Molecular-taxonomic study of two species of *Pseudonymus* Diesing, 1857 (Oxyuridomorpha: Thelastomatoidea: Pseudonymidae) from water beetles (Coleoptera: Hydrophilidae) of Volga estuary

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Summary. Two species of the genus *Pseudonymus* Diesing, 1857, *P. spirotheca* (Györy, 1856) and *P. islamabadi* (Basir, 1941), were obtained from the hindgut of water scavenger beetles (family Hydrophilidae), caught in the estuary of the River Volga on the territory of the Astrakhan State Biosphere Reserve (June, 2012). For both species, new morphological characters were discovered to supplement the original descriptions, and the conspecificity for both males and females was accepted on the basis of comparative study of nucleotide sequences. The nucleotide difference between the two *Pseudonymus* species for 28S rDNA was 44 bp. Females of *P. spirotheca* are characterised by enlarged first cuticular annulus and 76–85×44–56 μm egg-shells; males are characterised by narrowed tail. In females of *P. islamabadi*, first anterior six cuticular annuli are modified and differ by its diameter; egg-shells 83–89×45–54 μm in size; males are distinguished by rounded tail. According to phylogenetic analysis, *Pseudonymus* is clustering with strong bootstrap support with representatives of the superfamily Oxyuroidea (parasites of vertebrates) and with the subclade *Mesidionema* + *Binema* (intestinal parasites of earthworms and mole crickets, respectively).

Key words: pinworms, arthropod hosts, female-male conspecificity, phylogeny.

Pseudonymids are oxyurid nematodes placed within the superfamily Thelastomatoidea Travassos, 1929 (Adamson & Van Waerebeke, 1992). They inhabit the hindgut of water scavenger beetles of the family Hydrophilidae Latreille, 1802, which are considered as a specific host for these nematodes (Adamson, 1989). Presently, the genus *Pseudonymus* Diesing, 1857 comprises ten nominal species registered in different regions of the world (Basir, 1941, 1956; Todd, 1944; Kloss, 1959; Leibersperger, 1960; Fotedar, 1964; Jarry, 1964; Farooqui, 1967; Shvetsova & Kakulia, 1973; Gupta & Kaur, 1978; Shah & Rizvi, 2004). The parasitism of two species, *Pseudonymus spirotheca* (Györy, 1856) and *P. islamabadi* (Basir, 1941), in the same hydrophilid host was reported by several authors (Leibersperger, 1960; Jarry & Théodoridès, 1961; Jarry, 1964; Shvetsova & Kakulia, 1973). In case, when both species are found in the same host, the attribution of males and females to the same species by means of morphology is difficult. Molecular data were obtained for these two species and used to solve this problem.

MATERIALS AND METHODS

Hydrophilid beetles (*Hydrophilus piceus* Linnaeus, 1758) were collected in the estuary of the River Volga on the territory of the Astrakhan State Biosphere Reserve (Russia) in June, 2012. Insects were narcotised with chlorophorm, and dissected by lateral incisions on both sides of the body. In the zone of attachment of the Malpighian tubules the hindgut was cut off, transferred into 0.9% saline solution and torn by needles to release nematodes. Nematodes found were fixed in hot (60–70°C) 6% formalin and processed to glycerin according to Seinhorst (1959) and then mounted on permanent slides. The following abbreviations are used: n – number of measured specimens; L – overall body length; V' – distance of vulva from anterior end/body length from anterior end to anal aperture (%); a, b, c – de Man indices. All measurements are in μm.

Several formalin-fixed specimens of each nematode species were used for SEM studies. Nematodes

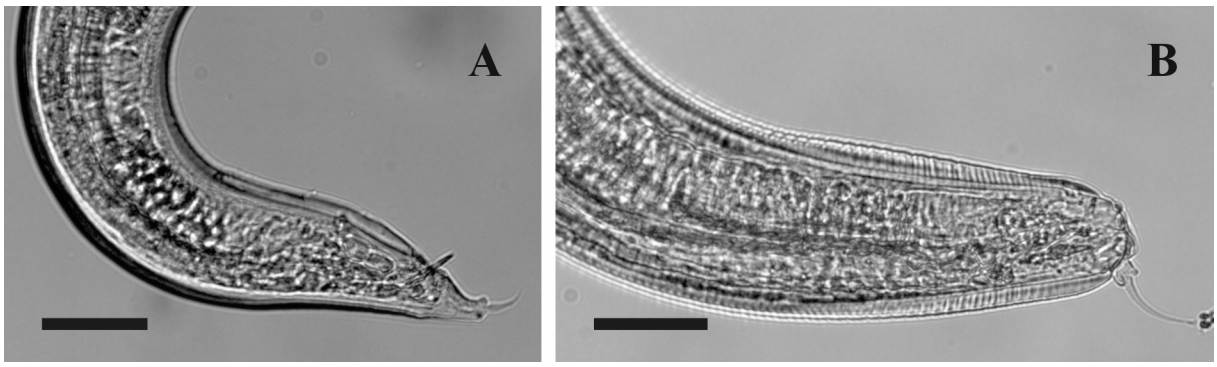

Fig. 1. LM photographs of the tail tip shapes of *Pseudonymus spirotheca* (A) and *P. islamabadi* (B) males. Scale bars: A = 200 μm; B = 25 μm.

