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Molecular Characterization of Gregarines from Sand Flies (Diptera: Psychodidae) and Description of *Psychodiella* n. g. (Apicomplexa: Gregarinida)

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ABSTRACT. Sand fly and mosquito gregarines have been lumped for a long time in the single genus *Ascogregarina* and on the basis of their morphological characters and the lack of merogony been placed into the eugregarine family Lecudinidae. Phylogenetic analyses performed in this study clearly demonstrated paraphyly of the current genus *Ascogregarina* and revealed disparate phylogenetic positions of gregarines parasitizing mosquitoes and gregarines retrieved from sand flies. Therefore, we reclassified the genus *Ascogregarina* and created a new genus *Psychodiella* to accommodate gregarines from sand flies. The genus *Psychodiella* is distinguished from all other related gregarine genera by the characteristic localization of oocysts in accessory glands of female hosts, distinctive nucleotide sequences of the small subunit rDNA, and host specificity to flies belonging to the subfamily Phlebotominae. The genus comprises three described species: the type species for the new genus—*Psychodiella chagasi* (Adler and Mayrink 1961) n. comb., *Psychodiella mackiei* (Shortt and Swaminath 1927) n. comb., and *Psychodiella saraviae* (Ostrovska, Warburg, and Montoya-Lerma 1990) n. comb. Its creation is additionally supported by sequencing data from other gregarine species originating from the sand fly *Phlebotomus sergenti*. In the evolutionary context, both genera of gregarines from mosquitoes (*Ascogregarina*) and sand flies (*Psychodiella*) have a close relationship to neogregarines; the genera represent clades distinct from the other previously sequenced gregarines.

Key Words. Accessory glands, *Ascogregarina*, *Lutzomyia*, neogregarines, parasite, *Phlebotomus*, SSU rDNA phylogeny.

GREGARINES represent an extremely large, diverse, and highly abundant group of early branching apicomplexans that are widely distributed in marine as well as in terrestrial invertebrates. They parasitize annelids, mollusks, nemerteans, phoronids, echinoderms, siphunculids, crustaceans, hemichordates, appendicularians, and insects. Traditionally, three gregarine groups are recognized according to differences in habitat, host range, and morphological features of the trophozoites: archigregarines, eugregarines, and neogregarines (Vivier and Desportes 1990).

The genus *Ascogregarina* Ward, Levine, and Craig 1982 (syn. *Monocystis* von Stein 1848; *Lankesteria* Mingazzini 1891, and *Ascocystis* Grassé 1953) belongs to the order Eugregarinida Léger, 1899 (class Gregarinida Duffour 1828; phylum Apicomplexa Levine 1970). Out of 16 named species of the genus (Clopton 2000), three species parasitize sand flies and nine species parasitize mosquitoes (Levine 1977, 1985, 1988; Ostrovska et al. 1990). The total number of species is, however, questionable, because Clopton (2000) did not respect the definition of Ormières (1965) who restricted the genus only to parasites of Diptera. The terminology of mosquito and sand fly gregarines is complicated and the history of final designations is quite long. The mosquito gregarine and type species of the genus is *Ascogregarina culicis* (Ross 1898), originally named as *Gregarina culicis*. Ross (1895) described this species from the yellow fever mosquito *Aedes aegypti* (Linnaeus) as *Gregarina culicidis*, but this is considered as a *lapsus calami*. Wenyon (1911) reclassified the species as *Lankesteria culicis* and Grassé (1953) proposed the name *Ascocystis* for gregarines of insects that had formerly been assigned to the genus *Lankesteria*, and reclassified the species as *Ascocystis culicis*. The genus *Ascocystis* was later reviewed by Ormières (1965), who accepted *Ascocystis* Grassé, 1953 for parasites of Diptera and restricted *Lankesteria* to parasites of ascidians. However, Ward et al. (1982) established the name *Ascogregarina* instead of *Ascocystis* because the name was pre-occupied by a fossil crinoid echinoderm.

