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Life Cycle and Morphology of *Steinina ctenocephali* (Ross 1909) comb. nov. (Eugregarinorida: Actinocephalidae), a Gregarine of *Ctenocephalides felis* (Siphonaptera: Pulicidae) in Taiwan

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Mauricio E. Alarcón, Chin-Gi Huang, Yi-Shang Tsai, Wei-June Chen, Anil Kumar Dubey, and Wen-Jer Wu (2011) Life cycle and morphology of *Steinina ctenocephali* (Ross 1909) comb. nov. (Eugregarinorida: Actinocephalidae), a gregarine of *Ctenocephalides felis* (Siphonaptera: Pulicidae) in Taiwan. *Zoological Studies* **50**(6): 763-772. Gregarines are endoparasitizing protozoa found across terrestrial invertebrate taxa including several insect species of medical and public health importance such as the cat flea, *Ctenocephalides felis* (Bouché). The finding of a gregarine infection in the cat flea may shed light on further pest control regimes of this pest insect species. To resolve taxonomic concerns (synonyms) and the life history of this cat flea-infecting gregarine, *Gregarina ctenocephali*, the morphology, life cycle, and phylogenetic status of this species in Taiwan were investigated and described. Virtually all lines of evidence showed that this gregarine is closely related to the genus *Steinina* and only distantly related to the genus *Gregarina* as described by Ross (1909) and thus should be transferred from *G. ctenocephali* to *S. ctenocephali* comb. nov. Results of this study provide baseline information for evaluation of future biological control practices.
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Key words: Cat flea, Gregarine, *Gregarina ctenocephali*, Life cycle, Morphology.

Gregarines are protozoa belonging to the phylum Apicomplexa that live in digestive tracts, Malpighian tubules, fat tissue, hemolymph, or reproductive organs of marine and terrestrial invertebrates (Chen et al. 1997, Field and Michiels 2006, Valigurová and Koudela 2006). It was proposed that each invertebrate harbors at least 1 species of gregarine; therefore, the exact number of these protozoans is hard to precisely estimate. In fact, gregarines are reported from only about 3124 invertebrate species, which is < 0.3% of the named invertebrate fauna (Levine 1988). For instance, of the 2575 species and subspecies of fleas known (Whiting et al. 2008), only 8 species (0.31%) are known to have gregarines. The 6 gregarine species currently well described from fleas are grouped in 6 genera (Ross 1909, Wellmer 1910, Ashworth and Rettie 1912, Strickland 1912, Dasgupta 1958, Mourya et al. 1996).

Flea-gregarine relationships could potentially be enlightening especially considering that fleas are important vectors of human and animal diseases (Lehane 1996). The cat flea, *Ctenocephalides felis* (Bouché), is the most common flea infesting dogs and cats worldwide, is an important pathogenic carrier which transmits

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Rickettsia spp. and *Bartonella* spp. (Tsai et al. 2011), and is an intermediate host of some tapeworms, such as *Dipylidium caninumi* and *Hymenolepis* spp. (Krämer and Mencke 2001, Krasnov 2008). In addition, the cat flea has developed resistance to certain insecticides (Krämer and Mencke 2001, Bossard et al. 2002). Because gregarines are generally non-pathogenic and host-specific (Valigurová et al. 2007), it was proposed to consider gregarines under integrated pest management practices. Therefore, the study of gregarines found in the cat flea may shed light on further pest control regimes. A septate gregarine was found in adult cat fleas in Taipei, Taiwan in 2004 (Tsai 2005). The gregarine from *C. felis* was first described by Ross (1909). He named the species *Gregarina ctenocephali canis*, but gave no details of other subspecies when proposing the name, "*canis*". So far, no other subspecies of *G. ctenocephali* have been described, and hence, "*canis*" was omitted from this article. Later on, gregarines with a cup-shaped epimerite were recovered from cat fleas in the US and Brazil. As the cup-shaped epimerite is a key feature of the genus *Steinina* Lèger and Duboscq, 1904, gregarines of cat fleas found in the US and Brazil were assigned to *Steinina* sp. (Beard et al. 1990, De Avelar et al. 2007, De Avelar and Linardi 2008). To the present, 2 different gregarines from cat fleas were described. Whether they belong to the same species or are indeed different species is important for both public health and taxonomic concerns. In this paper, the morphology and life cycle of a gregarine found in cat fleas in Taiwan were investigated and described. In addition, its taxonomic position was established by a phylogenetic analysis based on small subunit ribosomal RNA (SSU rRNA) sequences. Combining the current results with previous data, we concluded that the gregarine of cat flea described by Ross (1909) actually belongs to the genus *Steinina*, and we thus transferred *G. ctenocephali* to *S. ctenocephali* comb. nov.

