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Morphological and molecular characterization of cereal cyst nematode (*Heterodera avenae*) populations from arid environments

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Abstract

The morphological and molecular characteristics of four cereal cyst nematode (*Heterodera avenae*) populations collected from the Qassim, Tabouk, Riyadh, and Hail regions, Saudi Arabia were comparatively investigated. A large number of soil samples were collected from a representative field (72 ha) in each region. The morphological and morphometric characteristics of the populations were determined. Morphometric data were subjected to multivariate canonical discriminant analysis to analyze the relationship between the studied populations and to identify the variables that show the highest multiple correlations with these populations. For molecular characterization, DNA was extracted and purified from five random white females from each population. The internal transcribed spacer (ITS1) regions were subjected to direct sequencing to study the diversity of these populations. Discriminant analysis of the morphometric traits indicated that the studied populations belong to one species (*H. avenae*). The ITS1 sequence alignments showed similarity between individuals, ranging from 87 to 99%. Based on the sequencing data, consensus parsimonious and maximum likelihood trees showed an overlap between the individuals of the four populations, suggesting that all four populations represented one species. However, based on the morphological and molecular analysis, the Hail population was somewhat different from the other three populations. Minor genetic and phenotypic differences between the four populations could indicate that these populations are heterogenic, probably mixed populations. This study also revealed the value of some J_2 morphometric traits such as J_2 midbody width, J_2 body width at the anus, J_2 head height and the J_2 ratios a, b, c and c′ in determining intraspecific variation between *H. avenae* populations.

Keywords: Discriminant analysis, *Heterodera avenae*, intraspecific variation, ITS, heterogenic, polymorphism, phylogenetic.
Abbreviations: CCN – cereal cyst nematode, CDA **-** canonical discriminant analysis, DNA – deoxyribonucleic acid, dNTP – deoxyribonucleotide triphosphate, Ha – hectare, ITS – internal transcribed spacer, J_2 – second-stage juveniles, PCR – polymerase chain reaction, rDNA – ribosomal DNA, RFLP – restriction fragment length polymorphism.

Introduction

The cereal cyst nematode (CCN), *Heterodera avenae* Woll. (1924) has been reported on wheat and other cereal crops in many countries with different climatic types throughout the world (Handoo, 2002). In Saudi Arabia, the nematode was reported in an irrigated wheat (*Triticum aestivum* L.) field in the Qassim region in 1987 (Youssif, 1987). The nematode has since spread quickly to become a major limiting factor to wheat production in Saudi Arabia (Al-Hazmi and Dawabah, 2009, Ibrahim et al., 1999). Saudi populations of cereal cyst nematodes were identified as *H. avenae*, based on morphological and morphometric features (Al-Hazmi et al., 1994), and DNA markers (Al-Rehiayani, 2007). Isoelectric focusing of protein pattern analysis has shown that Saudi populations are typically *H. avenae* (Sturhan and Rumpenhorst, 1996; Subbotin et al., 1996; 1999; 2002). The pathotype of the nematode was characterized as being very close to the European pathotype Ha21 (Al-Hazmi et al., 2001). *Heterodera avenae* is one of several species comprising the *H. avenae* group, which contains 11-12 valid species plus other species (Handoo, 2002; Subbotin et al., 2003). These species can be differentiated from each other by some morphological and morphometric features (Abidou et al., 2005; Handoo, 2002; Subbotin et al., 1999). However, with the increasing number of species in this group, reliable identification based on morphology is becoming more difficult (Rivoal et al., 2003; Subbotin et al., 2003). DNA sequence variation in the internal transcribed spacer (ITS) regions of ribosomal DNA can be used to identify many nematode taxa and phylogenetic relations (Subbotin et al., 2001). The ribosomal genes flanking the ITS region are highly conserved, allowing for the construction of PCR primers. However, the ITS region is less conserved than the flanking regions, which allows for the detection of polymorphisms (Kumar et al., 2009; Madani et al., 2004).

Developed in recent years, PCR-ITS-rDNA diagnostics are a reliable tool for a precise and quick identification of cyst nematodes. ITS-rDNA sequences can facilitate the quick identification of most species of cyst nematodes (Abidou et al., 2005; Sabo et al., 2001; Subbotin et al., 1999; 2000; 2001; Szalanski et al., 1997; Tanha Maafi et al., 2003). In Saudi Arabia, PCR-RFLP analysis of ITS-rDNA sequences of six populations of *H. avenae*, collected from different parts in central Saudi Arabia, were studied by Al-Rehiayani (2007). The smallest genetic similarity was found between the populations from Unizah and Riyadh. However, the populations from Zolfy, Cherry and Buraydah were the most similar. The dendrogram constructed using UPGMA analysis distinguished three groups of *H. avenae* populations in central Saudi Arabia (Al-Rehiayani, 2007). Both molecular and classical methods can also be used to classify the nematode species within an *H. avenae* group. The results inferred from multivariate analysis applied to the morphometric characters of cysts and juveniles has shown good agreement between genetic and phenotypic classifications (Rivoal et al., 2003). This study aims to clarify the possible diversity of Saudi *H. avenae* populations collected from four major wheat-producing regions (Qassim, Tabouk, Riyadh and Hail) in Saudi Arabia, utilizing phenotypic and molecular tools.

