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ORIGINAL ARTICLE

Morphological and molecular identification of cyst nematode species (*Heterodera* spp.) in Algerian cereal fields

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Abstract

Cereal cyst nematodes (*Heterodera* spp.) are distributed globally and cause severe production losses of small grain cereals. To investigate the occurrence of cereal cyst nematodes in wheat-growing areas of Algeria, a survey was conducted and 27 cereal cyst nematode populations were collected. The populations were initially identified based on their morphological and morphometric characters, followed by molecular methods using species-specific primers, complemented by ITS-rDNA sequences. The morphological and morphometric features of second-stage juveniles (J2s) and cysts supported the presence of three *Heterodera* species: *H. avenae*, *H. filipjevi* and *H. hordecalis*. All morphological values of these distinct populations were very similar to those previously described for these species. Using species-specific primers for *H. avenae* and *H. filipjevi*, the specific bands of 109 bp and 646 bp confirmed the morphological identification of both species, respectively. In addition, the internal transcribed spacer (ITS) regions were sequenced to study the diversity of the 27 populations. These sequences were compared with those of *Heterodera* species available in the GenBank database (www.ncbi.nlm.nih.gov) and re-confirmed the identity of the species. Nineteen sequences of ITS-rDNA were similar (99–100%) to the sequences of *H. avenae* published in the GenBank, six sequences were similar (99–100%) to *H. hordecalis*, and two were similar (98–99%) to *H. filipjevi*. The results of this study are of great value to breeding programs and extension services, where they will contribute to the design of control measures to keep damaging nematodes in check.

Keywords: cereals, *Heterodera* spp., cyst nematode, ITS-rDNA, wheat

Introduction

Cereals are the largest source of food in the world. More than 70% of the land used for food crops is devoted to cereal crops (Riley *et al.* 2009). Wheat (bread = = *Triticum aestivum* and durum = *T. durum*), maize (*Zea mays*) and barley (*Hordeum vulgare*) are of the most important crops, making up 58% of annual crops in terms of area and production (Nicol *et al.* 2011). By 2030, cereal production is estimated to reach eight billion tons, globally (Fischer *et al.* 2009). An estimated 750 million tons of grain was grown on more than 220 million hectares worldwide in 2017 (Wuletaw *et al.* 2016; Dababat and Fourie 2018). In Algeria, grain farming is practiced exclusively in semi-arid areas where it occupies an average area of 3 million hectares (Minagri 2017). In 2017, total cereal production reached 3.5 million tons, 5% above the 2016 harvest and 17%

below the previous 5-year average (2012–2016) (FAO 2017). Unfortunately, this crop is still insufficient and does not cover the demand of the growing population (Hoover *et al.* 2010). Cereal cultivation in Algeria is often faced with various constraints that limit its production, such as the availability of water, status of soil nutrients, as well as incidence of insect pests and phytoparasitic nematodes (Shroyer *et al.* 1990).

Plant-parasitic nematodes are some of the harmful bio-aggressors that threaten the global yield of annual worldwide wheat (Nicol and Rivoal 2008; Dababat and Fouire 2018). Cereal cyst nematodes (CCNs) of the genus *Heterodera* contain a group of very similar species (Dababat *et al.* 2015). *Heterodera avenae*, *H. filipjevi* and *H. latipons* are among the most economically important cyst nematode pests of small grain cereals (Rivoal and Cook 1993; Smiley and Nicol 2009). *Heterodera avenae* is considered to be the most damaging species limiting cereal production in North Africa (Mokrini *et al.* 2009; Dababat *et al.* 2015; Mokrini *et al.* 2017). The presence of this nematode was first discovered and reported by Scotto La Massese (1961) and by Lamberti *et al.* (1975). However, recent studies have reported that this species was detected in various cereal growing areas of Algeria (Mokabli *et al.* 2001; Valette *et al.* 2002; Tirchi *et al.* 2016). *Heterodera filipjevi* appears mostly in Mediterranean and European countries (Rumpenhorst *et al.* 2003; Dababat 2019). Recently, Smaha *et al.* (2018a) reported the presence of *H. filipjevi* for the first time in Algeria. *Heterodera latipons* occurs in Mediterranean regions including Algeria (Valette *et al.* 2002) and Morocco (Mokrini *et al.* 2013). A recent survey of cereal fields in the Algerian provinces of Setif, Batna, Tiaret and Tlemcen showed the presence of *H. hordecalis* for the first time in Algeria (Smaha *et al.* 2018b).

Small morphological and morphometrical characteristics differentiate the species of *H. avenae* group (Molokanova *et al.* 1999). Identification of *Heterodera* species by morphological and morphometrical methods is becoming more complex and less accurate due to the increase in species number (Rumpenhorst *et al.* 2003). However, molecular techniques used in CCN identification is more convenient and gives accurate outcomes such as the polymerase chain reactions (PCR) based on species-specific primers (Toumi *et al.* 2013; Yan *et al.* 2013). Subbotin *et al.* (2001) indicated that many nematode taxa, including cyst nematodes, can be identified by DNA-sequence variation using the internal transcribed spacer (ITS) region of ribosomal DNA.

To the best of our knowledge, so far, Algerian CCN populations have only been described by using morphological methods (Mokabli *et al.* 2001; Valette *et al.* 2002; Haddadi *et al.* 2013); consequently, there is a lack of information on the morphometrics and genetic diversity of the cyst nematodes. Hence, a survey was conducted in the wheat-growing areas of Algeria to achieve the following objectives: i) collect and characterize *Heterodera* species and populations from Algeria by morphological and morphometrical methods; and ii) confirm the identity of collected populations of *Heterodera* spp. using PCR and sequencing of the ITS-rDNA expansion segments.

Materials and Methods

Nematode population and extraction

This study was conducted during the wheat growing season in 2015–2016 from 27 different localities covering the major wheat growing areas of Algeria. A total of 83 samples containing soil and root parts was collected from the wheat fields. Sampling involved collecting materials from the nematode affected areas that showed symptoms of poor growth and chlorosis. A soil sample of one kg was composed of 10 randomized subsamples obtained from each grid intersection, while root samples were obtained by carefully lifting plants at the same points. Each soil sample was carefully mixed and a representative sample of 250 g soil was used for cyst extraction by using the Fenwick can technique (Fenwick 1940). Extracted cysts were kept in a refrigerator at 4°C.

