Istituto Sperimentale per la Zoologia Agraria, MiPAF - 50125 Firenze, Italy

IDENTIFICATION OF *HETERODERA SCHACHTII* GROUP SPECIES IN ITALY BY MORPHOMETRICS AND RAPD-PCR

by

LAURA AMBROGIONI and TIZIANA IRDANI

Summary. Nine Italian populations of the *Heterodera schachtii* group, including *H. schachtii, H. trifolii* from carnation, *H. trifolii* from sugarbeet and *H. daverti*, were compared using both morphometrical and RAPD-PCR techniques. *H. trifolii* f. sp. *beta* differed markedly from the other populations because of its greater cyst and juvenile dimensions. The size of the tail is statistically the only highly significant morphometric character valid to separate one *H. schachtii* from another, *H. trifolii* from carnation, *H. trifolii* from sugarbeet and *H. daverti.* Cluster analysis of morphometric characters of cyst cones and juveniles distinguish *H. trifolii* associated with sugarbeet from the remaining cyst-forming populations. The dendrogram constructed with molecular data separates *H. schachtii* populations from the *H. trifolii* complex members.

Heterodera schachtii Schmidt, *Heterodera trifolii* Goffart and *Heterodera daverti* Wouts *et* Sturhan have been placed in the *Heterodera schachtii* group (Baldwin and Mundo-Ocampo, 1991). The characteristics of this lemon-shaped cyst nematode group, based on vulval structures, are ambifenestrate terminal regions, a long vulval slit (> 35 µm), bullae and underbridge generally strongly developed. *H. schachtii* and the *H. trifolii* are cosmopolitan whereas *H. daverti*, to the best of our knowledge, occurs only in the Netherlands, Germany, Tunisia and Italy (Wouts and Sturhan, 1978; Nordmeyer, 1979; Ambrogioni *et al.*, 1985).

In 1975 a race of *H. trifolii*, characterised by a distinct yellow phase and its attack on sugarbeet, was described in the Netherlands and called "the yellow beet cyst nematode" (YBCN) (Maas and Heijbroek, 1982). This most distinctive race of *H. trifolii* is commonly distributed in other European countries, namely Sweden, Switzerland, Germany, France (Corsica) and Italy (Andersson, 1984; Vallotton, 1985; Schlang, 1990; Bossis *et al.*, 1997; Porte *et al.*, 1997; Ambrogioni *et al.*, 1999).

Since all these cyst-forming nematodes have a similar host range within Chenopodiaceae, Caryophillaceae and Leguminosae (Maas and Heijbroek, 1982; Ambrogioni *et al.*, 1985; Tacconi, 1997), it is possible that they may occur in the field as mixed populations. Consequently, proper identification is necessary in selecting control measures. In this regard, there are some biological characters which are useful for differentiating these species. The main feature of *H. trifolii* populations, including "the yellow beet cyst nematode", is the lack of males, in contrast with *H. daverti* and *H. schachtii* which have bisexual reproduction (Triantaphyllou and Hirschmann, 1978; Wouts and Sturhan, 1978; Hirschmann and Triantaphyllou, 1979; Sikora and Maas, 1985). Another distinctive character in *H. schachtii* is the lack of a yellow phase in the female, typical of *H. trifolii*, including populations

from sugarbeet, and *H. daverti* (Hirschmann, 1956; Mulvey, 1959; Wouts and Sturhan, 1978; Maas and Heijbroek, 1982; Ambrogioni and Caroppo, 1988). However, this yellow phase is sometimes difficult to observe and not all cysts pass through it (Gerdermann and Linford, 1953). In *H. trifolii* many degrees of yellowing have been noted, from very intense to very faint, and it seems that this yellow coloration is related to the host plant (Norton, 1967). Lastly, all these cyst-forming nematodes produce an eggsac. The size and number of eggs, probably influenced considerably by the host and the environment, are considered of little diagnostic value. The number of eggs varies from a few to more than 100 in *H. schachtii* (Raski, 1950), up to about 200 in *H. trifolii* and in *H. daverti* (Gerdermann and Linford, 1953; Ambrogioni and Caroppo, 1988) and to very few in YBCN (unpublished data).

Most morphometrical characters that are useful for *Heterodera* species identification are often insufficient for differentiating populations of *H. schachtii*, *H. trifolii* and *H. daverti*. Consequently, other alternative methods have been considered in addition to morphometrics.

Biochemical approaches and DNA analysis have proved of value in the separation of some *Heterodera* populations which were difficult to distinguish morphologically (Radice *et al.*, 1988; Williamson, 1991; Ganguly *et al.*, 1992; Ferris *et al.*, 1993; Caswell-Chen *et al.*, 1992; Nobbs *et al.*, 1992; Ibrahim and Rowe, 1995; Subbotin *et al.*, 1999; 2000).

The objective of this work described here is to compare and discuss morphometrical and biological data pertaining to Italian populations of *H. schachtii* Schmidt, *H. trifolii* Goffart and *H. daverti* Wouts *et* Sturhan of different geographical origins with data obtained with the use of RAPD-PCR markers which assess genetic variability.

Material and methods

Morphometrical analysis

Nine Italian cyst-forming nematode populations belonging to three valid species of the *H. schachtii* group are studied; their designation, sources and originating host are listed in Table I.

