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First record of a cold-active entomopathogenic nematode *Steinernema kraussei* (Steiner) (Rhabditida: Steinernematidae) in Slovenia

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ABSTRACT

In preceding researches on occurrence of entomopathogenic nematodes in Slovenia, which started in 2007, we already recorded *Steinernema affine* (Bovien), *S. feltiae* (Filipjev) and *S. carpocapsae* (Weiser). In April 2008, 120 soil samples from 24 locations were collected in Gorenjska, Notranjska and Primorska regions as well as in Ljubljansko barje. The presence of entomopathogenic nematodes was confirmed in 9 samples from 6 locations. Only the sample C46, which was taken in the village Podbrezje in Gorenjska region, was sent to genetic analysis. Molecular biological analysis have proved the identity of the sample with the species *Steinernema kraussei* (Steiner). This was the first record of *Steinernema kraussei* in Slovenia.

Key words: entomopathogenic nematodes, Slovenia, *Steinernema affine*, *Steinernema feltiae*, *Steinernema carpocapsae*, *Steinernema kraussei*, biological control

IZVLEČEK

PRVA NAJDBA ENTOMOPATOGENE OGORČICE *Steinernema kraussei* (Steiner) (Rhabditida: Steinernematidae) V SLOVENIJI

V predhodnih raziskavah preučevanja razširjenosti entomopatogenih ogorčic v Sloveniji, ki potekajo od leta 2007, smo ugotovili zastopanost vrst *Steinernema affine* (Bovien), *Steinernema feltiae* (Filipjev) in *Steinernema carpocapsae* (Weiser). V aprilu 2008 smo na območjih Gorenjske, Notranjske, Primorske in Ljubljanskega barja na 24 lokacijah nabrali 120 talnih vzorcev. Zastopanost entomopatogenih ogorčic smo ugotovili v 9 vzorcih z 6 lokacij. V nadaljnjo genetsko analizo smo poslali le vzorec C46. Ta je bil odvzet v vasi Podbrezje na Gorenjskem. Z molekulsko analizo smo identificirali vrsto *Steinernema kraussei* (Steiner). Gre za prvo odkritje omenjene vrste entomopatogene ogorčice pri nas.

Ključne besede: entomopatogene ogorčice, Slovenija, *Steinernema affine*, *Steinernema feltiae*, *Steinernema carpocapsae*, *Steinernema kraussei*, biotično varstvo

1 INTRODUCTION

Entomopathogenic nematodes (EPNs) from genera *Steinernema* Travassos and *Heterorhabditis* Poinar are obligatory parasites of numerous insects (Ishibashi and Choi, 1991). They live in symbiotic relationship with bacteria from genera *Xenorhabdus* and *Photorhabdus* (Forst *et al*., 1997). Momentarily, 70 species of EPNs

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are classified into families Steinernematidae (56 species) and Heterorhabditidae (14 species) (Yilmaz et al., 2008).

EPNs are cosmopolitan animals, while we can find them on all continents, with the exception of Antarctica (Griffin et al., 1991; Hominick, 2002). Although EPNs are pathogenic to many pest insect species (Poinar, 1979), their successful commercial application is limited to relatively low number of insects (Grewal and Georgis, 1999; Shapiro-Ilan et al., 2002).

Application of EPNs in biological control was traditionally engaged in controlling soil pests until some years ago (Ishibashi and Choi, 1991). Results from researches in the last two decades indicate also their potential against foliar pests, but only under special conditions (Arthurs *et al*., 2004). Poorer efficacy of EPNs in controlling foliar pests is a consequence of unsuitable (too low) moisture (Lello *et al*., 1996), exposure to extreme temperatures (Grewal *et al*., 1994), and ultraviolet radiation (Gaugler and Boush, 1978). These factors are known as crucial for survival of the nematodes (Kaya, 1990). For this reason the efficacy of EPNs in the open is therefore often worser as expected, although predecessor laboratory tests shows rather better efficacy (Buitenhuis and Shipp, 2005).

In the last period biological potential of EPNs has influenced the large number of new investigations, in which scientists want to find new species of EPNs and their symbiotic bacteria (Hominick et al., 1996; Mráček et al., 2006; Nguyen et al., 2006; Tóth and Lakatos, 2008); with a desire to also study other topics, which are indirectly or directly connected with the efficiency of EPNs in biological control of insect pests: biodiversity, ecology, evolution, biochemistry, symbiosis and molecular genetics (Burnell in Stock, 2000; Li et al., 2007).

