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First Record of *Steinernema kraussei* (Rhabditida: Steinernematidae) from Turkey and Its Virulence against *Agrotis segetum* (Lepidoptera: Noctuidae)

Cihan Gokce,[1] Huseyin Yilmaz,[2] Zeynep Erbas,[1] Zihni Demirbag,[1] Ismail Demir[1]

Abstract: During a survey of entomopathogenic nematodes (EPNs) in the eastern Black Sea region of Turkey in 2009–2012, a steinernematid species was recorded and isolated using the *Galleria*-baiting method. The isolate was identified as *Steinernema kraussei* based on its morphological and molecular properties. The analysis of the ITS rDNA sequence placed the Turkish population of *S. kraussei* in the "feltiae-kraussei" group in the clade that contains different isolates of the species. This is the first record of *S. kraussei* from Turkey. The efficacy of *S. kraussei* was tested on *Agrotis segetum* (Lepidoptera: Noctuidea) larvae at different densities (100, 300, and 500 infective juveniles (IJs) g^{-1} dry sand) in laboratory conditions at 25 °C. The highest mortality (98%) was obtained with 500 IJs g^{-1} dry sand within 7 d after inoculation. Our results indicate that the new isolate is a highly promising biological control agent against *A. segetum*, one of the most serious soil pests of agricultural area and fruits worldwide.

Key words: Entomopathogenic nematode, *Steinernema kraussei*, biological control, *Agrotis segetum*, Turkey.

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are widely distributed in soils throughout the world (Hominick, 2002; Adams et al., 2006). They are obligate parasites of soil-inhabiting insects and have a great importance as biological control agents of many insect pests (Ehler, 1990; Koppenhöfer, 2000). EPNs possess many desirable characteristics, such as searching ability of hosts, safety to nontarget organisms and the potential to survive in the environment. Nematode-based commercial products have become available for use against several pests all over the world during the past decade (Gaugler, 2002; Arthurs et al., 2004). EPNs are found in a variety of habitats, and the various species and isolates exhibit considerable variation in terms of host range, reproduction, infectivity, and conditions for survival (Bedding, 1990). To further advance the use of EPNs as biological control agents in pest management, locally adapted species or isolates from native habitats need to be identified and their unique characteristics documented.

The eastern Black Sea region of Turkey has suitable climatic conditions for cultivation and a broad range of insect pests on plants. That part has also a large diversity of different climates suggesting the possibility of a large diversity of EPNs. Recently a few surveys on EPNs in Turkey and survey studies from dead insects have demonstrated the presence of the *Steinernema affine*, *Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema weiseri*, *Steinernema anatoliense*, and *Steinernema websteri* from the genus *Steinernema* (Ozer et al., 1995; Kepenekci, 2002; Hazir et al., 2003a; Hazir et al., 2003b; Unlu et al., 2007; Erbas et al., 2013), and *Heterorhabditis bacteriophora* (Susurluk et al., 2001; Hazir et al., 2003b; Susurluk and Toprak, 2006; Erbas et al., 2013) and *Heterorhabditis megidis* (Yilmaz et al., 2009) from *Heterorhabditis*.

In addition to the survey for EPNs in the eastern Black Sea region of Turkey, we also tested the isolated nematode against cutworms, common pests that destroy a number of agricultural and horticultural crops and forestry around the world (Zethner, 1980). The common cutworm (larvae of the turnip moth) *Agrotis segetum* Schiff (Lepidoptera: Noctuidae) occurs throughout Europe, Asia, and parts of Africa. It generally lives in the ground, where it feeds on seedlings of nearly all vegetable and field crops such as corn, potatoes, beans, peppers, eggplants, okra, lettuce, tobacco, sugar beets, cabbage, and many other plants (Ministry of Agriculture of Turkey, 2008). Because of their soil-dwelling habits, cutworms are generally difficult to control. They are often detected only when the plants are already severely damaged. Herein, we report the occurrence of *S. kraussei* in Turkey for the first time, and we investigated the potential for the use of this nematode isolate as a biological control agent against *A. segetum*.

