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Molecular Characterization of Cyst Nematode Species (*Heterodera* spp.) from the Mediterranean Basin using RFLPs and Sequences of ITS-rDNA

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Abstract

Fifteen populations of cyst-forming nematodes belonging to 11 known and one unidentified species collected in countries bordering the Mediterranean Sea were studied using polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) and internal transcribed spacer (ITS)-rDNA sequences. RFLP profiles generated by the restriction enzymes *Alu*I, *Ava*I, *Bsh*1236I, *Hae*III, *Hin*6I, *Mva*I, *Pst*I and *Rsa*I are presented for *Heterodera carotae*, *H. ciceri*, *H. fici*, *H. filipjevi*, *H. goettingiana*, *H. hordecalis*, *H. humuli*, *H. mediterranea*, *H. ripae* and *H. schachtii*. Molecular data support the first detection of *H. filipjevi* from wheat in Italy and *H. ripae* from nettle in Greece. A relative high level of sequence divergence between populations of *H. hordecalis* was observed. This suggests that two species might presently be grouped under this taxon. The phylogenetic relationship between the Mediterranean cyst-forming nematode species is analysed based on the ITS-rDNA sequences.

Introduction

Cyst-forming nematodes are highly specialized and economically important soil-borne parasites attacking numerous agricultural crops worldwide. Injury to crops by these nematodes is probably second in importance to damage caused by root-knot nematodes. Parasitism of cyst nematodes is characterized by the establishment of permanent feeding sites (syncitia) in the cortex, endodermis, pericycle and vascular parenchyma of the host tissues, acting as a metabolic sink to which photosynthesis are mobilized. Consequently plant growth and yield may be suppressed (Baldwin and Mundo Ocampo, 1991).

Currently, the genus *Heterodera* contains more than 60 species (Wouts and Baldwin, 1998). In the Mediterranean Basin several cyst nematode species have been reported on herbaceous and woody plants (Greco and Brandonisio, 1981; Di Vito and Inserra, 1982; Vovlas et al., 1985; Ferris and Greco, 1992; Castillo et al., 1999). These species belong to several taxonomic groups within the genus *Heterodera*: Avenae group (*H. filipjevi*, *H. hordecalis*, *H. latipons*); Goettingiana group (*H. carotae*, *H. goettingiana*, *H. mediterranea*); Humuli group (*H. humuli*, *H. ripae*); and Schachtii group (*H. ciceri*, *H. fici*, *H. schachtii*). Some of these species have been demonstrated to be highly pathogenic to legumes (Greco et al., 1988, 1991), wheat (Greco and Brandonisio, 1987), sugar beet (Greco et al., 1982), carrot (Greco et al., 1993), fig seedlings (Di Vito and Inserra, 1982) and olive planting stocks (Castillo and Vovlas, 2002).

Within each *Heterodera* group, only minor morphological and morphometrical differences can distinguish all of these species from each other. Identification based on morphology and morphometrics of cysts and second-stage juveniles is time-consuming and requires a lot of skill. However, accurate identification and pathogenic characterization of *Heterodera* spp. attacking crops in the Mediterranean Basin are needed as an initial step in designing effective control measures. This is especially important when searching for possible sources of host-plant resistance against *Heterodera* species (Cook and Noel, 2002). In addition, rapid and accurate identification is highly significant for quarantine purposes of important crop plants.

The internal transcribed spacer region (ITS) of the ribosomal DNA repeat unit is a good candidate for molecular taxonomic and phylogenetic studies. The ribosomal genes flanking this region are highly conserved allowing the construction of primers that enable polymerase chain reaction (PCR) amplification of the highly variable ITS region between them. Sequence variation in this region yields restriction fragment length polymorphisms (RFLPs), which can be used for taxonomic goals. Developed during recent years, PCR-ITS-rDNA diagnostics are a reliable tool for a precise and quick identification of cyst nematodes. Comparisons of RFLP profiles and sequences of the ITS-rDNA of unknown nematodes with those published or deposited in GenBank (Ferris et al., 1994; Thiéry and Mugniéry, 1996; Orui, 1997; Szalanski et al., 1997; Subbotin et al., 1999, 2000, 2001; Sabo et al., 2001; Tanha Maafi et al., 2003) facilitate quick identification of most species of cyst nematodes.