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Phlebotomine sand flies (Diptera: Psychodidae) of the genera *Phlebotomus* Rondani and Berté and *Lutzomyia* França are important vectors of human diseases, namely leishmaniasis, bartonellosis, and sand fly fever virus infections (Adler and Theodor 1957). Their larvae develop in soil rich in humus and microorganisms. Gregarines have been reported from more than 20 sand fly species (Ayala 1971; Levine 1977; Lisova 1962; Ostrovska et al. 1990; Tuzet and Rioux 1966; Warburg and Ostrovska 1991; Wu and Tesh 1989; Young and Lewis 1977), but only a few of them were denominated. The first sand fly gregarine was described as *Monocystis mackiei* Shortt and Swaminath 1927 from *Phlebotomus argentipes* (Annandale and Brunetti) in India. A few years later, presumably the same gregarine species was found in *Phlebotomus papatasi* (Scopoli) in Italy and renamed by Missiroli (1929, 1932) as *Lankesteria phlebotomi mackiei*. Ormières (1965) and 1 year later, Tuzet and Rioux (1966) reclassified the species as *Ascocystis mackiei*. However, the current name should be *Ascogregarina mackiei*, according to Ward et al. (1982).

The well-known sand fly gregarine described as *Monocystis chagasi* Adler & Mayrink, 1961 was found first in the hemocoel and accessory gland of *Lutzomyia longipalpis* (Lutz and Neiva) in Brazil and later in four other Neotropical sand fly species (Brazil and Ryan 1984; Coelho and Falcao 1964; Lewis, Lainson, and Shaw 1970; Scorza and Carnevali 1981). Tuzet and Rioux (1966) reclassified the species as *Ascocystis chagasi* and according to Ward et al. (1982) the current name should be *Ascogregarina chagasi*. The third ascogregarine species from sand flies was described by Ostrovska et al. (1990) as *Ascogregarina saraviae* Ostrovska, Warburg, & Montoya-Lerma, 1990 from *Lutzomyia lichyi* (Floch and Abbonenct).

Ascogregarina chagasi and other members of the genus were placed among the eugregarines (Apicomplexa: Conoidasida: Gregarinida: Eugregarinida: Aseptatorina: Lecudinidae) exclusively on the basis of morphological features and part of their developmental biology in the host. Their phylogenetic position has not been analyzed yet, despite Roychoudhury et al. (2007) having published sequences of four mosquito ascogregarines, including the type species *A. culicis*. DNA sequences generated by the present work enable phylogenetic analysis of the sand fly gregarines and provide more depth to our understanding of relatedness among gregarine groups.

MATERIALS AND METHODS

Sand flies. Sand fly colonies were maintained independently in the Czech Republic and in the United States. The colonies of *L. longipalpis* originated from Jacobina village, Bahia, Brazil (11°11′S, 40°32′W). The colonies of *Phlebotomus sergenti* originated from SanliUrfa city, Turkey (37°11′N, 38°48′E). Standard maintenance of colonies was described by Benkova and Volf (2007) for the Czech colonies and by Modi and Tesh (1983) for the colonies that were maintained at the Walter Reed Army Institute of Research, Silver Spring, MD.

Gregarine isolation and identification. All the molecular work was performed independently and in parallel in the Czech Republic and the United States. Two to 5-day-old adult flies of both sexes from colonies of *L. longipalpis* and *P. sergenti* were washed by 1.5% (v/v) Triton X-100 to remove any microorganisms and body hairs from the surface and by distilled water and phosphate-buffered saline (PBS). Approximately 50 gametocysts of each gregarine species were dissected under the stereo microscope (SZH-ILLD, Olympus Optical Co. Ltd., Tokyo, Japan) in NET-50 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 5 mM EDTA) and stored in −70 °C for DNA extraction. To confirm that gregarines found in both our colonies of *L. longipalpis* are of the species *Psychodiella* (formerly *Ascogregarina*) *chagasi*, adult sand flies of both sexes were dissected in PBS under the stereo microscope connected to a digital camera (DP-70, Olympus) for photo documentation of native preparations. Morphological characters of gregarine gamonts, gametocysts, and oocysts were evaluated using light microscope (BX-50, Olympus Optical Co. Ltd.).

