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Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs

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Summary – Canonical discriminant analysis of four morphometric characters of juveniles and restriction enzymes analysis of ribosomal DNA sequences were used to distinguish *Heterodera arenaria*, *H. aucklandica*, *H. avenae*, *H. filipjevi*, *H. hordecalis*, *H. iri*, *H. latipons*, *H. litoralis*, *H. schachtii* and an undescribed species from grasslands. The results of unweighted pair group cluster analysis showed that *H. avenae* populations formed three groups and *H. filipjevi* two groups at the 80% level of similarity. Intraspecific polymorphism was revealed by rDNA-RFLP studies and two types of ITS regions within *H. avenae* populations can be distinguished. The pattern of restriction bands obtained with *Bsu*RI, *Pst*I and *Taq*I clearly distinguished populations of *H. filipjevi* from other species of the *H. avenae* group. Further enzymes and their combinations distinguished the other species. There are no enzymes which differentiate European populations of *H. avenae* from *H. arenaria.* Morphometrics, restriction endonuclease cleavage maps of ITS regions and a dendrogram of putative phylogenetic relations of several cyst-forming nematode species are given.

Résumé – ***Identification des espèces du groupe*** **Heterodera avenae** ***par la morphometrie et les rDNA-RFLP*** **–** L'analyse canonique discriminante sur quatre caractères morphométriques des juvéniles et l'analyse des enzymes de restriction de l'ADN ribosomal ont été utilisées pour identifier *Heterodera arenaria*, *H. aucklandica*, *H. avenae*, *H. filipjevi*, *H. hordecalis*, *H. iri*, *H. latipons*, *H. litoralis*, *H. schachtii* et une nouvelle espèce originaire de prairies. Les résultats de l'analyse UPGMA ont montré que les populations d'*H. avenae* formaient trois groupes et celles d'*H. filipjevi* deux groupes à un niveau de similarité de 80%. Le polymorphisme intraspécifique a été révélé par des études de rDNA-RFLP et deux types de régions de l'ITS peuvent être mis en évidence dans les populations d'*H. avenae.* Les modèles de bandes de restriction obtenus avec *Bsu*RI, *Pst*I et *Taq*I ont identifié clairement les populations d'*H. filipjevi* des autres espèces du groupe *H. avenae.* D'autres enzymes et leurs combinaisons ont identifié les autres espèces. Aucun enzyme n'a différencié les populations européennes d'*H. avenae* de *H. arenaria.* Les caractères morphométriques, les cartes de clivage des régions des ITS par l'endonucléase de restriction et un dendogramme des relations phylogénétiques supposées sont donnés.

Keywords: canonical discriminant analysis, cyst nematodes, *Heterodera avenae*, *H. filipjevi*, identification, ITS-rDNA, morphometrics, RFLP.

The *Heterodera avenae* group *sensu lato* presently contains eleven valid species: *H. arenaria*, *H. aucklandica*, *H. avenae*, *H. bifenestra*, *H. filipjevi*, *H. hordecalis*, *H. iri*, *H. latipons*, *H. mani*, *H. spinicauda* and *H. turcomanica* and several undescribed species (Sturhan & Wouts, 1995; Wouts & Sturhan, 1995; Robinson *et al.*, 1996). Comparative morphology and morphometrics (Stone & Hill, 1982; Valdeolivas & Romero, 1990; Subbotin *et al.*, 1996), protein electrophoresis (Rumpenhorst, 1985; Ferris *et al.*, 1989, 1994; Bossis & Rivoal, 1996; Sturhan & Rumpenhorst, 1996; Subbotin *et al.*, 1996) and random amplified polymorphic DNA (López-Braña *et al.*, 1996) allow separation of several species.

The analysis of coding and non-coding regions of ribosomal DNA has become a popular tool for species and subspecies identification of several nematode species (Vrain *et al.*, 1992; Wendt *et al.*, 1993; Powers *et al.,* 1997). ITS regions were found to be useful to differentiate species within the *H. avenae* group (Ferris *et al.*, 1994; Bekal *et al.*, 1997). This article presents a comparative analysis of the morphometrics of juveniles and cysts and of the ITS regions, including the 5.8S rDNA gene plus flanking areas of the 18S and 26S genes, of several populations of seven species of the *H. avenae* group.

Materials and methods

Nematode species and populations

Twenty-four populations belonging to seven valid species of the *H. avenae* group and three populations of unknown species from the same group were studied (Table 1). To these were added one population of *H. schachtii* and one of *H. litoralis*. Cysts were isolated from soil by routine methods of sieving and flotation. Some cyst populations were allowed to dry, others were used freshly extracted from the soil. A minority was transferred to slides. Second-stage juveniles (J2s) were isolated from cysts.

Light microscopy

J2s were killed by gentle heat, fixed in TAF and embedded in glycerol as permanent slides following Seinhorst (1959). Cyst vulval cones were mounted in glycerine-gelatine. The specimens were examined and measured with a JENAVAL light microscope. Four morphometrical characters of the J2s (body length, stylet length, tail length and hyaline part of tail length) and two J2 morphological characters (stylet knob shape and shape of tail terminus) along with four morphometrical characters of the cysts (fenestra length, mean semifenestral width, vulval bridge width and vulval slit length) were studied. All these characters are considered taxonomically important for this group (Wouts & Weischer, 1977; Sturhan, 1982; Wouts & Sturhan, 1995).

Sample preparation for molecular studies

A single cyst was placed in 10 μl of double distilled water on a glass slide and crushed under a dissecting microscope. J2s and eggs were transferred into a sterile Eppendorf tube containing 8 μl lysis buffer (500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatin) and homogenised. Two μl of proteinase K (600 μg/ml) were then added. After freezing (– 80°C, at least 10 min) the tubes were incubated at 65°C for 1 h and then at 95°C for 10 min.

PCR reaction

After centrifugation (1 min; 16 000 g) 10 μl of the DNA suspension was added to the PCR reaction mixture containing 10 μl 10× *Taq* incubation buffer with 25 mM $MgCl_2$ (Appligene, B&L Systems, Boechout, Belgium); 4 μl *dNTP*-mixture 5 mM each (Eurogentec, Seraing, Belgium), 1 μl (1.5 M) of each primer (synthesised by Eurogentec), 0.8U *Taq* Polymerase (Appligene, B&L Systems) and double distilled water to a final volume of 100 μl. Primers AB 28 (5′ATATGCTTAAGTTCAGCGGGT 3′) and TW 81 (5′GTTTCCGTAGGTGAACCTGC 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 62°C, and 2 min 72°C; and 5 min 72°C. After DNA amplification, 5 μl product was run on a 1% agarose gel. The remainder was stored at – 20°C.

