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Research Note

First report of entomopathogenic nematode *Steinernema feltiae* (Rhabditida: Steinernematidae) from Croatia

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Summary

A survey of entomopathogenic nematodes was conducted in Croatia between 2016 and 2017. The steinernematids were recovered in two out of 100 soil samples from agricultural land characterized as loamy soils with acidic reaction. Molecular and morphological identification was used to distinguish the nematodes. The isolates were identified as two different strains conspecific with *Steinernema feltiae.* The variations in morphometrical characteristics of infective juveniles (IJs) and males were observed among Croatian strains and with the original description. The analysis of ITS region revealed the greatest similarity of Croatian strains with Slovenian B30 and English A2 strains, which together comprised a monophyletic group in evolutionary analysis. This is the first record of steinernematids, namely *S. feltiae* in Croatia**.**

Keywords: *Steinernema feltiae;* strain; Croatia; morphological variations*;* survey

Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* Travassos and *Heterorhabditis* Poinar are effective biological control agents against wide range of insect pests (Lacey & Georgis, 2012). Entomopathogenic nematodes actively seek for their host and leave no ecological footprint. These are the main advantages of EPNs as biocontrol agents over chemical pesticides (Kaya *et al*., 2006). The infective or third stage juvenile (IJs) carry in its intestine symbiotic bacteria which are released into the insect hemocoel once the nematode is inside the host. The bacteria produce highly virulent insecticidal toxins which kill the host within 24 to 72 h by septicaemia. Bacteria of the genus *Xenorhabdus* Boemare, Akhurts and Mourant are associated with steinernematids, while bacterial species of genus *Photorhabdus* Thomas and Poinar are symbionts *of* heterorhabditids (Koppenhöfer, 2007). They are globally distributed, but their species biodiversity and distribution is still unrevealed in some countries and regions (Adams *et al*., 2006). In Southeastern Europe there are records of EPNs from several countries only: Slovenia (Laznik *et al*., 2009), Bosnia and Herzegovina (Iqbal & Ehlers, 2016), Serbia (Tallosi *et al*., 1995), Bulgaria (Shishiniova *et al*., 1997), Greece (Menti *et al*., 1997), and Albania (Tarasco & Poliseno, 2005). An increasing trend in research and commercialization of EPNs has been observed throughout the world (Kaya *et al*., 2006). Primarily due to removal of chemical pesticides from the market due to the toxicological issues and need for the efficient biocontrol agent. Accordingly, it is important to isolate nematodes from different geographical regions, and also to evaluate and compare native with commercial strains for their potential in biocontrol programs in specific area (Laznik *et al*., 2010). Species from different regions are locally adapted and potentially different in terms of reproduction, infectivity, host range, and conditions for survival which have to be documented (Hazir *et al*., 2003). The aim of this study was to conduct survey in Croatia and report indigenous EPNs species.

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Material and Methods

Soil samples were collected in the eastern part of continental Croatia in 2016 and 2017. Sampling was conducted during spring and autumn months from diverse habitats. In total, 100 soil samples were collected. Soil characteristics and habitats of the positive sampling sites are presented in Table 1. Soil was collected from the upper 3 – 30 cm layer, and within each site a sample consisted of 1 kg of soil were randomly taken from five subsamples. Samples were kept in polyethylene bags and refrigerated until extraction. The samples were processed within two days upon collection. Each sample was thoroughly mixed and subsample of soil was taken from each sample, for analyses of soil type, organic matter content and pH. The insect baiting technique was used as described by Kaya and Stock (1997) with modification of insect host. *Achroia grisella* Fab., the lesser wax worm was used as baiting insect. The lesser wax moth larvae were placed in pierced Eppendorf tubes in each soil sample. In total, each soil sample received five baited tubes. The baited samples were kept in dark at room temperature (20 – 22 °C) for a period of 14 days. At two days interval, insect cadavers were collected and placed in a modified White trap to harvest emerging nematodes. The harvested IJs were stored in culture flasks in saline solution (M9 buffer) at 4 °C in refrigerator. In order to establish culture, ten live larvae of the lesser wax moth were placed in Petri dish lined with filter paper and infected with 50 IJs per insect larvae from each positive sample. The Petri dishes were incubated at room temperature in dark. Nematode progeny was used for molecular and morphologically based identification (Kaya & Stock, 1997). Twenty individuals of first generation males and IJs were fixed and transferred to anhydrous glycerin. Nematodes were examined under an Olympus BX50 (Japan) microscope equipped with differential interference contrast optics and digital image software (Olympus LCmicro 2.1, Japan). The morphometrics of nematode body measurements are presented in Table 2. Polymerase chain reaction (PCR) was performed to multiply ITS (internal transcribed spacer) region from genomic DNA extracted from single individual using primers TW81 and AB28 after Hominick *et al*. (1997). The PCR products were re-isolated from 1 % TAE-buffered agarose gel using E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA). Reisolated sample was se-