were dehydrated in a graded ethanol and acetone series, dried in a Hitachi HCP–2 critical point dryer, coated with gold and observed under a CamScan S2 electron microscope (accelerating voltage 20 kV). SEM photographs were taken in the Electron Microscopy Laboratory of Biological Faculty, Moscow State University (Moscow, Russia).

Molecular studies were carried out as described in Spiridonov & Guzeeva (2009): primers D2A (ACA AGT ACC GTG AGG GAA AGT TG) and D3B (TCG GAA GGA ACC AGC TAC TA) were used to amplify and sequence the D2-D3 LSU rDNA expansion segments (Nunn, 1992). Nucleotide sequences obtained for pinworms of hydrophilid beetles (*Pseudonymus islamabadi* – KF771648 and *P. spirotheca* – KF771649) and some other representatives of the superfamily Oxyuroidea Cobbold, 1864 used for comparison during this study (*Aspiculuris tetraptera* (Nitzch, 1821) – KF771644, *Spauligodon* sp. – KF771645, *Tachygonetria* sp. – KF771646 and *Thelandros* sp. – KF771647) were deposited in NCBI GenBank. Several sequences were also used for comparative purposes and phylogeny construction (accession numbers indicated on the phylogram – Fig. 6). The obtained nucleotide data were analysed under Bayesian Inference (BI), Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbour Joining (NJ) algorithms (Swofford, 1998; Huelsenbeck & Ronquist, 2001; Tamura *et al.*, 2011).

RESULTS

Supplementary descriptions. The original descriptions were supplemented by newly discovered morphological characters, provided by SEM microphotographs. Partial sequences of 28S rDNA were obtained to define the conspecificity of males and females in these two species. Only light microscopy images were available for *P. islamabadi*, taken from a living specimen, which was further used for molecular study.

Pseudonymus spirotheca (György, 1856) (Table 1, Figs 1A, 2, 3A, B & 4)

Male. In earlier reports these males with narrowed tail bearing short spine appendage were identified as *P. islamabadi* (Leibersperger, 1960; Jarry, 1964; Shvetsova & Kakulia, 1973). *Area rugosa* prominent in precloacal region. Seven pairs of genital papillae present: two pairs of precloacal papillae (anteriormost subventral and mid-ventral just anterior to cloaca), two pairs of adcloacal papillae, a pair of fused postcloacal papillae and two pairs of caudal papillae, placed at the base of tail terminus (on the border with spine appendage). Spicule straight and slender. Capitulum with hook-like appendage. Gubernaculum present.

Female. Anteriormost cuticular annulus enlarged (*ca* 47–69 μm in diameter) and filled with fibrous material. On dorsal and ventral sides, posterior edge of this annulus overhanging next annulus reaching third cuticular ring. Second annulus mostly hidden under the first one. Distinct lateral fields in 88–108 μm from anterior end. Perioral disc 32–43 μm in diam. Mouth opening submerged. Eight submedian oval elevations around mouth opening. Amphids tubular, with slit-like apertures. Stoma cylindrical. Cheilostom prominent. Gymnostom strongly cuticularised, 12–14 μm long, ring with widened base. Excretory pore slightly posterior to proventriculus level, followed by a sclerotised duct and then sac-like vesicle with several folds on outer wall. Inner wall with numerous vacuoles. From pseudocoel side, vesicle adjoined with large cells of irregular shape. Intestine with slightly developed proventricular part, *ca* 100 μm wide. Reproductive system didelphic, amphideiphic. Vulva post-

equatorial, as transverse slit with slightly protruding lips. Anterior lip inflated, covering vulva opening and anterior half of posterior lip. Vagina directed anteriorly, *vagina vera* with thick muscular wall near vulva. Uteri filled with ellipsoid eggs (mean number, 35), containing double-fold first-stage juveniles with distinct pharynx and hook-like tail appendage. The egg-shell, *ca* 2 μm thick, braided by two spirally-coiled filaments, issued from a roundish button-like swelling (mean dimensions, 6×4×3 μm), positioned at one-third of egg length from one of the poles. Ovaries divergent, dorsally positioned: anterior branch usually reflexed, posterior branch outstretched with distinct cap cell. Phasmids pore-like, 103–130 μm from anal aperture.

Table 1. Morphometry of *Pseudonymus spirotheca* (Györy, 1856) and *P. islamabadi* (Basir, 1941).

