due to the increase in the density of the fungal parasites that totally destroyed females on roots or resulted in the formation of small cysts, which are often empty. It is worth to observe whether this phenomenon also happens in Syria and what the outcome might be.

To maintain the population densities of cereal cyst nematodes below damaging levels, appropriate management measures such as crop rotation and the use of resistant varieties, are necessary. A number of resistance sources for breeding purposes have been detected in domestic cereals and their wild relatives, acting against both *H. avenae* and *Pratylenchus* spp. (Nicol *et al.,* 2004). Preliminary studies indicate that several resistance genes of barley or wheat are, to some extent, also active against populations of *H. filipjevi* or *H. latipons* originating from different sites in North Africa, Europe and Asia (Bekal *et al.,* 1998; Rivoal *et al.,* 2001; Mokabli *et al.,* 2002). Further efforts should be made to characterise existing varieties and identify resistance amongst existing cultivars as well as new sources to be used in cereal breeding programmes (see Chapter 5).

To develop a long-term strategy for CCN management, cultivars from other countries or from the local breeding programme should be screened for resistance to the prevailing CCN species. Also, comprehensive and regional surveys in Turkey, Syria, Lebanon and Jordan are needed for establishing the mapping population densities in order to define potential economic damage rates and determining more accurately the distribution of CCN. Also, to increase the understanding of the geographical distribution of CCN, and clarify the relation with different hosts and different cultivars of barley or wheat.

Chapter 4

Molecular identification and quantitative detection of the three-major species of cereal cyst nematodes *Heterodera avenae, H. latipons* and *H. filipjevi*[2]

[2] Chapter published in following references:

Fateh Toumi, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie M. Nicol, Francis Ogbonnaya & Maurice Moens (2013). Development of a species-specific PCR to detect the cereal cyst nematode *Heterodera latipons*. *Nematology* 15, 709-717.

Fateh Toumi, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie M. Nicol, Francis Ogbonnaya & Maurice Moens (2013). Development of two species-specific primer sets to detect the cereal cyst nematodes *Heterodera avenae* and *Heterodera filipjevi*. *European Journal of Plant Pathology* 136, 613–624.

Fateh Toumi, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie M. Nicol, Francis Ogbonnaya & Maurice Moens (2015). Development of qPCR assays for quantitative detection of *Heterodera avenae* and *H. latipons*. *European Journal of Plant Pathology* 10, 658-681.

4.1 Introduction

Accurate and rapid identification of nematodes is considered of major importance in nematode control strategies. The traditional identification of *Heterodera* species using morphological and morphometrical characteristic is time-consuming and requires special skills and training by the observer (Yan & Smiley, 2009). However, the development of molecular tools (PCR-RFLP, sequencing, PCR and qPCR) has contributed to the identification and discrimination between some cyst nematodes species (see Chapter 2).

Several genes of rDNA have been investigated to develop such tools (see Chapter 2). However, none of them was suitable for the development of molecular tools for the identification and/or quantification of the cereal cyst nematodes (CCN). Several alternative protein-coding genes might be used to develop molecular identification methods instead of the ITS-rDNA: genes coding for actin 1, aldolase, β-tubulin, pectate lyase, annexin, chorismate mutase, mitochondrial cytochrome oxidase subunit 1 (COI) and the heat shock protein 90 (hsp90). The actin 1 gene has been investigated and characterised in *H. glycines, H. schachtii, Globodera rostochiensis* and the free-living nematode *Panagrellus redivivus*, and once was used as an internal control of the PCR reaction (Matthews *et al.,* 2004; Tytgat *et al.,* 2004; Kovaleva *et al.,* 2005; Mundo-Ocampo *et al.,* 2008), but has never been used for diagnostic purposes. The COI gene was successfully used to discriminate between many species of free-living marine nematodes (Derycke *et al.,* 2010). This gene has also been used to generate DNA-barcodes for a variety of biological species, from bacteria to mammals (Hebert *et al.,* 2003). In addition, it was reported that the COI gene was useful in discriminating between plant-parasitic quarantine nematode species (Kiewnick *et al.,* 2011).

Using this background, I decided to investigate the above-mentioned genes to determine their usefulness for the development of species-specific PCR and qPCR for the detection and quantification of *H. avenae, H. latipons* and *H. filipjevi*.

4.2 Material and Methods

4.2.1 Nematodes

A collection of 73 populations belonging to 15 *Heterodera* species and 1 population of *Punctodera punctata* were obtained from 11 hosts and 18 countries particularly from Syria, Iran and Turkey (Table 4.1). For several species of the Avenae group, *viz. H. avenae, H. filipjevi, H. latipons* and *H. hordecalis,* more than one isolate was collected to investigate and verify the specificity of the developed PCR and qPCR assay. Most of the suppliers provided information regarding the identity of the species, their host and origin. The species identity was confirmed or determined molecularly by amplification and sequencing the ITS-rDNA (see below) and by comparing the obtained sequences with those available in GenBank (BlastN option, www.ncbi.nlm.nih.gov/).

Table 4.1 Cyst nematode species and populations used in this study with their origin, provider and Genbank accession number (AN) of their ITS, actin 1 and COI sequences

Code	Species	Host	Country	Source	AN ITS sequence	AN Actin 1 sequence	AN COI sequence
Did29	*Heterodera sturhani* (formerly *H. avenae*)	wheat	China	D. Peng	JX024193	-	-
Did33	*H. avenae*	wheat	France	R. Rivoal	JX024192	-	-
Did11	*H. avenae*	wheat	Saudi Arabia (Hail)	A. Dawabah	JX024191	-	-
Fa19	*H. avenae*	wheat	Syria – Deir Al-Zor	G. Hassan	JX024198	-	KC172908
Fa5	*H. avenae*	wheat	Syria – Al-Hasakah	G. Hassan	JX024199	-	-
Fa1	*H. avenae*	wheat	Syria – Al-Hasakah	F. Toumi	JX024197	JX024223	KC172909
Tuni6	*H. avenae*	wheat	Tunisia	N. Kachouri	JX024190	-	-
Mus21	*H. avenae*	wheat	Turkey – Hatay	M. Imren	JX024196	-	-
Did12	*H. avenae*	wheat	Turkey – Kilis	D. Saglam	JX024194	-	-
Did49	*H. avenae*	wheat	USA	R. Smiley	JX024195	-	-
DCP1248	*H. betae*	pea	Belgium	ILVO	JX024200	JX024224	-
Elsd2	*H. betae*	sugar beet	Germany	B. Niere	JX024221	-	-

Code	Species	Host	Country	Source	AN ITS sequence	AN Actin 1 sequence	AN COI sequence
DCP1734	*H. carotae*	carrot	France	ILVO	-	-	-
FaC3	*H. ciceri*	chickpea	Syria - Aleppo	S. Hajjar	JX024201	JX024225	KC172919
HD11	*H. daverti*	alfalfa	the Netherlands	G. Karssen	JX024202	-	KC172915
Did15	*H. filipjevi*	wheat	Iran - Aligoudarz	Z.T. Maafi	JX024208	-	KC172910
E88	*H. filipjevi*	wheat	Russia	R. Rivoal	JX024209	-	-
Fa125	*H. filipjevi*	wheat	Syria – Al-Hasakah	F. Toumi	-	-	KC172911
Fa126	*H. filipjevi*	wheat	Syria – Al-Hasakah	F. Toumi	-	-	-
Did23	*H. filipjevi*	wheat	Turkey - Ankara	D. Saglam	JX024207	-	-
Did23b	*H. filipjevi*	wheat	Turkey - Ankara	D. Saglam	JX024205	JX024226	-
Did23d	*H. filipjevi*	wheat	Turkey - Ankara	D. Saglam	JX024206	-	-
Did42b	*H. filipjevi*	wheat	Turkey – Eskisehir	D. Saglam	JX024204	-	-
Did42c	*H. filipjevi*	wheat	Turkey – Eskisehir	D. Saglam	JX024203	-	-
HFUSA	*H. filipjevi*	wheat	USA -Oregon	R. Smiley	-	-	-
HGHar	*H. glycines*	soybean	Canada	R. Riggs	JX024212	-	-
Did38	*H. glycines*	soybean	Iran - Mazan	Z. T. Maafi	JX024210	-	KC172914
HG10	*H. glycines*	soybean	USA	G. Karssen	JX024211	-	-
HGRiggs	*H. glycines*	soybean	USA	R. Riggs	JX024213	JX024227	-

Code	Species	Host	Country	Source	AN ITS sequence	AN Actin 1 sequence	AN COI sequence
MP1	*H. goettingiana*	pea	Germany	J. Hallmann	JX024214	-	-
E69	*H. hordecalis*	wheat	Israel	R. Rivoal	JX024215	JX024228	-
TuniB	*H. hordecalis*	wheat	Tunisia	N. Kachouri	JX024216	-	KC172912
MP5	*H. humuli*	hop	Germany	J. Hallmann	JX024217	-	-
HLCyp	*H. latipons*	wheat	Cyprus	M. Christoforou	JX024187	-	-
HL50	*H. latipons*	barley	Iran	Z. T. Maafi	JX024186	-	-
HLIran	*H. latipons*	wheat	Iran	Z. T. Maafi	JX024189	-	-
HL5	*H. latipons*	barley	Jordan	L. Al-banna	JX024188	-	-
HLMorc	*H. latipons*	wheat	Morocco	F. Mokrini	JQ319037	-	-
Fa3	*H. latipons*	wheat	Syria – Al-Hasakah	F. Toumi	JX024175	JX024222	-
Fa7A3	*H. latipons*	wheat	Syria – Al-Hasakah	G. Hassan	JX024178	-	-
Fa7B1	*H. latipons*	barley	Syria – Al-Hasakah	K. Assas	JX024181	-	-
Fa7A4	*H. latipons*	wheat	Syria – El-Raqqa	G. Hassan	JX024179	-	-
Fa7A1	*H. latipons*	wheat	Syria – Deir Al-Zor	F. Toumi	JX024176	-	KC172913
Fa7A2	*H. latipons*	wheat	Syria – Deir Al-Zor	G. Hassan	JX024177	-	-
Fa7A5	*H. latipons*	wheat	Syria – Aleppo	F. Toumi	JX024180	-	-

Code	Species	Host	Country	Source	AN ITS sequence	AN Actin 1 sequence	AN COI sequence
Fa7B2	*H. latipons*	barley	Syria –Al-Hasakah	K. Assas	JX024182	-	-
Mus2	*H. latipons*	wheat	Turkey – Gaziantep	M. Imren	JX024184	-	-
Mus1	*H. latipons*	wheat	Turkey – Gaziantep	M. Imren	JX024180	-	-
Mus17	*H. latipons*	wheat	Turkey – Kilis	M. Imren	JX024185	-	-
DCP1041A	*H. pratensis*	grass	Belgium	ILVO	-	-	KC172916
HSPol	*H. schachtii*	sugar beet	Poland	S. Kornobis	JX024219	JX024229	KC172918
HSC9872	*H. schachtii*	sugar beet	the Netherlands	G. Karssen	JX024220	-	-
HSNDL	*H. schachtii*	sugar beet	the Netherlands	HZPC	JX024218	-	-
HT9	*H. trifolii*	clover	the Netherlands	G. Karssen	FJ040402	-	-
DCP1041B	*Punctodera punctata*	grass	Belgium	ILVO	-	-	KC172917

4.2.2 DNA extraction and identification of nematode isolate

Five second-stage juveniles (J2) from one cyst per population (Table 4.1) were transferred to a 0.5-ml tube containing 25 µl of double distilled water (ddH_2O). Twenty-five µl lysis buffer (Holterman *et al.,* 2006) was added (final concentration 200 mM NaCl, 200 mM Tris-HCl (pH 8), 1% β-mercaptoethanol and 800 µg/ml Proteinase K). Samples were incubated for 2 h at 65°C followed by 10 min at 99°C in a thermomixer with a rotation speed of 300 rpm. From each DNA extract, amplification of the rDNA-ITS region was performed by adding 1 µl DNA to a PCR reaction mixture containing 23 µl ddH_2O, 25 µl of Dream *Taq* PCR Master Mix (2×) (Fermentas Life Sciences, Germany), 1µM of forward primer 5'-CGT AAC AAG GTA GCT GTA G-3' and 1µM of the reverse primer 5'-TCC TCC GCT AAA TGA TAT G-3' (Ferris *et al.,* 1993). The PCR-programme was as follows: initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 49°C for 45 sec and 72°C for 60 sec, and an additional amplification step at 72°C for 8 min. After electrophoresis of 5 µl PCR product in a 1.5% TAE buffered agarose gel (1 h, 100 V), the gel was stained in an ethidium bromide bath (1 mg l^{-1}) for 30 min and photographed under UV light. In the case of a positive result, the remainder of the PCR product was purified after electrophoresis in a 1% TAE buffered agarose gel (1 h, 100 V) following the instructions included in the Wizard SV Gel and PCR Clean-Up System kit (Promega Benelux, Leiden, The Netherlands). Subsequently, the concentrations of the purified PCR products were measured using a UV spectrophotometer (Nanodrop ND-1000, Isogen Life Sciences). The purified PCR products were sequenced in both directions to obtain overlapping sequences of the forward and reverse DNA strand. Finally, the sequences were visualised, edited and analysed using software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall, 1999), and compared with sequences in GenBank (BlastN option, www.ncbi.nlm.nih.gov/) to reveal the identity of all isolates in the collection.

4.2.3 Development of species-specific primer sets

4.2.3.1 Selection of species-specific primers

Eight DNA regions (Table 4.2) were screened and amplified using DNA of 9 *Heterodera* spp. (Table 4.1) along with 1 population of *P. punctata*. Amplification of

those regions was done with suitable primers in the PCR reaction mixture described above, and programme with an adaptation of the annealing temperature. The obtained PCR products were purified and sequenced. To identify putative species-specific DNA fragments that could be used as primers for the identification of *H. latipons*, *H. avenae* and *H. filipjevi*, sequences were aligned and compared visually using Clustal X 1.64 (Thompson *et al.,* 1997). For the selection of the primer fragments, software AlleleID 7.73 was used. The potential species-specific primers were also screened for their presence in sequences stored in GenBank (BlastN option, www.ncbi.nlm.nih.gov/). Finally, one primer set belonging to the actin 1 gene to detect *H. latipons*, and two primer sets belonging to COI to detect *H. avenae* or *H. filipjevi* were selected.

Table 4.2. DNA regions and primers used in this study, including primer sequences, annealing temperature (Ta) and expected length of the target fragments.

DNA-region	Primer code	Primers sequence	Fragment length (bp)	Ta (°C)	Sources
Actin 1	Hs-ACTF Hs-ACTR	5-ACT TCA TGA TCG AGT TGT AGG TGG ACT CG-3 5-GAC CTC ACT GAC TAC CGA TGA AGA TTC-3	376	55	Tytgat *et al.*, 2004
Pectate Lyase	PectLyaseF PectLyaseR	5-CCA TCA CAG TAC AAG C-3 5-GGT TGG TCT GAA TTT CGG AT-3	681	45-48-50	Boer *et al.*, 2002
Annexins	AnnexF AnnexR	5-ATG CTC CAA AAC GGC CTT ACC ATT-3 5-TCA CTG CTC CGT GTT GCC CTT-3	1023	50-52-55	Patel *et al.*, 2010
Chorismate mutase	ChorMutF ChorMutR	5-GCC ATG GGA CAA TGC GAG AAA CAT TGC AC-3 5-GGC CAA CAA TTT CTT TGC-3	796-1167	50	Vanholme *et al.*, 2009
Aldolase	AldoF AldoR	5-ATG GCA GAG GTC GGA AAC-3 5-GCT TTG TAG GTG TAG GC-3	1250-1350	45-48-50	Kovaleva *et al.*, 2005
β-Tubulin	TubulinF TubulinR	5-CTT TAC GAC ATT TGT TTC CGC AC-3 5-GCG GGT CAC AKG CGG CCA TCA TG-3	251-382	50	Sabo & Ferris, 2004
Hsp90	Hsp90F Hsp90R	5-GAY ACV GGV ATY GGN ATG ACY AA-3 5-TCR CAR TTV TCC ATG ATR AAV AC-3	900-1500	50-55-60-65	Skantar & Carta, 2004
COI	JB3F JB5R	5-TTT TTT GGG CAT CCT GAG GTT TAT-3 5-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3	470	41	Derycke *et al.*, 2005

4.2.3.2 **Optimisation of the annealing temperature (Ta) and controlling the specificity of the species-specific PCRs**

A gradient PCR was performed to determine the optimum Ta for each primer set. DNA of one population of *H. latipons* (Fa3), *H. avenae* (Fa1) and *H. filipjevi* (Did15) was used as template DNA. One µl DNA was added to the PCR master mix described above (4.2.2) for nematode identification with the exception of the primers. Instead, the newly designed primers *H. latipons*-specific actin 1 primer forward (HLAT-ACTF) (5′-ATG CCA TCA TTA TTC CTT-3′) and *H. latipons*-specific actin 1 primer reverse (HLAT-ACTR) (5′-ACA GAG AGT CAA ATT GTG-3′); or *H. avenae*-specific COI forward primer (AVEN-COIF) (5′-GGG TTT TCG GTT ATT TGG-3′) and *H. avenae*-specific COI reverse primer (AVEN-COIR) (5′-CGC CTA TCT AAA TCT ATA CCA-3′); or *H. filipjevi*-specific COI forward primer (FILI-COIF) (5′-GTA GGA ATA GAT TTA GAT AGT C-3′) and *H. filipjevi*-specific COI reverse primer (FILI-COIR) (5′-TGA GCA ACA ACA TAA TAA G-3′) were used. The gradient Ta for *H. latipons* ranged from 43-60°C, while for the other two species it varied between 53-65°C.

To check the specificity of the selected species-specific primers for *H. latipons*, the PCR was performed with an initial denaturation step at 95°C for 5 min, 50 cycles at 94°C for 30 sec, 50°C for 45 sec and 72°C for 45 sec, followed by 72°C for 8 min, and applied with DNA extracted from all populations (Table 4.1). The same test was performed to check the specificity of the *H. avenae* primer set (AVEN-COI) and the *H. filipjevi* (FILI-COI) primer set as well, with DNA from all *Heterodera* spp. and *P. punctata* (Table 4.1) using an initial denaturation step at 95°C for 5 min, 30 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec, followed by 72°C for 8 min. An additional test was performed to ensure that the primer sets HLAT-ACT, AVEN-COI and FILI-COI were valid for all *H. latipons*, *H. avenae* and *H. filipjevi* populations in the collection (Table 4.1), respectively.

4.2.3.3 Sensitivity test of species-specific PCRs

To estimate the sensitivity of the species-specific PCR to detect *H. latipons*, a crude DNA sample extracted from 5 J2 of *H. latipons* (Fa3), two dilutions (1/2 and 1/5) of the same DNA, and DNA extracted from 100 J2 of *H. avenae* (Fa19) using 300 µl lysis

buffer in a 0.5 ml tube (Holterman *et al.,* 2006), were prepared. Then, PCR was done using HLAT-ACT primers and 1 µl crude DNA extracted of 5 J2 of *H. latipons* (Fa3) or the two dilutions (1/2 and 1/5) mixed with 1 µl DNA extracted from 5 or 100 J2 of *H. avenae* (Fa19). Furthermore, 1 µl DNA of four dilutions (1/2, 1/5, 1/10 and 1/50) of DNA extracted from 5 J2 of *H. latipons* (Fa3) were included in the sensitivity test. The sensitivity of the selected species-specific primers to detect *H. avenae* and *H. filipjevi* was checked in a separate test. For this, DNA was extracted from 100 J2 obtained from one cyst of *H. latipons* (Fa7A1) using 300 µl lysis buffer (as above). The sensitivity test of the species-specific PCR to detect *H. avenae* was performed using AVEN-COI primers and 1 µl DNA extracted from 5 J2 of *H. avenae* (Fa1) mixed with 1 µl DNA extracted from 5 or 100 J2 *H. latipons*. Similarly, the sensitivity test of the FILI-COI primers set involved a species-specific PCR using 1 µl DNA extracted from 5 J2 of *H. filipjevi* (Fa125) mixed with 1 µl DNA extracted from 5 or 100 J2 *H. latipons*. Also, 1 µl of four dilutions (1/5, 1/10, 1/50 and 1/100) of DNA extracted from 5 J2 of both species was used in a species-specific PCR. Equally, 1 µl of two dilutions (1/5 and 1/10) of DNA extracted from 5 J2 of both species was mixed with 1 µl DNA extracted from 100 J2 of *H. latipons* and used in a species-specific PCR.

4.2.4 Development of primers and probe for the qPCR

4.2.4.1 Selection of primers and probe

The previously generated alignment of COI sequences was used to identify two qPCR primer sets, each comprising two primers and a probe, for detecting *H. avenae* and *H. latipons* in separate qPCRs. The primers and probe were designed using the software AlleleID 7.73. The sequences of the potential qPCR primer sets were further screened by investigating their presence in sequences stored in GenBank (BlastN option, www.ncbi.nlm.nih.gov/). Additionally, the primer sequences were imported in OligoAnalyzer to check the potential of secondary structures, self-primer-dimer and hetero primer-dimer formation within and between the different primer sets (www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Finally, one qPCR primer set was selected for each species. The forward primer, reverse primer and probe selected to detect and quantify *H. avenae* were AVENF-COI 3'-CTG GTT TGA GCA CAT CAT A-5',

AVENR-COI 3’-CCG GTA GGA ATT GCA ATA -5’ and AVENProbeCOI 3’-CCG CCT ATC TAA ATC TAT ACC AAC CAC-5’, respectively. The AVENProbeCOI-probe was labelled with the fluorescent dye VIC (Life Technologies Europe). The selected forward primer, reverse primer and probe to detect and quantify *H. latipons* were LATF-COI 3’-TTG GGC TCA TCA TAT ATT TG-5’, LATR-COI 3’-GTT GGA ATT GCA ATA ATT ATA GTA-5’ and LATProbeCOI 3’-TAG GCT CGT CTA TCC AAA TCT ATT CCA-5’, respectively. The LATProbeCOI-probe was labelled with the fluorescent dye 6-FAM (Life Technologies Europe).

4.2.4.2 Real-time PCR assay

All real-time PCRs were performed using the SensiFAST Probe Hi-ROX and SensiFAST SYBR Hi-ROX qPCR kits on a 7900HT ABI Sequence Detection System (Applied Biosystems, California, USA). Both kits 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. The SensiFAST SYBR Hi-ROX kit was only used to optimise the annealing temperature of the PCR reaction itself by investigating the melting curves generated. Melting curves showing one peak suggest that primers bind their specific DNA-target and not any other DNA-targets or do not form secondary structures or primer-dimers. All other tests (specificity, sensitivity, and construction of standard curve) were done with the SensiFAST Probe Hi-ROX kit.

4.2.4.3 Optimisation of the annealing temperature (Ta)

For the optimisation of the annealing temperature (Ta) of the primer sets for *H. avenae* and *H. latipons*, one population of *H. avenae* (Fa19) and one population of *H. latipons* (HLIran) (Table 4.1) were used. Different temperatures ranging from 60°C to 64°C were applied to a 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 1 J2 of HLIran or 1 J2 of Fa19. The qPCR-programme settings were as follows: initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 60°C, 61°C, 62°C, 63°C or 64°C for 15 sec and 72°C for 30 s. DNA melting curve analysis of the resulted amplicons was performed at the end of amplification by fast cooling the amplicons till 60ºC, and then

increasing the temperature to 95ºC at 0.03ºC/s. The fluorescence emission was measured during this stage over 20 min.

Each qPCR reaction was run with three replications for each sample. A no-template control (NTC) was included in each experiment using sterile de-ionized water instead of template DNA. The Sequence Detection Software SDS 2.4 was used to generate the amplification and dissociation curves. The threshold cycle number (Ct) was determined at a threshold set on 0.2.

4.2.4.4 Specificity test of primers and probe

For the specificity test, all reactions were performed 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 the extracted DNA. The cycling conditions for both species were as follows: 5 min at 95°C, followed by 40 cycles at 95°C for 15 sec; four different Ta were used (61, 62, 63 and 64°C) for 30 sec and followed by 72°C for 30 sec. In both tests, DNA from all populations (Table 4.1) was used in two replicates. Two NTC samples were included as well. The Sequence Detection Software SDS 2.4 was used to generate the amplification curves. The threshold cycle number (Ct) was determined at a threshold set on 0.2.

4.2.4.5 Detection sensitivity of primers and probe

DNA was prepared from single J2 (Holterman *et al.,* 2006) of *H. avenae* and *H. latipons* in 4 replicates. Using the obtained DNA, a qPCR was run in 3 repeats for each of the 4 replicates. For each of the 4 replicates, the Ct-values obtained in the 3 repeats were averaged and compared. Because the averages of the Ct-values were similar, the DNA of each replicate was pooled and used to determine the detection sensitivity (Mokrini *et al.,* 2014). The DNA samples from *H. avenae* and *H. latipons* (1 J2) were used in a qPCR separately or mixed with DNA extracted from 1, 10, 50 or 100 J2 of *H. filipjevi*. The performance and analysis of the qPCR assays were as described above (specificity primer sets). The Sequence Detection Software SDS 2.4 was used to generate the

amplification curves. The threshold cycle number (Ct) was determined at a threshold set on 0.2.

4.2.4.6 Construction of a standard curve

To construct the standard curve for both above-mentioned species, the stability of the DNA extraction method was checked (see above) using 120 J2 instead of 1 J2. Because the averages of the Ct-values were similar, DNA of each species was pooled (120 J2), diluted (1/10, 1/50, 1/100, 1/500 and 1/1000) and used to run qPCR in three replications. Two NTC samples were included in those tests.

4.3 Results

4.3.1 Nematode identification

Amplification of the rDNA-ITS region was successful for all isolates and produced a single band with a fragment size of 1100 bp. No PCR products were obtained in the negative control without nematode DNA template. Sequencing of both DNA strands of the purified PCR products confirmed the morphological identification. The sequences were deposited in GenBank; the accession numbers are presented in Table 4.1.

4.3.2 Development of species-specific PCR primer sets

4.3.2.1 Species-specific primers selection

Parts of the actin 1 and COI genes were amplified. PCR products were obtained for all used species and yielded a fragment of 420 bp (Figure 4.1) and 470 bp (Figure 4.2), respectively. No PCR products were obtained in the negative control without nematode DNA template. Also, PCR products were obtained from Hsp90 and β-tubulin genes but not for all used species. However, the amplifications revealed additional bands of 251-382 and 900-1500 bp, respectively (data not shown). No gene-specific bands or no PCR products at all were obtained from genes pectate lyase, annexin, chorismate mutase and aldolase.

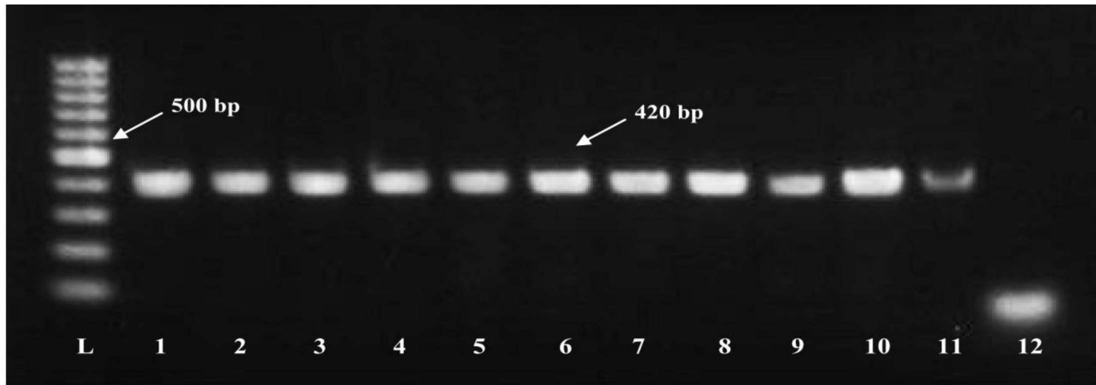

Figure 4.1. PCR amplification of DNA from eight different *Heterodera* species with the universal actin 1 gene primers (Hs-ACTF and Hs-ACTR) revealing a 420 bp fragment. L: 100 bp DNA ladder (Fermentas Life Sciences), 1-2: *H. latipons* (Fa3), 3-4: *H. avenae* (Fa1), 5: *H. hordecalis* (E69), 6-7: *H. filipjevi* (Did23b), 8: *H. glycines* (HGRiggs), 9: *H. schachtii* (HSPol), 10: *H. betae* (DCP1248), 11: *H. ciceri* (FaC3), 12: Negative control. (see Table 4.1 for codes).

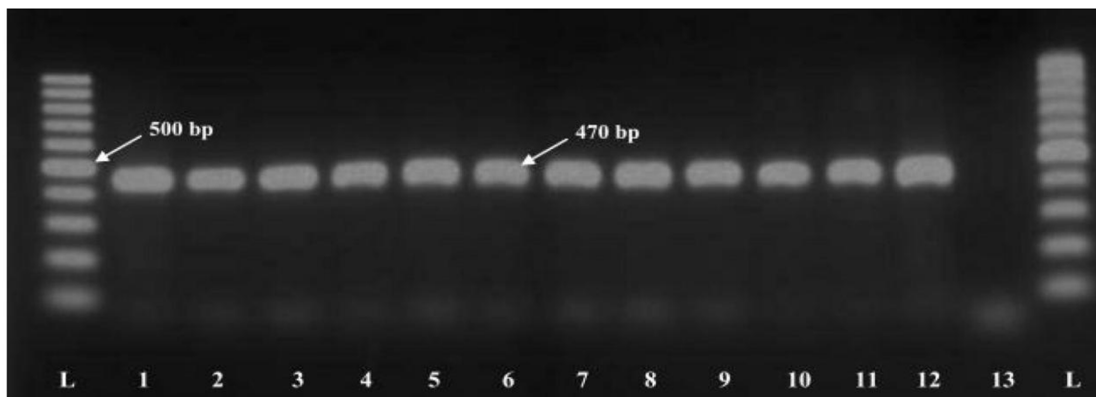

Figure 4.2. Amplification results with the COI gene primers (JB3 & JB5, see Table 4.2) on a selection of *Heterodera* spp. and *Punctodera punctata*. L: 100 bp DNA ladder (Fermentas Life Sciences), 1: *H. pratensis* (DCP1041A), 2: *P. punctata* (DCP1041B), 3 & 4: *H. filipjevi* (Did15 & Fa125), 5: *H. glycines* (Did38), 6: *H. daverti* (HD11), 7: *H.* latipons (Fa7A1), 8 & 9: *H. avenae* (Fa1 & Fa19), 10: *H. ciceri* (Fac3), 11: *H. hordecalis* (TuniB), 12: *H. schachtii* (HSPol), 13: Negative control.

After purifying and sequencing the PCR products of actin 1 and COI regions, the sequences were subjected to BlastN. This confirmed that parts of both genes were amplified. Results were also obtained for both tubulin and Hsp90. BlastN confirmed that amplified fragments belonged to tubulin and Hsp90 genes. After blasting the sequences of some of the unexpected bands, it became clear that tubulin and Hsp90 genes from

various origins were amplified as well (data not shown). The alignment and comparison of the obtained actin 1 sequences with those deposited in GenBank (Figure 4.3) showed moderate (83%) to very high (94%) similarities between the species, with the highest similarity between *H. latipons* and *H. filipjevi*. Nevertheless, the software AlleleID 7.73 allowed identifying species-specific primers for *H. latipons* (see above 4.2.3.1). The positions of the forward and reverse primers are visualised in the alignment (Figure 4.3). The forward primer was located in intron 5 of the actin 1 gene, the reverse primer in intron 6 (Figure 4.4). A BlastN-search with the newly designed primers revealed no match with any of the nematode sequences available in GenBank. The same was true when looking for potential primer binding sites within the *Heterodera* sequences obtained, with the obvious exception of the *H. latipons* sequences. Therefore, I retained this primer set for further experiments.

