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The life cycle and host specificity of *Psychodiella sergenti* n. sp. and *Ps. tobbi* n. sp. (Protozoa: Apicomplexa) in sand flies *Phlebotomus sergenti* and *Ph. tobbi* (Diptera: Psychodidae)

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ABSTRACT

Two new gregarines in the recently erected genus *Psychodiella* (formerly *Ascogregarina*), *Psychodiella sergenti* n. sp. and *Psychodiella tobbi* n. sp., are described based on morphology and life cycle observations conducted on larvae and adults of their natural hosts, the sand flies *Phlebotomus sergenti* and *Phlebotomus tobbi*, respectively. The phylogenetic analyses inferred from small subunit ribosomal DNA (SSU rDNA) sequences indicate the monophyly of newly described species with *Psychodiella chagasi*. *Ps. sergenti* n. sp. and *Ps. tobbi* n. sp. significantly differ from each other in the life cycle and in the size of life stages. The sexual development of *Ps. sergenti* in sp. (syzygy, formation of gametocysts and oocysts) takes place exclusively in blood-fed *Ph. sergenti* females, while the sexual development of *Ps. tobbi* n. sp. takes place also in males and unfed females of *Ph. tobbi*. The susceptibility of *Phlebotomus perniciosus*, *Phlebotomus papatasi*, *Ph. sergenti*, *Ph. tobbi*, and *Phlebotomus arabicus* to both gregarines was examined by exposing 1st instar larvae to parasite oocysts. High host specificity was observed, as both gregarines are considered as serious pathogens in laboratory-reared colonies of Old World sand flies.

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1. Introduction

The gregarine parasites have monoxenous life cycles and inhabit the intestines and other organs of invertebrates, particularly insects (Perkins et al., 2000). They are widespread in numerous hosts, but are currently not given the attention they deserve. Therefore, most gregarine species remain unknown and undescribed. The systematic status of gregarines has not been resolved and would greatly benefit from the addition of DNA sequences as molecular characters (Leander, 2008). Molecular phylogenetic data demonstrate that gregarines are more divergent than previously assumed and new lineages have emerged from older taxa (Leander, 2008; Votýpka et al., 2009).

Nematoceran Diptera are usually considered as rare hosts of gregarines and only 12 named species of two relatively morphologically uniform genera inhabit mosquitoes (*Ascogregarina*) and sand flies (*Psychodiella*) (Warburg and Ostrovska, 1991; Chen,

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1999; Perkins et al., 2000; Votýpka et al., 2009). The genus Psychodiella encompasses three named species of the aseptate gregarines (order Eugregarinorida according to Perkins et al. (2000)) that parasitize sand flies. Its type species, Ps. chagasi, was described by Adler and Mayrink (1961) as Monocystis chagasi in the hemocoel and accessory glands of the New World sand fly Lutzomyia longipalpis in Brazil. All life stages, including gametocysts and oocysts, occur in larvae and both adult sexes. Larvae become infected by feeding on oocysts either attached to the chorion of eggs or released into larval habitats following the death and decay of infected adults. Sporozoites are released from the oocysts after ingestion by sand fly larvae, attach to the gut epithelial cells, and develop into trophozoites (Adler and Mayrink, 1961; Coelho and Falcao, 1964; Wu and Tesh, 1989). Natural infections by this gregarine species have also been recorded in four other Neotropical sand fly species: Lutzomyia sallesi, Lutzomyia flaviscutellata, Lutzomyia townsendi, and Lutzomyia evandroi (see Wu and Tesh, 1989; Ostrovska et al., 1990). The second Neotropical species, Psychodiella saraviae, was described by Ostrovska et al. (1990) as Ascogregarina saraviae from blood-fed females of Lutzomyia lichyi with gametocysts attached to accessory glands and oocysts in the lumen.

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The only Old World species, *Psychodiella mackiei*, was described as *Monocystis mackiei* by Shortt and Swaminath (1927) from *Phlebotomus argentipes* in India and 2 years later was isolated from *Ph. papatasi* in Italy (Missiroli, 1929, 1932).

Contradictory results have come from studies focused on host specificity of gregarines in nematoceran hosts. Levine (1977) suggested a broad host range of Ps. chagasi among the New World sand fly species, while Wu and Tesh (1989) demonstrated strict host specificity of this species. Low host specificity was suspected for the sand fly gregarine Ps. mackiei, as it was described from Indian Ph. argentipes (Shortt and Swaminath, 1927) and Italian Ph. papatasi (Missiroli, 1929). Garcia et al. (1994) have shown that Ascogregarina species are not host-specific parasites in mosquitoes, as oocysts from Aedes albopictus were infectious to both Aedes aegypti and Ochlerotatus taeniorhynchus larvae. Similar results were obtained by Jacques and Beier (1982) infecting various Aedes species with Ascogregaring lanvuensis (Lien and Levine, 1980). On the other hand, a high degree of host specificity of these mosquito gregarines was demonstrated in cross-infection studies by Lien and Levine (1980) and Reyes-Villanueva et al. (2003).