Characters	*P. spirotheca* (Györy, 1856)			*P. islamabadi* (Basir, 1941)
	Male 1 (juvenile)	Male 2	Females (n = 11)	Females (n = 7)
L	560	810	2351±231 (2000–2790)	2381±358.2 (2060–3000)
Width	42	61	169±17.6 (150–200)	190±20.8 (170–220)
Pharynx	163	195	363±32 (293–390)	372±25.6 (350–420)
Nerve ring	110	125	207±11.6 (185–220)	252±17.2 (235–275)
Excretory pore	Not visible	223	475±95.2 (380–658)	453±34.5 (420–520)
Vulva	–	–	1383±142.3 (1184–1624)	1439±253 (1190–1783)
Tail	33	35	266±41.8 (225–373)	268±16 (250–290)
a	13.3	13.3	14.0±1.1 (11.8–15.4)	12.5±0.7 (11.8–13.6)
b	3.4	4.2	6.5±0.5 (6.0–7.4)	6.3±0.8 (5.7–7.6)
c	17	23	9.0±0.8 (7.5–10.5)	8.7±0.9 (7.8–10.3)
V', %	–	–	68±3.6 (64.8–75.8)	68.3±4.3 (64.7–76.8)
Eggs	–	–	76–85×44–56	83–89×45–54
Spicule length	25	25	–	–

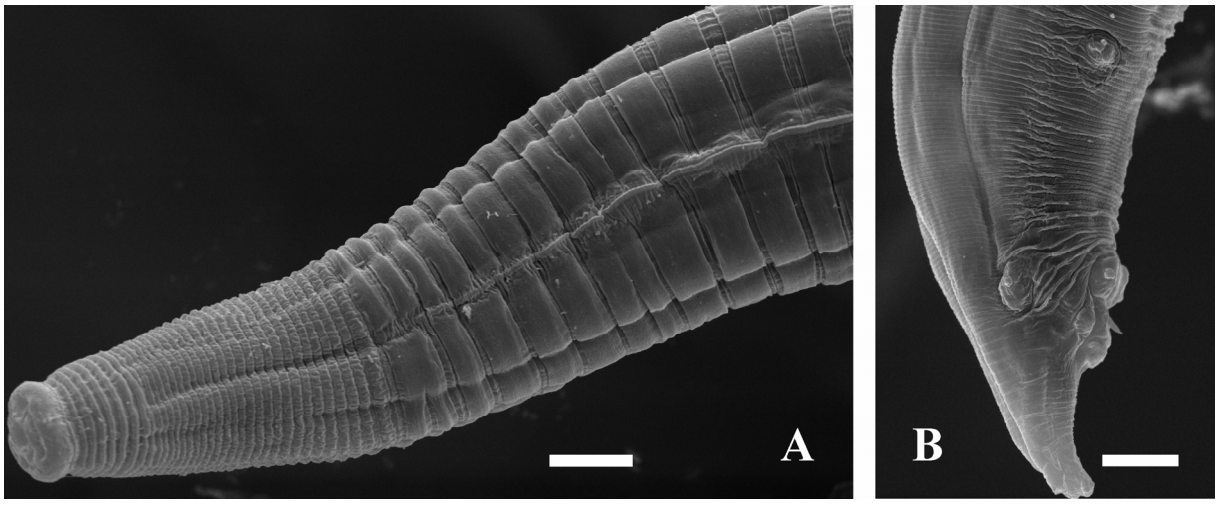

Fig. 2. SEM microphotographs of *Pseudonymus spirotheca*, male. A: anterior end; B: posterior end (cloacal area). Scale bars: A & B = 10 μm.