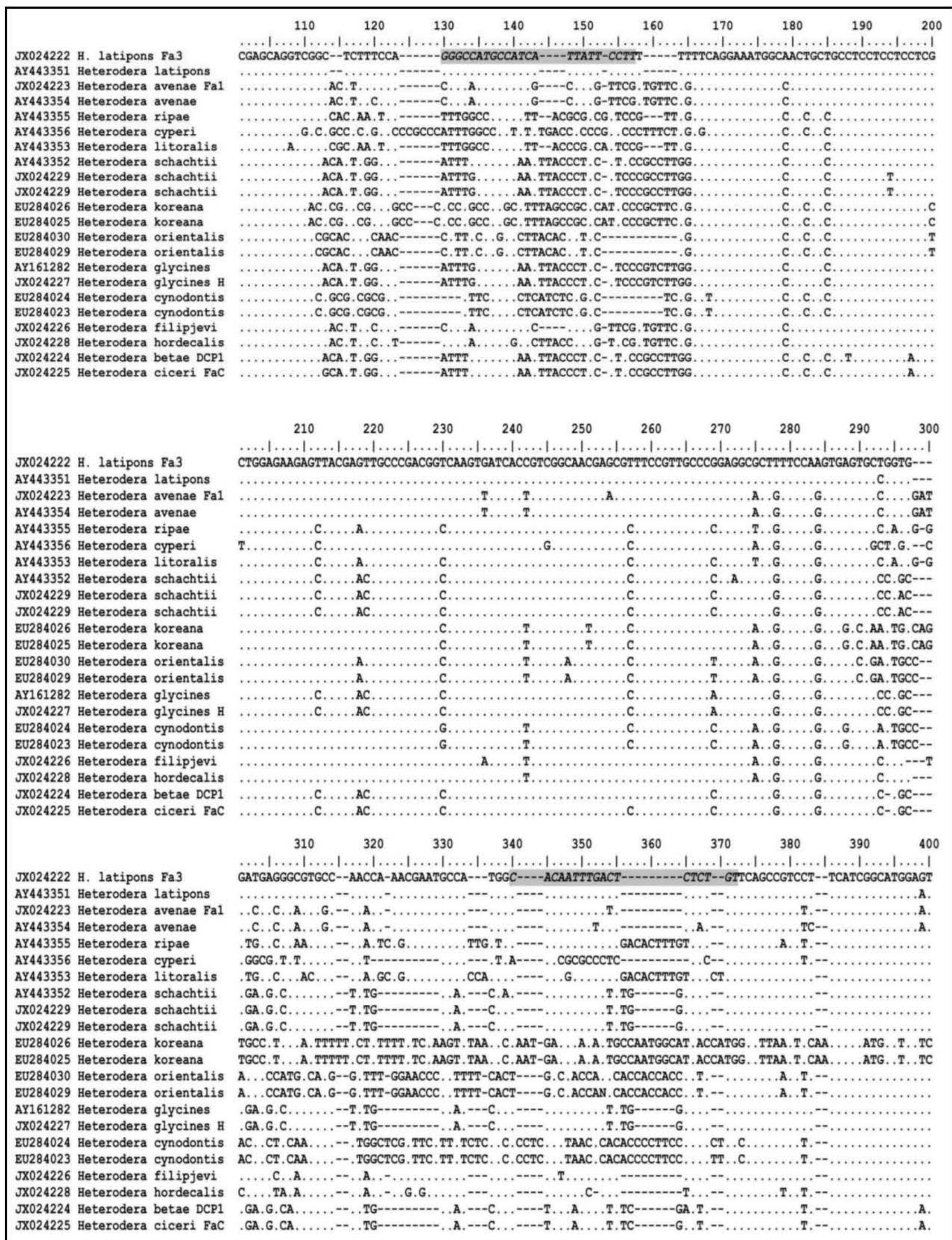

Figure 4.3. Alignment of a selection of the obtained actin 1 sequences (see Table 4.1 for codes). Position for the species-specific primer set for *Heterodera latipons* (HLAT-ACT) is highlighted, bold and italic.

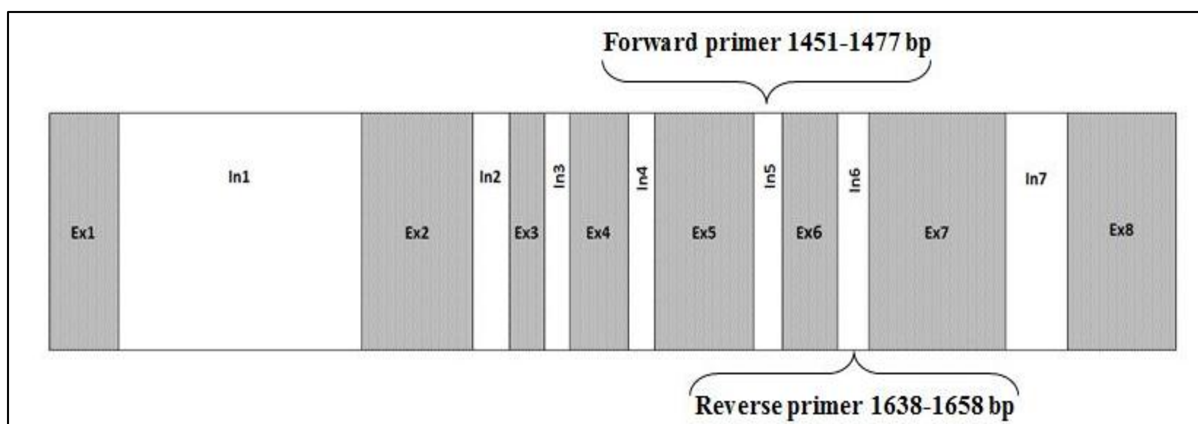

Figure 4.4. A graphical map of the actin 1 gene showing the positions of the designed species-specific primers (HLAT-ACT). The shaded and white boxes represent the exons (Ex) and introns (In), respectively. The sizes of the boxes correspond to the lengths of the exons and introns.

Alignment and pairwise sequence-comparison using the software CLC bio genomics workbench 8.0 of the obtained COI sequences showed moderate similarities (80.80 – 85.14%) between the *H. avenae* sequences and other species (Figure 4.5). Also, the comparison between the *H. filipjevi* sequences and other species resulted in moderate similarities (82.25 – 85.87%) (Figure 4.5). The software AlleleID 7.73 enabled selection of two species-specific primer sets for *H. avenae* and *H. filipjevi*, separately. Both sets were visualised in the alignment. This revealed both primer sets to be specific for the species for which they were designed within the given dataset (Figure 4.5). Hence, both sets were retained for advanced experiments.

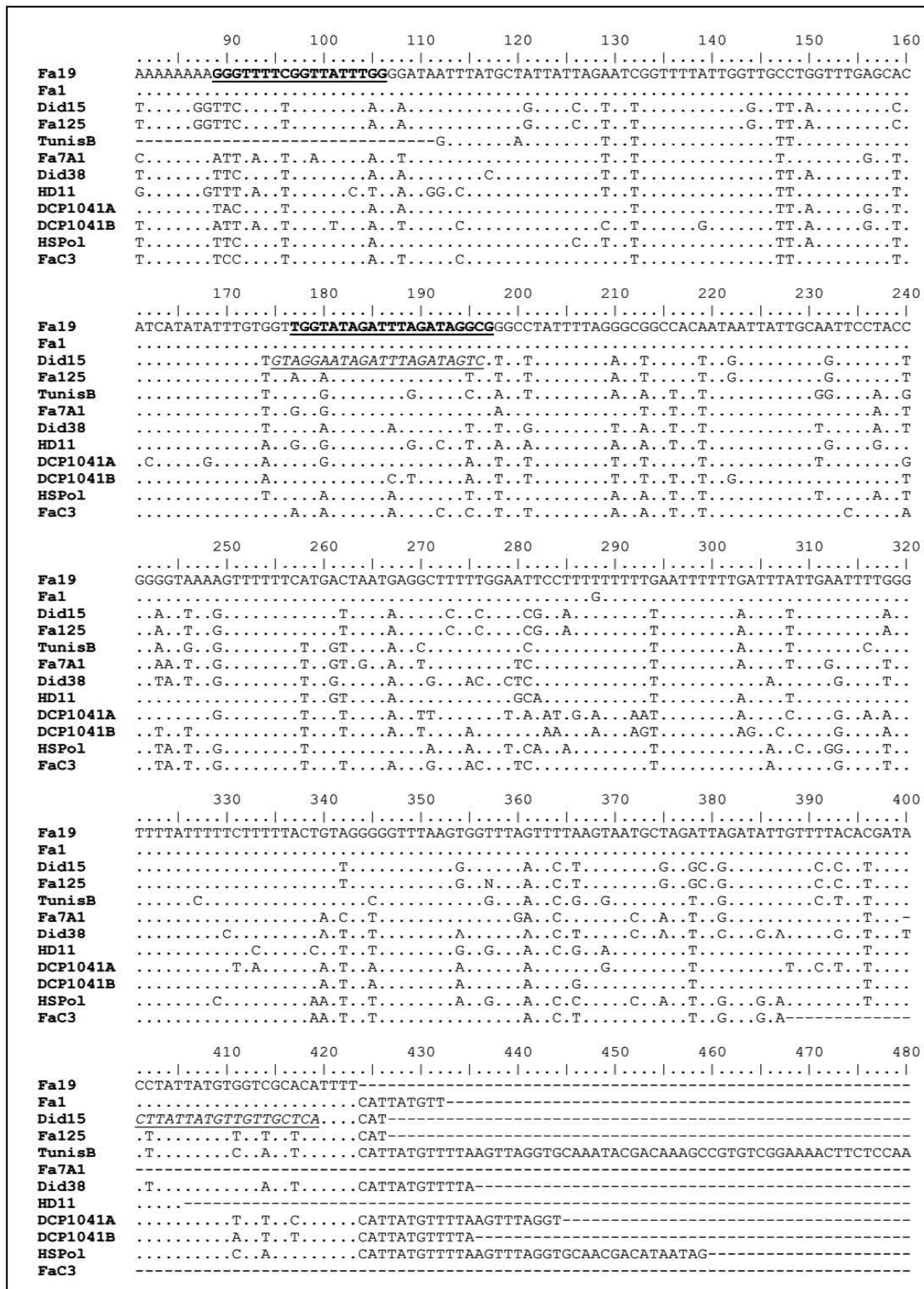

Figure 4.5. Alignment of a selection of the COI-mtDNA sequences (see Table 4.1 for codes). Position for the species-specific primer set for *Heterodera avenae* (AVEN-COI) is underlined and bold, and for *H. filipjevi* (FILI-COI) underlined and italic.

4.3.2.2 Optimisation (Ta) and validation of species-specific PCRs

The gradient PCR enabled to set the optimal annealing temperature to be set for the retained primers for HLAT-ACT between 47-55°C (Figure 4.6), and for AVEN-COI and FILI-COI (Figures 4.7-4.8), between 53-60°C. A Ta within this range resulted in clear bright bands; weaker bands for each primer set were obtained at temperatures out of those ranges. No primer-dimers or additional band(s) were noticed within the used temperature range. Because higher temperatures normally increase the specificity of amplification yielding the brightest specific band and high amount of PCR product, I fixed the annealing temperature at 50°C for HLAT-ACT, and 58°C for both AVEN-COI and FILI-COI.

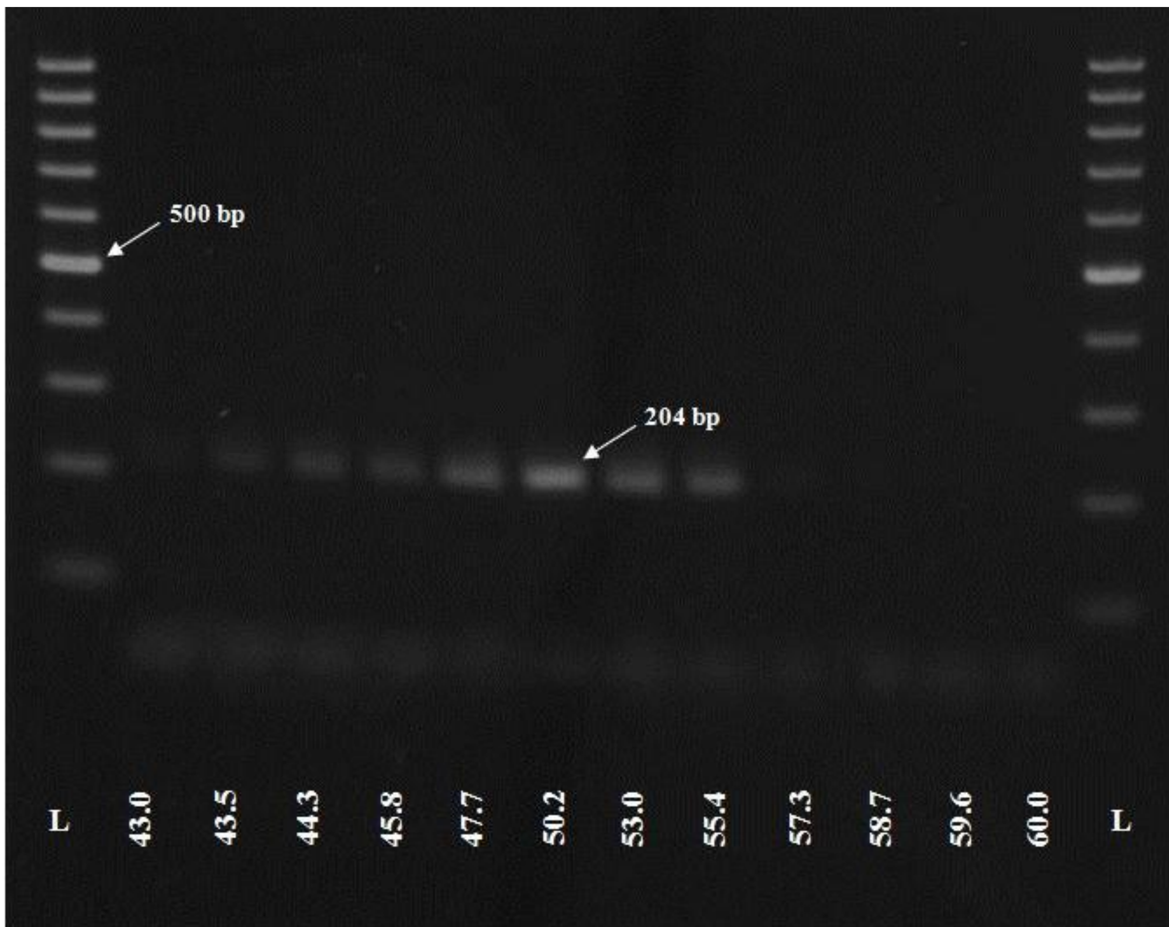

Figure 4.6. Gradient PCR with the *Heterodera latipons* - specific PCR (HLAT-ACT primer set) using DNA of *H. latipons* (Fa3). Temperature ranges from 43-60°C. L: 100 bp DNA ladder (Fermentas Life Sciences).

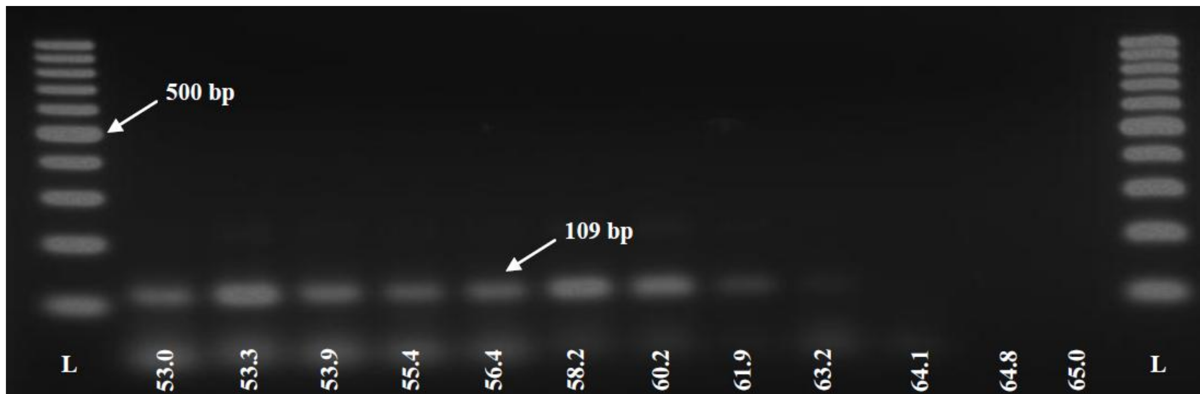

Figure 4.7. Gradient PCR (temperatures indicated) with the AVEN-COI primer set using *Heterodera avenae* (Fa1). Temperature ranges from 53 to 65°C. L: 100 bp DNA ladder (Fermentas Life Sciences).

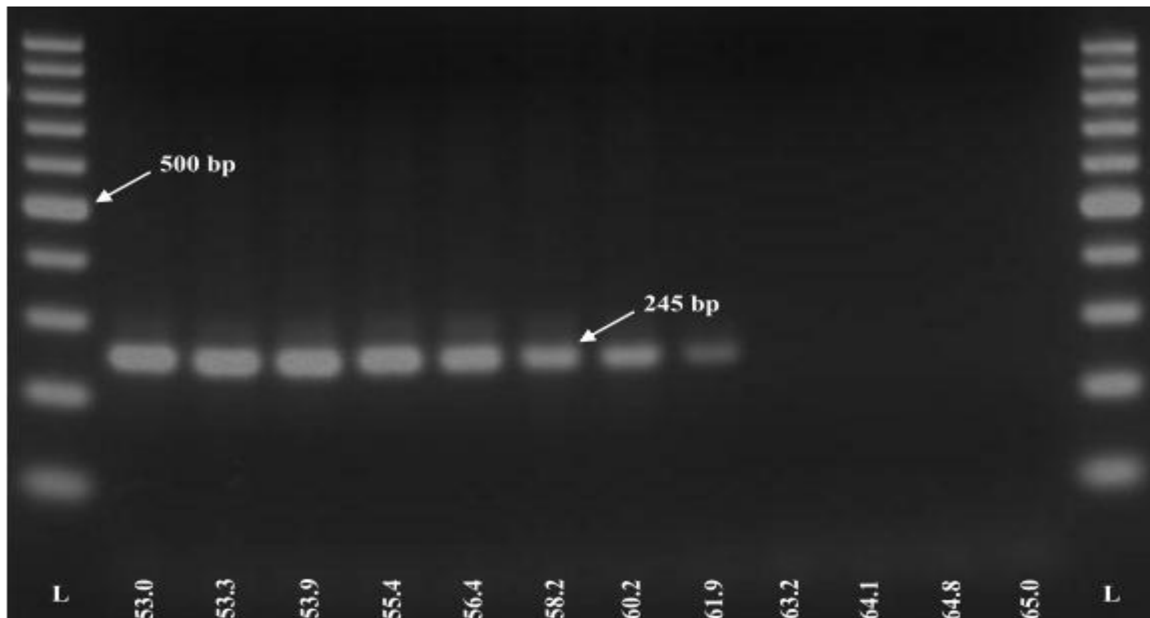

Figure 4.8. Gradient PCR (temperatures indicated) with the FILI-COI primer set using *Heterodera filipjevi* (Did15). Temperature ranged from 53°C to 65°C. L: 100 bp DNA ladder (Fermentas Life Sciences).

The optimised PCRs repeatedly resulted in a single band of 204 bp for all *H. latipons* populations (Figure 4.9; eight populations are shown) and resulted in a single band of 109 bp for all *H. avenae* populations (Figure 4.10), and of 245 bp for all *H. filipjevi* populations (Figure 4.11). The three PCR assays resulted with no appearance of any

additional band and did not generate a band for any of the other tested *Heterodera* species, nor for *P. punctata*.

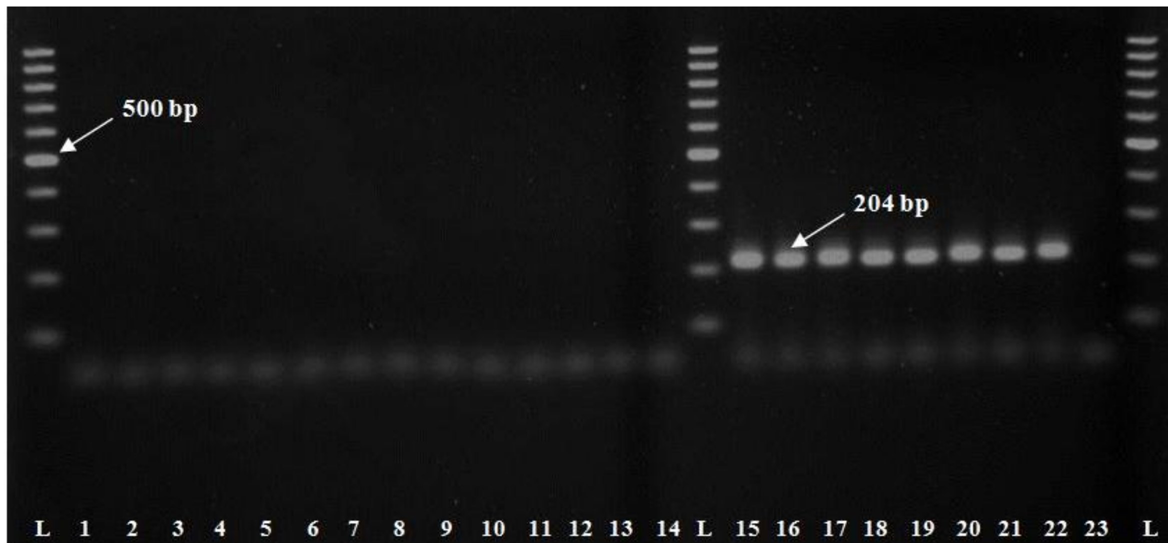

Figure 4.9. Results of the *Heterodera latipons*-specific PCR (HLAT-ACT primer set) using DNA from all samples (see Table 4.1 for codes). L: 100 bp DNA ladder (Fermentas Life Sciences), 1: *Heterodera pratensis* (DCP1041A), 2: *Punctodera punctata* (DCP1041B), 3: *H. avenae* (Fa1), 4: *H. hordecalis* (E69), 5: *H. glycines* (HGRiggs), 6: *H. schachtii* (HSPol), 7: *H. betae* (DCP1248), 8: *H. filipjevi* (Fa125), 9: *H. goettingiana* (MP1), 10: *H. humuli* (MP5), 11: *H. ciceri* (FaC3), 12: *H. trifolii* (HT9), 13: *H. carotae* (DCP1734), 14: *H. daverti* (HD11), 15-22: different populations of *H. latipons*: 15: HL5, 16: HLCyp, 17: Fa7A1, 18: Fa3, 19: HLMorc, 20: Mus1, 21: HLIran, 22: HL50, 23: Negative control.

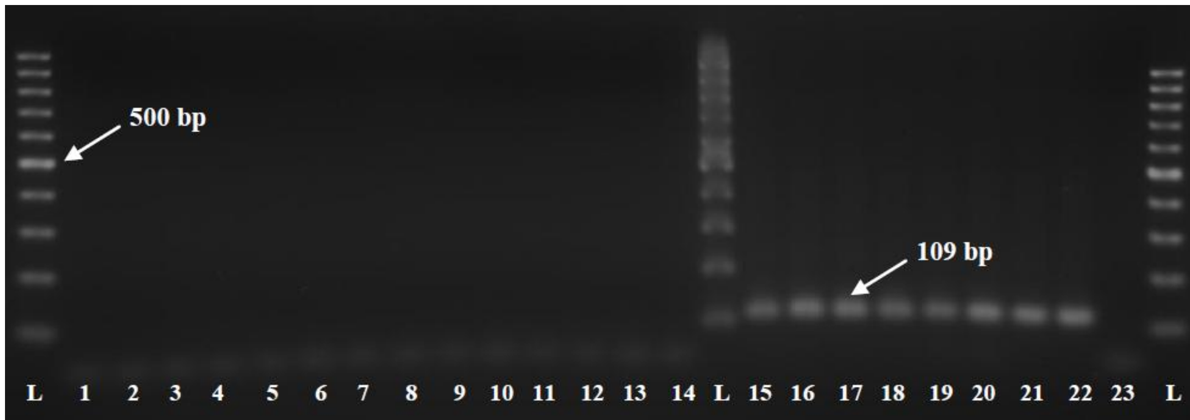

Figure 4.10. Results of the *Heterodera avenae*-specific PCR using the AVEN-COI primer set. L: 100 bp DNA ladder (Fermentas Life Sciences). 1: *Heterodera pratensis* (DCP1041A), 2: *Punctodera punctata* (DCP1041B), 3: *H. filipjevi* (Did23b), 4: *H. hordecalis* (E69), 5: *H. glycines* (HGRiggs), 6: *H. schachtii* (HSPol), 7: *H. betae* (DCP1248), 8: *H. daverti* (HD11), 9: *H. goettingiana* (MP1), 10: *H. humuli* (MP5), 11: *H. ciceri* (FaC3), 12: *H. trifolii* (HT9), 13: *H. latipons* (Fa3), 14: *H. carotae* (DCP1734), 15-22: different populations of *H. avenae* 15: Fa1, 16: Did29, 17: Did33, 18: Did11, 19: Tuni6, 20: Did49, 21: Mus21, 22: Fa19, 23: Negative control.

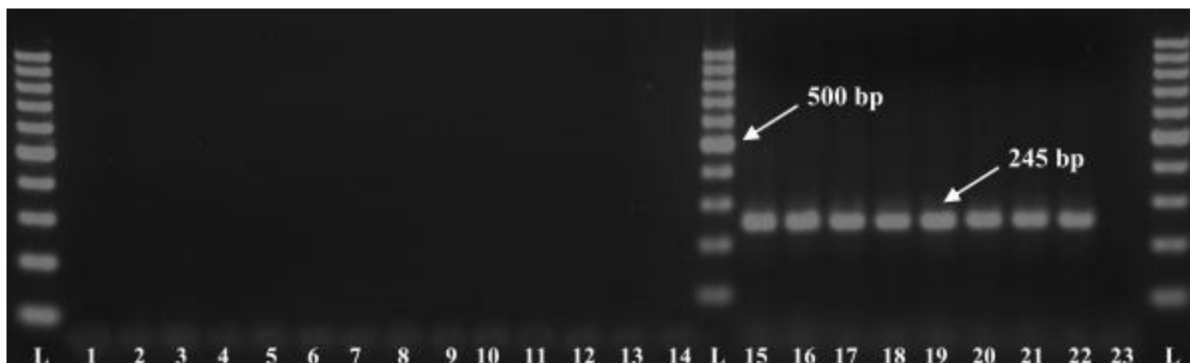

Figure 4.11. Results of the *Heterodera filipjevi*-specific PCR using the FILI-COI primer set. L: 100 bp DNA ladder (Fermentas Life Sciences). 1: *Heterodera pratensis* (DCP1041A), 2: *Punctodera punctata* (DCP1041B), 3: *H. avenae* (Fa1), 4: *H. hordecalis* (E69), 5: *H. glycines* (HGRiggs), 6: *H. schachtii* (HSPol), 7: *H. betae* (DCP1248), 8: *H. latipons* (Fa3), 9: *H. goettingiana* (MP1), 10: *H. humuli* (MP5), 11: *H. ciceri* (FaC3), 12: *H. trifolii* (HT9), 13: *H. carotae* (DCP1734), 14: *H. daverti* (HD11), 15-22: different populations of *H. filipjevi* 15: Did15, 16: Did23, 17: E88, 18: Did42b, 19: Did42c, 20: Fa125, 21: Fa126, 22: HFUSA, 23: Negative control.

4.3.2.3 Sensitivity test of species-specific PCRs

The sensitivity of the PCR assay to detect *H. latipons* using the HLAT-ACT primer set was acceptable since it was possible to detect five J2 of *H. latipons* when mixed with 100 J2 of *H. avenae.* The detection was still possible with a 1/10 dilution of DNA of *H. latipons* while the 1/50 dilution yielded a weaker band (Figure 4.12). Also, the sensitivity of the PCR assays using the AVEN-COI and FILI-COI primer sets was satisfactory. Both assays were able to detect DNA extracted from 5 J2 of *H. avenae* (Figure 4.13) or 5 J2 of *H. filipjevi* (Figure 4.14) when mixed with DNA obtained from 100 J2 of *H. latipons*. The assays were also able to detect DNA in four dilutions (1/5, 1/10, 1/50 and 1/100) from DNA originating from 5 J2 of *H. avenae* or *H. filipjevi.* Moreover, detection of both species was still possible when 1 µl of the diluted (1/5 and 1/10) target DNA was mixed with 1 µl DNA extracted from 100 J2 of *H. latipons.*

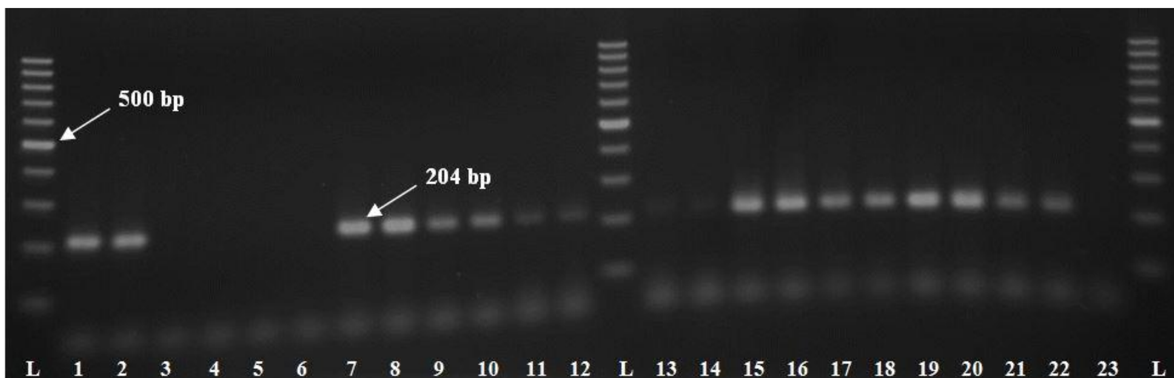

Figure 4.12. Results of the sensitivity test of the HLAT-ACT primer set in a PCR with 1 µl undiluted or diluted DNA of *Heterodera latipons* (Hl) mixed with 1 µl DNA from *H. avenae* (Ha). 1-2: 1/2 dilution of five second-stage juveniles (J2) of Hl, 3-4: five J2 of Ha, 5-6: 100 J2 of Ha, 7-8: five J2 of Hl, 9-10: 1/5 dilution of five J2 of Hl, 11-12: 1/10 dilution of five J2 of Hl, 13-14: 1/50 dilution of five J2 of Hl, 15-16: five J2 of Hl and 100 J2 of Ha, 17-18: 1/2 dilution of five J2 of Hl and 100 J2 of Ha, 19-20: five J2 of Hl and 5 J2 of Ha, 21-22: 1/5 dilution of five J2 of Hl and 100 J2 of Ha, 23: negative control, L: 100 bp DNA ladder (Fermentas Life Sciences).

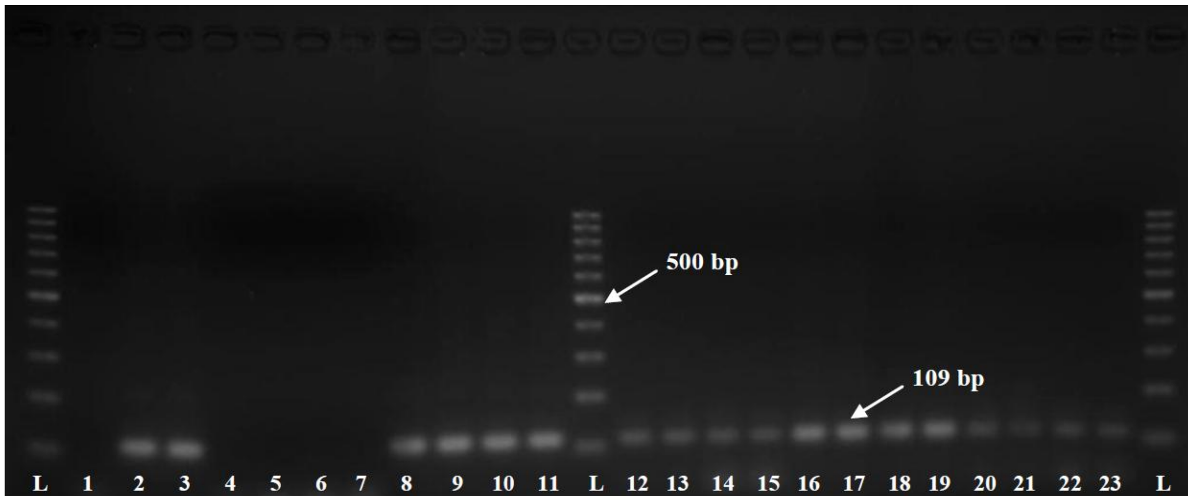

Figure 4.13. Results of the sensitivity test using the AVEN-COI primer set in a PCR with 1 µl undiluted or diluted DNA of *Heterodera avenae* (HA) mixed with 1 µl DNA from *H. latipons* (HL). 1: negative control, 2-3: 5 J2 of HA, 4-5: 5 J2 of HL, 6-7: 100 J2 of HL, 8-9: 1/5 dilution of 5 J2 of HA, 10-11: 1/10 dilution of 5 J2 of HA, 12-13: 1/50 dilution of 5 J2 of HA, 14-15: 1/100 dilution of 5 J2 of HA, 16-17: 5 J2 of HA and 5 J2 of HL, 18-19: 5 J2 of HA and 100 J2 of HL, 20-21: 1/5 dilution of 5 J2 of HA and 100 J2 of HL, 22-23: 1/10 dilution of 5 J2 of HA and 100 J2 of HL, L: 100 bp DNA ladder (Fermentas Life Sciences).

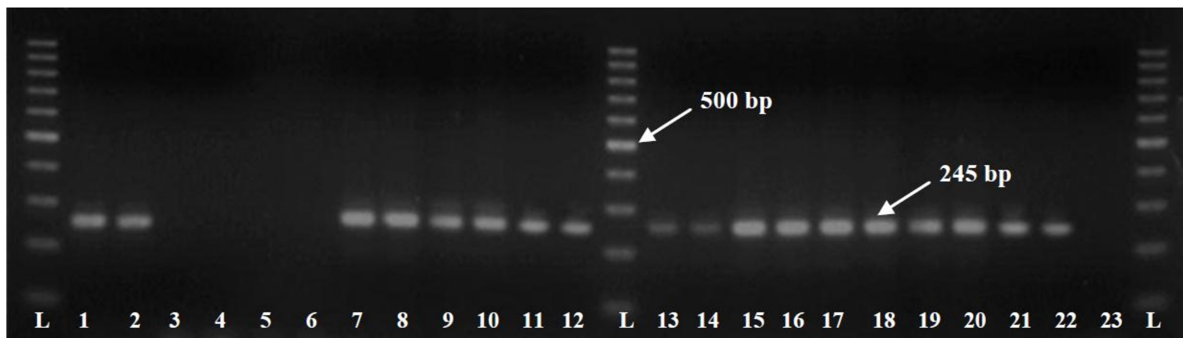

Figure 4.14. Results of the sensitivity test of the FILI-COI primer set in a PCR with 1 µl undiluted or diluted DNA of *Heterodera filipjevi* (HF) mixed with 1 µl DNA from *H. latipons* (HL). 1-2: 5 J2 of HF, 3-4: 5 J2 of HL, 5-6: 100 J2 of HL, 7-8: 1/5 dilution of 5 J2 of HF, 9-10: 1/10 dilution of 5 J2 of HF, 11-12: 1/50 dilution of 5 J2 of HF, 13-14: 1/100 dilution of 5 J2 of HF, 15-16: 5 J2 of HF and 5 J2 of HL, 17-18: 5 J2 of HF and 100 J2 of HL, 19-20: 1/5 dilution of 5 J2 of HF and 100 J2 of HL, 21-22: 1/10 dilution of 5 J2 of HF and 100 J2 of HL, 23: negative control, L: 100 bp DNA ladder (Fermentas Life Sciences).

