Description of Ganymedes yurii sp. n. (Ganymedidae), a New Gregarine Species from the Antarctic Amphipod Gondogeneia sp. (Crustacea)

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ABSTRACT
A novel species of aseptate eugregarine, Ganymedes yurii sp. n., is described using microscopic and molecular approaches. It inhabits the intestine of Gondogeneia sp., a benthic amphipod found along the shore of James Ross Island, Weddell Sea, Antarctica. The prevalence of the infection was very low and only a few caudo-frontal syzygies were found. Morphologically, the new species is close to a previously described amphipod gregarine, Ganymedes themistos, albeit with several dissimilarities in the structure of the contact zone between syzygy partners, as well as other characteristics. Phylogenetic analysis of the 18S rDNA from G. yurii supported a close relationship between these species. These two species were grouped with other gregarines isolated from crustacean hosts (Cephaloidophoroidea); however, statistical support throughout the clade of Cephaloidophoroidea gregarines was minimal using the available dataset.

THE Antarctic is an exceptional continent, with extreme climates and environmental conditions above and below the water surface. While not many species are thought to survive in these high latitudes, the diversity of marine fauna in this part of the world is enormously rich, especially in the plankton, nekton, and benthos. A number of reviews are dedicated to the climatology, geology, palaeontology, and fauna of vertebrates (fish, birds, and mammals) from the Antarctic region (Barbosa and Palacios 2009; Eastman 2005). Similarly, many investigations were devoted to apicomplexans parasitising fish, birds, and krill (Avdeev 1985, 1987; Barber and Mills Westermann 1988; Golemansky 2011; Takahashi et al. 2003, 2004, 2008, 2009). To date, only one study has been devoted to crustacean gregarines occurring in littoral/sublittoral invertebrates (Lipa and Rakusa-Suszczewski 1980).

Gregarines, in contrast to coccidia, largely exist as extracellular parasites of a broad range of invertebrate groups, for example, terrestrial insects, aquatic annelids, and crustaceans. Most gregarines inhabit the host intestinal lumen, are elongated and heteropolar, cylindrical, or vermiform in shape. Feeding stages (=trophozoites) generally develop attached to the host cell via their modified anterior end. Usually, the trophozoites are subdivided into three parts: epimerite (attachment function), and protomerite followed by a deutomerite containing a large nucleus. The last two regions are separated by a fibrillar septum. Gregarines with such organisation are classified as being “tricystid” or “septate” gregarines. On the other hand, gregarines that are not subdivided represent the “monocystid” form, and are known as aseptate gregarines. It should also be mentioned that, generally, trophozoites, as well as subsequent sexual stages (=gamonts), exhibit gliding motility and possess a unique organisation of the cell cortex. The
pellicle of eugregarines forms longitudinal epicytic folds equipped with special sets filamentous structures in their apex, represented by rippled-dense structure and 12-nm filaments. Before gametogenesis, the gamonts join into a sexual association called syzygy. Later on, the paired gregarines form a common envelope (gametocyst), under which further processes, including gametogenesis, fertilisation and formation of invasive stages (sporogenesis), take place (Desportes and Schrèvel 2013; Frolov 1991; Grassé 1953; Long 1982; Perkins et al. 2000; Simdyanov 2007).

Many septate and aseptate eugregarines have been described from different crustacean hosts from different marine and terrestrial aquatic localities, and have traditionally been distinguished based on their general morphology using light microscopy. These gregarines have been separated into different families including the Cephalodiodoridae, Porosporidae, Uradiophoridae, Ganymididae, and others. However, this system of families and nomenclature at the level of genus and species remains unsettled (Desportes and Schrèvel 2013; Grassé 1953; Levine 1977a,b; Perkins et al. 2000; Simdyanov 2007). Furthermore, the data collected from each group are not uniform, for example, only some of these groups have been investigated using electron microscopic approaches and/or molecular techniques (Desportes and Théodoridès 1969, 1985; Rueckert et al. 2011; Simdyanov et al. 2015; Takahashi et al. 2009; Théodoridès and Desportes 1975). Recent phylogenetic analyses of SSU rDNA sequences showed that gregarines from different crustacean hosts clustered in a single clade, together with a number of environmental sequences (Rueckert et al. 2011). This finding was confirmed by phylogenetic analyses of LSU rDNA and whole ribosomal operon (SSU + 5.8S + LSU) (accession numbers HQ891113.2 – HQ891115.2) (Simdyanov et al. 2015).