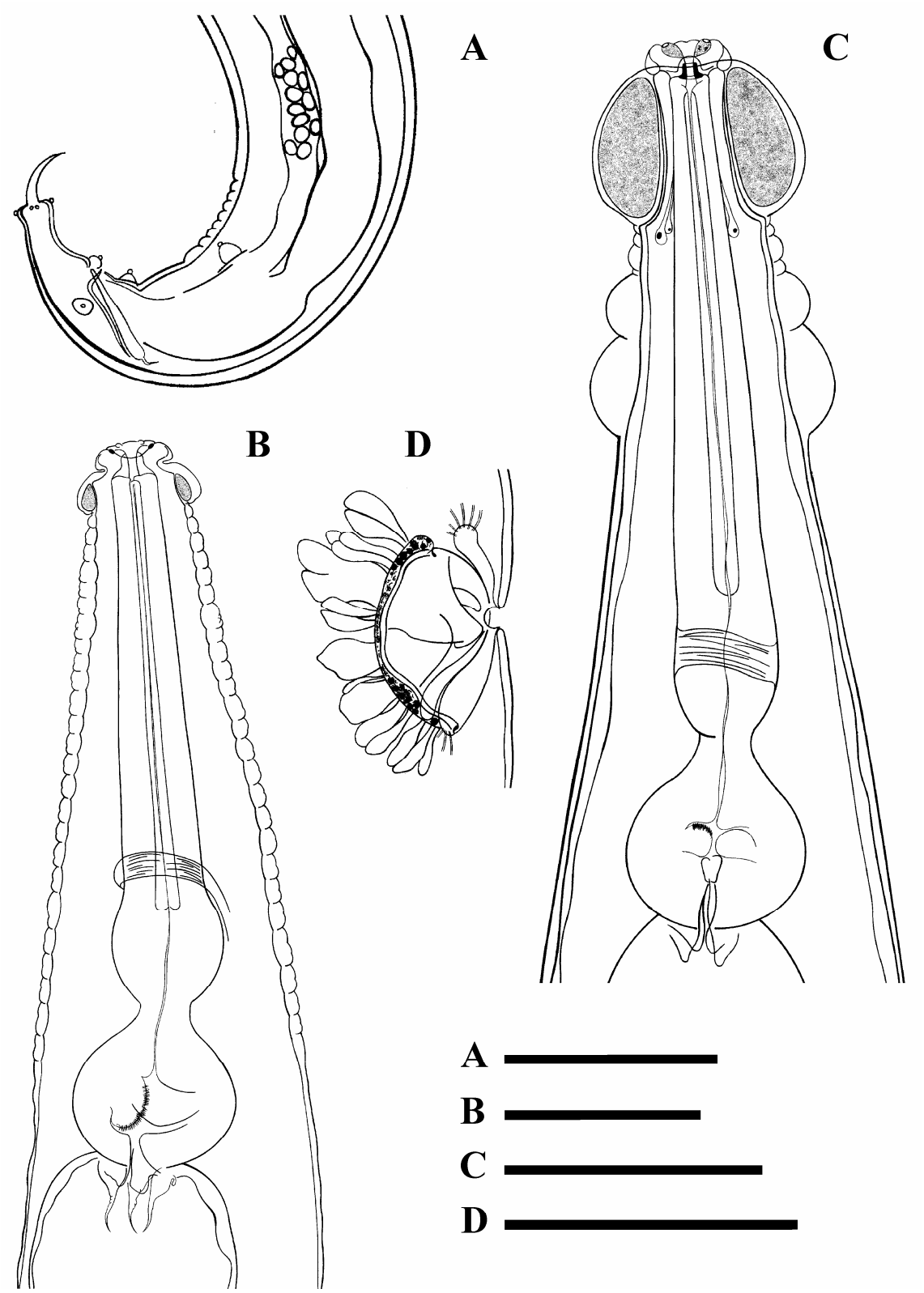

Fig. 3. *Pseudonymus spirotheca*. A: male, posterior end; B: female, anterior end. *P. islamabadi*. C: female, anterior end; D: female, excretory system. Scale bars: A = 50 μm; B–D = 100 μm.

