Description and phylogenetic affinities of two new species of *Nomadolepis* (Eucestoda, Hymenolepididae) from Eastern Palearctic

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Abstract

Two new species of hymenolepidid cestodes belonging to the genus *Nomadolepis* are described from small mammals in western Siberia and the Far East, Russian Federation. *Nomadolepis fareasta* n. sp. is described from the striped dwarf hamster *Cricetulus barabensis* in Amurskaya Oblast' and *Nomadolepis shiloi* n. sp. is described from the Eurasian harvest mouse *Micromys minutus* from Novosibirskaya Oblast' and Amurskaya Oblast'. *Nomadolepis fareasta* n. sp. differs from *N. merionis*, *N. ellobii* and *Nomadolepis shiloi* n. sp. in having a substantially longer strobila, longer cirrus-sac and wider ovary. Furthermore, *N. fareasta* n. sp. can be readily distinguished from its congeners by the arrangement of the testes and the number and size of rostellar hooks. *Nomadolepis shiloi* n. sp. differs from *N. merionis*, *N. ellobii* and *N. fareasta* n. sp. by the number and length of the rostellar hooks, the presence of irregular transverse anastomoses as well as the length of the cirrus-sac and position of the cirrus-sac in relation to the poral ventral osmoregulatory canal. Morphological differentiation of the two new species from morphologically similar Palearctic species of the related genus *Rodentolepis* (sensu lato) from rodents is also provided. Phylogenetic affinities of *Nomadolepis* were studied for the first time using partial sequences of the nuclear ribosomal 28S DNA gene. Phylogenetic analysis strongly supported the status of *Nomadolepis* as a separate genus closest to *Pararodentolepis*.

Key words: *Nomadolepis fareasta* n. sp., *Nomadolepis shiloi* n. sp., Hymenolepididae, Russia, molecular phylogeny, nuclear 28S gene.

1. Introduction

The majority of hymenolepidid cestodes with an armed scolex parasitic in rodents were until recently included in the broadly defined genus *Rodentolepis* (*sensu lato*) Spassky, 1954 that also contained several species from bats and shrews [1-3]. Spassky [1] chose *R. straminea* (Goeze, 1782) (syn.: *Taenia straminea* Goeze, 1782; *Hymenolepis straminea* (Goeze, 1782) Blanchard, 1891; *Vampirolepis straminea* (Goeze, 1782) Schmidt, 1986) as the type of the genus. Broad inconsistent morphological characteristics as well as an unusually broad range of rodent definitive hosts of *R. straminea* reported by different authors [e.g., 4-11] suggest that this taxon likely represents a species complex. Originally, *Rodentolepis* included 12 species [1]. However, due to the somewhat loose generic diagnosis and the lack of clear morphological features defining species boundaries, a number of additional, sometimes poorly described species from rodents, bats, insectivores, marsupials and primates have been added to the genus [1-3, 9, 12-18]. At least some of these species were not morphologically consistent with typical *Rodentolepis* which resulted in growing taxonomic and systematic confusion. The exact number of species in *Rodentolepis* cannot be defined at this point because the genus is in a dire need of a thorough taxonomic revision.

Czaplinski & Vaucher [17] emended the generic diagnosis of *Rodentolepis* (*sensu lato*) and proposed new important differential criteria (i.e. a labyrinthine uterus extending laterally beyond the osmoregulatory canals). However, they did not provide a formal revision of the genus and *Rodentolepis* remained a composite taxon.

The situation started to change recently. Several species formerly included in *Rodentolepis* have been transferred into the recently established genus *Pararodentolepis*

Makarikov et Gulyaev, 2009 which also includes at least one cestode species parasitic in shrews, namely *Pararodentolepis gnoskei* (Greiman et Tkach, 2012) [3, 19, 20]. In addition, Makarikov et al. [21] have established another new genus, *Nomadolepis* Makarikov, Gulyaev et Krivopalov, 2010, to accommodate two hymenolepidid species from cricetid rodents, *N. merionis* (Tokobajev et Erculov, 1966) and *N. ellobii* Makarikov, Gulyaev et Krivopalov, 2010. Both *Nomadolepis* and *Pararodentolepis* differ from *Rodentolepis* in having a two-chambered uterus developing in the middle field of the proglottid and typical fraternoid hooks. *Nomadolepis* differs from *Pararodentolepis* in the position of the genital pores (dextral in *Nomadolepis*, sinistral in *Pararodentolepis*) and the arrangement of the testes (triangular in *Nomadolepis* and linear in *Pararodentolepis*) [19, 21].

Concurrently, recent phylogenetic studies on systematic interrelationships among species belonging to the “*Rodentolepis* clade” [3, 22] agreed with the results of the morphological analysis. It has been shown that the genus *Rodentolepis* (*sensu lato*) is a composite, non-monophyletic taxon that requires a revision. Some species of *Rodentolepis* (*sensu lato*) for which molecular data are available demonstrated very high levels of divergence from the type species of the genus, *R. straminea*. This is particularly true for *R. asymmetrica* (Janicki, 1904), *R. fraterna* (Stiels, 1906) (*sensu lato*) and *R. microstoma* (Dujardin, 1845). The generic position of these species as well as other species previously assigned to *Rodentolepis* (*sensu lato*) remains unresolved. It is clear that further improvement in the classifications of hymenolepidid cestodes of mammals depends on the combined use of morphological and molecular criteria [3, 22]. For instance, based on available morphological and molecular evidence, *R. fraterna* (Stiles, 1906) (*sensu lato*) is now considered a member of *Pararodentolepis* [20]. Since *Nomadolepis* was erected based on morphological evidence only, , no members of the genus have been included in

phylogenetic analyses so far. Thus, the phylogenetic relationships of this genus with other members of the “*Rodentolepis* clade” remain unknown.

