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Redescription of *Steinernema arenarium* (Artyukhovsky, 1967) topotypes from Central Russia and a proposal for *S. anomalae* (Kozodoi, 1984) as a junior synonym

Anatolii K. Artyukhovsky*, Eugene M. Kozodoi, Alex P. Reid*** and Sergei E. Spiridonov******

***Department of Nature Conservation, Voronezh Institute of Forestry, Voronezh, 394012, Russia,**
****Nematological Laboratory of the State Quarantine Service, Bet-Dagan, PO78, 50250, Israel,**
*****International Institute of Parasitology, Hatfield Road 395A, St. Albans, Herts, AL4 0XU, UK,**
******Institute of Parasitology of Russian Academy of Sciences, Leninskii Prospect 33, 117071, Moscow, Russia,**
e-mail: spiridon@rjnem.msk.ru

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Summary. A redescription of *Steinernema arenarium* (Artyukhovsky, 1967) Poinar, 1990 is presented based on topotypes collected from the type locality in the Voronezh region, Central Russia. The synonymization of *S. anomalae* (Kozodoi, 1984) Curran, 1989, originally described from nematodes collected in the Ryazan region, Central Russia, with *S. arenarium* is proposed based on morphometrics, protein electrophoresis and DNA analysis.
Key words: entomopathogenic nematodes, *Steinernema arenarium*, *S. anomalae*, synonymization, Central Russia, redescription.

The number of described species of the family Steinernematidae has increased during the last decade. Also, many strains of steinernematids are referred to in literature by "laboratory" names or codes, even when they probably represent new species. Thus, a further proliferation in the number of steinernematid species being described can be predicted. The specific status of several steinernematid species described during the initial stages of steinernematid research remain unresolved with the absence of laboratory cultures of such species being an impediment for their classification, especially as modern steinernematid taxonomy is mainly based on molecular techniques. Recently, we collected specimens of the species *Steinernema arenarium* (described as *Neoaplectana arenaria* almost thirty years ago) from the type locality and here we use these topotypes to prepare a redescription of the species and include molecular taxonomic data. From comparison of taxonomic data collected from these specimens with similar data obtained from specimens of *S. anomalae* we conclude that *S. anomalae* should be considered a junior synonym of *S. arenarium*.

MATERIAL AND METHODS

Reisolation. Eleven soil samples of 1-2 kg were collected by two of us (A.K.A., S.E.S.) from the type locality of *Steinernema arenarium*. The soil was transported in plastic bags to the Voronezh Institute of Forestry where aliquots of approximately 200 cm^3 from each bag were used for nematode extraction by a flotation-sieving method (Spiridonov & Voronov, 1995). Slender juveniles, 700-800 μm long, were extracted from nine samples and from two of these nine samples much larger, 1 mm long, and comparatively thick juveniles were also recovered. Further soil samples of 3-4 kg were collected from the two sampling sites from which the larger juveniles had been recovered. These samples were transported to the Institute of Parasitology, Russian Academy of Sciences, Moscow. The short, slender steinernematid juveniles recovered from *Galleria* bait traps were identified as *S. feltiae*. The larger, thicker juveniles were identified as *S. arenarium* based on comparison of the new specimens with the description of *Neoaplectana arenaria* Artyukhovsky, 1967.

Slide preparation. Last instar caterpillars of *Galleria mellonella* were infected with 10-30 *S. arenarium* IJs and adult nematodes were collected during dissection of the caterpillars 3-4 days later. Adult nematodes without eggs or progeny in the gonadal tubes were not measured. Specimens were fixed in hot (70-80 °C) TAF, dehydrated and transferred to glycerine by Seinhorst standard technique (1959) and mounted on glass slides.

Protein disc-electrophoresis. Homogenates of invasive juveniles were prepared from filtered infective juvenile suspensions. The sediment was frozen and then crushed by spatula five times repetitively. Supernatant was collected after centrifugation under 3 °C (20000 g, 30 min) and diluted 1:1 with 40% sucrose solution with 0.01% Bromthymol Blue (Osterman, 1981). Davis's system of buffer solutions for electrophoresis was used, apart from cathode buffer (1% TRIS, 0.5% Boric acid and 0.1% EDTA). Electrophoresis was conducted at 200 V with 80 mA current and was terminated 1 hr after the exit of the leading dye from the gel. The malate dehydrogenase, acid phosphatase, leucine aminopeptidase and esterase were stained in gels according to Korochkin *et al.* (1977). The laboratory culture of *Steinernema glaseri* from North Carolina, USA, used for comparison, was supplied by Dr. R.J. Akhurst, CSIRO, Australia.

