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Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP

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Summary – Amplified ITS region products of rDNA from 25 valid species and one unidentified species from the genus *Heterodera* and from *Meloidodera alni* were digested by 26 restriction enzymes. A combination of seven enzymes clearly separated the agriculturally most important species from each other and from their sibling species. Species specific digestion profiles of ITS regions and a table with approximate sizes of digested fragments for several identification enzymes are given. Heterogeneity of ITS regions was revealed for some cyst forming nematode species.

Résumé – ***Identification de nématodes à kystes du genre* Heterodera *(Nematoda: Heteroderidae) basée sur les RFLP du DNA ribosomal –*** Des fragments amplifiés de la région de l'ITS du rDNA de 25 espèces valides et d'une espèce non identifiée du genre *Heterodera* et de *Meloidodera alni* ont été soumis à une digestion par 26 enzymes de restriction. La combinaison de sept enzymes a permis une séparation nette des espèces les plus importantes en agriculture, tant les unes par rapport aux autres que par rapport aux espèces jumelles. Sont donnés les profils spécifiques de digestion des régions de l'ITS et un tableau regroupant les tailles approximatives des fragments digérés pour plusieurs enzymes d'identification. L'hétérogénéité des régions de l'ITS a été révélée chez quelques espèces de nématodes à kyste.

Keywords – heterogeneity, ITS regions, *Meloidodera alni*.

The genus *Heterodera* belongs to the family Heteroderidae and contains 62 species (Wouts & Baldwin, 1998). Some of them, *H. avenae*, *H. cajani*, *H. cruciferae*, *H. filipjevi*, *H. glycines*, *H. goettingiana*, *H. medicaginis*, *H. oryzicola*, and *H. schachtii*, are important agricultural pests for European and global agriculture (Baldwin & Mundo-Ocampo, 1991; Evans & Rowe, 1998). Most of the *Heterodera* species are classified into so-called 'species complexes' each grouping several sibling species. Identification of these cyst forming nematodes based on differences in morphological and morphometric characters requires a lot of skill, is time consuming and often inconclusive for individuals. However, as most of the modern plant protection measures are species specific, accurate, fast and reliable identification of nematode populations at the species and subspecies level becomes more and more important.

Developed during the last decade, DNA-based diagnostics provide an attractive solution to problems associated with traditional identification methods. Comparative analyses of coding and non-coding regions of ribosomal DNA (rDNA) became a popular tool for species and subspecies identification of plant-parasitic nematodes from many genera (Vrain *et al.*, 1992; Wendt *et al.*, 1993; Zijlstra *et al.*, 1995). RFLP analyses of ribosomal internal transcribed spacer regions (ITS) were useful for the identification of *Globodera* species parasitic to solanaceous plants (Thiéry & Mugniéry, 1996). At least three restriction site differences were identified to discriminate *Globodera pallida* and *G. rostochiensis*, the two potato cyst nematode species (Fleming & Powers, 1998; Subbotin *et al.*, 1999a). Ferris *et al.* (1993, 1994), on the basis of sequence data from the ITS region, showed that the genus *Heterodera* exhibited a rather wide range of genetic distances. However, within the *H. schachtii* group, the clover cyst nematode, *H. trifolii*, and the soybean cyst nematode, *H. glycines*, differed by only a few nucleotide substitutions. RFLP analysis of rDNA was also used for the identification of *H. cruciferae*, *H. glycines*, *H. trifolii*,

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H. schachtii, *H. goettingiana* and *H. zeae* (Szalanski *et al.*, 1997). With the same technique, Subbotin *et al.* (1997) differentiated species from the *H. humuli* group, whereas Bekal *et al.* (1997) and Subbotin *et al.* (1999b) separated species within the *H. avenae* group, and Orui (1997) and Fleming *et al.* (1998) some other cyst nematodes.

In the present paper we report on the use of rDNA RFLP in distinguishing *Heterodera* species, on the creation of species-specific digestion profiles, and on our study of the variation of RFLP patterns between populations of some species.

Materials and methods

Nematode isolates

The present study comprised 25 valid *Heterodera* species (Table 1) all identified by their morphology and morphometrics. One *Heterodera* population collected from *Cynodon dactylon* and similar to *H. cardiolata* was also included. To determine the degree of intraspecific variation some of the species were represented by several populations. The species were categorised into four groups: *H. avenae*, *H. schachtii*, *H. humuli* and *H. goettingiana* according to the criteria and classifications of Mathews (1971), Baldwin and Mundo-Ocampo (1991), and Wouts and Sturhan (1995). The *H. avenae* group was further split into two subgroups: *H. avenae sensu stricto* and *H. latipons*. The *H. schachtii* group contained a subgroup *H. schachtii s. str.* composed of *H. schachtii*, *H. trifolii*, *H. glycines*, *H. ciceri*, and *H. medicaginis*. Previously obtained RFLP data of populations from the *H. humuli* group (Subbotin *et al.*, 1997) and the *H. avenae* group (Subbotin *et al.*, 1999b) were included in the analysis. Two populations of *Meloidodera alni* were used for comparison.

Sample preparation

For each population, one to four cysts were transferred into 10 μl of double distilled water in an Eppendorf tube and crushed with a microhomogenisator. Eight μl of nematode lysis buffer (125 mM KCl, 25 mM Tris-Cl pH 8.3, 3.75 mM $MgCl_2$, 2.5 mM DTT, 1.125% Tween 20, 0.025% gelatine) and 2 μl of proteinase K (600 μg/ml) were added. The tubes were incubated at 65°C (1 h) and 95°C (10 min) consecutively.

PCR reaction

After centrifugation (1 min; 16000 *g*) 10 μl of the DNA suspension was added to the PCR reaction mixture containing 10 μl 10X *Taq* incubation buffer, 20 μl 5X Q-solution, 200 μM of each dNTP (*Taq* PCR Core Kit, Qiagen, Germany), 1.5 μM of each primer (synthesised by Life Technologies, Merelbeke, Belgium), 0.8U *Taq* Polymerase (*Taq* PCR Core Kit, Qiagen, Germany) and double distilled water to a final volume of 100 μl. Primers AB 28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) and TW 81 (5′-GTT-TCCGTAGGTGAACCTGC-3′) as described by Joyce *et al.* (1994) were used in the PCR reaction. The DNA-amplification profile carried out in a GeneE (New Brunswick Scientific, Wezembeek-Oppem, Belgium) DNA thermal cycler consisted of 4 min 94°C; 35 cycles of 1 min 94°C, 1.5 min 55°C, and 2 min 72°C; followed by a final elongation step of 10 min 72°C. After DNA amplification, 5 μl product was run on a 1% agarose gel. The remainder was stored at – 20°C.

