European Journal of Plant Pathology **109:** 227–241, 2003.

Genetic and phenotypic diversity in the graminaceous cyst nematode complex, inferred from PCR-RFLP of ribosomal DNA and morphometric analysis

Roger Rivoal[1], Sylvie Valette[1], Sadia Bekal[1,*], Jean-Pierre Gauthier[1] and Amor Yahyaoui[2]
[1]*UMR INRA/ENSAR, Biologie des Organismes et des Populations appliquée à la Protection des Plantes (BiO3P), BP 35327, 35653 Le Rheu, France (Fax: +33-0-223485157; E-mail: rivoal@rennes.inra.fr);*
[2]*ICARDA, Cereal Pathology, P.O. Box 5466, Aleppo, Syria; *Present address: University of Illinois, Department of Crop Sciences, AW-101 Turner Hall, 1102 South Goodwin Avenue, Urbana, IL 61801-4798, USA*

Accepted 14 October 2002

Key words: classification, *Heterodera*, identification, ITS, morphometrics, multivariate analysis

Abstract

Graminaceous cyst nematodes form a group of eleven valid species including *Heterodera avenae*, *Heterodera filipjevi* and *Heterodera latipons* and constitute major pests to cereals. They are widely spread in circum-mediterranean areas where they are presumed to cause yield losses on bread and durum wheat. The objective was to document the diversity of these cereal cyst nematodes, in particular samples from Mediterranean regions, in comparison to species which develop on cultivated or wild grasses (*H. arenaria*, *H. hordecalis*, *H. mani*) and on rice or sugarcane (*H. sacchari*). Studies involved PCR-RFLP of ITS and morphometrics of the juvenile and cyst characters. UPGMA analysis of molecular data showed that the isolates segregated in two main clusters which seem to represent different evolutionary lineages. The *H. avenae sensu lato* cluster (I) contained *H. arenaria*, *H. avenae*, *H. filipjevi* and *H. mani*. The second cluster (II) contained isolates of *H. hordecalis* and *H. latipons*. Within *H. avenae sensu lato*, *H. filipjevi* was separated from the other related species with significant bootstrap value. The differentiation of *H. arenaria*, recognized for the first time based on molecular data, and *H. mani* with few restriction enzymes were the least significant. Intraspecific polymorphism allowed differentiation of isolates originating from Australia within *H. avenae sensu stricto*. The group *H. hordecalis–H. latipons* showed the greatest genetic variability between and within isolates. Two representatives of *Heterodera sacchari*, taxonomically included in the '*schachtii*' group, were genetically as distant to this group as to the other graminaceous species belonging to either *H. avenae sensu lato* or *H. hordecalis–H. latipons* group. Results inferred from multivariate analysis applied on morphometrics of the cysts and juveniles showed agreement between genetic and phenotypic classifications. This study demonstrates the utility of combined molecular and classical methods to enhance our knowledge about the diversity within the complex of graminaceous cyst nematodes and to establish robust techniques to identify a wider set of nematode species.

Introduction

Graminaceous cyst nematodes (*Heterodera* spp.) represent an important group of sedentary endoparasites which are found on various wild grasses and cultivated cereals. They are represented mainly by the *Heterodera avenae* group which contains eleven valid and several other undescribed species (Wouts et al., 1995; Subbotin et al., 1999). The main species attacking cultivated cereals are *H. avenae*, *H. filipjevi* and *H. latipons* (Ritter, 1982; Rivoal and Cook, 1993; Nicol, 2002). *Heterodera avenae* is the more widely distributed, known principally in Western Europe, Australia, but scarcely in Northern America. *Heterodera filipjevi* occurs in Eastern Europe whereas *H. latipons* is considered to be prevalent in Mediterranean regions.

These species can cause considerable damage and have a devastating impact, particularly, in semi arid regions where they dramatically increase natural moisture stress (Sikora, 1987; Rivoal and Cook, 1993). Economical and environmentally safe plant protection measures are increasingly based on resistant plants that require reliable identification of the isolates at the specific or infraspecific level (Cook and Rivoal, 1998).

The examination of polymorphism from amplified coding and noncoding regions (PCR-RFLP) of ribosomal DNA has provided useful tools for species and subspecies segregation of isolates based on digestion patterns and the genetic distances calculated (Powers et al., 1997; Blok et al., 1998; Iwahori et al., 1998; Waeyenberge et al., 2000). Such information could strengthen conventional identification based on the analysis of differences in morphology which is time consuming and often inconclusive (Subbotin et al., 2000). The PCR-RFLP technique enabled characterization of the main species belonging to the *H. avenae* group and demonstrated that the Gotland strain of *H. avenae* found in Spain and Sweden was very close to *H. filipjevi* (Bekal et al., 1997). These results were further confirmed by Subbotin et al. (1999) who showed that in this group of nematodes morphological and morphometrical divergence between species and populations correspond generally with genetic differences. PCR-RFLP has also established the phylogenetic relationships among species and isolates that are more or less clearly identified (Thiéry and Mugniéry, 1996).

The purpose of this study was to enhance our knowledge about the molecular variation through PCR-RFLP analysis of a portion of rDNA, among a range of isolates of graminaceous cyst nematodes originating mainly from cereal fields or wild grasses over wide areas in Europe, North Africa, East and Middle East regions and also from rice and sugarcane in Central Africa. Species and isolates of these nematodes were characterized by digestion patterns of their rDNA. In addition, a comparative study of morphological traits was performed on a selected set of nematode isolates.

Materials and methods

Nematode species and isolates

Fifty-three isolates of graminaceous cyst nematodes originating from 17 countries were studied. The typical or presumed species *H. avenae*, *H. filipjevi* and *H. latipons* were sampled mainly from the circum-mediterranean areas, except for the isolates from Australia, China, India and Russia. *Heterodera avenae* was represented by 30 isolates (Table 1). The samples analyzed were taken from the field or previously reared on the same susceptible host *Triticum aestivum* cv. Arminda, except for *H. avenae* E79 which developed on *Lolium perenne* cv Lipo. The graminaceous cyst nematode *H. mani* originated from France (E54), Germany (E87) or Great Britain (A36). *Heterodera arenaria* (E167, E183) and *H. hordecalis* (E166) were sampled from sandy coastal dunes of Northern and Southern Brittany (France). Unidentified *Heterodera* species from a wheat field in Algeria (E143-2) or from turf grass in California (E170) were also examined. Two isolates of *H. sacchari* from sugarcane (E175) and rice (E177) from Chad and Ivory Coast, respectively were added to the molecular analysis. Outgroup species used for the establishment of the genetic similarities between the nematode isolates were *H. ciceri* and *H. schachtii*, pathogenic to chick pea and beet, respectively. When necessary, the cyst samples were identified on morphometrics of the juveniles and the cyst characters with the keys to the species established by Mulvey (1972) and Wouts et al. (1995).

PCR-RFLP procedure

Species characterization of a set of graminaceous cyst nematodes was previously assessed using PCR-RFLP of the rDNA (Bekal et al., 1997). In the present study, a second set of isolates was processed through the same technique with the following minor modifications (Table 1). For each nematode isolate, total genomic DNA was extracted from three individual gravid cysts, washed and moistened overnight in distilled water at 4 °C. The three cysts were individually processed as following. Cysts were cut and eggs were squashed between two glass slides under a stereomicroscope, placed into a laminar hood to avoid contamination. The squashed cyst content was recovered into 150 µl of lysis buffer (200 mM Tris–HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Proteins were removed with 75 µl of sodium acetate (3 M, pH 5.2) at −20 °C for 10 min. After centrifugation, the DNA precipitated from the supernatant with 100% isopropanol. The DNA pellet was washed in 70% ethanol, air-dried and resuspended in 20 µl of TE (Tris–HCl 10 mM, EDTA 1 mM).

