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Molecular Polymorphism and Morphometrics of Species of the *Heterodera avenae* Group in Syria and Turkey

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Abstract: Molecular characterization of the three most common cereal cyst nematode species of the *Heterodera avenae* group (*H. avenae, H. filipjevi,* and *H. latipons*), originating from various locations in major cereal-cultivating areas in Syria and Turkey, showed distinct restriction fragment patterns of the ITS-rDNA following PCR amplification and RFLP digestion with four endonucleases (*Hae* III, *Hin*f I, *Ita* I, and *Pst* I). Genetic dissimilarity within *H. avenae* group populations increased in comparison with *H. avenae* and other species; it was 0.164 with *H. filipjevi* and 0.354 with *H. latipons* populations. No intraspecific polymorphism was observed within *H. latipons* or *H. filipjevi* populations. Principal component analysis revealed contrasted correlations among 12 morphological parameters of cysts and juveniles of the three *Heterodera* species that separated them and distinguished differences within populations of *H. latipons*. Our results showed a clear separation of the three cyst nematode species on cereal using a conventional method for classification and molecular tests, and confirmed the congruence between genetics and morphological traits.

Key words: cereal, cyst nematodes, *Heterodera, H. avenae, H. filipjevi, H. latipons,* morphology, PCR, rDNA polymorphism, RFLP.

The *Heterodera avenae* group *sensu lato* suggested by Subbotin et al. (1999) and Handoo (2002) contains at least 11 valid species and several other undescribed species. The three main species recognized among the most important cyst nematode pests to cereals are *H. avenae* Wollenweber, *H. latipons* Franklin, and *H. filipjevi* (Madzhidov) Stone (Evans and Rowe, 1998; Nicol, 2002; Rivoal and Cook, 1993). *Heterodera avenae* has worldwide distribution, *H. latipons* is located essentially in the Mediterranean regions, and *H. filipjevi* is found mainly in the Eastern European and West Asian regions (Rivoal and Cook, 1993; Sikora, 1988; Sturhan and Rumpenhorst, 1996; Subbotin et al., 1996).

Recent surveys of cereal fields in Syria and Central Anatolian Plateau of Turkey for *H. avenae* group nematodes showed that these three species are widely distributed in major wheat and barley cultivating areas, with *H. latipons* being dominant in Syria whereas both *H. latipons* and *H. filipjevi* were common in Turkey (Abidou et al., 2002; Abidou, unpubl. data). In this Western Asiatic region, *H. avenae* was limited in its distribution, but it has been found in Syria (Abidou et al., 2002; Rivoal et al., 2003) and reported previously in Turkey by Yuksel (1973) and Subbotin et al. (2003).

As reliable identification based on morphology becomes more difficult, several biochemical tests have proved to be useful for nematode identification. Isozyme analysis was used to differentiate species of *Heterodera* (Mokabli et al., 2001; Nobbs et al., 1992). RAPD analysis distinguished between *Heterodera* species and detected polymorphism among *H. avenae* populations (Lopez-Brana et al., 1996). The polymorphism of amplified coding and non-coding regions of rDNA provides a useful and popular tool for species and subspecies identification (Bekal et al., 1997; Iwahori et al., 1998; Powers et al., 1997; Tanha Maafi et al., 2003; Waeyenberge et al., 2000). The rDNA contains two internal transcribed spacer regions (ITS1 and ITS2) located between the 18S and 26S genes thought to be more variable (Chen, 1992; Ibrahim et al., 1995). Ferris et al. (1993) showed that the ITS1 and ITS2 regions of rDNA were highly conserved among species of the *Heterodera schachtii* group. This region of rDNA was found to be useful for differentiating between species of the *H. avenae* group (Bekal et al., 1997; Rivoal et al., 2003). Subbotin et al. (1999) revealed intraspecific polymorphism in this group and identified two types of ITS regions among *H. avenae* populations: Type A for most European populations and Type B for the Indian populations. Further, a unique RFLP profile with heterogeneity in ITS region of Australian *H. avenae* populations was observed (Subbotin et al., 2002) and confirmed based on sequences of the ITS-rDNA and Random Amplified Polymorphic DNA data (Subbotin et al., 2003).

Because the genetic distances between species correlates with specific morphological characters in the graminaceous cyst nematode complex (Rivoal et al., 2003; Subbotin et al., 1999), the combination of molecular and classical methods of systematics might steadily enhance the knowledge about the diversity of groups of these closely related species (Ferris, 1994). Therefore, the objectives of this study were to identify species of cereal cyst nematodes from populations sampled in Syrian and Turkish cereal fields, and to clarify the possible variability among them by analyzing the digestion patterns of their rDNA using PCR-RFLP technique. Additionally, comparative analysis of morphology and morphometrics of the cysts and the juveniles was also conducted.

Materials and Methods

Sample collection: Surveys of cereal cyst nematodes were conducted over a 4-year period (2000–2003) in major barley and wheat-growing areas in 11 provinces

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in Syria and 19 sites in the Central Anatolian Plateau of Turkey. Two kilograms of randomly sampled soil was collected in each visited field. Soil samples were processed through Kort elutriator (Kort, 1960). Extracted cysts caught on the 250-µm-pore sieve were picked with a brush and gathered under a stereomicroscope. Differentiation of presumed species *H. avenae, H. falipjevi,* and *H. latipons* was based on the color of the cyst (dark or light brown) and presence or absence of an underbridge in the vulval cone.

The characterization covered 10 *H. latipons* populations, three of *H. filipjevi,* and one of *H. avenae* (Table 1). Populations of *H. avenae* (Ha11) from France, *H. filipjevi* (E84) from India, and *H. latipons* (E156) from Syria were used as controls. The population (E146) of *H. ciceri* was included in the study as an out-group species.