Morphological and morphometric characterization

In addition to the morphometric properties of the second-stage juvenile (J2), vulval cyst cone structures were examined and measurements were taken. The vulval cone of the cysts was removed and prepared to examine under a microscope according to Hooper (1986). For each population, 10 vulval cones of matures were placed in glycerin gel media. The identification of the cysts was done on the basis of the underbridge structure, the semifenestra shape, and the development of the bullae (Handoo 2002). J2s were removed from the same mature cyst, killed by applying gentle heat that killed them but maintained the tissues undestroyed, fixed in triethanolamine formalin solution (TAF) and placed in glycerol. Permanent slides were made immediately, and measurements were taken at 100x magnification under a compound microscope.

Molecular identification

DNA extraction

For each population, DNA was recovered from the same individual cysts that were brought from field samples. The cysts were transferred into (0.5 ml)

Eppendorf tubes that contained 80 µl ddH_2O. Then, the cysts were crushed, and the contents were further stirred with a micro vibromixer for 30 sec. One to five J2s were transferred into a PCR tube (0.2 ml) using a micropipette containing 40 µl ddH_2O. The lysis buffer [50 mM KCl, 10 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 1 mM Dithiothreitol (DTT), 0.45% Tween 20 (Sigma, UK)], and 10 µl proteinase K (600 µg · ml^{-1}) were added to each tube. Tubes were centrifuged at 13,500 rpm for 2 min at 25°C, then placed at –80°C for 10 min, incubated at 65°C for 1 h, and heated at 95°C for 10 min. The samples were kept at –20°C until use (Waeyenberge *et al.* 2000).

PCR procedures

The species-specific primer 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.* 2013) 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 reverse primer 5'-TCC TCC GCT AAA TGA TAT G-3', were used to detect *H. avenae* in the DNA extracts of the 27 populations. Extracts that were not identified as belonging to *H. avenae* were used in a sequence characterized amplified region (SCAR) PCR primer set HFF2 (5'-CAG GAC GAA ACT CAT TCA ACC AA-3') and HFR2 (5'-AGG GCG AAC AGG AGA AGA TTA GA-3') to characterize *H. filipjevi* (Peng *et al.* 2013).

Amplification, sequencing and phylogenetic analysis

The ITS-rDNA region was amplified using 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 performed following the manufacturer's instructions (Wizard SV Gel and PCR Clean-Up System Kit, Promega). DNA from each sample was sequenced (Macrogen and Seoul, South Korea) in both directions to obtain overlapping sequences of both DNA strands. To analyze and edit the sequences the software package Chromas 2.00 was used (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-seven ITS sequences of *H. avenae*, *H. hordecalis*, and *H. filipjevi* (27 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 neighbor-joining tree. To determine statistical consistency of the classification, bootstrap analysis using 1,000 bootstrapped data sets was performed.

Results

Morphological and morphometric analysis

Twenty-seven *Heterodera* populations were identified (Table 2, Fig. 1). Nineteen populations were monospecific for *H. avenae*, six for *H. hordecalis*, and two populations for *H. filipjevi*.

Heterodera avenae (Wollenweber 1924)

Heterodera avenae was detected in 19 populations (C1, C2, C4, C6, C7, C8, C13, C14, C15, C16, C17, C19, C21, C23, C24, C25, C28, C30, and C31) (Tables 2, 3, 4). The morphological and morphometrical identification of these populations resembled the previously described populations (Handoo 2002; Rumpenhorst *et al.* 2003).

Cysts

Vulval cone morphology of 19 populations displayed typical properties of *H. avenae*, such as bifenestrate vulval cone, no underbridge and the presence of bullae. Cysts were mostly lemon-shaped, and dark brown with a protruding neck. The cyst wall was in a spectrum of dark brown to black in color, with a zigzag pattern. Median bulb was rounded with a distinct valvular apparatus. The cysts of populations C1, C14, C16 and C21 were slightly larger than other populations (Table 3).

Second-stage juveniles

The J2s were cylindrical and vermiform in shape with a sharply pointed tail, a slightly offset head, and a tapering round tail tip. Stylets were slender and strong

Table 1. Primers used for molecular identification of *Heterodera* spp.

Primer name	Sequence (5'–3')	Fragment (bp)	Species	Reference
AVEN-COIF AVEN-COIR	GGGTTTTCGGTTATTTGG CGCCTATCTAAATCTATACCA	109	*H. avenae*	Toumi *et al.* (2013)
HFF2 HFR2	CAGGACGAAACTCATTCAACCAA AGGGCGAACAGGAGAAGATTAGA	646	*H. filipjevi*	Peng *et al.* (2013)

Table 2. Species and populations of *Heterodera* collected during a survey in wheat producing areas of Algeria. (+) – positive for the mentioned species, (–) – negative for the mentioned species, (/) – not checked

Code	Localities	Province	Morphological identification	Identification using PCR		Result of sequencing
				H. avenae	*H. filipjevi*	
C1	Tighennif	Mascara	*H. avenae*	+	–	*H. avenae*
C2	Mouzaia	Blida	*H. avenae*	+	–	*H. avenae*
C3	Guellal	Sétif	*H. hordecalis*	/	–	*H. hordecalis*
C4	Tipaza-ville	Tipaza	*H. avenae*	+	–	*H. avenae*
C5	Hammam Sokhna	Sétif	*H. hordecalis*	/	–	*H. hordecalis*
C6	Oued Smar	Alger	*H. avenae*	+	–	*H. avenae*
C7	Dahmouni	Tiaret	*H. avenae*	+	–	*H. avenae*
C8	Sidi Amar	Tipaza	*H. avenae*	+	–	*H. avenae*
C12	Sebaine	Tiaret	*H. hordecalis*	/	–	*H. hordecalis*
C13	El Fedjoudj	Guelma	*H. avenae*	+	–	*H. avenae*
C14	Djendel	Ain Defla	*H. avenae*	+	–	*H. avenae*
C15	Hmadna	Relizane	*H. avenae*	+	–	*H. avenae*
C16	Chréa	Tebessa	*H. avenae*	+	–	*H. avenae*
C17	Oum El Bouaghi	Oum El Bouaghi	*H. avenae*	+	–	*H. avenae*
C18	Lamtar	Sidi Bel Abbes	*H. filipjevi*	–	+	*H. filipjevi*
C19	Guelb El Kebir	Médéa	*H. avenae*	+	–	*H. avenae*
C21	Khroub	Constantine	*H. avenae*	+	–	*H. avenae*
C22	Telagh	Sidi Bel Abbes	*H. filipjevi*	–	+	*H. filipjevi*
C23	Hachimia	Bouira	*H. avenae*	+	–	*H. avenae*
C24	Mechira	Mila	*H. avenae*	+	–	*H. avenae*
C25	Hennaya	Tlemcen	*H. avenae*	+	–	*H. avenae*
C26	Maghnia	Tlemcen	*H. hordecalis*	/	–	*H. hordecalis*
C27	Ain Arnat	Sétif	*H. hordecalis*	/	–	*H. hordecalis*
C28	Remchi	Tlemcen	*H. avenae*	+	–	*H. avenae*
C29	Seriana	Batna	*H. hordecalis*	/	–	*H. hordecalis*
C30	Draâ El Mizan	Tizi Ouzou	*H. avenae*	+	–	*H. avenae*
C31	El Fehoul	Tlemcen	*H. avenae*	+	–	*H. avenae*

