

# Entomoparasitic nematodes of the genus *Skarbilovinema*: *S. laumondi* and *S. lyoni* (Nematoda: Tylenchida), parasites of the flies of the family Syrphidae (Diptera), with phylogeny of the suborder Hexatylinea

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**Summary.** Morphological description of two entomoparasitic nematodes, *Skarbilovinema laumondi* and *S. lyoni*, parasitising flies from the genus *Helophilus* and *Eristalis* of the family Syrphidae (Diptera) are given. The life cycle of these nematodes is also presented. Phylogenetic analysis using the D2-D3 expansion fragments of the 28S rRNA gene and partial 18S rRNA gene sequences showed that *Skarbilovinema* has a sister relationships with *Fergusoria* sp. Sequence analysis revealed an anomalous mode of evolution for the ribosomal RNA genes in *Skarbilovinema*, which have long insertions, strong A-T nucleotide content bias and a high mutation rate. Difference in the D2-D3 expansion segments of 28S rRNA gene sequences between *S. laumondi* and *S. lyoni* was 23%, which is higher than usual sequence variation for nematode species belonging to same genus. Several long insertions make 18S rRNA gene and the D2-D3 of 28S rRNA gene the longest ribosomal rRNA genes in nematodes.

**Key words:** 18S rRNA, 28S rRNA, *Eristalis*, *Helophilus*, life cycle, morphology, phylogeny.

In 1991 a new genus of entomoparasitic nematode *Skarbilovinema* (Tylenchida: Iotonchiidae) with two species: *S. laumondi* and *S. lyoni* parasitising hoverflies of the genera *Helophilus* and *Eristalis* (Syrphidae) was described by Chizhov and Zakharenkova (1991) and Zakharenkova and Chizhov (1991). These authors also studied the life cycle and biological peculiarities of these species. After analyses of the ultrastructure of the body wall of parasitic and infective females of *S. laumondi*, Subbotin *et al.* (1993) found that the parasitic female is covered with numerous interwoven microvilli uptaking nutrients from the haemocoel of the host insects. The study of phylogenetic relationships of *S. lyoni* with other tylenchids using the D2-D3 expansion fragments of 28S rRNA gene sequences showed that *Skarbilovinema* was closely related to entomoparasitic nematode from the genus *Wachitylenchus*. In this paper we provide

additional data on morphology and phylogenetic positions of *Skarbilovinema* species within the suborder Hexatylinea.

## MATERIALS AND METHODS

**Nematode samples.** Entomoparasitic nematodes of the genus *Skarbilovinema* were collected for morphological and molecular studies from the flies of the family Syrphidae (Fig. 1) in the European part of Russia: Moscow and Yaroslavl regions, and foothills of the North Caucasus in the Stavropol Territory. Several other entomoparasitic nematode species were collected for molecular study: *Howardula phylotretae* from flea beetles *Phyllotreta* spp., *Howardula* sp, from an unidentified beetle and *Allantonema mirabile* from *Eylohius* sp. in Moscow region, Russia. Several unidentified hexatylin nematodes collected from soil samples in California and Florida, USA were also included in the phylogenetic analyses.

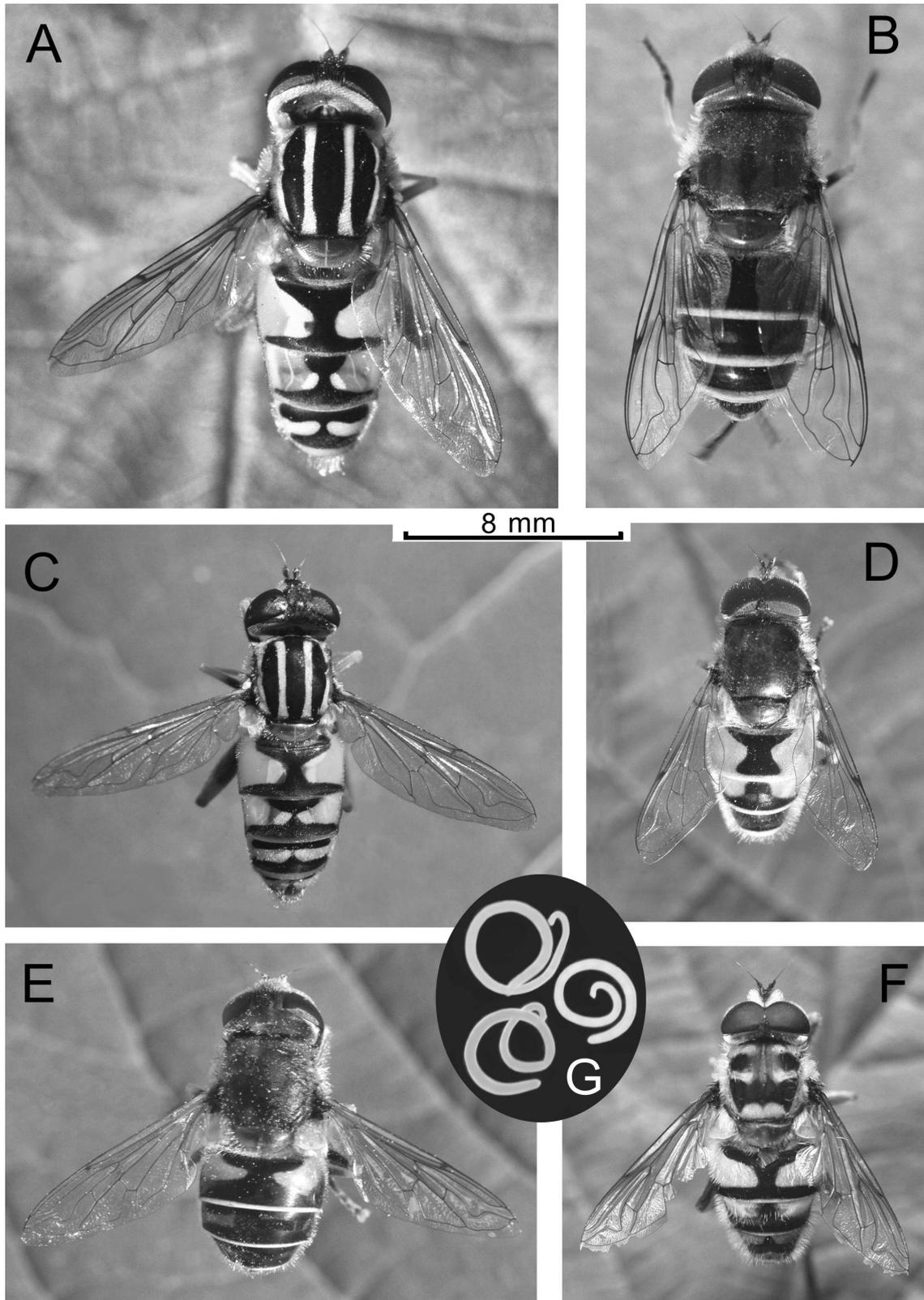
**Morphological and biological studies.** *Skarbilovinema* nematodes were extracted from the flies and initially fixed with the 4% solution of TAF under conditions of heating up to 55 °C. Nematodes were processed in glycerol and embedded on permanent slide using standard methods. Drawings and measurements of nematodes were made from permanent slides employing a Carl Zeiss microscope with Axio Image AI objective. Both immobilized and live nematodes were photographed. The life cycle of *Skarbilovinema* was

studied in a natural infected area, where flies were collected and dissected every 10-14 days during spring, summer and autumn.