The family Ganymididae was established by Huxley (1910) and comprises intestinal aseptate gregarines possessing ball-like and cup-like structures at the anterior and posterior ends of the cell, respectively. He described the type species, Ganymedes anaspidis, from a mountain shrimp, Anaspides tasmaniae Thomson, 1892 (Huxley 1910). Later many aseptate gregarines were described from different freshwater and marine crustacean hosts (Cirrhipedia, Amphipoda, Decapoda etc.) (Jones 1968, 1969; Jones et al. 1994; Prokopowicz et al. 2010; Théodoridès and Desportes 1972, 1975). Subsequently, Levine (1977a, b) made a taxonomical revision of this genus; he placed all species lacking the above-mentioned ball-like and cup-like structures into a new genus, Paraphoiodina, and only one species was retained in the genus Ganymedes, namely G. anaspidis. Perkins et al. (2000) and Simdyanov (2007) followed this opinion. In the latest revision of gregarine species, this point of view was rejected and many species were returned and assigned to the genus Ganymedes (Desportes and Schrèvel 2013).

In this study, we describe the general morphology and molecular phylogeny, based on SSU sequence data, of a new Antarctic gregarine, Ganymedes yurii sp. n. For this, we used a combined approach of transmission and scanning electron microscopy, and molecular phylogenetic analyses.

**MATERIALS AND METHODS**

**Gondogeneia** sp. Barnard, 1972, an amphipod, was collected in January and February 2013 in the littoral and upper sublittoral zone of Cape Lachman (63°47′32″S, 57°46′36″W), James Ross Island, Weddell Sea, Antarctica. The amphipods were transported to the laboratory and kept in cold conditions. About 400 crustaceans were dissected under a stereomicroscope (MST 131, Poland). Parasites released from the host intestine were collected using a thin glass pipette. Light microscopic observations of living parasites were performed using an Olympus CX41 Microscope (Olympus Corp., Tokyo, Japan) equipped with phase contrast and connected to an Olympus C-7070 Digital Camera (Olympus Corp.).

For electron microscopy, parasites were fixed in 2.5% glutaraldehyde in Millipore-filtered sea water (SW) (Milli-Grade 0.22 µm). For transmission electron microscopy (TEM), gregarines were then postfixed with 1% OsO4 (Os) in 0.2 M cacodylate buffer, dehydrated in an ethanol series and embedded in Epon blocks. Ultra-thin sections were made using Reichert Ultracut E and Leica UTC ultramicrotomes, stained according to a standard protocol (Reynolds 1963), and observed under a JEOL-1010 transmission electron microscope (JEOL Ltd., Peabody, MA, USA). For scanning electron microscopy (SEM), fixed trophozoites were postfixed for 2 h in 2% osmium tetroxide in 0.2 M cacodylate buffer, dehydrated with CO2 using Ermitech K850, and then coated with gold using Ermitech K560 sputter coaters. The samples were observed under a JEOL JSM-7401F field emission scanning microscope (JEOL Ltd.).

For molecular analysis, gamonts in syzygy were fixed in 100% ethanol and stored at room temperature. Genomic DNA was later extracted with the standard protocol provided in the MasterPure Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). However, the final elution step was lowered to 4 µl. Outside primers, PF1 5′-GGCCTACCTCGTATCCTGCC-3′ and SSUR4 5′-GATCCCTTCTGAGGTTACCTAC-3′ (Leander et al. 2003), were used in a 25 µl PCR with EconoTaq 2X Master Mix (Lucigen Corp., Middleton, WI). The following programme was used on a thermocycler for the initial amplification: Initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, anneal at 52 °C for 30 s, extension at 72 °C for 1 min 50 s, final extension 72 °C 5 min. Subsequently, internal primers F2 5′-GCTTGAAAAGGTGACDATCTG-3′ and R2 5′-CAGTCTGCTAAGGTTCGT-3′ were paired with outside primers in a nested PCR using the following programme on a thermocycler: Initial denaturation for 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 30 s, anneal at 52 °C for 30 s, extension at 72 °C for 1 min 30 s; final extension at 72 °C for 7 min.