RFLP

Five to 7 μl of each PCR product was digested with one of the following 26 restriction enzymes: *Alu*I, *Ava*I, *Bam*HI, *Bgl*I, *Bsi*ZI, *Bsu*RI, *Bsh*1236I, *Bsp*143I, *Cfo*I, *Dde*I, *Eco*RI, *Hpa*II, *Hin*dIII, *Hin*fI, *Kpn*I, *Mva*I, *Pst*I, *Pvu*II, *Rsa*I, *Sal*I, *Sfu*I, *Ssp*I, *Scr*FI, *Taq*I, *Tru*9I, and *Xba*I in the buffer stipulated by the manufacturer. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis (100V, 2.5 h), stained with ethidium bromide, visualised on a 2011 Macrovue UV transilluminator, and photographed with a Polaroid MP4+ Instant Camera System. Procedures for obtaining PCR amplified products and endonuclease digestion of these products were repeated several times to verify the results. Poorly visible fragments less than 100 bp and some weak additional restriction fragments have not been taken into account.

Results

Amplification of the rDNA-ITS regions with the present primers was successful for all species. Most species yielded a single fragment of approximately 1060 bp. However, the PCR amplified product of *H. cyperi* was near 1100 bp and that of *H. oryzicola* near 1010 bp. No PCR products were obtained in the negative control lacking DNA template.

Table 1. *Nematode species and populations used in this study.*

Species	Group or subgroup	Location	Hosts	Source	Code	Tested enzymes
H. avenae[1)] (type A)	*avenae* s. *st.*	Rinkam, Bavaria, Germany	Cereals	D. Sturhan, Germany	H1	All
H. avenae (type B)	*avenae* s. *st.*	Unknown, India	Cereals	J. Rowe, UK	H2	All
H. arenaria	*avenae* s. *st.*	Lincolnshire, England	*Ammophila arenaria*	J. Rowe, UK	H3	All
H. filipjevi[2)]	*avenae* s. *st.*	Vad, Russia	*Avena sativa*	L. Nasonova, Russia	H4	All
H. aucklandica	*avenae* s. *st.*	One Tree Hill, Auckland, New Zealand	*Microlaena stipoides*	W. Wouts, New Zealand	H5	All
H. iri	*avenae* s. *st.*	Forfar, Scotland, UK	Grasses	S.A. Subbotin, Russia	H6a	All
H. iri	*avenae* s. *st.*	Near, Belgium	Grasses	S.A. Subbotin, Russia	H6b	*Alu*I, *Bsp*1431, *Bsi*ZI, *Cfo*I, *Scr*FI, *Tru*9I
H. latipons	*latipons*	Rostov region, Russia	*Elytrigia repens*	S.A. Subbotin, Russia	H7	All
H. hordecalis	*latipons*	Montrose, Scotland, UK	Grasses	S.A. Subbotin, Russia	H8a	All
H. hordecalis	*latipons*	Unknown, Sweden	Cereals	A. Ireholm, Sweden	H8b	*Alu*I, *Bsi*ZI, *Bsp*1431, *Bsu*RI, *Cfo*I, *Dde*I, *Hpa*II, *Mva*I, *Pvu*II, *Rsa*I, *Scr*FI, *Ssp*I
H. schachtii	*schachtii* s. *st.*	Unknown, Germany	*Beta vulgaris*	D. Sturhan, Germany	H9a	All
H. schachtii	*schachtii* s. *st.*	Hem, The Netherlands	*Beta vulgaris*	B. Schoemaker, The Netherlands	H9b	*Hpa*II, *Mva*I, *Rsa*I, *Pvu*II
H. schachtii	*schachtii* s. *st.*	Unknown, Belgium	*Beta vulgaris*	M. Moens, Belgium	H9c	*Hpa*II, *Mva*I, *Rsa*I, *Pvu*II
H. trifolii	*schachtii* s. *st.*	Unknown, UK	*Trifolium* sp.	J. Rowe, UK	H10a	All
H. trifolii	*schachtii* s. *st.*	Brussegem, Belgium	*Trifolium* sp.	S.A. Subbotin, Russia	H10b	*Alu*I, *Cfo*I, *Hpa*II, *Mva*I, *Rsa*I, *Scr*FI
H. medicaginis	*schachtii* s. *st.*	Stavropol region, Russia	*Medicago sativa*	S.A. Subbotin, Russia	H11	All
H. ciceri	*schachtii* s. *st.*	Unknown, Italy	*Cicer* sp.	N. Vovlas, Italy	H12	All
H. salixophila	*schachtii*	Kherson, Ukraine	*Salix album*	S.A. Subbotin, Russia	H13a	All
H. salixophila	*schachtii*	Nieuwpoort, Belgium	*Salix* sp.	S.A. Subbotin, Russia	H13b	*Alu*I, *Bsp*1431, *Bsu*RI, *Cfo*I, *Hin*fI, *Mva*I, *Pst*I, *Rsa*I, *Taq*I
H. oryzicola	*schachtii*	Kerala, India	*Oryza sativa*	J. Rowe, UK	H14	All
H. glycines	*schachtii* s. *st.*	Arkansas, USA	*Glycine max*	R. Robbins, USA	H15	All
H. cajani	*schachtii*	Unknown, India	*Cajanus cajan*	J. Rowe, UK	H16	All
H. humuli[3)]	*humuli*	Poperinge, Belgium	*Humulus lupulus*	S.A. Subbotin, Russia	H17	All
H. riparia[4)]	*humuli*	Moscow region, Russia	*Urtica dioica*	S.A. Subbotin, Russia	H18a	All
H. riparia	*humuli*	St. Albans, UK	*Urtica* sp.	S.A. Subbotin, Russia	H18b	*Alu*I, *Cfo*I, *Pst*I, *Rsa*I, *Tru*9I
H. fici	*humuli*	Sukhumi, Georgia	*Ficus carica*	S.A. Subbotin, Russia	H19	All
H. litoralis	*humuli*	Glen Innes, Auckland, New Zealand	*Sarcocornia uinqueflora*	W. Wouts, New Zealand	H20	All
H. carotae	*goettingiana*	Sion, Wallis, Switzerland	unknown	J. Grunder, Switzerland	H21a	All
H. carotae	*goettingiana*	Créances, France	*Daucus* sp.	M. Bossis, France	H21b	*Alu*I, *Bsh*1236I, *Cfo*I, *Hpa*II, *Rsa*I *Tru*9I
H. cruciferae	*goettingiana*	Brielle, The Netherlands	unknown	B. Schoemaker, The Netherlands	H22	All
Heterodera sp.	*goettingiana*	Kherson, Ukraine	*Cynodon dactylon*	S.A. Subbotin, Russia	H23	All
H. cyperi	*goettingiana*	Unknown, Spain	*Cyperus* sp.	M. Romero, Spain	H24	All
H. goettingiana	*goettingiana*	Unknown, Germany	*Pisum* sp.	J. Rowe, UK	H25a	All
H. goettingiana	*goettingiana*	Vieille-Eglise, France	*Pisum sativum*	M. Moens, Belgium	H25b	*Alu*I, *Bsh*1236I, *Cfo*I, *Hpa*II, *Rsa*I
H. urticae	*goettingiana*	Luxembourg province, Belgium	*Urtica* sp.	S.A. Subbotin, Russia	H26a	All
H. urticae	*goettingiana*	Diksmuide, Belgium	*Urtica* sp.	S.A. Subbotin, Russia	H26b	*Alu*I, *Bsh*1236I, *Cfo*I, *Hpa*II, *Rsa*I, *Tru*9I
H. urticae	*goettingiana*	Near, Belgium	*Urtica* sp.	S.A. Subbotin, Russia	H26c	*Alu*I, *Bsh*1236I, *Cfo*I, *Hpa*II, *Rsa*I
Meloidodera alni		Luxembourg province, Belgium	*Alnus* sp.	S.A. Subbotin, Russia	M27a	All
Meloidodera alni		Moscow region, Russia	*Alnus incana*	V.N. Chizhov, Russia	M27b	*Alu*I, *Bsh*1236I, *Bsp*1431, *Bsu*RI, *Cfo*I, *Eco*RI, *Hpa*II, *Hin*dIII, *Hin*fI, *Mva*I, *Pst*I, *Rsa*I, *Taq*I