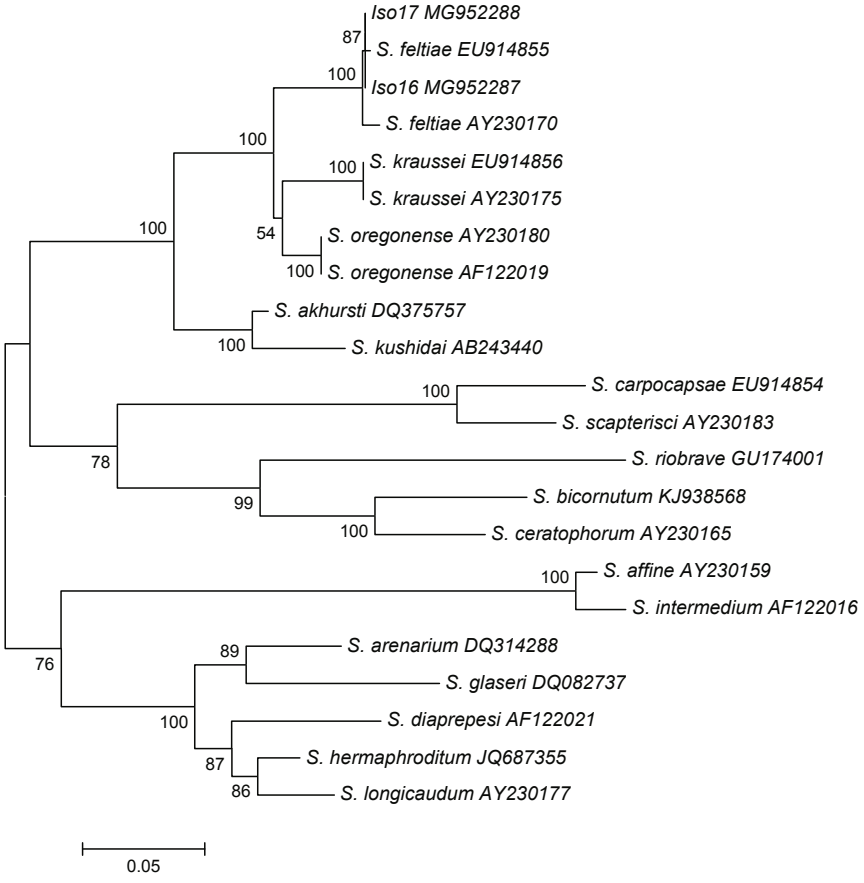

Fig. 1. Evolutionary relationships of new Croatian EPN isolates (ISO16 and ISO17). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

Table 1. Locality, soil characteristics, habitats and sampling time of the positive sites of *Steinernema feltiae*.

