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ORIGINAL PAPER

A survey for gregarines (Protozoa: Apicomplexa) in arthropods in Spain

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Abstract Gregarines thrive in the digestive tract of arthropods and may be deleterious to their hosts, especially when present in high densities. The impact of parasites on these invertebrates may affect both the ecosystem equilibrium and human economic activities. However, information available on gregarines in Spain is limited. Therefore, a microscopic study on prevalence of gregarine infection in 560 insects and crustaceans was undertaken in Madrid and Tarragona.*Gregarina ormierei* (78 % prevalence), *Stylocephalus gigas* (56 %), *Oocephalus hispanus* (13 %) and *Actinocephalus permagnus* (only one infected out of six beetles examined) were found in coleopteran hosts. *Gregarina ovata* and *G. chelidurellae* showed moderate frequency of infection (35 %) in dermapterans. An undescribed *Gregarina* sp. (76 % prevalence) was observed for the first time in freshwater decapod crustaceans. Interestingly, *G. ormierei* showed a noticeable phenotypic dimorphism, which justifies its redescription based on modern taxonomic criteria. Sequences of the 18S rRNA gene could be obtained only in the presence of highly prevalent gregarines. *G. ormierei* and *Gregarina* sp. were related (85 and 94 % identity by BLASTN, respectively) to *G. basiconstrictonea* and *G. cloptoni*, respectively, whereas *S. gigas* was closely related to both *Xiphocephalus ellisi* and *S. giganteus* (>97 % identity). Phylogenetic trees based on ribosomal sequences unequivocally grouped these new isolates either with the Gregarinidae (*G. ormierei* and *Gregarina* sp.) or the Stylocephalidae (*S. gigas*).

Keywords Insects · Crustaceans · Gregarines · Small ribosomal subunit gene

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Introduction

Many arthropod species are economically important: crustaceans are a traditional food source for humans, and some beetles are used in coleotherapy for treatment of certain ailments in traditional medicine (Cupul-Magaña 2010). In addition, arthropods also play an important role in trophic webs, where detritivore species contribute to matter cycling in ecosystems by decomposing organic material in soil (Cartagena and Galante 2003). Despite their importance in ecology, human nutrition and welfare, current knowledge on invertebrate pathogens is limited (Clopton 2009; Desportes and Schrevel 2013; Criado-Fornelio et al. 2013). Many parasites infect invertebrates, although gregarines—apicomplexan protozoa inhabiting the intestine, coelom and reproductive vesicles of marine, freshwater and terrestrial organisms—are quite common and sometimes detrimental for host survival (Purrini and Kel 1989). In fact, the impact of parasites on the biological fitness of arthropods may shape entire ecosystems (Prokopowicz et al. 2010). In shrimp aquaculture, heavy gregarine infections may have economic implications by reducing growth and causing stock mortality (Jiménez et al. 2002).

Nearly 2000 gregarines have been described to date. However, these represent a minority of the total number of species, since less than 1 % of known invertebrates have been examined for parasitic infections (Desportes and Schrevel

2013). Few studies on gregarines in Spain have been conducted. Theodorides (1958a, 1960) surveyed two small beetle populations in the Canary Islands and Murcia, respectively, finding frequent infections by stylocephalid gregarines in some tenebrionid beetles. Recently, a preliminary survey conducted in Alcalá de Henares did not detect gregarines in a small sample of insects by molecular methods, but a possible gregarine infection was found in *Armadillidium vulgare* (Crustacea) by Criado-Fornelio et al. (2013). Other studies conducted in Spain (Cordero del Campillo et al. 1994) reported absence of gregarines in the freshwater decapod crustacean *Atyaephyra desmaresti*. Species like *Uradiophora cuenoti* and *Heliospora longissima*, however, were reported in that Decapod host in a study carried out in southern France by Tuzet and Ormieres (1956).

The present work is aimed to further investigate gregarine biogeography in Spain. Two taxonomic groups of arthropods have been selected to survey the presence of gregarines in different geographical areas: insects in Alcalá de Henares (Madrid) and freshwater crustaceans in the Ebro River in the province of Tarragona. Gregarines were diagnosed by microscopy and, whenever feasible, characterised at the molecular level by amplification and sequencing of the 18S rRNA gene.