TABLE I - *Nematode populations and sources of* Heterodera schachtii *group species used in this study.*

Species	Originating host	Region	Locality (province)	Code population
Heterodera schachtii	*Beta vulgaris* (field)	Emilia Romagna	Ferrara (FE)	HS1
Heterodera schachtii	*Beta vulgaris* (field)	Abruzzo	Avezzano (AQ)	HS2
Heterodera trifolii f. sp. *beta*	*Beta vulgaris* (field)	Piedmont	Montagnino S. Michele (AL)	HT1
Heterodera trifolii	*Dianthus caryophyllus* (glasshouse)	Liguria	Sanremo (IM)	HT2
Heterodera trifolii	*Dianthus caryophyllus* (glasshouse)	Liguria	Riva Ligure (IM)	HT3
Heterodera trifolii	*Dianthus caryophyllus* (glasshouse)	Tuscany	Castellare di Pescia (PT)	HT4
Heterodera daverti	*Dianthus caryophyllus* (glasshouse)	Campania	Ercolano (NA) Az. Borrelli	HD1
Heterodera daverti	*Dianthus caryophyllus* (glasshouse)	Campania	Ercolano (NA) Az. Gentile	HD2
Heterodera daverti	*Dianthus caryophyllus* (glasshouse)	Campania	Torre del Greco (NA)	HD3

All the populations were grown in a glasshouse, 20-24 °C, on their host of origin, namely sugarbeet (*Beta vulgaris* L.) cv. Asso and Mediterranean carnation (*Dianthus caryophyllus* L.) cv. Violetto di Romano.

After two or three generations, brown cysts, extracted and collected from the soil of each of the nine populations, were placed in tap water and incubated at 25 °C in the dark. Second stage juveniles emerging were killed by gentle heat, fixed in FP 4:1 and mounted in anhydrous glycerol on permanent slides. Cyst vulval cones, cut around the perineal area from the brown cysts, previously sonicated and fixed in FP 4:1, were prepared and mounted in Canada balsam.

Statistical analysis

Morphometrical studies were conducted of twenty cyst cones and twenty second stage juveniles from each population; mean, standard deviation and range were calculated for all morphometric characters (Tables II, III). The standard test of means was used to determine the differences between species and populations of the same species. Variance was used to estimate the significant differences among the species and populations (P = 0.05); Duncan's new multiple-range test was used to compare the means of each morphological character for the nine populations. Hierarchical cluster analysis was carried out using SPSS 6.1 programme to assess the relative similarity of the nine populations. This was based on all available morphometric characters of cyst cones; for juveniles, the four taxonomically most important characters (body length, stylet length, tail length and hyaline part of tail length) and all twentyone measured characters were considered separately. The dendrogram (Fig. 1), was obtained using the single linkage method (nearest neighbour) with the four most important morphometric characters of the second stage juveniles (Norusis, 1994).

RAPD-PCR analysis

For each population, DNA was extracted from cysts according to Pastrik *et al.* (1995). DNA concentrations were measured fluorimetrically using the fluorescent dye Hoechst 33257 and a DNA-fluorometer TKO-100 (Hoefer, San Francisco). Random primers (ten oligonucleotides with 50-70% G+C content and random sequence) were purchased from Operon Kit A (Operon technologies, Atlanta, GA). The results reported here were obtained with random primers OPA1 (5'-CAG GCC CTT C-3'), OPA3 (5'-AGT CAG CCA C-3'), OPA7 (5'-GAA ACG

TABLE II - *Morphometrics of cyst cones of nine populations of* H. schachtii *group species (expressed in µm).*

CHARACTERS	POPULATIONS								
	HS1	HS2	HT1	HT2	HT3	HT4	HD1	HD2	HD3
Body length (without neck)	768.6±145.1 abc (480.0-960.0)	815.4±85.9 bc (684.0-960.0)	960.6±97.6 d (744.0-1080.0)	760.8±126.8 abc (576.0-1080.0)	847.2±137.8 cd (576.0-1152.0)	795.0±115.6 bc (516.0-996.0)	654.0±90.1 a (504.0-816.0)	706.8±110.1 ab (540.0-984.0)	748.8±96.3 abc (576.0-924.0)
Body width	529.8±87.9 ab (396.0-696.0)	512.4±63.0 ab (444.0-648.0)	581.4±120.3 b (168.0-720.0)	498.6±88.2 ab (336.0-696.0)	525.6±90.0 ab (384.0-744.0)	520.2±81.9 ab (312.0-672.0)	474.6±81.9 a (360.0-672.0)	495.0±89.9 ab (360.0-720.0)	490.8±71.1 ab (360.0-588.0)
a	1.5±0.2 abc (0.9-2.0)	1.6±0.1 bcd (1.3-1.8)	1.6±0.1 bcd (1.4-1.9)	1.5±0.2 abcd (1.3-1.9)	1.6±0.1 abcd (1.4-2.0)	1.6±0.1 bc (1.3-1.8)	1.4±0.2 a (0.9-1.7)	1.4±0.1 ab (1.2-1.7)	1.5±0.1 abcd (1.3-1.9)
Fenestra length	35.1±5.3 a (29.0-50.8)	38.7±6.2 ab (27.8-48.4)	44.6±6.0 bc (30.3-54.5)	52.3±7.6 d (30.3-70.2)	48.1±5.1 cd (37.5-55.7)	50.7±6.7 cd (38.7-62.9)	53.7±7.1 d (43.6-72.6)	50.7±6.8 cd (41.1-64.1)	53.1±7.3 d (43.6-65.3)
Fenestra width	27.7±3.5 a (21.8-32.7)	31.1±3.6 a (26.6-39.9)	38.3±4.6 b (30.3-48.4)	42.0±6.2 bc (33.9-54.5)	38.3±5.2 b (29.0-48.4)	45.5±6.7 c (30.3-58.1)	40.5±4.8 bc (31.5-48.4)	39.3±4.0 b (30.3-46.0)	40.2±8.3 bc (30.3-60.5)
Vulval bridge width	4.8±0.9 a (3.6-6.1)	6.6±1.2 c (4.8-8.5)	6.7±1.1 c (4.8-8.5)	6.2±1.0 bc (4.8-7.3)	5.7±1.1 abc (4.8-8.5)	5.9±1.1 bc (3.7-7.3)	7.0±0.6 c (6.1-7.3)	5.7±0.7 abc (4.8-7.3)	5.1±1.4 ab (3.6-8.5)
Vulval slit length	40.7±4.8 a (32.7-47.2)	44.1±4.6 ab (36.3-54.5)	48.7±4.3 bc (39.9-56.9)	51.3±7.8 c (33.9-67.8)	50.3±4.6 c (42.4-60.5)	58.0±6.1 d (48.4-78.2)	51.9±4.1 c (42.4-58.1)	49.6±2.9 c (44.8-56.9)	47.5±4.2 bc (39.9-54.5)
Underbridge length	107.8±8.8 ab (90.8-127.1)	109.9±10.4 ab (84.7-124.1)	110.4±10.4 ab (90.8-127.1)	121.3±18.3 bc (90.8-151.3)	119.9±15.4 bc (84.7-151.37)	133.6±14.7 c (102.9-159.7)	113.7±14.7 ab (90.8-139.2)	115.2±9.4 ab (96.8-127.1)	113.3±10.3 ab (90.8-127.1)