4.3.3 Development of primers and probe for the qPCR

4.3.3.1 Species-specific primers and probes selection

A BlastN-search for the newly designed primers and probes to develop two qPCR of both species (4.2.4.1) showed specific results, and no match with any of the sequences of other nematode species available in GenBank. Visualising the two qPCR primer sets in the alignment confirms the obtained results (Figure 4.15).

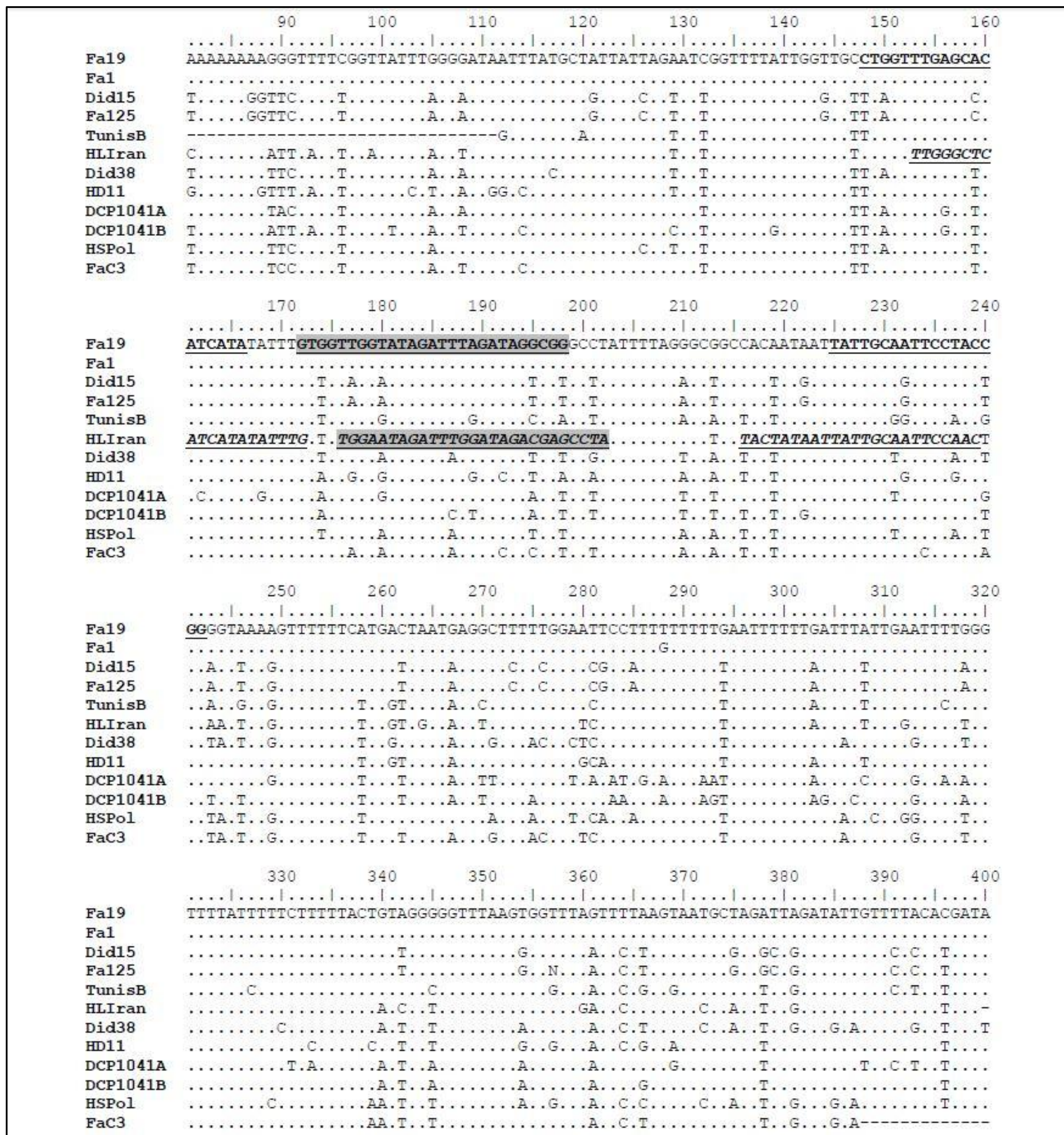

Figure 4.15. Alignment of a selection of the COI-mtDNA sequences (see table 4.1 for codes). Positions of the primers for *Heterodera avenae* are underlined and bold, the probe is bold, underlined and highlighted, for *H. latipons* the primers are underlined, bold and italic, the probe underlined, bold, italic and highlighted.

4.3.3.2 Optimisation of Ta

At all tested Ta, the dissociation curves for both species *H. latipons* and *H. avenae* (Figures 4.16-4.17) showed a single melting peak. Both assays were able to detect a single J2. For both assays, non-specific fluorescence due to amplification of primer-

dimers or another non-specific amplification product was not observed. No signals were observed in the NTC samples.

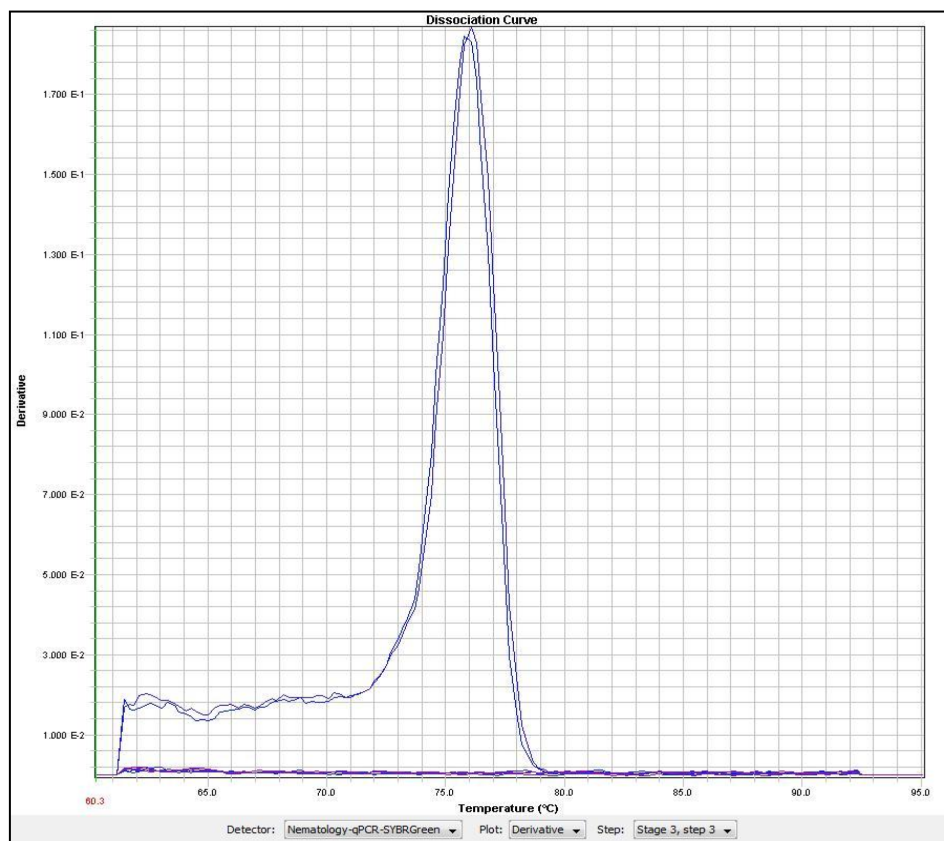

Figure 4.16. Melting curve (fluorescence versus temperature) of the qPCR test (SensiFAST SYBR Hi-ROX) resulted in a specific amplicon for *Heterodera latipons* (HLIran) with the annealing temperature set at 62°C.

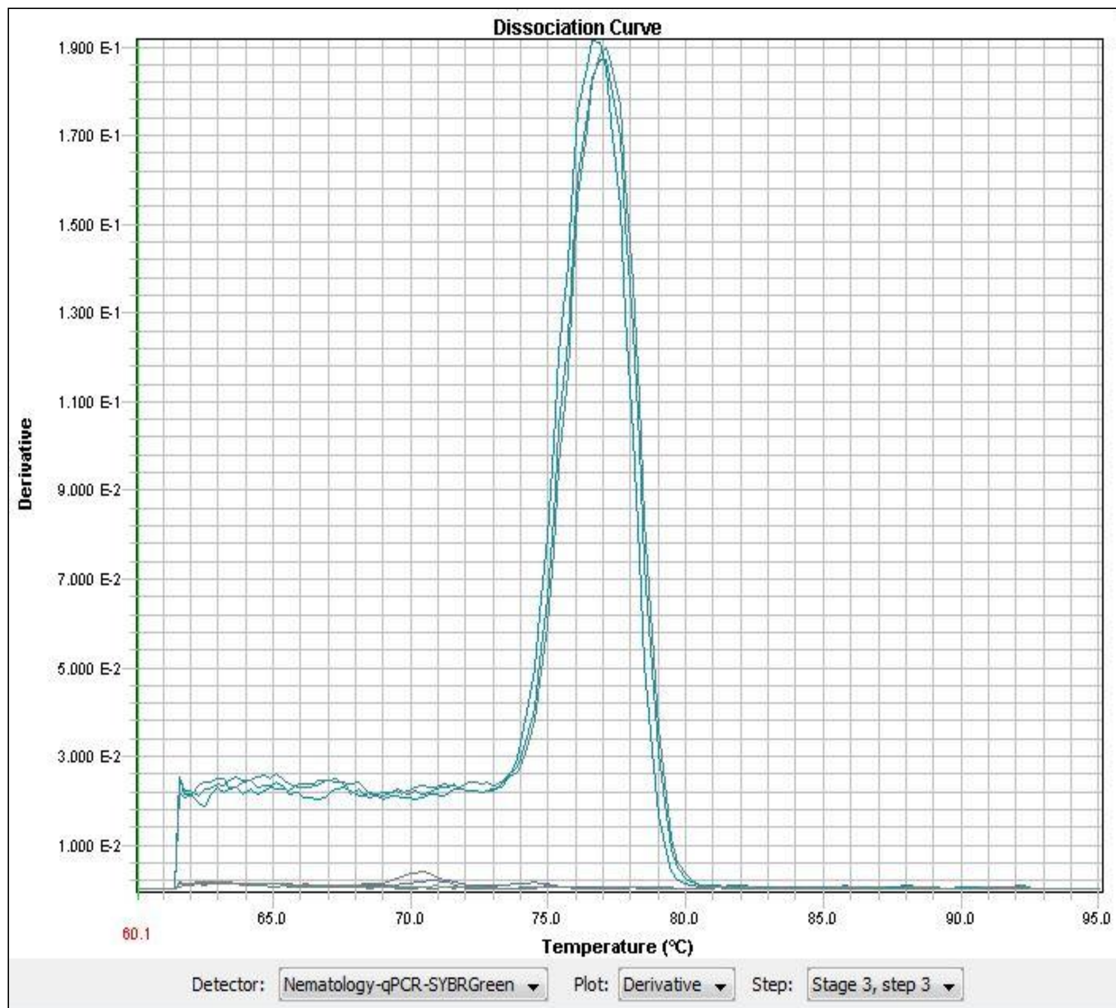

Figure 4.17. Melting curve (fluorescence versus temperature) of the qPCR test (SensiFAST SYBR Hi-ROX) resulted in a specific amplicon for *Heterodera avenae* (Fa19) with the annealing temperature set at 64°C.

4.3.3.3 Specificity of primer and probe set

The *H. latipons* qPCR assay conducted at different Ta (61, 62, 63 and 64°C) successfully amplified a specific fragment and resulted in mean Ct-values of 21.83, 22.13, 23.24 and 27.39, respectively. The qPCR assay showed a positive signal for all populations of *H. latipons* while no such signal was observed from other *Heterodera* spp. and *P. punctata*. Only at Ta = 61°C a positive signal for *H. hordecalis* was observed. As a consequence 62°C was retained as Ta for the *H. latipons* qPCR assay.

The qPCR assay of *H. avenae* with different Ta (61, 62, 63 and 64 °C) resulted in an average Ct-value of 22.37, 22.80, 23.05 and 23.15, respectively. The assay showed

a positive signal for all *H. avenae* populations used in the test. A positive signal was also obtained for *H. hordecalis, H. betae, H. pratensis* and *H. trifolii* at Ta equalling 61, 62 and 63°C (Table 4.3); positive signals were not observed when Ta equalled 64°C. For this reason, 64°C was retained as annealing temperature for the *H. avenae* qPCR assay. Both assays did not show any amplification of DNA in the NTC samples.

Table 4.3 Mean of Ct-values obtained at different annealing temperatures of the species showing a positive signal during the test to optimise the qPCR assay for *Heterodera avenae* (DNA extracted from 1 J2).

Sample	Mean Ct of *H. avenae* qPCR			
	61°C	62°C	63°C	64°C
H. hordecalis (E69)	35.35	35.95	37.42	N/A*
H. betae (DCP1248)	36.27	37.72	N/A	N/A
H. pratensis (DCP1041A)	34.12	35.15	36.53	N/A
H. trifolii (HT9)	36.96	N/A	N/A	N/A

* N/A: Not Applicable

4.3.3.4 Sensitivity of primers and probe

The qPCR assays with *H. avenae* and *H. latipons* successfully detected a single J2. The averaged Ct-value of the three technical repeats of the four DNA extraction replications with *H. latipons* was 26.79, 27.36, 26.76 and 27.38*,* while for *H. avenae* the Ct-value was 28.03, 28.24, 27.75 and 27.92, respectively. The qPCR using DNA from a single J2 of one of both species mixed with DNA extracted from 1, 10, 50 or 100 J2 of *H. filipjevi,* resulted in a Ct-value averaging 27.73, 27.65, 29.21 and 30.54, respectively, for *H. latipons*; for *H. avenae* the mean Ct-value was 27.84, 27.88, 27.87 and 27.95, respectively. In both tests, no signal was detected in the NTC samples.

4.3.3.5 Construction of a standard curve

Checking the stability of the DNA extraction of 120 J2 via qPCR resulted in stable Ct-values of 21.85 ± 0.3 and 22.34 ± 0.4 for *H. latipons* and *H. avenae*, respectively. For

both species, the dilution series of the DNA from 120 J2 resulted in a standard curve (Figure 4.18, 4.19) showing a highly significant linearity between the Ct-value and the dilution rate (Table 4.4) (R^2 = 0.99; slope = -3.03; E = 113.81% and R^2 = 0.99; slope = -3.28; E = 101.78% for *H. avenae* and *H. latipons*, respectively).

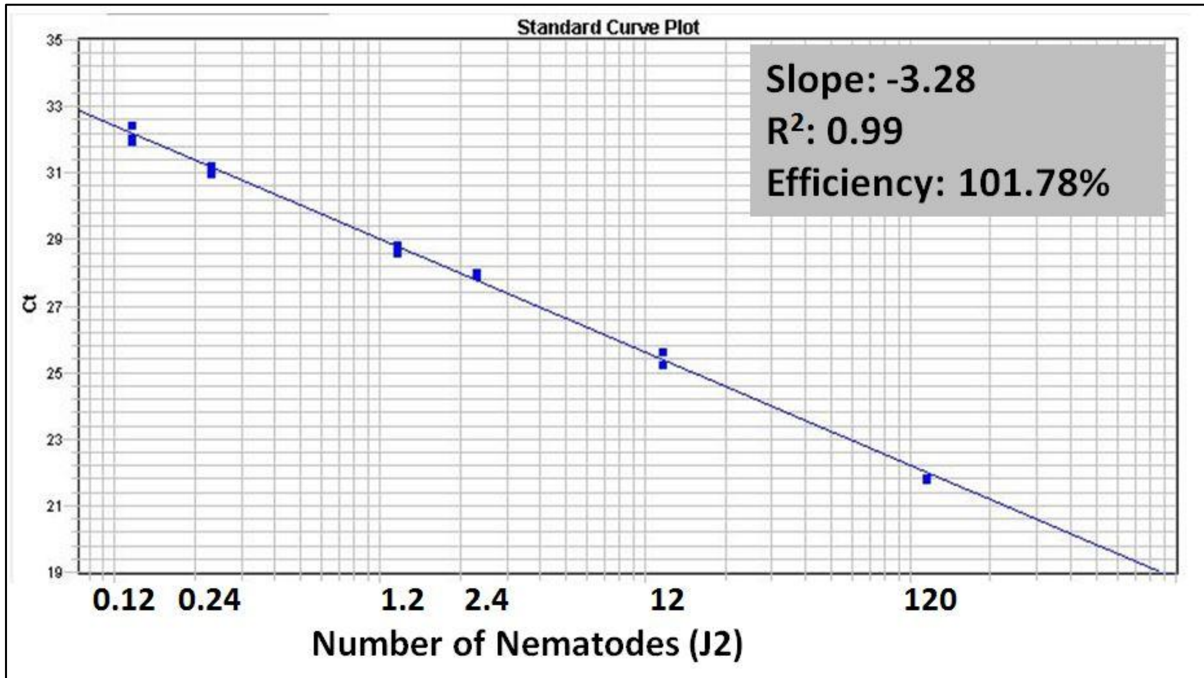

Figure 4.18. A standard curve of the qPCR assay (SensiFAST Probe Hi-ROX) for *Heterodera latipons*: threshold cycle number (Ct) plotted against the dilution series (1/10, 1/50, 1/100, 1/500 and 1/1000) of DNA extracted from 120 J2.

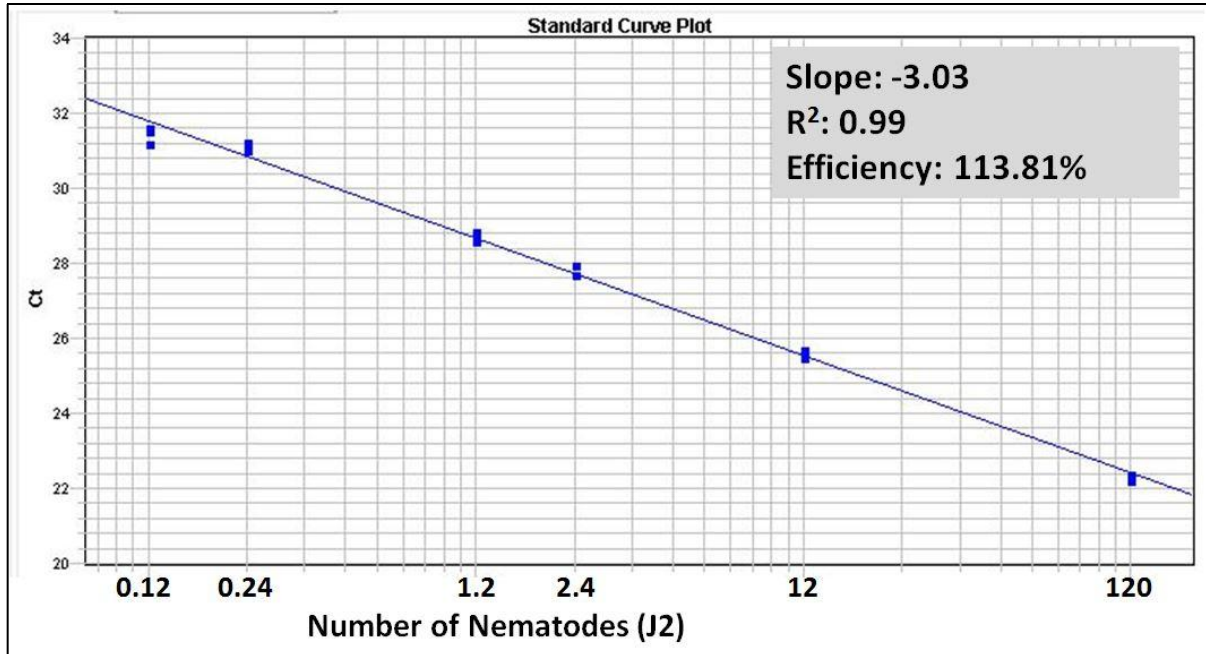

Figure 4.19. A standard curve of the qPCR assay (SensiFAST Probe Hi-ROX*)* for *Heterodera avenae*: threshold cycle number (Ct) plotted against the dilution series (1/10, 1/50, 1/100, 1/500 and 1/1000) of DNA extracted from 120 J2.

Table 4.4 Cycle threshold (Ct) values of the dilution series of *Heterodera avenae* (Ta= 64°C) and *H. latipons* (Ta= 62°C).

Dilution	**Ct mean**	
	***H. avenae* (Ta= 64°C)**	***H. latipons* (Ta= 62°C)**
undiluted DNA (120 J2)	22.31	21.83
1/10	25.56	25.85
1/50	27.85	27.99
1/100	28.71	28.80
1/500	31.16	31.08
1/1000	31.44	32.17

4.4 Discussion

In the last decade, several surveys assessed the distribution of CCN in the main wheat and barley growing areas in Syria (Abidou *et al.,* 2005a; Hassan, 2008; chapter 3)

and Turkey (Sahin *et al.,* 2009; Yavuzaslanoglu *et al.,* 2012). The result showed that mixtures of two *Heterodera* species (*H. avenae* with *H. latipons* or *H. avenae* with *H. filipjevi*) and sometimes even three species coexist in the same field. Species-mixtures increase the difficulties to reach a precise identification, which is very important for planning of successful plant protection measures against these pests. Unfortunately, lack of specific skills on morphological identification is frequently one of the major problems. Nevertheless, the use of molecular tools having the capacity to identify accurately and separate the three species offers an attractive option. Moreover, qPCR is a rapid and reliable diagnostic tool for quantitative detection of organisms (Fusco *et al.,* 2011).

Up to date, one publication reported the development and use of species-specific primers for the molecular identification of *H. avenae* and *H. filipjevi* based on the ITS-rDNA. However, the assays were developed based on an US population of both species (Yan *et al*., 2013). Two other publications reported the development and use of species-specific primers for the molecular identification of *Heterodera* spp., *viz. H. glycines* (Subbotin *et al.,* 2001b) and *H. schachtii* (Amiri *et al.,* 2002), based on the ITS-rDNA region. Amiri *et al.* (2002) designed a species-specific primer (SHF6) to detect *H. schachtii* based on a sequence polymorphism within the ITS-rDNA sequences. The authors were able to detect all 35 collected populations of *H. schachtii* without a positive result for other cyst nematode species, including two isolates of *H. betae*. Moreover, they stated that, due to the high similarity of the ITS-sequences within the Schachtii group, only this *H. schachtii* sequence polymorphism was suited for species-specific primer design. However, Chemeda *et al.* (2012) found that the SHF6 primer did not detect some populations of *H. schachtii* suggesting that this polymorphism is not present in all *H. schachtii* populations. This result could be explained by the heterogeneity of the ITS-sequences caused by the incomplete concerted evolution of multi-copy gene families such as rDNA, as described by Hillis & Dixon (1991). Polymorphism was also observed by Rivoal *et al.* (2003) who detected great genetic variability between and within *H. latipons* isolates. Moreover, Ferris *et al.* (1999) suggested the existence of sibling species in *H. latipons* when they compared the sequence of ITS rDNA in two morphologically similar but geographically separated isolates from Israel (Gilat) and Russia (Rostov). Thus, designing a species-specific primer for end-point PCR or qPCR against *H. latipons*

based on ITS-sequences could turn out to be problematic because of this polymorphism. Also, designing species-specific primers or a qPCR primer set for *H. avenae* and *H. filipjevi* starting from ITS-sequences was difficult because of sequence polymorphism present within each species. In addition, the *in silico* comparative analysis of all the sequences obtained in this study separately with the already available ITS-sequences in GenBank (BlastN) showed sometimes high similarity, *e.g.* 99% similarity between *H. avenae* (Fa1) and *H. mani* (AY148377), *H. arenaria* (AF274396) and *H. australis* (AY148395), 97% between *H. filipjevi* (Did15) and *H. ustinovi* (AY148406), and 97% between *H. filipjevi* (E88) and *H. avenae* (HM560755). Hence, I explored other DNA regions. Because it was impossible to obtain amplification products for all investigated *Heterodera* species from the DNA regions of Hsp90 and β-tubulin, the actin 1 gene was the only option to establish a species-specific PCR assay to detect the three species. The sequences of the coding regions of the actin genes are known to be highly conserved. However, many intron positions of the actin genes have been reported to be variable between different species. Hence, the intron sequences were used reliably as discriminating markers for phylogenetic analysis (Ohresser *et al*., 1997; Donnelly *et al*., 1999; Lee & Gye, 2001; Kovaleva *et al.,* 2005). In this study, both forward and reverse primers of the species-specific PCR (HLAT-ACT), were located in two different introns (Figure 4.4).

The species-specific PCR (HLAT-ACT) detected all *H. latipons* populations used in this study, originating from different countries and regions. No positive reaction was observed with any of the 14 other cyst nematode species examined. Gradient PCR revealed that the primers are useful and specific over a wide range of the annealing temperatures between 47-55°C. This simplifies the use of the PCR assay at different labs without further optimisation. 50°C was selected as the annealing temperature because a very clear and bright specific band was obtained in these conditions.

The assay is capable of detecting five J2 of *H. latipons* either alone or in a mixture with DNA extracted from five or 100 J2 of *H. avenae*, confirming the efficiency of the primer set. The sensitivity of the PCR was high: up to 1/10 dilution of DNA obtained from five J2 of *H. latipons* showed a clear DNA band (204 bp), indication a detection limit of at

least 0.5 J2. However, the detection limit is above 0.1 J2, as the 1/50 dilution of 5 J2 did not provide a visible band.

The actin 1 gene was screened to establish a species-specific PCR for *H. avenae* and *H. filipjevi* detection. However, the *in silico* comparison showed high similarities in actin 1 gene sequences to occur between *Heterodera* species; actin 1 gene sequences of *H. filipjevi, H. avenae* and *H. hordecalis* are 95% to 96% similar. Hence, the actin 1 gene could not be used to design species-specific primers detecting *H. avenae* or *H. filipjevi*. Subsequentially, COI was screened and used for the PCR assay to detect *H. avenae* and *H. filipjevi*, and for the qPCR assay as well. However, only two COI sequences, one from *H. glycines* and one from *H. cardiolata* could be found in GenBank. Other COI sequences (*H. glycines* and *H. filipjevi*) were found in the sequence database of the Quarantine organisms Barcoding Of Life (QBOL). When comparing the obtained sequences with those sequences from the GenBank or from QBOL, the results showed a clear possibility for the selection of primers and probes. However, heteroplasmy of mtDNA, the existence of multiple mtDNA types within an individual, has been detected in the root-knot nematodes (*Meloidogyne* spp.) (Okimoto *et al.,* 1991), and was recently reported for *M. chitwoodi* (Humphreys-Pereira & Elling, 2013). It was demonstrated that mismatch on these polymorphic sites can severely reduce PCR efficiency (Stadhouders *et al*., 2010). Therefore, the heteroplasmy can cause the primer to fail to recognise its binding place or cause it to bind a non-target species. However, heteroplasmy was not found in the populations under investigation, and during this study, I did not encounter any problem as the two primers sets were species-specific and were able to detect all representatives of the targeted species originating from different countries and regions. Hence, it can be concluded that the selected COI primer binding places are present in all the *H. avenae* and *H. filipjevi* populations. Also, Kiewnick *et al.* (2014) did not detect heteroplasmy in the mtDNA region during their investigation of a *Meloidogyne* species complex, *M. enterolobii*, *M. hapla*, and *M. maritima*. So, they suggested the use of mtDNA region for the identification of root-knot nematodes.

Both newly designed primer sets were able to detect successfully all *H. filipjevi* and *H. avenae* populations that were used in this study. No positive reaction was observed for

any of the other cyst nematode species examined. PCR optimisation showed that both primers sets are useful and specific on a range of annealing temperatures (56-60°C). Because of the very clear and unambiguous specific band obtained at 58°C, and since this temperature is suitable for PCR with both primers sets, I selected this temperature as annealing temperature making it possible to detect both species separately in one run.

The *in silico* study showed no reason to anticipate that the mixing of the two species-specific primer sets would have the potential for hetero dimer-primer formation when used in the same reaction. However, primer-dimers were observed when the two primers sets were mixed in one PCR using different primer concentrations (data not shown). Because primer-dimers can influence the efficiency and hence also the sensitivity of the PCR, it is not recommended to use both primers sets in a duplex PCR. However, when used in separate PCRs, both primers sets were able to detect successfully five J2 of *H. avenae* or *H. filipjevi* either alone or in a mixture with 100 J2 of *H. latipons.* The sensitivity is even higher than five J2 since the equivalent of 1/10 of the DNA of five J2 yielded a clear band. From these results, it can be concluded that 0.5 J2 of *H. avenae* or *H. filipjevi* can be detected among 100 J2 of *H. latipons*.

The qPCR assay is a rapid and reliable diagnostic tool for quantitative detection of many organisms including nematodes (Fusco *et al.,* 2011). Several articles reported on the use of qPCR for the quantitative detection of potato cyst nematodes (Madani *et al.,* 2004; Toyota *et al.,* 2010; Nakhla *et al.,* 2010; Christoforou *et al.,* 2014; Reid *et al.*, 2015). While, so far, two papers reported the successful development and use of qPCR for the molecular quantitative detection of *Heterodera* spp., *i.e. H. schachtii* (Madani *et al.,* 2004) and *H. glycines* (Goto *et al.,* 2009). Both qPCR assays were based on the ITS-rDNA.

Due to the successful use of the COI gene for species-specific PCR assay to detect *H. avenae* and *H. filipjevi* (Toumi *et al.,* 2013b), the COI gene was a good candidate to be checked and used for the development of the qPCR assay. To my knowledge, this gene has never been used for quantitative detection (qPCR) of plant-parasitic nematodes. I succeeded in establishing two qPCR assays for the quantitative detection of *H. avenae* and *H. latipons*. The two assays resulted not only in high amplification efficiency, they

were also highly specific. They showed a single amplicon in melting curve analysis, which suggests that no undesired amplicons or primer-dimers were generated, even at lower annealing temperatures. No non-specific amplification was generated when using DNA from other species of the genus *Heterodera* using the optimised Ta for *H. latipons* at 62ºC and for *H. avenae* at 64ºC. Also, both assays were able to detect successfully all *H. latipons* and *H. avenae* populations that were used in this study. If the sample is composed of only the three-main species of CCN, then for both assays a lower Ta equalling 61ºC and 62ºC can be safely used. In that case, the assay for both species can be done in one run. However, mixing both qPCR primers sets in one reaction was not successful (data not shown). Therefore, it is not recommended to use both sets in a duplex qPCR (Oberhänsli *et al.,* 2011). Also, using the Ta at 61ºC and 62ºC when different additional *Heterodera* species is to be expected, is recommended as I observed positive signals, although with high Ct-values, for some *Heterodera* species including *H. pratensis*, *H. hordecalis* and *H. betae* (data not shown). Both qPCR assays were sensitive as they detected DNA of a single J2 of *H. latipons* and *H. avenae* when mixed with DNA from 100 J2 of *H. filipjevi*. However, mixing one J2 of *H. latipons* with DNA from 100 J2 of *H. filipjevi* resulted in a high Ct-value indicating a drop of efficiency of the qPCR assay. To assure the reliability of the assay, I recommend using samples representing no more than 50 J2 in the *H. latipons* qPCR assay. The result obtained in the sensitivity test compares well with findings reported for *H. schachtii* (Madani *et al.,* 2005). The qPCR assays run with the serial dilutions of DNA samples of both species, showed a highly significant linearity between the Ct-value and the dilution rate (R^2=0.99). Also, the particular strength of both qPCR assays is that they were able to detect an amount of DNA less than the amount of DNA in a single J2. The high qPCR efficiencies for both species can suggest the presence of inhibitors, originate either from the nematode sample; or from sample preparation prior to the qPCR; or both, in the concentrated DNA sample (see Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, www6.appliedbiosystems.com/support/tutorials/pdf/performing_rq_gene_exp_rtpcr.pdf). The DNA signal can be converted into an accurate estimation of the number of individuals involved. As the number of cells in an individual nematode increases during growth

(Cunha *et al.,* 1999), it is recommended to use the assay after the maturation of the cyst, when the majority of eggs contain second-stage juveniles (J2s).

It can be concluded that the designed qPCR assays are reliable, fast and sensitive. However, the two assays are designed and optimised to be used only on J2 released out of collected cysts and not on J2 free in soil samples. The second-stage juveniles can be easily released out of cysts or eggs by vigorously shaking the cysts in the presence of a few glass beads for 1 min at a frequency of 30 Hz in a bead-beater (RETSCH MM301). To be able to use the assays directly on soil samples, another validation test should be conducted. I have chosen not to do so because it is rather easy to extract cysts from soils than to isolate DNA from J2 from chemically complex soil samples. The method is particularly important for agricultural extension services where the skills to collect cysts are present, but the skills to identify the species might be limited. Additionally, the qPCR machine and probe are relatively expensive.

