FIRST REPORT OF THE ENTHOMOPATHOGENIC NEMATODE *STEINERNEMA ARENARIUM* (STEINERNEMATIDAE: RHABDITIDA) IN BULGARIA

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Summary. *Steinernema arenarium* Artyukhovsky, 1967 (Steinernematidae: Rhabditida) was isolated from alluvial soil at a riverside location near the town of Kresna, at an altitude of 200 m. This is the first report of steinernematids of the *glaseri*-group for Bulgaria and the Balkan Peninsula. The average ratio *c* of the infective juveniles of the "Kresna" isolate is 13.8. Such high values of the ratio *c* (with an average of more than 12) are characteristic of the "*glaseri*-group" within the genus *Steinernema* Travassos, 1923, comprising species such as *S. diaprepesi* Nguyen *et* Duncan, 2002 [*c* = 12.1 (10.4-13.2)], *S. puertoricense* Roman *et* Figueroa, 1994 (*c* = 14.2), *S. cubanum* Mráček, Hernández *et* Boëmare, 1994 (*c* = 19), *S. glaseri* Steiner, 1929 [*c* = 14.7 (13.6-15.7)], *S. boemarei* Lee, Sicard Skeie *et* Stock [*c* = 13 (12-14)] and *S. arenarium*. These six species form a distinct monophyletic group within clade V of the genus *Steinernema*. The ratio *c* appears to be quite significant for the differentiation of this group. It seems that *S. arenarium* is widely distributed in some regions of Europe. The species is genetically and phenotypically polymorphic, with preferences for riverside alluvial soils. The establishment of *S. arenarium* in Bulgaria extends the known area of the species to the southeast. According to the chorotype classification, the distribution of *S. arenarium* resembles Turano-European. Larvae and adults of insects, mainly of the families Melolonthidae, Rutelidae, Aphodiidae (Coleoptera: Scarabaeoidea), Carabidae and Elateridae (Coleoptera), which probably are among the natural hosts of the species, were isolated from the investigated site. The morphological identification of the species was confirmed by molecular methods.

Keywords: Description, systematics, Steinernematid of *glaseri*-group.

The entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are obligate insect parasites. Their pathogenic effect is mostly due to the symbiotic bacteria of the genera *Xenorhabdus* Thomas *et* Poinar and *Photorhabdus* Boemare, Akhurst *et* Mourant (Enterobacteriaceae), located in the intestine of the infective juveniles. Entomopathogenic nematodes are subject to intensive investigation because of their potential as biological control agents and their specific biology. They are widely distributed in natural ecosystems and, in many countries worldwide, these two families are subjects of faunistic research. Until now, six species of the genus *Steinernema* Travassos, 1927 and one of the genus *Heterorhabditis* Poinar, 1976 have been reported from Bulgaria (Shishiniova *et al.*, 2000). During our investigations in the region of the Kresna Gorge, one *Steinernema* species belonging to the *glaseri*-group was found.

Therefore, the objectives of the present study were *i*) to identify and describe the morphology of the obtained isolate, *ii*) to compare the morphometric characters with data on the species in the literature, and *iii*) to analyze the geographical distribution and habitat preferences of *S. arenarium* in Europe.

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MATERIALS AND METHODS

The soil samples used in the present study were collected during November 2000-November 2002, from an arid region of Kresna Gorge (the Struma River Valley, SW Bulgaria, at altitudes of 200-700 m). Each sample included four subsamples, which were taken from the corners of a (2 × 5) metre rectangle to a depth of 20 cm. In total, 58 samples were collected.

Nematode juveniles were isolated from the soil using the "*nematode-bait*" method with larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) according to Bedding and Akhurst (1975). After 4-5 days, all dead host larvae were transferred to Petri dishes on moist filter paper to allow for development of nematodes. The nematodes were subsequently reared on *G. mellonella* in order to obtain the life stages necessary for morphological identification. Adult nematodes were obtained by dissection of parasitized *Galleria* larvae in 0.9% NaCl and were fixed in 4% formaldehyde. The nematodes were transferred to glycerol (simple evaporation method, after Poinar, 1975), mounted within paraffin rings on microscope slides and measured at microscope magnifications of 12×40 and 12×100. The morphological identification was performed on the basis of characteristics of infective juveniles and males of the first parasitic generation, according to Nguyen and Smart (1993) and Adams and Nguyen (2002).