MATERIALS AND METHODS

Collection and morphological observations

Adult cat fleas were collected from cats and dogs in the Taipei Animal Shelter, Taiwan from May 2005 to May 2008. In the laboratory, adult fleas were observed under a Leica Zoom 2000 stereomicroscope (Buffalo, NY, USA) to confirm gregarine infections. The infected fleas were dissected in phosphate-buffered saline (PBS, pH 7.4). The identity of the gregarines was established based on morphological characters described in the literature (Ross 1909, Beard et al. 1990, Clopton 2002, De Avelar et al. 2007, De Avelar and Linardi 2008). Gregarines were micropipetted and transferred to Eppendorf tubes with 90% alcohol and stored at 4°C for subsequent processing. We prepared frozen tissue sections of 3rd instar flea larvae infected with gregarines following the method of Huang et al. (2006). Terminologies of morphological characters follow Clopton (2004 2009). Fleas were observed under an Olympus BH2 microscope (Tokyo, Japan), and microphotographs were taken with a Nikon Cool Pix 5000 digital camera (Tokyo, Japan) attached to it. For scanning electronic microscopy (SEM), samples were dehydrated using a 30%, 50%, 70%, 95%, and 100% ethanol series followed by 1: 2 and 1: 1 acetone: ethanol series, then stored in 100% acetone prior to critical-point-drying. Specimens were critical-point-dried using CO_2 as the transfer fluid, then mounted on stubs and sputter-coated with a gold-palladium alloy, and pictures were taken using an SEM (JEOL JSM-5600, Tokyo, Japan) located at National Taiwan Univ., Taipei, Taiwan.

DNA extraction, polymerase chain reaction (PCR) amplification, cloning, and sequencing

About 100 trophozoites and 50 gametocysts of *S. ctenocephali* were pooled for genomic DNA extraction. DNA was extracted using a DNA Extraction Mini Kit (Watson, Kaohsiung, Taiwan). The SSU rRNA gene was amplified using the universal eukaryotic primers 5'-CGAATTCAACC TGGTTGATCCTGCCAGT-3' and 5'-CCGGATCC TGATCCTTCTGCAGGTTCACCTAC-3' (Leander et al. 2003). The PCR was performed as follows: an initial denaturation at 95°C for 2 min; 35 cycles of 92°C for 45 s, annealing at 45°C for 45 s, and extension at 72°C for 1.5 min; followed by a final extension at 72°C for 5 min (Leander et al. 2003). PCR products were electrophoresed and visualized on a 1.2% agarose gel (1x TBE buffer), and the expected size of ~2000 bp was excised for purification using the Gel-M Clean Up Kit (Viogene, Taipei, Taiwan). Purified DNA was then cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Two vector primers, T7 and SP6, and 2 newly designed primers, cat flea gregarine primers F (5'-CCATGTCTGGACCTGCTAAG-3')

and R (5'-AACTTTGCTCGTGGAGCTGG-3'), were used for sequencing. Sequences were determined using dye terminator cycle sequencing reactions that were subsequently loaded onto an Applied Biosystems 377A automatic sequencer (Foster City, CA, USA) using standard protocols. Sequences were assembled using the BioEdit program (Hall 1999).

Phylogenetic analysis

The newly determined SSU sequence from *S. ctenocephali* was aligned with selected gregarina sequences (shown in Fig. 4) downloaded from GenBank using default parameters of the ClustalX 1.83 program (Thompson et al. 1997). The Neighbor-joining (NJ) tree was constructed using MEGA (Tamura et al. 2007) with the Kimura two-parameter model of nucleotide substitution method. The maximum-parsimony (MP) tree was constructed using PAUP* vers. 4.0b10 with heuristic searches and 20 replications of random stepwise additions. Bootstrap replications for nodal support were 500 for the MP and 1000 for the NJ analysis.

RESULTS

Taxonomy

Steinina ctenocephali (Ross 1909) Alarcón comb. nov.

Gregarina ctenocephali canis Ross (1909): 359-363.

Remarks: Ross (1909) described various stages of *G. ctenocephali*. Although he did not state the term "new species", the species name implied that it was a new species. As to the authors' point of view, the cup-shaped (digitiform) epimerite is one of the most important characteristics of the genus *Steinina*. Based on current understanding and available information, we propose that this cat flea gregarine belongs to the genus *Steinina*. Therefore, *G. ctenocephali* should be *S. ctenocephali* comb. nov.

Morphology and life cycle of the cat flea gregarine

The general morphology of gregarines in cat fleas has only been briefly described in some previous work (Ross 1909, Beard et al. 1990, De Avelar et al. 2007, De Avelar and Linardi 2008). We provide the morphology and life cycle of *S. ctenocephali* in detail below.