with large anteriorly flattened to concave basal knobs. Tails were conoid, gradually tapering to a finely rounded terminus. The hyaline portion of the tail was irregularly annulated and was three to four times longer than its maximum diameter. Measurements of J2s (n = 10) included the length (range = 503–643 µm, mean = 591 µm) and width (20.5–30 µm, 22.6 µm) of body, stylet length (23–25 µm, 24 µm), and tail (55–78 µm, 65.3 µm). The lateral field of J2s had four incisures (Table 4).

Heterodera hordecalis

Six populations of *H. hordecalis* were detected in Guellal (Sétif), Hammam Sokhna (Sétif), Ain Arnat (Sétif), Seriana (Batna), Sebaine (Tiaret) and Maghnia (Tlemcen) localities. The morphometrics and morphological characters were identical to those reported by Handoo (2002).

Cysts

Cysts were rounded, deep yellow to brown, with thick sub crystalline layers, small vulval cones, bifenestrate, and two almost circular semifenestrates separated from each other by a rather wide vulval bridge. The vulval slits were obviously covered by a flap, and bullae were present at the underbridge level. Cysts also displayed a strong underbridge with pronounced thickening in the middle and bifurcation at both ends (Table 5).

Second-stage juveniles

The criteria of J2s (n = 10) showed a cylindrical head with a tapering, slightly offset, round tail tip. Bodies were slightly shorter and had short hyaline terminal tails when compared with *H. avenae* (Table 6). The lateral field of J2s had four incisures. The phasmids were situated at two to four annules behind the anus. Stylets

Table 3. Morphometric characters of cysts and vulval cones of *Heterodera avenae* populations from Algeria (n = 10). Measurements in µm and in mean form: mean ± SD (range). Codes for populations: see Table 1

Code	Length without neck	Neck length	Width	L : W*	Semifenetra width	Fenestra length	Vulval slit length	Vulval bridge width	Underbridge length	Bullae
C1	801 ± 76 (663–904)	34 ± 7 (20–48)	590 ± 46 (503–712)	1.35 ± 0.4	21 ± 2.1 (19–25)	47 ± 1.8 (44–49)	9 ± 1.5 (7–12)	5 ± 0.8 (4–6)	absent	present
C2	797 ± 51 (702–882)	52 ± 12 (26–76)	608 ± 65 (490–680)	1.31 ± 0.2	22 ± 1.5 (19–24)	45 ± 3.2 (39–49)	10 ± 0 .7 (9–11)	6 ± 0.5 (5–7)	absent	present
C4	664 ± 85 (549–729)	59 ± 14 (42–79)	496 ± 38 (420–595)	1.33 ± 0.3	22 ± 1.3 (19–24)	48 ± 1.5 (44–50)	10 ± 0.6 (9–11)	6 ± 0.4 (5–7)	absent	present
C6	582 ± 75 (482–682)	43 ± 8 (31–55)	429 ± 32 (377–495)	1.35 ± 0.3	19 ± 1 (18–22)	45 ± 3.1 (40–49)	19 ± 1 (18–22)	9 ± 1.1 (7–11)	absent	present
C7	564 ± 71 (423–637)	41 ± 12 (27–65)	414 ± 51 (337–496)	1.36 ± 0.2	20 ± 1.7 (18–23)	44 ± 2.2 (40–47)	10 ± 0.5 (9–12)	5 ± 0.6 (4–6)	absent	present
C8	682 ± 75 (587–787)	41 ± 11 (25–64)	507 ± 38 (440–578)	1.34 ± 0.3	22 ± 1.2 (19–24)	46 ± 1.7 (41–50)	10 ± 0.6 (9–11)	6 ± 0.5 (5–7)	absent	present
C13	640 ± 68 (542–728)	40 ± 10 (28–56)	480 ± 32 (425–531)	1.33 ± 0.2	19 ± 0.8 (18–22)	47 ± 1.5 (41–51)	9 ± 0.9 (7–11)	7 ± 0.8 (6–8)	absent	present
C14	826 ± 71 (683–910)	60 ± 12 (43–75)	617 ± 65 (521–699)	1.34 ± 0.3	20.2 ± 1.6 (18–22)	49 ± 2 (45–51)	10.2 ± 0.5 (9–11)	6 ± 0.5 (5–7)	absent	present
C15	691 ± 78 (611–765)	55 ±1 2 (38–71)	524 ± 31 (480–562)	1.32 ± 0.2	22 ± 1.1 (19–24)	43.7 ± 2.9 (38–50)	9.2 ± 0.6 (8–11)	5 ± 0.7 (4–6)	absent	present
C16	823 ± 88 (699–811)	70 ± 12 (45–91)	627 ± 58 (529–698)	1.31 ± 0.2	19 ± 0.7 (18–21)	46 ± 1.4 (40–51)	9.2 ± 0.7 (8–11)	6 ± 0.6 (5–7)	absent	present
C17	763 ± 45 (663–828)	67 ± 10 (48–82)	581±42 (512–643)	1.31 ± 0.2	21.2 ± 2 (18–24)	49 ± 1.9 (45–52)	9 ± 1 (7–11)	5 ± 0.8 (4–6)	absent	present
C19	744 ± 52 (674–810)	50 ± 11 (35–69)	569 ± 38 (499–627)	1.31 ±0.2	21 ± 1.8 18–23)	49 ± 2 (44–51)	9 ± 0.8 (8–11)	7 ± 0.7 (6–8)	absent	present
C21	839 ± 65 (744–924)	44 ± 7 (33–61)	635 ± 48 (568–713)	1.32 ± 0.3	24.5 ± 2.4 (22–27)	52.3 ± 3.5 (43–60)	10 ± 2 (8–14)	8.5 ± 2.3 (6–11)	absent	present
C23	674 ± 58 (554–752)	71 ± 16 (45–92)	520 ± 41 (461–598)	1.30 ± 0.2	19 ± 0.6 (18–22)	45 ± 3 (39–50)	10 ± 0.6 (8–11)	6 ± 0.4 (5–7)	absent	present
C24	682 ± 67 (558–775)	65 ± 11 (49–88)	510 ± 25 (464–571)	1.33 ± 0.2	21 ± 1.7 (19–24)	48 ± 2.8 (42–52)	9 ± 1 (7–11)	5 ± 0.5 (4–6)	absent	present
C25	783 ± 52 (705–865)	65 ± 12 (48–86)	584 ± 38 (506–652)	1.34 ± 0.3	22 ± 0.9 (19–24)	46 ± 2.9 (39–50)	10 ± 0.5 (8–11)	5 ± 0.6 (4–6)	absent	present
C28	716 ± 44 (638–788)	74 ± 17 (45–98)	530 ± 38 (468–595)	1.35 ± 0.3	21 ± 1.7 (19 – 23)	45 ± 2.9 (38–50)	10 ± 0.6 (9–11)	6 ± 0.5 (5–7)	absent	present
C30	647 ± 59 (545–718)	38 ± 9 (27–49)	496 ± 32 (435–575)	1.3 ± 0.20	19 ± 0.5 (18–22)	45 ± 2.8 (39–50)	10 ± 0.5 (9–11)	5 ± 0.5 (4–6)	absent	present
C31	606 ± 45 (523–705)	41 ± 10 (30–58)	461 ± 29 (408–512)	1.31± 0.2	20 ± 1.5 (18–23)	44 ± 2 (40–49)	9 ± 0.9 (8–11)	5 ± 0.6 (4–6)	absent	present