PCR product were digested by *Alu*I, *Bsh*1236I, *Bsp*143I, *Bsu*RI, *Cfo*I, *Hin*dIII, *Hin*fI, *Hpa*II, *Mva*I, *Pst*I, *Rsa*I and *Taq*I, from [1)]: eight additional populations of *H. avenae*; [2)]: seven of *H. filipjevi* (Subbotin *et al*., 1999b) and [3)]: two of *H. humuli*; [4)]: four of *H. riparia* (Subbotin *et al*., 1997).

Digestion with 23 out of the 26 enzymes gave RFLPs for all species studied; *Bam*HI, *Hin*dIII, and *Kpn*I did not restrict any of the amplified products. No single enzyme could distinguish all cyst forming species in this study, although some yielded much more taxonomic information than others (Table 2). For example, *Cfo*I (Fig. 1G) yielded 16 polymorphic patterns distinguishing 12 out of the 26 species. *Alu*I (Fig. 1A), *Bsu*RI (Fig. 1F), *Bsh*1236I (Fig. 1C), and *Scr*FI (Fig. 2F) each produced 15 polymorphic patterns and distinguished nine, ten, 11, and nine species, respectively. The combination of the patterns obtained by seven individual enzymes allowed differentiation of most species under study (Table 3). In some cases the sum of restricted fragments length was less than 1060 bp. This could be due to production of several fragments with similar sizes or/and of fragments smaller that 100 bp which were poorly visible on the agarose gels. Sometimes the sum of restricted fragment lengths was more than the length of the unrestricted amplified product. Repeated digestion with an extended digestion period suggested heterogeneity of ITS regions was present in the genome of these species.

RFLP of ITS regions allowed clear differentiation of most agriculturally important cyst forming nematode species from each other and from their sibling species. Some restriction enzymes produced a RFLP pattern specific for a species group. For example the patterns obtained after restriction with *Alu*I (Fig. 1A), *Bsh*1236I (Fig. 1C), *Bsu*RI (Fig. 1F), *Dde*I (Fig. 1H), *Hin*fI (data not shown) and *Eco*RI (Fig. 2A) distinguished the *H. schachtii s. str.* group from the other groups, whereas *Eco*RI restricted only ITS regions of species from this group. *Bgl*I (data not shown), *Bsi*ZI (Fig. 1D), *Bsp*143I (Fig. 1E), *Bsu*RI (Fig. 1F), and *Pst*I (Fig. 2D) separated the *H. goettingiana* group species having juveniles with four incisures (*H. carotae*, *H. cruciferae*, *H. goettingiana* and *H. urticae*) from other species. Some enzymes proved to be of little use for identification of larger numbers of the species but were extremely adequate for individual species. *Ssp*I (data not shown), for example, only restricted PCR products from *H. latipons* and *M. alni*, whereas the ITS from *Heterodera* sp. was only cut by *Xba*I (data not shown). *Bsi*ZI (Fig. 1D) and *Dde*I (Fig. 1H) restricted the ITS region of all species, except *H. litoralis.*

As we observed earlier (Subbotin *et al.*, 1997), European populations of *H. avenae* (type A) were easily separated from other species with *Alu*I (Fig. 1A) which digested the ITS regions of all studied nematodes, except those of *H. arenaria.* No enzymes distinguished European populations of *H. avenae* from *H. arenaria. H. filipjevi* was easily distinguished from all species by *Pst*I (Fig. 2D), *Bsu*RI (Fig. 1F) and *Taq*I (Fig. 2H), which produced unique RFLP patterns for this species. *H. aucklandica* was separated by *Cfo*I (Fig. 1G) from other species. *Bsh*1236I (Fig. 1C), *Cfo*I (Fig. 1G) and *Scr*FI (Fig. 2F) produced unique patterns for *H. iri. H. latipons* was separated from the others by *Alu*I (Fig. 1A), *Bsh*1236I (Fig. 1C), *Bsu*RI (Fig. 1F), *Cfo*I (Fig. 1G), *Dde*I (Fig. 1H), *Pvu*II, *Rsa*I (Fig. 2E), and *Ssp*I. *H. hordecalis* was distinguished by *Bsh*1236I (Fig. 1C), *Bsu*RI (Fig. 1F), *Mva*I (Fig. 2C), and *Scr*FI (Fig. 2F).