Although most gregarines are often considered as non-pathogenic to their natural hosts (Henry, 1981), their impact on infected insects is not always clear (Clopton, 1995), notably when infection levels within a population are high as sometimes happens in reared colonies (e.g., Klingenberg et al., 1997). In these situations, the overall number of sand flies produced drops (personal observations, Rowton and Lawyer). In mosquitoes, however, some gregarine species are clearly pathogenic (Sulaiman, 1992; Comiskey et al., 1999) and could potentially serve as disease agents for biological control (Perkins et al., 2000). In sand flies, negative impact on adult longevity was described for *Ps. chagasi* (Wu and Tesh, 1989). This species is known as common pathogen in laboratory-reared colonies of *L. longipalpis* (Dougherty and Ward, 1991).

It is notable that descriptions of sand fly or mosquito gregarines lack a molecular phylogenetic approach and all descriptions of *Psychodiella* species from sand flies lack cross-infection studies. The present work describes not only the life cycle and morphology of the two newly described gregarine species from *Ph. sergenti* and *Ph. tobbi*, but also reveals their phylogenetic position and their ability to infect other sand fly species in cross-infections.

2. Materials and methods

2.1. Parasites

Gregarines were obtained from laboratory-reared sand fly colonies maintained using the methods of Benkova and Volf (2007). However, these colonies were established from naturally infected sand fly females originated from different places. Gregarines of the species *Ps. chagasi* were obtained from *L. longipalpis* collected in Jacobina, Bahia, Brazil, *Ps. sergenti* n. sp. from *Ph. sergenti* collected in Sanli Urfa, South-East Anatolia, Turkey, and *Ps. tobbi* n. sp. from *Ph. tobbi* collected in Tepecikoren, near Adana city, South Anatolia, Turkey. Prevalence of gregarines in wild-caught females was determined based on dissection of sand flies in the frame of leishmania-detection studies.

2.2. Light microscopy

Up to 30 specimens each of: 4th instar larvae, 1–10 day-old males, 1–7 day-old unfed females (no previous blood meal), and females 3–7 days after a blood meal were dissected in phosphatebuffered saline (PBS) under a stereomicroscope (SZH-ILLD, Olympus). The shape and the size of gamonts, gametocysts, and oocysts of gregarines from adult sand flies were measured under an optical microscope (SBX-50, Olympus). Light micrographs were produced with a DP-70 digital camera (Olympus) and measurements were processed with QuickPHOTO MICRO 2.2 software (Olympus). The statistical evaluation and difference of measurements among gregarine stages from the three sand fly species studied was determined using Statistica 6.0 (StatSoft).

2.3. Experimental infections

Development of both newly described gregarine species was studied in five laboratory-reared sand fly species (see Table 1). Oocysts were obtained by homogenization of 25–30 adults in 500 µl of PBS. In the case of Ps. sergenti, blood-fed Ph. sergenti females after oviposition were used, whereas in the case of Ps. tobbi, oocysts were acquired from 4 to 7 day-old Ph. tobbi males. The homogenate was filtered through gauze and centrifuged (1700g) for 5 min, the supernatant was discarded, and the pellet was re-dissolved in 200 µl of water. The oocysts were counted using a Bürker counting chamber. For each species tested, 10-15 non-infected gravid sand fly females were placed into a rearing pot and allowed to oviposit; the number of eggs was counted under a stereomicroscope and the amount of gregarine oocysts corresponding to an infectious dose of 50 oocysts per egg were added to the food given to 1st instar larvae. Fourth-instar larvae, emerged adults, and gravid females after a blood meal were examined for evidence of gregarine infection as described above. Two different forms of 4th instar larvae were distinguished: actively feeding with gut filled with larval diet (further referred as "before defecation") and those ready to pupate with a "milky" gut, because of defecated midgut content (further referred as "after defecation").

2.4. DNA extraction, PCR amplification, sequencing, and phylogenetic analysis of SSU rDNA

DNA was isolated from *Ps. tobbi* n. sp. mature gametocysts dissected from *Ph. tobbi* adults. The procedures of DNA extraction, PCR

Table 1

Design of experimental infections of larvae of five sand fly species by two newly described gregarines.

Gregarine species	Natural sand fly host	Experimentally infected sand fly host
Ps. sergenti n. sp.	Ph. sergenti ^a (Paraphlebotomus)	Ph. sergenti (Paraphlebotomus) ^b Ph. papatasi (Phlebotomus) Ph. tobbi (Larroussius) ^b Ph. arabicus (Adlerius)
Ps. tobbi n. sp.	Ph. tobbi ^a (Larroussius)	Ph. sergenti (Paraphlebotomus) ^b Ph. perniciosus (Larroussius)

^a Infected colony.

