Comparative morphological and molecular study of Iranian populations of *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984 and other members of '*H. avenae* group'

A. M. Hesar[1*], E. M. Moghadam[1], Z. T. Maafi[2] and J. Karimi[1]

Summary.- Populations of '*H. avenae* group' were collected from sugar beet fields in Khorasan Razavi province (Iran) and identified on the basis of both morphological and morphometrical characters. PCR-RFLP and sequencing of rDNA-ITS regions were used to confirm the previous identification. Principal Components Analysis (PCA) of four morphometric characters of second-stage juveniles and vulval cones showed the overlapping between different species and populations, while UPGMA cluster analysis was used for grouping. Several populations of *H. filipjevi*, *H. mani* and *H. avenae* were grouped close together and PCA analysis demonstrated high overlapping between some populations of these species. The morphology of stylet knobs in *H. filipjevi* presented high intraspecific variability, hence this character is not useful to separate it from other closely related forms.

Key words: '*Heterodera avenae* group', identification, ITS-rDNA, morphometrics, PCA, RFLP.

Resumen.- Numerosas poblaciones del 'grupo *H. avenae*' se recolectaron en campos de remolacha azucarera en la provincia Khorasan Razavi (Irán) y se identificaron a partir de rasgos morfológicos y morfométricos. Las identificaciones se confirmaron mediante PCR-RFLP y la secuenciación de regiones ITS-ADNr. Se utilizó un análisis de componentes principales (PCA) para estudiar los solapamientos entre diferentes poblaciones y especies, en tanto que un análisis UPGMA proporcionó las asociaciones. Varias poblaciones de *H. filipjevi*, *H. mani* y *H. avenae* se agruparon estrechamente y el análisis PCA mostró elevado solapamiento entre algunas poblaciones de estas especies. La morfología de los nudos del estilete en *H. filipjevi* presentó amplia variabilidad intraespecífica, de tal suerte que este carácter no es útil para separar dicha especie de otras muy próximas a ella.

Palabras clave: 'Grupo *Heterodera avenae*', identificación, ITS-ADNr, morfometría, PCA, RFLP.

Introduction

The '*Heterodera avenae* group' currently contains 12 valid species (Nicol & Rivoal, 2008) which differ from each other by small morphological and morphometrical characters but show extensive overlapping between them (Wouts, *et al.*, 1995; Wouts & Baldwin, 1998; Handoo, 2002). Principal co-ordinate and stepwise discriminate analyses of morphometrical characters of cysts and J_2s have been successfully used to separate species of this complex (Stone & Hill, 1982; Valdeolivas & Romero, 1990; Subbotin *et al.*, 1999, 2003).

Traditional identification of cyst-forming nematodes based on morphological and morphometrical characters of cysts and second-stage juveniles (J_2s) is time consuming and demands careful study to distinguish sibling species. The increasing number of species in this group makes more difficult a reliable identification based on morphology (Subbotin *et al.*, 2003). Regarding this point, traditional identification, especially for morphologically closely related species, needs to be confirmed by molecular works. Several biochemical and molecular techniques have been used for the separation of species and populations of '*H. avenae* group', namely Random Amplified Polymorphic DNA (RAPD) (Sturhan & Rumpenhorst, 1996; Romero *et al.*, 1996), sequencing of ITS-rDNA (Subbotin *et al.*, 2003), and Restriction Fragment Length Polymorphism (RFLP) of ITS-rDNA (Subbotin *et al.*, 1999, 2003).

Sugar beet is one of the most important crops in Khorasan Razavi province of Iran, where some fields are suffering with the infection by sugar beet cyst nematode, *H. schachtii* Schmidt, 1871. Crop rotation with non-host crops is one of the control measures being applied and cereal are more in demand. The species of '*H. avenae* group' can reproduce on graminaceous plants in the absence of cereal and may cause yield losses in the next cereal cultivation. The most economically important

[1] Department of Plant Pathology, College of Agriculture, Ferdowsi University of Mashhad, Iran. Email: abasmokaram@gmail.com, mahdikhani_e@yahoo.com, javadkarimi10@gmail.com
[2] Iranian Research Institute of Plant Protection, Tehran, Iran. tanhamaafi@yahoo.com
* Corresponding author.

Table I. Populations of *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984 collected from different parts of Khorasan Razavi province in Iran.

Location	Data	Code	Studies
Fariman-sefid sangh	2009	H1	M, PCA, RFLP, SEQ
Fariman-asadie	2009	H2	M, RFLP
Mashhad-abkoh	2009	H3	M, RFLP
Saraks	2009	H4	M, RFLP
Kashmar	2009	H5	M, RFLP
Neyshabour	2009	H6	M, RFLP
Chenaran-mahmod abad	2009	H7	M, PCA, RFLP, SEQ

(M: Morphometrical, PCA: Principal Components Analysis, RFLP: Restriction Fragment Length Polymorphism and SEQ: Sequencing).

species (Rivoal & Cook, 1993; Nicol, 2002) of this group are *H. avenae* Wollenweber, 1923, *H. filipjevi* (Madzhidov, 1981) Stelter, 1984 and *H. latipons* Franklin, 1969. There are some reports showing the presence of five species of '*H. avenae* group' in sugar beet fields in Iran (Talachian *et al.*, 1976; Moghadam & Kheiri, 1995): *H. avenae*, *H. filipjevi*, *H. latipons*, *H. hordecalis* Andersson, 1975 and *Heterodera* sp, Among them, *H. filipjevi* is the most frequent form in wheat fields (Maafi *et al.*, 2007).

