Nematology, 2003, Vol. 5(1), 99-111

Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences

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Received: 17 April 2002; revised: 30 September 2002
Accepted for publication: 24 October 2002

Summary – RFLP and sequences of ITS-rDNA of 45 populations of cyst-forming nematodes collected from different parts of Iran were analysed and identified as representatives of 21 species. Eight enzymes generated RFLP for all studied populations. Comparison of RFLP profiles and sequences of the ITS regions with published data confirmed the presence of *Heterodera avenae*, *H. filipjevi*, *H. glycines*, *H. hordecalis*, *H. latipons*, *H. schachtii* and *H. trifolii* in Iran. RFLP patterns and ITS sequences for *H. elachista*, *H. turcomanica*, *H. mothi* and *C. cacti* were obtained for the first time in this study. *Heterodera humuli*, *H. goettingiana*, *H. fici*, *H. elachista*, *H. turcomanica* and *Cactodera cacti* are recorded for the first time in Iran. These results correspond with morphological and morphometric identification of the populations. Several populations were not identified at the species level and are attributed to *Heterodera* sp.; some of these may correspond to new species. Twenty-one new sequences from Iranian cyst-forming nematodes and 36 known sequences were used for the phylogenetic analyses. The cyst-forming nematodes formed several clades corresponding to their morphological features. *Heterodera mothi* and *H. elachista* clustered with high support with other *Cyperi* group species and *H. turcomanica* formed a moderately to highly supported clade with the *Humuli* group.

Keywords – diagnostics, *Heterodera*, PCR, phylogeny, RFLP.

The first evidence of the presence of cyst-forming nematodes in Iran was reported by Esmailpour and Schäffer (1970) who detected *Heterodera schachtii* Schmidt, 1871 in samples from sugar beet fields in Khorasan province, eastern Iran. Since then *H. avenae* Wollenweber, 1924, *H. carotae* Jones, 1950, *H. cruciferae* Franklin, 1945, *H. filipjevi* (Madzhidov, 1981) Stelter, 1984, *H. galeopsidis* Goffart, 1936, *H. glycines* Ichinohe, 1952, *H. hordecalis* Anderson, 1975, *H. iri* Mathews, 1971, *H. latipons* Franklin, 1969, *H. mani* Mathews, 1971, *H. mothi* Khan & Husain, 1965, *H. oryzae* Luc & Berdon Brizuela, 1961, *H. rosi* Duggan & Brennan, 1966 and *H. trifolii* Goffart, 1932 have been recorded from different provinces and various crops (Talachian *et al.*, 1976; Noori *et al.*, 1980; Barooti & Loof, 1990; Hojat-Jalali, 1991; Mehdikhani Moghadam & Kheiri, 1995; Mehdikhani Moghadam *et al.*, 1996; Sturhan, 1996; Mehdikhani Moghadam, 1998; Pedramfar *et al.*, 1998; Tanha Maafi *et al.*, 1999). The identification of *H. galeopsidis*, *H. iri*, *H. latipons*, *H. mothi* and *H. rosi*, was made on the basis of vulval cones without examination of the morphology and morphometrics of second stage juveniles (J2) (Talachian *et al.,* 1976; Noori *et al.*, 1980). Further investigations showed that some species of the *Avenae* group were not correctly identified and needed re-examination (Sturhan, 1996).

Traditional identification of cyst-forming nematodes based on morphological and morphometric characters of cysts and J2 is time consuming and demands careful study to separate the sibling species. Developed during recent years, ITS-rDNA diagnostics are a reliable tool for identification of cyst-forming nematodes. Comparison of RFLP

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profiles and sequences of the ITS-rDNA region of unknown species with those published and deposited in GenBank (Ferris *et al.*, 1994, 1999; Thiéry & Mugniéry, 1996; Orui, 1997; Szalanski *et al.*, 1997; Subbotin *et al.*, 1999, 2000a, b, 2001; Eroshenko *et al.*, 2001) facilitates fast identification of most species of cyst-forming nematode.

The goals of the present study were: *i*) to identify Iranian cyst-forming nematode populations based on RFLP and sequences of the ITS region of rDNA; *ii*) to present RFLP profiles along with information on the exact sizes of the restriction fragments for Iranian cyst-forming nematode species; *iii*) to analyse the phylogenetic relationship between these species based on their ITS-rDNA sequences.

Materials and methods

Nematode populations

During 1998-2000, about 1200 soil and root samples were collected from cultivated and wild plants and soil in natural areas, agricultural fields and orchards in different provinces of Iran. 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 glycerine jelly. Second stage juveniles from the same cyst were fixed in TAF and transferred to glycerine (De Grisse, 1969). Remaining cysts were air dried and kept at room temperature for molecular work. Primary identification was carried out on the basis of morphometrics plus morphological characters of cysts and juveniles (Mulvey, 1972; Mulvey & Golden, 1983; Golden, 1986; Wouts & Baldwin, 1998). A total of 45 cyst-forming nematode populations belonging to 21 species were collected (Table 1).

DNA extraction

Several cysts of each population were soaked overnight in double distilled water. One to four cysts were put in 8 μl ddH_2O on a glass slide and punctured under a dissecting microscope. Second stage juveniles and eggs were transferred to an Eppendorf tube containing 12 μl worm lysis buffer (500 mM KCl, 100 mM Tris-Cl pH 8, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20) and crushed with a microhomogeniser Vibro Mixer (Zürich, Switzerland). Two microlitres proteinase K (600 μg/ml) (Promega Benelux, Leiden, The Netherlands) were added and the tubes were frozen at −80°C for at least 10 min and then incubated at 65°C (1 h) and 95°C (10 min) consecutively. After incubation, the tubes were centrifuged for 2 min at 14 000 rpm and kept at −20°C until use.

PCR

Two μl of extracted DNA was transferred to an Eppendorf tube containing: 2.5 μl 10X *Taq* incubation buffer, 5 μl Q solution, 0.5 μl dNTPs mixture (*Taq* PCR Core Kit, Qiagen, Hilden, Germany), 0.15 μl of each primer (1.0 μg/μl) (synthesised by Life Technologies, Merelbeke, Belgium), 0.2 μl *Taq* Polymerase and double distilled water to a final volume of 25 μl. The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′), as described by Joyce *et al.* (1994), were used in PCR. The PCR amplification profile consisted of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1.5 min at 55°C and 2 min at 72°C, followed by a final step of 10 min at 72°C. Two μl of the PCR product was run on a 0.8% TBE buffered agarose gel (100 V, 40 min). The remaining PCR product was stored at −20°C until use.

