Research Article

Phylogenetic Analysis of Entomoparasitic Nematodes, Potential Control Agents of Flea Populations in Natural Foci of Plague

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Entomoparasitic nematodes are natural control agents for many insect pests, including fleas that transmit *Yersinia pestis*, a causative agent of plague, in the natural foci of this extremely dangerous zoonosis. We examined the flea samples from the Volga-Ural natural focus of plague for their infestation with nematodes. Among the six flea species feeding on different rodent hosts (*Citellus pygmaeus*, *Microtus socialis*, and *Allactaga major*), the rate of infestation varied from 0 to 21%. The propagation rate of parasitic nematodes in the haemocoel of infected fleas was very high; in some cases, we observed up to 1,000 juveniles per flea specimen. Our study of morphology, life cycle, and rDNA sequences of these parasites revealed that they belong to three distinct species differing in the host specificity. On SSU and LSU rRNA phylogenies, these species representing three genera (*Rubzovinema*, *Psyllotylenchus*, and *Spilotylenchus*), constitute a monophyletic group close to Allantonema and Parasitylenchus, the type genera of the families Allantonematidae and Parasitylenchidae (Nematoda: Tylenchida). We discuss the SSU-ITS1-5.8S-LSU rDNA phylogeny of the Tylenchida with a special emphasis on the suborder Hexatylina.

1. Introduction

More than 150 species of fleas feeding on different mammalian hosts, primarily rodents, are vectors of the bacterium *Yersinia pestis*, a causative agent of plague [1, 2]. In natural foci of plague, the dynamics of flea populations are among the main factors controlling the incidence of epizootics that pose a threat to humans inhabiting the areas [3–5]. Entomoparasitic nematodes of the order Tylenchida are known to control populations of various insect hosts [6–9]. The rate of tylenchid infestation in fleas reaches 50–60% in some cases [10, 11], when the nematodes cause castration and early death of the flea hosts [9, 12, 13].

Despite high importance of the Tylenchida as a nematode order harboring entomoparasites and notorious crop pests, their reliable phylogeny is still a challenge. Tylenchid nematodes differ widely in life cycle, parasitic strategies, and the host range that spans plants, fungi, and invertebrates. Phylogenies obtained from SSU and partial LSU rDNA data often disagree with classifications based on morphology and life cycle [14–21]. Phylogenetic resolution inside the order is far from being clear, which in many respects results from the insufficiency of data available to adequately describe its diversity. As for tylenchid parasites of fleas, only 31 species are described to date [9, 22–31], with no molecular vouchering. Here we present a study of parasitic nematodes isolated from fleas sampled from different rodent hosts in a natural focus of plague.

2. Materials and Methods

2.1. Collection of Samples. Samples were collected in 2012 (spring and autumn) and 2013 (spring) in the Volga-Ural natural focus of plague (Figure 1). The sampled rodents included soussliks (*Citellus pygmaeus*), mouse-like rodents (*Microtus socialis* and *Apodemus uralensis*), and jerboas (*Allactaga*...
Two species (Amphipsylla rossica and Ctenopthalmus secundus) were on M. socialis voles; and one species (Mesopsylla hebes) was on jerboas. Fleas were examined for nematode infestation (Table 1). Examination and dissection of fleas were carried out using the dissecting microscope MBS-2 (LOMO, Russia). A half of parasitic nematodes sampled from each flea was preserved for subsequent DNA extraction, and another half was used for morphological analysis. Live fleas infected with nematodes were placed in glass flasks with river sand to obtain free-living forms. Insects were kept in a KBF 720 (E5.2) climate chamber (Binder, Germany) at 26°C and 80% humidity.

2.2. Morphological Analysis. Fixation and clarification of nematode preparations were performed using standard techniques described by De Grisse [32]. Material was mounted on slides in a drop of glycerin, bound by a paraffin circlet (http://pest.cabweb.org). Color staining of preparations was not performed. Morphometric analysis was conducted using the light microscope “Leica DM 1000” (Leica, Germany) with an eyepiece micrometer. Pictures of nematodes were taken with the microscope “DFC 425” (Leica, Germany). Published data on morphometrics [23, 25, 26] were used for comparison.

2.3. DNA Extraction, PCR, and Sequencing. DNA samples were extracted with a Diatom DNA Prep (IsoGen Lab, Russia). rDNA fragments were amplified using an Encyclo PCR kit (Evrogen, Russia) and primers given in Table 2. The amplified rDNA fragments were sequenced using an Applied Biosystems 3500xL DNA analyzer. Sequence reads were assembled with the CAP contig assembly program [33] and proofread with the BioEdit software [34]. For three isolates, almost complete sequences of 18S and 28S rRNA and complete sequences of 5.8 rRNA, internal transcribed spacers ITS1 and ITS2 were assembled. The sequences were submitted to GenBank under accession nos. KF155281–KF155283. For the rest of isolates, partial (750–800 bp) sequences of 18S and 28S rRNA genes were submitted to GenBank under accession nos. KF373731–KF373740.

2.4. Phylogenetic Analysis. The newly obtained rDNA sequences of tylenchid parasites of fleas were aligned with a selected set of other tylenchid sequences obtained from the GenBank. The main selection criterion was to sample representatives of all clades that occur in published SSU and LSU rDNA phylogenies of the Tylenchida [16–21, 39]. Apart from the D2-D3 LSU rDNA expansion segment commonly used in previous studies, we included all LSU rDNA sequence data available for the Tylenchida, with the exception of Basiria sp. SAN-2005 (accession nos. DQ145619, DQ145667) that in our preliminary analyses (data not shown) demonstrated a disputable affinity to the Tylenchida. For the species Anguina tritici, Globodera pallida, Heterodera glycines, Pratylenchus vulnus, and Radopholus similis the nearly complete rDNA sequences were assembled with appropriate cDNA fragments identified with BLAST [40]. Partial LSU rDNA sequence of Ditylenchus dipsaci was combined with the soil environmental clone NTS.28S.061A.2_b4 (accession no. KC558346), as the clone sequence appeared to represent a close tylenchid relative of D. dipsaci. Chimeric sequences were also created in some cases when closely related partial rDNA sequences were found in the database. All sequences and their accession numbers are listed in Table 3. Cephalobidae and Chambersiellidae were chosen as the outgroup. Alignments were constructed with the MUSCLE program [41] and refined manually using the MEGA 5.0 software package [42]. Three alignments were generated: (1) SSU rDNA, (2) D3 region of LSU rDNA, and (3) concatenated rDNA data including SSU, LSU, 5.8S rDNA, and highly conserved regions of ITS1. After discarding ambiguously aligned positions, the alignments length was 1,723, 592, and 4,930 positions, respectively. Bayesian reconstruction of phylogeny was done with the PhyloBayes software, version 3.2 [43] under the GTR + CAT + DP model [44]. Eight independent runs were performed with 4,000,000 cycles each; the first 3,000,000 cycles were discarded. A consensus tree with Bayesian posterior probabilities was constructed for the remained tree sample. Bayesian reconstruction was also performed using the MrBayes software [45] under the GTR + G8 + I model [46] in two independent runs, each with four Markov chains. The chains were run for 5,000,000 generations, with trees sampling every 1,000th generation. The consensus posterior probabilities were calculated after discarding the first 3,000,000 generations. Partitioning “by genes” was used for the concatenated alignment with all parameters unlinked, except for the topology and branch lengths. In addition, node support was estimated with maximum likelihood bootstrap as implemented in the RAxML software, version 7.2.6 [47], under the GTR + G + I model with 1,000 bootstrap replicates. Alternative topologies were tested using the approximately unbiased (AU) [48] and Kishino and Hasegawa [49] tests implemented in the CONSEL software [50] and the expected likelihood weight test [51] implemented in the TREE-PUZZLE software [52].
Table 1: Number of fleas studied and the percentage of fleas infected with nematodes.

| Time of sampling | Host rodent species | Flea species                  | Number of collected fleas | Number of infected fleas | Percentage of infected fleas |
|------------------|---------------------|-------------------------------|---------------------------|---------------------------|------------------------------|
| April 2012       | Citellus pygmaeus    | Citellophilus tesquorum       | 41                        | 7                         | 17.1%                        |
|                  |                     | Neopsylla setosa              | 73                        | 5                         | 6.8%                         |
|                  |                     | Frontopsylla semura           | 54                        | 7                         | 13%                          |
| October 2012     | Microtus socialis   | Amphipsylla rossica           | 135                       | 9                         | 6.7%                         |
|                  |                     | Ctenophthalmus secundus       | 88                        | 1                         | 1.1%                         |
| April 2013       | Citellus pygmaeus    | Citellophilus tesquorum       | 34                        | 0                         | 0                            |
|                  |                     | Neopsylla setosa              | 271                       | 22                        | 8.1%                         |
|                  | Microtus socialis and Apodemus uralensis | Ctenophthalmus secundus | 19                        | 4                         | 21%                          |
|                  | Allactaga major      | Mesopsylla hebes             | 34                        | 2                         | 5.9%                         |