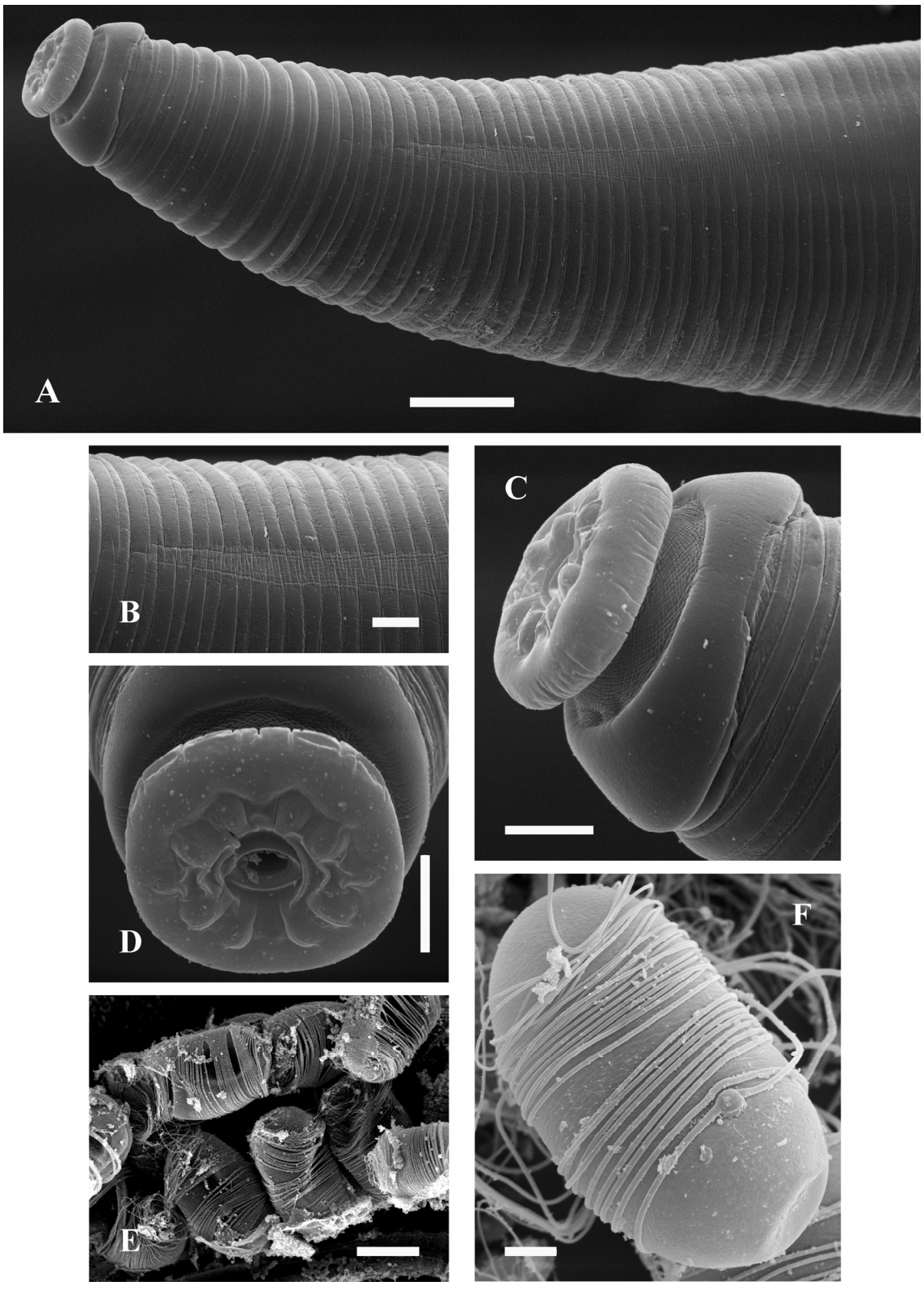

Fig. 4. SEM microphotographs of *Pseudonymus spirotheca*, female. A: anterior end; B: lateral field; C & D: perioral disc (lateral and frontal views, correspondingly); E: eggs in the uteri; F: egg-shell in details. Scale bars: A = 30 μm; B–D & F = 10 μm; E = 40 μm.

Pseudonymus islamabadi (Basir, 1941) (Table 1, Figs 1B, 3B, C & 5)

Male. Individuals with rounded tail and short spine appendage were earlier described within the species *P. spirotheca* (Leibersperger, 1960; Jarry, 1964; Shvetsova & Kakulia, 1973).

Female. Anterior end bearing six modified cuticular annuli (*ca* 90, 63, 70, 70, 96 and 115 μm in diameter, correspondingly). The first, third, fifth and the sixth annuli strongly inflated, with fibrous filling prominent especially in the first one. Rest of body cuticle with faint transverse striations. Lateral fields inconspicuous. Perioral disc (35–38 μm diam.) set off by deep constriction. Mouth opening enricled with eight submedian pseudolabia. Amphids tubular, with slit-like apertures. Stoma cylindrical. Cheilostom prominent. Gymnostom surrounded by a thick strongly cuticularised, 7–8 μm long, ring-like structure with widened base. Pharyngeal corpus gradually increases in diameter toward isthmus (up to 43–50 μm). Basal bulb 73–98 μm diam. Excretory pore at proventriculus level, with 6–11 μm wide sclerotised duct leading into excretory sac-like vesicle. Prominent folds on outer vesicle wall. Vesicle inner wall with numerous vacuoles. Intestine with slightly developed proventriculus of 120 μm diam. Reproductive system didelphic, amphidelphic. Vulva post-equatorial as transverse slit. Vulval lips only slightly protruding. Anterior lip covers vulva opening and anterior half of posterior lip. Vagina directed anteriorly, *vagina vera* with massively thickened walls. Uteri filled with ellipsoid eggs (mean number, 55), containing embryos in late stages of embryogenesis (from late gastrulation to bean-shaped). Egg-shell *ca* 3 μm