The objectives of this study were to identify the species of '*H. avenae* group' attacking sugar beet fields in Khorasan Razavi province of Iran on the basis of both morphological and molecular data, and to study the overlapping between the species by means of PCA and clustering methods.

Materials and Methods

Nematode populations and light microscopy: Soil samples were collected from sugar beet fields and the graminaceous weeds grown in the fields (Table I). The cysts were extracted by a combination of Cobb's sieving and decanting method and sugar flotation methods (Caveness & Jensen, 1955; Dunn, 1969). For each population, vulval cones of several cysts were mounted in glycerin jelly, and second stage juveniles from the same cyst were fixed in TAF and transferred to glycerin (De Grisse, 1969). Primary identification was carried out on the basis of morphometrics and morphological characters of cysts and juveniles (Wouts & Baldwin, 1998; Handoo, 2002; Subbotin *et al.*, 1999, 2003).

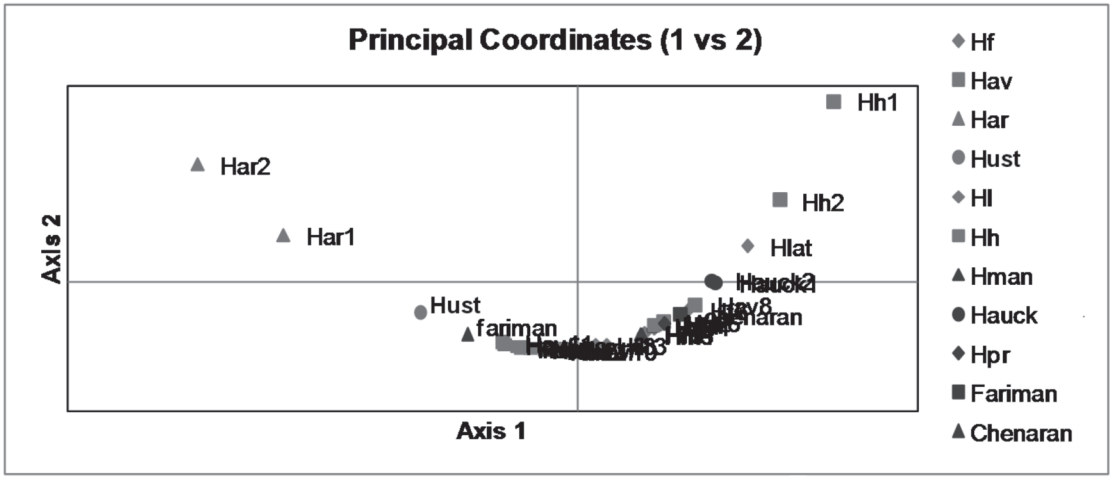

Figure 1. Overlapping between different species and populations of '*Heterodera avenae* group' revealed by first *vs.* second variables in PCA analyses.

Table II. Species and populations of the '*Heterodera avenae* group' used in the STATISTICAL analyses. (Measurements in μm and in the form of average).

Population	FL	SFW	VBW	VSL	L	Stylet	Tail	Hyaline
H. filipjevi,(Baimak,Russia). **Hf1**	53	30	8.1	10.9	552	25.4	60	39
H. filipjevi,(Gorodets, Russia), **Hf2**	51	28	9.4	10.3	526	24.5	55	33
H. filipjevi, (Pushkin, Russia), **Hf3**	52	27	7.9	14	539	25.2	60	37
H. filipjevi, (Chabany, Ukraine), **Hf4**	55	29	10.5	11.9	520	24.9	54	35
H. filipjevi, (Akenham, UK), **Hf5**	54	29	13	9.3	522	24	59	35
H. filipjevi, (Etelhem, Sweden), **Hf6**	52	28	9.9	10.1	509	24.3	56	33
H. filipjevi, (Dushanbe), **Hf7**	54	28	8.8	10.9	519	24.8	55	31
H. filipjevi, (Selçuklu, Turkey), **Hf8**	59	28	12	9.5	543	25	62	37
H. filipjevi, (Spain), **Hf9**	50	27	11.8	11	526	26.4	57	36
H. avenae, (Taaken, Germany), **Hav1**	48	25	7.3	10.1	566	26.3	69	45
H. avenae, (Rinkam, Germany), **Hav2**	48	23	7.1	8.9	557	26.5	69	44
H. avenae, (Argentan, France), **Hav3**	50	25	8.6	9.6	568	26.2	66	44
H. avenae, (St. Georges, France), **Hav4**	47	24	9.4	9.8	519	26.6	70	48
H. avenae, (Knokke, Belgium), **Hav5**	45	22	7.4	10.2	571	27.5	69	47
H. avenae, (Nuisement, France), **Hav6**	46	21	10.8	9.8	516	26.4	66	45
H. avenae, (Spain), **Hav7**	45	23	10.4	10	553	26.4	67	41
H. avenae, (desert reg. India), **Hav8**	48	21	10.6	8.7	505	26.1	61	38
H. avenae, (Villasavary, France), **Hav9**	46	24	10	9.7	563	26.9	69	44
H. avenae, (Unknown, Saudi Arabia), **Hav10**	45	24	12	8.1	552	27	66	41
H. avenae, (Çukurova plain, Turkey), **Hav11**	55	21	12	10	572	27	70	47
H. avenae from China, (Fonshu county sample1), **H"av"1**	46	23	8.3	7.9	537	25	66	42
H. avenae from China, (Fonshu county sample2), **H"av"2**	46	23	8.3	7.9	537	25	66	42
H. arenaria, (England), **Har1**	53	26	8.4	11.4	633	29.4	77	51
H. arenaria, (Unknown, The Netherlands), **Har2**	51	26	11	10	654	29	81	55
H .ustinovi, (Scotland), **Hust**	48	27	6.6	12.6	593	26.9	85	56
H. latipons, (Rostov, Russia), **Hlat**	60	23	28	7.3	485	23.4	53	32
H. hordecalis, (Sweden), **Hh1**	57	22	28	20	442	24.4	51	33
H. hordecalis, (Scotland), **Hh2**	63	25	26	22	470	25.6	54	35
H. mani, (Hamminkeln, Germany), **Hm1**	52	25	8.2	9.1	526	26	59	37
H. mani, (Andernach, Germany), **Hm2**	52	27	12	8.6	559	26	68	42
H. mani, (Heinsberg, Germany), **Hm3**	55	27	9.7	7.8	526	26	61	40
H. aucklandica, (Zarren, West Vlaanderen, Belgium), **Hauck1**	49	24	9.4	6.4	494	25	69	46
H. aucklandica, (St Albans, UK), **Hauck2**	47	28	8.9	7.4	494	24	76	48
H. pratensis, (Otterndorf, Lower Saxony, Germany), **Hpr**	45	22	8.5	7.6	516	25	65	42
H1	46	25	9.4	7	486	24	53	31
H7	52	23	9	10.1	516	26	50	28