Strain	Location	Coordinates	Soil type	Organic matter (%)	pH (H_2O)	Habitat	Vegetation	Sampling date
ISO16	Arduševac	45°18'46.58"N 18°30'58.68"E	Loam	2.10	5.26	Agricultural	Potato	October 2016
ISO17	Arduševac	45°8'45.89"N 18°30'53.19"E	Loam	3.69	5.95	Agricultural	Fallow	

quenced in the laboratory of Agricultural Biotechnology Centre in Gödöllő, Hungary. Sample DNA sequences represented species of all the five main clades inside the *Steinernema* genus (Spiridonov *et al*., 2004) were used to phylogenetic analysis. ITS1, IT2 and 5.8S rRNA gene sequences were aligned using CLUSTAL_X 2.0 (Larkin *et al*., 2007). The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 495 positions in the final dataset. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2011).

Ethical Approval and/or Informed Consent

This article does not contain any studies with human participants or animals by any of the authors.

Results

The steinernematids were recovered in two out of 100 soil samples, in fall from agricultural land used for growing potatoes and fallow, respectively. Soil characteristics of the positive sites are classified as loam with slightly acidic reaction, pH varied from 5.26 to 5.96. There was no obvious influence of organic matter content in recovery of steinernematids, since the nematodes were extracted from soils with differing organic matter content (2.10 % and 3.69 %), (Table 1).

Morphometric data obtained for males and IJs showed that nematodes belong to the family Steinernematidae, and both isolates (ISO16 and ISO17) are conspecific with *Steinernema feltiae* Filipjev 1934. The Croatian strains of *S. feltiae* are morphologically similar to known species, with variations of IJs of ISO17 strain. Compared to the ISO16 strain and known species, IJs of ISO17 appeared smaller for all measured characteristics. Values obtained for first generation males lie with the ranges measured of previously described *S. feltiae* (Poinar, 1990). The morphological characteristics presented in Table 2 are considered as the most reliable for *S. feltiae* (Adams & Nguyen, 2002).

The phylogenetic relationship of the studied isolates of *S. feltiae* and homologous sequences of the same genus from the GenBank is presented in Figure 1. The phylogenetic tree revealed five major clades. Both Croatian isolates were grouped with two strains *S. feltiae*. Multiple sequence alignment of the Croatian strains *S. feltiae* with sequences of other populations of the same species showed 99 % similarity with Slovenian strain *S. feltiae* B30 (GenBank EU914855) (Laznik *et al*., 2009). Overall, five major clades are supported by a high bootstrap value ranging from 99 to 100 %. The sequences were deposited in GenBank under accession numbers MG952287 (ISO16) and MG952288 (ISO17).

Discussion

This is the first record of EPNs of family Steinernematidae, namely *S. feltiae* in Croatia. The efficiency of recovery of steinernematids in our study was 2 %, similarly to earlier reports by Laznik *et al*. (2009). *Steinernema feltiae* was recovered from agricultural fields only, and both positive samples were taken at close locations. Depending on the climate and the region, the members of family Steinernematidae show prevalence for specific habitats. Results

Table 2. Comparative morphometric data of *Steinernema feltiae* (Croatian isolates and known species). All measurements represent mean and range in µm.

Isolate	IJs				Male		
	L[a]	EP	T	E%	SL	D%	MUC
ISO16	842 (733 – 929)	61 (50 – 69)	81 (72 – 90)	76 (65 – 86)	69 (59 – 77)	62 (48 – 70)	P
ISO17	801 (699 – 937)	60 (47 – 71)	77 (69 – 86)	76 (68 – 88)	70 (61 – 76)	62 (50 – 71)	P
S. feltiae[b]	849 (736 – 950)	62 (53 – 67)	81 (70 – 92)	78 (69 – 86)	70 (65 – 77)	60	P

