Characterization of Juvenile Stages of *Bursaphelenchus crenati* Rühm, 1956 (Nematoda: Aphelenchoidoidea)

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This paper was edited by Zafar Ahmad Handoo.  
Received for publication June 23, 2018.

The nematode species *Bursaphelenchus crenati* Rühm, 1956 belonging to *Sexdentati* group was originally described from the galleries of *Hylesinus crenati* Fabricius in bark of ash *Fraxinus excelsior* L. in Bavaria, Germany and later was re-described by Gu et al. (2017) using the populations from Poland. This species was recently detected in several localities of Russia and Belarus (Ryss et al., unpublished). Transmissive dauer juveniles of *B. crenati* were found in senior larvae, pupae, and female imagos of the beetle species. From these dauer juveniles, adult nematodes were grown in cultures of the spore-less strain of the fungus *Botrytis cinerea* on the 2% potato dextrose agar (PDA). All records revealed associations of *B. crenati* with wilt symptoms of the ash *Fraxinus excelsior*. In the same beetle vector, the fungi belonging to the family Ophiostomataceae were extracted and cultured in 2% PDA and 2% MEA (malt agar). Nutrition relations between the nematodes and fungi were established, it has been shown that the nematode population grown in slow rate (5 wk) comparing to that culture with *Botrytis cinerea* (7 d at room temperature). To study a functional role of different juvenile stages of *B. crenati* in the adaptations to vector and plant and fungus host, as well as in survival of winter seasonal stress, the identification of the life cycle stages would be necessary.

Special attention was given to observe the genital primordium, because according to previous studies this organ is most useful in identification of stage and

**Abstract**

In this study juvenile stages of *Bursaphelenchus crenati* (*Sexdentati* group) were distinguished based mainly on the genital primordium structure. Dauer stage juveniles were sampled under elytra of *Hylesinus crenatus* in galleries in bark of the wilted *Fraxinus excelsior* in the Voronezh region of Russia and were multiplied in laboratory cultures on the fungus *Botrytis cinerea*. Individual development included five stages (J1–J4 and adults) separated by molts. The first molt J1 to J2 occurred inside the egg and the J2 was at the hatching stage. Sex of juvenile stages can be identified using the morphology and size of the genital primordia and a body size of nematodes. Sex of juveniles may be identified from the J3 stage by the presence of the cloacal primordium in male juvenile and orientation of the germinal zone of the genital primordium. A tabular key to developmental stages of *B. crenati* is given. The body grows during molts and within each stage. The body increases rapidly after J3 stage combined with the most active cellular differentiation and elongation of the genital primordium. The dauer juveniles collected from elytra of vector beetles were similar to the third juvenile stage of the propagative generation by genital primordium structure and body size.

**Key words**  
*Bursaphelenchus crenati*, Life cycle, Dauer stage, Postembryogenesis, Diagnostics, Saproxylic nematodes, *Fraxinus, Hylesinus*, Russia, Ash wilt.
sex of juveniles (Hirschmann, 1962, 1971; Hirschmann and Triantaphyllou, 1968; Ryss, 1981, 1988; Ryss and Chernetskaya, 2009, 2010; Ryss and Polyanina, 2017). The purpose of this research is to characterize the juvenile stages of the species.

Materials and methods

Nematode samples

The bark and wood samples with the beetle bark tunnels of *Hylesinus crenatus* Fabricius, in the inner bark layer and sapwood in wilted ash tree, *Fraxinus excelsior* L. were collected by A.V. Petrov in the forestry plantation “Tellermanovskoe Opytnoye Lesnichestvo ILAN RAS,” Tellermanovsky settlement, Gribanovskiy District, Voronezh Oblast, Russia, June 2017, in a location with the GPS. 51°21’46.8″N 42°02’40.3″E. Samples were sent to Zoological Institute RAS. In the laboratory, the nematode *Bursaphelenchus crenati* dauer were collected from beetle imagoes, where they formed semi–dry spot–like assemblages (“nematangia”) on the inner surface of elytra.

