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Heterorhabditis bacteriophora (Poinar) – the first member from Heterorhabditidae family in Slovenia

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ABSTRACT

In August 2008, we examined 95 soil samples for the occurrence of entomopathogenic nematodes in eastern part of Slovenia. 11 samples from 9 different locations were positive to entomopathogenic nematodes, but to this time only sample D54 was analysed. This soil sample was collected in Dravograd. Genetic studies proved that the nematode species in this sample was *Heterorhabditis bacteriophora*. This is the first record of *Heterorhabditis* nematode in Slovenia. Until now we confirmed the presence of four entomopathogenic nematode species in Slovenia; *Steinernema affine, Steinernema carpocapsae, Steinernema feltiae* and *Steinernema kraussei*. We expect that in Slovenia the use of these biological agents against insect pests will become important alternative to insecticides as it is known in many other countries of the world.

Key words: biological control, entomopathogenic nematodes, indigenous species, Slovenia, *Heterorhabditis bacteriophora*, Heterorhabditidae, first record

IZVLEČEK

ENTOMOPATOGENA OGORČICA *Heterorhabditis bacteriophora* (Poinar) – PRVI PREDSTAVNIK IZ DRUŽINE HETERORHABDITIDAE, NAJDEN V SLOVENIJI

V avgustu 2008 smo preučili 95 talnih vzorcev, da bi ugotovili zastopanost entomopatogenih ogorčic v vzhodnem delu Slovenije. 11 vzorcev z devetih različnih lokacij je bilo pozitivnih na zastopanost entomopatogenih ogorčic. Doslej smo analizirali le vzorec D54, ki je bil odvzet blizu Dravograda. Genetska analiza je potrdila, da gre za vrsto *Heterorhabditis bacteriophora*. Gre za prvo najdbo ogorčice iz rodu *Heterorhabditis* v Sloveniji. Doslej smo v Sloveniji potrdili zastopanost 4 vrst entomopatogenih ogorčic, in sicer: *Steinernema affine, Steinernema carpocapsae, Steinernema feltiae* in *Steinernema kraussei*. Pričakujemo, da bo v Sloveniji uporaba omenjenih naravnih sovražnikov škodljivih žuželk postala pomembna alternativa insekticidom, kar je sicer že znano v številnih drugih državah sveta.

Ključne besede: biotično varstvo, entomopatogene ogorčice, domorodna vrsta, Slovenija, *Heterorhabditis bacteriophora*, Heterorhabditidae, prva najdba

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1 INTRODUCTION

The entomopathogenic nematode *Heterorhabditis bacteriophora* was first described in 1975 as a new species as well as a member of new genus, and family (Heterorhabditidae) of Rhabditida (Poinar, 1976). The infective juvenile (IJ) stage was found to transmit a specific Gram-negative bacterium in the anterior intestine to the hemocoel of insect hosts (Poinar *et al*., 1977). This symbiotic bacteria is *Photorhabdus luminescens* subspecies *luminescens* (Fischer-Le Saux *et al*., 1999). Until now several *Heterorhabditis* species have been described (Adams *et al*., 2006) and studied for their biological control potential (Selvan *et al*., 1993; Koppenhöfer *et al*., 2004).

Transmission of symbiotic bacteria by the IJ of entomopathogenic nematodes is significant for EPN to successfully parasitize insect host and to reproduce (Han and Ehlers, 2000). This relationship can also be described as an obligate (for nematode and bacteria) vector-born disease of insects. It is discussed upon symbiotic-mutualistic relationship because nematodes provide shelter and protection for bacteria in an exchange for killing the attacked insects (Han and Ehlers, 2000). Latter, bacteria also produce antibiotics, which prevent the development of intra- and interspecific competitors (Hu and Webster, 2000), which would also feed on cadavers. Bacteria transform the content of the host into feed, suitable for nematodes and also bacteria themselves are feed for nematodes (Kaya and Koppenhöfer, 1999).

Heterorhabditis bacteriophora life cycle includes an egg, four juvenile stages and the adult (Poinar, 1976). The third-stage juvenile is the only free-living form, which is able to attack and infect the insect. The infective third-stage juveniles move through the soil in search of an insect host. This stage is adapted to live without feeding for a prolonged time. When the host is found, the nematode can enter into it through natural openings, or uses a dorsal tooth or hook, to break the insect cuticle. After entrance the nematode releases the symbiotic bacteria (Milstead, 1979). The bacteria multiply in the insect hemocoel and in the period from 24 to 72 hours after the entrance of the entomopathogenic nematode insect usually dies (Ciche and Ensign, 2003).

