New data on morphology and molecular affiliations of two species of *Dicelis* Dujardin, 1845 (Cephalobomorpha, Drilonematoidea) parasitic in lumbricids

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Summary. Recent finding of two species of *Dicelis* Dujardin, 1845 parasitising lumbricids has added information on morphology and molecular affiliations of *Dicelis* members. The morphology of *D. ussuriensis* from the type locality and newly recovered *D. rubidi* and *D. kimmeriensis* was examined by SEM and the three species compared. The structure of the head end appeared to be very similar in all three species. Molecular analysis showed that *D. kimmeriensis* separated more strongly from the rest of the genus, which is in agreement with its morphological peculiarities. In pairwise nucleotide differences in the D2-D3 LSU rDNA region, *D. kimmeriensis* was at equal distance from congeners and free-living cephalobids. *Dicelis rubidi* from Khabarovsk Krai differed from the type locality specimens in 7 bp, which was regarded as intraspecific variation. On the phylogenetic tree, the species of *Dicelis* from the Western part of Palearctic were in the basal position in relation to Asian species, which formed the well supported subclade.

Key words: coelomic parasites, earthworms, nucleotide differences, SEM.

The nematodes of the genus *Dicelis* Dujardin, 1845 are parasites of the coelomic cavity of earthworms. So far, only 4 out of 15 valid species of the genus (*D. lovatiana* Ivanova, 1993, *D. caledoniensis* Spiridonov, Ivanova & Wilson, 2005, *D. ussuriensis* Spiridonov, Ivanova & Wilson, 2005 and *D. rubidi* Ivanova, 1994) were molecularly characterised. Except for one species, all *Dicelis* species parasitise lumbricids distributed throughout the Palearctic. *Dicelis eudrilii* Ivanova & Hope, 2009 was found in a host from the Eudrilidae family of African origin and possessed several traits characteristic also for the closely related genus *Adieronema* Timm, 1967 (Ivanova & Hope, 2009). Despite its differing morphology, the species was placed in the genus *Dicelis* because identification was possible only by means of morphological studies and the placement in *Dicelis* was considered more appropriate. The intermediate position of this species was discussed (Ivanova & Hope, 2009) and the possibility of different generic allocation once molecular characterisation became available was expressed. Given that *Dicelis* is separated from the rest of Drilonematoidea phylogenetically and represents a terminal branch of cephalobid phylogeny (Spiridonov *et al.*, 2005), the clarification of relationships within the genus is important to the phylogeny of the whole Drilonematoidea. Below we present additional data on two valid species of the genus, which were recovered recently and examined using molecular techniques.

MATERIAL AND METHODS

Parasitological procedures. *Dicelis kimmeriensis* Ivanova, 1993 was recovered from the coelomic cavity of *Dendrobaena veneta* (Rosa, 1886) collected by E. Ivanova in April 2014 in three localities in Crimea: the type locality, the juniper (*Juniperus excelsa*) forest (nature reserve) near Novyi Svet, 2 out of 31 specimens examined were infected by between 1 to 2 *D. kimmeriensis*; the Botanical garden in Simferopol, 1 out of 11 earthworms infected by 2 nematodes; the city park in Alushta, 1 out of 10 specimens infected by 5 nematodes.

Dicelis rubidi was recovered from *Eisenia nordenscioldi nordenscioldi* (Eisen, 1879) collected by G. Ganin by Manoma river, Aniuy National Park, Nanaysky district, Khabarovsk Krai, in September 2014 (4 out of 7 infected, range 1-14).

Except for two-three specimens reserved for DNA study, nematodes were fixed and preserved in hot 4-5% formaldehyde for light and scanning microscopy. For light microscopic studies using Nikon Eclipse, the formaldehyde-fixed nematodes were processed to anhydrous glycerol and mounted on slides (Seinhorst, 1959). For SEM studies on *D. kimmeriensis* and *D. rubidi*, nematodes were re-hydrated after formaldehyde, dehydrated in a graded ethanol series, critical-point dried using a HCP-2 Hitachi dryer, mounted on aluminium stubs and coated with gold in a Bio-Rad SC502 sputter coater; specimens were studied in a JCM-6380 LA SEM and CamScan S2 (Cambridge Instruments, UK) in the Laboratory for electron microscopic studies of Moscow State University. For SEM of *D. ussuriensis*, nematodes were rehydrated, stained with OsO_4, dehydrated in a graded ethanol series, critical-point dried in the critical-point dryer Bal-Tec CPD 030, mounted on aluminium stubs and coated with gold in a Polaron SC 7640 sputter coater; specimens were studied in a LEO VP 1540 SEM in the Museum für Naturkunde in Berlin.

