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FIRST REPORT OF *STEINERNEMA BICORNUTUM* (NEMATODA, RHABDITIDA, STEINERNEMATIDAE) FROM THE NORTH CAUCASUS

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Первая находка *Steinernema bicornutum* (Nematoda, Rhabditida, Steinernematidae) на Северном Кавказе. Иванова Е. С., Спиридонов С. Э. — Нематоды *Steinernema bicornutum* Tallosi, Peters & Ehlers, 1995 были выделены, с использованием гусениц *Galleria mellonella* в качестве приманок, из образцов почвы, собранных около Краснодара (Северный Кавказ). Обнаружены различия между краснодарской и типовой (югославской) культурами *S. bicornutum* в средней длине инвазионных личинок и длине спикул самцов. Все эти различия (за исключением длины спикул у самцов второй генерации) не являются статистически достоверными. Рестрикционный анализ рибосомальной ДНК дифференцирует эти две популяции *S. bicornutum* лишь по спектрам эндонуклеазы Cfo I (один сайт рестрикции у типовой, и два — у краснодарской культуры). Проведено секвенирование 715 пар оснований фрагмента рибосомального гена (ITS1 + 5.8S + ITS2). Сравнение с последовательностью типового изолята показало различие в 18 парах нуклеотидов, включая 17 замен и одну делецию в типовой культуре. Выявленные морфологические и молекулярные различия оцениваются как межпопуляционные, а обнаруженные в Краснодаре нематоды как еще один изолят *S. bicornutum*.

Ключевые слова: энтомопатогенные нематоды, Rhabditida, Steinernema, *S. bicornutum*, новый изолят, морфология, rDNA.

First report of *Steinernema bicornutum* (Nematoda, Rhabditida, Steinernematidae) from the North Caucasus. Ivanova E. S., Spiridonov S. E. — *Steinernema bicornutum* Tallosi, Peters & Ehlers, 1995 was isolated by *Galleria baiting* from soil samples collected near Krasnodar (North Caucasus). The differences were found between the type (Yugoslavian) population and Krasnodar isolate in the average length of infective juveniles and spicules. All these differences (except spicule length in the second generation males) are not statistically significant. The RFLP analysis was able to distinguish nematodes from these two populations of *S. bicornutum* only in the Cfo I profiles (one restriction site in the type culture and two restriction sites in the Krasnodar isolate). The 715 bp long fragment of ribosomal gene (ITS1 + 5.8S + ITS2) of the Krasnodar isolate of *S. bicornutum* was sequenced and compared with similar sequence of the type strain. In accordance with RFLP data, one additional Cfo I restriction site was found in the Krasnodar *S. bicornutum*. Totally, the difference in 18 pairs of nucleotides per fragment of 715 bp was found between Krasnodar and type cultures, including 17 substitutions and one deletion (in type culture). Reported morphological and molecular differences are considered as inter-populational ones, and described nematodes from Krasnodar as another isolate of *S. bicornutum*.

Key words: entomopathogenic nematodes, Rhabditida, Steinernema, *S. bicornutum*, new isolate, morphology, rDNA.

The studies in the taxonomy of Steinernematidae — the family of entomopathogenic soil-inhabiting nematodes — were very active in Western Europe because of expected economic potential of these nematodes as new tool for pest management. Unusual species of this family was found in the province of Vojvodina (Northern Yugoslavia) and described, as *Steinernema bicornutum* (Tallosi, Peters & Ehlers, 1995). The peculiarity of this species which was not known before among steinernematids is the presence of two horn-like appendages on the anterior end of infective juveniles. Since then, this species was reported throughout the Europe, though nowhere it was found common or widespread. Recently, the nematodes with morphology of infective juveniles similar to *S. bicornutum*, were isolated during Galleria baiting survey of Krasnodar region in Southern Russia (Kuban river on the north slope of Caucasus). After study and comparison these nematodes were considered as conspecific with Yugoslavian *S. bicornutum*. Short description of differences between type and Krasnodar cultures of this species are presented below.