(FL: Fenestral length, SFW: Semi fenestral width, VBW: Vulval bridge width, VSL: Vulval slit length, L: length)

Table III. Factor structure for principle variables of '*Heterodera avenae* group' using vulval cone and juveniles characters from 36 populations.

	Factor 1	Factor 2	Factor 3
FL	-0.651	-0.444	0.45
SFW	-0.161	0.328	**0.893**
VBW	**-0.702**	-0.56	-0.207
VSL	-0.407	**-0.743**	0.187
L	**0.793**	-0.098	0.386
Stylet	**0.783**	-0.482	0.065
Tail	**0.895**	-0.218	0.034
Hyaline	**0.874**	-0.322	-0.023
Expl.Var	3.919	1.567	1.234
Prp.Totl	0.489	0.195	0.154

(FL: Fenestral length, SFW: Semi fenestral width, VBW: Vulval bridge width, VSL: Vulval slit length, L: length)

Statistical analyses: Morphometrical data were statistically analyzed with the STATISTICA (version 5.0) computer package. Distance matrix and cluster analyses were done by this software too. Cluster analyses were used to assess the relative variations of 36 populations of nine species based on four morphometric characters of second stage juveniles and four morphometric characters of vulval cones (Table II). The unweighted pair group cluster analysis was used to compile a dendrogram clustering the populations at different levels on a scale of variation. And principal components analysis was used to examine overlapping between different populations of this group, performed with GenAlex6.1 (Orloci, 1978) (Fig. 1).

DNA extraction: One full cyst was put in 8 µl ddH_2O on a glass slide and punctured under a dissecting microscope. Second stage juveniles and eggs were divided in two parts; some were transferred to an Eppendorf tube and remaining juveniles fixed for morphological study. DNA was extracted according to Maafi *et al.* (2003) with some modifications: tubes were frozen at -80°C for at least 15 min and crushed by vortex, and 12 µl worm lysis buffer (500 mM KCl, 100 mM Tris-Cl pH 8, 15 mM $MgCl_2$, 0.05% Mercaptoethanol, and 4.5% Tween 20) and 2µl proteinase K (600 µg/ml) were added, respectively. The samples were incubated at 65 °C for 1 hour and 95 °C for 10 minutes. After incubation, the tubes were centrifuged for 2 min at 13000 rpm and kept at -20 °C until use.

PCR: 2 µl of the DNA template was added to the PCR reaction mixture containing 2.5 µl 10X buffer, 1 µl $MgCL_2$ (25mM), 0.5 µl dNTPs(10mM), 1 µl of each primers, 0.3 µl Taq DNA polymerase and 17.7 µl ddH_2O to a final volume of 25 µl (Maafi *et al.*, 2003). The forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT- 3') were used for amplification as described by Joyce *et al.* (1994). The PCR amplification profile consisted of 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 1.5 min at 62 °C and 2 min at 72 °C, followed by a final step of 5 min at 72 °C. Four µl of the PCR product was run on a 1.5% TBE buffered agarose gel (75 V, 80 min). The remaining PCR product was stored at -20 °C until use.

RFLP: 10-15 µl of PCR product was digested by one of the following restriction enzymes: *Alu*I, *Hinf*I, *Taq*I, *Bst*HI, *Pst*I, and *Tru*9I in the buffer stipulated by the manufacturer. The digested product was loaded on a 1.7% TBE buffered agarose gel, separated by electrophoresis (100V, 1.5h), stained with ethidium bromide, visualized on UV transilluminator and photographed. Procedure for obtaining PCR amplified products and endonuclease digestions of these products were repeated two times to verify the results. The exact length of each restriction fragment from the PCR products was obtained by visual and by a virtual digestion of the sequences using NebCutter 2.0.

Direct sequencing: PCR products were purified using Bioneer purification kit and sequenced in both directions using primers TW81 and AB28 with the BigDye 1.1 chemistry (PE Applied Biosystems) on an ABI 3100 (Foster City, CA) automated sequencer. The obtained sequences have been submitted to the GenBank (NCBI) database. Accession numbers are GU565574 and GU565575.