^b Non-infected colony.

amplification, and sequencing of SSU rDNA were described by Votýpka et al. (2009). The primers used for sequencing were: the vector primers M13 (F) 5'-GTAAAACCACGGCCAG-3' and M13 (R) 5'-CAGGAAACAGCTATGAC-3' and internal primers oriented in both directions 5'-AAGACGATCAGATACCG-3', 5'-TCGATTCCGGAGAGG-GA-3', 5'-CGTCAATTCCTTTAAG-3', 5'-GCTGGCACCAGACTTGC-3'. The obtained SSU sequence of Ps. tobbi was deposited in GenBank under accessional number GQ329865. SSU rDNA sequences of Ps. chagasi and Ps. sergenti (marked as Psychodiella sp. from Ph. sergenti) originated from our previous study (Votýpka et al., 2009) and are deposited in GenBank under accessional numbers FJ865354 and FJ865355, respectively. Data set containing all gregarine sequences of small subunit ribosomal DNAs accessible at the time of the study was used to establish the phylogenetic position of both newly described species of sand fly gregarines. Phylogenetic analvsis was performed following the same procedure as described previously by Votýpka et al. (2009).

3. Results

3.1. Description of morphology and life cycles

Different life stages of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi* are shown in Fig. 1. Their size characteristics and comparison are given in Tables 2 and 3.

3.1.1. Psychodiella sergenti n. sp.

Gamonts (Fig. 1A and J) are round or oval, aseptate, with distinct nucleus and nucleolus. Cytoplasm contains brown granules.

In young adults or when the infection intensity was high (over 30 gamonts per adult), some of the gamonts were smaller or prolonged. Gamonts were found in all examined stages of their host (4th instar larvae, adult males, and both, unfed and blood-fed females). Gamonts were mostly located in the intestine of 4th



Fig. 1. Native preparations of different life cycle stages of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi*. Gamonts of *Ps. sergenti* (A), *Ps. tobbi* (B), and *Ps. chagasi* (C), scale bars = 50 μm; gametocysts of *Ps. sergenti* (D), *Ps. tobbi* (E), and *Ps. chagasi* (F), scale bars = 50 μm; oocysts of *Ps. sergenti* (G), *Ps. tobbi* (H), and *Ps. chagasi* (I) from the adult body cavity of appropriate sand fly species, scale bars = 10 μm. Gamonts of *Ps. sergenti* in the lumen of *Ph. sergenti* 4th instar larva after defecation (J), scale bar = 150 μm. Gamonts of *Ps. tobbi* outside the intestine of *Ph. tobbi* 4th instar larva after defecation (K), scale bar = 150 μm.

Table 2

Mean length of gamonts (T) and gametocysts (G) and length (O-L), width (O-W), and length/width ratio (O-LW) of oocysts (all in μ m) with basic statistical characteristics of the three different gregarines from adults of *Phlebotomus sergenti*, *Ph. tobbi*, and *Lutzomyia longipalpis*.

Stage/species	Ν	Mean	Median	SD	Min.	Max.
Т						
Ps. sergenti	408	114.6	112.8	21.5	59.0	188.0
Ps. tobbi	188	123.6	123.0	27.2	54.5	204.1
Ps. chagasi	133	80.6	84.0	21.9	25.5	168.0
G						
Ps. sergenti	86	128.2	131.5	17.3	84.6	169.2
Ps. tobbi	77	137.2	132.6	22.0	102.6	189.0
Ps. chagasi	58	105.0	103.4	14.7	84.0	150.0
O-L						
Ps. sergenti	231	9.6	9.6	0.3	8.7	10.3
Ps. tobbi	194	9.6	9.6	0.3	8.8	10.7
Ps. chagasi	113	12.7	12.7	0.3	12.0	13.3
0-W						
Ps. sergenti	231	6.7	6.7	0.2	6.2	7.1
Ps. tobbi	194	7.5	7.5	0.3	6.8	8.5
Ps. chagasi	113	8.3	8.3	0.3	7.3	8.9
O-LW						
Ps. sergenti	231	1.44	1.44	0.05	1.30	1.56
Ps. tobbi	194	1.28	1.28	0.04	1.19	1.40
Ps. chagasi	113	1.52	1.52	0.06	1.41	1.68

Table 3

Size comparison among different stages of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi.* Stages: T = gamont; G = gametocyst; O = oocyst. Measurements: L = length; W = width; LW = length/width ratio. Gregarines: PS = *Ps. sergenti* n. sp.; PT = *Ps. tobbi* n. sp.; PC = *Ps. chagasi.*