The characters recorded were (Table I and II): body

length (L), greatest body width (W), distance to excretory pore (EP), body width at excretory pore (Wep), distance to nerve ring (NR), oesophagus length (OES), body width at oesophagus base (Woes), tail length (T), cloacal body width (WCL), spicule length (SP), gubernaculum length (GB), anal body width (ABW), hyaline part of tail (Lhcd), ratios *a* (L/W), *b* (L/ES), *c* (L/T), *d* (EP/OES), *e* (EP/T), GS (GB/SP), SW (SP/WCL), body length to oesophagus length (L/EP), greatest body width to tail length (W/T), and hyaline part of tail to tail length (Lhcd/T). The microscope slides prepared during this investigation and the isolated live culture of *S. arenarium* are deposited in the collection of the Department of Zoology and Anthropology, Faculty of Biology, Sofia University "St. Kliment Ohridski" (culture BG_Ks-4/2001 and slides BG_Ks-4/1-17).

Molecular identification of the isolate was performed at the Institute for Agricultural and Fisheries Research (ILVO), Merelbeke, Belgium, to confirm the results of the morphological identification. For this, four infective juveniles were cut and placed in an Eppendorf tube containing 10 µl of worm lysis buffer (10 mM Tris-HCl, pH 8.50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.45% Tween 20), 8 µl of double distilled water (dd H_2O) and 2 µl Proteinase K (600 µg/ml) (Spiridonov and Moens, 1999). After freezing at -80 °C for 15 min, the mix was incubated at 65 °C for 1 hr and at 95 °C for 10 min and centrifuged at 14 000 rpm (18 300 ×g) for 2 minutes. The resulting DNA extract in the supernatant was used in PCR reactions with two sets of primers to amplify both the rDNA ITS-regions and the D2D3 expansion segment of the 28S rRNA gene: TW81 (5' GTTTCCG-

Table I. Measurements of *Steinernema arenarium* (in µm). First generation males.

Character	"Kresna" isolate (n=30)			After Kozodoi, 1984 (n = 10)	After Artyukhovsky *et al.*, 1997 (n = 50)
	Mean (range)	± SD	CV	Mean (range)	Mean (range)
L	1791 (1475-2045)	168.2	9.39	2282 (2091-2550)	1845 (1280-2320)
W	129 (86-179)	30.26	23.54	188 (184-219)	117 (70-165)
EP	123 (95-150)	12.25	9.94	164 (153-187)	-
Wep	59 (45-77)	9.18	15.6	-	-
NR	130 (110-144)	7.93	6.1	-	-
OES	173 (153-193)	8.18	4.73	176 (173-184)	168 (125-209)
Woes	67 (50-87)	11.04	16.38	-	
T	32 (24-37)	3.37	10.5	49 (41-57)	32 (14-40)
WCL	46 (37-54)	3.85	8.34	-	-
SP	76 (66-86)	5.76	7.57	84 (81-91)	76 (63-93)
GB	52 (44-59)	4.56	8.7	55 (49-60)	53 (45-63)
a	14.52 (10.46-20.10)	2.82	19.41	12.1 (11.4-12.1)	16 (12.1-23.4)
b	10.36 (8.58-11.64)	0.77	7.47	13 (12.1-13.9)	10.9 (85-15.5)
c	56.09 (42.75-71.76)	5.17	9.23	46.6 (44.7-51)	60.1 (38.4-126.7)
d	0.71 (0.58-0.85)	0.07	9.89	-	0.78 (0.52-0.96)
e	3.87 (3.12-5.27)	0.46	11.88	-	-
GS	0.69 (0.59-0.74)	0.04	5.34	-	-
SW	1.65 (1.42-1.86)	0.11	6.79	-	-
L/EP	14.58 (12.56-17.32)	3.85	26.42	-	-
W/T	4.00 (2.41-5.33)	0.8	19.96	-	-

Table II. Measurements of *S. arenarium* (in µm). Infective juveniles.