DNA extraction, polymerase chain reaction (PCR) amplification, and cloning. Extraction of the total DNA from the pool of gregarine parasites was performed using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. The small subunit (SSU) rRNA genes were amplified as a single fragment using universal eukaryotic primers (Medlin et al. 1988). The PCR reactions were performed in 25 µl total volumes of reaction mix (Combi PPP Master Mix, Top-Bio, Prague, Czech Republic; Promega, Fitchburg, WI) using the following conditions: initial denaturation at 95 °C for 5 min followed by 30 amplification cycles (95 °C for 60 s, 55 °C for 90 s, and 72 °C for 90 s) and 72 °C for 7 min.

The PCR products corresponding to the expected size were gel isolated (Gel extraction kit, Qiagen, Valencia) and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD) using the manufacturer's protocol. Three clones from each gregarine species were sequenced with Terminator Ready Reaction Mix (Promega, Fitchburg, WI) using the vector primers and four internal primers oriented in both directions. The sequencing reaction was carried out on an automated DNA sequencer (310 Genetic Analyzer; ABI Prism, Foster City, CA) using the BigDye 3.1 kit (Applied Biosystems, Foster City, CA). Gregarine sequences were deposited in GenBank under the following accession numbers: FJ865354 (*P. chagasi* n. comb.) and FJ865355 (*Psychodiella* sp. from *P. sergenti*).

Phylogenetic analysis. The data set containing 38 nominal SSU rRNA gene sequences was used to establish the phylogenetic position of both newly sequenced sand fly gregarines (Table 1). The DNA sequences were compared with those in the GenBank database using the BLAST algorithm. All sequences of the SSU rRNA genes of gregarines available in public databases except incomplete sequences were used in our analysis. Appropriate sequences were aligned using the program CLUSTAL X 1.81. Alignment was manually edited using the program BioEdit 5.0.9.; gaps, as well as ambiguously aligned regions, were omitted from further analysis. The alignment is available from the corresponding author upon request. Phylogenetic analysis was performed using maximum parsimony (MP; PAUP *4.0b10; Swofford 2002) by 10 replicates of heuristic search, maximum likelihood (ML; PhyML; Guindon and Gascuel 2003), and Bayesian method (MrBayes; Huelsenbeck and Ronquist 2001). The MP bootstrap analyses were performed with 1,000 replicates. The ML trees were constructed using the GTR model for nucleotide substitutions with γ-distribution in 8+1 categories. The models of

Table 1. Origin of SSU rRNA gene sequences analyzed.

Species	Order[a]	Accession number	Host species
Selenidium serpulae	A	DQ683562	*Serpula vermicularis*
Selenidium terebellae	A	AY196709	*Thelepus* sp.
Selenidium vivax	A	AY196708	*Phascolosoma agassizii*
Ascogregarina armigerei	E	DQ462459	*Armigeres subalbatus*
Ascogregarina culicis (Thailand)	E	DQ462456	*Aedes aegypti*
Ascogregarina culicis (Viet Nam)	E	DQ462457	*Aedes aegypti*
Ascogregarina taiwanensis (Japan)	E	DQ462454	*Aedes albopictus*
Ascogregarina taiwanensis (India)	E	DQ462455	*Aedes albopictus*
Ascogregarina sp.	E	DQ462458	*Ochlerotatus japonicus*
Gregarina caledia[b]	E	L31799	
Gregarina chortiocetes[b]	E	L31841	
Gregarina niphandrodes	E	AF129882	*Tenebrio molitor*
Gregarina polymorpha	E	AF457129	*Tenebrio molitor*
Lankesteria abbotti	E	DQ093796	*Cnemidocarpa* sp.
Lankesteria chelyosomae	E	EU670240	*Chelyosoma columbianum*
Lankesteria cystodytae	E	EU670241	*Cystodytes lobatus*
Lecudina tuzetae	E	AF457128	*Nereis vexillosa*
Lecudina polymorpha type 1	E	AY196706	*Lumbrineris* sp.
Lecudina polymorpha type 2	E	AY196707	*Lumbrineris* sp.
Leidyana migrator	E	AF457130	*Gromphadorhina portentosa*
Lithocystis sp.	E	DQ093795	*Brisaster latifrons*
Monocystis agilis	E	AF457127	*Lumbricus terrestris*
Pseudomonocystis lepidiota[b]	E	L31843	
Psychodiella chagasi	**E**	**FJ865354**	***Lutzomyia longipalpis***
***Psychodiella* sp.**	**E**	**FJ865355**	***Phlebotomus sergenti***
Pterospora floridiensis	E	DQ093794	*Axiothella mucosa*
Pterospora schizosoma	E	DQ093793	*Axiothella rubrocincta*
Mattesia geminata	N	AY334568	*Solenopsis geminata*
Mattesia sp.	N	AY334569	*Solenopsis invicta*
Ophriocystis elektroscirrha	N	AF129883	*Danaus plexippus*
Syncystis mirabilis	N	DQ176427	*Nepa cinerea*
Cryptosporidium baileyi		L19068	birds
Cryptosporidium muris		L19069	rodents
Cryptosporidium parvum		AF112569	primates
Apicomplexan pathogen		AY490099	*Acarus siro*
Environmental sample		AY179988	water and sediments
Environmental sample		AY821921	water and sediments
Environmental sample		EF100358	water and sediments

