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Phylogeny of stenophorid gregarines from millipedes (Diplopoda)

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ABSTRACT: The gregarines from millipedes are diverse and understudied parasites. They are distributed across five families of eugregarines, but the majority of their species belong to family Stenophoridae, where their phylogenetic relationships are not well understood. Here we obtain 20 new gregarine sequences from millipede hosts and employ environmental sequence data to examine the phylogeny within the family. Phylogenetic analyses with SSU rDNA and combined SSU, 5.8S and LSU rDNA datasets recover all novel sequences as a monophyletic group consisting of six clades, five of which have not been recognized before. The stenophorids are sister to Gregarinoidea in the phylogenies, hence we redefine the group as Stenophoroidea Clopton, 2009. We describe four new species of *Stenophora* and show that the phylogeny of these gregarines does not follow the phylogeny of their millipede hosts.
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KEY WORDS: gregarine, phylogeny, parasites of millipedes, *Stenophora*, Stenophoroidea.

Филогения грегарин-стенофорид из двупарноногих многоножек (Diplopoda)

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РЕЗЮМЕ: Грегарины двупарноногих многоножек – разнообразная, но малоизученная группа паразитов. Они встречаются в пяти семействах эугрегарин, но большинство относится к семейству Stenophoridae. В данном исследовании мы получили 20 новых нуклеотидных последовательностей рДНК грегарин из диплопод и использовали метагеномные данные, чтобы изучить филогению группы. Филогенетический анализ 18S рДНК и конкатенированных 18S, 5.8S и 28S рДНК показал, что все новые последовательности образуют на сконструированном дереве монофилетичную группу, состоящую из шести клад, пять из которых ранее не были известны. Грегарины диплопод — сестинская группа клады Gregarinoidea, поэтому мы отождествили ее с надсемейством Stenophoroidea Clopton, 2009. Мы описываем четыре новых вида рода *Stenophora* и показываем, что филогения этих грегарин не совпадает с филогенией многоножек-хозяев.
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КЛЮЧЕВЫЕ СЛОВА: грегарины, филогения, паразиты многоножек, *Stenophora*, Stenophoridae.

Introduction

The millipedes (Myriapoda: Diplopoda) represent the most diverse and abundant myriapod group with more than 12,000 described species (Brewer *et al*., 2012), while the number of parasitic gregarine species described from millipedes reaches 130. Several gregarine species in the families Actinocephalidae, Dactylophoridae, Cnemodosporidae, and Monoductidae were described from millipedes, but the vast majority of these parasites belong to the family Stenophoridae, with more than 100 described species (Desportes, Schrével, 2013).

Systematics of the gregarines from millipedes is mainly based on the morphological

data such as the type of association, the gametocyst and oocyst structure, the gametocyst dehiscence and the oocyst expulsion mechanisms (Desportes, Schrével, 2013). For the majority of the gregarines from millipedes these characteristics were rarely observed or are not known at all. The only molecular-phylogenetic study on these gregarines was performed with a single species *Stenophora robusta* Ellis, 1912 (Clopton, 2009). According to the phylogeny obtained in that study, *S. robusta* grouped with *Pyxinia crystalligera* Frenzel, 1892 within the clade sister to the Gregarinoidea. On the basis of that result the genus *Pyxinia*, comprising parasites of beetles, was moved from the Actinocephalidae to the amended superfamily Stenophoroidea, family Monoductidae (Clopton, 2009), and then to the Stenophoroidea: Pyxiniidae (Desportes, Schrével, 2013).

In this study, we describe four new *Stenophora* species and five new gregarine rDNA sequences from the Caucasian and Vietnamese millipedes. We assemble another fourteen gregarine SSU rDNA sequences from the available transcriptomic data of seven species of Diplopoda and survey the available metagenomic data for related environmental sequences. The rDNA phylogenetic trees for terrestrial gregarines outline 5 new clades within the Stenophoroidea. The phylogenies also reveal that *Pyxinia crystalligera* is not related to stenophorids, as previously claimed.

Material and methods

Millipede hosts were collected from the litter layer in various locations in 2017-2022. Twenty individuals of *Pachyiulus krivolutskyi* Golovatch, 1977 (Julida) were collected in the vicinity of Nickel, Adygea Republic, Russia (44°10′38.2″N 40°09′21.4″E). Three individuals of *Thyropygus carli* Attems, 1938 (Spirostreptida) and three individuals of *Hylomus* (*Desmoxytes*) *pilosus* Attems, 1937 (Polydesmida) were collected in the Cat Tien National Park, Socialist Republic of Vietnam (11°24′27.0″N 107°22′53.8″E). One individual of *Sphaerobelum* sp. (Sphaerotheriida) was collected in the vicinity of Suoi Phai village (20°33′40.0″N 104°36′20.0″E), Muong Lat District, Thanh Hoa, Socialist Republic of Vietnam.

Millipedes were dissected in the insect Ringer's solution (0.650% NaCl, 0.025% KCl, 0.025% $CaCl_2$, 0.025% $NaHCO_3$). Gregarine parasites were isolated from the hosts' intestines with fine tip needles and plastic pipettes under a MBS-10 stereomicroscope (LOMO, Russia). Isolated gregarines were washed in three changes of the fresh insect Ringer's solution. Live parasites from *P. krivolutskyi* and *T. carli* were photographed with a Nikon DS-Fi1 digital camera (Nikon Corporation, Japan) connected to a Leica DM2500 light microscope (Leica Microsystems, Germany) by means of differential interference contrast (DIC) and bright-field (LM) microscopy. Gregarines from *H. pilosus* were photographed with iPhone 12 camera (Apple Inc., USA) under a Nikon Labophot-2 microscope (Nikon Corporation, Japan) by means of bright-field (LM) microscopy. The following measurements were taken: TL — total length; PL — length of protomerite; DL — length of deutomerite; PWM — maximum width of protomerite; PWE — width of protomerite at equatorial axis; DWM — maximum width of deutomerite; DWE — width of deutomerite at equatorial axis (Clopton, 2004; Devetak *et al*., 2019).

Gregarines from *P. krivolutskyi*, *T. carli*, and *H. pilosus* were fixed with 2.5% (v/v) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4 (4 °C, 2 h), then rinsed with the cacodylate buffer and post-fixed with 1% (w/v) osmium tetroxide in the cacodylate buffer (4 °C, 2 h). After fixation, the sample was dehydrated in an ethanol series and critical point dried in liquid CO_2. The dried cells were sputter-coated with gold and investigated with a Tescan MIRA3 LMU scanning electron microscope (TESCAN, Czech Republic).

The first parasite from *P. krivolutskyi* (*Stenophora nickeli* sp.n., 20 cells) and gregarines from *T. carli* (*Stenophora cattiensis* sp.n., 20 cells) and *H. pilosus* (*Stenophora draconis* sp.n., 5 cells) were fixed with the RNA-later reagent (Life Technologies, USA). The cDNA libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio, Japan). Four to five million paired-end 100 bp reads were generated with Illumina HiSeq for the cDNA libraries of *Stenophora nickeli* sp.n. and *Stenophora cattiensis* sp.n., and 23 million paired-end 150 bp reads were generated for the cDNA library of *Stenophora draconis* sp.n. The rDNA operon sequences (SSU rDNA, ITS1, 5.8S rDNA, ITS2, LSU rDNA) of the gregarines were assembled from the transcriptome sequencing data using Trinity (Grabherr *et al*., 2011) and SPAdes (Nurk *et al*., 2013) assemblers.