At the moment there are some companies on the market, which generate bioproducts on the basis of EPNs (Willmott et al., 2002). In biological control of pests in plant protection limited number of EPNs is commercialy available: *Steinernema feltiae, S. carpocapsae, S. kraussei, S. riobrave, S. scapterisci, Heterorhabditis bacteriophora in H. megidis H. marelata, H. zealandica*). Due to before mentioned facts, it is important to study domestic soil fauna on the occurrence of EPNs, while in many countries these organisms are treated as exotic organisms and therefore their application is limited to laboratory researches.

In Slovenia, momentarily only entomopathogenic nematodes *Steinernema feltiae* and *S. carpocapsae* have a status of indigenous species (MAFFab, 2008, Laznik et al., 2008bc); therefore only this two nematodes can be applied in the field. With the researches, which results we also present in this paper, we want to enlist as more species of entomopathogenic nematodes as it is possible, while in foreign countries they worth as alternatives to insecticides in controling pest insects. The strain C46 (*Steinernema kraussei*), which we present in a current paper, we plan to use in extensive experiments in the future; first in the laboratory and afterward, when its status will be administratively entrenched, also in the field.

2 MATERIALS AND METHODS

In April 2008, we examined 120 soil samples from 24 different locations on the occurrence of EPNs in Slovenia. The soil samples, five from each sampling place, were taken in Gorenjska, Notranjska and Primorska regions as well as in Ljubljansko barje. We used »*Galleria* bait method«, which is the most frequently used method for EPNs detection from soil. After the death of greater wax moth (*Galleria mellonella* [Linnaeus]) larvae, we dried cadavers for 12 days and put them in so-called »white trap« (Bedding and Akhurst, 1975) to separate the nematodes from death larvae. The suspension, which was acquired in this way, was used for artificial infection of the larvae of greater wax moth. Following procedure contained the use of centrifuge and 5 % concentration of sodium hypochlorate. The aim of this process was to acquire infective juveniles from the suspension. We confirmed the presence of the nematodes in 9 soil samples from 6 locations. Only 1 positive sample, C46 (taken in the forest near village Podbrezje in Gorenjska region [N Slovenia, 46°17′N, 14°16′E, 403 m alt.] was identified to this time.

3 RESULTS

To confirm the identification of isolated nematodes from larvae of wax moth, a selected sample was analysed by molecular biological approach. Genomic DNA was extracted from individual nematodes and PCR was performed to multiply ITS region using primers TW81 and AB28 after Hominick et al. (1997). PCR product were reisolated from 1 % TAE-buffered agarose gel using QIAquick Gel Extraction Kit (Qiagen, USA) (Fig. 1). Reisolated sample was sequenced in the laboratory of Biological Research Centre in Szeged,

Hungary. The sequence was submitted into GenBank public database (Accession Number: EU914856). Sample DNA sequence was compared to sequences of species *Steinernema* using BLAST search in National Centre for Biotechnology Information (NCBI) web site (www.ncbi.nlm.nih.gov). The sequences producing significant alignments and at least 99 % identity were derived from *Steinernema kraussei*: GenBank Accession No. AY230175 and AY171264 (Spiridonov *et al*., 2004) (Fig. 2).