RFLP

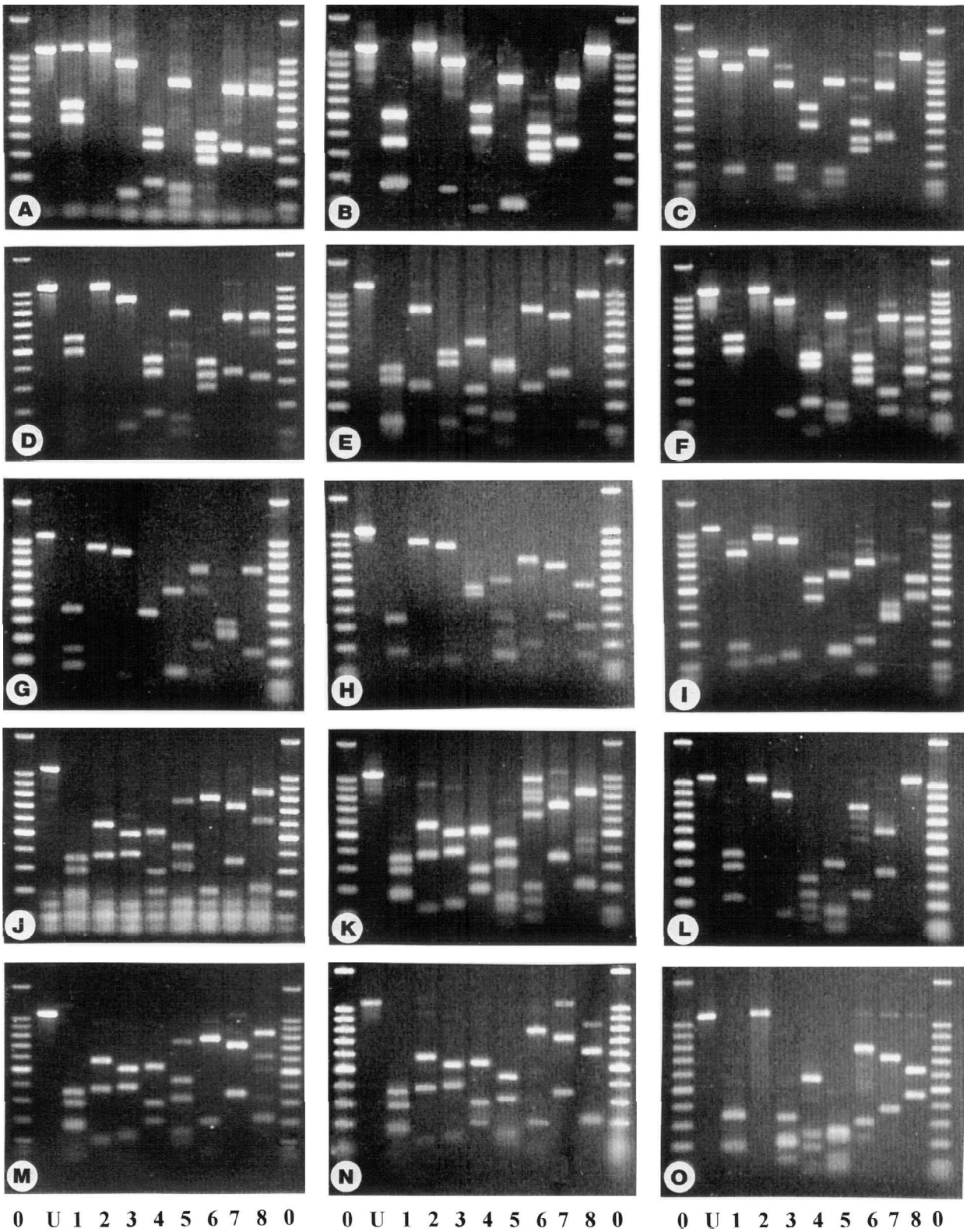
The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen). 2-8 μl of purified DNA was digested by one of following restriction enzymes: *Alu*I, *Ava*I, *Bsh*1236I, *Bsu*RI, *Cfo*I, *Mva*I, *Pst*I, or *Rsa*I in the buffer stipulated by the manufacturer. The PCR-product of the *Avenae* group populations was also restricted by *Hin*fI or *Taq*I to obtain higher discrimination. The digested DNA was run on a 1.5% TBE buffered agarose gel, stained with ethidium bromide, visualised on UV transilluminator, photographed and analysed using Kodak Scientific Imaging Systems (Kodak, Rochester, NY, USA). The exact length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html).

Cloning and sequencing

As the initial direct sequences of *Heterodera goettingiana* Liebscher, 1892, *H. humuli* Filipjev, 1934, *H. mothi*, *H. schachtii*, *H. trifolii*, *H. turcomanica* Kirjanova & Shagalina, 1965 and *Heterodera* spp. 3, 4 and 7 showed some ambiguous positions, the PCR products of these species were cloned and re-sequenced. DNA was excised from