Table 1. Origin of cyst nematodes (*Heterodera*) used in this study

Species and isolate code	Location	PCR/RFLP analysis[1]	Morphometrics analysis[2]	Country	Source[3]
H. arenaria					
E167	Ste Barbe	+[b]	+	France	S. Valette
E183	Vilde la Marine	+[b]	+	France	S. Valette
H. avenae					
B96	Horsham	+[b]	−	Australia	R.H. Brown
E45	Djeudel	+[b]	−	Algeria	A. Mokabli
E46	Meknès	+[b]	−	Morocco	G. Caubel
E50	South Australia	+[a]	+	Australia	J.M. Fischer
E56	Bet Dagan	+[b]	−	Israel	Y. Spiegel
E57	Nir Oz	+[a,b]	+	Israel	Y. Spiegel
E70	Nir Oz	+[b]	−	Israel	Y. Spiegel
E71	Erez	+[b]	−	Israel	Y. Spiegel
E79	La Barbière	+[b]	+	France	R. Rivoal
E83	Najafgarh	+[a,b]	+	India	K.K. Kaushal
E84	Ludhiana	+[b]	+	India	K.K. Kaushal
E124-1	Boueidar	+[b]	+	Syria	R. Rivoal
E125	Denish/Bayaa	+[b]	−	Syria	R. Rivoal
E126	El Bab	+[b]	−	Syria	R. Rivoal
E128	Bejaia	+[b]	−	Algeria	A. Mokabli
E129	Tiaret	+[b]	−	Algeria	A. Mokabli
E130	Relizane	+[b]	−	Algeria	A. Mokabli
E131	Oued-Smar	+[b]	−	Algeria	A. Mokabli
E132	Birtouta	+[b]	−	Algeria	A. Mokabli
E142	Ain Defla	+[b]	−	Algeria	A. Mokabli
E143-1	Staoueli	+[b]	−	Algeria	A. Mokabli
E144	Bouira	+[b]	−	Algeria	A. Mokabli
E147	Beaucaire	+[b]	−	France	R. Rivoal
E171	Fanshan	+[b]	+	China	D. Peng
E172	Horsham	+[b]	−	Australia	J. Jahier
E173	St Aubin de Baubigné	+[b]	−	France	A. Buisson
Ha12 (Fr4)	Nuisement/Coole	+[a]	+	France	R. Rivoal
Ha41 (Fr1)	Villasavary	+[a,b]	+	France	R. Rivoal
IA	Argentan	+[b]	−	France	R. Rivoal
IB	Argentan	+[b]	−	France	R. Rivoal
H. ciceri					
E146	Idleb	+[b]	−	Syria	N. Greco
H. filipjevi					
A26	Karnobat	+[a]	+	Bulgaria	D. Stoyanov
E49	Torralba de Calatrava	+[a,b]	+	Spain	M. Romero
E55	Etelhem	+[a]	+	Sweden	A. Ireholm
E88	Pushkin	+[a,b]	+	Russia	S.A. Subbotin
H. hordecalis					
E166	Ste Barbe	+[b]	+	France	S. Valette
H. latipons					
E69	Gilat	+[a,b]	+	Israel	Y. Spiegel
E99	Aimargues	+[b]	−	France	H. Marzin
E100	Breda	+[a]	+	Syria	U. Scholz
E123	Tel Hadya	+[b]	−	Syria	R. Rivoal
E124-2	Boueidar	+[b]	+	Syria	R. Rivoal
E137	Breda	+[b]	−	Syria	R. Rivoal
E156	Homs	+[b]	−	Syria	R. Rivoal
H. mani					
A36	Morfa Maur	+[b]	+	Great Britain	R. Cook

Table 1. (*Continued*)

Species and isolate code	Location	PCR/RFLP analysis[1]	Morphometrics analysis[2]	Country	Source[3]
E54	Saint Germain	+[a]	+	France	H. Marzin
E87	Hamminkeln	+[a]	+	Germany	D. Sturhan
H. sacchari					
E175	Sarh	+[b]	−	Chad	G. Reversat
E177	Bouaké	+[b]	−	Ivory Coast	G. Reversat
H. schachtii					
Hs	Laon	+[b]	−	France	G. Caubel
Unidentified species					
E143-2	Staoueli	+[b]	−	Algeria	A. Mokabli
E170	California	+[b]	+	USA	S. Bekal

[1]+: Assessed by Bekal et al. (1997) (a) or present study (b).
[2]+: Tested; −: untested.
[3]S. Valette, INRA, Le Rheu, France. R.H. Brown, Plant Research Institute, Burnley, Australia; A. Mokabli, INA, El Harrach, Algeria; G. Caubel, INRA, Le Rheu, France; J.M. Fischer, University of Adelaide, Australia; Y. Spiegel, The Volcani Center, Bet Dagan, Israel; R. Rivoal, INRA, Le Rheu, France; K.K. Kaushal, IARI, New Dehli, India; D. Peng, Academy of Agricultural Sciences, Beijing, China; J. Jahier, INRA, Le Rheu, France; A. Buisson, LNPV, Le Rheu, France; N. Greco, CNR, Bari, Italia; D. Stoyanov, Plant Protection Institute, Kostinbrod, Bulgaria; M. Romero, CSIC, Madrid, Spain; A. Ireholm, Swedish University of Agricultural Sciences, Alnarp, Sweden; S.A. Subbotin, Academy of Sciences, Moscow, Russia; H. Marzin, LNPV, Le Rheu, France; U. Scholz, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany; R. Cook, IGER, Aberystwyth, Great Britain; D. Sturhan, Institut für Nematologie, Münster, Germany; Reversat, IRD, Bondy, France; S. Bekal, University of Illinois, Urbana, USA.

PCR amplification of the ITS region was performed using the 18S and 26S primer pair (Vrain et al., 1992). The primer sequences used were 5′-TTG-ATT-ACG-TCC-CTG-CCC-TTT-3′(18S) and 5′-TTT-CAC-TCG-CCG-TTA-CTA-AGG-3′ (26S). PCR was carried out in a 110 µl reaction volume containing 1X Taq buffer, 1.94 mM $MgCl_2$, 0.25 µM of each primer, 0.1 mM of each dNTPs, 1.6 U Taq DNA polymerase and 12 µl of DNA solution. PCR cycles consisted of an initial denaturation step at 94 °C for 1 min followed by 30 cycles of 1 min at 94 °C (denaturation), 50 s at 60 °C (annealing) and 1 min at 72 °C (polymerization). A final extension cycle (72 °C, 5 min) was used to terminate the reaction. This PCR program was performed in a 480 Perkin Elmer DNA thermal cycler. PCR reactions were visualized in a 1% agarose gel, stained with BET.

For each isolate, PCR products from the three individual cysts were digested separately with a set of 10 restriction endonucleases: *Dde* I, *Hae* III, *Hin*f I, *Hpa* I, *Ita* I, *Mae* III, *Pst* I, *Rsa* I, *Taq* I and *Tru* 9I. A 10 µl aliquot was digested with 2U of the enzyme overnight following the manufacturer's recommendations. The fragments generated were separated in a 2% agarose gel (with 50% of standard agarose and 50% of high resolution agarose) in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) for 3 h at 120 V.

Morphometric analysis

Morphometric analysis was conducted on 23 isolates of the species belonging to the *H. avenae* complex using a subsample of the isolate analyzed or, specifically, the three cut cysts and an aliquot of the same juveniles processed through the PCR-RFLP. Juveniles were fixed in boiling TAF (Hooper, 1970), mounted and examined with an Orthoplan Leitz light microscope. Morphometric traits used of the juveniles involved the lengths of the body (J-Lto), the stylet (J-Ls), the tail (J-Lt) and the hyaline part of the tail (J-Lht) plus the ratios J-C = J-Lto/J-Lt) and J-B = J-Lht/J-Ls. Cysts were prepared according to Hooper (1970) and their morphological study included cone structure, fenestra length (C-Lf), vulval bridge width (C-Wvb), vulval slit length (C-Lvs) as well as the importance of bullae (C-Bul) and the absence or presence of an underbridge, linked or separated to the vulval bridge (C-ub). Numbers of

