Angiostoma meets *Phasmarhabditis*: a case of *Angiostoma kimmeriense* Korol & Spiridonov, 1991

Elena S. Ivanova and Sergei E. Spiridonov

Centre of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskii Prospect 33, 119071, Moscow, Russia
e-mail: elena_s_ivanova@rambler.ru

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Summary. *Angiostoma kimmeriense* (= *A. kimmeriensis*) Korol & Spiridonov, 1991 was re-isolated from the snail *Oxyhilus* sp. in the West Caucasus (Adygea Republic) and characterised morphologically and molecularly. The morphology of the genus *Angiostoma* Dujardin, 1845 was discussed and vertebrate-associated species suggested to be considered as species *insertae sedis* based on the head end structure (3 *vs* 6 lips). Phylogenetic analysis based on partial sequences of three RNA domains (D2-D3 segment of LSU rDNA and ITS rDNA) did not resolve the relationships of *A. kimmeriense*, as the most similar sequences of these loci were found between members of another gastropod associated genus, *Phasmarhabditis* Andrássy, 1976. However, such biological traits of *A. kimmeriense* as its large size, limited number of parasites within the host and the site of infection, point to a parasitic rather than pathogenic/necromenic way of life typical for *Phasmarhabditis*.

Key words: description, D2-D3 LSU sequences, ITS RNA sequences, Mollusca, morphology, morphometrics, phylogeny, taxonomy.

The family Angiostomatidae comprises two genera, *Angiostoma* Dujardin, 1845 with its 18 species, and monotypic *Aulacnema* Pham Van Luc, Spiridonov and Wilson, 2005. The representatives of the family are generally associated with terrestrial gastropods but four species of *Angiostoma* have been described from vertebrate (amphibian and reptile) hosts. It had been shown that the gastropod-associated angiostomatids belong to the clade V of Nematoda together with gastropod-associated representatives of *Agfa* Dougherty, 1955 and *Phasmarhabditis* Andrássy, 1976 (Ross *et al.*, 2010). The position of the *Angiostoma* species from vertebrate hosts remains unclear as no molecular data for them are yet available. As to the gastropod-associated species, only half of species have been molecularly characterised so far. Below we present the molecular-phylogenetic analysis for angiostomatids from gastropods based on the recent discovery of *A. kimmeriense*. Originally, *A. kimmeriense* Korol & Spiridonov, 1991 (= *A. kimmeriensis* in original description, spelled here according to the gender of the genus following Ivanova & Wilson, 2009) was recovered from the oesophagus of an endemic snail *Oxychilus deilus* (Bourguignat, 1857) in Crimea (Korol & Spiridonov, 1991). The surveys carried in Crimea in 2008, 2011 and 2014 did not reveal the presence of the nematode in this or other gastropods examined (Vorobjeva *et al.*, 2008; Ivanova *et al.*, 2013). Several species of snails collected in Adygea (West Caucasus) were examined for the presence of nematodes. *Oxychilus* sp. about 20 mm in diam. (most probably, *O. difficilis*) was found infected by *Angiostoma* sp. together with two other nematode species, *Phasmarhabditis* sp. and *Alloionema* sp. Morphological examination of *Angiostoma* sp. showed that it represents *A. kimmeriense*. Three RNA domains were examined and partial sequences obtained hereby giving the molecular characteristics of this species. The description of *A. kimmeriense* from the West Caucasus is given together with illustrations.

MATERIAL AND METHODS

Nematode material. Snails, *Oxychilus* sp., were collected at the field research station of the South Federal University in Adygea, North Caucasus, Russian Federation in June 2016 and April 2018. Each snail collected was found to contain a mixed parasite infection. The mixed infection of *Phasmarhabditis* sp. with *Angiostoma* sp., *Phasmarhabditis* sp. and *Alloionema* sp.,

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Phasmarhabditis sp. and unidentified trematodes was observed as well as other combinations including more than two parasites.

Angiostoma kimmeriense were isolated from the snail oesophagus and anterior portions of intestine and preserved as follows: for morphological studies, in hot 4-5% formalin, and for molecular studies, by freezing at –20°C. Formalin-preserved nematodes were then processed to glycerin following the method of Seinhorst (1959) and mounted on permanent slides using the wax ring method. Measurements and drawings were obtained with Nikon Eclipse E200 microscopes with drawing attachments. Illustrations were finalised with a WACOM Intuos A4 USB drawing tablet and Adobe Illustrator CS5. Abbreviations in the text are: a, b, c, c' – de Man indices; L – body length; V% – distance from anterior to vulva as the percentage of the body length; GP – male genital papillae. For the SEM studies, formalin-preserved material was dehydrated and dried out at critical point in a Hitachi Critical Point Dryer HCP-1 and coated with gold in a S150A sputter coater. Images were taken on a Tescan Cam Scan MV 2300.