Table 2: Nucleotide sequences of primers used in this study.

| Primer | Sequence | Orientation | References |
|--------|----------|-------------|------------|
| Nik22  | tmycyggtgtagtyctgc   | F           | This study |
| A      | gtacgttttgatatccgactgt | F       | [35]        |
| Q5nemCh| ggcgcgaaggtcattayaac | F           | This study |
| G18SU  | gtgctctcaagattaaagcc | F           | [36]        |
| Ves18-d9| tgtctaaaggtatccgtaggtgaac | F          | This study |
| R18Ty1l| gttccaaatgttaccttc    | R           | [36]        |
| B      | gtaaggtaacctcagagaagataca | R      | [35]        |
| Q39nem| gaaacctgttagacacctttrcbygg | R      | This study |
| 58di   | rctagctgaagaagcywgg   | F           | [37]        |
| 58r nem| gcwgctttcttcagacgcyc   | R           | This study |
| 28d3   | gttcgaacagcagaaagcag  | F           | [37]        |
| 28d6   | gttcyggtggatccctcgagtc | F       | [37]        |
| D2A    | cacaagttcggaggaaggttg | F           | [38]        |
| 28r4   | gctatctcggaggaacctcgg  | R          | [37]        |
| 28r2nem| cggtagttcgactgctagc   | R           | This study |
| 28r7   | agccataaatttcctccgaaggta | R      | [37]        |
| 28r12  | ttctagcttacgtagcgagc  | R           | [37]        |
| D3B    | tcgagaagaaaccagcctaca  | R           | [38]        |

[53] was used as the tree viewer and editor, and site-wise log-likelihoods were computed with TREE-PUZZLE under the GTR + G8 + I model with substitution matrix parameters estimated by MrBayes.

3. Results

3.1. Infestation of Fleas with Nematodes. The infestation rate is shown in Table 1 (in total, 807 flea specimens were studied). Among the six flea species studied, the population size and the percentage of infected fleas varied depending on the season. Three flea species sampled on sousliks (Citellophilus tesquorum, Neopsylla setosa, and Frontopsylla semura) exhibited a stable population density. In the two species, N. setosa and F. semura, the infestation rate was moderate to high in the spring seasons of 2012 and 2013. In C. tesquorum, no infected fleas were detected in spring 2013, whereas in spring 2012 the fleas were highly infested (17.1%). The vole flea Amphipsylla rossica was abundant and moderately infested in autumn, whereas being less abundant in spring, which may explain the absence of infected fleas in the spring sample. Another vole flea, Ctenophthalmus secundus, exhibited a consistently high population density and low infestation rate in both spring and autumn samples.

Adult parasitic females and their progeny were found in the haemocoel of infected fleas. In the infected fleas C. tesquorum, A. rossica, C. secundus, and Mesopsylla hebes, only one generation of parasitic females was observed. Their amount in a flea specimen is determined by the number of free-living infective females that penetrate into the flea larva. We observed 1 to 2 or 1 to 4 adult parasitic females per flea specimen in spring and autumn, respectively. An additional parthenogenetic generation of parasitic females was found in some fleas of N. setosa and F. semura, where
Table 3: List of OTUs and accession numbers of sequences.

| Name                        | I 18S rRNA | ITS1-5.8SrRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8] |
|-----------------------------|------------|---------------|----------|-------------------------|-----------|---------------|
| **Chambersiellidae**        |            |               |          |                         |           |               |
| *Fescia grossa*             | KC242218   | —             |          |                         | DQ145636  | [54] Chambersiellidae |
| *Geraldia sp. SAN-2010a*    | —          | —             | GU062821 | 17.8/—                  |           | [56] Chambersiellidae |
| **Cephalobidae**            |            |               |          |                         |           |               |
| *Acroboloides maximus*      | EU196016   | JX026706      | EU195987 | 94.8/—                  |           | [57]          |
| *Cephalobus cubensis*       | AF202161   | AF202161      | EU253570 | 89.8/—                  |           | [57] Cephalobidae |
| *Panagrolobus sp. SN-2010*  | —          | —             | HM439771 | 51.9/—                  |           | [60]          |
| *Cephalobidae Gen. sp. MHMH-2008* | FJ040406 | —             | —        |                         | Holterman et al., 2008, unpublished. |
| *Zeldia punctata*           | —          | DQ146426      | EU195988 | 96.6/—                  |           | [57]          |
| *Zeldia sp.*                | AY284675   | —             | —        |                         |           |               |
| **Aphelenchidae**           |            |               |          |                         |           |               |
| *Aphelenchus avenae*        | JQ348399   | AF199048      | —        | 96.9/—                  |           | [63] Aphelenchidae |
| *Aphelenchus sp.*           | —          | —             | DQ145664 |                         |           | [55]          |
| *Paraphelenchus aconioides* | —          | —             | HQ18822  | 45.5/—                  |           | [64]          |
| *Paraphelenchus sp.*        | AY284642   | —             | —        |                         |           | [18]          |
| **Hexatylinia + "Anguinata (part)"**: Iotenchioidea** | | | | | | |
| *Allantonema mirabile*      | —          | —             | JX291132 | 10.6/85.8               |           | [39]          |
| *Bradynema listronoti*      | DQ95805    | —             | DQ95804  | 45.6/96.8               |           | [65]          |
| *Bradynema rigidum*         | —          | —             | DQ328730 | 10.4/86.3               |           | [20]          |
| *Contortylenchus sp.*       | —          | —             | DQ328731 | —/85.4                  |           | [20]          |
| *Deladenus durus*           | JQ957898   | —             | —        | 34.0/—                  |           | [66]          |
| *Deladenus proximus*        | JF304744   | JF304744      | —        | 35.2/—                  |           | [67]          |
| *Deladenus siricidicola isolate 354* | AY633444 | —             | AY633444 | 45.8/98.1               |           | [68]          |
| *Deladenus siricidicola isolate 466* | FJ004890 | FJ004890      | —        | 41.7/—                  |           | [69]          |
| *Deladenus siricidicola isolate 1093* | FJ004889 | FJ004889      | —        | 42.0/—                  |           | [69]          |
| *Fergusobia camaldulensae*  | AY589294   | —             | AY589346 | 45.7/98.0               |           | [68]          |
| *Fergusobia sp. 444*        | EF01667    | —             | EF01675  | 45.7/97.3               |           | [68]          |
| *Fergusobia sp. SBG*        | FJ39270    | —             | FJ386996 | 45.7/98.3               |           | [70]          |
| cf. Gymnotylenchus sp. TSH-2005 | AY912040   | —             | —        | 12.9/—                  |           | Powers et al., unpublished. |
| Name                                | 18S rRNA     | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8]       |
|-------------------------------------|--------------|----------------|----------|------------------------|-----------|---------------------|
| Howardula aoronymphium             | AY589304     | AY589304       | AY589395 | 49.7/96.1              | [68]      |                     |
| Howardula dominicki                | AF519234     | AF519234       | —        | 37.4/—                 | [71]      |                     |
| Howardula neocosmis                | AF519226     | AF519226       | —        | 38.2/—                 | [71]      |                     |
| Howardula phyllostreta              | JX291137     | —              | DQ328728 | 41.9/86.1              | [20]      | Allantonematidae    |
| Howardula sp. CD353                | —            | —              | JX291131 | —/93.9                 | [39]      |                     |
| Howardula sp. SP-A                 | AF519232     | AF519232       | —        | 37.7/—                 | [71]      |                     |
| Howardula sp. SP-F                 | AF519222     | AF519222       | —        | 38.2/—                 | [71]      |                     |
| Howardula sp. SP-MA                | AF519233     | AF519233       | —        | 38.1/—                 | [71]      |                     |
| Howardula sp. SP-PS                | AF519231     | AF519231       | —        | 38.1/—                 | [71]      |                     |
| Parasitylenchus bifurcatus         | KC875397     | —              | DQ328729 | 44.0/85.3              | [20]      |                     |
| Parasitylenchus sp.                | —            | —              | —        | —/93.9                 | [39]      |                     |
| Psylotylenchus sp. ex Frontopsylla semura | KF373734     | —              | KF373739 | 27.1/93.7              | This study | Parasitylenchidae |
| Psylotylenchus sp. ex Neopsylla setosa | KF373733     | —              | KF373738 | 27.1/93.7              | This study | Parasitylenchidae |
| Rubzovinema sp. ex Amphisylla rossica | KFI55281     | KFI55281       | KFI55281 | 90.0/100.0             | This study | Neotylenchidae     |
| Rubzovinema sp. ex Ctenophthalmus accundus | KFI55282     | KFI55282       | KFI55282 | 89.8/100.0             | This study | Neotylenchidae     |
| Rubzovinema sp. ex Citellophilus tesquorum | KFI55283     | KFI55283       | KFI55283 | 93.2/100.0             | This study | Parasitylenchidae |
| Rubzovinema sp. ex Frontopsylla semura | KF373732     | —              | KF373737 | 27.1/93.7              | This study | Parasitylenchidae |
| Rubzovinema sp. ex Neopsylla setosa | KF373731     | —              | KF373736 | 27.1/93.7              | This study | Parasitylenchidae |
| Skarbilovinema laumondi             | —            | —              | JX291136 | 10.9/91.0              | [39]      | Itonchioidea       |
| Skarbilovinema lyoni                | JX291138     | —              | DQ328733 | 41.8/86.3              | [20]      |                     |
| Spilotylenchus sp. ex Mesopsylla hebes | KF373735     | —              | KF373740 | 27.1/93.4              | This study | Parasitylenchidae |
| cf. Sychnotylenchus sp. CSP1-09     | DQ080531     | —              | —        | 12.9/—                 | Powers et al., unpublished. | Sychnotylenchidae |
| Wacheckitylenchus bovieni           | —            | —              | DQ328732 | —/85.9                 | [20]      | Parasitylenchidae  |
| Unidentified Allantonematidae       | —            | —              | —        | 18.5/—                 | Rhule, unpublished. | Allantonematidae |
| HaMW                                | JQ941710     | —              | —        | 18.5/—                 | [73]      |                     |
| Unidentified Allantonematidae       | —            | —              | —        | —/—                   | [73]      |                     |
| NK2011_2                            | AB663183     | —              | —        | 12.0/—                 | [73]      |                     |
| Unidentified Allantonematidae       | —            | —              | —        | 12.0/—                 | [73]      |                     |
| NK2011_3                            | AB663184     | —              | —        | 12.0/—                 | [73]      |                     |
| Unidentified nematode               | EU880149     | —              | —        | 12.0/—                 | [74]      |                     |
| 804U-025                            | —            | —              | —        | —/—                   | [74]      |                     |
| Name                                      | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8] |
|-------------------------------------------|----------|----------------|----------|-------------------------|-----------|---------------|
| Unidentified nematode CD289               | —        | —              | JX291133 | —/84.1                  | [39]      |               |
| Unidentified nematode RGD591T12           | AB455970 |                | —        | 12.0/—                   | [73]      |               |
| Unidentified nematode WY2009_BAR-1        | —        | —              | FJ661075 | —/96.3                  | [75]      |               |
| Unidentified parasite ex Chrysothrix affinis | —        | —              | DQ202658 | —/5.0                   | Hunt et al., unpublished. |               |