In the course of helminthological studies of rodents in Siberia and the Far East (Russian Federation) we have discovered two new species of cestodes belonging to *Nomadolepis* from the striped dwarf hamster *Cricetulus barabensis* (Pallas, 1773) and the Eurasian harvest mouse *Micromys minutus* (Pallas, 1771). The descriptions of the new species and their morphological differentiation from closely related species taxa are provided herein.

We used partial sequences of the nuclear ribosomal 28S gene to analyze relationships of *Nomadolepis* with other members of the *Rodentolepis* clade sensu Haukisalmi et al. (2010). The results of the phylogenetic analysis are provided and discussed below.

2. Materials and methods

Specimens of *Nomadolepis fareasta* n. sp. were found in 6 out of 12 striped dwarf hamster (*Cricetulus barabensis*) collected in August 2012 near Dolgoe Lake, Khinganskiy State Nature Reserve, Arkharinskiy Raion, Amurskaya Oblast', Russian Federation.

Two specimens of *Nomadolepis shiloi* n. sp. were collected during July 2003 from 1 of 4 Eurasian harvest mice (*Micromys minutus*) in Bol'shekhekhtsirskiy Nature Reserve (ca., 48°16'N, 134°45'E), Khabarovskiy Kray, Russian Federation. Another specimen of *N. shiloi* n. sp. was found in a single specimen of Eurasian harvest mouse collected in April 2014 from the vicinity of Karasuk Scientific Field Station (53°43'49"N, 77°52'06"E), Karasuksky Raion, Novosibirskaya Oblast', Russian Federation. Additional two specimens morphologically consistent with this species were found among materials previously deposited in the helminthological collection at the Institute of Systematics and Ecology of Animals, Novosibirsk,

Russia (ISEA). These specimens were collected by K.P. Fedorov in 1985 from *M. minutus* near Village of Ust'-Urgul'ka (56°19'N, 77°47'E), Severny Raion, Novosibirskaya Oblast', Russian Federation, and originally identified as *R. straminea*.

Cestodes were isolated, rinsed in saline, relaxed in water and preserved in 70% ethanol. They were stained with Ehrlich's haematoxylin, dehydrated in an ethanol series, cleared in clove oil and mounted in Canada balsam. Some of the scoleces and fragments of strobilae were mounted in Berlese's medium to facilitate detailed examination of the rostellar hooks, cirrus armature and eggs structure. The type specimens of the new species have been deposited in the Zoological Museum at the Institute of Systematics and Ecology of Animals, Novosibirsk, Russia (ISEA) and the Natural History Museum, Geneva, Switzerland (MHNG).

Genomic DNA for molecular phylogenetic analysis was extracted from *N. fareasta* n. sp. collected in the type locality from *Cricetulus barabensis*, *Nomadolepis shiloi* n. sp. collected in the type locality from *Micromys minutus* and *Pararodentolepis* sp. obtained from Johnston's Forest Shrew *Sylvisorex johnstoni* (Dobson, 1888) in the Kibale National Park, Uganda, following the protocol of Tkach & Pawlowski [23]. A fragment of a single adult worm was used for each DNA extraction upon preliminary morphological identification. Scoleces and the rest of the strobilae have been mounted on slides as vouchers. DNA fragments approximately 1,400 base pairs long at the 5' end of the nuclear large ribosomal subunit (28S) gene were amplified by PCR on a an Eppendorf EP Gradient thermal cycler using OneTaq Quick-load Mastermix from New England Biolabs (Ipswich, MA) according to manufacturer's instructions. Forward primer cestl2 (5'- AAGCATATCAATAAGCGG-3') and reverse primer 1500RC (5' - GACGATCGATTTGCACGTC - 3') designed by Vasyl Tkach, were used in PCR reactions. PCR protocol included 40 cycles with annealing temperature 53°C. PCR products were purified

using ExoSap PCR clean-up enzymatic kit from USB (now Affimetrix, Santa Clara, CA), cycle-sequenced using ABI BigDye kit 3.1 chemistry, alcohol-precipitated, and run on an ABI Prism 3100 Genetic Analyzer (Life Technologies, Grand Island, New York). The original PCR primers as well as an internal reverse primer c1100r (5' - GCGCATCACCGGCCCGTC- 3') designed by Vasyl Tkach, were used for sequencing. Contiguous sequences were assembled using Sequencher™ ver. 4.2 (GeneCodes Corp.) and submitted to GenBank under accession numbers KT161962- KT161964.

For phylogenetic analysis, the newly obtained sequences were aligned with previously published [3, 20, 22] sequences of 15 other mammalian hymenolepidids belonging to closely related genera. Sequence of *R. asymmetrica* was used as an outgroup in accordance with the results of the phylogenetic analysis published by Greiman & Tkach [3]. The sequences from the GenBank were as follows: *Hymenolepis sulcata* (Linstow, 1879) (GU166277); *R. asymmetrica* (HM138528); *Rodentolepis fraterna* (GU166241); *R. microstoma* (GU166267); *R. microstoma* (GU166278); *Rodentolepis* sp. (GU166239); *Rodentolepis* sp. (GU166243); *R. straminea* (GU166238); *Pararodentolepis fraterna* (GU166268); *P. gnoskei* (JQ260806); *Staphylocystis brusatae* (Vaucher, 1971) (JQ260805); *S. clydesengeri* Tkach, Makarikov et Kinsella, 2013 (KF257898); *S. furcata* (Stieda, 1862) (KF257897); *S. schilleri* (Rausch et Kuns, 1950) (KF257896); *Vampirolepis* sp. (GU969051) and *Vampirolepis* sp. (JQ260802). Sequences were aligned using BioEdit software, version 7.0.1 [24].