Material and methods

Steinernema bicornutum culture was isolated from the garden soil on the grounds of the All-Russian Institute for Biological Protection of Plants in the vicinities of Krasnodar in June 2000. Culture was maintained in Moscow on Galleria. Adults for morphological examination were obtained from infected caterpillars. All measurements of nematodes given in text are in micrometers. Infective juveniles were stored in the refrigerator and used for molecular studies. For comparative studies the isolate of *S. bicornutum* was also obtained in April 2000 from Dr. Igor Kramer (then at Federal Agricultural Centre in Wadenswil, Switzerland).

Juveniles were collected in 8 mkl of worm-lysis buffer (100 mM KCl, 20 mM Tris-HCl pH 8.3, 3 mM MgCl2, 2 mM DDT and 0,9% Tween 20) and homogenized. The contiguous sequence including 2 non-transcribed regions and 5.8S gene (ITS1 + 5.8S + ITS2) was amplified for compared Krasnodar and Swiss populations of *S. bicornutum* with the use of Vrain et al. (1992) primers. The RFLP analysis of obtained PCR products was performed as described by Hominick et al. (1997) with 7 endonucleases (Dde I; Eco RI; Cfo I; Hind III; Hinf I; Rsa I and Pvu II). To obtain product for sequencing of Krasnodar isolate a pair or primers TW81 (5'–GTTTCAGTAGGTGAACCTGA-3') and AB28 (5'–ATATGATTAAGTTCAGCGGGT-3') as described by Joyce et al. (1994) was used. Amplifications were carried out in a 23–25 mkl reaction volume, containing 2.5 mkl of 10x PCR-buffer solution, 5 mkl of Q-solution, 0.5 mkl of dNTP mix, 0.15 mkl of each primer and 0.1 mkl of Taq polymerase (from standard "Taq PCR Core Kit" of Qiagen Ltd, Crawley, UK) with 2–4 mkl of the single juvenile homogenate and 12.6 mkl of autoclaved bidistilled water. PCR products were cleaned using Qiaquick PCR or Gel Purification Kit (Qiagen Ltd, Crawley, UK). Attempt was made to use purified PCR products for direct sequencing using AB28, TW81, but as direct sequencing of two samples of *S. bicornutum* from Krasonodar produced electrophoregrams with multiple peaks, cloning of PCR products was performed. PCR product from single juvenile was cloned in pGEM-T vector and transformed into JM109 competent cells according to the instructions of the manufacturer (Promega, Benelux b. v. Leiden, The Netherlands). PCR products from two colonies were used for sequencing. For the sequencing reaction the mixture of 8 mkl BigDye Terminator v3.0 cycle sequencing ready reaction mix (Applied Biosystems, Warrington, UK) and 0.5 mkl of each primer were used with 1–3 mkl of PCR product. Then the total volume of sequencing mix was adjusted to the total volume of 20 mkl with double distilled water. Nucleotides and other DNA remnants were removed from sequencing Gel Filtration cartridges (Edge Biosystems, Inc., Gaithersburg, USA).

The contiguous sequences including 2 non-transcribed regions (ITS) and 5.8S gene obtained from two colonies of transformants with Krasnodar *S. bicornutum* were found to be identical. The sequence was deposited in the GenBank (accession number AY 171 279). Similar sequence for type (Yugoslavian) culture of *S. bicornutum* was obtained from GenBank (Accession number AF 121 048). These sequences were compared using GeneDoc 2.5.000 program.

Results

General morphology. Body robust. Head end bluntly rounded or truncate. Six partly fused lips bearing very short labial papillae. Four distinct cephalic papillae. Amphids indistinct. Stoma short and wide consisting from two cuticularized rings. Oesophagus muscular with cylindrical procorpus, slightly swollen metacorpus a little shorter than procorpus, short isthmus as wide as procorpus and basal bulb a bit wider than metacorpus. Excretory pore past halfway from anterior to oesophageal base, 2–4 wide. Cuticularized excretory duct 1–3 wide and 10–20 long. Excretory cell prominent. Cardia conical. Intestine well developed, expanded posterior to oesophagus. Intestinal walls 10–30 thick.