Table 1. *List of cyst nematode species and populations used in present study.*

Species	Province and locality	Plant host	Study
Heterodera avenae	Ilam, Mehran-Reza Abad	Wheat (*Triticum* sp.)	Sequencing (AF498378), RFLP including *Hin*fI, *Taq*I
H. elachista	Mazandaran, Tonekabon, Soleyman Abad	Rice (*Oryza sativa*)	Sequencing (AF498391), RFLP
H. fici	Kordestan, Sanandaj	*Ficus elastica*	Sequencing (AF498385), RFLP
H. filipjevi	Fars, Abaadeh, Tashak	Wheat (*Triticum* sp.)	RFLP including *Hin*fI, *Taq*I
	Kordestan, Ghorveh	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Western Azarbayejan, Salmas	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Yazd, Marvast	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Esfahan	Wheat (*Triticum* sp.)	Sequencing (AF498380), RFLP with *Hin*fI, *Pst*I, *Taq*I
	Hamadan, Kaboutar Ahang	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Golestan, Gonbad Kavous	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Mazandaran, Sari Sourbon	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Kerman, Bardsir	Wheat (*Triticum* sp.)	RFLP with *Hin*fI, *Pst*I, *Taq*I
	Eastern Azarbayejan, Marand	Wheat (*Triticum* sp.).	RFLP
H. glycines	Golestan, Baghoo Kenareh	Soybean (*Glycines max*)	Sequencing (AF498387), RFLP
	Mazandaran, Dashtenaz	Soybean (*Glycines max*)	RFLP
H. goettingiana	Lorestan, Doroud	Unknown	RFLP
	Lorestan, Doroud, Akbar Abad	Clover (*Trifolium repens*)	Sequencing (AF498374), RFLP
H. hordecalis	Ardabil, Meshkin shahr	Wheat field and grasses	Sequencing (AF498381), RFLP
H. humuli	Mazandaran, near Amol	Nettle (*Urtica dioica*)	Sequencing (AF498384), RFLP
H. latipons	Western Azarbayejan, Salmas	Cereals field	Sequencing (AF498382), RFLP
	Zanjan, Abhar	Cereals field	RFLP
	Eastern Azarbayejan, Marand	Cereals field	RFLP
	Lorestan, Doroud	Cereals field	RFLP
	Golestan, Agh Ghaleh	Cereals field	RFLP
H. mothi	Khuzestan, Ahvaz	*Cyperus* sp.	Sequencing (AF498392), RFLP
	Systan and Blouchestan, Iranshahr	*Cyperus* sp.	RFLP
H. schachtii	Fars, Marvdasht	Sugar beet (*Beta vulgaris*)	Sequencing (AF498389), RFLP
	Khorasan, Chenaran	Sugar beet (*Beta vulgaris*)	RFLP
	Western Azarbayejan, Khoy	Sugar beet (*Beta vulgaris*)	RFLP
	Ardabil, Ardabil	Sugar beet (*Beta vulgaris*)	RFLP
	Esfahan, Ghohab	Sugar beet (*Beta vulgaris*)	RFLP
H. trifolii	Lorestaan, Broujerd	Clover (*Trifolium* sp.)	Sequencing (AF498388), RFLP
H. turcomanica	Ardabil, Meshkin shahr	Unknown	Sequencing (AF498386), RFLP
Heterodera sp. 1	Gilan, Bandar Anzali	Grasses	Sequencing (AF498379), RFLP including *Hin*fI, *Taq*I
	Ardabil, Meshkin shahr	Grasses	RFLP including *Hin*fI, *Taq*I
	Tehran, Gachsar	Grasses	RFLP including *Hin*fI, *Taq*I
Heterodera sp. 2	Mazandaran, Nashtaroud	*Phragmites* sp.	Sequencing (AF498383), RFLP
	Gilan, Bandar Anzali	*Phragmites* sp.	RFLP
Heterodera sp. 3	Tehran, Nivaran Park	Unknown	Sequencing (AF498390), RFLP
Heterodera sp. 4	Ardabil, Moghan	*Phragmites* sp.	Sequencing (AF498373), RFLP
Heterodera sp. 5	Tehran, Dizin	Unknown	Sequencing (AF498377), RFLP
Heterodera sp. 6	Mazandaran, Khoram-Abad	Unknown	Sequencing (AF498375), RFLP
	Mazandaran, Amol	Unknown	RFLP
Heterodera sp. 7	Western Azarbayejan, Urumieh	Unknown	Sequencing (AF498376), RFLP
Cactodera cacti	Markazi, Mahalat	Unknown	Sequencing (AF498393), RFLP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0.8% TAE buffered agarose gels using the QIAquick Gel Extraction Kit (Qiagen), cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega, Leiden, The Netherlands). Several clones of each species were isolated using blue/white selection and submitted to PCR with vector primers. PCR products from each sample or clone were sequenced using primers TW81, AB28 or internal reverse primer 5.8SM5 (5′-GGCGCAATGTGCATTCGA-3′) as described by Zheng *et al.* (2000) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems Benelux, The Netherlands) according to the manufacturer's instructions. The programme used for sequencing reactions was: 30 s at 94°C, 30 s at 50°C and 3 min 30 s at 60°C, repeated for 25 cycles. The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems Inc., Gaithersburg, MD, USA). Sequences were run on a 377 DNA Sequencer (PE Applied Biosystems, Warrington, UK). The sequences obtained have been submitted to the GenBank database.

Sequence and 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 region sequences of several species of the genera *Heterodera, Cactodera, Punctodera* and *Globodera* were obtained from the GenBank database (Ferris *et al.,* 1999; Subbotin *et al.,* 2001) or from published data (Subbotin *et al.*, 2000b; Zheng *et al.*, 2000). *Meloidodera alni* Turkina & Chizhov, 1986 and *Cryphodera brinkmani* Karssen & van Aelst, 1999 were designated as outgroup taxa (Subbotin *et al.*, 2001). Several sequences of ITS1, ITS2 or ITS1-5.8S-ITS2 deposited in GenBank (Szalanski *et al.,* 1997; Clapp *et al.,* 2000; Sui *et al.,* unpubl.) were also used for comparison, but were not included in the full sequence and phylogenetic analysis.

Sequence alignment was analysed with an equally weighted maximum parsimony (MP) method and minimum evolution (ME) method using PAUP* 4.0b4a (Swofford, 1998). We used heuristic search setting with ten replicates of random taxon addition, tree bisection-reconnection branch swapping to seek for the most parsimonious trees. Gaps were treated as missing data. For ME analysis, the appropriate substitution model of DNA evolution that best fitted the data set was determined by the Akaike Information Criterion with ModelTest 3.04 (Posada & Crandall, 1998). To obtain an estimation of the support for each node, a bootstrap analysis (100 replicates, heuristic search, and simple addition of sequence) was also performed.