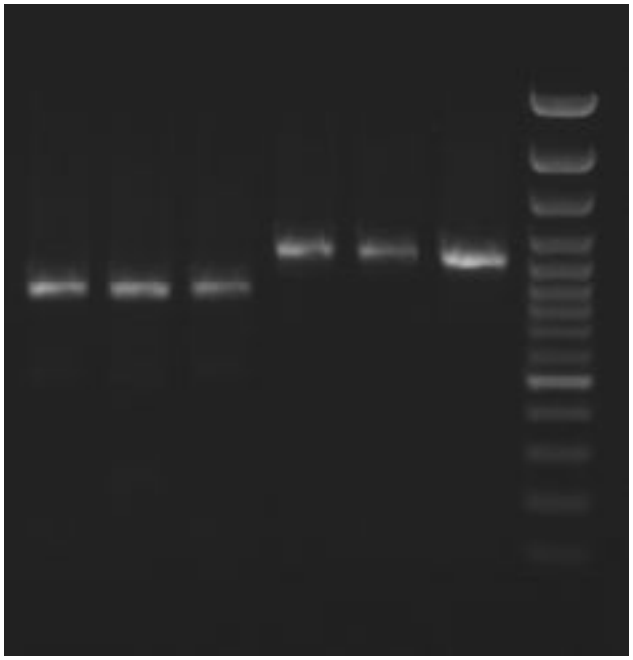

Figure 1*: 1% TAE buffered agarose gel, in the 7th lane: GeneRuler 100 bp DNA Ladder Plus (Fermentas), in the 4th, 5th and 6th lane: PCR product of our sample C46, using the primer pair specified in the text. The two most strength fragment in the ladder are 500 and 1000 bps length.*

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EU914856  1    GAGCTTATCCATTT-CTTGGCTTCAAATGAATCGAGCTGAATC-TTTGCTG-TCTGTTTC  57
AY230175  195  ..............A.....A.....................-.......-.T......  252
AY171264  3    ..............A.....A.....................-.......-.T......  60
DQ375757  175  ..............A.....A.....................G.......-.T...C..  233

EU914856  58   GAAGCGATGTATTCTCTCAACAAACGGCTATGAAGGGTTTCTGTAGGTGTCTGGAGCAGT  117
AY230175  253  ............................................................  312
AY171264  61   ............................................................  120
DQ375757  234  ..G..A............T.T...........T.......A.................  293

EU914856  118  TGTATGTGCGTGACTGTGGTGATG-GACATTTGAGTTCTTCTGGAACTAGAATTAAAGAA  176
AY230175  313  ......................-.............................. ..  371
AY171264  121  ......................-.............................. ..  179
DQ375757  294  ......A.................-.............G.-.-.............  350

EU914856  177  GTCTGTTACGACTCGCCGTTCTT-AAAAAACTTCAATTAACGTTTGAACAATTTGACTGC  235
AY230175  372  ......................-.............................. ..  430
AY171264  180  .....C................-.............................. ..  238
DQ375757  351  .....A..............A.....................T...........  410

EU914856  236  ACCAGCCGTAGGTGTAATTAAAGATTTATCAAGTCTTGTCGGTGGATCACTCGGTTCGTA  295
AY230175  431  ............................................................  490
AY171264  239  ............................................................  298
DQ375757  411  ..............C.............................................  470

EU914856  296  GTTCGATGAAAAACGGGGCAAAAACCGTTATTTGGCGTGAATTGCAGACATATTGAACGC  355
AY230175  491  ............................................................  550
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AY171264  299  ..........................................................  358
DQ375757  471  ..........................................................  530

EU914856  356  TAAAATTTTGAACGCAAATGGCACTATCAGGTTTATATCTGTTAGTATGTTTGGTTGAGG  415
AY230175  551  ..........................................................  610
AY171264  359  ..........................................................  418
DQ375757  531  ..........................................................  590

EU914856  416  GTCGATTAATTCGTAACTTGCAGTCCGCCGTGNCTGTTCTTTC-GATCAGCTACTTGATC  474
AY230175  611  ............................A.........-.................  669
AY171264  419  ............................A.........-.................  477
DQ375757  591  ......................T..T...A.....-....C...T..T....C...-  648

EU914856  475  TG--------C---ATTGCTGATCGAGTACCTGT-TAGGTATGTGAACTTTTGATAGTCT  522
AY230175  670  ..---------.---...................-.......................  717
AY171264  478  ..---------.---...................-.......................  525
DQ375757  649  ..G-------.-----.C.................-.C.............-.......  695

EU914856  523  AATTCGTTTCTTA---A--T------GT-----A---A--CGAGCTATCTTTGAATTCTG  561
AY230175  718  ..............---.--.------..-----.---.--.....................  756
AY171264  526  ..............---.--.------..-----.---.--.....................  564
DQ375757  696  ..........C..---.--.C-----.A-----.---.--.....................  735

EU914856  562  -TGCTTTGTATA-TTTGGTGTTT------CGGCGCGTTTCTTGCCGACTGAAT-TGTACG  612
AY230175  757  -...........-.........------.........................-......  807
AY171264  565  -...........-.........------.........................-......  615
DQ375757  736  G...G......C-.........------.........................-.C.....  787