The assays I developed are the first species-specific PCRs for detecting any of the three species of the cereal cyst nematodes, and the first qPCRs for quantitative detection of *H. latipons* and *H. avenae*. I believe they are particularly important for agricultural extension services where the skills to identify the species are often limited. They allow a reliable and fast processing of several nematode samples in one day including DNA extraction, sensitive identification and, for *H. latipons* and *H. avenae*, also quantification. Due to the global distribution and the importance of the yield loss caused by *H. filipjevi,* it is recommended to develop a qPCR assay for its quantification and detection as well.

Chapter 5

Screening of Synthetic Wheat Lines for Resistance to the Cereal Cyst Nematode *Heterodera filipjevi*

5.1 Introduction

Many attempts have been made to control cereal cyst nematodes (*Heterodera* spp.; CCN) including agricultural practices, use of nematicides or biological control agents, and development of resistant cultivars (Smiley & Nicol, 2009; Dababat *et al.,* 2011). The use of resistant hosts for nematode management is considered cost-efficient, environmentally safe and user-friendly (Dababat *et al.,* 2014). The usefulness of resistance to CCN depends on the effectiveness and durability of the sources of resistance, the interaction of the specific putative resistant accessions and on the correct identification of the prevailing nematode species and/or pathotype(s) (Dababat *et al.,* 2014). Various species of *Triticum*, *Aegilops* and *Secale* have been screened through the wheat-breeding programmes around the world and were used as potential sources of resistance to CCN (Ogbonnaya *et al.,* 2001). Nine resistance genes to CCN, the *Cre* genes, have been reported from different sources. These genes confer resistance to different CCN pathotypes (Dababat *et al*., 2014).

Synthetic hexaploid wheat (SHW) genotypes were recreated from their two progenitor species: the tetraploid *T. turgidum* and its diploid wild relative *Ae. tauschii*. *Aegilops tauschii* is a useful resource of new genes for hexaploid wheat improvement. These include genes coding for many productivity traits such as resistant/tolerant for abiotic (drought, heat, salinity, waterlogging) and biotic (rusts, barley yellow dwarf virus, crown rot, nematodes and powdery mildew) stress factors, as well as novel grain quality traits (Ogbonnaya *et al*., 2013). On the other hand, many studies have indicated inconsistency of the traits in diploid (*Ae. tauschii*) and allohexaploid (SHW) levels, suggesting the difficulty of selection of useful traits at diploid level. Furthermore, the expected traits of SHWs may not always appear in the synthetic back-cross-derived lines produced by backcrosses with elite wheat cultivars. This is because the genetic background of SHWs is largely different from that of elite cultivars (Ogbonnaya *et al*., 2013).

The International Winter Wheat Improvement Programme (IWWIP; www.iwwip.org) is a cooperative breeding programme of the Turkish Ministry of Food Agriculture and Livestock, the International Maize and Wheat Improvement Centre

(CIMMYT), and the International Centre for Agricultural Research in the Dry Areas (ICARDA). IWWIP aims to develop broadly adapted lines for irrigated and semi-arid areas of Central and West Asia. The new advanced lines from IWWIP and lines submitted by co-operators are distributed annually to more than 150 breeding programmes in 50 countries including Syria, where *H. filipjevi*, one of the major CCN, is expanding in the major areas of cereal production (Abidou *et al.*, 2005a; see Chapter 3).

In view of this expansion of *H. filipjevi*, it is very important to devise strategies for nematode management with the main emphasis on breeding for nematode resistant cultivars. Therefore, the objectives of this study were: (1) to phenotype synthetic winter wheat lines obtained from IWWIP for root traits and host suitability to *H. filipjevi* (2) to assess the genetic structure of these synthetic wheat lines using AFLP markers; and (3) to identify lines with resistance to *H. filipjevi* and place them within the framework of the observed genetic diversity. Ultimately, this should lead to identifying new sources of resistance to *H. filipjevi* in wheat lines, which will be beneficial to the breeding programmes around the globe.

5.2 Materials and methods

5.2.1 Screening assay of wheat lines

5.2.1.1 Nematode inoculum

Cysts of *H. filipjevi* were extracted from an infested field in Yerkoy, Kirsehir, Turkey (39°39′709″N, 34°25′515″E) in July 2014 at the end of the wheat-growing season. The pathotype of the population was identified as Ha33 (Toktay *et al.*, 2013). Cyst nematodes were extracted using Cobb’s decanting and sieving method (Cobb, 1918). Their identity was confirmed using species-specific PCR (chapter 4). Extracted cysts were surface sterilised with 0.5% NaOCl for 10 min and rinsed 3-5 times with distilled water (modified from Nitao *et al.*, 1999). The cysts were kept in distilled water and placed in a refrigerator at 4°C for 4-5 weeks before being used as a source of inoculum. To enhance hatching, cysts were transferred between fridge (16 h) and room temperature (8 h) during 3 days before collecting the freshly hatched (<7 days old) second-stage juveniles (J2) which were used as inoculum in the screening tests (Sahin *et al.,* 2010).

5.2.1.2 Wheat lines

A total of 217 synthetic winter wheat lines from three separate groups, located in Turkey, were obtained from IWWIP (Appendix 1). Group 1 consisted of 102 lines, which were primary hexaploid synthetics. They originated from crosses between Ukrainian and Romanian winter durum wheat varieties/breeding lines as female parents and *Ae. tauschii* accessions from Iran and Azerbaijan as male parents. In 2004, six winter durum genotypes were crossed with 11 *Ae. tauschii* accessions in Mexico (Appendix 1). Both F1 and F2 were grown in Mexico in bulk, and the F2 seeds were made available to IWWIP (Eskisehir, Turkey). Individual selections of the plants with high fertility and disease resistance were made in F3 and F4. Resulting F5 progenies of individual spikes were bulked to obtain constant lines representing 15 crosses (Appendix 1), which were used in this study as group 1.

Genotypes from group 2 contained 69 lines. These were developed from single crosses between primary synthetics (group 1) and winter bread wheat varieties commonly grown in Turkey and the surrounding countries (varieties: Demir, Adyr, Gerek, Mezgit-6, Sonmez, Ekiz, Bagci and Katea). The crosses were made in 2009, F1 was grown in Izmir, and F2 populations were grown in Eskisehir and bulked. Individual plant selections were made in Diyarbakir in F3 in 2012 and F4 headrows were bulk harvested in Eskisehir in 2013 to obtain constant lines representing 8 crosses that were used in this study.

Group 3 consisted of 46 lines; all lines were represented by primary hexaploid synthetics developed at Kyoto University in Japan as described by Matsuoka *et al.* (2007). The durum parent in these synthetics was the variety ‘Langdon’ from the USA. *Aegilops tauschii* parents were selected based on a study of the genetic diversity of the species collection at Kyoto University (Dreisigacker *et al.*, 2008). There were 46 different accessions of *Ae. tauschii* used to develop synthetics; they represented a wide geographic area including Afghanistan, Armenia, China, Georgia, Iran, Kyrgyzstan, Syria, Turkey and Turkmenistan.

Two susceptible varieties (Bezostaya and Kutluk) and two moderately resistant varieties (Katea and Sonmez) were included in each group as references for classification (Dababat *et al.*, 2014).

5.2.1.3 Screening assay of wheat lines and their root phenotyping

All 217 lines were screened twice for resistance to *H. filipjevi* under the same growth chamber conditions at the Transitional Zone Agricultural Research Institute (TZARI), Eskisehir, Turkey. Standardised small tubes (16 cm high × 2.5 cm diam., Ray Leach Cone-tainer™; Stuewe & Sons, USA) filled with a sterilised mixture of sand, field soil, and organic matter of plant residues (70:29:1, v/v/v), were used in the screening experiment. The field soil and sand were sieved and sterilised at 110°C for 2 h and for 2 successive days, respectively; the organic matter was sterilised at 70°C for 5 h (Dababat *et al.*, 2014). Seeds were germinated for 3 days at 22°C in Petri dishes lined with moistened tissue. Eventually, a single pre-germinated seed was planted per tube. Each tube was inoculated with 250 freshly hatched J2 (4 J2 cm^{-3}) of *H. filipjevi* in 1 ml distilled water injected into 3 holes made around the stem base (Pariyar *et al.*, 2016a). Plants were gently watered after nematode inoculation and were watered whenever needed during the experiment. The plants were kept in a growth chamber at 22 ± 3°C, a photoperiod of 16 h, and RH = 70%. Three plants of each line including both reference varieties were assessed; tubes were arranged in a randomised complete block design and kept in a 200-tubes rack (RL200; Ray Leach Cone-tainer™). The experiment was terminated 9 weeks after nematode inoculation. Cysts were then extracted from soil by Cobb's decanting and sieving method (Cobb, 1918). Dry soil was collected from each tube in a 0.5-L beaker filled with water. The soil suspension was stirred and left for about 30 s to allow the heavy sand and soil debris to settle. Eventually, the content was poured through 850- and 250-μm sieves. This process was repeated three times to ensure all cysts were collected. Also, roots were washed gently on the upper sieve to free cysts attached to the roots; roots were examined under the microscope to confirm removed of cysts. Cysts from both roots and soil were retained on a 250-μm sieve and counted under a dissecting microscope.

Washed roots were scanned (WinRHIZO 2009c, Regent Instruments Inc.) and the following data were collected for each plant: root length (cm), root surface (cm^2), root volume (cm^3) and the number of root tips. Based on the total number of cysts collected from both roots and soil per plant, all lines were classified into 5 categories using the following ranking on a per plant basis: resistant (R) = <5 cysts; moderately resistant (MR) = 5-10 cysts; moderately susceptible (MS) = 11-15 cysts; susceptible (S) = 16-19 cysts; and highly susceptible (HS) ≥ 20 cysts (Pariyar *et al*., 2016a). Additionally, all lines were classified in another way into 2 categories using the following ranking on a per plant basis: resistant (R) = <3 cysts; and susceptible (S) ≥ 3 cysts (Andersen & Andersen, 1982).

5.2.2 Molecular analysis of the genetic diversity of the wheat lines

5.2.2.1 DNA preparation

The plants used for DNA extraction were grown in a greenhouse using pots filled with 500 g sieved and sterilised field soil (as in 5.2.1.3). Each pot was planted with a single seed of one of the wheat lines. When plants reached approximately 25 cm, 1 g fresh young leaves of each line was cut and immediately immersed in liquid nitrogen. Leaves were kept at -80ºC until lyophilisation. DNA extraction was done according to a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). DNA concentration and quality were determined using a UV spectrophotometer (Nanodrop ND-1000, Isogen Life Sciences). The extracted DNA was stored at –20°C for future use.

5.2.2.2 AFLP analysis

The analysis of AFLP markers were performed according to Vos *et al.* (1995) and Peng *et al*. (2000) with some modifications (De Riek *et al.,* 2001). Genomic DNA was digested for 2 h at 37°C with two combinations of restriction enzymes: *Eco*RI + *Mse*I and *Pst*I + *Mse*I. The final digestion volume of 25 µl contained 300 ng DNA, 1 x reaction buffer, 2.5 U *Mse*I (New England Biolabs), and 2.55 U *Eco*RI (Invitrogen) or 2.5 U *Pst*I (Invitrogen). Next, the fragments were ligated with adapters for 2 h at 37°C. The 25-µl

adapter ligation mixture contained 1 U of T4 DNA-ligase (Invitrogen) and 10 mM MgAc, 50 mM KAc, 10 mM Tris-HCl pH 7.5, 0.4 mM ATP (Invitrogen), 50 pmol *Mse*I-adapter and 5 pmol *Eco*RI- or *Pst*I-adapter. The quality of the restriction-adapter ligation was checked by loading 2 µl on a 1.5% agarose gel in 1 x Tris Acetate EDTA (TAE) along with a λ*Pst* marker for 1 h. Pre-amplification was done using 5 µl of the undiluted (*Pst*I) or 1/10 diluted (*EcoR*I) in TE (10/0.1) (TE: 10 mM Tris-Cl, pH 8.0; 0.1 mM EDTA) restriction-adapter ligation mixture. Pre-amplification was done using primers with one selective nucleotide (*Eco*RI+A, *Pst*I+G and *Pst*I+A in combination with *Mse*I+C) in a final volume of 50 µl containing 1 x GoTaqG2 Flexi Buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs, 0.1 µM of each primer, 0.025U GoTaq® G2 Flexi DNA Polymerase (Promega) on an Applied Biosystems thermocycler (GeneAmp® 9700). The pre-amplification settings were: 25 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min. To check the quality of the pre-amplification results, 5 µl of each sample was loaded again on a 1.5% agarose gel. To lower the cost and save time, 10 samples were selected randomly, and the AFLP analysis was completed using 13 primer combinations (PC) for the selective amplification. The 13 primer combinations were: *E*-AGG/*M*-CTT, *E*-AGC/*M*-CTT, *E*-ACG/*M*-CAA, *E*-AAC/*M*-CAT, *E*-ACC/*M*-CAT, *P*-GCT/*M*-CAG, *P*-GCT/*M*-CAA, *P*-GTT/*M*-CAT, *P*-ACA/*M*-CTT, *P*-ACG/*M*-CAT, *P*-ACG/*M*-CAA, *P-GGT/M-CAA,* and *P-GGT/M-CAT.* Finally, and based on the level of polymorphism detected, 4 primer combinations (PC1: *E*-AGG/*M*-CTT, PC2: *E*-ACG/*M*-CAA, PC3: *P*-GCT/*M*-CAA and PC4: *P*-GCT/*M*-CAG) were selected for the selective amplification on the entire dataset. The *Pst*I primers were labelled with 6-carboxyfluorescein (6-FAM), and the *Eco*RI primers were labelled with hexachloro-6-carboxy-fluorescein (HEX). The selective amplification was performed in 20-µl reaction volumes that contained 3 µl 1/10 diluted (*EcoR*I) or undiluted (*Pst*I) pre-amplification product, 1 x PCR-buffer (Applied Biosystems), 0.2 mM dNTPs, 0.0675 µM *Eco*RI- or *Pst*I-primer, 0.25 µM *Mse*I-primer and 0.03 U ampliTaq-polymerase (Applied Biosystems). The selective amplification was carried out on a normal Applied Biosystems thermocycler (GeneAmp® 9700) with 1 cycle at 94°C for 2 min, 65°C for 30 s, 72°C for 2 min; followed by 8 cycles of a “touchdown” profile with the annealing temperature decreasing at 1°C $cycle^{-1}$, and a final step of 23 cycles of 94°C for 1 s, 56°C for 30 s, 72°C for 2 min. AFLP

fragments were separated on an ABI3130*xl* Genetic Analyzer (Applied Biosystems) as described by De Keyser *et al.* (2010).

5.2.3 Statistical analysis

The normality of the root data and number of cysts was checked before analysis of variance (ANOVA) with SPSS 22 (IBM Corp, 2012), and the non-normally distributed data were transformed into a normal distribution using log(x+1). Then, the normalised data of the two runs were combined, resulting in 6 replications for each line, and a one-way ANOVA was carried out for each group separately, each time including the references. Whenever there was a significant difference ($P \leq 0.05$) in the number of cysts, root length, root surface, root volume or the number of root tips between lines of the same group, a post hoc-test was performed for mean separation using the Student-Newman-Keuls method.

Within each group separately, the pairwise Pearson correlation between the number of cysts and the root parameters (root length, root surface, root volume and the number of root tips) was evaluated. Additionally, the pairwise correlation between all root parameters was evaluated.

For the collected AFLP data, presence and absence of bands were scored as 1 and 0, respectively. A selection was made towards the most polymorphic set of markers, i.e. polymorphic markers lower 15% presence in the population and above than 85% were deleted. In order to understand the genetic diversity between all lines, Jaccard similarity was measured between all lines, and the Principal Coordinate Analysis (PCoA) based on molecular data was designed as well. Also, to measure the quality or informativeness of a polymorphism as a genetic marker, the polymorphic information content (PIC) values were calculated for each primer combination over all genotypes. Based on Ward 's method, a dendrogram of genetic distance (dissimilarity) was established.

5.3 Results

5.3.1 Screening assay evaluation

5.3.1.1 Evaluation of resistance to *H. filipjevi*

Depending on the rating scale of Pariyar *et al.* (2016a), the screening of 217 lines of synthetic winter wheat resulted in identifying 16 phenotypes (7%) as resistant (R), 63 phenotypes (29%) as moderately resistant (MR), 82 phenotypes (38%) as moderately susceptible (MS), 41 phenotypes (19%) as susceptible (S), and 15 phenotypes (7%) as highly susceptible (HS) to *H. filipjevi* (Figure 5.1). The number of cysts produced on the 217 breeding lines ranged between 0-57 cysts plant^{-1}. On the two moderately resistant reference varieties (Katea and Sonmez) between 2-17 cysts plant^{-1} were counted, and on the susceptible varieties (Bezostaya and Kutluk) 11-37 cysts plant^{-1} were found.

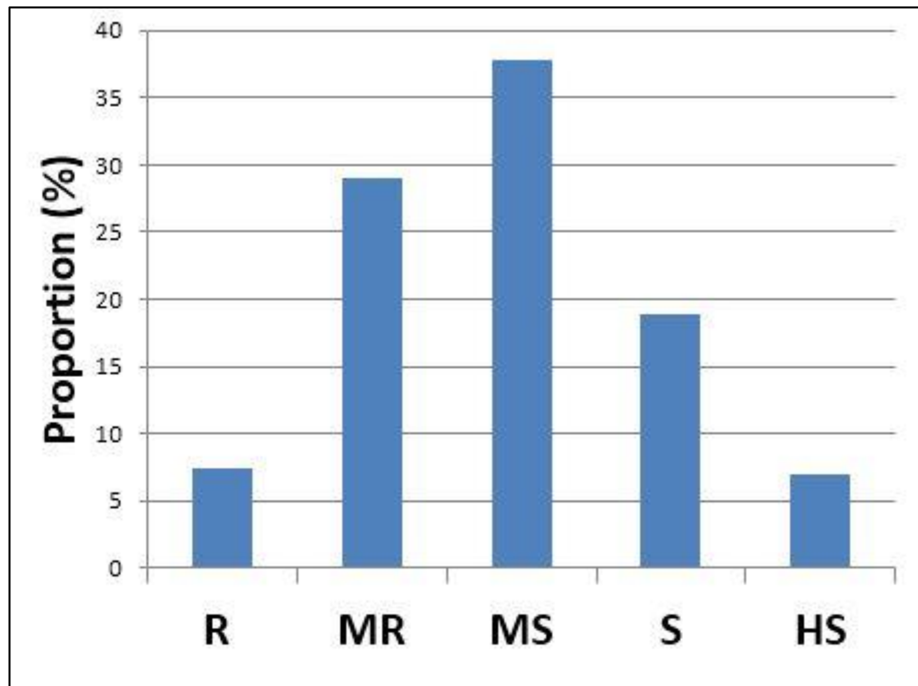

Figure 5.1. The proportion (%) of synthetic winter wheat lines' resistance response to *Heterodera filipjevi* classified as resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S), and highly susceptible (HS) based on the scoring scale of Pariyar *et al.* (2016a) (see 5.2.1.3).

Most of the genotypes showing R (15) and MR (27) response were observed in the lines of group 3 (46 lines). Similarly, less R (1) and MR (17) genotypes were found in group 2 (69 lines), and in group 1 (0 R and 19 MR out of 102 lines) (Figure 5.2). The proportion of both R and MR lines for the three groups 3, 2, and 1 was thus 91%, 26% and 16%, respectively (Figure 5.2).

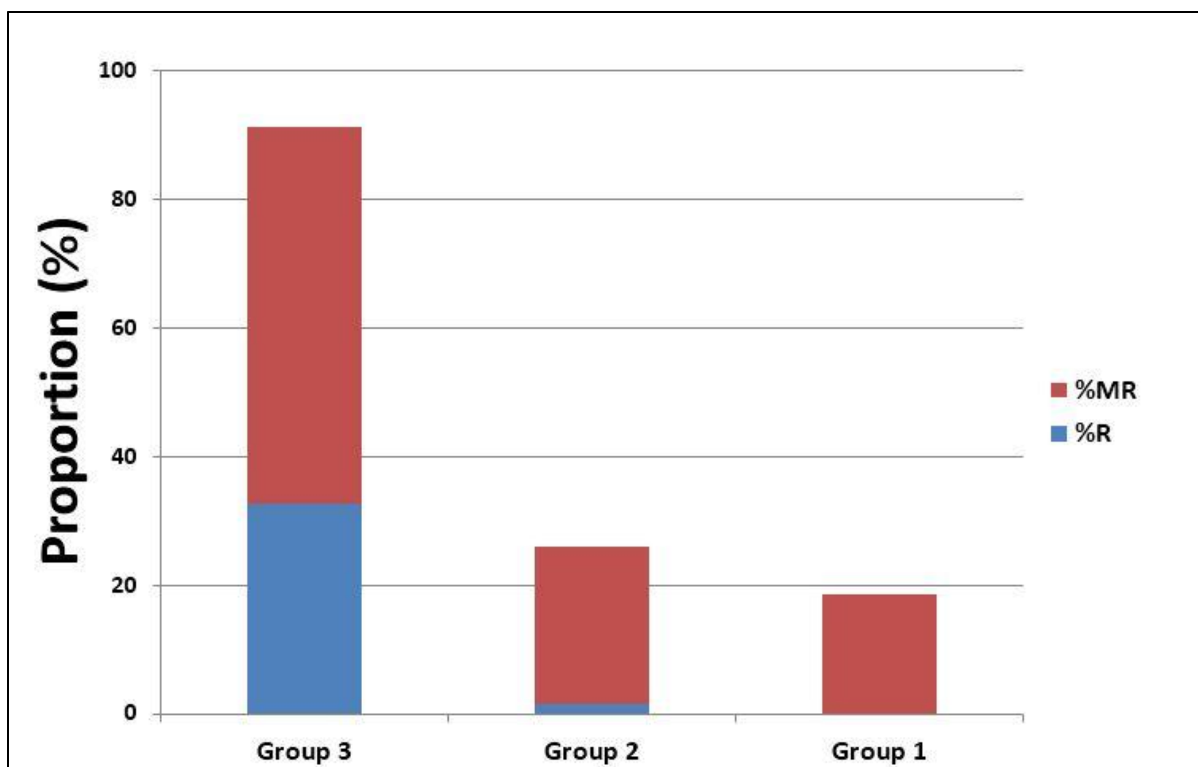

Figure 5.2. The cumulative proportion (%) of lines resistant (R) and moderately resistant (MR) to *Heterodera filipjevi* in the three groups of synthetic winter wheat (see 5.2.1.3).

5.3.1.2 Statistical analysis of the screening assay of synthetic wheat lines

The screening result of the 217 lines according to the rating score of Andersen & Andersen (1982) showed only 3 (1.4%) lines (75, 202 and 203) as resistant (R) and all other lines (98.6%) as susceptible (S) (Appendix 2). Scoring the synthetic wheat lines for resistance to *H. filipjevi* based on Pariyar *et al.*, (2016a), revealed different levels of susceptibility among the tested genotypes. There was variation in the number of cysts amongst lines of the three groups. Among all lines of group 1, line 75 contained the lowest number of cysts per plant (1.3±2.2) and differed significantly from the majority of all lines of group 1 (Appendix 2). Also, other three lines 7, 64 and 104 had significantly lower number of cysts per plant than the other lines of the same group (6±4.9, 5.8±5.7 and 7.2±6.3, respectively). The number of cysts per plant of all lines of group 2 was not significantly different (Appendix 2). The greatest average number of cysts per plant (28±6) was found in lines 70 in group 2, which was greater than the susceptible references Bezostaya (25±9 cysts $plant^{-1}$) and Kutluk (23±7 cysts $plant^{-1}$), although not statistically

significant (Appendix 2). In group 3, line 230 had significantly more cysts (24.8±9.5) than the other 24 lines (range: 0.5±1.2 - 5.7±3.3) in this group whereas line 202 (0.5±1.2) had a significantly lower number of cysts compared to the other 24 lines of group 3 (range: 5.7±3.3 - 24.8±9.5). In group 1, the root length of line 28 (268±234 cm) was significantly shorter than lines 111 (956±138 cm) and 113 (959±190 cm) (Appendix 2). Line 84 had a significantly higher root surface (94±18 cm^2) than both lines 22, 28 and 97 (24±4, 17±13 and 25±1 cm^2). While, line 28 (17±13 cm^2) had a significantly lower root surface than 82, 83, 111, 112 and 113 (range: 87±43 cm^2 – 91±18 cm^2). Also, line 28 had significantly smaller (0.1±0.1 cm^3) root volume than the other three lines 83, 84 and 112 (0.8±0.3 cm^3).

Regarding the collected root data of group 2, the root length of the lines 68, 73 and 74 (334±114 cm, 429±100 cm and 415±13 cm, respectively) had significantly shorter roots compared with almost half of other lines of the same group (range: 733±92 cm - 955±150 cm). However, the line 127 had significantly longer root length (955±150 cm) than 71, 138, 154 and 181 (range: 524±104 cm - 540±128 cm). Lines 68, 73, 74 and 178 (range: 22±10 cm^2 - 41±20 cm^2) had significantly less roots surface than lines 123, 127, 133, 161, 166 and 171 (range: 89±5 cm^2 - 100±17 cm^2). In the same group, the three lines (68, 73 and 74) which showed less root surface, also showed smaller root volume (0.1±0.1 cm^3 0.3±0 cm^3 0.2±0.1 cm^3, respectively) when compared with 123, 127, 133 and 166 (range: 0.8±0.1 cm^3- 0.8±0.2 cm^3). However, lines 123, 127 and 133 (0.8±0.2 cm^3, 0.8±0.2 cm^3 and 0.8±0.2 cm^3, respectively) had significantly bigger root volume than 138, 144, 147, 154, 178 and 181 (range: 0.3±0.1 cm^3 - 0.3±0.1 cm^3). In group 2, the number of root tips in lines 176 (2320±673) was significantly higher than the number of root tips in lines 149, 165 and 173 (951±149, 986±244 and 954±519, respectively), while the other lines did not differ significantly from each other.

Within group 3, root length of line 229 (969±156 cm) differed significantly from 191, 211 and 213 (360±79, 384±65 and 294±147 cm, respectively). Root volume of the line 224 (0.8±0.2 cm^3) was significantly bigger than lines 191, 192, 207, 208, 211, 212, 213, 215, 216 and 228, which ranged between (0.2±0.1 cm^3) and (0.33±0.1 cm^3). More differences of the root volume were found between 199, 218, and 222 (0.8±0.4 cm^3) and lines 191, 192, 208, 211, 212, 213, 215, 216 and 228 (range: (0.2±0.1 cm^3) - (0.31±0.1

cm^3)). However, lines 191 (0.2±0.1 cm^3) and 213 (0.2±0.2 cm^3) had significantly less root volume compared to 199, 201, 218, 220, 222, 223 and 224 (Range: (0.7±0.1 cm^3) - (0.8±0.2 cm^3)). Two lines (202 and 229) showed significantly more root tips (3231±2235 and 2947±809, respectively) than any other lines in group 3. The number of root tips for other lines ranged between 849 and 1943.

5.3.1.3 Evaluation of relation between number of cysts and root variables

There was a significant correlation between the number of cysts and the root length, root surface and root volume in group 1 ($r = 0.11$, 0.14 and 0.15, respectively), number of cysts and the number of root tips in group 2 ($r = 0.21$), and between the number of cysts and the root length and root volume in group 3 ($r = 0.25$ and 0.19, respectively) (Table 5.1). However, the correlation in the three groups was low; r equalled 0.25 at most. In group 1, a highly significant positive correlation was between both the root surface and root length ($r = 0.87$), and between the root surface and root volume ($r = 0.95$). Separately, both variables of root volume and root length were positively linearly related to the root surface. Also, a positive and significant correlation was between root volume and root length ($r = 0.67$). In the same group, a high significant correlation was shown between the number of root tips and other root parameters of root length, root surface and root volume ($r = 0.23$, 0.19 and 0.16, respectively) (Table 5.1). In group 2, a highly significant positive correlation was between root length and root surface ($r = 0.9$), and between the root length and root volume ($r = 0.76$), also a significant low correlation between the root length and the number of tips ($r = 0.14$). Additionally, significantly high correlation was between the root volume and the root surface ($r = 0.96$), and the correlation was a strong uphill linear relationship (Table 5.1). A highly significant correlation in group 3 was found between root volume and root length ($r = 0.47$) and between root volume and root surface ($r = 0.61$) (Table 5.1).

The statistical analysis of the screening for resistance to *H. filipjevi* divided all lines in the three groups into different classes labelled with different letters (Appendix 2). However, these components of these classes differed from those in the five categories of susceptibility (R, MR, MS, S and HS).

Table 5.1 Pairwise Pearson correlation coefficients (r) and their significance level (p-value) between the collected root data and the number of cysts in the three groups.

Pearson Correlation Coefficients						
Group 1		**Cysts number**	**Root length**	**Root surface**	**Root volume**	**Number of tips**
Cysts number	**r***	1.00				
	P**					
Root length	**r**	**0.11*****	1.00			
	P	0.05				
Root surface	**r**	**0.14**	**0.87**	1.00		
	P	0.01	<.0001			
Root volume	**r**	**0.15**	**0.67**	**0.95**	1.00	
	P	0.008	<.0001	<.0001		
Number of tips	**r**	-0.05	**0.23**	**0.19**	**0.16**	1.00
	P	0.37	<.0001	0.0006	0.005	
Group 2		**Cysts number**	**Root length**	**Root surface**	**Root volume**	**Number of tips**
Cysts number	**r**	1.00				
	P					
Root length	**r**	0.005	1.00			
	P	0.94				
Root surface	**r**	-0.02	**0.9**	1.00		
	P	0.54	<.0001			

Root volume	**r**	-0.03	**0.76**	**0.96**	1.00	
	P	0.69	<.0001	<.0001		
Number of tips	**r**	**0.21**	**0.14**	-0.007	-0.07	1.00
	P	0.003	0.05	0.9	0.3	
Group 3		**Cysts number**	**Root length**	**Root surface**	**Root volume**	**Number of tips**
Cysts number	**r**	1.00				
	P					
Root length	**r**	**0.25**	1.00			
	P	0.003				
Root surface	**r**	0.06	0.11	1.00		
	P	0.51	0.18			
Root volume	**r**	**0.19**	**0.47**	**0.61**	1.00	
	P	0.03	<.0001	<.0001		
Number of tips	**r**	0.12	0.15	0.10	0.01	1.00
	P	0.17	0.07	0.24	0.88	

*r: correlation coefficient.
**P: Probability
***Values in bold are significantly correlated at the 0.05 level.

5.3.2 Genotyping diversity of the lines AFLP analysis

All 4 AFLP primer combinations detected polymorphisms between the studied wheat lines. A total of 82, 65, 56 and 34 fragments were amplified for the 4 primer combinations and the polymorphic bands between all lines were 23%, 17%, 22% and 16% for the 4 primer combinations (PC1, PC2, PC3 and PC4, respectively), where PC1 resulted the highest proportion of the polymorphic bands (Table 5.2).

Table 5.2 The number and genomic distribution of AFLP fragments generated from each of the 4 primer combinations on all the 217 synthetic winter wheat lines.

primer combination	Number of polymorphic fragments	Number of scored bands	% of polymorphic bands
PC1: *E*-AGG/*M*-CTT	82	357	23
PC2: *E*-ACG/*M*-CAA	65	383	17
PC3: *P*-GCT/*M*-CAA	56	257	22
PC4: *P*-GCT/*M*-CAG	34	212	16

Results of the principal coordinate analysis based on molecular data are shown in a biplot on the first 2 factors of the PCoA of the AFLP DNA markers (Figure 5.3). Factor 1 and factor 2 explained 42.55% and 19.30% of the variation, respectively. Clear evidence of the genetic diversity among the resistant lines in the three groups can be observed. Moreover, the total number of R and MR lines derived from the group 3 were the most distinct from other two groups (Appendix 2). Some lines belonging to group 1 (5, 7, 8, 15, 21, 31, 59, 63, 64, 75, 82, 107, 108 and 118), group 2 (142, 144, 148, 149, 152, 160, 163 and 174) and group 3 (185, 186, 188,187, 190, 191, 192, 195, 196, 197, 198, 203, 204, 205, 206, 207, 211, 213, 215, 217, 220, 224, 225, 226) showed fewer developed cysts, and they were excluded from the group in the biplot (Figure 5.3).