Nematode culturing

Nematodes were multiplied from dauer in the sterile laboratory cultures on the lawn of spore–less strain of the fungus *Botrytis cinerea*, inoculated in the 2% potato dextrose agar media in petri dish (Ryss, 2015). Over 2 wk after 20 nematode specimen inoculation, nematodes occupied all the petri dish space (diam. 6cm), devastating the fungus lawn, showing active oviposition and population growth. To maintain the nematode isolate, approximately 100 nematode specimens were transferred to the fresh culture of *B. cinerea*, multiplying population occupied all the petri dish space in 5 to 7 d at 20 to 22°C.

Morphological study

To study the morphology, nematodes were washed out from petri dish with 1ml of distilled water and fixed in hot TAF as described by Ryss (2017b). Over 2 d after fixation, nematodes were stained with acetic orcein or methylene blue. A modification of the staining technique (Ryss, 1988; Ryss and Chernetskaya, 2009; Ryss and Polyanina, 2017) was used.

Figure 1: *Bursaphelenchus crenati*. Development in egg. A. Cleavage; B. Gastrula; C,D. First stage juvenile (J1) within egg shell, at different focal planes; E,F,G. Second stage juvenile (J2) molting with shed cuticle, at different focal planes; H. Hatching J2, preparing to leave the egg shell. Scale 20 µm.
to stain nematodes with acetic orcein. A 1.5 ml Eppendorf tube with nematodes in TAF was placed in upright position for 10 min to settle nematodes down, and the upper layer of fixative was removed with a syringe up to the two fold level of the nematode suspension height (usually 100 µL). Then 200 µL of 70% acetic acid was added into the tube. After shaking the tube, two drops of saturated acetic orcein solution in 65% acetic acid were added, which is usually used for chromosome staining. The mixture was kept overnight for staining procedure and then a drop of staining suspension was placed into the glycerin in a cavity slide. The stained nematodes picked out by needle and transferred to 5 µL drop of glycerin.

Figure 2: *Bursaphelenchus crenati*. Methylene blue staining. Junior juveniles. A,B. Second stage juvenile (J2): A. Genital primordium; B. Tail; C,D. Genital primordium of J3 female juveniles (arrows: anterior germinal zone); E,F. Tails of female J3; G, H. Genital primordium of J3 male juveniles (arrows: posterior germinal zone); I,J. Tails of male J3 (arrows: cloaca primordium). Scale 20 µm.

To stain with methylene blue, the nematodes were fixed in TAF and processed to glycerin according to Ryss (2017a). Then, nematodes were picked out and transferred from glycerin suspension into a small 1 µL drop of glycerin on a glass slide, and a 5 µL drop of saturated methylene blue water solution was added from above. The drop was placed for 12 hr in open air at room temperature for water evaporation and staining. Then nematodes were transferred in another small glycerin drop on glass slide, heated softly and quickly for stain contrasting and differentiation.

Stained nematodes were picked out with needle under a stereomicroscope and placed in a minute drop of glycerin on a microscopic slide. The drop was covered with 20 x 20 mm coverslip. The drop was spread under coverslip to press nematodes to obtain flattened worm bodies for detailed study of nematode genital primordia structures. After drop spreading, coverslip was sealed with nail polish along borders. All stained nematodes in slides were studied, measured, and photographed under the automated microscope system Leica DM5000 B with differential interference contrast (DIC) and with the Leica DFC320 (R2) digital camera with Leica DFC Twain Software for PC and Leica IM50 Image Manager for PC. All measurements were made using the software ImageJ 1.48 v (National Institute of Health, USA, http://imagej.nih.gov/ij).

**Measurements and values used in morphometry of the individual development stages**

Measures: L: body length; BW: body width; pharynx length till the pharyngo–intestinal valve; pharynx length till the end of the pharyngeal gland lobe; tail length; tail width at anus (ABW); GP: genital primordium length;
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GW: genital primordium width; spicule length along arc; hyaline part of tail in male juveniles (future bursal flap of male). Ratios: a: body length divided to body width; b: body length divided to pharynx length till the pharyngo-intestinal valve; b': body length divided to pharynx length till the end of pharyngeal glands; c: body length divided to tail length; c': tail length divided to tail width; V or (V): distance from anterior body end to vulva, or to the center of the genital primordium, divided to the body length, in %; GP/GW: genital primordium length divided to its width; GP/L: genital primordium length divided to body length, in %.