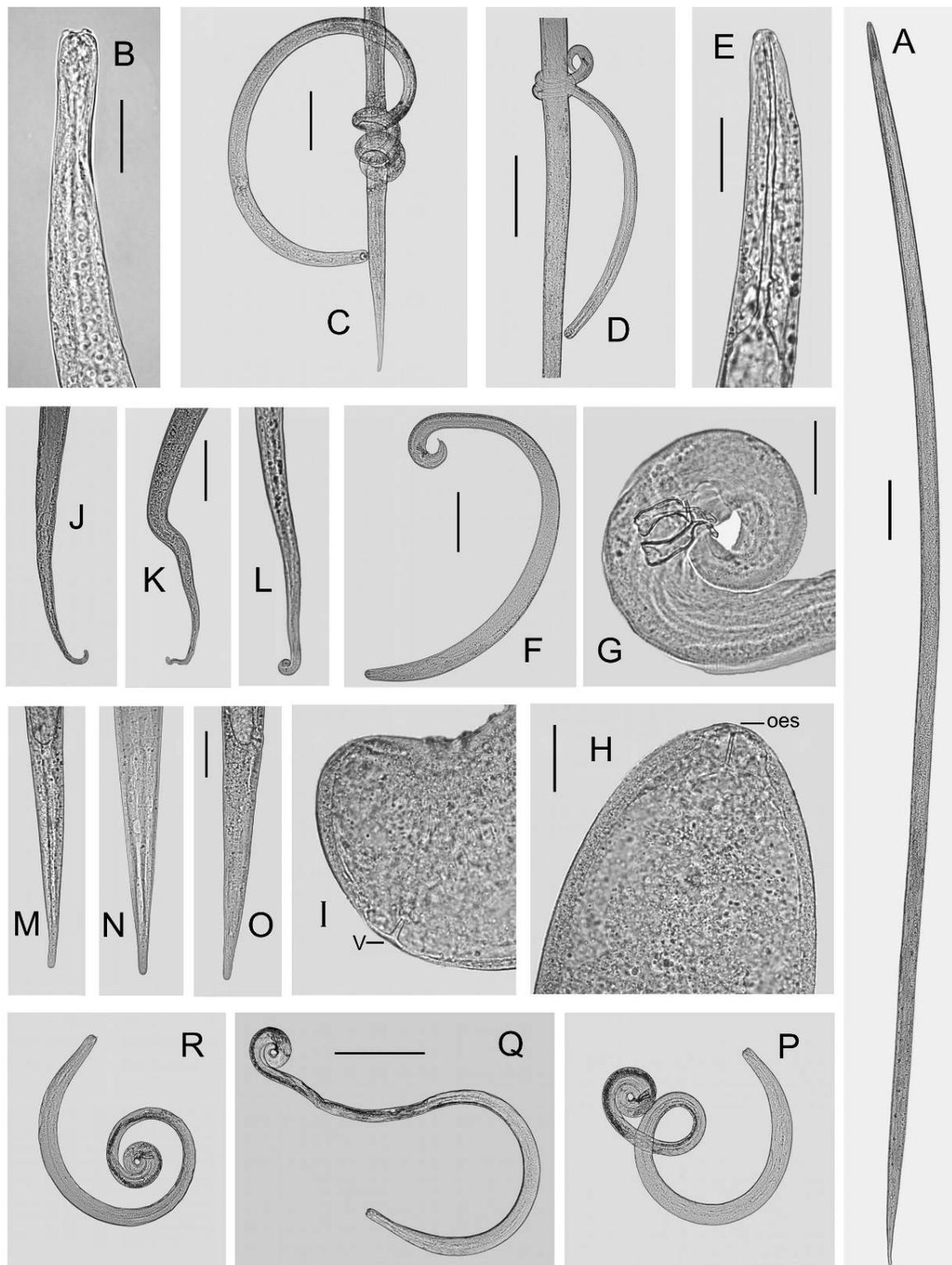
**DNA extraction, PCR, cloning and sequencing.** Nematode DNA was extracted from several individuals using proteinase K following protocols for DNA extraction and PCR as described by Tanha Maafi *et al.* (2003). The forward D2A (5'-ACA AGT ACC GTG AGG GAA AG TTG-3') or forward D2Tyl (5'-GAG AGA GTT AAA NAG BAC GTG A -3') primers and reverse D3B (5' -

**Table 1.** Morphometrics of *Skarbilovinema laumondi* и *S. lyoni*; all sizes are in  $\mu\text{m}$  and in the form: range (mean and standard error).

Stage	Infective female of <i>S. laumondi</i>	Infective female of <i>S. lyoni</i>	Parasitic female of <i>S. laumondi</i>	Parasitic female of <i>S. lyoni</i>	Male of <i>S. laumondi</i>	Male of <i>S. lyoni</i>	4th stage female juvenile of <i>S. laumondi</i>	4th stage female juvenile of <i>S. lyoni</i>
Characters								
n	25	23	15	15	20	20	26	25
L	2870-3340 (3160±97)	2090-2630 (2350±114)	3000-15850 (8150±1470)	1380-10530 (6040±1205)	1080-1300 (1170±45)	689-1040 (870±78)	1770-2510 (2040±165)	1150-1710 (1470±140)
a	72.0-103.8 (83.7±7.3)	61.7-76.4 (64.1±2.1)	8.8-39.0 (25.3±6.8)	7.2-30.6 (14.3±5.2)	28.1-46.4 (36.2±4.1)	36.1-60.0 (48.7±5.6)	46.4-67.3 (55.1±4.8)	25.5-45.7 (38.6±5.0)
b	2.8-4.0 (3.5±0.3)	3.0-3.7 (3.4±0.2)	–	–	–	–	2.4-3.8 (3.3±0.3)	2.5-3.4 (3.0±0.2)
c	17.0-23.4 (20.9±1.1)	16.6-20.5 (18.5±0.7)	–	–	11.1-16.2 (13.5±0.6)	12.0-16.1 (14.0±0.5)	13.7-18.6 (15.3±0.3)	7.2-11.5 (9.4±0.5)
Body width	32-41 (38±1.4)	34-41 (37±1.2)	275-450 (327±33.8)	110-470 (230±74.4)	25-42 (33±3.8)	16-21 (18±1.0)	32-43 (37±2.8)	34-42 (38±2.1)
Tail length	135-175 (151±8.8)	113-141 (127±7.0)	62-125 (93±12.4)	30-105 (70±16.9)	73-106 (87±6.2)	58-78 (65±4.1)	99-175 (135±17.6)	130-188 (155±12.9)
V%	91-93 (92±0.2)	91-93 (92±0.2)	96-99 (98±0.6)	95-99 (97±0.8)	–	–	89-93	85-95
Stylet	22-28 (25±1.1)	19-25 (22±1.3)	–	–	–	–	–	–
Oesophageal lumen	74-90 (83±3.5)	70-85 (80±3.2)	–	–	–	–	–	–
Anterior end to excr. pore	246-293 (265±8.8)	220-265 (240±10.5)	–	–	168-204 (186±7.9)	126-176 (151±9.6)	178-222 (190±8.4)	143-210 (177±12.7)
Uterus length	690-1083 (886.5±78.2)	550-850 (730±65.5)	–	–	–	–	–	–
Vulva-anus distance	58-88 (72±5.5)	37-56 (48±4.8)	62-188 (135±26.0)	30-130 (78±22.6)	–	–	–	–
Spicules	–	–	–	–	25-34 (29±1.4)	16-21 (19±0.8)	–	–



**Fig. 1.** Insect-hosts of nematodes of the genus *Skarbilovinema*. A: *Helophilus pendulus*; B: *Eristalis tenax*; C: *Helophilus* sp.; D: *Eristalis arbustorum*; E: *Eristalis horticola*; F: *Myathropa flaræ*; G: Parasitic female adults of *S. laumondi*.