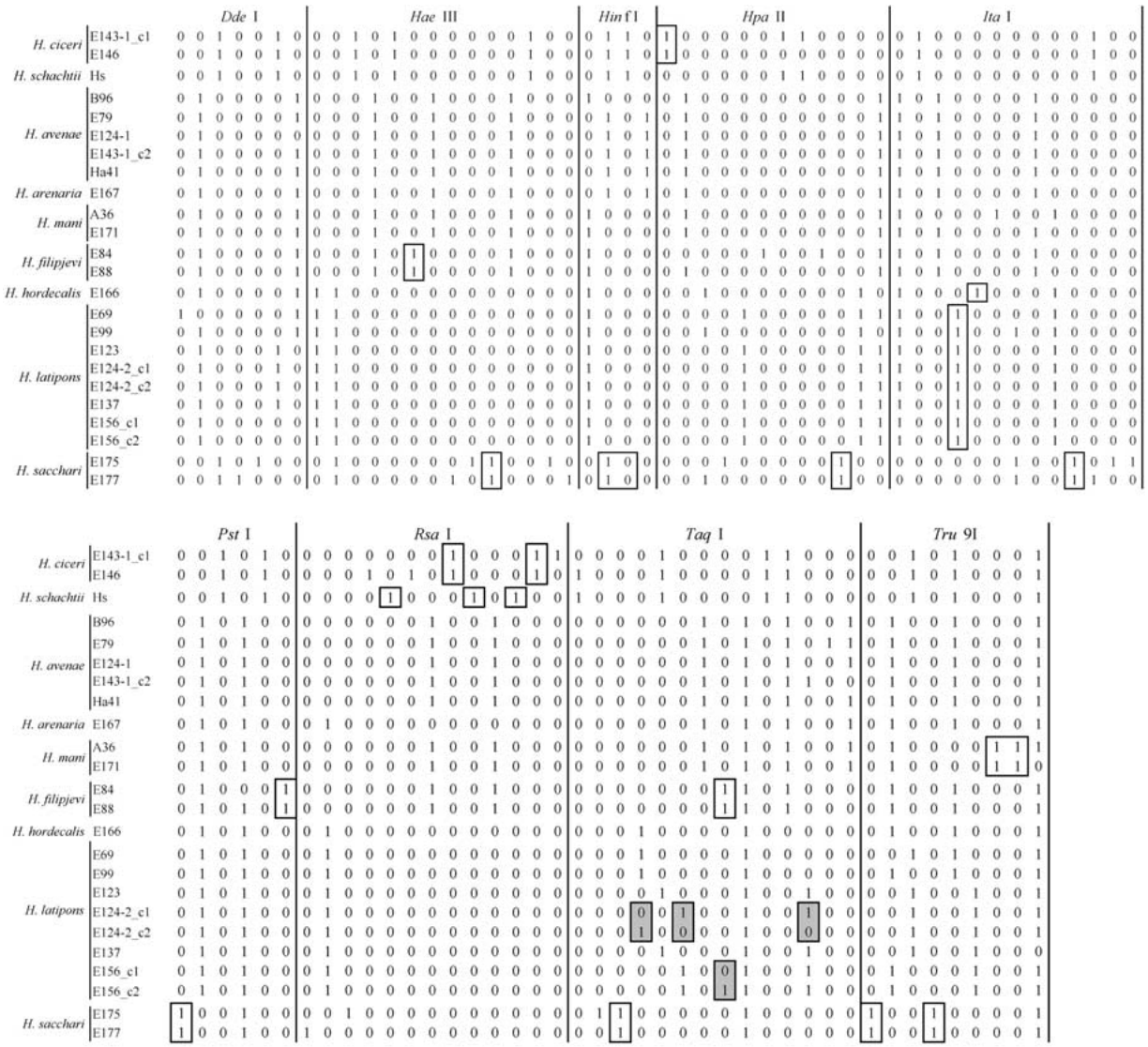

Figure 1. Matrix of PCR-RFLP fragments of the ribosomal DNA region, digested by nine endonucleases *Dde* I, *Hae* III, *Hinf* I, *Hpa* II, *Ita* I, *Pst* I, *Rsa* I, *Taq* I and *Tru* 9I, observed for 24 isolates of different cyst nematodes (*Heterodera*). The presence of a band is coded by 1 and its absence by 0. Only one representative is given when nematode isolates showed monomorphic patterns. The white boxes correspond to markers which differentiate nematode species; grey boxes indicate genetic polymorphism between cysts (c1 and c2) of the same isolate.

cysts and juveniles analyzed per nematode population are given in Table 4.

Data analysis

The patterns of DNA bands inferred from restriction endonuclease digestion of the ITS were compiled in a binary matrix coded as '1' or '0', referring to the presence or absence of the band, respectively. The genetic similarities between isolates and species were established using the Nei's genetic distance (Nei and Li, 1979). Cluster analysis with the unweighted pair group method using arithmetic averages (UPGMA) was applied using the NEIGHBOR

program. Bootstrap analysis (Felsenstein, 1985) was performed to determine statistical consistency of the classification. Both analysis were performed using PHYLIP software (Felsenstein, 1993).

The mean values of the 11 morphometric parameters were processed through a principal component analysis to highlight the relationships between 23 isolates belonging to *H. arenaria*, *H. avenae*, *H. filipjevi*, *H. latipons*, *H. mani* and unidentified *Heterodera* sp. Euclidean distances inferred from this analysis allowed groupings of isolates and species by direct hierarchical classification using Ward's method (Lebart et al., 2000). These analysis were performed with SPAD (Système pour l'analyse de données) release 4.01 (CISIA-CERESTA, Montreuil, France).

Results

PCR amplification and RFLP analysis

For all nematode populations and each cyst analyzed, the amplification of the rDNA region produced a single fragment of approximately 1200 bp. Except for *Mae* III, all the endonucleases tested gave reliable patterns which were to score. Figure 1 describes the PCR-RFLP patterns generated by the nine endonucleases in 24 genotypes of the cyst nematodes tested. No single enzyme was able to separate all the nematode isolates and the species studied. The endonuclease *Dde* I differentiated the graminaceous cyst isolates of *H. arenaria*, *H. avenae*, *H. filipjevi*, *H. hordecalis* and *H. mani* from *H. sacchari* and from the outgroup *H. ciceri* and *H. schachtii*. *Hae* III, *Ita* I and *Rsa* I endonucleases separated all the species (Table 2; Figure 2A,B). *Heterodera arenaria* E167 and E183 were differentiated from *H. avenae* species only with the *Rsa* I. Intraspecific differentiation between isolates of the same species was observed in *H. avenae* with *Hin*f I (Figure 2C) and in *H. latipons* with a higher number of endonucleases as *Dde* I, *Hpa* II, *Taq* I and *Tru*9 I. Genetic polymorphism was demonstrated between cysts within E124 and E156 isolates of *H. latipons* with *Taq* I.

The Nei and Li genetic distances (d) among the isolates ranged from 0.021 to 0.956, which was observed between the Chinese E171 and the outgroup *H. schachtii*. The smallest distance resulted between isolates of *H. avenae*, in particular, between E79 and Ha41. Genetic distances increased when Ha41 was compared to *H. arenaria* E167 and E183 ($d = 0.067$), *H. filipjevi* E88 ($d = 0.230$), *H. hordecalis* E166 ($d = 0.561$) and *H. latipons* E69 ($d = 0.619$). The genetic distance between *H. ciceri* and *H. schachtii* was low ($d = 0.217$). But these two outgroup species, isolates of *H. avenae* and those of *H. sacchari*, were equally distant from each other with d values ranging from 0.804 to 0.867. The analysis showed a sound genetic variability between isolates of the same species, particularly in *H. avenae* or *H. latipons*, ranging from $d = 0.021$ to $d = 0.111$ and $d = 0.026$ to $d = 0.405$, respectively (Table 3). This variability was documented also within two isolates of *H. latipons* where two cysts differed by $d = 0.026$ in E156 or by $d = 0.077$ in E124.

The dendrogram inferred from the UPGMA analysis illustrated the genetic relatedness of the 47 nematode isolates (Figure 3). At a distance of 0.177, four main clusters were delineated, supported by bootstrap values above 95%. Cluster I grouped all isolates of

Table 2. Segregation of species of cyst nematodes (*Heterodera*) according to their rDNA ITS/RFLP patterns

Restriction enzyme	Species grouping[1]				
Dde I	Har + Hav + Hf + Hh + Hm	Hl	Hc + Hsch	Hsac	
Hae III	Har + Hav + Hm	Hf	Hh + Hl	Hsac	Hc + Hsch
*Hin*f I	Har + Hav	Hf + Hh + Hl + Hm	Hc + Hsch	Hsac	
Hpa II	Har + Hav + Hf + Hm	Hl	Hc	Hsch	Hsac
Ita I	Har + Hav + Hf	Hh	Hl	Hc + Hsch	Hsac
Pst I	Har + Hav + Hh + Hl + Hm	Hf	Hc + Hsch	Hsac	
Rsa I	Hav + Hf + Hm	Har + Hh + Hl	Hc	Hsch	
Taq I	Har + Hav + Hm	Hf	Hc + Hsch	Hsac	
Tru 9I	Har + Hav + Hf + Hh	Hm	Hc + Hsch	Hsac	

[1]Har: *H. arenaria*; Hav: *H. avenae*; Hc: *H. ciceri*; Hf: *H. filipjevi*; Hh: *H. hordecalis*; Hl: *H. latipons*; Hm: *H. mani*; Hsac: *H. sacchari*; Hsch: *H. schachtii*.