Stage/species	t-Value	D.f.	Р	Comparison
Т				
PS-PC	15.75	539	<0.01	PS > PC
PT-PC	15.12	319	< 0.01	PT > PC
PT-PS	4.37	594	<0.01	PT > PS
G				
PS-PC	8.36	142	< 0.01	PS > PC
PT-PC	9.63	133	< 0.01	PT > PC
PT-PS	2.93	161	<0.01	PT > PS
O-L				
PS-PC	99.67	342	< 0.01	PS < PC
PT-PC	84.25	305	< 0.01	PT < PC
PT-PS	-0.39	423	>0.05	$\text{PT}\approx\text{PS}$
0-W				
PS-PC	70.70	342	< 0.01	PS < PC
PT-PC	27.81	305	< 0.01	PT < PC
PT-PS	-38.42	423	< 0.01	PT > PS
O-LW				
PS-PC	15.09	342	< 0.01	PS < PC
PT-PC	44.40	305	< 0.01	PT < PC
PT-PS	37.21	423	<0.01	PT < PS

instar larvae and in the body cavity of adults. In one larva, gamonts were found in the body cavity and in two adults in the fat body. In larvae before defecation, gamonts (as well as gametocysts) were situated more often in the posterior part of the midgut, usually in the ectoperitrophic space. However, in larvae after defecation, gamonts were located within the full length of the midgut lumen (Fig. 1]).

Gametocysts (Fig. 1D) have a distinct wall and are usually round or oval. Young gametocysts are formed by two gamonts with visible nuclei. Older gametocysts are filled with granules that suggest sporogony.

Gametocysts were only found in the midgut of 4th instar larvae and in blood-fed females, where they were attached to the accessory glands or lying freely in the body cavity. No sexual development occurred in males or unfed females.

Oocysts (Fig. 1G) are broad spindle-shaped (hesperidiform) with flattened, rather indistinctive ends, containing eight sporozoites. The length/width ratio is 1.44 ± 0.05 (range: 1.30-1.56).

They were observed exclusively in blood-fed females. During dissection, some gametocysts ruptured, adopting a characteristic horseshoe-like shape and releasing oocysts into the body cavity. In several blood-fed females, oocysts were observed in the lumen of accessory glands.

3.1.2. Psychodiella tobbi n. sp

Gamonts (Fig. 1B and K) similar to Ps. sergenti gamonts.

Gamonts were found in the body cavity of all examined adult sand fly stages. In 4th instar larvae before defecation, gamonts were usually located in the ectoperitrophic space of the hindgut of younger individuals and/or the body cavity of older individuals. In larvae after defecation, they were usually found in the body cavity and occasionally in the intestine (Fig. 1K).

Gametocysts (Fig. 1E) are round or oval, brownish, with a distinct wall.

Gametocysts were located in the intestine, the ectoperitrophic space of the hindgut, or the body cavity of 4th instar larvae. In adults, they were found in the body cavity. Sexual development of gregarines appeared 1 day after emergence in females and 4 days after emergence in males and it was not induced by a blood meal in the females.

Oocysts (Fig. 1H) are broad spindle-shaped (hesperidiform) with distinctive "button-like" ends, containing eight sporozoites. The length/width ratio is 1.28 ± 0.04 (range: 1.19-1.40).

Oocysts with eight sporozoites were only found in the body cavity of adults and in the lumen of accessory glands of females. Both gametocysts and oocysts appeared earlier in females than in males.

3.2. Size differences among stages of Psychodiella species

The size and differences between stages of the three studied gregarine species (*Ps. sergenti, Ps. tobbi*, and *Ps. chagasi*) are presented in Tables 2 and 3, and Fig. 2.

Both Old World species, *Ps. sergenti* and *Ps. tobbi*, had significantly bigger gamonts (114.6/123.6 μ m > 80.6 μ m; *P* < 0.01) and gametocysts (128.2/137.2 μ m > 105.0 μ m; *P* < 0.01) and significantly shorter oocysts (9.6/9.6 μ m < 12.7 μ m; *P* < 0.01) than the New World species *Ps. chagasi. Ps. tobbi* had significantly bigger gamonts (123.6 > 114.6 μ m; *P* < 0.01) and gametocysts (137.2 > 128.2 μ m; *P* < 0.01) than *Ps. sergenti*. The difference in the length of oocysts between *Ps. tobbi* and *Ps. sergenti* was not significant (9.6 μ m and 9.6 μ m; *P* > 0.05).

All three gregarine species significantly (P < 0.01) varied in the width of oocysts from each other with *Ps. chagasi* having the widest ones (8.3 µm), *Ps. tobbi* with 7.5 µm wide oocysts and *Ps. sergenti* possessing the narrowest oocysts of 6.7 µm. In order to sufficiently distinguish between *Ps. sergenti* and *Ps. tobbi* oocysts, a length/width ratio was evaluated. *Ps. sergenti* length/width ratio was significantly bigger than the one of *Ps. tobbi* (1.44 > 1.28; P < 0.01). Graphical representation of individual oocyst measurements is shown in Fig. 2.