Results and discussion

Morphological and morphometric characteristics of H. avenae populations

All morphological features of white females, cysts and second-stage juveniles (J_2) of the four Saudi populations were approximately similar. An example of the morphological characters of Saudi *Heterodera avenae* stages and structures is presented in Fig. 2. Nevertheless, morphometric data analysis showed some variability among the studied populations (Table 1). In general, females were lemon-shaped, white in color, with a protruding neck (Fig. 2 A) and vulva. The cuticle bears a zig-zag pattern, and a subcrystalline layer is sloughed off as females transform into cysts (Fig. 2 B). The head is offset and the stylet shape is slender, with posteriorly sloping basal knobs (Fig. 2 A). The brown cysts of all populations were variable in size and mostly lemon-shaped, with a protruding neck and vulvar cone structure (Fig. 2 C). The cyst wall is dark brown to black in color, and bears a zig-zag pattern. New cysts are mostly enveloped with a chalk-like bloom. The vulvar cone is bifenestrated (Figs 2 D-G) with a short vulvar slit (8-10 µm) (Figs. 2 E & F) and heavily crowded protruding bullae (Fig. 2 E, F & H). No underbridge was found in the vulvar cone structures of any of the examined populations. Second-stage juveniles are cylindrical in shape, with a slightly offset head (Fig. 2 I) and a tapering round tail tip (Fig. 2 J). The stylet is strong with shallow anteriorly concave basal knobs (Fig. 2 I). Males were also present (Fig. 2 K). Species of *H. avenae* group can be differentiated from each other by some morphological and morphometric features including six characters of J_2 (body length, stylet length, tail length, hyaline tail length, stylet knob shape and tail terminus shape) and four characters of the brown cyst (fenestral length, semi-fenestral width, width of the vulvar bridge and vulvar slit length) (Subbotin et al., 1999). Other morphological and morphometric features such as the b and c ratios of J_2, the length of cysts excluding the neck (L'), the width of cysts (B), the L'/B ratio, the presence, size and shape of bullae inside the vulvar cone of cysts and cyst color are also important characters (Handoo, 2002; Rivoal et al., 2003; Subbotin et al., 1996; 2003). Based on these morphological and morphometric characters, the four Saudi populations were found to be similar, and were identified as *H. avenae*. Previous morphological and electrophoretic studies have also characterized some Saudi populations as *H. avenae* (Al-Hazmi et al., 1994; Sturhan and Ruhmpenhorst, 1996). A pathotype characterization study by Al-Hazmi et al. (2001) showed that populations from Riyadh, Qassim and Hail are one pathotype which is very close, but not similar, to the European pathotype Ha21, suggesting that these populations might have arisen indigenously or have been imported from a single source (Al-Hazmi et al., 2001). The hierarchical cluster based on 37 morphometric characters was examined, and the distance index (Table 2 and Fig. 3) showed that the four populations can be grouped into two main clusters at 10 units of genetic distance (Fig. 3). The first main cluster included a combination of the Tabouk, Riyadh and Qassim populations. On the other hand, the Hail population represents another main cluster. However, at 25 units of genetic distance, the four populations were grouped in one cluster. The genetic distance coefficients ranged from 8.15 between the Tabouk and Riyadh populations to 9.32 between the Qassim and Tabouk populations (Table 2). The canonical discriminant analysis, performed with the standardized canonical discriminant function coefficients for the 37 morphometric characters, showed that the first three functions accounted for 100% of the total variation (76.9, 16.60, and 6.50%, respectively). The standardized canonical discriminant functions coefficients (Table 3) for the studied morphometric characters revealed that the first function was strongly influenced by the J_2 midbody width, J_2 c ratio, cyst body width, J_2 body width at the anus and J_2 c′ ratio. The second canonical discriminate function was strongly influenced by the J_2 midbody width, J_2 b ratio, J_2 c ratio and J_2 caudal ratio A. The third canonical discriminate function was strongly influenced by the J_2 c ratio, J_2 b ratio and J_2 head height. A graphical representation of the distribution of the four populations in the space of the two discriminate functions (Fig. 4) showed that function 1 clearly separated the Hail population from the other populations. However, the second function discriminated the Qassim and Riyadh populations from the Tabouk and Hail populations; 96.0% of the original grouped cases were correctly classified. These results reveal the value of some characters that can be utilized for the separation of different populations within *H. avenae* and in determining the intraspecific variations between these populations. These characters include cyst body dimensions, J_2 midbody width, J_2 body width at the anus, J_2 head height and J_2 ratios; a, b, c, c′, and caudal ratio A.

ITS1 sequence analysis

Sequence alignments of the selected ITS1 region showed that the similarity between individuals within each population ranged from 87% between Q1 and Q2 to 99% between Q1 and Q7. Sequence similarities for the Hail population were 97% between H1 and H4 and 98% between H1 and H7. Sequence similarity for the Qassim population ranged from 85% between Q2 and Q4 to 99% between Q1 and Q7. Within different populations, sequence similarity ranged from 79% between Q2 and R6 to 97% between Q7 and R6.