Phylogenetic analyses: DNA sequences were edited with Chromas 1.45 aligned with Clustal X 1.64 with default options (Thompson *et al.*, 1997). Only sequences of ITS1-5.8S-ITS2 were used for phylogenetic analysis. The full ITS regions sequences of several species of the *H. avenae* group, were obtained from the GenBank database. Sequence alignment was analyzed with an equally weighted maximum parsimony (MP) method using PAUP* 4.0b4a (Swofford, 1998). Gaps were treated as fifth nucleotide.

Results

The morphometric characters of cysts and J_2s of seven collected populations of '*H. avenae* group' are presented in Table IV. The data of other species and populations, which were compiled from literature, are presented in Table II. All morphometric characters showed extensive variations and overlapping between the species and populations. Based on morphological identification, populations of H1 and H2 are similar to

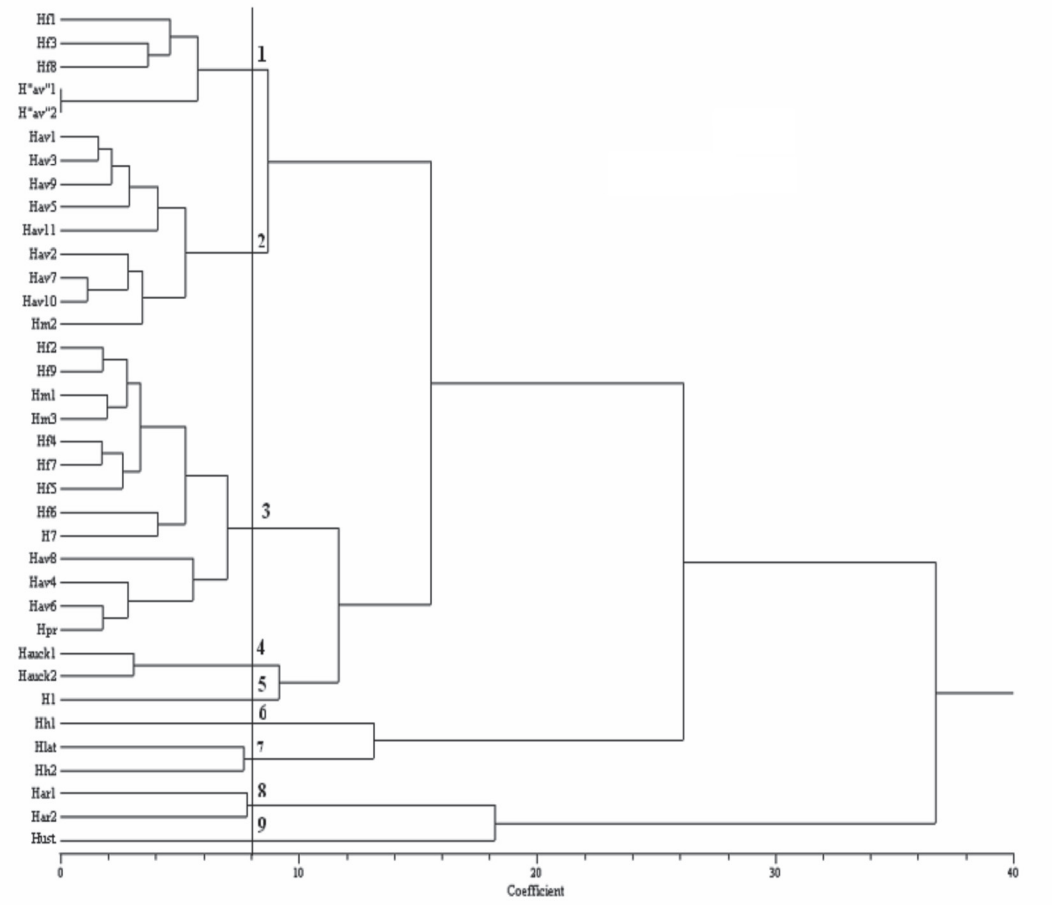

Figure 2. Similarity dendrogram of 36 populations of the '*Heterodera avenae* group' as computed by principal components analysis of eight morphometrical characters (for codes see Table I).

H. mani Mathews, 1971 in morphometric characters, shape of stylet knobs (indented anteriorly), four incisures in lateral field and presence of a weak underbridge in some vulval cones (Figs 5 & 6). Other populations are similar to *H. filipjevi* in morphometrics, shape of stylet knobs which is concave and presence of a strong underbridge (Figs 5 & 6). Amplification of the rDNA-ITS regions yielded a single PCR fragment for each of seven populations. Digestion with the six enzymes showed identical profiles for all populations. Restriction profiles for H1 and H7 are shown in Fig. 2. H1 and H7, which showed different morphological characters, were selected for STATISTICAL analysis and sequencing. The PCA analysis of the 36 populations calculated three variables. The vulval bridge width of vulval cone and body length, stylet length, tail and hyaline length of J_2s had the highest correlation with the first variable; vulval slit length with the second variable; and fenestral width with the third one (Table III). PCA was used to examine overlapping between some species of '*H. avenae* group'. Fig. 1 presents the two-dimensional scatter plot generated from mean values of variables. PCA analyses showed good separation for *H. arenaria* Cooper, 1955, *H. hordecalis*, *H. latipons* and to some extent for *H. ustinovi* Kirjanova, 1969, but revealed overlapping between other species and populations. The results of the unweighted pair group cluster analysis are presented in a dendrogram (Fig. 3): the populations of *H. avenae*, *H. mani* and *H. filipjevi* were grouped together; H7, however, clustered with populations of *H. filipjevi*; H1 clustered with populations of *H. aucklandica* Wouts & Sturhan, 1995, although H1 can be distinguished from *H. aucklandica* in the shape of stylet knobs (indented anteriorly *vs* flat) and by its shorter tail and length of its hyaline portion. Similar to PCA, good separation for *H.*

Table IV. Morphometrics of cyst, vulva cone and second stage juvenile of seven populations of *H. filipjevi* (Madzhidov, 1981) Stelter, 1984. Measurements in μm and in the form mean ± standard deviation (range).