RFLP

Seven μl of each PCR-product was digested with one of the following twelve restriction enzymes: *Alu*I, *Bsu*RI, *Bsh1236*I, *Bsp143*I, *Hin*dIII, *Hin*fI, *Hin6*I, *Msp*I, *Mva*I, *Pst*I, *Rsa*I and *Taq*I, in the buffer stipulated by the manufacturer. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis, stained with ethidium bromide and visualised and photographed under UV light. Digested PCR products loaded on a polyacrylamide gel were separated by electrophoresis on a Multiphor II Electrophoresis Unit (Pharmacia Biotech, Roosendaal, The Netherlands), silver stained (Pharmacia Biotech) and photographed. Procedures for obtaining PCR amplified products and endonuclease digestion of these products were repeated at least three times to verify the results.

Statistical analyses

Morphometrical data were statistically analysed with the STATISTICA (version 5.0) computer package. Canonical discriminant analysis (CDA) was used to assess the relative similarity of 24 populations based on four morphometrical characters of the juveniles. We used the unweighted pair group cluster analysis, as recommended by Wishart (1978) and Orloci (1978), to compile a dendrogram clustering the populations at different levels on a scale of similarity. The similarity values were calculated as $S = (1 - D/10)^*100$ (Brown & Topham, 1985), where D is the Mahalanobis distance calculated in the CDA. The number of clusters was estimated and the cophenetic correlation calculated by methods described by Aldenderfer and Blashfield (1989).

All digested DNA bands separated by electrophoresis on a 1.5% agarose gel were recorded as a binary matrix

Table 1. *Nematode populations of the genus* Heterodera *used in this study.*

Species	Location	Country	Isolate number	Source	Studies
H. arenaria	Lincolnshire	England	Har	J. Rowe, IACR-Rothamsted, Harpenden, UK	CDA, RFLP
H. aucklandica	One Tree Hill, Auckland	New Zealand	Hac	W. Wouts, Auckland, New Zealand	RFLP
H. avenae	Taaken, Lower Saxony	Germany	Hav1	D. Sturhan, BBA, Münster, Germany	CDA, RFLP
	Rinkam, Bavaria	Germany	Hav2	D. Sturhan, BBA, Münster, Germany	CDA, RFLP
	Argentan, Fr3 strain	France	Hav3	R. Rivoal, INRA, France	CDA, RFLP
	St. Georges-du-Bois, Fr2 strain	France	Hav4	R. Rivoal, INRA, France	CDA, RFLP
	Knokke, West Vlaanderen	Belgium	Hav5	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
	Nuisement-sur-Coole, Fr4 strain	France	Hav6	R. Rivoal, INRA, France	CDA, RFLP
	Santa Olalla	Spain	Hav7	D. Romero, Centro de Ciencias Medioambientales, Madrid, Spain	CDA, RFLP
	Desert region	India	Hav8	J. Rowe, IACR-Rothamsted, Harpenden, UK	CDA, RFLP
	Villasavary, Fr1 strain	France	Hav9	R. Rivoal, INRA, France	CDA, RFLP
H. filipjevi	Baimak, Bashkiria	Russia	Hf1	V.P. Balakhnina, Institute of Helminthology, Moscow, Russia	CDA, RFLP
	Gorodets, Nizhnii Novgorod region	Russia	Hf2	L. Nasonova, Nizhnii Novgorod, Russia	CDA, RFLP
	Pushkin, Leningrad region	Russia	Hf3	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
	Chabany, Kiev region	Ukraine	Hf4	V. Termino, Kiev, Ukraine	CDA, RFLP
	Saratov	Russia	Hf5	E. Osipova, Institute of Helminthology, Moscow, Russia	RFLP
	Etelhem	Sweden	Hf6	A. Ireholm, Swedish University of Agriculture Sciences, Sweden	CDA, RFLP
	Dushanbe	Tadzhikistan	Hf7	A.R. Madzhidov, Dushanbe, Tadzhikistan	CDA
	Vad, Nizhnii Novgorod region	Russia	Hf8	L. Nasonova, Nizhnii Novgorod, Russia	RFLP
	Torralba de Calatrava	Spain	Hf9	D. Romero, Centro de Ciencias Medioambientales, Madrid, Spain	CDA, RFLP
H. hordecalis	Montrose	Scotland	Hh1	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
	Sk. Fagerhult (paratypes)	Sweden	Hh2	J. Rowe, IACR-Rothamsted, Harpenden, UK	CDA
H. iri	Forfar	Scotland	Hir	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
H. latipons	Rostov region	Russia	Hlat	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
H. spp.	Putilovo, Leningrad region	Russia	Hsp1.1	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
	Kurilovo, Moscow region	Russia	Hsp1.2	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA
	Zarren, West Vlaanderen	Belgium	Hsp2	S.A. Subbotin, Institute of Parasitology, Moscow, Russia	CDA, RFLP
H. litoralis	Glen Innes, Auckland	New Zealand	Hlit	W. Wouts, Auckland, New Zealand	RFLP
H. schachtii	Unknown	Belgium	Hsh	M. Moens, Agricultural Research Centre, Merelbeke, Belgium	RFLP

Table 2. *Morphometrics of cysts and juveniles (means in μm ± S.E.) of studied populations of seven species from* Heterodera avenae *group.*