Molecular characterisation and DNA analysis. DNA extraction, amplification and direct sequencing were performed following previously established protocols (Spiridonov *et al.*, 2007). Partial sequence of D2-D3 expansion segment of 28S rDNA was obtained with primers D2A (ACA AGT ACC GTG AGG GAA AGT TG) and D3B (TCG GAA GGA ACC AGC TAC TA) for two samples: *Dicelis kimmeriensis* from Crimea and *D. rubidi* from Khabarovsk region. Sequences were deposited in GenBank: for *D. kimmeriensis*, under accession number KY126844 and for *D. rubidi* under accession number KY126843. DNA extraction of *D. ussuriensis* from *E. n. pallida* was described in Spiridonov *et al.* (2005). For the rest of the material, DNA was extracted from deep frozen samples.

For comparative purposes and reconstruction of the phylogeny, the nematode D2-D3 rDNA sequences deposited in GenBank were used, including those obtained earlier for *Dicelis* species: AY 967866 for *D. rubidi*; AY 967867 for *D. caledoniensis*; AY 967868 for *D. lovatiana*; and AY 967869 for *D. ussuriensis*. Related forms of free-living nematodes were included after BLAST searches (Altschul *et al.*, 1990). The NCBI accession numbers of sequences of these nematode species are cited on the phylogenetic trees. Bootstrap or posterior probabilities values are presented near the nodes. Sequence alignments were generated using Clustal X using default values for gap opening and gap extension penalties. Alignments were analysed using maximum parsimony (MP) with PAUP* 4.0b10 (Swofford, 1998) and also maximum parsimony, maximum likelihood (ML) and neighbour joining (NJ) analyses with MEGA7 (Kumar *et al.*, 2016).

RESULTS

Morphology. SEM studies have revealed details of morphology which were not detected by light microscopy or were incorrectly interpreted at the time of the species description.

Dicelis ussuruiensis
Figs 1A & B, 2A, B & G, 3A-C

SEM studies carried out on the material obtained at the type locality (Primorsky Krai) at the time of the species description.

Cuticle transversally densely striated. Lateral fields distinct, with 10-12 flattened, thinly striated, even ridges divided by shallow narrow incisions, *ca* 15 wide at mid-body, starting at short distance from anterior extremity and expanding nearly to distal tail tip (Fig. 2A & B). Deirid papilliform, prominent, situated at level of basal pharyngeal expansion in middle of lateral field (Fig. 2A & B). Head bluntly rounded, lips absent (Fig. 1A). Mouth aperture circular, *ca* one third of head diameter wide. Four pairs of sensilla (*vs* 4 papillae in original description, papilliform sensilla not recognised) located very closely to each other at some distance from mouth aperture. Each pair (2 subdorsal and 2 subventral) consisting of short setiform sensilla and large (*ca* 2 in diam.) and nearly flat button-like papilla situated very slightly posterior to a setiform sensillum and very slightly displaced from it in lateral direction (Fig. 1A). Amphids large elliptical apertures *ca* 7 max in diam. without visible pouch (*vs* pore-like in original description), situated between large papillae of each pair (Fig. 1A). Tail in both sexes conical with one or two digital tips *ca* 3 long (*vs* bifurcated tail tip in original description). Caudal fimbriate organs situated in a pit at mid-line of lateral field at mid-length of tail or anterior to it; longitudinally elongated to almost circular in shape, with raised narrow rim (Fig. 1B).

Prominent, inflated precloacal flap in males. Three pairs of precloacal papillae and a pair of adanal papillae in sublateral position, on the edge of a lateral field, all evenly spaced and of similar appearance; a pair of lateral papillae at mid-tail in