Because the bodies were flattened, the re-calculation of the body and tail diameter was used: the flattened width was considered as a half of the circle (\(\pi R\) or \(\pi R\)), thus the diameter (2R) was calculated as the flattened body width (BW) \(\times (2/\pi) = 0.64 \times BW\).

**Results**

The stained nematodes and eggs were studied. The first molt took place inside the egg shell; thus during hatching the second stage juvenile (J2) broke the egg-shell and came out (Fig. 1E,F,G).

Other stages may be classified as J2, J3, J4, and adult males and females, separated by three molts. Molts were detected by cuticle separation from the body at body extremities, the head and tail tips. The sex of J3 and J4 may be recognized, whereas it cannot be determined in J2. The stage and sex of the juvenile may be determined in the genital primordium structure.

**Figure 3:** *Bursaphelenchus crenati*. Methylene blue staining. Genital primordium and tails of the fourth stage juveniles and adults. A. Genital primordium of the fourth stage female juvenile (J4); B,C. Molting female juvenile J4–adult; B. Genital primordium; C. Tail; D. Genital system of adult female; E, F. Fourth stage male juvenile; E. Genital primordium; F. Tail and cloacal primordium; G. Genital system of adult male. Scale 50 µm.

**Figure 4:** Position of genital primordia in bodies of male juveniles and adults. Scale 50 µm.
and its position in the body. The body size and the ratio between genital primordium length and the body are useful to identify the stage. Genital primordia in every stage and sex are described and illustrated below.

**Second stage juveniles (J2)**
(Figs. 2A,B, 4,5; Table 1)

The genital primordium consists of four cells: two large germinal cells in the central part and two small somatic nuclei at extremities (Fig. 2A). Primordium is in the middle of the intestine part of the body (Fig. 4).

**Differences from other stages**

J2 differs from senior stages by the smallest number of cells in primordium as well as in the body size less than 200µm vs body length of 300µm or longer in juveniles of senior stages.

**Third stage juveniles**

**Third stage female juvenile**
(Figs. 2C,D, 5; Table 1)

In the genital primordium, 2 large germinal cells in the anterior part and 12 somatic nuclei in the posterior primordium part are present together with a single somatic apical nucleus on every extremity of the primordium (Fig. 2C,D). A circle of six somatic nuclei interrupts the ventral hypodermal cord chain of somatic nuclei. It is a primordium of vulva.

**Differences of the third stage juveniles**

Differences of male juvenile of the third stage from female juvenile are manifested by the presence of cloaca primordium and the position of germinal cells in the posterior part of the primordium. In female juveniles, germinal cells are located in the anterior part of the primordium. Third stage juveniles differ from J2 in the number of cells of the primordium (more than 10 vs 4 in J2). From J4 juveniles, the third stage juveniles differ by the length of primordium occupying 6% of the body length or less vs 11% or more in J4 and adults.

**Fourth stage juveniles**

**Fourth stage male juveniles**
(Figs. 3G, 4; Table 1)

Cloaca primordium is massive, with numerous somatic nuclei around rectum and a transparent cavity anterior to rectum with rudimentary spicules. The genital primordium is distinctly divided into an anterior germinal part of 30 to 60 large cells and a
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### Table 1. Measurements (in µm) and ratios of the development stages of *Bursaphelenchus crenati*.