*Alu*I, *Ava*I, *Cfo*I, *Hpa*II, *Mva*I, *Rsa*I and *Scr*FI separated the closely related and morphologically poorly distinguished species from the *H. schachtii s. str.* group (*H. schachtii*, *H. glycines*, *H. trifolii*, *H. medicaginis* and *H. ciceri*) from each other and all other species. *Mva*I (Fig. 2C), *Pvu*II, *Rsa*I (Figs 2E, 3) and *Scr*FI (Fig. 2F) produced a specific pattern for *H. schachtii*, which separated this species from others. Restrictions with *Mva*I, *Rsa*I, *Hpa*II, or *Pvu*II did not show intraspecific variation in restriction patterns between the three *H. schachtii* populations under study (Fig. 3). Totalling the fragment sizes produced by *Mva*I (Figs 2C, 3), *Rsa*I (Figs 2E, 3), *Pvu*II (Fig. 3), or *Scr*FI (Fig. 2F) yielded a sum of more than 1060 bp, indicating a heterogeneity of ITS regions present in the genome of *H. schachtii* populations. *H. glycines*, another member of the *H. schachtii s. str.* group, was distinguished within this group by *Ava*I, which produced a pattern identical to that for *H. cajani* (Fig. 1B). *H. cajani* was easily separated from others by many enzymes: *Alu*I (Fig. 1A), *Bsh*1236I (Fig. 1C), *Bsi*ZI (Fig. 1D), *Bsu*RI (Fig. 1F), *Cfo*I (Fig. 1G), *Dde*I (Fig. 1H), *Hin*fI, *Hpa*II (Fig. 2B), *Scr*FI (Fig. 2F), *Taq*I (Fig. 2H) and *Tru*9I. RFLP patterns produced by *Cfo*I (Fig. 1G) separated *H. ciceri* from others. Heterogeneity of the ITS region was observed for *H. ciceri* with *Alu*I (Fig. 1A), *Cfo*I (Fig. 1G), *Rsa*I (Fig. 2E). Combinations of at least two restriction enzymes separated other species from this *H. schachtii s. str.* group. For example *Rsa*I (Fig. 2E) distinguished *H. trifolii* and *H. ciceri* from other species, whilst *Cfo*I (Fig. 1G) separated these two species. *Rsa*I (Fig. 2E) demonstrated heterogeneity of ITS regions in two populations of *H. trifolii*, and *Alu*I in one population of this species. *H. medicaginis* was distinguished from other species by a combination of three enzymes: *Hpa*II (Fig. 2B), *Mva*I (Fig. 2C), and *Ava*I (Fig. 1B; Table 3). *H. salixophila* belonging to the *H. schachtii* group, was separated from all species studied by *Bsh*1236I (Fig. 1C),

Table 2. *Number of different RFLP profiles*[1)] *yielded by a single enzyme digestion of the ITS regions of cyst nematodes.*

Species	*Alu*I	*Ava*I	*Bgl*I	*Bsh*1236I	*Bsi*ZI	*Bsp*143I	*Bsu*RI	*Cfo*I	*Dde*I	*Eco*RI	*Hin*fI	*Hpa*II	*Mva*I	*Pst*I	*Pvu*II	*Rsa*I	*Sal*I	*Scr*FI	*Sfu*I	*Ssp*I	*Taq*I	*Tru*9I	*Xba*I
H. avenae (type A)	1*	1	1	1/1a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H. avenae (type B)	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1
H. arenaria	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H. filipjevi	2	1	1	1	1	1	2	1	1	1	2	1	1	2	1	2	1	1	1	1	2	2	1
H. aucklandica	2	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	1	1	1	3	1	1
H. iri	2	1	1	2	2	2	1	3	1	1	2	2	2	1	1	2	1	2	1	1	3	2	1
H. latipons	3	1	1	3	3	2	3	4	2	1	2	3	2	1	2	3	1	3	1	2	3	3	1
H. hordecalis	4	1	1	4	3	1	4	1	1	1	2	3	3	1	1	1	1	4	1	1	4	1	1
H. schachtii	5	2	1	5	4	1	5	5	3	2	3	4	4	1	1a	4a	1	5a	1	1	3	1	1
H. trifolii	5/5a	2	1	5	4	1	5	5	3	2	3	2	5	1	3	4b	1	6	1	1	3	1	1
H. medicaginis	5	2	1	5	4	1	5	5	3	2	3	4	5	1	3	4	1	5	1	1	3	1	1
H. ciceri	5a	2	1	5	4	1	5	5a	3	2	3	2	5	1	3	4b	1	6	1	1	3	1	1
H. salixophila	2	3	1	6	5	1	6	6	4	1	4	5	1	1	1	5/7	1	3	2	1	5	4	1
H. oryzicola	6	1	1	7	6	3	7	7	5	1	5	5	6	3	4	6	1	7	3	1	6	5	1
H. glycines	5	4	1	5	4	1	5	5	3	2	3	4	5	1	3	4	1	5	1	1	3	1	1
H. cajani	7	4	1	8	7	1	8	8	6	1	6	6	7	1	3	7	1	8	2	1	7	6	1
H. humuli	8	3	1	1	8	1	9	9	7	1	7	7	7	4	5	8	1	9	1	1	4	7	1
H. riparia	9	3	1	1	8	1	9	10	7	1	7	7	7	1	5	8	1	9	1	1	4	2	1
H. fici	10	3	1	1	9	1	10	9	7	1	7	8	8	4	3	9	1	10	1	1	4	2	1
H. litoralis	4	3	1	9	10	1	11	11	8	1	7	7	9	4	3	10	1	11	1	1	4	2	1
H. carotae	11	1	2	10	11	4	12	12/12a	9	1	7	2	7	5	1	11	1	12	1	1	4	8a	1
H. cruciferae	11	1	2	10	11	4	12	12	9	1	7	2	7	5	1	11	1	12	1	1	4	8a	1
Heterodera sp.	12	1	3	11	12	5	13	13	10	1	3	9	10	6	1	7	2	13	1	1	8	9	2
H. cyperi	13	1	1	12	13	6	14	14	11	1	8	10	11	7	6	12	3	14	4	1	9	10	1
H. goettingiana	14	1	2	13	11	4	12	15	12	1	7	2	7	5	1	13	1	12	1	1	10	11	1
H. urticae	11	1	2	14	11	4	12	12	9	1	7	3	7	5	1	11/11a	1	12	1	1	4	8	1
Meloidodera alni	15	1	1	15	14	7	15	16	13	1	9	11	12/12a	1	1	14	1	15	1	3	4	3	1

[1)]: numbers represent identical restriction patterns; patterns with a or b have additional fragments; patterns divided by / differed between populations of a single nematode species.