Table 3. Genetic distances established from PCR-RFLP of the rDNA between and within isolates in the cereal cyst nematodes *Heterodera avenae* and *Heterodera latipons*

H. avenae	B96	E79	E124-1	E143-1-c2	Ha41				
B96	—								
E79	0.087	—							
E124-1	0.091	0.043	—						
E143-1-c2	0.111	0.064	0.067	—					
Ha41	0.067	0.021	0.022	0.043	—				
H. latipons	E69	E99	E123	E124-2-c1	E124-2-c2	E137	E156-c1	E156-c2	E166
E69	—								
E99	0.211	—							
E123	0.231	0.333	—						
E124-2-c1	0.231	0.333	0.050	—					
E124-2-c2	0.158	0.263	0.077	0.077	—				
E137	0.263	0.368	0.026	0.077	0.105	—			
E156-c1	0.263	0.263	0.128	0.077	0.158	0.158	—		
E156-c2	0.282	0.282	0.150	0.100	0.179	0.179	0.026	—	
E166	0.243	0.081	0.368	0.368	0.297	0.405	0.297	0.316	—

H. avenae sensu stricto but a subgroup represented by the Australian B96, E172 and at a lesser extent, the French E79, the Syrian E124-1 and the unidentified *Heterodera* E143-1_c2 from Algeria was slightly differentiated. *Heterodera arenaria* and *H. mani* were included in this cluster with branching supported by low values of bootstrap, 47% and 61%, respectively. In contrast, isolates of *H. filipjevi* from Russia (E88), Spain (E49) and India (E84) previously given as *H. avenae*, were discarded from the three former species with 99% of bootstrap consistency. Cluster II grouped *H. hordecalis* (E166) with isolates of *H. latipons* from France (E99), Israel (E69) and Syria (E123, E124-2, E137, E156) which showed a clear genetic diversity according to their geographical origin. The remaining two clusters grouped either isolates of *H. ciceri*, *H. schachtii* plus the unidentified Algerian E143-2 (cluster IV) or those of *H. sacchari* (cluster III). A significant separation was observed between clusters III, IV and those from I and II, representing *H. avenae sensu lato* and *H. hordecalis*/*H. latipons* isolates. Isolates distributed in different clusters demonstrated the mixture of species such as for E143-1 and E183, presumably representatives of *H. avenae* and *H. arenaria*, respectively.

Morphometric data

Measurements of cysts and juveniles from 23 isolates of graminaceous cyst nematodes are given in Table 4. The correlation circle inferred from a principal component analysis applied to these data plus the characteristics of the bullae, the underbridge, the ratios B and C showed three groups of parameters which were highly correlated (Figure 4A). On the left side are grouped cyst parameters as the length of fenestra (C-Lf) and vulval slit (C-Lvs), the width of the vulval bridge (C-Wvb), and at a lesser extent the variation observed into the underbridge depth (C-ub). These parameters were negatively correlated with the total length of juveniles (L-to) and the importance of bullae (C-Bul). The third group was composed essentially of parameters associated with the juvenile stage such as the lengths of the tail (entire or hyaline part) and the ratio J–B. The length of stylet (J-Ls) supported differentiation between isolates on the third axis of the analysis (not shown). The ratio J–C expressed a low correlation in this analysis.

The plan defined by axis 1 and 2 which accounts for 82% of the total variation demonstrated separations between groups of isolates which showed a certain level of variability (Figure 4B). The main group included all the isolates of identified and presumed *H. avenae* and *H. mani* species, but the French E79 and the Chinese E171 isolates were separated from the rest of the group, essentially by the reduced length of juveniles (Table 4). A second group of isolates was composed of *H. arenaria* (E167, E183), *H. avenae* (E124-1) and the unidentified E170 which showed some variation in their juvenile morphometrics. *H. arenaria* E167 was differentiated by a lower C ratio (7.6) compared to values from 8.1 to 8.3 observed in the three other

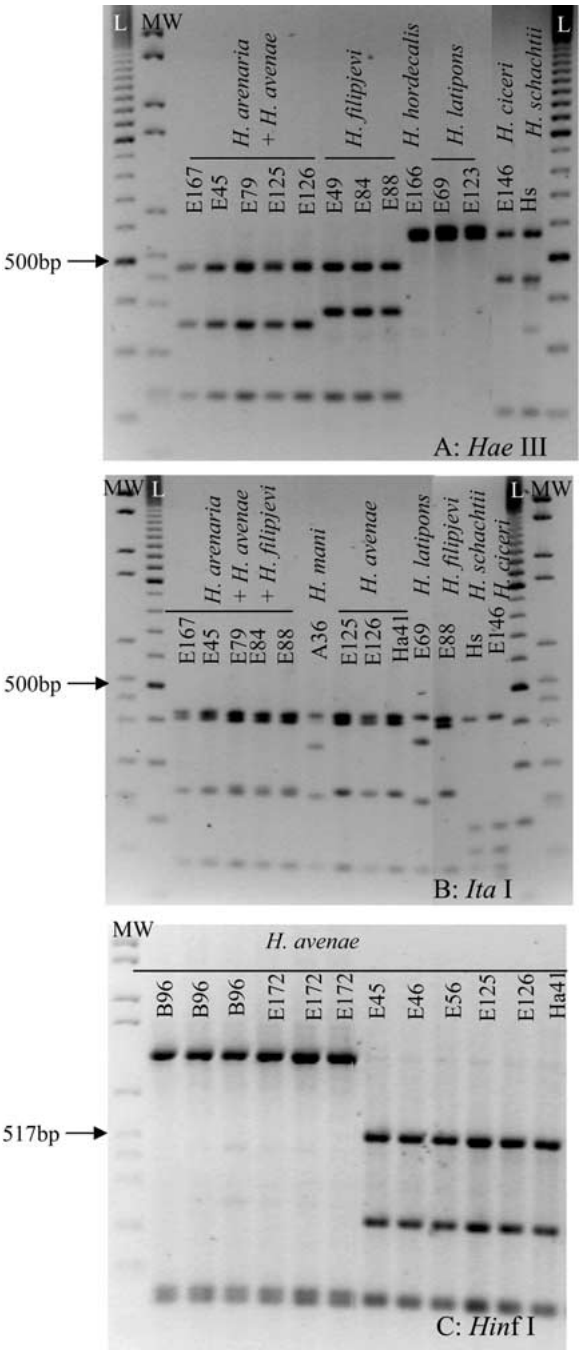

Figure 2. PCR-RFLP patterns inferred from three restriction endonucleases applied to the rDNA ITS amplicon of different species of cyst nematodes (*Heterodera*). MW: molecular weight marker VI (Roche); L: 100 bp DNA ladder (Tebu). See Table 1 for the origin of nematode isolates.

isolates and by the higher length of juveniles (596 μm) in contrast to 564 μm measured for E170. The third group was composed of typical *H. filipjevi* and the presumed *H. avenae* E84 which showed higher measurements of the juvenile tail.

Isolates of *H. hordecalis* and *H. latipons* were clearly differentiated from the three previous groups as juveniles, by the shorter length of the body, the values of ratio B lower or equal to 1.3. The isolates were also differentiated as cysts, by the presence of a deep and strong underbridge and the greater length of fenestra. Nevertheless, they showed a dramatic variation in morphometrics, which seemed greater between the isolates E100 and E124-2 originating both from Syria than between the Syrian E100 and the Israeli E69. *Heterodera latipons* E124-2 was morphologically closer to *H. hordecalis* E166, differing only by the width of the vulval bridge, the length of the vulval slit or the stylet and, to a lesser extent, by the ratios B and C with upper values as 1.6 against 1.3 in the first case, 8.2 against 7.1 in the second.

The direct hierarchical classification demonstrated groupings of isolates and species, which were quite in agreement with the previous relationships based on molecular markers (Figure 5). Under distance index 1, are grouped the representatives of *H. arenaria*, *H. avenae*, *H. mani* and the unidentified E170 (cluster A). All the isolates of *H. filipjevi* and the presumed *H. avenae* E84 from India differed slightly from the previous group at a distance index close to 2 (cluster B). The group including *H. hordecalis* and *H. latipons* isolates with the distance index above 5 differed clearly from the other nematode populations (cluster C).

Discussion

The rDNA intergenic spacer region has been useful for diagnostics and phylogenetic relationships between isolates and taxa of plant parasitic nematodes (Ferris et al., 1993; 1994). Our PCR-RFLP results demonstrated that, apart from the representatives of *H. sacchari*, two divergent groups were readily observed in this complex of graminaceous nematodes. The first group includes the isolates of the closely related species *H. arenaria*, *H. avenae*, *H. mani* and *H. filipjevi*. The second was composed of the isolates of *H. hordecalis* and *H. latipons*.