Molecular characterisation and phylogenetic analysis. Individual nematodes identified under light microscope were used for DNA extraction according to the protocol of Holterman *et al.* (2006). PCR amplification was performed as described by Ivanova & Spiridonov (2017). The partial sequence of A. *kimmeriense* D2-D3 expansion segment of LSU rDNA was deposited in GenBank under MH627383, and partial ITS rDNA as MH627382. BLAST-search was used to identify sequences similar to these in NCBI GenBank, which were then used for comparison in phylogenetic analysis. The sequences were aligned using the Clustal_X program (Thompson *et al.*, 1997). Subsequently, the sequences were edited using the Genedoc 2.7 program (Nicholas *et al.*, 1997), to prepare a file for the analysis in MEGA5 (Tamura *et al.*, 2011). Phylogenetic trees were obtained using three different methods (MP – maximum parsimony, NJ – neighbour joining and ML – maximum likelihood) and pairwise nucleotide differences were calculated.

RESULTS

The prevalence of *Angiostoma* infection was 83% (10 out of 12) and the mean intensity 6 (3-16) specimens. Morphological examination using light and scanning microscopy has shown that the discovery represents *A. kimmeriense* as the specimens from Adygea were similar to that in the original description in metric characteristics and general morphological traits.

DESCRIPTION

Angiostoma kimmeriense Korol & Spiridonov, 1991 (Figs 1 & 2)

General. Body long, cylindrical, tapering to both ends, tail short. Cuticle thinly annulated. Lateral field expressed as simple thin band less than 1 µm wide (Fig. 2G).

Anterior portion of head *ca* 13 µm long and *ca* 25-30 µm wide, hemi-spherical, lacking annulations, slightly offset from body contour. Tissue layer just beneath cuticle thickened (*ca* 3-4 µm thick) and enforced by fine, densely packed fibres arranged perpendicularly to the body surface. Six elevations situated at the start of annulated region just beneath the margin of the non-annulated part. Each elevation bearing a salient papilla of internal circle and smaller one just posterior to it of external circle. Amphids tiny, located slightly farther back. Mouth aperture triangular, moderately sized. Stoma 4-5 µm wide, *ca* 15-18 µm long, stoma walls not sclerotised, cylindrical at anterior and expanding at bottom. Each stoma sector at bottom bearing 3 prominent cuticular denticles, one *ca* 3 µm long and other two *ca* 1.5 µm long. Pharynx long, finely muscular, club-like. Procorpus two thirds of pharynx length; isthmus moderately narrow, poorly marked; basal bulb as wide as procorpus. The anterior part of procorpus slightly narrower than middle and posterior parts forming a kind of a collar surrounding the posterior half of stoma. Valves in the basal bulb very fine, haustrulum present. Cardia large, conical, protruding into intestine lumen.

The position of a nerve ring varies from anterior part of isthmus to anterior part of bulb and the position of an excretory pore from bulb base to two body diameters posterior to bulb base. The distance between the nerve ring and the excretory pore is nearly constant and the more anterior position of the former corresponds with the similar position for the latter. Excretory duct *ca* 35-45 µm long leading to ampoule-like chamber. Intestine with thick walls forming diverticula in some specmens.

Male. n = 4. L = 3643 ± 593 (3180-4460) µm; a = 38.3 ± 3.7 (35.2-43.3); b = 11.5 ± 0.7 (11.1-12.4); c = 60.4 ± 6.9 (51.9-66.1); c' = 1 ± 0.2 (0.7-1.3); pharynx length = 316 ± 75 (257-400) µm; distance from anterior extremity to nerve ring = 230 ± 34 (208-270) µm; distance from anterior extremity to excretory pore = 305 ± 65 (262-380) µm; tail length = 62 ± 17 (49-86) µm; spicule length measured on arc = 202 ± 8 (191-211) µm; spicule length measured on chord = 186 ± 13 (175-198) µm; gubernaculum length = 59 ± 5 (54-66) µm. Stoma