Results

RFLP of the ITS-rDNA for Iranian populations of cyst-forming nematodes

Amplification of the ITS-rDNA region, including flanking parts of the 18S and 28S genes, yielded a single fragment varying from 981 bp in *Cactodera cacti* (Filipjev & Schuurmans Stekhoven, 1941) Krall & Krall, 1978 to 1087 bp in *H. mothi.* No PCR products were obtained in the negative control without nematode DNA template. Eight enzymes generated RFLP for all studied populations (Figs 1, 2). Sizes of restriction fragments generated by eight enzymes are given in Table 2. Additional restriction of PCR product by enzymes *Hin*fI and *Taq*I allowed discrimination of the species within the *Avenae* group: *Hin*fI distinguished *Heterodera* sp. 1. (817, 187, 41 bp) from *H. avenae* (499, 318, 191, 41 bp) and *Taq*I clearly differentiated *H. filipjevi* from the other *Avenae* group species (data not shown). Substantial ITS heterogeneity was revealed for *H. schachtii* individuals using *Mva*I. Because only a single clone was sequenced among several ITS haplotypes, the sizes of restriction fragments were not calculated for this species. For some species, the weak additional bands observed in the RFLP profiles were not taken into account.

Intraspecific variation in RFLP patterns was not revealed for the species studied. Heterogeneity of the ITS was observed in *H. trifolii*, *H. turcomanica*, *H. avenae*, *H. glycines*, *H. schachtii*, *H. filipjevi* and *Heterodera* sp. 3 and sp. 6.

Fig. 1. *Restriction fragments of amplified ITS regions of Iranian cyst-forming nematodes. A:* Heterodera avenae*; B:* H. latipons*; C:* H. hordecalis*; D:* Heterodera *sp. 1; E:* Heterodera *sp. 2; F:* H. filipjevi*; G:* H. humuli*; H:* H. turcomanica*; I:* H. fici*; J:* H. trifolii*; K:* H. schachtii*; L:* Heterodera *sp. 4; M:* H. glycines*; N:* Heterodera *sp. 3; O:* H. elachista *(0: 100 bp marker; U: unrestricted fragment; 1:* Alu*I; 2:* Ava*I; 3:* Bsh*1236I; 4:* Bsu*RI; 5:* Cfo*I; 6:* Mva*I, 7:* Pst*I, or 8:* Rsa*I).*

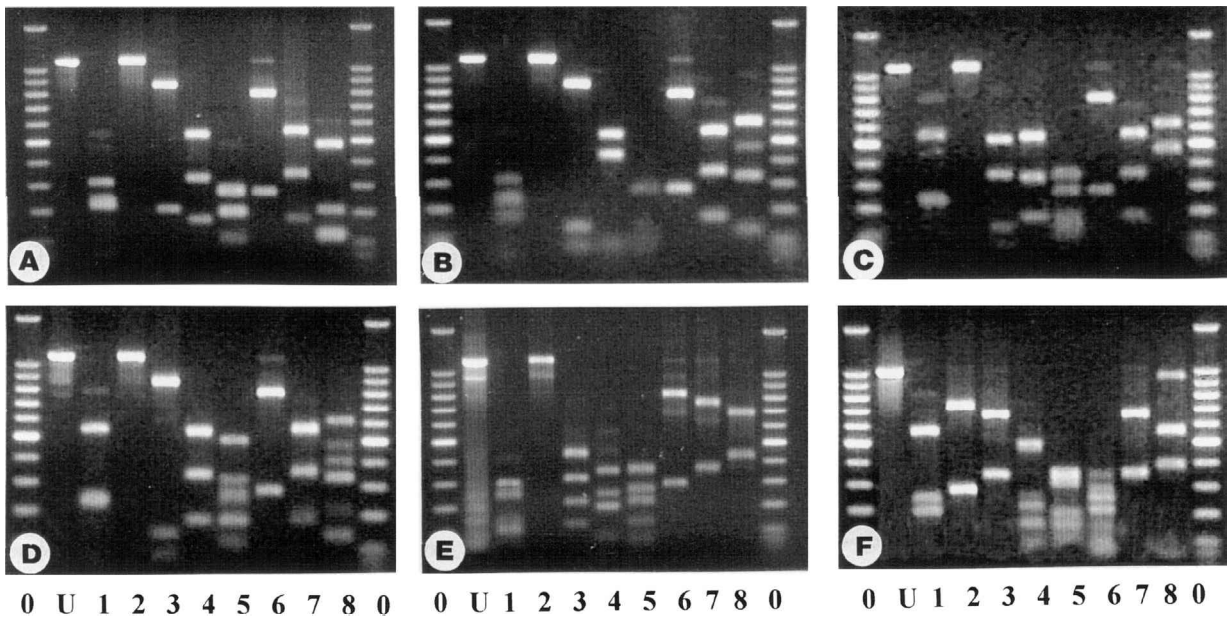

Fig. 2. *Restriction fragments of amplified ITS regions of Iranian cyst-forming nematodes. A:* Heterodera goettingiana*; B:* Heterodera *sp. 5; C:* Heterodera *sp. 6; D:* Heterodera *sp. 7; E:* H. mothi*; F:* Cactodera cacti *(0: 100 bp marker; U: unrestricted fragment; 1:* Alu*I; 2:* Ava*I; 3:* Bsh*1236I; 4:* BsuR*I; 5:* Cfo*I; 6:* Mva*I, 7:* Pst*I, or 8:* Rsa*I).*

Sequence analysis of the ITS region

The total length of the alignment including 57 sequences was 1182 bp. Sequence divergence for Heteroderidae ranged from 0.0-38.0%, for Heteroderinae from 0.0-30.2% and for Punctoderinae from 1.6-16.5%, as observed by pairwise comparisons of sequences with adjustment for missing data. Sequences of Indian and Iranian *H. avenae* populations were identical. Sequences of Georgian and Iranian *H. fici* Kirjanova, 1954 were also similar, with the exception of one (A) insertion. Sequences of other Iranian species showed some differences from those of same species collected from other regions. Except for the deletion/insertion of A, or a single or several, T after $(T)_n$ repeats in some sequences, the following nucleotide differences were detected between pairs of studied populations: three for *H. humuli* (sequence divergence: 0.3%), three for *H. glycines* (0.3%), five for *H. hordecalis* Andersson, 1975 (0.5%), six for *H. goettingiana* (0.6%), ten for *H. schachtii* (1.1%), 12 for *H. latipons* (1.3%), and 12 for *H. trifolii* (1.3%). The populations of the same species always clustered together, except for *H. trifolii* (Fig. 3). Morphological identification of several other species was not well supported by molecular data, these species being considered as *Heterodera* spp. The sequence of the unidentified Iranian species showed a high level of similarity with that of some known species. The sequence of *Heterodera* sp. 1 from the *Avenae* group differed by a single T deletion after T_n repeats and by three and one nucleotides from those of the ITS Hav1 and Hav3 clones of Chinese *H. avenae*, respectively (Zheng *et al.,* 2000). The sequences of *Heterodera* sp. 2 and *H. sacchari*, and of *Heterodera* sp. 4 and *H. cynodontis* Shahina & Maqbool, 1989 differed from each other by 22 (2.2%) and 19 (2.0%) nucleotides, respectively. *Heterodera* spp. 6 and 7 showed high similarities with *H. cruciferae*, *H. carotae* and *H. urticae* Cooper, 1955. Differences between these species ranged from five to 13 nucleotides.