The multiple alignment revealed point mutations between sequences. The consensus for the ITS1 stretch 5'TCTGTGCTTTGGGgTGTTCT-3 was similar for all analyzed Saudi samples and international clones (NCBI,

Table 1. Diagnostic data on white females, cysts and second-stage juveniles (J_2) of four *Heterodera avenae* populations collected from the Qassim, Tabouk, Riyadh and Hail regions, Saudi Arabia.

Traits	*Heterodera avenae* population			
	Qassim Mean ± SD (Range)	Tabouk Mean ± SD (Range)	Riyadh Mean ± SD (Range)	Hail Mean ± SD (Range)
	White females			
Body length including neck (L)	649 ± 63 (518 – 777)	659 ± 75 (549 – 808)	661 ± 64 (539 – 766)	657 ± 65 (559 – 798)
Body length excluding neck (L′)	523 ± 64 (414 – 632)	522 ± 65 (430 – 642)	523 ± 64 (404 – 653)	522 ± 67 (414 – 673)
Neck length	138.5 ± 21.0 (104 – 187)	136.8 ± 25.0 (104 – 187)	138.5 ± 21.0 (104 – 177)	134.7 ± 24.8 (104 – 187)
Stylet length	28.6 ± 1.5 (27 – 31)	28.7 ± 1.6 (27 – 31)	28.6 ± 1.5 (27 – 31)	28.8 ± 1.5 (26 – 31)
Body width across median bulb valve	67.00 ± 8.0 (51 – 92)	67.8 ± 10.4 (38 – 87)	66.98 ± 8.0 (51 – 81)	69.0 ± 12.6 (51 – 89)
Midbody width	371.5 ± 51.0 (269 – 466)	365.4 ± 48.9 (280 – 467)	371.5 ± 51.0 (269 – 466)	365.6 ± 47.9 (269 – 487)
Base of stylet to DGO	5.0 ± 0.2 (4.6 – 5.1)	5.0 ± 0.2 (4.6 – 5.1)	5.0 ± 0.2 (4.6 – 5.1)	5.1 ± 0.2 (4.6 – 5.1)
L′/B ratio	1.41 ± 0.07 (1.24 – 1.58)	1.43 ± 0.1 (1.24 – 1.59)	1.41 ± 0.1 (1.26 – 1.56)	1.43 ± 0.1 (1.25 – 1.59)
	Cysts			
Cyst body length excluding neck (L′)	760 ± 58 (675 – 896)	749 ± 43 (675 – 844)	751 ± 96 (688 – 857)	761 ± 54 (649 – 870)
Cyst body width	515 ± 36 (455 – 584)	511 ± 42 (418 – 623)	508 ± 32 (442 – 597)	524 ± 35 (442 – 584)
L′/B ratio	1.48 ± 0.13 (1.18 – 1.76)	1.47 ± 0.11 (1.27 – 1.73)	1.48 ± 0.11 (1.28 – 1.69)	1.46 ± 0.13 (1.24 – 1.66)
Fenestral length	48.1 ± 2.1 (46 – 54)	49.6 ± 2.5 (46 – 54)	48.5 ± 2.3 (44 – 54)	48.0 ± 1.7 (46 – 51)
Semi-fenestral width	21.4 ± 1.2 (20 – 24)	22.3 ± 1.7 (20 – 24)	21.3 ± 1.4 (19 – 23)	21.0 ± 1.3 (19 – 23)
Vulva slit length	9.6 ± 0.9 (8 – 10)	9.7 ± 0.7 (8 – 10)	9.7 ± 0.7 (8 – 10)	9.5 ± 0.7 (8–10)
Width of bridge	5.8 ± 1.0 (5 – 8)	5.8 ± 1.2 (5 – 8)	5.5 ± 0.9 (5 – 8)	5.6 ± 0.9 (5 – 8)
	Second-stage juveniles (J_2)			
Body length	592 ± 24 (532-649)	619 ± 12 (597-649)	580 ± 20 (545-610)	596 ± 24 (519-623)
Midbody width	23.3 ± 1.6 (20-26)	23.0 ± 0.3 (23-24)	22.7 ± 0.5 (22-23)	23.2 ± 1.0 (22-26)
Head height	5.0 ± 0.6 (4-6)	5.1 ± 0.2 (4-5)	5.0 ± 0.3 (4-5)	5.0 ± 0.3 (4-5)
Head width	9.8 ± 0.6 (9-10)	10.1 ± 0.4 (9-10)	9.8 ± 0.6 (9-10)	9.9 ± 0.6 (9-10)
Stylet length	26.3 ± 0.9 (24-28)	26.7 ± 0.4 (26-27)	26.7 ± 0.9 (24-28)	26.7 ± 0.9 (24-29)
	Heterodera avenae population			
Stylet blade length	9.1 ± 0.9 (8-10)	9.6 ± 0.6 (9-10)	8.9 ± 0.7 (8-10)	9.3 ± 0.9 (9-12)
Stylet knob height	2.9 ± 0.6 (2.6-3.8)	2.7 ± 0.4 (2.6-3.8)	2.7 ± 0.4 (2.6-3.8)	2.7 ± 0.4 (2.6-3.8)
Stylet knob width	5.3 ± 0.5 (5-6)	5.1 ±0.2 (5-6)	5.2 ± 0.3 (5-6)	5.1 ± 0.3 (5-6)
Base of stylet to DGO	5.0 ± 0.3 (4-5)	5.0 ± 0.2 (4-5)	5.0 ± 0.3 (4-5)	5.0 ± 0.3 (4-5)
Head tip to median bulb valve	79.1 ± 2.8 (75-85) 127.0 ± 1.7	84.8 ± 1.8 (82-88) 129.5 ± 1.4	81.2 ± 1.3 (79-84) 129.3 ± 1.1	81.1 ± 3.1 (75-83) 125.8 ± 5.2