| Stage/Character | J2 | J3 male | J3 female | J4 male | J4 female | Male | Female |
|-----------------|----|---------|----------|---------|-----------|------|--------|
| n               | 20 | 20      | 20       | 15      | 18        | 15   | 15     |
| L               | 166 ± 22 | 367 ± 21 | 375 ± 26 | 455 ± 21 | 489 ± 14 | 698 ± 69 | 843 ± 94 |
| Body width      | 7 ± 1 | 10 ± 2 | 11 ± 2 | 14 ± 4 | 12 ± 0.2 | 17 ± 3 | 16 ± 3 |
| Pharynx         | 68 ± 4 | 111 ± 5 | 115 ± 6 | 106 ± 0.7 | 125 ± 12 | 122 ± 12 | 136 ± 22 |
| Pharyngeal gland| 30 ± 5 | 50 ± 5 | 52 ± 5 | 49 ± 6 | 50 ± 6 | 50 ± 6 | 59 ± 14 |
| Stylet          | 7 ± 1 | 8 ± 1 | 8 ± 0.8 | 10 ± 1 | 10 ± 1 | 10 ± 0.1 | 11 ± 1 |
| Lip region width| 4.0 ± 0.3 | 5.0 ± 2 | 5.0 ± 4 | 5.0 ± 2 | 5.8 ± 0.2 | 7.0 ± 0.6 | 7.0 ± 0.5 |
| Lip region height| 2.3 ± 0.5 | 3.0 ± 0.3 | 2.0 ± 0.2 | 3.0 ± 0.2 | 2.5 ± 0.1 | 4 ± 0.5 | 3.6 ± 0.3 |
| Median bulb from Anterior| 30 ± 2 | 48 ± 2 | 48 ± 1.4 | 40 ± 3 | 56 ± 6 | 58 ± 5 | 60 ± 9 |
| Median bulb length| 9.4 ± 1 | 12 ± 1 | 12 ± 1.3 | 12 ± 0.3 | 12.7 ± 0.7 | 15 ± 2 | 18 ± 4 |
| Median bulb width| 5.7 ± 0.3 | 9 ± 2 | 9 ± 1.2 | 9 ± 2 | 10.3 ± 1.3 | 12 ± 1 | 12 ± 1 |
| Excretory pore from anterior| 35 ± 3 | 53 ± 2.1 | 54.0 ± 3.9 | 46 ± 4 | 61.3 ± 0.9 | 63 ± 2 | 70 ± 11 |
| Tail            | 9 ± 3 | 22 ± 3 | 25 ± 2 | 23 ± 3 | 27.5 ± 1.4 | 29 ± 3 | 31 ± 5 |
| Anal body width | 4 ± 1 | 9 ± 1.1 | 9 ± 2 | 8 ± 1 | 10 ± 2 | 17 ± 2 | 11 ± 2 |
| Genital primordium| 7 ± 1 | 20 ± 5 | 15 ± 2 | 78 ± 25 | 132 ± 40 | 472 ± 68 | 526 ± 90 |
| length (GPL)    | (6–8) | (14–28) | (12–17) | (60–95) | (77–160) | (355–581) | (413–630) |
| Genital primordium| 2.7 ± 0.1 | 5 ± 0.4 | 6 ± 1 | 5 ± 1 | 8 ± 2 | 14 ± 3 | 16 ± 3 |
| Width (GPW)     | (2.5–3.0) | (4–5) | (4–7) | (4–6) | (6–10) | (11–14) | (12–21) |
| Spicule length along arc| – | – | – | – | – | 17 ± 1 | – |
| a               | 23 ± 2 | 40 ± 8 | 36 ± 4 | 31.6 ± 1.2 | 40 ± 7 | 40 ± 5 | 53 ± 8 |
| (22–24) | (32–58) | (32–41) | (31–33) | (33–49) | (34–50) | (41–60) |
Differences between male juveniles of the fourth stage from female juveniles are manifested by the presence of cloaca primordium. In female juveniles, the lens–like transparent vulva primordium at the level of uterus primordium part is present. In J4 male juveniles, the vulva primordium is absent. The fourth stage juveniles differ from J3 in the larger genital primordium (11% of body length or more vs 6% or less in J3) and in numerous cells of the primordium: more than 50 in J4 vs 20 or less in J3. From adult nematodes, J4s differ in the absence of copulative organs vs spicules or vulva is present in adult nematodes.

**Adults (Figs. 3,4,5; Table 1)**

Staining did not reveal any new morphological features in addition to detailed description (Gu et al., 2017).

**Molting individuals**

Molting individuals are characterized by the separation of molting cuticle at extremities: head and tail tip (Figs. 3C, 6B,D,E,H,J). The genital primordium structure of molting specimens is intermediate between structures of primordia described above for the juveniles of J2 to J4 stages.