Species (Population)	Vulval areas of cyst					Juveniles				
	n	Fenestra length	Semi-fenestral width	Vulval bridge width	Vulval slit length	n	Body length	Stylet length	Tail length	Hyaline part of tail length
H. filipjevi	18	53± 0.9	30± 0.8	8.1± 0.4	10.9± 0.4	20	552± 3.7	25.4± 0.2	60± 0.9	39± 0.6
(Baimak, Russia)*, Hf1		(48-60)	(28-38)	(5.0-10.0)	(7.5-15.0)		(514-573)	(23.5-26.5)	(55-67)	(36-45)
H. filipjevi	15	51± 0.9	28± 0.6	9.4± 0.4	10.3± 0.3	20	526± 4.9	24.5± 0.1	55± 0.9	33± 0.7
(Gorodets, Russia)*, Hf2		(45-58)	(25-33)	(7.5-11.3)	(9.3-12.5)		(485-570)	(23.9-25.5)	(50-62)	(28-38)
H. filipjevi	6	52± 1.8	27± 1.6	7.9 ± 0.8	14.0± 0.6	15	539± 4.5	25.2± 0.2	60± 0.6	37± 0.7
(Pushkin, Russia)*, Hf3		(45-58)	(20-30)	(5.0-10.0)	(12.5-15.0)		(504-568)	(24.5-26.0)	(56-62)	(34-41)
H. filipjevi	21	55± 0.9	29± 0.6	10.5± 0.3	11.9± 0.3	20	520± 7.1	24.9± 0.2	54± 0.9	35± 0.5
(Chabany, Ukraine)*, Hf4		(48-63)	(25-33)	(7.5-12.5)	(9.5-14.3)		(478-577)	(23.5-26.0)	(50-60)	(31-39)
H. filipjevi	8	52± 1.4	28± 1.1	9.9± 0.5	10.1± 0.5	14	509± 5.2	24.3± 0.1	56± 1.1	33± 0.6
(Etelhem, Sweden), Hf6		(45-58)	(21-33)	(7.5-12.5)	(7.5-12.5)		(477-552)	(24.5-24.8)	(46-60)	(30-37)
H. filipjevi	16	54± 1.1	28± 0.8	8.8± 0.4	10.9± 0.6	16	519± 4.7	24.8± 0.2	55± 0.6	31± 0.6
(Dushanbe)*, Hf7		(50-61)	(24-32)	(6.9-10.9)	(7.9-15.8)		(494-537)	(23.2-25.6)	(52-59)	(29-36)
H. filipjevi	6	50± 1.1	27± 0.5	11.8± 0.7	11.0± 0.6	20	526± 6.0	26.4± 0.2	57± 0.7	36± 0.6
(Spain), Hf9		(48-55)	(25-29)	(9.3-13.3)	(9.3-12.5)		(484-572)	(25.5-27.5)	(52-63)	(31-41)
H. avenae	20	48± 0.7	25± 0.6	7.3± 0.2	10.1± 0.3	20	566± 4.8	26.3± 0.3	69± 1.0	45± 0.6
(Taaken, Germany)*, Hav1		(43-53)	(20-30)	(6.3-7.5)	(8.0-12.5)		(522-598)	(23.3-29.3)	(59-80)	(40-49)
H. avenae	20	48± 1.1	23± 0.5	7.1± 0.4	8.9± 0.3	20	557± 4.8	26.5± 0.3	69± 0.9	44± 0.7
(Rinkam, Germany)*, Hav2		(40-55)	(18-27)	(5.0-10.8)	(6.3-10.8)		(491-595)	(23.5-29.1)	(61-76)	(39-50)
H. avenae	11	50± 1.1	25± 0.6	8.6± 0.3	9.6± 0.2	20	568± 4.5	26.2± 0.1	66± 0.8	44± 0.8
(Argentan, France), Hav3		(45-58)	(23-30)	(7.5-10.0)	(8.8-10.2)		(538-602)	(25.5-27.5)	(57-71)	(39-51)
H. avenae	8	47± 1.0	24± 0.7	9.4± 0.4	9.8± 0.3	24	519± 3.0	26.6± 0.1	70± 0.7	48± 1.0
(St. Georges, France), Hav4		(43-50)	(20-25)	(7.5-10.8)	(7.5-10.5)		(488-541)	(25.5-27.5)	(66-77)	(41-56)
H. avenae	3	46± 1.9	21± 0.8	10.8± 0.8	9.8± 0.2	20	516± 8.8	26.4± 0.2	66± 0.9	45± 0.9
(Nuisement, France), Hav6		(44-50)	(20-23)	(10.0-12.5)	(9.5-10)		(449-570)	(24.5-27.5)	(56-75)	(36-50)
H. avenae	10	45± 1.4	22± 1.1	7.4± 0.2	10.2± 0.3	20	571± 13	27.5± 0.3	69± 1.0	47± 1.1
(Knokke, Belgium), Hav5		(38-53)	(18-28)	(6.3-8.8)	(9.3-12.5)		(423-644)	(25.5-29.6)	(59-77)	(38-57)
H. avenae	5	46± 0.6	24± 0.6	10± 0.4	9.7± 0.6	20	563± 5.5	26.9± 0.2	69± 0.8	44± 0.5
(Villasavary, France), Hav9		(45-48)	(23-26)	(8.8-11.3)	(7.5-10.8)		(505-560)	(25.5-28.6)	(62-76)	(40-48)
H. avenae	5	48± 1.1	21± 0.6	10.6± 0.6	8.7± 0.5	15	505± 7.4	26.1± 0.2	61± 0.8	38± 1.3
(desert reg. India), Hav8		(45-50)	(20-23)	(9.3-12.5)	(7.5-10.0)		(453-559)	(24.5-27.5)	(56-65)	(31-46)
H. avenae	5	45± 3.8	23± 0.6	10.4± 1.5	10± 0.1	20	553± 6.0	26.4± 0.2	67± 0.6	41± 0.5
(Spain), Hav7		(38-50)	(23-24)	(7.5-12.5)	(9.8-10.2)		(478-597)	(24.5-28.6)	(61-74)	(37-44)
H. arenaria	4	53± 1.8	26± 1.6	8.4± 0.8	11.4± 0.6	20	633± 8.0	29.4± 0.2	77± 1.3	51± 0.7
(England), Har		(50-58)	(23-30)	(6.8-10.0)	(10-13)		(536-671)	(27.5-30.6)	(63-84)	(46-56)
H. iri	5	48± 1.0	27± 0.8	6.6± 0.7	12.6± 0.8	18	593± 4.1	26.9± 0.1	85± 1.3	56± 0.7
(Scotland), Hir		(45-50)	(25-30)	(5.0-8.8)	(10-15)		(562-639)	(25.8-27.5)	(77-97)	(47-61)
Heterodera sp1.1	13	43± 0.7	23± 0.8	7.0± 0.5	10.6± 0.4	20	504± 4.5	24.9± 0.2	65± 0.9	42± 0.7
(Putilovo, Russia)*		(38-48)	(18-28)	(5.0-10.0)	(8.8-12.5)		(471-532)	(22.4-26.0)	(59-69)	(39-45)
Heterodera sp1.2	11	43± 1.3	25± 0.4	9.8± 0.3	8.6± 0.3	20	522± 5.2	24.4± 0.2	69± 1.1	45± 0.8
(Kurilovo, Russia)		(40-50)	(23-28)	(8.8-11.8)	(7.5-10.8)		(464-563)	(22.9-25.5)	(53-77)	(39-53)
Heterodera sp2	9	49± 1.8	24± 1.0	6.4± 0.4	9.4± 0.3	20	494± 5.8	24.9± 0.1	69± 1.2	46± 1.1
(Zarren, Belgium)		(38-55)	(20-28)	(5.0-7.5)	(7.5-10.0)		(434-545)	(24.0-26.5)	(62-78)	(38-52)
H. latipons	12	60± 1.6	23± 0.5	28± 1.2	7.3± 0.3	20	485± 6.4	23.4± 0.1	53± 0.7	32± 0.7
(Rostov, Russia), Hlat		(50-70)	(20-25)	(23-38)	(5.9-9.3)		(421-552)	(22.4-24.5)	(43-59)	(26-38)
H. hordecalis	14	57± 0.9	22± 0.5	28± 0.6	20± 0.4	11	442± 4.1	24.4± 0.1	51± 0.4	33± 0.6
(Sweden), Hh1		(52-66)	(20-26)	(24-32)	(18-22)		(417-462)	(24.0-24.8)	(50-54)	(30-36)
H. hordecalis	10	63± 1.8	25± 0.9	26± 1.1	22± 1.1	20	470± 5.5	25.6± 0.2	54± 0.9	35± 0.8
(Scotland), Hh2		(58-75)	(20-30)	(18-30)	(18-28)		(407-507)	(24.5-27.5)	(48-62)	(30-42)

*Measurements made by Subbotin *et al.* (1996).