The newly obtained DNA sequence from Ganymedes yurii was initially identified by BLAST (http://
blast.ncbi.nlm.nih.gov). This sequence was then aligned with 89 additional alveolate sequences selected from NCBI/GenBank (to cover the diversity of apicomplexans, including some dinoflagellates as an outgroup), using MUSCLE 3.8.31 (Edgar 2004). The alignment of 90 OTUs was subsequently edited and fine-tuned using MacClade 4.08 (Maddison and Maddison 2005). Garli0.951-GUI (www.bio.uteexas.edu/faculty/antisense/garli/Garli.html) was used to analyse the 90-sequence alignment (1,170 unambiguously aligned positions; gaps excluded) with maximum-likelihood (ML). Jmodeltest 0.1.1 selected a general-time reversible (GTR) model of nucleotide substitutions (Posada and Crandall 1998) that incorporated invariable sites and a discrete gamma distribution (eight categories) (GTR + I model: $\alpha = 0.6430$ and fraction of invariable sites = 0.2160) under Akaike Information Criterion (AIC) and AIC with correction (AICc.). ML bootstrap analyses were performed on 500 pseudo-replicates, with one heuristic search per pseudo-replicate (Zwickl 2006), using the same programme set to the GTR model $+ \Gamma + I$. Bayesian analysis of the 90 OTU-dataset was performed using the programme MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The programme was set to operate with GTR, a gamma-distribution, and four Monte Carlo Markov Chains (MCMC; default temperature = 0.2). A total of 10,000,000 generations were calculated with trees sampled every 100 generations and with a prior burn-in of 1,000,000 generations (10,000 sampled trees were discarded; burn-in was checked manually). When the average split fell below 0.01, the programme would terminate. All other parameters were left at the default. A majority rule consensus tree was constructed from 90,000 postburn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the postburn-in trees.

RESULTS

The prevalence and abundance of gregarines within the host were low; approximately 10% of crustacean hosts were parasitised with no more than 2–5 syzygies per host. Mostly, head-to-tail syzygies with two partners were found, while no solitary trophozoites, gametocysts or other stages were observed. Both partners in syzygy were of monocystid form, with the nucleus situated in the middle of the cell. Primites (anterior syzygy partner) were usually slightly curved, longer and more slender than the satellite (posterior syzygy partner) (190–160 µm vs. 140–150 µm, respectively) (Fig. 1A, B, 2A). The cytoplasm of both partners was packed with a granular content, corresponding to the grains of amylopectin. The thin transparent cortical layer was seen on the lateral sides of the cells; however, on the side of anterior ends of the primate and satellite, and posterior end of the satellite this zone was thicker (Fig. 1A, B). In contrast to the satellite, the anterior end of the primate possessed a transparent vacuolar zone (Fig. 1A–C). The nuclei of both partners were ovoid in shape and each contained one round nucleolus (Fig. 1D). The contact between the primate and satellite was simple, without any visible interdigitations at LM level (Fig. 1E). Only once we observed a multiple association, when three differently sized satellites attached to the posterior end of the primate (Fig. 1F). All syzygies exhibited unidirectional gliding motility.

Under transmission electron microscopy, the cells were round in cross-section. The bulk of cytoplasm (~endoplasm) was packed with amylopectin granules and the zone of ectoplasm was very thin (Fig. 2B, C). The nucleus possessed granular karyoplasm and an uneven nuclear envelope (Fig. 2D, E). The parasites were covered with numerous epicytic folds (about 1 µm in length and 0.1 µm in width), which ran along the surface of the both partners (Figs. 2A, C, F, 3A, D, E), and most of these folds were wavy (Fig. 2F, 3H). The gregarine surface exhibited specific areas where some folds formed a loop; i.e. these folds went towards one of the ends, but reversed in the
opposite direction (Fig. 2F). Such patterns were found in all observed cells.

The three-layered pellicle was of typical apicomplexan organisation, having a plasma membrane, underlain by two closely adjacent membranes of the inner membrane complex (imc) (Fig. 3A, B). The base of the folds was underlain with a thick internal lamina, which formed bridges with dense triangular rods (Fig. 3A, I). The internal lamina, located underneath the pellicle, continued into the folds, where it became thinner and more dense (Fig. 3A, B). At the top of each epicytic fold, four to five rippled-dense structures could be observed, situated between the plasma membrane and imc. An electron-dense rod was also seen in the apex of epicytic folds, underlying the imc (Fig. 3A, B). Typical micropores were rarely found between the epicytic folds, they appeared as cylindrical invaginations of the plasma membrane (approximately 40 nm in diameter, and 50 nm in length) ending with a vesicle (approximately 50 nm in diameter). The cylindrical part of micropore was enforced by the internal lamina; however, no typical collar formed by imc was observed (Fig. 3C).