Trophozoite: Found attached singly to host gut epithelium by means of cup-shaped epimerite with secondary structure with flattened bottom at its attachment site to protomerite (possibly providing mechanical support for initial attachment), which disappears in mature gamonts, epimerite measure 19.37 ± 3.1 μm in length and 54.52 ± 10.78 μm in width (Table 1); protomerite and deuteromerite obpyriform-shaped, those measure 104.39 ± 20.42 μm in length and 63.18 ± 14.22 μm in width maximum (Table 1). Color brown; nucleus posterior and always under septum in deuteromerite (Figs. 1A, B, 2A).

Mature trophozoite and gamonts: Mature trophozoites becoming broadly obpyriform, gradually elongated toward posterior apices without constriction. Epimerite vestigial or completely

Table 1. Morphological measurements (μm) of various stages of *S. ctenocephali* comb. nov.

Measurement	*S. ctenocephali* comb. nov. stage					
	Oocyst	Trophozoite		Gamont	Gametocyst	
		Proto. + Deuteromerite	Epimerites		Immature	Mature
Length	11.99 ± 0.33 (12.32 - 11.66)	104.39 ± 20.42 (84.83 - 147.00)	19.37 ± 3.1 (16.27 - 22.47)	163.63 ± 34.84 (112.00 - 200.00)	151.72 ± 17.38 (125.50 - 188.27)	157.49 ± 13.36 (146.45 - 188.50)
Width	9.74 ± 0.30 (10.04 - 9.44)	-	54.52 ± 10.78 (43.74 - 65.3)	-	-	-
Width maximum	-	63.18 ± 14.22 (51.51 - 97.67)	-	98.49 ± 19.25 (76.50 - 126.20)	-	-
Width equatorial	-	51.92 ± 6.01 (43.75 - 63.16)	-	70.83 ± 23.43 (43.65 - 104.76)	-	-
Width constriction	-	39.24 ± 5.49 (29.69 - 45.67)	-	-	-	-
Sample size (*n*)	15	9	8	7	8	8