Due to low amount of collected cells, gregarines from *Sphaerobelum* sp. were only used for the molecular analysis. Gregarines from *Sphaerobelum* sp. (5 cells), *Stenophora nickeli* sp.n. (20 cells), and the second parasite from *P. krivolutskyi* — *Stenophora pachyiuli* sp.n. (50 cells) were fixed with 96% ethanol. DNA was extracted with Arcturus PicoPure DNA Extraction Kit (Thermo Fisher Scientific, USA).

The rDNA fragments were amplified with Encyclo PCR kit (Eurogen, Russia) in a total volume of 20 ìl in the following PCR steps: 95 °C for 2.5 min (initial denaturation); 40 cycles of 95 °C for 30 s (denaturation), 60–65 °C for 30 s (annealing), and 72°C for 1.5 min (elongation); 72 °C for 10 min (final extension). The following pairs of forward and reverse primers were used for the parasite of *Sphaerobelum* sp.: 5'-GTATCTGGTTGATCCTGCCAGT-3' (Medlin *et al*., 1988) and 5'-GATCCTTCTGCAGGTTCACCTAC-3' for SSU rDNA, 5'-GTACACACCGCCCGTCGCTC-3' and 5'-GACTCCTTGGTCCGTGTTTCAAGACG-3' for ITS1, ITS2, and 5.8S rDNA, and 5'-ACCCGCTGAAYTTAAGCATAT-3' and 5'-ACATTCAGAGCACTGGGCAG-3' for 28S rDNA (part). The same primers were used for *Stenophora pachyiuli* sp.n., except the forward primer for 28S rDNA which was constructed specifically to separate the gregarine DNA from contamination: 5'-CGGAACCTAGTGAAAAGAGTAAG-3'. The ITS1, ITS2, and 5.8S rDNA region of *Stenophora nickeli* sp.n. was amplified with the same primers to check the transcriptomic assembly in ITS region.

In order to increase the sampling of gregarine sequences, the transcriptomic data of seven millipedes were examined for the presence of gregarine rDNAs: *Trigoniulus corallinus* Eydoux et Souleyet, 1842 (Spirobolida), *Cylindroiulus* sp., *Uroblaniulus* sp. (Julida), *Helicorthomorpha holstii* Pocock, 1895 (Polydesmida), *Eudigraphis taiwaniensis* Ishii, 1990, *Eudigraphis takakuwai* Miyosi, 1947, and *Polyxenus lagurus* Linnaeus, 1758 (Polyxenida). The sequencing data were obtained from the NCBI Sequence Read Archive, accessions: SRR10160968, SRR10160983 (*T. corallinus*), SRR3458645 (*Cylindroiulus* sp.), SRR6767050 (*Uroblaniulus* sp.), SRR10161405 (*H. holstii*), SRR3458640 (*E. taiwaniensis*), SRR1653191 (*E. takakuwai*), SRR3485994 (*P. lagurus*). Assembled rDNA contigs and scaffolds were aligned using MAFFT (Katoh, Standley, 2013) and inspected manually for assembly errors and chimeric sequences. To further improve the sampling, we performed BLAST (Camacho *et al*., 2009) searches in the nr database (NCBI) and the metagenomic data generated by Heger *et al*. (2018; NCBI Sequence Read Archive, accession: PRJNA396681) and Jamy *et al*. (2020; NCBI Sequence Read Archive, accession: PRJEB25197) for environmental sequences related to these gregarines. The 5.8S and LSU rDNA sequences of *Monocystis agilis* were assembled from available transcriptomic data (SRR8980208).

An SSU rDNA alignment with 200 different apicomplexan OTUs, 5.8S and LSU rDNA alignments both comprising 98 apicomplexan OTUs were generated using the MAFFT online service employing the E-INS-i alignment method (Katoh *et al*., 2019). Unaligned regions and columns containing few nucleotides were removed from the alignments for the phylogenetic inference using a custom mask resulting in three alignments: 1,530 bp for the SSU, 145 bp for the 5.8S, and 1,805 bp for the LSU rDNA. The SSU rDNA preliminary dataset was used to verify the grouping of collected environmental sequences with gregarines from millipedes. The tree reconstruction was performed with the IQ-TREE web server (Trifinopoulos *et al*., 2016) using the GTR+I+G12+F model, and ultrafast bootstrap approximation (Minh *et al*., 2013) with 1,000 replicates for estimation of branch support. The main datasets for phylogenetic inference were constructed after the verification, and included sequences of terrestrial gregarines, environmental sequences, cryptosporidians, and coccidians: a total of 84 OTUs for the SSU rDNA dataset (1,530 bp), 28 OTUs for the LSU rDNA dataset (1,805 bp), and 28 OTUs for the combined SSU, 5.8S, and LSU rDNA dataset (3,480 bp).

Bayesian inference (BI) analyses were performed with MrBayes 3.2.7a (Ronquist *et al*., 2012) utilizing the resources of the CIPRES web server (Miller *et al*., 2010). The analyses were performed under the GTR+Ã+I model with 8 rate categories for the SSU and LSU rDNA datasets and utilizing partitions for the combined dataset. For the 5.8S rDNA partition GTR+Ã+I model with 4 rate categories were used. The chain heating coefficient (temp) was set to 0.2. For all datasets, the inference was done with two independent runs of four MCMC, and the consensus trees were built with a 50% burn-in after 10 million generations, and the tree sampling frequency of 0.001. The average standard deviations of split frequencies at the end of computations were 0.006796 for the SSU rDNA dataset, 0.005060 for the LSU rDNA dataset, and 0.001419 for the combined dataset. ML analyses were performed with the IQ-TREE 2.1.2 (Minh *et al*., 2020) using the GTR+I+G8+F model for the SSU and LSU rDNA datasets and the same partitions in the combined dataset. For the 5.8S partition in the combined dataset the GTR+I+G4+F model was used. All computations were performed with 1000 nonparametric bootstrap (Felsenstein, 1985) replicates for estimation of branch support. The ML support values were assigned to the Bayesian trees using 1000 non-parametric bootstrap trees from the ML analyses via the –sup option of IQ-TREE 2.1.2.