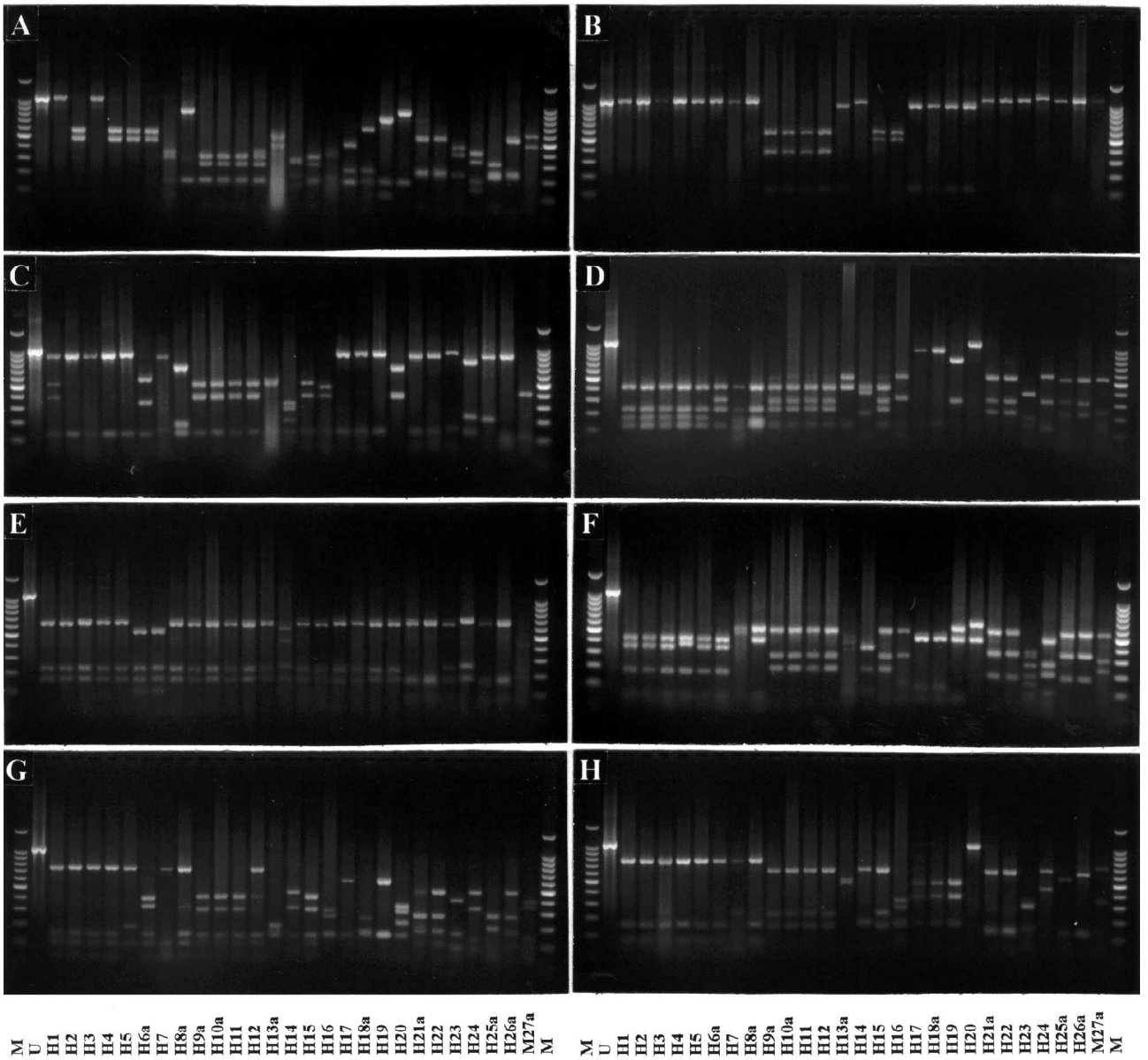

Fig. 1. *Restriction fragments of amplified ITS regions of cyst forming nematodes. A:* Alu*I; B:* Ava*I; C:* Bsh*1236I; D:* Bsi*ZI; E:* Bsp*143I; F:* Bsu*RI; G:* Cfo*I; H:* Dde*I. (For species code see Table 1; lanes U: unrestricted PCR product, M: 100 bp DNA ladder).*

*Bsi*ZI (Fig. 1D), *Bsu*RI (Fig. 1F), *Cfo*I (Fig. 1C), *Dde*I (Fig. 1H), *Hin*fI, *Taq*I (Fig. 2H), and *Tru*9I.

H. humuli, *H. riparia* and *H. fici* were differentiated from other species and from each other by *Alu*I (Fig. 1A). Three other enzymes *Cfo*I (Fig. 1G), *Pst*I (Fig. 2D) and *Tru*9I (Fig. 4) separated *H. humuli* from its sibling species *H. riparia. H. litoralis* was distinguished by RFLP generated by *Bsh*1236I (Fig. 1C), *Bsi*ZI (Fig. 1D), *Bsu*RI (Fig. 1F), *Cfo*I (Fig. 1G), *Dde*I (Fig. 1H), *Mva*I (Fig. 2C), *Rsa*I (Fig. 2E), and *Scr*FI (Fig. 2F).

