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ORIGINAL ARTICLE

Characterization of cereal cyst nematodes (*Heterodera* spp.) in Morocco based on morphology, morphometrics and rDNA-ITS sequence analysis

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Abstract

Morphological and molecular diversity among 11 populations of cereal cyst nematodes from different wheat production areas in Morocco was investigated using light microscopy, species-specific primers, complemented by the ITS-rDNA sequences. Morphometrics of cysts and second-stage juveniles (J2s) were generally within the expected ranges for *Heterodera avenae*; only the isolate from Aïn Jmaa showed morphometrics conforming to those of *H. latipons*. When using species-specific primers for *H. avenae* and *H. latipons*, the specific bands of 109 bp and 204 bp, respectively, confirmed the morphological identification. In addition, the internal transcribed spacer (ITS) regions were sequenced to study the diversity of the 11 populations. These sequences were compared with those of *Heterodera* species available in the GenBank database (www.ncbi.nlm.nih.gov) and confirmed again the identity of the species. Ten sequences of the ITS-rDNA were similar (99–100%) to the sequences of *H. avenae* published in GenBank and three sequences, corresponding with one population, were similar (97–99%) to *H. latipons*.

Key words: *Heterodera avenae, Heterodera latipons*, molecular

Introduction

Cereal cyst nematodes (CCN) form a group of several closely related species. Three species (*Heterodera avenae, H. filipjevi* and *H. latipons*) are among the economically most important cyst nematode pests of cultivated cereals (Smiley and Nicol 2009). *Heterodera avenae* is widely distributed in temperate wheat-producing regions throughout the world (Smiley and Nicol 2009). *Heterodera latipons* is found in the Mediterranean regions, eastern and northern Europe, the Middle and Near East, North and South Africa, Asia and North America (Greco *et al.* 2002; Abidou *et al.* 2005; Smiley and Nicol 2009), whilst *H. filipjevi* has been reported from eastern and northern Europe, Central and West Asia, the Middle East, the Indian subcontinent and North America (Rumpenhorst *et al.* 1996; Rivoal *et al.* 2003; Holgado *et al.* 2004). Earlier reports from Morocco mention only *H. avenae* as representative of the CCN. The nematode was detected for the first time in 1951 in an irrigated wheat (*T. aestivum*) field in the Gharb region (Ritter 1982). More populations of *H. avenae* were found during later surveys (Ammati 1987; Mokrini *et al.* 2009). In all of these Moroccan studies, cysts were identified using only morphological features; morphometrical and molecular identification were not considered.

The taxonomy of the *H. avenae* group and its members has been the object of several review papers (Ferris *et al*. 1994; Handoo 2002). Species belonging to this group form a complex, and invade and reproduce only in roots of cereals and grasses (Subbotin *et al.* 1999). Within the *H. avenae* group, only minor morphological and morphometrical differences distinguish the species from each other (Subbotin *et al.* 1999). The increasing number of species in this group makes morphological and morphometrical identification more difficult because it is time consuming and requires appropriate skills (Subbotin *et al*. 2003). Nevertheless, accurate identification of members of the *H. avenae* group is needed as an initial step in designing effective control measures. This is especially important when searching for potential sources of host-plant resistance against *Heterodera* species (Dababat *et al*. 2015). Furthermore, rapid and accurate identification is highly significant for quarantine purposes. For these reasons, the development of molecular methods to identify members of the *H. avenae* group has been the goal of numerous studies. The internal transcribed spacer regions of ribosomal genes (ITS-rDNA) were found to be useful to differentiate species within the *H. avenae* group (Subbotin *et al*. 2000; Zheng *et al*. 2000). Additionally, the comparison of sequences of the ITS-rDNA region of unknown species with those published and deposited in GenBank has facilitated fast identification of most species of cyst-forming nematodes (Subbotin *et al*. 1999, 2000).

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

Materials and Methods

Collection of populations of the *Heterodera avenae* group

Sampling was carried out during the wheat-growing season (May to June 2011) in four different regions representing the main wheat growing areas of Morocco. Soil and root samples were taken from 75 cereal fields. Sixty-nine samples were taken from wheat fields; the remaining six samples were obtained from barley fields. Samples were taken where wheat or barley plants showed chlorotic, yellowing leaves and poor growth. Each sample (soil and root) was composed of 15 subsamples randomly collected per field. Cysts were extracted from each soil sample using the modified Cobb decanting and sieving method (Cobb 1918). After extraction, cysts were stored at 4°C.