Materials and Methods

Origin and isolation of the nematode: An EPN isolate was obtained in June 2009 from a soil sample collected from a bank of spruce forest (*Piceaorientalis* L.) around Sumela Monastery (N 40° 39′ E 39° 40′) of Trabzon in the eastern Black Sea region of Turkey. The soil sample, a composite of five random subsamples (in total ca. 1 kg), was taken in an area of 100 m^2 and at a depth of 20 cm in sandy-clay-loam soil. A subsample of 200 g was baited with 10 last instar *Galleria mellonella* (L.) larvae (Bedding and Akhurst, 1975). One week later, dead larvae were placed onto White traps (White, 1927) and emerging IJs were collected and stored at 15 °C in aerated water (Kaya and Stock, 1997). To confirm their pathogenicity to insects, the IJs were transferred onto moist filter paper in petri dishes to which living *G. mellonella* larvae were added. The new generation IJs were collected in a beaker and rinsed three times with sterile distilled water and stored

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[1]Department of Biology, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon-Turkey.
[2]Department of Biology, Faculty of Arts and Sciences, Giresun University, 28049 Giresun-Turkey.
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E-mail: idemir@ktu.edu.tr
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at 11 °C. Each nematode isolate was passed through *G. mellonella* every 6 mon (Rosa et al., 2000).

Morphological observation: For the identification of the isolate, 20 specimens from each developmental stage (first generation female, male, and IJ) were randomly selected from different *G. mellonella* cadavers. Nematodes were killed and fixed in 4% hot formalin (50 to 60 °C) and kept in this solution for 48 h. Fixed nematodes were transferred to anhydrous glycerine according to Seinhorst's (1959) rapid method as modified by De Grisse (1969) and mounted on slides using cover-glass supports to avoid flattening. All measurements were made using a drawing tube attached to an Olympus BX50 light microscope. The nematodes were identified according to a selection of morphological and morphometric criteria summarized by Hominick et al. (1997).

Molecular identification and phylogeny of the nematode isolate: DNA was extracted from a single hermaphroditic female using a modified method described by Joyce et al. (1994). The nematode specimen was cut in 8-µl double-distilled H_2O. The fragments were transferred into an Eppendorf tube containing 8 µl of worm lysis buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.3, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatine) and 2 µl of proteinase K (600 µg ml^{-1}). After freezing (−70 °C for 10 min) the tubes were incubated at 65 °C for 1 h, and then at 95 °C for 10 min.

After centrifugation (1 min; 13,000 g) of the tubes, 5 µl of the DNA suspension were added to a PCR reaction mixture containing 5 µl of 10X PCR buffer, 2 µl of $MgCl_2$ (25 mM), 1 µl of dNTP mixture (10 mM each), 0.3 µl (500 nM) of each primer, 1.5 U of Taq DNA polymerase, and 36 µl of double-distilled water to a final volume of 50 µl. The forward primer TW81 (5' - GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5' - ATATGCTTAAGTTCAGCGG GT-3') were used in the PCR reaction for amplification of the complete ITS region (Joyce et al., 1994). The amplification profile was carried out using a PTC-100 thermal cycler (BIO-RAD, Foster City, California), which was preheated at 95 °C for 2 min followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1 min, and then 72 °C for 8 min. Amplified products were separated by electrophoresis on 1% agarose gel and excised from the gel by using the NucleoSpin® Extract II Kit (Macherey-Nagel, Duren, Germany). Gel purified PCR products were cloned directly into the pGEM-T vector cloning system and transformed into *E. coli* DH5α strain. After selection of transformed colonies, plasmid isolation was performed and plasmid DNA samples were digested by restriction enzymes to confirm whether the gene cloned into the vector or not. Plasmid DNA samples that had the right clone were sent to Macrogen (Korea) for sequencing. The evolutionary relationship of the nematode isolate and 24 closely related species were evaluated. Comparisons of the sequences of these steinernematid species were conducted using the GeneDoc bioinformatics software (Nicholas et al., 1997).

Insecticidal activity of the nematode isolate: Experimental inoculations were conducted using the new nematode isolate to determine its virulence to *A. segetum*. Healthy *A. segetum* larvae were collected on a daily basis from different tobacco fields throughout the vicinity of Trabzon, Turkey, and were grown under ambient conditions as laboratory culture approximately up to three to four generations. Healthy third-instar larvae were used for testing the insecticidal effect of the nematode in a dose response experiment.