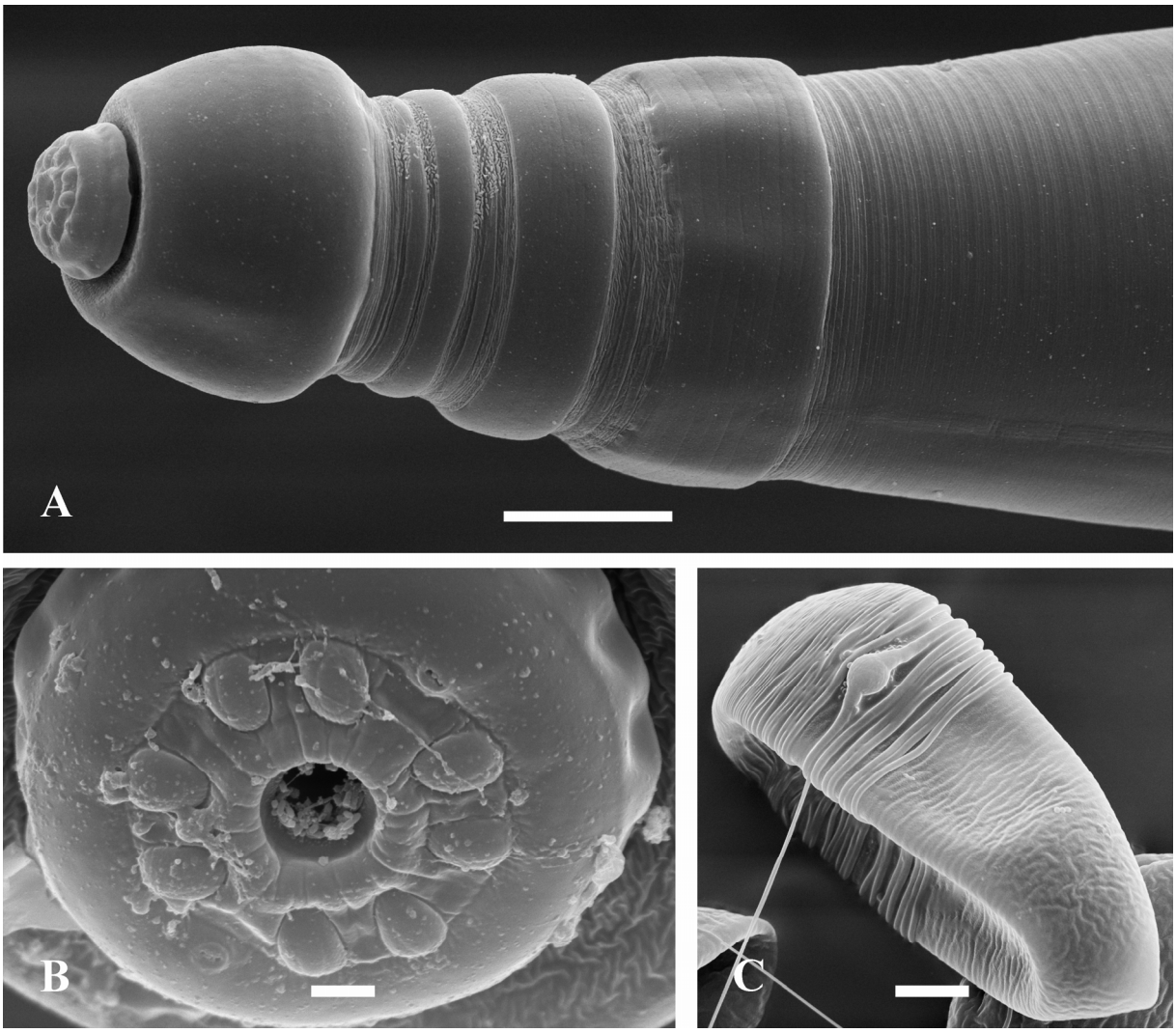

Fig. 5. SEM microphotographs of *Pseudonymus islamabadi*, female. A: anterior end; B: perioral disc (frontal view); C: egg-shell in details. Scale bars: A = 30 μm; B = 3 μm; C = 10 μm.

thick. From outside, it is encircled by two spirally-coiled filaments (*ca* 1 μm in thickness), issued from a roundish button-like swelling (mean dimensions, 6×5×3 μm), positioned at one-third egg length from one of the poles. Ovaries divergent, both branches positioned dorsally. Posterior branch protruding behind anus level. Phasmids pore-like, 55–79 μm from anal aperture.

Molecular characterisation. The sequences of the D2-D3 segment of 28S rDNA were obtained for both *Pseudonymus* species. The alignment of these 686 bp long sequences with other available Oxyuridomorpha (*sensu* De Ley & Blaxter, 2002) sequences (including newly obtained ones for *Aspiculuris tetraptera*, *Spauligodon* sp., *Tachygonetria* sp. and *Thelandros* sp.) was 740 bp long and included 227 constant characters, 107 uninformative characters and 406 parsimony-informative characters. The difference between two *Pseudonymus* species was 44 bp. Bayesian Inference and other methods of phylogenetic analysis (ML, MP and NJ – data not shown) produced similar phylogenetic trees with *Pseudonymus* species, clustering under strong support (posterior probability, bootstrap) with *Mesidionema* Timm, 1959 (intestinal parasites of earthworms) and *Binema* Travassos, 1925 (intestinal parasites of mole crickets) (Fig. 6).