The objectives of this study were: (i) to molecularly characterize cyst-forming nematode populations collected in countries bordering the Mediterranean Sea based on RFLPs and sequence of the ITS region, (ii) to study the phylogenetic relationships of some species based on maximum parsimony and minimum evolution analyses of the alignment of the ITS sequences.

Materials and Methods

Nematode populations

The study comprised a total of 16 isolates of cyst-forming nematodes belonging to 10 known and one unidentified species (Table 1). Cysts were either obtained from the nematode collections maintained in the Istituto per la Protezione delle Piante, Sezione di Bari, Italy, and the Instituto de Agricultura Sostenible, Cordoba, Spain, or were directly collected in orchards and agricultural fields. After their extraction from soil cysts were kept in dry condition at room temperature. Identification was carried out on the basis of morphometrics and morphological characteristics of cyst vulval cones and second-stage juveniles (Wouts and Baldwin, 1998). *Heterodera latipons* from Rostov region, Russia (Ferris et al., 1999) was re-sequenced and also included in this study.

DNA extraction, PCR, RFLP and sequencing

The total DNA from one or several cysts was extracted using proteinase K in the worm lysis buffer as described by Subbotin et al. (2000). The ITS region of rDNA was amplified by PCR with the forward TW81 primer (3′-GTTTCCGTAGGTGAACCTGC-5′) and reverse AB28 primer (3′-ATATGCTTAAGTTCAGCGGGT-5′). Two to eight microlitres of the PCR product was digested by one of the following restriction enzymes: *Alu*I, *Ava*I, *Bsh*1236I, *Hae*III, *Cfo*I, *Mva*I, *Rsa*I and *Pst*I in the buffer stipulated by the manufacturer. The digested DNA was run on a 1.5% TAE buffered agarose gel, stained with ethidium bromide and photographed and analysed using Kodak Scientific Imaging System (Rochester, NY, USA) under UV light. PCR products were sequenced using the TW81, AB28 primer and internal reverse 5.8SM5 primer (Zheng et al., 2000) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Benelux, the Netherlands). The resulting products were purified and sequences were run on a 377 DNA Sequencer (PE Applied Biosystems, Warrington, UK). The sequences obtained in this study were deposited in GenBank under accessions numbers AY347917–AY347927). The exact lengths of restriction fragments from the PCR products were obtained by virtual digestion of the sequences using WebCutter 2.0.

Sequence and phylogenetic analysis

Only sequences of ITS1-5.8S-ITS2 were used for analyses. Newly obtained sequences and the sequences obtained from publications and the GenBank (Ferris et al., 1999; Sabo et al., 2001; Subbotin et al., 2001, 2003; Tanha Maafi et al., 2003) were aligned with ClustalX1.64 with default options. *Meloidodera alni* and *Cryphodera brinkmani* were used as outgroup taxa. Sequence alignment was analysed with an equally