[a]A, Archigregarinida; E, Eugregarinida; N, Neogregarinida.

[b]These three taxa have been included as names of environmental samples, they do not represent named species. The authors of those sequences did not provide any hosts.

New sequences reported in this work are in bold.

nucleotide substitution for maximum likelihood was chosen by hierarchical nested likelihood ratio tests implemented in Modeltest 3.06 and bootstrap analysis was computed in 1,000 replicates using the same model with Γ-distribution in four categories and all parameters (the proportion of invariant sites, γ shape parameter, TS/TV ratio for purines and pyrimidines) estimated from the data set. The Bayesian analysis was performed using MrBayes 3.1.2. Base frequencies, rates for six different types of substitution, proportion of invariant sites, and shape parameter of the γ correction for the rate heterogeneity with four discrete categories were allowed to vary. The covarion model was used to allow rate heterogeneity along the tree. The number of generations of Markov chain Monte Carlo was 5×10^6 and the trees were sampled every 100th generation. The first 12,500 trees were discarded as burn-in.

RESULTS

Features of SSU rDNA gene sequences. The identical SSU rRNA gene sequences of *P. chagasi* n. comb. from the colony of *L. longipalpis* (Jacobina village, Brazil) and the identical sequences of *Psychodiella* sp. from the colony of *P. sergenti* (SanliUrfa City, Turkey), were independently obtained from the USA and from the Czech Republic, respectively. The full length of the SSU rRNA gene sequences deposited in GenBank is 1,749 base pairs for *P. chagasi* (under the accession number FJ865354) and 1,752 base pairs for *Psychodiella* sp. from *P. sergenti*, respectively.

Phylogenetic analysis. Comparison of available gregarine sequences in GenBank revealed that the sequences of *P. chagasi* n. comb. and the most closely related *Psychodiella* sp. from *P. sergenti* form well supported clade (Fig. 1). Sequence divergence between the New World *P. chagasi* from *L. longipalpis* and the Old World *Psychodiella* sp. from *P. sergenti* is about 2% (35 changes). In our analysis, all tree topologies inferred using MP, ML, and Bayesian method were basically congruent. Cryptosporids were used as an outgroup. Within gregarine lineage, *Psychodiella* spp. and *Ascogregarina* spp. formed monophyletic clades supported by high bootstrap values (MP, 100%; ML, 100%; BA, 1.00). Phylogenetic analyses clearly demonstrate disparate position of gregarines parasitizing mosquitoes (genus *Ascogregarina*) and gregarines from sand flies (genus *Psychodiella*).