Mean values followed by a letter in common are not significantly different (P = 0.05) according to Duncan's new multiple range-test.

TABLE III - *Morphometrics of second stage juvenile of nine populations of* H. schachtii *group species (expressed in μm).*

CHARACTERS	POPULATIONS								
	HS1	HS2	HT1	HT2	HT3	HT4	HD1	HD2	HD3
Body length (L)	436.3±16.8 a (405.4-481.6)	444.5±14.2 a (418.7-471.9)	586.0±31.3 e (525.1-671.6)	495.3±21.4 cd (458.6-533.6)	515.9±30.1 d (457.4-565.1)	521.2±29.1 d (467.1-566.3)	463.9±38.1 ab (405.4-551.8)	454.0±37.5 ab (407.8-550.6)	476.6±31.3 bc (429.6-556.6)
Body width (max)	19.8±0.9 ab (18.2-20.6)	20.2±0.9 ab (18.2-21.8)	22.3±1.7 d (16.9-24.2)	19.2±1.3 a (14.5-20.6)	20.2±1.9 ab (16.9-23.0)	21.3±1.7 bcd (19.4-24.2)	20.7±2.2 abc (15.7-25.4)	20.3±1.9 abc (18.2-24.2)	21.1±1.3 bcd (19.4-24.2)
Lip height	4.2±0.6 a (3.6-4.8)	4.7±0.3 bc (3.6-4.8)	5.6±0.6 e (4.6-6.1)	4.8 const. c	4.8 const. c	4.8 const. c	4.3±0.6 ab (3.6-4.8)	4.8±0.04 c (4.7-4.8)	5.2±0.3 d (4.8-5.4)
Lip width	9.7 const. abc	9.6±0.3 ab (8.5-9.7)	10.9 const. de	10.1±0.7 bc (9.1-11.5)	10.3±0.6 cd (9.7-10.9)	10.3±0.6 cd (9.7-10.9)	9.2±0.6 a (8.5-9.7)	11.0±1.6 e (8.5-12.1)	9.7 const. abc
Stylet length (STY)	25.5±0.7 ab (24.2-26.6)	26.0±0.8 b (24.2-27.8)	29.6±0.8 d (27.8-30.3)	27.9±0.9 c (26.0-29.0)	28.8±1.2 cd (27.8-31.5)	28.6±1.1 cd (26.6-30.3)	25.5±0.9 ab (24.2-26.6)	24.7±0.7 a (24.2-26.6)	26.0±1.1 b (24.2-27.8)
DGO	4.4±0.8 abc (3.6-6.1)	4.0±0.7 a (2.4-4.8)	4.1±0.4 a (3.6-4.8)	4.8±1.0 bc (3.6-6.1)	5.1±0.7 c (3.6-6.1)	4.8±0.5 bc (3.6-5.4)	4.4±0.6 ab (3.6-5.4)	4.4±0.4 ab (3.6-4.8)	4.4±0.5 abc (3.6-4.8)
Median bulb valve to head end (MB)	71.0±5.2 a (59.3-79.9)	73.6±5.9 a (60.5-85.9)	90.8±6.4 c (76.2-102.9)	83.8±4.4 b (76.2-90.8)	85.2±6.4 b (75.0-96.8)	82.8±3.6 b (76.2-89.5)	69.9±5.1 a (62.9-84.7)	70.6±6.9 a (60.5-84.7)	70.4±3.1 a (65.3-73.8)
Oesophagus intestine junction to head end	87.9±5.4 a (75.0-96.8)	90.6±5.7 a (76.2-98.0)	111.5±6.7 c (94.4-123.4)	103.7±5.1 b (92.0-113.7)	102.1±7.1 b (89.5-111.3)	100.3±4.3 b (93.2-108.9)	86.0±4.8 a (79.9-99.2)	88.1±6.8 a (76.2-102.9)	89.4±4.7 a (82.3-98.0)
Median bulb base to head end	184.0±15.0 bcde (157.3-206.9)	158.5±16.5 a (124.6-182.7)	190.1±9.5 bcdef (171.8-205.7)	183.8±13.0 bc (163.4-205.7)	197.9±13.6 def (175.5-217.8)	184.1±11.2 bc (158.5-204.5)	180.0±16.8 b (148.8-203.3)	194.3±10.1 cdef (176.7-215.4)	199.5±11.5 def (175.5-220.2)
Excretory pore to head end	99.3±3.8 a (89.5-106.5)	98.4±1.2 a (84.7-108.9)	129.6±5.3 d (119.8-144.0)	115.4±4.3 c (104.1-121.0)	116.8±6.1 c (104.1-124.6)	116.6±4.6 c (107.7-124.6)	101.4±6.3 a (92.0-115.0)	102.8±9.5 ab (93.2-127.1)	107.9±6.4 b (96.8-121.0)
Tail length	44.9±2.4 a (41.1-48.4)	47.3±4.3 a (42.4-55.7)	72.4±4.5 f (65.3-81.1)	60.8±3.3 de (54.5-66.6)	62.3±4.3 e (55.7-69.0)	60.2±3.4 cde (53.2-66.6)	57.4±2.8 bc (49.6-62.9)	52.8±3.4 b (47.2-58.1)	56.9±5.0 c (47.2-64.1)
Body width anus	12.6±0.8 a (10.9-14.5)	13.2±0.9 ab (10.9-14.5)	15.0±1.1 e (13.3-16.9)	13.7±0.7 bcd (12.1-14.5)	13.5±0.4 abc (13.3-14.5)	14.3±1.1 cde (13.3-16.9)	14.0±1.6 bcde (10.9-18.2)	13.4±1.1 bcde (12.1-15.7)	14.1±0.9 abc (13.3-16.9)
Hyaline part length (H)	24.4±1.8 a (20.6-27.8)	25.4±2.2 a (20.6-29.0)	40.7±3.7 e (35.1-47.2)	33.4±1.9 cd (31.5-37.5)	35.9±3.9 d (31.5-42.4)	34.5±2.6 cd (29.0-41.1)	33.0±3.0 cd (24.2-37.5)	30.0±2.0 b (25.4-31.5)	32.9±4.0 c (27.8-38.7)
a	22.1±1.0 a (20.9-24.2)	22.0±0.9 a (20.2-23.7)	26.5±2.4 d (22.9-35.2)	25.9±2.0 bc (23.6-32.7)	26.0±2.0 bc (22.2-29.3)	24.6±1.2 b (22.6-26.6)	22.5±1.6 a (19.5-26.2)	22.5±1.0 a (19.8-24.5)	22.6±1.3 a (19.7-24.9)
b	5.0±0.3 ab (4.7-5.6)	4.9±0.2 ab (4.6-5.5)	5.3±0.4 cd (4.8-6.2)	4.8±0.3 a (4.1-5.2)	5.1±0.2 abc (4.6-5.5)	5.2±0.2 bcd (4.9-5.5)	5.3±0.4 d (4.8-6.3)	5.2±0.3 bcd (4.8-5.5)	5.3±0.2 cd (4.8-5.7)
b'	2.5±0.7 abc (2.1-4.8)	2.8±0.3 cd (2.4-3.4)	3.1±0.3 de (2.3-3.9)	2.7±0.2 bc (2.4-3.1)	2.6±0.3 abc (2.2-3.0)	2.8±0.2 cde (2.5-3.1)	2.6±0.4 abc (2.2-3.6)	2.3±0.1 a (2.1-2.7)	2.4±0.2 ab (2.2-3.2)
L/MB	6.2±0.4 abc (5.4-7.5)	6.1±0.4 ab (5.5-6.9)	6.4±0.7 bcde (4.5-8.2)	5.9±0.2 a (5.5-6.2)	6.1±0.3 ab (5.5-6.7)	6.3±0.2 abcd (5.9-6.6)	6.7±0.4 de (5.7-7.6)	6.4±0.4 bcd (5.8-7.1)	6.8±0.4 e (6.1-7.7)
c	9.8±0.5 d (8.9-10.6)	9.5±0.8 d (7.7-10.6)	8.1±0.5 ab (7.0-8.9)	8.2±0.4 ab (7.3-9.0)	8.3±0.3 abc (7.6-8.8)	8.7±0.5 c (8.0-9.9)	8.1±0.5 a (7.1-9.3)	8.6±0.6 bc (7.8-10.0)	8.4±0.5 abc (7.2-9.1)
c'	3.6±0.3 a (3.1-4.0)	3.6±0.4 a (3.2-4.6)	4.8±0.3 e (4.1-5.4)	4.5±0.2 cd (4.1-4.8)	4.6±0.3 de (4.2-5.2)	4.2±0.3 bc (3.6-4.6)	4.1±0.3 b (3.4-4.6)	4.0±0.2 b (3.6-4.5)	4.0±0.3 b (3.5-4.7)
H/STY	1.0±0.1 a (0.8-1.1)	1.0±0.1 a (0.8.-1.1)	1.4±0.1 cd (1.3-1.6)	1.2±0.1 b (1.1-1.4)	1.2±0.1 b (1.1-1.5)	1.2±0.1 b (1.0-1.4)	1.2±0.1 b (1.0-1.5)	1.3±0.1 bc (1.1-1.3)	1.3±0.1 b (1.1-1.5)
Tail length/H	1.9±0.1 ab (1.7-2.2)	1.9±0.2 b (1.7-2.5)	1.8±0.2 ab (1.6-2.2)	1.8±0.1 ab (1.7-2.1)	1.7±0.1 ab (1.6-1.9)	1.8±0.1 ab (1.5-2.0)	1.8±0.2 ab (1.5-2.4)	1.8±0.1 ab (1.5-2.0)	1.7±0.1 a (1.5-2.0)