3.3. Prevalence in wild caught sand flies

The prevalence in wild caught *Ph. sergenti* was 15 gregarine infected females out of 96 and in wild caught *Ph. tobbi* was 21 out of 125 females.



Fig. 2. Characteristics of Psychodiella sergenti n. sp., Ps. tobbi n. sp., and Ps. chagasi oocysts: length (A); width (B); length/width ratio (C); frequency scatterplot of oocyst measurements (D).

Table 4

Presence of *Psychodiella sergenti* n. sp. stages in four experimentally infected sand fly species. L4 = 4th instar larvae; M = males; F = females; FB = blood-fed females; T = gamonts; G = gametocysts; O = oocysts; + = gregarine found; (+) = gregarine found in low number and in less than 10% of individuals; - = gregarine not found.

	Ph. sergenti			Ph. pa	Ph. papatasi			Ph. arabicus				Ph. tobbi				
	L4	М	F	FB	L4	М	F	FB	L4	М	F	FB	L4	М	F	FB
Т	+	+	+	+	+	+	(+)	(+)	_	-	-	_	+	+	+	+
G	+	_	_	+	+	_	-	(+)	_	_	_	_	_	_	_	_
0	_	-	-	+	_	-	_	-	-	_	-	-	-	-	-	(+)

3.4. Experimental infection of various sand fly species

Testing four sand fly species belonging to different subgenera, the gregarine *Ps. sergenti* was able to complete the life cycle and fully develop only in its natural host, *Ph. sergenti* (Table 4 and Fig. 3). No infection occurred in any of the stages of *Ph. arabicus*. In *Ph. papatasi*, gamonts were found in all life cycle stages, gametocysts were found in 4th instar larvae and blood-fed females, but oocysts were not found in any of the stages. *Ph. tobbi* was also fairly refractory to *Ps. sergenti* infection. No gametocysts were found in any of the studied *Ph. tobbi* stages and few oocysts were found only in the body cavity of one blood-fed female out of 76 dissected. The number of gamonts per individual of *Ph. tobbi* was usually one or two, unlike in its natural host *Ph. sergenti*, where the intensity of infection was about three to 20 gamonts per individual. Testing two sand fly species, *Ps. tobbi* was not able to mature and produce oocysts in *Ph. sergenti* (Table 5 and Fig. 4). No gregarine infection occurred in 4th instar larvae or unfed and bloodfed females. Out of 120 dissected *Ph. sergenti* males, only two carried a single gamont of *Ps. tobbi*. The life cycle of *Ps. tobbi* in *Ph. perniciosus* was similar to the one seen in *Ph. tobbi*; sexual development was not induced by blood meal of females and occurred also in males and unfed females. In *Ph. perniciosus*, oocysts were found in the lumen of accessory glands of only two females out of 176; gamonts and gametocysts were found in low numbers (1–4) in adults and 4th instar larvae.

3.5. Phylogenetic analysis

We sequenced 1752 base pairs of the SSU rRNA gene of the new *Psychodiella* species from *Ph. tobbi*. The final data set contained all



Fig. 3. Proportion of infected sand flies (*Phlebotomus sergenti*, *Ph. papatasi*, *Ph. tobbi*, and *Ph. arabicus*) with *Psychodiella sergenti* n. sp. Numbers above the columns represent the number of examined individuals.

Table 5

Presence of *Psychodiella tobbi* n. sp. stages in two experimentally infected sand fly species. L4 = 4th instar larvae; M = males; F = females; FB = blood-fed females; T = gamonts; G = gametocysts; O = oocysts; + = gregarine found; (+) = gregarine found in low number and in less than 10% of individuals; - = gregarine not found.

	Ph. se	rgenti			Ph. per			
	L4	М	F	FB	L4	М	F	FB
Т	_	(+)	-	_	+	+	+	+
G	_	_	_	_	(+)	_	(+)	_
0	-	-	-	-	-	-	(+)	(+)



Fig. 4. Proportion of infected sand flies (*Phlebotomus sergenti* and *Ph. perniciosus*) with *Psychodiella tobbi* n. sp. Numbers above the columns represent the number of examined individuals.

sequenced species of named gregarines including the *Ps. sergenti* sequence from the previous work of Votýpka et al. (2009). The tree was rooted using cryptosporids as an outgroup and all major branches were well supported (data not shown) and correspond with the phylogenetic tree recently published by Votýpka et al. (2009). The tree revealed the relationship of both newly described gregarines (*Ps. sergenti* and *Ps. tobbi*) and *Ps. chagasi* and the monophyly of a separate genus *Psychodiella* is supported by high bootstrap values for all methods used (MP, 100%; ML, 100%; BA, 1.00; Fig. 5). The Old World sand fly gregarines, *Ps. sergenti* and *Ps. tobbi*,

are closer to each other (genetic uncorrected p-distance was 1%, 17 nucleotide changes) than to the New World *Ps. chagasi* (2%, 35 and 30 changes, respectively).