**Hexatylina + “Anguinata (part)”: Sphaerularioidea**

| Name                                      | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8] |
|-------------------------------------------|----------|----------------|----------|-------------------------|-----------|---------------|
| Deladenus sp. PDL-2005                    | AJ966481 |                | —        | 35.0/—                   | [16]      | Neotylenchidae |
| cf. Helionema sp. MHMH-2008               | EU669913 |                | —        | 34.0/—                   | [19]      | Parasitylenchidae (genera dubia in Hexatylina) |
| cf. Hexatylus sp. Westplace               | AY912050 |                | —        | 12.9/—                   | Powers et al., unpublished. | Neotylenchidae |
| Nothotylenchus acris                      | AY593914 |                | —        | 34.0/—                   | [76]      | Anguinida     |
| Sphaerularia bombi                       | AB250212 |                | DQ328726 | 56.7/100.0              | Takahashi, unpublished. | Sphaerularioidea |
| Sphaerularia vespe                        | AB300595 | AB300595       | AB300596 | 54.7/100.0              | [77]      |               |
| Unidentified nematode 801L-022            | EUS80129 |                | —        | 12.1/—                   | [74]      |               |

**Anguinata**

| Name                                      | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8] |
|-------------------------------------------|----------|----------------|----------|-------------------------|-----------|---------------|
| Anguina tritici                           | AY593913 | JF826515       | H0058555 | DQ328723                | 57.6/92.9 |               |
| Ditylenchus adasi                         | EU669909 |                | —        | 34.6/—                   | [19]      |               |
| Ditylenchus angustus                      | AJ966483 |                | —        | 34.6/—                   | [16]      |               |
| Ditylenchus destructor                     | JX162205 |                | —        | 50.0/99.5               | [78]      | Anguinida     |
| Ditylenchus dipsaci                       | AY593911 | AY593911       | JF327759 | 60.9/100.0              | Zhao 2011, unpublished. |               |
| done NTS.28S.061A.2_b4                    |          |                |          |                         | [79]      |               |
| Ditylenchus drepanocercus                 | JQ429768 | JQ429774       | JQ429772 | 48.7/89.3               | [80]      |               |
| Ditylenchus halicus                       | AY589297 |                |          | 52.8/97.3               | [68]      |               |
| Ficoylus congestae                        | EU018049 |                |          | 45.6/97.5               | [81]      |               |
| Halenchus fucicola                        | EU669912 |                |          | 34.6/—                   | [19]      |               |
| Pseudhalenchus minutus                    | AY284638 |                |          | 34.6/—                   | [19]      |               |
| Unidentified entomoparasitic nematode SAS-2006 | —        | —              | DQ328725 | —/85.6                   | [20]      |               |

**“Tylenchina”: Tylenchidae**

| Name                                      | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8] |
|-------------------------------------------|----------|----------------|----------|-------------------------|-----------|---------------|
| Aglenchus agricola                        | FJ969113 |                | —        | 46.0/—                   | van Megen et al., unpublished. | Tylenchidae |
| Aglenchus sp.                             | —        | —              | JQ004996 |                         | [82]      |               |
| Name                           | 18S rRNA | ITS-5.8S rRNA | 28S rRNA | %, SSU-ITS-5.8S-LSU/D3 | Reference | Family by [8] |
|-------------------------------|----------|---------------|----------|------------------------|-----------|--------------|
| Coslenchus costatus           | AY284581 | —             | —        | 45.5/-                 | [18]      |              |
| Coslenchus sp.                | —        | —             | JQ005007 | 46.4/-                 | [82]      |              |
| Filenchus annulatus           | JQ814880 | —             | JQ005017 | 44.1/-                 | [82]      |              |
| Tylenchus davainei            | AY284588 | —             | —        | 33.9/-                 | [18]      |              |

**“Tylenchina”: Tylodoridae**

| Eutylenchus excretorius       | EU915487 | EU915500      | EU915490 | 35.8/-                 | [83]      | Atylenchidae |
| Cephalenchus hexalineatus     | AY284594 | —             | —        | 44.1/-                 | [18]      | Tylenchidae  |

**“Tylenchina”: Boleodoridae**

| Basiria gracilis             | EU130839 | —             | —        | 44.6/-                 | [84]      |              |
| Basiria sp. 3 TJP-2012       | —        | —             | JQ004998 | 12.0/-                 | [82]      |              |
| Boleodorus thy lactus         | AY993976 | —             | —        | 46.7/-                 | [16]      | Tylenchidae  |
| Boleodorus sp.               | —        | —             | JQ005001 | 46.7/-                 | [18]      |              |
| Neopsislenchus magnidens     | AY284585 | —             | —        | 45.6/-                 | [18]      |              |
| Neopsislenchus sp. 3 TJP-2012 | —      | —             | JQ005020 | 11.9/-                 | [82]      |              |
| Neopsislenchus sp. 1 TJP-2012 | —      | —             | JQ005018 | 11.9/-                 | [82]      |              |

**“Hoplolaimina”: Merliiidae**

| Nagelus leptus               | —        | —             | —        | 45.2/-                 | [20]      | Telotylenchidae |
| Nagelus obscurus             | EU306350 | —             | —        | 45.2/-                 | [17]      | Telotylenchidae |
| Pratylenchoides ritteri     | AJ966497 | —             | JX261964 | 48.7/-                 | [16]      | Pratylenchidae |
| Psilenchus cf. hilarus       | AY284593 | —             | EU915489 | 44.1/-                 | [18]      | Psilenchidae  |
| Scutylenchus quadriser       | AY284599 | —             | —        | 41.5/-                 | [18]      | Telotylenchidae |
| Scutylenchus sp.             | —        | JQ069956      | —        | 41.5/-                 | [86]      | Telotylenchidae |