Our results are in accordance with Bekal et al. (1997), and we confirmed that the graminaceous

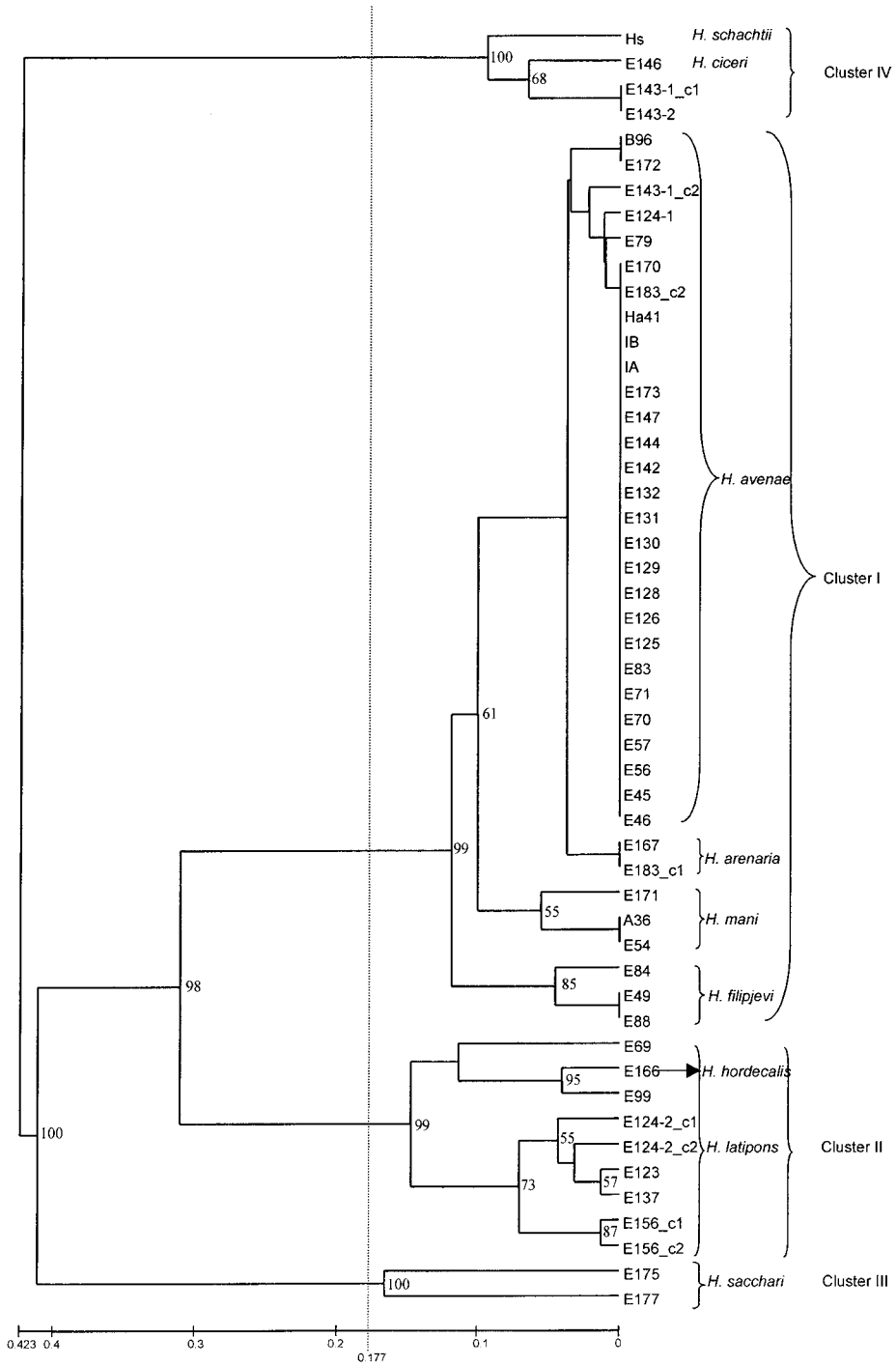

Figure 3. UPGMA dendrogram between 47 isolates of cyst nematodes (*Heterodera*), based on the genetic distances calculated from PCR-RFLP data. See Table 1 for the origin of nematode isolates; c1 and c2 indicated the cyst number which corresponds to individual PCR-RFLP reactions. Bootstrap values (%) were obtained after 1000 resamplings.

Table 4. Measurements (means in μm ± SD; number of individuals measured in brackets) of morphological parameters in cysts and juveniles of graminaceous cyst nematodes (*Heterodera*)

Species and isolates[1]	Vulval cone				Second stage juvenile		
	Fenestra length	Vulval bridge width	Vulval slit length	Total length	Stylet length	Tail length	Hyaline part of tail length
H. arenaria							
E167	49 ± 3.9 (10)	7 ± 1.9 (10)	9 ± 0.7 (10)	596 ± 20.2 (25)	28 ± 0.6 (20)	78 ± 6.7 (23)	53 ± 6.7 (23)
E183	51 ± 1.2 (3)	6 ± 1.2 (3)	8 ± 1.8 (3)	584 ± 22.4 (29)	29 ± 0.8 (20)	72 ± 2.7 (21)	47 ± 3.1 (21)
H. avenae							
E50	47 ± 3.9 (23)	5 ± 0.8 (23)	7 ± 1.1 (23)	610 ± 50.2 (20)	25 ± 1.0 (20)	67 ± 6.1 (20)	43 ± 3.4 (20)
E57	48 ± 5.0 (16)	5 ± 1.6 (18)	8 ± 0.8 (19)	600 ± 63.0 (31)	25 ± 0.6 (19)	64 ± 5.6 (31)	40 ± 3.5 (19)
E79	51 ± 2.2 (2)	7 ± 1.1 (2)	6 (2)	523 ± 25.4 (25)	26 ± 0.4 (13)	64 ± 2.9 (13)	38 ± 2.1 (13)
E83	43 ± 4.0 (20)	5 ± 1.1 (20)	8 ± 0.9 (20)	600 ± 45.0 (30)	25 ± 1.2 (20)	67 ± 4.2 (30)	40 ± 3.4 (20)
E84	53 ± 1.6 (2)	9 ± 0.6 (3)	9 (3)	579 ± 12.8 (19)	26 ± 1.0 (12)	66 ± 2.2 (12)	38 ± 2.7 (12)
E124-1	49 ± 1.6 (2)	9 ± 1.1 (2)	9 ± 1.1 (2)	579 ± 16.0 (26)	27 ± 0.9 (13)	70 ± 3.3 (13)	46 ± 1.4 (13)
E171	44 ± 2.7 (3)	5 ± 1.8 (3)	6 ± 0.5 (3)	508 ± 32.1 (21)	25 ± 0.7 (15)	64 ± 4.7 (15)	39 ± 2.5 (15)
Ha12 (Fr4)	48 ± 4.5 (19)	4 ± 0.5 (20)	8 ± 0.5 (20)	650 ± 26.3 (27)	26 ± 1.0 (20)	70 ± 4.4 (27)	43 ± 3.2 (20)
Ha41 (Fr1)	42 ± 2.2 (19)	5 ± 0.9 (19)	8 ± 0.7 (19)	620 ± 43.0 (30)	26 ± 0.9 (20)	71 ± 4.9 (30)	45 ± 3.0 (20)
H. filipjevi							
A26	51 ± 11.9 (13)	12 ± 4.0 (22)	10 ± 1.7 (22)	550 ± 40.5 (31)	25 ± 0.7 (22)	58 ± 3.8 (31)	37 ± 2.2 (22)
E49	48 ± 4.1 (20)	4 ± 0.7 (25)	9 ± 1.9 (25)	510 ± 33.2 (30)	24 ± 0.9 (25)	57 ± 5.5 (30)	34 ± 4.7 (25)
E55	46 ± 6.8 (19)	5 ± 0.1 (22)	8 ± 1.0 (22)	535 ± 20.0 (31)	24 ± 0.9 (22)	59 ± 2.7 (31)	34 ± 2.9 (22)
E88	52 ± 4.2 (20)	9 ± 2.8 (19)	9 ± 1.0 (17)	550 ± 18.7 (18)	25 ± 0.9 (18)	60 ± 3.2 (18)	37 ± 2.5 (18)
H. hordecalis							
E166	71 ± 6.4 (13)	26 ± 5.0 (13)	16 ± 2.6 (13)	447 ± 4.2 (25)	26 ± 0.6 (25)	63 ± 9.5 (26)	34 ± 3.2 (26)
H. latipons							
E69	61 ± 4.9 (20)	12 ± 5.2 (15)	12 ± 2.9 (19)	470 ± 33.0 (19)	24 ± 0.6 (18)	49 ± 5.9 (19)	28 ± 3.1 (19)
E100	66 ± 9.5 (19)	31 ± 5.0 (18)	11 ± 2.6 (19)	475 ± 23.0 (23)	24 ± 0.7 (19)	50 ± 2.7 (23)	30 ± 2.9 (19)
E124-2	70 ± 2.2 (2)	35 ± 3.8 (3)	30 ± 2.5 (3)	467 ± 15.1 (23)	22 ± 0.7 (10)	57 ± 3.7 (16)	35 ± 3.8 (16)
H. mani							
A36	42 ± 3.4 (18)	5 ± 0.7 (22)	7 ± 1.1 (22)	590 ± 24.0 (31)	24 ± 0.6 (22)	67 ± 3.6 (31)	44 ± 3.4 (22)
E54	37 ± 3.3 (19)	5 ± 1.6 (19)	8 ± 0.8 (19)	555 ± 64.5 (29)	23 ± 0.6 (19)	64 ± 6.0 (29)	38 ± 2.8 (19)
E87	47 ± 2.7 (14)	5 ± 1.4 (19)	7 ± 1.4 (19)	580 ± 30.0 (32)	24 ± 0.8 (19)	64 ± 4.7 (33)	40 ± 4.4 (19)
Unidentified species							
E170	52 ± 1.9 (3)	6 ± 0.8 (3)	7 ± 0.9 (3)	564 ± 25.8 (26)	26 ± 2.5 (20)	68 ± 2.9 (20)	43 ± 3.2 (20)