Inoculations were conducted in plastic cups that were 4-cm deep with a diameter of 3.4 cm. Ten grams of sterilized, air-dried sand, obtained from a field where the *A. segetum* population density was very high, was adjusted to 7% (w/v) moisture by adding distilled water. Nematodes were added to the cups in three densities: 100, 300, and 500 IJs g^{-1}dry sand. Controls were treated with water only. The final moisture content was 10% after the water-suspended nematodes were added to the cups that include one healthy larva. Each dose of nematode was tested against 20 larvae and repeated for three times at different days under same conditions, yielding total of 20 larvae × 4 densities × 3 d = 240 cups. The cups were incubated at room temperature (23 to 24 °C) for 1 h. Then, a single third-instar *A. segetum* larva was placed on the sand surface and the cups were capped with a lid. Seven days after inoculation, each *A. segetum* larva was inspected for survival. All dead larvae were placed individually onto White traps and the emergence of IJs from the larval corpses was recorded.

Statistical analysis: The mortalities in the control and in the treatments were determined 7 d after the beginning of the experiment. The mortalities in the treatment were corrected against the mortality in the control according to Abbott (1925).

To determine differences among different densities, the data were subjected to ANOVA and subsequently to LSD multiple comparison test. Finally, LC50 values were calculated with Probit analysis. All analyses were performed by using SPSS 16.0 statistical software.

Results

Morphological identification: The new isolate is characterized by the infective juvenile maximum body width of 948.1 (848.3−1021.3) µm, excretory pore position 61.4 (55.5−65.3) µm, D% value 45.6 (44.2−47), and E% value 74.9 (70.8−78.2); see Table 1. First-generation males are distinguished by the position of the excretory pore (average: 83.9 µm), anal body width (average: 26.4 µm), tail length (average: 39.2 µm), and D% (average: 54.1 µm); see Table 1. Taxonomic investigations revealed the population to be conspecific with *S. kraussei* (Mracek, 1994).

TABLE 1. Morphometrics (in μm) of *Steinernema kraussei* (new Turkish isolate and redescription of topotype). Data are expressed as mean ± SD (range).

	S. kraussei Turkish			*S. kraussei* (after Mracek, 1994)		
Characters[a]	1st generation female	1st generation male	Infective juvenile	1st generation female	1st generation male	Infective juvenile
n	20	20	20	-	-	-
L	4510.3 ± 698.1 (2948.7-5224.8)	1491.1 ± 89.1 (1307.5-1629.0)	948.1 ± 38.5 (848.3-1021.3)	4,200 (2500-5400)	1,400 (1200-1600)	950.5 (796.7-1101.8)
EP	91.2 ± 10.3 (68.3-103.9)	83.9 ± 8.8 (69.8-101.0)	61.4 ± 3.0 (55.5-65.3)	87.0 (66.0-99.0)	80.9 (72.6-99.0)	62.7 (56.1-66.0)
NR	143.6 ± 5.6 (132.5-151.7)	110.6 ± 11.2 (90.3-128.7)	103.8 ± 2.8 (97.8-109.9)	136.9 (127.2-146.1)	104.7 (94.9-122.1)	104.9 (98.5-111.3)
ES	198.9 ± 8.3 (182.3-209.5)	154.8 ± 11.7 (131.8-169.8)	134.9 ± 7.5 (121.3-144.7)	191.7 (178.2-204.6)	152.8 (138.6-178.2)	134.0 (118.8-145.2)
T	50.7 ± 9.6 (37.3-66.4)	39.2 ± 2.5 (34.1-42.9)	82.1 ± 6.2 (72.2-92.2)	47.9 (33.0-59.4)	38.6 (36.3-43.5)	78.5 (69.3-85.8)
ABW	93.4 ± 13.2 (70.2-114.0)	26.4 ± 2.6 (22.4-31.4)	19.8 ± 1.2 (17.9-22.2)	90.7 (66.4-113.3)	44.5 (39.1-50.4)	19.9 (18.6-21.7)
W	255.3 ± 36.5 (168.5-293.7)	129.9 ± 13.8 (106.7-158.8)	32.1 ± 2.2 (28.6-35.8)	240.3 (152.6-288.2)	128.0 (110.2-144.1)	33.1 (29.7-36.3)
SL	NA[b]	59.6 ± 4.7 (49.6-67.6)	NA	NA	49.0 (42.4-53.0)	NA
GL	NA	33.7 ± 2.9 (28.7-39.5)	NA	NA	33.1 (29.2-37.1)	NA
GW	NA	7.4 ± 0.7 (6.2-8.6)	NA	NA	7.2 (6.6-8.0)	NA
Mucro	NA	2.1 (0.7-4.4)	NA	NA	1.8 (0.0-4.9)	NA
V%	54.2 ± 0.5 (53.1-55.0)	NA	NA	53.8	NA	NA
a	17.6 ± 0.3 (17.0-18.0)	11.5 ± 0.6 (10.3-12.4)	29.6 ± 1.2 (28.5-29.7)	17.4	10.9	28.8
b	22.6 ± 2.7 (16.2-25.1)	9.6 ± 0.2 (9.3-10.0)	7.0 ± 0.2 (6.8-7.6)	21.9	9.2	7.1
c	89.6 ± 7.7 (78.7-102.8)	38.0 ± 0.6 (36.9-39.4)	11.6 ± 0.5 (10.9-12.7)	87.7	37.3	12.1
D%	45.7 ± 3.5 (37.5-49.6)	54.1 ± 2.1 (51.2-59.5)	45.6 ± 0.7 (44.2-47.0)	45	53	47
E%	182.4 ± 16.7 (156.5-208.9)	213.3 ± 10.3 (200.3-231.7)	74.9 ± 2.2 (70.8-78.2)	182	210	80
f	5.1 ± 0.5 (4.4-5.9)	3.3 ± 0.2 (3.0-3.7)	0.4 ± 0.0 (0.4-0.4)	-	-	-

