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Two new species of Ungellidae and Homungellidae (Drilonematoidea; Rhabditida) from Vietnamese earthworms and the phylogenetic links of these families

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Summary. Two new species of the families Ungellidae and Homungellidae (Drilonematoidea) are described from the coelomic cavity of Vietnamese earthworms. *Siconema ovispicatum* sp. n. (from *Amynthas robustus* (Perrier, 1872) collected in Cuc Phuong Natural Park) is characterized by fused cephalic hooks, egg-shells covered with widely set acute pyramid-shaped spikes and polar caps comprised of tightly set small rounded or pointed tubercles around 2-3 μm high. *Perodira minuta* sp. n. (collected from *Pheretima hawayana* (Rosa, 1891) in Ba Vi National Park) is characterized by its small size; membrane on a head end; cephalic sensilla in two circles; pin-shaped sperm in male gonad; tapered not coiled male tail; lack of caudal bursa/membrane; symmetrically disposed caudal organs. According to the phylogenetic analysis based on 18S rDNA partial sequences of described nematodes both genera *Siconema* (Ungellidae) and *Perodira* (Homungellidae) are related to free-living cephalobid genera.

Key words: Drilonematoidea, earthworm parasites, new species, *Perodira, Siconema*, 18S rDNA.

Nematodes of the superfamily Drilonematoidea are coelomic parasites of earthworms. Recently, DNA sequence data had been used for estimation of the taxonomic position and phylogenetic links for a genus of this superfamily, *Dicelis* (Spiridonov *et al.*, 2005). This genus belongs to the type family of the superfamily, Drilonematidae, where phylogenetic links of other families within this superfamily are still not clear. Hypotheses of possible phylogenetic relationships with oxyurids (Poinar, 1978) and cephalobids have been proposed (De Ley & Blaxter, 2002). The sequence data for two newly described representatives of Drilonematoidea belonging to the families Ungellidae and Homungellidae, together with results of the phylogenetic analysis are presented in this contribution.

MATERIAL AND METHODS

The earthworms were collected by the authors in April 2005 in two national parks near Hanoi, Vietnam. All earthworms were dissected alive and nematodes removed. The majority of nematodes were fixed in hot formaldehyde for morphological study and the rest in 70% ethanol for molecular study. Formaldehyde-fixed nematodes were processed into glycerol (Seinhorst, 1959) and mounted on slides. De Man indices and absolute measurements are given, where D is mid-body diameter, Ph is pharynx length and NR is distance from anterior to nerve ring. All measurements are in micrometers. The recently proposed terminology (Ivanova & Hope, 2004) for ungellid morphology was used.

The processing of material for molecular taxonomy was the same as used to study *Dicelis* nematodes (Spiridonov *et al.*, 2005). Primer pairs 'Aleshin-left' (TMY-CYC-RTT-GAT-YCT-GYC) and 26R (GCT-TTC-GTA-AAC-GGA-AGA-ATG) were used to amplify the most informative part of the 18S rDNA (Floyd *et al.*, 2002). The 889 bp long 18S sequence was obtained for *Perodira minuta* sp. n. and 889 bp long for

Siconema ovispicatum sp. n. These sequences were deposited in the NCBI GenBank under №№ EU287477 and EU287478, respectively. For comparative purposes and phylogeny construction, the following 18S rDNA sequences deposited in GenBank were used: *Plectus acuminatus* – AF037628; *Teratocephalus lirellus* – AF036607; *Myolaimus* sp. – U81585; *Pristionshus lheritieri* – AF036640; *Rhabdias buffonis* – AJ417022; *Rhabditophanes* sp. – AAF202151; *Pelodera strongyloides* – U12932; *Panagrolaimus subelongatus* – AY284681; *Turbatrix aceti* – AF202165; *Subanguina radicicola* – AF202164; *Pseudacrobeles variabilis* – AF202150; *Acrobeloides bodenheimeri* – AF202162; *Cephalobus oryzae* – AF034390; *Zeldia punctata* – U61760; *Cervidellus alutus* – AF202152; *Acrobeles* sp. – U81576; *Drilocephalobus* sp. – AY284680 and also a sequence for 18S rDNA of *Dicelis rubidi* Ivanova, 1994 – AY967862.