**Fig. 2.** *Scarbilovinema*. A: Free-living infective female; B: Anterior end of male; C-D: Copulation; E: Anterior end of infective female; F: male third- to fourth-stage juvenile; G: Posterior end of male; H: Anterior end of young parasitic female; I: Posterior end of young parasitic female; J-L: Tails of female fourth-stage juvenile of *S. lyoni*; M-O: Tails of female fourth-stage juvenile of *S. laumondi*; P-R: Males. Scale bars: A, R, Q, P – 120 µm; B, G – 35 µm; C – 100 µm; D – 110 µm; E – 40 µm; F – 85; H, I – 25 µm; J-O – 80 µm.

TCG GAA GGA ACC AGC TAC TA- 3') were used for amplification and sequencing of the D2-D3 expansion fragments of the 28S rRNA gene (Subbotin *et al.*, 2006); the forward G18SU (5' - CGT TGT CTC AAA GAT TAA GCC- 3') and the reverse R18Ty11 (5'- GGT CCA AGA ATT TCA CCT CTC - 3') were used for amplification of the partial 18S rRNA gene fragment (Chizhov *et al.*, 2006). PCR products were purified using QIAquick (Qiagen, USA) gel extraction kits and then cloned using pGEM-T Vector System II kit (Promega, Madison, WI, USA). From each sample either PCR products were directly sequenced, or one or two clones were sequenced employing a DNA sequencer at the University of California, Riverside, Genomics Center. The newly obtained sequences were submitted to the GenBank database under accession numbers as indicated in Figures 5 & 6.

**Sequence and phylogenetic analysis.** The newly obtained sequences of D2-D3 of 28S rRNA and partial 18S rRNA genes were aligned using ClustalX with corresponding gene sequences for selected tylenchids (Perlman *et al.*, 2003; Powers *et al.*, 2005; Subbotin *et al.*, 2006; Ye *et al.*, 2007; Bert *et al.*, 2008; van Megen *et al.*, 2009; Hazir *et al.*, 2010; Poiras *et al.*, unpublished) and representatives of Cephalobidae used as outgroup taxa. Phylogenetic analyses of the datasets were performed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the GTR + I + G model. BI was initiated with a random starting tree and was run with four chains for  $1.0 \times 10^6$  generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log likelihood values of the sample points stabilised after approximately  $10^3$  generations. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) were given on appropriate clades. Sequence differences between samples were calculated with PAUP\* 4b10 (Swofford, 2002) as an absolute distance matrix and the percentage was adjusted for missing data.

## DESCRIPTIONS

### *Skarbilovinema laumondi* Chizhov & Zakharenkova, 1991 (Figs. 2 & 3)

**Measurements.** Table 1.

**Free-living infective female.** Body straight. Cuticle finely annulated with very fine longitudinal striations. Lateral field with four incisures. Two outer incisures in posterior part of body 5-11  $\mu$ m

from each other. Large nuclei of hypoderm, 8-11  $\mu$ m diam., uniformly distributed 25-56  $\mu$ m from each other. Head capsule not sclerotised. Lip region set off, slightly projected. Stylet protractors reduced. Oesophagus cylindrical. Oesophagus lumen almost straight, about 3  $\mu$ m in width and slightly widening towards base. Oesophagus lumen walls sclerotised. Stylet formed by the extension of oesophagus lumen walls. Total length of oesophagus lumen together with stylet is 99-111  $\mu$ m. Stylet without basal knobs. Dorsal oesophageal gland orifice of star-like shape, located 15-25  $\mu$ m from stylet base. Subventral gland orifice opens at base of oesophagus lumen. Probably, there are three oesophageal glands. Only dorsal and one of subventral glands distinctly visible. Subventral gland extends posteriorly to base of dorsal gland. Nuclei of oesophageal glands are not visible. Oesophagus narrows at nerve ring area. Nerve ring located under sharp angle to a longitudinal axis of body. Excretory pore distinctly visible. Hemizonid small, located anteriorly 35-42  $\mu$ m from the excretory pore. Intestine as narrow strip without internal cavity filled with a dark, granulated mass. Intestinal nuclei in a row with uniform intervals between them. Gonad well-developed and consists of ovary and uterus, occupying 60-70% of body length. Ovary straight with ovary apex, which may sometimes extend up to excretory pore. Uterus has an appearance of tube, whose width equals two-thirds of body width, and its length equals about one-third of body length. Uterus usually with short blind appendage up to 30  $\mu$ m long located behind vulva. Uterus often filled with a very fine sperm. Vulva lips are slightly protruded. Anus and rectum not clearly visible. Tail elongated and conical, protruded, with blunt and slightly expanded tip.

**Parasitic female.** After penetrating into a larva of the insect host (rat-tailed maggot), the infective female moults and starts growing rapidly, turning into an adult parasitic female. The moulting female with typical short constriction at junction of ovary with uterus. The constriction divides body of female into two uneven parts: longer and wider anterior part with ovary, and short and slender posterior part. At this stage deformed lumen of oesophagus and stylet are still visible at the anterior end of female body. Oesophageal glands and nerve ring completely reduced. Excretory pore is not observed. Ovary elongates and gets reflexed 1-3 times. Tail is rounded.

Adult parasitic females with sausage-like, usually C-shaped seldom spirally twisted, body. Anterior part of body wider than other parts and is gradually tapering towards tail. Head rounded in old

females, and often trapeze-form in young females. Thickness of hypoderm varies considerably depending on age of females. In young parasitic females hypoderm 5-20  $\mu\text{m}$  thick. In old females hypoderm up to 40  $\mu\text{m}$  thick in the middle part of body and up to 85  $\mu\text{m}$  thick at the anterior end of body. Annulation of cuticle and lateral field not evident. In old females, that are turning yellow in colour, hypoderm forms thick annulation folds along the entire length of body. In younger females the lumen of oesophagus sometimes considerably deformed, and all other structures of oesophagus reduced. Narrow strip of intestine extending along body and filled with a granulated mass containing uniformly distributed large nuclei. Ovary considerably hypertrophied. Anterior part of body of adult females contains parts of former ovary, eggs and juveniles of first and second stages. Posterior part of body and uterus area contain juveniles of third and fourth stages, males, infective females and occasionally copulating individuals. Posterior part of body including vulva usually turned on the dorsal side. Tail terminus bluntly rounded. Anus present. In younger females vulva lips protracted considerably, but in old females turning yellow vulva lips usually flat and do not project above body surface. Viviparous.

**Male.** Body C-shaped and twisted in a spiral in its posterior part, which is slightly expanded close to spicules. Annulation of cuticle not observed. Lateral field with two incisures, 2-3  $\mu\text{m}$  wide. Head area expanded, pin-shaped, 16-24  $\mu\text{m}$  in diam., with an oval internal cavity. Four head papillae located symmetrically and have an appearance of small knobs up to 2  $\mu\text{m}$  in height. Stylet and structures of oesophagus including oesophageal glands reduced. Intestine visually detectable as a narrow strip, which begins at base of internal cavity of head and ends at anus. Nerve ring located at a sharp angle to the longitudinal axis of body. Walls of excretory pore sclerotised. Hemizonid dot-like, located 20-28  $\mu\text{m}$  in front of excretory pore. Testis usually straight, may reach the base of head capsule and occupies up to 90% of body length. Spicules L-shaped with a tubular and straight tips and a short bend at the end and triangular internal protuberances. Spicule base expanded, gubernaculum absent. Tail strongly twisted in the ventral direction and has a stretched out tip 16-24  $\mu\text{m}$  long. Tail terminus blunt. Bursa with a narrow band, 5-7  $\mu\text{m}$  wide, which starts slightly anteriorly to spicules outlet and reaches base of the tip of tail, but without overlapping.

