MOLECULAR CHARACTERISATION OF ENTOMOPATHOGENIC NEMATODES ISOLATED IN ITALY BY PCR-RFLP ANALYSIS OF THE ITS REGION OF THE RIBOSOMAL DNA REPEAT UNIT

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Summary. The ITS region of 44 Italian strains of entomopathogenic nematodes (EPNs) belonging to two species isolated during the last ten years in southern Italy was amplified by PCR and the resulting products were digested with nine different enzymes; the fragments generated were then separated by agarose gel electrophoresis. The strains were previously identified by morphological examination using morphometric data. For many of the strains, RFLP analysis confirmed morphological identification (as *Steinernema feltiae* and *Heterorhabditis bacteriophora*) but, for some, RFLP analysis revealed differences when compared with the results from morphometric examinations. In particular, the RFLP profiles of three strains from Sardinia showed differences when compared to the profiles of *S. feltiae* and *H. bacteriophora* and were the same as that of *Steinernema litorale*, which until now has only been found in Japan. We tried to interbreed the Sardinian strains with *S. litorale* and *S. feltiae*; mating attemps were observed but no progeny resulted. The symbiotic bacteria of Sardinian strains belong to the species *Xenorhabdus bovienii*.

Key words: DNA analysis, Italian entomopathogenic nematodes, *Steinernema*, *Heterorhabditis, Xenorhabdus bovienii.*

The entomogenous nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae are able to infect a wide range of insect hosts. The infective juveniles (IJs) of these families enter the host larva, release their symbiont bacteria, *Xenorhabdus* or *Photorhabdus* spp., and kill the insect larvae within 36-48 hours (Poinar, 1975; Griffin *et al.*, 1991; Brown and Gaugler, 1997; Adams and Nguyen, 2002); they are used to control numerous species of soil-borne insect pests (Ehlers, 1996). New species and strains of EPNs are always being discovered. Unfortunately, when new isolates are discovered, their identification is not always straightforward. The identification of nematodes by standard morphological methods is rarely straightforward, and this is why many researchers have turned to molecular techniques, based on the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP). The ribosomal DNA (rDNA) repeat unit is an ideal choice for identification purposes because it is present as a multi-copy tandem repeat in the genome of most organisms. The repeat unit contains highly conserved regions and potentially highly variable regions. The most useful region is the internal transcribed spacer (ITS1+ITS2: between 18S and 26S), which separate the genes. RFLP profiles of the ITS region exist for several species of *Heterorhabditis* and *Steinernema*. The aim of this study was to identify 44 strains of the entomopathogenic nematodes isolated in the last ten years in southern Italy by using the PCR-RFLP molecular technique. The strains had been previously identified as *Steinernema feltiae* (Filipjev) and *Heterorhabditis bacteriophora* Poinar, based on morphometric data (Tarasco and Triggiani, 1997; Triggiani and Tarasco, 2000).

MATERIALS AND METHODS

Nematode isolation and storage

The 44 isolates of EPNs (Table I) were collected using the *Galleria* baiting technique (Bedding and Akhurst, 1975) during a soil survey in different biotopes of the southern Italian regions Apulia, Basilicata, Sardinia, Campania, Molise and Calabria (Tarasco and Triggiani, 1997; Triggiani and Tarasco, 2000). Each soil sample was of nearly 1,000 g and was taken from 0-12 cm depth. Nematodes were cultured in last-instar stages of wax worm *Galleria mellonella* L. larvae at 22-24 °C. To obtain fresh infective juveniles (IJs), ten wax worms in a 100 × 10 mm Petri dish containing one 90 mm filter paper were treated with ca. 2,000 IJs in 1.5 ml of tap water. Two weeks after treatment, wax worms were put on modified White traps (White, 1927) for the recovery of new generations of IJs. The recovered IJs were kept in standard Ringer solution at 8 °C until use.

Molecular identification of the nematodes

The identification of the nematode isolates was made by PCR-RFLP of the ITS region (Internal Transcribed Spacer) of the ribosomal DNA.