Table 3. *Factor structure for canonical variables of* Heterodera *species juveniles from 24 populations.*

	Axis 1	Axis 2	Axis 3	Axis 4
Body length	0 .457	0 .612	0 .634	0 .141
Stylet length	0 .597	0 .672	–0 .436	–0 .030
Hyaline part of tail length	0 .818	–0 .193	0 .183	0 .520
Tail length	0 .845	–0 .134	0 .433	–0 .283
Canonical correlation	0 .921747	0 .812422	0 .694518	0 .405525
Cumulative	0 .647918	0 .870565	0 .977429	1 .00000

of 0 and 1 corresponding to the absence or presence of individual bands. The matrix was given as input data to the Phylogeny Inference Package (PHYLIP, version 3.572). Gendist was the Phylip program used to compute the genetic Nei's distance. Cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA) was performed by the Neighbor program. Bootstrap analysis, using 1000 bootstrapped data sets, was performed to determine statistical constancy of the classification.

Results

Morphometrical and morphological characteristics of species

The J2 and cyst morphometrical data are presented in Table 2. Measurements are means followed by their standard error; the range is placed in parenthesis. The CDA of the 24 populations calculated four canonical variables. Cumulated, the first two variables accounted for 87% of the variance and the first three for almost 98% of the variance. The tail length and the length of the hyaline part of the tail had the highest correlation with the first variable; the stylet length was best correlated with the second variable and body length with the third one (Table 3). Two-dimensional scatterplots of population means of canonical variables were generated (Fig. 1A, B). They allow the population grouping to be compared with species identification and the similarities among species to be estimated. The results of the unweighted pair group cluster analysis are presented as a dendrogram (Fig. 2). The populations were clustered in nine distinct groups at a similarity level of 80% (cophenetic correlation = 0.87).

H. filipjevi populations were grouped in two clusters. Six populations(Hf1, Hf2, Hf3, Hf4, Hf6 and Hf7) formed a cluster at 83% similarity; Hf9 (Torralba de Calatrava, Spain) was clustered with the Indian population of *H. avenae* (Hav8). J2 of *H. filipjevi* were easily distinguished from the closely related European *H. avenae* populations by a shorter tail (average 54-60 *vs* 66-70 μm) and a shorter hyaline part of tail (31-39 *vs* 44-48 μm). They were also distinguished from *H. avenae* by the shape of the stylet knobs (distinctly concave anteriorly) and the rounded tail terminus. The Spanish population of *H. filipjevi* differed from other populations by a longer stylet length (Table 2). Fenestra length and mean semifenestral width of vulval areas of cysts of *H. filipjevi* were larger than of *H. avenae* cysts (average 50-55 *vs* 45-50 μm, 27-30 *vs* 22-25 μm, respectively). Vulval cyst cones of *H. filipjevi* had an underbridge.

H. latipons clustered with six *H. filipjevi* populations, the two *H. hordecalis* (Hh1 and Hh2) populations clustered with to Hf9 and Hav8. *H. latipons* and *H. hordecalis* were easily distinguished from other species and from each other by differences in vulval slit length, the vulval bridge width, the strong underbridge and the lack of distinct bullae in the vulval cone of the cyst.

H. avenae populations were grouped in three clusters. The two French populations (Hav4 and Hav6) were separated from the other European populations (Hav1, Hav2, Hav3, Hav5, Hav7, and Hav9); the Indian population (Hav8) was grouped with *H. filipjevi* from Spain (Hf9). A shorter J2 body length, tail length and hyaline part of tail length distinguished the Indian population from the other *H. avenae* populations. The French populations (Hav4 and Hav6) differed from the other European populations by a shorter J2 body length (Table 2). A weak underbridge was present in some specimens of these two populations. Stylet knobs of J2s from all *H. avenae* populations were flat or sometimes slightly concave anteriorly and the tail terminus was usually narrowly rounded.

The *Heterodera* spp. populations all from grasslands formed a distinct cluster at almost 68% similarity with the large *H. avenae* group. The body length, the fenestral length and the vulval bridge width differed slightly between the populations (Table 2). Shapes of stylet knobs and tail terminus, however, were similar. Stylet knobs were flat or sometimes slightly concave anteriorly and the tail terminus was narrowly rounded. J2s of these species were distinguished from the similar European *H. avenae* populations by a shorter stylet length (24.4-24.9 *vs* 26.2-27.5 μm) and a shorter hyaline part of tail length (42-46 *vs* 44-48 μm).

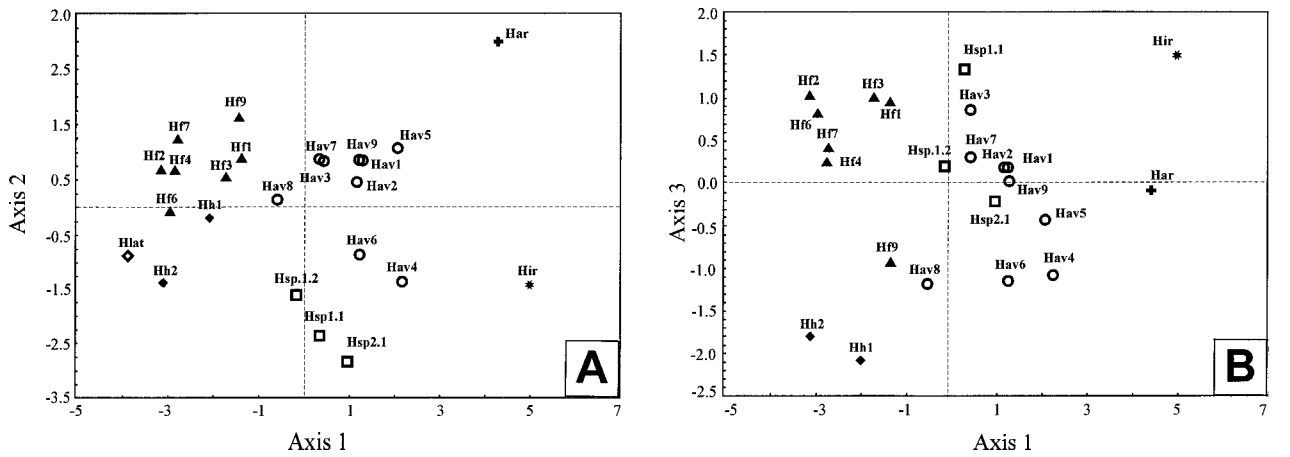

Fig. 1. *Canonical discriminant analysis. Scatterplots of means of 24 populations of the* Heterodera avenae *group on (A) the first and the second axes and on (B) the first and third canonical axes (for species codes, see Table 1).*