We found high levels of similarity between our sequences of *H. schachtii*, *H. goettingiana* and *H. hordecalis* and sequences of these species published in Genbank (Szalanski *et al.,* 1997; Clapp *et al.,* 2000; Sui *et al.,* unpubl.). The *H. glycines* sequences used in our study differed by several deletions or insertions from known sequences (Szalanski *et al.,* 1997; Sui *et al.,* unpubl.).

Analyses of phylogenetic relationships within some cyst-forming nematodes

Twenty-one new and 36 known sequences were used in the phylogenetic analyses. Minimum evolution and max-

Table 2. *Restriction fragment sizes (bp) of rDNA-ITS regions of cyst-forming nematodes based on RFLP and sequence data.*

Species/Enzyme	*Alu*I	*Ava*I	*Bsh*1236I	*Bsu*RI	*Cfo*I	*Mva*I	*Pst*I	*Rsa*I
Heterodera avenae	(*1049*), 566, 483	1049	897, 131, 21	420, 353, 176, 52, 24, 24	745, 152, 108, 44	400, 332, 276, 22, 19	708, 341	729, 320
H. elachista	338, 325, 187, 179, 28, 17	1074	319, 220, 205, 201, 129	552, 236, 183, 79, 24	324, 248, 215, 129, 94, 64	778, 296	713, 361	618, 456
H. fici	756, 174, 105	922, 113	883, 131, 21	550, 434, 51	585, 152, 151, 147	688, 199, 76, 72	384, 336, 315	561, 474
H. filipjevi	569, 484	1053	901, 131, 21	422, 379, 176, 52, 24	749, 152, 108, 44	402, 334, 276, 41	712, 211, 130	708, *600*, 324, 21
H. glycines	341, 283, 192, 166, 44, 15	552, 365, 124	498, 380, 142, 21	524, 285, 197, 24, 11	(*732*), 421, 311, 146, 117, 46	760, 196, 85	695, 346	818, (*620*), 217, 6
H. goettingiana	304, 239, 222, 218, 56	1039	837, 202	521, 324, 170, 24	281, 267, 194, 193, 104	769, 270	533, 335, 171	470, 203, 130, 119, 117
H. hordecalis	867, 172	1039	706, 180, 131, 22	529, 410, 52, 24, 24	735, 153, 108, 43	431, 332, 276	698, 341	1018, 21
H. humuli	451, 241, 175, 171	925, 113	886, 131, 21	447, 438, 103, 50	586, 152, 152, 148 148	766, 272	385, 337, 316	748, 243, 26, 21
H. latipons	484, 343, 170, 25, 18	1040	886, 131, 23	531, 408, 77, 24	734, 108, 107, 47, 38, 6	410, 332, 278, 20	697, 343	1019, 21
H. mothi	291, 246, 145, 117, 110, 109, 69	1087	430, 316, 212, 129	350, 246, 195, 191, 81, 24	354, 258, 214, 129, 71, 56, 5	801, 286	736, 351	666, 421
H. trifolii	338, 282, 180, 166, 44, 15	549, 364, 112	495, 379, 130, 21	511, 282, 197, 24, 11	*730*, 420, 308, 119, 105, 73	756, 196, 73	691, 334	*814*, 597, 217, 205, 6
H. turcomanica	347, 327, 174, 174, 13	924, 111	885, 129, 21	525, 486, 24	585, 151, 150, 149	764, (*580*), 199, 72	699, 336	569, (*439*), 289, 150, 18, 9
Heterodera sp. 1	562, 483	1045	893, 131, 21	416, 353, 176, 52, 24, 24	741, 152, 108, 44	396, 276, 232, 100, 22, 19	704, 341	729, 316
Heterodera sp. 2	373, 311, 141, 132, 101, 26	806, 278	465, 402, 129, 67, 21	538, 269, 179, 98	402, 378, 150, 58, 54, 42	808, 276	743, 341	965, 119
Heterodera sp. 3	383, 281, 180, 166, 15	550, 363, 112	496, 351, 130, 48	510, 283, 197, 24, 11	419, 309, 146, 105, 46	756, 196, 73	1025, *691, 334*	*814*, 597, 217, 205, 6
Heterodera sp. 4	441, 381, 227	1049	899, 150	319, 247, 182, 138, 79, 36, 24, 13, 11	402, 166, 159, 98, 88, 84, 44, 8	811, 238	623, 353, 73	1028, 21
Heterodera sp. 5	304, 240, 222, 168, 48, 38, 18	1038	835, 129, 74	521, 417, 76, 24	270, 266, 144, 108, 87, 61, 59, 43	767, 271	532, 336, 170	589, 321, 128
Heterodera sp. 6	527, 239, 220, 37, 18	1041	504, 335, 129, 73	522, 325 , 170, 24	335, 268, 171, 140, 104, 23	771, 270	534, 335, 172	590, 451
Heterodera sp. 7	527, 239, 220, 37, 18	1041	839, 129, 73	522, 325, 170, 24	475, 268, 171, 104, 23	771, 270	534, 335, 172	590, (*450, 400*), 321, 130
Cactodera cacti	552, 239, 190	706, 275	644, 337	458, 214, 151, 72, 40, 24, 22	343, 301, 185, 145, 7	331, 249, 198, 128, 75	643, 338	*924,* 545, 379, 36, 21

Italics: additional fragments; ( ): additional restriction fragments with approximate sizes for some populations or cysts.

imum parsimony analyses yielded similar basic relationships within Heteroderidae (Figs 3, 4). The MP and ME trees differed with respect to relationships within *Cactodera* species and species composing clade VI and V. *Cactodera cacti* occupied a basal position in the genus. *Heterodera mothi* and *H. elachista* Ohshima, 1974 clustered with high support with other *Cyperi* group species. *Heterodera turcomanica* formed a moderate to highly supported clade with the *Humuli* group.