Phylogenetic analysis was done using Bayesian inference as implemented in MrBayes 3.1.2 software [25, 26]. The best nucleotide substitution model was estimated using jModelTest Version 0.1.1 [27-30]. The best fitting model was the general time reversible model including estimates of gamma distributed among site-rate variation and the proportion of invariant sites

(GTR+G+I). The following model parameters were used in the MrBayes analysis: lset nst = 6, rates = invgamma with 3,000,000 generations and sample frequency set at 1,000 generations. Burn-in value was 750 (equal to 750,000 generations) and was estimated by plotting the log-probabilities against the generations and visualizing plateau in parameter values (sump burnin=750), and nodal support was estimated by posterior probabilities (sumt) [25], with all other settings left at default values.

3. Results

Nomadolepis fareasta n. sp.

3.1. Description

(Figures 1–3)

Description (based on 6 stained mounted specimens and 2 scoleces cleared in Berlese's medium); measurements of the holotype are followed by the range, average values and number of measured specimens in parentheses): Strobila 155 mm (85–155; 112; 4) mm long, with maximum width 1200 (700–1200; 1017; 4) mm at level of gravid proglottids. Strobila consisting of 1680 (1100–1680; n = 3) craspedote proglottids.

Scolex conical, slightly flattened dorso-ventrally, longer than wide, very narrow, 134 (134–168, 146, n = 3) wide, not clearly distinct from neck (Fig. 1A, B). Suckers small, very muscular, thick-walled, unarmed, rounded or oval, cup-shaped, 64–75 × 52–61 (64–76 × 47–62; 70 × 54; n = 14). Rostellar pouch 107 × 63 (107–164 × 63–106; 144 × 82; n = 4), with muscular walls, entirely filled with intensely staining cells, its bottom extending beyond level of posterior margin of suckers. Rostellum conical, sac-like, very muscular, apex not invaginable, 73 × 53

(73–102 × 53–65; 90 × 59; n = 4). Rhynchus with well developed circular musculature, armed by a single crown of 22 (22–24; n = 5) rostellar hooks of fraternoid type (Figs. 1C–D; 3B). Rostellar hooks with curved, long handle, straight blade approximately equal in length with guard, the last one is narrow in anterior surface. Hook measurements: total length 19–20 (19–22; 20.3; n = 16), handle 9.5–10 (8.5–11.5; 10.2; n = 16), blade 6–6.5 (5.5–6.5; 6; n = 16) and guard 5–5.5 (5–7; 6; n = 16).

Neck 60 (60–130; 91; n = 3), narrower than scolex (Fig. 1A, B). Ventral osmoregulatory canals 20–41 (20–50, 32, n = 14) wide, at level of hermaphroditic proglottids, transverse anastomoses not observed. Dorsal osmoregulatory canals very thin, 2–3 (2–4, 3, n = 10) wide, at level of hermaphroditic proglottids, usually situated directly above ventral canals. Genital pores unilateral, dextral (Fig. 1E, F, G). Genital ducts pass dorsally to both ventral and dorsal longitudinal osmoregulatory canals. Development of proglottids gradual, protandrous. All segment in the strobila transversely elongated.

Mature proglottids 75–85 × 615–675 (50–90 × 490–675; 67 × 598; n = 25), trapezoid (Fig. 1F). Testes relatively small, three, almost equal in size, 69–87 × 40–56 (60–87 × 39–58; 74 × 48; n = 30), spherical or somewhat elongated, situated in elongated triangle with obtuse angle, one poral and two antiporal. Cirrus-sac thin-walled, elongate, 138–153 × 26–34 (128–157 × 26–35; 140 × 31; n = 25), in hermaphroditic proglottids, distinctly crossing poral osmoregulatory canals dorsally to them (Fig. 1F, G). Genital atrium simple, infundibular, deep, opens laterally approximately in middle of lateral proglottid margin. Cirrus 28–36 × 3–5 (27–37 × 3–5, 37 × 7, n = 10), cylindrical, armed with minuscule (in distal part 1.5 long, in basal and middle part less than 1 long) spines (Fig. 2A). Internal seminal vesicle elongate, 81–103 × 23–28 (75–107 × 21–30; 86 × 25; n = 25), occupying more than half of cirrus-sac length. External seminal vesicle

ovoid, $68-89 \times 40-50$ ($55-91 \times 26-50$; 75×39 ; $n = 20$), clearly distinguishable from vas deferens, distinctly smaller than seminal receptacle (Fig. 1F, G).

Ovary $249-290$ ($195-290$; 237 ; $n = 27$) wide, median, usually consisting of three large lobes, sometimes with secondary smaller lobes, ventral to male genital organs, occupying two-thirds of median field, commonly overlapping external seminal vesicle and poral and first antiporal testes (Fig. 1F). Vitellarium irregularly shaped, sometimes lobed $40-51 \times 80-115$ ($30-51 \times 65-116$; 38×86 ; $n = 27$), postovarian, median or slightly shifted aporally. Copulatory part of vagina $27-35 \times 5-7$ ($27-38 \times 5-8$, 23×6 , $n = 8$), tubular, clearly distinct from seminal receptacle; ventral to cirrus-sac (Fig. 2A). Seminal receptacle relatively large, $235-284 \times 51-69$ ($200-284 \times 38-69$; 242×52 ; $n = 24$) pear-shaped (Fig. 1F, G).

Uterus first appears as transversely elongated tube with enlarged ends, not extending beyond osmoregulatory canals, situated ventrally genital ducts and testes and dorsally to ovary. With proglottid development, uterus grows and forms two lobes (Fig. 2D). Testes and vitellarium persist in postmature proglottids; cirrus-sac and vagina persist in gravid proglottids. Gravid proglottids transversely elongate, $140-193 \times 980-1120$ ($110-193 \times 850-1140$; 137×964 , $n = 21$). Fully developed uterus saccate, bilobed, not extending into both lateral fields (Fig. 2E). Uterus contains numerous (up to $130-250$) small eggs. Eggs $60-71 \times 48-58$ (66×52 ; $n = 12$), oval or subspherical, with very thin, easily deformed outer coat (less than 1); oncosphere oval, $29-37 \times 25-28$ (33×26 ; $n = 12$). Embryophore thin, $35-40 \times 27-30$ (38×29 ; $n = 12$), lemon-shaped, with symmetrical polar protrusions and with about 2 pairs of polar filaments on each side (Fig. 2B; 3A). Embryonic hooks of different shape and length (Fig. 2C), antero-lateral embryonic hooks ($15-15.5$) much more robust than slender postero-lateral ($14.5-15$) and median hooks ($16.5-17$).