Population	H1	H2	H3	H4
Cysts (n)	**11**	**12**	**10**	**7**
Character				
Length excl. neck	699±16.6 (680-735)	675.33±47.26 (630-770)	774±21.05 (750-810)	775.71±17.89 (750-805)
Width	509±9.59 (495-525)	461.41±27.82 (430-510)	507.6±19.34 (470-530)	508.28±9.77 (495-520)
Length/width	1.37±0.03 (1.3-1.42)	1.45±0.08 (1.32-1.68)	1.52±0.06 (1.44-1.64)	1.52±0.04 (1.44-1.56)
Vulval areas (n)	7	9	10	7
Fenestral length	45.85±2.60 (42-49)	46±2.73 (42-51)	49.2±1.81 (47-52)	53.42±1.90 (50-55)
Mean semifenestral Width	25.28±1.25 (23-27)	27.55±0.72 (27-29)	27.4±0.84 (26-29)	29.57±0.78 (29-31)
Vulval bridge width	9.42±1.27 (8-12)	9±0.43 (8.5-10)	9±0.66 (8-10)	7.78±0.69 (7-9)
Vulval slit length	7±1.15 (6-9)	8.22±0.97 (7-10)	7.4±1.07 (5-9)	8.57±0.78 (8-10)
Underbridge length	78.57±3.73 (74-85)	81.22±3.19 (75-86)	73.1±5.66 (60-81)	75.57±7.72 (60-85)
Juveniles (n)	21	22	21	18
L	486.25±16.85 (460-515)	498.18±29.51 (460-540)	550.90±25.96 (520-590)	417.5±14.88 (405-440)
a	23.04±1.24 (20.9-24.52)	24.45±1.18 (23-26)	24.84±1.39 (22.6-26.81)	19.95±0.69 (19.09-20.75)
b	4.29±0.29 (4.04-4.62)	4.12±0.09 (4.04-4.23)	4.36±0.03 (4.34-4.41)	4.04±0.04 (4-4.1)
c	9.13±0.37 (8.7-9.71)	9.44±1.11 (8.07-11.97)	9.06±0.30 (8.66-9.58)	9.30±0.63 (8.61-10.12)
Stylet length	24.44±0.72 (24-26)	24.4±0.69 (23-25)	25.81±0.75 (25-27)	25.25±0.46 (25-26)
Lip region height	4.2±0.42 (4-5)	4.36±0.50 (4-5)	4.27±0.46 (4-5)	4.37±0.51 (4-5)
Lip region width	9.4±0.51 (9-10)	9.36±0.50 (9-10)	9.63±0.50 (9-10)	9.06±0.41 (8.5-10)
DGO	5.25±0.5 (5-6)	6.5±0.70 (6-7)	5.1±0.56 (4-6)	5.5±0.70 (5-6)
Anterior end to valve of median bulb (MB)	77.55±2.96 (72-81)	75.62±3.06 (72-81)	73.3±5.18 (66-83)	58.2±2.28 (55-61)
Anterior end to excretory pore	108.8±1.78 (106-111)	107.8±8.55 (94-116)	110.45±5.66 (98-117)	89.25±4.57 (84-94)
Pharynx length (cardia)	112±11.31 (104-120)	129±3.60 (125-132)	134±2.94 (130-137)	131.33±4.16 (128-136)
Body diam.at mid-body	21.09±0.53 (20-22)	20.41±0.51 (20-21)	22.18±0.60 (21-23)	21±1.19 (20-23)
Body diam. at level of anus (BWA)	16.14±0.69 (15-17)	16.11±0.60 (15-17)	16.63±0.67 (16-18)	12.85±0.37 (12-13)
Tail length	53.42±1.27 (52-55)	52.77±5.23 (43-61)	60.72±2.05 (57-64)	45.14±2.26 (41-48)
Hyaline part of tail length (H)	31.75±1.66 (28-33)	33.2±4.77 (28-45)	35.18±1.53 (32-38)	25.57±1.90 (23-29)
Tail length/BWA	3.30±0.10 (3.17-3.46)	3.26±0.27 (2.86-3.91)	3.65±0.17 (3.35-3.93)	3.51±0.23 (3.15-3.91)
H/Stylet length	1.27±0.07 (1.16-1.37)	1.37±0.19 (1.2-1.8)	1.35±0.05 (1.28-1.44)	1.30±0.35 (0.96-1.92)
L/MB	6.26±0.27 (5.94-6.6)	6.90±0.33 (6.29-7.29)	7.52±0.36 (6.92-8.08)	7.15±0.49 (6.72-8)
Incisures in lateral field	3	3	3	4

Table IV. (Cont)