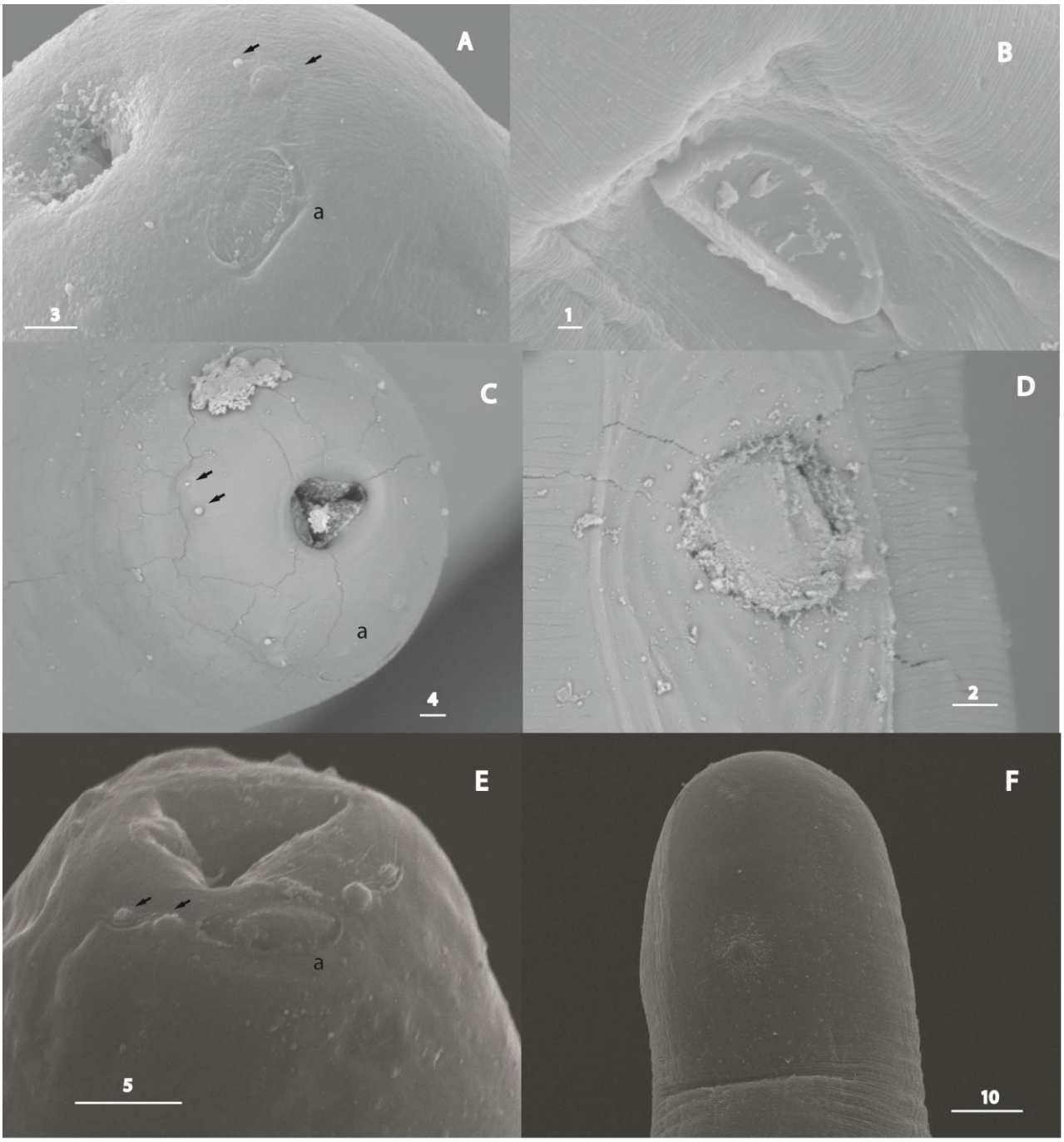

Fig. 1. A-B; *Dicelis ussuriensis*, Primorsky Krai, A, head, B, caudal organ; C-D; *D. rubidi*, Khabarovsky Krai, B, head, D, caudal organ; E-F; *D. kimmeriensis*, Crimea, E, head, caudal organ. Arrows, papillae; a, amphid. Scale-bars in micrometers.