[1]See Table 1 for the origin of species and isolates.

cyst nematodes showed an extensive genetic diversity resulting most likely from elaborate evolutionary events which have occurred in this complex of species. The comparative morphometric study showed morphological divergences between species and isolates, however an agreement was observed between genetic and morphometric data for the nematode samples utilising molecular and conventional systematic analyses. These results support the previous findings regarding the strength of the relative relationship between molecular polymorphism and morphological traits (Reed and Frankham, 2001).

The principal component analysis showed that these two groups are basically separated by the morphological characters of the vulval cone (e.g. bullae, underbridge and vulval bridge width) which, in the present study, revealed more reliable and significant information than previously discussed by Ferris (1979). The female attributes revealed contrasting features according to the separated groups. Strong bullae were associated with a narrow vulval bridge in the *H. arenaria–H. avenae–H. mani* group and are replaced by few bullae, a strong and deep underbridge and a wider vulval area in the *H. hordecalis–H. latipons* group (Wouts et al., 1995).

Within the *sensu lato H. avenae* group (cluster I), our results show a clear genetic differentiation of *H. arenaria* isolates which was supported

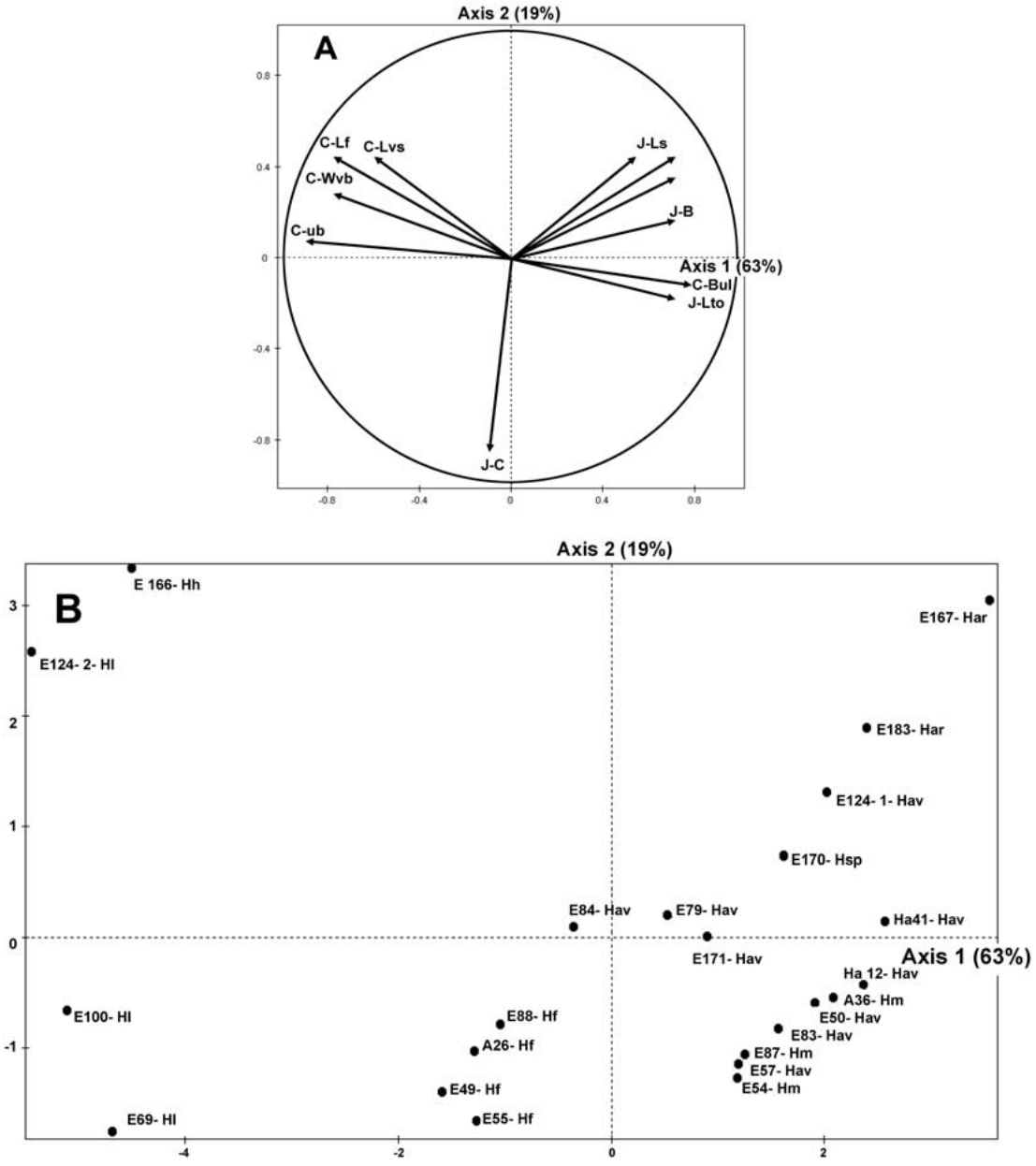

Figure 4. Correlations (A) between morphometric parameters of cysts: fenestra length (C-Lf), vulval bridge width (C-Wvb), vulval slit length (C-Lvs), underbridge (C-ub), bullae size (C-Bul), juveniles: total length (J-Lto), tail length (J-Lt), hyaline tail length (J-Lht), stylet length (J-Ls) and ratios J–C (J-Lto/J-Lt) or J-B (J-Lht/J-Ls) and distribution (B) of isolates of graminaceous cyst nematodes *Heterodera arenaria*: Har; *Heterodera avenae*: Hav; *Heterodera filipjevi*: Hf; *Heterodera hordecalis*: Hh; *Heterodera latipons*: Hl; *Heterodera mani*: Hm; unidentified *Heterodera*: Hsp, inferred from a principal component analysis. See Table 1 for the origin of isolates.

by morphometrics of the tail in juveniles. Although Ibrahim and Rowe (1995) have already separated *H. arenaria* from closely related species with esterases, neither Subbotin et al. (1999) nor Clapp et al. (2000) using PCR-RFLP and PCR/SSCP, respectively have succeeded in making such differentiation. Our success could arise from the amplification of a longer part of the ribosomal DNA, 1200 bp instead of 1060 bp for