Character	"Kresna" isolate (n=30)			After Kozodoi, 1984 (n = 10)	After Poinar & Kozodoi, 1988 (n = 15)	After Artyukhovsky *et al.*, 1997 (n = 50)
	Mean (range)	± SD	CV	Mean (range)	Mean (range)	Mean (range)
L	1157.6 (994-1282)	78.65	6.79	1097 (724-1408)	970 (928-1088)	1217 (930-1580)
W	35.2 (31-39)	2.07	5.89	63 (41-77)	29 (28-35)	37 (31-44)
EP	88.7 (81-95)	3.76	4.24	-	83 (76-86)	-
NR	106.5 (94-113)	5.15	4.83	-	109 (100-120)	-
OES	152.2 (138-161)	7.41	4.87	125 (123-131)	151 (138-160)	156 (132-187)
T	83.7 (72-93)	5.73	6.85	80 (77-84)	70 (64-77)	81 (65-95)
Lhcd	41.9 (32-49)	4.15	9.89	-	-	-
ABW	23.4 (21-26)	1.23	5.23	-	20 (16-22)	-
a	32.84 (30.59-35.00)	1.04	3.17	-	30.6 (26.4-34.4)	33.3 (24.7-39.6)
b	7.61 (6.63-8.20)	0.36	4.74	8.8 (5.9-10.8)	6.4 (5.9-7.0)	7.8 (5.9-9.2)
c	13.85 (12.54-15.83)	0.63	4.55	13.7 (9.4-16.9)	13.9 (13.2-15.0)	15.1 (12.2-17.9)
d	0.58 (0.52-0.63)	0.03	4.31	-	0.55 (0.52-0.59)	0.63 (0.53-0.68)
e	1.06 (0.95-1.27)	0.06	5.91	-	1.19 (1.06-1.3)	-
Lhcd/T	0.50 (0.44-0.54)	0.03	5.56	-	-	-

TAGGTGAACCTGC 3') and AB28 (5' ATATGCTTAAGTTCAGCGGGT 3') as described by Joyce *et al.* (1994); and D2A (5' ACAAGTACCGTGAGGGAAAGTTG 3') and D3B (5' TCGGAAGGAACCAGCTACTA-3') as described by De Ley *et al.* (1999). The PCR mix for one reaction contained 5 µl of PCR Taq buffer 10×, 2 mM $MgCl_2$, 200 µM dNTPs, 1 µM of each primer, 2U Taq Polymerase, 5 µl DNA extract and ddH_2O to a final volume of 50 µl. The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Leiden, The Netherlands). They were cloned in the pGEM-T vector and transformed into JM 109 High Efficiency Competent Cells (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. Plasmid DNA from competent cells was purified using the Wizard® *Plus* SV Minipreps DNA purification system (Promega, Leiden, The Netherlands) and two colonies were sent for sequencing to Macrogen's Laboratory for Genomics and Bioinformatics.