DNA isolation. Total genomic DNA was isolated as described by Reid & Hominick (1992). DNA purified by this method was used to produce RFLP profiles for the *S. anomalae*. The RFLP profiles of the *S. arenarium* isolate were obtained from adult female lysates (Joyce *et al.*, 1994).

PCR primers. Primers for the ITS region were those described by Vrain *et al.* (1992). Primers were synthesized by Pharmacia Biotech.

PCR amplification. Reaction and cycling conditions were identical for both the purified DNA and nematodes lysate reactions. Amplifications were carried out in a volume of 100 µl, containing 50 mM KCl, 10 mM TRIS (pH=9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.5 M of each primer, 100 ng of purified DNA (or 5 µl of nematode lysate) and 8 units of *Taq* polymerase (Promega Corporation). Amplifications were carried out using a PHC-3 thermocycler with a heated lid. Samples were placed in the thermocycler (preheated to 95 °C) and incubated at 94 °C for 2 min followed by 40 cycles of 94 °C for 30 seconds, 45 °C for 1 min and 72 °C for 1 min 30 seconds. A final step of 5 min at 72 °C was included to ensure that all of the final amplification products were full length. Amplified products were immediately digested with a range of restriction endonucleases.

Restriction digestion and electrophoresis of PCR products. Restriction enzymes were purchased from Amersham International or Promega and used with the manufacturer buffers. All digestions were carried out using 4 µl of amplified product at 37 °C for a minimum of 12 hours. The resulting fragments were separated on 1.5% (w/v) agarose gels in TBE at 5V/cm for 3 hours. Fragments were visualized by ethidium bromide staining (Maniatis *et al.*, 1989). Each sample was digested with the following enzymes *Alu*I, *Bst*OI, *Dde*I, *Eco*RI, *Hae*III, *Hha*I, *Hind*III, *Hinf*I, *Hpa*II, *Kpn*I, *Pst*I, *Pvu*II, *Rsa*I, *Sal*I, *Sau*3AI, *Sau*96I, *Xba*I.

RESULTS AND DISCUSSION

Redescription of *Steinernema arenarium* (Artyukhovsky, 1967) (Fig. 1)

Measurements. Table 1.

Males. Body of yellowish colour. Rounded anterior end with six labial and four prominent cephalic sensillae (Fig. 1G). Stoma bowl-shaped with 1-1.5 µm thick ring (protostome ?) on the border with body surface cuticle and 3 µm long narrowing tube behind anterior ring (mesostome and/or metastome). Telostome about 10 µm long. Oesophagus with muscular fibers, especially prominent in metacorpal widening and in bulb. Slightly cuticularized valves discernible in bulb. Cardia 5-7 µm long. Excretory pore 2 µm wide. Deirids visible, 2-3 µm high with 4-6 µm wide base, situated 80-90 µm posterior to the bulb. Spicules of reddish-brown colour. Distal end of spicula usually with swollen tip having a small pore on the anterior surface of the spicular body. Median precloacal papillae, 4-5 µm high with 8-9 µm dia. base. Two pairs of papillae situated close to tail terminus ventrally, and one pair dorsally. Two pairs of submedian papillae situated near cloaca; one additional pair laterally at the same level. Five pairs of precloacal papillae situated in two oblique rows; with anteriormost pair nearly lateral and posteriormost pair nearly ventral in position.

Females. Body with yellowish intestine and colourless gonads. Anterior end broadly rounded with six pointed labial papillae and four rounded cephalic papillae. In stoma walls an anterior cuticular ring 1.5 µm thick can be distinguished on the border with the body surface cuticle joined posteriorly with narrowing 2-3 µm long cuticularized tube. Prominent metacorpal swelling of oesophagus. Thin valves present in bulb as a plate with undulating surface. Excretory channel close to pore 3 µm wide. Anterior branch of