Table 1
Species and populations of *Heterodera* used in the present study

Species	Host-plant	Location	Study
Heterodera carotae	*Daucus carota*	Zapponeta, Italy	RFLP, sequencing
Heterodera ciceri	*Cicer arietinum*	Aleppo, Syria	RFLP
Heterodera fici	*Ficus carica*	Acores, Portugal	RFLP
Heterodera fici	*Ficus carica*	Epirus, Greece	RFLP
Heterodera fici	*Ficus carica*	Potenza, Italy	RFLP
Heterodera filipjevi	*Triticum* sp.	Foggia, Italy	RFLP, sequencing
Heterodera goettingiana	*Pisum sativum*	Ostuni, Italy	RFLP
Heterodera hordecalis	*Triticum* sp.	Zapponeta, Italy	RFLP, sequencing
Heterodera hordecalis	*Triticum* sp.	Israel	sequencing
Heterodera humuli	*Humulus lupulus*	Bragança, Portugal	RFLP, sequencing
Heterodera latipons	*Elymus repens*	Rostov, Russia	sequencing
Heterodera mediterranea	*Pistacia lentiscus*	Torre Canne, Italy	RFLP, sequencing
Heterodera mediterranea	*Olea europaea* ssp. *europaea*	Utrera, Spain	RFLP, sequencing
Heterodera mediterranea	*Olea europaea* ssp. *sylvestris*	Vejer, Spain	RFLP, sequencing
Heterodera ripae[1]	*Urtica dioica*	Epirus, Greece	RFLP, sequencing
Heterodera schachtii	*Beta vulgaris*	Fucino, Italy	RFLP
Heterodera sp.	Unknown	Morocco	RFLP, sequencing

[1]*Heterodera riparia* is considered as a synonym of *H. ripae* (Subbotin et al., 2003).
RFLP, restriction fragment length polymorphism.

weighted maximum parsimony (MP) using PAUP*4.0 (Swofford, 2002). Gaps were treated as missing data. We used heuristic search setting with 10 replicates of random taxon addition, tree bisection-reconnection branch swapping to seek for the most parsimonious trees. For minimum evolution (ME) analysis, the appropriate substitution model of DNA evolution that best fitted the data set, was determined by the Akaike Information Criteria with ModelTest 3.04 (Posada and Crandall, 1998). Bootstrap analysis with 100 replicates was conducted to assess the degree of support for each branch on the MP and ME trees.

Results and Discussion

PCR–RFLP

Amplification of the ITS region including the flanking parts of the 18S and 28S genes yielded a single fragment of approximately 1 kb for all studied populations. No PCR products were seen in the negative control without nematode DNA template. The eight restriction enzymes generated RFLPs for all studied populations (Fig. 1). The exact length of the restriction fragments for some species is given in Table 2. The RFLP patterns obtained from the studied populations did not reveal any difference with those previously reported for *H. schachtii*, *H. ciceri*, *H. humuli*, *H. fici*, *H. ripae*, *H. carotae* and *H. goettingiana* (Subbotin et al., 2000; Eroshenko et al., 2001; Tanha Maafi et al., 2003). RFLP patterns of *H. mediterranea* were similar for the three studied populations, except for an additional fragment generated by *Bsh*1236I in the Italian population (Fig. 1j,k). *Heterodera mediterranea* showed RFLPs quite similar with those for *H. ciceri* but can be distinguished from the latter species by the restriction enzyme *Cfo*I (Fig. 1). The *Alu*I pattern of the Italian *H. hordecalis* population (438, 433 and 172 bp) was different from that for the North European and Iranian *H. hordecalis* populations (867 and 172 bp) (Subbotin et al., 2000; Tanha Maafi et al., 2003). The *Cfo*I pattern obtained from the Italian *H. filipjevi* population (750, 108, 106, 46 and 44 bp) differed from earlier reported patterns of this