4. Discussion

The life cycle of *Ps. sergenti* n. sp. differs markedly from *Ps. chagasi*. In adult *Ph. sergenti*, mature oocysts occur only in blood-fed females, unlike *Ps. chagasi*, whose sexual development occurs also in males and unfed females. Morphological differences as well as molecular phylogenetic analysis bring further evidence that *Ps. sergenti* n. sp. and *Ps. tobbi* n. sp. are different from *Ps. chagasi*. Broad-spindle oocysts with wider midsections of both newly described species differ from longer oocysts of *Ps. saraviae* that have narrower midsections and thicker walls (Ostrovska et al., 1990).

The only Old World sand fly gregarine species described previously, *Ps. mackiei*, was found in larvae, pupae, and adults of *Ph. argentipes* and *Ph. papatasi*, respectively (Shortt and Swaminath, 1927; Missiroli, 1929, 1932). It is the only sand fly gregarine where intracellular stages in the larval gut epithelium were observed. Previously reported observations that the sexual cycle of *Ps. mackiei* occurs in males and unfed females, that gametocysts attach to oviducts when oocysts are injected into the lumen, and the differences in the size of the gregarine stages provide strong evidence that *Ps. sergenti* and *Ps. tobbi* are well differentiated from *Ps. mackiei*.

Ps. sergenti varies from Ps. tobbi in the size of gamonts and gametocysts, but not in the length of oocysts. However, oocysts of these two gregarines clearly differ in the width and length/ width ratio, which is bigger for Ps. sergenti (1.44) compared to Ps. tobbi (1.28). The length/width ratio intersection of the two species is only 0.1 and using this feature, Ps. sergenti can be clearly distinguished from Ps. tobbi. The length/width ratio can then serve as an unambiguous species characteristic. Besides morphological and phylogenetic differences and strict host specificity, both newly described species Ps. sergenti and Ps. tobbi differ from each other in their life cycles. In adults, sexual development of Ps. sergenti occurs exclusively in females after a blood meal unlike Ps. tobbi, where development also takes place in males and unfed females. For Ps. sergenti, gamonts were found mostly in the intestinal lumen of larvae, while for Ps. tobbi, they were also found in the body cavity.

How gregarines get from the larval intestine to the body cavity of sand fly adults was discussed by Shortt and Swaminath (1927). The authors propose the hypothesis that gregarines passively enter into the body cavity of pupae during tissue reconstitution in the early stage of pupation. However, our results may suggest that gregarines actively leave the larval intestine since they are found in the hemocoel of older *Ph. tobbi* larvae before body reconstitution occurred. In *Ps. tobbi*, the "transfer" to the host body cavity occurs earlier in the larval development, while gregarines of *Ps. sergenti* are in the intestine of all larval stages and emerge later in the body cavity of pupae.

Not only the morphological and the life cycle differences, and the phylogenetic position of *Ps. tobbi* and *Ps. sergenti*, but also the inability of *Ps. tobbi* to produce gametocysts or oocysts in *Ph. sergenti* and very low susceptibility of *Ph. tobbi* to *Ps. sergenti* confirm that the two newly described gregarines are distinct species. The full development of *Ps. sergenti* and *Ps. tobbi* takes place only in their natural hosts. In preliminary studies on *Phlebotomus* (*Phlebotomus*) duboscqi, sand flies can become accidentally infected with *Ps. sergenti*, however in all cases the number of gregarines found in foreign hosts was substantially smaller than in their natural hosts. Figs. 3 and 4, showing the proportions of infected sand fly



Fig. 5. Part of a maximum likelihood (ML) phylogenetic tree inferred from SSU rRNA gene sequences. The figure shows the topology 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 ML, MP (1000 replicates), and Bayesian posterior probabilities (number of generations was 5×10^6) are shown above branches. Asterisks (*) at the nodes denote Bayesian posterior probabilities and bootstrap percentages of 95% or higher. Bar represents 0.1 substitutions per site.

stages, suggest that the bottleneck for gregarines in an artificial host is larval development and pupation, because the percentage of infected sand flies decreases rapidly in adults. The results of cross-infections are in agreement with findings of Wu and Tesh (1989), but contradict studies demonstrating broad host range of gregarines from nematoceran Diptera (Shortt and Swaminath, 1927; Missiroli, 1929; Levine, 1977; Garcia et al., 1994). The conspecificity of the gregarine *Ps. mackiei* found in *Phlebotomus* (*Euphlebotomus*) *argentipes* (Shortt and Swaminath, 1927) and *Ph.* (*Phlebotomus*) *papatasi* (Missiroli, 1929) is also questioned, since the two phlebotomine genera do not form a monophyletic clade (Aransay et al., 2000).