Head tip to esophago-intestinal junction	(122-131)	(128-131)	(126-131)	(125-128)
Head tip to excretory pore opening	115.5 ± 1.7 (112-119)	121.4 ± 3.0 (115-126)	118.1 ± 1.9 (115-124)	118.3 ± 3.0 (115-124)
Tail length	66.6 ± 3.8 (59-77)	69.4 ± 3.1 (64-74)	65.9 ± 2.7 (64-71)	67.3 ± 3.4 (60-74)
Body width at the anus	16.6 ± 1.1 (15-19)	17.7 ± 0.4 (17-18)	17.1 ± 0.9 (15-18)	16.9 ± 1.1 (15-19)
Hyaline tail length	42.4 ± 2.3 (38-49)	42.5 ± 3.1 (38-47)	40.0 ± 2.6 (36-46)	41.8 ± 2.8 (38-47)
Hyaline tail width at its beginning	9.6 ± 1.4 (8-13)	9.8 ± 1.4 (8-13)	9.5 ± 1.2 (8-13) (8-13)	9.8 ± 1.4 (9-13)
a	25.5 ± 2.4 (21.4-30.6)	26.9 ± 0.4 (26.0-28.3)	25.5 ± 0.8 (23.8-27.6)	25.8 ± 1.6 (21.4-28.8)
b	4.7 ± 0.2 (4.3-5.1)	4.8 ±0.1 (4.6-5.1)	4.5 ± 0.2 (4.2-4.7)	4.7 ± 0.3 (4.5-5.4)
c	8.9 ± 0.4 (7.8-9.8)	8.9 ± 0.3 (8.4-9.6)	8.8 ± 0.3 (8.3-9.4)	8.9 ± 0.4 (8.0-10.2)
c′	4.0 ± 0.4 (3.3-5.0)	3.9 ± 0.2 (3.6-4.3)	3.9 ± 0.3 (3.6-4.6)	4.0 ± 0.3 (3.3-4.8)
Caudal ratio A	4.5 ± 0.7 (3.1-5.8)	4.4 ± 0.7 (3.0-5.8)	4.3 ± 0.6 (2.8-5.3)	4.4 ± 0.7 (3.0-5.3)
Hyaline tail length/stylet length	1.6 ± 0.1 (1.4-1.8)	1.6 ± 0.1 (1.4-1.8)	1.5 ± 0.1 (1.3-1.8)	1.6 ± 0.1 (1.4-1.8)

Data are the average of fifty measurements.
a= total body length/maximum body width; b= total body length/pharyngeal length; c= total body length/tail length; c′= tail length/body width at the anus; caudal ratio A= hyaline tail length/hyaline tail width.

Fig 1. A map of Saudi Arabia showing the four regions from where the samples were collected.

2012). However, only sample T6 had a point mutation in this stretch (the lowercase "g" was mutated into "T"). In fact, point mutations are the most recorded variation for ITS1 sequences (Baldwin et al., 1995). On the other hand, only a few sites were affected by insertions/deletions (indels). This includes a single "T" after (T)n repeats as a deletion or insertion in samples Q6 and Q7 or in samples R6 and R7, respectively. Similar indel variations were recorded for *Heterodera* spp. (Tanha Maafi et al., 2003; Tanha Maafi et al., 2007).