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Table I. Strains of EPNs identified and characteristics of the site of isolation in southern Italy.

Isolate code	Nematode species	Geographical origin	Altitude (m) a.s.l.	Habitat	Soil texture
ItS-MA12	*S. feltiae*	Matera (MT)	400	Cave	Loamy sand
ItS-MO1	*S. feltiae*	Melfi (PZ)	500	Orchard (Walnut)	Sand
ItS-MF1	*S. feltiae*	Martina Franca (TA)	350	Woodland (Oak)	Silty loam
ItS-GR1	*S. feltiae*	Grassano (MT)	300	Woodland	Clay loam
ItS-SAR2	*S. litorale*(?)	Platamona (SS)	20	Woodland (Pine)	sand
ItS-SAR4	*S. litorale*(?)	Platamona (SS)	0	Sandy beach	sand
ItS-SAR6	*S. litorale*(?)	Platamona (SS)	20	Woodland (pine)	sand
ItS-G16	*S. feltiae*	Gravina (BA)	380	Woodland (Pine)	Silty loam
ItS-CE2	*S. feltiae*	Cerignola (FG)	120	Orchard (Olive)	Loamy sand
ItS-SA1	*S. feltiae*	Sammichele (BA)	280	Orchard (Cherry)	Loamy sand
ItS-MSA3	*S. feltiae*	Monte S. Angelo (FG)	790	Woodland (Oak)	Silt
ItS-MSA4	*S. feltiae*	Monte S. Angelo (FG)	700	Woodland (Oak)	Silt
ItS-TE1	*S. feltiae*	Terlizzi (BA)	200	Orchard (Cherry)	Clay loam
ItS-LE1	*S. feltiae*	Tricase (LE)	50	Woodland (Oak)	Silty loam
ItS-Q1	*S. feltiae*	Quasano (BA)	150	Woodland (Oak)	Silty loam
ItS-TG4	*S. feltiae*	Torre Guaceto (BR)	20	Moist zone	Loamy sand
ItS-CL2	*S. feltiae*	Celsi (AV)	650	Orchard (Hazelnut)	Loamy sand
ItS-MU1	*S. feltiae*	Mugnano del Cardinale	550	Orchard (Kaki)	Clay
ItS-MV1	*S. feltiae*	Montevergine (AV)	700	Orchard (Chestnut)	Sand
ItS-CS6	*S. feltiae*	Brindisi (BR)	0	Sandy beach	Sand
ItS-CZ19	*S. feltiae*	Gianberga (CS)	800	Woodland (Pine)	Sandy loam
ItS-CZ23	*S. feltiae*	Gianberga (CS)	1100	Woodland (Pine)	Loamy sand
ItS-BQ1	*S. feltiae*	Monte S. Angelo (FG)	780	Woodland (Oak)	Silty loam
ItS-CO1	*S. feltiae*	Corato (BA)	230	Uncultivated land	Silt
ItS-OT3	*S. feltiae*	Otranto (LE)	20	Woodland (Pine)	Sand
ItH-MS6	*H. bacteriophora*	Margherita di Savoia (FG)	40	Orchard (Vineyard)	Clay
ItH-CE1	*H. bacteriophora*	Cerignola (FG)	120	Field (Artichoke)	Loamy sand
ItH-MR4	*H. bacteriophora*	Manfredonia (FG)	50	Uncultivated land	Sandy loam
ItH-CS9	*H. bacteriophora*	Brindisi (BR)	20	Uncultivated land	Sandy clay
ItH-CS15	*H. bacteriophora*	Cellino S. Marco (LE)	70	Uncultivated land	Silty loam
ItH-CS17	*H. bacteriophora*	S. Pietro Vernotico (BR)	20	Orchard (Vineyard)	Silty loam
ItH-LA3	*H. bacteriophora*	Lavello (PZ)	650	Field (Turnip)	Silt
ItH-LU1	*H. bacteriophora*	Lucera (FG)	70	Uncultivated land	Clay
ItH-C3	*H. bacteriophora*	Castellaneta Marina (TA)	50	Field (Maize)	Clay
ItH-C6	*H. bacteriophora*	Castellaneta Marina (TA)	50	Woodland (Pine)	Sand
ItH-C13	*H. bacteriophora*	Castellaneta Marina (TA)	50	Orchard (Olive)	Silt
ItH-C38	*H. bacteriophora*	Castellaneta Marina (TA)	0	Sea coast	Sand
ItH-C53	*H. bacteriophora*	Castellaneta Marina (TA)	30	Woodland	Sand
ItH-C57	*H. bacteriophora*	Castellaneta Marina (TA)	30	Uncultivated land	Sandy loam
ItH-OF3	*H. bacteriophora*	Canosa (FG)	200	Uncultivated land	Sandy loam
ItH-OF5	*H. bacteriophora*	Canosa (FG)	250	Orchard (Vineyard)	Silt
ItH-OF36	*H. bacteriophora*	S. Ferdinando di Puglia	180	Orchard (Peach)	Silty loam
ItH-OF37	*H. bacteriophora*	S. Ferdinando di Puglia	180	Orchard (Vineyard)	Silty loam
ItH-OF40	*H. bacteriophora*	Canosa (FG)	200	Field (Artichoke)	Loamy sand