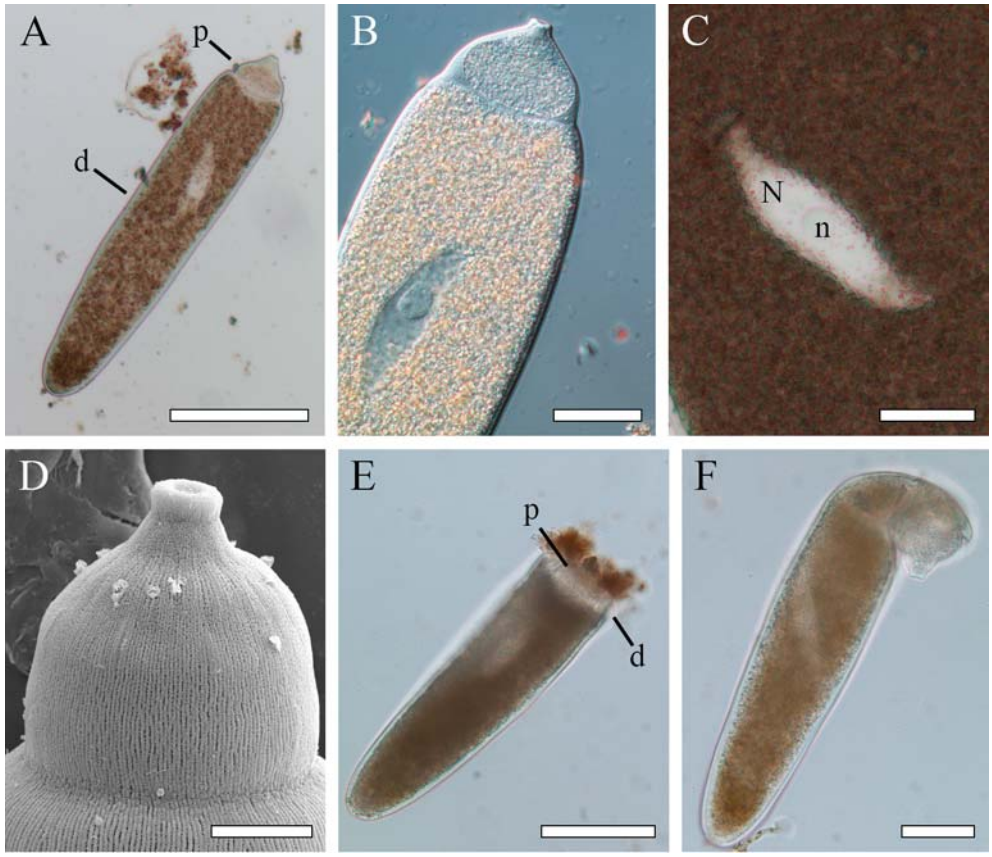

Fig. 1. Morphology of *Stenophora cattiensis* sp.n. A — trophozoite; B — anterior end of trophozoite, DIC; C — boat-shaped nucleus with centrally located oval nucleolus; D — protomerite; E — trophozoite with retracted anterior end; F — trophozoite with recurved front end.
Abbreviations: d — deutomerite; N— nucleus; n — nucleolus; p — protomerite. Scale bars: A — 200 μm; B, C — 50 μm; D — 10 μm; E — 100 μm; F — 50 μm.
Рис. 1. Морфология *Stenophora cattiensis* sp.n. A — трофозоит; B — передний конец трофозоита, DIC; C — ядро в форме лодочки с центральным овальным ядрышком; D — протомерит; E — трофозоит с втянутым передним концом; E — трофозоит с загнутым назад передним концом.
Обозначения: d — дейтомерит; N — ядро; n — ядрышко; p — протомерит. Масштаб: A — 200 μm; B, C — 50 μm; D — 10 μm; E — 100 μm; F — 50 μm.

Results

Taxonomy

Phylum Apicomplexa Levine, 1970
Subphylum Sporozoa Leuckart, 1879
Class Gregarinomorpha Grassé, 1953; Simdyanov *et al*., 2017
Order Eugregarinida Léger, 1900; Simdyanov *et al*., 2017
Superfamily Stenophoroidea Clopton, 2009 (= Stenophoricae Chakravarty, 1959)
Family Stenophoridae Léger et Duboscq, 1903
Stenophora (Labbé, 1899) (= *Stenocephalus* A. Schneider, 1875); Gasc, Ormières et Bouix, 1975; Desportes et Schrével, 2013

Stenophora cattiensis Miroliubova **sp.n.**
Fig. 1.

Trophozoites and gamonts were found in the intestine of three individuals of *Thyropygus carli* Attems, 1938. The relatively large (up to 764 μm long, av. 465.6 ± 23.5 μm and 200 μm wide, av.

Table 1. Measurements of gregarines (in μm).
Таблица 1. Измерения грегарин (в мкм).

Stenophora cattiensis sp.n.							
n=25	TL	PL	DL	PWM	PWE	DWM	DWE
Mean	465.6	42.7	422.8	61.1	50.2	128.5	114.9
Standard deviation	117.3	12.7	108.7	11.3	9.8	27.7	25.6
Standard error of the mean	23.5	2.5	21.7	2.3	2.0	5.5	5.1
Min	285.7	26.4	246.2	39.6	33.0	76.9	70.3
Max	764.4	88.9	702.2	97.8	80.0	200.0	173.3
Stenophora draconis sp.n.							
n=25	TL	PL	DL	PWM	PWE	DWM	DWE
Mean	635.8	24.9	610.9	34.0	29.7	45.8	29.3
Standard deviation	69.1	3.2	69.0	4.4	4.3	10.2	7.7
Standard error of the mean	13.8	0.6	13.8	0.9	0.9	2.0	1.5
Min	500.2	19.1	476.4	27.8	23.8	35.7	19.9
Max	817.8	31.8	794.0	43.7	39.7	79.4	47.6
Stenophora nickeli sp.n.							
n=40	TL	PL	DL	PWM	PWE	DWM	DWE
Mean	400.6	35.6	364.9	41.4	37.0	70.1	49.5
Standard deviation	174.6	5.7	169.7	12.3	9.6	26.1	19.0
Standard error of the mean	27.6	0.9	26.8	1.9	1.5	4.1	3.0
Min	52.8	19.2	33.6	19.2	19.2	24.0	20.0
Max	696.0	48.0	657.6	62.4	52.8	134.4	96.0
Stenophora pachyiuli sp.n.							
n=25	TL	PL	DL	PWM	PWE	DWM	DWE
Mean	337.0	23.6	313.3	31.3	28.4	70.7	44.5
Standard deviation	82.2	2.4	81.5	3.7	4.1	18.4	11.2
Standard error of the mean	16.4	0.5	16.3	0.7	0.8	3.7	2.2
Min	177.6	19.2	153.6	24.0	19.2	33.6	24.0
Max	451.2	28.8	427.2	38.4	33.6	100.8	64.0

Legend: TL — total length; PL — length of protomerite; DL — length of deutomerite; PWM — maximum width of protomerite; PWE — width of protomerite at equatorial axis; DWM — maximum width of deutomerite; DWE — width of deutomerite at equatorial axis; n — number of individuals.

Обозначения: TL — общая длина; PL — длина протомерита; DL — длина дейтомерита; PWM — максимальная ширина протомерита; PWE — ширина протомерита по экваториальной оси; DWM — максимальная ширина дейтомерита; DWE — ширина дейтомерита по экваториальной оси; n — количество особей.

