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Natural occurrence of *Steinernema carpocapsae*, Weiser, 1955 (Rhabditida: Steinernematidae) in Belgian turf and its virulence to *Spodoptera exigua* (Lepidoptera: Noctuidae)

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Summary. During a routine survey for the white grub, *Hoplia philanthus* Füessly (Coleoptera, Scarabaeidae), in turf fields, we isolated entomopathogenic nematodes from one out of 40 soil samples. The morphology and morphometrics identified the isolate as *Steinernema carpocapsae*. The ITS-rDNA sequence confirmed the identification. This is the first report of *S. carpocapsae* from Belgium. In laboratory assays the isolate demonstrated a virulence to *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) comparable to two commercial strains and two other strains kept in laboratories. All tested isolates including the Belgian strain caused high mortality to *S. exigua* larvae.
Key words: Biological control; entomopathogenic nematode; ITS regions, natural occurrence, soil survey, virulence.

Entomopathogenic nematodes (EPN) from the families Heterorhabditidae and Steinernematidae are lethal parasites occurring in various soil habitats around the world (Hominick, 2002). Isolating EPN from soil or naturally infected insects provides insight into the biodiversity of these naturally occurring pathogens and provides a pool of potential biocontrol agents.

In the late 1990s Belgian soils were intensively sampled for the presence of EPN (Miduturi *et al.*, 1996a; 1996b; 1997; Spiridonov & Moens, 1999). As a result, *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, *S. affine* (Bovien, 1937) Wouts, Mráček, Gerdin & Bedding, 1982, *S. kraussei* (Steiner, 1923) Travassos, 1927 and *Heterorhabditis megidis* Poinar, Jackson & Klein 1987 were isolated using the *Galleria*-baiting technique (Bedding & Akhurst, 1975) or by direct extraction from soil (*S. kraussei*). Later, *H. bacteriophora* Poinar, 1976 (Ansari *et al.*, 2003a) and *S. glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding, 1982 (Ansari *et. al.* 2005) were isolated from naturally infected third-instar *Hoplia philanthus* Füessly grubs (Coleoptera: Scarabaeidae). These latter EPN species are widely used biocontrol agents against various insect pests (Ansari *et al.*, 2003b; Georgis *et al.*, 2006).

Despite the progress that has been made in the use of EPN, knowledge about their natural host range and their effect on insect populations is still limited. Therefore, the objectives of this study were to identify EPN occurrence in Belgian turf, as well as to determine their virulence against the beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), an important polyphagous pest of cultivated crops primarily in the tropical and subtropical regions.

MATERIAL AND METHODS

Soil sampling. During the summer of 2004, a total of 40 soil samples (approximately 1500 g each) were collected under the grass lawn (*Festuca rubra* L.) of a sport field at Eeklo, province of East-Flanders, Belgium. The soil was a sandy loam (88.0 % sand, 5.6% loam, 6.4% clay, 5.8 % organic matter). These samples were taken as a part of a routine field survey for white grubs, *H. philanthus*, which is an important pest of sports turf, lawns and pastures throughout the country (Ansari *et al.*, 2003b).

Nematode identification. Each sample was homogenised and divided into 500g subsamples. EPN were extracted from these subsamples by the insect baiting method. Ten *Galleria mellonella* (Lepidoptera: Pyralidae) larvae were placed in 500

ml plastic containers (3 containers/sample) with moist soil from each sample. Containers were covered with a lid, turned upside down and kept at 23 ± 2°C. After 7-8 days, all *Galleria* were recovered and the parasitized cadavers were individually placed in White traps (White, 1927) to allow the emergence and collection of the infective juveniles (IJ) from the cadaver. To verify their pathogenicity, collected IJ were transferred onto moist filter paper in Petri dishes to which living *G. mellonella* larvae were added. After infection, *G. mellonella* larvae were dissected in Ringer's solution at 3- or 4-day intervals to obtain the characteristic developmental stages of the nematodes. For the identification of EPN, 25 first-generation males and 25 IJs were randomly selected from different *G. mellonella* cadavers. The nematodes were identified according to a selection of morphological and morphometrical criteria summarised by Hominick *et al.* (1997).