Sequence alignments (about 1000 bp long) were generated using Clustal X under default values for gap opening and gap extension penalties. All alignments were analyzed using PAUP* 4.0b10 (Swofford, 1998) for maximum parsimony (MP), distance method (NJ) and maximum likelihood (ML). The model for ML-analysis was selected with the use of ModelTest 5.0. The programme MtGui by Pablo Nuin was used as an interface to prepare Modeltest results for ML analysis in PAUP* 4.0b10. The program 'MrBayes' (Huelsenbeck & Ronquist, 2001) was used for Bayesian analysis (Bayesian inference – BI).

RESULTS AND DISCUSSION

Siconema ovispicatum sp. n. (Fig. 1)

Medium-sized ungellids. Males not much smaller than females. Sturdy cephalic hooks fused at base. Pharynx curved. Pharyngeal bulb displaced dorsally. Tail region swollen in both sexes and ends in off-set long spike.

Holotype female: L = 1413 μm; mid-body diameter = 60 μm; Ph = 130 μm (when extended); nerve ring = 75 μm; EP = 73 μm; V% = 64.5; a = 23.55; b = 10.87.

Head hooks strong, distal and proximal ends widely distributed, situated on the surface of head end and shifted dorsad. Total length of hooks 22 μm, single base 7 μm max. width with fused blades divided by clefts 5 μm long. Blade tips pointed, bent dorsad. Mouth opens in the centre of hook base, mouth aperture encircled by cuticular rim. No cephalic sensilla present. Amphids in a shape of half-moon; 7 μm wide and 3 μm deep. No stoma. Pharynx from muscular curved procorpus 9 μm wide at anterior and 12 μm at the middle, with no distinct isthmus, and big glandular pear-shaped bulb. Posterior part of procorpus in front of bulb encircled by thick nerve ring 8 μm wide. Bulb 45 μm long and 25 μm wide, contains three large nuclei of pharyngeal glands. Pharynx surrounded by numerous coelomocytes. Excretory pore 1 μm wide situated in front of nerve ring; excretory duct thin, weakly cuticularized, 52 μm long. Excretory cell reaches at least middle of the body. Cardia inconspicuous, intestine present. Anus indistinct, group of large cells visible at 145-205 μm behind the vulva. Tip cell of gonad in tail; genital tube makes three loops in wider portion of tail before running straight to anterior. Gonad reflexes in 222 μm from anterior, where not-offset spermatheca 18 μm long and 30 μm wide situated, containing several dozens of spermatozoa 2-3 μm in diameter. Descending branch of gonad represents long oviduct, whose walls formed by large cells 4-5 x 7-10 μm in size. Oviduct contains several immature eggs and leads to thin walled uterus with post-vulval portion 150 μm long. Four mature eggs 61-62 x 28-30 μm in size in uterus, egg-shells 2 thick, covered with widely set acute pyramid-shaped spikes about 3 μm high and bearing on both ends polar caps 5-6 μm high and 10 μm in diameter comprised of tightly set small rounded or pointed tubercles around 2-3 high. General appearance of eggs being slightly lemon-shaped due to polar caps. Vagina 25 μm long, inclined, directed posteriad, vulva lips protuberant. Posterior portion of tail spindle-shaped and widened up to 90 due to the presence of large caudal organs ('suckers' according to Timm (1967), fimbriate organs according to Ivanova & Hope (2004). Caudal organs 110 μm long and 60 μm wide, consist of spongy-like tissue with central part 65 μm in diameter with somewhat different tissue structure. No visible cavity or chamber presents. Terminal portion of tail set-off, 65 μm long and 4 μm wide.

Paratype male: L = 1019 μm; mid-body diameter = 45 μm; Ph = 109 μm (when extended); nerve ring = 70 μm; EP = 70 μm; tail = 187 μm; a = 22.64; b = 9.35; c = 5.45.