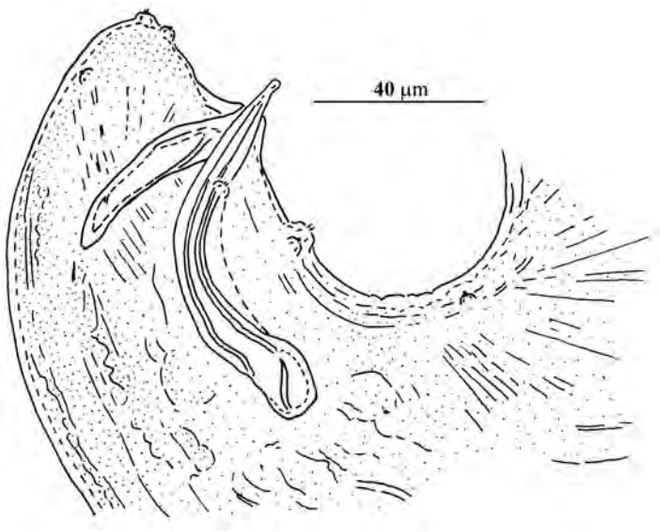

Fig. 1. *Steinernema arenarium*, "Kresna" isolate. Tail region of first generation male.

RESULTS

EPNs were detected in six out of the 58 collected samples. Three EPN species previously reported from Bulgaria (Shishiniova *et al.*, 2000), two belonging to the genus *Steinernema*, namely *S. feltiae* Filipjev, 1934 and *S. intermedium* Poinar, 1985, as well as one of the genus *Heterorhabditis*, *H. bacteriophora* Poinar, 1976, were found. These species are reported for the first time from this region of SW Bulgaria.

Also, a species from the *glaseri*-group of the genus *Steinernema* was isolated from one sample. Morphological analyses identified it as *S. arenarium* Artyukhovsky, 1967, syn. *Neoaplectana arenaria* Artyukhovsky, 1967, *Neoaplectana anomali* Kozodoi, 1984, *Steinernema anomalae* (Kozodoi, 1984) Curran, 1989. This species was found in an alluvial soil sample, collected in May, 2001 from the bank of Struma River in Kresna Gorge (longitude 23°09'22"E, latitude 41°45'44"N, altitude 200 m). The vegetation at the sample site was composed mainly of *Populus nigra* L., *Platanus orientalis* L., *Rubus caesius* L., *Agrostis capillaris* L., *Cynodon dactylon* (L.) Pers., *Aristolochia clematitis* L., *Chenopodium glaucum* L., *Euphorbia niciciana* Borb., *Artemisia vulgaris* L. and other herbaceous plant species.

The following characteristics were decisive for the morphological identification of the species: body length, ratios *c* and *d* of infective juveniles, spicule and gubernaculum morphology, especially the spicule tip, which is typical for *S. arenarium* (Figs 1 and 2), and ratio *d* of first generation males (Poinar and Kozodoi, 1988; Nguyen and Smart, 1993; Artyukhovsky *et al.*, 1997). The morphometrics of males from the first generation (n = 30) and the infective juveniles (n = 30) of the Kresna isolate are presented in Table I and Table II, respectively, in comparison with data from reference sources.

The morphometric identification of the species was confirmed by molecular analysis. Using the sequences obtained with each pair of primers (Genbank accession numbers HM160092 to HM160095), nucleotide queries were submitted in the BLAST database at GenBank. The comparison of the products obtained with the first and the second set of primers to the database sequences yielded top similarity values of 93% and 98% for *S. arenarium*, respectively.

Insect larvae and adult individuals were isolated from soil at the site of isolation of *S. arenarium* in Kresna Gorge. Among them were *Geotomus* sp. (Cydnidae: Heteroptera), *Harpalus pumilus* Sturm, 1818, *H. anxius* Duftschmid, 1812, *Amara aenea* De Geer, 1774, *A. anthobia* Villa *et* Villa, 1833, *Ophonus cribricollis* Dejean, 1829 (Carabidae: Coleoptera), *Platynychus* sp. (Elateridae: Coleoptera), species from three genera of the superfamily Scarabaeoidea (Coleoptera): *Omaloplia* sp. (Melolontidae), *Anomala* sp. (Rutelidae), *Rhyssemus* sp. (Aphodiidae), *Chlorophanus* sp. (Curculionidae: Coleoptera), Tenebrionidae g.sp. (Coleoptera) and Therevidae g.sp. (Diptera).