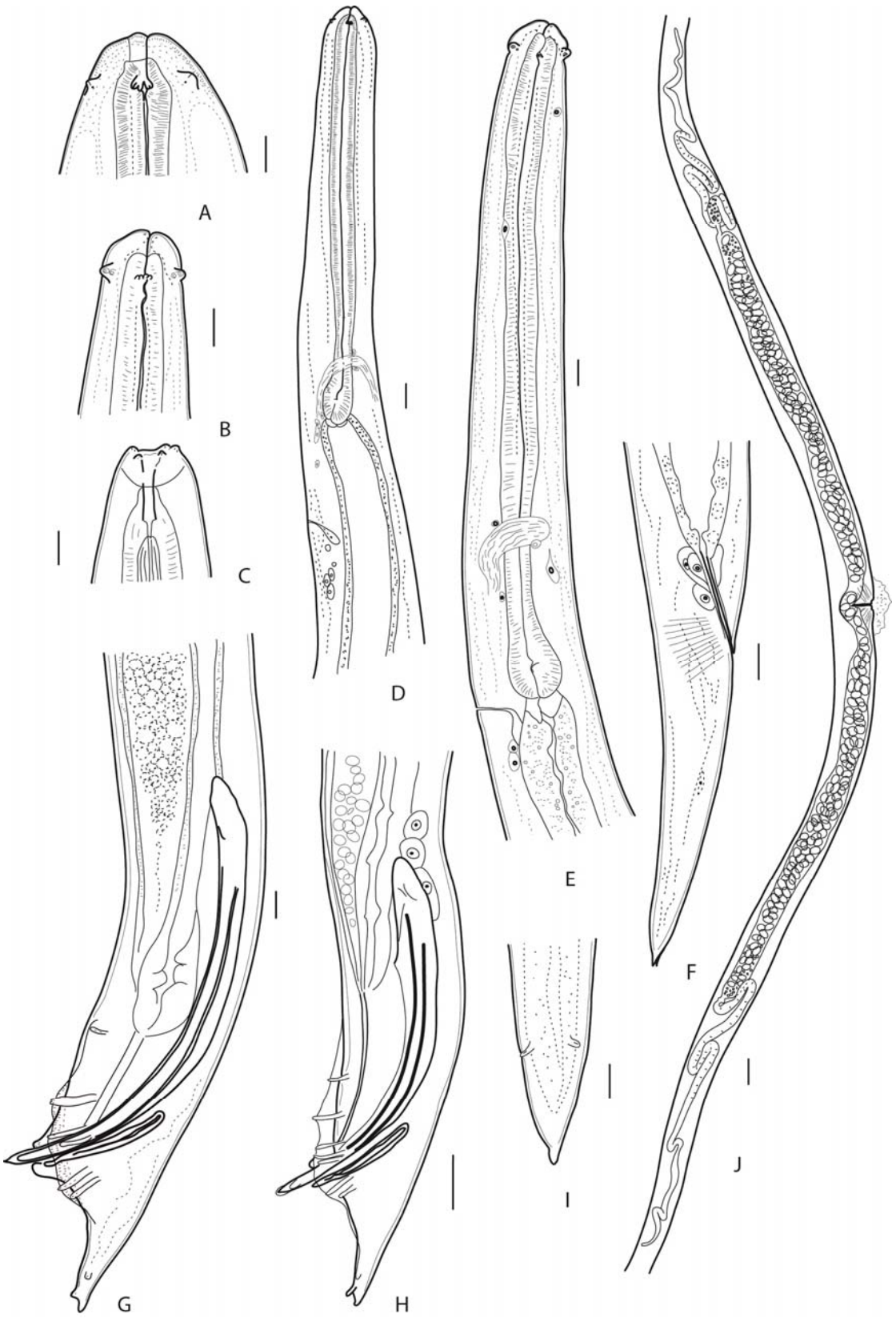

Fig. 1. Line drawings of *Angiostoma kimmeriense* from Adygea. A-C: head (A, female; B, male; C, juvenile female); D, E: anterior end of female with different position of excretory pore; F: female tail; G, H: male tail; I: tail of juvenile female; J: female gonad. Except I, all in lateral position. Scale bars in μm.