First generation females. Cuticle with prominent broad annulations at anterior. Stoma 9–11 wide and 4–5 deep. Oesophagus 181 (150–212) long. Procorpus 50–60 long and 20–25 wide, metacorpus 45–53 long and 31–34 wide, isthmus 18–25 long and 21–23 wide, basal bulb 50–60 long and 37–42 wide. Excretory pore at 90 (55–115) from anterior. Flexure of anterior ovary in 480-600 from oesophageal base. Oviducts of four cells in cross-section. Mature oocytes 30–32 in diameter. Up to 16 giant amoeboid cells, round or elliptical in shape, 36–60 x 27–40 in size, in both uteri and oviducts (fig. 1, *D, E*). Vulva at mid-body: V% = 52 (49–57). Vagina straight, cuticularized, about 20 long. Vulva lips 3–5 long slightly protruded. Tail round or rarely truncate, short (10–19) and wide (anal diameter 80–90). Small conical mucron 2–3 long.

Second generation females. Much shorter than first generation females. Body C-shaped. Lips more prominent. Stoma 8–9 wide and 3–5 deep. Oesophagus 137–150 long. Procorpus 45–50 long and 15–17 wide, metacorpus 40–45 long and 22–25 wide, isthmus 15–20 long and 15–20 wide, basal bulb 30–40 long and 26–28 wi-

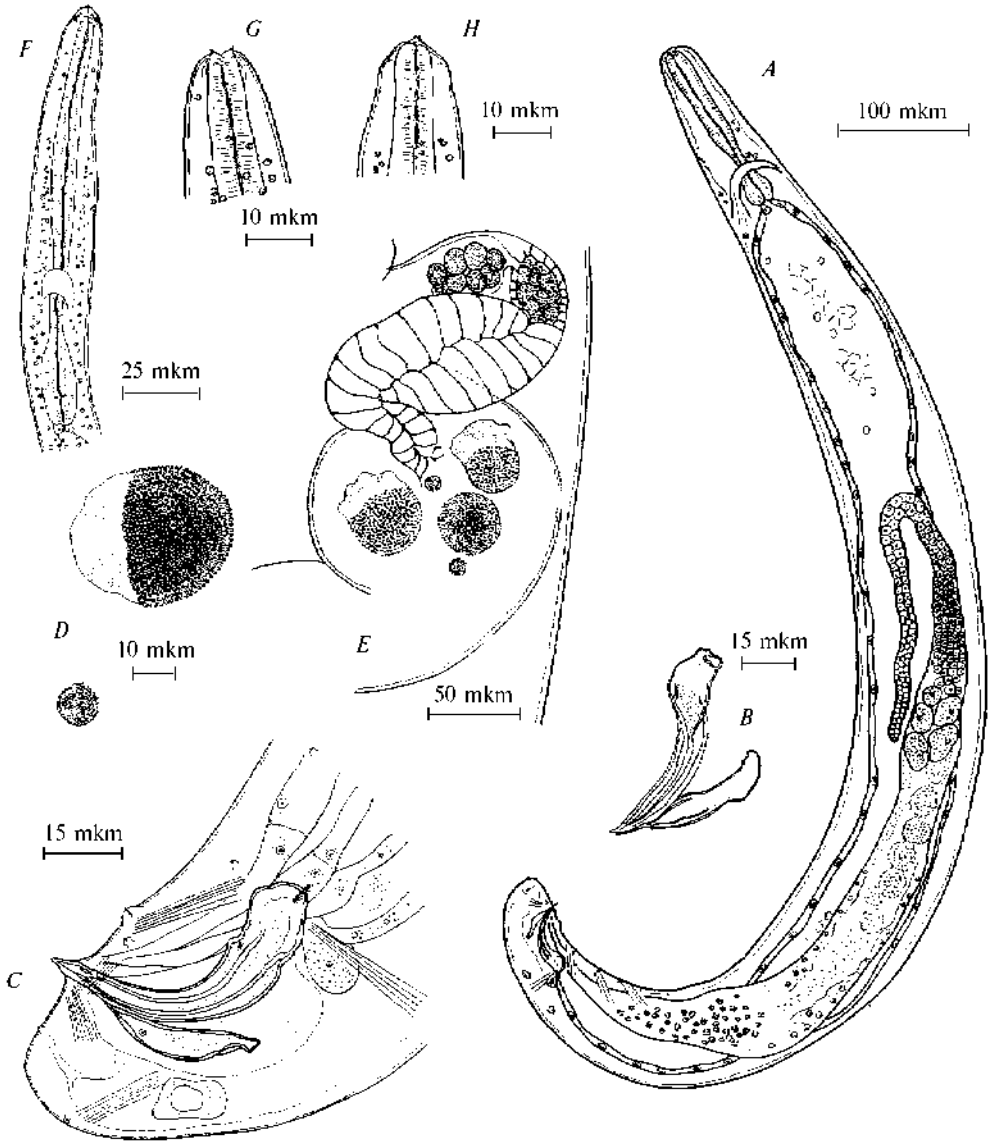
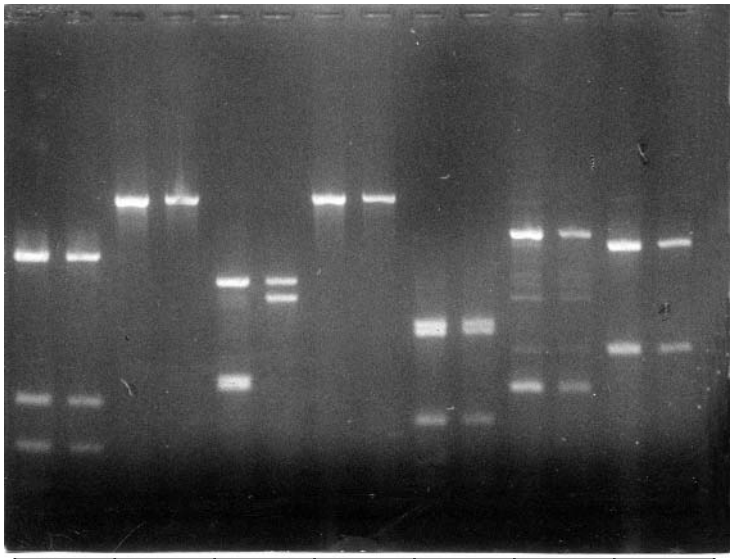