During the laboratory cultivation of the Kresna isolate on *G. mellonella* larvae, we observed elements typical for the life cycle of another nematode of the *glaseri*-group, *S. glaseri* Steiner, 1929, quick development (first generation adults were observed by the third day of the invasion) and gray colouration of the *Galleria* larvae (Poinar and Kozodoi, 1988), caused by the symbiotic bacteria of this group, *Xenorhabdus poinarii* Akhurst, 1983 (Boemare, 2002). The host tissues, decomposed by the symbiotic bacteria, were completely consumed by the nematodes and subsequently only the cuticles of the cadavers remained.

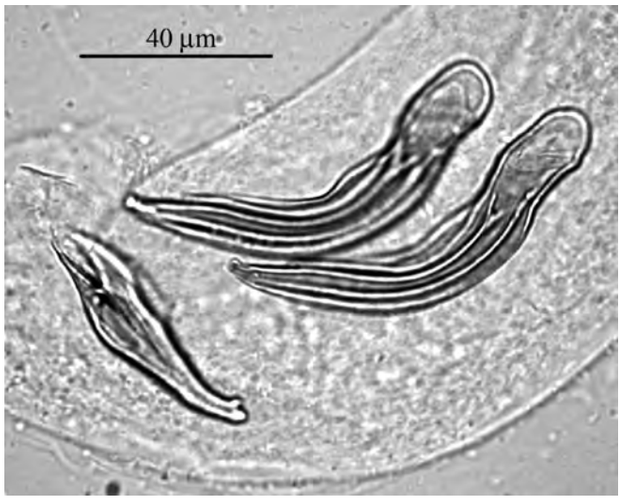

Fig. 2. *Steinernema arenarium*, "Kresna" isolate. Spicules and gubernaculum of first generation male.

DISSCUSSION

The species of the genus *Steinernema* are grouped in five major clades, based on cluster analysis of the sequences of ITS1-5.8S-ITS2 of rDNA and morphometric characteristics, with clade V corresponding to the species of the *glaseri*-group (Spiridonov *et al*., 2004; Lee *et al*., 2009). The most extensively investigated members of the *glaseri*-group are distributed in Europe, the Mediterranean region, North and Central America (Sturhan and Mráček, 2002). In the continental part of North America, the group is represented by the species *S. glaseri* (Poinar and Kozodoi, 1988) and on the island of Cuba by *S. cubanum* Mráček, Hemandes *et* Boemare, 1994. These two species may be a result of geographical divergence. Poinar and Kozodoi (1988) proposed a hypothesis that their presence in the Americas resulted from migration of *S. arenarium* across the Bering Strait and subsequent isolation. However, the species *S. glaseri* was also reported from Spain (Doucet and Gabarra, 1994). The subsequent establishment of *S. glaseri* on the Azores (Hominick, 2002) and in Belgium (Ansari *et al*., 2005) and Iran (Karimi *et al*., 2009) casts doubt on the hypothesis of Poinar and Kozodoi (1988). Therefore, the questions on the centre of origin and directions of spread of the species of the *glaseri*-group remains open.

So far, *S. arenarium* has been reported from central Russia (Voronezh and Ryazan regions) (Kozodoi, 1984; Artyukhovsky *et al*., 1997), Slovakia (Sturhan and Lišková, 1999), Switzerland (Kramer *et al*., 2001), Italy and Spain (Hominick, 2002), Czech Republic (Mráček *et al*., 2005) and Poland (Sturhan and Mráček, 2002). Some reports of isolates from Europe probably refer to other species of the *glaseri*-group (Sturhan and Mráček, 2002). Thus, two new species from the group have been described from Italy: *S. apuliae* Triggiani, Mráček *et* Reid, 2004 (Triggiani *et al*., 2004) and from France: *S. boemarei* Lee, Sicard, Skeie *et* Stock, 2009 (Lee *et al*., 2009).

Mráček *et al*. (2005) considered the distribution of *S. arenarium* as Pannonian and Mediterranean. The establishment of this species in Bulgaria is of considerable interest as it extends the known area of the species to the southeast. According to the classification of chorotypes proposed by Taglianti *et al*. (1999), the distribution of *S. arenarium* resembles Turano-European.