[a] n: Number of specimens. L: Total body length. EP: Distance from anterior end to base excretory pore. NR: Distance from anterior end to nerve ring. ES: Distance from anterior end base of basal bulb. T: Tail length. ABW: Anal body width. W: Maximum body width. SL: Spicule length. GL: Gubernaculum length. GW: Gubernaculum width. V%: (Distance from anterior end to vulva/total body length) × 100. a: L/W. b: L/ES. c: L/T. D%: (EP/ES) × 100. E%: (EP/T) × 100. f: W/T.

[b] NA: Not available.

Molecular identification: The full sequence length of the ITS1-5.8S-ITS2 region including the partial sequence of 18S and 28S rRNA genes of the Turkish isolate of *S. kraussei* was 758 bp. The BLAST search indicated a 99% similarity among the *S. kraussei* CG-81 isolate sequence (JX872515) and isolates from Japan (AB243442), Iceland (AY171248), Germany (AY230175), and Russia (AY171270). Additionally, the Turkish isolate had a sequence similarity between 98% and 96% with some different *S. kraussei* isolates available in GenBank.

The evolutionary relationship of the isolate and closely related other species were evaluated. The 758bp segment of ITS rDNA was used for phylogenetic analysis by MEGA. Phylogenetic analysis of ITS rDNA sequence data placed our *S. kraussei* isolate in a clade with other isolates of *Steinernema* species (see Fig. 1).

Insecticidal activity: In the dose response tests of *S. kraussei*, larval mortality reached 97.8% within 7 d after application of density of 500 IJs g^{-1} dry sand. Treatment with different densities of nematodes resulted in different mortality rates ($F = 628{,}25$; $df = 3, 8$; $P < 0.05$) (see Fig. 2). Additionally, applications of 100 IJs g^{-1} and 300 IJs g^{-1} sand of isolate CG-81 during the same period resulted in 52.2% and 68.9% larval mortality, respectively. The LC50 of this nematode against *A. segetum* larvae 7 d after treatment was 99.039 (lower bound; 0.997 and upper bound; 176.211) IJs. The results show that the concentration of IJs affected the mortality of *A. segetum* larvae differently ($P < 0.05$).

Discussion

A new entomopathogenic nematode was isolated from the eastern Black Sea region of Turkey. Based on the taxonomical characteristics; the nematode isolate was identified as *S. kraussei*. According to morphologic data, *S. kraussei* CG-81 isolate was similar to the European isolates. Stock et al. (2000) reported that the morphological differences between the populations of *S. kraussei* could be related to their geographic origins. Although the European isolates were shown to be distinctly different from the North American isolates, there were no significant differences between the European isolates.