Inside the *H. goettingiana* group species were separated by several enzymes. *Bsh*1236I (Fig. 1C) differentiated all species, except *H. carotae* and *H. cruciferae.* None of the enzymes separated these two species from each other. *H. goettingiana* was distinguished from other species by *Alu*I (Fig. 1A), *Bsh*1236I (Fig. 1C), *Cfo*I (Fig. 1G), *Dde*I

Table 3. *Approximate sizes of restriction fragments of rDNA ITS regions for cyst forming nematodes.*

Species	*Alu*I	*Ava*I	*Bsh*1236I	*Bsu*RI	*Cfo*I	*Mva*I	*Rsa*I
H. avenae (type A)	1060	1060	880, (*500*, *380*), 140	420, 360, 180, 50	750, 160, 110	400, 330, 290	1040
H. avenae (type B)	560, 500	1060	880, 140	420, 360, 180, 50	750, 160, 110	400, 330, 290	720, 320
H. arenaria	1060	1060	880, 140	420, 360, 180, 50	750, 160, 110	400, 330, 290	1040
H. filipjevi	560, 500	1060	880, 140	435, 370, 180, 50	750, 160, 110	400, 330, 290	720, 320
H. aucklandica	560, 500	1060	880, 140	420, 360, 180, 50	750, 200, 110	400, 330, 290	720, 320
H. iri	560, 500	1060	540, 340, 140	420, 360, 180, 50	410, 340, 160, 110	420, 330, 290	720, 320
H. latipons	420, 350, 180	1060	880, 160	530, 510	750, 110	420, 330, 290	900, 160
H. hordecalis	880, 180	1060	700, 180, 140	530, 435, 50	750, 160, 110	440, 330, 290	1040
H. schachtii	350, 280, 180, 170	560, 370, 130	520, 380, 140	530, 300, 210	430, 320, 150, 110	*1010*, *840*, 760, *630*, 220, *150*, 80	830, *460*, *380*, 230
H. trifolii	(*390*), 350, 280, 180, 170	560, 370, 130	520, 380, 140	530, 300, 210	430, 320, 150, 110	760, 220, 80	830, 600, 230
H. medicaginis	350, 280, 180, 170	560, 370, 130	520, 380, 140	530, 300, 210	430, 320, 150, 110	760, 220, 80	830, 230
H. ciceri	*390*, 350, 280, 180, 170	560, 370, 130	500, 380, 140	530, 300, 210	750, *430*, *320*, 150, 110	760, 220, 80	830, 600, 230
H. salixophila	560, 500	930, 130	530	450, 435, 80	200, 160, 150	400, 330, 290	770, 290 [1060]
H. oryzicola	330, 295, 200, 150	1010	320, 270, 200, 130	360, 210, 80	470, 330, 150, 60	470, 300, 210	870, 90
H. glycines	350, 280, 180, 170	560, 510	520, 380, 140	530, 300, 210	430, 320, 150, 110	760, 220, 80	830, 230
H. cajani	360, 200, 180, 140, 100	560, 510	470, 380, 140	530, 310, 120	320, 270, 160, 150	760, 300	1060
H. humuli	460, 250, 180, 170	930, 130	880, 140	450, 110, 50	600, 160, 150	760, 300	760, 260
H. riparia	630, 250, 180	930, 130	880, 140	450, 110, 50	250, 180, 170, 160, 150	760, 300	760, 260
H. fici	780, 180, 100	930, 130	880, 140	560, 450, 50	600, 160, 150	690, 200, 80	560, 480
H. litoralis	880, 180	930, 130	670, 390	610, 450	350, 310, 250, 150	560, 310, 290, 240	800, 260
H. carotae	530, 250, 230	1060	830, 140, 70	530, 330, 170	480, 270, (*220*), 170, 110	760, 300	600, 330, 130
H. cruciferae	530, 250, 230	1060	830, 140, 70	530, 330, 170	480, 270, 170, 110	760, 300	600, 330, 130
Heterodera sp.	450, 400, 240	1060	900, 160	360, 250, 180, 150, 90	410, 160, 110, 80	800, 260	1060
H. cyperi	410, 360, 200, 160	1100	710, 240, 150	450, 250, 200, 100, 50	480, 330, 130, 100	780, 320	950, 150
H. goettingiana	350, 250, 230	1060	830, 230	530, 330, 170	280, 270, 190, 110	760, 300	480, 210, 130, 120
H. urticae	530, 250, 230	1060	830, 120	530, 330, 170	480, 270, 170, 110	760, 300	600, (*460*), 330, 130
Meloidodera alni	590, 470	1060	410, 140	530, 300, 230	400, 350, 160, 100	(*780*), 570, 210	880, 180

Italic letters: additional fragments; ( ): additional restriction fragments for some populations; [ ] restriction for some populations only.