Morphology and morphometrics of populations

Species identification was based on cyst vulval cone structures and measurements, as well as morphometric features of the J2s. The vulval cone of the cysts was cut and prepared for microscopic examination according to Hooper (1986). For each population, cones of 10 mature cysts were mounted in glycerine jelly. For each population, juveniles were obtained from the same cysts, killed by gentle heat (warming up enough to kill the nematode but not too long not to deform or destroy it), fixed in triethanolamine formalin solution (TAF), embedded in glycerol; permanent slides were made immediately. Ten J2s of the selected cyst populations were examined and measured using an Olympus BX51 compound microscope.

Molecular characterization

DNA extraction

For each population, a single J2 isolated from a single cyst was transferred into an Eppendorf tube containing 25 µl double distilled water (ddH_2O) and 25 µl nematode lysis buffer [final concentration: 200 mM NaCl, 200 mM Tris-HCl (pH 8), 1% mercaptoethanol and 800 µg Proteinase K]. The tubes were incubated at 65°C for 1.5 h and at 99°C for 5 min, consecutively (Holterman *et al.* 2006). The extracted DNA suspension was stored at −20°C or used immediately for DNA amplification.

PCR with species-specific primers

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

with the universal reverse primers (Ferris *et al.* 1993) to eventually characterise *H. latipons*.

The PCR used to detect *H. avenae* was run as follows: 2 µl DNA extract (see above) were added to the PCR reaction mixture containing 21 µl ddH_2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany) and 1 µM of each of the primers AVEN-COI (Toumi *et al.* 2013a) and Ferris *et al.* (1993). The thermal cycler programme consisted of 5 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C, followed by a final elongation step of 8 min at 72°C. For the detection of *H. latipons*, 2 µl of the DNA extract was added to the PCR reaction mixture containing 21 µl ddH_2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany), and 1 µM of each of the primers Hla-acti (Toumi *et al.* 2013b) and Ferris *et al.* (1993). The programme of the thermal cycler consisted of 5 min at 95°C; 50 cycles of 30 s at 94°C, 45 s at 50°C and 45 s at 72°C, followed by a final elongation step of 8 min at 72°C.

Amplification, sequencing and phylogenetic analysis

The ITS-rDNA region was amplified using the primers 5'-CGT AAC AAG GTA GCT GTA G-3' and 5'-TCC TCC GCT AAA TGA TAT G-3' (Ferris *et al.* 1993). The purification process was done as described by the manufacturer's instructions (Wizard® SV Gel and PCR Clean-Up System Kit, Promega). DNA from each sample was sequenced (Macrogen, Seoul, South Korea) in both directions to obtain overlapping sequences of both DNA strands. The sequences were edited and analysed using software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall 1999). Finally, all sequences were blasted in GenBank (Sequin v. 9.00, http://www.ncbi.nlm.nih.gov/). Twenty-nine ITS sequences of *H. avenae* and *H. latipons* (13 new and 16 from GenBank) were aligned using Clustal W (Thompson *et al.* 1994) and visually checked. Differences between sequences were estimated using the DNA distance option provided by BioEdit sequence alignment editor (Hall 1999). The alignment was imported into the software package Mega 5.0; after checking 24 different nucleotide substitution models, the model with the lowest BIC score (Bayesian Information Criterion) was retained for constructing a 60% consensus Neighbour-joining tree. To determine statistical consistency of the classification, bootstrap analysis using 1,000 bootstrapped data sets was performed.

Results

Morphology and morphometrics

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

Heterodera avenae (Wollenweber, 1924)

Cysts

Mostly lemon-shaped, with a protruding neck and vulvar cone. Cyst wall – dark brown, bearing a zig-

Table 1. Species and populations of *Heterodera* collected during a survey in wheat producing areas of Morocco

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

'+' positive for the mentioned species
'–' negative for the mentioned species
Ha – *Heterodera avenae*; Hl – *Heterodera latipons*
DW – Durum Wheat ; W – Wheat

zag pattern. Vulval cone bifenestrate. No underbridge. Bullae in all populations. The cyst of populations H01, H03, H07 and H08 – slightly bigger than other populations (Table 2).