Anterior region as in females. Cephalic hooks with a base 11 μm long and 6 μm thick and blades 19 μm long, clefts 4 μm deep. Procorpus 9 μm wide at anterior. Bulb 36 μm wide and 30 μm long. Intestine wide. Excretory channels can be traced until testis flexure. Testis short, reflexes at the level of posterior quarter of body length. Flexure 85 μm long. Ejaculatory duct as long as region of growing

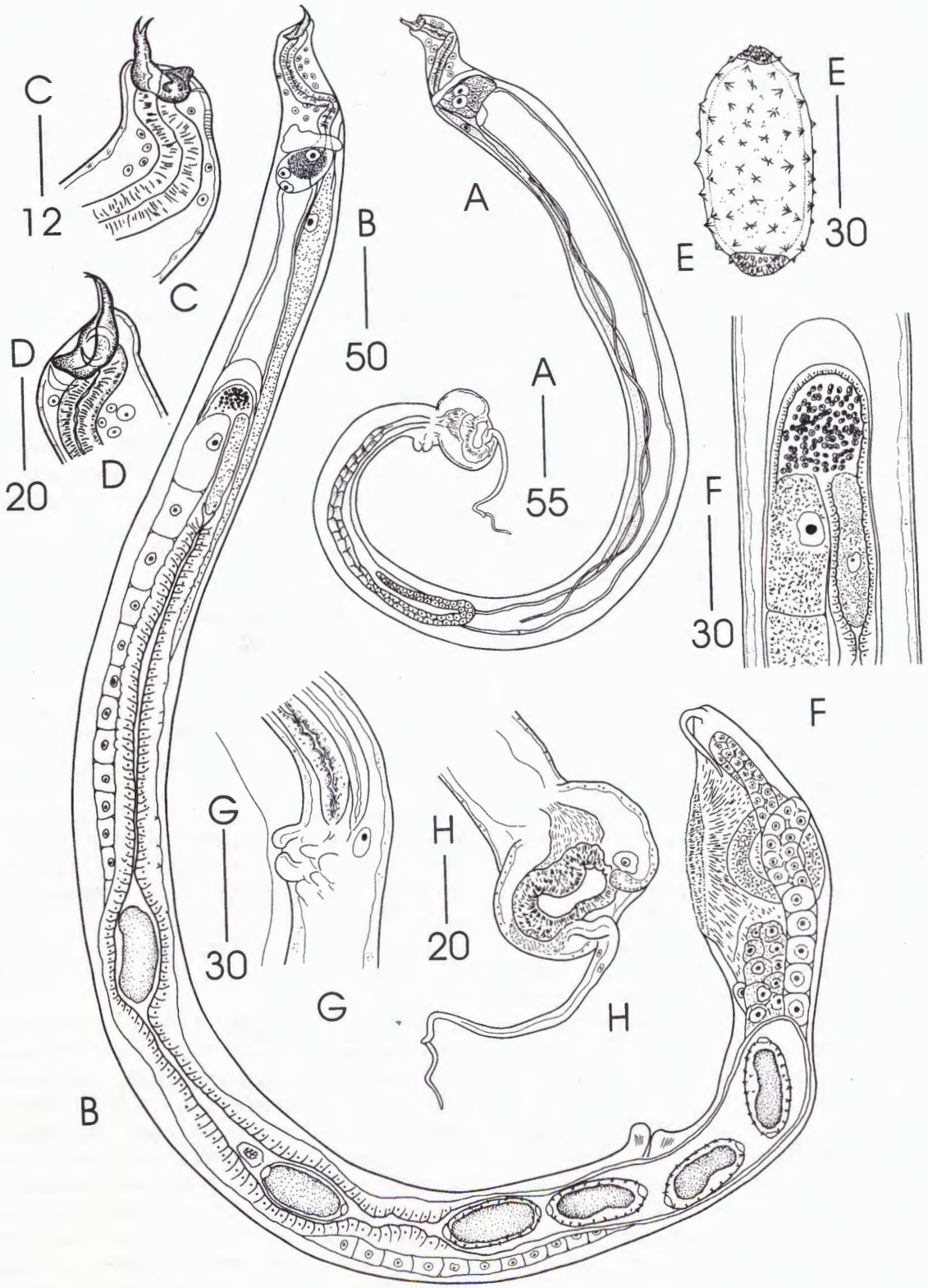

Fig. 1. *Siconema ovispicatum* sp. n. A: entire male, laterally; B: entire female, laterally; C: male head, laterally; D: female head, subdorsally; E: egg; F: spermatheca; G: male cloaca, laterally; H: male tail, laterally. Scales in µm.

spermatocytes. Anal aperture with two equal lips in front of it and behind. No caudal sensilla present. Thin membrane (bursa?) extends on 80 μm anteriad from anus and posteriad nearly to terminal portion of tail. Tail swollen, bearing a pair of lateral caudal organs each with chamber opening on the surface. Aperture of caudal organ chamber 20 μm long and 6 μm wide and surrounded by a concave rim up to 8 thick. Behind caudal organs, tail rounds bearing set-off terminal portion 112 μm long 4 μm wide.