Due to the differences in the life cycle, morphology, and dimensions of gamonts, gametocysts and particularly oocysts, and the results of cross-infection studies and molecular phylogenetic analysis, we clearly demonstrated that gregarines from *Ph. tobbi* and *Ph. sergenti* are two new species of the genus *Psychodiella*.

5. Taxonomic summary

Phylum: Apicomplexa Levine, 1970. Order: Eugregarinorida Léger, 1900. Suborder: Aseptatorina Chakravarty, 1960. Genus: *Psychodiella* Votýpka, Lantová and Volf, 2009.

5.1. Psychodiella sergenti n. sp. Lantová, Volf and Votýpka

Type specimens: Ps. sergenti n. sp.

Type host: Ph. sergenti (Diptera: Psychodidae)

Type locality (origin of the sand fly colony): Turkey, South-East Anatolia, SanliUrfa (37°11′N, 38°48′E)

Site of infection: Midgut of 4th instar larvae and body cavity of adults. Oocysts in the lumen of accessory glands and in the body cavity, exclusively in blood-fed females.

Type material: Slides (No. PsSerF2d.1B1.2007/01) stained with PAS reaction followed by Ehrlich's Hematoxylin have been deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic. *Etymology*: The species name has been adopted from the species name of the host sand fly, *Ph. sergenti* named in honour of the doctors Edmond and Etienne Sergent.

Diagnosis in the native: Gamonts $(114.6 \pm 21.5 \,\mu\text{m})$ round or oval, aseptate, with distinct nucleus and nucleolus. Gametocysts $(128.2 \pm 17.3 \,\mu\text{m})$ round or oval, sometimes with original two gamonts, older gametocysts with "granules" suggesting sporogony. Oocysts (length: $9.6 \pm 0.3 \,\mu\text{m}$, $8.7-10.3 \,\mu\text{m}$; width: 6.7 ± 0.2 , $6.2-7.1 \,\mu\text{m}$) broad spindle-shaped (hesperidiform) with flattened, rather indistinctive ends, containing eight sporozoites. The length/width ratio is $1.44 \pm 0.05 (1.30-1.56)$.

- 5.2. Psychodiella tobbi n. sp. Lantová, Volf and Votýpka
 - Type specimens: Ps. tobbi n. sp.

Type host: Ph. tobbi (Diptera: Psychodidae)

Type locality (origin of the sand fly colony): Turkey, South Anatolia, Tepecikoren (37°36′N, 35°62′E)

Site of infection: Body cavity and intestine of 4th instar larvae, body cavity of adult sand flies. Oocysts in the body cavity and lumen of accessory glands of adult sand flies of both sexes.

Type material: Gregarine-infected *Ph. tobbi* males and females placed in a tube with AFA fixative (alcohol-formalin-acetic acid) (No. PsTobFM2d.2008/01) deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic.

Etymology: The species name has been adopted from the species name of the host sand fly, *Ph. tobbi* dedicated to Dr. Agha Khan Tobb.

Diagnosis in the native: Gamonts $(123.6 \pm 27.2 \mu m)$ round or oval, aseptate, with distinct nucleus and nucleolus. Gametocysts $(137.2 \pm 22 \mu m)$ round or oval, sometimes with original two gamonts, older gametocysts with granules suggesting sporogony. Oocysts (length: $9.6 \pm 0.3 \mu m$, $8.8-10.7 \mu m$; width: 7.5 ± 0.3 , $6.8-8.5 \mu m$) broad spindle-shaped (hesperidiform) with distinctive "button-like" ends, containing eight sporozoites. The length/width ratio is $1.28 \pm 0.04 (1.19-1.40)$.

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References

- Adler, S., Mayrink, W., 1961. A gregarine, Monocystis chagasi n. sp., of Phlebotomus longipalpis. Remarks on the accessory glands of P. longipalpis. Rev. Inst. Med. Trop. Sao Paulo 3, 230–238.
- Aransay, A.M., Scoulica, E., Tselentis, Y., Ready, P.D., 2000. Phylogenetic relationships of phlebotomine sandflies inferred from small subunit nuclear ribosomal DNA. Insect Mol. Biol. 9, 157–168.
- Benkova, I., Volf, P., 2007. Effect of temperature on metabolism of *Phlebotomus papatasi* (Diptera: Psychodidae). J. Med. Entomol. 44, 150–154.
- Chen, W.J., 1999. The life cycle of *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae). Parasitol. Today 15, 153–156.
- Clopton, R.E., 1995. Leidyana migrator n. sp. (Apicomplexa: Eugregarinida: Leidyanidae) from the Madagascar hissing cockroach, Gromphadorhina portentosa (Insecta: Blattodea). Invertebr. Biol. 114, 271–278.