Fig. 1. Morphology of *Steinernema bicornutum* from Krasnodar, Russia: *A* — 1st generation male, total view, laterally; *B* — spicules and gubernaculum of type II, laterally; *C* — male tail and spicules of type I, laterally; *D* — giant amoeboid cell and smaller cell of similar structure; *E* — amoeboid cells in female oviduct; *F* — infective juvenile, anterior end, laterally; *G* — infective juvenile, head end, ventrally; *H* — infective juvenile, head end, laterally.

Рис. 1. Морфология *Steinernema bicornutum* 1995 из Краснодара, Россия: *A* — самец 1-го поколения, общий вид, латерально; *B* — спикулы и рулек второго типа, латерально; *C* — хвост самца и спикулы первого типа, латерально; *D* — гигантская амебоидная клетка и небольшая клетка сходной структуры; *E* — амебоидные клетки в яйцеводе самки; *F* — передний конец инвазионной личинки, латерально; *G* — головной конец инвазионной личинки, вентрально; *H* — головной конец инвазионной личинки, латерально.

de. Intestinal walls thinner than in first generation females. Anterior ovary flexure at 155–400 from oesophageal base. Eggs thin-shelled, 40–46x30 in size. Vulva lips protruded. Up to 4 giant amoeboid cells in uteri. Tail broadly conical or rarely truncate, longer (26–30) and narrower (anal diameter 35–55) than in first generation females. Small mucron often presents.