Differential diagnosis. *S. ovispicatum* is characterized by swollen posterior with thin and long set-off terminal portion of tail, cephalic hooks fused at base, relatively weak excretory duct, tip ovary cell position in tail, non-set-off spermatheca, eggs with spiky shells bearing polar caps on each end, presence of post-vulval sac, very short testis, pre- and post-cloacal processes in males, male caudal organ with inner chamber causing swelling of tail. The most prominent features of the new species are fused cephalic hooks, characteristic appearance of egg-shells and male tail shape which differs it from the rest of *Siconema*. In the shape of cephalic hooks the present species strongly resembles *S. mucrorimae* Ivanova & Spiridonov, 1987, although hooks in the latter are smaller in size. Also, *S. mucrorimae* is clearly distinguished from *S. ovispicatum* sp. n. by having a longer tail with shorter mucron, particularly in males, much longer caudal organs without inner chamber and smaller eggs ornamented with tubercles instead of spikes. The present species differs from all other species of the genus resembling it in tail shape and general body proportions (*S. aculeatum* Spiridonov, 1993, *S. laotense* Spiridonov, 1993, *S. micrurum* Timm, 1966, *S. ovicostatum* Timm, 1966 and *S. saccaturum* Timm, 1966) by having a much longer set-off terminal portion of tail. From *S. aculeatum* it also differs by larger size, three times larger cephalic hooks, other type of ornamentation of egg-shells (in *S. aculeatum* egg-shells are without polar caps and bearing rod-like tubercles), presence *vs* absence cloacal processes in males, presence *vs* absence of post-vulval branch of uterus and male caudal organs with chamber *vs* without chamber. From *S. laotense*, it can be distinguished also by less elongated pharyngeal bulb, spiky egg-shells *vs* ones covered by rod-like tubercles underneath membrane, sturdier cephalic hooks, and more posterior vulva position (71% *vs* 52-60%). From *S. micrurum*, the present species is distinguished by fused *vs* separated cephalic hooks and heavily ornamented egg-shells *vs* finely punctuated ones. From *S. ovicostatum*, it also differs by fused *vs* separated cephalic hooks and different size (62-64 x 28-30 μm *vs* 48 x 22 μm) and ornamentation of egg-shells which are much thicker (5 *vs* 2) and having an irregular hexagonal pattern on the surface. From *S. saccaturum*, the present species differs by wider and shorter pharynx, larger hooks, non-set-off spermatheca, more posterior vulva position, presence *vs* absence of post-vulval branch of uterus, tip ovary cell in tail *vs* between vulva and anus, spiky egg-shells *vs* moderately mammillate ones.

Etymology: The species name is derived from its egg-shell appearance.

Type host: *Amynthas robustus* (Perrier, 1872), collected by authors in April 12, 2005.

Type locality: Cuc Phuong Natural Park, Vietnam.

Type habitat: Body cavity.

Type material: Holotype female № 1045 is in the collection of nematodes of invertebrates at the Centre of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, and paratype male in the author's collection.

Perodira minuta sp. n. (Fig. 2)

Holotype male: L = 657 μm; mid-body diameter = 34 μm; anal diameter = 15 μm; Ph = 130 μm (when extended); nerve ring = 70 μm; EP = 173 μm; tail = 86 μm; a = 19.3; b = 5.1; c = 7.6

Paratype males (n = 5): L = 582 ± 153 (449-793) μm; mid-body diameter = 36 ± 2.5 (33-40) μm; anal diameter = 16 ± 0.9 (15-17) μm; Ph = 126 ± 17 (112-149) μm (when extended); nerve ring = 72 ± 11 (53-78) μm; EP = 138 ± 34 (103-184) μm; tail = 77 ± 8 (69-89) μm; a = 16.4 ± 5 (12.5-22.7); b = 4.6 ± 0.6 (4.0-5.3); c = 7.5 ± 1.4 (6.4-9.1)