Phylogenetic analysis

The phylogenetic relationship of *H. avenae* Saudi populations was compared to international genotypes. Based on sequencing of the ITS1 region, two phylogenetic trees were generated. The first was a consensus parsimonious tree (Fig. 5). This tree showed strong clustering of R7 with both R2 and Q2 samples. This could indicate a common ancestor, which would have presumably spread through transportation between the Riyadh and Qassim regions. These two regions are geographically adjacent to each other as compared to the other two regions assessed in this study (Fig. 1). In addition, Q6 and Q7 samples were tightly clustered in one clade (Fig. 5). Furthermore, they were grouped with international isolates from Europe and China. All other samples were dispersed along the tree. Although they were weakly clustered (based on bootstrap values), isolates from Morocco, Turkey and India were grouped with some Saudi samples (T6, AY148361 and R6, respectively). The second tree was a more robust and reliable phylogenetic analysis, the consensus maximum likelihood tree (Fig. 6). The tree was generated from 100 bootstrapped sequence alignments, which were subjected to global rearrangement and an additional five random replications. According to this tree, a clean separation of the outgroup species *H. latipons* AF274402 was found. In addition, the international isolate from China, AY148381, was separated from all international and Saudi samples of *H. avenae*. The maximum likelihood tree conveyed a similar message as the parsimony tree. For example, R7 was strongly clustered with R2 and Q2 (97%). On the other hand, some Saudi samples were weakly clustered with international counterparts, e.g. T6 and AY148369 from Morocco, or Q4 and AY148364 from Turkey. This could shed light on the potential ancestor(s) of the Saudi samples. Saudi *H. avenae* AY148361 was the only available ITS1 sequence in the Genbank before conducting this study. This entry did not cluster tightly with any of the Saudi samples characterized in this investigation. Therefore, it is not a representative sample for *H. avenae* populations available in Saudi Arabia, as was earlier presumed (Subbotin et al., 2003). In this study, Saudi population AY148361 was clustered with the AY148373 population from France (Fig. 6). However, Saudi *H. avenae* AY148361 was found to cluster with Australian and another populations from the Middle East in a previous study (Bekal et al., 1997). However, another report has grouped this Saudi population with populations from Turkey and India based on ITS-RFLP profile and RAPD markers (Subbotin et al., 2003). Generally, the resolved maximum likelihood tree in this study (Fig. 6) showed a clean separation of Saudi *H. avenae* populations from those of eastern origin such as AY148362 from India and AY148381 from China. Thus, the appearance of Saudi population AY148361 in the same clade with several European *H. avenae* isolates demonstrated a possible dissemination origin. This agrees with the findings of Subbotin et al. (2002), who reported that the Saudi *H. avenae* AY148361 isolate showed an isoelectric focusing (IEF) protein pattern similar those from European isolates. The inconsistent positioning of Saudi populations in the cladogram between this study and previous reports (Al-Rehiayani, 2007; Bekal et al., 1997; Subbotin et al., 2002) could indicate that these populations are mostly hetrogenic or mixed populations. A previous pathotype characterization study by Al-Hazmi et al. (2001) showed that three *H. avenae* populations collected from Riyadh, Qassim and Hail appeared to have the same novel virulence pathotype, suggesting that these populations might be heterogeneous for wheat virulence. These findings and previous reports are also supported by Bekal et al. (1997) in that there is intraspecific genetic variation between *H. avenae sensu stricto* populations. Noticeably, some of the investigated samples were not grouped in the phylogenetic tree according to geographical region (Fig. 5 and Fig. 6). These data describe a more complex population of *H. avenae* available in the four investigated geographical areas of Saudi Arabia. Some of the phylogenetic analysis results in this study coincided with the morphometric data analysis. The morphometric analysis indicates a unique grouping for the Hail samples. Likewise, three out of four Hail samples (H7, H4, and H6), used for ITS1 sequence analysis (Fig. 6), were clustered in one group. On the other hand, the canonical discriminant analysis, based on morphometric measurements, revealed an overlap between some samples from the Riyadh and Qassim regions. Similarly, the samples from Riyadh were occasionally grouped (based on ITS1 sequences) with the samples from Qassim, i.e. (R2, Q2 and R7) and (Q4, R6, and R8), suggesting that these two populations are generally similar on the species level, and that the minor differences between them in morphometric and molecular traits might be due to genetic variation arising from an interaction between environmental and genetic factors. The minor genetic and phenotypic differences that occurred among the four populations could indicate that these populations are heterogenic, probably mixed populations. Furthermore, it implies a threatening spread from one region to another in the country, either by farmers or farm machines, or even by the soil particles (containing cysts) which are carried on equipment or potato seed tubers produced in *H. avenae*-infested fields (Dawabah and Al-Hazmi, 2007; Smiley, 2005). This occurs especially when wheat is rotated with potato in Saudi Arabia (Dawabah and Al-Hazmi, 2007). The general agreement between our morphometric and molecular data is supported by previous reports that revealed the strength of the relative relationship between morphometric and molecular traits (Reed and Franklin, 2001; Rivoal et al., 2003; Subbotin et al., 2003).

Materials and methods

Nematode populations

Four populations of the cereal cyst nematode, *H. avenae*, were collected from the four major wheat-producing regions (Qassim, Tabouk, Riyadh and Hail) in Saudi Arabia (Fig. 1) during the 2009 wheat growing season. Soil samples were collected from one representative field in each region. Nematodes were extracted using sieving and floatation methods (Shepherd, 1986). Monoxenic cultures of *H. avenae* and wheat, *T. aestivum* (cv. Yecora Rojo) were maintained under controlled conditions.

Table 2. Coefficient of the Euclidean distance* between four *Heterodera avenae* populations collected from the Qassim, Tabouk, Riyadh and Hail regions, Saudi Arabia, based on 37 morphometric characters.

Heterodera avenae population	*Heterodera avenae* population			
	Qassim	Tabouk	Riyadh	Hail
Qassim				
Tabouk	9.32			
Riyadh	8.76	8.15		
Hail	8.48	9.05	9.18	

*The Euclidean distance between two populations (A and B) is the length of the line segment connecting them $\overline{AB}$.