In Slovenia, momentarily only two species of entomopathogenic nematodes, *Steinernema feltiae* and *S. carpocapsae*, have a status of indigenous species (MAFF, 2008ab); therefore only this species 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 (Schroer *et al*., 2005). The strain D54, 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 August 2008, we examined 95 soil samples from 19 different locations on the occurrence of EPNs in Slovenia. The soil samples, five from each location, were taken in Prekmurje, Koroška and Štajersko region (eastern part of the country). We used »Galleria bait method«, which is the most frequently used method of 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 nematodes in 11 soil samples from 9 locations. Only 1 positive sample, D54 (taken in the meadow near the city Dravograd (NW Slovenia, 46°35′N, 15°01′E, 390 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 nematode 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 the Agricultural Biotechnological Research Centre, Gödöllő, Hungary. The sequence was

submitted into GenBank public database (Accession Number: FJ477060).

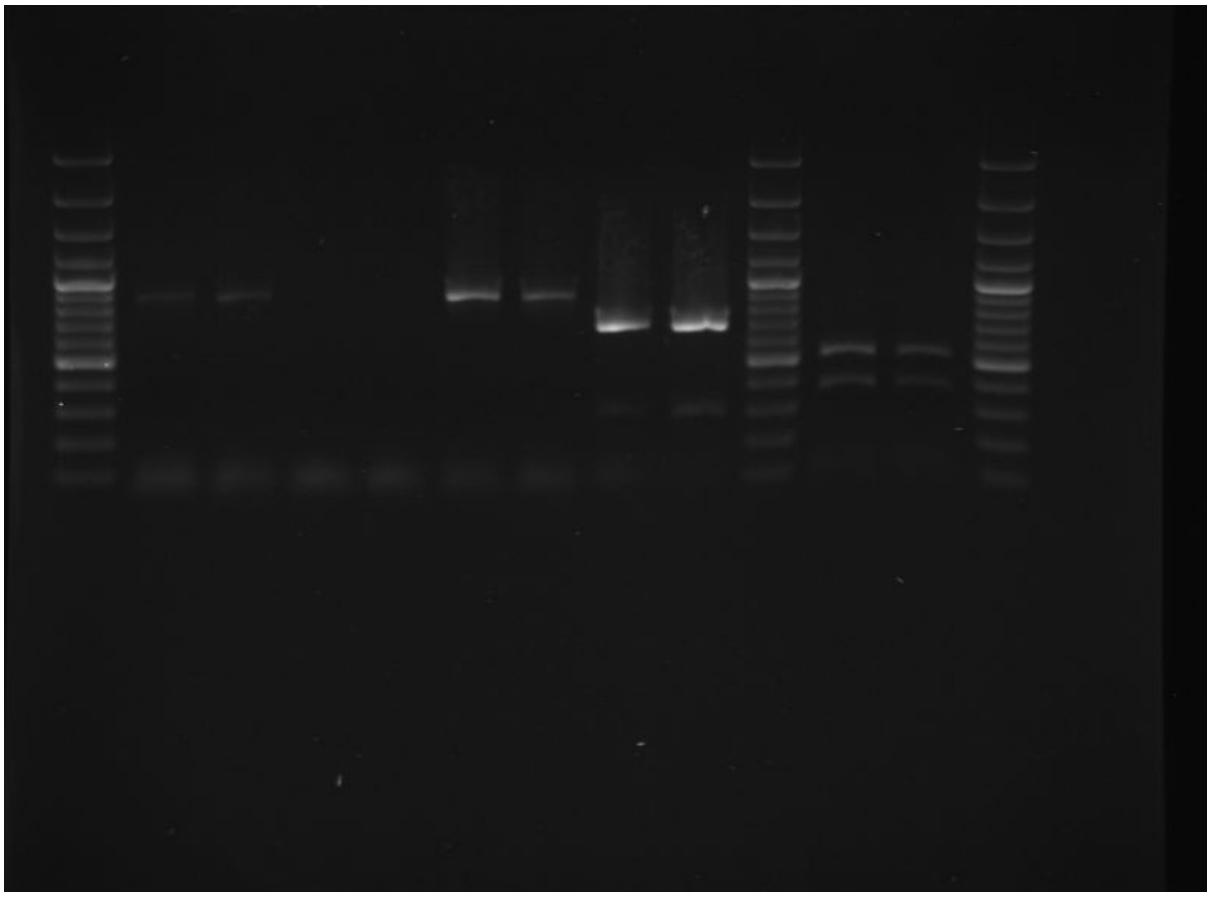

***Figure 1**: 1 % TAE buffered agarose gel, in the 1st, 10th and 13th lanes: GeneRuler 100 bp DNA Ladder Plus (Fermentas), in the 6th and 7th lane: PCR product of our sample D54, using the primer pair specified in the text, 8th and 9th lane: PCR product of sample slug nematode – Alloionema appendiculatum. The two most strength fragment in the ladder are 500 and 1000 bps length.*

Sample DNA sequence was compared to sequences of species *Heterorhabditis* 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 *Heterorhabditis bacteriophora*: GenBank Accession No. FJ346825 and EU921445 (Fig. 2).