**“Tylenchina”: Ecyphadophoridae**

| Ecphyadophora sp. JH-2004     | AY593917 | —             | —        | 33.7/-                 | [76]      | Ecyphadophoridae |
| “Ditylenchus” brevicauda     | AY284635 | —             | —        | 33.9/-                 | [18]      | Anguinidae     |
| Malenchus andrassi          | AY284587 | —             | —        | 32.3/-                 | [18]      | Tylenchidae    |
| Ottolenchus discrepans       | AY284590 | —             | —        | 33.7/-                 | [18]      | Tylenchidae    |

**Criconematina**

| Hemicricemonoides gaddi     | —        | KC520471      | KC520470 | 55.6/-                 | [87]      | Criconematidae |
| Hemicricemonoides pseudobrachyurus | AY284622 | —             | —        | 33.7/-                 | [18]      |              |
| Hemicyclophora latosa       | —        | GQ406237      | GQ406240 | 53.2/-                 | [88]      | Hemicyclophoridae |
| Hemicyclophora thienemanni  | AY284628 | —             | —        | 53.2/-                 | [18]      |              |
Table 3: Continued.

| Name                              | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference                           | Family by [8]                |
|-----------------------------------|----------|----------------|----------|-------------------------|-------------------------------------|-------------------------------|
| *Meloidoderita kirjanovae*       | —        | DQ768427       | —        | 50.8/—                  | van Megen, unpublished. [89]         | Sphaeronematidae             |
| *Sphaeronema alni*               | FJ969127 | —              | —        | —                       | [90]                                 |
| *Meloidoderita sp.*              | GU253916 | GU253917       | JQ771954 | 50.8/—                  | Cudejkova and Cermak, unpublished. [91] [92] |
| *Tylenchulus semipenetrans*      | AJ966511 | FJ588909       | FJ969710 | 57.5/—                  |                                     | Tylenchulidae                |

**“Hoplolaimina”: Belonolaimidae**

| *Belonolaimus longicaudatus*     | AY633449 | DQ672366       | GQ896548 | 55.8/—                  | [93] [94]                           | Belonolaimidae               |
| *Ihiosoma lolii*                 | JQ771535 | —              | —        | 30.9/—                  | [95]                                 |

**“Hoplolaimina”: Hoplolaimidae**

| *Carphodorus sp.*                | JQ771538 | —              | JQ771550 | 41.3/—                  | [95]                                 |
| Name           | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference                     | Family by [8] |
|---------------|----------|---------------|----------|-------------------------|-------------------------------|--------------|
| *Globodera pallida* | EU855119 | EU85511      |          | 93.6/—                  | Nowaczyk et al., unpublished. | Heteroderidae |
|               |          |              | BM415342 |                         |                               |              |
|               |          |              | BM415248 |                         |                               |              |
|               |          |              | CV577211 |                         |                               |              |
|               |          |              | CV577977 |                         |                               |              |
|               |          |              | CV579301E|                         |                               |              |
|               |          |              | U85511   |                         |                               |              |
|               |          |              | AF133304 |                         |                               |              |
|               |          |              | AF216579 |                         |                               |              |
|               |          |              | BI704144 |                         |                               |              |
|               |          |              | BI704144 |                         |                               |              |
|               |          |              | BI749520 |                         |                               |              |
|               |          |              | CA940190 |                         |                               |              |
|               |          |              | CA940212 |                         |                               |              |
|               |          |              | CA940243 |                         |                               |              |
|               |          |              | CA940406 |                         |                               |              |
|               |          |              | CA940424 |                         |                               |              |
|               |          |              | CA940429 |                         |                               |              |
|               |          |              | CA940589 |                         |                               |              |
|               |          |              | CB238697 |                         |                               |              |
|               |          |              | CB279977 |                         |                               |              |
|               |          |              | CB299455 |                         |                               |              |
|               |          |              | CB373844 |                         |                               |              |
|               |          |              | CB373981 |                         |                               |              |
|               |          |              | CB379125 |                         |                               |              |
|               |          |              | CB379140 |                         |                               |              |
|               |          |              | CB379219 |                         |                               |              |
|               |          |              | CB379312 |                         |                               |              |
|               |          |              | CB379439 |                         |                               |              |
|               |          |              | CB379505 |                         |                               |              |
|               |          |              | CB379696 |                         |                               |              |
|               |          |              | CB379707 |                         |                               |              |
|               |          |              | CB379996 |                         |                               |              |
|               |          |              | CB380091 |                         |                               |              |
|               |          |              | CB380241 |                         |                               |              |
|               |          |              | CB38041  |                         |                               |              |
|               |          |              | CB824788 |                         |                               |              |
|               |          |              | CB824878 |                         |                               |              |
|               |          |              | CB825995 |                         |                               |              |
|               |          |              | CB934877 |                         |                               |              |
|               |          |              | CB934931 |                         |                               |              |
|               |          |              | CB934950 |                         |                               |              |
|               |          |              | CB934954 |                         |                               |              |
|               |          |              | CK348525 |                         |                               |              |
|               |          |              | CO036619 |                         |                               |              |
|               |          |              | HM560850 |                         |                               |              |
|               |          |              | JN684906 |                         |                               |              |
| *Heterodera glycines* |          |              | AF216579 | 98.3/—                  | Yan and Davis, unpublished.  |              |
|               |          |              | BI704127 |                         |                               |              |
|               |          |              | BI748392 |                         |                               |              |
|               |          |              | CA940548 |                         |                               |              |
|               |          |              | CB379240 |                         |                               |              |
|               |          |              | CB379263 |                         |                               |              |
|               |          |              | CB379850 |                         |                               |              |
|               |          |              | CB380242 |                         |                               |              |
|               |          |              | CB825296 |                         |                               |              |
|               |          |              | CB825409 |                         |                               |              |
|               |          |              | CB825970 |                         |                               |              |
|               |          |              | CB935610 |                         |                               |              |
|               |          |              | CK348871 |                         |                               |              |
|               |          |              | CK348904 |                         |                               |              |
|               |          |              | CK349775 |                         |                               |              |
|               |          |              | CK352112 |                         |                               |              |
| *Morulaimus sp.*   | JQ771540 | —             | —        | 31.5/—                  | [95] Belonolaimidae            |              |
| Name            | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference              | Family by [8] |
|-----------------|----------|---------------|----------|-------------------------|------------------------|---------------|
| Radopholus similis | AJ966502 | AY912509      | EF384224 | 97.5/—                  | [16] Longet al., unpublished. | Pratylenchidae |
|                 | EY191076 | EY191197      | EY191883 |                         | [100] Holterman et al., unpublished. |
|                 | EY192786 | EY193123      | EY193253 |                         | [102] Zhao unpublished. |
|                 | EY194340 | EY194464      | EY194646 |                         | [86]                   |
|                 | EY195472 | EY195408      | EY195406 |                         |                        |

*Table 3: Continued.*
Table 3: Continued.

| Name                        | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference                      | Family by [8]        |
|-----------------------------|----------|----------------|----------|-------------------------|--------------------------------|----------------------|
| Rotylenchulus reniformis    | JX406356 | FJ374686       | HML31884 | 59.4/—                  | [103] Rahman et al., unpublished. | Rotylenchulidae     |
| "Hoplolaimina": Pratylenchidae |          |                |          |                         |                                |                      |
| Dolichodorus sp. WY-2006    | DQ912918 | —              | —        | 33.9/—                  | [105] Dolichodoridae           |                      |
| Hirschmanniella loofi       | EU306353 | EU620472       | EU620469 | 51.6/—                  | [17] Pratylenchidae            |                      |
| Macrotrophurus arbusticola  | AY284595 | —              | —        | 33.9/—                  | [18] Telotylenchidae           |                      |
| Meloidogyne arenaria        | U42342   | U42342         | AF023855 | 99.2/—                  | Georgi and Abbott, unpublished.| Meloidogynidae       |
| Meloidogyne artiellia       | AF248477 | AF248477       | AF248477 | 99.2/—                  | [107]                          |                      |
| Name                      | 18S rRNA | ITS1-5.8S rRNA | 28S rRNA | %, SSU-ITS1-5.8S-LSU/D3 | Reference | Family by [8] |
|---------------------------|----------|----------------|----------|-------------------------|-----------|---------------|
| *Nacobbus aberrans*      | AJ966494 | DQ017473       | U47557   | 49.0/—                  | [16]      | Pratylenchidae |
|                           |          |                |          |                         |           |               |
|                           |          |                |          |                         | [108]     |               |
|                           |          |                |          |                         | [109]     |               |
|                           | BQ580554 | CV198923       | CV198995 | CV199233                |           |               |
|                           | CV199349 | CV199490       | CV200036 | CV200423                |           |               |
|                           | CV200464 | CV200467       | CV200471 | CV200530                |           |               |
|                           | CV200687 | CV200896       | CV201004 | CV201135                | [19]      |               |
|                           |          |                |          |                         | [110]     |               |
|                           |          |                |          |                         | [96]      |               |
|                           |          |                |          |                         | [111]     |               |
| *Pratylenchus vulnus*    | EU669955 | JQ966892       | 100.0/—  |                         | [17]      | Zhao, unpublished. |
|                           |          |                |          |                         |           |               |
|                           |          |                |          |                         | [19]      |               |
|                           |          |                |          |                         | [110]     |               |
|                           |          |                |          |                         | [96]      |               |
|                           |          |                |          |                         | [111]     |               |
|                           |          |                |          |                         |           |               |
| *Tylenchorhynchus dubius*| EU306352 | —              | DQ328707 | 53.2/—                  | [17]      | Telotylenchidae |
| *Tylenchorhynchus zeae*  | —        | EF519711       | —        |                         | [20]      |               |