In addition to the aforementioned typical micropores, two different structures were observed in contact with the pellicle of the gregarine (Fig. 3D–G). The first was membranous vesicles. These were round, with a central, and electron-dense inclusions. At the point of contact with the pellicle, the plasma membrane did not form an invagination, but the imc formed a cone-shaped, electron-dense collar, instead of...
cylindrical collar present in typical micropores (Fig. 3D, F). The same membranous structures were also found in the ectoplasm (Fig. 3E). The second structures (gi) that came in contact with the pellicle contained granular content and were round or teardrop in shape; no membrane surrounded these structures. No invaginations of the plasma membrane or the imc collar were observed in this region (Fig. 3E, G).

Fused epicytic folds were observed in cross-sections of the cells. Commonly two or three folds fused along their lateral surfaces (Fig. 3D, E, G–J). The fusion of the fold was discontinuous throughout the length of the cell; some folds were fused across a longer distance, while others were only fused across a short distance (Fig. 2F, 3H). At the point of fusion, only the imc membranes of two adjacent folds were present. Usually, a triangular canal was seen at the base of fused folds, while one or two narrow canals in the middle were observed (Fig. 3D, E, G, I). Occasionally, a mucus-like substance could be observed in the space between fused folds (Fig. 3J).

During preparation of samples for SEM, some cells previously associated in syzygy disassociated, so it was possible to compare the surface morphology of anterior and posterior ends of both the primite and satellite (Fig. 4A, B). The anterior end of primite was convex and had an oval-shaped attachment area with a wrinkled surface (Fig. 4A, C). The epicytic folds near this attachment tip were straight (Fig. 4C). Many small pores were visible on the surface of attachment area (Fig. 4D). In longitudinal sections through the anterior part of primite, the cytoplasm was subdivided into several zones along the longitudinal cell axis (Fig. 4E). The most anterior area (zone 1) was packed with heterogeneous electron-dense roundish inclusions; this was presumed to be condensed mitochondria. It was followed by a concaved zone (zone 2) filled with various opaque and transparent round inclusions (Fig. 4F). These two zones were generally located eccentrically to the longitudinal axis of the cell. The next zone was thicker and packed with various vacuolar and granular inclusions, while the rest of the cell was filled with numerous amylopectin granules (Fig. 4E–G). The apical part of the primite was covered with a typical three-membrane pellicle, underlain by a dense, thick internal lamina. The internal lamina and imc were interrupted at some points (Fig. 4F, inset), which corresponded to the pores observed under SEM (Fig. 4D). At the free posterior end of the satellite, wavy epicytic folds converged in the centre, where a light depression was usually observed (Fig. 4H–I). At the posterior end of the satellite, a lentil-shaped granular zone was situated without amylopectin granules (Fig. 4J).

While under light microscopy the contact site between the primite and satellite appeared simple (Fig. 1E), electron microscopic observations revealed its complex organisation (Fig. 5, 6). Two collars were observed at the posterior end of the primite. The innermost collar had an uneven edge, and tightly adhered to the anterior most part of the satellite. The outermost collar did not adhere to the innermost one, and the epicytic folds running along the surface of the primite merged to this collar (Fig. 5A, B, 6A, B). The junction site of the partners from the side of the primite was crateriform, and was surrounded by the aforementioned collars. The radial ridges were observed on the surface of this area (Fig. 6A). The anterior end of the satellite, which contacted the primite, was cap-like and not surrounded by any collar (Fig. 6C, D). Usually, in the centre, it had a light depression from which many narrow grooves radiated. Small round and shallow caverns were found on the surfaces between these grooves (Fig. 6D).
The junction site of both syzygy partners was covered with three-membrane pellicle, underlain with a thick internal lamina. Occasionally, membranous structures resembling "mv" (aforementioned) were found in the contact with the pellicle of the junction area (Fig. 5E). The profile of the ridges of the primate crateriform posterior end and the grooves at the satellite anterior end corresponded to each other (Fig. 5D, E). The cytoplasm of both ends, in general, had the same organisation as what was described previously. The posterior end of the primate exhibited the same granular zone without any amylopectin granules, as seen in the free posterior end of the satellite. However, in the satellite, there was only a zone of dense granules (zone 2), immediately followed by a cytoplasm filled with amylopectin granules (Fig. 5C, D). As described previously, the innermost collar tightly adjoined to the satellite and possessed the rod consisting of fibrillar-like material.