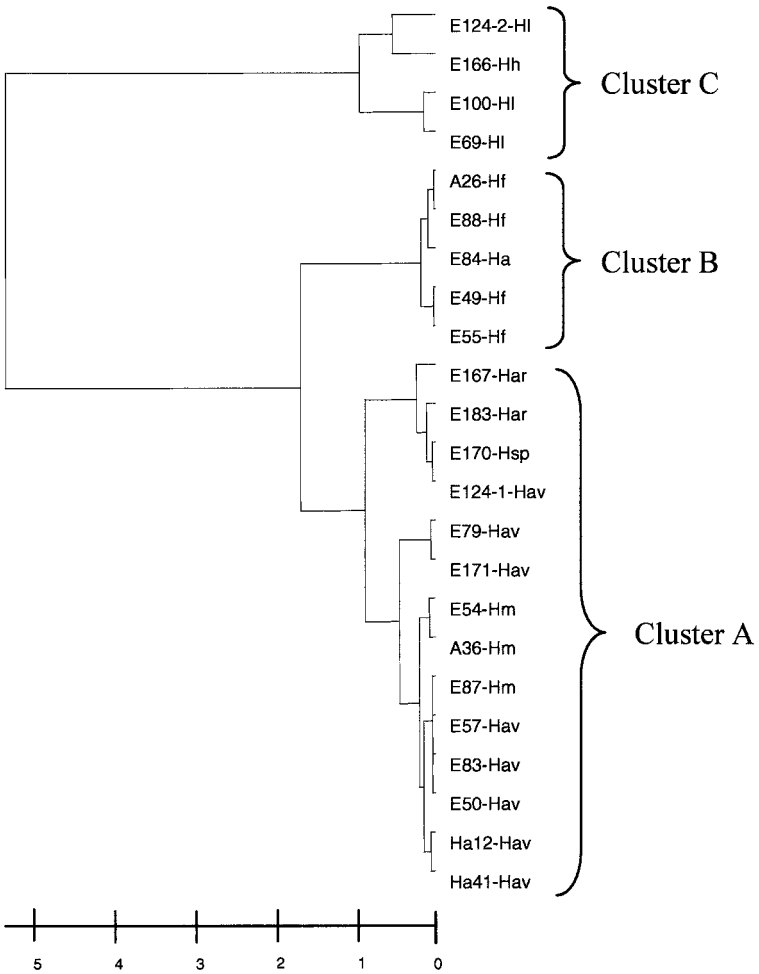

Figure 5. Dendrogram showing the groupings of isolates of graminaceous cyst nematodes *(Heterodera arenaria*: Har; *Heterodera avenae*: Hav; *Heterodera filipjevi*: Hf; *Heterodera hordecalis*: Hh; *Heterodera latipons*: Hl; *Heterodera mani*: Hm; unidentified *Heterodera*: Hsp) inferred from a principal component analysis applied on morphometric parameters. Scale values indicated distances between nodes. See Table 1 for the origin of isolates.

Subbotin et al. (1999), suggesting that the targeted restriction site was located in the supplement amplicon. The true identity of isolates from Syria (E124-1) or from California (E170) was nevertheless questionable.

Our results indicated also that the morphological differentiation between *H. avenae sensu stricto* and *H. mani* is not as clear as previously described (Mathews, 1971; Cook, 1982; Sturhan, 1982). Recently, analysis of IEF enzyme patterns (Andrés et al., 2001) concluded that the German isolate of *H. mani* from Hamminkeln (E87), already characterized by Bekal et al. (1997), was not significantly differentiated from *H. avenae sensu stricto*. The genetic and phenotypic variation in this group included also the French E79 and the Chinese E171. Zheng et al. (2000) have also observed that RFLP profiles of PCR-ITS products by *Hin*f I and *Tru*9 I differentiated unexpectedly Chinese populations, from typical *H. avenae*.

Based on our genetic data, the Indian E84 was more related to *H. filipjevi* than to *H. avenae*. Conversely, this isolate was morphologically close to the *H. avenae sensu lato* grouping, but presented the typical underbridge described in *H. filipjevi* (Madzhidov, 1981). Subbotin et al. (1999) have found that an Indian population sampled in the desert differed genetically from all others isolates of *H. avenae*, and suggested that this

discrimination resulted in an intraspecific polymorphism revealed by the *Alu* I and *Rsa* I digestion of the PCR amplified product. Our study enabled the separation of two isolates sampled in the same field cropped with barley in Syria, belonging to either *H. avenae* (E124-1) or to *H. latipons* (E124-2). The mixture of both species in this region was expected as *H. avenae sensu stricto* (E125) was sampled in the vicinity of the former field. Cysts belonging to *H. avenae* or close to *H. ciceri* were also diagnosed in an Algerian field (E143).

Genetic infraspecific variation in *H. avenae sensu stricto* has been previously revealed through the *Hae* III restriction pattern which enabled the separation of Australian, Israeli and Saudi Arabian isolates from the rest of *H. avenae* isolates tested (Bekal et al., 1997). Our data again demonstrated the characterization of two other Australian isolates of *H. avenae* originating from different locations, with another enzyme *Hin*f I. They suggest that a variant has spread through different isolates as E50 studied by Bekal et al. (1997) or both B96 and E172 from the present work in concerted evolution as described by Solignac (1995). However, the genetic variability expressed within *H. latipons* isolates as E124-2 and E156, which was detected only with the *Taq* I endonuclease, could be explained by an ITS heterogeneity from uncompleted concerted evolution of multicopy gene families such as rDNA described by Hillis and Dixon (1991). The presence of an extra fragment inferred from digestions with *Alu* I and *Rsa* I in several isolates, as *H. arenaria* E183, suggests the existence of different types of ITS as previously reported in Heteroderidae (Szalanski et al., 1997; Subbotin et al., 1999; 2000).

The *H. hordecalis–H. latipons* cluster showed the largest genetic diversity observed among and within the isolates studied. They appear to represent an evolutionary lineage which differs from the *H. avenae sensu lato* group by both genetic and phenotypic traits. Ferris et al. (1999) suggested the existence of sibling species in *H. latipons* when they compared the sequence of ITS rDNA in two morphologically similar and geographically separated isolates from Israel (Gilat) and Russia (Rostov). Nevertheless, strong differences in morphology between Syrian isolates as E100 and E124-2 may presume the existence in this country of *H. hordecalis*, mainly known in Northern and Central Europe, which was recently presumed to occur with *H. latipons*, in the Gilat site (E69) from Israel (Mor and Sturhan, 2000). However, our PCR-RFLP data showed that the *H. hordecalis* isolate from France was more closely realted to the CCN group than an isolate from Italy which, upon phylogenetic relationships inferred from the rDNA sequence, appeared unexpectedly to be only distantly related to other *Heterodera* species (Sabo et al., 2001).

Our molecular data were in agreement with the taxonomical classification of *H. ciceri* and *H. schachtii* which are both placed in the large '*H. schachtii*' group (Mulvey, 1972; Volvas et al., 1985). In contrast, it was not expected that the representatives of *H. sacchari*, which is morphologically included in the *H. schachtii* group and develops on graminaceae as rice and sugarcane, were genetically so distant from both *H. ciceri–H. schachtii* or from the other graminaceous cyst nematodes. These isolates of *H. sacchari* seem to represent another separate evolutionary lineage of the cyst nematodes (Luc and Merny, 1963; Nobbs et al., 1992).

Combined molecular and classical methods of systematics might enhance knowledge of the diversity of restricted groups of closely related species (Ferris, 1994). Our results demonstrated a genetic diversity in the graminaceous cyst nematodes which was greater than previously observed in the comparison of *H. avenae* isolates through the partial sequence variation in spacer ribosomal DNA (Ferris et al., 1994). They indicate that further investigations are required on this complex of species which have relatively narrow host ranges but are broadly distributed. Attention should be focused on the comparative diversity of isolates of these nematodes originating from different parts of the world and more particularly from the fertile crescent and border areas where they might have evolved along with the cropping of their obligatory hosts. Recent surveys conducted in Iran and Turkey have showed that the main cereal cyst nematodes *H. avenae*, *H. filipjevi* and *H. latipons* occurred frequently and have been previously confused with other entities specific to wild graminaceae as *H. mani* (Rumpenhorst et al., 1996; Sturhan, 1996). Any information gathered about phylogeographic relationships in this nematode complex should contribute to a knowledge base on how the genus *Heterodera* has evolved (Baldwin, 1992; Ferris, 1985; 1998).

This study further expands the catalogue of RFLP in the ITS region for a more confident and easier identification of a wider set of nematode species, as recommended by Subbotin et al. (2000). In addition, the wide diversity in this complex of graminaceous cyst nematodes, which could occur in mixed populations,

must be taken into account for their integrated control based on the resistance of plants (Rivoal et al., 2001).

