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First report of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) from continental Portugal

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Summary

Until recently, the only entomopathogenic nematode (EPN) species reported from continental Portugal, was of the genus *Steinernema.* Following a national survey of EPNs in continental Portugal, several natural and managed habitats have been surveyed in the southern part of the country. From 57 soil samples collected using the *Galleria mellonella* trapping method, three samples yielded EPN. Morphological characterization and sequence analysis of the ITS regions of ribosomal DNA allowed the identification of EPN isolates as *Heterorhabditis bacteriophora*, representing the first report of this genus for continental Portugal.

Key words: *Heterorhabditis bacteriophora*; entomopathogenic nematode; Portugal

Introduction

The recognition of entomopathogenic nematodes (EPN) as successful biological agents in controlling important insect pests has promoted current knowledge on species biodiversity and their distribution within distinct geographical areas. These species belong to the families Steinernematidae Chitwood & Chitwood, 1937 and Heterorhabditidae Poinar, 1976. Although obligatory insect parasites, these nematodes are capable of exploiting a wide range of soil habitats, such as cultivated fields, grasslands, dry areas, forests or ocean beaches and because of that they are widely distributed throughout the world (Hominick, 2002).

The presence and distribution of EPNs in continental Portugal is poorly known. The first report of an EPN species was made recently (Valadas *et al.*, 2007), with the detection of *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 from the Alentejo and Tejo valley regions. Following the initial surveyed habitats (oak stands and rice paddies), other natural and managed habitats have been selected and surveyed in Alentejo, and extended to some areas of the Algarve.

Material and methods

Soil sampling and nematode isolation

A total of 57 soil samples were collected from different types of natural and cultivated habitats distributed within five different habitats (Table 1). Each composite sample (3 – 4 subsamples), with a final volume of 2L of soil, was collected at a depth of 0 – 20 cm and randomly taken from an area of 200m^2. In order to obtain EPN from the soil, the *Galleria mellonella* L. (Lepidoptera: Pyralidae) trapping method (Bedding & Akhurst, 1975) was used. After mixing the soil, a 1L subsample was placed in a plastic pot (12 cm diameter and 15 cm depth), with 20 last instar larvae of *G. mellonella*. The pots were stored at 25 ºC, and after 6 – 12 days, the dead *G. mellonella* larvae were collected and transferred to White traps (White, 1929). Every 3 – 4 days, dead larvae were removed from the soil. Harvested infective juveniles (IJ's) were stored in an incubator at 10 ºC in distilled water.

Molecular characterization

For morphological observations, several specimens of each isolate were collected from dead *G. mellonella*, mounted in agar 2 % with 0.01 % sodium azide temporary slides, and observed with an Olympus BX-51 light microscope. For molecular analyses, a single adult pregnant female of each isolate was used to extract genomic DNA using the JETQUICK Tissue DNA Spin Kit (GENOMED). PCR reactions were performed to amplify the complete ITS (Internal Transcribed Spacer)-rDNA region, using the forward primer TW81 (5´-GTTTCCGTAGGTGAACC TGC-3´) and the reverse primer AB28 (5´-ATATGCTTA AGTTCAGCGGGT-3´) (Joyce *et al.,* 1994). A 50 µL PCR reaction was prepared with 20 µL of extracted DNA, 2,5U

Table 1. Surveyed areas: habitat from the different soil samples, positive to the presence of *H. bacteriophora*. (The number of samples with EPN specimens is shown in brackets).

Habitats	Region	Number of soil samples	EPN's species
Corn field	Alentejo	5 (1)	*H. bacteriophora*
Mediterranean grasslands ("*esteva* and *giesta"*)	Alentejo Algarve	4 (1) 5	*H. bacteriophora* -
Pinus pinea stand	Alentejo Algarve	8 (1) 8	*H. bacteriophora* -
Dunes	Alentejo	3	-
Olive trees field	Alentejo	24	-

Taq polymerase (BioPortugal), 1X reaction buffer, 1.25 mM $MgCl_2$, 200 µM each dNTP and 16 pmol each primer. The amplification programme was: one cycle at 95 ºC for 3 min followed by 30 cycles at 95 ºC for 1 min; 65 ºC 1 min 30 sec; and 72 ºC 2 min. The last step was at 72 ºC for 5 min. Products were run on 0.8 % agarose gels with 0.5 X TBE buffer. PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) following the manufacturer's instructions. PCR products were sequenced in both directions by a contract sequencer (Macrogen Inc). For sequencing reaction, the primers used were: TW81 and AB28, with two additional internal primers, 58P (5´-ACGAATTGCAGACGCTTAG-3´) (forward) and H58R (5´-GTGCGTTCAAAACTTC