Kr Sw	Kr Sw	Kr Sw	Kr Sw	Kr Sw	Kr Sw	Kr Sw
Dde I	Eco RI	Cfo I	IIind III	IIind I	Rsa I	Pvu II

Fig. 2. The restriction patterns of *Steinernema bicornutum* from Krasnodar and Switzerland. Each pair of lanes represents digestion of rDNA (about 1000 bp) of two cultures under comparison. Kr — *S. bicornutum* from Krasnodar, Sw — *S. bicornutum* from Switzerland.

Рис. 2. Спектры рестрикции для *Steinernema bicornutum* из Краснодара и Швейцарии. Каждая пара дорожек представляет результат рестрикции рибосомальной ДНК сравниваемых культур: Kr — *S. bicornutum* из Краснодара и Sw — *S. bicornutum* из Швейцарии.

First generation males. Body J-shaped (fig. 1, *A*). Cuticle annulations not visible. Stoma 6–8 wide and 3–5 deep. Oesophagus 145–161 long with procorpus 52–55 long and 12–14 wide, metacorpus 40–45 long and 17–21 wide, isthmus 23–25 long and 13–16 wide and basal bulb 27–35 long and 23–24 wide. Excretory pore 2–3 wide, located in 90–110 from anterior, more distant than in females. Excretory cell less prominent. Testis flexure at 550–680 from oesophageal base. Testis sometimes forms one or two loops. Spicules (fig. 1, *C*) curved, yellow-brownish in colour. Capitulum well separated from lamina, bowl-shaped, 13–18 long and 11–15 wide, calamus 9–12 wide with protrusion, lamina curved 5–7 thick. Two cuticularized ridges 1–1.5 thick run from calamus along lamina. Spicule tips straight, pointed, 2 thick with shallow transverse incision. Velum 4–5 wide. Another type of spicules (fig. 1, *B*) occasionally seen: Capitulum less separated from lamina and not much broader than latter one, spicule tips a little wider. Gubernaculum spindle-shaped, flattened or convex, much variable in size and direction of proximal end, which can be more or less separated, straight or bent inwardly or outwardly. Muscles of tail end strongly developed. Six pairs of subventral precloacal papillae and a single ventral adanal papillae, 3 pairs of subventral postcloacal papillae, 2 pairs near tail tip and 1 pair on dorsal side of tail tip (fig. 1, *A, C*). No mucron presents.

Second generation males. Body thinner, without swelling at mid-part as in first generation males. Oesophagus shorter and thinner (total length 127–130, width of metacorpus 14–16, of bulb 19–21). Testis flexure at 280–330 from oesophageal base. Spicules light yellow in colour. Velum thinner or absent. Both types of spicules reported above for first generation males present but type II more common.

Infective juveniles (fig. 1, *F-H*). Cuticle with distinct annulation. Two horn-like structures on head end around mouth opening oriented in lateral position. Head very slightly constricted at cervical region. Four prominent cephalic papillae in 1.5–2 back

from head end. Stoma about 5–7 long, collapsed. Oesophagus thin, metacorpus very slightly swollen (up to 7), isthmus long and thin, basal bulb 25–27 long and 10–12 wide. Valve ridges of bulb poorly visible. Excretory pore 1 thick, cuticularized duct about 10 long, situated halfway from anterior to oesophageal base. Vesicle containing bacterial cells distinct in few specimens, 12–30x5–17 in size in three-month-old juveniles. Lateral field 6 wide.

Analysis of rDNA sequences. The RFLP comparison of type and Krasnodar populations of *S. bicornutum* revealed marked differences only in Cfo I profiles. When type culture of *S. bicornutum* is characterized with the profile of two bands (i. e. one restriction site for compared rDNA sequence), the nematodes from Krasnodar population are characterized by three bands in Cfo I profile (fig. 2). The alignement of partial rDNA sequences for both populations demonstrate molecular basis of observed RFLP differences: two restriction sites for Cfo I were found in the sequence of Krasnodar population, when only single Cfo I restriction site for type Yugoslavian culture (fig. 3).