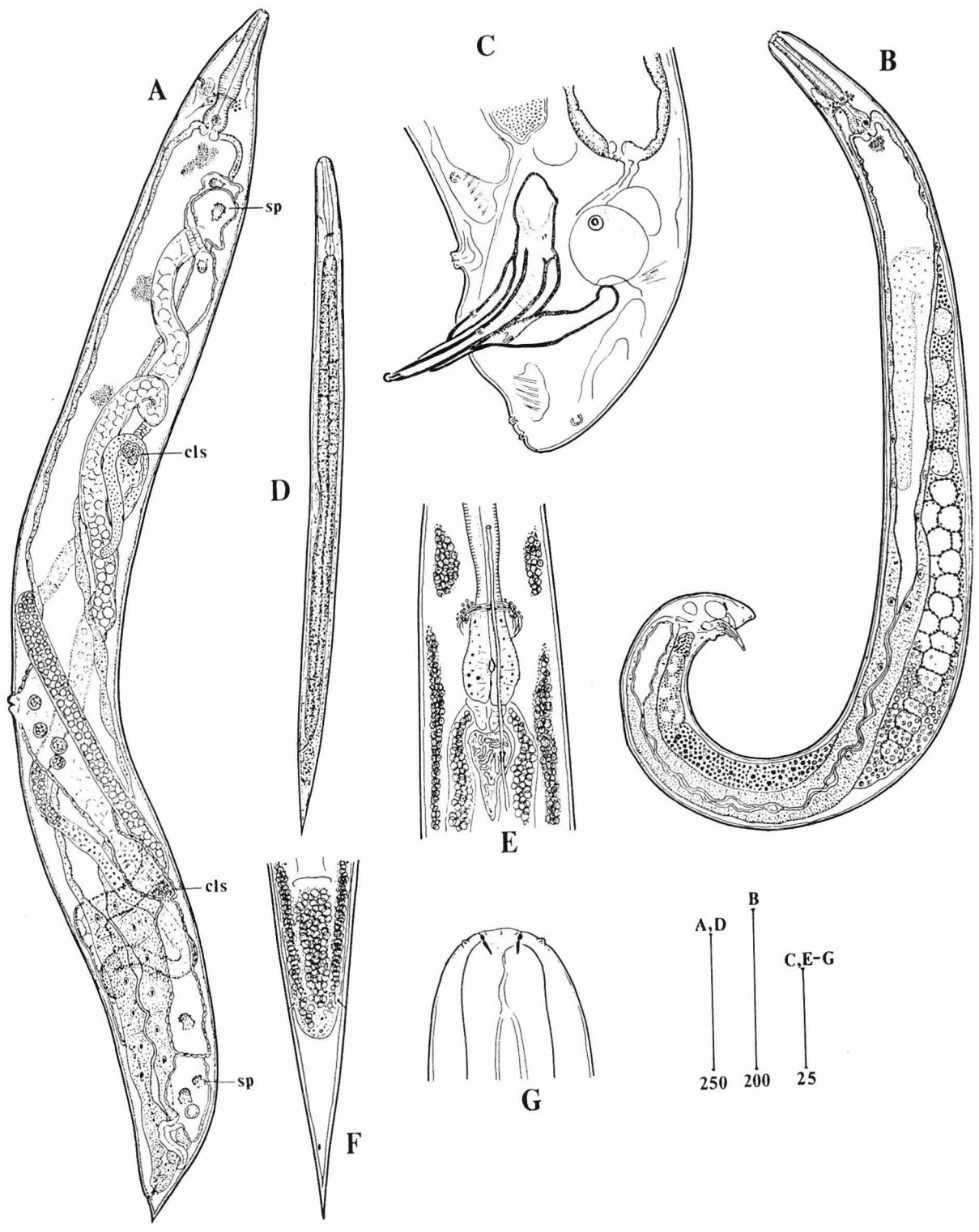

Fig. 1. Morphology of *Steinernema arenarium* (Artyukhovsky, 1967). A: Female, lateral view, distribution of gonad tubes; sp - spermatozoa, cls - clusters of immature spermatozoa; B: Male, lateral view; C: Male tail, lateral view; D: Infective juvenile, lateral view; E: Infective juvenile, oesophageal bulb and vesicle, ventral view; F: Infective juvenile tail, lateral view; G: Male stoma, lateral view. Bars in μm.

Table 1. Measurements (in μm) and ratios of *Steinernema arenarium*.