Mean values followed by a letter in common are not significantly different (P = 0.05) according to Duncan's new multiple range-test.

GGT G-3'). PCR was carried out using a Perkin-Elmer 9600 thermocycler. The reaction mixture (40 μl) contained 20-40 ng of template DNA, 200 μM of each dNTP (Boehringer-Mannheim), 100 μM of a single random primer, 2.5U of Taq-polymerase (Boehringer-Mannheim), 4 μl 10X reaction mixture, autoclaved ultra-pure distilled water. DNA thermal cycler consisted of 5 min 94 °C; 45 cycles of 94 °C for 1 min., 35 °C for 2 min., 72 °C for 2 min. and a final elongation step at 72 °C for 5 min. After DNA amplification, 10 μl of product was run on a 2% agarose gel and stained with ethidium bromide. The patterns of stained DNA fragments were examined on a U.V. transilluminator and recorded by polaroid film.

Data from RAPD patterns were scored for the presence (1) or the absence (0) of similar bands in each nematode population. Only reproducible bands were taken into account, and band-sharing was analysed in a pair-wise comparison according to Nei and Li (1979). Simple matching was used to calculate a genetic similarity matrix (Sneath and Sokal, 1973). The matrix was used to perform hierarchical cluster analysis based on the unweighted pair-group

method using arithmetic averages (UPGMA) implemented in the NTSYS vers. 1.3 package (Rohlf, 1987).