*length : width ratio

were well developed (22–26 µm) with strong forward--projecting knobs.

Heterodera filipjevi

Only two populations of *H. filipjevi* were detected (Lamtar and Hachimia). The morphometrics and morphological characters were similar to those reported by Handoo (2002).

Cysts

The cysts were lemon shaped with a posterior protuberance. The vulval cone was bifenestrate with horseshoe--shaped semifenestra and had strongly developed bullae, and underbridge. Measurements (range, mean ± SD) of cysts (n = 10) were: body length without neck 780 µm (670 to 862 µm), neck length 95 µm (76 to 116 µm), fenestra length 52 µm (42 to 60 µm), and width 24.5 µm (21 to 27 µm) (Table 7).

Second-stage juveniles

Second-stage juveniles were cylindrical in shape, with a slightly offset head and a tapering round tail tip. The stylet was strong with shallow anteriorly concave basal knobs. The juvenile body length of *H. filipjevi* varied

Table 4. Morphometrics of *Heterodera avenae* second-stage juvenile populations collected in Algeria (n = 10). Measurements in µm and in form: mean ± SD (range)

Populations	Second stage juveniles					
code	body length	body width	stylet length	tail length	hyaline length	terminal DGO*
C1	590 ± 25 (546–621)	22.4 ± 0.5 (21.4–23.1)	24.1 ± 1.4 (22.3–26.1)	67.3 ± 5.7 (60.1–74.4)	43.1 ± 4.6 (37.6–52.0)	5.6 ± 0.1 (5.2–5.8)
C2	566 ± 27.5 (520–596)	22.23 ± 0.42 (21.0–23.8)	24.76 ± 0.36 (22.0–28.0)	68.1 ± 2.8 (60.4–77.0)	44 ± 3.9 (37.4–50.0)	5 ± 0.13 (4.0–6.0)
C4	590 ± 25.2 (545–620)	22.5 ± 0.5 (21.4–23.1)	24.21 ± 1.3 (22.2–26.1)	67.3 ± 5.4 (60.1–74.4)	43.2 ± 4.7 (37.7–52.0)	5.7 ± 0.2 (5.2–5.9)
C6	576 ± 30.8 (540–630)	22.2 ± 0.6 (21.3–24.0)	24.9 ± 0.6 (22.1–27.0)	66 ± 5.8 (59.0–77.0)	42.2 ± 4.6 (37.5–52.0)	5 ± 0.13 (4.0–6.0)
C7	559.6 ± 27.2 (500–620)	22 ± 0.5 (21.0–28.0)	26.2 ± 0.7 (24.9–27.5)	62.3 ± 0.35 (60.0–66.0)	43 ± 4.5 (38.0–50.4)	5.6 ± 0.14 (5.3–6.0)
C8	575.6 ± 14.5 (550–610)	22.5 ± 0.7 (20.5–23.4)	25.6 ± 0.32 (23.0–28.0)	66.4 ± 0.59 (62.0–71.0)	44 ± 3.8 (39.0–51.0)	5.9 ± 0.14 (5.0–7.0)
C13	566 ± 27.8 (520–596)	23.05 ± 0.4 (21.5–23.6)	24.2 ± 1.3 (23.7–27.0)	68.1 ± 2.8 (60.5–78.0)	44 ± 4.6 (38.0–50.2)	5.4 ± 0.4 (4.9–6.1)
C14	591.3 ± 4.41 (550–630)	23.7 ± 0.3 (23.0–30.0)	24.4 ± 0.3 (22.0–27.0)	69 ± 0.44 (65.0–73.0)	42.5 ± 4.1 (38.0–50.2)	5.96 ± 0.13 (5.0–7.0)
C15	577 ± 30.6 (540–629)	22.75 ± 0.6 (21.0–24.3)	25.5 ± 0.9 (24.3–26.5)	61 ± 4.5 (57.0–71.5)	43.1 ± 4.1 (39.0–51.1)	5.2 ± 0.3 (4.9–5.7)
C16	575 ± 43.5 (503–637)	23.1 ± 0.5 (22.1–23.9)	26.1 ± 1 (24.7–27.8)	66 ± 5.9 (59.1–77.0)	43.1 ± 3.9 (38.3–51.0)	5.3 ± 0.3 (5.0–5.9)
C17	581 ± 40 (522–643)	22.4 ± 0.7 (21.0–23.5)	25.1 ± 0.8 (24.1–26.5)	64.2 ± 5.1 (57.1–72.0)	43.4 ± 3.7 (39.0–50.0)	5.6 ± 0.3 (5.0–6.1)
C19	575 ± 17.6 (557–611)	22.6 ± 0.7 (20.9–23.6)	24.6 ± 0.5 (24.0–25.8)	60.2 ± 4.1 (57.0–68.6)	43 ± 3.3 (39.0–50.5)	5.3 ± 0.1 (5.0–5.6)
C21	564 ± 28.1 (521–614)	21.9 ± 0.6 (20.9–23.1)	26.2 ± 0.7 (25.4–27.6)	65.5 ± 3 (59.2–69.5)	41.3 ± 3.1 (38.1–48.0)	5.3 ± 0.2 (5.0–5.7)
C23	590 ± 16.1 (570–621)	22.4 ± 0.8 (21.4–24.1)	26.1 ± 0.6 (25.4–27.4)	63.1 ± 5 (57.2–71.1)	43 ± 3.8 (39.0–51.0)	5.3 ± 0.3 (4.8–5.7)
C24	561 ± 34.5 (525–620)	22 ± 0.6 (21.2–23.4)	25.3 ± 0.8 (24.4–27.5)	66.4 ± 4.1 (60.5–73.3)	41 ± 3.5 (34.0–47.0)	5.3 ± 0.2 (5.0–5.8)
C25	573 ± 30 (538–623)	23.01 ± 0.4 (22.1–24.0)	25.8 ± 1.2 (24.4–27.2)	68 ± 4.8 (62.5–76.9)	44 ± 3.9 (38.3–50.0)	5.1 ± 0.2 (4.7–5.8)
C28	564±16.3 (532–584)	23.4± 1.2 (21.3 –25.4)	26.2 ± 1 (25.0–28.0)	65.2 ± 4 (60.4–72.4)	41 ± 1.9 (37.0–43.0)	5.4 ± 0.6 (4.5–6.1)
C30	561 ± 34.7 (525–621)	23.4 ± 1.2 (21.3–25.2)	24.6±0.9 (23.0–26.0)	67.5 ± 1.2 (65.1–71.1)	42 ± 3.1 (38.0–47.0)	5.2 ± 0.45 (4.6–7)
C31	576 ± 17.5 (556–609)	22.6 ± 0.7 (20.5–23.6)	24.6 ± 0.4 (23.9–25.7)	60.1 ± 4.1 (55.0–68.6)	44 ± 3.2 (39.0–50.3)	5.3 ± 0.1 (5.0–6.1)