	Males	Females	Juveniles
n	50	50	50
Length	1845±236 (1280-2320)	5807±1682 (2600-9720)	1217±105 (930-1580)
Greatest width	117±23 (70-165)	279±52 (170-400)	37±2.8 (31-44)
Oesophagus length	168±15 (125-209)	228±23 (180-280)	156±9.3 (132-187)
Tail length	32±6.6 (14-40)	68±14.3 (40-105)	81±4.5 (65-95)
Anterior end to testis flexure	441±116 (230-690)	–	
Spicula	76±6.6 (63-93)	–	
Gubernaculum	53±4.1 (45-63)	–	
A	16.0±2.6 (12.1-23.4)	21.0±5.7 (9.7-38.9)	33.3±3.5 (24.7-39.6)
B	10.9±1.5 (8.5-15.5)	25.2±5.9 (11.8-37.4)	7.8±0.6 (5.9-9.2)
C	60.1±15.6 (38.4-126.7)	87.2±23.7 (39.4-140.9)	15.1±1.2 (12.2-17.9)
D%	78±10 (52-96)	72±0.07 (47-98)	63±0.08 (53-68)
V%	–	50±2.2 (48-60)	
H%	–	–	41±0.05 (23-57)

gonad tube in some specimens with few eggs in the uteri (Fig. 1A) situated mainly in the dorsal and lateral sectors of the body; the posterior branch having more loops and passing through all sectors of the body. Uteri of females in two parts: that nearest the vulva having walls consisting of swollen cells with two-three nuclei, and the postovarial part having thinner walls. In young females these parts of the uterus are divided by a constriction. Rounded tail terminus usually without mucron though a 2-3 μm long spike is present on the tail of a quarter of all females, and two such spikes were observed in one female.

Infective juveniles. Comparatively long and thick juveniles, with lateral field of 8 equal bands (9 lines). Anterior end rounded, occasionally with slightly offset cephalic capsule, 12-14 μm dia. Two refractive rings present in stoma walls. Excretory channel 1 μm wide close to pore. Refractive tube 3 μm long connected with the excretory channel behind the bulbus. Tail conical, with spherical or irregular border between hypodermal and hyaline parts.

Features of live *S. arenarium*. In the postovarial part of the uterus large moving spermatozoa can be seen. Immobile cells with coarse surface (probably spermatocytes) can be seen close to the constriction both in the anterior and posterior branches of young female gonad tubes. Structures resembling bacterial vesicles can be seen behind the cardia in some juveniles as a transparent sack-like body with numerous folds inside. No separate bacterial cells can be distinguished inside it.

Neotype deposition. According to the International Code of Zoological Nomenclature (ICZN, 1985) the designation of a neotype can be justified when specific conditions have been fulfilled. In our opinion a neotype of *Steinernema arenarium* (Artyukhovsky, 1967) should be designated because: all the type slides have been lost (ICZN, 75a); the first author of the present publication is the author of the original description and confirms that the isolation of the new culture of *S. arenarium* was from the type locality (condition 5, ICZN, 75d). Only *S. arenarium, S. feltiae* and a clearly different *Heterorhabditis* sp. were recovered from the type locality of *Neoaplectana arenaria*. The topotypes of *S. arenarium* are identical in their morphology to the information of the original type specimens contained in the drawings and photographs, still stored in Voronezh by the first author (condition 4, ICZN, 75d). Thus, a neotype male of *S. arenarium* (Artyukhovsky, 1967) has been deposited in the collection of the Zoological Museum of Moscow State University (slide n° Ic-396).

Position of *S. arenarium* in the genus *Steinernema*. *S. arenarium* was originally distinguished from *S. glaseri* by the absence of a hook-like structure on the distal end of the spicule and from other species by the shape of the spicules, and the tail ends of males and females. The length of infective juveniles reported in original description (1143-1288 μm) indicates that *S. arenarium* belongs to the group of steinernematids with long juveniles (Nguyen & Smart, 1996). Another species with long juveniles – *S. anomalae* (Kozodoi, 1984) was described from Central Russia, but a comparison with *S. arenarium* was not given in the differential diagnosis of *S. anomalae*. The comparison of the descriptions of these two species, both from Central Russia, reveals several features in common: size of adults and juveniles, shape of spicules and a short period of