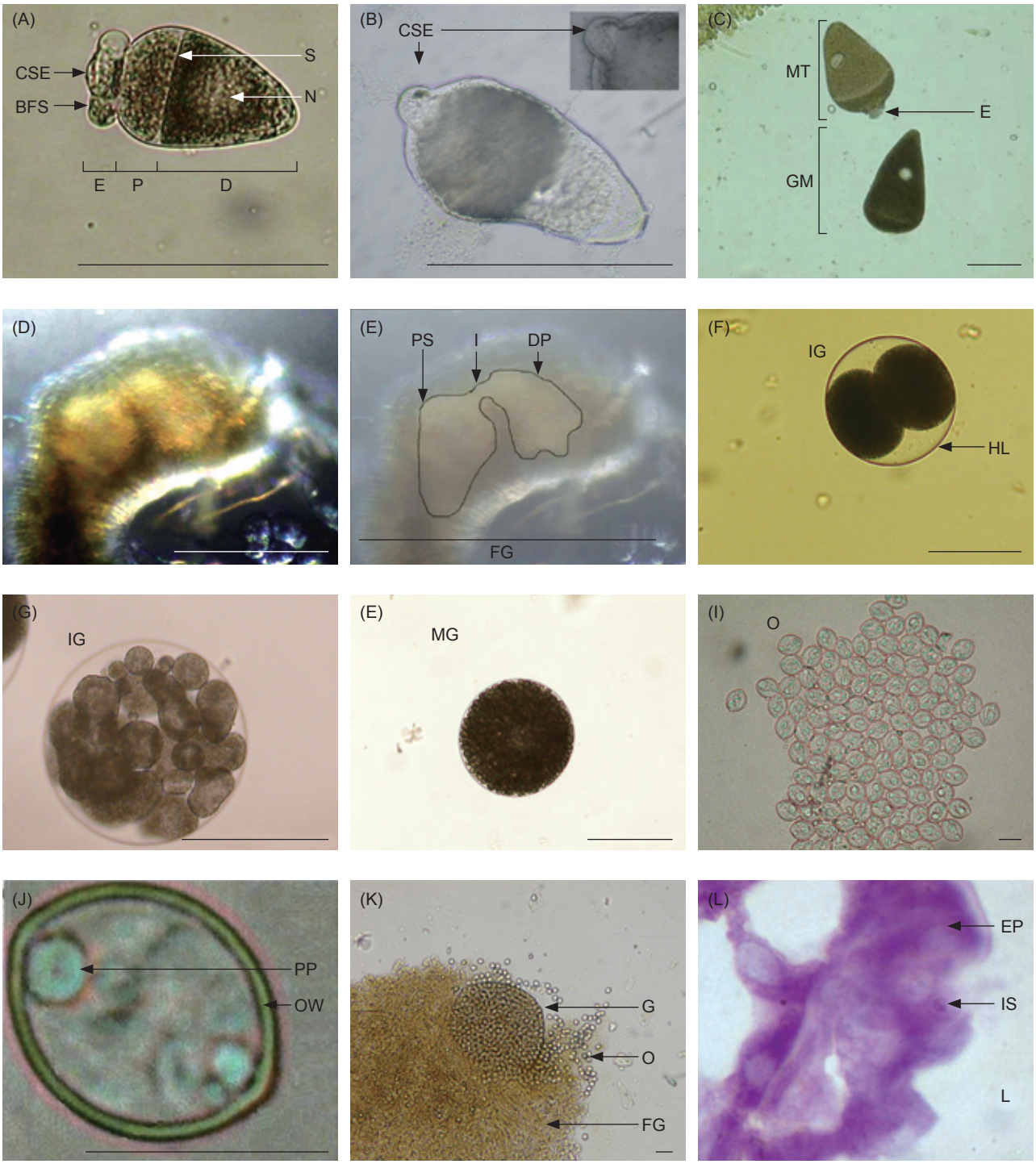

Fig. 1. Life stages of *Steinina ctenocephali* comb. nov. (A) Tropozoite when fresh. (B) Close-up of the cup-shaped epimerite. (C) Mature trophozoite and gamonts. (D) Gamonts in caudofrontal association. Microphotograph of paired gamonts, in situ stage. (E) Same, diagrammatic representation (drawn by author). (F) Early gametocyst with 2 spherical bodies. (G). Daughter nuclei produced through the process of cell division. (H) Mature gametocyst with oocysts. (I) Mass of oocysts in the cat flea gut. (J) Magnified view of an oocyst. (K) Oocyst released by simple rupture in *Ctenocephalides felis* gut. (L) Infected 3rd instar larvae of flea. BFS, bottom flattened structure; CSE, cup-shaped epimerite; D, deuteromerite; DP, deuteromerite primate; E, epimerite; EP, epithelium; FG, flea gut; G, gametocyst; GM, gamont; HL, hyaline layer; I, interlock; IG, immature gametocyte; IS, intracellular stage; L, lumen; MG, mature gametocyte; MT, mature trophozoite; N, nucleus; O, oocyst; OW, oocyst wall; P, protomerite; PP, polar plugs; PS, protomerite satellite; S, septum. Scale bars: A-H = 100 μm; I and J = 10 μm; K = 30 μm; L = 20 μm.

absent. Septum absent or vestigial. Gamonts usually found swimming freely in gut (Fig. 1C). Gamonts measure 163.63 ± 34.84 μm in length and 98.49 ± 19.25 μm in width maximum (Table 1). Association caudofrontal and biassociative. Protomerite of satellite engulfing posterior end of primite deuteromerite to interlock (Fig. 1D, E).

Immature and mature gametocysts (zygote formation): Immature gametocysts containing 2 or more spherical bodies (Fig. 1F, G). Mature gametocysts spherical, white, yellowish in highly infected fleas (with more than 10 gametocysts per infected flea). Gametocysts enclosed in a thin hyaline layer (hyaline epicyst), containing many oocysts, evenly distributed (Figs. 1H, 2B, C). Mature gametocyst without sporoducts. Mature gametocysts measure 157.49 ± 13.36 μm in diameter and immature gametocysts measure 151.72 ± 17.38 μm in diameter (Table 1).

Oocyst: Fusiform (lemon-shaped) surrounded by a thick wall at high-magnification (Fig. 1J). Polar plugs always distinguishable. Oocysts discharged singly by simple rupture (Figs. 1I-K, 2D). Oocysts measure 11.99 ± 0.33 μm in length and 9.74 ± 0.30 μm in width (Table 1).

Biology (Fig. 3): Development of *S. ctenocephali* initiated by entry of sporozoites released from ingested oocysts into gut of 1st instar flea larvae (Fig. 3A, B). The *S. ctenocephali* develops intracellularly during larval and pupal stages of cat flea (Fig. 3C). Once the cat fleas emerge and start feeding blood, the gregarine move out to lumen of flea gut (Fig. 3D). As cell grows, it turns into pear-shaped trophozoite which attaches to membrane of flea gut by its epimerite (Figs. 1A, 3E). Then a horizontal septum formed separating trophozoite, resulting in 2 equal halves, protomerite and deutomerite (Fig. 1A). Separation (septum) gradually becoming less clear as it begins to lose its pyriform shape (Fig. 1B). After