Material and methods

Arthropod field sampling

Beetles and earwigs were captured in the vicinity of the Faculty of Pharmacy at the University de Alcalá (Alcalá de Henares, Madrid, Spain), approximate geographic coordinates: 40° 30′ 35.089″ N; 3° 20′ 27.015″ W. Specimens were obtained either by hand or pitfall traps, as described by Greenslade (1964). Identification of insects was based on keys published by Chinery (1988) and Harde and Severa (1984) and further confirmed by Dr. Arturo Baz-Ramos (Professor of Entomology at the University of Alcalá, Zoology laboratory, Departamento de Ciencias de la Vida). Likewise, specimens of the decapod *A. desmaresti* (Decapoda) were collected in the Ebro river delta, in Tarragona (40° 43′ 20.007″ N; 0° 41′ 49.247″ E). Specimens were captured with the help of funnel nets following the method described by Larraz et al. (2005). Crustaceans were brought to the laboratory in refrigerated plastic tanks containing stream water and identification was done according to Mateus (1963).

In this study, protected species of arthropods were not included in the collection. In total, 560 specimens were studied: 240 insects and 320 crustaceans. Detailed description of the sampled species is included in the "Results" section.

Laboratory maintenance of *Scaurus* beetles for recovery of gametocysts/oocysts

In order to complete the study on gregarines of *Scaurus punctatus* (namely *G. ormierei*), individual beetles were placed separately in 8-cm Petri dishes. They were fed with apples, decaying leaves of *Platanus hispanica* trees and occasionally with preserved pork (Spam® or similar). Insects were kept in a controlled environmental chamber (Ibercex C-3, Madrid, Spain) at 24 °C under a light-dark schedule of 12:12. Petri dishes were examined daily with a stereo microscope. Gametocysts were recovered later, washed with sterile saline solution (0.9 % NaCl) and transferred for dehiscence to concave slides and kept at 24 °C either in a humid chamber or in saline. Gametocysts were observed by microscopy to confirm and record dehiscence. In addition, some of the gametocytes recovered were transferred to Eppendorf tubes until dehiscence for genetic analysis.

Microscopic studies

Using a scalpel, the insect gut was removed and longitudinally sliced on a microscopic glass slide with a drop of insect saline solution at 1.8 % NaCl, 1.88 % KCl, 0.16 % $CaCl_2$ and 0.004 % $NaHCO_3$ *w*/*v* in distilled water. This was done to facilitate microscopic observation of the intestinal content. Microscopically, gregarines were examined in wet mount or stained with eosin or acetocarmine and mounted as permanent preparation (Criado-Fornelio et al. 2014). Morphologic features were measured in microphotographs obtained in a Motic BA300 microscope equipped with a digital camera and employing the Motic Images Plus software version 2.0. Terms used in morphometric measurements followed those by Clopton (2004) and Janovy et al. (2007) as proposed and on live specimens (whenever feasible) as recommended by Clopton et al. (1992). Denominations of biological stages follow the terminology by Levine (1971). For each stage, usually, 30 sample specimens are taken for measurements using the mean ± standard deviation and range, expressed in micrometers.

For scanning electron microscopy (SEM), direct observation was made mainly on the gregarine oocysts, without subjecting to fixation and acetone dehydration, as recommended by Janovy et al. (2007). Other gregarine stages were fixed in Milloning's solution containing 2 % glutaraldehyde, washed in Milloning's solution with 0.5 % glucose and dehydrated first through an increasing series of ethanol concentrations and finally with anhydrous acetone. All samples were sputter-coated with 200 Å gold-palladium using a Polaron E5400. Scanning electron microscopy was performed at 5–20 kV in a Zeiss DSM 950 SEM.

DNA extraction, amplification and sequencing of the 18S rRNA gene

Gregarine material used for DNA extraction consisted in pooled trophozoites for *Stylocephalus gigas* and *Gregarina* sp. For *G. ormierei*, pooled (trophozoites and gametocysts) and unpooled samples (two gametocysts) from each phenotype were taken for DNA extraction. DNA was extracted using the Blood and Tissue Isolation kit (Qiagen, Hilden, Germany) for pooled samples. Extraction for unpooled individual gametocysts was done only by boiling. In brief, a single gametocyst was placed in an Eppendorf tube to which 150 μl of distilled water was added. The tube was heated at 100 °C for 2 min in a thermal block. Thus, oocysts burst and gregarine DNA was released.