Molecular study: Species characterization of different graminaceous cyst nematodes was assessed previously using PCR-RFLP of the rDNA (Bekal et al., 1997; Rivoal et al., 2003). The same technique was used in this study and the following protocol applied: For each cyst nematode population, four gravid cysts were moistened overnight in distilled water at 4 °C. Individual cysts were cut near the anterior part to liberate the eggs and juveniles. Part of the juveniles was squashed between two cover slides and recovered in 150 µl of lysis buffer (200 mM Tris-HCI pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and kept in tubes on ice. The rest of the juveniles and the posterior part of each cyst were used for the morphological and morphometrical study.

The DNA was purified by removing proteins with 75 µl sodium acetate (3M, pH 5.2) at –20 °C for 10 minutes. After centrifugation for 5 minutes at 12000×g, the DNA from the supernatant was precipitated with 100% isopropanol and the pellet washed with 70% ethanol, then dried and re-suspended in 20 µl of Tris-EDTA buffer (10:1 mM) (pH 8) overnight at 4 °C.

Amplification of ITS-DNA: A pair of 21-bp primers localized at the extremities of the 18S and 26S ribosomal genes (18S 5′ TTGATTACGTCCCTGCCCTTT 3′ and 26S 5′ TTTCACTCGCCGTTACTAAGG 3′) were used to amplify a fragment of DNA, including the two internal transcribed regions (ITS1 and ITS2) and the 5.8S gene (Vrain et al., 1992). PCR amplification was carried out in 5 µl including 10× Taq buffer (Promega, Madison, WI), 2 mM of $MgCl_2$, 0.25 µM of each primer, 0.2 mM of each dNTPs, 1.25U of Taq DNA polymerase (Promega), and 4.5 µl of DNA. PCR cycles consisted of an initial denaturation step at 94 °C for 1 minute followed by 30 cycles of 1 minute at 94 °C (denaturation), 50 seconds at 60 °C (annealing), and 1 minute at 72 °C (elongation). The reaction was terminated by a final extension cycle (72 °C, 5 minutes), and the PCR product was stored at 4 °C. The PCR program was performed in a Perkin Elmer DNA thermal cycler. Ten µl of each PCR product mixed with 3 µl of loading buffer was loaded in 1% agarose gel with 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and ethidium bromide. PCR products were visualized after electrophoresis (120 V) under ultraviolet light (260 nm).

RFLP procedure: The restriction enzymes *Hae* III, *Hin*f I, *Ita* I, and *Pst* I were used in a reaction mixture containing 1 mM $MgCl_2$, 1× appropriate enzyme buffer, 4 units of the enzyme, and 10 µl of PCR product, then incubated overnight at optimum temperature for each enzyme as recommended by the manufacturer. The DNA fragments that were generated were then separated by electrophoresis in 2% agarose gel (50% standard agarose and 50% high-resolution agarose) in 1× TBE buffer and ethidium bromide for 3 hours at 120 V, then observed using UV light.

Morphometric analysis: The perineal portion of the cysts of 16 populations (Table 1) were cut and prepared for microscopic examination (Hooper, 1970). The juveniles were killed with heat, and fixed and mounted in TAF. Twelve characters of cyst and juveniles (J2), known to be important for taxonomic diagnosis in this group (Wouts et al., 1995), were included in the analysis. The cyst characters measured were the fenestra length (c-fl), semifenestra width (c-sfw), vulval bridge width (c-vbw), and vulval slit length (c-vsl). Other morphological traits related to bullae and underbridge were displayed as numeric data. Bullae size (c-bul) had three scale levels, where 0.5 expressed small or absent bullae, 1 expressed slightly prominent bullae, and 2 expressed big and heavy bullae. Underbridge presence and its depth from vulval bridge (c-udb) had three scale levels with 0 for the absence of underbridge, 1 for a light underbridge close to vulval bridge, and 2 for a

TABLE 1. Populations of cyst nematodes of the *Heterodera avenae* group tested for rDN polymorphism and morphometrics.

Heterodera species	Population code	Origin and locality	PCR-RFLP[a]	PCA[b]
H. avenae	Ha11[c]	France	+	+
=	Syr77	Syria (Aleppo)	+	+
H. filipjevi	Tk5	Turkey (Adana-Konya)	+	+
=	Tk7	Turkey (Cumra)	–	+
=	Tk23	Turkey (Ankara)	+	+
=	E84[c]	India	+	–
H. latipons	Syr6	Syria (Darra)	+	+
=	Syr8	Syria (Darra)	+	+
=	Syr13	Syria (Hama)	+	+
=	Syr2l	Syria (Aleppo)	–	+
=	Syr26	Syria (Aleppo)	+	+
=	Syr28	Syria (Idleb)	–	+
=	Syr76	Syria (Hama)	–	+
=	Tk20	Turkey (Eskisehir)	–	+
=	Tk26	Turkey (Termalli)	–	+
=	Tk29	Turkey (Haymana)	–	+
=	E156[c]	Syria (Homs)	+	–
H. ciceri	E146[d]	Syria (Idleb)	+	+

[a] PCR-RFLP technique used to analyze molecular polymorphism.
[b] PCA (Principal component analysis) applied to morphological traits.
[c] Populations used as control.
[d] Out-group species.

strong and deep underbridge (Table 2). The J2 characters analysed were total length (j-tll), tail length (j-tl), stylet length (j-sl), hyaline part of tail length (j-htl), ratio B (j-B = hyaline part of tail length/stylet length), and ratio C (j-C = total length/tail length) (Table 3).

Morphometrics of cyst characters were performed using a bright field microscope. Juvenile characters were measured using a computer-assisted image analysis (Videomet, release 4.5, from MICROVISION Instruments, Evry, France) at magnifications up to 1000×. For each nematode population, the cyst characters were evaluated on specimen numbers between 4 and 29, including those used for the molecular study. The juvenile characters were measured on specimen numbers between 7 and 28.

Data analysis: The patterns of rDNA fragments produced after endonuclease digestion were converted into a binary matrix according to the presence (1) or absence (0) of fragments. The genetic relationships among species and populations were shown using Nei's genetic distances (Nei and Li, 1979). Cluster analysis with the unweighted pair-group method using arithmetic averages (UPGMA), and Bootstrap analysis (Felsenstein, 1985) were applied to prove the statistical consistency of the classification. Both analyses were performed using PHYLIP software (Felsenstein, 1993).