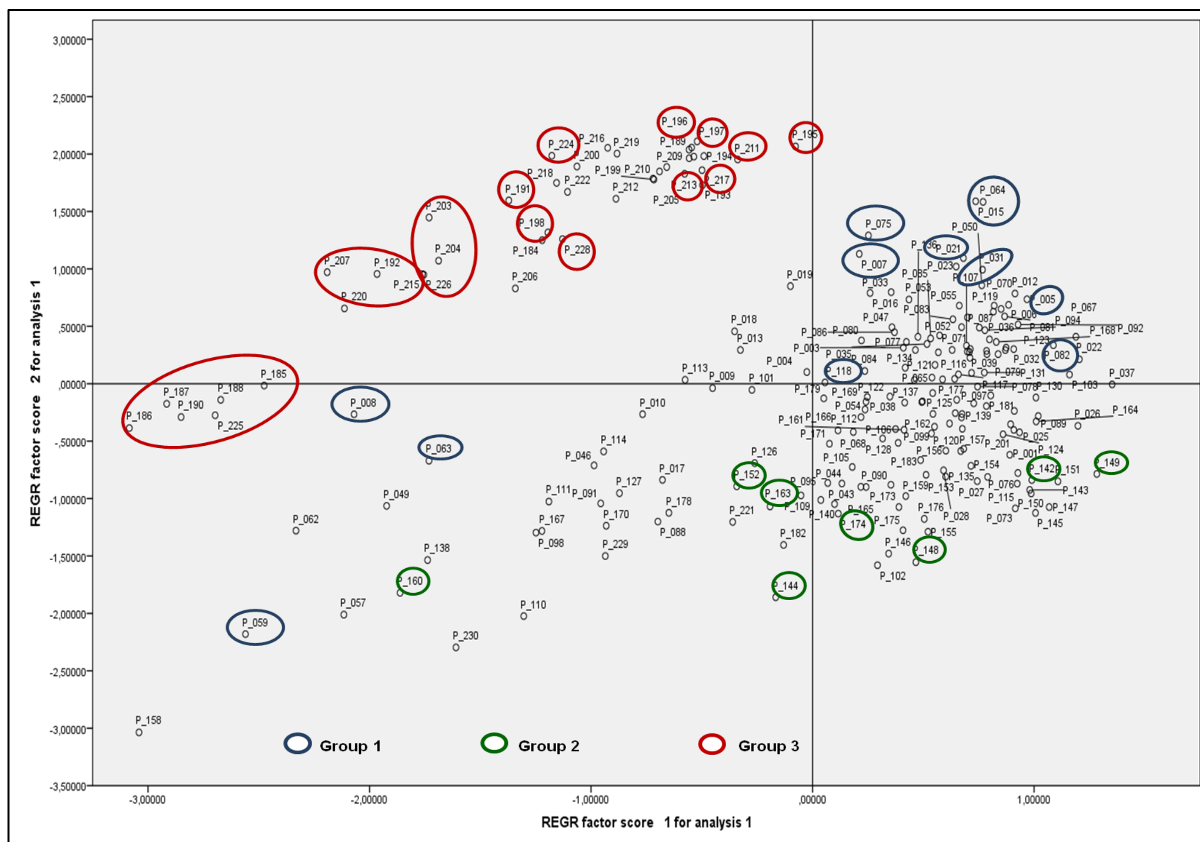

Figure 5.3. Biplot of principal coordinate analysis (PCoA) of the 217 lines of synthetic wheat based on AFLP data. Circled lines in different colours were classified as Resistant (R) and Moderately Resistant (MR) to *Heterodera filipjevi* in the three groups (see Appendix 1 for codes).

The polymorphic information content values for the four primers combinations (PIC1, PIC2, PIC3 and PIC4) were 0.34, 0.41, 0.40 and 0.36, respectively.

A dendrogram, established using the genetic distance index between the different lines, showed that all samples were regrouped into four well-separated clusters (Figure 5.4). The first cluster (cluster 1) included all lines from group 3 having in common the Langdon parent. However, three lines (229, 230 and 183) were clustered separately in the other clusters. Lines from group 3 of *Ae. tauschii* from Iran closely came together in subgroups in cluster 1. However, the majority of the lines from other countries, irrespective of their geographical origin were clustered together in subgroups and the geography did not have much effect on the clustering of lines originated from Turkey,

Syria, Kyrgyzstan and Afghanistan. Cluster 2 and 4 have the majority of lines belong to group 1, and cluster 3 contain the majority of lines belong to group 2. All lines of the cross 03 of group 1 and the majority of cross 05 lines of the same group tend to group together in cluster 4 (Appendix 1). Nevertheless, some lines of the cross (05) were spread in both clusters 3 and 2. Similarly, the lines of Ukrainian durum parent LEUC (cross 09 – 10 – 12), and the lines of another Ukrainian durum parent UKR.OD. (cross 71 – 73 – 74 – 76 – 78 – 22 – 61 – 68) were mainly clustered together in cluster 3, and rarely in cluster 2 and 4. Additionally, it was expected for lines derived from both crosses 22 and 71 to be similar since they have same common *Aegilops* accession (*Ae. tauschii* 392), but they did not come in one cluster and were distributed in the other three clusters 2, 3 and 4. While an opposite response was observed for the parent Pandur (cross 79 and 81) which is different from Ukrainian parents originated from Romania. The dendrogram shows that lines were appeared close in cluster 2 and 4.

The lines of group 2 of the Ukrainian durum parent AISBERG (cross 54) were grouped in sub-cluster in cluster 4 with lines of group 1 of the same parent (cross 03) including the line (75) which showed an interesting level of resistance (R) among the other lines of both crosses. However, lines of the same parent cross (05) were not present along. In the same group 2, the lines of the parent LEUC (cross 59 and 61) came together in cluster 4, and the lines of the parent LEUC (cross 12) of group 1 join them in the same cluster. The same remark was noticed for the Ukrainian durum parent UKR.OD (cross 71) of group 1 with lines of the same parent (cross 71) of group 2 where the lines located in cluster 3. The Ukrainian durum parent UKR.OD had similar lines (cross 64 – 66 – 71 – 72 – 74), and the majority of the lines closely appeared in cluster 3.

In the three groups, markers were not able to clearly differentiate the lines according to the resistance level as in the scoring scale (R, MR, MS, S, and HS) in a cluster or in sub-clusters. Additionally, sometime some lines of the same cross did not locate in the same cluster or in sub-clusters as well.

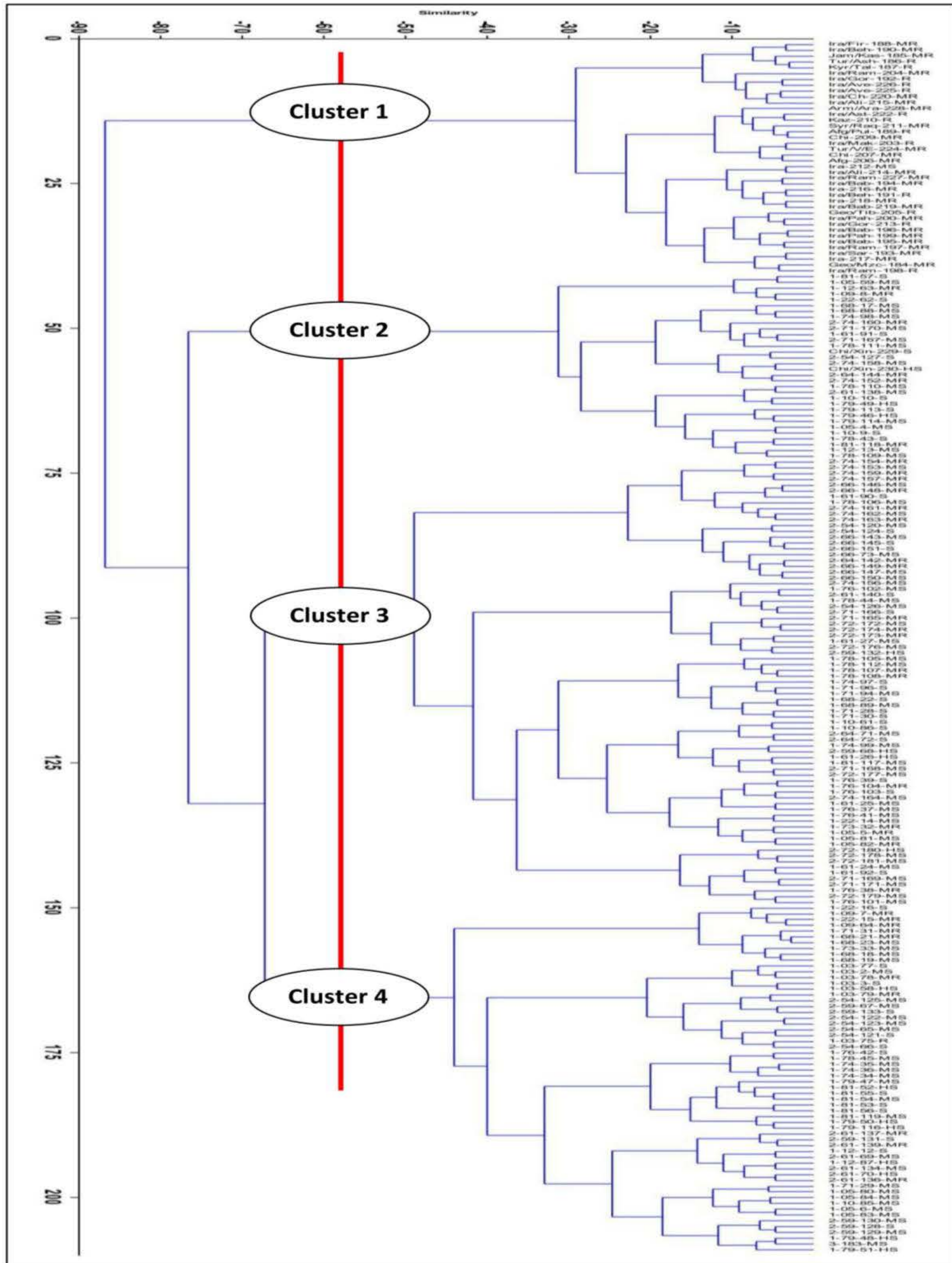

Figure 5.4. Dendrogram of all lines of the three groups based on the Ward's method (see Appendix 1 for the code). The lines belong to group 1 and 2 were named: group number-cross ID-number of the entry-Resistance level. The lines belong to group 3 were named: Country/site-number of the entry-Resistance level).

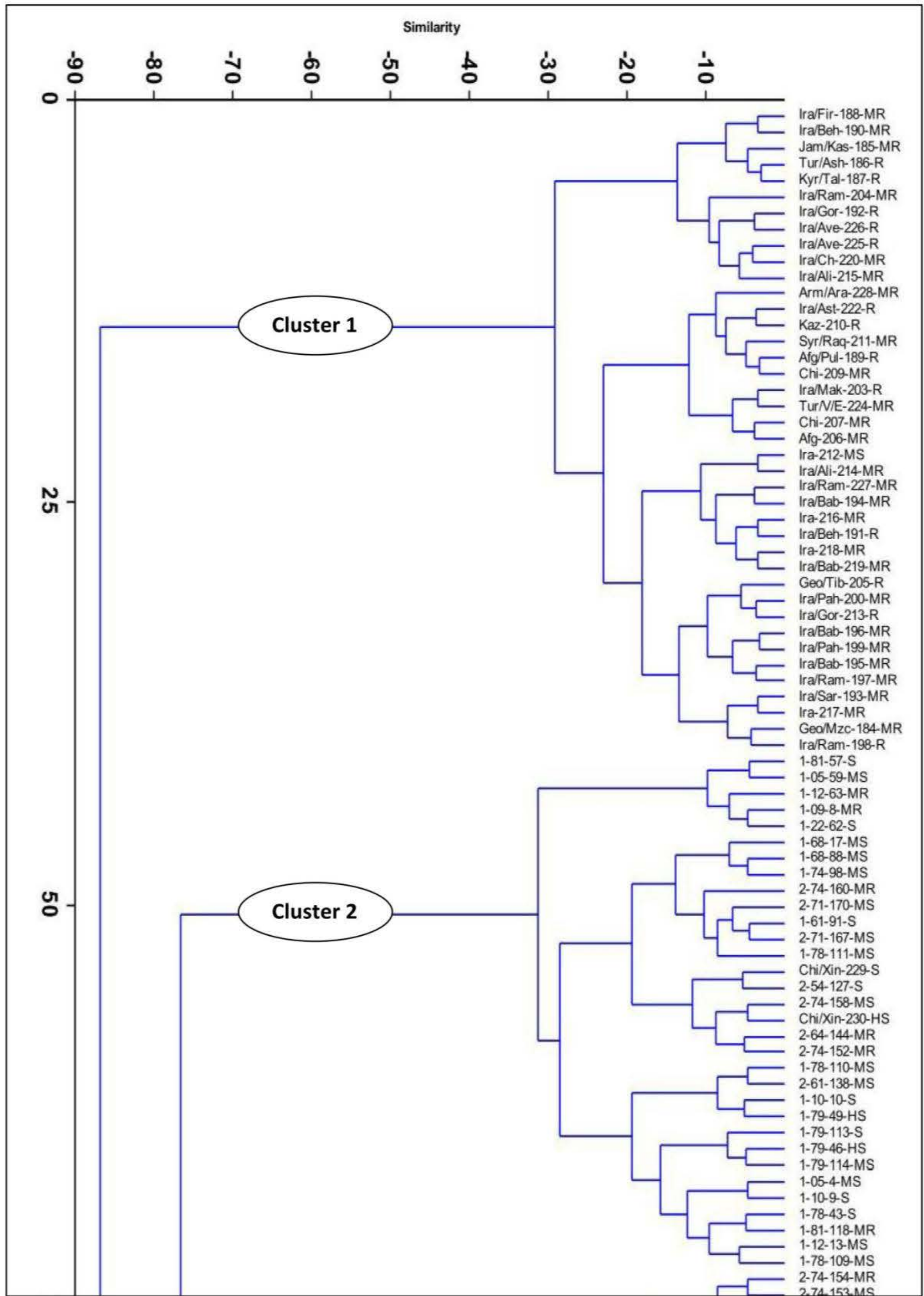

Figure 5.4. continued

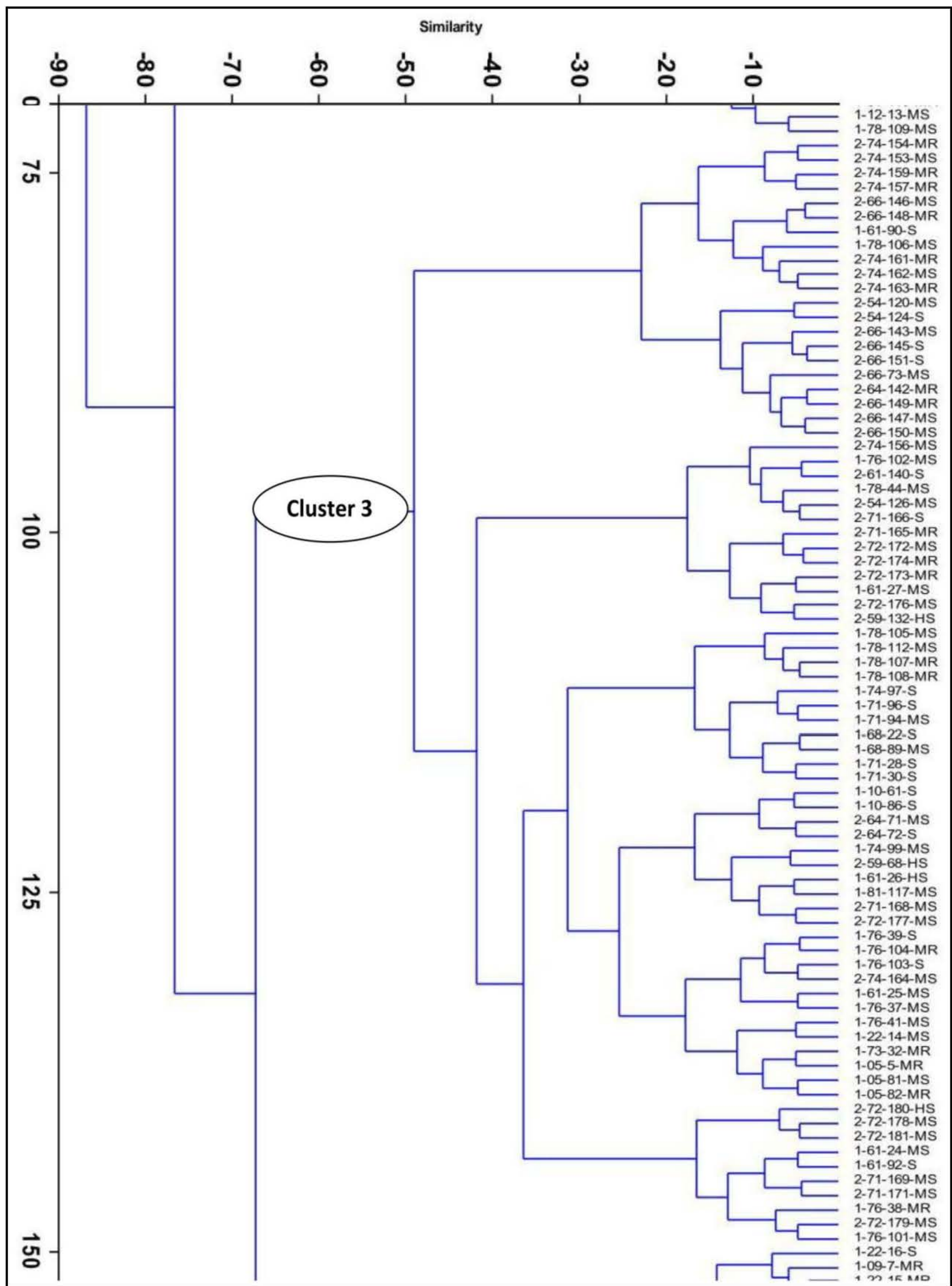

Figure 5.4. continued

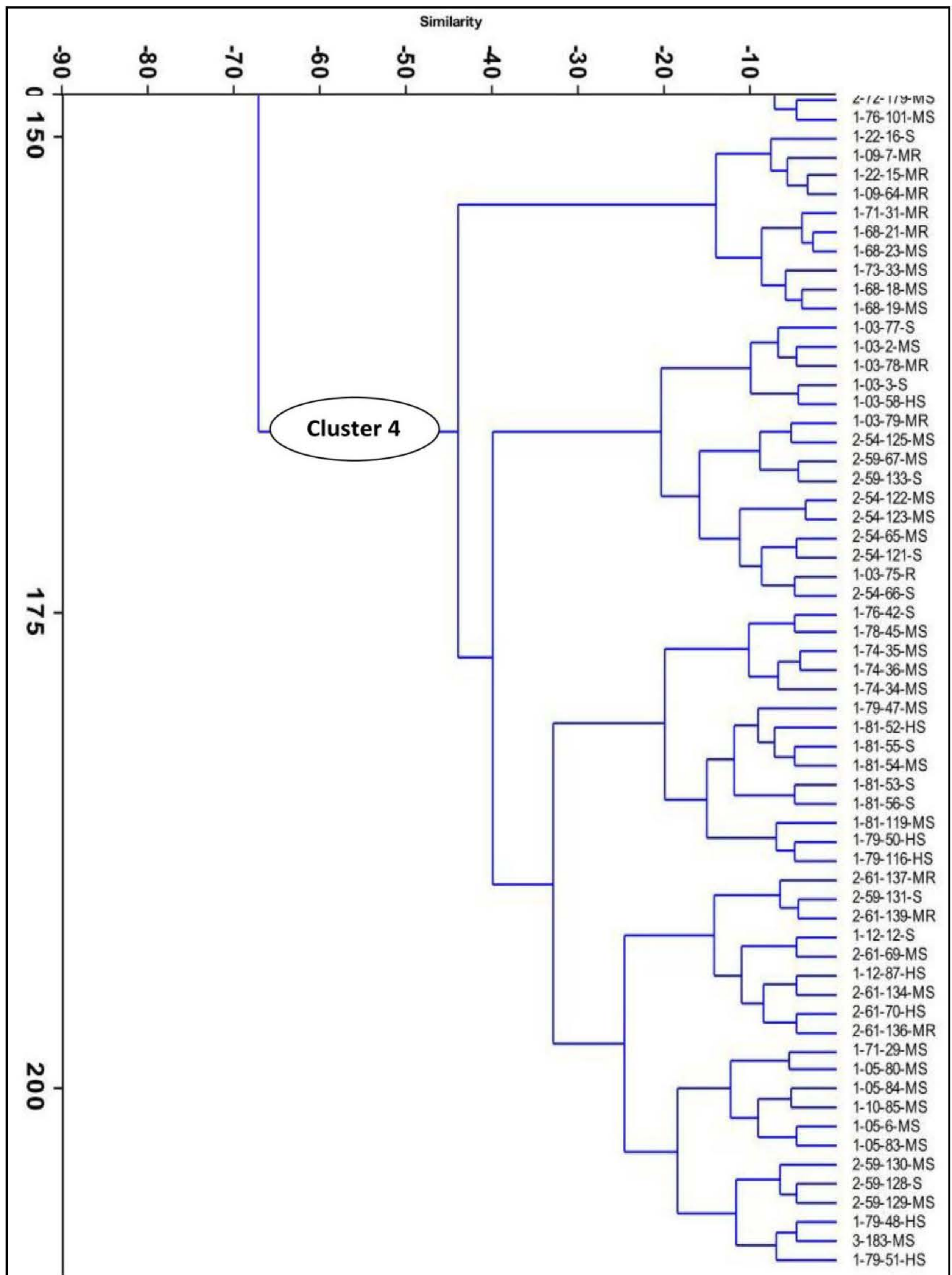

Figure 5.4. continued

5.4 Discussion

Attempts to control cyst nematodes by resistant commercial cultivars have been made to keep the nematode populations below the threshold level (Turner & Subbotin, 2013). Resistant cultivars remain the most economically worthy practice for managing cyst nematodes, although such cultivars are not always available (Turner & Subbotin, 2013). Sometimes only a low level of resistance is known (Riggs & Schuster, 1998).

In this chapter, wheat lines with wide geographical and original distribution with diverse genetic background were examined to identify new sources of resistance that might be introduced into the wheat-breeding programmes. The 217 lines of winter wheat responded differently to *H. filipjevi* and represented 5 levels of nematode resistance. Seventy-nine phenotypes were classified as resistant and moderately resistant. More than half of these (42) belonged to the group 3. This is most likely due to recycling of resistance genes of the *Ae. tauschii* accessions representing a wide geographic area and a wide genetic base (Eastwood *et al*., 1991; Ogbonnaya *et al*., 2001). In group 1 and 2, the proportion of resistant and moderately resistant phenotypes was lower than group 3; this might be attributed to the fact that in these two groups *Ae. tauschii* was involved in the crossing in F1 and F2 only. In view of this, the use of *Ae. tauschii* and its diverse accessions as a genome donor species are highly recommended for enhancement of resistance against CCN. This was reported for many of the synthetic hexaploid wheat lines obtained from different crosses with *Ae. tauschii*, which showed resistance or tolerance to various biotic and abiotic stresses, indicating the importance of the *Ae. tauschii* gene pool for stress breeding purposes (Ogbonnaya *et al*., 2001; Dreisigacker *et al*., 2008). Additionally, previous studies reported four *Cre* genes in wheat to code for resistance to *H. filipjevi*; *i.e. Cre8* and *CreR* in winter wheat (Dababat *et al.,* 2014), and also *Cre1* and *Cre3* in spring wheat (Toktay *et al.,* 2012; 2013), and this explained by the potential existence and expression of one or more of the *Cre* genes in the evaluated lines as resistant (R) and moderately resistant (MR). In those research, the alleles were defined based on microsatellite loci linked to the *Cre* locus.

The results of counting the average of the developed cysts on all reference susceptible varieties (Bezostaya and Kutluk) and the two moderately resistant varieties

(Katea and Sonmez) were in agreement with results obtained by two previous studies (Dababat *et al.,* 2014; Pariyar *et al*., 2016a) using the same growth chamber conditions and different nematode populations; but, the pathotype of those two populations used in the other two studies were not identified unfortunately. However, the population used in this study and the other two populations were collected from the same region of Central Anatolian Plateau (Dababat *et al.,* 2014; Pariyar *et al*., 2016a). The used population in this research is being used since 2010 in the wheat screening programme, and the population has kept its virulence to overcome the resistance of the reference varieties included in this study (G. Erginbas-Orakci, pers. com.).

Using the scale of Andersen & Andersen (1982) was very strict since lines with more than 2 cysts were susceptible and were advised to be eliminated out of the breeding programme, and probably those eliminated lines with relatively fewer cysts could be promising and useful as a source for resistance genes and included in further breeding programme. However, the comparison between the two scales used in this research was difficult and unfair since the experimental factors of CCN species, soil containers, soil mixture and initial inoculum density were different. On the other hand, the used scale in this research (Pariyar *et al*., 2016a) to evaluate the host response to *H. filipjevi*, is successfully used for the same purpose in other two previous studies (Dababat *et al.,* 2014; Pariyar *et al*., 2016a), and also used for evaluation of the host response to *H. filipjevi* and *H. avenae* (Nicol *et al*., 2009; Cui *et al*., 2016), and it is considered the only score for *H. filipjevi.* Therefore, I recommend it as a suitable rating scale to evaluate the host response to *H. filipjevi*. For further experiments, it is also advised to calculate the final nematode population density (Pf) based on egg counts of the cysts on roots and in the post-harvest soil (Pf = the number of eggs gram^{-1}), and to determine the reproductive factor (Rf) (Rf = Pf/Pi, Pi: initial population density), especially for those interesting lines with fewer developed cysts. Moreover, lines rated resistant or moderately resistant to *H. filipjevi* in this study should be screened with other pathotypes of *H. filipjevi*, and later under field conditions.

The significant and low correlation which was found in the three groups between the number of cysts and other root parameters was in agreement with a study that showed

a greater root system (length and volume) correlated with a greater number of developed cysts as it provides more root area for the establishment of nematodes infection and feeding sites (Seah *et al*., 2000). Moreover, a study in corn showed that addition of more fertilisers resulted in the increase of length, surface and volume of the roots which led to more nematode infection sites for *H. zeae* as compared to unfertilised control (Hashmi & Krusberg, 1995). Unfortunately, in this study, this correlation between cysts number and root variables in wheat increases challenges for breeding against both drought and nematode stresses, because breeding against drought aims to select for wide, long and deep root (Berry *et al*., 2003; Comas *et al*., 2013). Similarly, the observed positive and logical correlation between the three variables of root surface, root length, and root volume in the three groups was in accordance with the studies recently reported in bread wheat (Bai *et al*., 2013).

Primer combination 2 (PC2) had the highest PIC value (0.41) than the other three primer combinations. Hence, PC2 was more informative than other primer combinations used to study the variation and genetic relationships between various lines of the synthetic winter wheat used in this set of experiments.

Due to the sharing of one parent or the two parents in different crosses, the cluster analysis based on AFLP diversity distinguishes group 3 but to less degree the groups 1 and 2 which probably shows similarity of D genome between *Aegilops* and modern varieties. This set of markers was found effective and useful to differentiate clearly all lines from group 3 only which were similar in their resistant response to *H. filipjevi* (R and MR). Also, two distinct lines (229 and 230) were the only susceptible and highly susceptible response and both were closely clustered with other lines belonging to group 2 with similar reaction to *H. filipjevi* (S and HS). Likewise, line 183 of group 3 was not present in cluster 1 and was grouped in cluster 4, probably due to the lack of the resistance genes to *H. filipjevi* since its response was moderately susceptible (12 cysts plant^{-1}). However, line 212 with moderately susceptible reaction (11 cysts plant^{-1}) still fell in cluster 1 with other lines of the same group. On the other hand, line 75 was resistant (1.7 cysts plant^{-1}) but did not locate in cluster 1, and it was found in a sub-cluster with a susceptible line (66), and this could be explained due to non-use or non-sharing of the

parent of line 75 *Ae. tauschii* (accession 365) as a parent in the group 3. Therefore, maybe the resistance genes are different from those in the lines in the cluster 1 (group 3), or maybe the markers were not linked to those genes in this case. In general, markers in the three groups were not able to clearly distinct lines according to the resistance level as in the scoring scale (R, MR, MS, S, and HS) in clusters or in sub-clusters. This observation could be explained due to incomplete coverage of the AFLP markers on the wheat genome or these markers were far from the genes which are responsible for the trait of resistance or susceptibility to *H. filipjevi*. Also, the lines of one cross (*i.e.* 81 and 71) had a variable resistance level of MR, MS or S. Additionally, sometimes lines of the same cross did not locate in the same cluster or in sub-clusters as well (*i.e.* 79 and 22). To avoid any miss link between the genotype and phenotype data, I would like to recommend collecting the data for phenotyping and genotyping purpose from the same plant to be able to link the resulted data accurately. The lines of *Ae. tauschii* spp. from Iran were closely clustered probably due to the abundant dominant markers in all Iranian *Ae. tauschii* accessions. While, the geographic distribution of different lines does not have much effect, as the lines from China, Syria and Afghanistan showed lack of sharing of existing dominant markers in this case. This led to the results that the genetic distance is not correlated with geographical distance. Similar lack of association of genetic distances with geographical distances was recently reported in different accessions of deciduous tree *Acer grosseri* Pax. using sequence-related amplified polymorphism (SRAP) markers (Zhang *et al*., 2015).

The majority of the wheat lines with resistance levels of R and MR were excluded from the group in the PCoA after AFLP analysis. Those lines should subsequently be crossed with higher-yielding varieties because many locally adapted wheat varieties are susceptible to CCN. New resistant wheat lines will enable breeders to generate new crosses with local varieties thereby improving their genetic resistance to the nematodes. Combining resistance to CCN and increasing grain yield might be obtained by pyramiding different resistance genes into single lines.

For further molecular characterisation of genetic resistance sources against CCN species, and to detect the inherited markers in close proximity to the genetic causatives

or genes controlling the target traits, genetic mapping can be done mostly using the diverse lines from the three groups that is called linkage disequilibrium (LD) mapping or “association mapping”. Moreover, research using microsatellite loci linked to the *Cre* locus, or advanced techniques such as next-generation sequencing (NGS) is needed to characterise and identify the location of the genes involved in the resistance lines to *H. filipjevi.* Ultimately, a comprehensive description of lines at the molecular level, coupled with extensive phenotyping information, would allow the breeders to evolve wheat lines with greater yield performance and nematode resistance.

Chapter 6

General Discussion

Small grain cereals, such as wheat, barley, oat, rice, triticale and rye, constitute the world's most important source of food. They are critical components of local economies in developed and developing countries (Alexandratos & Bruinsma, 2012). Small grain production is often restricted by economic, agronomic and/or climatic factors, which cause cereals to be repeatedly planted on the same tracts of land (Dababat *et al*., 2015). In such circumstances, plant-parasitic nematodes that invade roots of cereals typically become most numerous in direct proportion to the frequency of host crops produced on an infested field. Three decades ago, it was estimated that nematodes reduced the global productivity of small grain cereals by nearly 750 million metric tonnes annually, with yield reductions of 7.0%, 6.3% and 4.2% for wheat, barley, and oat, respectively (Sasser & Freckman, 1987). A more recent estimate indicates yield losses equivalent to about 10% of global production (Dixon *et al*., 2009). Among the plant-parasitic nematodes, the cereal cyst nematodes (CCN) are major constraints to production of small grain cereals. These nematodes are spread worldwide and cause significant damage. Losses caused by CCN are strongly influenced by environmental conditions; in some environments, they can be in excess of 90% (Nicol *et al*., 2011). Once CCN become established in the field, and due to the protection provided by both the cyst wall and the eggshell, the efficacy of many management strategies will be reduced. Eradication is nearly impossible. Therefore, it was very important to investigate and confirm the CCN distribution in previously surveyed areas, especially in countries like Syria where the available skills for an accurate and reliable identification are limited. For the same reason, it was essential to extend the survey to previously unexamined regions.

During this study, *Heterodera latipons* was reported in high frequency in Syrian rain-fed regions where barley production was dominant. Barley, oat, rye and wheat are excellent hosts for *H. latipons* (Franklin, 1969; Mor *et al.,* 1992; Scholz, 2001; Greco *et al.,* 2002). Due to the very limited demand for seeds, domestic oat and rye lines are not common in that area. However, the wild species of oat are very common in the same fields of barley and wheat due to the little use of herbicides or/and the use of non-specific herbicides. During the survey, I found many fields infested with CCN, and compared to previous surveys, the increased incidence of *H. latipons*, *H. avenae* and *H. filipjevi* illustrated their rapid dissemination. This rapid spread is probably due to the farmers'

ignorance of the CCN problem as they spread the cysts through the irrigation system and unclean farm equipment and farm machinery. Also, the monoculture practice can increase CCN populations and result in severe damage and yield loss as reported in Saudi Arabia (Al-Hazmi *et al*., 2015) and in Tunisia (Namouchi-Kachouri *et al*., 2007). However, long-time monoculture of cereals declined the *H. avenae* population due to the increase of biological control agents (Kerry, 1982b). Many times, during the survey, I had the opportunity to discuss the CCN symptoms and the resulting damage with farmers. Surprisingly, none was aware of the CCN problem and crop rotation was not practised. Spots of weak and stunted plants were presumed to be due to a shortage of fertilisers. Fortunately, collaboration between regional and national research programmes and centres has proven to be highly beneficial for improving the researcher's skill in all steps from sampling to the control of CCN. In this joint effort, resistance is a major and durable strategy to manage CCN; wheat-breeding programmes screen local and commercial cultivars (see below). The benefits of the collaboration and the joint effort can end with valuable advantages to help farmers through illustration of the problem, advice on possible and emerging agricultural practices, and finally making available resistant cultivars for CCN control and reduction of yield losses. Next to CCN, root lesion nematodes (RLN) cause serious damage on cereals worldwide, and damage caused by these nematodes has been reported in the neighbouring countries of Syria, *viz.* Turkey (Elekçioğlu *et al*., 2004; Sahin *et al*., 2009) and Jordan (Al-Banna *et al*., 2015). Regrettably, RLN had never been investigated in Syria. It is highly recommended to include this group in future research aiming at the improvement of wheat production.