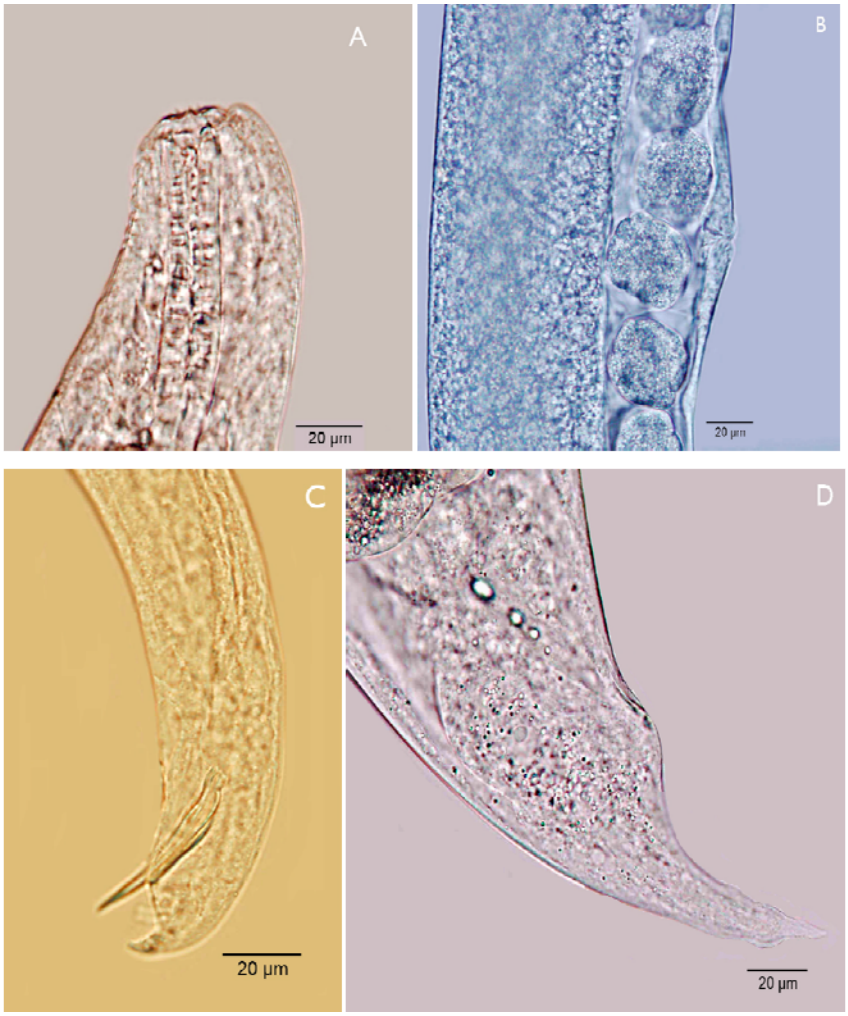

Fig. 1. *Heterorhabditis bacteriophora*. A: Light micrograph (LM) of the anterior region of a hermaphroditic female. B: LM of vulval region. C: LM of male tail region. D: LM of female tail region

ACC-3′) (reverse) (Nguyen *et al.,* 2004). Nucleotide sequences were analysed and edited using BioEdit Sequence Alignment Editor (version 7.0.4.1). The DNA sequences of the Portuguese isolates were compared with other sequences deposited in the GenBank database. Multiple alignments of the ITS rDNA sequences were obtained using Clustal W (Thompson *et al.,* 1994). Phylogenetic analyses were performed by the Neighbour-Joining method, with bootstrap analysis based on 1000 resamplings using MEGA4 software (Tamura *et al.,* 2007).

Results and discussion

EPNs were recovered from three samples from the Alentejo region, corresponding to three different sampled habitats (Table 1). The baited larvae infected from these samples displayed the typical red coloration, lack of putrefaction and retention of shape that is characteristic for *G. mellonella* cadavers infected with heterorhabditids. All the specimens found associated with the different sampled areas displayed the same main morphological characters (Fig. 1).