The mean values of the 12 morphological and morphometric characters examined on the cysts and on the juveniles were analyzed through principal component analysis (PCA) to establish the relationships among the 16 populations representing expected and control *H. avenae, H. filipjevi, H. latipons,* and *H. ciceri* species. Euclidean distances inferred from this analysis allowed grouping populations and species by direct hierarchical classification using Ward's method (Lebart et al., 2000). These analyses were performed with SPAD (Système pour l'analyse de données) release 4.01 (CISIA-CERESTA, Montreuil, France).

Results

Molecular study (PCR amplification and RFLP analysis): PCR amplification of ITS regions of rDNA produced a single fragment of approximately 1.2 kb for all populations and species tested. Polymorphic PCR-RFLP patterns inferred from the four restriction endonucleases

Table 2. Morphology and morphometrics of cyst characters (means in µm ± SE and range) in Syrian (Syr) and Turkish (Tk) populations of cyst nematodes of the *Heterodera avenae* group.

Heterodera species[a]	Population code	N[b]	c-bul[c]	c-udb	c-fl	c-sfw	c-vbw	c-vsl
H.a.	Ha11	8	2	0	43.32 ± 1.33 39.52–49.4	22.61 ± 0.87 19–27.36	6.56 ± 0.25 6.08–7.6	7.13 ± 0.35 6.08–8.36
H.a.	Syr77	7	2	0	50.41 ± 1.55 47.12–57	25.3 ± 1.77 20.9–34.58	7.27 ± 0.76 5.32–9.88	6.95 ± 0.31 6.08–8.36
H.f.	Tk5	16	1	1	51.21 ± 1.63 38–61.56	23.39 ± 0.9 19–33.44	8.08 ± 0.39 6.08–11.4	7.6 ± 0.17 6.08–9.12
H.f.	Tk7	9	1	1	47.88 ± 2.32 40.28–3.8	21.58 ± 1.13 15.2–25.84	7.85 ± 0.44 6.08–9.12	6.84 ± 0.4 6.08–9.88
H.f.	Tk23	23	1	1	45.7 ± 1.14 30.4–57	20.37 ± 0.61 13.3–25.08	8.46 ± 0.38 6.08–11.4	7.63 ± 0.44 3.8–11.4
H.l.	Syr6	9	0.5	2	68.48 ± 1.89 60.8–77.52	17.82 ± 0.87 14.82–22.8	36.14 ± 1.88 30.4–48.64	8.59 ± 0.59 6.08–11.4
H.l.	Syr8	12	0.5	2	63.84 ± 2.26 53.2–76	18.15 ± 0.91 12.16–22.8	34.01 ± 2.01 22.8–41.8	8.65 ± 0.38 6.08–11.4
H.l.	Syrl3	8	0.5	2	63.84 ± 4.14 53.2–90.44	21.52 ± 0.98 18.24–25.1	30.12 ± 2.73 20.52–44.8	8.55 ± 0.7 6.84–12.16
H.l.	Syr21	13	0.5	2	68.93 ± 2.31 46.36–84.4	21.95 ± 0.51 18.24–24.7	30.69 ± 1.81 19–40.28	8.54 ± 0.53 5.32–11.4
H.l.	Syr26	15	0.5	2	67.59 ± 1.67 57–81.32	19.61 ± 0.78 15.2–26.6	31.67 ± 1.58 19–41.8	7.6 ± 0.43 5.32–10.64
H.l.	Syr28	10	0.5	2	63.84 ± 2.14 60.8–70.68	18.92 ± 0.89 14.06–23.2	31.54 ± 1.85 22.04–41.8	7.9 ± 0.38 6.08–9.88
H.l.	Syr76	12	0.5	2	60.23 ± 2.44 44.8–72.96	15.64 ± 0.61 11.4–18.62	30.02 ± 1.68 19.76–38	7.47 ± 0.23 6.08–8.63
H.l.	Tk20	29	0.5	2	61.35 ± 1.79 45.6–91.2	19.62 ± 0.56 14.06–25.8	29.35 ± 1.45 15.2–42.56	8.15 ± 0.18 6.08–9.88
H.l.	Tk26	12	0.5	2	59.66 ± 1.49 50.16–66.88	19.79 ± 10.76 16.34–26.22	34.62 ± 1.46 26.6–39.52	10.32 ± 0.45 7.6–12.92
H.l.	Tk29	15	0.5	2	65.36 ± 1.85 53.2–77.52	19.6 ± 0.77 15.2–26.98	36.89 ± 2.23 22.8–53.2	8.31 ± 0.27 6.84–10.64
H.c.	E146	4	2	2	45.14 ± 2.42 38–51.68	28.41 ± 1.18 26.6–31.54	8.55 ± 2.16 3.8–12.92	65 ± 5 60–70

[a] H.a. = *H. avenae,* H.f. = *H. filipjevi,* H.l. = *H. latipons,* and H.c. = *H. ciceri.*
[b] Numbers analyzed.
[c] c-bul = bullae size, c-udb = underbridge development, c-fl = fenestra length, c-sfw = semifenestra width, c-vbw = vulval bridge width, and c-vsl = vulval slit length.

TABLE 3. Morphometrics of juveniles (means in µm ± SE and range) in Syrian (Syr) and Turkish (Tk) populations of cyst nematodes of the *Heterodera avenae* group.