3.2. Taxonomic summary

Site in the host: small intestine.

Type host: *Cricetulus barabensis* (Pallas, 1773) (Rodentia, Cricetidae).

Symbiotype: Male subadult, skull only, No. S-191983 (field collection 1612/20), 15.08.2012, collection of the Zoological Museum of M.V. Lomonosov State University, Moscow, Russia.

Type locality: Dolgoe Lake, Khinganskiy State Nature Reserve, Arkharinskiy Raion, Amurskaya Oblast', Russia (49°23'13"N, 129° 41'13"E).

Type-material: Holotype: ISEA JM12-20#1, single slide, from type host species and type locality, collected by Y. Mel'nikova, 15 August 2012. Paratypes from type host species and type locality, collected by Y. Mel'nikova: ISEA JM12-14#1, 15 August 2012; ISEA JM12-14#2, two slides, 15 August 2012; MHNG-PLAT-91136 (field number JM12-20#2, ex 121/ 28S sequence, GenBank KT161962), two slides, 15 August 2012; ISEA JM12-20#4, 15 August 2012; ISEA JM12-21#1, two slides, 15 August 2012; ISEA JM12-21#2, 15 August 2012. Voucher ISEA JM12-20#3.

Etymology: The species name refers to the distribution in the Russian Far East.

3.3. Remarks

Nomadolepis fareasta n. sp. has morphological characteristics of *Nomadolepis*, namely the scolex armed with rostellar hooks of fraternoid type, dextral genital pores, testes arranged in triangle, tri-lobed ovary, copulatory part of vagina clearly distinct from seminal receptacle, bi-lobed uterus not extending beyond osmoregulatory canals, and embryophore with polar filaments.

Currently, the genus *Nomadolepis* includes two species, namely *N. merionis* from *Cricetulus migratorius* (Pallas, 1773) and *Meriones tamariscinus* (Pallas, 1773) and *N. ellobii*

from *Ellobius talpinus* (Pallas, 1770) [14, 21]. A third species, *Nomadolepis shiloi* n. sp., is described in the present work.

Nomadolepis fareasta n. sp. is readily distinguishable from all three congeners in having a substantially longer strobila, substantially longer cirrus-sac and wider ovary (Table 1). Additionally, *N. fareasta* n. sp. differs from *N. merionis* and *N. ellobii* in the number of rostellar hooks and very significantly differs from *N. ellobii* and *N. shiloi* in rostellar hook size (Table 1). We did not find transverse anastomoses in *N. fareasta* n. sp. while *N. ellobii* and *N. shiloi* have irregular transverse anastomoses. Finally, the testes in *N. fareasta* n. sp. are arranged in an elongated triangle at an obtuse angle, while in *N. merionis* and *N. ellobii* the testes are arranged at a right angle. We consider the latter character suitable for diagnosis because the relative position of the testes does not seem to vary significantly in members of *Nomadolepis* unlike some other hymenolepidid genera.

Species of *Nomadolepis* significantly differ from species of *Rodentolepis* (*sensu stricto*) and its type species *R. straminea* in several important morphological characters: rostellar hooks of fraternoid type (vs rostellar hooks of cricetoid type); ovary consisting of three lobes (vs fan-shaped) saccate bilobed uterus (vs labyrinthine uterus); uterus not extending beyond osmoregulatory canals (vs uterus extending laterally beyond osmoregulatory canals) [7, 8, 10, 11, 17, 19, 21]. However, due to the lack of a formal revision of *Rodentolepis* we provide here a differentiation between *N. fareasta* n. sp. and some cestodes currently included in *Rodentolepis* (*sensu lato*).

Among species of *Rodentolepis* (*sensu lato*), *Nomadolepis fareasta* n. sp. is morphologically most similar to *R. sinensis* (Oldham, 1929) (syn.: *Hymenolepis sinensis* Oldham, 1929) described from the Chinese hamster (*Cricetulus griseus* Milne-Edwards, 1867) in

China. The two species have fraternoid hooks, close number and length of rostellar hooks (22–24 vs 20 and 19–22 vs 22–24) and testes situated in a triangle at an obtuse angle. The new species differs from *R. sinensis* in having dextral genital pores (vs sinistral genital pores), the longer cirrus-sac (128–157 vs 100) and wider ovary (195–290 vs 130–140). The ovary of *N. fareasta* n. sp. occupies two thirds of the median field and overlaps the external seminal vesicle and poral and first antiporal testes, whereas in *R. sinensis* it occupies less than one third of the median field and does not reach the external seminal vesicle and poral testis [see 31]. In addition, the embryophore of *N. fareasta* has polar filaments while the embryophore of *R. sinensis* lacks them. The substantial morphological similarity between *R. sinensis* and *Nomadolepis* suggests that the former species may belong to this genus. However, in addition to the above-mentioned differences one critically important distinctive character of *Nomadolepis* is currently unknown in *R. sinensis*, namely the morphology of gravid proglottids. Therefore we refrain from establishing a new combination for *R. sinensis* for now. The generic allocation of this species requires an additional study.