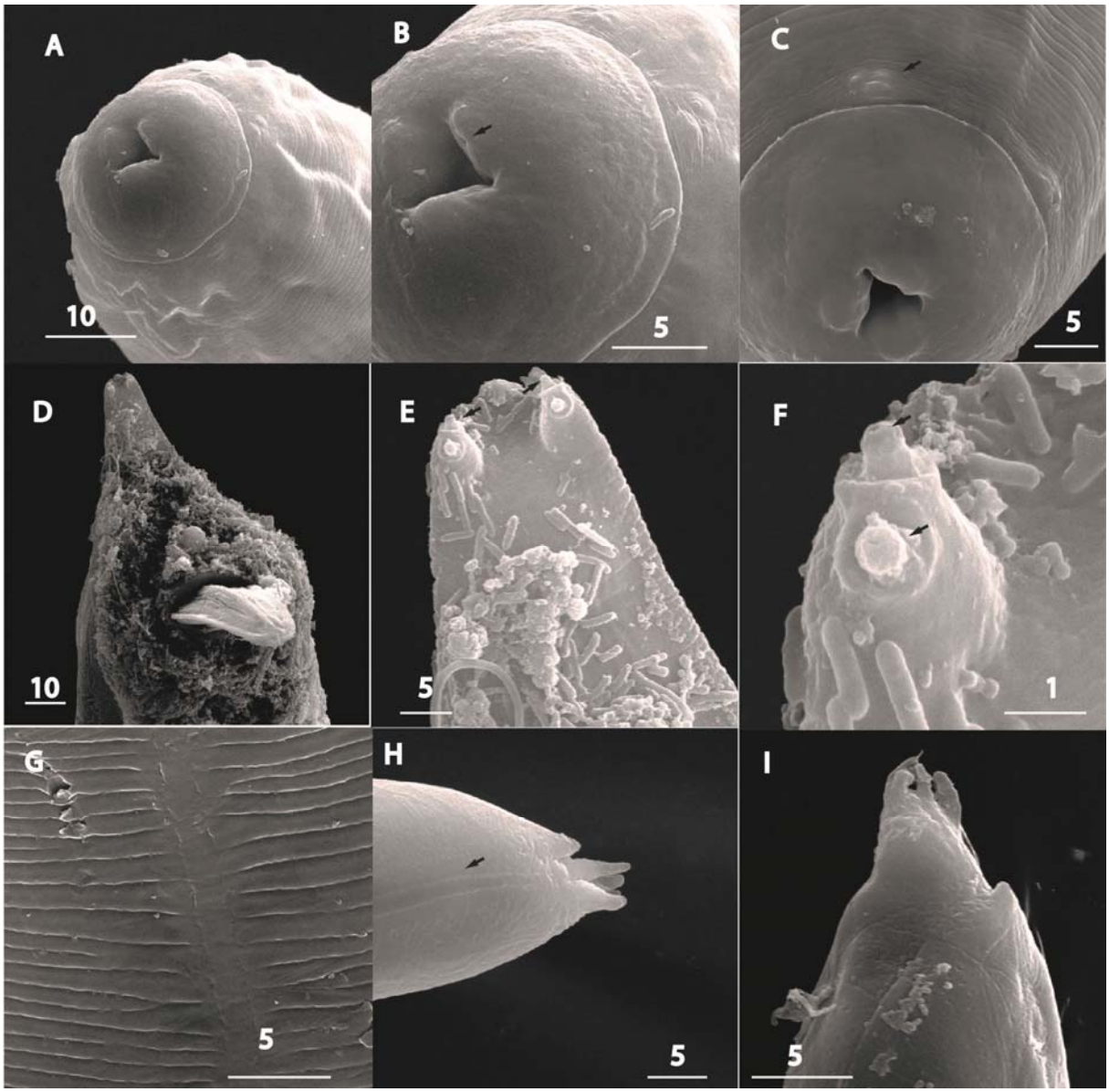

Fig. 2. SEM images of *Angiostoma kimmeriense* from Adygea. A-C: male head; D: male tail; E: male tail, posterior part; F: posteriormost GP and phasmid; G: cuticle of mid-body region of female; H, I: tail tip of female. Arrow indicating opening of pharyngeal gland (B); reduced lip (C); phasmids (E); GP and phasmid (F); lateral field (H). Scale bars in μm.

16 ± 1 (14-17) μm long and 4-5 μm wide. Cardia *ca* 10 μm long. Testis narrow (*ca* 1/4 of body diam.), distal portion of testis contains about 15-20 spermatids 15 μm in diam. *Vas deferens* and seminal vesicle not differing morphologically from ejaculatory duct. Sperm in an ejaculatory duct 3-6 μm in diam. and morphologically similar to sperm cells in female spermathecae. Anal glands prominent.