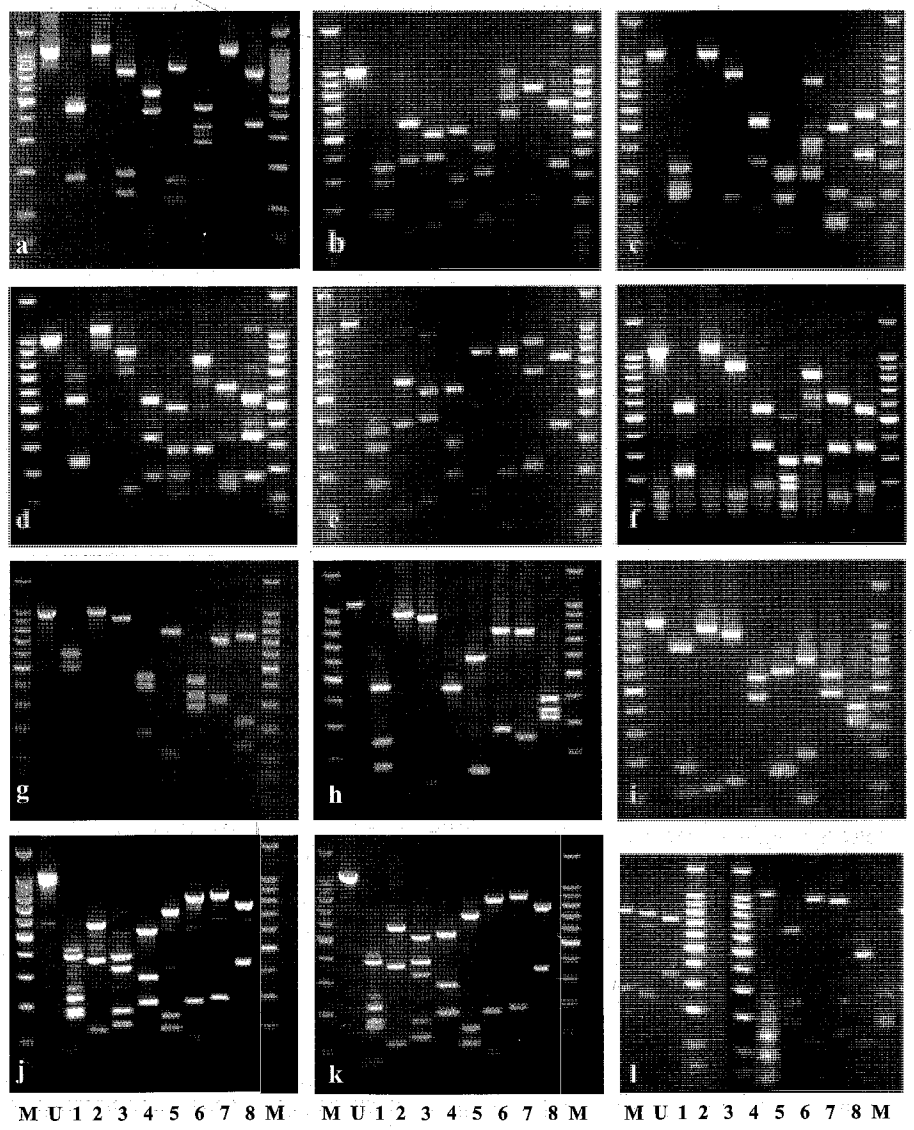

Fig. 1 Restriction fragment of amplified internal transcribed spacer (ITS) region of cyst-forming nematodes. (a) *Heterodera hordecalis*; (b) *H. schachtii*; (c) *H. goettingiana*; (d) *H. carotae*; (e) *H. ciceri*; (f) *Heterodera* sp. (Morocco); (g) *H. filipjevi*; (h) *H. humuli*; (i) *H. fici*; (j) *H. mediterranea* (Spain, *Olea europaea*); (k) *H. mediterranea* (Italy, *Pistacia lentiscus*); (l) *H. ripae*. (M, 100 bp DNA marker (Promega, Leiden, The Netherlands); U, unrestricted fragment; 1, *Alu*I; 2, *Ava*I; 3, *Bsh*1236I; 4, *Hae*III; 5, *Cfo*I; 6, *Mva*I; 7, *Rsa*I; 8, *Pst*I)

Table 2
Length (bp) of restriction fragments of rDNA-internale transcribed spacer (ITS) regions for some cyst nematodes of the genus *Heterodera* based on restriction fragment length polymorphisms (RFLPs) and sequence data