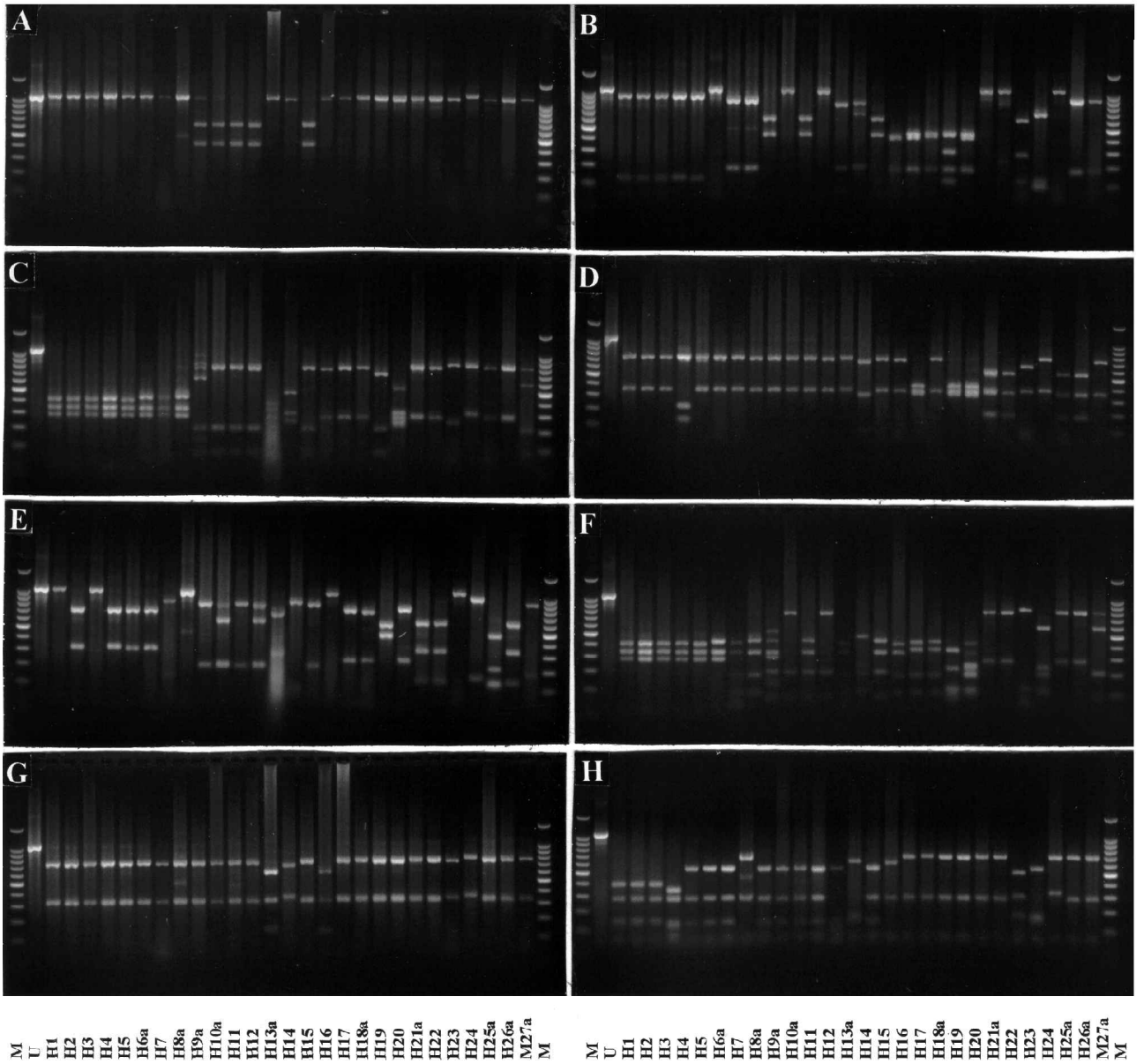

Fig. 2. *Restriction fragments of amplified ITS regions of cyst forming nematodes. A:* Eco*RI; B:* Hpa*II; C:* Mva*I; D:* Pst*I; E:* Rsa*I; F:* Scr*FI; G:* Sfu*I; H:* Taq*I. (For species code see Table 1; lanes U: unrestricted PCR product, M: 100 bp DNA ladder).*

(Fig. 1H), *Rsa*I (Fig. 2E), *Taq*I (Fig. 2H), and *Tru*9I (Fig. 4). *H. urticae*, parasite of nettle in many West European countries, differed from *H. carotae* and *H. cruciferae* by RFLP patterns produced by three enzymes: *Bsh*1236I (Fig. 1C), *Hpa*II (Fig. 2B), and *Tru*9I (Fig. 4). *Hpa*II clearly differentiated *H. urticae* from these two species. It did not digest the PCR product of *H. carotae* and *H. cruciferae*, but did restrict the ITS regions of *H. urticae* producing two fragments (870 and 190 bp).

PCR amplified product obtained from *H. oryzicola* and *H. cyperi* differed from other species by its size. The digestion with different enzymes usually produced unique RFLP profiles for these two species (Figs 1, 2, 4; Table 3). The unidentified *Heterodera* sp. from *Cynodon dactylon*

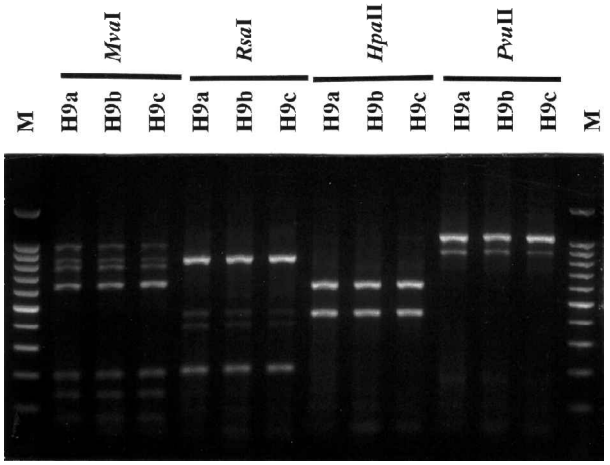

Fig. 3. *Restriction fragments of amplified ITS regions of sugar beet cyst nematode* Heterodera schachtii. *(For species code see Table 1; M: 100 bp DNA ladder).*

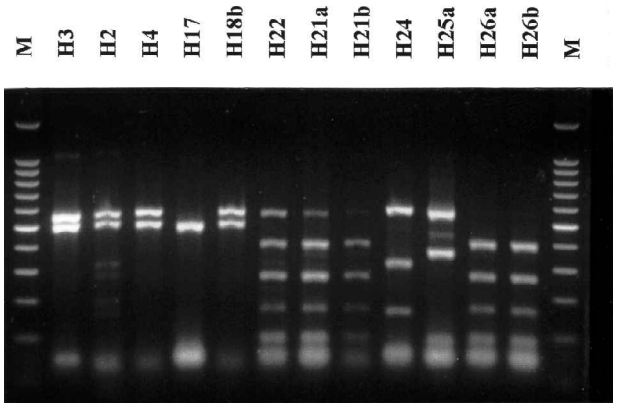

Fig. 4. *Restriction fragments of amplified ITS regions of cyst forming nematodes digested by* Tru9I. *(For species code see Table 1; M: 100 bp DNA ladder).*

was also distinguished from all other species by several enzymes (Table 2).

Intraspecific variation was revealed within some species. *Alu*I (Fig. 1A) and *Rsa*I (Fig. 2E) distinguished European populations of *H. avenae* (type A) from the Indian population (type B). These enzymes partly digested ITS regions of three French populations indicating the heterogeneity in rDNA (Subbotin *et al.*, 1997). *Bsh*1236I (Fig. 1C) produced additional restriction fragments for the Rinkam (Bavaria) population of *H. avenae*. These fragments, however, were not found in other *H. avenae* populations (Subbotin *et al.*, 1999b). The two populations of *H. salixophila* differed by their *Rsa*I restriction patterns. This enzyme restricted the ITS regions of the Belgian population, but not that of the Ukrainian one (Table 3). Two populations of *M. alni* differed by the *Mva*I restriction pattern; the enzyme partly digested the ITS regions of the Belgian population indicating a heterogeneity in this region of the species (Table 3). Intraspecific variation in RFLP patterns was not revealed within *H. humuli*, *H. riparia* (Subbotin *et al.*, 1997), *H. filipjevi* (Subbotin *et al.*, 1999b), *H. iri*, *H. hordecalis*, *H. goettingiana* and *H. schachtii.*

Discussion

The present work confirms that rDNA-RFLP allow clear differentiation of agriculturally important cyst forming nematode species from each other and from their sibling species. RFLP produced by only seven enzymes separated 21 species of cyst forming nematodes (Table 3).