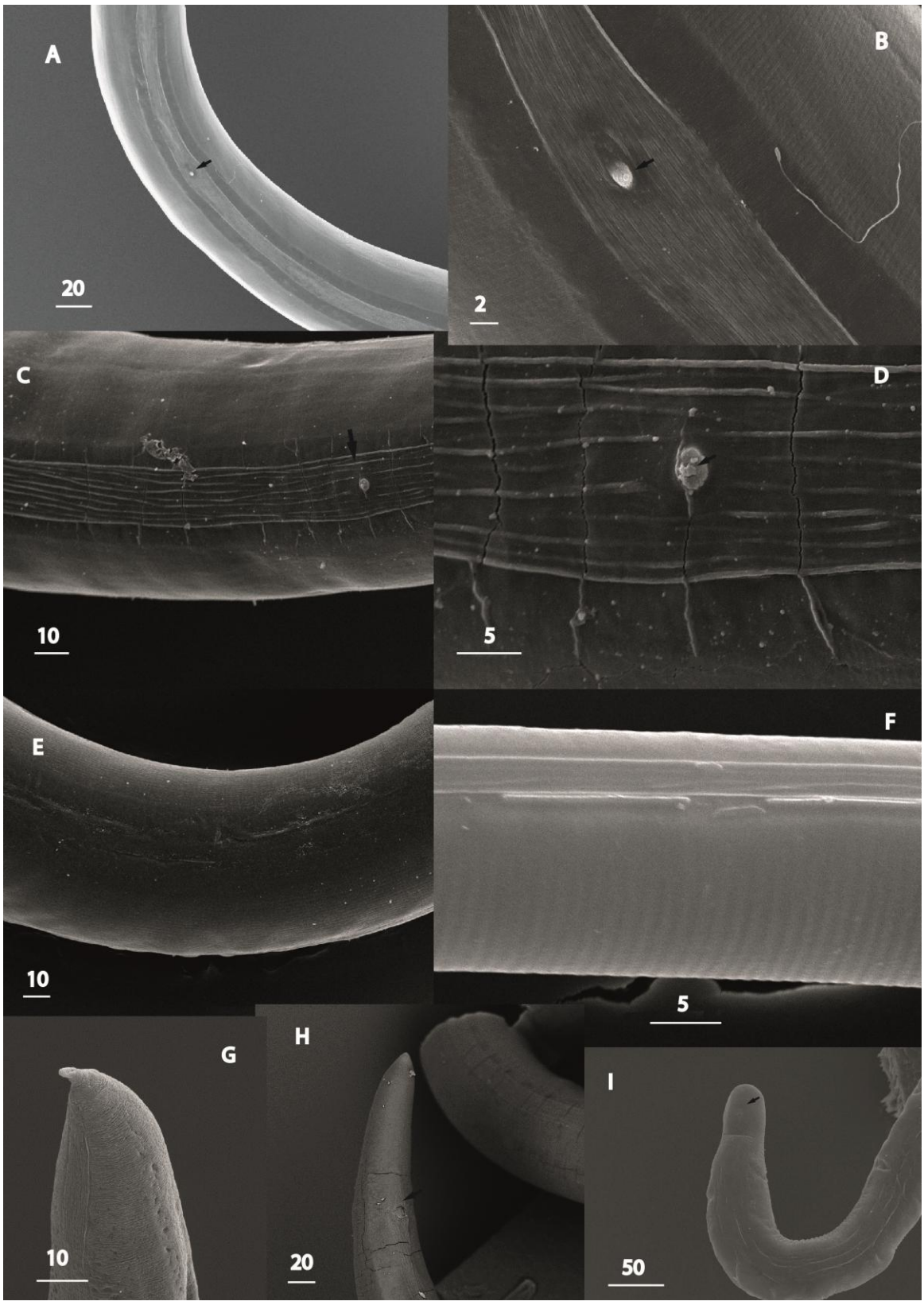

Fig. 2. A-B, G; *Dicelis ussuriensis*, Primorsky Krai, A, lateral field with deirid, B, same, at higher magnification, G, tail appendage, female; C-D, H; *D. rubidi*, Khabarovsky Krai, C, lateral field with deirid, D, same, at higher magnification, H, tail region with caudal organ, female; E-F, I; *D. kimmeriensis*, Crimea, E, lateral field of female, F, lateral field of juvenile, I, tail region with caudal organ, female. Arrows, deirid. Scale-bars in micrometres.

front of caudal organs and a pair of sublateral papillae posterior to caudal organs, largest in size. The variant of different disposition of postcloacal papillae was observed when the 5th pair was subventral and located posterior to caudal organs and the 6th pair of papillae was positioned laterally at lateral field closely to tail extremity. Distal tips of spicule blunt either acute (Fig. 3A-C).

Dicelis rubidi
Figs 1C&D; 2C, D, H; 3D-F

Very similar to *D. ussuriensis* in organisation of the head end, lateral field, deirid location and male genital papillae. Lateral field with at least 10 longitudinal, discontinuous, evenly spaced, narrow, protruding ridges instead of flattened ridges divided by incisions in *D. ussuriensis* (Fig. 2C & D). Mouth aperture circular to triquetrous, smaller than in *D. ussuriensis* (Fig. 1C). *Dicelis rubidi* and *D. ussuriensis* are differentiated mainly by the shape of caudal organs and a tail (Fig. 1D). The difference of caudal organs structure is better demonstrated by light microscopy showing the deeper cavity and smaller aperture in *D. rubidi* and the shallow cavity with a larger aperture in *D. ussuriensis*. SEM studies showed that its external structures are circular in *D. rubidi vs* oval in *D. ussuriensis* (Fig. 2H). Tail in both sexes wider and less tapering than in *D. ussuriensis* and lacking appendages (Fig. 2H, Fig. 3D-F). Tail extremity with 1 or 2 tiniest mucrons. Also, eggshells in *D. rubidi* with smaller and less protruberant tubercles.