Discussion

Our comparison of RFLP-ITS profiles and sequences from Iranian populations of cyst-forming nematodes with published data (Orui, 1997; Szalanski *et al.,* 1997; Subbotin *et al.,* 1997, 2000a, 2001; Clapp *et al.,* 2000) confirms the presence of *H. avenae*, *H. filipjevi*, *H. glycines*, *H. hordecalis*, *H. latipons*, *H. schachtii* and *H. trifolii* in Iran. RFLP patterns and ITS sequences for *H. elachista*,

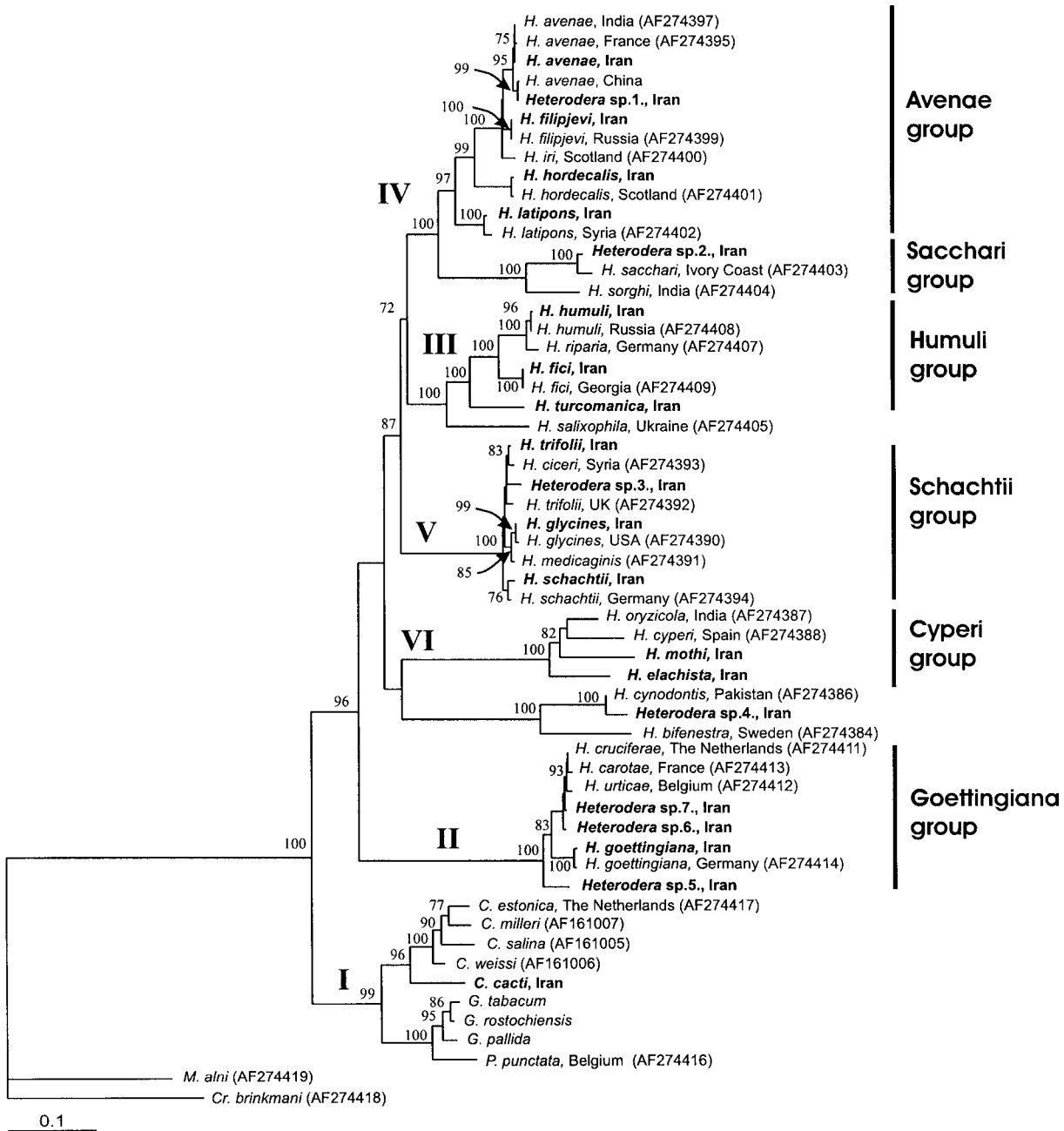

Fig. 3. *Minimum evolution (GTR + I + G model and neighbour-joining method) tree constructed from the ITS sequence alignment for 57 species of cyst-forming nematodes. Bootstrap values (more than 70%) are given in the appropriate clades. New sequences are indicated by bold.*

H. turcomanica, *H. mothi* and *C. cacti* were obtained for the first time in this study. *Heterodera humuli*, *H. goettingiana*, *H. fici*, *H. elachista*, *H. turcomanica* and *C. cacti* are recorded for the first time in Iran.