Bursa narrow, leptoderan. GP formula as follows 1 + 1 + 3/2 + 1 + ph, with papillae in pairs 4 & 5 and 6 & 7 very close. GP 4 & 5 not staggered. Except GP 5 & 7, all papillae reaching margin of bursa. Both GP5 and GP7 opened on dorsal side of bursa. GP8 not incorporated into bursa and situated closely to tail tip in subventral position. Close to GP8, a pair of slightly smaller than GP8 phasmids situated in ventral position observed in SEM only (Fig. 2E & F). The precloacal unpaired papillae and a pair of circumcloacal papillae present. Spicule distal tips sharply pointed with a narrow ala surrounded. Spicules strong, arcuate, manubria hardly demarcated. Spicular velum short, extending to mid-spicule length. A variation was observed in the

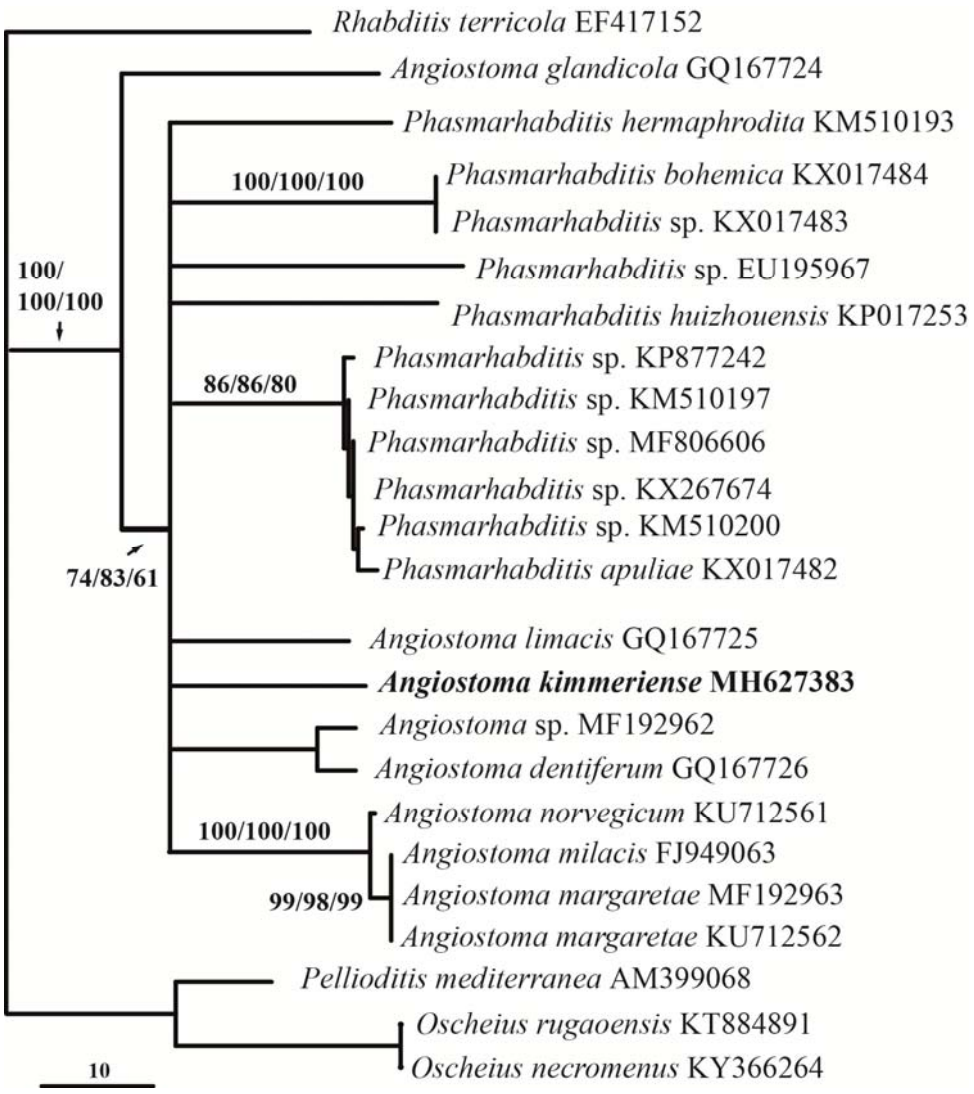

Fig. 3. Phylogenetic relationships of *Angiostoma kimmeriense* based on D2-D3 LSU rDNA. Bootstrap support values are presented near nodes as MP/NJ/ML. ML analysis (500 bootstrap replications), K2 + G model. For MP and NJ – 1000 bootstrap replications.

number of precloacal papillae in a single specimen (see Fig. 1G) where papillae 2 and 3 were merged retaining two sensillar endings. Denticles on tail tip absent.