Results

Morphometrical comparisons

Table II lists the morphometrics of the cyst cones of the nine Italian populations of the *H. schachtii* group. The mean cyst size of *H. trifolii* from sugarbeet (HT1) is significantly larger than the other populations. The means of fenestra dimensions (length and width) and vulval slit length of two *H. schachtii* populations (HS1 and HS2) are significantly smaller than the same characters in the *H. trifolii* and *H. daverti* populations. Also in the *H. trifolii* Tuscan population from carnation (HT4) the mean lengths of vulval slit and underbridge are the greatest. Compared to the ratio a, there is a difference for the two *H. daverti* isolates (HD1 and HD2) whose cysts appear more spheroidal than others.

Between the two *H. schachtii* populations, the mean of vulval bridge width of specimens of HS2 from Avezzano appears significantly greater than those of HS1 from Ferrara. In the *H. trifolii* population from sugarbeet (HT1), with the exception of cyst dimension, all cyst features are generally smaller than those reported for either *H. trifolii* from Liguria (HT2, HT3) or from Tuscany (HT4).

As to the second stage juveniles, the body length of *H. trifolii* from sugarbeet (HT1), lip height, distances from the anterior end to the median bulb valve, to the oesophagus intestine junction and to the excretory pore besides the tail length, the hyaline part of the tail length and ratio a, are significantly the largest among those of the other populations (Table III). The mean stylet length and ratio c' of *H. trifolii* juveniles from sugarbeet (HT1) are also significantly larger than those of *H. schachtii* and *H. daverti* juveniles; they do not differ significantly from those of *H. trifolii* populations from carnation (HT2, HT3 and HT4). As to the stylet length, distances from the anterior end to the median bulb valve, to the oesophagus intestine junction and to the excretory pore and ratio a, the means of *H. schachtii* and *H. daverti* populations (HS1, HS2, HD1, HD2 and HD3) are very close and smaller

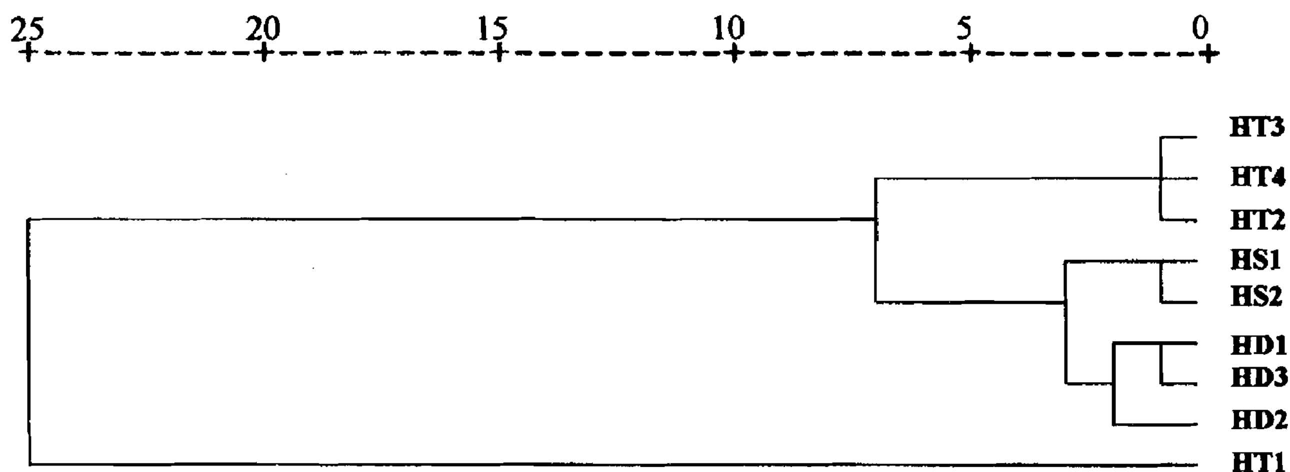


Fig. 1 - Similarity dendrogram of nine populations of *Heterodera schachtii* group species, as computed by SPSS 6.1 programme, of the four most distinctive juvenile morphometrical characters (body length, stylet length, tail length and hyaline part of tail length); (for code populations, see Table I).

than those of *H. trifolii* from carnation (HT2, HT3 and HT4) or sugarbeet (HT1).

The cluster analysis based on morphometrical characters of cyst cone clearly differentiates the *H. trifolii* population reproducing on sugarbeet from the other cyst nematodes of the *H. schachtii* group. In fact this population constitutes a distinct main cluster separated from the group of the eight remaining populations (the dendrogram is not given since it is essentially similar to that of Fig. 1). This suggests that *H. trifolii* f. sp. *beta* is well differentiated according to its larger cyst size.

From the result of the cluster analysis based on the four most distinctive characters of 180 juveniles examined (body length, stylet length, tail length and hyaline part of tail length), which is the same as that based on all twenty-one characters, the juveniles of YBCN are placed in a main cluster at 75% of similarity with that of the remaining populations, which at 93% of similarity constitute two subclusters (Fig. 1). Juveniles of *H. trifolii* from carnation are clustered together according to their degree of similarity based on the mean values of some characters (body length, distances from the anterior end to the median bulb valve, to the oesophagus intestine junction and to the excretory pore, tail length and hyaline part of tail length) (Table III); juveniles of *H. daverti* and *H. schachtii* are included in another cluster at 97% of similarity based on the mean values of some significantly similar characters (stylet length, DGO, distances from the anterior end to the median bulb valve, to the oesophagus intestine junction and ratio a (Table III).

RAPD-PCR comparisons

In RAPD analysis, polymorphisms are scored for the presence versus absence of a particular band; the proportion of DNA fragments shared between two populations of the *H. schachtii* group members is correlated with the degree of genetic divergence of DNA.