**Fourth-stage female juvenile.** Body straight. Cuticle with thin longitudinal striation. Posterior one-third of body usually finely annulated. Head

capsule expanded, pin-shaped, 16-24  $\mu\text{m}$  in diam., with an oval internal cavity and four head papillae. During the period of moulting it acquires a cylinder shape. Stylet and structures of oesophagus not developed. They start forming during the period of moulting into a free-living infective female. Oesophageal glands well-developed, but their borders are undetectable. Nerve ring clear. Hemizonid located 28-37  $\mu\text{m}$  from excretory pore, closer to the anterior end of body. Distance from undeveloped vulva to anus is 46-59  $\mu\text{m}$ . Gonad about 50% of length of the body. Border between ovary and uterus fuzzy, differentiated by the end of moulting. Tail conical, elongated in shape, with a blunted tip.

**Fourth-stage male juvenile** (n=15): L = 900-1070 (990)  $\mu\text{m}$ ; a = 22.0-31.2 (26.0); c = 11.0-16.0 (13.0); body width = 29-46 (38)  $\mu\text{m}$ ; tail length = 63-86 (76)  $\mu\text{m}$ ; anterior end to excretory pore = 129-187 (156)  $\mu\text{m}$ . Body slightly curved ventrally, expanded considerably in the area of rudiment spicules. Developed spicules frequently observed. Head capsule cylindrical. Stylet absent. Structures of oesophagus and oesophageal glands reduced. Genital tube may extend to excretory pore. Tail terminus blunt.

**Third-stage female juvenile** (n=15): L = 1330-1970 (1570)  $\mu\text{m}$ ; a = 39.1-54.2 (44.6); c = 12.9-17.7 (15.3); body width = 30-39 (35)  $\mu\text{m}$ ; tail length = 84-126 (102)  $\mu\text{m}$ ; anterior end to nerve ring = 135-157 (145)  $\mu\text{m}$ . Head capsule pin shaped, 18-25  $\mu\text{m}$  in diam., with internal cavity and four head papillae. Stylet and structures of oesophagus undeveloped. Oesophageal glands clearly observed and extend downwards to one-third of the body. Gonad occupies approximately similar portion of body length.

**Eggs** (n=25): Located within internal cavity of the parasitic female body. Oval or almost round, 40-68 (55)  $\times$  30-52 (40)  $\mu\text{m}$ .

**Insect hosts:** *Helophilus pendulus*, *H. trivittatus* and *Helophilus* sp. (Syrphidae).

***Skarbilovinema lyoni* Zakharenkova &  
Chizhov, 1991  
(Figs. 2 & 3)**

**Measurements.** Table 1.

**Free-living infective female.** Body straight. Cuticle finely annulated with a very gentle longitudinal striation. Lateral field 5-8  $\mu\text{m}$  wide. Four incisures arranged at equal intervals in the central part of body. Nuclei of hypoderm large, 7-10  $\mu\text{m}$  in diam., uniformly distributed at a distance of 27-51  $\mu\text{m}$ . Head capsule not sclerotised. Lip region slightly set off and projected. Stylet protractor reduced.

Oesophagus cylindrical. Lumen of oesophagus almost straight, about 3  $\mu\text{m}$  wide, slightly extended posteriorly at the base. Walls of the oesophagus lumen sclerotised. Stylet without basal knobs. Walls of stylet thickened, formed by an extension of walls of oesophagus lumen. Total length of oesophagus lumen together with stylet is 90-110  $\mu\text{m}$ . Dorsal oesophageal gland orifice located 15-25  $\mu\text{m}$  away from stylet base. Three oesophageal glands visible. Subventral glands extend backwards beyond base of dorsal gland. Nuclei of the oesophageal glands not evident. Lumen of oesophageal glands very wide, occupies up to 25% of the body width, become gets narrower at the area of nerve ring. Nerve ring located at a sharp angle to the longitudinal axis of the body. Excretory pore distinctly visible. Intestine begins in front of nerve ring at the base of oesophagus. It has an appearance of a narrow stripe without internal cavity, filled with a granulated mass and contains a single row of nuclei spaced at uniform intervals. Genital tube well-developed and differentiated into ovary and uterus occupying 60-70% of the body length. Ovary straight and may reach the excretory pore. Uterus has an appearance of a hollow tube, whose width equals two-thirds of the body, and its length equals to about one-third of the length of body. Short blind appendage up to 30  $\mu\text{m}$  on uterus, located behind vulva. Front part of it filled with very fine sperm. Vulva lips slightly protruded. Anus and rectum poorly visible. Tail elongated, conical, and protruded, with a blunt and slightly expanded tip.

**Parasitic female.** Adult parasitic females immobile. Their body sausage-shaped, usually C-shaped, seldom spirally twisted, widest at the anterior part, gradually narrowing towards the tail. Head rounded in old females, and often with a trapeze-shape projection in young females. Thickness of hypoderm varies from 5 to 70  $\mu\text{m}$  depending on the age of females and body part. Nuclei of hypoderm uniformly distributed. In old, yellowing females hypoderm forms thick annular folds along the entire length of the body. All oesophagus structures usually reduced. Considerably reduced oesophageal lumen may remain in young females. Excretory pore and nerve ring not evident. Intestine looks like a narrow strip along the body. Ovaries considerably hypertrophied, reflexed several times. Anterior part of body of adult females contains eggs and juveniles of first- and second-stages, and posterior part contains third- and fourth-stage juveniles, males and, occasionally, copulating individuals. Posterior part of the body, including vulva usually turned on the dorsal side. Anus obvious. In young females lips of vulva

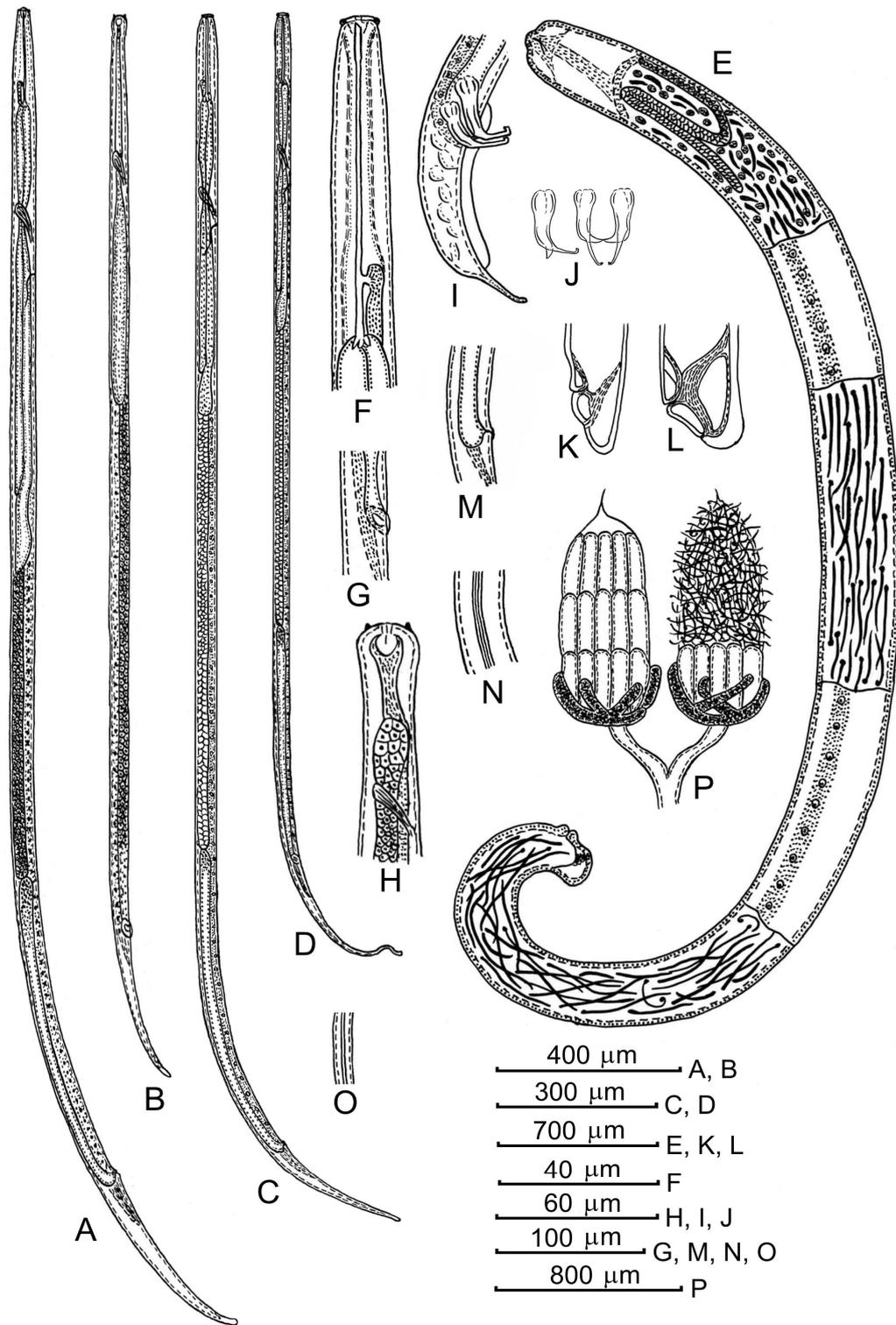
protracted and flat in old females. Tail terminus rounded. Viviparous.