DNA Isolation. Total genomic DNA was isolated with a DNeasy® Kit supplied by Qiagen GmbH (Hilden, Germany). For each strain, 200 µl of nematode sample (ca. 2,500-3,000 IJs) was used for DNA extraction in a 2 ml Eppendorf reaction tube. After extraction, isolated DNA was stored at -27 °C until use.

PCR Amplification. The amplification of the ITS region was carried out in a reaction volume of 100 µl for each strain, containing 75.6 µl of H_2O, 2 µl of dNTPs (2 mM), 10 µl of 10× PCR-Buffer, 1 µl of Primer Forward (200 µM), 1 µl of Primer Reverse (200 µM), 0.4 µl of Taq polymerase (5U/µl) and 10 µl of purified DNA. The primers 18S (5'-TTGATTACGTCCCTGCCCTTT-3') and 26S (5'-TTTCACTCGCCGTTACTAAGG-3') were used as forward and reverse primers, respectively (Vrain *et al.*, 1992). The amplification was carried out using a DNA thermal cycler (Perkin Elmer Cetus, Emeryville, CA, USA). The samples were placed in the thermal cycler, which was pre-heated to 95 °C and incubated at 94 °C for 3 min. Amplification was started at an annealing temperature for 1 min of 55 °C, followed by an extension period at 72 °C for 1 min 30 s and a denaturation period at 94 °C for 30 s. After 40 of these cycles, there was a final step of 1 min at 94 °C, 3 min at the annealing temperature of 55 °C and 5 min at 72 °C for extension, to ensure that all of the final amplification products were full length (Reid and Hominick, 1998). After amplification the samples were stored at 4 °C.

RFLP Restriction. The digestions with different restriction enzymes to produce RFLPs (Restriction Fragment Length Polymorphisms) were carried out at 37 °C for 3 hours in the thermal cycler, using the corresponding restriction buffers with approximately 200 µl of PCR product. Nine different enzymes were used: Alu I, Hae III, Hind III, Dde I, Hha I, Hinf I, Hpa II, Rsa I and Sau3A I (Amersham Biosciences, Freiburg, Germany). Each reaction tube contained 1 µl enzyme, 3 µl enzyme buffer and 4 µl aqua dest., except for Hind III, which was used at 0.5 µl, 3 µl of enzyme buffer and 4.5 µl aqua dest., and Hinf I, used at 1.25 µl enzyme, 3 µl enzyme buffer and 3.75 µl aqua dest.

Electrophoresis. The resulting fragments were separated by electrophoresis on a 2% agarose gel containing ethidium bromide (0.2%) in 0.5× TBE buffer (54.0 g Tris base + 27.5 g boric acid + 20 ml 0.5 M EDTA (pH 8.0) to 1 l aqua dest). The fragments were photographed under UV light. RFLP patterns were compared with published information on described species (Reid and Hominick, 1998).