Restriction enzymes	*Heterodera ripae*	*Heterodera hordecalis*	*Heterodera carotae*	*Heterodera humuli*	*Heterodera mediterranea*	*Heterodera filipievi*
*Alu*I	628, 243, 178	438, 433, 172	527, 236, 220, 37, 18	451, 241, 175, 171	384, 224, 180, 166, 58, 15	571, 483
*Ava*I	934, 115	1043	1041	925, 113	551, 364, 112	1054
*Bsh*1236I	895, 133, 21	710, 180, 131, 22	839, 126, 42, 31	886, 131, 21	(497), 379, 323, 174, 130, 21	902, 131, 21
*Hae*III	448, 446, 105, 50	533, 410, 52, 24, 24,	519, 320, 170, 24	438, 447, 103, 50	511, 283, 198, 107, 24, 11	424, 378, 176, 52, 24
*Cfo*I	250, 175, 167, 155, 154, 148	739, 153, 108, 43,	475, 268, 171, 104, 23	586, 152, 152, 148	630, 146, 105, 100, 46	750, 108, 106, 46, 44
*Mva*I	775, 274	431, 276, 236, 100	861, 177	766, 272	758, 196, 73	404, 333, 276, 22, 19
*Rsa*I	765, 246, 26, 21	1022, 21	587, 169, 152, 130	748, 243, 26, 21	785, 205, 58, 6	709, 326, 22
*Pst*I	710, 339	702, 341	534, 332, 172	385, 337, 316	693, 334	713, 211, 130