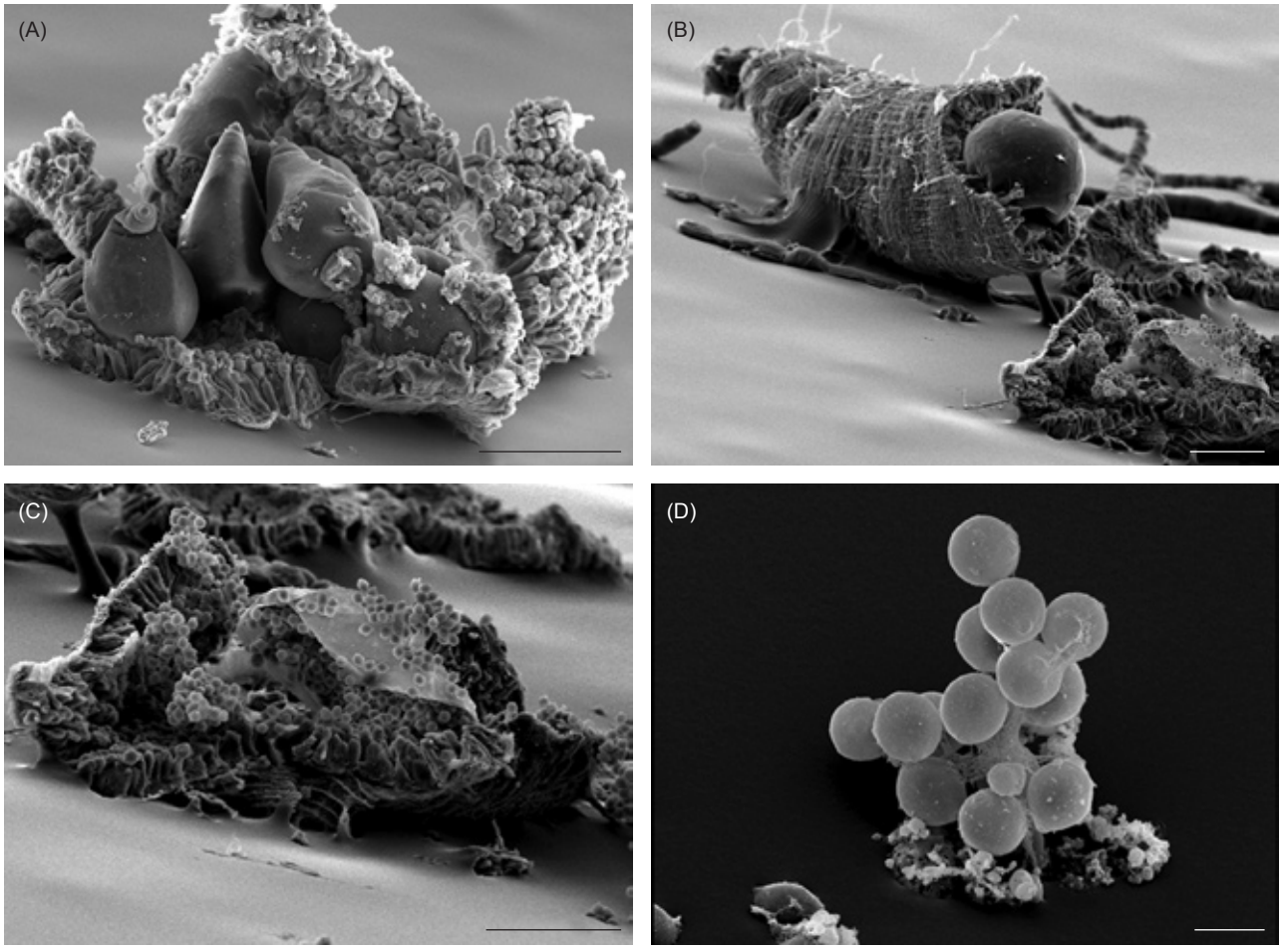

Fig. 2. *Steinina ctenocephali* comb. nov. (A) Trophozoites attached to the gut wall of *Ctenocephalides felis*. (B) Spherical gametocyst in *C. felis* gut. (C) Same, ruptured gametocyst releasing oocysts. (D) Same, clumped oocysts. Scale bars: A-C = 100 μm; D = 10 μm.

epimerite completely detached from trophozoite, parasite turns darker to transmitted light and develops into gamonts (Figs. 1C, 3F). Two gamonts then become associated and connected as seen in figure 1D and E, developing into early stage of gametocysts (which tend to be circular in shape) embedded in hyaline layer (Figs. 1F, 3G). Daughter nuclei produced through process of cell division (Figs. 1G, 3H) until a large number of oocysts bud off from mature gametocysts (Figs. 1H-J, 3I). Oocysts then discharged and released along with feces of adult flea (Figs. 1K, 3J). Those oocysts ingested by developing *C. felis* larvae and infect epithelium of larval midgut (Fig. 1L) where intracellular developmental cycle starts over (Fig. 2A-D).