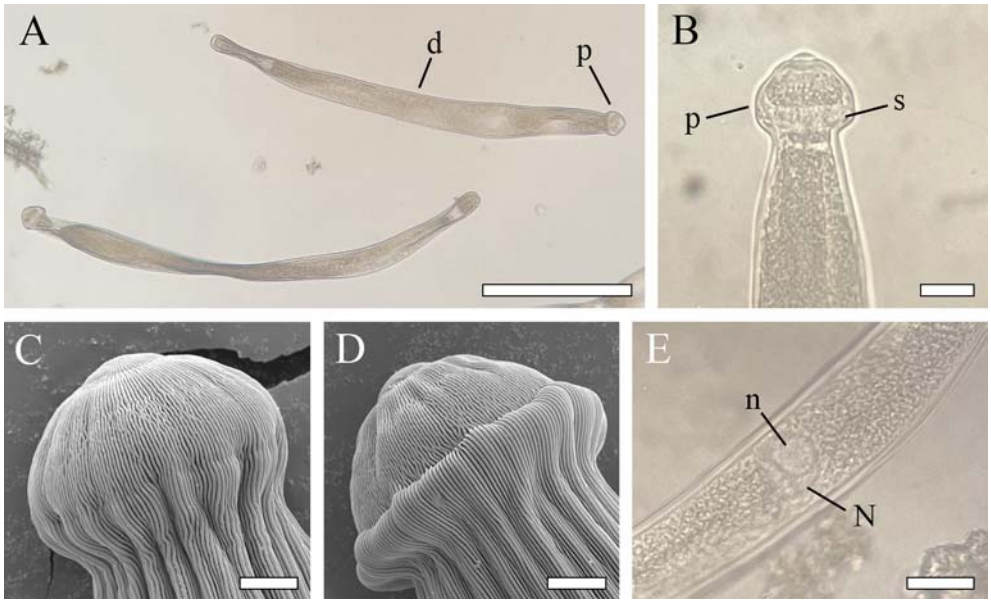

Fig. 2. Morphology of *Stenophora draconis* sp.n. A — gamonts; B — anterior end of gamont; C — protomerite, SEM; D — retracted protomerite, SEM; E — spherical nucleus with an eccentric round nucleolus.

Abbreviations: d — deutomerite; N — nucleus; n — nucleolus; p — protomerite; s — septum. Scale bars: A — 200 μm; B — 20 μm; C — 20 μm; D — 5 μm; E — 5 μm.

Рис. 2. Морфология *Stenophora draconis* sp.n. A — гамонты; B — передний конец гамонта; C — протомерит, SEM; D — втянутый протомерит, SEM; E — сферическое ядро с эксцентричным округлым ядрышком.

Обозначения: d — дейтомерит; N — ядро; n — ядрышко; p — протомерит; s — septum. Масштаб: A — 200 μm; B — 20 μm; C — 20 μm; D — 5 μm; E — 5 μm.

128.5 ± 5.5 μm) gregarines had a dome-shaped protomerite with a prominent papilla at its apex. Trophozoites were oblong (Fig. 1A, B). Gamonts were narrowly obovoid or ellipsoid. Detailed measurements of gregarines are given in Table 1. Live parasites had a boat-shaped nucleus with a centrally located oval nucleolus (Fig. 1B, C). The nucleus might be located in any part of the deutomerite. The cell surface was organized in the epicytic folds that start from the apical pole on the protomerite papilla (Fig. 1D). In addition to the gliding motility, the parasites were able to retract the protomerite into the deutomerite (Fig. 1E) and recurve the front end of the cell (Fig. 1F). Gametocysts and oocysts are unknown.

DNA SEQUENCE. GenBank OP390085.

TYPE LOCALITY. Cat Tien National Park, Socialist Republic of Vietnam (11°24′27.0″N 107° 22′53.8″E).

TYPE HABITAT. Terrestrial.

TYPE HOST. *Thyropygus carli* Attems, 1938 (Diplopoda: Spirostreptida).

LOCATION IN HOST. Intestine.

TYPE (syntype) MATERIAL: A gold sputter-coated SEM stub with several protists, specimen of parasite cells and host material fixed in ethanol have been deposited in the collection of The Center for Parasitology IPEE RAS; cells fixed in 96% ethanol deposited in the collection of the Department of evolutionary biochemistry, Belozersky Institute for Physico-Chemical Biology, Lomonosov Moscow State University; Fig. 1 (this publication) shows some of the syntypes.

LSID: urn:lsid:zoobank.org:act:9E718297-B2EC-4AE9-AFE0-F4EE885DF705

ETYMOLOGY: From the type locality.

Stenophora draconis Miroliubova **sp.n.**
Fig. 2.

Gamonts were found in the intestine of two out of three dissected dragon millipedes *Hylomus* (*Desmoxytes*) *pilosus* Attems, 1937. Oblong gregarines reached 817.8 μm in length (av. 635.8 ± 13.8 μm) and 79.4 μm in width (av. 45.8 ± 2 μm) with elongate deutomerite and relatively short protomerite (Fig. 2A). Shallowly pyriform or dome-shaped protomerite had a small papilla at its apex (Fig. 2B, C).

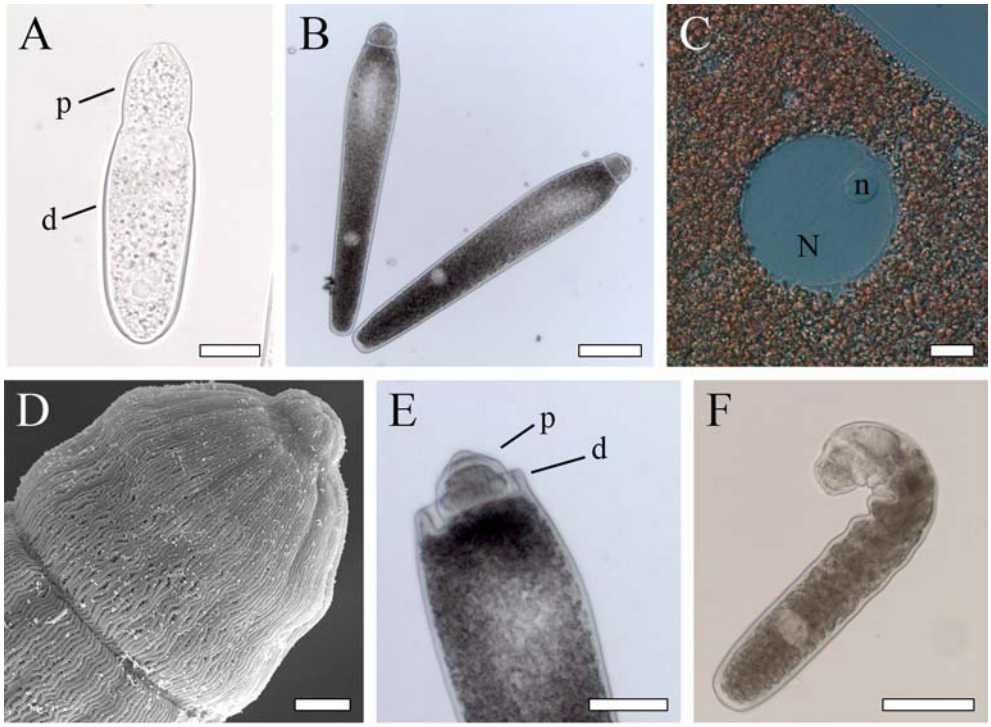

Fig. 3. Morphology of *Stenophora nickeli* sp.n. A — young trophic stage; B — gamonts; C — spherical nucleus with an eccentric round nucleolus, DIC; D — anterior end — protomerite, SEM; E — gamont with retracted anterior end; F — gamont with recurved front end.
Abbreviations: d — deutomerite; N — nucleus; n — nucleolus; p — protomerite. Scale bars: A — 20 μm; B — 100 μm; C — 20 μm; D — 5 μm; E, F — 50 μm.
Рис. 3. Морфология *Stenophora nickeli* sp.n. A — молодой трофозоит; B — гамонты; C — сферическое ядро с эксцентричным округлым ядрышком, DIC; D — передний конец — протомерит, SEM; E — гамонт с втянутым передним концом; F — гамонт с загнутым назад передним концом.
Обозначения: d — дейтомерит; N — ядро; n — ядрышко; p — протомерит. Масштаб: A — 20 μm; B — 100 μm; C — 20 μm; D — 5 μm; E, F — 50 μm.