Identification of Iranian cyst-forming nematodes in this work has been made based on combined analyses of molecular (sequence similarity, autopomorphic character) results with morphological, morphometric (J2 and cysts)

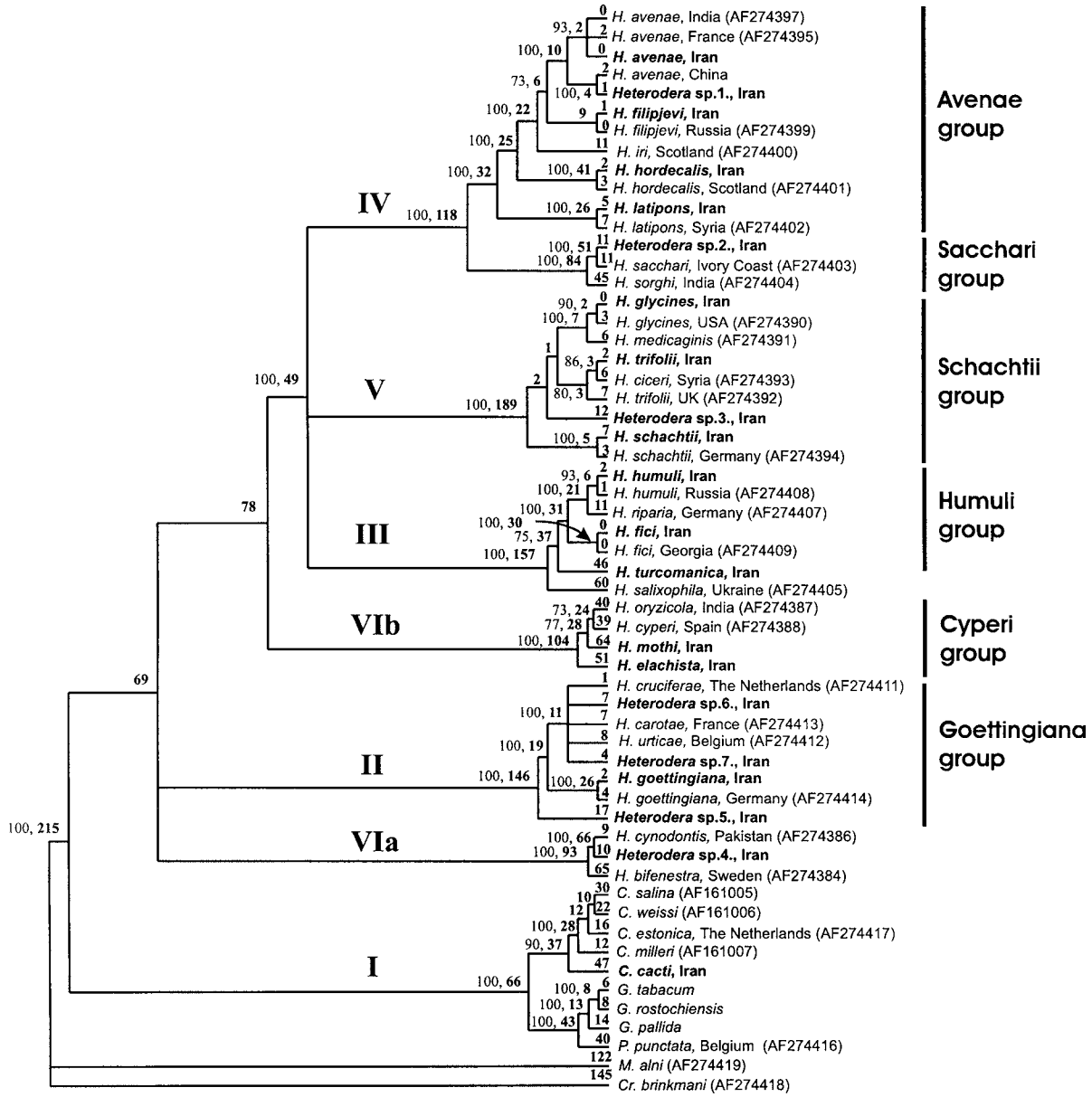

Fig. 4. *Strict consensus of 24 maximum parsimonious trees constructed from the ITS sequence alignment for 57 species of cyst-forming nematodes. Bootstrap values (more than 70%) and numbers of nucleotide changes (bold) are given in the appropriate clades. (Tree length = 2886, number of parsimony-informative characters = 681, CI = 0.5274, HI = 0.4726, RI = 0.8199, RC = 0.4324.) New sequences are indicated by bold.*

and biological (host-plant) data for each population. If the identification result of any of these approaches could be considered as indicating a significant difference with the characteristics of known species, we considered this population as *Heterodera* sp. Seven unidentified and possibly new species were recognised amongst the studied Iranian populations. Further comparative taxonomic studies, however, are required to clarify their relationships with known cyst nematodes.

Our study confirms the earlier identification in Iran of *H. filipjevi* (Sturhan, 1996; Damadzahed & Ansaripour, 2001). Although the earlier report of *H. avenae* (Barooti & Loof, 1990; Hojat-Jalali, 1991) in Iran was revealed to be *H. filipjevi* (Sturhan, 1996), our study supports the pres-

ence of *H. avenae* and provides additional information on the variability of RFLP profiles for the latter species generated by *Alu*I. This enzyme reveals heterogeneity of the ITS region among several specimens from some populations of *H. avenae.* These RFLP profiles should be considered for identification of populations of this species.

Our data further indicate the presence of another species from the *Avenae* group, *Heterodera* sp. 1, morphologically and genetically very similar to *H. pratensis* Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000 (see Gäbler *et al.,* 2000) and *H. avenae* from China. It was found on grasses in three different provinces. However, because of the presence of some minor differences in the ITS sequences we could neither attribute these populations to *H. pratensis*, nor to the Chinese *H. avenae*, because of the difference in host-plants (Subbotin *et al.*, unpubl.).

The ITS sequences and RFLP profiles of the Iranian *H. latipons* populations differ from those for the Rostov population (Ferris *et al.,* 1999; Subbotin *et al.,* 2000a) and from the sequence of the Syrian population (Subbotin *et al.,* 2001). More detailed studies should be conducted to clarify the taxonomic status of the *H. latipons* populations.

Heterodera sp. 2 was collected on *Phragmites* sp. in two Iranian provinces. These populations are morphologically similar to *H. sacchari* Luc & Merny, 1963 (finger-like projections on the underbridge, many bullae, three lateral lines, stylet knobs deeply forwardly concave and three annules in the lip region). However, morphometric study showed differences between these populations and *H. sacchari* in body, stylet, tail and hyaline part of the tail of the second stage juveniles (Tanha Maafi *et al*., unpubl.). Comparison of the ITS sequence of one population with the published sequence for *H. sacchari* (Subbotin *et al.,* 2001) revealed rather variable nucleotide substitutions between them. Therefore, we consider these populations as representing a new species.