EU914856  613  GACGTAACAGTACGTATAT-GCTTCAATTT-AT-T---CAGATG-CCCT-AATG-TTACA  663
AY230175  808  ..................-.........-..-.---.....-....-....-.....  858
AY171264  616  ..................-.........-..-.---.....-....-....-.....  666
DQ375757  788  ----------.....-.........G..----------....-T.-.C....T  830

EU914856  664  TCACTCGACACAACACGTTTCGTTTGTTGAATAATTGCGCAAGAAA--G-AAACTTTT-C  719
AY230175  859  ..................................................--.-.....-.  914
AY171264  667  ..................................................--.-.....-.  722
DQ375757  831  ............................G....C......-...TT.T........T.  889

EU914856  720  G-TT---ACGACCTCAACTCAAGCAAG  742
AY230175  915  .-..TT-...................  939
AY171264  723  .-..TT-...........          739
DQ375757  890  .-..TTT...................  915
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Figure 2: *Multiple sequence alignment of the ITS rDNA region (including partial fragments of the 18S and 28S rDNA genes) of 4 Steinernema species. Code EU914856 correspond to the Slovenian isolate of Steinernema kraussei (C46). Codes AY230175 and AY171264 are Steinernema kraussei strains from Germany and Russia. Code DQ375757 correspond to Steinernema akhursti strain from China.*

4 DISCUSSION

Genetic studies proved that the nematode species is *Steinernema kraussei* (Steiner, 1923). The ITS1-5.8S-ITS2 region, including the partial 18S and 28S rRNA genes (flanked by above primers) of Slovenian isolate C46, is 742 bp long. BLAST searches (Altschul *et al*., 1990) in GenBank showed that Slovenian isolate C46 (Fig. 1) has a high similarity (99 %) with those sequences available for *S. kraussei* populations (e.g. accession numbers AY230175 and AY171264). Sequence of other species from *feltiae* group, namely *S. akhursti* was obtained from GenBank searches that exhibited a lesser degree of similarity with the Slovenian isolate and other *S. kraussei* populations (e.g. accesion number DQ375757) (Fig. 1). The present study constitutes the first report of *S. kraussei* in Slovenia. In Europe, until now *S. kraussei* was already recorded in Austria, Belgium, Great Britain, Czech Republic, Germany, Slovakia, Switzerland, Island, Norway, Spain in Bulgaria and in many other parts of the world (for a detailed EPN species distribution see Hominick, 2002).

We can place mentioned species into »*feltiae* group« of nematodes from genus *Steinernema* (Nguyen, 2006); for infective juveniles it is known that they are between 700 and 1000 µm long. This nematode lives in symbiosis with bacterium *Xenorhabdus bovienii* (Boemare and Akhurst, 1988; Fischer-Le Saux et al., 1998). It is *Steinernema kraussei*, the first recorded EPN species (Glaser and Fox, 1923), when attack and death by this EPN was observed in Japanese beettle (*Popillia japonica* Newman). In the same year Steiner renamed this species to *Aplectana kraussei*, but in 1927 Travassos changed the original name of the genus and used the name *Steinernema* (Laznik in Trdan, 2008a).

Numerous researches showed that *S. kraussei* is efficient at low temperature (from 6 to 10 ºC) (Long et al., 2000). It was mainly studied when controlling black vine weevil (*Otiorhynchus sulcatus* [Fabricious]) and they have found out also over 80 % efficiency at low temperature; meanwhile some other species (*S. carpocapsae, S. feltiae* and *H. megidis*) have not shown satisfying efficiency (Long et al., 2000; Willmott et al., 2002; Haukeland, 2007). Efficacy of *S. kraussei* in low temperature has a big importance in plant protection, specially when applying in the open, while temperature, beside UV radiation and moisture, represents the most important limiting factor aforesaid biological agents (Kaya, 1990).

After the first record of *Steinernema kraussei* in Slovenia, we expect that the use of these biological agents against insect pests will become important alternative to insecticides. These will be especially desired against the pests, which is not easy to control with insecticides, due to their massive occurrence in the period of harvesting, against the pests, which are resistant to insecticides etc. In the future experiments, C46 strain of *S. kraussei*, will be used against different agricultural pests under laboratory conditions as well as in the open field.

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