Short spindle-shaped nematodes; anterior end wider than posterior one. Cuticle thin and smooth. Head end bluntly rounded bearing two circles of short bristle-like cephalic sensilla, inner circle of 6 smaller and outer of 4 longer ones. Thin membrane covering cephalic sensilla. No buccal cavity present. Amphids in 5 ± 1.7 (4-7) μm from anterior with transversally elongated or horse-shoe-shaped cuticularized rim 5.5 ± 1.2 (4-7) μm wide or about 2/3 of corresponding body diameter and shallow pouch. Pharynx with long thin muscular procorpus and enlarged glandular bulb. Nerve ring encircles posterior part of procorpus. Numerous coelomocytes in pharynx region. Excretory pore shortly behind

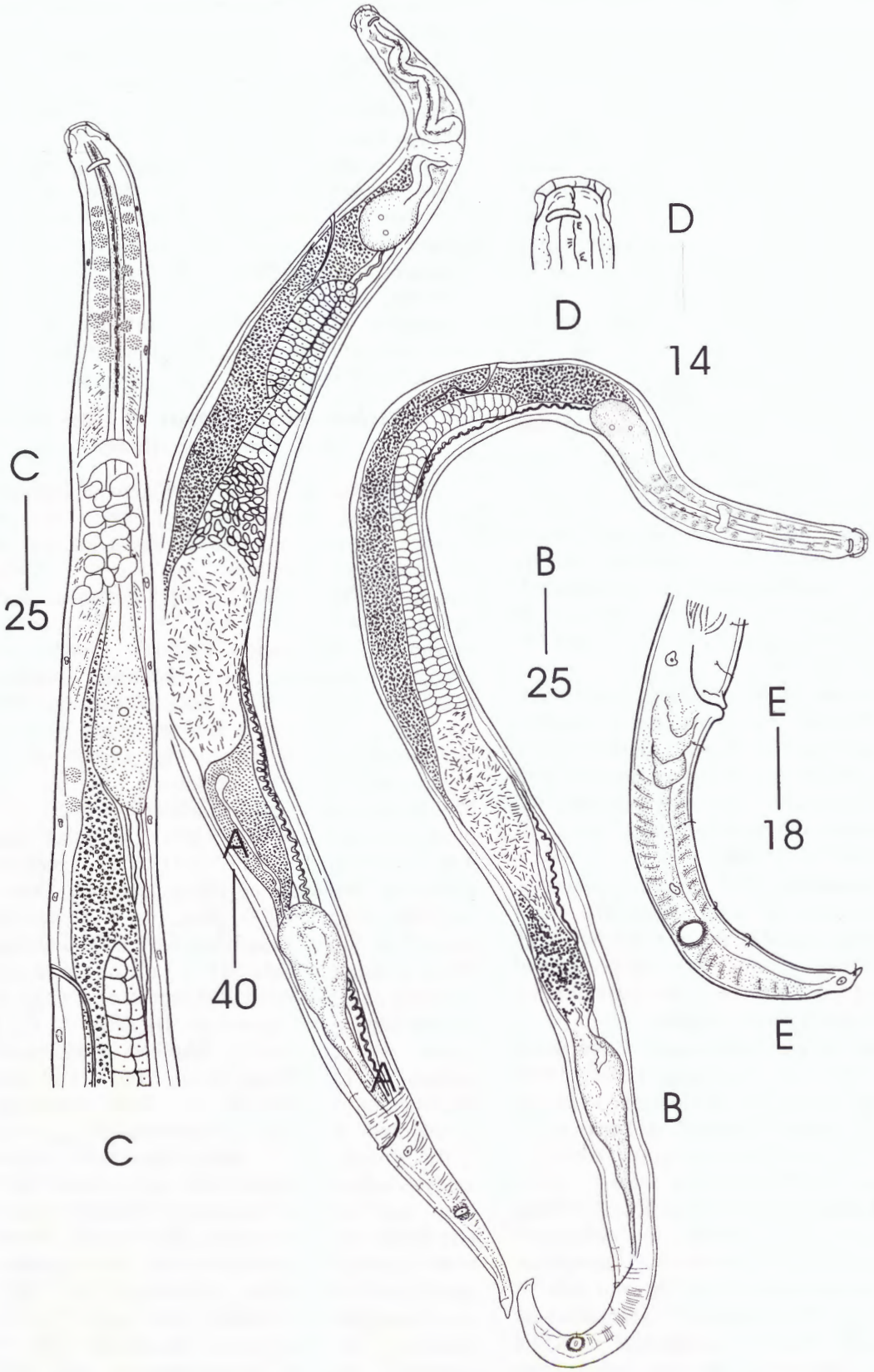

Fig. 2. *Perodira minuta* sp. n. males. A: shorter male, entire lateral view; B: longer male, entire lateral view; C: anterior part, laterally; D: head, laterally; E: tail, laterally. Scales in µm.

the pharyngeal bulb. Excretory duct 25-50 μm long, cuticularized, funnel-shaped proximally. Excretory cell occupies most part of the body from nerve ring to seminal vesicle. No cardia. Intestine with wavy, heavily-sclerotized lumen. Testis reflexes in 143 ± 29 (108-177) μm from anterior, testis flexure 68 ± 21 (43-92) μm long. Spermatocytes in 2 rows, transversally elongated; spermatids bean-shaped, 3-4 x 6-7 μm in size; vast seminal vesicle filled with pin-shaped immature sperm; *vas deferens* and ejaculatory duct set off by constriction. Tail tapers and ends in a blunt conical tip. Prominent copulatory and caudal muscles. No bursa or membrane on tail end present. Caudal sensilla whip-like; 2 sublateral pairs situated pre-cloacally, 1 sublateral adanally and 5 subventrally post-cloacally: first pair shortly posteriorly to anus, second one between cloaca and caudal organs, third one in front of caudal organs, fourth at mid-distance between caudal organs and tail tip, fifth at the tail tip. Caudal organs situated symmetrically at mid-tail; sucker-like, circular or slightly transversely elongated; single sensillum protruding from the aperture of each organ. Size of each organ 5-7 x 6-7, aperture half the size.