**Male.** Body C-shaped with posterior part twisted in a spiral and slightly expanded in spicule area. Head area pin-shaped, 16-22  $\mu\text{m}$  in diam., with oval internal cavity. Four head papillae arranged symmetrically and have a shape of small knobs up to 2  $\mu\text{m}$  in height. Stylet and structures of the oesophagus reduced. Narrow strip of the middle intestine extends from the base of internal cavity of head to anus. Nerve ring located at a sharp angle to the longitudinal axis of the body. Hemizonid dot-like, located 20-24  $\mu\text{m}$  in front of the excretory pore. Testis usually straight, occupies up to 90% of the length of body. Spicules L-shaped, with an expanded base, hollow straight tips, which end with a short bend, and the internal triangular protuberance on the body of every spicules. Gubernaculum absent. Tail strongly twisted in the ventral direction, with a stretched out tip 16-22  $\mu\text{m}$  long. Tail terminus blunt. Bursa has a shape of a narrow band 5-7  $\mu\text{m}$  wide, which begins slightly anteriorly to spicules and extends to the base of the tip without its enveloping.

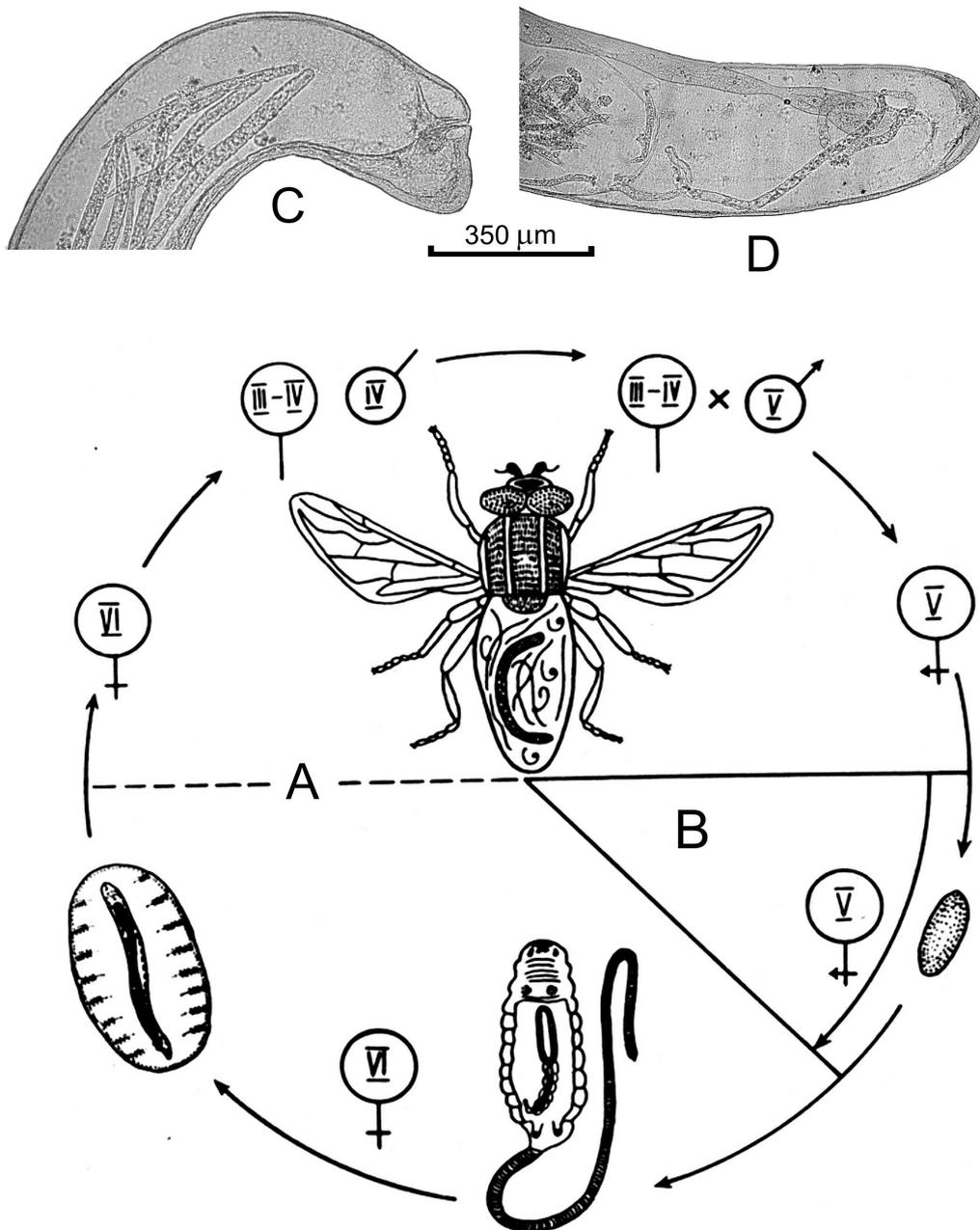
**Fourth-stage female juvenile.** Body straight, with posterior part curved ventrally. Cuticle has a fine longitudinal striation. Posterior one-third of body finely annulated. Head capsule cylindrical. Lip area set off and slightly protuberant. Edges of lumen of oesophagus sclerotised. Lumen 2.0-2.5  $\mu\text{m}$  wide. The orifice of dorsal gland opens 5-7  $\mu\text{m}$  from the base of the lumen of oesophagus. Length of the oesophageal lumen including the stylet is 45-65 (65)  $\mu\text{m}$ . Total length of the oesophagus lumen including oesophageal glands is 440-600  $\mu\text{m}$ . Subventral glands extend downwards beyond dorsal gland. Hemizonid located 20-30  $\mu\text{m}$  from excretory pore towards the anterior end of body. Genital tube occupies about 50% of the body length, differentiated into ovaries and uterus. Uterus hollow and seldom filled with fine sperm (copulation may occur in fourth-stage juveniles). Rudiment of the vulva located 36-58  $\mu\text{m}$  from the anus. Tail considerably elongated, with a thin and curved tip.

**Fourth-stage male juvenile** (n=15): L = 620-750 (700)  $\mu\text{m}$ ; a = 25.9-33.5 (29.4)  $\mu\text{m}$ ; c = 9.3-12.6 (10.8); body width = 22-26 (24)  $\mu\text{m}$ ; tail length = 56-79 (65)  $\mu\text{m}$ ; anterior end to excretory pore = 110-120  $\mu\text{m}$ .

