DOI 10.2478/s11687-007-0037-1

HELMINTHOLOGIA, 44, 4: 226 – 229, 2007

Research Note

First report of an entomopathogenic nematode from continental Portugal

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Summary

In continental Portugal no information is available concerning the occurrence of entomopathogenic nematodes. During a survey in several different habitats from the southern regions of the country, several isolates where identified as *Steinernema feltiae*. This represents the first report of an entomopathogenic nematode genus for continental Portugal.

Key words: *Steinernema feltiae*; Portugal; sequence

Entomopathogenic nematodes (EPN) of the families Steinernematidae Chitwood & Chitwood, 1937 and Heterorhabditidae Poinar, 1976 are parasites of insects, and can be found under diverse ecological conditions, including cultivated fields, grasslands, deserts, forests and oceanic beaches (Hominick *et al.*, 1996). The need to find new alternatives that may reduce the use of harmful insecticides has promoted the exploitation of such organisms as biological agents. In the past decades, the recognition of EPN as successful biological agents to control important agricultural insect pests, has led to an increase in the research and use of these nematodes for this purpose. Additionally, extensive surveys for the detection and isolation of new useful species/strains of EPN, in distinct geographic areas were also undertaken (Gaugler, 2002).

In Portugal, the only region where studies of EPN have been made is in the Azores archipelago (Simões *et al.*, 1994; Medeiros *et al.*, 2000; Rosa & Simões, 2004). In these Atlantic islands, 1500 km west of Lisbon, several surveys have been undertaken, as part of a wide program to find endemic biological agents to control insect pests of pastures, such as the Japanese beetle, *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae), and the armyworm, *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae) (Rosa *et al.*, 2000). Presently, the EPN species reported from the Azores archipelago correspond to *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, *S. glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding, 1982 and *Heterorhabditis bacteriophora* Poinar, 1976. To date these species have been found only in the eastern and central groups of islands, the closest to the European and African continents (Rosa *et al.*, 2000). However, in continental Portugal there are no known reports of this group of nematodes.

In 2006 a national survey was initiated in order to study the presence and distribution of EPN in all continental territory of Portugal, especially in areas that are considered suitable habitats (e.g. sandy soils, cultivated fields, grasslands) for the presence of steinernematids and heterorhabditids. During a random survey in Alentejo (oak stands) and southern Tejo valley (rice paddies) regions, soil samples from different habitats were collected and assayed for the presence of EPN. At each sampling site several sub samples were taken, totalling approximately 2 L of soil from a depth between 3 and 20 cm, placed in a plastic bag and transported to the laboratory. In order to extract EPN from the soil, the *Galleria mellonella* L. (Lepidoptera: Pyralidae) trapping method (Bedding & Akhurst, 1975) was used. After soil homogenisation, a 1 L sub sample was placed in a plastic pot (12 cm diameter and 15 cm depth), with 20 last instar larvae of *G. mellonella*. The boxes were stored at 25 ºC, and after 6 – 12 days the dead *G. mellonella* larvae were collected and transferred to White traps (White, 1929). Harvested infective juveniles (IJs) were stored at 10 ºC in distilled water.

To confirm the identification of the nematodes harvested from the *G. mellonella* cadavers, a molecular characterization was conducted. DNA was extracted from many