There are two types of males: longer and shorter ones. Shorter males possess a shorter pharynx with bent procorpus and rounded bulb, while in longer males procorpus is straight and pharyngeal bulb elongated. Excretory pore situated closer to pharyngeal bulb than in longer males; 'c' index 6.4-6.7 μm *vs* 7.6-9.1 μm.

Differential diagnosis. The present species is characterized by its small size; membrane on a head end; cephalic sensilla in two circles; pin-shaped sperm in male gonad; tapered not coiled male tail; lack of caudal bursa/membrane; symmetrically disposed caudal organs.

Three *Perodira* species are presently described: *P. alata* Baylis, 1943, *P. pheretimae* Timm, 1960, and *P. lignophilae* Ivanova & Spiridonov, 1987. By its small size the present species is close to *P. pheretimae*: 449-793 μm *vs* 610-668 μm. Two other species are longer: 840-1100 μm (*alata*), 1510-1730 μm (*lignophila*). From all species *P. minuta sp. n.* differs by its nearly straight not coiled tail and lack of bursa; from *P. alata* and *P. lignophilae* by conical *vs* widely rounded tail tip and lack of buccal cavity; from *P. alata* and *P. pheretimae* by thinner tail region. From *P. pheretimae* it differs also by lack of constriction in neck region and pin-shaped *vs* amoeboid sperm; from *P. lignophilae* it can be distinguished by lack of cuticularized rim around mouth aperture.

Etymology: The species name is derived from its small size.

Type host: *Pheretima hawayana* (Rosa, 1891) collected by authors in April 6, 2005.

Type locality: Ba Vi National Park, Vietnam.

Type habitat: Body cavity.

Type material: Holotype male and 2 paratype males on the single slide № 1046 are in the collection of nematodes of invertebrates at the Centre of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, one paratype male in the collection of Laboratory of Parasitology, Institute of ecology and biological resources, Vietnamese Academy of Science and Technology.