Thickened septum provided retraction of protomerite (Fig. 2B, D). Gregarines had a spherical nucleus with an eccentric round nucleolus (Fig. 2E). The cell surface was organized in the epicytic folds that start from the apical pole on the protomerite papilla and divide twice on the protomerite (Fig. 2C, D). Parasites demonstrated gliding motility. Detailed measurements of the gregarines are given in Table 1. Gametocysts and oocysts are unknown.

DNA SEQUENCE. GenBank OP390087.

TYPE LOCALITY. Cat Tien National Park, Socialist Republic of Vietnam (11°24′27.0″N 107°22′53.8″E).

TYPE HABITAT. Terrestrial.

TYPE HOST. *Hylomus* (*Desmoxytes*) *pilosus* Attems, 1937.

LOCATION IN HOST. Intestine.

TYPE (syntype) MATERIAL: A gold sputter-coated SEM stub with several protists, specimen of parasite cells and host material fixed in ethanol have been deposited in the collection of The Center for Parasitology IPEE RAS; cells fixed in 96% ethanol deposited in the collection of the Department of evolutionary biochemistry, Belozersky Institute for Physico-Chemical Biology, Lomonosov Moscow State University; Fig. 2 (this publication) shows some of the syntypes.

LSID: urn:lsid:zoobank.org:act:328F4964-7A59-4998-9A9D-7A0264A71535

ETYMOLOGY: From the host, a dragon millipede.

Stenophora nickeli Miroliubova et Kudriavkina **sp.n.**
Fig. 3.

Trophozoites and gamonts were found in the intestine of 18 out of 20 dissected millipedes *Pachy-*

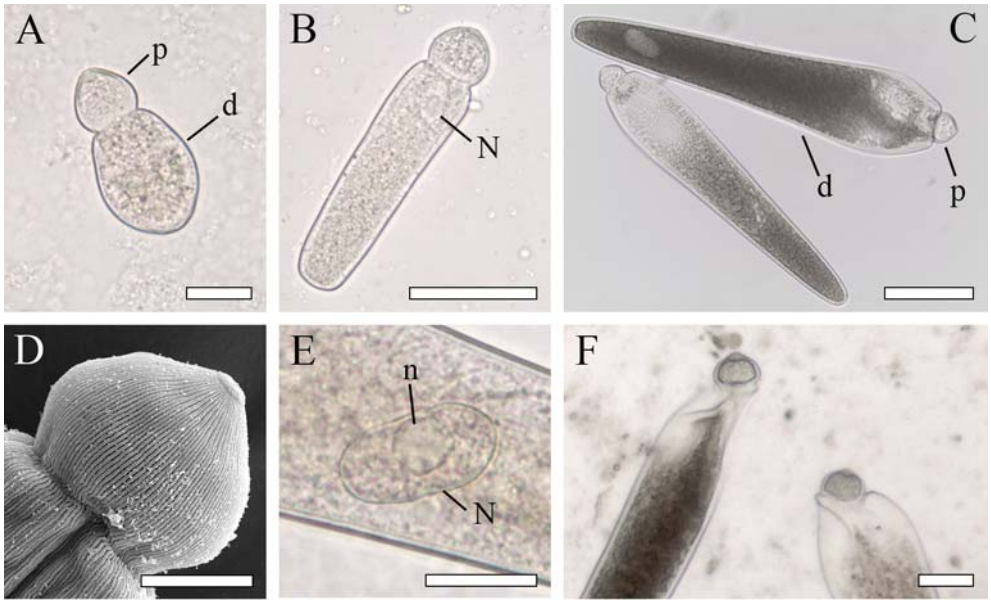

Fig. 4. Morphology of *Stenophora pachyiuli* sp.n. A — young trophozoite; B — trophozoite; C — gamonts; D — protomerite, SEM; E — oval nucleus with an eccentric round nucleolus; F — gamonts with contracted anterior part of deutomerite.
Abbreviations: d — deutomerite; N — nucleus; n — nucleolus; p — protomerite. Scale bars: A — 20 μm; B — 50 μm; C — 100 μm; D — 10 μm; E — 20 μm; F — 20 μm.
Рис. 4. Морфология *Stenophora pachyiuli* sp.n. A — молодой трофозоит; B — трофозоит; C — гамонты; D — протомерит, SEM; E — овальное ядро с эксцентричным округлым ядрышком; F — гамонты с сокращенной передней частью дейтомерита.
Обозначения: d — дейтомерит; N — ядро; n — ядрышко; p — протомерит. Масштаб: A — 20 μm; B — 50 μm; C — 100 μm; D — 10 μm; E — 20 μm; F — 20 μm.

iulus krivolutskyi Golovatch, 1977 (Fig. 3A, B). The relatively large (696 μm long, av. 400.6 ± 27.6 μm and 134.4 μm wide, av. 70.1 ± 4.1 μm) gregarines had a beanie-shaped protomerite (Fig. 3A, B) and an elongated deutomerite that gradually narrowed towards its truncate posterior end (Fig. 3A, B). Detailed measurements of the gregarines are given in Table 1. Live parasites had a spherical nucleus with an eccentric round nucleolus (Fig. 3C). The nucleus might be located in any part of deutomerite. The cell surface was organized in the epicytic folds that start from the apical pole on the protomerite papilla (Fig. 3D). Gregarines demonstrated gliding motility, the ability to retract protomerite into deutomerite (Fig. 3E), and to recurve the front end of the cells (Fig. 3F). Gametocysts and oocysts are unknown.

DNA SEQUENCE. GenBank OP390086.

TYPE LOCALITY. Nickel, Adygea Republic, Russia (44°10′38.2″N 40°09′21.4″E).

TYPE HABITAT. Terrestrial.

TYPE HOST. *Pachyiulus krivolutskyi* Golovatch, 1977 (Diplopoda: Julida).

LOCATION IN HOST. Intestine.

TYPE (syntype) MATERIAL. A gold sputter-coated SEM stub with several protists, specimen of parasite cells, and host material fixed in 96% ethanol have been deposited in the collection of The Center for Parasitology IPEE RAS; extracted DNA used for obtaining of rDNA sequences deposited in the collection of the Department of evolutionary biochemistry, Belozersky Institute for Physico-Chemical Biology, Lomonosov Moscow State University; Fig. 3 (this publication) shows some of the syntypes.

LSID: urn:lsid:zoobank.org:act:B6541780-573C-4203-AD00-51C431B7ACD8

ETYMOLOGY: From the type locality.

Stenophora pachyiuli Miroliubova et Kudriavkina **sp.n.**

Fig. 4.