Out of 13 primers used for screening polymorphic markers, six show faint bands or the complete absence of fragments with all tested species and therefore they have not been included in further observations. The remaining seven primers (AGI, AI2, AH29, AH30, OPA1, OPA3, OPA7) produce distinct RAPD patterns that can be scored for both *H. schachtii* and *H. daverti* populations but not always visible banding patterns for *H. trifolii* ones. Only three of those seven primers (OPA1, OPA3, OPA7) enable us to analyse the nine Italian cyst-forming nematode populations in their entirety.

A total of 31 consistent bands obtained by these ten-mer primers has been employed to generate a similarity matrix (Fig. 2A). The average percentage similarity for pair-wise comparison between the examined populations, based on shared DNA fragments, reveals a dendrogram with two main separate clusters (Fig. 2B). In the first cluster only the *H. schachtii* populations (HS1 and HS2) are located whereas in the second one both the *H. trifolii* and *H. daverti* populations are included. Between these clusters there is at least 45% of similarity. The second cluster is split into two subclusters (the third and the fourth) at 70% of similarity; the third contains the *H. trifolii* isolates associated to carnation, the fourth, besides *H. trifolii* associated to sugarbeet, all the *H. daverti* populations.

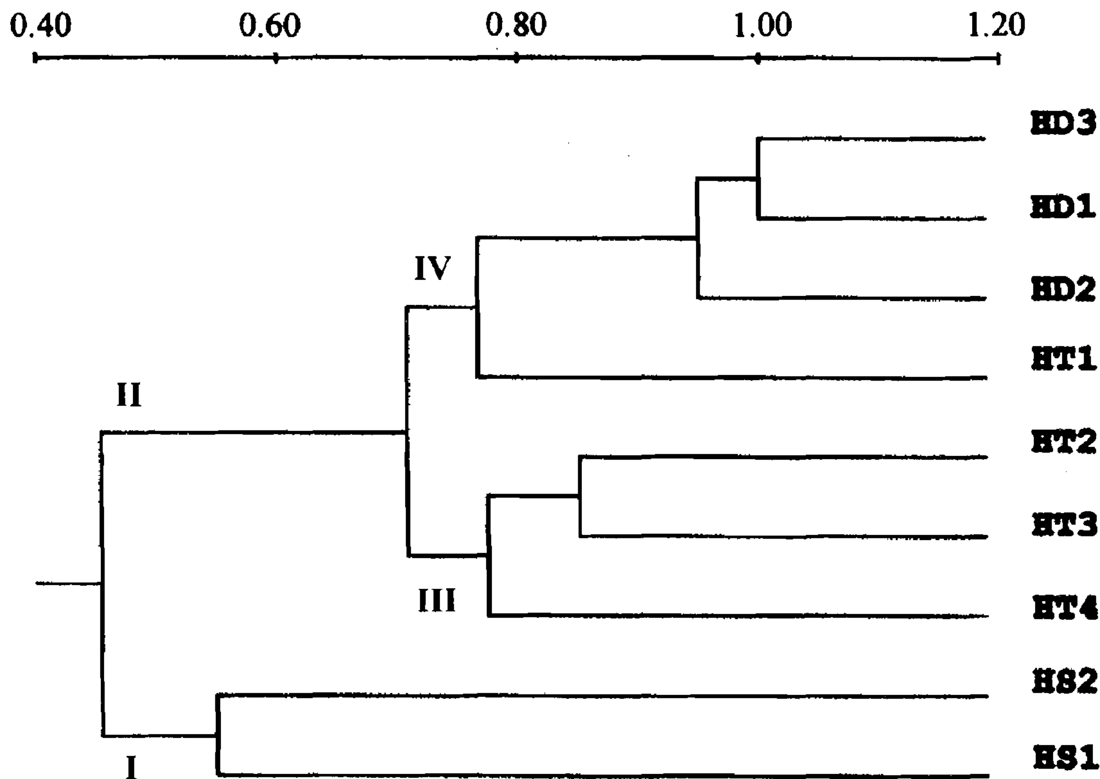
The dendrogram of genetic distances on the basis of 55 bands scored for *H. daverti* and *H. schachtii* has not been reported since it does not change substantially from what is already shown in Fig. 2B.

Discussion

The morphometric analysis (linear measurements and ratios of characters) of the cyst cones and juveniles of the nine cyst-forming Italian populations of the *Heterodera schachtii* group used in the present study, indicates that *H. tri-*

	HD3	HD1	HD2	HS2	HT1	HS1	HT2	HT3	HT4
HD3	1.000								
HD1	1.000	1.000							
HD2	0.950	0.950	1.000						
HS2	0.500	0.500	0.450	1.000					
HT1	0.750	0.750	0.800	0.450	1.000				
HS1	0.450	0.450	0.400	0.550	0.400	1.000			
HT2	0.700	0.700	0.750	0.500	0.750	0.450	1.000		
HT3	0.750	0.750	0.800	0.450	0.800	0.500	0.850	1.000	
HT4	0.600	0.600	0.650	0.500	0.650	0.350	0.800	0.750	1.000

A



B

Fig. 2 - Relationships of nine populations of *H. schachtii* species group in RAPD-PCR analysis using primers OPA 1, OPA 3 and OPA 7: A, average percent similarity matrix; B, dendrogram.