Population	H5	H6	H7
Cysts (n)	8	8	7
Character			
Length excl. neck	723.75±53.1 (650-810)	785±52.30 (740-875)	755.71±34.20 (690-795)
Width	491.87±23.59 (450-520)	519.62±14.91 (500-542)	507.14±7.55 (495-515)
Length/width	1.46±0.05 (1.37-1.55)	1.50±0.07 (1.41-1.63)	1.48±0.07 (1.33-1.55)
Vulval areas (n)	8	8	7
Fenestral length	52.12±1.64 (50-55)	51±2.61 (45-53)	51.85±1.77 (49-54)
Mean semifenestral Width	25.12±0.99 (24-27)	23.75±1.03 (22-25)	23.14±1.34 (21-25)
Vulval bridge width	11.75±0.70 (11-13)	8.62±0.74 (8-10)	9±0.57 (8-10)
Vulval slit length	7.75±0.70 (7-9)	8.37±0.74 (7-9)	10.14±0.89 (9-11)
Underbridge length	76.5±5.70 (70-86)	76.25±5.17 (70-85)	81.42±4.11 (75-87)
Juveniles (n)	22	22	21
L	505.41±13.39 (490-530)	544.54±27.24 (500-580)	516±15.92 (492-530)
a	23.97±0.63 (22.72-24.76)	24.22±1.56 (21.73-26.36)	23.80±0.62 (23.04-25)
b	3.97±0.15 (3.75-4.15)	4.15±0.05 (4.09-4.21)	4.14±0.04 (4.1-4.21)
c	9.10±0.36 (8.62-9.71)	9.29±0.38 (8.68-10.08)	10.17±0.57 (9.7-11.18)
Stylet length	24.58±0.51 (24-25)	25.27±0.64 (25-27)	25.75±0.46 (25-26)
Lip region height	4.08±0.28 (4-5)	4.18±0.40 (4-5)	4.25±0.5 (4-5)
Lip region width	9.5±0.52 (9-10)	9.72±0.46 (9-10)	10.25±0.5 (10-11)
DGO	4.66±0.81 (4-6)	4.33±0.57 (4-5)	4.2±0.27 (4-4.5)
Anterior end to valve of median bulb (MB)	70±4.82 (63-76)	84.57±7.56 (74-95)	79.2±3.63 (75-85)
Anterior end to excretory pore	102.54±4.92 (97-114)	103.2±12 (91-120)	109.33±8.96 (99-115)
Pharynx length (cardia)	131.25±2.98 (128-135)	132.2±2.58 (129-135)	124±7.78 (115-131)
Body diam.at mid-body	21.08±0.51 (20-22)	22.54±0.93 (21-24)	21.27±0.90 (20-23)
Body diam. at level of anus (BWA)	15.83±0.57 (15-17)	16.09±0.70 (15-17)	15.33±0.57 (15-16)
Tail length	55.58±2.84 (51-59)	58.36±2.73 (54-63)	50.28±3.14 (44-53)
Hyaline part of tail length (H)	33±2.13 (29-36)	34.90±1.75 (33-38)	28.12±2.85 (22-31)
Tail length/BWA	3.50±0.24 (3.05-3.93)	3.62±0.12 (3.43-3.81)	3.17±0.21 (2.93-3.33)
H/Stylet length	1.34±0.10 (1.16-1.5)	1.41±0.06 (1.32-1.52)	1.07±0.14 (0.84-1.19)
L/MB	7.24±0.48 (6.75-8.25)	6.42±0.58 (5.73-7.16)	6.62±0.36 (6.22-6.93)
Incisures in lateral field	3	4	4