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FJ477060  1    CGCCGA-AACCTTAT--GGGT-AATGCTT-TG-AT-CACGAGAG-ATCGGTACCACT-GG  51
FJ346825  100  .....-.........--....-.......-..-..-........-.........A.-..  150
EU921445  1              ..--....-..-..CC.-..-........-.........A.-..  38
EF043440  190  .....-.........--....-.......-..-..-.......T-.........A.-..  240

FJ47706   52   -AAT-CAG-GCT-T-G-TTCTT-GATTT-C-AATCGGTTT---CTCA-CCCCATCTAAGC  98
FJ346825  151  -...-...-...-.-.-.....-....-.-.........---....-...........  197
EU921445  39   -...-...-...-.-.-.....-....-.-.........---....-...........  85
EF043440  241  -...-...-...-.-.-.....-....-.-.........---....-...........  287

FJ477060  99   -TCAT-GGAG-A-GGTGT-CTAGT-CCCAAT-TGGAGTCGCTTTGAGTGA-C-GGCTAT-  148
FJ346825  198  -.....-....-.....-.....-.....-.....................-.-.....-  247
EU921445  86   -.....-....-.....-.....-.....-.....................-.-.....-  135
EF043440  288  -.....-....-.....-.....-.....-.....................-.-.....-  337

FJ477060  149  G-AAAATTGGGTATG---T-TCCC---CGTGAGGGTCGAGCATAGACTTTATGAACAGCT  200
FJ346825  248  .-...............---.-....---.............................-.  298
EU921445  136  .-...............---.-....---.............................-.  186
EF043440  338  .-...............---.-....---.............................-.  388

FJ477060  201  GCT-GG-AGCTGTCGCCTCACCAAAAAATCATC-GATAACT-GGTGGCTAT-G-TGTGAC  254
FJ346825  299  ...-.-.............................-.....-.........-.-.....  352
EU921445  187  ...-.-.............................-.....-.........-.-.....  240
EF043440  389  ...-.-.............................-.....G.-.........-.-.....  442

FJ477060  255  ATT-AGTCACAT-AG-GTA-TC-TG-C-TGATGCAG-AGAG-CCTCTAATGAGTTGTT--  303
FJ346825  353  ...-.........-.-...-..-..-.-.........-....-...-............--  400
EU921445  241  ...-.........-.-...-..-..-.-.........-....-...-............--  288
EF043440  443  ...-.........T.-...-..-..-.-.........-....-...-............--  490

FJ477060  304  C-GTGTCATC-TGACC-TACAA-CCGCCAG-TATCGGT--AAA-T-C--AACCCAA-TTA  351
FJ346825  401  .-.........-.....-.....-.......-.........--...-.-.--.......-...  448
EU921445  289  .-.........-.....-.....-.......-.........--...-.-.--.......-...  336
EF043440  491  .-.........-.....-.....-......C-.........--...-.-.--.......-...  538

FJ477060  352  ACTTGTTTC-T-TG-TGTCGTGT-TAATACATAC-TGGCA-AAGTGTATTAGCTTTAGCG  405
FJ346825  449  .........-.-..-........-.........-.....-..................  502
EU921445  337  .........-.-..-........-.........-.....-..................  390
EF043440  539  .........-.-..-........-.........-.....-..................  592

FJ477060  406  ATGG-ATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGC--TGCGTTATTTACCAC  462
FJ346825  503  ....-.....................................--.............  559
EU921445  391  ....-.....................................--.............  447
EF043440  593  ....-.....................................--.............  649

FJ477060  463  GAATTGCAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCT  521
FJ346825  560  ..................-.....................................  618
EU921445  448  ..................-.....................................  506
EF043440  650  ..................-.....................................  708


FJ477060  522  TCGGCACGTCTGGCTCAGGGTTGTTTA-ATAAGCGAAAGTGTTGAAAGTTCATTAAACGA  580
FJ346825  619  ........................-.............................  677
EU921445  507  ........................-.............................  565
EF043440  709  ........................-.....-..-.............G.....  765

FJ477060  581  GAGTTCGGTGATACTGACAACACTACGTCGAGCGGTGTACTGTTGAAAGTACCCCGTTCA  640
FJ346825  678  ............................................................  737
EU921445  566  ............................................................  625
EF043440  766  A.....A...........G..T.G......T...C.......................  825