***Nomadolepis shiloi* n. sp.**

3.4. Description

(Figures 4–6)

Description (based on 3 stained mounted specimens and 2 scoleces cleared in Berlese's medium); measurements of the holotype are followed by the range, average values and number of measured specimens in parentheses): Strobila 55 mm (50–55; 52; n = 3) mm long, with maximum width 865 (700–865; 758; n = 3) mm at level of gravid proglottids. Strobila consisting of 704–732 craspedote proglottids.

Scolex conical, slightly flattened dorso-ventrally, 158 (158–162, $n = 2$) wide, not clearly distinct from neck (Fig. 4A, B). Suckers small, thick-walled, unarmed, rounded or oval, cup-shaped, $56\text{--}66 \times 55\text{--}63$ ($56\text{--}66 \times 40\text{--}63$; 61×52 ; $n = 7$). Rostellar pouch 134×70 ($115\text{--}134 \times 70\text{--}85$; $n = 2$), with muscular walls, its bottom extending beyond level of posterior margin of suckers. Rostellum saclike, muscular, apex not invaginable, 77×42 ($65\text{--}77 \times 42\text{--}55$; $n = 2$). Rhynchus with well developed circular musculature, armed by a single crown of 19 (19–23; $n = 3$) rostellar hooks of fraternoid type (Fig. 4C, D). Rostellar hooks with curved, relatively long handle, straight blade approximately equal in length with guard, the last one is narrow in anterior surface. Hook measurements: total length 13.5–14 (13.5–15; 13.9; $n = 10$), handle 6–6.5 (6–7; 6.3; $n = 10$), blade 4–5 (4–5.5; 4.4; $n = 10$) and guard 5–5.5 (4–5.8; 5.3; $n = 10$). Neck 98 (98–155; $n = 2$), narrower than scolex (Fig. 4A, B).

Ventral osmoregulatory canals 26–32 (25–28, 31, $n = 25$) wide, rarely connected by irregular transverse anastomoses. Dorsal osmoregulatory canals very thin, 1.5–3 (1.5–3.2, 2.3, $n = 25$) wide, at level of hermaphroditic proglottids, usually situated directly above ventral canals. Genital pores unilateral, dextral (Fig. 4E, F, G). Genital ducts pass dorsally to both ventral and dorsal longitudinal osmoregulatory canals, however irregular transverse anastomoses always situated above vagina. Development of proglottids gradual, protandrous. All segment in the strobila transversely elongated.

Mature proglottids $81\text{--}88 \times 484\text{--}538$ ($80\text{--}112 \times 395\text{--}538$; 85×460 ; $n = 25$), trapezoid (Fig. 4F). Testes relatively small, three, almost equal in size, $48\text{--}65 \times 28\text{--}40$ ($44\text{--}65 \times 25\text{--}45$; 54×35 ; $n = 20$), oval, situated in triangle with an right or sharp angle, one poral and two antiporal, not always clearly separated by female gonads. Cirrus-sac thin-walled, pyriform, $80\text{--}92 \times 18\text{--}22$ ($78\text{--}95 \times 17\text{--}22$; 86×19 ; $n = 25$). Antiporal part of cirrus-sac rarely overlapping ventral

longitudinal canal but not crossing it (Fig. 4F, G). Genital atrium simple, infundibular, deep, opens laterally approximately in middle of lateral proglottid margin. Cirrus $22\text{--}30 \times 3\text{--}4$ ($22\text{--}30 \times 3\text{--}5$, 26×3 , $n = 10$), cylindrical, armed with minuscule (less than 1 long) spines (Fig. 5A). Internal seminal vesicle elongate, $45\text{--}60 \times 16\text{--}19$ ($40\text{--}60 \times 15\text{--}19$; 48×17 ; $n = 25$), occupying more than half of cirrus-sac length. External seminal vesicle ovoid, $47\text{--}70 \times 20\text{--}32$ ($40\text{--}70 \times 20\text{--}33$; 50×25 ; $n = 18$), clearly distinguishable from vas deferens, distinctly smaller than seminal receptacle (Fig. 4F, G).

Ovary $166\text{--}201$ ($163\text{--}203$; 180 ; $n = 18$) wide, median, usually consisting of three large lobes, sometimes with secondary smaller lobes, ventral to male genital organs, occupying more than half of median field, commonly overlapping testes (Fig. 4F). Vitellarium compact $36\text{--}39 \times 63\text{--}72$ ($30\text{--}39 \times 50\text{--}72$; 35×60 ; $n = 18$), postovarian, median or slightly shifted aporally. Copulatory part of vagina $27\text{--}33 \times 3\text{--}4$ ($27\text{--}33 \times 3\text{--}4$, 29×3.6 , $n = 9$), tubular, clearly distinct from seminal receptacle; ventral to cirrus-sac (Fig. 5A). Seminal receptacle relatively large, $92\text{--}116 \times 18\text{--}26$ ($80\text{--}120 \times 18\text{--}39$; 101×27 ; $n = 18$) pear-shaped (Fig. 4F, G).

Uterus first appears as transversely elongated tube with enlarged ends, not extending beyond osmoregulatory canals, situated ventrally genital ducts and testes and dorsally to ovary. With proglottid development, uterus grows and forms two lobes (Fig. 5D). Testes and vitellarium persist in postmature proglottids; cirrus-sac and vagina persist in gravid proglottids. Gravid proglottids transversely elongate, $103\text{--}116 \times 790\text{--}863$ ($103\text{--}163 \times 600\text{--}863$; 132×732 , $n = 21$). Fully developed uterus saccate, bilobed, not extending into both lateral fields (Fig. 5E). Uterus contains numerous (up to $90\text{--}150$) small eggs. Eggs $60\text{--}70 \times 43\text{--}55$ (63×45 ; $n = 13$), oval, with very thin, easily deformed outer coat (less than 1); oncosphere oval, $22\text{--}29 \times 20\text{--}26$ (25×22 ; $n = 13$). Embryophore thin, $30\text{--}45 \times 24\text{--}29$ (32×26 ; $n = 13$), lemon-shaped, with symmetrical polar

protrusions and polar filaments on each side of protrusions (Figs. 5B; 6A, B). Embryonic hooks of different shape and length (Fig. 5C), antero-lateral embryonic hooks (13.5–14) much more robust than slender postero-lateral (13–13.2) and median hooks (14.2–14.5).