In contrast, the outermost collar did not possess a rod, but some epicytic folds merged with it (Fig. 5B, F, 6D).

The new SSU rDNA sequence generated from *Ganymedes yurii* sp. n. had a 77% similarity to a closely related species, *Ganymedes themistos*; 354 sites were mismatched across their pairwise alignment of 1,553 bp.

Maximum-likelihood and Bayesian analysis (Fig. 7) generally agreed with previous work in this field. Clades comprising Cephalidophoroidea (Rueckert et al. 2011), Lecudinoidea (Simdyanov and Diakin 2013) (=Uroporoidea and Cavalier-Smith 2014), Gregarinoidea (Clopton 2009) and Actinocephaloidea (Cavalier-Smith 2014) were recovered. Other selected gregarines including archigregarines, as well as eugregarines from sipunculids and polychaetes, were dispersed between these main clades. Additional alveolate sequences were also grouped into the main clades, namely cryptosporidia, rhytidocystids, coccidia, and piroplasmids. The relationships between these clades were unresolved with this current dataset.

The novel SSU rDNA sequence obtained from *G. yurii* sp. n. was strongly affiliated with the SSU rDNA sequence from a previously investigated species, namely *Ganymedes themistos*. Both of these sequences were grouped with robust support in the family Ganymedidae, which is incorporated into the clade Cephalidophoroidea, comprising gregarines from crustaceans, as well as phylogenetically related environmental sequences. The sequences of two *Ganymedes* spp. branched early within the entire clade Cephalidophoroidea, albeit, with low posterior probability and bootstrap support (Fig. 7).

**TAXONOMIC SUMMARY**

Family *Ganymedidae* Huxley 1910
Genus *Ganymedes* Huxley 1910
*Ganymedes yurii* sp. n. (Fig. 1A, B, 2A)
Figure 7 Combined maximum-likelihood (ML) and Bayesian interference tree derived from phylogenetic analyses of the 90 OTUs dataset. 1.170 unambiguously aligned sites) of small subunit (SSU) rDNA sequences. This tree was inferred using the GTR + I substitution model \((\text{ln} L = 16,738.83829, \text{gamma shape} = 0.62738, \text{proportion of invariable sites} = 0.2381)\). Numbers at the nodes denote the ML bootstrap percentage (numerator) and Bayesian posterior probabilities (denominator). Bootstrap support values are listed above Bayesian posterior probabilities. Black dots on branches denote the bootstrap support values and Bayesian posterior probabilities of 95/0.95 or higher, respectively. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree, the Bayesian values lower than 0.9 marked ‘–’. The novel sequence generated in this study is highlighted in a black box. Some branches were shortened by multiples of the length of the substitutions/site scale bar.
Diagnosis. Syzygy caudo-frontal, the primite being slightly curved and longer than satellite. Vacular region present at the anterior end of the primite. Nucleus situated in the centre of the cell. Dimensions of the primate varied between 190–160 µm and satellite between 140–150 µm (Fig. 1, 2A). The posterior end of the primate is crateriform (cup-like), with two peripheral collars at the point of satellite attachment (Fig. 4A, 5A, B, 6A). Satellites possess cap-like anterior end (Fig. 4B, 6C). GenBank accession number KU726617 of small subunit rDNA distinguishes G. yurii from other investigated species.


Type host habitat. Cape Lachman, James Ross Island, Weddell Sea, Antarctica (63°47′32″S, 57°46′86″W). Littoral and upper sublittoral zone.

Type material deposition. Parasites on gold sputter-coated SEM stubs have been deposited at the Dept. of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic (Fig. 2A, 4A, B, 5A, B, 6A, C).

Etymology. The name of this species refers to a Russian name Yury (in the honour of first author’s father name).

Localisation in host. Intestinal lumen.

Gene sequence. Sequence of SSU RNA GenBank accession number KU726617.