Second-stage juveniles

Body – cylindrical, head – slightly offset, tapering round tail tip. Stylet – strong with shallow anteriorly concave basal knobs. Body length – 503 to 640 µm; stylet length – 22.3–27.9 µm; anteriorly concave basal knobs. Lateral field with four incisures (Table 3).

Remarks

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

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

Cysts

The cysts (n = 10) had the following characteristics: lemon-shaped; cyst wall partially transparent, between light and dark brown; ridges with zigzag pattern. Bifenestrate vulval cone, body length without neck 590 µm (551 to 632 µm), body width – 393 µm (310 to 490 µm), neck length – 75 µm (65 to 90 µm), fenestra length – 64 µm (60 to 72 µm) and width – 21 µm (18 to 25 µm), underbridge length – 96 µm (85 to 115 µm), vulval slit length – 8 µm (7 to 9 µm), vulva bridge width – 27 µm (24 to 33 µm), and bullae absent. The bifenestrate cysts with a strong underbridge and no bullae.

Second-stage juveniles

The J2s (n = 10) had the following characteristics: cylindrical head slightly offset, round tail tip tapering. Compared with *H. avenae* bodies – slightly shorter and short hyaline terminal tail. Body length – 445 µm (412 to 472 µm), body width – 19 µm (19 to 21 µm), stylet length – 24 µm (23 to 25 µm), four lateral lines, tail length – 50 µm (46 to 54 µm), and hyaline terminal tail – 28 µm (24 to 31 µm).

Remarks

Only one population of *H. latipons* was detected (Ain Jmaa, Saiss). The morphometrics and morphological characters corresponded to those reported by Handoo (2002).

Molecular characterization

Species-specific PCR and sequencing

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

ITS sequence and analysis

A comparison of ITS-rDNA sequences of *H. avenae* and *H. latipons* populations among themselves and with sequences of *Heterodera* species available in GenBank is presented in Figure 2. The comparison confirmed the identification of the species using morphological features and species-specific PCR. Ten sequences of the ITS-rDNA were similar (99–100%) to the sequences of *H. avenae* published in GenBank (AY148363, AY148364, AY148360, AY148359, AY148361, AY148362, AY148354, AY148358, AY148367, AY148368, AY148369) and three sequences (JQ319035, JQ319036 and JQ319037) were similar (97–99%) to *H. latipons.* On the basis of the topology of the calculated majority rule, 60% consensus Maximum Likelihood tree for all the Moroccan populations

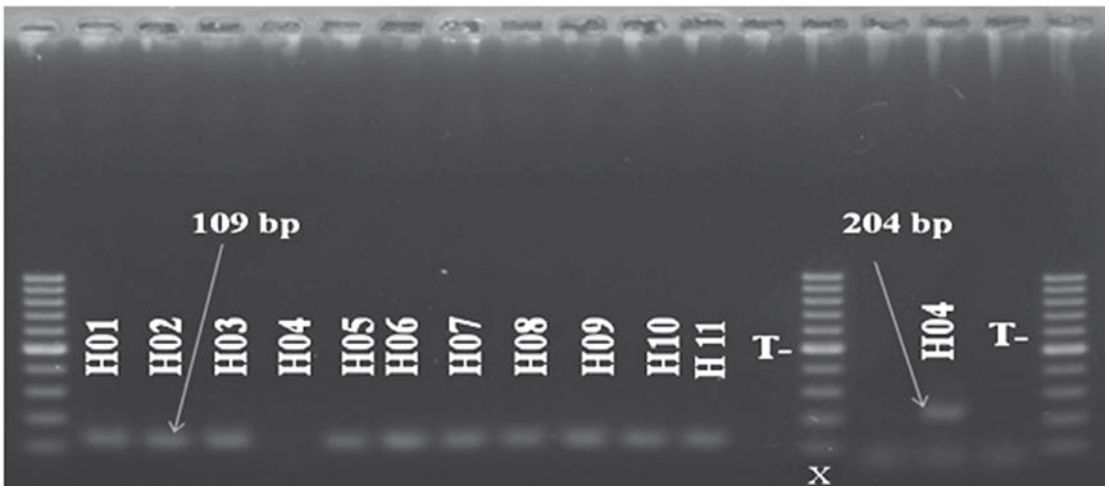

Fig. 1. Results of specific PCR for *Heterodera avenae* (H01, H02, H03, H05, H06, H07, H08, H09, H10, H11) and *H. latipons* (H04). X = 100 bp DNA ladder (Promega Benelux). Codes for populations: see Table 1