Body slightly curved ventrally, expands considerably in the spicules part. Head capsule cylindrical. Stylet absent. Oesophagus and oesophageal glands degenerated. Testis, filled with spermagonia, reaches excretory pore with its apex. Tail terminus blunt.



**Fig. 3.** *Skarbilovinema*. A: Free-living infective female of *S. laumondi*; B: Female fourth-stage juvenile of *S. laumondi*; C: Free-living infective female of *S. lyoni*; D: Female fourth-stage juvenile of *S. lyoni*; E: Parasitic female of *S. laumondi*; F: Anterior end of infective female; G: Part of female fourth-stage juvenile body at vulva level; H: Anterior end of male; I: Posterior end of male; J: Spicules; K, L: Parasitic female at vulva level; M: Infective female at vulva level; N: Lateral field of infective female in the middle of body; O: Lateral field of infective female in tail; P: Ovaries of *Helophilus* infected by nematodes.



**Fig. 4.** Life-cycle of *Skarbilovinema*. A: Inside host; B: Outside host, in water. C: Posterior end with vulva region of adult parasitic female; D: Anterior end of adult parasitic female.

**Third-stage female juvenile** (n=18): L = 1130-1740 (1240) µm; a = 25.3-33.8 (31.1); body width = 33-56 (41) µm; anterior end to nerve ring = 120-130 µm; anterior end to excretory pore = 134-174 (154) µm.

Body straight. Head capsule pin-shaped, 18-24 µm in diam., with the internal cavity and four head papillae about 2 µm in height. Stylet and oesophagus non-developed. Oesophageal glands clearly observed, occupy up to one third of the body length. Gonads occupy about 30% of the body

length. Tail elongated and conical, with protruded tip.

**Eggs** (n=25): Located within the internal cavity of the parasitic female body, 40-65 (50) × 30-50 (40) µm in size.

**Insect hosts:** *Eristalis tenax*; *E. arbustorum*, *E. horticola* and *Myathropa flora* (Syrphidae).

**Differential diagnosis.** Two *Skarbilovinema* species differ by the following characters: (i) female fourth-stage juvenile of *S. lyoni* has stylet and sclerotised oesophagus, whereas these structures are

absent in *S. laumondi* at the same stage; (ii) female third- and fourth-stage juveniles of *S. lyoni* have a long thin and curved tail terminus, whereas in *S. laumondi* at the same stages the tail terminus is elongate-conical with expanded blunted terminus; (iii) parasitic female and male adults, infective female, and female fourth-stage juvenile of *S. lyoni* have smaller body size than those of *S. laumondi*.

**Biology.** The biological peculiarities of these two species are similar. Both are obligate parasites of flies, belonging to the genera *Helophilus*, *Eristalis* and *Myathropa* (family Syrphidae), the larvae of which develop in the bottom sediments of lentic waters. The life cycle of nematodes is shown in Figure 4. In the Central region of the European part of Russia both species produce only one heterosexual generation with fertilisation inside a host insect. Infection of a new host occurs by free-living infective female, which develops inside abdomen of flies, and escapes out into the external environment via the genital ducts of the host together with fly eggs. Usually it occurs in the locations where flies regularly lay eggs. The infective female is very mobile in humid environment; it actively searches for a host larvae and penetrates it. After penetration, the juvenile moults, begins to grow rapidly and then turns into a parasitic female. By the time of the flight of the host imago, parasitic females acquire the maximum size and, usually, concentrate near the ovaries or at their base. Parasitic females are viviparous. Juveniles of the initial stages develop inside parasitic females. Then, female third- and fourth-stage juveniles and male fourth-stage juveniles of the next generation of the parasite enter the host's body cavity. After moulting to the infective stage, they copulate. Inseminated infective females stay in the abdominal cavity of the host for a long period, sometimes for a month or more. Then, during the egg laying, they move out into the surrounding environment. In Central and Western regions of the European part of Russia the first individuals of infective females in the fly abdomen are observed in early August to the first half of September. In Southern regions they are detected in mid-summer. The possibility of development of two generations per year can not be excluded for these regions. It is possible that they are able to overwinter at the bottom of water bodies near eggs of the host, and infect the young larvae of flies in spring of the succeeding year.

The intensity of invasion varies considerably from 1 to 30 infective females of the parasite per a host individual. Under intense invasion conditions that exceeded eight nematodes per fly, a suppression

of growth of a portion of parasitic females, which penetrated into the host later than others, was observed. Only a certain portion of parasitic females developed to the maximum size. Growth of some of them slowed down so markedly, that only several juveniles could develop. The percentage of infected flies varied usually from 2 to 10%, but in certain areas was as high as 100%. The infestation rate of flies belonging to the genus *Helophilus* was considerably higher than of representatives of the genus *Eristalis*. Male flies seldom became infected, but sometimes the rate of their infection may exceed 50%. The possible infection of females during copulation was not observed. Nematodes of the species *S. laumondi* were never found in flies of the genus *Eristalis* and nematodes of the species *S. lyoni* were never found in flies of the genus *Helophilus*. Egg production of the infested flies decreased approximately by a factor of 2-3, but complete female sterility was never observed. Eggs developing in ovaries of infected flies did not differ in morphology from those developing in healthy specimens. Nematodes did not cause any visually detectable anomalies in morphology and behaviour of infected flies.

**Distribution.** These nematodes were found in the territory of the European part of Russia in Moscow, Vladimir, Voronezh, Nizhniy Novgorod, Vologda, Tver and Yaroslavl regions, and in the foothill zone of North and Western Caucasus. Apparently the distribution area of these nematodes matches with the area of distribution of the host insect and both species occur together in one and the same biocenosis.

#### MOLECULAR CHARACTERISATION OF *SCARBILOVINEMA* AND ITS PHYLOGENETIC RELATIONSHIPS TO OTHER HEXATYLINA

**Partial 18S rRNA gene.** The partial 18S rRNA gene was amplified for *S. lyoni*. Comparison of this sequence with those of other nematodes revealed several long insertions in *S. lyoni* 18S rRNA. The sequence length used for the analysis was 1807 bp and it did not include the first six (1-6) and last two (49 and 50) helices of the 18S RNA structure. Taking in consideration that non-sequenced regions with nearly 250 bp, the total length of 18S rRNA gene of *S. lyoni* could be estimated as 2050 bp, that made it the longest known 18S rRNA for the phylum Nematode. The A-T nucleotide content for *S. lyoni* was 60%.

The 18S rRNA alignment contained 46 taxa including two outgroups and was 1972 bp in length. The BI tree is given in Figure 5. The suborders Hoplolaimina and Criconematina showed a sister

relationship, whereas relationships between other tylenchid suborders were not well resolved. Majority of entomoparasitic nematodes were distributed within two main clades of Hexatyliina and one unidentified entomoparasitic nematode clustered with Anguinoidea. The suborder Hexatyliina was divided into two major clades: (i) *Deladenus* spp., *Howardula* spp., *Bradynema listronotum*, *Skarbilovinema lyoni* and cf. *Gymnotylenchus* sp.; (ii) cf. *Hexatylus* sp., *Deladenus* sp., *Sphaerularia* spp., cf. *Helionema* sp. The relationships of *Skarbilovinema lyoni* with *Fergusoria* sp. and *Howardula* spp. were not resolved.

#### D2-D3 expansion segments of 28S rRNA gene.

The D2-D3 regions of 28S rRNA gene sequences for *S. lyoni* and *S. laumondi* were 817 and 939-941 bp, respectively. They can be considered the longest known fragments for this region for the phylum Nematoda according to our knowledge. Two obtained sequences of *S. laumondi* differed by 3 bp or 0.3%. Differences between *S. laumondi* and *S. lyoni* equalled 189 bp or 23%. The A-T nucleotide content for *S. lyoni* and *S. laumondi* was about 64%.