Heterodera species[a]	Population code	N[b]	j-tl[c]	j-htl	j-sl	j-tll	j-B	j-C
H.a.	Ha11	18	62.91 ± 1.02 57–72.2	41.29 ± 1.34 31.16–51.68	22.84 ± 0.18 21.28–24.32	507.58 ± 3.14 439–555	1.81 ± 0.04	8.07 ± 0.09
H.a.	Syr77	25	68.37 ± 0.81 61.56–80.56	45.99 ± 0.63 40.28–53.96	25.08 ± 0.2 23.56–28.12	545.31 ± 3.19 485.4–576.1	1.83 ± 0.02	7.98 ± 0.06
H.f.	Tk5	23	62.92 ± 1.14 53.2–70.68	40.31 ± 1.30 30.4–51	24.19 ± 0.23 21.28–26.6	523.32 ± 3.16 458–589.3	1.67 ± 0.04	8.32 ± 0.1
H.f.	Tk7	21	57.34 ± 0.66 52.44–64.6	37.52 ± 1.09 30.4–50.92	24.47 ± 0.23 22.04–25.84	477.92 ± 3.19 427–523	1.53 ± 0.04	8.34 ± 0.08
H.f.	Tk23	25	61.2 ± 0.6 57–66.88	40.86 ± 0.49 38–45.6	23.53 ± 0.18 22.04–25.08	499.61 ± 3.02 427–561.3	1.74 ± 0.02	8.16 ± 0.07
H.l.	Syr6	22	56.24 ± 0.53 52.44–61.56	33.48 ± 0.89 26.6–41.8	24.01 ± 0.22 22.04–25.84	474.35 ± 4.65 387.3–546.6	1.39 ± 0.03	8.43 ± 0.09
H.l.	Syr8	23	51.68 ± 0.4 47.88–54.72	29.11 ± 0.62 24.32–34.96	22.30 ± 0.27 19.76–24.32	449.21 ± 3.7 414.7–516.7	1.31 ± 0.02	8.69 ± 0.06
H.l.	Syr13	18	52.4 ± 0.85 44.84–58.52	30.65 ± 1.2 24.32–41.8	22.17 ± 0.31 19.76–23.56	441.67 ± 6.81 385.4–525.3	1.38 ± 0.04	8.43 ± 0.14
H.l.	Syr21	7	55.91 ± 1.36 50.16–60.04	33.98 ± 1.6 30.4–42.56	23.34 ± 0.43 22.04–25.08	479.06 ± 17.4 413.5–541.1	1.46 ± 0.04	8.57 ± 0.29
H.l.	Syr26	25	54.93 ± 0.7 47.12–60.8	30.92 ± 0.49 25.84–36.48	22.19 ± 0.19 19.76–23.56	506.35 ± 3 448–558.1	1.39 ± 0.02	9.22 ± 0.1
H.l.	Syr28	15	52.95 ± 0.61 48.64–57	28.53 ± 0.84 22.04–31.922	22.09 ± 0.29 20.52–23.56	471.01 ± 2.74 438.4–498.6	1.29 ± 0.03	8.9 ± 0.07
H.l.	Syr76	17	55.93 ± 0.66 51.68–60.8	33.31 ± 0.72 28.88–39.52	22.44 ± 0.24 20.52–23.56	484.43 ± 3.33 373.4–520.3	1.48 ± 0.03	8.66 ± 0.06
H.l.	Tk20	28	54.37 ± 0.7 45.6–60.8	32.94 ± 0.79 21.28–41.8	24.59 ± 0.55 19–27.36	467.48 ± 4.91 421.5–586.1	1.34 ± 0.04	8.6 ± 0.07
H.l.	Tk26	24	55.91 ± 0.9 45.6–64.6	37.37 ± 0.9 25.08–44.08	25.84 ± 0.29 22.04–27.36	485.29 ± 3.9 429–535	1.45 ± 0.03	8.68 ± 0.13
H.l.	Tk29	19	52.96 ± 0.71 47.88–57.76	34.12 ± 0.95 28.12–43.32	23.88 ± 0.26 21.28–25.08	477.2 ± 2 428.6–512.4	1.43 ± 0.04	9.01 ± 0.08
H.c.	E146	17	63.23 ± 1.5 53.2–72.96	37.11 ± 0.79 30.4–44.08	29.64 ± 0.37 25.08–31.16	554.68 ± 10.8 452.1–657.2	1.25 ± 0.02	8.77 ± 0.18

[a] H.a. = *H. avenae,* H.f. = *H. filipjevi,* H.l. = *H. latipons,* and H.c.= *H. ciceri.*
[b] Numbers analyzed.
[c] j-tl = tail length, j-htl = hyaline part of the tail length, j-sl = stylet length, j-tll = total length, j-B = hyaline part of the tail length/stylet length, and j-C = total length/tail length.

allowed differentiation of the *H. avenae, H. filipjevi,* and *H. latipons* and the outgroup *H. ciceri* (Figs. 1,2). The missing fragment upper 800 bp and the presence of two fragments in the vicinity of 550 bp and 350 bp generated by *Hin*f I characterized the Ha11 (control) and Syr77 populations of *H. avenae* (Fig. 1A). All populations of *H. latipons* from Syria and Turkey were obviously differentiated by the 380-bp fragment resulting from *Pst* I digestion (Fig. 1B). *Ita* I digestions allowed for the differentiation of *H. latipons* from both *H. avenae* and *H. filipjevi*. *Heterodera latipons* populations were characterized fragments of 350 bp and 240 bp, respectively. In contrast, populations of *H. avenae* and *H. filipjevi* showed RFLP patterns with two fragments at ca. 400 bp and a third at 260 bp (Fig. 2A). *Hae* III digestion also differentiated *H. latipons* from both *H. filipjevi* and *H. avenae* by two fragments near 580 bp. But, *H. filipjevi* and *H. avenae* differentiated by a specific band at 380 bp and 360 bp, respectively (Fig. 2B). *Heterodera ciceri* differed from the cereal cyst nematodes in all RFLP patterns generated by the four endonucleases.

The dendrogram constructed from Nei's genetic distances clearly displayed four main clusters relevant to each species tested and supported by bootstrap values above 95% (Fig. 3). The highest genetic distance (d = 0.464) was observed between the out-group *H. ciceri* and *H. avenae sensu stricto*. Genetic dissimilarity inside the *H. avenae* group populations increased in comparison between *H. avenae* populations and other species (d = 0.164 with *H. filipjevi* and d = 0.354 with *H. latipons*).