Table 2. Morphometric characters of cysts and vulval cones of *Heterodera avenae* populations from Morocco (n = 10). Measurements in µm and in form: mean±standard deviation (range). Codes for populations: see Table 1

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

*length : width ratio

Table 3. Morphometrics of *Heterodera avenae* second-stage juvenile populations collected in Morocco (n = 10). Measurements in µm and in form: mean±standard deviation (range)

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

*dorsal esophageal gland orifice

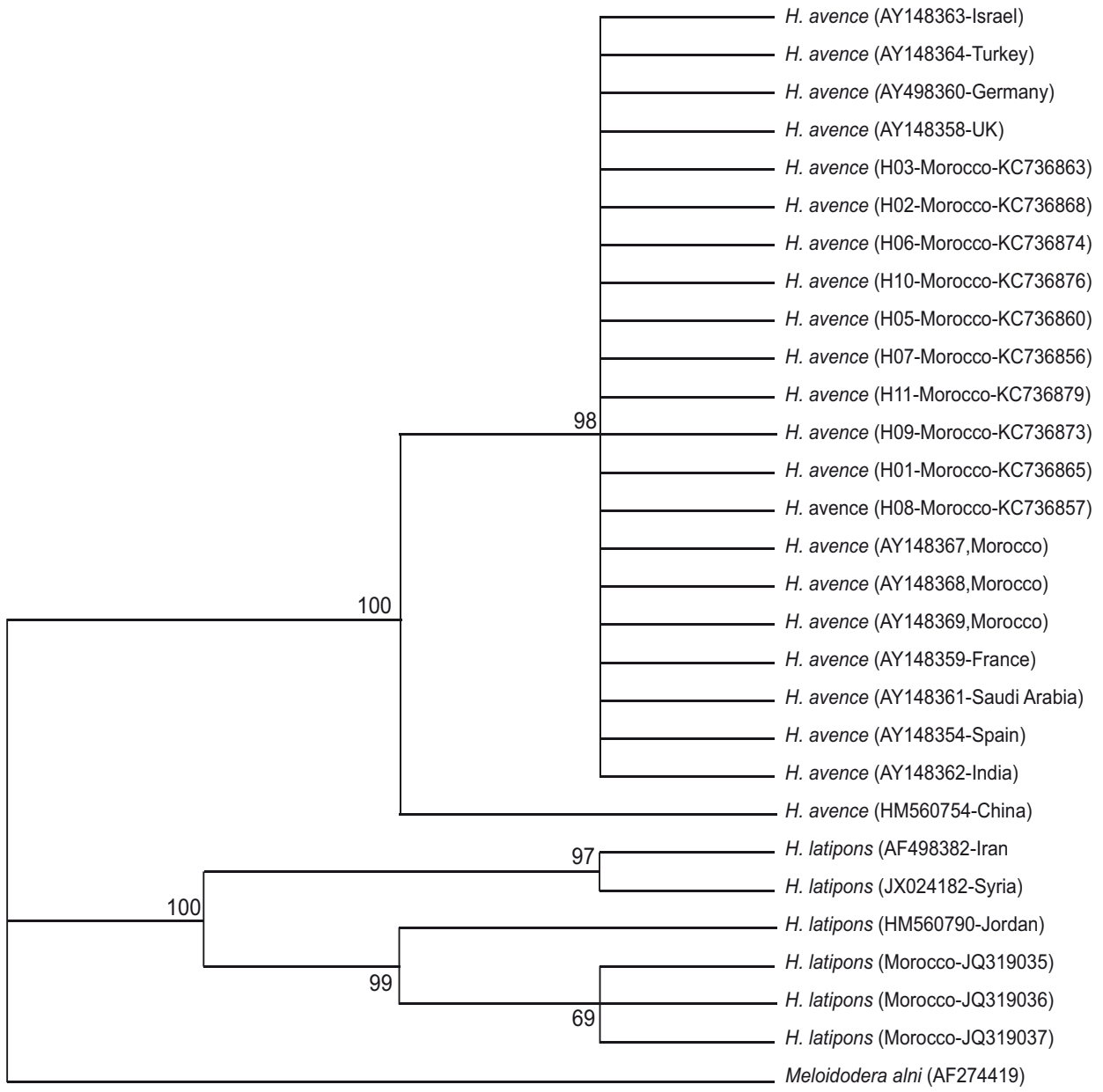

Fig. 2. The topology of the majority rule 60 consensus Maximum Likelihood tree for all populations studied with the addition of *Heterodera* populations obtained from GenBank based on the sequence alignment of the ITS-rDNA. For the list with the abbreviations of the population codes see Table 1