The partial 28S rRNA alignment contained 44 ingroup and two outgroup taxa and was 1029 bp in length. The BI is presented in Fig. 6. The relationships between tylenchid suborders were not well resolved. Entomoparasitic nematodes were distributed within two main clades of the suborder Hexatyliina and one unidentified entomoparasitic nematode clustered with Anguinoidea. The suborder Hexatyliina was divided into two major clades, one of them with several highly supported subclades. *Skarbilovinema* spp. formed highly supported subclade with *Fergusobia* sp., *Howardula aoronymphium* and *Wachitylenchus bovienii*. *Skarbilovinema* spp. had sister relationships with *Fergusobia* sp.

## DISCUSSION

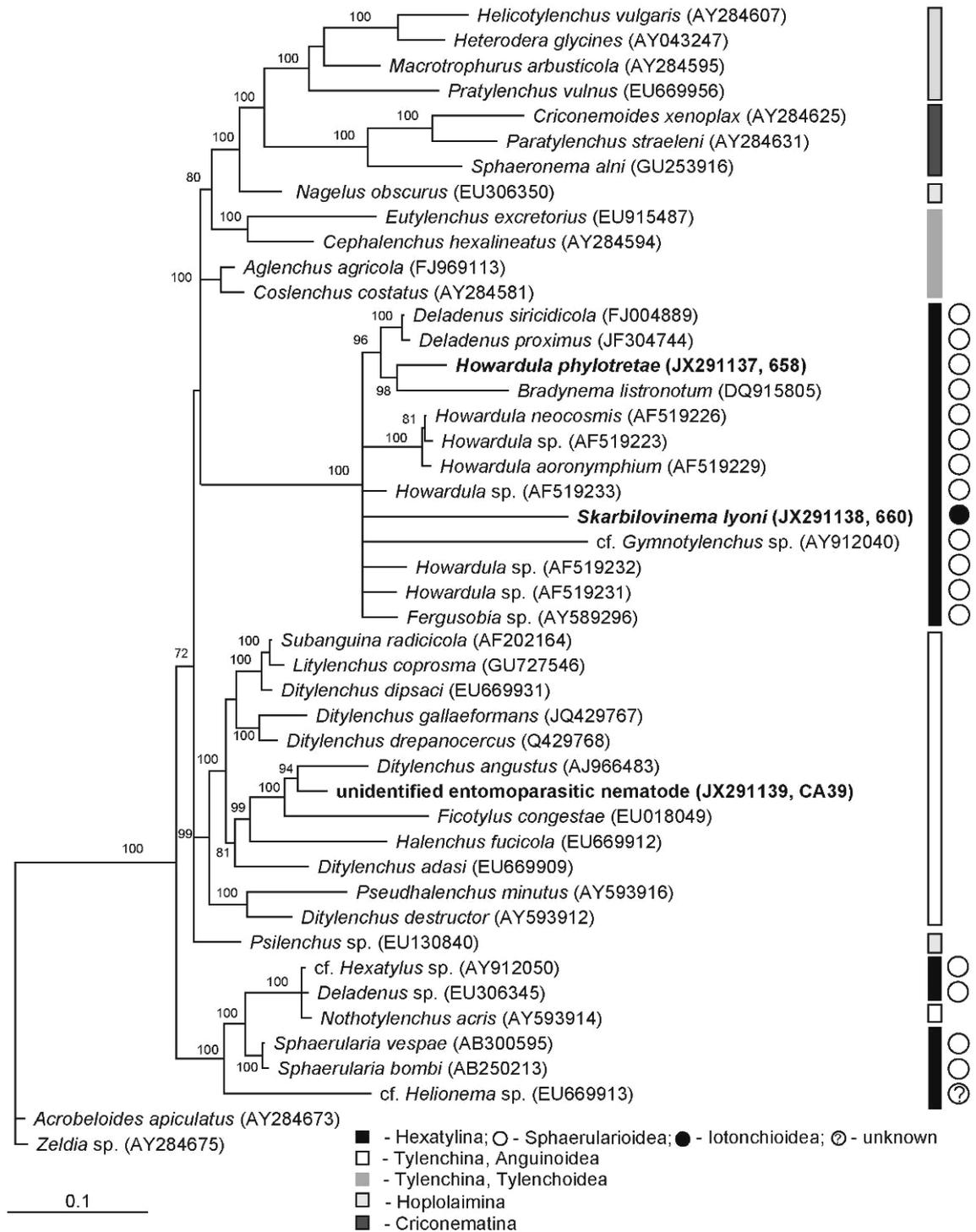
Probably, the first report on *Skarbilovinema* nematodes was made in the mid-seventies of the last century. Nematodes from the order Tylenchida parasitising flies of the genus *Helophilus* (Syrphidae: Diptera) were described by Laumond and Lyon (1975). The authors gave a description of all stages of these nematodes localised in the fly abdomen. However, they believed that they belonged to the known genus *Iotonchium* Cobb, 1920. After discovery and morphological study of two morphologically similar nematode species from hoverflies of the genera *Helophilus* and *Eristalis* collected in several regions of the European part of

Russia, Chizhov and Zakharenkova (Chizhov & Zakharenkova, 1991; Zakharenkova & Chizhov, 1991) concluded that they did not belong to any known genera and established a new genus *Skarbilovinema*. These authors also described all stages of development of the parasites and their life cycle and found that it included the only heterosexual generation. Peculiar morphological features, such as the structure of spicules and body shape of a free-living infective female, and unique life cycle including the only heterosexual generation with copulation in the fly hemocoel made these nematodes different from others and gave a reason to establish a new subfamily Skarbilovinematinae (Chizhov & Zakharenkova, 1991) within the family Iotonchiidae. However, Siddiqi (2000) did not recognise this subfamily, but also considered the genus *Skarbilovinema* as belonging to the family Iotonchiidae.