Morphometric analysis: Contrasted relationships between values and features of cyst and juvenile characters were demonstrated by the correlation circle inferred from the Principal Component Analysis (PCA) (Fig. 4A). In a same grouping, the analyzed characters are highly correlated; the closer the arrow relevant to each character is to the circle, the greater the involvement of the character in discriminating the populations presented on the PCA plan (Fig. 4B). On Axis 1, accounting for 63% of the variation, two opposite groups of characters are shown; on the left side, c-udb, c-fl, and c-vbw in cysts and the ratio j-C in juveniles; on the right, j-tl, j-htl, and the ratio j-B in juveniles plus c-bul in cysts. Axis 2, which accounted for 23% of the variation, re-

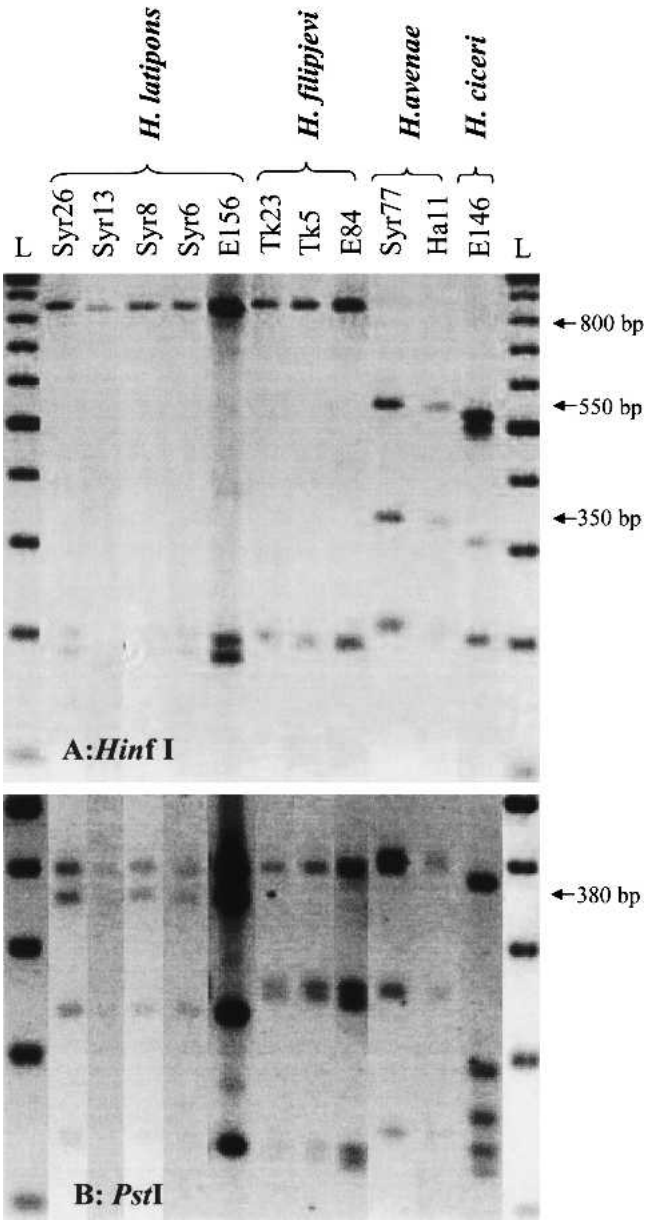

FIG. 1. RFLP patterns from *Hinf* I and *Pst* I digestion of the rDNA amplicon of different presumed species of cereal cyst nematodes of the *Heterodera avenae* group. L = 100 bp DNA ladder (Tebu). See Table 1 for origin of nematode populations.

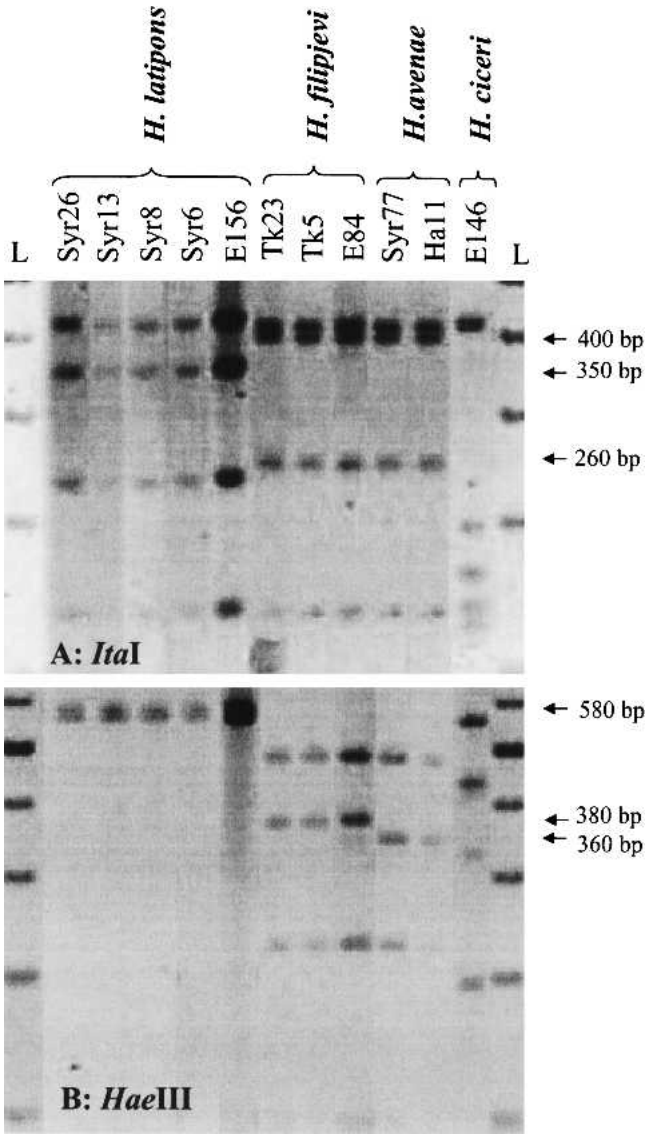

FIG. 2. RFLP patterns from *Ita* I and *Hae* III digestion of the rDNA amplicon of different presumed species of cereal cyst nematodes of the *Heterodera avenae* group. L = 100 bp DNA ladder (Tebu). See Table 1 for origin of nematode populations.

flected the effect of lengths of both j-sl and c-vsl. The c-sfw and j-tll showed an intermediate position on the correlation circle and were not really discriminated for the populations.