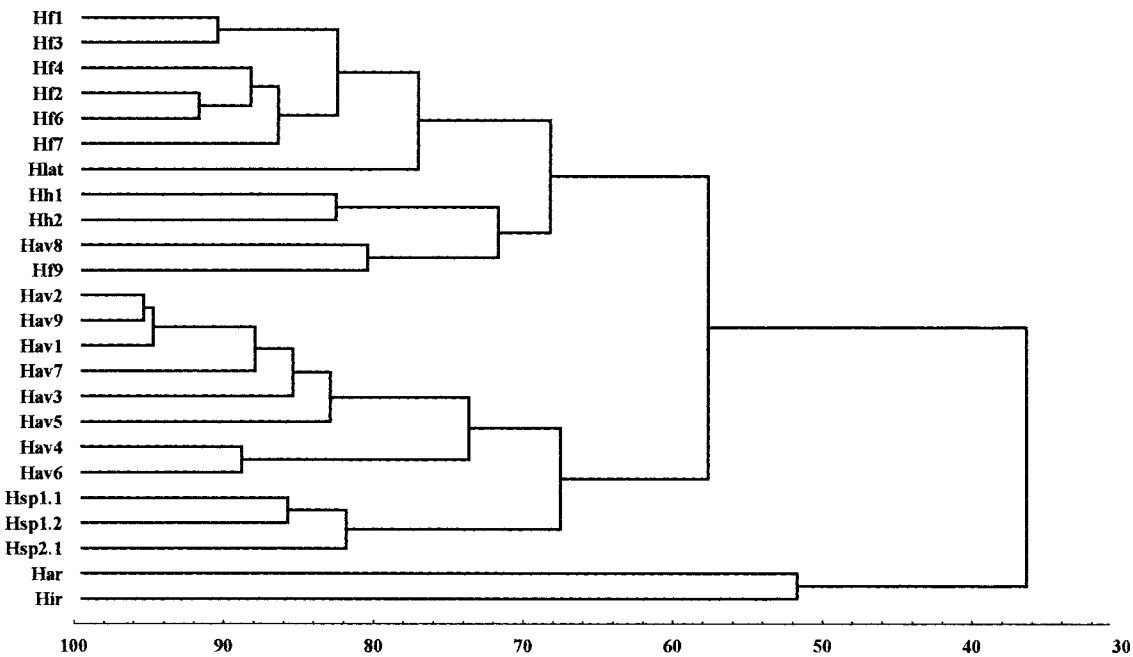

Fig. 2. *Similarity dendrogram of 24 populations from the* Heterodera avenae *group as computed by canonical discriminant analysis of four morphometrical characters (for isolate numbers, see Table 1).*

H. arenaria and *H. iri* constituted a very distinct group with only 36% similarity with the other populations. *H. arenaria* significantly differed from all other species by a longer body and stylet length of the J2s and a larger fenestra length of the cysts. The stylet knobs of the J2s were distinctly concave anteriorly and the tail terminus rounded. *H. arenaria* had only 52% similarity to *H. iri*. This latter species was distinguished from the other species by a longer tail and hyaline part of the tail. The stylet knobs of the J2s were flat or sometimes slightly concave anteriorly; the tail terminus was narrowly rounded.

PCR AMPLIFICATION AND RFLP ANALYSIS

The amplification of the rDNA ITS regions of each population yielded one fragment of approximately 1060

bp. No PCR products were obtained in the control lacking the DNA template. *Hin*dIII was the only enzyme that did not restrict any of the ITS. Most enzymes separated all the species of the group. The restriction pattern obtained with *Bsu*RI (data not shown), *Pst*I (Fig. 3A) and *Taq*I (Fig. 3B) clearly distinguished *H. filipjevi.* There was no intraspecific polymorphism within *H. filipjevi. Alu*I (Fig. 3C) separated European *H. avenae* populations and *H. arenaria* from the other species of the group; no tested enzyme allowed the differentiation of these two species from each other. *Alu*I (Fig. 3C), *Hin6*I (Fig. 3D), *Bsh1236*I (data not shown), *Bsu*RI (data not shown) and *Rsa*I (Fig. 3E) yielded RFLP distinguishing *H. latipons. Bsu*RI, *Bsh1236*I and *Mva*I separated *H. hordecalis. Msp*I distinguished *H. latipons* and *H. hordecalis* from others. Digestion with *Hin6*I (Fig. 3D) distinguished *Heterodera* sp2. (Hsp2) and *H. aucklandica* from the other species; *Taq*I (Fig. 3B) and *Hin*fI (Fig. 3F) distinguished these species from *Heterodera* sp1. (Hsp1.1). No enzyme enabled the differentiation of *H. aucklandica* from *Heterodera* sp.2 (Hsp2). *H. iri* was separated by RFLP generated by *Bsh1236*I (data not shown), *Hin6*I (Fig. 3D) and *Msp*I (data not shown). For the distinction of *Heterodera* sp1. (Hsp1.1) at least two enzymes were necessary. Table 4 groups the data obtained from all RFLPs and shows the enzymes that can be used to separate species.

Intraspecific polymorphism was revealed within *H. avenae*. *Alu*I (Fig. 3C) and *Rsa*I (Fig. 3E) did not digest PCR amplified products of European populations, but did digest PCR products of the Indian population (Hav8). Digestion by both enzymes also showed heterogeneity in ITS regions of the three French populations (Hav4, Hav6 and Hav9). For these populations two additional bands were obtained. The sum of the three fragments was approximately 2120 bp, *i.e.*, about twice the size of the undigested amplified product. These additional bands were of the same length as those in the restriction patterns of the Indian population. Different intensity of these bands was observed between cysts from the St. Georges-du-Bois population (Hav4). *Alu*I digestion of PCR products of each of three cysts showed clear additional bands for all three cysts; *Rsa*I digestion of these products, however, produced distinct additional bands for only two cysts (Fig. 4). Very weak additional bands were repeatedly obtained in some *H. avenae* populations after digestion by *Bsh1236*I.

A total of 90 scored fragments were obtained with eleven enzymes and used for analysis. The dendrogram constructed from Nei's distances with UPGMA analysis revealed seven main clusters (Fig. 5). Cluster I contained all populations of *H. filipjevi*; cluster II contained populations of *H. avenae*, *H. arenaria* and the *Heterodera* spp. from grasses. The remaining five clusters were each composed of single species: *H. iri*, *H. latipons*, *H. hordecalis*, *H. schachtii* and *H. litoralis.*

Restriction endonuclease cleavage maps

Restriction patterns obtained after digestion of the PCR product of *H. avenae* populations Hav1, Hav2, Hav3, Hav5 and Hav7 and of the *H. filipjevi* populations corresponded to ITS sequences of the Swedish strict *H. avenae* isolate and Swedish East Gotland strain isolate, respectively, as published by Ferris *et al.* (1994). Based on our ITS-RFLP data and the sequence data published by Ferris *et al.* (1994) restriction endonuclease cleavage maps of ITS region of *H. aucklandica*, *H. avenae*, *H. iri*, *H. filipjevi* and *Heterodera* spp. were constructed for five enzymes (Fig. 6).