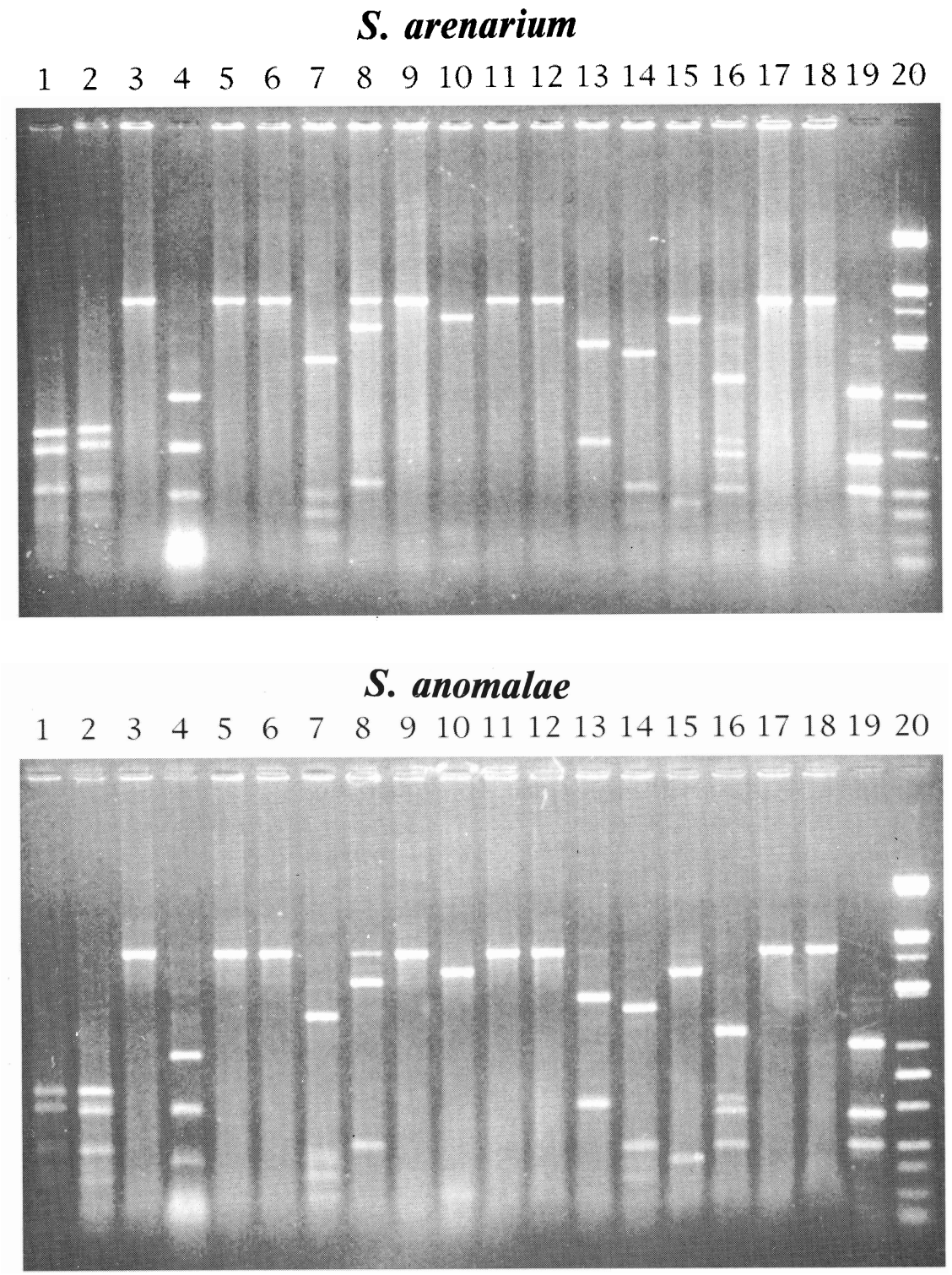

Fig. 2. PCR amplified products from the internal transcribed spacer (ITS) region digested with 17 restriction enzymes. Fragments were separated on ethidium bromide stained 1.5% (w/v) agarose gels. In each gel lanes 2-18 are individual digests of the respective species for that gel with the following restriction enzymes; 2, *Alu*I; 3, *Bst*OI; 4, *Dde*I; 5, *Eco*RI; 6, *Hae*III; 7, *Hha*I; 8, *Hind*III; 9, *Hinf*I; 10, *Hpa*II; 11, *Kpn*I; 12, *Pst*I; 13, *Pvu*II; 14, RsaI; 15, *Sal*I; 16, *Sau*3AI; 17, *Sau*96I; 18, *Xba*I. Lane 1 is an *Alu*I digest of *S. anomalae* in the *S. arenarium* gel and *vice versa.* Lane **19** is an *Alu*I digest of *S. feltiae* for comparative purposes. Lane 20 is the molecular weight marker with band sizes of 2,000, 1,200, 1,000, 800, 750, 500, 400, 300, 200, 150, 100, 100 and 50 base pairs.