Discussion

The data on morphometry of these two *S. bicornutum* isolates are presented in the table 1. The values for type specimens of the species were obtained from original description (Tallosi et al., 1995). The mean value for spicule length is higher in Krasnodar specimens than in Yugoslavian ones, though gubernaculum length ranges are quite close for these two isolates. The ranges for spicule length are not overlapping in second generation males of both isolates. The mean length of infective juveniles from Krasnodar was found to be lower than in type isolate (701 vs. 770), though the data for *S. bicornutum* infective juveniles reported by D. Sturhan (personal communication) for juvenile body length — 717(608–873) — are closer to those of Krasnodar isolate.

The restriction analysis of ribosomal DNA sequences of both *S. bicornutum* populations revealed difference in the profiles of Cfo I endonuclease. The comparison of

Table 1. Comparative morphometrics of *Steinernema bicornutum* of type culture and Krasnodar isolate, Russia, mean value ± standard deviation (range)

Таблица 1. Сравнительные данные по морфометрии типовой культуры и краснодарского изолята *S. bicornutum*

Character	Type specimens from Strazilovo, Vojvodina, Yugoslavia (Tallosi, Peters, Ehlers, 1995)	Specimens collected near Plant Protection Institute, Krasnodar, Russia
Body length of infective juvenile	770 ± 52 (648–873)	701 ± 94 (548–840)
Oesophagus length of infective juveniles	124 ± 6 (113–135)	129 ± 8 (112–145)
Body length of first generation males	1353 ± 150 (945–1539)	1530 ± 97 (1418–1675)
Distance between anterior end and excretory pore of first generation males	82 ± 8 (68–98)	90 ± 15 (62–110)
Spicule length of first generation males	65 ± 4.3 (53–70)	84 ± 9.6 (66–94)
Gubernaculum length of first generation males	48 ± 3.5 (38–50)	44 ± 1.9 (40–46)
Spicule length of second generation males	51 ± 1.5 (48–53)	71 ± 9.7 (59–82)
Gubernaculum length of second generation males	33 ± 2.9 (25–38)	37 ± 2.8 (31–40)