Phylogenetic analysis based on SSU rRNA sequences

The recovered SSU rRNA sequence of the gregarine from cat flea was 1793 bp long with a GC content of 43.3%. After alignment, the sequence matrix was 2058 bp long including gaps. The NJ tree shows phylogenetic relationships of different gregarines (Fig. 4). The phylogenetic tree constructed by the MP method yielded an essentially identical topology; we, therefore, only present the NJ tree with bootstrap values derived from both the NJ and MP methods at the nodes. The monophyly of the Gregarinoidea including the genera, *Gregarina*, *Protomagalhaensia*, *Amoebogregarina*, and *Leidyana*, was strongly

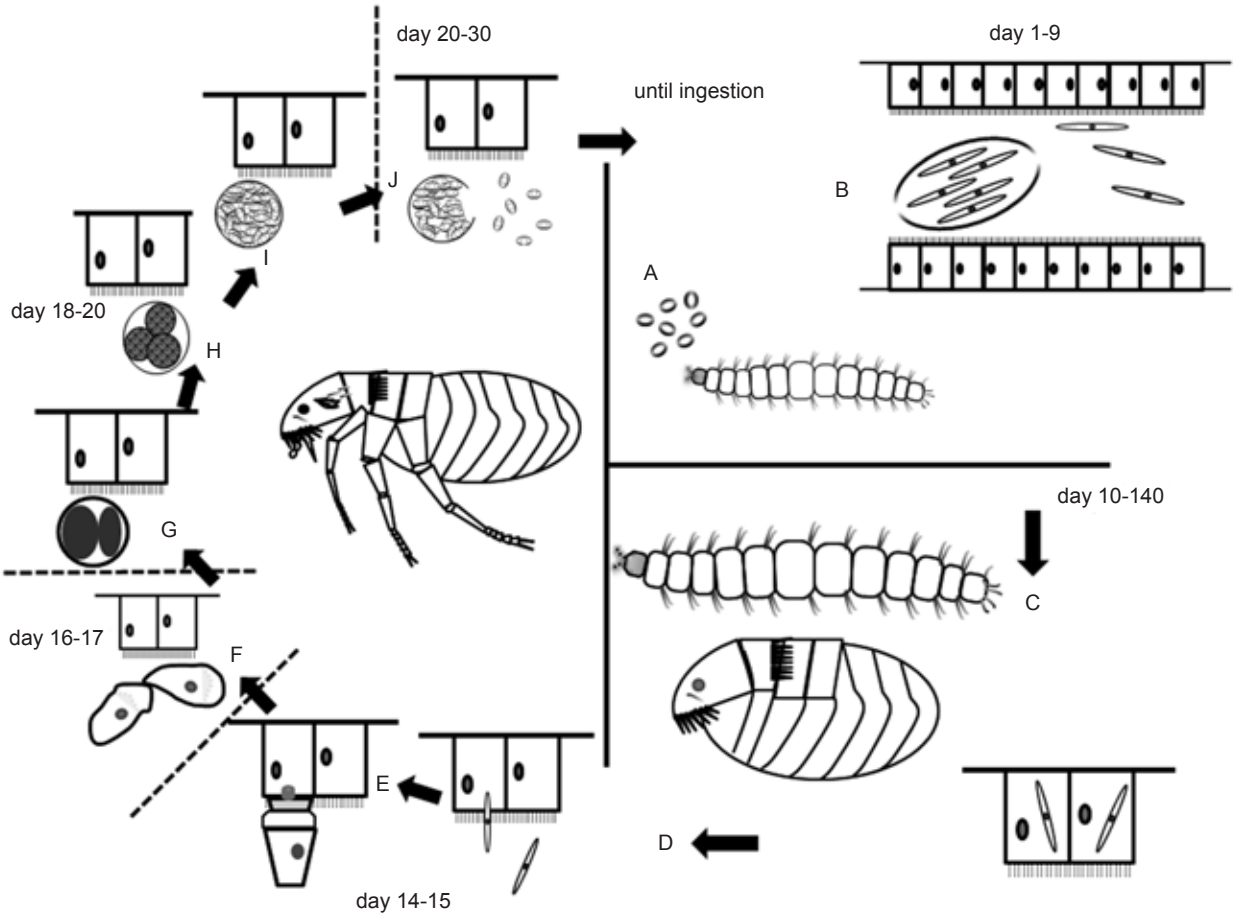

Fig. 3. Schematic representation of the life cycle of *Steinina ctenocephali* comb. nov. in *Ctenocephalides felis*. (A) A flea larva ingesting an oocyst. (B) Release of sporozoites in the gut of a flea larva. (C) Third instar flea larva, cocoon, and pupa formation, intracellular stage. (D) Exit of sporozoite into the lumen of the adult flea gut, extracellular stage. (E) Trophozoites in the gut of an adult flea. (F) Trophozoites mature and become gamonts which undergo syzygy. (G, H) An early gametocyst, consisting 2 and more spherical bodies. (I) A mature gametocyst filled with oocysts. (J) A gametocyst releasing oocysts to the environment along with flea feces.