* Clades of the tree, marked by boldface.
up to 16 specimens per flea were observed. As in other entomoparasitic nematodes, the propagation rate depends on the host age. Thus, in young fleas up to 10 juveniles was found per flea specimen, whereas up to 1,000 juveniles of different stages were contained in some old fleas (Figure 2). After the 2nd molt the number of juveniles is maximal, and 3rd stage juveniles massively migrate to the rectal section of the flea intestine for exit to the environment. In some cases, the observed infestation level was so high that nematodes penetrated distal segments of the flea legs, from where they have no way to the environment.

3.2. Morphological Analysis of Entomoparasitic Stages in Nematode Isolates and Their Taxonomic Identification. Analysis of morphology of entomoparasitic stages suggests that the studied nematode isolates from three distinct groups. A single generation of parasitic females was observed in the first two groups and an additional parthenogenetic generation—in the third group. According to morphometric data on adult parasitic females (Tables 4–6), the first two groups belong to the genera Rubzovinema or Spilotylenchus and the third group to the genus Psyllotylenchus. Photographs of parasitic females of Rubzovinema sp., Spilotylenchus sp., and Psyllotylenchus sp. are depicted in Figure 3. Figure 4 shows their distribution among flea samples studied.

According to morphometric evidence, parasitic females and juveniles of the genera Rubzovinema and Spilotylenchus are very similar. However, in the first two groups of isolates we found characters bearing discriminative and identification value. In particular, the oesophageal glands in juveniles III of the first group are poorly developed. This is a distinctive feature of the genus Rubzovinema, where males and females have shortened oesophageal glands located close to the nerve ring. In the second group of isolates, oesophageal glands are well developed and elongated, which is characteristic of the genus Spilotylenchus. In the first group, the stylet possesses a heavily sclerotized distal spear with a length of approximately half the total stylet length and has a stem with a weaker sclerotization and widening to the base. This stylet structure is characteristic of the genus Rubzovinema, and stylet length (18.5 (14–22) μm) is in accordance with morphometrics given in the description of this genus [26]. In the genus Spilotylenchus, the stylet varies in shape but always possesses a shortened conical distal spear. In the second group of isolates, the stylet structure was similar to that of Spilotylenchus. Also, the vulval lips of the first group are more protruded than in Spilotylenchus. Other features, including the morphometrics, vary widely in both genera, which hampers taxonomic identification. Nevertheless, based on distinctive traits, we identified the first and second group of isolates as Rubzovinema sp. and Spilotylenchus sp., respectively.

In the genus Rubzovinema, the single species described to date is Rubzovinema ceratophylla [26]. This species is known to parasitize exclusively the flea Citellophilus tesquorum that feeds on soulsiks. The specimens of Rubzovinema studied in this work were isolated from five flea species, C. tesquorum, Neopsylla setosa, Frontopsylla semora, Amphipsylla rossica, and Ctenophthalmus secundus, of which the latter two were sampled on mouse-like rodents. Also, the parasitic females of Rubzovinema sp. differed from R. ceratophylla by morphology; they have a shorter tail and more protruded vulval lips. A morphometric comparison of Rubzovinema sp. and R. ceratophylla is given in Table 4.

The parasitic females of Spilotylenchus sp. were isolated from the flea Mesopsylla hebes associated with jerboas. The females were not identified to the species level because of a small number of available specimens and the lack of a free-living stage. A morphometric comparison of Spilotylenchus sp. and the morphologically closest species Spilotylenchus maisonabei [23] is given in Table 5.

In the genus Psyllotylenchus, descriptions of most species are fragmentary and incomplete, which precluded the species identification of the Psyllotylenchus isolates from the fleas N. setosa and F. semura feeding on soulsiks. A morphometric comparison of Psyllotylenchus sp. and the type species of this genus, Psyllotylenchus viviparous [25], is given in Table 6.

The 18S and 28S rDNA sequences of Rubzovinema sp. specimens from A. rossica and C. secundus were 100% identical, which indicates that the isolates belong to the same species. The sequences of Rubzovinema sp. ex C. tesquorum, Rubzovinema sp. ex N. setosa, and Rubzovinema sp. ex F. semura diverged from one another and from the gene sequences of Rubzovinema sp. ex A. rossica and Rubzovinema sp. ex C. secundus by 0.4–0.7%, which corresponds to the levels of intraspecific variation [14, 114–119]. The 18S and 28S rDNA sequences of Spilotylenchus sp. ex N. setosa and Psyllotylenchus sp. ex F. semura were 100% identical, indicating that they belong to the same species. The 18S and 28S rDNA sequences of Rubzovinema sp. and Psyllotylenchus sp. diverge by 1.2% and 1.9%, respectively. Those of Spilotylenchus sp. ex M. hebes were found to be more divergent. The degree of divergence of the 18S rDNA sequence of Spilotylenchus sp. ex M. hebes from those of either Rubzovinema sp. or Psyllotylenchus sp. was 2.4%; the D3 expansion segment of 28S rDNA diverged by 13.1% and 12.0%, respectively. The observed divergence rate of rDNA sequences agrees well with published evidence on entomoparasitic nematodes [14, 114–118]. Thus, intraspecific divergence of 18S rDNA in Deladenus siricidica is 1% [120], of D2 and D3 expansion segments in the phytoparasite Bursaphelenchus xylophilus is from 0% to 0.6%, and the interspecific variation between the
Figure 3: Parasitic females of the studied nematode species. (a) *Rubzovinema* sp., heterogeneous female; (b) *Spilotylenchus* sp., heterogeneous female; (c) *Psillotylenchus* sp., heterogeneous female of the first generation; (d) (c): *Psillotylenchus* sp., parthenogenetic female of the second generation. Scale bar—200 μm.

Table 4: Comparison of morphometrics in parasitic females of *Rubzovinema* sp. and *Rubzovinema ceratophylla*.

| Character                              | *Rubzovinema* sp. (this study) | *Rubzovinema ceratophylla* [26] |
|----------------------------------------|--------------------------------|---------------------------------|
| N                                      | 29                             | 27                              |
| L                                      | 1278,6 (840–1570)              | 1265,1 (810–1840)               |
| D                                      | 120,8 (85–145)                 | 137,3 (62–200)                  |
| A                                      | 11,19 (7,9–16,1)               | 9,51 (6,4–16,8)                 |
| C                                      | 65,4 (31,4–100)                | 44,10 (10–86,4)                 |
| V%                                     | 96,4 (93,1–97,9)               | 95,44 (92–98,9)                 |
| Total length of stylet (St)            | 18,5 (14–22)                   | 19,5 (18–21)                    |
| Length of distal edge of stylet        | 7,2 (5–8,7)                    | —                               |
| Distance between anterior end and excretory pore (Ex) | 20,7 (10–31) | —                               |
| Distance between anterior end and nerve ring | 61,2 (50–74,5) | —                               |
| Total length of tail (Cd)              | 21,9 (10–42)                   | 26,35 (14–47,5)                 |
| Distance between vulva and tail end    | 46,1 (23–75)                   | —                               |
| Distance between vulva and anus (V–A)  | 26,9 (13–40)                   | —                               |

All measurements are in μm and in the form mean (range).
Table 5: Comparison of morphometrics of parasitic females in *Spilotylenchus* sp. and *Spilotylenchus maisonabei*.

| Characters | *Spilotylenchus* sp. (this study) | *Spilotylenchus maisonabei* [23] |
|-----------|----------------------------------|---------------------------------|
| N         | 2                                | 6                               |
| L         | 1,600–1,840                      | 1,244 (1,200–1,320)             |
| D         | 155–160                          | 125 (107–160)                   |
| A         | 10.3–11.5                        | 10.3 (7.5–12)                   |
| C         | 167.3–177.8                      | 84.4 (64.5–121)                 |
| V%        | 97.4–97.7                        | 96.2 (95.8–96.5)                |
| Total length of stylet (St) | 9.5–9.8 | 9.10 |
| Distance between anterior end and excretory pore | 1.5–15.5 | 23.3 (20–28) |
| Distance between anterior end and nerve ring | — | 52–54 |
| Total length of tail (Cd) | 9–11 | 15.4 (10–19) |
| Distance between vulva and tail end | 41.5–43 | 47 (42–52) |
| Distance between vulva and anus (V–A) | 32–33 | — |

All measurements are in μm and in the form mean (range).