Cross breeding tests

The reproductive compatibilities of five *Steinernema* strains were examined in a series of individual diallelic crosses using the "Hanging drop" technique (Poinar, 1975). In this method a sterile drop of insect haemolymph seeded with surface-disinfected nematode juveniles is used. A haemolymph drop obtained from a *Galleria* larva by cutting a leg was released onto a cover slip, which was kept on a cavity microscope slide inside a Petri dish with moistened filter papers. Before immersing in the haemolymph drop, IJs were kept for 15-20 sec in Hyamine® solution (0.1%), which was also used to sterilize the larval leg surface before cutting. Between two and four IJs were released in each haemolymph drop and, after their development, males and females were separated and crosses among different strains assessed using the same haemolymph drops in which the IJs developed. The development of IJs was observed and checked continuously and males and females separated before mating could occur. The nematode isolates tested were: ItSAR2, ItSAR4, ItSAR6, *S. litorale* Yoshida, 2004 (original Japanese strain, choosen for the test because the Sardinian strains showed the same RFLP profile) and *S. feltiae* (ItS-MA12, Italian strain). Each Sardinian strain was crossed with *S. litorale* and *S. feltiae* in a series of six individual diallelic crosses. The Sardinian strains were also crossed among each other and the selfings served as controls.

Characterization of bacterial strains

The symbiotic bacteria were isolated by Patrick Tailliez (INRA, Université Montpellier II, France) from the infective stages of the nematodes by the hanging-drop technique (Poinar, 1966). Total bacterial genomic DNA and the 16S rRNA gene sequence (GenBank accession number EF219400) were obtained as previously described (Tailliez *et al.*, 2006).

RESULTS

The restriction fragment analysis of 22 isolates previously identified as *S. feltiae* resulted in restriction patterns identical to those known for *S. feltiae* strains (Fig. 1A). The restriction fragment analysis of the 19 isolates previously identified as *H. bacteriophora* gave restriction patterns identical to those known for *H. bacteriophora* strains (Fig. 1B). The restriction fragment analysis of 3 isolates (ItS-SAR2, ItS-SAR4, ItS-SAR6) previously identified as *S. feltiae* resulted in restriction patterns different from those obtained with the *S. feltiae* strain. According to the results, ItS-SAR isolates and *S. feltiae* differ in the profiles of the following restriction enzymes: Dde I, Hha I, Hinf I, Rsa I and Sau3A I. For *S. feltiae*, restriction digestion of the ITS region of the rDNA with Dde I yielded fragments of ca 650 and 250 basepairs (bp), for ItS-SAR isolates (ItS-SAR2-4 and 6) ca 900 bp. The rDNA of *S. feltiae* with Hha I yielded fragments of ca 900 and 100 bp, that of ItS-SAR isolates ca 800 bp. For *S. feltiae* with Hinf I fragments of ca 750, 250 and 150 bp; for ItS-SAR isolates ca 300 and 200 bp. Rsa I yielded fragments for *S. feltiae* of ca 400 and 200 bp, for ItS-SAR isolates ca 700 bp. Sau3A I yielded fragments

for *S. feltiae* of ca 500 and 200 bp, for ItS-SAR isolates ca 350 and 250 bp (Fig. 1C). Dde I, Hha I, Hinf I, Rsa I and Sau3A I gave completely different restriction patterns for the isolates, thus provided the characterization of these isolates. These enzymes showed undigested fragments (Fig. 1). The RFLP profiles of the three strains from Sardinia (ItS-SAR2, ItS-SAR4 and ItS-SAR6) were the same of that of *Steinernema litorale* Yoshida, 2004, a species that until this study had only been found in Japan (Yoshida, 2004).

During the individual cross-breeding experiments, mating attempts were observed between the Sardinian strains and *S. litorale* and *S. feltiae*, but no fertile offspring occurred in any of the crosses while the crosses between the Sardinian strains gave fertile progeny. Reproductive incompatibility between *S. litorale* and *S. feltiae* was also observed, confirming the results obtained by Yoshida (2004).