A cyst-forming nematode population was collected from roots and soil from the rhizosphere of *Urtica dioica* L. in Mazandaran province. Comparison of its RFLP profile and the ITS sequence of this population with published data (Subbotin *et al.,* 1997, 2000a, 2001; Eroshenko *et al.,* 2001) allowed us to conclude that the species was *H. humuli.* The presence of numerous nematodes of different developmental stages on the roots and in the rhizosphere indicates that common nettle is a suitable host for this species.

Heterodera turcomanica, which was morphometrically identical to data taken from the redescription of this species made by Sturhan and Wouts (1995), was found in Ardabil province in the northern west part of country. The RFLP and ITS sequence easily distinguish this species from the morphologically similar *H. latipons*, which has a similar cyst cone structure, but lacks an underbridge. Attempts to determine the host plants failed, but the following plants were identified in the sampling area: *Setaria viridis* (L.) Beauv., *Daucus carota* L., *Thesium arvense* Horv. and *Euphorbia boissieriana* (Woronow) Prokh.

Heterodera trifolii has previously been reported from Iran in sugar beet fields (Talachian *et al.,* 1976). In the present study, a population from clover (*Trifolium repens* L.) was studied. In molecular trees it clustered with *H. ciceri* Vovlas, Greco & Di Vito, 1985. Another population collected from the rhizosphere of *Oxalis* sp. and clover was initially identified morphologically as *H. trifolii.* As it showed sequence dissimilarity with *H. trifolii* and *H. ciceri* it is herein regarded as *Heterodera* sp. 3. Phylogenetic and sequence analyses of the ITS clones obtained from the genetically and morphologically closely related species *H. trifolii*, *H. ciceri*, *H. galeopsidis*, *H. daverti* Wouts & Sturhan, 1979 and unidentified *Heterodera* populations from *Plantago* and *Rumex* failed to distinguish these species and populations and other genes should be used to discriminate this species complex (Subbotin *et al*., unpubl.).

For the differentiation of *H. elachista* and *H. oryzicola* Rao & Jayaprakesh, 1984, both infecting rice, Wouts and Baldwin (1998) proposed the shape of stylet knobs of the second stage juvenile as a key character whereas Nobbs *et al.* (1992) indicated differences in morphometrics and esterase isoenzyme profiles between these species. Comparisons of our results with the published RFLP profile of *H. oryzicola* (Subbotin *et al.*, 2000a) support the use of the enzymes *Bsu*RI, *Cfo*I, *Mva*I, *Rsa*I for separation of these species from each other. Comparison of RFLP profiles obtained for the Iranian *H. elachista* population with that from Japan (Orui, 1997) confirms their conspecific status.

Heterodera mothi was first recorded in Iran by Talachian *et al.* (1976). Plants of the genus *Cyperus*, which are hosts for this species, are dominant weeds in Iranian rice fields and the probability of joint occurrence of *H. mothi* and *H. elachista* in rice fields is therefore high. The ITS-RFLP of *Bsh*1236I and *Bsu*RI separates these species.

Heterodera sp. 4, found on the roots of *Phragmites* sp., was morphologically identified as a member of the *H. cardiolata* Kirjanova & Ivanova, 1969 complex, including *H. cardiolata*, *H. graminis* Stynes, 1971, *H. cynodontis* parasitising *Cynodon dactylon* and *H. phragmitidis* Kazachenko, 1986 parasitising *Phragmites australis* (Cav.) Trin. ex Steud. Further morphometric analysis of the J2 and cysts revealed differences between the Iranian population and these species (Tanha Maafi, unpubl.). The sequence of *Heterodera* sp. 4 was different from to that of *H. cynodontis*. Based on the molecular and morphometric results, this species can be considered as an undescribed species.

Heterodera sp. 5, morphologically identified as an unknown member of the *Goettingiana* group, shows genetic similarity with species from this group. Detailed morphological and morphometric study of the J2 and cysts revealed differences between this species and *H. goettingiana*, *H. cruciferae*, *H. carotae* and *H. urticae* (Tanha Maafi, unpubl.). Molecular analysis also suggests that this population belongs to an undescribed species.

Another member of the *Goettingiana* group, *Heterodera* sp. 6, collected from soil around the root of kiwi and peach trees, morphologically and morphometrically resembles *H. cruciferae*. However, RFLP generated by *Bsh*1236I, *Cfo*I and *Rsa*I distinguished these populations from a Dutch population of *H. cruciferae* (Subbotin *et al.*, 2000a). *Heterodera* sp. 7 found in soil samples from apple orchards was morphometrically and morphologically similar to *H. urticae*, but *Bsh*1236I and *Rsa*I digestion resulted in different patterns for these nematodes.

Phylogenetic relationships within cyst-forming nematodes based on the ITS sequences together with the influence of alignment procedures and methods of phylogenetic inference were recently analysed (Subbotin *et al.,* 2001). The phylogenetic trees obtained in the present study are in accordance with these published results. The fact that *H. turcomanica* clusters with high bootstrap support with the *Humuli* group and does not belong to the *Avenae* group is a novel conclusion from our study. It supports the opinion of Sturhan (pers. comm.) that this species is clearly not related to the *H. avenae* and *H. latipons* species complex. Present data do not support the validity of the genus *Ephippiodera* proposed by Shagalina and Krall (1981) for the species *H. turcomanica* and *H. latipons* and justify the synonymisation of *Ephippiodera* with *Heterodera* (Luc *et al.,* 1988; Sturhan & Wouts, 1995).

Nobbs *et al.* (1992) suggested that an additional species group was appropriate for *H. cyperi* Golden, Rau & Cobb, 1962, *H. elachista*, *H. oryzicola* and *H. mothi*, species characterised by the presence of bullae and a weak or absent underbridge. Subbotin *et al.* (2001), based on phylogenetic analyses of sequences from the ITS region of rDNA and morphological characters of cyst and J2, erected a new group, the *Cyperi* group, for *H. cyperi* and *H. oryzicola*. Clustering in our trees of the newly sequenced *H. elachista* and *H. mothi* with *H. oryzicola* and *H. cyperi* therefore fully justify the erection of the *Cyperi* group.

Acknowledgements

The first author is grateful for the awarding of a scholarship from the Agricultural Research, Education and Extension Organisation and Plant Pests and Diseases Research Institute, Tehran, Iran. S.A. Subbotin gratefully acknowledges financial support from a NATO Research Fellowship.

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