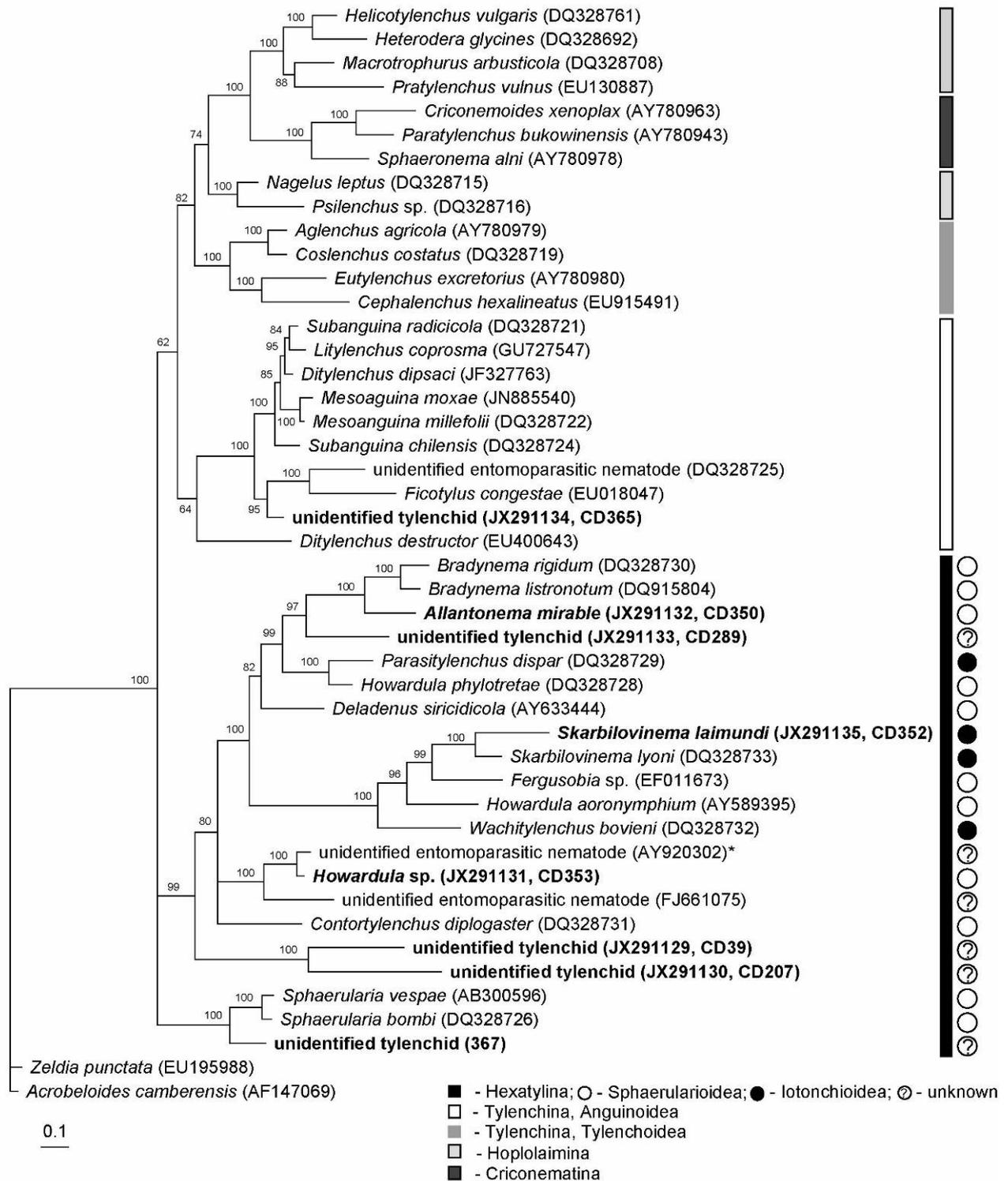
Using the D2-D3 of 28S rRNA gene sequences, Subbotin *et al.* (2006) were the first to analyse the position of *Skarbilovinema lyoni* within the order Tylenchida and showed that this species has a sister relationship with *Wachekitylenchus bovienii*. Only a few sequences from entomoparasitic nematodes were included in that analysis and other studies (Ye *et al.*, 2007; Davies *et al.*, 2010), and, unfortunately, the relationships within Hexatyliina and between *Skarbilovinema* and entomoparasitic nematodes lineages remained uncertain.

Our present analyses give a more complicated picture of entomoparasitic nematode evolution and paraphyly and polyphyly for the superfamilies Sphaerularioidea and Iotonchioidea, genera *Howardula* and *Deladenus*. It also shows the presence of a nematode having entomoparasitic stage within Anguinoidea and the close relationship of *Sphaerularia* with a nematode inhabiting soil. The genus *Skarbilovinema* with morphological features typical for the superfamily Iotonchioidea is attributed to the superfamily Sphaerularioidea, which indicates the importance of using morphological characters and life cycle types for the Hexatyliina systematics.

Our phylogenetic analysis of the D2-D3 of 28S rRNA gene sequences revealed that *Skarbilovinema* species form a highly supported clade with *Fergusobia*. The genus *Fergusobia* represents a large radiation of nematode species that are currently classified in a monogeneric subfamily (Fergusobiinae) within the suborder Hexatyliina, based upon morphology and life history (Siddiqi, 2000; Giblin-Davis *et al.*, 2004). The sister relationship of *Skarbilovinema* and *Fergusobia* conflicts with current systematics and phylogenies of



**Fig. 5.** Bayesian 50% majority rule consensus tree as inferred from the analysis of the partial 18S rRNA gene sequence alignment under the GTR + G + I model. Posterior probabilities more than 60% are given for appropriate clades. New sequences obtained in this study are marked by bold font.



**Fig. 6.** Bayesian 50% majority rule consensus tree as inferred from the analysis of the D2–D3 of 28S rRNA gene sequence alignment under the GTR + G + I model. Posterior probabilities more than 60% are given for appropriate clades. \* - submitted as a sequence of insect *Spathius generosus* (Hymenoptera) by Yang *et al.* (2005). New sequences obtained in this study are marked by bold font.

entomoparasitic nematodes based on morphological features. Subbotin *et al.* (1993, 1994, 1996) showed that females of different entomoparasitic tylenchids parasitising insect hemocoel have different types of modifications of body walls. Parasitic females of *Skarbilovinema* and *Fergusoria* from the adult flies share similar structures. Nematodes of both genera have an enlarged epidermis covered by modified microvilli forming a dense 'spongy' layer (Subbotin *et al.*, 1993; Giblin-Davis *et al.*, 2001). This is only one prominent common character shared by these genera.

Our present study enabled the position of *Fergusobia* within Tylenchida to be clarified, and confirmed the hypothesis that *Fergusobia* evolved from entomoparasitic nematodes similar to modern *Howardula* (Ye *et al.*, 2007), and rejected the hypothesis that *Fergusobia*, anguinids and neotylenchids have common ancestors (Giblin-Davis *et al.*, 2004). Thus, *Fergusobia* may present a remarkable example of tylenchid evolution to plant parasitism via entomoparasitism, whereas it has been shown that all other known plant-parasitic tylenchids originated from mycophageous nematode ancestors.

The present analysis also revealed an anomalous mode of the ribosomal RNA evolution in *Skarbilovinema*. The ribosomal RNA genes of these nematodes have long insertions, A-T nucleotide content bias and high mutation rate. The D2-D3 expansion segments of the 28S rRNA gene sequences of *S. laumondi* and *S. lyoni* differed by 23%, which considerably exceeded any known variation for nematode species belonging to the same genus. Several long insertions also make 18S rRNA gene and the D2-D3 of 28S rRNA gene to be the longest ribosomal rRNA genes among the phylum Nematoda, according to our knowledge. The unusual rRNA evolution might potentially lead to an anomalous position of *Skarbilovinema* on phylogenetic trees. Additional taxa as well as other gene fragments should be included in subsequent analyses to create a more reliable phylogeny for entomoparasitic tylenchids as well as to test their origin.

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**В.Н. Чижов, Н. Н. Буторина, С.А. Субботин.** Энтомопаразитические нематоды рода *Skarbilovinema*: *S. laumondi* и *S. lyoni* (Nematoda: Tylenchida), паразиты мух семейства Syrphidae (Diptera), и филогения подотряда Hexatyliina.

**Резюме.** Дано морфологическое описание двух энтомопаразитических нематод, *Skarbilovinema laumondi* и *S. lyoni*, являющихся паразитами мух родов *Helophilus* и *Eristalis* семейства Syrphidae (Diptera). Описан жизненный цикл этих нематод. Филогенетический анализ с использованием D2-D3 сегмента последовательности большой субъединицы рибосомальной ДНК и частичной 18S-последовательности рибосомальной ДНК показал близкое родство этих нематод с *Fergusoria* sp. Анализ последовательностей выявил аномальный характер эволюции генов рибосомальной ДНК *Skarbilovinema*, характеризующихся наличием длинных вставок, высоким содержанием А-Т оснований и высокой скоростью мутаций. Различия в D2-D3 участке последовательности большой субъединицы рибосомальной ДНК у *S. laumondi* и *S. lyoni* составляли 23%, что превышает различия по этой последовательности, обычно наблюдаемые у видов нематод, принадлежащих к одному роду. Присутствие нескольких вставок в 18S последовательности рибосомальной ДНК, а также в D2-D3 сегменте последовательности большой субъединицы рибосомальной ДНК делает эти рибосомальные фрагменты самыми длинными среди нематод.

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