No significant difference in morphometrics compared with the type material from Magadan was observed except for shorter tails (mean 162 *vs* 253 males, 250 *vs* 358 females) and slightly smaller body size (mean 2569 *vs* 2778 males, 3246 *vs* 3575 females).

Dicelis kimmeriensis
Figs 1E&F; 2E, F, I; 3G-H

Cuticle thinly transversally and longitudinally striated giving the tesselated appearance. Head extremity slightly narrowed, lips absent. Mouth aperture wide (more than half head diameter), triangular (Fig. 1E). Four pairs of cephalic papillae (2 subdorsal and 2 subventral), each pair consisting of a larger, slightly raised papilla and smaller one with protruding sensillum ending, located side by side. Large amphids without pouches (*vs* pore-like in original description), situated between larger papillae of a subventral and a subdorsal pair (Fig. 1E). Lateral field poorly expressed, starting in 25 from head extremity and terminating anterior to caudal region; 2 wide in juvenile, 3 to 10 in adult, single discontinuous ridge present in the middle (Fig. 2E & F). Male with a flat, circular pericloacal disc (Fig. 3H). Genital papillae prominent, button-like: 4 pairs precloacal subventral (*vs* 8 pairs in original description), a pair adanal subventral, 2 pairs postcloacal ventral: largest at the level of caudal organs and smallest closer to tail tip (Fig. 3G). Caudal organs in both sexes situated at posterior third of tail; small, circular (Fig. 2I). Tail very gradually tapered, tail extremity wide, rounded (Fig. 2I).

Molecular studies. The phylogenetic relationships between the studied species of *Dicelis* were inferred from the analysis of 630 bp long alignment of D2-D3 28S rDNA sequences. Several free-living nematode species (*Acrobeloides*, *Cephalobus*, *Chiloplacus*, *Placodira*, *Zeldia* have been used in the analysis after the BLAST search has revealed their affinity to *Dicelis* species. In the MP analysis of D2-D3 LSU rDNA, the *Dicelis* species formed a single clade with a high level of bootstrap support (Fig. 4). Equally a high level of bootstrap support for *Dicelis* monophyly were observed under NJ and ML analyses (data not shown). Inside the *Dicelis* clade, the sequences of *Dicelis* species from Western part of Palearctic were in the basal position, whilst the Asian species formed the well supported terminal subclade. The pairwise nucleotide differences were compared between *Dicelis* species. The newly sequenced isolate of *D. rubidi* differed from the type one in 7 bp, which can be considered as the intraspecific variation for these nematodes. The level of difference between species was significantly higher: from 23-24 bp between the closest *D. rubidi vs D. ussuriensis* and up to 45-51 between European and Asian *Dicelis* (Table 1). The greatest difference was observed between *D. kimmeriensis* and all the remaining species, reaching 76-77 bp with Asian species and 84 bp with European ones. Surprisingly, the level of difference between *D. kimmeriensis* and some congeners (*D. caledoniensis*, *D. lovatiana*) was higher than that between *D. kimmeriensis* and the majority of free-living cephalobids that were included in the analysis.

DISCUSSION

Three species-groups (*'hyrcanus'*, *'filaria'* and *'nira'*) were distinguished within the genus *Dicelis* (Spiridonov & Ivanova, 2005) based on morphology. On morphological grounds only, *D. ussuriensis* is considered to be a member of the *'nira'* group and *D. kimmeriensis* to be a member of the *'hyrcanus'* group, while *D. rubidi* has an intermediate position between *'filaria'* and *'nira'*