Discussion

Our comparative study showed that morphological and morphometrical divergences between species and populations correspond generally with genetic differences. *H. arenaria*, however, has to be considered as an exception. J2s of this species were easily distinguished from juveniles of other species by their larger size. The *H. arenaria* restriction patterns, however, did not differ from those of *H. avenae*. Different authors found good separations of *H. arenaria* from other species of the *H. avenae* group. Stone and Hill (1982), when using principal coordinate analysis of six J2s' numerical characters found *H. arenaria* well separated from *H. avenae* and *H. mani*. Ibrahim and Rowe (1995) showed that *H. arenaria* could be separated from closely related species by non-specific esterases.

The dendrogram constructed with molecular data showed two distinct clusters within the *H. avenae* group, which correspond with the two morphological groups of Wouts and Sturhan (1995): the *H. avenae* group *sensu stricto* (*H. avenae*, *H. mani*, *H. filipjevi*, *H. iri*, *H. aucklandica*) and the *H. latipons* group (*H. latipons* and *H. hordecalis*) supporting their view and adding evidence to the observations of Krall and Krall (1978), Shagalina and Krall (1981) and Sturhan and Wouts (1995) that there is an extensive variation within the *H. avenae* group and confirms the view that this group is not monophyletic.

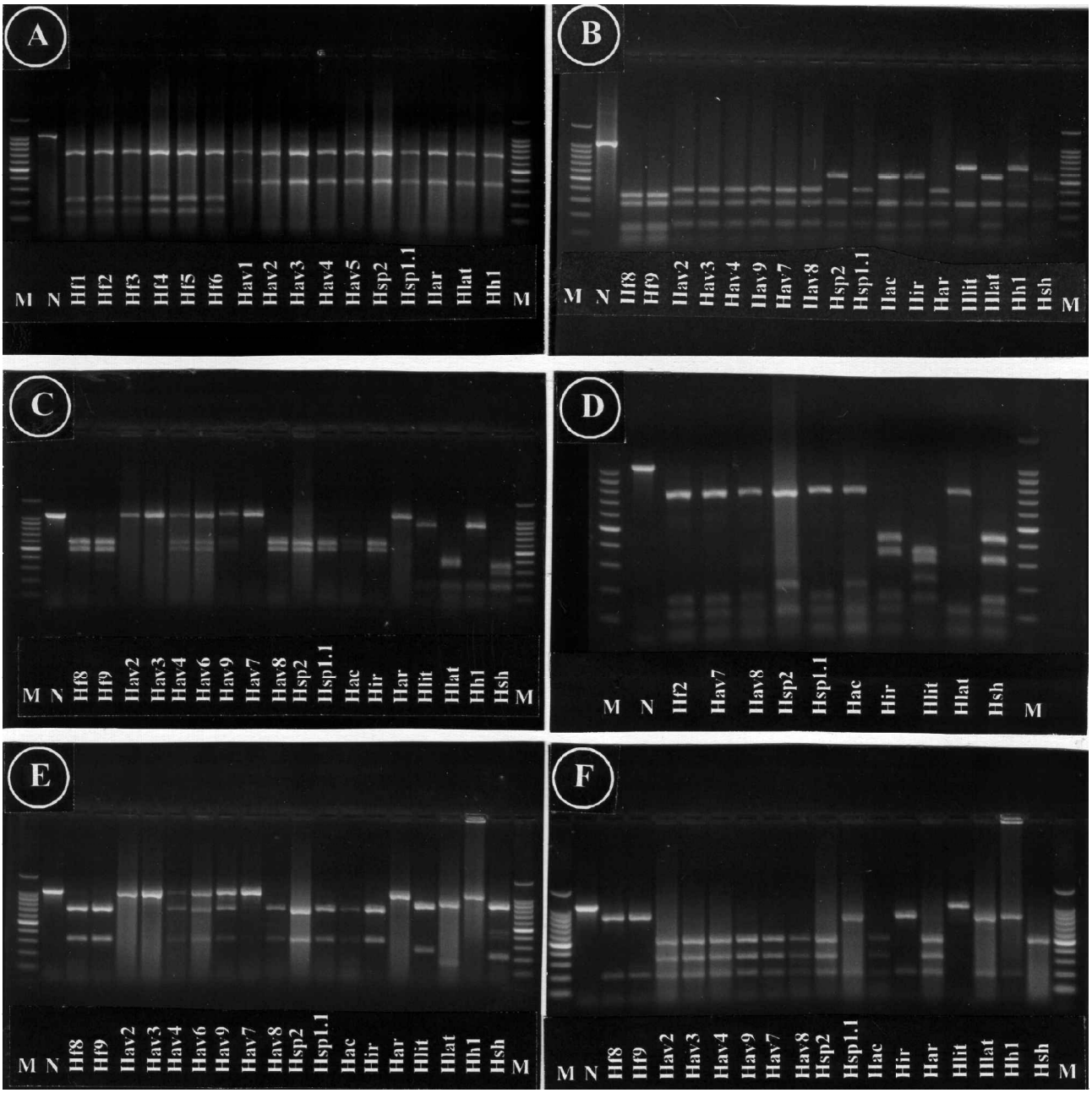

Fig. 3. *Restriction fragments of amplified ITS regions of species belonging to the* Heterodera avenae *group. A: PstI; B: TaqI; C: AluI; D: Hin6I; E: RsaI; F: HinfI (for species codes, see Table 1, lane M: 100 bp DNA ladder).*

Table 4. *Number of different RFLP profiles yielded by a single enzyme of the ITS regions of cyst nematodes.*

Species	Restriction enzymes										
	*Alu*I	*Bsu*RI	*Bsh1236*I	*Bsp143*I	*Hin*fI	*Hin6*I	*Msp*I	*Mva*I	*Pst*I	*Rsa*I	*Taq*I
H. avenae (type A)	1(*)	1	1	1	1	1	1	1	1	1	1
H. arenaria	1	1	1	1	1	1	1	1	1	1	1
H. avenae (type B)	2	1	1	1	1	1	1	1	1	2	1
H. avenae (types A+B)	3	1	1	1	1	1	1	1	1	3	1
H. filipjevi	2	2	1	1	2	1	1	1	2	2	2
Heterodera sp.1	2	1	1	1	2	1	1	1	1	2	1
Heterodera sp.2	2	1	1	1	1	2	1	1	1	2	3
H. aucklandica	2	1	1	1	1	2	1	1	1	2	3
H. iri	2	1	2	2	2	3	2	2	1	2	3
H. latipons	4	3	3	2	2	4	3	2	1	4	3
H. hordecalis	5	4	4	1	2	1	3	3	1	1	4
H. schachtii	6	5	5	1	3	5	4	4	1	5	3
H. litoralis	5	6	6	1	4	6	5	5	3	6	4

(*) a same number indicates species with identical patterns.