Steinernema kraussei was originally isolated from the body cavity of the web spruce sawfly (*Cephaleia abietis*) in the Egge Mountains, Westphalia, Germany (Steiner, 1923) and is the first-recorded EPN species. This nematode

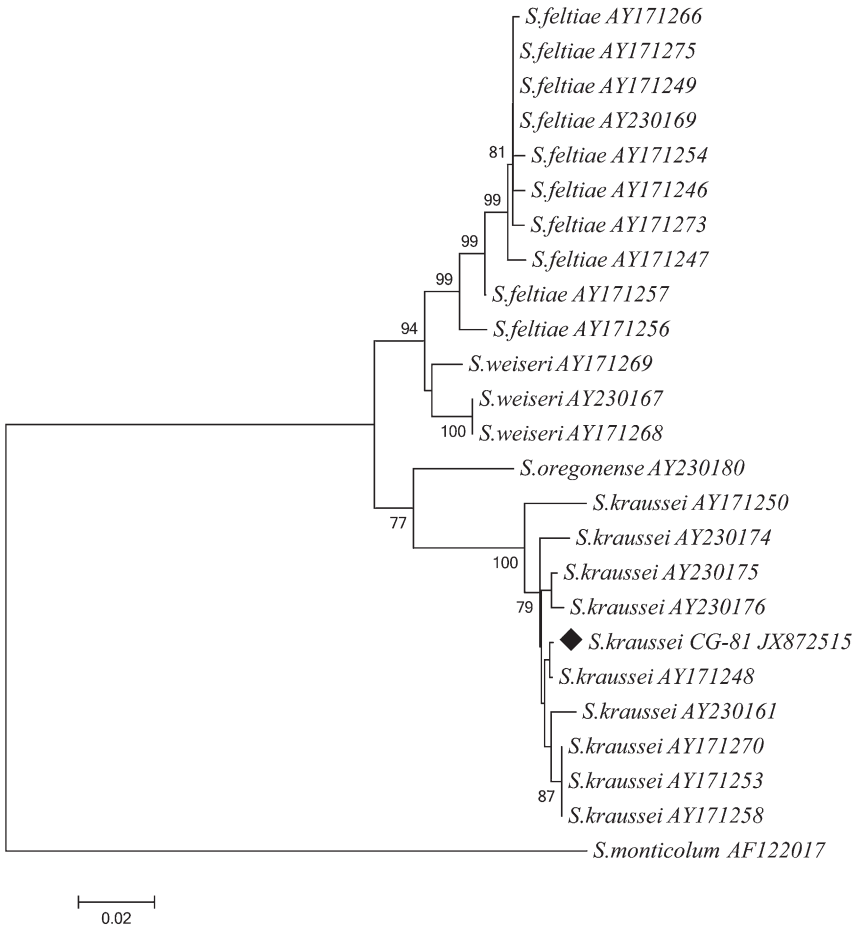

Fig. 1. Phylogenetic relationships of the *Steinernema* species based on analysis of ITS rDNA regions. Number on branches > 70% indicates the percentage of 1,000 bootstrap replicates.

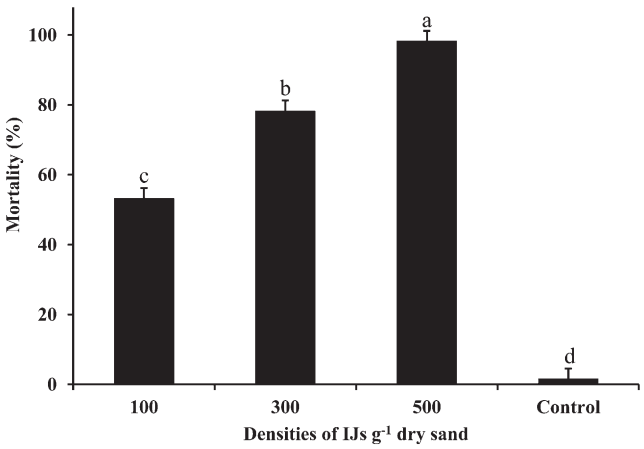

FIG. 2. Percentage mortality (means ± SE) of third instar *Agrotis segetum* at different densities of *Steinernema kraussei* CG-81 recorded 7 d after inoculation. Different letters above bars indicate statistical significance (LSD test, $P < 0.05$). Control = water only.