Morphological and morphometric characterization

The specimens used for the morphological and morphometric studies were obtained from fresh material. Brown cysts were extracted from the soil of the monoxenic cultures using the floatation method (Shepherd, 1986). White females were picked out from infected wheat roots by maceration under a dissecting microscope. Eggs were obtained by squashing cysts that had been soaked in water for two days. Second-stage juveniles (J_2) were obtained by incubating the eggs in hatching cups at 5/15°C (18 h dark, 6 h light) (Al-Hazmi et al., 2001). Temporary mounts of juveniles (gently heat-killed), females and cysts were made in water (to avoid shrinking) on glass slides, and then immediately examined and measured under a compound microscope, using a calibrated ocular micrometer. Cone tops (the posterior ends of brown cysts) were prepared as described by Mulvey (1972), examined and photographed using an E600 Nikon® system equipped with a monitor and a Nikon® digital camera. The obtained morphological and morphometric data of the four populations were compared to each other and referenced to related published data (Golden 1986; Handoo, 2002; Mulvey and Golden, 1983; Robinson et al., 1996; Subbotin et al., 2003).

Morphometric data analysis

Based on Euclidean distance coefficients, a phenological dendrograms were constructed to evaluate the level of phenotypic variation among the four populations. Data were then run through discriminant multivariate analysis to investigate the separability of the four populations based on their morphometric characters. Canonical discriminant analysis (CDA) was performed using SPSS 15 (SPSS Inc., 2006).

Nematode DNA extraction

White females from each population (Qassim, Tabouk, Riyadh and Hail) were picked out from wheat roots (cv. Yecora Rojo) using a dissecting microscope. Nematode females were placed individually in Eppendorf PCR tubes and stored at -80˚C until used. For DNA extraction, five single white females from each population were ground in liquid nitrogen, and DNA extraction was performed in 20 µl of nematode lysis buffer [2 µl of 10X PCR buffer, 8 µl of double distilled water and 0.06 µl of proteinase K (20 mg/ml, Qiagen)] (Subbotin et al., 2000). The reaction was incubated at 60˚C for 1 h followed by enzyme inactivation by incubating the reaction at 95˚C for 15 min. The lysate was centrifuged at 12000 rpm for 1 min.

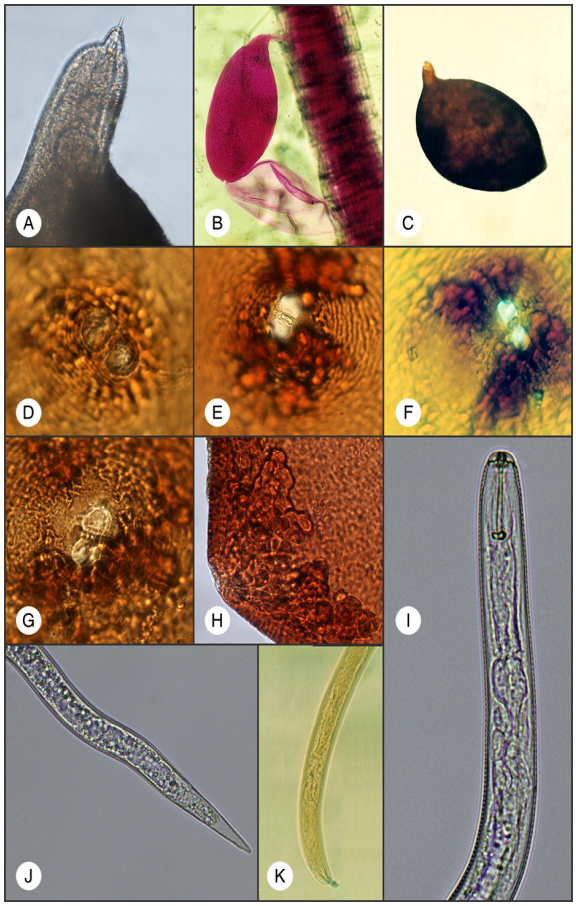

Fig 2. Morphological characters of some Saudi *Heterodera avenae* stages and structures: A) Female anterior end showing head offset, stylet and basal knobs and esophagus, B) subcrystalline layer sloughed-off as females transform into cysts, C) brown cysts, D-G) vulvar cones of specimens from the Tabouk, Qassim, Hail and Riyadh populations, respectively, showing the identity of the internal structures (fenestra, vulva slit, bridge and bullae) between the four studied populations. H) lateral view of the protruding bullae, I) J_2 anterior end showing the head, stylet, basal knobs and esophagus, J) J_2 tail, K) posterior end of a male showing spicules.

Table 3. Standardized canonical discriminant function coefficients* for four *Heterodera avenae* populations collected from the Qassim, Tabouk, Riyadh and Hail regions, Saudi Arabia, based on 37 morphometric characters.