Trophozoites and gamonts were found in the intestine of 15 out of 20 dissected millipedes *Pachyiulus krivolutskyi* Golovatch, 1977. Young trophozoites had a pyriform deutomerite, growing trophozoites were oblong, and gamonts were very narrowly

obdeltoid in shape up to 451.2 μm in length (av. 337 ± 16.4 μm) and 100.8 μm in width (av. 70.7 ± 3.7 μm) (Fig. 4A, B, C). All forms had a dome-shaped protomerite with a small papilla at its apex. The cell surface was organized in the epicytic folds that start from the protomerite papilla (Fig. 4D). An oval nucleus with an eccentric round nucleolus might be located in any part of deutomerite (Fig. 4B, C, E). Gregarines demonstrated gliding motility and the ability to contract the anterior part of deutomerite to move the protomerite (Fig. 4F). Gametocysts and oocysts are unknown.

DNA SEQUENCE. GenBank OP423031.

TYPE LOCALITY. Nickel, Adygea Republic, Russia (44°10′38.2″N 40°09′21.4″E).

TYPE HABITAT. Terrestrial.

TYPE HOST. *Pachyiulus krivolutskyi* Golovatch, 1977 (Diplopoda: Julida).

LOCATION IN HOST. Intestine.

TYPE (syntype) MATERIAL. A gold sputter-coated SEM stub with several protists, specimen of parasite cells and host material fixed in ethanol have been deposited in the collection of The Center for Parasitology IPEE RAS; extracted DNA used for obtaining of rDNA sequences deposited in the collection of the Department of evolutionary biochemistry, Belozersky Institute for Physico-Chemical Biology, Lomonosov Moscow State University; Fig. 4 (this publication) shows some of the syntypes.

LSID: urn:lsid:zoobank.org:act:9E240BB8-917D-41E3-A5E4-373AE432736C

ETYMOLOGY: From the host genus name.

Molecular phylogeny

Near-complete sequences of the rRNA operon (SSU rDNA, ITS1, 5.8S rDNA, ITS2, and LSU rDNA) were obtained for *Stenophora cattiensis* (5,436 bp), *S. nickeli* (5,438 bp) and *S. draconis* (5,469 bp). The SSU rDNA, ITS1, 5.8S rDNA, ITS2, and partial LSU rDNA sequence was obtained for the gregarine ex *Sphaerobelum* sp. (3,770 bp). Partial SSU rDNA, ITS1, 5.8S rDNA, ITS2, and partial LSU rDNA sequence was obtained for *Stenophora pachyiuli* n. sp. (2,300 bp). Fourteen partial SSU sequences of gregarines were assembled from the transcriptomic data of Diplopoda: *Trigoniulus corallinus* (three scaffolds), *Cylindroiulus* sp. (two scaffolds), *Uroblaniulus* sp. (one contig), and *Helicorthomorpha holstii* (one scaffold), *Eudigraphis taiwaniensis* (two contigs), *E. takakuwai* (two contigs and one scaffold) and *Polyxenus lagurus* (one contig and one scaffold).

Bayesian inference (BI) and Maximum Likelihood (ML) analyses recovered almost identical tree topologies for the SSU and the combined SSU-5.8S-LSU datasets (Figs 5 and 6). The Bayesian tree for SSU inferred from the dataset of 84 sequences and 1,530 aligned sites showed the monophyly of major terrestrial gregarine groups: the Actinocephaloidea, the Stylocephaloidea, the Gregarinoidea with high posterior probabilities (PP) and ML support values (bootstrap percentages, BP). The 20 new gregarines from Diplopoda together with *Stenophora robusta* and related environmental sequences formed the monophyletic Stenophoroidea with low support (PP = 0.62 and BP = 52). The Gregarinoidea appeared as a sister clade to Stenophoroidea with PP = 1 and BP = 81. The analyses subdivided the Stenophoroidea into four clades comprising multiple sequences, and three lineages represented by a single sequence. The earliest-diverging clade consisted of gregarines parasitizing millipedes from the order Polyxenida. We designate this clade as Stenophoroidea clade I. Within clade I six OTUs groped together in a robust clade (PP = 1 and BP = 100%). The sequence "gregarine 2 ex *Polyxenus lagurus*" grouped with clade I as a deeply diverging sister lineage with insufficient support values. The Stenophoroidea clade II (PP = 1 and BP = 99%) contained a large number of environmental sequences and 6 gregarines from millipedes: *Stenophora cattiensis*, *S. draconis*, *S. nickeli*, gregarine 1 ex *Trigoniulus corallinus*, gregarine 1 ex *Cylindroiulus* sp., and a parasite from *Helicorthomorpha holstii*. The Stenophoroidea clade III (PP = 1 and BP = 99%) mainly consisted of the short V4 region sequences from Heger *et al*. (Heger *et al*., 2018) and included gregarine 2 from *Cylindroiulus* sp. The Stenophoroidea clade IV (PP = 1 and BP = 100%) contained *Stenophora robusta*, *Stenophora pachyiuli* sp.n., gregarine 3 ex *Cylindroiulus* sp., a parasite of *Uroblaniulus* sp., and several environmental sequences mainly from Jamy *et al*. (Jamy *et al*., 2020). In the phylogenies, the clades branch sequentially from the Stenophoroidea stem, forming assemblages of clades III and IV (PP = 1 and BP = 81%), and clades II–IV (PP = 0.97 and BP = 53%). Peculiarly, two gregarines isolated from *Trigoniulus corallinus* did not fall within either of the well-supported clades. One gregarine sequence from