Table 6: Comparison of morphometrics of parasitic females in *Psyllotylenchus* sp. and *Psyllotylenchus viviparous*.

| Character | *Psyllotylenchus* sp. (this study) | *Psyllotylenchus viviparous* [25] |
|-----------|----------------------------------|---------------------------------|
| Gamogenetic | Parthenogenetic | Gamogenetic | Parthenogenetic |
| N         | 3                                | 7                                | 8                  | 10                |
| L         | 1,016.7 (900–1,100)              | 446 (420–500)                    | 1,000 (840–1,480)  | 500 (360–840)     |
| D         | 81.3 (79–84)                     | 70 (60–80)                       | 77 (62–115)        | 60 (54–100)       |
| A         | 12.5 (11.1–13.3)                 | 6.25 (5.6–7)                     | —                  | —                 |
| C         | 64.3 (60–68.2)                   | 40.15 (37.1–43.5)                | —                  | —                 |
| V%        | 95.1 (95–95.4)                   | 93.3 (90–95.3)                   | —                  | —                 |
| Total length of stylet (St) | 17.5 (17–18.5) | 5.25 (4–6) | 17 (15–20) | 7 (5–8) |
| Length of the distal edge of stylet | 8.6 (8–9) | — | — | — |
| Distance between anterior end and excretory pore | 26.5 (25–31.5) | 17.5 (15–19.5) | 23 (13–33) | 22 (14–46) |
| Distance between anterior end and nerve ring | — | 51.7 (50–55) | — | — |
| Total length of tail (Cd) | 15.8 (15–17) | 11.1 (10.5–11.5) | 25 (17–35) | 9 (1–17) |
| Distance between vulva and tail end | 48 (45–51) | 30.5 (19.7–55) | 56 (37–71) | 52 (40–104) |
| Distance between vulva and anus (V–A) | 30.8 (29–31.5) | 13.5 (11.7–21.6) | — | — |

All measurements are in μm and in the form mean (range).

phytoparasites *B. xylophilus* and *Bursaphelenchus mucronatus* is from 1.7% to 3.7%. The spacers ITS1 and ITS2 are generally more diverged; the intra- and interspecific variation for these species is from 0 to 3.1% and 11.2 to 13.4%, respectively [121–123].

Molecular vouchering is proved to efficiently complement morphological species identification in nematodes [73, 122, 124–128]. Combining the rDNA and morphological data confirms the species identity within each of the three studied groups of isolates.

3.3. Phylogenetic Analysis. In phylogenetic analyses of rDNA we used a dataset with extensive species and gene sampling (SSU-ITS1-5.8S-LSU) compared to earlier published tylenchid phylogenies, most of which were based on SSU rDNA or D2-D3 expansion segments [17, 19–21, 39, 129]. The SSU-ITS1-5.8S-LSU rDNA tree topology (Figure 5) is highly similar to other published phylogenies of tylenchids. In this tree, tylenchomorphs are represented by the sister groups Aphelenchidae and Tylenchida. Most of the tylenchid clades occur in published trees but often contradict classifications based on morphology, as it was also noted by other authors [17, 19–21, 39, 129]. The three robust major branches in the SSU-ITS1-5.8S-LSU rDNA tree (Bayesian posterior probabilities of 0.99–1.0) are (1) the clade includes representatives of the suborders Holoilaimina, Criconematina, and Tylenchina (excluding Anguinoidae); (2) the majority of classic Anguinata; (3) the suborder Hexatylina. The studied parasites of fleas form a monophyletic group (bootstrap support of 100%) within the Hexatylina.

The nonredundant rDNA data on the Hexatylina in GenBank mostly represents the D2-D3 expansion segments of LSU rDNA. To maximize species sampling of the Hexatylina, we chose the D3 expansion segment as the molecular marker. The phylogenetic tree with the Anguinoidea as an outgroup is shown in Figure 6. In this tree, the suborder Hexatylina consists of two well-supported clades, in accordance with previously published D2-D3 rDNA phylogenies [19, 20, 39]. The clade of the studied flea parasites is placed within the
largest branch of the Hexatylina, similarly to the result of the concatenated rDNA analysis.

The three alternative relationships between the three major branches of Tylenchida (Figure 5) are not discriminated by the AU and Kishino and Hasegawa tests, and only the basal position of the Hexatylina is rejected by the expected-likelihood weights test (Table 7). All three tests do not discriminate between the alternative placement of the flea parasites as closest to the Allantonema, Parasitylenchus, or Deladenus branches; however, its positioning outside this grouping is not rejected only by a less conservative Shimodaira-Hasegawa test [50].

4. Discussion

4.1. Ribosomal DNA Phylogeny of the Tylenchida and Relationships within the Suborder Hexatylina. Phylogenetic analyses of SSU [16, 17, 19, 39] and D2-D3 [20, 39] rDNA data using various methods and species sampling generally agree on the monophyly of most tylenchid clades and contradict classic morphology based classifications. In the SSU-ITS-5.8S-LSU tree (Figure 5), the monophyletic Tylenchida consists of three major robust clades. The first clade diverges into six groups: (1) the “Tylenchidae (part 2)” (by [17]), (2) the Tylodoridae (represented by the two genera, Cephalenchus and Eutylenchus [83]), (3) Boleodorinae + “Tylenchidae (part 1)” (by Bert), (4) the Merliniidae [130], (5) Criconematina + Sphaeronomatidae + selected Tylenchina, and (6) Belonolaimidae + “Hoplolaimina.” The Merliniidae group corresponds to Clade C in [19] and includes partially the polyphyletic “Telotylenchinae” [131], “Pratylenchidae”, and “Hoplolaimina” (Psilenchus cf. hilarulus). Group (5) corresponds to Clade 12A in [129], where Sphaeronomatidae (Sphaeronomes and Meliododerita) were earlier shown to be closely related to Criconematina [20, 89], and selected Ephydorphoridae + Otollenchus + Malenchus were found to represent a monophyletic clade within the paraphyletic Tylenchina likely to be related to the Criconematina [18, 82]. Group (6) corresponds to Clade VII in [20], Clade 12B in [129], and Clade A + Clade B in [19]. Belonolaimidae (the genera Belonolaimus and Ibiporta) tend to occupy the basal position. Clade A in [19] contains a “long branch” of the burrowing nematode Radopholus similis (“Pratylenchidae”) in sister position to the Hoplolaimidae [17, 19]. This nematode occurs a similar position relative to the Hoplolaimidae in the SSU-ITS-5.8S-LSU tree, and we consider this unlikely to be an LBA artefact. Similarly to [95], Carphodorus and Morulaimus that belong to the classic Belonolaimidae comprise the basal branch of Clade A sensu [19]. The clade corresponding to Clade B in [19] contains Meloidogyneidae, Dolichodoridae, paraphyletic Pratylenchidae, and a part of Telotylenchidae.

The second major clade of the Tylenchida includes representatives of the classic infraorder Anguinata, with a well-supported monophyletic origin, except for a few species. They belong outside the second clade and may initially have been wrongly identified.

The third major clade includes representatives of the classic suborder Hexatylina and consists of two groups. The smaller one unites the three species of Sphaerularia, Helionema sp., cf. Hexaylus sp., Deladenus sp. PDL-2005, and Nothotylenchus acris (Anguinata: Nothothylenchidae). It is further referred to as the Sphaerulariioidea according to the type genus. The larger group contains the clade of studied flea parasites and members of the superfamilies lotonchioidea (Skarbilovinema spp., Parasitylenchus spp., and Wachekitylenchus bovieni) and Sphaerulariioidea (Allantonema mirabile, Brarynema spp., Howardula spp., and Contortylenchus sp. (fam. Allantonematidae); Deladenus durus, Deladenus proximus, Deladenus siricidica, Fergusobia spp., and Gymnotylenchus sp. (fam. Neotylenchidae)). One species of the Anguinata, Sychnotylenchus sp., also joins the larger group. Our study renders the genera Howardula and Deladenus paraphyletic, as was earlier shown in [19, 39, 71, 119].