The morphological characteristics and the measurements of the males and the infective juveniles of the isolate from Kresna are in most cases close to those given for the species by Artyukhovsky *et al*. (1997) (Tables I and II). Among them are some important taxonomic features, such as the size and morphology of the spicules (including the characteristic spicule tip) and the gubernaculum of the first generation males.

The deviations in some of the morphometric characters and ratios established in the isolated culture of *S. arenarium* are not greater than those reported for the species by Artyukhovsky *et al*. (1997) and Poinar and Kozodoi (1988). The comparison of the morphometric characters of first generation males of the Kresna isolate with the reference data from the literature (Table I) leads to the following conclusions: *i*) there are no significant differences in the characters OES (173, 176 and 168 µm respectively for the three cultures), SP (76, 84 and 76 µm) and GB (52, 55 and 53 µm); *ii*) the values of characters W and T and ratios *a*, *b* and *c* of the Kresna isolate are in the range of the variations observed for the other two cultures; *iii*) there are some deviations in the values of characters L (1791, 2282 and 1845 µm, respectively) and EP (123 µm for our culture and 164 µm according to Kozodoi, 1984); *iv*) most of the taxonomic characters of the examined male individuals show high coefficients of variation (frequently above 9), which limits their possible use for identification purposes; *v*) the characters OES and SP and ratio *b* show relative stability (with coefficients of variation of 4.3, 7.7 and 7.47, respectively).

The characters of the infective juveniles of the cultures of *S. arenarium* compared in this study show greater stability and lower variability. The values of all investigated characters of the Kresna isolate are in the range of the data used for reference (Table II). The coefficients of variation of the characters and ratios of our culture are relatively low. The lowest coefficients of variation were found for the character EP and the ratios *a*, *c* and *d* (4.24, 3.17, 4.55 and 4.31, respectively). However, some differences were observed for the characters L, W, EP, OES, T, etc. This can be explained by the biological characteristics of the different cultures, as well as by the different sample sizes used in the separate studies.

It seems that some of the morphometric characters of both males and infective juveniles may vary considerably among the different isolates of this species. According to Sturhan and Mráček (2002), the ratios *a*, *b* and *c* demonstrate relatively little variation among the infec-

tive juveniles of the *glaseri*-group in Europe. However, we considered that ratio *c* of the infective juveniles of this group deserves special attention. For our isolate its average value is 13.8. For comparison, for the cultures of species investigated by Kozodoi (1984), Poinar and Kozodoi (1988) and Artyukhovsky *et al*. (1997), this ratio has values of 13.7, 13.9 and 15.1, respectively (Table II). For the Spanish isolate of the *glaseri*-group (Doucet and Gabarra, 1994) it is 14.8. Such a high value of ratio *c* (>12 on the average) within the genus *Steinernema* is typical for the *glaseri*-group, represented by species such as *S. diaprepesi* Nguyen *et* Duncan, 2002 [*c* = 12.1 (10.4-13.2)], *S. puertoricense* Roman *et* Figueroa, 1994 (*c* = 14.2), *S. cubanum* Mráček, Hernández *et* Boëmare, 1994 (*c* = 19), *S. glaseri* [c = 14.7 (13.6-15.7)], *S. boemarei* Lee, Sicard Skeie *et* Stock [c = 13 (12-14)] and *S. arenarium* (Adams and Nguyen, 2002; Nguyen and Hunt, 2007; Lee *et al*., 2009). These six species form a distinct monophyletic group within clade V of the genus *Steinernema* (Lee *et al*., 2009). The ratio *c* appears to be quite significant for the differentiation of this group within the genus. For comparison, the ratio *c* of infective juveniles of species from the adjacent clades III and IV rarely exceeds 10-11 (Adams and Nguyen, 2002; Nguyen and Hunt, 2007).