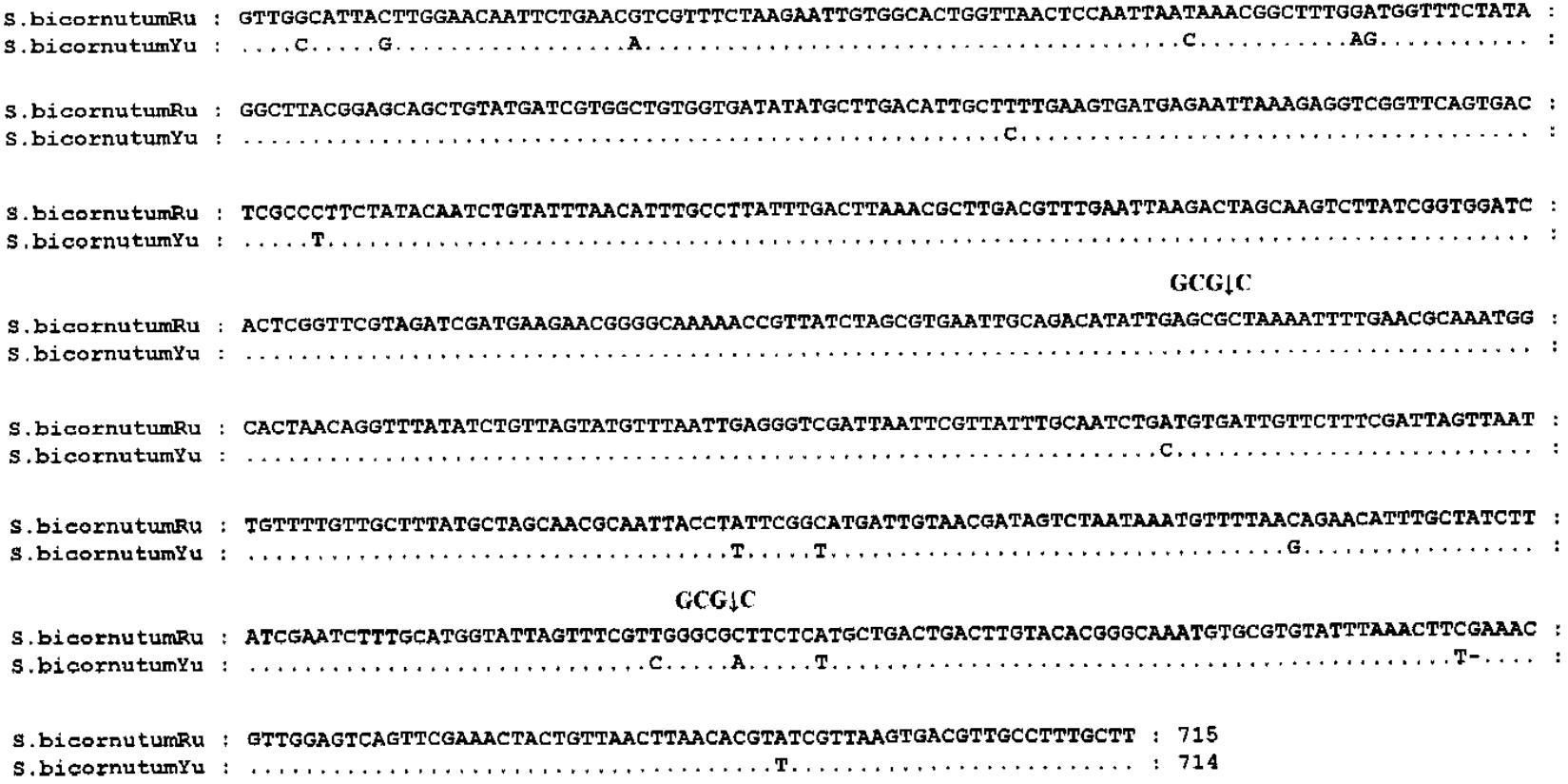


Fig. 3. Alignement of ribosomal DNA partial sequence of *Steinernema bicornutum* from Krasnodar with similar sequence of *S. bicornutum* of Yugoslavian culture. *S. bicornutum* Yu — sequence for type Yugoslavian culture, *S. bicornutum* Ru — sequence for Krasnodar isolate. Cfo I restriction sites indicated.

Рис. 3. Выравнивание неполных последовательностей рибосомальной ДНК краснодарского изолята и типовой культуры *S. bicornutum* из Югославии. *S. bicornutum* Yu — последовательность типовой культуры (Югославия), *S. bicornutum* Ru — последовательность краснодарского изолята. Сайты рестрикции для Cfo I обозначены стрелками.

715 bp long sequence of rDNA of Krasnodar isolate with the similar sequence of Yugoslavian *S. bicornutum* (Nguyen et al., 2001) revealed the molecular basis for observed RFLP difference: because of the single nucleotide C-A substitution (fig. 3) the nematodes of type culture lack second Cfo I restriction site. Totally differences in 18 pairs of nucleotides were found between these two isolates including 17 substitutions and one indel in Yugoslavian strain. Thus, 2.2% of nucleotides from this 715 bp part of ribosomal DNA are different in the isolates under comparison. We have found the difference in 2.4% of rDNA nucleotides between the populations of another steinernematid species — *S. feltiae*. The fertile crosses between the steinernematid populations with such level of nucelotide differences were obtained (own unpublished observations). Much higher differences (more than in 15% of nucleotides) were found between *S. bicornutum* and the closest species of the genus — *S. ceratophorum*, which was described from China (Jian et al., 1997; A. Reid, personal communication, unpublished). The absence of clear morphological differences between studied isolates persuades us to consider these as conspecific. This finding is the first report of *S. bicornutum* for the territory of former USSR. Our results indicate that European *S. bicornutum* is splad at least up to northern slope of the Caucasus. Infective juveniles of *S. bicornutum* from Krasnodar were found to be highly invasive for *Galleria mellonella* caterpillars. Both these nematodes and their symbiotic bacteria represent promising object for the research in biocontrol.

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