3.5. Taxonomic summary

Site in the host: small intestine.

Type host: *Micromys minutus* (Pallas, 1771) (Rodentia, Muridae, Murinae).

Type locality: Karasuk Scientific Field Station (53°43'49"N, 77°52'06"E), Karasuksky Raion, Novosibirskaya Oblast', Russia.

Other localities: Village of Ust'-Urgul'ka (ca., 56°19'N, 77°47'E), Severny Raion, Novosibirskaya Oblast'; Bol'shekhekhtsirskiy Nature Reserve (ca., 48°16'N, 134°45'E), Khabarovskiy Kray, Russia.

Type-material: Holotype: ISEA AM14-22 (ex 220/ 28S sequence, GenBank KT161963), from type host and locality, 27 April 2014. Paratypes: ISEA JM2003-12#1 from type host species, Bol'shekhekhtsirskiy Nature Reserve, collected by Y. Mel'nikova, 08 July 2003; MHNG-PLAT-91722 (field number JM2003-12#2) from type host species, Bol'shekhekhtsirskiy Nature Reserve, collected by Y. Mel'nikova, 08 July 2003. Vouchers: ISEA KP115 from type host species, Village of Ust'-Urgul'ka, collected by K. Fedorov, 1985; ISEA KP619 from type host species, Village of Ust'-Urgul'ka, collected by K. Fedorov, 1985.

Etymology: This species is named in honour of Dr. Vladimir A. Shilo, the director of Karasuk Scientific Field Station (ISEA SB RAS) for his outstanding contribution in preservation and development of the Karasuk Scientific Field Station and his help to one of the authors (AAM) during his field work in Karasuk in April 2014.

3.6. Remarks

Nomadolepis fareasta n. sp. has morphological characteristics of *Nomadolepis* (see above in remarks on *N. fareasta* n. sp.). It is readily distinguishable from congeners *N. merionis* and *N. fareasta* in having substantially smaller rostellar hooks, cirrus-sac and seminal receptacle (Table 1). The cirrus-sac of *Nomadolepis shiloi* n. sp. rarely overlaps the poral ventral osmoregulatory canal. In contrast, the cirrus-sac in *N. merionis* and *N. fareasta* crosses the ventral canal. In addition, *Nomadolepis shiloi* n. sp. has irregular transverse anastomoses, while in *N. merionis* and *N. fareasta* these anastomoses are absent.

Nomadolepis shiloi n. sp. can be distinguished from *N. ellobii* by having a substantially wider strobila and ovary, and greater number of rostellar hooks (Table 1). Furthermore, the cirrus-sac in *Nomadolepis shiloi* n. sp. rarely overlaps the poral ventral osmoregulatory canal while in *N. ellobii* the cirrus-sac crosses the ventral canal.

Interestingly, the new species is a parasite of the Muridae while all other currently known *Nomadolepis* are associated with the Cricetidae.

4. Molecular phylogenetic analysis

The alignment was 1271 nucleotides long and required only a few gaps. Thirty-six ambiguously aligned positions were excluded from the phylogenetic analysis.

The tree resulting from the Bayesian analysis (3,000,000 generations) showed a very high support for the majority of branches (Fig. 7). Importantly, it demonstrated 100% support for the monophyletic clade of *Nomadolepis* and the cluster uniting *Nomadolepis* and *Pararodentolepis* (Fig. 7). Within the *Nomadolepis* clade, *N. shiloi* clustered together with the sequence GU166241 identified as *Rodentolepis fraterna* by Haukisalmi et al. [22] while *N. fareasta* clustered with the sequence GU166243 identified as *Rodentolepis* sp. by Haukisalmi et al. [22].

All these topologies were supported by 100% posterior probability values. The clade of *Pararodentolepis* included previously sequences *P. fraterna*, *P. gnoskei* and the newly sequenced *Pararodentolepis* sp.

The second major cluster in the phylogenetic tree was rather weakly supported by posterior probability values. However, all internal clades corresponding to genera, were 99-100% supported (Figure 7). The topologies in this group were essentially the same as in the tree published by Greiman & Tkach [3]. The only additions were represented by recently published sequences of *S. clydesengeri* and *S. schilleri* which clustered together with the closely related *S. furcata*.

5. Discussion

Our study has doubled the number of named species of *Nomadolepis* by adding two new species to previously described *N. ellobii* and *N. merionis*. It has also expanded the taxonomic diversity of the members of the “*Rodentolepis* clade” represented in previous phylogenetic analyses [3, 22] and allowed the assessment of the phylogenetic relationships of *Nomadolepis* for the first time.

Recent molecular analysis showed that *Rodentolepis fraterna* from *Mus musculus* Linnaeus, 1758 and *R. gnoskei* from a shrew *Suncus varilla* (Thomas, 1895) formed a clade separate from *R. straminea*, the type species of *Rodentolepis* [3]. Based on combined morphological and molecular evidence, these two species were transferred to *Pararodentolepis* [20]. Two additional genetically distinct species were associated with the *Pararodentolepis* clade, namely *Rodentolepis* sp. from *C. barabensis* (GU166243, isolate U29, Russia: Republic of Buryatia) and *R. cf. fraterna* from *M. minutus* (GU166241, isolate AV0, Finland). Both these

forms were considered undescribed species by Haukisalmi et al. [22]. However, these authors did not present any morphological descriptions or almost any morphological details of these species, except for providing the number of fraternoid rostellar hooks and stating the presence of polar filaments in the embryophore. These characters fit diagnoses of both *Pararodentolepis* and *Nomadolepis* and thus did not provide conclusive evidence of the generic position of these species.