DISCUSSION

The genus Ganymedes contains several species of aseptate eugregarines infecting various groups of crustacea. All known gregarine species within the genus Ganymedes are aseptate and form caudo-frontal syzygy. On the anterior end of the primite, they have a transparent zone (ball-like structure), and most have a cup-like structure at the posterior end, as it is described in this study. However, some possess cup-like anterior ends of the satellite, as it was described for G. themistos. Under light microscopy, G. yurii differs from previous described species by its form and size of the cells/partners in syzygy. Most of the described species possess elongated and flexible cells (Huxley 1910; Jones 1968, 1969; Prokopowicz et al. 2010; Théodoridès and Desportes 1972, 1975), in case of species studied in this work, the syzygy partners are shortened and are more or less rigid.

Surface morphology of G. themistos and G. yurii is similar; both gregarines are covered by longitudinal epicystic folds that run from one end of the cell to the other (as is the case with most of eugregarines). However, G. yurii possesses unique “looped folds” on the surface, the function of which remains unclear. The ultrastructure of epicystic folds was studied in several eugregarines from crustacean hosts, namely Porospora portunidarum, Thriotypia pisae, G. vibiliae, G. eucopiae, Uradiphora maetzii, Cephalodiophora cf. communis, and Heliospora cf. longissima (Desportes and Théodoridès 1985; Desportes et al. 1977, 1977, Simdyanov et al. 2015). All of them show similar morphology among folds, which appear club-shaped in cross-section. At the top of each fold, there are several rippled-dense structures (3–6), and in some species, the 12-nm filaments were observed. Many of the aforementioned species, except C. cf. communis and H. cf. longissima, have electron-dense rods in the apical part of the fold; this has also been found in G. yurii. Similar structures were also found in other eugregarines, for example, in Gregarina steini, G. polymorpha and G. cuneata, parasites of mealworm larvae (Valigurová et al. 2013), urosporids Gonospora beloneides (Corbel et al. 1979), Urospora ovalis and U. travisiae (Diakin et al. 2016). Therefore, it can be assumed that this structure appeared (or was lost) in different lineages of eugregarines independently. The function of this dense rod is unknown; however, Valigurová et al. (2013) suggested that in mentioned Gregarina spp. the half-moon-shaped rod underlying the 12-nm filaments could serve as a ‘skeleton’ reinforcing the apex of the folds, which is in contact with the substrate while the gregarine is gliding.

In G. yurii, we observed micro pores typical for apicomplexans, and in addition, two types of structures that differed in their morphology making contact with the cortex of the cell. However, there is no consensus regarding the function of these structures (typical micro pores and two additional structures described in this study). Some authors assume that micro pores could take part in the process of nutrient acquisition (Chobotar and Scholtyseck 1982; Scholtyseck 1973; Scholtyseck and Mehlhorn 1970; Vivier et al. 1970), while others suppose that they are in fact extrusomes, and function more in the process of secreting mucus (Desportes and Schrével 2013; Philippe and Schrével 1982; Valigurová et al. 2013; Vegni Talluri and Dallai 1983). In the light of these studies, and our recent observations, the following could be taken into consideration: (i) typical micro pores could serve in the process of nutrient acquisition, and (ii) different structures observed to be in contact with the pellicle correspond to phases of mucous excretion. Previously, structures similar to teardrop granular inclusions (gi) were described in several species (Gregarina spp. and Urospora spp.). It is possible that at different stages of the life cycle or during the excretion process, these structures could look like vesicles or ducts in transverse sections of the cells (Diakin et al. 2016; Valigurová et al. 2013). However, it is important to mention that we did not observe any droplets of mucus on the surface of the cell; nonetheless, mucus-like substances between the laterally fused folds were detected.

Lateral fusions of epicystic folds are not unique to G. yurii. In Porospora portunidarum, for example, some folds are fused as well; however, it could be caused by the presence of bacteria disposed between the folds (Desportes et al. 1977). This phenomenon was also reported in the septate gregarine Leidyana tinei (Leidyaniidae) (Vivier et al. 1970); in which 2–3 folds were fussed (similar to G. yurii). Similar fusion has been reported in Monocystis agilis and M. herculea, (Monocystidae), aseptate eugregarines found in the seminal vesicles of Oligochaetes. In the case of M. herculea, 2–7 folds are fused at their tips; in M. agilis folds are fused at their tips, and 2–3 lateral points (Vinckier 1969; Vinckier and Vivier 1968). In all
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mentioned cases, the canals between the folds were formed. It was proposed that for monocystids the fusion of the folds serves to reduce the friction force during metabolic movement of the parasite between seminal cells (Frolov 1991). In the case of intestinal gregarines, the functionality of the fused folds remains unknown.