Trait	Function		
	1	2	3
Brown cysts			
Body length	0.17	-0.12	-0.04
Body length excluding neck (L')	-0.95	0.59	-0.61
Body width	-5.53	3.99	0.80
L'/B ratio	0.33	-0.78	2.40
Fenstral length	0.33	-0.82	2.53
Semi-fenestral width	-0.07	-0.10	-0.13
Vulva slit length	-0.01	-0.03	0.27
Width of bridge	0.04	-0.05	-0.11
White females			
Body length	2.15	-0.24	-0.31
Neck length	2.17	-1.24	1.13
Stylet length	-0.80	0.45	-0.43
Body width at median bulb valve	0.13	-0.02	-0.08
Midbody width	-0.11	0.05	0.13
Dorsal esophageal gland orifice (DGO)	-2.15	1.26	-1.13
L/B ratio	-0.07	0.13	-0.02
Second-stage juveniles (J2)			
Body length	1.00	2.00	3.00
Midbody width	-7.81	-6.82	3.33
Head height	-0.12	3.19	6.34
Head width	0.02	-0.03	-0.04
Stylet length	-0.11	-0.15	0.08
Stylet blade length	1.12	-0.01	-0.63
Stylet knob height	0.19	-0.38	0.48
Stylet knob width	0.09	0.14	0.01
Dorsal esophageal gland orifice (DGO)	0.02	0.18	0.25
Head tip to median bulb valve	-0.14	0.07	-0.14
Head tip to esophago-intestinal junction	-0.07	-0.67	-0.02
Head tip to excretory pore	1.22	1.72	-3.58
Tail length	0.17	-0.51	-0.20
Body width at the anus	3.75	-3.48	2.38
Hyaline tail length	-0.52	3.30	-1.25
Hyaline tail width	-2.15	-0.07	0.37
a	0.21	0.17	0.26
b	-0.51	5.03	9.00
c	5.84	4.20	-9.05
c′	3.20	-0.48	1.29
Caudal ratio A	-0.42	5.00	-1.97
Hyaline tail length/stylet length	0.34	0.43	0.30

*Values reflect the contribution of each trait in the score of each function discrimination between the studied populations.
a= total body length/maximum body width; b= total body length/pharyngeal length; c= total body length/tail length; c′= tail length/body width at the anus; caudal ratio A= hyaline tail length/hyaline tail width.

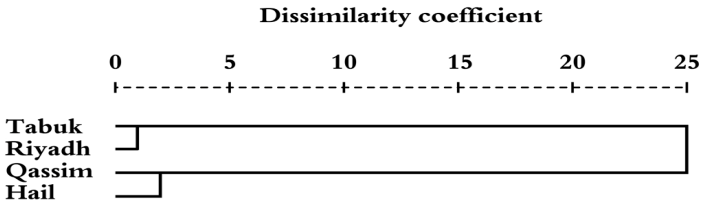

Fig 3. Hierarchical cluster showing the dissimilarity coefficients between four *Heterodera avenae* populations collected from the Qassim, Tabouk, Riyadh and Hail regions, Saudi Arabia, based on 37 morphological characters.

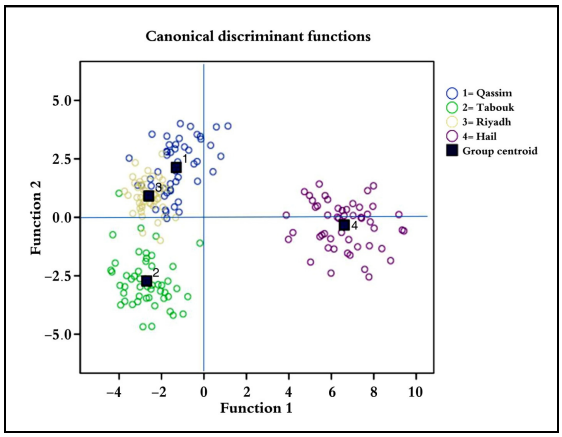

Fig 4. The two main canonical discriminant functions between the four *Heterodera avenae* populations collected from the Qassim, Tabouk, Riyadh and Hail regions, Saudi Arabia, based on 37 morphological characters. Functions 1 and 2 accounted for 76.9 and 16.6%, respectively, in the total variation between the studied populations.

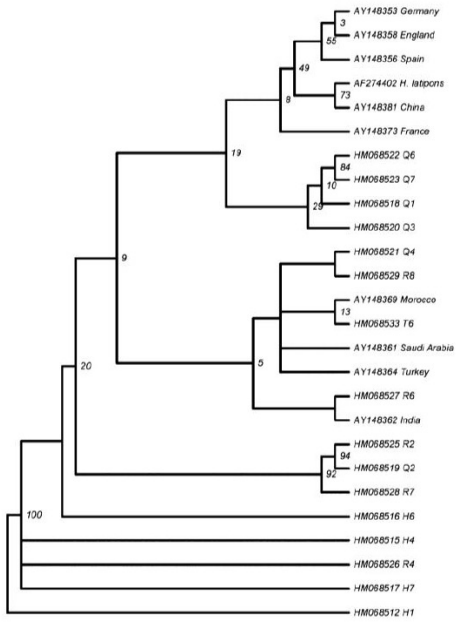

Fig 5. Phylogenetic relationships between four *Heterodera avenae* populations collected from the Qassim, Tabouk, Riyadh and Hail regions, Saudi Arabia, and international samples. A consensus of parsimonious trees. Nodes show the percentage bootstrap values (out of 100). All abbreviations are available in the Materials and Methods section.