Heterodera avenae group populations were distributed into three groups by PCA (Fig. 4B). The two populations of *H. avenae* discriminated with heavy prominent bullae completely surrounding the vulval cone and absent underbridge. Both populations showed the lowest average ranges in c-fl (43.32–50.41 µm), c-vbw (6.56–7.27 µm), and ratio j-C (7.98–8.07) (Tables 1 and 2). Highest values of ratio j-B (1.81–1.83), j-tl (62.91–68.37 µm), and j-htl (41.29–45.99 µm) also characterized these populations. The close, but separated, populations of *H. filipjevi* were distinguished by less prominent bullae and a clear underbridge thick and fine in the center, sometimes bifurcated at the periphery, and located near the vulval bridge. Intermediate values of c-vbw (7.85–8.46 µm), j-tl (57.34–62.92 µm), j–htl (37.52–40.86 µm), ratio j-B (1.53–1.74) and ratio j-C (8.16–8.34) characterized the *H. filipjevi* populations. The populations relevant to presumed *H. latipons* differed from both *H. avenae* and *H. filipjevi* by a strong and deep underbridge without bullae and increased c-fl (59.66–68.93 µm), c-vbw (29.35–36.89 µm), and the highest c-vsl (7.47–10.32 µm). These populations were also distinguished by the lowest j-tl (51.68–56.24 µm), j-htl (28.53–37.37 µm), and ratio j-B (1.29–1.48). Nevertheless, the Turkish population, Tk26, deviated from this group by increased c-vsl (10.32 µm) of cyst and j-sl

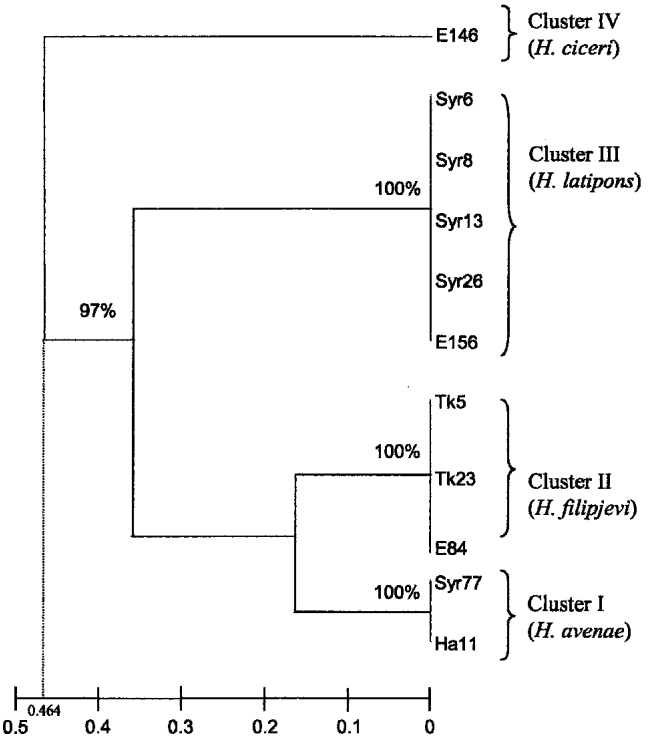

FIG. 3. Unweighted pair-group method using arithmetic averages (UPGMA) dendrogram between 15 populations of cereal cyst nematodes of the *Heterodera avenae* group and the out-group *H. ciceri*, based on the genetic distances calculated from PCR-RFLP data. See Table 1 for origin of nematode populations. Bootstrap values (%) were observed after 1000 resembling.

(25.84 µm) of juvenile. The out-group *H. ciceri* segregated normally with uncommon lengths of j-sl (29.64 µm) and c-vsl (65 µm).

Two distinct clusters were displayed by the direct hierarchical classification (Fig. 5). The first cluster contained *H. avenae* and *H. filipjevi* populations, which differed by distance values less than 1. The second cluster represented all populations of *H. latipons* that differed from the previous cluster by distance value upper to 6, and showed a variation between populations. *Heterodera ciceri* was located between the two clusters but was closer to the *H. avenae*/*H. filipjevi* grouping than to *H. latipons* (distance value = 2.41).

Discussion

Surveys of the *H. avenae* group nematodes in Syria and Turkey showed a wide distribution of both *H. latipons* and *H. filipjevi* in major cereal-cultivating areas with high population densities in specific regions (Abidou, unpubl. data). Therefore, the establishment of economic importance of these pests is essential to understand their real role on yield losses of cereals. Such studies need reliable techniques for the accurate identification of species in order to develop effective integrated control measures because of the diversity in this group of nematodes that can occur in mixed populations (Rivoal et al., 2003).

The PCR-RFLP analysis allowed a rapid identification of cereal cyst nematode species. Analysis of genomic DNA with RFLP has been used to differentiate species, races, and populations of several groups of plant-parasitic nematodes (Castagnone-Sereno et al., 1991). It helped verify the relationship among the populations of three species of the stem nematode *Ditylenchus* (Wendt et al., 1993) and has proved to be a valuable tool in accurately differentiating species of root-knot nematodes in mixtures (Zijlstra et al., 1997). Our analysis detected polymorphism among the three most common species of the *H. avenae* group by using four endonucleases. We could easily distinguish three *Heterodera* species (*H. avenae, H. filipjevi*, and *H. latipons*) using *Hae* III, which resulted in clearly defined patterns for each species. Additional digesting enzymes are needed for differentiation among more species of the *H. avenae* group. Subbotin et al. (2000) clearly separated 21 species of agriculturally important cyst-forming nematode from each other and from their sibling species according to specific RFLP profiles of amplified ITS region of the rDNA produced from seven digesting enzymes. More genetic dissimilarities were revealed by applying a wider set of restriction enzymes on the same or extra chromosomal (mitochondrial) DNA (De Giorgi et al., 1994).