Morphological features of *Psychodiella chagasi* parasitizing *Lutzomyia longipalpis*. All life cycle stages of the gregarine from *L. longipalpis* were clearly identified as or conformed to the species *P.* (formerly *Ascogregarina*) *chagasi* originally described by Adler and Mayrink (1961). The life cycle of the gregarine in adult sand flies is identical to this of *P. chagasi* (Adler and Mayrink 1961; Warburg and Ostrovska 1991); syzigies, gametocysts, and oocysts were found in the body cavity of both female and male adults while in females gametocysts were attached to accessory glands (Fig. 2). Ocysts are released from gametocysts into the lumen of the glands and when laying eggs, the content of accessory glands including oocysts are excreted on the eggshells providing transovarial transmission.

DISCUSSION

Molecular phylogeny of *Ascogregarina* and *Psychodiella* n. g. as inferred from SSU rDNA. Sand fly and mosquito gregarines have been considered for a long time as a single genus *Ascogregarina*. Up to now, nine species were described from mosquitoes and three from sand flies. It should be mentioned that all available gregarine sequences of the fly-infecting species are from nematoceran flies, a relatively small group within the Diptera. Even though sand fly and mosquito gregarines have been considered as eugregarines for a long time, phylogenetic analyses revealed that the SSU sequences of both genera (*Ascogregarina* and *Psychodiella*) do not show similarity to the Eugregarinida but to the Neogregarinida. Monophyly of both genera in our trees is supported by 100% bootstrap by all methods used, and the genera seem to represent two separate and dissimilar clusters within the gregarines.

The phylogenetic analyses of all available sequences of gregarines correspond with the findings of other authors (Leander 2007, 2008; Leander, Clopton, and Keeling 2003a; Leander, Harper, and Keeling 2003b; Leander et al. 2006; Rueckert and Leander 2008, 2009). The genera *Selenidium* and *Lecudina* form paraphyletic groups and monocystids (e.g. *Monocystis* that infect terrestrial annelids), traditionally considered to be aseptate eugregarines, tend to be included in the group of neogregarines (e.g. *Syncystis* and *Mattesia*). The monophyly and composition of the order Eugregarinida are uncertain, and this is especially because the SSU rDNA sequences of eugregarines tend to be highly divergent, forming long branches in our molecular phylogenetic analyses.

Gregarines are important from an evolutionary perspective because of their suspected early diverging position within the Apicomplexa. Their molecular phylogenetic data have added additional complexity (and uncertainty) to the deepest relationships among apicomplexans. An internal topology of the apicomplexans is just beginning to emerge from comparisons of morphological characteristics and gene sequences (e.g. Beck et al. 2008; Ellis, Morrison, and Jeffries 1998; Kuo, Wares, and Kissinger 2008; Šlapeta et al. 2003). However, most of this work has focused on representatives from three of the four major groups: coccidians, haemosporidians, and piroplasms. Most studies of the phylogenetic relationships of gregarines are based on a relatively restricted data set of SSU rDNA sequences (Carreno, Martin, and Barta 1999; Leander 2007, 2008; Leander et al. 2003a,b, 2006; Roychoudhury et al. 2007; Rueckert and Leander 2008; Valles and Pereira 2003). With the advancement of DNA technology as more genes or whole genomes are sequenced and more data become available, there will be a need in the future for revisiting the systematics of gregarines, a neglected but extremely numerous branch of apicomplexan parasites.

As determined from the SSU rRNA gene sequence-based analyses, the genus *Psychodiella* n. g. is a member of the class Gregarinida Dufour, 1828. However, it cannot be associated with any valid genus. The morphological appearance, overall shape and cell size of species belonging to this genus resembles that of the genus *Ascogregarina*, although in adult sand flies gregarines are not localized in Malpighian tubules like gregarines from mosquitoes. Moreover, the genus *Psychodiella* is distinguished from all other related gregarine genera in having characteristic localization of oocysts in the accessory glands of the female host, its distinctive nucleotide sequences of SSU rDNA (FJ865354 and FJ865355), and its host specificity to phlebotomine sand flies. The phylogenetic analyses indicate that *Psychodiella* and *Ascogregarina* evolved independently of each other.