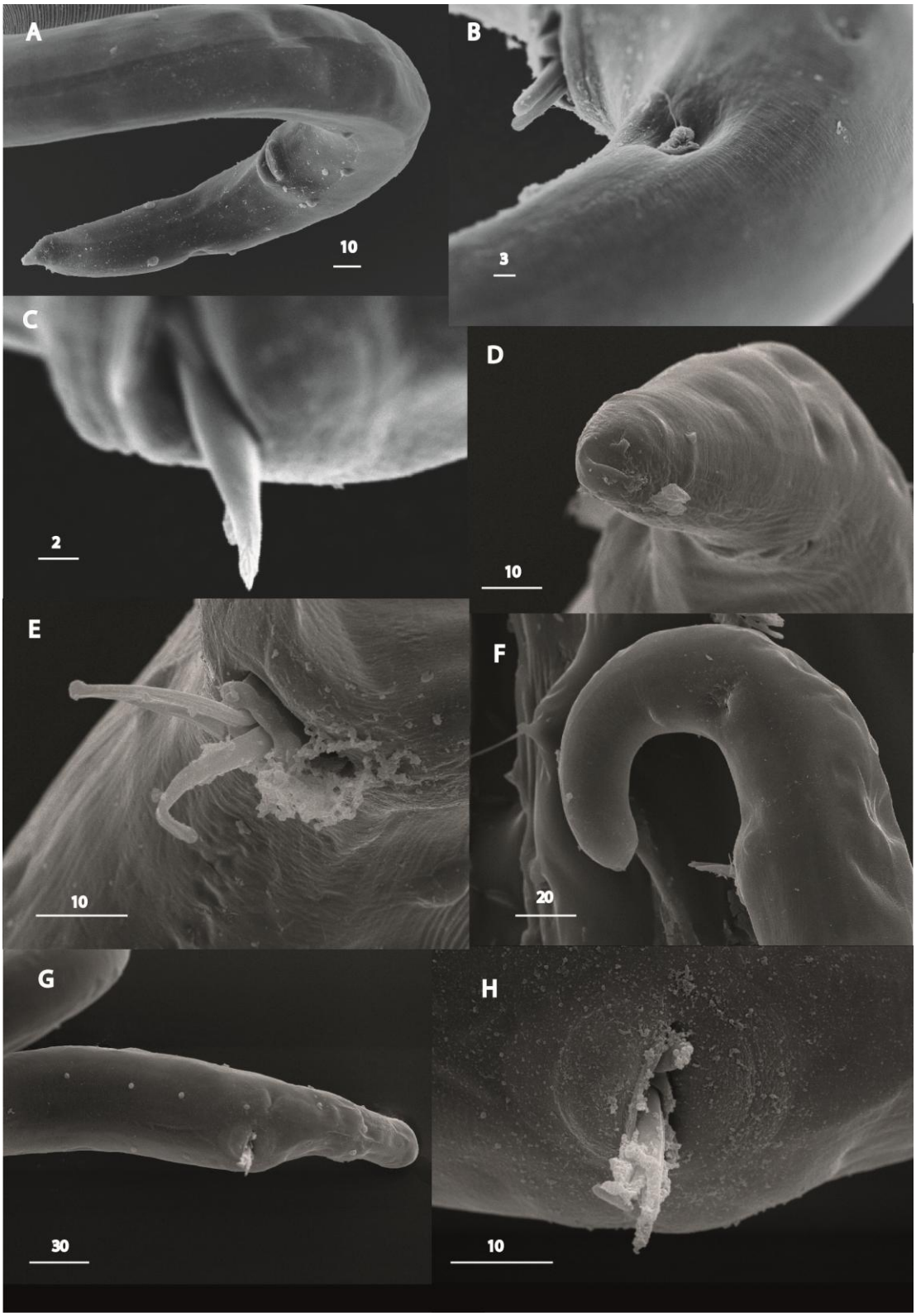

Fig. 3. A-C; *Dicelis ussuriensis*, Primorsky Krai, A, male tail; B, blunt spicule tips; C, acute spicule tips; D-F; *D. rubidi*, Khabarovsky Krai, D, male tail tip with appendage, E, blunt spicule tips; F, male tail without appendage and with acute spicule tips; G-H, *D. kimmeriensis*, Crimea, G, male tail, H, pericloacal area of male. Scale-bars in micrometres.