Molecular characterization. To confirm the identification, the nematodes isolated from *Galleria* cadavers were characterized using molecular techniques. DNA was extracted from single nematodes and amplified as described by Waeyenberge *et al.* (2000). Primers TW81 and AB28 (Joyce *et al.*, 1994) were used for amplification of the ITS1-5.8S-ITS2 region of the rDNA cistron. After electrophoresis, the amplified products were excised from 1% TAE-buffered agarose gels using the Qiaquick Gel Extraction Kit (Qiagen Benelux B.V., Venlo, The Netherlands), cloned into the pGEM-T vector and transformed

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                          10         20         30         40         50         60         70         80
DQ302092.carpocapsae* ATCAAGTTTT CGCTGTTCGT TTCTAAGCTT TAACTTGATC TCTAACGGCT TTGAAAGGTT TCTACAGATG TTTGGAGCAG
AY171282.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY230164.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY170334.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY487919.sasonense    ....G..... .......... ........G. .G........ .......... .......... ....T..... ..........
AY487920.cumgarense   ....G..C.. T......... ........A. .G.....T.T C.C----... .....T.... ....G...C. ..........
AY171280.tami         ....G..C.. T......... ........A. .G...C...- CT.----... .....T.... ....T...C. ..........

                           90         100        110        120        130        140        150        160
DQ302092.carpocapsae* TTGTATGAGC GTGACTGTGC TGATGAACAT TGTACATTGT TATCTAAGCG TTTCGATGTT TCTAGAATGC TTAGTGATGA
AY171282.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY230164.carpocapsae  .......... .......... .......... .......... .......... .....TC... ...T...... ..........
AY170334.carpocapsae  .......... .......... .......... .......... .......... .....TC.C. ...T...... ..........
AY487919.sasonense    ........A. .........T .......... .T.....C.. .......... .....TC... ...T...... ..........
AY487920.cumgarense   .C..TCA... .C.......A ....A.G.G. .T...T...- ---..T...A A....T.... ...T...... ....C.....
AY171280.tami         .CA.TCA... .........G ....A.G.G. .T...T...- ---..TT..A G....TC... ...T...... ....C.....

                          170        180        190        200        210        220        230        240
DQ302092.carpocapsae* GAATTAAAGA GGTCTGCTGA CTCGCCATTC TTTGATTGCT AACAAAAACG TTTTGTTTCG ATAATTGTGT CACTCGTTGA
AY171282.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY230164.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY170334.carpocapsae  .......... .........G .......... .......... .......... .....G.... .......... ..........
AY487919.sasonense    .......... .......... .......... .......... .......... ........T. .........A. ..T.......
AY487920.cumgarense   .......... A......... .........- ...A...... .......... ........T. ..TT...... ....T.....
AY171280.tami         .......... A......... .........- .......... .......... ........T. ..TT...... ....T.....

                          250        260        270        280        290        300        310        320
DQ302092.carpocapsae* TGCATTTTTT AATTATCAA- GTCTTATCGG TGGATCACTC GGTTCGTAGG TCGATGAAAA ACGGGGCAAA AACCGTTATT
AY171282.carpocapsae  .......... .........- .......... .......... .......... .......... .......... ..........
AY230164.carpocapsae  .......... .........- .......... .......... .......... .......... .......... ..........
AY170334.carpocapsae  .......... .........A .......... .......... .......... .......... .......... ..........
AY487919.sasonense    ......A..C .T.......- .......... .......... .......... .......... .......... ..........
AY487920.cumgarense   ......A..A .........- .......... .......... .......... .......... .......... ..........
AY171280.tami         .A....A..C .........- .......... .......... .......... .......... .......... ..........

                          330        340        350        360        370        380        390        400
DQ302092.carpocapsae* TGGCGTGAAT TGCAGACATA TTGAGCGCTA AAATTTTGAA CGCAAATGGC ACTAACAGGT TTTTATCTGT TAGTATGTTC
AY171282.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY230164.carpocapsae  .......... .......... .......... .......... .......... .......... .......... ..........
AY170334.carpocapsae  .......... .......... .......... .......... .......... .......... ......T... ..........
AY487919.sasonense    .......... .......... .......... .......... .......... .......... ..C......G ..........
AY487920.cumgarense   .......... .......... .......... .......... .......... .........G ..--...... ..........
AY171280.tami         .......... .......... .......... .......... .......... .........G ..--...... ..........

                          410        420        430        440        450        460
DQ302092.carpocapsae* AATTGAGGGT CTTTTGACTA GAATCTGGCA ATCGGCTGTG ATTGCTTTTT CGGTAAGCTA CTTT
AY171282.carpocapsae  .......... .......... .......... .......... .......... .......... ....
AY230164.carpocapsae  .......... .......... .......... .......... .......... .......... ....
AY170334.carpocapsae  .......... .......... .......... .......... .......... .......... ....
AY487919.sasonense    .......... .......... .......... .......... .......... .......... ....
AY487920.cumgarense   .......... .......... .......... .......... .......... ...A...... ....
AY171280.tami         .......... .......... .......... .......... .......C.. ...A...... ....
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Fig. 1 Alignment of the ITS-rDNA sequence of the Belgian isolate of *Steinernema carpocapsae* with the sequences of three other *S. carpocapsae* populations and three most closely related *Steinernema* species.

into JM 109 High Efficiency Competent Cells (Promega, Leiden, The Netherlands). Per individual, five colonies were isolated using blue/white selection and used for PCR with vector primers. Amplified products were purified using a Qiaquick PCR Purification Kit (Qiagen Benelux B.V, Venlo, The Netherlands). Purified fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit and purified according to manufacturer's instructions (PE Applied Biosystems, Foster City, CA, USA). The resulting products were analysed using an ABI Prism 310 Genetic analyser.