### Table 1

| b | 4.5 ± 0.4 | 6.1 ± 0.3 | 6.0 ± 0.3 | 8.0–1.2 | 6.6 ± 0.6 | 9.7 ± 1.2 | 11.1–2.0 |
| b’ | 2.5 ± 0.5 | 3.3 ± 0.2 | 3.3 ± 0.2 | 4.3 ± 1.1 | 3.9 ± 0.4 | 5.8 ± 0.6 | 6.3 ± 1.0 |
| c | 19.0 ± 4.3 | 16.6 ± 2.1 | 15.2 ± 0.5 | 20.0 ± 2.4 | 17.9 ± 1.5 | 24.3 ± 2.9 | 27.7 ± 5.5 |
| c’ | 2.1 ± 0.4 | 2.7 ± 0.4 | 2.8 ± 0.4 | 2.8 ± 0.1 | 2.8 ± 0.4 | 1.8 ± 0.2 | 2.9 ± 0.3 |

V % or [V]: genital primordium center from anterior, to body length, in %

| GPL/GPW | 2.6 ± 0.4 | 4.3 ± 1.0 | 2.4 ± 0.2 | 17 ± 10 | 16.8 ± 3.0 | 34 ± 7 | 34 ± 8 |
| GPL/L, % | 4.3 ± 0.04 | 5.4 ± 1.1 | 4 ± 0.3 | 18 ± 10 | 27 ± 8 | 67 ± 4 | 62 ± 6 |

Lip region length to its Width

| Median bulb length to Its width | 1.6 ± 0.1 | 1.4 ± 0.3 | 1.4 ± 0.1 | 1.4 ± 0.3 | 1.3 ± 0.2 | 1.3 ± 0.1 | 1.4 ± 0.2 |

All values are given as mean ± SD (minimum-maximum).

Somatic part of two rows of somatic nuclei with 15 to 16 nuclei in every row. The somatic part is not divided into sections (Fig. 3G). Genital primordium occupies 11 to 25% of the body length (Fig. 4). Tail tip with narrowly conical 6 (4–9) µm hyaline zone is curved ventrally, corresponding to bursal flap of adult male.

Fourth stage female juvenile (Figs. 3,5; Table 1). Genital primordium is divided into the anterior germinal part, consisting of 20 to 30 large cells and the posterior somatic part consisting of more than 60 somatic nuclei. On the ventral side of body wall in the middle of somatic primordium part, the lens–like invagination surrounded by massive structure attached to the ventral body wall, is distinct. It is the vulva primordium. Genital primordium occupies 16 to 33% of the body length (Fig. 5).
Molt from J2 to J3 (Fig. 6A,B; Table 2)

In the center of primordium, 3 to 4 mitoses of somatic nuclei are distinct between germinal cells (Fig. 6A).

Molt from J3 to J4, male juvenile (Figs. 4, 6C–H; Table 2)

The genital primordium is initially straight, then transforming to loop–shaped (Fig. 6F,G), with the anterior apex and both somatic and germinal parts reversed posteriorly; the somatic part is set off by constriction from germinal part and its tip is curved hook–like (Fig. 6G). The cloaca primordium is enlarged with a transparent inner cavity anterior to the rectum (Fig. 6H). This molting phase is an indication of the orientation change of the somatic part, which in male juvenile J3 is located anteriorly and in J4 is shifted posteriorly, thus pulling a germinal part to the anterior direction (Fig. 6F,G).

Molt from J3 to J4, female juvenile (Figs. 5,6I,J; Table 2)

The somatic part of the genital primordium in its posterior part possesses more than 20 somatic nuclei, the germinal part in the anterior part of the primordium with 5 to 10 large germinal cells.

Molt from J4 to male (Fig. 4; Table 2)

The somatic part of the genital primordium reaches in some specimens the massive cloaca primordium, in which inner cavity transparent spicules outlines are distinct. The bursal flap is not formed, the tail is narrowly conical with 9 µm long hyaline zone.
Table 2. Measurements (in µm) and ratios of the development stages of *Bursaphelenchus crenati*.