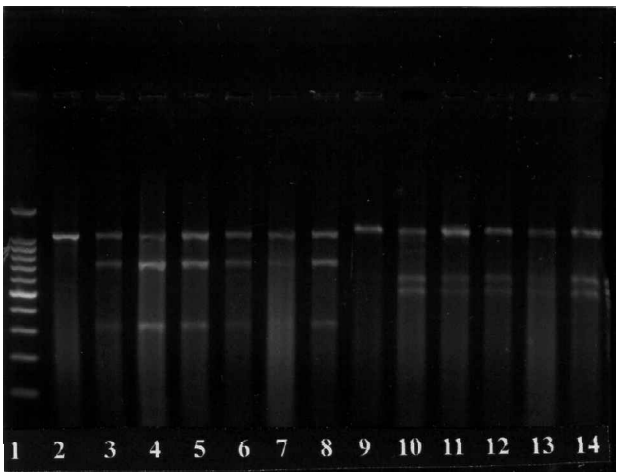

Fig. 4. *Restriction fragments of amplified ITS regions of individuals from two populations of* Heterodera avenae*. Lane 1: 100 bp DNA ladder; lane 2: unrestricted PCR product; lanes 3-8: digestion with RsaI; lanes 9-14 digestion with AluI; lanes 3 and 9: cyst 1 from St. Georges-du-Bois, 4 and 10: cyst 2 from St. Georges-du-Bois, 5 and 11: cyst 3 from St. Georges-du-Bois; lanes 6 and 12: cyst 1 from Nuisement-sur-Coole, 7 and 13: cyst 2 from Nuisement-sur-Coole, 8 and 14: cyst 3 from Nuisement-sur-Coole.*

H. litoralis — with a bifenestral vulval cone but not belonging to the *H. avenae* group because of its vulval slit of about 40 μm — has a greater genetic distance from the *H. avenae* group than from *H. schachtii* (Wouts & Sturhan, 1996). This fact supports Krall and Krall (1978) who stated that the bifenestrate configuration of the vulval area evolutionary developed in different cyst nematode groups independently. Our dendrogram of genetic diversity of the *H. avenae* group (Fig. 5) constructed with RFLP data corresponds to a previously published dendrogram by Bekal *et al.* (1997), but shows additional relationships with other species of this group.

Our results support those obtained by Sturhan and Rumpenhorst (1996) and Bekal *et al.* (1997) that the 'Gotland strain' from Sweden is identical to populations of *H. filipjevi.* Similarities of ITS regions sequences of Pushkin (Hf3) and Vad (Hf8) populations of *H. filipjevi* were found to be over 99% similar to the East and West Gotland strain isolates from Sweden (V. Ferris, pers. comm.). Differences in protein patterns and in rDNA between different populations of *H. avenae* and the 'Gotland strain' or *H. filipjevi* have been reported by several authors (Ferris *et al.*, 1989, 1994; Bossis & Rivoal, 1996; Rumpenhorst *et al.*, 1996; Sturhan, 1996; Sturhan & Rumpenhorst, 1996; Subbotin *et al.*, 1996; Bekal *et al.*, 1997). At present, *H. filipjevi* is found in Tadzhikistan, Uzbekistan, Iran, Turkey, Bulgaria, Russia, Ukraine, Estonia, Poland, Germany, England and Sweden. The main centre of its distribution is considered to be the East European-orient region (Rumpenhorst *et al.*, 1996; Sturhan & Rumpenhorst, 1996; Subbotin *et al.*, 1996). Using twelve restriction enzymes we did not find any intraspecific polymorphism within the ITS region of *H. filipjevi* populations. The Spanish population from Torralba de Calatrava (Hf9) did not differ in our rDNA restriction analysis; however, it was clearly distinguished by CDA.

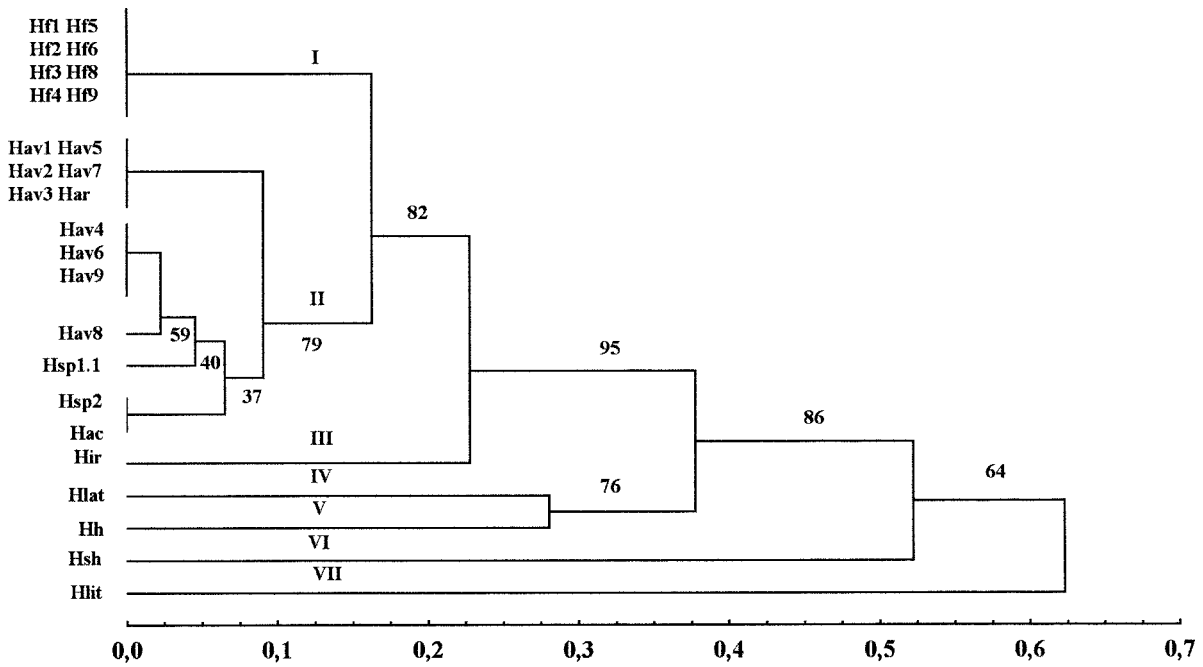

Fig. 5. *Dendrogram constructed from Nei's distance and showing the clustering of 26 populations of species from the* Heterodera avenae *group based on rDNA-RFLP data. Bootstrap values (%) based on 1000 resamplings are given on appropriate clusters.*

Our study and data published by Valdeolivas and Romero (1990) show that the J2s of this population have a longer stylet than those of typical *H. filipjevi* populations (average 26.4, 26.9 *vs* 24.3-25.4, respectively). Protein electrophoresis also revealed differences between the Spanish population and other *H. filipjevi* populations (Bossis & Rivoal, 1996; Sturhan & Rumpenhorst, 1996).