Before this study started, identification of CCN was done on the basis of morphological and morphometric characters (Yan & Smiley, 2009). This is time-consuming and problematic due to the high phenotypic plasticity among populations, and the absence of clear diagnostic characteristics for cryptic species (Subbotin *et al*., 2000). Biochemical techniques using protein profiles of a single protein or small subsets of proteins, and molecular markers (RAPD and PCR-RFLP of rDNA-ITS) have been used for CCN identification. However, because each of these methods have some restrictions (see Chapter 2), there was a need for a rapid and reliable identification of CCN up to species level, which is important when monitoring both their distribution and density,

especially when developing control measures relying on rotation or resistance. The traditional way of microscopic identification should be complemented with molecular identification techniques. These techniques make diagnostic procedures more effective and accessible, also to scientists not specialised in taxonomy. Moreover, they have the advantages of increased sensitivity and high detection throughput processing of samples. PCR assays developed to detect singly the three-major species of CCN will enable research and commercial laboratories to diagnose efficiently and facilitate systematic nematode surveys to investigate the geographic distribution of CCN. Species-specific PCR and qPCR are currently used for identification and quantification of *H. avenae* in the commercial diagnostic laboratory at the South Australia Research and Development Institute (Ophel-Keller *et al.*, 2008); unfortunately, the primers and probe sequences (PreDicta B) used in that lab are unpublished. To establish and design reliable species-specific conventional PCR assays as well as quantitative qPCR assays, I investigated the possibility of using different DNA regions instead of the commonly used ITS-rDNA gene, like genes coding for actin, aldolase, β-tubulin, pectate lyase, annexin, chorismate mutase, mitochondrial cytochrome oxidase subunit 1 (COI), and the heat-shock protein 90 (hsp90). It was impossible to obtain amplification products for all investigated *Heterodera* species from the DNA regions of Hsp90, aldolase, pectate lyase, annexin, chorismate mutase and β-tubulin. However, I obtained useful amplification products from both the actin gene and the COI gene. Based on the actin 1 gene, I successfully developed a species-specific PCR assay to detect *H. latipons* (Toumi *et al.,* 2013a). Also, based on the COI gene, two species-specific PCR assays to detect *H. avenae* and *H. filipjevi* were designed (Chapter 4). Similarly, based on COI, two qPCR assays for quantitative detection of *H. avenae* and *H. latipons* were developed (Chapter 4).

In 2013, the species-specific PCR assay to detect *H. latipons* was the first molecular assay for any species of the Avenae group. In the same year, two other assays to detect *H. avenae* and *H. filipjevi* were developed (Chapter 4). All three assays enabled detecting the respective target species amongst 14 other *Heterodera* species and *Punctodera punctata*. The assays were also able to detect 1 second-stage juvenile (J2) of the respective target species mixed with 100 J2 of a non-target *Heterodera* species. The three assays provided a rapid, robust, sensitive and easily applicable method for the

molecular detection of any of the three CCN species separately. Due to the fruitful collaboration and network between ILVO scientists and other scientists worldwide in CIMMYT, ICARDA, and other institutes, I was able to assemble a valuable collection of isolates of the three CCN species. This collection allowed me to validate and check the specificity of each of the assays carefully. However, after reporting and publishing these three assays (Toumi *et al*., 2013a,b), other research groups published species-specific PCR protocols to detect *H. avenae* and *H. filipjevi* using the ITS-rDNA region (Yan *et al*., 2013), and *H. filipjevi* by species-specific SCAR-PCR assays (Peng *et al*., 2013).

The survey reported here (Toumi *et al*., 2015b) as well as previous surveys (Chapter 2) conducted during the last decade showed that mixtures of 2 and sometimes even 3 CCN species can coexist in the same field. qPCR assays can be applied for the accurate detection and quantification of the economically most important species of CCN coexisting in mixtures or singly, whereas this is much more difficult with end-point PCR. qPCR assays can be used for advisory purposes but also to document a negative correlation between the density of any species of CCN and the yield of wheat (Smiley *et al*., 2005). Estimating the density accurately is needed to provide breeders, nematologists and farmers with reliable information for each species separately. Repeated cropping of cultivars with the same resistance gene(s) induces a selection pressure on the nematode population leading to an increase in virulence of CCN and a progressive 'loss' of resistance. This was reported from pot tests in the greenhouse with *Meloidogyne incognita*, where the virulence to resistance genes *Rk* and Rk^2 in cowpea was increased after less than 5 years of continuous growing of both *Rk* cowpea and *Rk2* cowpea plants (Petrillo *et al*., 2006). Additionally, it is wise to take into account the phenomenon of shifting species in a CCN species mixture due to the continuous use of the resistant cultivars to a single species of the CCN. This was reported in fields infected with *Pratylenchus neglectus* and *P. thornei* during a three-year rotation (Smiley *et al*., 2016). A similar and well-known shift was reported in potato fields infested with *G. pallida* and *G. rostochiensis*, in which continuous use of potato cultivars resistant to *G. rostochiensis* resulted in the increase of *G. pallida* (*e.g.* Marshall, 1989; Den Nijs, 1992).

The qPCR is more sensitive than end-point PCR due to the alternative way of signal detection. It is also faster since it eliminates the time-consuming post-PCR agarose gel electrophoresis (Waeyenberge & Viaene, 2015). The qPCR assays for *H. avenae* and *H. latipons*, which were reported in this thesis, were specific, efficient and sensitive. There was a highly significant linearity for the standard curve between their Ct-values and the tested dilution rates. The assays allowed detecting DNA extracted of a single J2 mixed with DNA extracted of 100 J2 from a non-target *Heterodera* species (Toumi *et al*., 2015a). At the time of the design of the qPCR assay for *H. avenae* and *H. latipons,* I started the establishment of a qPCR assay to detect *H. filipjevi*. However, I did not succeed to develop the assay using the COI region due to DNA polymorphism in the sequences of this region in some populations. Hence, and due to the wide distribution and the importance of the yield loss caused by *H. filipjevi* in Turkey and Iran, I highly recommend screening other DNA regions to be used for the qPCR development. Further developing and improving qPCR assays for the most economic important CCN, will assist in the correct identification of the nematode and hence, facilitate the breeder's tasks in designing resistant lines for the target CCN species.

During this research, I noticed that the sequences of the ITS region of *H. filipjevi* populations from Iran showed a slightly low similarity (97% *H. filipjevi* – data not shown) when compared with those available in the GenBank. Also, the alignment of the *H. filipjevi* sequences for the tested populations showed in three positions a distinct difference between the Iranian *H. filipjevi* samples and the other *H. filipjevi* samples (insertion of one nucleotide, 1 SNP, and a deletion of two nucleotides on another position; see appendix 5 for the alignment). In addition, deviating morphological characteristics for some of the *H. filipjevi* populations sampled from different provinces in Iran were noticed. These included a wide range of the underbridge types (from very weak to strong), bullae being very conspicuous and numerous in some populations, but very weak, few and hard to find in others (Dr Zahra Maafi, pers. comm.). In view of the above-mentioned molecular and morphological differences, I would suggest further investigation because the populations of *H. filipjevi* in Iran might be another species of *Heterodera.*

In general, sources of genetic resistance to some nematodes have been identified, but due to the difficulties in transferring the resistance factor(s) into cultivars that have agronomic traits and productive capabilities of importance to agriculturalists, the rate of incorporating effective genes into commercial cultivars has been slow, *e.g.* more than 50 years of research were needed to produce an acceptable sugar beet (*Beta vulgaris*) resistant to *H. schachtii* (Roberts, 1992). Disease management strategies other than using the resistance lines are effective for some nematode species but those strategies are often neither environmentally nor economically acceptable (Smiley & Nicol, 2009). The use of genetic resistance is an important control strategy because it is environmentally and socially most acceptable for the management of nematode populations thereby minimising yield losses caused by plant-parasitic nematodes (Dababat *et al.*, 2015). Breeding the synthetic hexaploid wheat could result in resistant/tolerant cultivars for abiotic (heat, salinity, drought, waterlogging) and biotic (rusts, nematodes, barley yellow dwarf virus, crown rot and powdery mildew) stress factors. However, difficulties were reported for selection of useful traits at the diploid level (Ogbonnaya *et al.*, 2013). Because of these advantages of the synthetic winter wheat, three groups (group 1, group 2 and group 3) of synthetic winter wheat lines were bred for use in areas where *H. filipjevi* is dominant (Matsuoka *et al.*, 2007; Dr Alex Morgunov, pers. com.). All lines were genotyped and phenotyped against *H. filipjevi* to check the possibility to control or lower the population density of this species under the threshold level. Phenotyping the lines showed that each group contained promising lines of which some can be considered resistant (R) or moderate resistant (MR). Lines belonging to group 3 showed the greatest number of R and MR phenotypes. This result could be explained by the presence of one or more *Cre* genes (*Cre1*, *Cre3*, *Cre8* and *CreR*) in this group (Toktay *et al.*, 2012, 2013; Dababat *et al.*, 2014). The clustering analysis of all lines showed four well-separated clusters without link and not depending on the resistance level (R, MR, MS, S, and HS). The promising lines (R and MR) should be screened with other populations and pathotypes of *H. filipjevi* to evaluate and validate their reaction for different conditions. Additionally, screening these lines for resistance to other diseases and investigating other traits would be of great value.

The two main international research institutes, CIMMYT and ICARDA, largely work on wheat with the aim at raising farm productivity in developing countries. The impact of the relationship between these two organisations and the National Agriculture Research is reflected in the fact that 78% of wheat cultivars grown in all developing countries are derived lines from CIMMYT and ICARDA wheat breeding programmes. To achieve its objective of yield improvement, its research has involved looking at the effects of biotic and abiotic factors constraining the attainment of optimum yield. To deliver to developing countries, both of the organisation have had a long history of partnering with national agricultural research systems around the world. Consequently, part of their research objectives includes (1) fostering capacity building of young scientists as well as international experts to identify nematodes attacking cereals, (2) accelerate the exchange of information, providing greater understanding of the complexity, economic importance, and control of CCN, (3) standardizing of screening methods and rating scale, and (4) assistance in extensive surveys. Collaboration between those international centres and national institutes will ultimately lead to better understanding of the disease and the best option to control it under the IPM strategy. While substantial progress has been made in the past, continuing funding which nurture and foster such collaborations are being threatened with potential to limit the ability to realise the aforementioned benefits. In 2009, CIMMYT launched the international cereal nematode initiative programme (ICNI), which is now known as the International Cereal Nematodes Symposium to cover the above-mentioned aims, however, more financial support is required.

One of the recent tools which are being used to find new sources of resistance to wheat diseases is association mapping (Mackay & Powell, 2007; Lipka *et al.*, 2015; Ogura & Busch, 2015; Poland, 2015; Dababat *et al.*, 2016; Pariyar *et al.*, 2016b). It is maximising recent advances in genomic tools and statistical methods by exploiting cumulative recombination and mutation events that occurred in a population and taking into account numerous alleles present in the population to identify significant marker-trait associations (MTAs). It is a powerful approach to detect associations between phenotypic variation and genetic polymorphisms; in this way, novel traits such as resistance to pathogens could be identified and mapped.

Once the quantitative trait loci (QTL) are confirmed, markers are developed and validated, marker-assisted selection could be used for quick deployment of resistance by combining and pyramiding different potential loci into elite wheat breeding lines (Mulki *et al.*, 2013; Dababat *et al.*, 2016). Therefore, the traditional phenotyping method are replaced by the use of those markers at early development stages for different filial generations and the susceptible lines can be eliminated earlier in the breeding cycle. This increases the rate of genetic gain. Eagles *et al.* (2001) reported extensive use of DNA markers for selection of resistance to *Cre1* and *Cre3* genes, in the wheat breeding program of Agriculture Victoria based at Horsham.

The availability of molecular markers linked to different species of nematode (*e.g.* CCN and RLN) makes it possible to pyramid different quantitative trait loci (QTL) and genes linked to resistance to different species and pathotypes (Mulki *et al.*, 2013, Dababat *et al.*, 2016); a difficult objective to achieve using the traditional bioassay in a classical backcrossing breeding program. However, with increasing advances in next-generation sequencing (NGS), it is conceivable that tapping the diversity of wheat accessions in gene banks will be quicker enhancing the discovery of novel alleles/genes for deployment in the control of nematodes. After identification of *Cre* genes from different genetic resources, they could be speedily incorporated into higher yielding elite adapted wheat cultivars to feed the ever-increasing population of the world (Lipka *et al.*, 2015). Despite its advantages and the advances made the use of marker-assisted selection for the improvement of quantitative traits in breeding programs will be supplanted by genomic selection. Genomic selection predicts the breeding values of lines in a population by analysing their phenotypes and high-density marker scores, making it more amenable for use in QTL deployment in breeding programs. A key to the success of genomic selection is that it incorporates all marker information in the prediction model, thereby avoiding biased marker effect estimates and captures more of the variation due to small-effect QTL.

Chapter 7

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Chapter 8

Curriculum Vitae

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Date of Birth: 5 February 1977

Marital Status: Married

Nationality: Syrian – Belgian

Education and Qualifications

2011 to date **PhD student**

Department of Crop Protection, Ghent University, Belgium,
Research: Cereal Cyst Nematodes in Syria: molecular identification and quantification, and screening for resistance in wheat.

2008-2010 **MSc student**

European Master of Science in Nematology (EUMAINE). Faculty of Sciences, Ghent University, Belgium. MSc degree obtained with distinction on 30.06.2010.
Thesis: Development of a species-specific PCR to detect the Cereal Cyst Nematode *Heterodera latipons*.

2006-2008 **MSc student**

Department of Plant Protection, Faculty of Agriculture, University of Aleppo, Syria in collaboration with the International Centre for Agricultural Research in the Dry Areas (ICARDA).
Thesis: The Interaction between *Fusarium* Wilt and Chickpea Cyst Nematode *Heterodera ciceri* on Chickpea in Aleppo and Idlib Governorates.

2004 **Post Graduate Diploma degree in Agriculture Sciences,**

Plant Protection, Faculty of Agriculture, Aleppo University, Aleppo, Syria.
Thesis: Investigation of four Nematicides to Control the Nematodes in the Olive Tree Orchard"

2001 **BSc degree in Agriculture Sciences, Plant Protection**,

Faculty of Agriculture, Al-Baath University, Homos, Syria.

Work Experience/ Employment

2006 **Head, Biocontrol lab in Hama, Ministry of Agriculture, Syria**

2005 **Head, Plant Quarantine Centre in Hama, Ministry of Agriculture, Syria**

2002 **Employee at Central Nematology lab in Hama, Ministry of Agriculture, Syria**

Attending meetings and presentation of research

2016 32nd International Symposium for the European Society of Nematologists (ESN), 28 August - 1 September 2016, University of Minho, Braga, Portugal. (Oral presentation: Screening of Synthetic Wheat Lines for Resistance to the Cereal Cyst Nematode *Heterodera filipjevi*).

2016 68th International Symposium on Crop Protection (ISCP), 17 May 2016 in Ghent University, Ghent – Belgium.

2016 5th International Symposium on Agricultural Sciences-AGRORES, 29 February-3 March 2016, Banja Luka, Bosnia. (Oral presentation: Cereal Cyst Nematodes: Identification, Quantification and Control)

2015 5th International Cereal Nematodes Initiative Workshop, 13-16 September 2015 in Ankara, Turkey. (Oral presentation: Resistance in synthetic bread wheat lines against the cereal cyst nematode *Heterodera filipjevi*)

2015 67th International Symposium on Crop Protection (ISCP), 19 May 2015 in Ghent University, Ghent – Belgium. (Oral presentation: Screening of synthetic wheat lines for resistance to the cereal cyst nematode *Heterodera filipjevi*).

2014 EUCARPIA meeting - Cereals Section: Cereals for Food, Feed and Fuel– Challenge for Global Improvement. Wernigerode, Germany, June 29 – July 4, 2014.

2014 66th International Symposium on Crop Protection (ISCP), 20 May 2014, Ghent University, Ghent – Belgium. (Oral presentation: Quantitative detection of *Heterodera avenae* and *H. latipons* using qPCR).

2014 6th International Congress of Nematology, 4 - 9 May 2014 in Cape Town, South Africa. (Poster presentation: Development of qPCR assays for quantitative detection of *Heterodera avenae, H. filipjevi* and *H. latipons*).

2014 The Borlaug summit on wheat for Food Security, 25 - 28 March 2014, Ciudad Obregón, Mexico. (Poster presentation: Control of Cereal Cyst Nematodes in Wheat).

2013 4**th** International Cereal Nematodes Initiative Workshop, 22 - 24 August 2013 in Beijing, China. (Oral presentation: Quantitative detection of *Heterodera avenae* and *H. filipjevi* using qPCR)

2013 10th International Congress of Plant Pathology (ISPP), August 25 - 30 2013 in Beijing, CHINA

2013 65th International Symposium on Crop Protection (ISCP), 21 May 2013 in Ghent University, Ghent – Belgium.

2013 Pioneering Plant Biotechnology in Europe, 22 April 2013, Technology park, Ghent, Belgium.

2013 Integrated Pest Management in Horticulture: Research for Practice, 07 March 2013 - Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit Caritasstraat 21, 9090 Melle, Belgium.

2012 The 26th World Food Prize Anniversary, October 16-19, 2012 – Des Moines, Iowa State – USA.

2012 31st International Symposium for the European Society of Nematologists (ESN) 23 - 27 September 2012, Adana, Turkey (Oral presentation: Development of two species-specific primer sets to detect the cereal cyst nematodes *Heterodera avenae* and *H. filipjevi*).

2012 3rd Cereal Cyst Nematode Biology and Management Workshop 21 - 22 September, Adana, Turkey (Oral presentation: Distribution of the cereal cyst nematodes (*Heterodera* spp.) in wheat and barley fields in north-eastern regions of Syria).

2012 64th International Symposium on Crop Protection (ISCP), 22 May 2012 in Ghent University, Ghent – Belgium.

2011 The 25th World Food Prize Anniversary, October 12-14, 2011 – Des Moines, Iowa State – USA.

2010 2nd Cereal Cyst Nematode Biology and Management Workshop on Friday 24th of September in Vienna – Austria (Oral presentation: Development of a species-specific duplex PCR to detect the cereal cyst nematode *Heterodera latipons*).

2010 62th International Symposium on Crop Protection (ISCP), 18 May 2010 in Ghent University, Ghent – Belgium.

2009 1st International Cereal Cyst Nematode Workshop held in Antalya, Turkey 21 - 23 October.

2009 61th International Symposium on Crop Protection (ISCP), 19 May 2009, Ghent University, Ghent – Belgium.

2006 9th Arab Congress of Plant Protection in Damascus – Syria.

Grants obtained

2016 SON/ONTA bursary to attend the meeting and give an oral presentation - Canada.

2016 European Society of Nematologists (ESN) bursary to attend the ESN meeting in 2016 in Portugal, and giving an oral presentation during the meeting.

2012 European Society of Nematologists (ESN) bursary to attend the ESN meeting in 2012 in Turkey, and giving an oral presentation during the meeting.

2011 Monsanto's Beachell-Borlaug International Scholarship Programme for three years and half to obtain PhD at Ghent University.

2008 ERASMUS MUNDUS Scholarship for European Master of Science in Nematology, (EUMAINE) for two years at Ghent University, Ghent, Belgium.

2006 Food Legume International Programme (FLIP) from the Centre for Agricultural Research in the Dry Areas in Syria (ICARDA) for two years (Master).

Publications

1. Distribution of the cereal cyst nematodes (*Heterodera* spp.) in wheat and barley fields in north-eastern regions of Syria. *Journal of Plant Diseases and Protection* (2015). 122, 255-263.

Fateh Toumi, Ghassan Hassan, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie Nicol, Francis Ogbonnaya, Khaled Al-Assas, Taissir Abou Al-Fadil & Maurice Moens.

2. Development of qPCR assays for quantitative detection of *Heterodera avenae* and *H. latipons*. *European Journal of Plant Pathology* (2015). 143, 305–316.
   Fateh Toumi, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie M. Nicol, Francis Ogbonnaya & Maurice Moens.

3. Distribution of the root-knot nematode *Meloidogyne* spp., in tomato greenhouses at Lattakia and Tartus province in Syria. *Pakistan Journal of Nematology* (2014), 32(2), 163-172.
   F. Toumi, L. Waeyenberge, R. Yousef, H. Khalil, K. Al-Assas & Maurice Moens.

4. Development of two species-specific primer sets to detect the cereal cyst nematodes *Heterodera avenae* and *Heterodera filipjevi*. *European Journal of Plant Pathology* (2013), 136, 613–624.
   Fateh Toumi, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie M. Nicol, Francis Ogbonnaya & Maurice Moens.

5. Development of a species-specific PCR to detect the cereal cyst nematode, *Heterodera latipons*. *Nematology* (2013). 15, 709-717.
   Fateh Toumi, Lieven Waeyenberge, Nicole Viaene, Abdelfattah Amer Dababat, Julie M. Nicol, Francis Ogbonnaya & Maurice Moens.

Languages

English: Good in writing and speaking

Netherlands: Fourth level

Arabic: Mother tongue

Additional Information

Good computer advanced skills for scientific research and training

Driving licence: Full, Clean.

Interests: travelling - reading

References

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Chapter 9

Appendices

9.1 **Appendix 1.** Lines of the three groups of winter wheat assessed for resistance to *Heterodera filipjevi* with their crossed parents, cross identification and selection history.

Code	Group	Cross	Cross Identification	Selection History*	Name for the Dendrogram
Group 1: 102 lines					
2	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-11E-0E	1-03-2-MS
3	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-14E-0E	1-03-3-S
58	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-2E-0E	1-03-58-HS
75	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-10E-0E	1-03-75-R
77	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-6E-0E	1-03-77-S
78	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-16E-0E	1-03-78-MR
79	1	AISBERG/AE.SQUARROSA (369)	CAWW04GH00003S	-0GH-0SE-030E-18E-0E	1-03-79-MR
4	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-5E-0E	1-05-4-MS

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
5	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-11E-0E	1-05-5-MR
6	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-16E-0E	1-05-6-MS
59	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-3E-0E	1-05-59-MS
80	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-1E-0E	1-05-80-MS
81	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-2E-0E	1-05-81-MS
82	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-8E-0E	1-05-82-MR
83	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-10E-0E	1-05-83-MS
84	1	AISBERG/AE.SQUARROSA (511)	CAWW04GH00005S	-0GH-0SE-030E-14E-0E	1-05-84-MS
11	1	LEUC 84693/AE.SQUARROSA (1026)	CAWW04GH00012S	-0GH-0SE-030E-6E-0E	1-12-11-MR
12	1	LEUC 84693/AE.SQUARROSA (1026)	CAWW04GH00012S	-0GH-0SE-030E-15E-0E	1-12-12-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
13	1	LEUC 84693/AE.SQUARROSA (1026)	CAWW04GH00012S	-0GH-0SE-030E-20E-0E	1-12-13-MS
63	1	LEUC 84693/AE.SQUARROSA (1026)	CAWW04GH00012S	-0GH-0SE-030E-3E-0E	1-12-63-MR
87	1	LEUC 84693/AE.SQUARROSA (1026)	CAWW04GH00012S	-0GH-0SE-030E-10E-0E	1-12-87-HS
7	1	LEUC 84693/AE.SQUARROSA (310)	CAWW04GH00009S	-0GH-0SE-030E-8E-0E	1-09-7-MR
8	1	LEUC 84693/AE.SQUARROSA (310)	CAWW04GH00009S	-0GH-0SE-030E-12E-0E	1-09-8-MR
64	1	LEUC 84693/AE.SQUARROSA (310)	CAWW04GH00009S	-0GH-0SE-030E-4E-0E	1-09-64-MR
9	1	LEUC 84693/AE.SQUARROSA (409)	CAWW04GH00010S	-0GH-0SE-030E-10E-0E	1-10-9-S
10	1	LEUC 84693/AE.SQUARROSA (409)	CAWW04GH00010S	-0GH-0SE-030E-16E-0E	1-10-10-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
61	1	LEUC 84693/AE.SQUARROSA (409)	CAWW04GH00010S	-0GH-0SE-030E-2E-0E	1-10-61-S
85	1	LEUC 84693/AE.SQUARROSA (409)	CAWW04GH00010S	-0GH-0SE-030E-11E-0E	1-10-85-MS
86	1	LEUC 84693/AE.SQUARROSA (409)	CAWW04GH00010S	-0GH-0SE-030E-15E-0E	1-10-86-S
46	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-4E-0E	1-79-46-HS
47	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-9E-0E	1-79-47-MS
48	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-10E-0E	1-79-48-HS
49	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-14E-0E	1-79-49-HS
50	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-16E-0E	1-79-50-HS
51	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-18E-0E	1-79-51-HS

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
113	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-2E-0E	1-79-113-S
114	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-13E-0E	1-79-114-MS
116	1	PANDUR/AE.SQUARROSA (223)	CAWW04GH00079S	-0GH-0SE-030E-17E-0E	1-79-116-HS
52	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-2E-0E	1-81-52-HS
53	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-4E-0E	1-81-53-S
54	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-11E-0E	1-81-54-MS
55	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-12E-0E	1-81-55-S
56	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-13E-0E	1-81-56-S
57	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-15E-0E	1-81-57-S
117	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-6E-0E	1-81-117-MS
118	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-7E-0E	1-81-118-MR

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
119	1	PANDUR/AE.SQUARROSA (409)	CAWW04GH00081S	-0GH-0SE-030E-17E-0E	1-81-119-MS
43	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-2E-0E	1-78-43-S
44	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-11E-0E	1-78-44-MS
45	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-18E-0E	1-78-45-MS
105	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-3E-0E	1-78-105-MS
106	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-5E-0E	1-78-106-MS
107	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-7E-0E	1-78-107-MR
108	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-9E-0E	1-78-108-MR

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
109	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-12E-0E	1-78-109-MS
110	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-15E-0E	1-78-110-MS
111	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-16E-0E	1-78-111-MS
112	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	CAWW04GH00078S	-0GH-0SE-030E-17E-0E	1-78-112-MS
17	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-4E-0E	1-68-17-MS
18	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-13E-0E	1-68-18-MS
19	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-9E-0E	1-68-19-MS
21	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-18E-0E	1-68-21-MR

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
22	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-21E-0E	1-68-22-S
23	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-22E-0E	1-68-23-MS
88	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-6E-0E	1-68-88-MS
89	1	UKR-OD 1530.94/AE.SQUARROSA (310)	CAWW04GH00068S	-0GH-0SE-030E-12E-0E	1-68-89-MS
28	1	UKR-OD 1530.94/AE.SQUARROSA (392)	CAWW04GH00071S	-0GH-0SE-030E-6E-0E	1-71-28-S
29	1	UKR-OD 1530.94/AE.SQUARROSA (392)	CAWW04GH00071S	-0GH-0SE-030E-7E-0E	1-71-29-MS
30	1	UKR-OD 1530.94/AE.SQUARROSA (392)	CAWW04GH00071S	-0GH-0SE-030E-15E-0E	1-71-30-S
31	1	UKR-OD 1530.94/AE.SQUARROSA (392)	CAWW04GH00071S	-0GH-0SE-030E-22E-0E	1-71-31-MR

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
94	1	UKR-OD 1530.94/AE.SQUARROSA (392)	CAWW04GH00071S	-0GH-0SE-030E-10E-0E	1-71-94-MS
96	1	UKR-OD 1530.94/AE.SQUARROSA (392)	CAWW04GH00071S	-0GH-0SE-030E-23E-0E	1-71-96-S
32	1	UKR-OD 1530.94/AE.SQUARROSA (446)	CAWW04GH00073S	-0GH-0SE-030E-9E-0E	1-73-32-MR
33	1	UKR-OD 1530.94/AE.SQUARROSA (446)	CAWW04GH00073S	-0GH-0SE-030E-14E-0E	1-73-33-MS
34	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-11E-0E	1-74-34-MS
35	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-12E-0E	1-74-35-MS
36	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-14E-0E	1-74-36-MS
97	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-4E-0E	1-74-97-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
98	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-5E-0E	1-74-98-MS
99	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-9E-0E	1-74-99-MS
100	1	UKR-OD 1530.94/AE.SQUARROSA (458)	CAWW04GH00074S	-0GH-0SE-030E-15E-0E	1-74-100-S
37	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-3E-0E	1-76-37-MS
38	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-4E-0E	1-76-38-MR
39	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-9E-0E	1-76-39-S
41	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-13E-0E	1-76-41-MS
42	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-14E-0E	1-76-42-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
101	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-1E-0E	1-76-101-MS
102	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-11E-0E	1-76-102-MS
103	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-15E-0E	1-76-103-S
104	1	UKR-OD 1530.94/AE.SQUARROSA (629)	CAWW04GH00076S	-0GH-0SE-030E-18E-0E	1-76-104-MR
14	1	UKR-OD 761.93/AE.SQUARROSA (392)	CAWW04GH00022S	-0GH-0SE-030E-8E-0E	1-22-14-MS
15	1	UKR-OD 761.93/AE.SQUARROSA (392)	CAWW04GH00022S	-0GH-0SE-030E-12E-0E	1-22-15-MR
16	1	UKR-OD 761.93/AE.SQUARROSA (392)	CAWW04GH00022S	-0GH-0SE-030E-15E-0E	1-22-16-S
62	1	UKR-OD 761.93/AE.SQUARROSA (392)	CAWW04GH00022S	-0GH-0SE-030E-2E-0E	1-22-62-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
24	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-6E-0E	1-61-24-MS
25	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-9E-0E	1-61-25-MS
26	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-15E-0E	1-61-26-HS
27	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-20E-0E	1-61-27-MS
90	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-8E-0E	1-61-90-S
91	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-11E-0E	1-61-91-S
92	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-16E-0E	1-61-92-S
93	1	UKR-OD 952.92/AE.SQUARROSA (1031)	CAWW04GH00061S	-0GH-0SE-030E-18E-0E	1-61-93-S

Group 2: 69 lines					
Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
65	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-22DYR-0E	2-54-65-MS
66	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-46DYR-0E	2-54-66-S
120	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-2DYR-0E	2-54-120-MS
121	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-5DYR-0E	2-54-121-S
122	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-8DYR-0E	2-54-122-MS
123	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-13DYR-0E	2-54-123-MS
124	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-18DYR-0E	2-54-124-S
125	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-27DYR-0E	2-54-125-MS
126	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-36DYR-0E	2-54-126-MS
127	2	AISBERG/AE.SQUARROSA (369)//DEMIR	TCI091254	-0SE-0E-58DYR-0E	2-54-127-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
69	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-3DYR-0E	2-61-69-MS
70	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-7DYR-0E	2-61-70-HS
134	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-18DYR-0E	2-61-134-MS
136	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-23DYR-0E	2-61-136-MR
137	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-26DYR-0E	2-61-137-MR
138	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-29DYR-0E	2-61-138-MS
139	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-30DYR-0E	2-61-139-MR
140	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	TCI091261	-0SE-0E-33DYR-0E	2-61-140-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
67	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-13DYR-0E	2-59-67-MS
68	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-52DYR-0E	2-59-68-HS
128	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-2DYR-0E	2-59-128-S
129	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-16DYR-0E	2-59-129-MS
130	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-18DYR-0E	2-59-130-MS
131	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-41DYR-0E	2-59-131-S
132	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-42DYR-0E	2-59-132-HS
133	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	TCI091259	-0SE-0E-58DYR-0E	2-59-133-S

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
165	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-4DYR-0E	2-71-165-MR
166	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-16DYR-0E	2-71-166-S
167	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-17DYR-0E	2-71-167-MS
168	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-18DYR-0E	2-71-168-MS
169	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-25DYR-0E	2-71-169-MS
170	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-35DYR-0E	2-71-170-MS
171	2	UKR-OD 1530.94/AE. SQUARROSA (311)//EKIZ	TCI091271	-0SE-0E-43DYR-0E	2-71-171-MS
172	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-4DYR-0E	2-72-172-MS
173	2	UKR-OD 1530.94/AE.	TCI091272	-0SE-0E-6DYR-0E	2-72-173-MR

		SQUARROSA (312)//BAGCI2002			
Code	**Group**	**Cross**	**Cross Identification**	**Selection History**	**Name for the Dendrogram**
173	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-6DYR-0E	2-72-173-MR
174	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-9DYR-0E	2-72-174-MR
176	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-19DYR-0E	2-72-176-MS
177	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-23DYR-0E	2-72-177-MS
178	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-31DYR-0E	2-72-178-MS
179	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-38DYR-0E	2-72-179-MS
180	2	UKR-OD 1530.94/AE.	TCI091272	-0SE-0E-48DYR-0E	2-72-180-HS

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
		SQUARROSA (312)//BAGCI2002			
181	2	UKR-OD 1530.94/AE. SQUARROSA (312)//BAGCI2002	TCI091272	-0SE-0E-59DYR-0E	2-72-181-MS
152	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-4DYR-0E	2-74-152-MR
153	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-10DYR-0E	2-74-153-MS
154	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-17DYR-0E	2-74-154-MR
156	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-22DYR-0E	2-74-156-MS
157	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-24DYR-0E	2-74-157-MR
158	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-30DYR-0E	2-74-158-MS
159	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-44DYR-0E	2-74-159-MR

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram
160	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-47DYR-0E	2-74-160-MR
161	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-48DYR-0E	2-74-161-MR
162	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-61DYR-0E	2-74-162-MS
163	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-66DYR-0E	2-74-163-MR
164	2	UKR-OD 1530.94/AE. SQUARROSA (446)//KATIA1	TCI091274	-0SE-0E-73DYR-0E	2-74-164-MS
71	2	UKR-OD 1871.94/AE. SQUARROSA (213)//MEZGIT-6	TCI091264	-0SE-0E-3DYR-0E	2-64-71-MS
72	2	UKR-OD 1871.94/AE. SQUARROSA (213)//MEZGIT-6	TCI091264	-0SE-0E-5DYR-0E	2-64-72-S
141	2	UKR-OD 1871.94/AE. SQUARROSA (213)//MEZGIT-6	TCI091264	-0SE-0E-7DYR-0E	AFLP data is missing (M)
142	2	UKR-OD 1871.94/AE.	TCI091264	-0SE-0E-8DYR-0E	2-64-142-MR