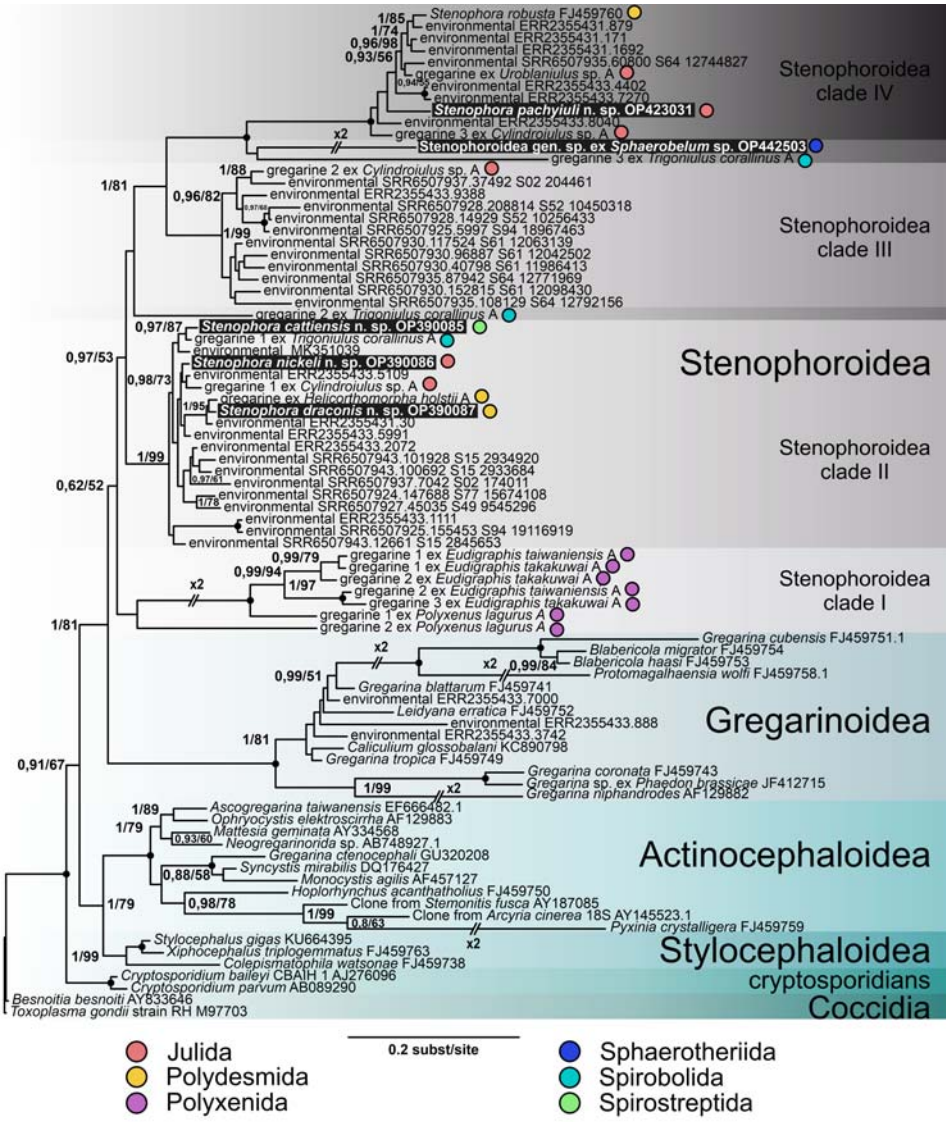

Fig. 5. Bayesian inference tree of terrestrial gregarines with an alignment of 84 SSU rDNA sequences (1,530 sites). Tree node support values: Bayesian posterior probabilities (PP, top) and ML bootstrap percentage (BP, bottom). Black dots indicate PP = 1 and BP = 100%. Support values of PP ≤ 0.89 and BP ≤ 69% are omitted (except the key node for Stenophoroidea). Oblique transverse lines on the branches mark how many times the latter were manually shortened. The newly obtained sequences are on black background; sequences assembled from the available transcriptomic data are marked by A. Colored circles mark the millipede orders the hosts of the gregarines belong to. See also Suppl. Fig. 5.

Рис. 5. Байесовское дерево наземных грегарин, построенное по 18S рДНК (84 OTE, 1530 нуклеотидных позиций). Числа обозначают апостериорные вероятности (PP, числитель) и ML проценты бутстрепа (BP, знаменатель). Черные точки обозначают PP = 1 и BP = 100%. Поддержки PP ≤ 0.89 и BP ≤ 69% не показаны (за исключением ключевого узла Stenophoroidea). Поперечные косые линии на ветвях указывают, во сколько раз была сокращена ветвь на рисунке для удобства визуализации дерева. Последовательности, полученные в данной работе, выделены черным фоном; последовательности, полученные из доступных транскриптомных данных, отмечены буквой А. Цветные кружки обозначают отряды, к которым относят многоножек-хозяев. См. тж. Suppl. Fig. 5.

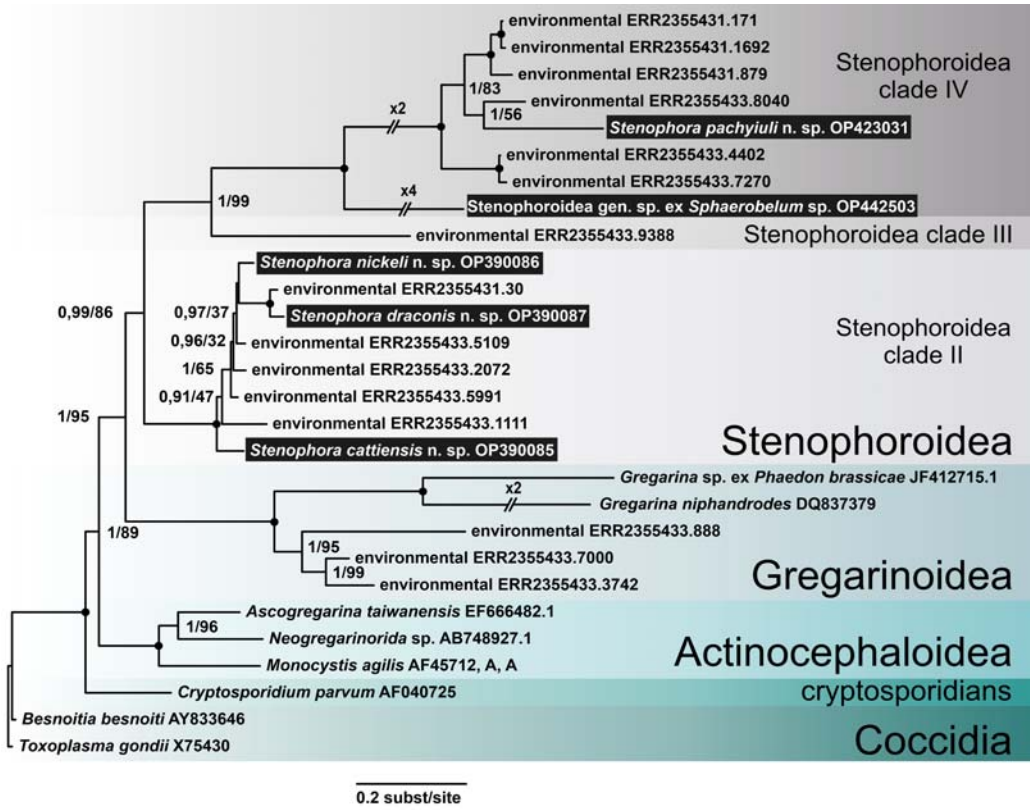

Fig. 6. Bayesian inference tree of terrestrial gregarines with an alignment of 28 concatenated SSU, 5.8S and LSU rDNA sequences (3,480 sites). Tree node support values: Bayesian posterior probabilities (PP, top) and ML bootstrap percentage (BP, bottom). Black dots indicate PP = 1 and BP = 100%. Support values of PP ≤ 0.89 and BP ≤ 69% are omitted. Oblique transverse lines on the branches mark how many times the latter were manually shortened. The newly obtained sequences are on black background; sequences assembled from the available transcriptomic data are marked by A. See also Suppl. Fig. 6.

Рис. 6. Байесовское дерево наземных грегарин, построенное по конкатенированным 18S, 5.8S и 28S рДНК (28 OTE, 3480 нуклеотидных позиций). Числа обозначают апостериорные вероятности (PP, числитель) и ML проценты бутстрепа (BP, знаменатель). Черные точки обозначают PP = 1 и BP = 100%. Поддержки PP ≤ 0.89 и BP ≤ 69% не показаны. Поперечные косые линии на ветвях указывают, во сколько раз была сокращена ветвь на рисунке для удобства визуализации дерева. Последовательности, полученные в данной работе, выделены черным фоном; последовательности, полученные из доступных транскриптомных данных, отмечены буквой A. См. тж. Suppl. Fig. 6.