Molecular intraspecific polymorphism was observed within *H. avenae* populations. Extended digestion period, mixing experiments with control DNA, repeated amplifications, and amplification with primers described by Vrain *et al.* (1992) suggest that this heterogeneity is not the result of partial digestion. At least two types of ITS regions were identified: type A for most European populations (Hav1, Hav2, Hav3, Hav5 and Hav7), type B for the Indian population (Hav8) and their combination (type A+B) for three French populations (Hav4, Hav6 and Hav9). These three genetic types generally corresponded to morphological types analogous to the same populations. The Indian population (Hav8) found in a desert region clearly differs from all others. *Alu*I and *Rsa*I digested the ITS region and permitted differentiation of this population from the other *H. avenae* populations. Ferris *et al.* (1994) published the ITS-sequence of the Australian Rainbow isolate of *H. avenae*. From this sequence, it can be concluded that *Alu*I and *Rsa*I digest this population at the same sites. ITS restriction patterns produced by *Taq*I, however, may distinguish the Australian from the Indian population. Protein electrophoretic studies also showed a great similarity between the Australian population and the population from Delhi (Sturhan & Rumpenhorst, 1996). Further investigations are needed to clarify the taxonomic status of Indian populations. The polymorphism observed by Bekal *et al.* (1997) between *H. avenae* populations was different from ours. The number of genetic population types, therefore, may be higher than as yet has been identified.

Three French populations (Hav4, Hav6 and Hav9) showed heterogeneity in the ITS1 region. The additional bands generated by *Alu*I and *Rsa*I were of the same length as those obtained for the Indian population. Therefore, we think that the French specimens we examined were composed of a mixture of ITS types A and B. These two additional bands produced an additional cluster for the French populations. Two of these populations (Hav4 and Hav6) belong to the second group of *H. avenae* pathotypes or Ha12 (Andersen & Andersen, 1982) and were separated by differences in morphometrics and CDA. ITS heterogeneity was revealed in many nematode species (Zijlstra *et al.*, 1995; Thiéry & Mugniéry, 1996; Cherry *et al.*,

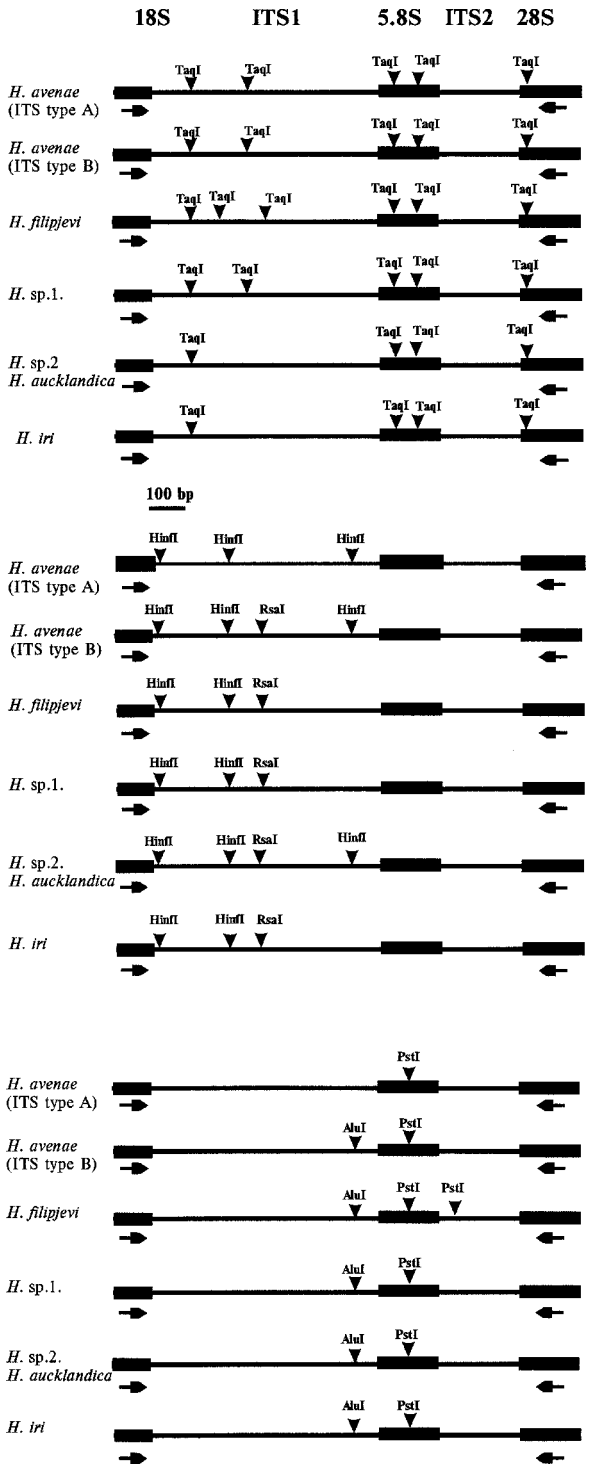

Fig. 6. *Restriction endonuclease cleavage maps of ITS regions of* Heterodera avenae *group species, constructed on the base of our RFLP data and sequence data published by Ferris* et al. *(1994). (▼— restriction site).*

1997) and, perhaps, reflects the result of evolutionary interactions between populations and between species.

The graminaceous cyst nematode populations used in our study (Hsp1.1, Hsp1.2 and Hsp2) formed a distinct separate cluster. They were morphologically and morphometrically easily distinguished from other species from the *H. avenae* group. Population Hsp1.1 from Putilovo is similar in morphology and morphometrics to the so-called 'German grassland *Heterodera* species' populations, which will be described as a new species (Sturhan, pers. comm.). This similarity is also supported by protein electrophoresis (Sturhan & Rumpenhorst, 1996; Subbotin *et al.*, 1996). Discriminant analysis of four J2 numerical characters showed conspecifity of populations from Putilovo and Kurilovo (Hsp1.1 and Hsp1.2).

The morphology of J2s and cysts of the Belgian grassland population (Hsp2), is very similar to that of the Russian grassland populations (Hsp1.1 and Hsp1.2) and the morphology detailed in the original description of *H. aucklandica*, but differs from the latter by a shorter tail (average 65-66 *vs* 76) (Wouts & Sturhan, 1995). The ITS regions of Hsp2 were similar to those of *H. aucklandica* but differed from Hsp1.1 by the RFLPs obtained with four enzymes: *Hin*fI, *Hin6*I, *Taq*I and *Tru9*I (data not published). The level of morphometric similarity between these European 'grassland populations' is rather high, suggesting that these populations should not be considered as belonging to a separate species, but only as belonging to a subspecies.

Our study showed that rDNA-RFLPs and multivariation analysis of morphometric characters can distinctly separate species and populations within the *H. avenae* group. The *H. avenae* group *sensu stricto* is a complex of more or less distinct populations differentiated at species or subspecies level. Further DNA observations, and more detailed morphological, morphometrical, biological, ecological and biogeographical studies are needed to identify at which taxonomic level populations of cyst-forming nematodes can be separated. The creation of a catalogue of RFLPs of the ITS region of cyst forming nematode species would facilitate the identification of species and population.

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