*dorsal esophageal gland orifice

from 477 to 516 µm and stylet length was 22–25 µm (Table 8) with moderately concave stylet knobs. Lateral fields had four lines, although often only the two inner lines were distinct.

Molecular characterization

Molecular identification of the populations from Algeria was reliably verified by species-specific PCR primers using ribosomal DNA from cysts and sequencing of the ITS-rDNA. The *H. avenae*-specific primers (AVEN-COI) amplified a band of 109 bp for 19 populations (C1, C2, C4, C6, C7, C8, C13, C14, C15, C16, C17, C19, C21, C23, C24, C25, C28, C30, and C31) (Fig. 2). This means that 19 out of 27 populations were *H. avenae*. For those not verified as *H. avenae*, the *H. filipjevi* specific primers (Hlat-act) amplified a specific band of 646 bp for two populations (Fig. 2).

The sequences of the ITS-rDNA region of the *H. avenae*, *H. filipjevi* and *H. hordecalis* populations collected from Algeria were compared as well as with sequences of other *Heterodera* species available in the GenBank (Fig. 3). Thus, both morphological properties and species-specific PCR confirmed the identification

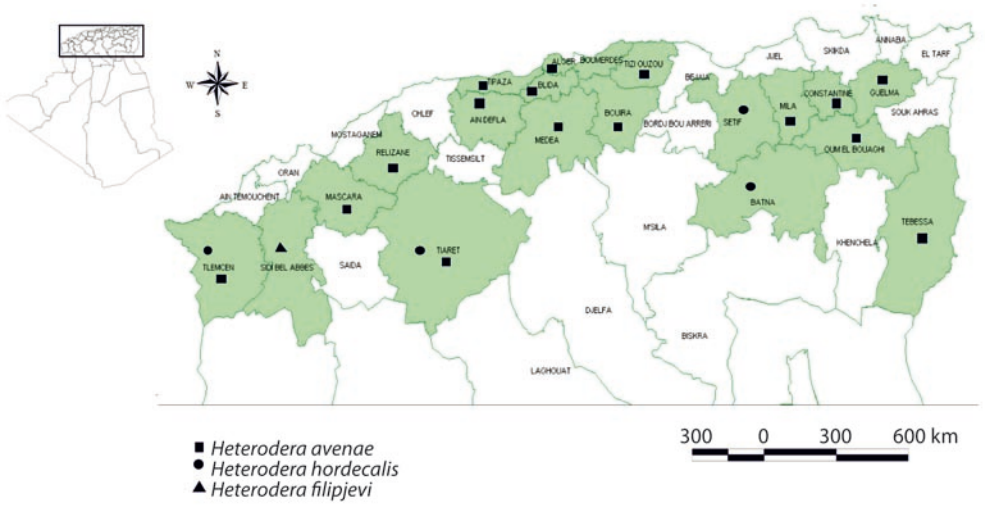

Fig. 1. Distribution of the three-major species of cereal cyst nematodes in Algeria

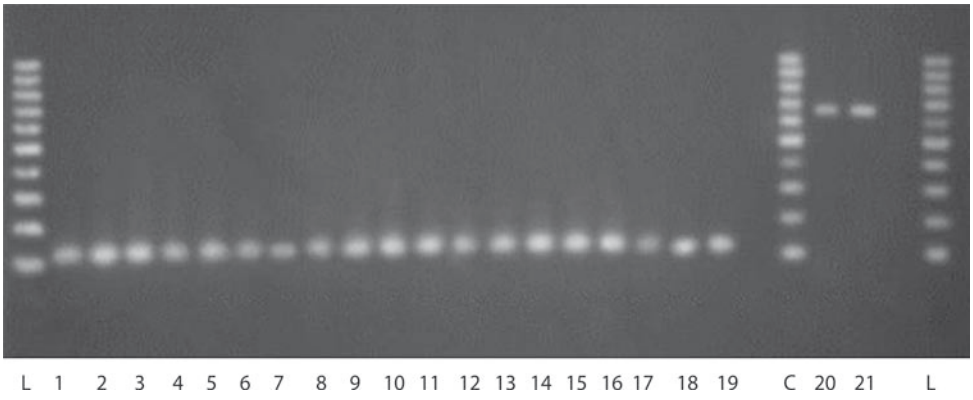

Fig. 2. Results of the *Heterodera avenae* and *H. filipjevi* specific PCR. L – 100 bp DNA ladder (Fermentas Life Sciences). *Heterodera avenae* is from number of 1 to 19, *H. filipjevi* is 20 and 21. The codes correspond with those in Table 2. C – negative control