Molecular and morphological characterization indicated that species of the *H. avenae* complex inside the *H. avenae* group *sensu lato* represent a paraphyletic taxon including extensive genetic variation between and within the species involved (Subbotin et al., 2003). Results of our study did not show any intraspecific polymorphism between populations of *H. filipjevi* or *H. latipons*. Also Bekal et al. (1997) did not detect any genetic variation among different populations of these species. However, intraspecific polymorphism was revealed between *H. filipjevi* populations (Subbotin et al., 2003). Differences in ITS sequences were observed between *H. latipons* populations (Ferris et al., 1999; Madani et al., 2004). Rivoal et al. (2003) also showed intraspecific differentiation between populations in *H. latipons* with a higher number of endonucleases and considered this species and *H. hordecalis* Andersson to form a separate species complex within the *H. avenae* group.

No intraspecific polymorphism was shown among French and Syrian populations of *H. avenae* in this study. But, polymorphism among different populations of this species was reported previously (Bekal et al., 1997; Rivoal et al., 2003; Subbotin et al., 1999). Bekal et al. (1997) hypothesized that the most likely site of origin of *H. avenae* is within the cereal areas in the Middle East and that this nematode could have been introduced to Australia from West Asia. Differences in sequences, RFLP of the ITS regions, and IEF patterns allowed for the description of a new species (*H. australis* Subbotin, Sturhan, Rumpenhorst and Moens), even

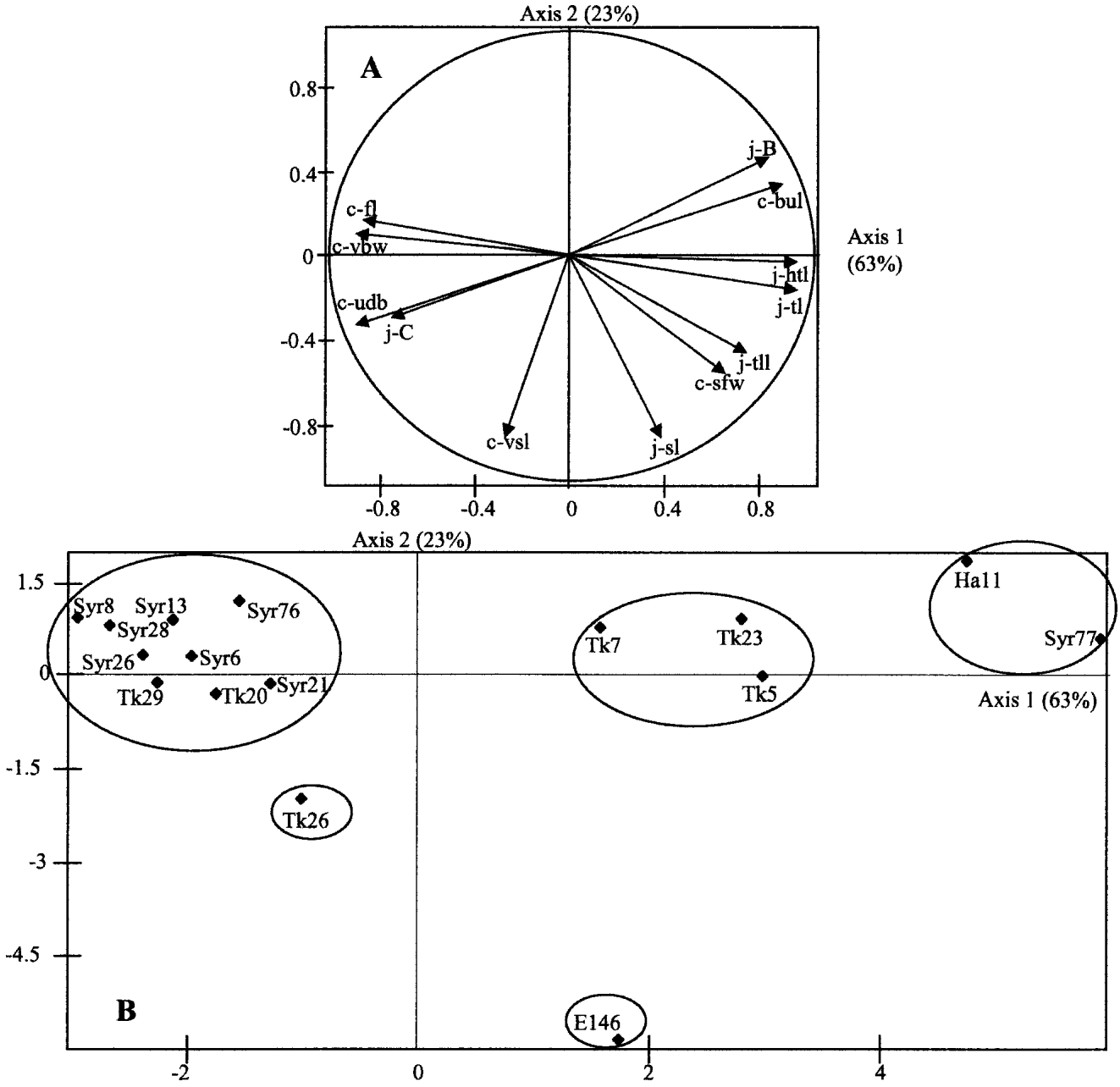

FIG. 4. Correlations. A) Between morphological characters and morphometrics in cyst vulval cones: c-bul = bullae size, c-udb = underbridge development, c-f = fenestra length, c-sfw = semifenestra width, c-vbw = vulval bridge width, c-vsl = vulval slit length. Juveniles: j-tl = tail length, j-htl = hyaline part of the tail length, j-sl = stylet length, j-tll = total length, j-B = hyaline part of the tail length/stylet length, j-C = total length/tail length. B) Distribution of populations of cereal cyst nematodes of the *Heterodera avenae* group inferred from the principal component analysis. See Table 1 for origin of nematode populations.

though *H. australis* is morphologically indistinguishable from *H. avenae* (Subbotin et al., 2002).