The phylogenetic tree obtained in our analysis generally conformed with the topology of the tree published by Greiman & Tkach [3]. The only meaningful changes were related to the inclusion of the two species of *Nomadolepis* and an additional species of *Pararodentolepis* in the present work. The tree has demonstrated 100% support for the monophyletic *Nomadolepis* clade as well as the two clades uniting each of the new species described in the present study with one of the above mentioned sequences published as *R. cf. fraterna* and *Rodentolepis* sp. [22].

Nomadolepis and *Pararodentolepis* proved to be most closely related genera as was expected based on some morphological characteristics shared by these genera such as the shape of the uterus, type of rostellar hooks, and the presence of polar filaments of the embryophore. Notably, both genera are phylogenetically distant from *R. straminea*, the type species of *Rodentolepis* (Figure 7). While the *Nomadolepis*-*Pararodentolepis* clade seems to be well resolved, the situation with the majority of the remaining members of the “*Rodentolepis* clade” clearly needs further attention, especially considering the unusual position of *R. straminea* that clustered together with members of *Staphylocystis* Villot, 1877 from shrews in all analyses published so far [3, 22, this study]. Two other highly supported clades, namely the clade including two different species published as *R. microstoma* and the clade of *Hymenolepis sulcata*+*Rodentolepis* sp., mostly likely correspond to additional genera which need to be formally described.

The partial 28S rRNA sequence of *N. fareasta* from *C. barabensis* in our study is identical to the sequence of *Rodentolepis* sp. (GU166243) from the same host. Likewise, our 28S sequence of *N. shiloi* from *M. minutus* is identical to that of *R. cf. fraterna* (GU166241) from the same host published by Haukisalmi et al. [22]. It is clear that the specimens identified by Haukisalmi et al. [22] as *Rodentolepis* spp. belong to *Nomadolepis*. However, the currently available morphological and molecular evidence is insufficient to confirm or refute conspecificity of the two forms sequenced by Haukisalmi et al. [22] with the two new species described in the present work. As mentioned above, only rostellar hook number and the presence of polar filaments of the embryophore were mentioned for the two *Rodentolepis* spp. Haukisalmi et al. [22], which is not enough for species differentiation. According to previously published data, the 28S gene has very low interspecific variability among mammalian hymenolepidids and, correspondingly, has a low utility for differentiation among closely related species. For instance, there were no differences in the approximately 1400 base pair long sequences of the same region of the 28S gene among *Staphylocystis clydesengeri* from US and *S. furcata* collected in the Ukraine, as well as between *Pararodentolepis gnoskei* from shrews in Malawi and *P. fraterna* from rodents [3, 20]. Additionally the differences of partial sequences (1,374 bp) of the 28S gene between the 2 species of *Staphylocystoides* Yamaguti, 1959, *S. gulyaevi* Greiman, Tkach et Cook, 2013 and *S. parvissima* Voge, 1953, differed in only 2 nucleotides [32]. At the same time, the pairs of species listed above showed substantial differences in more variable genes as well as in several morphological characteristics

It should be mentioned that a technical error was made in the original differential diagnosis of *Nomadolepis*. The statement that *Nomadolepis* is characterized by sinistral genital

pores was erroneous because both species attributed to the genus in that publication [21] have dextral genital pores. This is also true for the two new species described herein.

There are a number of records of hymenolepidids with an armed scolex from *M. minutus* which were designated as *R. fraterna* [e.g. 4, 10, 33-37] or *R. straminea* [e.g., 9, 38, 39].

However, none of these records contain morphological descriptions. We assume that some of these records may represent *N. shiloi* or yet unrecognised related species of *Nomadolepis*.

Nomadolepis shiloi is readily distinguishable from *R. fraterna* by the testes situated in a triangle as opposed to a linear arrangement of the testes, dextral genital pores vs sinistral genital pores, and ventral osmoregulatory canals having irregular transverse anastomoses vs ventral osmoregulatory canals without anastomoses. The situation with *Rodentolepis*, *Nomadolepis* and associated taxa once again demonstrates the importance of voucher deposition in museum collections during survey and inventory studies as well as molecular taxonomic and phylogenetic studies. This provides basis for comparative research as new tools become available and our knowledge on certain taxa advances.

The generic diagnosis of *Rodentolepis* by Czaplinski & Vaucher [17] mentions hooks of various shapes, often fraternoid. In our opinion, it should be amended by the inclusion of cricetoid hook shape because the type species of the genus, *R. straminea*, possesses cricetoid rather than fraternoid, rostellar hooks [see 6-8, 10, 11, 17, 22, 33, 40-43]. We consider the records of *R. straminea* with fraternoid rostellar hooks as misidentifications [e.g., 4, 5]. The shape of rostellar hooks has been traditionally used as a generic level character in the systematics of mammalian hymenolepidids [44]. So far the results of several phylogenetic analyses of the “*Rodentolepis* clade” were generally consistent with this notion. In part, the cestodes of rodents with fraternoid rostellar hooks (i.e., *Pararodentolepis*, *Nomadolepis*) proved to be

phylogenetically very closely related and distinct from *R. straminea* which has cricetoid rostellar hooks. On the other hand, *R. straminea* is found nested among members of *Staphylocystis* that have hooks of a different shape. In addition, *R. microstoma* has cricetoid hooks, but is not closely related to *R. straminea*. This most likely is a result of parallelism in the evolution of scolex armature within two different branches of the “*Rodentolepis* clade”. Thus, the relative importance of some morphological characters traditionally used in the systematics of the group, including the shape of rostellar hooks, needs to be carefully re-evaluated.