| Stage/Character | J2→J3 | J2→J3 | J3→J4 | J3→J4 | J4→male | J4→female | Dauer J3D |
|-----------------|-------|-------|-------|-------|---------|-----------|----------|
|                 | male  | female| male  | female|         |           |          |
| n               | 16    | 5     | 10    | 20    | 5       | 17        | 20       |
| L               | 347 ± 7 | 323 ± 86 | 370 ± 3 | 377 ± 10 | 596 ± 91 | 544 ± 69 | 413 ± 20 (338 – 354) (181 – 410) (366 – 374) (364 – 398) (532 – 660) (433 – 614) (392 – 463) |
| Body width      | 9 ± 1 | 10 ± 2 | 11 ± 2 | 12 ± 1 | 16 ± 0.3 | 13 ± 3 | 8 ± 1 (8 – 10) (8 – 14) (9 – 13) (11 – 13) (14 – 17) (9 – 17) (6 – 9) |
| Pharynx         | 107 ± 4 | 103 ± 21 | 110 ± 5 | 110 ± 5 | 112 ± 9 | 117 ± 6 | 119 ± 9 (104 – 112) (65 – 116) (105 – 115) (102 – 117) (105 – 118) (109 – 123) (101 – 128) |
| Pharyngeal gland| 49 ± 5 | 51 ± 1 | 49 ± 4 | 48 ± 4 | 46 ± 1 | 48 ± 6 | 43 ± 7 (44 – 56) (49 – 53) (44 – 55) (41 – 54) (45 – 47) (40 – 55) (35 – 55) |
| Stylet          | 7 ± 1.2 | 8 ± 1 | 7 ± 1 | 9 ± 0.7 | 9 ± 0.4 | 9 ± 1.4 | 9.4 ± 0.4 (6 – 9) (6 – 9) (6 – 8) (8 – 10) (9 – 10) (8 – 11) (9 – 10) |
| Lip region width| 5.0 ± 0.3 | 5 ± 0.6 | 5.6 ± 0.5 | 5 ± 0.6 | 5 ± 0.4 | 5.4 ± 0.4 | 4.8 ± 1.0 (4 – 5) (4 – 6) (4 – 6) (4 – 6) (5 – 6) (5 – 6) (4 – 7) |
| Lip region height| 3 ± 0.3 | 2.4 ± 0.2 | 2.4 ± 0.1 | 3 ± 0.3 | 3 ± 1 | 2.9 ± 0.4 | 2.9 ± 0.3 (2 – 3) (2 – 3) (2.3 – 2.5) (2 – 3) (2 – 3) (2 – 3) (2.5 – 4) |
| Median bulb from anterior | 46 ± 1 | 45 ± 8 | 47 ± 0.1 | 47 ± 3 | 46 ± 12 | 53 ± 3 | 49 ± 2 (45 – 47) (31 – 50) (46 – 48) (40 – 50) (38 – 54) (51 – 58) (45 – 51) |
| Median bulb length | 11 ± 1.2 | 11 ± 0.9 | 12 ± 1 | 12 ± 1 | 13 ± 1 | 13 ± 0.2 | 11 ± 2 (10 – 13) (10 – 12) (11 – 13) (10 – 13) (12 – 14) (12.5 – 13.5) (9 – 15) |
| Median bulb width | 9 ± 1.1 | 8 ± 1.5 | 10 ± 1 | 10 ± 1 | 9.9 ± 0.2 | 10 ± 1 | 8 ± 2 (8 – 10) (6 – 10) (9 – 11) (7 – 10) (9.5 – 10.0) (9 – 11) (6 – 10) |
| Excretory pore from anterior | 51 ± 1 | 50 ± 8 | 53 ± 2 | 54 ± 4 | 52 ± 12 | 58 ± 3 | 40 ± 3 (50 – 52) (37 – 56) (51 – 54) (45 – 59) (44 – 61) (54 – 62) (37 – 44) |
| Tail | 22 ± 4 | 21 ± 6 | 22 ± 1 | 26 ± 1.5 | 28 ± 5 | 28 ± 3 | 37 ± 3 (20 – 28) (11 – 27) (21 – 24) (24 – 28) (25 – 32) (23 – 31) (30 – 41) |
| Anal body width | 8 ± 0.4 | 8 ± 3 | 11 ± 2 | 11 ± 1.1 | 11 ± 3 | 10.1 ± 0.9 | 8 ± 1 (7 – 8) (5 – 12) (8 – 13) (10 – 13) (9 – 13) (9 – 11) (5 – 9) |
| Genital primordium | 18 ± 4 | 10 ± 3 | 20 ± 2 | 18 ± 5 | 158 ± 138 | 197 ± 105 | 14.8 ± 2.1 |
| Length (GPL) | (14 – 23) | (6 – 14) | (17 – 22) | (13 – 28) | (60 – 255) | (122 – 369) | (11 – 18) |
| Genital primordium Width (GPW) | 5 ± 1 | 5 ± 1 | 4.8 ± 0.5 | 5 ± 1 | 10 ± 5 | 9 ± 3 | 3.9 ± 0.4 (4 – 5) (3 – 7) (4.0 – 5.3) (3 – 6) (6 – 13) (7 – 14) (3 – 5) |
Molt from J4 to female (Fig. 5; Table 2)