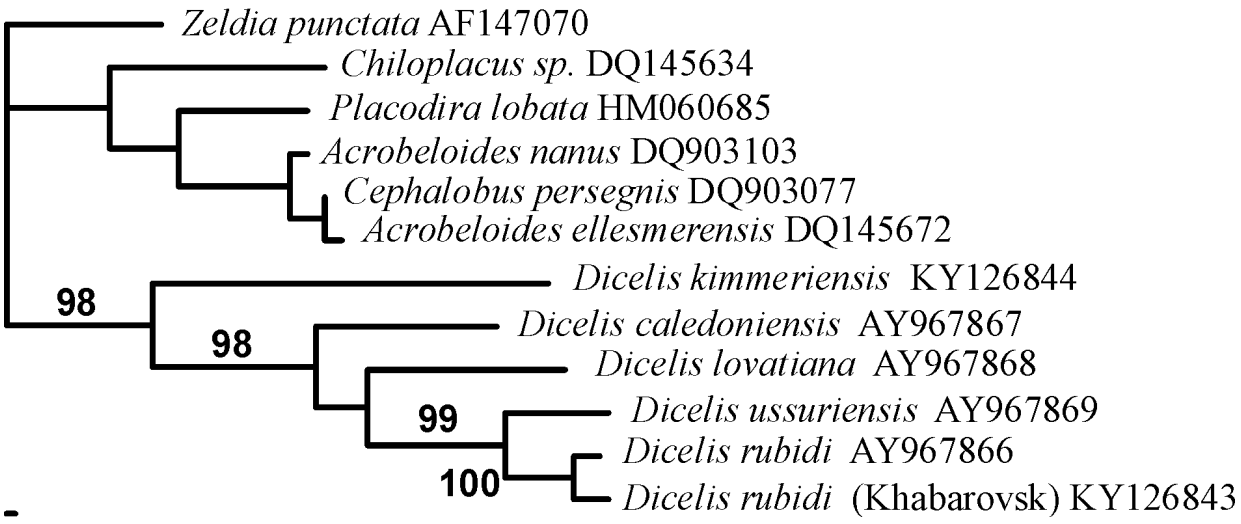

Fig.4. Phylogram of the relationships between nematodes of the genus *Dicelis* and other nematode genera based on the MP analysis of the sequences of D2-D3 region rDNA. Bootstrap 50% majority-rule consensus tree, based on 811 total characters with 219 parsimony informative. Gaps are treated as "missing". 1000 bootstrap replicates. Scale – 1 bp difference.

Table 1. Pairwise nucleotide differences between several cephalobid species and 6 species of *Dicelis*.

		1	2	3	4	5	6	7	8	9	10	11	12
1	*Zeldia punctata* AF147070	-											
2	*Cephalobus persegrinus* DQ903077	35	-										
3	*Acrobeloides ellesmerensis* DQ145672	37	2	-									
4	*Acrobeloides nanus* DQ903103	29	6	8	-								
5	*Placodira lobata* HM060685	40	32	34	30	-							
6	*Chiloplacus* sp. DQ145634	49	50	52	46	46	-						
7	*Dicelis rubidi* AY967866	66	71	69	65	75	75	-					
8	*Dicelis rubidi* (Khabarovsk) KY126843	67	72	70	66	76	76	7	-				
9	*Dicelis ussuriensis* AY967869	67	69	69	63	73	74	23	24	-			
10	*Dicelis lovatiana* AY967868	66	69	69	63	71	77	46	45	51	-		
11	*Dicelis caledoniensis* AY967867	61	64	64	61	65	68	47	48	49	48	-	
12	*Dicelis kimmeriensis* KY126844	72	73	73	71	83	86	76	77	77	84	84	-

with more properties of *'nira'* (Spiridonov & Ivanova, 2005). The *'nira'* and *'hyrcanus'* groups can be distinguished mainly by the pharynx shape and position of caudal organs: a cylindrical pharynx with an anterior expansion in both *'hyrcanus'* species (*D. hyrcanus* Belostotzkaja, Kozodoi & Spiridonov, 1987 and *D. kimmeriensis*) and club-like in *'nira'* and small caudal organs positioned at the last third of a tail in *'hyrcanus'* and anterior to mid-tail in *'nira'* (*D. nira* Chitwood & Lucker, 1934, *D. keymeri* Morand, Ivanova & Vausher, 1996, *D. sibirica* Ivanova, 1994, *D. ussuriensis*), whereas members of *'filaria'* and *'nira'* groups of species differ from each other by the set of characters present in each of the groups (Belostotzkaja *et al.*, 1987; Ivanova, 1993; 1994; Chitwood & Lucker, 1934; Morand *et al*., 1996; Spiridonov *et al*., 2005). However, the present study employing SEM has shown that all three species are characterised by the very similar structure of the head end. Hence, the classification partly based on the head structure of representatives of each group has to be re-examined and revised.

Previous molecular analysis based on four *Dicelis* species (Spiridonov *et al*., 2005) showed the separation of the European species from the Asian one rather than separation of the morphological groups. In the present analysis, the nucleotide difference between two species of '*filaria'* group, *D. caledoniensis* and *D. lovatiana* (both European), was similar to the nucleotide distance between these species and Asian *D. rubidi* and *D. ussuriensis* (intermediate position between *'nira'* and *'filaria'*; *'nira'*, respectively). These Asian species formed the subclade under all the methods of analysis. By contrast, European *D. caledoniensis* and *D. lovatiana* did not form a clade, but the former was always in the basal position for the latter plus two Asian species. The examination of a new finding of *Dicelis rubidi* in *E. n. nordenscioldi* from Khabarovsk Krai has demonstrated the level of intraspecific differences (7 bp). The collection site

for *D. rubidi* was more than 1,000 km from the type locality and the only morphological difference observed was in the tail length of both males and females.