- Coelho, M. de V., Falcao, A.L., 1964. Aspects of the life-cycle of Monocystis chagasi Adler and Mayrink, 1961, in *Phlebotomus longipalpis*. Rev. Bras. Biol. 24, 417– 421.
- Comiskey, N.M., Lowrie, R.C., Wesson, D.M., 1999. Effect of nutrient levels and Ascogregarina taiwanensis (Apicomplexa: Lecudinidae) infections on the vector competence of Aedes albopictus (Diptera: Culicidae) for Dirofilaria immitis (Filarioidea: Onchocercidae). J. Med. Entomol. 36, 55–61.
- Dougherty, M.J., Ward, R.D., 1991. Methods of reducing Ascogregarina chagasi parasitaemia in laboratory colonies of Lutzomyia longipalpis. Parassitologia 33 (Suppl. 1), 185–191.
- Garcia, J.J., Fukuda, T., Becnel, J.J., 1994. Seasonality, prevalence and pathogenicity of the gregarine Ascogregarina taiwanensis (Apicomplexa: Lecudinidae) in mosquitoes from Florida. J. Am. Mosq. Control Assoc. 10, 413–418.
- Henry, J.E., 1981. Natural and applied control of insects by protozoa. Annu. Rev. Entomol. 26, 49-73.
- Jacques, P.A., Beier, J.C., 1982. Experimental infections of Ascogregarina lanyuensis (Apicomplexa, Lecudinidae) in Aedes (Stegomyia) sp. mosquitoes. Mosq. News 42, 438–439.
- Klingenberg, C.P., Leigh, R.H.B., Keddie, B.A., Spence, J.R., 1997. Influence of gut parasites on growth performance in the water strider *Gerris buenoi* (Hemiptera: Gerridae). Ecography 20, 29–36.
- Leander, B.S., 2008. Marine gregarines: evolutionary prelude to the apicomplexan radiation? Trends Parasitol. 24, 60–67.
- Levine, N.D., 1977. Revision and checklist of the species (other than *Lecudina*) of the aseptate gregarine family Lecudinidae. J. Protozool. 24, 41–52.
- Lien, S.-M., Levine, N.D., 1980. Three new species of Ascocystis (Apicomplexa: Lecudinidae) from mosquitoes. J. Protozool. 27, 147–151.
- Missiroli, A., 1929. I protozoi parasiti del Phlebotomus. Ann. Ig. 39, 481-487.
- Missiroli, A., 1932. Sullo sviluppo di una gregarina del Phlebotomus. Ann. Ig. 42, 373-377.
- Ostrovska, K., Warburg, A., Montoya-Lerma, J., 1990. Ascogregarina saraviae n. sp. (Apicomplexa: Lecudinidae) in Lutzomyia lichyi (Diptera: Psychodidae). J. Protozool. 37, 69–70.
- Perkins, F.O., Barta, J.R., Clopton, R.E., Peirce, M.A., Upton, S.J., 2000. Phylum Apicomplexa (Levine, 1970). In: Lee, J.J., Leedale, G.F., Bradbury, P. (Eds.), The Illustrated Guide to the Protozoa. Allen Press, Inc., Lawrence Kansas, pp. 190– 369.
- Reyes-Villanueva, F., Becnel, J.J., Butler, J.F., 2003. Susceptibility of Aedes aegypti and Aedes albopictus larvae to Ascogregarina culicis and Ascogregarina taiwanensis (Apicomplexa: Lecudinidae) from Florida. J. Invertebr. Pathol. 84, 47–53.
- Shortt, H.E., Swaminath, C.S., 1927. Monocystis mackiei n. sp. parasitic in Phlebotomus argentipes. Ann. Brun. Indian J. Med. Res. 15, 539–553.
- Sulaiman, I., 1992. Infectivity and pathogenicity of Ascogregarina culicis (Eugregarinida: Lecudinidae) to Aedes aegypti (Diptera: Culicidae). J. Med. Entomol. 29, 1–4.
- Votýpka, J., Lantová, L., Ghosh, K., Braig, H., Volf, P., 2009. Molecular characterization of gregarines from sand flies (Diptera: Psychodidae) and description of *Psychodiella* n. Gen. (Apicomplexa: Gregarinida). J. Eukaryot. Microbiol. 56, 583–588.
- Warburg, A., Ostrovska, K., 1991. Host-parasite relationships of Ascogregarina chagasi (Eugregarinorida: Aseptatorina: Lecudinidae) in Lutzomyia longipalpis (Diptera: Psychodidae). Int. J. Parasitol. 21, 91–98.
- Wu, W.-K., Tesh, R.B., 1989. Experimental infection of Old and New World phlebotomine sandflies (Diptera: Psychodidae) with Ascogregarina chagasi (Eugregarinida: Lecudinidae). J. Med. Entomol. 26, 238–242.