The genus Howardula is paraphyletic in published rDNA and mitochondrial COI phylogenies [71]. Such characters of Howardula as the degeneration of oesophagus, tail shape, and the absence of stylet in males seem to have evolved independently by convergence. The paraphyletic genus Deladenus is more closely related to either ancestral forms of the Hexatylina or forms typical to the Anguinata. The infraorder Anguinata includes soil-dwelling nematodes, mostly mycetophagous or parasitizing various parts of plants. However, an unidentified entomoparasitic nematode was also grouped within the Anguinoida [39]. The life cycle of Deladenus spp. is an irregular alternation of free-living and entomoparasitic forms. The nematode D. siricidica is able of producing an unlimited number of free-living generations in the absence of the host larvae of siricid
Primarily entomoparasitic, most with free-living mycetophagous or plant-parasitic generation
Obligate parasites of plant roots
Feeding on algae, mosses, and fungi; parasites of plants
Mostly mycetophagous
Bacterial feeding

Figure 5: Phylogenetic tree of Tylenchida, inferred from SSU-ITS1-5.8S-LSU rDNA sequences. Topology was inferred using the PhyloBayes software (maxdiff = 0.36). Node support values are shown as follows: the first two values are Bayesian posterior probability assessed using the PhyloBayes and MrBayes software, respectively, and the third is bootstrap support assessed by the ML method. Thick lines lead to the nodes, in which at least one support value of posterior probability is 0.95 and higher. Names of clades (framed) are mainly given by type genus included in them (with the exception of Itonchioidea). Formal taxonomic position (family by [8]) is shown on the right to the color bar. Colors indicate the ecologies (see the legend). Names of the species of Hexatylinea that have a mycetophagous stage in their life cycle are shown in blue. The three robust major branches of Tylenchida are marked by gradient.
Primarily entomoparasitic, most with free-living mycetophagous or plant-parasitic generation

Feeding on algae, mosses, and fungi; parasites of plants

Type genera

Rubzovinema sp. ex Neopsylla setosa
Rubzovinema sp. ex Ctenophthalmus cecundus
Rubzovinema sp. ex Frontopsylla semura
Rubzovinema sp. ex Amphipsylla rossica
Splotylenchus sp. ex Mesopsylla hbees
Psilomylenchus sp. ex Frontopsylla semura
Psilomylenchus sp. ex Neopsylla setosa

Bradynema listronoti
Bradynema rigidum
Allantonema mirabile

entomoparasitic nematode CD289

Howardula phyllocretae
Parasitylenchus sp.

Deladenus siricidicola isolate 354

Skarbolvinema laumondi
Skarbolvinema lyoni

entomoparasitic nematode sp. WY2009 BAR-1

Contortylenchus sp.

entomoparasitic nematode from Chrysobothris affinis

Figure 6: Phylogenetic tree of Hexatylina, inferred from D3 expansion segment of LSU rDNA. Topology was inferred using the PhyloBayes software. Node support values are shown as follows: Bayesian posterior probability/bootstrap support assessed by the ML method. Thick lines indicate the nodes supported at the level of 0.95 and higher. Color of lines indicates the ecologies (see the legend). Names of species were shown in different colors indicating their taxonomic position. Three families that include their type genera (shown as circles) are marked by gradient.
pine-killing wood wasps [132]. Like in Anguinata, the free-living forms of Deladenus spp. are fungal feeding. Such characters of Deladenus asthe mycetophagy, enlargement of subventral glands in entomoparasitic females versus their reduction in free-living forms, the hypertrophy of dorsal glands, and stylet reduction in free-living forms seem to be symplesiomorphic. Resemblance with the Anguinata is also typical of other mycetophagous free-living forms: Hexatylus (Neotylenchidae), Rubzovinema (Neotylenchidae), Prothallonema (Sphaerularioidae) Helionema (Hexatylina dubia), and Pauroidontidae. For the latter, the entomoparasitic stage is expected but has never been observed. The relationship between the Hexatylina and Anguinata was earlier hypothesized based on morphology [7, 8, 130, 133, 134]. On rDNA phylogenies of tylenchids, the monophyly of the Hexatylina + Anguinata is either supported [19] or not rejected [20]. In the SSU-ITS-5.8S-LSU rDNA tree obtained in this study, the monophyly of the Hexatylina + Anguinata has the Bayesian posterior probability of 0.91, but the maximum-likelihood bootstrap support is low; the AU and Kishino and Hasegawa tests did not discriminate between alternative hypotheses.

According to our SSU-ITS-5.8S-LSU rDNA phylogeny (Figure 5), the major robust branches of the Tylenchida are incongruent with morphology-based classifications suggesting three rather than four suborders (the rank is adopted from morphological systems of tylenchids). Among them, the Hexatylina and Anguinata (both are monophyletic) are likely to be sister groups. The third emerged suborder includes representatives of three classic suborders: Tylenchina, Hoplo-laimina, and Criconematina, among which only the latter does not contradict morphology-based classifications.

Considering ecological traits coded in Figure 5, the mycetophagy and/or facultative ectophytoparasitism are likely to be ancestral in the Tylenchida. Sedentary phytoparasites (root-knot species of Meloidogyne, the false root-knot genus Nacobbus, and cyst-forming Heteroderas and Globodera) and other obligate endoparasites of plants evolved several times from free-living or facultative sedentary forms, as it was previously hypothesized in accordance with the concept of evolutionary trend to endoparasitism in phytonematodes [135]. Similarly, obligate endoparasites of insects from the Hexatylina are likely to have evolved from mycetophagous forms, with some species retaining the ancestral mycetophagous stage in the life cycle (e.g., species of the paraphyletic genus Deladenus and flea nematodes of the genus Rubzovinema). An interesting specific case in the Hexatylina is the genus Fergusobia that includes plant parasites associated with insects [68, 70], which may have transited to plant parasitism via entomoparasitism [39].

### Table 7: Results of tree topology tests for alternative hypotheses on (1) the initial divergence of Tylenchida (Figure 4) and on (2) the relationships within the monophyletic branch that includes the studied group of nematodes parasitizing fleas (designated by asterisk).

| Topology | Rank | obs | au | np | bp | pp | kh | sh | c-ELW |
|----------|------|-----|----|----|----|----|----|----|-----|
| (((H,An),T),o) | 1 | -1.8 | 0.787 | 0.415 | 0.402 | 0.804 | 0.663 | 0.969 | 0.4197 |
| (((An,(H,T)),o),o) | 2 | 4.1 | 0.326 | 0.198 | 0.205 | 0.013 | 0.254 | 0.623 | 0.1848 |
| (((H,An,T)),o) | 3 | 6.9 | 0.061 | 0.013 | 0.014 | 0.001 | 0.101 | 0.492 | 0.0186 |
| (((*,(Al,P),Ds),o),o) | 1 | -1.8 | 0.787 | 0.415 | 0.402 | 0.804 | 0.663 | 0.969 | 0.4197 |
| (((*,(Al,P),Ds),o),o) | 2 | 1.8 | 0.495 | 0.242 | 0.247 | 0.130 | 0.337 | 0.813 | 0.2249 |
| (((*,(Al,P),Ds),o),o) | 3 | 2.7 | 0.371 | 0.110 | 0.105 | 0.052 | 0.243 | 0.824 | 0.1209 |
| (((*,(Al,P),Ds),o),o) | 6 | 15.7 | 0.063 | 0.024 | 0.025 | 1e−007 | 0.053 | 0.153 | 0.0272 |
| (((*,(Al,P),Ds),o),o) | 7 | 18.3 | 0.013 | 0.002 | 0.002 | 9e−009 | 0.020 | 0.096 | 0.0028 |

Al: Allantonematidae, An: Anguinata, Ds: Deladenus siricidicola—D. proximus group, H: Hexatylina, P: Parasitylenchidae, T: Tylenchina, o: outgroup.

4.2. Ribosomal DNA Phylogeny of the Flea Nematodes and Their Classification. The nematodes of fleas do not group with the families known as their relatives in morphology-based systems, as these families do not form monophyletic groups in the tree. However, they do group with both type genera of the families Parasitylenchidae and Allantonematidae (Parasylenchus and Allantonema, resp.). This grouping is preceded by a successive divergence of Deladenus dierus and Deladenus siricidicola (Figure 5). As mentioned above, the pronounced free-living form in Deladenus seems to be ancestral to this group.