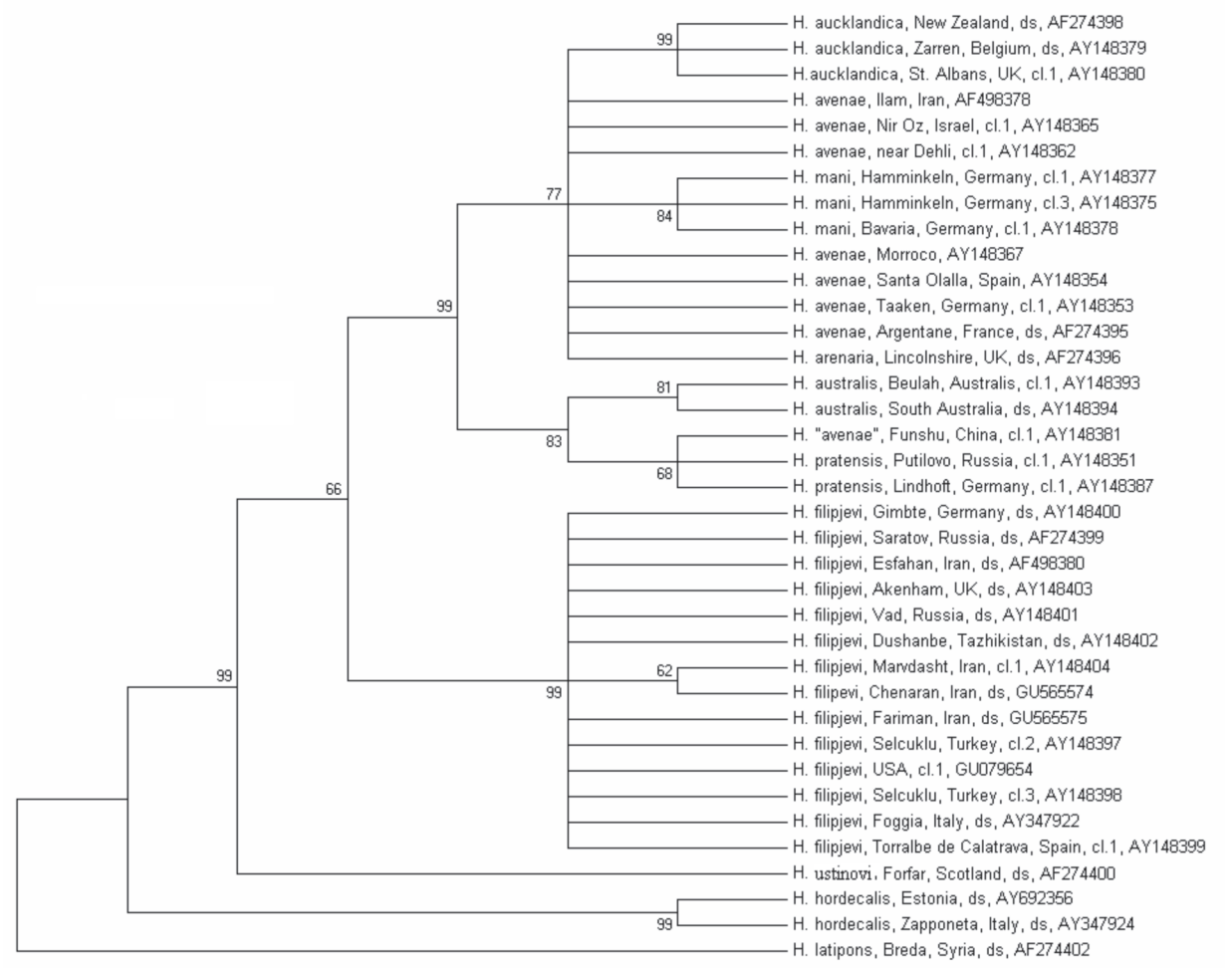

Figure 3. Phylogenetic relationships within the '*Heterodera avenae* group' inferred from analyses of the full ITS data set based of MP method.

arenaria, *H. hordecalis*, *H. latipons* and *H. ustinovi* is visible with this method as well.

Amplification of the ITS-rDNA region, including flanking parts of the 18S and 28S genes, yielded a single fragment in 1053 bp in all studied populations. Six enzymes generated RFLP for all studied populations (Fig. 4). Sizes of restriction fragments generated by the enzymes are given in Table V. *Hin*fI and *Taq*I could clearly distinguish *H. filipjevi* from *H. mani* and other morphologically similar species of this group. Intraspecific variation in RFLP patterns was not revealed for the studied populations.

The phylogenetic tree constructed from the sequences of the studied populations and the sequences deposited in the Genbank showed clustering of H1 and H7 with other *H. filipjevi* populations in the same clade (Fig. 4).

Discussion

The present study revealed that *H. filipjevi* is the only species of the '*H. avenae* group' found in sugar beet fields in Khorasan Razavi province (Iran), which suffer high infestation by this cyst nematode species, and is a dominant form in other provinces (Maafi *et al.*, 2003, 2007). Shape of stylet knobs and number of incisures in lateral field were documented as the main differences for distinguishing *H. filipjevi* from *H. mani* (Handoo, 2002), but our results showed many variations in these characters and that should not be used to properly distinguish both taxa.

Good separation of *H. arenaria* with PCA and clustering method (Fig. 1) is in agreement with Stone and Hill (1982), who studied six J_2s' numerical characters.

Table V. The ITS-rDNA-RFLP profiles yielded by a single enzyme for the studied populations

Enzymes	*Alu*I	*Bst*HHI	*Hinf*I	*Pst*I	*Taq*I	Tru9I
Fragments	569,484	749,152,108,44	820,192,41	712,211,130	340,274,134,118,79,65,43	551,486,9,7

Using additional features of vulval cone resulted a good differentiation of *H. latipons* from *H. hordecalis* as well. These results were confirmed by phylogenetic analysis, and *H. latipons*, *H. hordecalis* and to some extent *H. ustinovi* were separated as out group. According to Subbotin *et al.* (2003), *H. filipjevi* and *H. mani* could be separated from other species on the basis of cyst characters; however, our study showed variations of morphological characters in different populations of *H. filipjevi*, which may result in misidentification of these species.

No intraspecific polymorphism within the ITS regions of Iranian populations of *H. filipjevi* was found by using six restriction enzymes. And the results obtained revealed high overlapping in morphometrics of some species of '*H. avenae* group', in particular *H. avenae*, *H. filipjevi* and *H. mani*, which could impact on the reliability of traditional identification.