FJ477060  641  AGTA--TCTTTATGGGGCAACATGTCTTCTATATGGAGACATGAAAGATATTAAGAGTAT  698
FJ346825  738  .....-......................................................  795
EU921445  626  .....-......................................................  683
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EF043440  826  ..G.AA.........A..............C....G............T.......  885

FJ477060  699  ATACCTGTGGATGCCCACGTATGAAATATGACGTGTCGTATAC-ACGGCTAGGAGGTATG  757
FJ346825  796  ..........................................-.................  854
EU921445  684  ..........................................-.................  742
EF043440  886  ..........................................-.................  944

FJ477060  758  TCTC-AGATGAA-TTT-G-TT-ATGCAACC-TGAGCTCAG  791
FJ346825  855  ....-.......-...-.-..T........-.........  889
EU921445  743  ....-...G...-.--.-..-........-.........  775
EF043440  945  ....-...-...A....A..-........-.........  979
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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 Heterorhabditis species. Code FJ477060 correspond to the Slovenian isolate of Heterorhabditis bacteriophora (D54). Codes FJ346825 and EU921445 are Heterorhabditis bacteriophora strains from South Africa and Hungary. Code EFO43440 correspond to Heterorhabditis zealandica strain from Ireland.*

4 DISCUSSION

Genetic studies proved that the nematode species is *Heterorhabditis bacteriophora* (Poinar, 1976) (Fig. 2). The ITS1-5.8S-ITS2 region, including the partial 18S and 28S rRNA genes (flanked by above primers) of Slovenian isolate D54, was 791 bp long (Fig. 1). BLAST searches (Altschul *et al*., 1990) in GenBank showed that Slovenian isolate D54 (Fig. 2) has a high similarity (99 %) with those sequences available for *H. bacteriophora* populations (e.g. accession numbers FJ346825 and EU921445). Sequence of other species from *Heterorhabditis* group, namely *H. zealandica* was obtained from GenBank searches that exhibited a lesser degree of similarity with the Slovenian isolate and other *H. bacteriophora* populations (e.g. accesion number EFO43440) (Fig. 2). The present study constitutes the first report of *H. bacteriophora* in Slovenia. In Europe, the occurrence of *H. bacteriophora* was up to now confirmed in Bulgaria, Czech Republic, France, Germany, Hungary, Italy, Moldova, Poland, Portugal, Spain and Switzerland (Hominick, 2002). Only 4 species from genus *Heterorhabditis* were found in Europe; *H. bacteriophora* (11 countries), *H. megidis* (13 countries), *H. downesi* (3 countries) and *H. zealandica* (1 country) (Hominick, 2002).

***Figure 3**: Infective juvenile (IJ) of Heterorhabditis bacteriophora from sample D54.*

The infective juveniles of *H. bacteriophora* are between 520 and 600 µm long (Fig. 3). Entomopathogenic IJs developed different foraging behaviours to infect insect host, cruiser and ambusher strategies (Lewis *et al*., 1995). *H. bacteriophora* is a cruiser forager, meaning that it actively searches its host. In addition to sensing CO_2 and volatile cues released by the host (O'Halloran and Burnell, 2002), IJs are attracted to ß-caryophyllene (Rasmann *et al*., 2005). This substance is a terpene, which is released, if the plant roots are damaged (Rasman *et al*., 2005). IJs developed chemosensory mechanisms not only to perceive the host, but also the locations where insect host are likely to be present.

H. bacteriophora IJs develop into hermaphrodites and this can lay eggs that develop into hermaphrodites, females or males. When the egg laying stops, nematodes can develop inside the maternal body with the process called *endotokia matricida* (Johnigk and Ehlers, 1999). Nematodes that are developed by *endotokia matricida* are predominantly hermaphroditic IJs (Dix *et al*., 1992).

Entomopathogenic nematode *H. bacteriophora* was proved to have big potential in biological control of insects (Selvan *et al*., 1993; Koppenhöfer *et al*., 2004). Some target pests that have been controlled by *H. bacteriophora* in field tests are white grubs, black vine weevil, strawberry root weevil, Colorado potato beetle, cucumber beetles and some others (Grewal *et al*., 2005). Some attempts have been made to test this nematode also against foliar pests, but the problem of desiccation, exposure to sunlight and high temperatures that are lethal to exposed nematodes have limited such applications (Grewal *et al*., 2005).

In Slovenia, momentarily only *Steinernema feltiae* and *S. carpocapsae* are on the domestic list and we are able to use them also in field experiments (Laznik *et al*., 2008ab). When also *H. bacteriophora* will shift from exotic agents list, we will test its activity against the pest insects in the open too. The most intensive experiments will be done against the insect pests for the control of which we do not have registered insecticides, their number is limited, and specially against the insects, which already developed the resistance to insecticides.

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