Female. n = 10. L = 5936 ± 1231 (3450-7530) µm; a = 35 ± 3.3 (30.3-41.8); b = 14.8 ± 2.5 (9.1-17.9); c = 27.4 ± 3.2 (21.3-31.4); c' = 3.8 ± 0.3 (3.4-4.3); pharynx length = 403 ± 53 (330-490) µm; distance from anterior extremity to nerve ring = 320 ± 56 (260-413) µm; distance from anterior extremity to excretory pore = 415 ± 85 (317-540) µm; V% = 48 ± 0 (42.7-53.3); tail length= 216 ± 31 (162-260) µm. Lateral field extending to tail extremity. Stoma 18 ± 1 (17-21) µm long and 4.3 ± 0.8 (3-5) µm wide. Cardia *ca* 13 µm long. Ovaries paired, opposed, distal parts not reversed. Developing oocytes arranged in one row. Spermathecae located between each ovary and uterus and filled with sperm. Sperm cells in spermathecae and uteri 5-7 µm in diam. Uteri long, filled with widely oval, thin-walled eggs on different stages of development; 64 ± 4 (57-70) µm long and 37 ± 4 (30-42) µm wide. Juveniles in uteri not observed. Vulva transverse slit, vulval lips not or very slightly inflated, vagina about two-third body diameter long or 134 ± 26 (87-176) µm long. Copulatory plug present in fertilised specimens. Musculature in vulval and anal regions well developed. Rectum 77 ± 10 (64-90) µm long, sometimes inflated; lining strongly cuticularised. Anal glands large. Phasmids papilliform, located at 35 ± 4 (30-40) µm from tail tip. Two-four denticles on tail tip present.

Young female. n = 2. Body 2009 µm and 2070 µm long and 61 µm and 78 µm wide. Lip region less reflected than in adult female, 4 µm and 6 µm long. Stoma retaining rhabditoid plan with separate rhabdions, 15 µm and 18 µm long and 3 µm wide; denticles in gymnostom indistinct. Pharynx 223 µm and 280 µm long, nerve ring at 157 µm and 213 µm from apex, excretory pore at 184 µm and 291 µm from apex. Cardia 11 µm and 7 long µm. Spermatheca region demarcated by morphologically different tissue connecting distal portions of ovary and uterus with distinct duct inside. Vulva at 52.2% of body length. Tail 98 µm and 130 µm long, phasmids at 30 µm and 35 µm from tail tip. Tail tip pointed without denticles.

Remarks. We observed the variations in the position of a nerve ring and excretory pore position and the correlation between a nerve ring position and an excretory pore position in our material which was not mentioned in the original description. We did not find whether such variations were correlated with the size and age of the nematode.

Specimens from Crimea and Caucasus are very similar in general body proportions and metric characteristics except for the longer spicules (average 186 µm *vs* 124 µm measured on chord) and gubernaculum (average 59 µm *vs* 44 µm) of males in the material from Caucasus. As the host preferences are similar in both cases we tend to believe that specimens from Adygea represent *A. kimmeriense*. Molecular characteristics of *A. kimmeriense* from the type locality are yet to be obtained and compared with the Caucasian ones.

Molecular analysis and phylogenetic position of *Angiostoma kimmeriense*. A matrix of nucleotide data with the length of 493 bp was obtained for D2-D3 LSU rDNA with 136 characters being parsimony-informative. A matrix of nucleotide data with the length of 783 bp was obtained for ITS rDNA with 209 parsimony-informative characters. The results inferred from the analysis of D2-D3 LSU rDNA sequences revealed that EM434 strain of *Phasmarhabditis* sp. (deposited as EU195967) was the most similar form with the difference in only 16 bp, while the nucleotide differences with all other nematodes (including species of *Angiostoma*) were above 21 bp. The relationships between species of *Phasmarhabditis* and *Angiostoma* were not resolved in D2-D3 LSU rDNA analysis (Fig. 3). Similarly, the phylogenetic relationships were not resolved in the ITS rDNA analysis (Fig. 4). The lowest level of nucleotide difference in ITS rDNA was found between *A. kimmeriense* and two unidentified strains of *Phasmarhabditis* from Czech Republic (VP '185' and VP 'CH1' deposited as KX017487 and KX017486, respectively).