Acknowledgements

We thank Dr. O. Plantard and Dr. E. Lagudah for their helpful comments and critical reading of the manuscript. The authors are grateful to all persons who have supplied nematode materials and to Dr. Dieter Sturhan for the identification of *H. hordecalis*. Part of this work was achieved in the program France/ICARDA 'Biotechnologies et Amélioration du blé dur et du blé tendre', funded by the Ministère des Affaires Etrangères, France.

References

Andrés MF, Romero MD, Montes MJ and Delibes A (2001) Genetic relationships and isozyme variability in the *Heterodera avenae* complex determined by isoelectrofocusing. Plant Pathology 50: 270–279

Baldwin JG (1992) Evolution of cyst and non cyst-forming Heteroderinae. Annual Review of Phytopathology 30: 271–290

Bekal S, Gauthier JP and Rivoal R (1997) Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. Genome 40: 479–486

Blok VC, Malloch G, Harrower B, Phillips MS and Vrain TC (1998) Intraspecific variation in ribosomal DNA in isolates of the potato cyst nematode *Globodera pallida*. Journal of Nematology 30: 262–274

Clapp JP, Van der Stoel CD and Van der Putten WH (2000) Rapid identification of cyst (*Heterodera* spp., *Globodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformational polymorphism (PCR-SSCP) in cultures and field samples. Molecular Ecology 9: 1223–1232

Cook R (1982) Cereal and grass hosts of some gramineous cyst nematodes. EPPO Bulletin 12: 399–411

Cook R and Rivoal R (1998) Genetics of resistance and parasitism. In: Sharma SB (ed) The Cyst Nematodes (pp 322–352) Chapman and Hall, London

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791

Felsenstein J (1993) PHYLIP (phylogeny inference package). Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

Ferris VR (1979) Cladistic approaches in study of soil and plant parasitic nematodes. American Zoology 19: 1195–1215

Ferris VR (1985) Evolution and biogeography of cyst-forming nematodes. EPPO Bulletin 15: 123–129

Ferris VR (1994) The future of nematode systematics. Fundamental and Applied Nematolology 17: 97–101

Ferris VR (1998) Evolution, phylogeny and systematics. In: Sharma SB (ed) The Cyst Nematodes (pp 57–82) Chapman and Hall, London

Ferris VR, Ferris JM and Faghihi J (1993) Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. Fundamental and Applied Nematology 16: 177–184

Ferris VR, Ferris JM, Faghihi J and Ireholm A (1994) Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. Journal of Nematology 26: 144–151

Ferris VR, Subbotin SA, Ireholm A, Spiegel Y, Faghhihi J and Ferris J (1999) Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates. Russian Journal of Nematology 7: 121–125

Hillis DM and Dixon MT (1991) Ribosomal DNA: Molecular evolution and phylogenetic inference. The Quarterly Review of Biology 66: 411–453

Hooper DJ (1970) Handling, fixing, staining and mounting nematodes. In: Southey JF (ed) Laboratory Methods for Work with Plant and Soil Nematodes. Technical Bulletin 2 (pp 39–54) Her Majesty's Stationery office, London

Ibrahim SK and Rowe JA (1995) Use of isoelectric focusing and polyacrylamide gel electrophoresis of nonspecific esterase phenotypes for the identification of cyst nematodes *Heterodera* species. Fundamental and Applied Nematology 18: 189–196

Iwahori H, Tsuda K, Kanzaki N, Izui K and Futai K (1998) PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. Fundamental and Applied Nematology 21: 655–666

Lebart L, Morineau A and Piron M (2000) Statistique exploratoire multidimensionnelle. 3rd edn, Dunod (ed) Paris, 439 p

Luc M and Merny G (1963) *Heterodera sacchari* n.sp. (Nematoda: Tylenchoidea) parasite de la canne à sucre au Congo-Brazzaville. Nematologica 9: 31–37

Madzhidov AR (1981) [Bidera filipjevi n.sp. (Heteroderina: Tylenchida) in Tadzhikistan.] Izvestiya Akademii Nauk Tadzhikskoĭ SSR, Biologicheskie Nauki 2: 40–44

Mathews HJP (1971) Two new species of cyst nematode, *Heterodera mani* n.sp. and *H. iri* n.sp., from Northern Ireland. Nematologica 17: 553–565

Mor M and Sturhan D (2000) On the identity of *Heterodera latipons* Franklin. Nematology 2: 776–777 (abst.)

Mulvey RH (1972) Identification of *Heterodera* cysts by terminal and cone structures. Canadian Journal of Zoology 50: 1277–1292

Nei M and Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences of the USA 76: 5269–5273

Nicol J (2002) Important nematode pests of cereals. Bread Wheat: Improvement and Production (pp 345–366) FAO Publisher, Rome, Italy

Nobbs JM, Ibrahim SK and Rowe JA (1992) A morphological and biochemical comparison of the four cyst nematode species, *Heterodera elasticha*, *H. oryzicola*, *H. oryzae* and *H. sacchari* (Nematoda: Heteroderidae) known to attack rice

(*Oryza sativa*). Fundamental and Applied Nematolology 15: 551–562

Powers TO, Todd TC, Burnell AM, Murray PCB, Fleming CC, Szalanski AL, Adams BAA and Harris TS (1997) The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. Journal of Nematology 29: 441–450

Reed DH and Frankham R (2001) How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. Evolution 55: 1095–1101

Rivoal R, Bekal S, Valette S, Gauthier JP, Bel Hadj Fradj M, Mokabli A, Jahier J, Nicol J and Yahyaoui A (2001) Variation in reproductive capacity and virulence on different genotypes and resistance genes of Triticeae, in the cereal cyst nematode complex. Nematology 3: 581–592

Rivoal R and Cook R (1993) Nematode pests of cereals. In: Evans K, Trudgill DL and Webster JM (eds) Plant Parasitic Nematodes in Temperate Agriculture (pp 259–303) CAB International, Wallingford

Ritter M (1982) Importance des nématodes à kystes des céréales. Bulletin OEPP 12: 307–316

Rumpenhorst HJ, Elekçioglu IH, Sturhan D, Öztürk G and Enneli S (1996) The cereal cyst nematode *Heterodera filipjevi* (Madzhidov) in Turkey. Nematologia Mediterranea 24: 135–138

Sabo A, Volvas N and Ferris VR (2001) Phylogenetic relationships based on ribosomal DNA data for four species of cyst nematodes from Italy and one from Syria. Journal of Nematology 33: 183–190

Sikora RA (1987) Plant parasitic nematodes of wheat and barley in temperate and temperate semiarid regions – a comparative analysis. In: Saxena MC, Sikora RA and Srivastava JP (eds) Nematodes Parasitic to Cereals and Legumes in Temperate Semi-arid Regions (pp 46–68) Proc. Workshop, Larnaca, Cyprus

Solignac M (1995) Evolution concertée des séquences répétées. In: Hermann (ed) Génétique et évolution, Tome II. L'espèce, l'évolution moléculaire (pp 237–255) Paris

Sturhan D (1982) Distribution of cereal and grass cyst nematodes in the Federal Republic of Germany. EPPO Bulletin 12: 321–324

Sturhan D (1996) Occurrence of *Heterodera filipjevi* (Madzhidov, 1981; Stelter, 1984) in Iran. Pakistan Journal of Nematology 14: 89–93

Subbotin SA, Waeyenberge L and Moens M (2000) Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. Nematology 2: 153–164

Subbotin SA, Waeyenberge L, Molokanova IA and Moens M (1999) Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. Nematology 1: 195–207

Szalanski AL, Sui DD, Harris TS and Powers TO (1997) Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. Journal of Nematology 29: 255–267

Thiéry M and Mugniéry D (1996) Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of Solanaceous plants. Fundamental and Applied Nematology 19: 471–479

Volvas V, Greco N and Di Vito M (1985) *Heterodera ciceri* sp.n. (Nematoda: Heteroderidae) on *Cicer arietinum* L. from Northern Syria. Nematologia Mediterranea 13: 239–252

Vrain TC, Wakarchuk DA, Lévesque AC and Hamilton RI (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundamental and Applied Nematology 15: 563–573

Waeyenberge L, Ryss A, Moens M, Pinochet J and Vrain TC (2000) Molecular characterization of 18 *Pratylenchus* species using rDNA restriction fragment length polymorphism. Nematology 2: 135–142

Wouts WW, Schoemaker A, Sturhan D and Burrows PR (1995) *Heterodera spinicauda* sp.n. (Nematoda: Heteroderidae) from mud flats in the Netherlands, with a key to the species of the *H. avenae* group. Nematology 41: 575–583

Zheng J, Subbotin S, Waeyenberge L and Moens M (2000) Molecular characterisation of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. Russian Journal of Nematology 8: 109–113