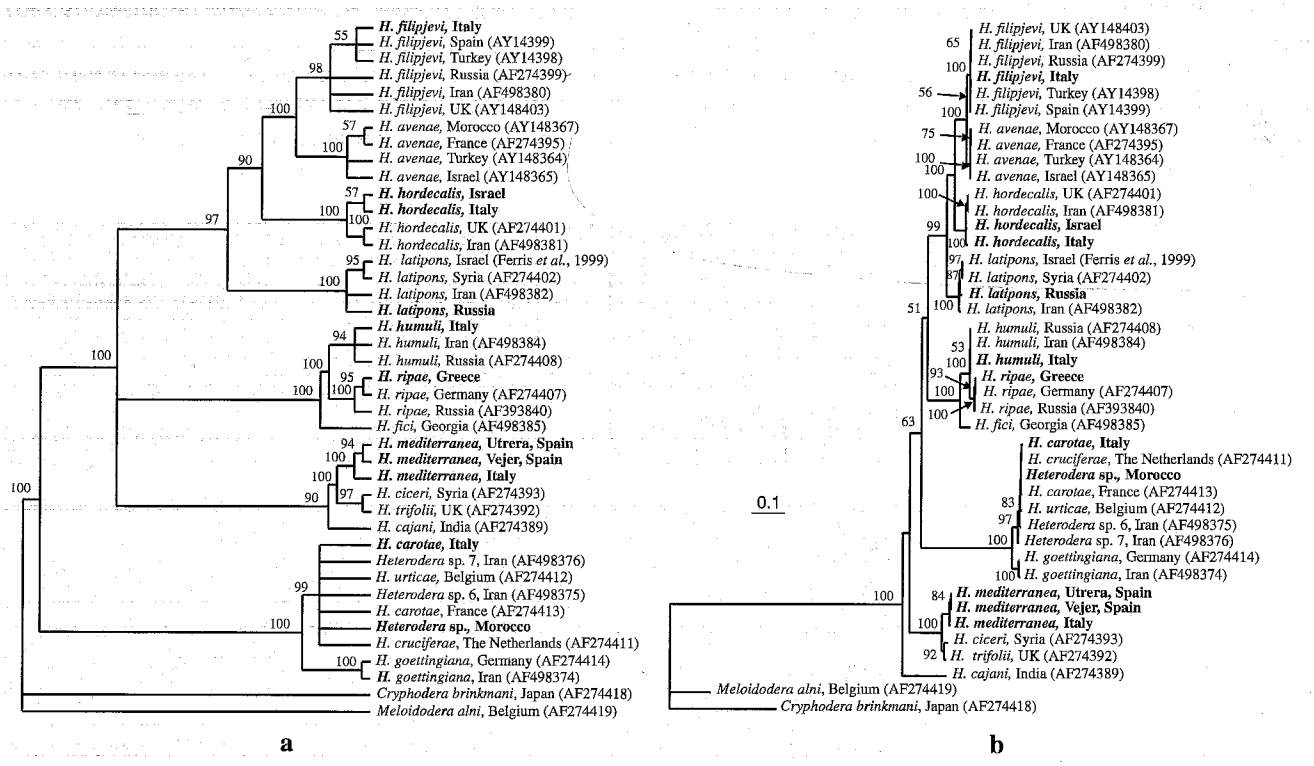

Fig. 2 (a) Strict consensus of 240 maximum parsimonious tree constructed from the internal transcribed spacer (ITS) alignment for 42 sequences of cyst-forming nematodes and two outgroup species (tree length = 1377). (b) Minimum evolution tree constructed using neighbor-joining method and GTR + I + G model of DNA evolution. Bootstrap value more than 50% are given in the appropriate clades

species from Iran and Russia (749, 152, 108 and 44 bp) (Subbotin et al., 2000, 2003; Tanha Maafi et al., 2003).

Sequence and phylogenetic analysis

Nine new and 21 known ITS sequences were used in the analyses. The sequences of the studied populations showed variable similarities with earlier published sequences of the same species: *H. avenae* 0.2%; *H. filipjevi* 0.3%, *H. mediterranea* 0.4%; *H. humuli* 0.3%, *H. ripae* 0.4%, *H. carotae* 0.6%, *H. hordecalis* 1.6%, *H. latipons* 1.8%.

The strict consensus of 240 MP trees and ME tree are presented in Fig. 2. Four main clades each corresponding to the Avenae, Schachtii, Humuli and Goettingiana group are observed on these trees.

Heterodera mediterranea originating from two countries and three different hosts (Vovlas et al., 1981; Castillo et al., 1999) clustered with the Schachtii group. It confirms earlier observation by Sabo et al. (2001). *Heterodera mediterranea* is the only species from the Schachtii group known parasitizing woody plants from the families Anacardiaceae and Oleaceae. It has been suggested that the Schachtii group species primarily coevolved with plants from the family Fabaceae and secondary colonized Chenopodiaceae (Subbotin et al., 2001). The present data also suggest that a host switch from the Fabaceae to the Anacardiaceae

and Oleaceae occurred during the evolution of this group.

Our data also confirm previous observation that *H. hordecalis* belongs to the Avenae group (Subbotin et al., 2001; Tanha Maafi et al., 2003). In the phylogram presented by Sabo et al. (2001), sequences of the Italian population of this species clustered with *Betulodera betulae* (=*Cactodera betulae*) and not with the Avenae group. The relative high level of sequence divergence of the Italian population of *H. hordecalis* from the Scottish and Iranian populations of the same species (1.6%) suggests the presence of two biological species presently grouped under this taxon.

The high sequence divergence was also observed between *H. latipons* populations. It cannot be excluded that the isolate from Russia could, in fact, be a sibling species of *H. latipons* as was early proposed by Ferris et al. (1999).

Two new sequences from an unidentified *Heterodera* species from Morocco and an Italian *H. carotae* clustered with the known sequence data of the Goettingiana group (Subbotin et al., 2001; Tanha Maafi et al., 2003). Our study did not allow to resolve the phylogenetic relationships between most species of this group; two populations of *H. carotae* did not even show clear sister relationships. Perhaps, other genetic markers should be used to discriminate the species of the Goettingiana group.

This is the first report of *H. filipjevi* in Italy. In the Mediterranean region, this species has been detected in Spain (Andres et al., 2001) and Turkey (Rumpenhorst et al., 1996) only. Probably, the species is wider spread than currently known. Therefore, it might be interesting to re-examine earlier findings of the cereal cyst nematode *H. avenae* in this region.

The rDNA-RFLPs distinctly separate species within the genus *Heterodera*. Further, DNA observations and more detailed biological, and ecological studies are needed to identify pathogenic populations of cyst-forming nematodes. Therefore, the creation of a catalogue of RFLPs of the ITS region of cyst-forming nematode species in the Mediterranean Basin would facilitate the identification of species and population, and consequently these data will be of great interest for control of these nematodes.

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