Molecular phylogeny of Ungellidae and Homungellidae

The estimation of the phylogenetic links of the two studied nematodes of the superfamily Drilonematoidea was mainly based on the results of maximum likelihood analysis and Bayesian inference. The GTR+G model was selected (K = 9; -lnL=6876.2725; AIC = 13770.5449) for maximum likelihood analysis. Both genera (*Perodira*+*Siconema*) were clustering together in ML-tree under moderate bootstrap support (Fig. 3 A). This pair of taxa is a sister group for a set of cephalobid genera (including *Dicelis* genus – earthworm parasitic nematodes of the family Drilonematidae) with moderate support of the node uniting these two clades. Bayesian analysis was performed with 1,000,000 generations (sampling frequency of 100 generations; Data type = DNA; Nucmodel = 4by4; Nst = 6; number of states = 4; State frequencies have a Dirichlet prior; Rates = Equal; -7243.547 – LnL of active chain). *Perodira* and *Siconema* also represent a single clade in the phylogram based on BI (Fig. 3, B). This group was completely resolved with posterior probability 1.0 (Erixon et al. 2003), and support for the monophyly of the clade consisting of cephalobids and three Drilonematoidea genera was quite strong (0.98). Also maximum parsimony analysis was performed with gaps treated as 'fifth base' with 918 total characters of equal weight: 246 characters were constant; 274 variable characters were parsimony-uninformative with number of parsimony-informative characters = 398. In neighbour-joining search with 'gaps treated as missing', of 918 total characters 285 were constant; 260 – uninformative and 365 – informative. The topologies of trees obtained through MP and NJ analysis were similar to those of ML analysis (data not shown).