folii from sugarbeet (HT1), termed YBCN, can be significantly differentiated from the other *Heterodera* populations since it has the greatest dimensions of cysts and juveniles. The dendrograms constructed with biometrical data of cyst cones or juveniles show two main clusters; the first is only *H. trifolii* reproducing on sugarbeet, the second includes all other remaining populations. Among eight cyst cone morphometric characters considered, only the fenestra dimensions and vulva slit length separate the *H. schachtii* populations from those of *H. daverti* and *H. trifolii*; they are the smallest values among those examined. From previous research the fenestra length is already considered to be useful in differentiating the New York populations of the two closely related species *H. schachtii* and *H. trifolii*, which often occur as mixed populations (Abawi *et al.*, 1973). The body length of juveniles in HT1, as well as lip height, distances from the anterior end to the median bulb valve, to the oesophagus intestine junction and to the excretory pore besides the tail length, the hyaline part of the tail length and ratio a, are useful to distinguish *H. trifolii* reproducing on beet from the other populations. The tail length is valid to separate one from another *H. schachtii*, *H. trifolii* from sugarbeet and carnation and *H. daverti*. The dimensions of body, length of tail and hyaline part of tail were previously considered valid to separate *H. trifolii* associated to sugarbeet populations from those of *H. schachtii* because they were significantly the largest (Steele and Whitehand, 1984). YBCN not yet reported in USA, is a tetraploid parthenogenetic form (Steele and Whitehand, 1984) and this biological condition can explain why the cysts and juveniles of *H. trifolii* from sugarbeet present the largest dimensions (Triantaphyllou and Riggs, 1979).

In the dendrogram constructed with molecular data of our RAPD analysis, there is a clear difference between *H. schachtii* and the remaining populations belonging to the *H. trifolii* complex (that consists of the bisexual species *H. daverti*, of several nominally parthenogenetic species, some considered as synonyms and of populations more or less morphologically distinct, more or less adapted to special host plants) (Hirschmann and Triantaphyllou, 1979; Sturhan 1983; Sikora and Maas, 1985). The similarity between the two *H. schachtii* populations is about 55%, while that among the *H. trifolii* complex members is about 70%. Wider molecular polymorphism appears between the *H. schachtii* population from Ferrara (HS1) and that from Avezzano (HS2) than among *H. trifolii* and *H. daverti* populations found in the other Italian regions. This may indicate that these two *H. schachtii* populations may have existed separately for a long time. However, preliminary experiments had already revealed that the geographical proximity of *H. schachtii* populations did not correlate with genetic similarity. In fact, two different California populations, approximately 5 km apart, had a similarity coefficient of only 45% (Caswell-Chen *et al.*, 1992). The *H. trifolii* populations from carnation are grouped in the same subcluster with a high similarity of 78%; HT2 and HT3 both coming from Liguria show a closer relationship (85%) than with HT4 from Tuscany. Finally, *H. trifolii* from sugarbeet (HT1) isolated in Piedmont shared a closer relationship (77%) with the very homogeneous group of the *H. daverti* populations from Campania (HD1, HD2 and HD3), which show the highest DNA homology.

The molecular analysis allows the differentiation of the *H. schachtii* populations and the *H. trifolii* complex members. The sugarbeet parasitic *H. trifolii* race differentiates from the others reproducing on carnation.

In conclusion, this study supports the existence of a relatively high genetic variability between two geographic isolates of *H. schachtii* not associated with distinct morphometric differences. On the contrary, it was observed a considerable genome similarity among *H. trifolii* complex members collected throughout Italy, not corresponding generally with morphometric similarities. However, a comparison between

morphometrical and genetic variations is always useful to support the identification and separation of many closely related species.

This preliminary data needs to be extended with additional molecular studies to improve the evolutionary status of the *H. schachtii* group and *H. trifolii* complex members.

Acknowledgements. The authors thank Dr. S. Caroppo and Dr. S. Simoni of the Istituto Sperimentale per la Zoologia Agraria, Florence, for their assistance with statistical analysis and Dr. M. Cavalli and Mr. D. Coniglio of the same Institute for their technical support.

Literature cited

ABAWI G. S., FOSTER J. and MAI W. F., 1973. Differentiation of cysts of *Heterodera schachtii* and *H. trifolii* by fenestral length. *Journal of Nematology, 5*: 74-75.

AMBROGIONI L. and CAROPPO S., 1988. Ciclo biologico di un nematode cistiforme affine a *Heterodera daverti* Wouts e Sturhan in radici escise di *Trifolium repens* L. *Redia, 71*: 439-454.

AMBROGIONI L., COTRONEO A., MORETTI F., TACCONI R. and IRDANI T., 1999. First record of the yellow beet cyst nematode (*Heterodera trifolii*) in Italy. *Nematologia Mediterranea, 27*: 151-158.

AMBROGIONI L., D'ERRICO F. P. and MARINARI PALMISANO A., 1985. Observations on a population of cyst forming nematodes from carnation. Pp. 315-320. *In*: *Cyst Nematodes* (F. Lamberti and C. E. Taylor, eds). Plenum Press, New York and London.

ANDERSSON S., 1984. First record of a yellow beet cyst nematode (*Heterodera trifolii*) in Sweden. *Växtskyddsnotiser, 48*: 93-95.

BALDWIN J. G. and MUNDO-OCAMPO M., 1991. Heteroderinae, cyst- and non-cyst forming nematodes. Pp. 275-362. *In*: *A manual of agricultural nematology* (W. R. Nickle, ed.). M. Dekker Inc., New York.

BOSSIS M., CAUBEL G. and PORTE C., 1997. Variabilité protéique observée par electrophorèse bidimensionelle sur neuf isolats d'*Heterodera schachtii* provenant de 6 pays européens et sur deux isolats d'*Heterodera trifolii* f. sp. *beta*. *Fundamental and Applied Nematology, 20*: 149-155.

CASWELL-CHEN E. P., WILLIAMSON V. M. and WU F. F., 1992. Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* populations. *Journal of Nematology, 24*: 343-351.

FERRIS V. R., FERRIS J. M. 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.

GANGULY A. K., DASGUPTA D. R. and RAJASEKHAR S. P., 1992. b-Esterase variation in three common species of *Heterodera*. *Indian Journal of Nematology, 20*: 113-114.

GERDEMANN J. W. and LINFORD M. B., 1953. A cyst-forming nematode attacking clovers in Illinois. *Phytopathology, 43*: 603-608.