Pores localised on the surface of the gregarine attachment site were described in several species: C. cf. communis, Gregarina cuneata and G. yurii (Rueckert et al. 2011; Simdyanov et al. 2015; Valigurová 2012; present study). In all cases, these pores exhibit similar morphology; e.g. inc and the internal lamina are interrupted in this region, while no obvious interruption or invagination of plasma membrane is observable. Interestingly, only C. cf. communis and G. yurii has heterogeneous inclusions situated under the pellicle. Simdyanov et al. (2015) considered these inclusions to be microne-like structures, due to their elongated shape. In contrast, the inclusions from G. yurii were round. We assume that this could be condensed mitochondria. Another possible explanation is that these structures could represent vesicles varying in shape and containing mucus-like substances or adhesive material, which can be extruded outside the cell, facilitating the parasites adhesion to the host, as it was shown in C. cf. communis, G. cuneata, and some actinocerapid eugregarines (Cook et al. 2001; Simdyanov et al. 2015; Valigurová 2012).

The ball-like structure described in all representatives of the genus Ganymedes obviously corresponds to the vacuolar zone found in G. yurii (most likely zone 3) (Fig. 4E). The fine structure of anterior end of primit of G. yurii appears simple in comparison to the previously described gregarines from crustaceans, despite all of them being septate. As it was described in this study, the cytoplasm in the anterior end is subdivided into three zones differing in structure, which are not separated from each other by any fibrillar material (septum). The apical end in septate species is also subdivided into three zones; however, these zones are separated by a septum. The number of septae varies in different species: e.g. one septum separates the protomerite and deutomerite in Heliospora cf. longissima, Callynthrochlamys phronima; while two septae divide the cell of Cephaloidophora cf. communis into the epimerite, protomerite, and deutomerite (Desportes and Théodoridès 1969; Simdyanov et al. 2015).

The fine structure of the contact site between the primitive and satellite of G. yurii is comparable to the previously described syzygy contact zone in Callynthrochlamys phronima (Desportes and Théodoridès 1969). Both of these species possess collars formed by the posterior end of primitive at the periphery of the contact site: two collars are present in G. yurii, whereas C. phronima has a single collar. The surface of the junction zone in the primitive and satellite are covered with a three-membrane pellicle lacking epicytic folds; however, in G. yurii, radial ridges (on the primitive) and grooves (on the satellite) were documented. In contrast to this, the anterior end of satellite in G. themistos forms the collar, and only a small area lacks epicytic folds, whereas the rest of the pellicle covering the junction site is folded (Prokopowicz et al. 2010). In Cephaloidophora phrosinae, only one of the partners (primit) possesses modified epicytic folds in the contact zone (with bifurcated or flattened tops), while the anterior end of satellite has a smooth surface. It is assumed that the role of these modifications is to increase the surface of the contact area (Desportes et al. 1977).

Phylogenetic analyses of the novel sequence generated from G. yurii supported a close relationship with G. themistos, a previously described gregarine that was also isolated from an amphipod. Both of these sequences formed a basal clade within the large clade (Cephaloidophoroidea), comprising other eugregarines from crustaceans and some environmental sequences. As in previous studies, this clade was long-branching, relative to other clades of gregarines and apicomplexans (Prokopowicz et al. 2010; Rueckert et al. 2011). It is generally assumed that all gregarines so far described from crustaceans are phylogenetically closely related to each other, despite the wide range and global distribution of the hosts. Nonetheless, the phylogenetic analyses of 18s rDNA were unable to resolve the relationships along the backbone and among many of gregarines isolated from crustaceans hosts (Simdyanov et al. 2015).

In conclusion, the new species described in this study, G. yurii, shares common features with G. themistos and with other investigated gregarine species from crustaceans. But, there exists specific regions throughout the sequence that clearly distinguish each species among members of the genus Ganymedes, and gregarines from other crustaceans, in general. While all crustacean gregarines share two common features: (i) all of them are intestinal parasites of crustaceans, and (ii) phylogenetic analyses group these isolates in a single clade – Cephaloidophoroidea. Despite this, it is difficult to resolve the phylogenetic backbone and morphological trends within this group. To solve this problem, further investigations focusing on molecular phylogeny in connection with ultrastructural studies are needed.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Morphology and Phylogeny of Ganymedes yurii sp. n.