Vulva primordium is a massive cellular agglomeration with an inner transversal slit, but without opening outside. All sections of the genital system are distinct: the ovary of 30 to 40 germinal cells, the oviduct, empty spermatheca, cristaformeria, and the uterus with inner empty cavity (Fig. 5).

Tabular key to juvenile stages of Bursaphelenchus crenati (Key is given in Table 3).

Morphometric ratios of juvenile development

Individual growth is illustrated in diagrams (Fig. 7) with two main parameters: the body length and the ratio between the genital primordium length and the body length. The elongation of the body is faster after the third stage (Fig. 7A). At the same period, the maximum elongation and differentiation of the genital primordium takes place in both male and female development lines (Fig. 7B). Juvenile body increases between molts and within developmental stages, which is evident in the size ranges of every stage (Tables 1, 2).

Dauer juveniles (Figs. 4,5,8,9; Table 2)

The dauer juveniles have the same genital primordium structure and size as J3. The differences are: the slender and straight body and straight long tail vs curved ventrally tail in the J3 of the propagative gen-

| Spicule length along arc | a | b | b' | c | c' | V % or (V): genital primordium center from anterior, to body length, in % |
|-------------------------|---|---|----|---|----|-----------------------------------------------|
|                         | 40±5 | 34±8 | 34.8±5.2 | 32±2 | 39±12 | 41.5±4.6 | 54±8.3 |
| (34–46) | (22–41) | (28.4–41.8) | (29–35) | (31–48) | (36.8–48.9) | (44.6–69.6) |
|                         | 6.0±0.3 | 5.6±0.6 | 6.1±0.2 | 6.1–0.2 | 9.1±0.3 | 7.8±0.6 | 5.5±0.4 |
| (5.7–6.4) | (4.8–6.4) | (5.9–6.3) | (5.8–6.5) | (8.9–9.2) | (6.7–8.3) | (5.1–6.4) |
|                         | 3.2±0.1 | 3.1±0.3 | 3.4±0.2 | 3.4±0.2 | 5.3±0.4 | 4.6±0.5 | 3.5±0.3 |
| (3.1–3.3) | (2.8–3.6) | (3.2–3.6) | (3.1–3.7) | (5.1–5.6) | (4.0–5.2) | (3.0–4.1) |
|                         | 16.0±2.5 | 15.6±0.6 | 16.6±0.9 | 14.8±0.8 | 21.1±0.9 | 19.7±1.7 | 11.2±0.9 |
| (12.4–17.9) | (14.7–16.2) | (15.1–17.3) | (13.8–1.58) | (20.5–21.7) | (18.0–22.3) | (9.9–13.2) |
|                         | 2.8±0.4 | 2.8±0.5 | 2.1±0.3 | 2.3±0.2 | 2.6±0.1 | 2.8±0.3 | 4.7±0.5 |
| (2.4–3.4) | (2.2–3.4) | (1.7–2.5) | (2.0–2.8) | (2.6–2.7) | (2.3–3.2) | (4.3–5.5) |
|                         | 61±2 | 64±3 | 62±1 | 66±2 | 52±3 | 73.2±1.6 | 62±5 |
| (59–64) | (59–68) | (60–64) | (63–68) | (49–54) | (70.5–74.3) |

All values are given as mean±SD (minimum-maximum).
eration. Lateral field with two closely located bands of equal width (three incisures). Dauers differ in hemispherical continuous head with hyaline inner cap vs set off head and well developed cephalic circular framework. Stylet is very thin capillary tube, conus and basal thickenings not distinct. Median bulb elongated, its length twice as its width, central valve is weakly developed. Pharyngointestinal junction is one half of the median bulb length posterior to median bulb, surrounded by nerve ring with excretory pore at the posterior third of median bulb. Pharyngeal glands form very narrow band, gland nuclei indistinct.