HIRSCHMANN H., 1956. Comparative morphological studies on the soybean cyst nematode, *Heterodera glycines*, and the clover cyst nematode, *H. trifolii* (Nematoda: Heteroderidae). *Proceedings of the Helminthological Society of Washington, 23*: 140-151.

HIRSCHMANN H. and TRIANTAPYLLOU A. C., 1979. Morphological comparison of members of *Heterodera trifolii* species complex. *Nematologica, 25*: 458-481.

IBRAHIM S. K. and ROWE J. A., 1995. Use of isoelectric focusing and polyacrylamide gel electrophoresis of non-specific esterase phenotypes for the identification of cyst nematode *Heterodera* species. *Fundamental and Applied Nematology, 18*: 189-196.

MAAS P. W. TH. and HEIJBROEK W., 1982. Biology and pathogenicity of the yellow beet cyst nematode, a host race of *Heterodera trifolii* on sugar beet in the Netherlands. *Nematologica, 28*: 77-93.

MULVEY R. H., 1959. Investigations on the clover cyst nematode *Heterodera trifolii* (Nematoda: Heteroderidae). *Nematologica, 4*: 147-156.

NEI M. and LI W. H., 1979. Mathematical model for studying genetic variation in terms of restriction endoglucanases. *Proceedings of National Academy of Science, 15*: 391-395.

NOBBS J. M., IBRAHIM S. K. and ROWE J., 1992. A morphological and biochemical comparison of the four cyst nematode species, *Heterodera elachista, H. oryzicola, H. oryzae* and *H. sacchari* (Nematoda: Heteroderidae) known to attack rice (*Oryza sativa*). *Fundamental and Applied Nematology, 15*: 551-562.

NORDMEYER D., 1979. Untersuchungen ueber einen Wurzelfaeulekomplex an *Trifolium subterraneum* L. in Nordwest-Tunesien unter besonderer Beruecksichtigung von Wechselbeziehungen zwischen den Erregern. *Ph. D. thesis, University of Bonn, Germany*, 123 pp.

NORTON DON C.,1967. Relationships of *Heterodera trifolii* to some forage legumes. *Phytopathology, 57*: 1305-1308.

NORUSIS M. J., 1994. *SPSS Professional Statistics 6.1*. SPSS Inc. Chicago, USA, 385 pp.

PASTRIK K. H., RUMPENHORST H. J. and BURGERMEISTER W., 1995. Random amplified polymorphic DNA analysis of a *Globodera pallida* population selected for virulence. *Fundamental and Applied Nematology, 18*: 109-114.

PORTE C., MAHFOUD A. and CAUBEL G., 1997. Reaction of geographically dispersed populations of *Heterodera schachtii* and *H. trifolii* to resistant lines of sugarbeet. *Nematologia Mediterranea, 25*: 113-120.

RADICE A. D., POWERS T. O., SANDALL L. J. and RIGGS R. D., 1988. Comparisons of mitochondrial DNA from the sibling species *Heterodera glycines* and *H. schachtii*. *Journal of Nematology, 20*: 443-450.

RASKI D. J., 1950. The life history and morphology of the sugar-beet nematode, *Heterodera schachtii* Schmidt. *Phytopathology, 40*: 135-152.

ROHLF F. J., 1987. NTSYS - pc. Numerical taxonomy and multivariate analysis system. Pp. 1-37. *In*: *Applied Biostatistics.* Inc. Exeter Publ., New York.

SCHLANG J., 1990. Erstnachweis des Gelben Rübenzcystennematoden (*Heterodera trifolii*) für die Bundesrepublik Deutschland. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, 42*: 58-59.

SIKORA R. A. and MAAS P. W. TH., 1985. An analysis of the *Heterodera trifolii* complex and other species in the *schachtii* group attacking legumes, pp. 293-313. *In*: *Cyst Nematodes* (F. Lamberti and C. E. Taylor, eds). Plenum Press, New York and London.

SNEATH P. H. and SOKAL R. R., 1973. *Numerical taxonomy* (W. H. Freeman and Co., eds). S. Francisco, USA, 573 pp.

STEELE A. E. and WHITEHAND L., 1984. Comparative morphometrics of eggs and second-stage juveniles of *Heterodera schachtii* and a race of *H. trifolii* parasitic on sugarbeet in the Netherlands. *Journal of Nematology, 16*: 171-177.

STURHAN D., 1983. The use of the subspecies and the superspecies categories in nematode taxonomy. Pp. 41-53. *In*: *Concepts in Nematode Systematics* (A.R. Stone, H. M. Platt and L. F. Khalil, eds). Academic Press Inc., London.

SUBBOTIN S. A., 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 S. A., WAEYNBERGE L., MOLOKANOVA I. A. and MOENS M., 1999. Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. *Nematology, 1*: 195-207.

TACCONI R., 1997. Riproduzione di *Heterodera schachtii* su piante coltivate (2° contributo). *Nematologia Mediterranea, 25*: 93-97.

TRIANTAPHYLLOU A. C. and HIRSCHMANN H., 1978. Cytology of the *Heterodera trifolii* parthenogenetic species complex. *Nematologica, 24*: 418-424.

TRIANTAPHYLLOU A. C. and RIGGS R. D., 1979. Poliploidy in an amphimitic population of *Heterodera glycines. Journal of Nematology, 11*: 371-376.

VALLOTTON R., 1985. Première observation en Suisse de la "forme spécialisée Betterave" du nématode à kyste *Heterodera trifolii* Goffart. *Revue Suisse d'Agriculture, 17*: 137-140.

WILLIAMSON V. M., 1991. Molecular techniques for nematode species identification. Pp. 107-123. *In*: *Manual in agricultural nematology* (W. R. Nickle, ed.). M. Dekker Inc., New York.

WOUTS W. M. and STURHAN D., 1978. The identity of *Heterodera trifolii* Goffart, 1932 and the description of *H. daverti* n. sp. (Nematoda: Tylenchida). *Nematologica, 24*: 121-128.

Accepted for publication on 28 May 2001.