[a]L = body length, EP = distance from anterior end to excretory pore, T = tail length, SL = spicule length, D% = EP/oesophagus length × 100, E% = EP/T × 100, MUC = mucron, P = present.
[b]After Poinar (1990)

from surveys in the UK, the Netherlands and Germany reveal that *S. feltiae* prefers fields and grassland (Sturhan & Liskova, 1999), and our findings support these results. However, *S. feltiae* has been recovered from the forest biotopes often (Spiridonov and Moens, 1999; Tarasco *et al*., 2015). Soil properties such as moisture level, pH, organic matter content, texture and others, affect EPNs dispersal and potential to find host (Stuart *et al.,* 2015). Entomopathogenic nematodes move more freely and find host in lighter soils. Their locomotion decreases with smaller size of soil particles (Glazer, 2002). However, for recovery of steinernematids, soil type is considered of minor importance (Sturhan, 1999). We recovered *S. feltiae* from loamy soils which contain balanced mixture of light (sand) and heavy (clay) particles with acidic reaction, similar to the results of survey in California (Stock *et al*., 1999). Koppenhöfer and Fuzzy (2006) found that infectivity of tested heterorhabditids and steinernematid species is lower in acidic conditions, while preference for soil texture was species specific. Organic matter content did not influence recovery of *S. feltiae* in our study. Our results support findings of Hazir *et al*. (2003) who also reported natural occurrence of *S. feltiae* in soils differing in organic matter content.

The Croatian *Steinernema* isolates (ISO16 and ISO17) were identified as strains *S. feltiae*. However the variability in morphometrics was observed within the strains and with the original descriptions (Poinar, 1990). Body length and other characters of IJs *S. feltiae* ISO17 were comparatively smaller, while strain ISO16, morphologically resembles more the originally described *S. feltiae* than Croatian ISO17 strain. Morphological and morphometric variations of EPNs can be host (Campos-Hererra *et al*., 2006) or temperature induced (Hazir *et al*., 2001). In this study we used different host (the lesser instead of greater wax moth) and this could be the reason of the observed variability with the original described by Poinar (1990). Furthermore, it has been suggested that IJs body length is longest when EPNs are reared at 8 °C and becoming shorter if reared at room temperature conditions (Hazir *et al*., 2001). The Croatian strains were reared at room temperature, and this could be another reason for shorter morphometric values of ISO17. The difference among the Croatian strains can be due to the intraspecific variability (Stock *et al*., 1999). Furthermore, the morphological differences can be expected when populations of different geographic origins are compared (Stock *et al*., 2000). According to the molecular characterization, both Croatian strains are closest to two European isolates, Slovenian strain *S. feltiae* B30 (GenBank EU914855; Laznik *et al*., 2009) and English strain *S. feltiae* A2 (GenBank AY230170; Reid & Hominick, 1993) (Fig.1). These four strains of *S. feltiae* comprise a monophyletic group by analysis of the ITS region. Groups of closely related isolates of steinernematids are often determined by specific geographical area of distribution (Spiridonov *et al*., 2004). From ITS region of Slovenian strain *S. feltiae* B30, both Croatian strains differ by 33 (ISO16) and 34 bp (ISO17). The strains (ISO16 and ISO17) differ from each other by 1 bp. We expected greater species diversity and more positive sites for steinernematids. Since this genus has more described species, and generally are recovered more often than heterorhabditids. *Steinernema feltiae* are found globally in all terrestrial habitats. However, they have a wider distribution in temperate regions (Adams *et al*., 2006). Our results extend the range of geographical distribution of *S. feltiae*, indicating the plasticity of the species to adapt to the conditions of southeastern parts of Europe. The Croatian strains *S. feltiae* should be tested for pathogenicity and possibly included in future biological or integrated pest management programs in Croatia and other countries with similar climates. This indicates need for further research on EPNs biodiversity in Croatia and host ranges.

Conflict of Interest

Authors state no conflict of interest.

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References

Adams, B.J., Fodor, A., Koppenhöfer, H.S., Stackebrandt, E., Stock, S.P., Klein, M.G. (2006): Reprint of "Biodiversity and systematics of nematode–bacterium entomopathogens" [(2006): *Biol. Control,* 37: 32 – 49]. *Biol. Control*, 38(1): 4 – 21. DOI: 10.1016/S1049-9644(06)00126-5

Adams, B. J., Nguyen, K. B. (2002): Taxonomy and systematics. In: Gaugler, R. (Ed) *Entomopathogenic nematology*. Wallingford, UK: CAB International, pp. 1 – 33.