Table 5. Morphometric characters of cysts and vulval cones of *Heterodera hordecalis* populations from Algeria (n = 10). Measurements in µm and in mean form: mean ± SD (range). Codes for populations: see Table 1

Populations	Cyst						Vulval areas of cyst			
code	length without neck	neck length	width	L : W*	semifenestra width	fenestra length	vulval slit length	vulval bridge width	underbridge length	bullae
C3	486 ± 50 (426–576)	44 ± 9 (31–58)	338 ± 42 (312–421)	1.43 ± 0.4	27 ± 1.5 (24–29)	60 ± 3 (57–64)	17 ± 2 (15–20)	10 ± 2 (8–15)	present	absent
C5	512 ± 48 (428–590)	44 ± 8 (32–56)	348 ± 44 (326–445)	1.47 ± 0.5	25 ± 1.2 (23–28)	62 ± 2 (58–64)	18 ± 1 (16–20)	11 ± 1.5 (9–14)	present	absent
C12	595 ± 24 (521–624)	49 ± 11 (35–61)	398 ± 33 (325–451)	1.49 ± 0.4	24 ± 1 (23–27)	57 ± 1.5 (55–61)	16 ± 1 (15–19)	10 ± 1.5 (8–13)	present	absent
C26	518 ± 41 (435–599)	56 ± 10 (41–68)	358 ± 35 (323–422)	1.44 ± 0.4	24 ± 0.9 (23–26)	62 ± 1.9 (57–64)	17 ± 2 (15–19)	12 ± 2 (9–14)	present	absent
C27	575 ± 29 (499–618)	51 ± 11 (37–64)	406 ± 29 (362–472)	1.41 ± 0.3	25 ± 1.1 (23–27)	61 ± 1.5 (57–63)	18 ± 1.1 (16–20)	11 ± 1.2 (9–13)	present	absent
C29	568 ± 26 (476–613)	59 ± 12 (42–73)	386 ± 31 (319–447)	1.47 ± 0.4	28 ± 1 (25–29)	64 ± 2.1 (59–68)	18 ± 0.9 (16–20)	12 ± 1.5 (9–14)	present	absent

*length : width ratio

Table 6. Morphometrics of *Heterodera hordecalis* second-stage juvenile populations collected in Algeria (n = 10). Measurements in µm and in form: mean ± SD (range)

Populations	Second stage juveniles					
code	body length	body width	stylet length	tail length	hyaline length	terminal DGO*
C3	450.0 ± 15.0 (423.0–475.0)	20.2 ± 1.4 (18.0–24.0)	24 ± 0.7 (23.0–26.0)	53.6 ± 2.2 (50.0–58.0)	34.3 ± 2.0 (31.0–37.0)	5.4 ± 0.6 (4.4–6.4)
C5	448.0 ± 10.4 (424.0–472.0)	20.9 ± 0.6 (19.5–23.8)	23.9 ± 0.6 (22.0–26.0)	52.1 ± 2.1 (49.6–57.0)	34.8 ± 1.9 (31.0–38.0)	5.2 ± 0.4 (4.2–6.1)
C12	452.0 ± 11.4 (429.0–478.0)	20.3 ± 1.2 (18.5–23.0)	23.8 ± 0.8 (22.8–25.0)	55.4 ± 1.9 (53.1–58.0)	35.1 ± 0.9 (33.5–37.0)	5.1 ± 0.3 (4.5–6.0)
C26	458.9 ± 12.4 (433.1–472.0)	20.8 ± 1.1 (19.4–22.9)	23.5 ± 0.5 (22.8–25.1)	54.6 ± 1.5 (52.5–57.0)	35.5 ± 1.5 (33.2–37.8)	5.3 ± 0.2 (4.8–6.0)
C27	456.1 ± 12.8 (435.9–471.0)	21.1 ± 0.5 (20.1–21.9)	24.1 ± 0.8 (22.9–25.5)	56.1 ± 1.2 (54.1–58.2)	34.1 ± 0.8 (32.8–36.2)	5.3 ± 0.3 (4.7–6.2)
C29	451.2 ± 13.1 (434.2–469.0)	20.7 ± 0.9 (19.1–22.5)	23.7 ± 0.5 (22.5–25.0)	54.1 ± 1.2 (51.8–57.4)	35.2 ± 0.8 (32.9–37.0)	5.2 ± 0.3 (4.5–6.2)

*dorsal esophageal gland orifice

Table 7. Morphometric characters of cysts and vulval cones of *Heterodera filipjevi* populations from Algeria (n = 10). Measurements in µm and in mean form: mean ± SD (range). Codes for populations: see Table 1

Populations	Cyst				Vulval areas of cyst					
code	length without neck	neck length	width	L : W*	semife-nestra width	fenestra length	vulval slit length	vulval bridge width	underbridge length	bullae
C18	769 ± 47 (670–851)	95 ± 15 (76–116)	55 ± 40 (375–505)	1.69 ± 0.5	24.0 ± 2.3 (21.1–27)	48.3 ± 4.6 (42–59)	9.6 ± 1.3 (7–11)	7.7 ± 1.6 (6–10)	present	absent
C22	780 ± 50 (684–862)	91 ± 14 (78–110)	450 ± 35 (393–500)	1.73 ± 0.4	24.5 ± 2.4 (22–27)	52.3 ± 3.5 (43–60)	10 ± 2 (8–14)	8.5 ± 2.3 (6–11)	present	absent

*length : width ratio

Table 8. Morphometrics of *Heterodera filipjevi* second-stage juvenile populations collected in Algeria (n = 10). Measurements in µm and in form: mean ± SD (range)