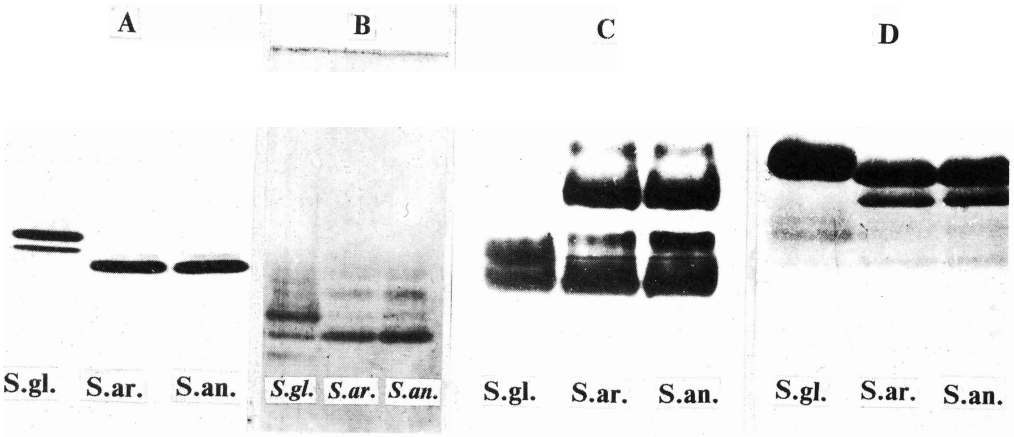

Fig. 3. Disc-electrophoresis patterns of *S. arenarium* culture from the type locality, *S. anomalae* type culture and *S. glaseri* from USA. A: Esterases, B: Leucine aminopeptidases; C: Malate dehydrogenases; D: Acid phosphatases. S. ar. – reisolated culture of *S. arenarium*; S. an. – type culture of *S. anomalae*, S. gl. – culture of *S. glaseri* from USA.

development in the insect cadaver. The culture of *S. arenarium* reisolated in Voronezh was compared with the type culture of *S. anomalae* from the Rjazan region. Comparison between the spectra of four enzymes (Fig. 3) in gels obtained from these two cultures demonstrated the similarity of *S. arenarium* with *S. anomalae*. Also, the spectra of enzymes of *S. glaseri* is substantially different from both these cultures (Fig. 3). Further evidence of *S. arenarium* and *S. anomalae* conspecificity was obtained by DNA analysis with the *S. anomalae* and *S. arenarium* samples yielding identical RFLP profiles with 16 of the 17 restriction enzymes tested (Fig. 2). The only exception was *Alu*I which yielded a minor additional band in the *S. arenarium* sample, a feature also found in different geographical isolates of *S. kraussei* (Reid *et al.*, 1997). The level of variation found between the two cultures in this study is indicative of intraspecific population variation. These results support the opinion of Poinar (1990) that *S. arenarium* may be a possible senior synonym of *S. anomalae,* and thus we propose that *S. anomalae* be considered a junior synonym of *S. arenarium*.

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Артюховский А. К., Козодой Е. М., Рейд А. П., Спиридонов С. Э. Переописание топотипов *Steinernema arenarium* (Artyukhovsky, 1967) из Центральной России и сведение в младший синоним *S. anomalae* (Kozodoi, 1984).

Резюме. Переописание *Steinernema arenarium* (Artyukhovsky, 1967) сделано на основании топотипов, выделенных в типовом месте обнаружения в Воронежской области. Предлагается свести в младший синоним *S. arenarium* вид *S. anomalae* (Kozodoi, 1984), первоначально описанный из Рязанской области. Идентичность этих видов обосновывается данными морфологии, электрофореза белков и анализа ДНК.