The complete genetic similarity found among the isolate Indian E84, previously identified as *H. avenae,* and three Turkish populations of *H. filipjevi* confirmed their adherence to this species, which is morphologically very close to *H. avenae* but differs mainly by the presence of a light underbridge located just under the vulval bridge. For a long time confused with the Gotland race and race 3 of *H. avenae, H. filipjevi* showed conversely a restricted distribution in Western and Southern Europe (Cook, 1982; Sturhan, 1982); this species may be more widespread than currently known. Therefore, it is necessary to determine the movement of this species from its Asiatic origin to neighboring areas of Western Asia and Eastern Europe (Rumpenhorst et al., 1996; Sturhan, 1996; Sturhan and Rumpenhorst, 1996; Subbotin et al., 1996, Tanha Maafi et al., 2003). More attention should be given to the comparative diversity of isolates of these nematodes originating from different parts of the world, and more especially from the Fertile Crescent and border areas where they

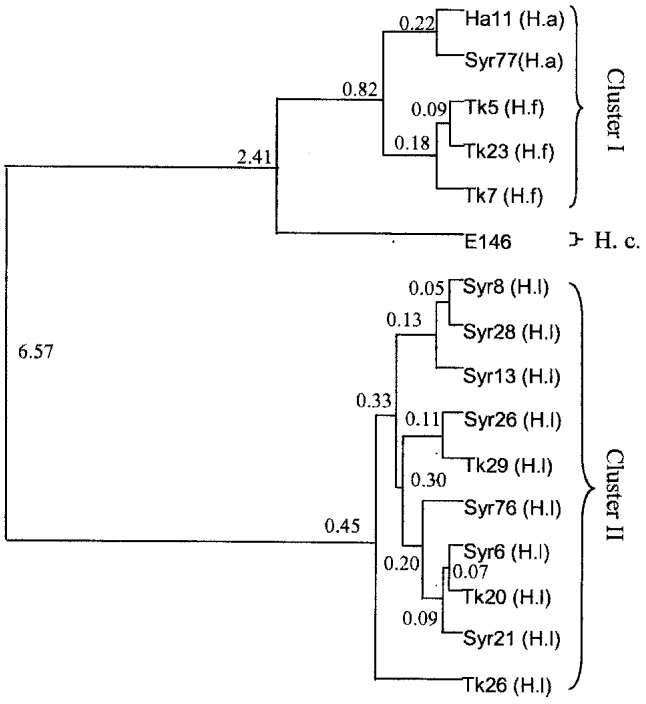

FIG. 5. Dendrogram showing the groupings of populations of cereal cyst nematodes of the *Heterodera avenae* group: *H. avenae* (H.a.), *H. filipjevi* (H.f.), *H. latipons* (H.l.), and the out-group *H. ciceri* (H.c.) inferred from the principal component analysis applied on morphological characters and morphometrics of cysts and juveniles. Values indicated distances between nodes. See Table 1 for the origin of nematode populations.

might have evolved along with the cropping of their obligatory hosts (Rivoal et al., 2003).

Correlations between several morphological characters of cysts and juveniles allowed for the restriction of the numbers of characters that differentiate these species of *H. avenae* group. For example, useful characters for distinguishing *H. avenae* from *H. filipjevi* were the presence of distinct underbridge in the vulval cone and small bullae situated below the fenestrae of cysts of *H. filipjevi,* as opposed to the absence of an underbridge and the presence of well-developed bullae surrounding the vulval cone of the cysts of *H. avenae* (Rivoal et al., 2003; Subbotin et al. 2003). The morphometric characters, such as hyaline part of the tail of the second-stage juvenile and the fenestra length of cysts, are also separating these species as pointed out by Madzhidov (1981) and Valdeolivas and Romero (1990). Differentiation between *H. avenae* and *H. latipons* could be readily achieved by using either the fenestra length or the vulval bridge width of the cyst and either the lengths of the tail or hyaline part of the tail of the juvenile, in addition to the underbridge and bullae presence in the vulval cone. These discriminating characters have been proposed for diagnosis of the *H. avenae* group (Wouts et al., 1995). In our experiment, the discrimination of specimens based on the development of bullae and presence of an underbridge was successful at low magnification, including populations of mixed species (Abidou, unpubl. data).

Using image analysis with automatic processes could have provided more accurate measurements instead of traditional methods based on drawings (Fortuner, 1991). This confirms the real morphological variations observed in the *H. latipons* grouping for the Turkish Tk26 population that showed a longer cyst vulval slit and juvenile stylet, which may relate to the presence of a different lineage with described or undescribed species and that should be confirmed by further intensive studies. Rivoal et al. (2003) already mentioned such morphological divergences in Syrian populations of *H. latipons* and hypothesized the existence of *H. hordecalis,* mainly known in Northern and Central Europe (Andersson, 1974; Sturhan, 1982).

Heterodera ciceri from chickpea was placed closer to the *H. avenae*/*H. filipjevi* grouping than to *H. latipons.* Most of its morphological and morphometrical features resemble those of *H. avenae* and *H. filipjevi* such as the big bullae, the short fenestra length and vulval bridge width in cysts, and the long tail and hyaline part of the tail in juveniles. Both molecular and morphological data agreed with the taxonomical classification of this outgroup species, which is a member of the *schachtii* group (Baldwin and Mundo-Ocampo, 1991).

An RFLP catalogue of the ITS region of cyst-forming nematode species is necessary in addition to morphological traits to accurately identify of *Heterodera* spp. attacking crops, especially when estimating pathogenic characterizations and searching for sources of host-plant resistance.

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