In conclusion, sequence data do not justify the inclusion of sand fly gregarines in the genus *Ascogregarina* and therefore we propose in accordance with the rules of the zoological nomenclature ICZN to separate sand fly gregarines into a newly erected genus *Psychodiella* n. g. In an evolutionary framework, both genera of gregarines from mosquitoes (*Ascogregarina*) and sand flies (*Psychodiella*) have close relationship to neogregarines, but represent a distinct clade from other previously sequenced gregarines. Studies on gregarines isolated from other sand fly species are underway (Lantová et al., unpubl. data) in order to examine if they are morphologically and genetically related to *P. chagasi*.

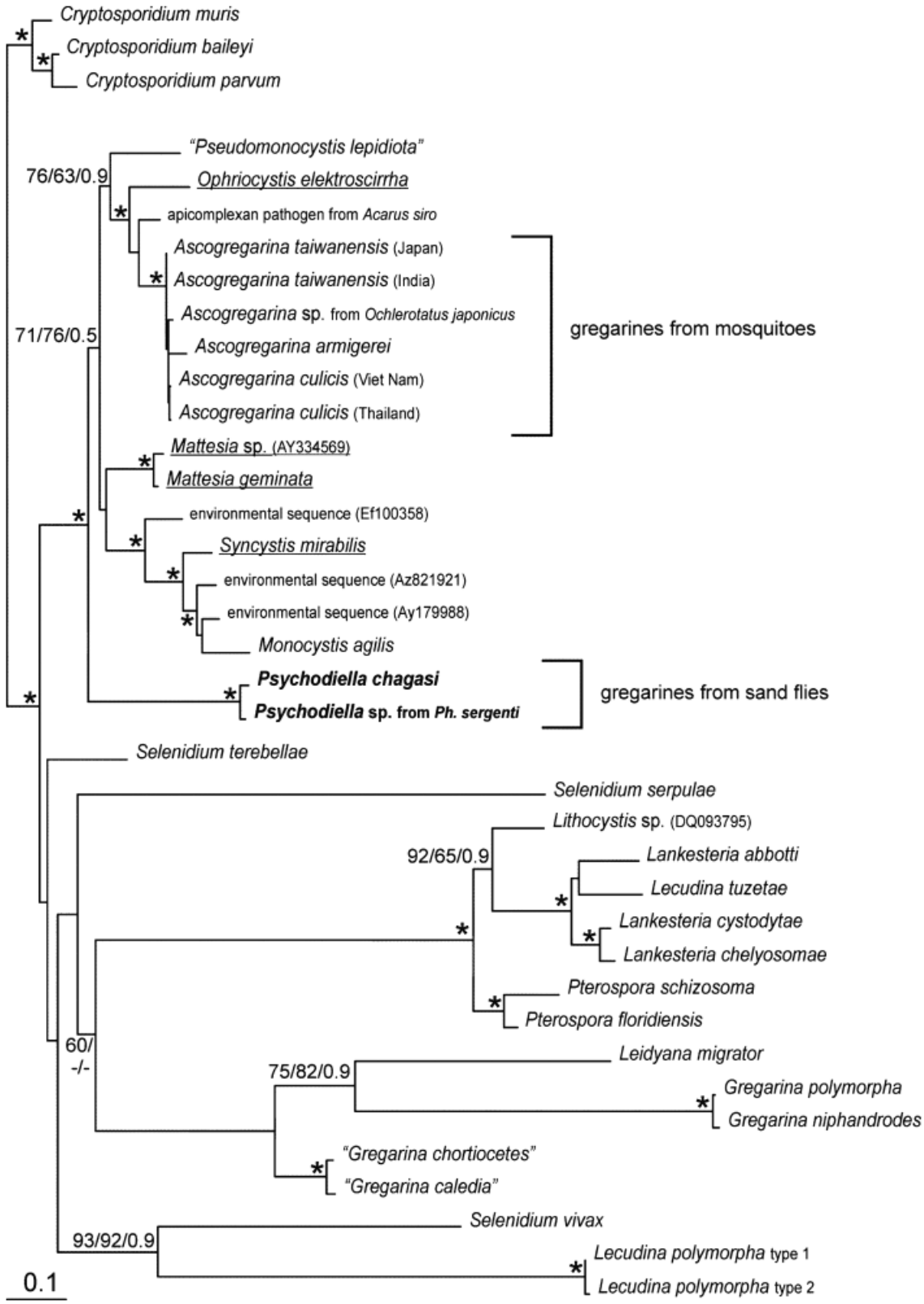

Fig. **1.** Maximum likelihood phylogenetic tree as inferred from small subunit rRNA gene sequences. The figure shows the topology for 38 taxa obtained by maximum likelihood using the GTR model for nucleotide substitutions with Γ-distribution in 8+1 categories as implemented in PhyML. Bootstrap values from maximum likelihood (100 replicates), maximum parsimony (1,000 replicates), and Bayesian posterior probabilities (number of generations was 5×10^6) are shown above branches, respectively. Asterisks (*) at the nodes denote Bayesian posterior probabilities and bootstrap percentages of 95% or higher. Dashes (-) indicate bootstrap support below 50 or posterior probability below 0.5 or different topology. The sequences of the species derived from this study are marked in bold. Gregarine species of the order Neogregarinida are underlined. Bars represent 0.1 substitutions per site. *Cryptosporidium* spp. served as an outgroup.