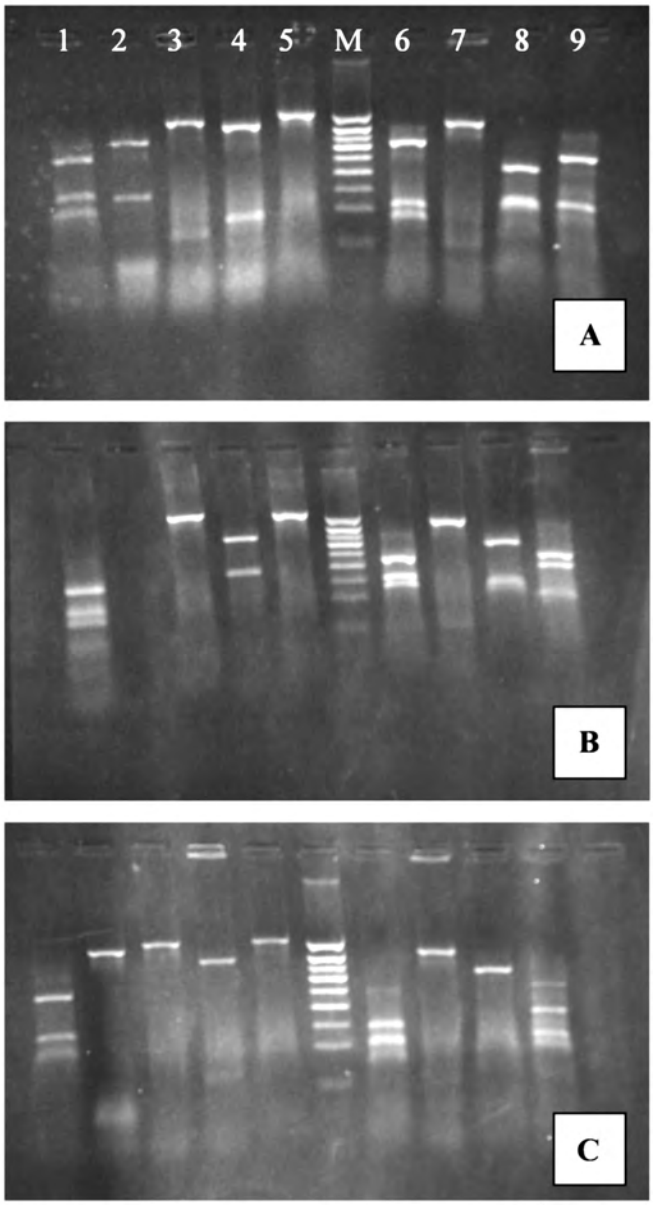

Fig. 1. PCR amplified products from the ITS region digested with 9 restriction enzymes. EPNs species are: A. *Steinernema feltiae* (ItS-GR1); B. *Heterorhabditis bacteriophora* (ItH-C3); C. Isolate ItS-SAR2. Lanes 1-9 indicate the following enzymes: 1. Alu I; 2. Dde I; 3. Hae III; 4. Hha I; 5. Hind III; M. Molecular weight markers (band sizes 1000, 800, 700, 600, 500, 400, 300, 200, 100 base pairs) 6. Hinf I; 7. Hpa II; 8. Rsa I (Afa I); 9. Sau3A I. No pattern for Dde I was obtained for 1B.

The almost complete (~ 90%) 16S rRNA gene sequences of bacterial strains isolated from the nematodes ItS-SAR2 (1429 bps; GenBank accession number EF219400) and ItS-SAR4 were compared with *Xenorhabdus* 16S rRNA gene sequences in the database (Tailliez *et al.*, 2006) and were aligned using the ClustalW multiple alignment programme of the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The 16S rRNA sequences of these bacteria are identical to that of *X. bovienii* type strain DSM4766^T (GenBank accession number X82252), indicating that these strains belong to the *X. bovienii* species.