Nematode species in the same genus are considered to have similar sized amplified products of the spacer regions, whereas size variation between genera is accepted to be common (Powers, 1996, 1997). Our study demonstrated that the size of amplified ITS products is rather stable within the genus *Heterodera.* Only for *H. cyperi* and *H. oryzicola* did we obtain a deviating length so that these species can easily be separated from other cyst forming nematode species. Variation in the size of the ITS regions has been reported for species from the genera *Aphelenchoides* (Ibrahim *et al.*, 1994), *Meloidogyne* (I. De Ley, pers. comm.), and *Pratylenchus* (Orui, 1996; L. Waeyenberge, pers. comm.).

In the present study we used primers amplifying an rDNA product including the ITS1, ITS2 regions and the 5.8S gene plus flanking areas of the 18S and 28S genes. Primers used for amplification of nematode ribosomal DNA were recently reviewed (Fleming & Powers, 1998; Powers & Fleming, 1998). For identification of some cyst nematodes species Szalanski *et al.* (1997) and Fleming *et al.* (1998) used only the ITS1 region. Although Ferris *et al.* (1993, 1994) and Blok *et al.* (1998), all studying cyst forming nematodes, reported more variation in the ITS1 region than in the ITS2, the use of both these regions looks to be more promising, because after its digestion the larger size of the PCR amplified fragment yields more informative patterns. Moreover, some restriction sites useful for identification of several agricultural important species are situated in the ITS2 region (unpubl.).

ITS regions are considered to be rather conservative and so not appropriate for separation of populations belonging to same species. However, intraspecific variations in these regions have been revealed within plant parasitic

nematode populations of *H. zeae* and *H. trifolii* (Szalanski *et al.*, 1997), *H. avenae* (Bekal *et al.*, 1997; Subbotin *et al.*, 1999b), and *G. pallida* (Blok *et al.*, 1998). Differences in RFLP between populations can be presented as the existence of differences in restriction sites in ITS sequence and/or the appearance of additional ITS haplotypes with different sequences. Heterogeneity in ITS regions, or presence of several ITS haplotypes within a single genome were found during the present work for populations of *H. avenae*, *H. ciceri*, *H. carotae*, *H. cruciferae*, *H. schachtii*, *H. trifolii*, *H. urticae*, *Heterodera* sp. and *M. alni*. ITS heterogeneity was reported for *Meloidogyne* (Zijlstra *et al.*, 1995), *Belonolaimus* (Cherry *et al.*, 1997), *Radopholus* (L. Waeyenberge, pers. comm.) and for several cyst forming nematode species: *H. zeae* (Szalanski *et al.*, 1997), *G. pallida* (Blok *et al.*, 1998) and *G. rostochiensis* (Subbotin *et al.*, unpubl.) and, perhaps, is widely distributed among nematodes. The mechanism supporting such a mixture of haplotypes in one genome is not clear.

Relationships between such changes in rDNA and changes in the morphological and biological peculiarities of populations has not been studied in detail yet. The Indian population of *H. avenae* (ITS type B) used in our study differed from most European populations by RFLP obtained by two restriction enzymes and can also be separated by morphometrics. Several French populations belonging to different groups of pathotypes contain a mixture of two ITS types (A+ B) (Subbotin *et al.*, 1999b). Molecular polymorphism has frequently been observed between geographically isolated populations. Szalanski *et al.* (1997) reported differences in *H. trifolii* from the USA and Australia and in *H. zeae* from the USA and India, as opportunities for gene exchange between these populations were restricted. Differences in ITS sequences were observed in two populations of *H. latipons*, one from Rostov region, Russia and one from Gilat, Israel (V. Ferris *et al.*, unpubl.). When using several restriction enzymes, the senior author was able to separate the Rostov population from a Syrian *H. latipons* population. For example, *Rsa*I and *Pvu*II did not digest the ITS regions of the Syrian population (Subbotin *et al.*, unpubl.). Bekal *et al.* (1997) also reported that *Rsa*I did not restrict ITS regions of populations from Israel and Syria. It is obvious that the taxonomic status the Rostov population of *H. latipons* needs clarification.

The *Heterodera* sp. sampled for the present study from the Ukraine belongs to the *H. cardiolata* complex. This complex contains the morphologically closely related species *H. cardiolata*, *H. graminis*, and *H. cynodontis* all infecting *Cynodon dactylon*, and found in various regions of the world: Australia, South Africa, Fiji Islands, Trinidad, India, Tadzhikistan, Uzbekistan and Pakistan (Kirjanova & Ivanova, 1969; Stynes, 1971; Luc, 1986; Shahina & Maqbool, 1993). Perhaps some of these species can be considered as synonyms or as geographical subspecies of *H. cardiolata*. However, we found that two (*Alu*I and *Bsu*RI) from the nine studied enzymes produced different RFLP profiles for the *Heterodera* sp. from the Ukraine and *H. cynodontis* from Pakistan (unpubl.). More detailed morphological and molecular studies of this species complex are needed for understanding the relationships between the populations and supporting the validity of some of these species.

We did not find restriction enzymes that enable the separation of *H. avenae* from *H. arenaria*, and *H. carotae* from *H. cruciferae*, proving that these species are closely related. These species can only be distinguished from each other by minor morphometrical and morphological characteristics (Baldwin & Mundo-Ocampo, 1991; Robinson *et al.*, 1996).

The amplification and analysis of the ITS has a lot of advantages. The rapidity to obtain profiles and the clarity of the results allow identification of most species very easily. This technique is relatively easy to operate and not expensive. It is particularly suited for determining the identity of quarantine nematodes, where it is often necessary to perform identification on very few individual specimens but where an incorrect identification can have major economical implications (Vrain & McNamara, 1994; Szalanski *et al.*, 1997). However, in order to be able to apply the techniques as a routine in quarantine inspections or plant protection services, it is necessary to make a catalogue of RFLP in the ITS region of widely distributed nematode species.

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