H. bacteriophora, the most geographically widespread species of this genus, is a common species in regions with continental and Mediterranean climates and it has been reported from different areas of Africa, Asia, Europe, and America (Hominick, 2002). In Southern Europe, this species has an abundant distribution, and it was found associated with several types of habitats in Northern Spain (Garcia del Pino and Palomo, 1996; Campos-Herrera *et al.*, 2007) and in Southern France (Emelianoff *et al.,* 2008). In the last case, two strains of *H. bacteriophora* were reported, based on small differences (4 nucleotides) in the ITS sequence, both strains being associated with the different types of sampled habitats (Emelianoff *et al.,* 2008). Although several habitats with the same soil and climate characteristics as those in Southern France were sampled in Alentejo and the Algarve, only specimens belonging to one of the strains (strain 2) were found, and only in non-coastal habitats (Table 2) as reported in southern France (Emelianoff *et al.,* 2008).

Based on the biodiversity and distribution of EPN's from the Iberian Peninsula and other Mediterranean regions, the presence of *H. bacteriophora* should be expected in continental Portugal. However, no species of this genus had been reported until now for continental Portugal, therefore this work represents the first report of the genus in this geographic area.

Table 2. Positive samples: geographical region and characteristics from soil with *H. bacteriophora*

Sample	Seasons	Soil Characteristics			Geographic location			Atmospheric conditions	
		PH	Texture	Total Nitrogen	Latitude	Longitude	Altitude	Tm anual (ºC)	Pp (mm)
I3	Autumn	6.58	Medium	0.2 %	38.77 N	8.68 W	77 m	21.15	3.0
R7	Winter	6.35	Medium	0.2 %	37.34 N	8.79 W	90 m	13.35	0
X7	Spring	5.48	Coarse	0.13 %	39.29 N	7.46 W	394 m	7.65	0

In the first generation, hermaphroditic females display a truncated head, with six well-developed conical lips (Fig. 1A), and median vulva without epiptygma (Fig. 1B). Second-generation amphimictic females are similar to the hermaphroditic females. Males displayed paired, separate spicules; bursa with nine pairs of papillae, a pair anterior to cloaca, two pairs adjacent to the spicules and six pairs distal to the anal opening, the latter six distributed in two sets of three (Fig. 1C).

The sequence flanked by the two primers yielded a total of 809 bp, composed of the partial 18S (nucleotides 1 – 14 in the alignment), ITS1 (15 – 403), 5.8S gene (404 – 557), ITS2 (558 – 785), partial 28S (786 – 809). The ITS1-5.8S-ITS2 region of the three Portuguese isolates displayed 100 % similarity to each other [accession numbers EU435138 (isolate I3), EU435139 (isolate R7), EU435140 (isolate X7)]. BLAST searches suggested that the isolates belong to the species *Heterorhabditis bacteriophora* Poinar, 1976. Phylogenetic relationships based on the sequence alignment of the ITS rDNA region confirmed that the Portuguese isolates group with other isolates of *H. bacteriophora*, when compared with other species of the genus (Fig. 2).

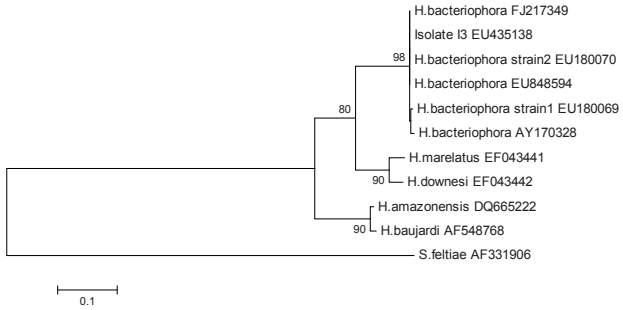

Fig. 2. Phylogenetic relationships of *Heterorhabditis bacteriophora* isolates from Portugal and other geographical regions, including other species of the genus, based on the sequence alignment of the ITS regions from ribosomal DNA. The dendrogram was generated by Neighbor-Joining analysis with 1000 bootstrap replication. Bootstrap values (%) are indicated at the nodes. The scale bar indicates 1% substitutions per site.

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