DISCUSSION

Accurate identification is fundamental to understand geographical distribution and habitat specificity of any organism. Many EPN identifications are based on traditional morphological methods, but recently some scientists have used molecular techniques for the identification of EPNs. The use of molecular methods can greatly reduce the amount of time needed for identification of unknown nematode isolates. PCR offers several advantages compared to more traditional methods of diagnosis: organisms do not need to be cultured prior to processing by PCR, the technique has sensitivity, and it is rapid and versatile (Lee *et al.*, 1993). Most recently, DNA sequence analysis of mitochondrial genes, i.e. cytochrome oxidase II (COII-16S) (Szlanski *et al.*, 2000), 12S rDNA and *cox* 1 genes (Nadler *et al.*, 2006) and nuclear genes, i.e. internal transcribed spacer region (ITS-1) of rDNA (Nguyen *et al.*, 2001) and the large subunit (28S) of rDNA (Stock and Hunt, 2005; Nadler *et al.*, 2006), have been used to assess evolutionary relationships among *Steinernema* spp. and *Heterorhabditis* spp.

In the present study, the RFLP profiles we obtained were compared with those known for the EPN reference species belonging to *S. carpocapsae*-group, *S. intermedium*-group, *S. feltiae*-group *S. glaseri*-group, *H. indica*-group, *H. bacteriophora*-group and *H. megidis*-group (Stock and Hunt, 2005); most importantly, the comparison was made with the species belonging to "*S. feltiae*-group", morphologically similar to Sardininan strains and including *S. feltiae* (Filipjev), *S. thanhi* Phan, Nguyen *et* Moens, *S. neocurtillae* Nguyen *et* Smart, *S. scarabaei* Stock *et* Koppenhöfer, *S. oregonense* Liu *et* Berry, *S. kraussei* Steiner, *S. litorale* Yoshida, *S. loci* Phan, Nguyen *et* Moens, *S. karii* Waturu, Hunt *et* Reid, and *H. bacteriophora* Poinar and *H. megidis* Poinar, Jackson *et* Klein (Miduturi *et al.*, 1996; Elawad *et al.*, 1997; Jian *et al.*, 1997; Waturu *et al.*, 1997; Reid and

Hominick, 1998; Pham Van *et al.*, 2000; Van Luc *et al.*, 2000; Susurluk *et al.*, 2001; Susurluk *et al.*, 2003; Yoshida, 2004; Susurluk, 2005; Stock and Hunt, 2005; Melo-Molina *et al.*, 2006). For many of the strains, the RFLP analysis confirmed the morphological identification (*S. feltiae* or *H. bacteriophora*), but for some of them the RFLP analysis gave different data. In particular the RFLP profiles of the three strains from Sardinia (ItS-SAR2, ItS-SAR4 and ItS-SAR6) were the same of that of *Steinernema litorale*, a species that until this study had only been found in Japan (Yoshida, 2004). As for *S. litorale* from Japan, the Sardinian strains were also isolated from sandy soil of coastal pine woodlands.

Regarding the symbiotic bacteria, the bacteria isolated from Sardinian strains belong to the species *X. bovienii*, but there are no data at the moment on the bacteria of *S. litorale*.

According to the RFLP patterns, the enzymes Alu I, Hae III, Hind III and Hpa II digested the ITS regions into the same size fragments for each isolate. However, the following five restriction enzymes digested the ITS regions into fragments of different sizes: Dde I, Hha I, Hinf I, Rsa I (Afa I) and Sau3A I. Our study has confirmed that the ITS region is ideal for such identification purposes and the isolates examined can be unequivocally distinguished from one another depending on the restriction enzyme used to generate species-specific RFLPs. The results indicate that *S. litorale* is present in Italy and this is the first record for the country.

The cross-breeding tests led to some important considerations. Because the Sardinian strains do not interbreed with *S. litorale*, despite having the same RFLP profile, they may be different races of the same species with a reproductive incompatibility likely to have evolved in two such widely separated countries as Italy and Japan. However, they could also be different species; to clarify this further research is needed.

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