		SQUARROSA (213)//MEZGIT-6			
Code	**Group**	**Cross**	**Cross Identification**	**Selection History**	**Name for the Dendrogram**
144	2	UKR-OD 1871.94/AE. SQUARROSA (213)//MEZGIT-6	TCI091264	-0SE-0E-13DYR-0E	2-64-144-MR
73	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-2DYR-0E	2-66-73-MS
74	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-42DYR-0E	M
143	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-8DYR-0E	2-66-143-MS
145	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-14DYR-0E	2-66-145-S
146	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-16DYR-0E	2-66-146-MS
147	2	UKR-OD 952.92/AE.	TCI091266	-0SE-0E-17DYR-0E	2-66-147-MS

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram	Complete name for the Dendrogram
		SQUARROSA (409)//SONMEZ				
148	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-21DYR-0E	2-66-148-MR	
149	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-23DYR-0E	2-66-149-MR	
150	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-26DYR-0E	2-66-150-MS	
151	2	UKR-OD 952.92/AE. SQUARROSA (409)//SONMEZ	TCI091266	-0SE-0E-36DYR-0E	2-66-151-S	
Group 3: 46 lines						
Code	**Group**	**Cross**	**Cross Identification**	**Selection History**	**Name for the Dendrogram**	**Complete name for the Dendrogram**
183	3	LANGDON/AE 454	-	13JAP-SYNT	3-183-MS	3-183
184	3	LANGDON/AE 929	-	13JAP-SYNT	Geo/Mzc-184-MR	3- Georgia/ Mzcheta-184
210	3	LANGDON/AE 1090	-	13JAP-SYNT	Kaz-210-R	3-Kazakhstan-210

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram	Complete name for the Dendrogram
207	3	LANGDON/AT 55	-	13JAP-SYNT	Chi-207-MR	3-China-207
208	3	LANGDON/AT 76	-	13JAP-SYNT	M	M
209	3	LANGDON/AT 80	-	13JAP-SYNT	Chi-209-MR	3-China-209
186	3	LANGDON/IG 126387	-	13JAP-SYNT	Tur/Ash-186-R	3- Turkmenistan/ Ashkhabad-186
187	3	LANGDON/IG 131606	-	13JAP-SYNT	Kyr/Tal-187-R	3- Kyrgyzstan/Talas-187
211	3	LANGDON/IG 47259	-	13JAP-SYNT	Syr/Raq-211-MR	3-Syria/Raqqa -211
185	3	LANGDON/IG 48042	-	13JAP-SYNT	Jam/Kas-185-MR	3- Jammu/Kashmir -185
212	3	LANGDON/KU-20-10	meyeri Griseb.	13JAP-SYNT	Ira-212-MS	3-Iran-212
189	3	LANGDON/KU-2039	var. Typica	13JAP-SYNT	Afg/Pul-189-R	3-Afghanistan/Pulikhumri-189
190	3	LANGDON/KU-2074	ssp. 193trangulate Eig	13JAP-SYNT	Ira/Beh-190-MR	3-Iran/Behshahr -190
191	3	LANGDON/KU-2075	ssp. 193trangulate Eig	13JAP-SYNT	Ira/Beh-191-R	3-Iran/Behshahr-191
213	3	LANGDON/KU-2076	ssp. 193trangulate Eig	13JAP-SYNT	Ira/Gor-213-R	3-Iran/Gorgan-213

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram	Complete name for the Dendrogram
214	3	LANGDON/KU-2078	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Ali-214-MR	3-Iran/Aliabad-214
215	3	LANGDON/KU-2079	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Ali-215-MR	3-Iran/Aliabad-215
188	3	LANGDON/KU-20-8	var. Typica	13JAP-SYNT	Ira/Fir-188-MR	3-Iran/Firuzkuh-188
192	3	LANGDON/KU-2080	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Gor-192-R	3-Iran/Gorgan-192
193	3	LANGDON/KU-2088	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Sar-193-MR	3-Iran/Sari-193
216	3	LANGDON/KU-20-9	ssp. 194trangulate Eig	13JAP-SYNT	Ira-216-MR	3-Iran-216
217	3	LANGDON/KU-2090	ssp. 194trangulate Eig	13JAP-SYNT	Ira-217-MR	3-Iran-217
218	3	LANGDON/KU-2091	ssp. 194trangulate Eig	13JAP-SYNT	Ira-218-MR	3-Iran-218
194	3	LANGDON/KU-2092	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Bab-194-MR	3-Iran/Babulsar-194
219	3	LANGDON/KU-2093	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Bab-219-MR	3-Iran/Babulsar/Chalus-219
195	3	LANGDON/KU-2096	ssp. 194trangulate Eig	13JAP-SYNT	Ira/Bab-195-MR	3-Iran/Babulsar-195

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram	Complete name for the Dendrogram
196	3	LANGDON/KU-2097	var. Typica	13JAP-SYNT	Ira/Bab-196-MR	3-Iran/Babulsar-196
197	3	LANGDON/KU-2098	var. Typica	13JAP-SYNT	Ira/Ram-197-MR	3-Iran/Ramsar-197
198	3	LANGDON/KU-2100	var. Meyeri	13JAP-SYNT	Ira/Ram-198-R	3-Iran/Ramsar-198
220	3	LANGDON/KU-2103	var. Typica	13JAP-SYNT	Ira/Ch-220-MR	3-Iran/Chalus-220
199	3	LANGDON/KU-2105	var. Typica	13JAP-SYNT	Ira/Pah-199-MR	3-Iran/Pahlavi-199
200	3	LANGDON/KU-2106	var. Typica	13JAP-SYNT	Ira/Pah-200-MR	3-Iran/Pahlavi-200
222	3	LANGDON/KU-2109	var. Meyeri	13JAP-SYNT	Ira/Ast-222-R	3-Iran/Astara-222
202	3	LANGDON/KU-2124	var. Typica	13JAP-SYNT	M	M
223	3	LANGDON/KU-2132	var. Typica	13JAP-SYNT	M	M
224	3	LANGDON/KU-2136	var. Typica	13JAP-SYNT	Tur/V/E-224-MR	3-Turkey/Van/Ercis-224
203	3	LANGDON/KU-2144	var. Typica	13JAP-SYNT	Ira/Mak-203-R	3-Iran/Maku-203
225	3	LANGDON/KU-2155	var. Typica	13JAP-SYNT	Ira/Ave-225-R	3-Iran/Avei-225

Code	Group	Cross	Cross Identification	Selection History	Name for the Dendrogram	Complete name for the Dendrogram
226	3	LANGDON/KU-2156	var. Typica	13JAP-SYNT	Ira/Ave-226-R	3-Iran/Avei-226
227	3	LANGDON/KU-2158	var. Meyeri	13JAP-SYNT	Ira/Ram-227-MR	3-Iran/Ramsar-227
204	3	LANGDON/KU-2159	var. Typica	13JAP-SYNT	Ira/Ram-204-MR	3-Iran/Ramsar-204
228	3	LANGDON/KU-2816	var. Typica	13JAP-SYNT	Arm/Ara-228-MR	3-Armenia/Aragaband-228
205	3	LANGDON/KU-2829A	var. Typica	13JAP-SYNT	Geo/Tib-205-R	3-Georgia/Tibilisi-205
206	3	LANGDON/PI 476874	-	13JAP-SYNT	Afg-206-MR	3- Afghanistan-206
229	3	LANGDON/PI 499262	-	13JAP-SYNT	Chi/Xin-229-S	3-China/Xinjian-229
230	3	LANGDON/PI 508262	-	13JAP-SYNT	Chi/Xin-230-HS	3-China/Xinjian-230

* Abbreviations used in selection were gaven in the Appendix 3.

9.2 **Appendix 2.** Number of cysts per plant, root length, root surface, root volume, number of root tips and resistance level to *Heterodera filipjevi* of 217 lines of winter wheat (means ± standard deviations of replications).

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
2	1	AISBERG/AE.SQUARROSA (369)	13±6.4 A***	614±156 AB	50±7 ABC	0.3±0.1 AB	2692±2333 A	MS	S
3	1	AISBERG/AE.SQUARROSA (369)	17.3±10 A	520±262 AB	41±16 ABC	0.3±0.1 AB	2738±2072 A	S	S
4	1	AISBERG/AE.SQUARROSA (511)	11.2±6.5 A	742±200 AB	61±11 ABC	0.4±0.1 AB	3632±1468 A	MS	S
5	1	AISBERG/AE.SQUARROSA (511)	9.5±5.1 A	728±215 AB	71±17 ABC	0.6±0.2 AB	3643±1918 A	MR	S
6	1	AISBERG/AE.SQUARROSA (511)	10.8±6.3 A	776±245 AB	72±27 ABC	0.5±0.2 AB	3681±1615 A	MS	S
7	1	LEUC 84693/AE.SQUARROSA (310)	6±4.9 AB	746±235 AB	64±18 ABC	0.4±0.1 AB	2593±1358 A	MR	S
8	1	LEUC 84693/AE.SQUARROSA (310)	7±4.1 A	674±283 AB	49±19 ABC	0.3±0.1 AB	2433±1097 A	MR	S
9	1	LEUC 84693/AE.SQUARROSA (409)	15.8±6.1 A	713±259 AB	57±25 ABC	0.4±0.2 AB	2480±1134 A	S	S
10	1	LEUC 84693/AE.SQUARROSA (409)	17.5±10.6 A	706±193 AB	53±22 ABC	0.3±0.2 AB	2393±875 A	S	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
11	1	LEUC 84693/AE.SQUARROSA (1026)	8.8±7.8 [A]	716±269 [AB]	60±14 [ABC]	0.4±0.1 [AB]	2046±1112 [A]	MR	S
12	1	LEUC 84693/AE.SQUARROSA (1026)	16.7±8.3 [A]	846±247 [AB]	71±29 [ABC]	0.5±0.3 [AB]	2414±531 [A]	S	S
13	1	LEUC 84693/AE.SQUARROSA (1026)	14.3±8.3 [A]	618±285 [AB]	51±27 [ABC]	0.3±0.3 [AB]	2035±775 [A]	MS	S
14	1	UKR-OD 761.93/AE.SQUARROSA (392)	13.8±10.5 [A]	733±300 [AB]	54±18 [ABC]	0.3±0.1 [AB]	2224±854 [A]	MS	S
15	1	UKR-OD 761.93/AE.SQUARROSA (392)	8.5±6 [A]	481±359 [AB]	30±22 [ABC]	0.2±0.1 [AB]	1686±949 [A]	MR	S
16	1	UKR-OD 761.93/AE.SQUARROSA (392)	19±9.5 [A]	769±215 [AB]	60±8 [ABC]	0.4±0.1 [AB]	2382±667 [A]	S	S
17	1	UKR-OD 1530.94/AE.SQUARROSA (310)	14±6.4 [A]	557±115 [AB]	38±3 [ABC]	0.2±0.1 [AB]	2242±239 [A]	MS	S
18	1	UKR-OD 1530.94/AE.SQUARROSA (310)	12.7±6.5 [A]	812±136 [AB]	63±10 [ABC]	0.4±0.1 [AB]	2444±669 [A]	MS	S
19	1	UKR-OD 1530.94/AE.SQUARROSA (310)	11.7±5 [A]	673±168 [AB]	53±9 [ABC]	0.4±0.2 [AB]	2184±896 [A]	MS	S
21	1	UKR-OD 1530.94/AE.SQUARROSA (310)	10.2±5.1 [A]	701±343 [AB]	49±21 [ABC]	0.3±0.1 [AB]	2170±946 [A]	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
22	1	UKR-OD 1530.94/AE.SQUARROSA (310)	16.5±7.3 ^{A}	366±65 ^{AB}	24±4 ^{BC}	0.1±0 ^{AB}	1755±91 ^{A}	S	S
23	1	UKR-OD 1530.94/AE.SQUARROSA (310)	12.3±6.6 ^{A}	689±158 ^{AB}	61±16 ^{ABC}	0.4±0.1 ^{AB}	2090±176 ^{A}	MS	S
24	1	UKR-OD 952.92/AE.SQUARROSA (1031)	14.3±5.2 ^{A}	624±182 ^{AB}	52±23 ^{ABC}	0.4±0.2 ^{AB}	2322±597 ^{A}	MS	S
25	1	UKR-OD 952.92/AE.SQUARROSA (1031)	14±6.4 ^{A}	687±193 ^{AB}	56±24 ^{ABC}	0.4±0.2 ^{AB}	2529±383 ^{A}	MS	S
26	1	UKR-OD 952.92/AE.SQUARROSA (1031)	20.2±9.2 ^{A}	624±137 ^{AB}	45±17 ^{ABC}	0.3±0.1 ^{AB}	2394±373 ^{A}	HS	S
27	1	UKR-OD 952.92/AE.SQUARROSA (1031)	13.8±5.8 ^{A}	424±11 ^{AB}	30±6 ^{ABC}	0.2±0.1 ^{AB}	1571±387 ^{A}	MS	S
28	1	UKR-OD 1530.94/AE.SQUARROSA (392)	18.5±9.1 ^{A}	268±234 ^{B}	17±13 ^{C}	0.1±0.1 ^{B}	1576±1141 ^{A}	S	S
29	1	UKR-OD 1530.94/AE.SQUARROSA (392)	13±12.3 ^{A}	693±235 ^{AB}	62±28 ^{ABC}	0.5±0.2 ^{AB}	2160±641 ^{A}	MS	S
30	1	UKR-OD 1530.94/AE.SQUARROSA (392)	15.5±11.6 ^{A}	673±158 ^{AB}	65±30 ^{ABC}	0.5±0.3 ^{AB}	2232±629 ^{A}	S	S
31	1	UKR-OD 1530.94/AE.SQUARROSA (392)	9±3.1 ^{A}	576±171 ^{AB}	50±18 ^{ABC}	0.4±0.1 ^{AB}	1916±404 ^{A}	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
32	1	UKR-OD 1530.94/AE.SQUARROSA (446)	8.3±8.9 A	539±297 AB	44±29 ABC	0.3±0.2 AB	1834±746 A	MR	S
33	1	UKR-OD 1530.94/AE.SQUARROSA (446)	14±5.2 A	601±170 AB	52±15 ABC	0.4±0.1 AB	1755±527 A	MS	S
34	1	UKR-OD 1530.94/AE.SQUARROSA (458)	14.3±7.1 A	565±166 AB	46±8 ABC	0.3±0 AB	1900±526 A	MS	S
35	1	UKR-OD 1530.94/AE.SQUARROSA (458)	15.2±9.7 A	719±153 AB	57±10 ABC	0.4±0.1 AB	2148±161 A	MS	S
36	1	UKR-OD 1530.94/AE.SQUARROSA (458)	14.2±6.7 A	675±167 AB	64±4 ABC	0.5±0.1 AB	2071±610 A	MS	S
37	1	UKR-OD 1530.94/AE.SQUARROSA (629)	14±8.8 A	631±279 AB	57±25 ABC	0.4±0.2 AB	2227±546 A	MS	S
38	1	UKR-OD 1530.94/AE.SQUARROSA (629)	8.7±8.8 A	511±244 AB	53±29 ABC	0.4±0.3 AB	1259±77 A	MR	S
39	1	UKR-OD 1530.94/AE.SQUARROSA (629)	17.8±10. A 5	627±181 AB	60±19 ABC	0.5±0.2 AB	1960±929 A	S	S
41	1	UKR-OD 1530.94/AE.SQUARROSA (629)	13.7±9.9 A	558±106 AB	48±2 ABC	0.3±0.1 AB	1858±938 A	MS	S
42	1	UKR-OD 1530.94/AE.SQUARROSA (629)	16.5±6.7 A	640±85 AB	58±1 ABC	0.4±0.1 AB	2525±593 A	S	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
43	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	17.3±8.3 ^{A}	702±42 ^{AB}	66±15 ^{ABC}	0.5±0.2 ^{AB}	2514±508 ^{A}	S	S
44	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	13.2±9.5 ^{A}	642±95 ^{AB}	51±8 ^{ABC}	0.3±0.1 ^{AB}	2081±936 ^{A}	MS	S
45	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	12.8±7.9 ^{A}	547±137 ^{AB}	44±11 ^{ABC}	0.3±0.1 ^{AB}	2324±724 ^{A}	MS	S
46	1	PANDUR/AE.SQUARROSA (223)	25.2±17.3 ^{A}	681±20 ^{AB}	69±14 ^{ABC}	0.6±0.2 ^{AB}	2456±625 ^{A}	HS	S
47	1	PANDUR/AE.SQUARROSA (223)	14.2±10.1 ^{A}	637±71 ^{AB}	56±7 ^{ABC}	0.4±0.1 ^{AB}	2044±1188 ^{A}	MS	S
48	1	PANDUR/AE.SQUARROSA (223)	19.8±7.8 ^{A}	637±80 ^{AB}	59±6 ^{ABC}	0.4±0 ^{AB}	2193±840 ^{A}	HS	S
49	1	PANDUR/AE.SQUARROSA (223)	19.5±6.9 ^{A}	690±104 ^{AB}	58±3 ^{ABC}	0.4±0.1 ^{AB}	2370±1157 ^{A}	HS	S
50	1	PANDUR/AE.SQUARROSA (223)	20±14.7 ^{A}	562±136 ^{AB}	63±30 ^{ABC}	0.6±0.5 ^{AB}	1780±773 ^{A}	HS	S
51	1	PANDUR/AE.SQUARROSA (223)	19.8±10.2 ^{A}	675±177 ^{AB}	68±29 ^{ABC}	0.6±0.3 ^{AB}	2307±657 ^{A}	HS	S
52	1	PANDUR/AE.SQUARROSA (409)	20.5±10.9 ^{A}	610±117 ^{AB}	63±24 ^{ABC}	0.5±0.3 ^{AB}	2212±804 ^{A}	HS	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
53	1	PANDUR/AE.SQUARROSA (409)	15.8±5 ^{A}	593±85 ^{AB}	54±10 ^{ABC}	0.4±0.1 ^{AB}	2384±573 ^{A}	S	S
54	1	PANDUR/AE.SQUARROSA (409)	11.2±8.8 ^{A}	507±229 ^{AB}	44±23 ^{ABC}	0.3±0.2 ^{AB}	2014±912 ^{A}	MS	S
55	1	PANDUR/AE.SQUARROSA (409)	18±9.2 ^{A}	543±108 ^{AB}	43±7 ^{ABC}	0.3±0 ^{AB}	2229±547 ^{A}	S	S
56	1	PANDUR/AE.SQUARROSA (409)	15.8±10.1 ^{A}	580±97 ^{AB}	55±23 ^{ABC}	0.4±0.3 ^{AB}	2268±968 ^{A}	S	S
57	1	PANDUR/AE.SQUARROSA (409)	15.8±7.4 ^{A}	584±68 ^{AB}	54±16 ^{ABC}	0.4±0.2 ^{AB}	2297±1065 ^{A}	S	S
58	1	AISBERG/AE.SQUARROSA (369)	20±10.6 ^{A}	453±138 ^{AB}	43±17 ^{ABC}	0.3±0.2 ^{AB}	1596±617 ^{A}	HS	S
59	1	AISBERG/AE.SQUARROSA (511)	7.3±2.4 ^{A}	467±127 ^{AB}	43±14 ^{ABC}	0.3±0.1 ^{AB}	1574±903 ^{A}	MR	S
61	1	LEUC 84693/AE.SQUARROSA (409)	18.7±11.1 ^{A}	502±175 ^{AB}	48±25 ^{ABC}	0.4±0.3 ^{AB}	1542±240 ^{A}	S	S
62	1	UKR-OD 761.93/AE.SQUARROSA (392)	17.2±5.7 ^{A}	537±104 ^{AB}	56±12 ^{ABC}	0.5±0.1 ^{AB}	1500±673 ^{A}	S	S
63	1	LEUC 84693/AE. SQUARROSA (1026)	10±6.8 ^{A}	482±66 ^{AB}	49±17 ^{ABC}	0.4±0.2 ^{AB}	1405±356 ^{A}	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
64	1	LEUC 84693/AE.SQUARROSA (310)	5.8±5.7 AB	633±0 AB	61±0 ABC	0.5±0 AB	2149±0 A	MR	S
75	1	AISBERG/AE.SQUARROSA (369)	1.3±2.2 B	M ****	M	M	M	R	R
77	1	AISBERG/AE.SQUARROSA (369)	17.2±7.9 A	752±81 AB	73±7 ABC	0.6±0.1 AB	2175±1485 A	S	S
78	1	AISBERG/AE.SQUARROSA (369)	9.2±5.4 A	684±206 AB	64±13 ABC	0.5±0.1 AB	2310±1321 A	MR	S
79	1	AISBERG/AE.SQUARROSA (369)	8.2±3.8 A	798±105 AB	79±12 ABC	0.6±0.1 AB	3223±3076 A	MR	S
80	1	AISBERG/AE.SQUARROSA (511)	12.8±8.9 A	462±398 AB	49±44 ABC	0.4±0.4 AB	9703±14851 A	MS	S
81	1	AISBERG/AE.SQUARROSA (511)	10.7±7.3 A	477±377 AB	47±43 ABC	0.4±0.4 AB	9026±13931 A	MS	S
82	1	AISBERG/AE.SQUARROSA (511)	9.7±7.1 A	862±101 AB	90±13 AB	0.7±0.1 AB	9062±13194 A	MR	S
83	1	AISBERG/AE.SQUARROSA (511)	11.3±5.8 A	813±116 AB	88±22 AB	0.8±0.3 A	9304±13631 A	MS	S
84	1	AISBERG/AE.SQUARROSA (511)	14±10.8 A	905±77 AB	94±18 A	0.8±0.3 A	7148±9453 A	MS	S

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85	1	LEUC 84693/AE.SQUARROSA (409)	14±12.3 A	583±165 AB	61±16 ABC	0.5±0.1 AB	994±219 A	MS	S
86	1	LEUC 84693/AE.SQUARROSA (409)	19±11.3 A	773±68 AB	81±3 ABC	0.7±0 AB	1231±88 A	S	S
87	1	LEUC 84693/AE.SQUARROSA (1026)	21.5±9.9 A	718±176 AB	72±13 ABC	0.6±0.1 AB	1115±110 A	HS	S
88	1	UKR-OD 1530.94/AE.SQUARROSA (310)	14.3±7.8 A	554±264 AB	47±27 ABC	0.3±0.2 AB	1821±502 A	MS	S
89	1	UKR-OD 1530.94/AE.SQUARROSA (310)	14.8±5.7 A	633±322 AB	57±29 ABC	0.4±0.2 AB	1541±403 A	MS	S
90	1	UKR-OD 952.92/AE.SQUARROSA (1031)	16.2±14.7 A	679±80 AB	56±4 ABC	0.4±0 AB	1519±160 A	S	S
91	1	UKR-OD 952.92/AE.SQUARROSA (1031)	15.5±8.8 A	814±138 AB	76±9 ABC	0.6±0 AB	1444±376 A	S	S
92	1	UKR-OD 952.92/AE.SQUARROSA (1031)	17±7.8 A	840±151 AB	71±12 ABC	0.5±0.1 AB	1782±461 A	S	S
93	1	UKR-OD 952.92/AE.SQUARROSA (1031)	18.5±6.7 A	782±286 AB	60±33 ABC	0.4±0.3 AB	2175±128 A	S	S
94	1	UKR-OD 1530.94/AE.SQUARROSA (392)	14.5±8.9 A	622±261 AB	45±22 ABC	0.3±0.2 AB	2706±834 A	MS	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
96	1	UKR-OD 1530.94/AE.SQUARROSA (392)	16.2±10 A	762±84 AB	65±12 ABC	0.4±0.1 AB	2022±289 A	S	S
97	1	UKR-OD 1530.94/AE.SQUARROSA (458)	17.5±11.6 A	355±78 AB	25±1 BC	0.1±0 AB	1545±430 A	S	S
98	1	UKR-OD 1530.94/AE.SQUARROSA (458)	13.2±8.7 A	892±98 AB	77±8 ABC	0.5±0.1 AB	2240±305 A	MS	S
99	1	UKR-OD 1530.94/AE.SQUARROSA (458)	13.7±8.3 A	916±245 AB	81±18 ABC	0.6±0.1 AB	1837±108 A	MS	S
100	1	UKR-OD 1530.94/AE.SQUARROSA (458)	17.8±9.4 A	840±191 AB	79±33 ABC	0.6±0.4 AB	1824±640 A	S	S
101	1	UKR-OD 1530.94/AE.SQUARROSA (629)	12.8±12 A	672±174 AB	63±23 ABC	0.5±0.2 AB	1181±240 A	MS	S
102	1	UKR-OD 1530.94/AE.SQUARROSA (629)	13.5±9.1 A	873±149 AB	81±19 ABC	0.6±0.2 AB	1320±85 A	MS	S
103	1	UKR-OD 1530.94/AE.SQUARROSA (629)	16.5±8.3 A	868±229 AB	73±12 ABC	0.5±0 AB	1306±354 A	S	S
104	1	UKR-OD 1530.94/AE.SQUARROSA (629)	7.2±6.3 AB	738±131 AB	68±17 ABC	0.5±0.2 AB	1479±205 A	MR	S
105	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	12.2±8.2 A	742±31 AB	66±4 ABC	0.5±0.1 AB	1531±110 A	MS	S

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106	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	14.5±7.7 ^{A}	770±202 ^{AB}	69±20 ^{ABC}	0.5±0.2 ^{AB}	1852±110 ^{A}	MS	S
107	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	9.2±7.4 ^{A}	554±64 ^{AB}	42±12 ^{ABC}	0.3±0.1 ^{AB}	1615±574 ^{A}	MR	S
108	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	7.7±7.3 ^{A}	816±118 ^{AB}	74±20 ^{ABC}	0.5±0.2 ^{AB}	1397±261 ^{A}	MR	S
109	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	14.5±10.8 ^{A}	593±173 ^{AB}	50±15 ^{ABC}	0.3±0.1 ^{AB}	1170±485 ^{A}	MS	S
110	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	10.7±6.8 ^{A}	751±117 ^{AB}	68±22 ^{ABC}	0.5±0.3 ^{AB}	1684±215 ^{A}	MS	S
111	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	15±7.1 ^{A}	956±138 ^{A}	91±18 ^{AB}	0.7±0.2 ^{AB}	1854±260 ^{A}	MS	S
112	1	UKR-OD 1530.94/AE.SQUARROSA (1027)	10.7±9.7 ^{A}	796±313 ^{AB}	87±43 ^{AB}	0.8±0.4 ^{A}	1457±694 ^{A}	MS	S
113	1	PANDUR/AE.SQUARROSA (223)	18.2±7.5 ^{A}	959±190 ^{A}	90±23 ^{AB}	0.7±0.3 ^{AB}	1678±429 ^{A}	S	S
114	1	PANDUR/AE.SQUARROSA (223)	13.3±9.6 ^{A}	656±176 ^{AB}	63±18 ^{ABC}	0.5±0.1 ^{AB}	1137±287 ^{A}	MS	S
116	1	PANDUR/AE.SQUARROSA (223)	20±9.2 ^{A}	737±99 ^{AB}	78±18 ^{ABC}	0.7±10.2 ^{AB}	1117±82 ^{A}	HS	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
117	1	PANDUR/AE.SQUARROSA (409)	15.3±7.8 ^{A}	810±60 ^{AB}	84±14 ^{ABC}	0.7±0.3 ^{AB}	1374±163 ^{A}	MS	S
118	1	PANDUR/AE.SQUARROSA (409)	9±3.8 ^{A}	708±71 ^{AB}	62±10 ^{ABC}	0.4±0.1 ^{AB}	1146±207 ^{A}	MR	S
119	1	PANDUR/AE.SQUARROSA (409)	10.8±6.3 ^{A}	688±120 ^{AB}	66±11 ^{ABC}	0.5±0.1 ^{AB}	1160±262 ^{A}	MS	S
231	**	Bezostaya	23.8±12.3 ^{A}	M	M	M	M	HS	S
232	**	Katea	8.5±5.2 ^{A}	M	M	M	M	MR	S
233	**	Kutluk	16.7±9.5 ^{A}	M	M	M	M	S	S
234	**	Sönmez	10.3±4.1 ^{A}	M	M	M	M	MR	S
65	2	AISBERG/AE.SQUARROSA (369)//DEMIR	12.2±13.2 ^{A}	670±75 ABCDEF	66±19 ABCDEFGHI	0.5±0.2 ABCDEFGH	1910±312 AB	MS	S
66	2	AISBERG/AE.SQUARROSA (369)//DEMIR	18.3±8.6 ^{A}	588±40 ABCDEF	66±15 ABCDEFGHI	0.6±0.2 ABCDEFGH	1633±549 AB	S	S
67	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	14.5±11.5 ^{A}	587±34 ABCDEF	60±12 ABCDEFGHI	0.5±0.2 ABCDEFGH	1682±348 AB	MS	S
68	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	27.7±15.9 ^{A}	334±114 ^{F}	22±10 ^{I}	0.1±0.1 ^{H}	1634±681 AB	HS	S