collected in the survey and the three Moroccon populations in GenBank (AY148367, AY148368, AY148369), Subbotin *et al*. 2003 and 16 *Heterodera* spp. from GenBank, two major groups of *Heterodera* were revealed (Fig. 2).

Discussion

Two species, *viz*. *H. avenae* and *H. latipons* were detected during the survey of cyst nematodes in the major cereal-cultivating areas of Morocco. The latter species was detected for the first time in the country; it was found in a wheat field in Ain Jmaa (Saiss) (Mokrini *et al*. 2012). Previous surveys in the area had revealed the presence of *H. avenae* only (Ammati 1987; Mokrini *et al*. 2009). In this study, cysts of *H. avenae* were found only in wheat fields (Saiss, Chaouia and Zaers regions of Morocco) with two species of root-lesion nematodes (*Pratylenchus penetrans* and *P. thornei*) (Mokrini *et al*. 2016). However, the absence of cysts in barley fields is probably related to the rotation with vegetables or food legumes, practiced in these fields. Moreover, farmers in the main cereal growing areas of Morocco prefer to grow wheat, so fewer fields of barley were sampled, hence reducing the chances for detecting infestations with cysts. Both species were distinguishable easily on the basis of the cyst morphology. All cysts of *H. avenae* had prominent bullae, but no underbridge; *H. latipons*,

however, had a strong underbridge and lacked distinct bullae in the vulval cone. Previous studies (Wouts and Sturhan 1995; Subbotin *et al*. 2003) reported the same morphological characteristics that separated *H. avenae* from *H. latipons*. Compared to *H. latipons*, J2s of *H. avenae* have a longer tail, stylet and hyaline part of tail. Abdollahi (2009) reported that the Indian populations of maize cyst nematodes were identified as *H. zaea* based on morphological and morphometric features.

Species-specific primers for PCR have been developed to complement the traditional species identification of *H. avenae* (Toumi *et al*. 2013a; Yan *et al*. 2013) and *H. latipons* (Toumi *et al*. 2013b). Several genes were successfully used to identify many species of *Heterodera* (Subbotin *et al.* 1999; Yan *et al*. 2013). When using the species-specific primers developed for both *H. avenae* and *H. latipons* (Toumi *et al*. 2013a, b) we obtained the characteristic bands of 109 bp and 204 bp, respectively, confirming their morphological identification. This confirms the specificity of the primer sets.

In addition to the morphology, morphometric, species-specific primers, the sequence comparison of the ITS region clearly separate the Moroccan *H. avenae* from *H. latipons*. This rDNA region has been commonly used to separate nematodes at the species level, including the genus *Heterodera* (Subbotin *et al*. 2003). The results reported here did not show any intraspecific polymorphism between Moroccan populations of *H. avenae* based on the ITS sequences. These results are in agreement with Baklawa *et al*. (2015) who found that *H. avenae* populations originating from different localities of Egypt clustered together in the same group and had high similarities to each other. However, polymorphism among different populations of both *H. avenae* and *H. latipons* had been reported previously (Subbotin *et al.* 1999; Madani *et al*. 2004). In our study, based on data of the ITS region, the Moroccan populations of *H. avenae* clustered with *H. avenae* populations from Europe and Asia. The data also confirmed previous results in the phylogram presented by Madani *et al.* (2004), in which a Moroccan population of *H. avenae* clustered with populations from France, Turkey and Israel. Moreover, the three *H. latipons* sequences from Morocco obtained from the same population were identical to each other and also to a sequence in GenBank from a *H. latipons* population from Jordan (HM560790). This is the first report providing the integrated morphometric, morphological and molecular characterization of cereal cyst nematode populations from Morocco. Further investigations are necessary to identify the pathotypes of the *H. avenae* and *H. latipons* populations of the Saiss, Gharb, Chaouia and Zaers regions of Morocco, as well as suitable resistance sources to be used in cereal breeding programmes.

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