Only 31 tylenchid species that parasitize in fleas have been described to date. They differ by morphology, life cycle, and the host specificity, and belong to the five genera: Spilotylenchus (8 species), Psyllotylenchus (20 species), Incurvinema (1 species) Kurochkintylenchus (1 species), and Rubzovinema (1 species). According to the classification of Siddiqi [8], the genera Spilotylenchus and Psyllotylenchus belong to the family Parasitylenchidae, whereas the genus Rubzovinema is a member of the Neotylenchidae. The two families represent two superfamilies, lotonchioidea and Sphaerularioidea, respectively. All rDNA phylogenies published to date suggest that these superfamilies are paraphyletic [19, 20, 39], which is also inferred in our study with an extensive gene and taxon sampling.

A high degree of rDNA similarity in the three studied species suggests a closer relationship of these species than that assumed by the accepted system of classification. Earlier, Slobodyanyuk proposed to unite all known flea parasites into one family, the Spilotylenchidae. Its four subfamilies, Spilotylenchinae, Rubzovinematinae, Psyllotylenchinae, and Kurochkintylenchinae, are discriminated based on the life
cycle features [28]. In Spilotylenchinae and Rubzovinemati-
nae, the entomoparasitic stage is represented by parasitic
females of one heterosexual generation. In Psyllotylenchinae,
in addition to the heterosexual generation, a parthenogenetic
generation occurs in the flea haemocoele. In Kurochkinity-
lenchinae, two heterosexual generations exist in the haemo-
coel: the first generation produces parasitic females and the
second generation produces both females and males [28].
Siddiqi also considered the unification of all flea tylenchids
into one family but observed the need for further evidence in
support [8].

Our results strongly suggest the inclusion of the three
genera, Rubzovinema, Psyllotylenchus, and Spilotylenchus, in
one family, the Spilotylenchidae [28]. The ribosomal DNA
genic distance within the family Spilotylenchidae is much
smaller than that of certain tylenchid genera, for example,
Meloidogyne (Figure 4) or Pratylenchus [19, 84].

4.3. Host Specificity of Flea Nematodes. The majority of
tylenchid nematodes are monoxenous or oligoxenous; in
particular, flea parasites were thought to be strictly host
specific. Earlier papers suggested the lack of strict host
specificity in Psyllotylenchus pawlowskyi and Psyllotylenchus
viviparous [13, 25]. However, later these species were found to
be heterogeneous and sustained revision [9, 27–29]. Spiloty-
lenchus pawlowskyi and Spilotylenchus caspius were referred
to as single-host parasites of the flea Coptosylla lamellifer
[27, 136]. Kurochkinitylenchus laevicepsi and Spilotylenchus
ivaskhini also share the same flea host, Nosopsylla laeviceps
[28, 29]. Before our study, the genus Rubzovinema was known
to contain a single species, Rubzovinema ceratophylla, which
parasitizes exclusively the flea Citellophilus tesquorum.

We found that at least two out of the three studied
species are not single-host parasites. Psyllotylenchus sp. was
shown to parasitize two flea species feeding on sousliks,
Frontopsylla semura and Neopsylla setosa. Rubzovinema sp.
was found on five flea species feeding on different rodent
hosts: C. tesquorum, F. semura, N. setosa (all sampled from
sousliks), Ctenophthalmus secundus, and Amphipsylla rossica
(all sampled from voles). A. rossica, F. semura, and C. tesque-
rum belong to different families of the superfamily Cerato-
philoidea (Leptopsyllidae and Ceratophyllidae), whereas C.
secundus and N. setosa belong to the superfamily Hystri-
chopsylloidea. Unlike the host-specific R. ceratophylla, the
studied Rubzovinema sp. parasitizes taxonomically distant
fleas feeding on different rodents. Thus, the common opinion
that flea nematodes are strictly host specific should be
revisited.

As the two species of Rubzovinema demonstrate, even
closely related parasites may exhibit different host range
size. Among other known examples are the entomoparasitic
nematodes of the genus Howardula parasitizing various
beetles and flies [71, 137, 138], many phytonematodes [8],
sibling species of parasitoid flies [128], and herbivorous
insects [139]. The host range of parasites is an indicator
of their evolutionary strategy in the ecosystem. Multihost
parasites can be considered ecological generalists, in contrast
to specialists that coevolve with a particular host. Generalists
and specialists play different roles in the ecosystem [140],
where they keep in balance, taking advantages and disadvan-
tages of the two strategies. The advantages of generalization
are yet to be explained by evolutionary biologists, whereas
advantages of specialization are obvious, and it is generally
accepted that evolution favors specialization [141, 142]. In
the flea parasites, this trend is demonstrated by a greater species
diversity of ecological specialists, the genera Spilotylenchus
and Psyllotylenchus.

Nevertheless, the generalist Rubzovinema sp. was most
abundant in the studied samples, which indicates that extend-
ing the host range may be evolutionarily successful. Besides
the need to combat the immune response of several hosts,
which is a requirement to widen the hosts range [143], the
free-living stage of Rubzovinema sp. is to adapt to diverse
microbioclimatic conditions of complex environments of
rodent habitats. Multihost parasites pay a cost of adapting
to alternative conditions [141, 144] compensated by stable
survival of the species. Considering the spatial and temporal
dynamics of flea populations feeding on a particular rodent
host (one or two flea species usually dominate over a sampling
season), multihost nematode parasites gain an advantage of
their relative independence of population waves of either flea
hosts or their rodent hosts. A higher infestation rate observed
for Rubzovinema sp., compared to the two other studied
species, may be an indicator of a greater ecological plasticity
of this multihost parasite.

4.4. Entomoparasitic Nematodes in Natural Foci of Plague. In
natural foci of plague, the epizootic dynamics are influenced
by numerous climatic and biotic factors. The spatial and
temporal population dynamics of the plague agent, Y. pestis,
afect the population dynamics of the flea vectors and their
mammalian hosts. Members of the transmission route of the
plague agent also closely interact with other living organisms.
For example, parasites of fleas that in turn feed on rodents
are hyperparasites that play the role of high-level control
agents on the ecosystem level, the role that entomoparasitic
nematodes share with the bacterial plague agent. High-
level control agents render the epidemiological state of a
natural focus of disease less predictable. On the one hand,
a lower density of the flea vector population reduces the
plague transmission rate; on the other, its growth causes
an exponential decay of the host rodent population [145]
below its epidemiological threshold, above which there is a
threat of spillover of plague infection into human population
[145]. Hypothetically, nematode-induced decrease of flea
population is able to increase the number of rodents above
the threshold and thus trigger an epidemic. The dual effect
of high-level control agents is well exemplified by cases,
when during plague episodes the extermination of rodents
are hyperparasites that play the role of high-level control
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level control agents render the epidemiological state of a
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a lower density of the flea vector population reduces the
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an exponential decay of the host rodent population [145]
below its epidemiological threshold, above which there is a
threat of spillover of plague infection into human population
[145]. Hypothetically, nematode-induced decrease of flea
population is able to increase the number of rodents above
the threshold and thus trigger an epidemic. The dual effect
of high-level control agents is well exemplified by cases,
when during plague episodes the extermination of rodents
by humans causes the return of infection through stimulating
the migration of fleas, the plague vectors [5].

The studied entomoparasitic nematodes possess high
potential as control agents of the flea vectors of plague
owing to their high propagation rate within the flea host (Figure 2) and high infestation level (up to 21% observed in
this study and from 50 to 60%, as estimated by other authors
One of the studied nematode species, *Rubzovinema* sp., is a multihost parasite. Host-specific parasites reach the optimal level of pathogenicity by maintaining the trade-off between pathogenicity and transmissibility. Adding of a new host to a multihost system makes the model more complicated [141]. The multihost parasite *Rubzovinema* sp. is expected to exhibit different levels of pathogenicity with respect to different flea hosts which, in turn, play different roles in the transmission of plague. Epizootics cause sporadic mortality in local populations of all members involved in the interaction with the plague agent, and their survival is contingent on migrations within a metapopulation. It is the interaction with the plague agent, and their survival is contingent on migrations within a metapopulation. Epizootics cause sporadic mortality in local populations of all members involved in the interaction with the plague agent, and their survival is contingent on migrations within a metapopulation. It is the case when the Cope’s law [139, 146] governs the extinction of specialists on a shorter time scale rather than a geological period, and evolution may favor the ecological generalists, such as *Rubzovinema* sp.

Some authors surmised the involvement of entomoparasitic nematodes in the transmission of the plague agent [4], as it was observed that biofilms of *Yersinia pestis* adhere to cuticle receptors of *Caenorhabditis elegans* [147–149]. In this perspective, nematodes parasitizing fleas in natural foci of plague take on greater importance, as they may provide for the transmission route that does not include a mammal [4]. Further studies will clarify the role of flea nematodes in the transmission of plague infection.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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