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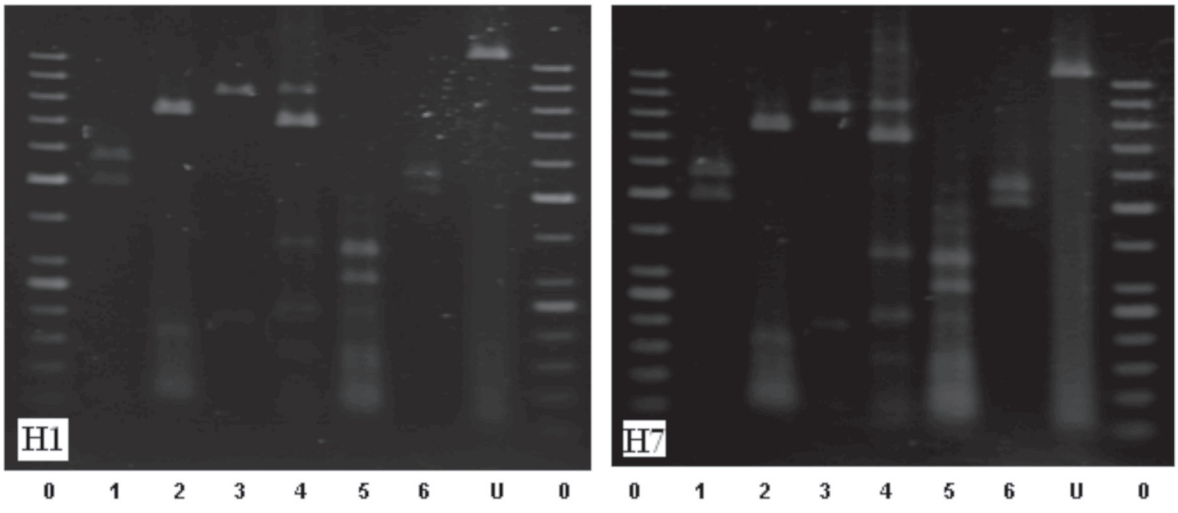

Figure 4: Restriction fragments of amplified ITS regions of *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984. H1: Fariman , H7: Chenaran. (0: Ladder 50bp; U: unrestricted fragment; 1: *Alu*I; 2: *Bst*HI; 3: *Hinf*I; 4: *Pst*I; 5: *Taq*I; 6: Tru9I).

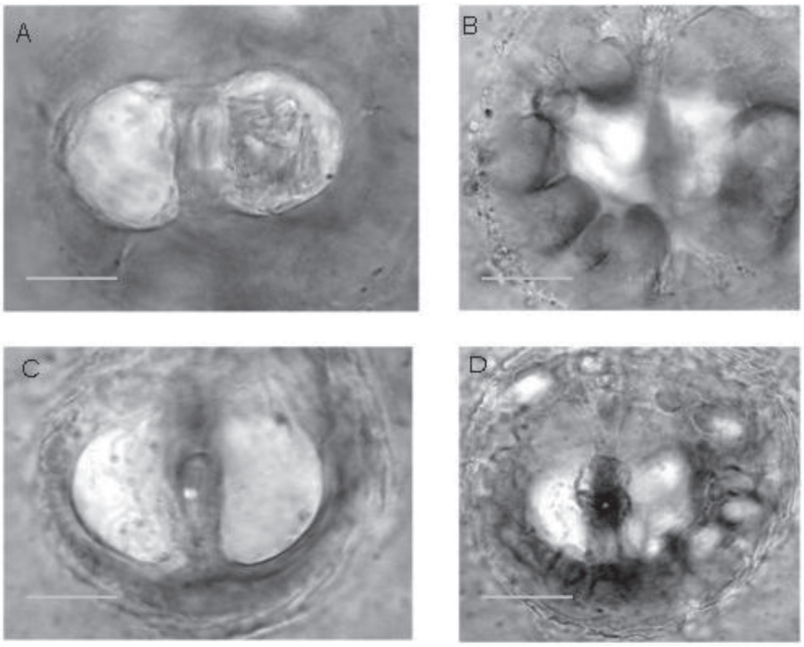

Figure 5. LM pictures of the terminal region of cysts of *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984. A, B: population H7; C, D: population H1. (Scale bar = 10 µm).

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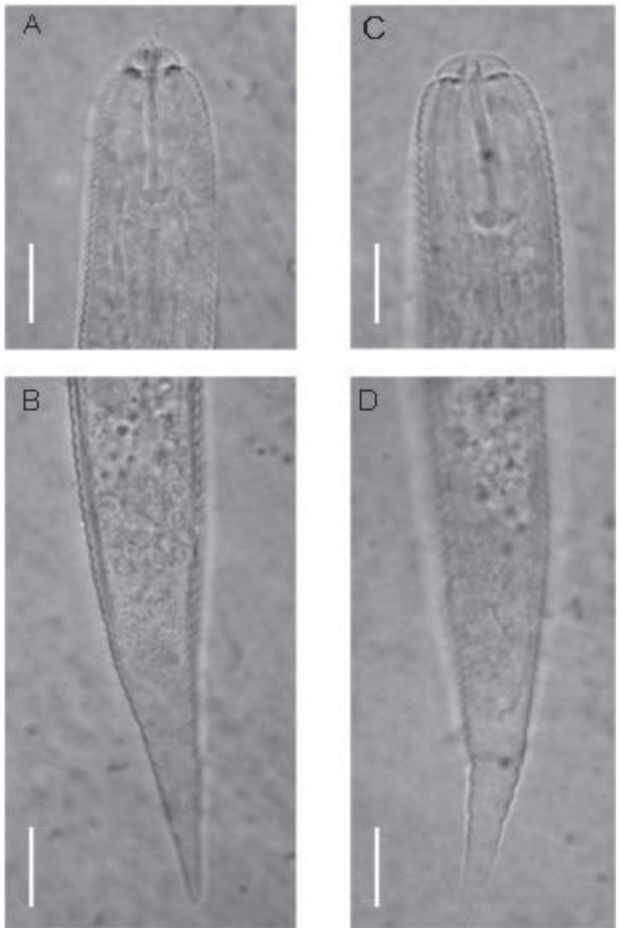

FIGURE 6: Light micrographs of the *second-stage juveniles of Heterodera filipjevi. A and B population of H7, C and D population of H1. (Scale bar* 10 µm*).*

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