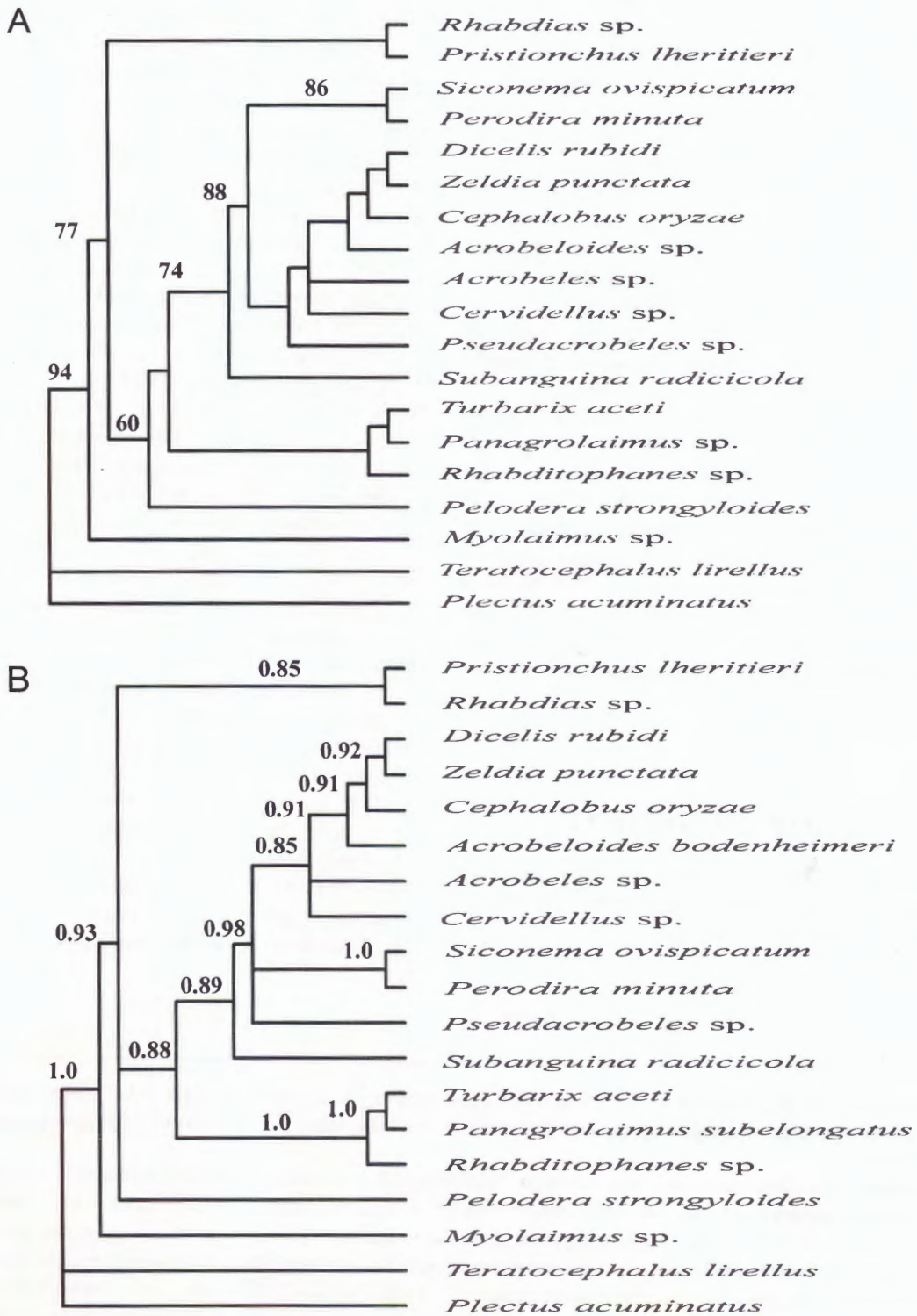

Fig. 3. Phylogram of the relationships between nematodes of the genera *Siconema* and *Perodira* and other nematode genera based on the sequences of 18S rDNA (ML and BI). A – Maximum likelihood analysis, Model selected: GTR+G; -lnL = 6756.5039; K = 9; AIC = 13531.0078; B – Bayesian inference. Bootstrap values and posterior probabilities greater than 50% are given for appropriate clades.

The close relationships of certain cephalobid genera with another representative of the superfamily Drilonematoidea, the genus *Dicelis*, were demonstrated earlier (Spiridonov *et al.*, 2005). In the analysis of the data presented above a similar set of genera belonging to the suborders Tylenchina and Rhabditida was involved, with *Plectus* and *Teratocephalus* used as outgroups. As it was a case with *Dicelis* nematodes, the representatives of these two genera, *Siconema* (Ungellidae) and *Perodira* (Homungellidae), were always clustering with cephalobid genera: either with inner nodes of cephalobid phylogeny or representing a sister group to the majority of cephalobid genera in some topologies. Surprisingly the genus *Dicelis* was not clustered together with the newly sequenced Drilonematoidea in all topologies, but retained the tight clustering with genera *Zeldia* and *Cephalobus,* as demonstrated earlier (Spiridonov *et al.*, 2005). The links demonstrated between three families of Drilonematoidea and cephalobid genera support the hypothesis of De Ley & Blaxter (2002) about phylogenetic interrelationships between the Drilonematoidea and cephalobids but, at the same time, they reveal the possible polyphyletic nature of Drilonematoidea.

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Spiridonov S. E., Ivanova E. S., Pham V. L. Два новых вида семейств Ungellidae и Homungellidae (Drilonematoidea; Rhabditida) от вьетнамских дождевых червей и филогенетические связи этих семейств.

Резюме. Описано два новых вида семейств Ungellidae и Homungellidae (Drilonematoidea) из полости тела дождевых червей Вьетнама. *Siconema ovispicatum* sp. n. из *Amynthas robustus* (Perrier, 1872), собранные в заповеднике Кук Фыонг, характеризуются слитыми головными крюками, и оболочками яиц, усеянными острыми пирамидоподобными шипиками, а также полярными шапочковидными крышечками, состоящими из плотно сидящих округлых выступов высотой около 2-3 μm. *Perodira minuta* sp. n. из *Pheretima hawayana* (Rosa, 1891), собранные в Национальном парке Ба Ви, характеризуются небольшими размерами, наличием мембраны на головном конце, головными сенсиллами в двух кругах, булавковидными спермиями в половой трубке самца, заостренным и не закрученным вентрально хвостовым концом самца, отсутствием бурсальной мембраны, симметично расположенными хвостовыми органами. Анализ филогенетических отношений, проведенный на основе сравнения части последовательности 18S-рибосомальной ДНК, выявил связь родов *Siconema* (Ungellidae) и *Perodira* (Homungellidae) с родами свободноживущих цефалобид.