Contemporary interbreeding experiments and molecular investigations of European isolates belonging to the *glaseri*-group sometimes show contradictory results (Sturhan and Mráček, 2002). *Steinernema arenarium* is probably a genetically and phenotypically polymorphic species that is widespread in some regions of Europe. Its comparatively rare establishment could be a result of preferences for specific habitats such as riverside alluvial soils, at least in regions with dry climates. *Steinernema arenarium* has been found by Mráček *et al*. (2005) in sandy soil habitats. The closely related species *S. boemarei* was also established in sandy soil (Lee *et al*., 2009). Similarly, the other European species belonging to the *glaseri*-group were isolated from salted silt soil in south Italy (Triggiani *et al*., 2004). In a following investigation (Gradinarov, 2003, 2005, unpublished) we obtained other isolates of the *glaseri*-group, morphologically belonging to *S. arenarium* (xerothermic alluvial pasture with similar conditions located in the area of Rupite, SW Bulgaria, longitude 23°15'49"E, latitude 41°27'28"N, altitude 93 m). However, molecular diagnostics of these isolates have not been performed.

At the Bulgarian site of isolation of *S. arenarium*, the climatic conditions resemble those of a semi-desert area. It is not a coincidence that the species *H. bacteriophora*, *S. feltiae*, and *S. intermedium* were found in the same type of habitat. In all cases, EPNs were found in riverside soils, showing that, under these climatic conditions, they inhabit localities in proximity to water.

In our investigations during the period 1994-2010, we found entomopathogenic nematodes of the genus *Steinernema* in approximately 260 samples from different regions of Bulgaria. We found nematodes of the *glaseri*-group only in three of these samples: Kresna isolate (one sample) and Rupite isolate (two samples). The regions where these nematodes were established are among the warmest and most arid ones within Bulgaria (mean annual temperature 13-14 °C, mean annual rainfall 500-550 mm). The establishment of *S. arenarium* in such climatic conditions contradicts the suggestion of Sturhan and Mráček (2002) that the species is adapted to cooler climate.

Similarly to the investigations of Mráček *et al*. (2005), our studies showed that the species can be found at altitudes lower than 500 m. This may be due to the more suitable climate, as well as to the formation of sandy soils, which are not typical of higher elevations. The phytocenosis at the points of establishment of *S. arenarium* in the investigation of Mráček *et al*. (2005) differ from the ones observed in our study. The authors report the species from a coniferous ecosystem, while we found it in a phytocenosis of *Populus nigra* and *Platanus orientalis*. It seems that the major factor for the presence of *S. arenarium* is rather the soil type and the insect hosts connected with it.

So far, larvae of *Anomala dubia* Scopoli, 1763 (Rutelidae: Scarabaeoidea) have been reported as natural hosts for *S. arenarium* (Peters, 1996). The host range of *S. glaseri* comprises mainly representatives of the superfamily Scarabaeoidea (Peters, 1996; Karimi *et al*., 2009). These facts and the life-cycle specifics of the species gave Poinar and Kozodoi (1988) a reason to propose that these nematodes are adapted to parasitizing insects belonging to this superfamily. In the soils of the site at Kresna Gorge, larvae and adults of Scarabaeoidea, including larvae of the genus *Anomala* Schoenherr, 1817, which are reported as hosts for the species (Kozodoi, 1984), have been found. The meadow near Rupite, where the nematodes from the *glaseri*-group were established, had been used as a pasture, which lead to an increased density of coprophagous scarabaeid larvae in the soil. It is possible that some of the natural hosts of *S. arenarium* are among the Scarabaeoidea found in the investigated habitat. However, other insects found in the soil may also be natural hosts for the species because it has a potentially broad host range, which is essential for most species of the genus *Steinenema* (Peters, 1996). For example, larvae of Carabidae, Curculionidae (Coleoptera) and Asilidae (Diptera) are reported as hosts for *S. bicornutum* Tallosi, Peters *et* Ehlers, 1995 in habitats with alluvial soil in Bulgaria (Gradinarov, 2003).

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