Bioassay. To obtain more information on its potential as a biocontrol agent, we compared the virulence of the Belgian strain with that of four other *S. carpocapsae* strains, which were obtained either from commercial companies Becker Underwood (UK) and Koppert B.V. (the Netherlands) or from different institutes (Drs R.-U. Ehlers, Christian-Albrechts-University, Kiel, Germany and Z. Mráček, Institute of Entomology, Czech Academy of Science, Česke Budějovice, Czech Republic). The first two strains were stored at 10°C and used following the instructions given on the label. The later two strains were cultured on last instar *G. mellonella* at 22°C (Kaya & Stock, 1997).

The virulence assays were conducted in 9-cm diameter Petri dishes lined with a Whatman No 1 paper disc. To each dish a piece of insect diet (Poitout *et al.*, 1972) was added before five 4th instar larvae of *S. exigua* were added and exposed to IJ of one isolates. Different concentrations, 763 and 1526 IJ per dish (corresponding to 1.2 and 2.5 $\times$ 10^9 IJs/ha), were pipetted to each Petri dish in a volume of 900 µl of tap water. The control treatments received water only. The dishes were incubated at 23 $\pm$ 1°C, 70 $\pm$ 5% relative humidity and a 16 : 8 h light/day cycle. Fresh food was added after 24 h. Percentage mortality data at 48 h were analysed using ANOVA and Tukey's test for separation of the means (SPSS 13). Differences among means were considered significant at $P < 0.05$. Experiments were repeated three times in the same conditions.

RESULTS AND DISCUSSION

One out of the 40 samples contained EPN belonging to the genus *Steinernema*. Taxonomic investigations revealed the population to be conspecific with *S. carpocapsae*.

The DNA sequence (deposited in Genbank under accession number DQ302092) was compared to sequences of *Steinernema* species available in Genbank. The BLAST search indicated a 98% similarity between the sequences of the PCR-product from our *Steinernema* isolate and that of three *S. carpocapsae* populations previously submitted to Genbank (accession numbers AY171282, AY230164 and AY170334) (Fig. 1). Other quite similar sequences were those from *S. sasonense* (AY487919), *S. cumgarense* (AY487920) and *S. tami* (AY171280) with 95% similarity. All other *Steinernema* species available in Genbank showed less than 95% similarity.

This is the first record of the natural occurrence of *S. carpocapsae* in Belgium. This species is geographically widespread in temperate regions of the world (Hominick, 2002). However, it is rare in central and northern European countries. The species has been isolated previously from turf (Campbell *et al.*, 1998; Alumai *et al.*, 2006). Although, *S. carpocapsae* was recorded only from one out of 40 samples collected in a restricted area, its recovery highlights the importance of conducting more surveys considering other natural areas and geographic regions.

The five *S. carpocapsae* strains tested showed virulence to fourth instar *S. exigua*, causing a significant higher mortality (95-100%) than in the untreated control ($F = 427.4$; df = 5, 12; $P < 0.001$). Obviously, *S. carpocapsae* is an efficient control agent of *S. exigua* and our results corroborate the finding of a previous study (Hara & Kaya, 1983). However, environmental factors such as temperature and host density under glasshouse and field conditions had a considerable impact on the efficacy of the tested EPN species/strains (Koppenhöfer, 2000; Georgis *et al.*, 2006). Presently we are testing the efficacy of the *S. carpocapsae* Belgian strain under glasshouse conditions.

More species may still be detected in future surveys adding significant information to the diversity and biogeography of this group of nematodes. Knowledge of the diversity, distribution of EPN is extremely valuable not only for future ecological and biocontrol studies but also from a bio-prospecting perspective.

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Резюме. В процессе изучения хрущей *Hoplia philanthus* Füessly (Coleoptera: Scarabaeidae) в газонах в Бельгии в одной из 40 проб были выявлены энтомопатогенные нематоды. Морфология и морфометрические данные этого штамма соответствовали *Steinernema carpocapsae*. Секвенирование ITS-rDNA подтвердило это определение. Это первое сообщение о *S. carpocapsae* из Бельгии. В сравнении с двумя коммерческими штаммами в лабораторных экспериментах была изучена вирулентность бельгийского штамма *S. carpocapsae* для *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). Все изученные штаммы *S. carpocapsae* патогенны для личинок *S. exigua*.