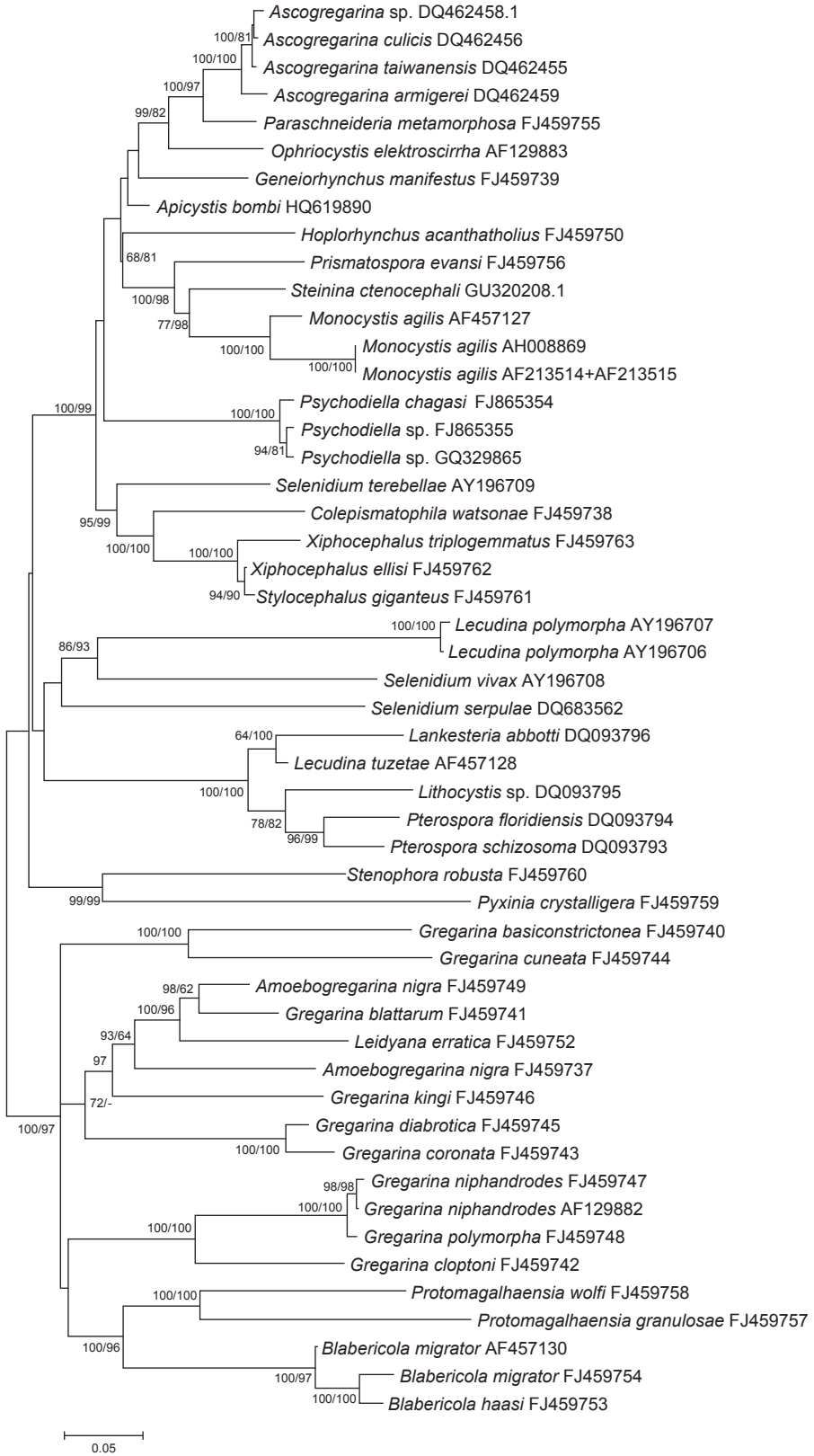

Fig. 4. Neighbor-joining (NJ) tree from an alignment of 51 SSU rRNA sequences, on the basis of the Kimura 2-parameter distance method. Numbers at the branches are bootstrap values derived from 1000 replicates of the NJ (above slash) and maximum-parsimony (below slash) methods.

supported with high bootstrap replications (99% in the NJ and 97% in the MP tree). Nevertheless, the gregarine collected from cat fleas was not close to the Gregarinoidea lineage. Instead, the sequence clustered with the families Stylocephalidae, Lecudinidae, Ophryocysidae, and Sphaerocystidae with high bootstrap support (100% in the NJ and 99% in the MP tree). Although phylogenetic relationships within this cluster were not resolved owing to low bootstrap values, a clear distinction between currently recovered gregarine and the genus *Gregarina* was evident. In addition, the average genetic distance between different species of the Gregarinoidea was 0.245 ± 0.010, while the average genetic distance between *S. ctenocephali* and the Gregarinoidea was 0.294 ± 0.010. Thus, the gregarine found in cat fleas is genetically distinct from *Gregarina*.