Populations	Second stage juveniles					
code	body length	body width	stylet length	tail length	hyaline length	terminal DGO*
C18	477.9 ± 13.2 (427–567)	21.9 ± 0.7 (20.5–24)	24.4 ± 0.23 (22.4–25.1)	57.3 ± 0.7 (52.4–64.6)	37.5 ± 1.1 (30.4–50.5)	6.2 ± 0.5 (5–7)
C22	516.3 ± 16.2 (476.8–547)	19.7 ± 0.5 (19–20)	24.2 ± 0.2 (23.2–25.6)	57.34 ± 0.7 (52.4–64.6)	33.8 ± 0.8 (25–39)	6.1 ± 0.6 (5–7)

*dorsal esophageal gland orifice

of *H. avenae*, *H. filipjevi* and *H. hordecalis*. Nineteen sequences of the ITS sequences were matched (99–100%) with the previously published sequences of *H. avenae* in the GenBank, six sequences (C3, C5, C12, C26, C27, and C29) were similar (99–100%) to *H. hordecalis*, and two sequences (C18 and C22) were similar (98–99%) to *H. filipjevi*. The relationship of the sequences of the ITS-rDNA region of *Heterodera* species from Algeria with other species *of Heterodera* were measured using Bayesian Inference (BI) analysis. Based on the topology of the calculated majority role, 60% consensus maximum likelihood tree for all the studied populations with the addition of 7 *Heterodera* spp. from the GenBank, two major groups of *Heterodera* were revealed (Fig. 3). In group I, (bootstrap value = 77%), two subgroups were found. The first sub-group comprised all *H. avenae* populations from Algeria including five other *H. avenae* populations available in the GenBank (bootstrap value = 99%), while the second sub-group contained two populations of *H. filipjevi*.

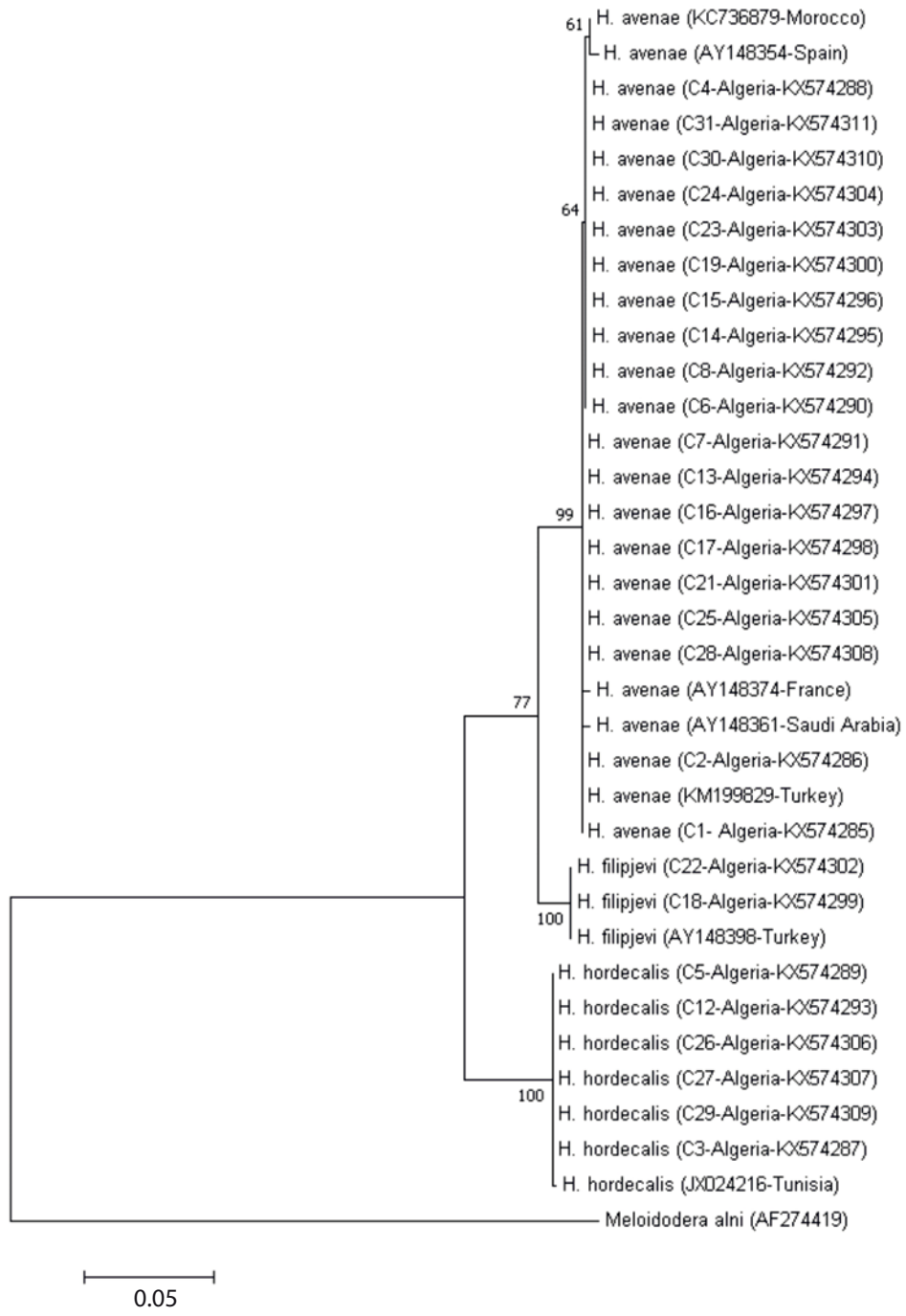

Fig. 3. The topology of the majority rule 60 consensus Maximum Likelihood tree for all populations studied with the addition of *Heterodera* populations obtained from the GenBank based on the sequence alignment of the ITS-rDNA. For the list with the abbreviations of the population codes see Table 1

Group II (bootstrap value = 100%) contained all the *H. hordecalis* (Algerian and other) populations.