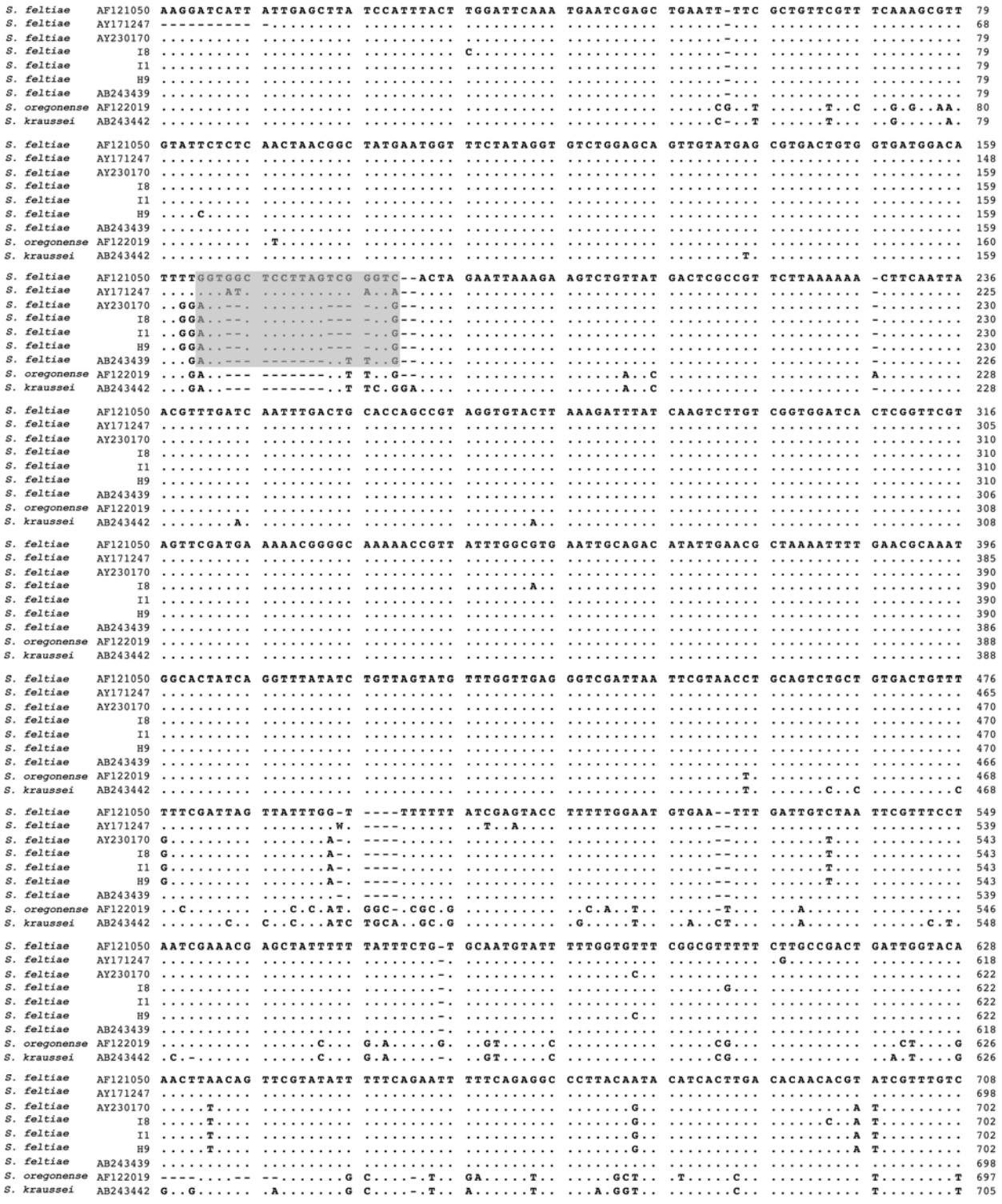

Fig. 1. Multiple sequence alignment of the ITS rDNA region (including partial fragments of the 18S and 28S rDNA genes) of three *Steinernema* species. Codes H9, I1 and I8 correspond to the Portuguese isolates of *Steinernema feltiae*. Highlighted grey correspond to different ITS1 sequences types of *Steinernema feltiae* isolates.

nematodes using the Tissue DNA Isolation Kit (Amersham Biosciences). PCR assays were set up in order to amplify the complete ITS-rDNA region, using the forward primer ITS1 [5´-TCCGTAGGTGAACCTGCGG-3'] and the reverse primer ITS4 [5´-TCCTCCGCTTATTGATATGC-3´] (Nasmith *et al.*, 1996). The PCR products were cloned and sequenced using the TOPO TA Cloning®Kit for Sequencing (Invitrogen). Specifically, PCR fragments were ligated into the pCR®4-TOPO plasmid, and transformed into TOP10 chemically competent *E. coli*. Successful recombinant colonies were cultured overnight in LB broth (Lab M), with 100µg/ml ampicillin (Sigma), at 37°C with gentle agitation. Plasmids were extracted from subsequent cultures using the StrataPrep® Plasmid MiniPrep Kit (Stratagene). The presence of ligated inserts was confirmed by *EcoR* I (Promega) digestions of plasmids followed by size comparisons between plasmid and insert using agarose gel electrophoresis. PCR inserts were sequenced directly from plasmids by a contract sequencer (Qiagen Inc.). Nucleotide sequences were determined in both directions using PCR M13 primers. The DNA base sequences obtained for the Portuguese isolates [accession numbers EF595633 (isolate I1), EF595634 (isolate I8), EF595635 (isolate H9)] were compared with other sequences deposited in the GenBank database.

Bait larvae infected with isolates from three sampled sites (I1 and H9 from Alentejo region, and I8 from southern Tejo valley) displayed the grey-brown colouration, lack of putrefaction and retention of shape that is characteristic of *Galleria* cadavers infected with steinernematids. The three strains isolated show the typical morphological characters within the genus *Steinernema* Travassos, 1927 (Adams & Nguyen, 2002).

The ITS1-5.8S-ITS2 region, including the partial 18S and 28S rDNA genes (flanked by the above primers) of the three Portuguese isolates are almost identical (2 – 6 nucleotide substitutions), and are 773 bp long. BLAST searches (Altschul *et al*., 1990) in GenBank showed that the three Portuguese isolates have a high similarity (96 – 99%) with those sequences available for *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 populations (e.g. accession numbers AF121050, AY1711247, AY230170, AB243439). Sequences of other species from the *feltiae* group, namely *S. oregonense* Liu & Berry 1996 and *S. krauseei* (Steiner, 1923) Travassos, 1927, were obtained from GenBank searches that exhibited a lesser degree of similarity with the Portuguese isolates and other *S. feltiae* populations (e.g. accession numbers AF122019, AB243442) (Fig. 1).

Alignment of the ITS 1 regions from the Portuguese isolates with *S. feltiae* ITS 1 sequences obtained from GenBank, suggests that the Portuguese isolates share a high degree of homology to a UK population (accession number AY230170) as characterised by six shared nucleotide deletions (Fig.1) classified previously by Spiridonov *et al.* (2004).

Unlike other European countries Portugal has not fully engaged with its soil biodiversity for the discovery of EPN. The present study constitutes the first report of an EPN genus for continental Portugal. *S. feltiae* has a wide distribution in temperate regions, being one of the most common species found in Europe, and in many other parts of the world (for a detailed EPN species distribution see Hominick, 2002). The preliminary results presented herein show that *S. feltiae* is distributed in a geographic medium-scale in central-south areas of Portugal, associated with distinct types of habitats (oak stands and rice paddies). In addition, this is the first time that *S. feltiae* is recorded for the Portuguese territory.

Acknowledgements

The authors kindly thank Francisca Figo for technical assistance. This short communication is a portion of the PhD dissertation of the first author, University of Évora, who is supported by a doctoral scholarship from Fundação para a Ciência e a Tecnologia (SFHR\BD\22086\2005).

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RECEIVED MAY 25, 2007

ACCEPTED JULY 10, 2007