Campos-Herrera, R., Escuer, M., Robertson, L., Gutiérrez, C. (2006): Morphological and ecological characterization of *Steinernema feltiae* (Rhabditida: Steinernematidae) Rioja strain isolated from *Bibio hortulanus* (Diptera: Bibionidae) in Spain. *J. Nematol.*, 38(1): 68 – 75

Glazer, I. (2002): Survival biology. In: Gaugler, R. (Ed) *Entomopathogenic nematology*. Wallingford, UK: CAB International, pp. 169 – 187

Hazir, S., Keskin, N., Stock, S.P., Kaya, H.K., Özcan, S. (2003.): Diversity and distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Turkey. *Biodivers. Conserv.*, 12(2): 375 – 386. DOI: 10.1023/A:1021915903822

Hazir, S., Stock, S.P., Kaya, H.K., Koppenhöfer, A.M., Keskin, N. (2001): Developmental temperature effects on five geographic isolates of the entomopathogenic nematode *Steinernema feltiae* (Nematoda: Steinernematidae). *J Invertebr Path*, 77(4): 243 – 250. DOI: 10.1006/jipa.2001.5029

Hominick, W.M., Briscoe, B.R., Del Pino, F.G., Heng, J., Hunt, D.J.,

Kozodoy, E., Mracek, Z., Nguyen, K.B., Reid, A.P., Spiridonov, S., Stock, P., Sturhan, D., Waturu, C., Yoshida, M. (1997): Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *J. Helminth.*, 71: 271 – 298. DOI: 10.1017/S0022149X00016096

Iqbal, M., Ehlers, R.U., Waeyenberge, L. (2016): Molecular characterisation of novel isolates of entomopathogenic nematodes. *Nematology*, 18(3): 277 – 291. DOI: 10.1163/15685411-00002959

Kaya, H.K., Aguillera, M.M., Alumai, A., Choo, H.Y., De La Torre, M., Fodor, A., Ganguly, S., Hazir, S., Lakatos, T., Pye, A., Wilson, M., Yamanaka, S., Yang, H., Ehlers, R.U. (2006): Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. *Biol. Control*, 38: 134 – 155. DOI: 10.1016/j.biocontrol.2005.11.004

Kaya, H.K., Stock, P. (1997): Techniques in insect nematology. In: Lacey, L.A. (Ed) *Manual of techniques in insect pathology*. London, UK: Academic Press, pp. 281 – 324

Koppenhöfer, A.M, Fuzy, E.M. (2006): Effect of soil type on infectivity and persistence of the EPNs *Steinernema scarabaei, Steinernema glaseri, Heterorhabditis zealandica,* and *Heterorhabditis bacteriophora. J. Invertebr. Path*, 92: 11 – 12. DOI: 10.1016/j.jip.2006.02.003

Koppenhöfer, A. M. (2007): Nematodes. In: Lacey, L.A., Kaya, H.K. (Eds) *Field manual techniques in invertebrate pathology: Application and evaluation of pathogens for control of insects and other invertebrate pests*. Dordrecht, The Netherlands: Springer, pp. 249 – 264

Lacey, L.A., Georgis, R. (2012): Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *J. Nematol.*, 44(2): 218 – 225.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., Mcgettigan, P.A., Mcwilliam, H., Valentin F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. (2007): CLUSTAL W and CLUSTAL_X version 2.0. *Bioinformatics*, 23: 2947 – 2948. DOI: 10.1093/bioinformatics/btm404

Laznik, Ž., Tóth, T., Lakatos, T., Vidrih, M., Trdan, S. (2009): First record of *Steinernema feltiae* (Filipjev)(Rhabditida: Steinernematidae) in Slovenia. *Helminthologi*a, 46(2): 135 – 138. DOI: 10.2478/s11687-009-0026-7