DISCUSSION

Initially, *P. spirotheca* was described as *Oxyuris spirotheca* by György (1856). Diesing (1857) had established the genus *Pseudonymus* for these pinworms of water beetles, but then (1861) proposed another name for the same taxon – *Ptychocephalus*. This latter generic name was found to be preoccupied, and the genus *Pseudonymus* was restored by Stiles & Hassall (1905). Basir (1941) had established a new genus *Galebiella* Basir, 1941 with two species, *G. galebiella* (= *P. spirotheca*) and *G. islamabadi* (= *P. islamabadi*). Todd (1944)

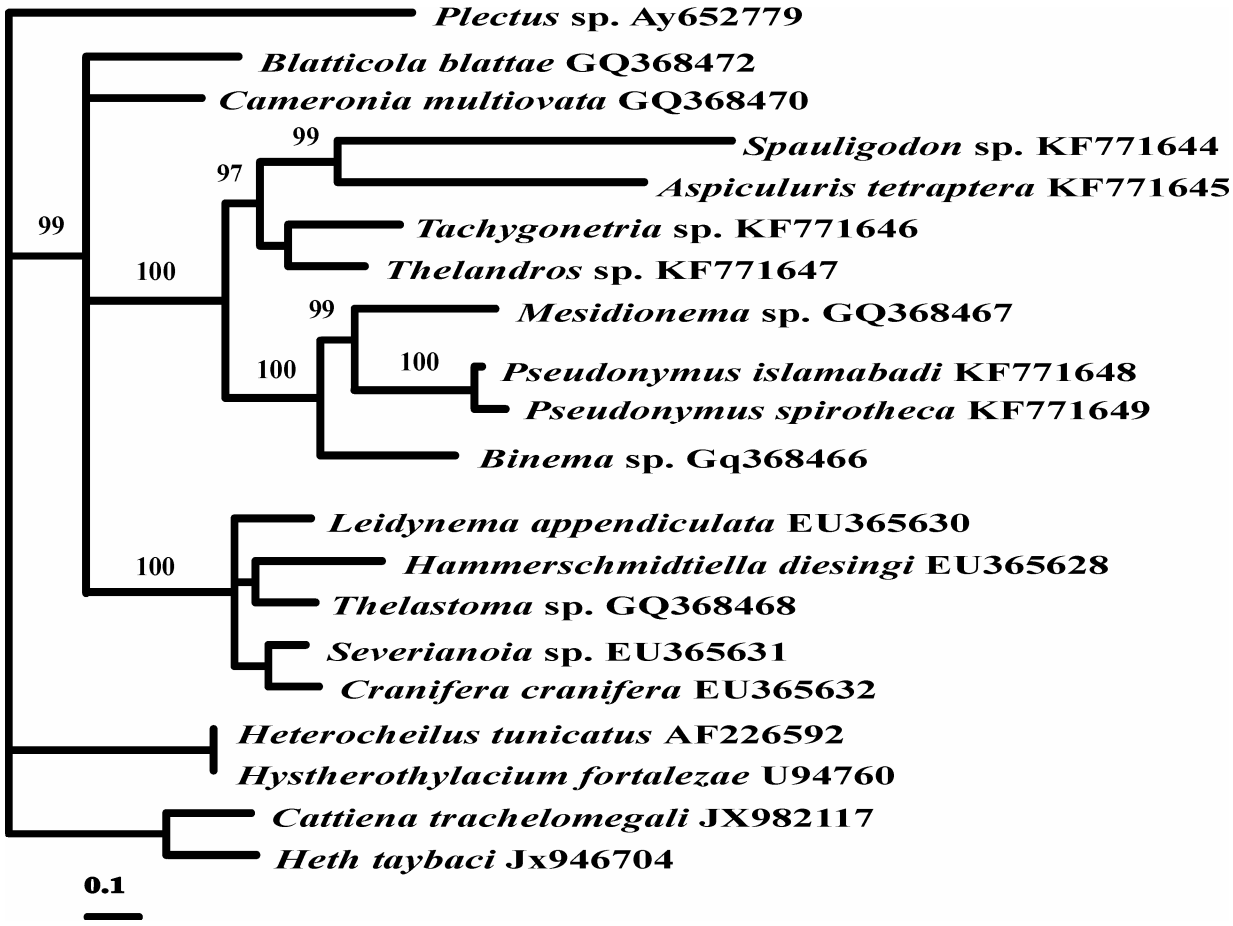

Fig. 6. Phylogenetic relationships among the studied species of the infraorder Oxyuridomorpha and related groups based on the partial sequences of 28S rDNA (50% majority-rule Bayesian Inference tree). Scale bar – expected changes per site.