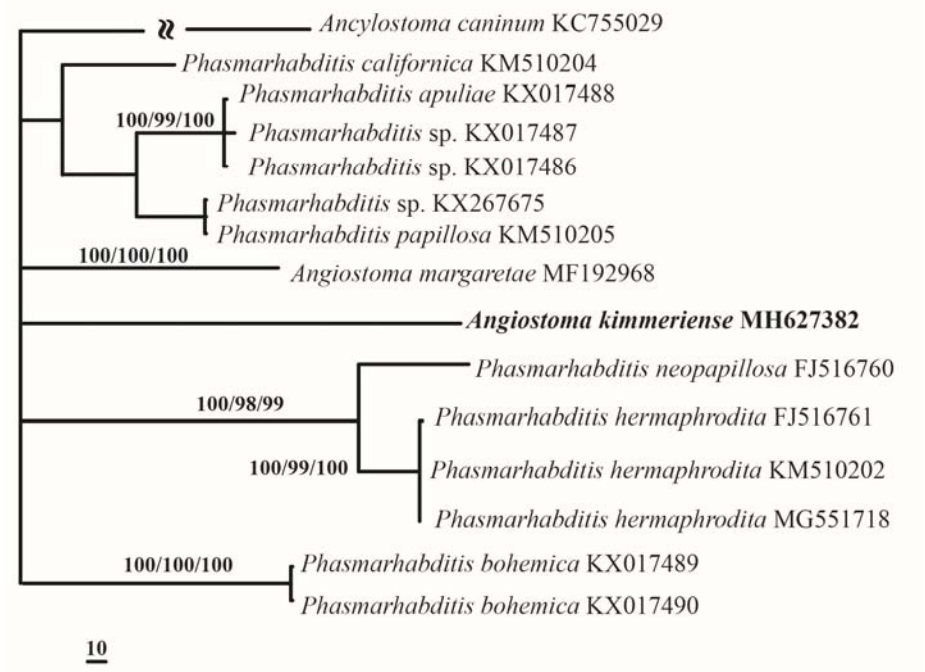

Fig. 4. Phylogenetic relationships of *Angiostoma kimmeriense* based on ITS rDNA. Bootstrap support values are presented near nodes as MP/NJ/ML. ML analysis (500 bootstrap replications), T92 + G model. For MP and NJ – 1000 bootstrap replications.

DISCUSSION

Four out of 19 species of Angiostomatidae were described from amphibian and reptile hosts: *A. plethodontis* Chitwood, 1933, *A. onychodactyla* Bursey & Goldberg, 2000, *A. carettae* Bursey & Manire, 2001 and *A. lamotheargumedoi* Falcón-Ordaz *et al.*, 2008. In the absence of molecular data on these and many gastropod-associated angiostomatids we have to recourse to the comparative analysis of their morphology.

The main morphological trait differentiating these four species from the rest of the genus is the head end structure with its three distinct lips. Here, each lip bears a pair of cephalic papillae. By contrast, the head end of the majority of *Angiostoma* species associated with gastropods is not differentiated into lips but has 6 elevations situated closely to a mouth aperture. The elevations are reduced, flattened lips.

Among gastropod-associated species, three retain the possession of 6 lip-like structures at the head end but differ in its organisation. These are *A. glandicola* Ivanova & Spiridonov, 2010 with 6 probolae apically bearing head sensillae and *A. kimmeriense* and *A. zonitidis* Ivanova & Wison, 2009 with 6 elevations displaced from the mouth aperture posterior to the margin of a cap-like structure of a head end (Ivanova & Wison, 2009; Ivanova & Spiridonov, 2010). Such general 6-elements scheme of a head end is characteristic for all known gastropod-associated members of the genus. Thus, the three-lipped structure of a head of vertebrate-associated *Angiostoma* spp. indicates that they may belong to a different genus (or genera, considering the variable vertebrate hosts, from amphibians to reptiles). Genetic distance between two parts of the genus (or between species) can be determined only with the use of the molecular data, which are not yet available. For the time being, we suggest that *A. plethodontis*, *A. onychodactyla*, *A. carettae* and *A. lamotheargumedoi* are to be considered as species *insertae sedis* (Chitwood, 1933; Bursey & Goldberg, 2000; Bursey & Manire, 2001; Falcón-Ordaz *et al.*, 2008).