DISCUSSION

Gregarines, usually tend to show a high degree of host specificity, and they may be restricted to a particular tissue (or site) of a specific life stage of a single host (Clopton et al. 1992, Clopton and Gold 1996, Rueckert and Leander 2008a, Clopton 2009).

Ross (1909) described a gregarine, *G. ctenocephali*, living in the alimentary canal of the adult dog flea, *C. felis* (= *C. serraticeps* (Gervais)). Nevertheless, the morphological and life history traits described by Ross (1909) have many aspects similar to those of the genus *Steinina*. For example, the morphologies of trophozoites in Ross (1909) varied from acorn- and pear- to obpyriform-shaped ones (Clopton 2004). Similar shapes of trophozoites were also found in *Steinina* and gregarines living in cat fleas (Beard et al. 1990, De Avelar and Linardi 2008).

While Ross's and current gregarines from cat fleas are alike, the genera *Gregarina* and *Steinina* actually greatly differ from each other. The genus *Gregarina* Dufour, comprises 317 species (Clopton 2002) and belongs to the Gregarinoidea, in which the association of gamonts is caudofrontal, and oocysts are discharged in monete chains by means of gametocyst spore tubes (Clopton 2009). The genus *Steinina*, on the other hand, is grouped under the superfamily Stylocephaloidea (Clopton 2009), the gamonts of which are frontal or frontolaterally associated, and oocysts are discharged solitarily by the simple rupture of the gametocyte.

In addition, species of *Steinina* have unique characteristics of epimerites including short retractile digitiform, a short-conical hyaline projection, and a small spherical to cup-shaped cone superimposed upon the protomerite (Gupta and Haldar 1987). All of the above features were observed in the gregarine of the cat flea studied herein. Furthermore, we observed that in *S. ctenocephali*, oocysts are discharged solitarily by simple rupture of the gametocyt. Based on the above information, we concluded that *G. ctenocephali* should be placed in the genus *Steinina*.

In addition to the morphological and life history traits mentioned above, the current genetic analysis also showed that this cat flea gregarine is only distantly related to *Gregarina*. The phylogeny constructed by SSU RNA sequences revealed that *S. ctenocephali* comb. nov. is phylogenetically distinct from all available *Gregarina*.

S. ctenocephali comb. nov. resembles neogregarines in which merozoites (trophozoites) are septate (Rueckert and Leander 2008b). Therefore, the position of *S. ctenocephali* comb. nov. was unexpected as it is close to *Monocystis agilis*, a common aseptate protozoan from the seminiferous vesicles of earthworms. Clopton (2009) revised the taxonomic state of septate gregarines using SSU rDNA sequences. The phylogeny showed that septate gregarines belong to Septatorina which includes 3 superfamilies: the Gregarinoidea, Stenophoroidea, and Stylocephaloidea. Our data generally agree with the monophyly of the Gregarinoidea and Stenophoroidea. However, some aseptate gregarines (e.g., *Ascogregarina*) share close phylogenetic relationships with the Stylocephaloidea (Fig. 4). Combined with the fact that septate and aseptate species are not reciprocally monophyletic, all evidence appears to indicate that either the formation of a septate might not be a good character to define different taxonomic groups or the current marker was not powerful enough to resolve the phylogenetic relationships between them.

S. ctenocephali comb. nov. is transmitted from adult fleas to their progenies by means of their oocysts. The necessity of feces of adult fleas for larval development, referred to as a kind of parental investment which is typical of *C. felis* (Hinkle et al. 1991, Silverman and Appel 1994, Krasnov 2008), promotes its perpetuation and survival. Initial intracellular stages of trophozoites (transformed from sporozoites) seem to be an interesting survival adaptation of *S. ctenocephali*

comb. nov. for 2 possible reasons: (a) to avoid being discharged from the gut when larvae void their gut contents in preparation for pupation, and (b) to remain quiescent during the pupal stage of the flea, which may be delayed for up to 140 d (Silverman and Rust 1985). The latter provides a better opportunity for gregarine survival, because if a multiplying phase (merogony) occurs, as in some neogregarines, it may kill the host (Pereira et al. 2002) before the flea reaches the adult stage.

S. ctenocephali comb. nov. is an intriguing gregarine associated with one of the most important pests (*C. felis*) worldwide. Further investigations of the ultrastructure of the various life stages and molecular data will shed light on its classification and evolutionary history. In addition, these gregarines may be explored as potential biocontrol agents.

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