ITS1-rDNA PCR amplification

For all nematode populations (Qassim, Tabouk, Riyadh and Hail), the ITS1 region was amplified using conserved primers located in the flanking regions of 18S and 5.8S genes. The 5.8S primer was rDNA1 (5'-ACGAGCCGAGTGAT-CCACCG-3') (Vrain et al., 1992) and the 18S primer was rDNA2 (5'- TTGATTACGTCCCTGCCCTTT-3') (Cherry et al., 1997). PCR reactions were carried out in a reaction mixture containing 1 µl of DNA, 1 µl (20 mM) of each primer, 2.5 µl of 10x reaction buffer, 0.5 µl (10 mM) of dNTP mix, 2 units of Taq DNA polymerase and double distilled water to 25 µl. The cycling program was as follows: initial denaturation at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 30 s, annealing at 50˚C for 30 s, extension at 72˚C for 30 s and a final extension at 72˚C for 5 min. For sequencing, the PCR amplified ITS1 products were gel purified and sent to a commercial company (RefGen Biyoteknologi, Turkey) for sequencing using the 5.8S rDNA1 primer. Sequences were used to study the diversity between and within the four populations [(H1, H4, H6, H7 from Hail), (Q1, Q2, Q3, Q4, Q6, Q7 from Qassim), (R2, R4, R6, R7, R8 from Riyadh), and (T6 from Tabouk)]. The sequences obtained from the four studied populations were deposited in Genbank (NCBI, 2012). The numbers (Lab: number: Genbank accession number) are: (H1: HM068512; H4: HM068515; H6: HM068516; H7: HM068517; Q1: HM068518; Q2: HM068519; Q3: HM068520; Q4: HM068521; Q6: HM068522; Q7: HM068523; R2: HM068525; R4: HM068526; R6: HM068527; R7: HM068528; R8: HM068529; T6: HM068533).

Phylogenetic analysis

All 341 *H. avenae* ribosomal sequences were downloaded from Genbank (NCBI, 2011). As reference samples, eight international entries of *H. avenae*, in addition to the only available *H. avenae* entry from Saudi Arabia, AY148361, and one related *H. latipons* species, AF274402, were used in the phylogenetic analysis. The international entries were; AY148353 from Germany, AY148364 from Turkey, AY148381 from China, AY148373 from France, AY148369 from Morocco, AY148358 from England, AY148356 from Spain and AY148362 from India. The ten reference sequences of these entries from Genbank were aligned with the sequenced population samples from Saudi Arabia, using the ClustalW multiple alignment function available in BioEdit (Hall, 1999). For the sequences generated in this study, a total of 581 nucleotides were selected from the consensus sequence represented in all samples. The selected region starts with the consensus sequence TGATTCCATT-CACCATCTAC and ends with TTCATTTATTTTTT-TGACCA. The aligned sequences were used to generate 100

replicates using the SEQBOOT function available in Phylip (Felsenstein, 1989). The 100 data sets were subjected to the parsimony method using the DNAPARS function in Phylip (Felsenstein, 1989), which carries out un-rooted parsimony (analogous to Wagner trees) (Eck and Dayhoff, 1966; Kluge and Farris, 1969) on DNA sequences.

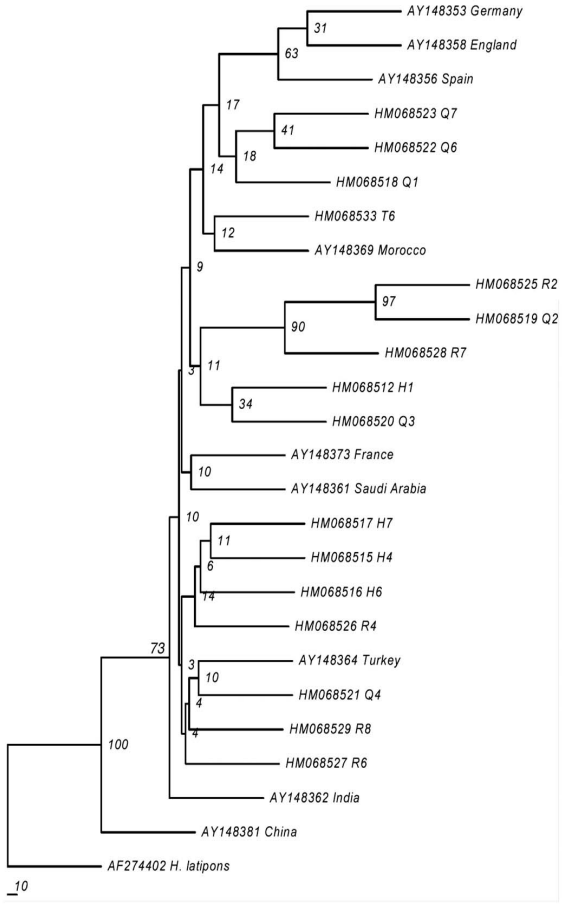

Fig 6. Phylogenetic relationships between four Saudi *Heterodera avenae* populations and international samples. A consensus of maximum likelihood trees. Nodes show the percentage bootstrap values (out of 100). All abbreviations are available in the Materials and Methods section.

The method of Fitch (1971) was used to count the number of base changes needed on a given tree. In addition, the bootstrapped data were subjected to the maximum likelihood method using the DNAML function (Felsenstein, 1989; Felsenstein and Churchill, 1996).

The transition/transversion ratio was 2. A global rearrangement was selected, which causes each possible group to be removed and re-added after the last species is added to the tree. Five randomized sequence input orders (Jumble) were also included. *H. latipons* AF274402 was selected as the outgroup species. The extended majority rule consensus tree was generated using the CONSENSE function. The consensus trees were plotted using TreeView software (Page, 1996).

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