Trigoniulus corallinus formed a sister lineage to the stenophorid clades III and IV, and the other formed a deep-diverging sister lineage to the Stenophoroidea clade IV (PP = 1 and BP = 100%) together with Stenophoroidea gen. sp. from *Sphaerobelum* sp.

Bayesian inference tree with the combined dataset was consistent with the tree obtained for the SSU alignment, although the position of gregarines from polyxenid millipedes could not be determined due to lack of LSU sequences for this clade. Both analyses show the monophyly of major terrestrial gregarine groups and recover the same relationship between the clades of the Stenophoroidea as the SSU tree (Fig. 6). The combined dataset tree shows higher support for the sisterhood of the Gregarinoidea and the Stenophoroidea (PP = 1 and BP = 95%). The affinity of Stenophoroidea clade II and clades III–IV also receives a boost in support values

with the combined dataset: 0.99 PP and 86% BP.

Discussion

The gregarine fauna of millipedes of the Indochinese Peninsula is almost unknown. The only described species is *Stenophora hoshidei* Théodoridès, Desportes et Jolivet, 1975 parasitising *Orthomorpha uncinata* Attems, 1931 in Thailand (Théodoridès *et al*., 1975). A similar situation is seen in the Caucasus, where no gregarines from millipedes were reported. However, both regions abound in millipede fauna and represent perfect sites to study millipede parasites. In the present study, we describe four new species from Vietnam and the Western Caucasus, Russia. The described gregarines have an appearance typical for *Stenophora*: lacking a large epimerite, lacking longitudinal myonemes or lateral bundles of myonemes, unlike the other genera of millipede parasites, *Cnemidospora*, *Chakravartiella*, *Monoductus* or *Hyalosporina* (Desportes, Schrével, 2013). Their trophozoites do not have a caliciform or crateriform protomerite as in *Amphoroides* (Simdyanov, 2007), and do not demonstrate shape polymorphism as in *Fonsecaia polymorpha* Pinto, 1918 (Pinto, 1922). Thus, despite the lack of information on the gametocyst dehiscence and the oocyst morphology, these gregarines were assigned to the genus *Stenophora*. The parasite of *Thyropygus carli* is very similar in appearance to *Stenophora conjugata* Rodgi et Ball, 1961 described from *Phyllogonostreptus nigrolabiatus* Newport, 1844 in Southern India (Rodgi, Ball, 1961). Both species have a dome-shaped protomerite, an elongated deutomerite and a boat-shaped nucleus, but gamonts of *S. conjugate* are smaller with the maximal size 460 μm (Rodgi, Ball, 1961), so *Stenophora cattiensis* sp.n. was assigned to a new species. *Stenophora draconis* sp.n. is similar to *Stenophora elongata* Ellis, 1912. However, *S. elongata* is less than half the size of *S. draconis*, parasitizes another millipede species (*Orthomorpha coarctata* Saussure, 1860) and was found in Central America (Watson, 1916). *Stenophora nickeli* sp.n. and *Stenophora pachyiuli* sp.n. were established as new species due to unique combinations of characteristics not found together in other stenophorids.

In our phylogenies we used 21 gregarines from 12 different millipede species belonging to 6 orders of Diplopoda (see M&M). All 21 gregarines and related environmental sequences grouped together within the likely monophyletic Stenophoroidea.

Gregarines from insects are more diverse as they are polyphyletic and belong to the Actinocephaloidea, the Stylocephaloidea, and the Gregarinoidea. The insect gregarine *Pyxinia crystalligera*, parasitizing the hide beetle *Dermestes maculatus* Fabricius, 1781, grouped strongly with actinocephalids and neogregarines (Fig. 5). Earlier phylogenies grouped *P. crystalligera* together with *Stenophora robusta* (Clopton, 2009), prompting the establishment of a new family Pyxiniidae within the Stenophoroidea (Desportes, Schrével, 2013). The previous grouping of *S. robusta* and *P. crystalligera* was likely a result of insufficient sampling and the long branch attraction artefact. In our analyses, new short-branching stenophorids and two gregarines mis-annotated as myxomycetes broke up the long branches of *S. robusta* and *P. crystalligera* alleviating the impact of excessive sequence divergence and rectifying the closer relationship of *P. crystalligera* to the Actincephaloidea.

The Stenophoroidea turned out to be divided into four sequence-rich clades and three single-sequence lineages (Fig. 5). Interestingly, the basal clade of Stenophoroidea consists of parasites of the earliest-diverging group of Diplopoda — Polyxenida (Fernández, Edgecombe, Giribet, 2016; Sierwald, Bond, 2007), although support for the union of gregarines from polyxenids with other members of Stenophoroidea was low in the analysis of the SSU dataset (Fig. 5). There is one described gregarine from polyxenids assigned to *Stenophora* (*Stenophora polyxeni* Léger et Duboscq, 1903) (Léger, Duboscq, 1904). According to our trees, one polyxenid species may host more than one gregarine species. However, we also cannot exclude the possibility that the presence of several similar sequences in one library might be a result of intragenomic polymorphisms in these gregarines. In other respects, the phylogeny of stenophorids does not correspond to the phylogeny of their millipede hosts. The phylogenies show that gregarines from the same millipede species may belong to different stenophor-

id clades. For instance, *Stenophora nickeli* sp.n. and *Stenophora pachyiuli* sp.n. from *Pachyiulus krivolutskyi* were placed in two different clades: Stenophoroidea clade II and Stenophoroidea clade IV. Moreover, a single clade may contain gregarines from millipedes belonging to different orders: Stenophoroidea clade II includes parasites of millipedes from at least four orders (Fig. 5).

The phylogenetic position of the stenophorid group as a whole has been understudied. After Clopton (Clopton, 2009), the sisterhood of the Gregarinoidea and the Stenophoroidea was shown with a large dataset of environmental sequences: many of "Putative novel gregarines" (Supplementary fig. 6–8 in Jamy *et al*., 2020) correspond to the Stenophoroidea as we revealed with our phylogeny. Metagenomic data give a great opportunity to improve phylogenies for groups with long branches and/or small amount of sequenced representatives. The short V4-region sequences of Heger and colleagues (Heger *et al*., 2018) help illustrate the fact that stenophorids are diverse and abundant. The long-read data of Jamy and colleagues (Jamy *et al*., 2020) further increase the resolution not only for SSU, but also for LSU and combined phylogeny.

The results obtained in this study show that the Stenophoroidea are a diverse group, whose systematics require further improvement using molecular data. Exploring this understudied segment of the gregarine tree is crucial for gaining insight into the evolution of gregarine oocyst morphology, gametocyst dehiscence, and adaptation to different hosts.

Supplementary data. The following figures are available online.

Supplementary Figure 5. Pdf version of text Figure 5.

Supplementary Figure 6. Pdf version of text Figure 6.

Acknowledgments. We thank Irina Semenyuk and Sergey Golovatch for the help with identification of vietnamese millipedes. We also acknowledge Sergei Spiridonov and Alexander Balakirev for obtaining sample from Suoi Phai village. This study was funded by the Russian Science Foundation (project No. 18-14-00123). The SEM study was conducted using the Joint Usage Center "Instrumental methods in ecology" at the IPEE RAS.

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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