In 3 hr after pouring beetle elytra with "nematania" in water or 0.9% NaCl solution, the J3D juveniles started to molt to J4 juvenile with long genital primordium and well developed stylet and median bulb (Fig. 9).

Table 3. Tabular key to juvenile stages of *Bursaphelenchus crenati*.

|              | C1     | C2     | C3           | C4                              | C5     | C6                      |
|--------------|--------|--------|--------------|---------------------------------|--------|-------------------------|
| Stage/Character | Copulative structures | Average body size, in µm | Ratio: genital primordium to body length, in % | Genital primordium cellular structure | Cloaca primordium or cloaca around rectum | Genital primordium somatic part: a division to sections |
| J2           | Absent | 170 (150–180) | 4 | Small with two large germinal cells in center | Absent | Absent                     |
| J3 male      | Absent | 370 (340–400) | 5 (4–7) | 2–5 large germinal cells in the posterior part of primordium, 12 small somatic nuclei in anterior part | Present | Absent                     |
| J3 female    | Absent | 380 (340–400) | 4 (3–4) | 2–5 large germinal cells in the anterior part of primordium, 12 small somatic nuclei in posterior part | Absent | Absent                     |
| J4 male      | Absent | 460 (380–530) | 18 (11–25) | 20–60 large germinal cells in anterior part of primordium and 30–60 somatic cells in posterior part | Present | Absent                     |
| J4 female    | Absent | 490 (480–510) | 27 (16–33) | 20–60 large germinal cells in anterior part of primordium and 30–60 somatic cells in posterior part | Absent | Absent                     |
| Male, adult  | Paired | 700 (610–800) | 67 (58–72) | More than 60 germinal cells in anterior part | Present | Absent                     |
| Female, adult| Vulva at 75% body length from anterior end | 840 (750–1100) | 62 (55–71) | More than 60 germinal cells in anterior part | Absent | Present                     |

All values are given as mean (minimum-maximum).
Characterization of Juvenile Stages of *Bursaphelenchus crenati* Rühm, 1956 (Nematoda: Aphelenchoidoidea)

**Discussion**

The embryonic development with hatching of J2 after the first molt within egg shell is the ovoviviparity. The sex of juvenile is distinctly observed from the third stage. It is an indication of the possible physiological sex determination at the second stage of juvenile (Ryss and Polyanina, 2017). The fastest growth and genital primordium differentiation takes place after the J3 to J4 molt. Earlier study of the development of *Bursaphelenchus mucronatus kolymensis* (Ryss and Chernetskaya, 2009) and *B. ulmophilus* (Ryss and Polyanina, 2017) gave similar results: the first molt occurring in the egg shell and four stages outside of egg are divided by molts: J2 to J4 and adult worms. The only difference is the dauer juvenile stage. In *B. mucronatus* it is the fourth stage juvenile (Ryss, 2008), but in *B. ulmophilus* and *B. crenati* dauers are J3D stage. Leaving the beetle surface, the *B. crenati* dauers start to molt to the fourth stage juvenile with elongated and differentiated genital primordium, just in 0.9% NaCl solution or in water. The same molting just after reviving in water was detected for dauer J3D of *B. ulmophilus*.
Figure 9: Dauer juveniles molted to J4D after 3 hr in 0.9% NaCl solution. A. Male juvenile; B. Female juvenile.

(Ryss et al., 2015). Difference between the Bursaphelenchus clades in the dauer juvenile stages (J3D or J4D) is the important character for the genus Bursaphelenchus phylogeny and intrageneric taxonomic diagnostics (Ryss and Subbotin, 2017). Dauers play the important role in the deciduous woody plant wilt spread, as the infective juveniles vectored by insects (Polyanina et al., 2016). The stage of dauers in nematode life cycles and their adaptations to insect vectors is of special interest.

Acknowledgments

The authors are grateful to the State Academic Programs FSR: AAAA–A17–117030310322–3, AAAA–A17–117080110040–3 and the grant RFBR 17–04–00360a. The authors are grateful to the forest entomologist Dr. Alexander V. Petrov (ILAN RAS) for wood and bark samples with tunnels of the bark beetle Hylesinus crenatus Fabricius.

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