Laznik, Ž., Tóth, T., Lakatos, T., Vidrih, M., Trdan, S. (2010): Control of the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) on potato under field conditions: a comparison of the efficacy of foliar application of two strains of *Steinernema feltiae* (Filipjev) and spraying with thiametoxam. *J. Plant Dis. Prot.*, 117(3): 129 – 135. DOI: 10.1007/BF03356348

Menti, H., Wright, D. J., Perry, R. N. (1997): Desiccation survival of populations of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the UK. *J. Helminth.*, 71(1): 41 – 46. DOI:10.1007/BF02981828

Nguyen, K.B., Smart Jr, G.C. (1995): Morphometrics of infective juveniles of *Steinernem*a spp. and *Heterorhabditis bacteriophora* (Nemata: Rhabditida). *J. Nematol.*, 27(2): 206 – 212

Poinar, G.0. Jr. (1990): Taxonomy and biology of Steinernematidae and Heterorhabitidae. In: Gaugler, R., Kaya, H.K. (Eds) *Entomopathogenic nematodes in biological control*. Boca Raton, USA: CRC Press, pp. 23 – 61

Reid, A.P., Hominick, W.M. (1993): Cloning of the rDNA repeat unit from a British entomopathogenic nematode (Steinernematidae) and its potential or species identification *Parasitology*, 107: 529 – 536

Shishiniova, M., Budurova, L., Gradinarov, D. (1997): Contribution to fauna of entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae) from Bulgaria. *Biotechnol. Biotechnol. Equip.*, 11(1 – 2): 45 – 52. DOI_ 10.1080/13102818.1998.10818975

Spiridonov, S.E., Moens, M. (1999): Two previously unreported species of steinernematids from woodlands in Belgium. *Russ. J. Nematol.*, 7: 39 – 42

Spiridonov, S.E., Reid, A.P., Podrucka, K., Subbotin, S.A., Moens, M. (2004): Phylogenetic relationships within the genus *Steinernema* (Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1-5.8S-ITS2 region of rDNA and morphological features. *Nematology*, 6: 547 – 566. DOI: 10.1163/1568541042665304

Stock, S.P., Pryor, B.M., Kaya, H.K. (1999): Distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in natural habitats in California, USA. *Biodivers. Conserv.*, 8(4): 535 – 549. DOI: 10.1023/A:1008827422372

Stock, S.P., Mrácek, Z., Webster, J.M. (2000): Morphological variation between allopatric populations of *Steinernema kraussei* (Steiner, 1923) (Rhabditida: Steinernematidae). *Nematology*, *2*(2): 143 – 152

Stuart, R.J., Barbercheck, M.E., Grewal, P.S. (2015): Entomopathogenic nematodes in the soil environment: distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Eds) *Nematode pathogenesis of insects and other pests. Sustainability in Plant and Crop Protection.* Switzerland: Springer International Publishing, pp, 97 – 137. DOI: 10.1007/978-3-319-18266-7_4

Sturhan, D., Liskova, M. (1999): Occurrence and distribution of entomopathogenic nematodes in the Slovak Republic. *Nematology*, 1(3): 273 – 277. DOI: 10.1163/156854199508261

Tallosi, B., Ehlers, R. (1995): *Steinernema bicornutum* sp. n. (Rhabditida: Steinernematidae) from Vojvodina, Yugoslavia. *Russ J. Nematol.*, 3: 71 – 80.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011): MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.*, 28: 2731 – 2739. DOI: 10.1093/molbev/msr121

Tarasco, E., Poliseno, M. (2005): Preliminary survey on the occurrence of entomopathogenic nematodes and fungi in Albanian soils. *IOBC/WPRS Bull.*, 28(3): 165 – 168

Tarasco, E., Clausi, M., Rappazzo, G., Panzavolta, T., Curto, G., Sorino, R., Oreste, M., Longo, A., Leone, D., Tiberi, R., Vinciguerra, M.T., Triggiani, O. (2015): Biodiversity of entomopathogenic nematodes in Italy. *J. Helminthol.*, 89: 359 – 366 DOI: 10.1017/S0022149X14000194