considered *Galebiella* as a junior synonym of *Pseudonymus*. Later, Basir (1956) supplemented the description of these two species with new morphological details and measurements based on the material from Indian *Hydrophilus piceus*. For all species he stated the presence of two spirally coiled filaments arising from a knob-like swelling on the eggshell. *Pseudonymus islamabadi* was also reported from Brazil but treated within the genus *Györyia* Kloss, 1958 (Kloss, 1958, 1959). Leibersperger (1960) reported the presence of both *P. spirotheca* (as *P. hydrophili*) and *P. islamabadi* from *Hydrous aterrimus* Eschscholtz, 1822 in Germany and described males for both species. Leibesperger noted that the correspondence of the males with two morphologically different females was only conditional. Later, Jarry (1964) followed this male-female correspondence for these two *Pseudonymus* species, when described both of them from France. At the same time she considered *O. spirotheca* (Galeb, 1878) as a synonym of *P. islamabadi*. Shvetsova & Kakulia (1973) identified *P. spirotheca* and *P. islamabadi* in hydrophilid beetles from Russian Far East.

The nucleotide sequences of the D2-D3 expansion segment of 28S rDNA are informative enough for construction of comparatively stable phylogeny of the infraorder Oxyuridomorpha. Earlier we demonstrated that the majority of Thelastomatidae Travassos, 1929 from cockroaches formed a separate, well supported clade (Spiridonov & Guzeeva, 2009). This clade is obvious on the Bayesian phylogram obtained in the course of this study (Fig. 6). Surprisingly, the high support is demonstrated in this Bayesian tree for the clade uniting Oxyuroidea from vertebrates and the subclade of forms parasitizing in intestines of different invertebrates including both *Pseudonymus* species studied. Similar topology [(Oxyuroidea sublcade) ((*Pseudonymus* + *Mesidionema*) *Binema*)] was obtained with NJ analysis, though it was not observed on ML and MP trees. The [(*Pseudonymus* + *Mesidionema*) *Binema*] subclade was observed with all four methods of analysis. Clearly, the more extensive sampling of Oxyuridomorpha nematodes is needed for the definite evolution reconstruction but some phylogenetic patterns can be discussed at this stage. Thus, there is no strict correspondence between taxonomic position of the host and the position of the Oxyuridomorpha nematode on the phylogenetic tree. For example, although cockroach nematodes usually form a single, well supported clade, the genus *Blatticola* Schwenk, 1926 (parasites of German cockroaches and other Blattellidae) is always positioned outside this cluster. The representatives of some genera from this cluster (*Thelastoma* Leidy, 1849 and *Severianoia* Schwenk, 1926) were reported from non-cockroach hosts such as diplopodes and scarabeids (Adamson, 1989). Such distribution patterns are most probably the result of 'lateral transfer' of parasitic nematodes. The possibility of exchange with these intestinal parasites between even more distant groups of hosts cannot be excluded.

The phylogenetic links between Oxyuridomorpha of vertebrates and invertebrates as revealed in our analysis, still are needed to be confirmed with multilocus analysis. At the same time, unique morphological features support the close relationships of *Pseudonymus* with *Binema* (presence of filaments of eggshells; 7–8 pairs of genital papillae on the male tail). So far, the only conclusion concerning the phylogenetic position of the *Pseudonymus* genus is that that these nematodes of water beetles are not closely related to the genera of Thelastomatidae family; instead they cluster with the genera belonging to other families of Oxyuridomorpha.

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Guzeeva, E.A. and Spiridonov, S.E. Молекулярно-таксономическое изучение двух видов рода *Pseudonymus* Diesing, 1857 (Oxyuridomorpha: Thelastomatoidea: Pseudonymidae) от жуков-водолюбов (Coleoptera: Hydrophilidae) дельты Волги.

Резюме. Два вида нематод рода *Pseudonymus* Diesing, 1857, *P. spirotheca* (Györy, 1856) и *P. islamabadi* (Basir, 1941), обнаружены в задней кишке жуков-водолюбов (семейство Hydrophilidae), пойманных в авандельте реки Волги на территории Астраханского государственного биосферного заповедника в июне 2012 г. Получены новые морфологические данные по этим видам, и на основе сравнения нуклеотидных последовательностей рибосомальной ДНК (28S) подтверждена конспецифичность для самцов и самок двух видов псевдонимусов. Нуклеотидные различия между видами составили 44 пары оснований. Самки *P. spirotheca* характеризуются сильно утолщенным первым кутикулярным кольцом и оболочками яиц размерами 76–85×44–56 мкм, самцы – резко сужающимся хвостовым концом. Самки *P. islamabadi* на головном конце имеют шесть модифицированных кутикулярных колец разного диаметра, оболочки яиц 83–89×45–54 мкм, самцы этого вида отличаются округлым хвостовым концом. Согласно молекулярно-филогенетическому анализу, нематоды рода *Pseudonymus* образуют отдельную ветвь с высоким уровнем поддержки вместе с представителями родов *Binema* и *Mesidionema*.