Discussion

The findings of this study are a very valuable source of new information on the occurrence and distribution of *H. avenae*, *H. hordecalis* and *H. filipjevi* in the main wheat growing regions of Algeria. Both of the latter species were detected for the first time in the country (Smaha *et al.* 2018a, b); they were found in wheat fields of Setif, Tiaret, Tlemcen, Batna and Sidi Bel Abbes. *H. avenae* was the most common species in 23% of the fields and was associated with wheat in 16 provinces. Previous studies in various cereal growing areas of Algeria indicated the presence of *H. avenae* (Valette *et al.* 2002; Tirchi *et al.* 2016). Valette *et al.* (2002) reported the presence of both species *H. avenae* and *H. latipons*

in other regions of Algeria such as Setif, Béjaia, Relizane, Dar El Beida and Mascara. In this study, *H. avenae* was found more spread out than previously reported and *H. latipons* was not detected in all surveyed fields. Recently, Tirchi *et al.* (2016) reported the presence of *H. avenae* in the Ain Defla region of Algeria. Due to the similarities among *Heterodera* species, identification based on morphological characters resulted in misidentification within species. The morphological features and vulval cone patterns of J2s have been recommended as the most definitive way to characterize *Heterodera* spp. (Handoo 2002); however, cysts and J2s, respectively, are only useful when they are present in a sample. The three species detected in this study were easily distinguishable based on the cyst morphology. All cysts of *H. avenae* had distinctive bullae that completely surrounded the vulval cone, and no underbridge. Compared to *H. avenae*, *H. hordecalis* had a strong underbridge, with the presence of bullae at the underbridge level. *H. filipjevi* cysts had strong bullae and underbridge. Similar studies have been reported by different researchers (Wouts and Sturhan 1995; Handoo 2002; Rivoal *et al.* 2003; Rumpenhorst *et al.* 2003), who indicated which morphological features distinguished *H. avenae* from both *H. filipjevi* and *H. hordecalis*. Recently, Mokrini *et al.* (2017) reported the discrimination of Moroccan *H. avenae* populations from *H. latipons* populations based on the presence of bullae without an underbridge. Furthermore, Imren *et al.* (2015) reported a clear discrimination of *H. avenae* from *H. filipjevi* and *H. hordecalis* based on cyst morphology, including the presence or absence of bullae and underbridge. Main morphometric characteristics of J2, listed in Tables 4, 6 and 8 for the examined populations, were similar to data obtained by Handoo (2002) and Rumpenhorst *et al.* (2003) and corresponded with data for *H. avenae, H. filipjevi* and *H. hordecalis*. The J2s of *H. avenae* have prominent stylets, tails and hyaline parts of the tail when compared to similar characteristics of both *H. hordecalis* and *H. filipjevi*. Other studies conducted by Handoo (2002) and Imren *et al.* (2015) reported that *H. avenae* populations have longer bodies, stylets and hyaline tail lengths than both *H. hordecalis* and *H. filipjevi.*

Recently, the traditional identification of *H. avenae* was supported by the development of species-specific primers for PCR (Toumi *et al.* 2013; Yan *et al.* 2013), and *H. filipjevi* (Toumi *et al.* 2013; Yan *et al.* 2013; Peng *et al.* 2013). Several markers have been found to be successful in discriminating many species of *Heterodera*. Yan *et al.* (2013) developed species-specific primer sets to detect *H. avenae* and *H. filipjevi* based on the ITS region of DNA and Peng *et al.* (2013) developed species-specific SCAR-PCR assay to detect *H. filipjevi*. When using the species-specific primers developed for both *H. avenae* and *H. filipjevi* (Toumi *et al.* 2013; Peng *et al.* 2013) bands of 109 bp and 646 bp were obtained, respectively, confirming their morphological characterization and the reliability of the developed primers. The same set of primers has been successfully used in other research (Imren *et al.* 2016, 2017; Cui *et al.* 2017) and seems to be universal. Confirming results were reported by Mokrini *et al.* (2017) who characterized some populations of Moroccan cyst nematodes. Peng *et al.* (2013) reported that SCAR primers HfF2/HfR2 were useful for identification of *H. filipjevi* populations.

Besides the morphology, morphometric, species-specific primers, the sequence comparison of the ITS region clearly separates the Algerian *H. avenae* from both *H. hordecalis* and *H. filipjevi.* The ITS region has been frequently used in separating nematode species, as well as the genus of *Heterodera* (Rumpenhorst *et al.* 2003; Ou *et al.* 2008; Fu *et al.* 2011; Hesar *et al.* 2012; Imren *et al.* 2015; Baklawa *et al.* 2015; Mokrini *et al.* 2017; Ciu *et al.* 2017). In this study, no intraspecific polymorphism among Algerian populations of *H. avenae* was found based on the ITS sequences. These results are in agreement with Mokrini *et al.* (2017) who reported that *H. avenae* populations collected from different parts of Morocco fell into the same group with high resemblance. Baklawa *et al.* (2015) indicated that the *H. avenae* complex clustered into one branch, with a bootstrap value of 100%. Likewise, Abidou *et al.* (2005) reported the absence of intraspecific polymorphism among French and Syrian populations of *H. avenae*. However, several studies have reported that there was a polymorphism among different *H. avenae* and *H. latipons* populations (Bekal *et al.* 1997; Molokanova *et al.* 1999; Rivoal *et al.* 2003; Madani *et al.* 2004; Imren *et al.* 2015). Rumpenhorst *et al.* (2003) reported that African, European and Asian isolates of *H. avenae* formed several groups based on phylogenetic analysis of the ITS-rDNA. Imren *et al.* (2015) found intraspecific polymorphism among *H. avenae* populations originating from the eastern Mediterranean section of Turkey based on the ITS sequences and did not show any genetic variation among different populations of this species. The results of the present study did not find any polymorphism in *H. filipjevi* populations. In another study Imren *et al.* (2012) reported that *H. filipjevi* populations from Iran and Turkey could cluster with a bootstrap value of 99%. Subbotin *et al.* (2003) reported that the ITS sequence alignment of *H. filipjevi* isolates from Iran and Russia (Acc. no. AF274399) clustered together with a nucleotide identity of 100%, using the minimum evolution method. In this study, all the *H. filipjevi* populations were clustered in the same group with a high bootstrap value of 100%.

The results reported here are the first to provide integrated morphometric, morphological and molecular characterization of CCNs populations from wheat-producing areas in Algeria. *H. avenae* is the most prevalent species and is distributed widely in Algeria.

In the future research should be carried out to study the pathotypes among the *H. avenae* and *H. filipjevi* populations in Algeria. Also, all local wheat cultivars should be screened against the main populations of *H. avenae* and *H. filipjevi* to identify and/or develop suitable resistance sources to limit damage caused by these cyst nematode species.

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