Surprisingly, the D2-D3 28S rDNA analysis has placed *A. kimmeriense* close to *Phasmarhabditis* sp. EU 195967. Another sequence of *Phasmarhabditis* sp. was the closest one to *A. kimmeriense* in the ITS rDNA analysis. It seems that two loci used are not informative enough to resolve the relationships between these two genera. In LSU rDNA phylogram (Fig. 3) the sequences of *Phasmarhabditis* and *Angiostoma* did not form separate clades but were resolved as a united clade with the morphologically and molecularly deviant *A. glandicola* from tropical gastropods as a basal form. This united *Phasmarhabditis* + *Angiostoma* clade was characterised by strong support.

Affinity of *Phasmarhabditis*, *Angiostoma* and *Agfa* (a parasite of salivary glands of gastropods) was shown by Ross *et al.* (2010) based on the analysis of 18S rDNA gene. The latter two genera have evidently undergone morphological transformations due to the parasitic way of life while *Phasmarhabditis* still retains the general morphological traits of free-living Rhabditidae. According to Sudhaus (2011) "There is hardly any apomorphic morphological character that can substantiate this relationship apart from GP4 and GP5 standing very close and staggered, and perhaps GP5 and GP9 opening on the dorsal surface of the bursa velum".

Considering the affinity of *A. kimmeriense* and, perhaps, *A. zonitidis* to *Phasmarhabditis* the following common traits should be noted: cylindrical stoma; denticles in stegostom; narrowed anterior part of corpus enveloping posterior of stoma similarly to pharyngeal collar of *Phasmarhabditis*, male phasmids situated close to the last pair of GP. The dissimilarities are more numerous and include the large size, head end papillae displaced, leptoderan bursa, outstretched ovaries and many more.

While the number of genital papillae in males of *Phasmarhabditis* is stable, the same feature in *Angiostoma* in general is variable (7 to 10) and differs even in the closest species of *A. kimmeriense* and *A. zonitidis* (8 *vs* 10). Whether the rule of 'GP4 and GP5 standing very close and staggered' within *Angiostoma* is usually observed, the other ('GP5 and GP9 opening on the dorsal surface of the bursa velum') is not.

The position of male phasmids within *Angiostoma* (Morand, 1986, 1992; Ivanova & Wilson, 2009; Ivanova & Spiridonov, 2010) was posterior to the last pair of genital papillae, which conforms to observations by Kiontke (1999) on *Angiostoma* spp. and supports the affinity with *Phasmarhabditis*.

On the other hand, such traits of *A. kimmeriense* as the large size, parasitising of adult nematodes in a host and the site of infection points to a parasitic rather than pathogenic way of life. At present, we refrain from transferring *A. kimmeriense* to *Phasmarhabditis*. Whether *A. kimmeriense* represents the case of emergence of parasitism within necromenic/pathogenic genus requires further research.

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Е.С. Иванова и С.Э. Спиридонов. *Angiostoma* или *Phasmarhabditis*: случай *Angiostoma kimmeriense* Korol & Spiridonov, 1991.

Резюме. *Angiostoma kimmeriense* (= *A. kimmeriensis*) Korol & Spiridonov, 1991, впервые описанную из Крыма, выделили из наземной улитки *Oxyhilus* sp. на западном Кавказе в Республике Адыгея и получили морфологические и молекулярные характеристики вида. В результате обсуждения морфологии видов *Angiostoma* Dujardin, 1845 пришли к решению рассматривать те из видов, которые ассоциированы с позвоночными, в качестве *insertae sedis* на основании структуры головного конца (3 *vs* 6 губ). Проведен филогенетический анализ на основе частичных последовательностей трех участков РНК (18S, 28S и ITS), который не смог определить взаимоотношения *A. kimmeriense* и других ангиостом, так как наиболее близкие последовательности по этим участкам РНК были найдены среди членов другого ассоциированного с гастроподами рода - *Phasmarhabditis* Andrássy, 1976. Однако такие биологические особенности *A. kimmeriense* как большие размеры тела, ограниченное число паразитов в хозяине и место локализации паразитов указывают скорее на паразитический образ жизни этих нематод, чем на типичные для *Phasmarhabditis* некромению или патогенное влияние.