The newly obtained molecular data for *D. kimmeriensis* have confirmed its isolated position within the genus *Dicelis*. The position of this species is in agreement with its morphological features. Whether the other species of *'hyrcanus'* group, *D. hyrcanus*, will belong to the same group genetically, is going to be investigated. Both known species of the *'hyrcanus'* group were recovered in geographic provinces with similar climatic characteristics (in Crimea and Azerbaijan) but in different hosts (*D. veneta* and *Eisenia fetida* Savigny, respectively). It should be noted here that the identity of several hosts for *Dicelis* species, and especially *E. fetida* for *D. hyrcanus*, is under question. The possibility of its confusion with *D. veneta* cannot be excluded, because both species of earthworm have similar lifestyles and are often found at the same sites.

The earthworm host for several *Dicelis* spp. including *D. rubidi* and *D. ussuriensis*, *E. nordenscioldi*, was considered as a species-complex by several authors (Blakemore, 2013; Shekhovtsov *et al.*, 2013). Nominally, it is subdivided to two subspecies (*E. n. nordenscioldi* and *E. n. pallida*) characterized by different level of ploidy and different ecological traits (Blakemore, 2013; Vsevolodova-Perel & Leirich, 2014). Currently, 14 lineages with high genetic diversity (up to 5%) have been isolated within the species, 9 of them representing *E. n. nordenscioldi* and 5 - *E. n. pallida* (Berman *et al*., 2016) based on analysis of mitochondrial (cox1) and nuclear ribosomal (ITS2) sequence markers. Based on the research of Shekhotsov *et al.* (2015), we can presume that the hosts of *D. rubidi* from Magadan region (Njuklja) belong to the lineage 9 while the hosts from Khabarovsk Krai belong to the different lineage, as lineage 9 was distributed to the north of Nanaysky district of Khabarovsk Krai. We do not know whether the earthworm hosts of these two strains of *D. rubidi* are going to be described as different species as suggested by Berman *et al.* (2016) in regard of *E. nordenscioldi*. In our analysis, their parasites clustered together and demonstrated insufficient genetic and morphological differences for their delimitation.

Thus, so far the molecular data obtained have confirmed the monophyletic status of *Dicelis*. A further sampling is necessary to test the relationships among the species and, specifically, to determine the position of the only non-lumbricid species, *D. eudrili*. It is still early to say whether the molecular data reflect the morphological peculiarities of three species groups within the genus. However, the stronger differences of *D. kimmeriensis* in comparison with the rest of the species examined correspond well with the stronger morphological separation of the *'hyrcanus'* group.

At the moment, the speculations on the possible relations of nematodes and their hosts are hindered by the problems with the species delimitation within Lumbricidae: different classifications indicate from 6-14 to 34-45 genera in the family and on the species level, morphological studies do not support molecular ones and *vice versa* (Pérez-Losada *et al.*, 2015). However, the different evolutionary history and different dispersal histories of earthworm hosts of *Dicelis* spp. are in some ways reflected in the relationships of their nematode parasites.

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Е.С. Иванова и С.Э. Спиридонов. Новые данные по морфологии и филогенетическим отношениям двух видов рода *Dicelis* Dujardin, 1845 (Cephalobomorpha, Drilonematoidea) - паразитов люмбрицид.

Резюме. Недавнее обнаружение двух видов рода *Dicelis* Dujardin, 1845, паразитирующих у дождевых червей-люмбрицид, позволило получить дополнительную информацию о морфологии и филогенетических связях членов рода. Морфологию *D. ussuriensis* из типового местообитания и вновь обнаруженных *D. rubidi* и *D. kimmeriensis* изучали с помощью сканирующего микроскопа. Анализ данных показал сходство строения головного конца у всех трех видов. Молекулярный анализ показал, что *D. kimmeriensis* сильнее обособлен от других видов рода, что находится в согласии с особенностями морфологии этого вида. Попарные нуклеотидные различия в участке Д2-Д3 рДНК у *D. kimmeriensis* и других видов рода были эквивалентны различиям с видами свободноживущих цефалобид. Нуклеотидные различия у *D. rubidi* из типового местообитания и Хабаровского края составляли 7 bp, что можно рассматривать как внутривидовую изменчивость. На филограмме виды *Dicelis* из западной части Палеарктики были в базальной позиции по отношению к видам из Азии, которые, в свою очередь, образовывали группу с хорошей поддержкой.