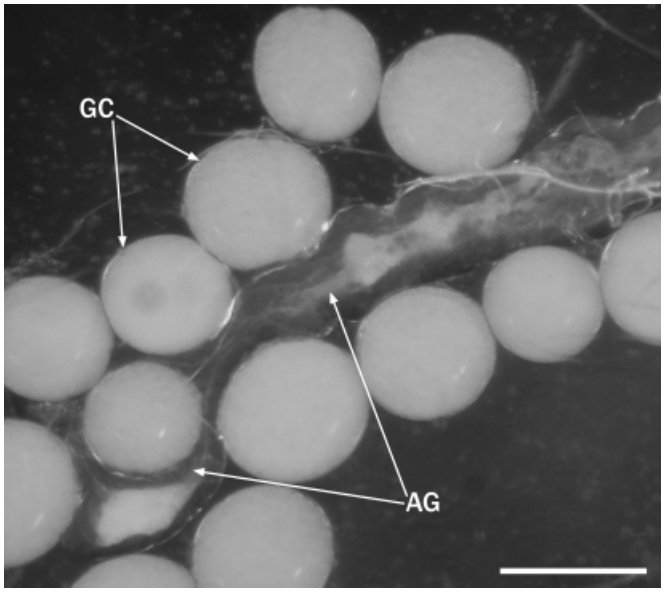

Fig. **2.** Gametocysts (GC) of *Psychodiella chagasi* n. comb. attached to accessory glands (AG) of an adult female of the phlebotomine sand fly *Lutzomyia longipalpis*. Native preparation. Scale bar = 100 μm.

TAXONOMIC SUMMARY

Phylum Apicomplexa
Order Eugregarinida
Suborder Aseptatorina

***Psychodiella* n. g. Votýpka, Lantová, and Volf**

Description. Monoxenous parasitic gregarine in Diptera. Gamonts oval, circular, or pear-shaped, aseptate, mucron not always apparent; gametocysts spherical or broad oval, in adults in the body cavity, in females usually attached to accessory glands; oocysts ellipsoidal or spindle shaped, often with a plug at each end, injected into accessory glands of female host.

Type species. *Monocystis chagasi* Adler & Mayrink 1961

Etymology. *Psychodiella*. The genus name has been derived from the name of the host family Psychodidae, the name is of feminine gender.

Remarks. The genus *Psychodiella* encompasses the following three species: *Psychodiella mackiei* (Shortt and Swaminath 1927) n. comb., *P. chagasi* (Adler and Mayrink 1961) n. comb., and *Psychodiella saraviae* (Ostrovska, Warburg, & Montoya-Lerma, 1990) n. comb.

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