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69	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	13.5±7.8 A	639±82 ABCDEF	59±7 ABCDEFGHI	0.4±0.1 ABCDEFGH	1706±345 AB	MS	S
70	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	27.8±6 A	583±147 ABCDEF	54±19 ABCDEFGHI	0.4±0.2 ABCDEFGH	1661±631 AB	HS	S
71	2	UKR-OD 1871.94/AE.SQUARROSA (213)//MEZGIT-6	13.8±9.8 A	534±51 BCDEF	51±9 BCDEFGHI	0.4±0.1 ABCDEFGH	1756±573 AB	MS	S
72	2	UKR-OD 1871.94/AE.SQUARROSA (213)//MEZGIT-6	16.3±13.9 A	704±132 ABCDEF	78±27 ABCDEFGH	0.7±0.3 ABCDEFGHI	1916±580 AB	S	S
73	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	11.5±7.2 A	429±100 DEF	37±5 GHI	0.3±0 FGH	1512±441 AB	MS	S
74	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	11.2±7.3 A	415±13 EF	33±4 HI	0.2±0.1 GH	1588±405 AB	MS	S
120	2	AISBERG/AE.SQUARROSA (369)//DEMIR	14.2±5.7 A	786±234 ABCDE	62±22 ABCDEFGHI	0.4±0.2 ABCDEFGH	1623±336 AB	MS	S
121	2	AISBERG/AE.SQUARROSA (369)//DEMIR	16.7±8.4 A	665±178 ABCDEF	53±16 BCDEFGHI	0.3±0.1 ABCDEFGH	1677±418 AB	S	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
122	2	AISBERG/AE.SQUARROSA (369)//DEMIR	10.5±6.5 ^{A}	769±222 ABCDE	74±21 ABCDEFGH	0.6±0.2 ABCDEFGH	1376±201 AB	MS	S
123	2	AISBERG/AE.SQUARROSA (369)//DEMIR	15.3±6.8 ^{A}	935±179 ^{AB}	97±21 ^{AB}	0.8±0.2 ^{AB}	1457±96 ^{AB}	MS	S
124	2	AISBERG/AE.SQUARROSA (369)//DEMIR	17.8±13.5 ^{A}	901±60 ^{AB}	75±10 ABCDEFGH	0.5±0.1 ABCDEFGH	1619±247 AB	S	S
125	2	AISBERG/AE.SQUARROSA (369)//DEMIR	10.7±5.5 ^{A}	802±154 ABCDE	79±20 ABCDEFGH	0.6±0.2 ABCDEFG	1369±348 AB	MS	S
126	2	AISBERG/AE.SQUARROSA (369)//DEMIR	11.3±4 ^{A}	806±77 ABCDE	66±7 ABCDEFGHI	0.4±0 ABCDEFGH	1589±99 ^{AB}	MS	S
127	2	AISBERG/AE.SQUARROSA (369)//DEMIR	19.2±7.8 ^{A}	955±150 ^{A}	100±17 ^{A}	0.8±0.2 ^{A}	1600±169 AB	S	S
128	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	16.5±9.1 ^{A}	854±104 ABC	81±12 ABCDEFG	0.6±0.1 ABCDEFG	1351±147 AB	S	S
129	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	11±5.9 ^{A}	774±82 ABCDE	80±7 ^{ABCDEFG}	0.7±0.1 ABCDEFG	1477±386 AB	MS	S
130	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	15.3±9.9 ^{A}	810±79 ABCDE	78±1 ABCDEFGH	0.6±0.1 ABCDEFG	1440±223 AB	MS	S
131	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	17.5±9.5 ^{A}	707±15 ABCDEF	55±2 ABCDEFGHI	0.3±0 ^{BCDEFGH}	1671±169 AB	S	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
132	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	19.7±8.8 ^{A}	928±58 ^{AB}	87±13 ^{ABCDEF}	0.7±0.2 ^{ABCDEFG}	1437±283 ^{AB}	HS	S
133	2	LEUC 84693/AE.SQUARROSA (310)//ADYR	16.5±13.9 ^{A}	873±66 ^{ABC}	93±15 ^{ABC}	0.8±0.2 ^{ABC}	1510±395 ^{AB}	S	S
134	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	12.3±9.1 ^{A}	847±104 ^{ABC}	86±18 ^{ABCDEF}	0.7±0.2 ^{ABCDEF}	1414±38 ^{AB}	MS	S
136	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	9.3±3.7 ^{A}	631±113 ^{ABCDEF}	56±17 ^{ABCDEFGHI}	0.4±0.2 ^{ABCDEFGH}	1160±361 ^{AB}	MR	S
137	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	11.2±5.6 ^{A}	778±120 ^{ABCDE}	67±13 ^{ABCDEFGHI}	0.5±0.1 ^{ABCDEFGH}	1273±165 ^{AB}	MS	S
138	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	14.8±4.8 ^{A}	530±146 ^{BCDEF}	41±11 ^{EFGHI}	0.3±0.1 ^{EFGH}	1582±284 ^{AB}	MS	S
139	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	7.3±5.5 ^{A}	713±102 ^{ABCDEF}	61±19 ^{ABCDEFGH}	0.4±0.2 ^{ABCDEFGH}	1251±339 ^{AB}	MR	S
140	2	LEUC 84693/AE.SQUARROSA (1026)//GEREK79	18.7±5.6 ^{A}	746±147 ^{ABCDE}	66±17 ^{ABCDEFGHI}	0.5±0.2 ^{ABCDEFGH}	1370±215 ^{AB}	S	S
141	2	UKR-OD 1871.94/AE. SQUARROSA (213)//MEZGIT-6	9.3±6.6 ^{A}	706±70 ^{ABCDEF}	63±2 ^{ABCDEFGHI}	0.5±0 ^{ABCDEFGH}	1332±427 ^{AB}	MR	S
142	2	UKR-OD 1871.94/AE.SQUARROSA (213)//MEZGIT-6	10.2±3.8 ^{A}	561±30 ^{ABCDEF}	49±3 ^{CDEFGHI}	0.3±0 ^{BCDEFGH}	1175±209 ^{AB}	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
143	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	13.5±6.7 A	681±123 ABCDEF	59±11 ABCDEFGHI	0.4±0.1 ABCDEFGH	1612±244 AB	MS	S
144	2	UKR-OD 1871.94/AE.SQUARROSA (213)//MEZGIT-6	10.2±6.3 A	646±113 ABCDEF	49±5 CDEFGHI	0.3±0 DEFGH	1532±138 AB	MR	S
145	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	17±5.9 A	801±154 ABCDE	68±17 ABCDEFGH	0.5±0.1 ABCDEFGH	1602±571 AB	S	S
146	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	12.8±13.7 A	764±134 ABCDE	66±15 ABCDEFGHI	0.5±0.1 ABCDEFGH	1348±120 AB	MS	S
147	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	14.5±11.9 A	647±155 ABCDEF	50±14 CDEFGHI	0.3±0.1 DEFGH	1276±314 AB	MS	S
148	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	7.7±2.9 A	712±43 ABCDEF	62±10 ABCDEFGHI	0.4±0.1 ABCDEFGH	1370±198 AB	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
149	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	7.7±5 A	687±47 ABCDEF	67±11 ABCDEFGHI	0.5±0.1 ABCDEFGH	951±149 B	MR	S
150	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	10.8±5 A	692±62 ABCDEF	66±8 ABCDEFGHI	0.5±0.1 ABCDEFGH	1149±239 AB	MS	S
151	2	UKR-OD 952.92/AE.SQUARROSA (409)//SONMEZ	16.2±7.4 A	774±62 ABCDE	69±6 ABCDEFGH	0.5±0 ABCDEFGH	1407±223 AB	S	S
152	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	9.3±4.3 A	625±38 ABCDEF	51±1 BCDEFGHI	0.3±0 BCDEFGH	1353±566 AB	MR	S
153	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	10.7±6.3 A	741±86 ABCDE	72±11 ABCDEFGH	0.6±0.1 ABCDEFGH	1681±307 AB	MS	S
154	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	8±4.4 A	524±104 BCDEF	43±9 DEFGHI	0.3±0.1 EFGH	1336±571 AB	MR	S
156	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	14.3±8.2 A	851±111 ABC	75±7 ABCDEFGH	0.5±0 ABCDEFGH	1863±447 AB	MS	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
157	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	9±5.8 A	722±107 ABCDEF	56±5 ABCDEFGHI	0.3±0 BCDEFGH	1843±216 AB	MR	S
158	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	12±3.3 A	675±237 ABCDEF	57±23 ABCDEFGHI	0.4±0.2 ABCDEFGH	1378±79 AB	MS	S
159	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	6.7±3.3 A	733±92 ABCDE	82±15 ABCDEFG	0.7±0.2 ABCDEF	1205±154 AB	MR	S
160	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	9.2±4.9 A	747±99 ABCDE	80±15 ABCDEFG	0.7±0.2 ABCDEFG	1648±326 AB	MR	S
161	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	8.3±3.7 A	894±126 AB	89±18 ABCD	0.7±0.2 ABCDEF	1523±177 AB	MR	S
162	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	11±6.1 A	707±144 ABCDEF	73±20 ABCDEFGH	0.6±0.2 ABCDEFGH	1426±356 AB	MS	S
163	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	9.7±6.9 A	810±100 ABCDE	86±14 ABCDEF	0.7±0.2 ABCDE	1708±395 AB	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
164	2	UKR-OD 1530.94/AE.SQUARROSA (446)//KATIA1	11.5±6.3 A	704±77 ABCDEF	63±3 ABCDEFGHI	0.4±0 ABCDEFGH	1278±308 AB	MS	S
165	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	7.8±5.5 A	558±66 ABCDEF	51±4 BCDEFGHI	0.4±0 ABCDEFGH	986±244 B	MR	S
166	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	16.3±9.1 A	897±31 AB	92±6 ABC	0.8±0.1 ABCD	1393±58 AB	S	S
167	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	14.7±10.3 A	626±166 ABCDEF	51±16 BCDEFGHI	0.3±0.1 CDEFGH	1518±318 AB	MS	S
168	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	15±9.4 A	900±173 AB	83±13 ABCDEFG	0.6±0.1 ABCDEFG	1534±346 AB	MS	S
169	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	14.2±7.1 A	741±162 ABCDE	72±18 ABCDEFGH	0.6±0.2 ABCDEFGH	1172±180 AB	MS	S
170	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	14.2±5.6 A	833±78 ABC	76±13 ABCDEFGH	0.6±0.1 ABCDEFGH	1540±75 AB	MS	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
171	2	UKR-OD 1530.94/AE.SQUARROSA (311)//EKIZ	12.3±8.7 A	880±81 ABC	89±5 ABCDE	0.7±0 ABCDEF	1611±75 AB	MS	S
172	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	13.8±6.2 A	838±57 ABC	67±9 ABCDEFGHI	0.4±0.1 ABCDEFGH	1655±350 AB	MS	S
173	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	9.7±6 A	605±336 ABCDEF	55±30 ABCDEFGHI	0.4±0.2 ABCDEFGI	954±519 B	MR	S
174	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	7±2.2 A	545±94 ABCDEF	46±10 CDEFGHI	0.3±0.1 CDEFGH	1383±36 AB	MR	S
176	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	14±7.5 A	607±105 ABCDEF	50±15 CDEFGHI	0.3±0.1 CDEFGH	2320±673 A	MS	S
177	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	12.7±10 A	773±104 ABCDE	68±7 ABCDEFGH	0.5±0.1 ABCDEFGH	1541±316 AB	MS	S
178	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	14.2±6.7 A	469±183 CDEF	41±20 FGHI	0.3±0.2 EFGH	1673±871 AB	MS	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
179	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	11.2±6.2 A	634±56 ABCDEF	63±17 ABCDEFGHI	0.6±0.2 ABCDEFGH	1196±183 AB	MS	S
180	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	19.5±7.5 A	657±34 ABCDEF	54±11 ABCDEFGHI	0.4±0.1 BCDEFGH	1395±463 AB	HS	S
181	2	UKR-OD 1530.94/AE.SQUARROSA (312)//BAGCI2002	11.7±7.1 A	540±128 BCDEF	42±10 DEFGHI	0.3±0.1 EFGH	1661±92 AB	MS	S
231	**	Bezostaya	24.7±9.6 A	M	M	M	M	HS	S
232	**	Katea	9±5 A	M	M	M	M	MR	S
233	**	Kutluk	22.8±6.8 A	M	M	M	M	HS	S
234	**	Sönmez	9.8±6 A	M	M	M	M	MR	S
183	3	LANGDON/AE 454	12.0±6 ABCD	962±169 ABCDEFG	92.1±17.8 B	0.7±0.2 ABCDE	1628±279 B	MS	S
184	3	LANGDON/AE 929	5.0±4.1 BCDEF	466±238 DEFG	45.9±20.7 B	0.4±0.2 ABCDEF	1120±231 B	MR	S
185	3	LANGDON/IG 48042	9.0±2.4 ABCD	582±32 ABCDEFG	58.3±8.8 B	0.5±0.1 ABCDEF	1128±207 B	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
186	3	LANGDON/IG 126387	3.3±1.2 CDEF	583±48 ABCDEFG	57.5±2.7 B	0.5±0.1 ABCDEF	1264±263 B	R	S
187	3	LANGDON/IG 131606	3.3±1.6 CDEF	580±173 ABCDEFG	53.5±16.5 B	0.4±0.1 ABCDEF	1269±357 B	R	S
188	3	LANGDON/KU-20-8	5.2±5.2 CDEF	707±58 ABCDEFG	65.9±7.1 B	0.5±0.1 ABCDEF	1267±107 B	MR	S
189	3	LANGDON/KU-2039	2.8±2.5 DEF	659±89 ABCDEFG	60.4±8.9 B	0.4±0.1 ABCDEF	1253±296 B	R	S
190	3	LANGDON/KU-2074	9.0±3.7 ABCD	643±1 ABCDEFG	59.3±11.7 B	0.5±0.2 ABCDEF	1355±294 B	MR	S
191	3	LANGDON/KU-2075	2.7±1.6 DEF	360±79 FG	31.7±8.8 B	0.2±0.1 F	1288±88 B	R	S
192	3	LANGDON/KU-2080	4.5±1.6 BCDEF	606±43 ABCDEFG	48.8±6.1 B	0.3±0.1 CDEF	1644±470 B	R	S
193	3	LANGDON/KU-2088	5.7±3.3 BCDE	700±31 ABCDEFG	60.5±6.3 B	0.4±0.1 ABCDEF	1621±435 B	MR	S
194	3	LANGDON/KU-2092	9.3±4.6 ABCD	712±45 ABCDEFG	58.8±5.2 B	0.4±0.1 ABCDEF	1642±508 B	MR	S
195	3	LANGDON/KU-2096	7.0±2.5 ABCD	717±56 ABCDEFG	60.7±5 B	0.4±0.1 ABCDEF	1685±94 B	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
196	3	LANGDON/KU-2097	6.8±5.1 ABCDE	799±101 ABCDEF	67.2±12. B 8	0.5±0.1 ABCDEF	1595±406 B	MR	S
197	3	LANGDON/KU-2098	6.0±2.4 ABCDE	747±26 ABCDEFG	65.3±8 B	0.5±0.1 ABCDEF	1434±334 B	MR	S
198	3	LANGDON/KU-2100	4.3±2.7 BCDEF	574±277 ABCDEFG	51.4±27. B 8	0.4±0.2 ABCDEF	1655±144 B	R	S
199	3	LANGDON/KU-2105	5.8±2.8 ABCDE	840±86 ABCDEFG	90.7±4.2 B	0.8±0.1 AB	1452±243 B	MR	S
200	3	LANGDON/KU-2106	6.2±1.7 ABCDE	501±209 BCDEFG	50.1±20 B	0.4±0.2 ABCDEF	1113±355 B	MR	S
202	3	LANGDON/KU-2124	0.5±1.2 F	911±487 ABCDEFG	80.7±35.8 B	0.6±0.2 ABCDEF	3231±2235 A	R	R
203	3	LANGDON/KU-2144	1.5±3.2 EF	461±24 DEFG	52.1±3.1 B	0.5±0.1 ABCDEF	849±74 B	R	R
204	3	LANGDON/KU-2159	6.0±2.6 ABCDE	781±138 ABCDEF	75.5±17 B	0.6±0.2 ABCDEF	1327±279 B	MR	S
205	3	LANGDON/KU-2829A	3.5±2.7 CDEF	709±69 ABCDEFG	67.4±16.2 B	0.5±0.2 ABCDEF	1550±225 B	R	S
206	3	LANGDON/PI 476874	5.8±3.7 ABCDE	602±177 ABCDEFG	53.1±22.2 B	0.4±0.2 ABCDEF	1571±167 B	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
207	3	LANGDON/AT 55	5.7±4.5 BCDEF	651±188 ABCDEFG	52.6±16.5 B	0.3±0.1 BCDEF	1580±305 B	MR	S
208	3	LANGDON/AT 76	9.0±3 ABCD	609±97 ABCDEFG	48.8±9.9 B	0.3±0.1 CDEF	1680±337 B	MR	S
209	3	LANGDON/AT 80	5.0±2.3 ABCDE	722±46 ABCDEFG	66.2±1.1 B	0.5±0.1 ABCDEF	1384±348 B	MR	S
210	3	LANGDON/AE1090	3.8±2.8 CDEF	634±70 ABCDEFG	53.1±4.2 B	0.4±0.1 ABCDEF	1528±441 B	R	S
211	3	LANGDON/IG 47259	5.2±5.6 CDEF	384±65 FG	34.5±7.6 B	0.3±0.1 EF	1374±298 B	MR	S
212	3	LANGDON/KU-20-10	11.8±6.8 ABCD	496±0 BCDEFG	40.8±1 B	0.3±0.1 EDF	1517±212 B	MS	S
213	3	LANGDON/KU-2076	3.5±3.5 DEF	294±147 G	29.7±14.3 B	0.2±0.2 F	861±334 B	R	S
214	3	LANGDON/KU-2078	10.2±3.3 ABCD	666±48 ABCDEFG	57.6±4.6 B	0.4±0.1 ABCDEF	1327±312 B	MR	S
215	3	LANGDON/KU-2079	8.8±3.5 ABCD	525±54 BCDEFG	40.7±4.2 B	0.3±0.1 EF	1396±159 B	MR	S
216	3	LANGDON/KU-20-9	5.7±5.9 BCDEF	717±83 ABCDEFG	50.5±8.3 B	0.3±0.1 EDF	1641±162 B	MR	S
217	3	LANGDON/KU-2090	5.7±2.6 ABCDE	750±198 ABCDEFG	70.1±17.9 B	0.5±0.2 ABCDEF	1543±380 B	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
218	3	LANGDON/KU-2091	5.2±4.2 BCDEF	849±30 ABCDEFG	90.3±23.4 B	0.8±0.4 AB	1607±82 B	MR	S
219	3	LANGDON/KU-2093	10.3±3.4 ABCD	837±78 ABCDEFG	84.0±8.2 B	0.7±0.1 ABCDEF	1523±427 B	MR	S
220	3	LANGDON/KU-2103	5.3±4.1 BCDEF	812±62 ABCDEF	84.9±4.2 B	0.7±0.1 ABCD	1447±263 B	MR	S
222	3	LANGDON/KU-2109	4.0±2.2 CDEF	690±74 ABCDEFG	82.7±4.1 B	0.8±0.1 AB	1506±346 B	R	S
223	3	LANGDON/KU-2132	3.7±3.3 CDEF	637±269 ABCDEFG	77.4±27.2 B	0.8±0.2 ABC	1232±443 B	R	S
224	3	LANGDON/KU-2136	6.3±2.7 ABCDE	834±37 ABCDEF	91.8±8.1 B	0.8±0.2 A	1342±140 B	MR	S
225	3	LANGDON/KU-2155	3.7±3.8 DEF	588±56 ABCDEFG	61.2±5.1 B	0.5±0.1 ABCDEF	1104±56 B	R	S
226	3	LANGDON/KU-2156	4.5±4.6 CDEF	813±90 ABCDEF	82.8±20.6 B	0.7±0.3 ABCDEF	938±828 B	R	S
227	3	LANGDON/KU-2158	7.7±4.9 ABCDE	704±148 ABCDEFG	59.5±10.5 B	0.4±0.1 ABCDEF	1424±425 B	MR	S
228	3	LANGDON/KU-2816	5.3±3.7 BCDEF	477±211 BCDEFG	42.7±19 B	0.3±0.1 CDEF	1520±278 B	MR	S

Code	Group	Cross	Number of cysts	Root length (cm)	Root surface (cm^2)	Root volume (cm^3)	Number of root tips	Resistance level *(1)	Resistance level *(2))
229	3	LANGDON/PI 499262	18.7±11.7 ABCD	969±156 AB	71.7±8.3 B	0.4±0.1 ABCDEF	2947±809 A	S	S
230	3	LANGDON/PI 508262	24.8±9.5 A	745±237 ABCDEFG	62.5±18.9 B	0.4±0.1 ABCDEF	1943±368 B	HS	S
231	**	Bezostaya	11.1± 6.1 ABC	M	M	M	M	MS	S
232	**	Katea	14.6± 11.8 ABC	M	M	M	M	MS	S
233	**	Kutluk	12.3± 7.8 AB	M	M	M	M	MS	S
234	**	Sönmez	13.5± 3.2 AB	M	M	M	M	MS	S

*(1) Scoring based on Pariyar *et al*. (2016) as follow:

*Resistant (R) = <5 females and cysts; moderately resistant (MR) = 5-10 females and cysts; moderately susceptible (MS) = 11-15 females and cysts; susceptible (S) = 16-19 females and cysts; and highly susceptible (HS) ≥ 20 females and cysts.

*(2) Scoring based on Andersen & Andersen (1982) as follow:

Resistant (R) = <3 cysts; and susceptible (S) ≥ 3 cysts

** Reference cultivars included in each group.

*** Means within a group followed by different letters are significantly different based on Student-Newman-Keuls analysis at ($P \leq 0.05$).

****M: Missing data.

9.3 **Appendix 3.** Most common abbreviations used in selection

abbreviations used to describe locations in selection history			
Abbreviation	**Full name**	**Abbreviation**	**Full name**
AP	Aleppo, ICARDA, Syria	YC	Cumra, Turkey
H	Hyslop, Oregon, USA	YM or YK	Merkez, konya
M	Toluca, CIMMYT, Mexico	YE or E	Eskisehir, Turkey
WM	Winter Mexico, CIMMYT	TE or T	Edirne, Turkey
YA	Ankara, Turkey	GD	Diyarbakır
SE	Izmir	R	Erzurum
SA	Adana	P	Adapazarı (sakarya)
Abbreviations used for the country or programme of origin of a cultivar			
Abbreviation	**Full name**	**Abbreviation**	**Full name**
AFG	Afghanistan	KS	Kansas, USA
ALG	Algeria	MOL	Moldova
ARG	Argentina	MOS	Moscow, Russia
ARM	Armenia	MV	Martonvasar, Hungary
AU	Australia	MX	Mexico MD
BD	Konya, Turkey	ND	North Dakota, USA
BG	Bulgaria	NE	Nebraska, USA
CH	Switzerland	NY	New York, USA
CHL	Chile	NL	Netherlands
CIT	CIMMYT/ICARDA/Turkey	NS	Novi Sad, Yugoslavia

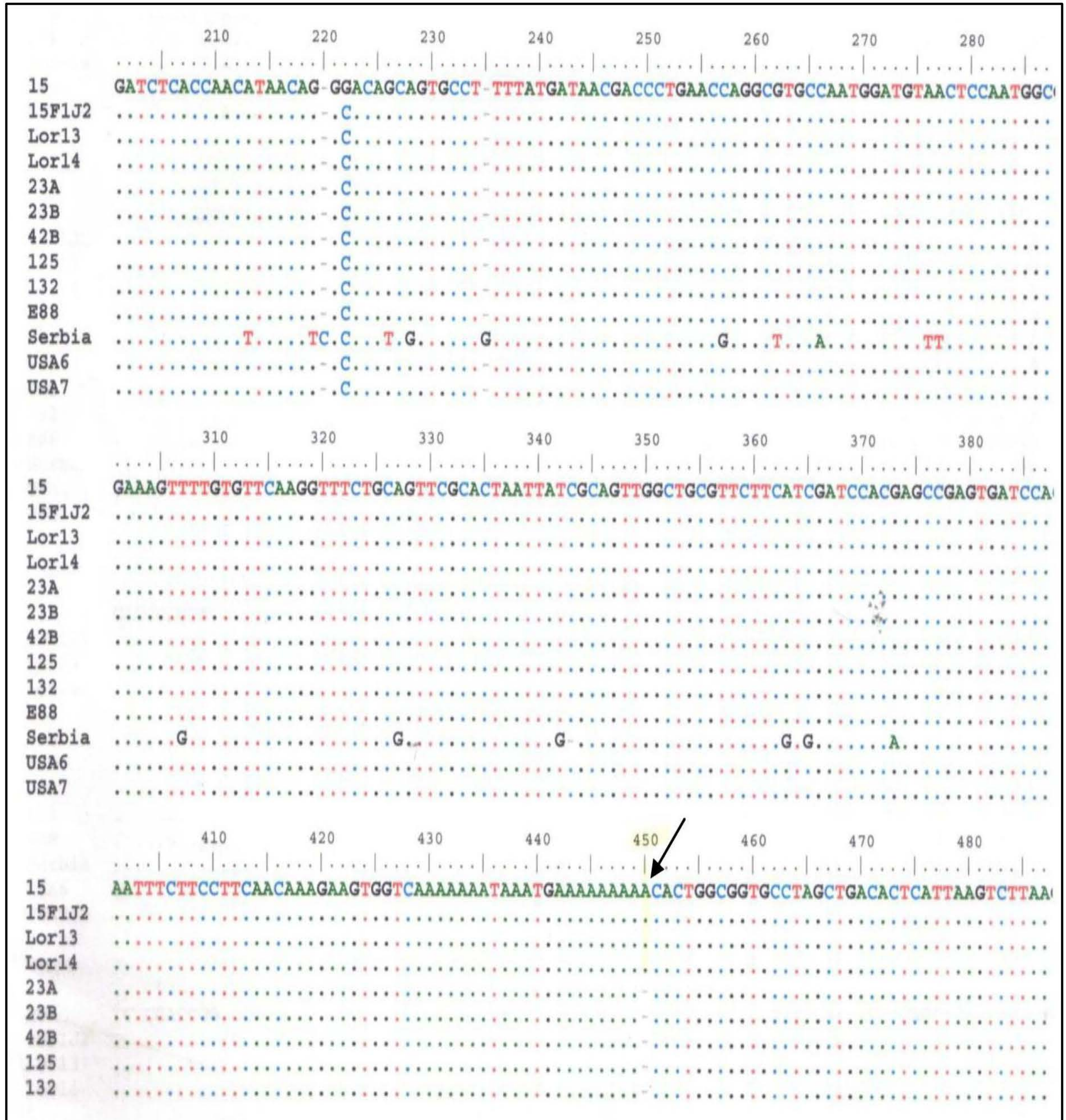
CN	China	OD	Odessa, Ukraine
CO	Colorado, USA	OK	Oklahoma, USA
CRO	Croatia	ON	Ontario, USA
CZ	Czech Rep.	OR	Oregon, USA
DE	Erzurum, Turkey	RO	Romania
DO	Dobrudja, Bulgaria	RUS	Russia
FL	Fundulea, Romania	SD	South Dakota, USA
FLO	Florida	SY	ICARDA, Syria
FR	France	SZ	Szeged, Hungary
GA	Georgia, USA	TCI	Turkey/CIMMYT/ICARDA
GE	Germany	TE	Edirne, Turkey
GEO	Republic of Georgia	TR	Turkey
HU	Hungary	TRM	Turkmenistan
IND	India	TX	Texas
IR	Iran	VA	Virginia, USA
IT	Italy	UK	United Kingdom
JAP	Japan	UKR	Ukraine
KAZ	Kazakstan	US	USA
KC	Sadovo, Bulgaria	YA	Ankara, Turkey
KR	Krasnodar, Russia	YE	Eskisehir, Turkey
LIT	Lithuania	YU	Yugoslavia
MIR	Mironovka, Ukraine	ZA	Bethlehem, S. Africa
KYR	Kyrgizstan	ZH	Zhengzhou, China

9.4 **Appendix 4.** Summary of the Cross

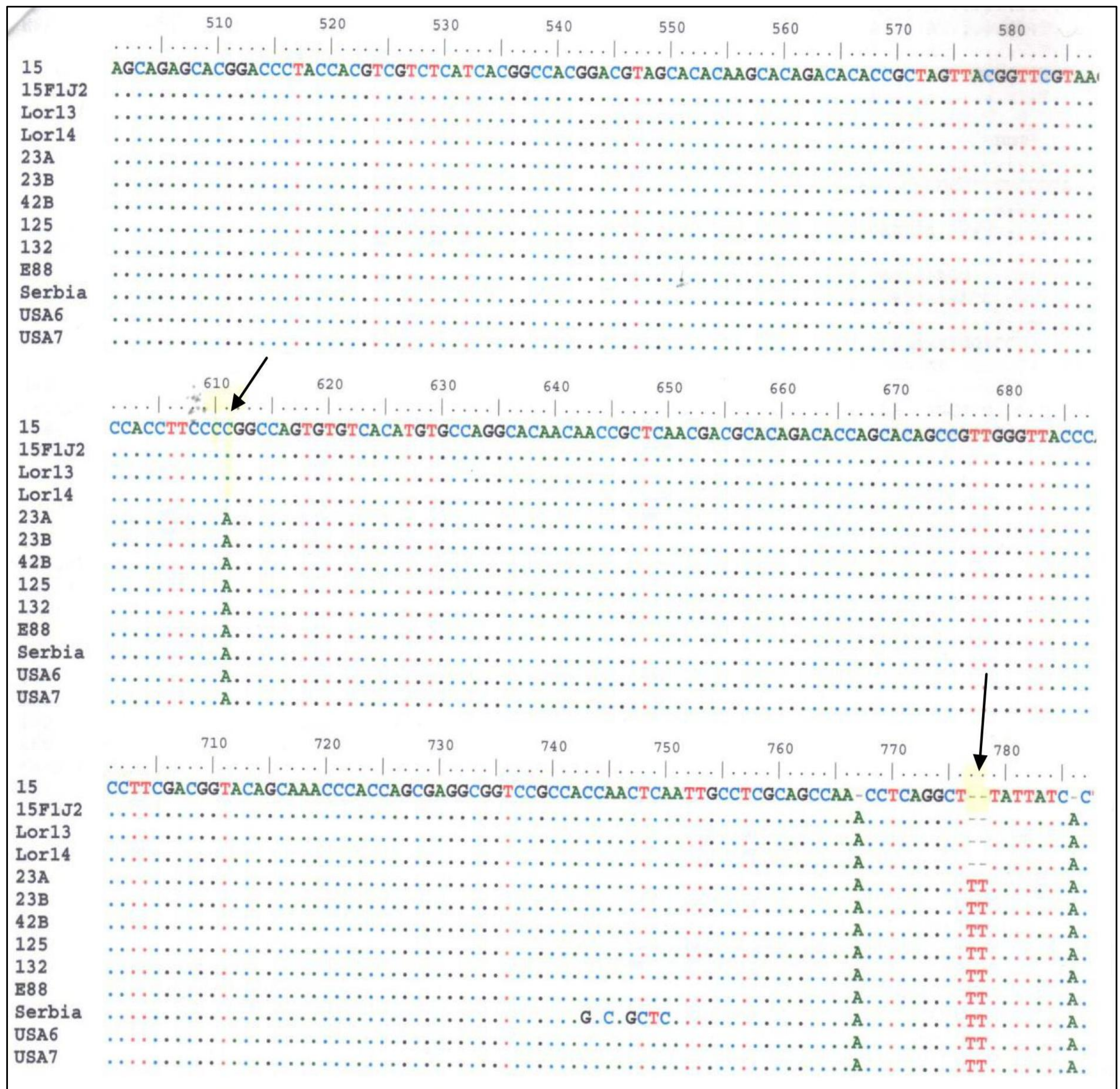
GROUP 1	AE.SQ (223)	AE.SQ (310)	AE.SQ (369)	AE.SQ (392)	AE.SQ (409)	AE.SQ. (446)	AE.SQ. (458)	AE.SQ. (511)	AE.SQ. (629)	AE.SQ. (1026)	AE.SQ. (1027)
AISBERG (Ukraine)			03-7 lines					05-9lines			
LEUC 84693 (Ukraine)		09-3lines			10-5lines					12-5 lines	
UKR-OD 761.93 (Ukraine)				22-lines							
UKR-OD 952.92 (Ukraine)											
UKR-OD 1530.94 (Ukraine)		68-8 lines		71-6 lines		73-2 lines	74-7 lines		76-9 lines		78-11 lines
PANDUR (Romania)	79-9 lines				81-9 lines						
GROUP 2	DEMIR	ADYR	GEREK	MEZGIT-6	SONMEZ	EKIZ	BAGCI	KATYA			
AISBERG/AE.SQ. (369)	54-10 lines										
LEUC 84693/ AE.SQ. (310)		59-8 lines									
LEUC 84693/ AE.SQ. (1026)			61-8 lines								
UKR-OD 1871.94/ AE.SQ. (213)				64-5 lines							
UKR-OD 952.92/ AE.SQ. (409)					66-10 lines						
UKR-OD 1530.94/AE.SQ. (311)						71-6 lines					

GROUP 2	DEM IR	ADY R	GER EK	MEZG IT-6	SONM EZ	EKIZ	BAG CI	KAT YA			
UKR-OD 1530.94/ AE.SQ. (312)							72-9 lines				
UKR-OD 1530.94/ AE.SQ. (446)								74-12 lines			

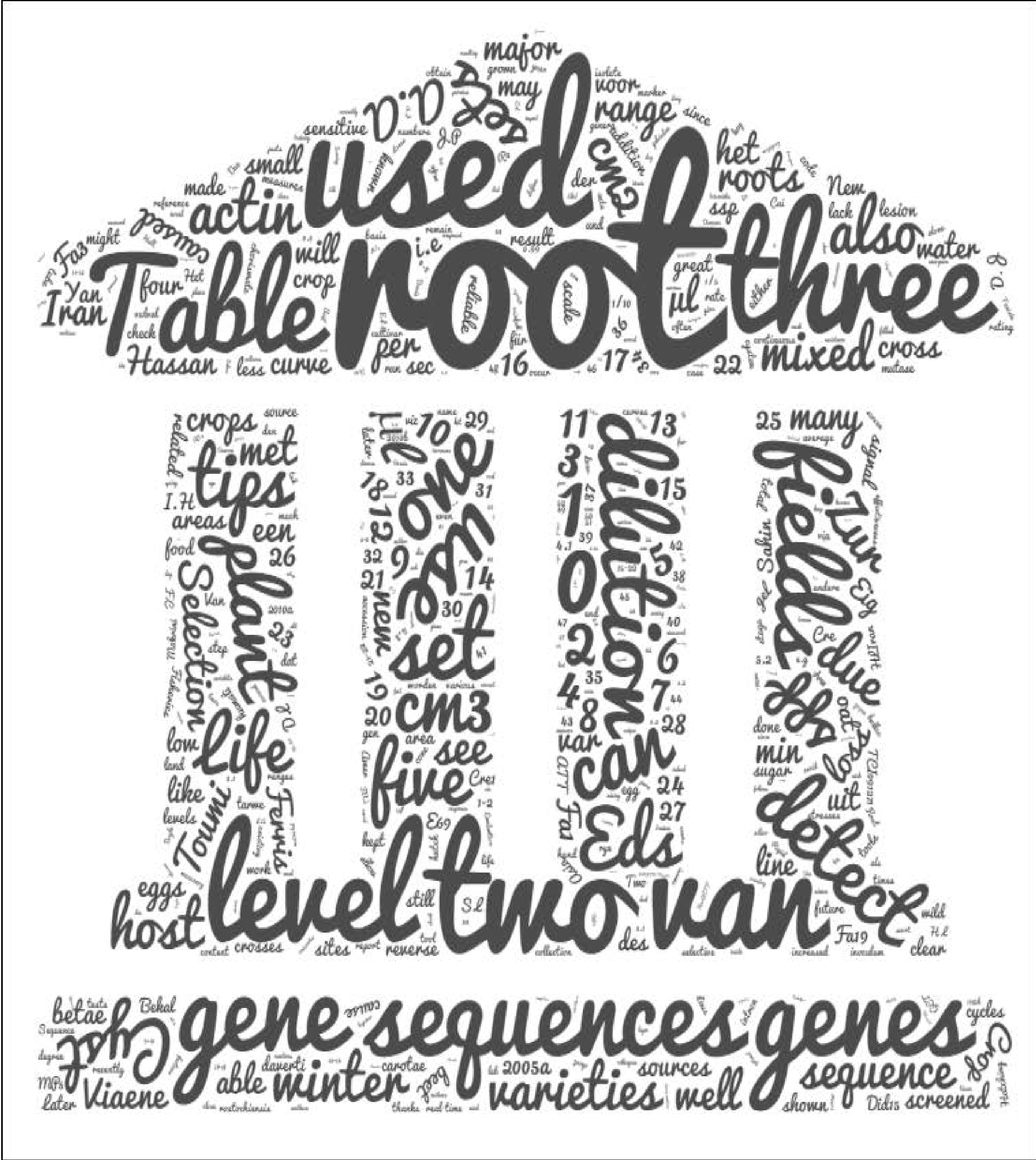
9.5 **Appendix 5.** Alignment of rDNA-ITS region for different *Heterodera filipjevi*



Appendix 5. Part of the alignment of rDNA-ITS region for different *Heterodera filipjevi* populations originated from different countries. Iran: 15, 15F1J2, Lor13 and Lor14; Turkey: 23A, 23B and 42B; Syria: 125 and 132; Russia: E88; Serbia: Serbia; USA: USA6 and USA7.



Appendix 5. continued.



Thesis words cloud