was subsequently isolated from other locations in Germany (Mracek et al., 1992; Mracek, 1994), and also from other European countries, such as Austria (Peters, 1996), Belgium (Spiridonov and Moens, 1999), Czech Republic (Mracek, 1977), Italy (Reid et al., 1992; Reid, 1994), The Netherlands (Hominick et al., 1995), Russia (Spiridonov et al., 2004), Slovakia (Sturhan and Liskova, 1999), Slovenia (Laznik et al., 2009), Switzerland (Steiner, 1996), the United Kingdom (Hominick et al., 1995), and Spain (Garcia del Pino and Palomo, 1996), suggesting that *S. kraussei* has a Palearctic distribution. Recently, however, the known geographic range of this species has been expanded to include Canada (Mracek and Webster, 1993), United States (Stock et al., 1999), and Japan (Yoshida, 2003), thus suggesting a Holarctic distribution. However, this is the first record of the natural occurrence of *S. kraussei* in Turkey. The isolation site of the new isolate is congruent with the described woodland habitats, both coniferous and deciduous (Spiridonov and Moens, 1999; Stock et al., 2000; Hominick, 2002; Mracek et al., 2005). It is rarely found in open habitats with the exception of alpine altitudes (Steiner, 1996; Shishinova et al., 1998). We found only one population of this species, but a more intensive survey, covering all parts of the Black Sea region of Turkey, is currently being done and may add important information to the distribution of this nematode in the region.

In the current study, the inoculation results showed that third instar *A. segetum* larvae were very susceptible to infection by the new isolate and showed a dose-dependent response. *A. segetum* larvae were highly susceptible to a low density of nematodes (100 IJs g^{-1}). These data suggest the potential of the new isolate as a microbial control agent for the control of *A. segetum*.

Studies have established the potential of using entomopathogenic nematodes for cutworm control. Lössbroek and Theunissen (1985) determined that *Neoaplectana* (*Steinernema*) *bibionis* was an effective biological control agent against *A. segetum*. The density of *S. carpocapsae* that Yokomizo and Kashio (1996) applied was comparable with our density of 100 IJs g^{-1} dry sand; however, the efficacy of their treatment was higher. When we used 300 IJs g^{-1} dry sand, the efficacy of the treatment was comparable with that of Yokomizo and Kashio (1996). Another study, Unlu et al. (2007), also compared the efficacy of *S. weiseri* (BEY), *S. feltiae* (TUR-S3), and *S. carpocapsae* (TUR) isolated in Turkey against last instar larva of *A. segetum*. Each nematode species was applied at 10, 25, 50, and 100 IJs per *A. segetum* larva in 10 ml of water. *S. weiseri* was more effective than *S. feltiae* (TUR-S3) at 50 and 100 IJs per larva; however, *S. weiseri* was less effective than *S. carpocapsae*. In the context of the reported potency of other entomopathogenic nematodes, our results show that virulence of our *S. kraussei* isolate is effective as a biological control agent against *A. segetum* larva.

In Europe, *H. megidis*, *H. bacteriophora*, and *S. kraussei* are commercialized for control of *Otiorhynchus sulcatus* in ornamentals and strawberry. *Heterorhabditis* species are not considered effective at low temperatures, whereas *S. kraussei* is cold active and considered effective at temperatures well below 10 °C (Long et al., 2000; Willmott et al., 2002). Haukeland and Lola-Luz (2010) reported results from field trials conducted in Norway and Ireland using two commercial nematode products, *H. megidis* and *S. kraussei*, against *O. sulcatus* larvae in field-grown strawberry plants. The overall results indicated that *H. megidis* was effective as long as temperatures were optimal.

Steinernema kraussei is active at low temperatures and is thought to adopt a "cruise" foraging strategy particularly suitable for finding subterranean sedentary insects (Long et al., 2000; Willmott et al., 2002; Torr et al., 2007). The efficacy of *S. Kraussei* at low temperature is important for plant protection, especially when applied in the open, whereas temperature, beside UV radiation and moisture, represents the most important limiting factor (Kaya, 1990).

There is an urgent need for the development of safe pesticides to control some noxious insects. To this aim, we demonstrate a native isolate of *S. kraussei*, an EPN from the eastern Black Sea region of Turkey.

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