Morphological data and recent phylogenetic studies have convincingly demonstrated that cestodes from rodents, insectivores and chiropterans comprising the “*Rodentolepis* clade” are closely related [3, 21, 22, 45]. As suggested by previous molecular phylogenetic studies [3, 22] and the present analysis the evolution of this group is characterized by multiple host switching events which is particularly evident in the genus *Pararodentolepis* and the lineage uniting the typical *Staphylocystis* and *R. straminea*. An obvious conclusion from these data is that the host specificity in this group of cestodes should not be used as the main criterion for the separation of taxa at a supraspecific level. A broader, more taxon dense molecular phylogenetic analysis combined with consideration of morphological characters, is needed in order to complete a taxonomic revision of *Rodentolepis* and closely related taxa belonging to this major lineage of hymenolepidids parasitic in mammals.

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FIGURE LEGENDS

FIGURE. 1. *Nomadolepis fareasta* n. sp. A, paratype (JM12-21#1), dorsoventral view of scolex; B, paratype (JM12-14#2) lateral view of scolex; C, paratype (JM12-14#1), rostellar hooks in profile; D, paratype (MHNG-PLAT-91136) rostellar hooks in profile and view from posterior surface showing narrow hook guard; E, holotype, male mature proglottid; F, holotype, hermaphroditic mature proglottid; G, holotype, genital ducts. (scale bars: A, B, G = 100 μ m; C, D = 10 μ m; E, F = 200 μ m).

FIGURE. 2. *Nomadolepis fareasta* n. sp. A, holotype, cirrus and vagina; B, paratype (M12-14#2A), egg; C, paratype (JM12-14#2A), embryonic hooks; D, holotype, pregravid proglottid, showing uterus development; E, holotype, gravid proglottid. (scale bars: A, B = 20; C = 10 μ m; D, E = 200 μ m).

FIGURE. 3. *Nomadolepis fareasta* n. sp. A, egg (from temporary slide, equal to paratype JM12-20#4) showing lemon-shaped embryophore with polar filaments; B, paratype (JM12-14#1) crown of rostellar hooks in profile. (scale bars: A, B = 20 μ m).

FIGURE. 4. *Nomadolepis shiloi* n. sp. A, holotype (AM14-22), dorsoventral view of scolex; B, paratype (MHNG-PLAT-91722) sublateral view of scolex; C, holotype, rostellar hooks in profile and view from anterior surface showing narrow hook guard; D, paratype (JM03-12#1), rostellar hooks in profile; E, holotype, male mature proglottids, dorsal view; F, holotype, hermaphroditic

mature proglottids, dorsal view; G, holotype, genital ducts, dorsal view. (scale bars: A, B, G = 100 μm ; C, D = 10 μm ; E, F = 200 μm).

FIGURE. 5. *Nomadolepis shiloi* n. sp. A, holotype, cirrus and vagina, ventral view; B, holotype, egg; C, holotype, embryonic hooks; D, holotype, pregravid proglottids from dorsal side, showing uterine development; E, holotype, gravid proglottids from dorsal side, showing bilobed uterus. (scale bars: A, B = 20 μm ; C = 10 μm ; D, E = 200 μm).

FIGURE. 6. *Nomadolepis shiloi* n. sp. A, B egg (from temporary slide, equal to holotype) showing lemon-shaped embryophore with polar filaments. (scale bars: A, B = 10 μm).

FIGURE. 7. Phylogenetic relationships among 18 taxa of mammalian hymenolepidids belonging to the “*Rodentolepis* clade” resulting from Bayesian analysis (3,000,000 generations) of partial sequences of 28s rDNA gene. *Rodentolepis asymmetrica* was used as an outgroup. Posterior probabilities are indicated as percentages above internodes (only values greater than 70% are shown). Branch length scale bar indicates number of substitutions per site.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1. Comparison of main morphometric characteristics of *Nomadolepis* spp. (measurements in micrometres unless stated otherwise).

Characters	<i>N. merionis</i> ¹	<i>N. ellobii</i> ¹	<i>N. fareasta</i> n. sp.	<i>N. shiloi</i> n. sp.
Strobila length	18–37 mm	30–42 mm	85–155 mm	50–55 mm
Strobila width	0.46–0.71 mm	0.21–0.34 mm	0.70–1.2 mm	0.70–0.86 mm
Scolex width	190–210	160–180	134–168	158–162
Sucker size	74–95 × 73–86	60–70 × 58–68	64–76 × 47–62	56–66 × 40–63
Rostellar hooks number	18–21	18	22–24	19–23
Rostellar hooks size	19–20	13.4–13.8	19–22	13.5–15
Hermaphroditic mature proglottid size	57–83 × 345–485	100–150 × 160–280	50–90 × 490–675	80–112 × 395–538
Transverse anastomoses	absent	irregular transverse anastomoses	absent	irregular transverse anastomoses
Testes size	55–95 × 43–56	45–65 × 42–50	60–87 × 39–58	44–65 × 25–45
Cirrus-sac size	95–112 × 22–30	74–82 × 14–17	128–157 × 26–35	78–95 × 17–22
Cirrus size	34–40 × 4–7	27–35 × 6–7.5	27–37 × 3–5	22–30 × 3–5
Ovary width	135–170	85–121	195–290	163–203
Vitellarium size	30–40 × 48–62	26–42 × 35–45	30–51 × 65–116	30–39 × 50–72
Seminal receptacle size	122–216 × 43–66	45–60 × 17–22	200–284 × 38–69	80–120 × 18–39
Eggs number	up to 80–110	up to 45	up to 130–250	90–150
Egg size	50–67 × 40–55	–	60–71 × 48–58	60–70 × 43–55
Oncosphere size	27–34 × 23–31	–	29–37 × 25–28	22–29 × 20–26
Embryonic hook size	12–16.5	–	14.5–17	13–14.5

¹Measurements from Makarikov et al. [21].

Graphical Abstract