Amplification of the 18S rRNA gene of gregarines was performed with primers GR1-F (forward 5′-GGTT GATCCTGCCAGTAGT-3′) and GR2-R (reverse 5′-CTTC TGCAGGTTCACCTACG-3′) that yield a fragment of approximately 1700 bp. The thermal cycling profile for GR1-F and GR2-R was as follows: hot-start 3 min at 94 °C; 40 cycles of 30 s at 95 °C, 45 s at 60 °C and 1 min 30 s at 72 °C, with a final extension step of 6 min at 72 °C. *Taq* DNA polymerase enzyme employed in amplifications was purchased from Biotools (Alcobendas, Madrid, Spain). The PCR mixture contained 1 mM dNTP mix, 25 pmol of each primer, 2.5 μl 10× buffer Biotools with 2 mM Mg, 0.8 U Biotools DNA polymerase, template DNA 0.5 to 5 ng and sterile distilled water up to 25 μl.

Amplified products obtained by PCR were analysed by electrophoresis in 1.5 % agarose gels, and electrophoretic runs were visualized by ethidium bromide staining with UV transillumination. Bands of interest were carefully excised from the agarose gels to isolate DNA with the UltraClean 15 kit from Mobio (Carslbad, CA, USA). DNA fragments from at least two separate amplifications were used for direct sequencing using the automated sequencer ABI 3130 (Applied Biosystems Inc., Foster City, CA, USA).

Phylogenetic analysis

DNA sequences were aligned with the CLUSTALOMEGA® software (Sievers et al. 2011). The MEGA6® software package (Tamura et al. 2013) was employed for distance calculation and tree construction. Tree-consistency was confirmed by bootstrap analysis (100 replications).

Results

Survey for gregarines in arthropods in Spain

Most gregarines found in Spain were morphologically identical to species described previously by other authors (Table 1). Therefore, showing micrographs of these protozoa in this section was considered unnecessary, except for two gregarine species: *G. ormierei* and *Gregarina* sp.-AD (see below). Tenebrionid coleopterans such as *S. punctatus* and *Blaps lusitanica* were frequently infected by gregarines (*G. ormierei* and *S. gigas*, respectively). *G. ormierei* was found as well in a *Cetonia aurata* beetle. Prevalence of gregarines was much lower in the dermapteran *Forficula auricularia* but showing infections mainly caused by *G. ovata* and less frequently by *G. chelidurellae*. In coleopterans such as *Carabus* sp and *Akis acuminata*, infection was caused by *Actinocephalus permagnus* and *Oocephalus hispanus*, respectively. Some coleopterans, however, were free of infection (*Tenebrio molitor*, *Ocypus olens*, *Silpha tristis*). Moreover, findings showed that a septate gregarine (Fig. 1a–d) with an orbicular epimerite-like structure of a diameter ranging from 6 to 23 μm, frequently parasitized the crustacean *A. desmaresti* in Tarragona. The epimerite-like structure often persists in associations (primite). The gregarine showed a shallow constriction between protomerite and deutomerite. Two parts could be distinguished in the protomerite: an apical, button-like smooth region and a basal granular region slightly protruding of the otherwise cylindrical protozoa cell body. Gamonts resembled those of *U. gammari*, *H. longissima* or *Ganymedes* spp. but were smaller (total length 101.83 ± 52.15 μm; range 43–209 μm; deutomerite width 11.5 ± 4.99 μm; range 6–23 μm). Nuclei in the crustacean gregarine were vesicular, with a single spherical nucleolus. No multiple associations were observed in this species. Based on the presence of septum and an orbicular epimerite-like structure, the isolate was provisionally named as *Gregarina* sp.-AD.

Emendation of the description of the species *G. ormierei*

Theodorides (1955) published an incomplete description of *G. ormierei*: first, two important biological stages of the protozoa (gametocysts and oocysts) were unreported and second, gregarine gamonts were described based on measurements obtained in just a few specimens. On the other hand, inquiries made in the Musée Nationale d'Histoire Naturelle in Paris (France) revealed that there is no voucher material for this species.

G. ormierei Theodorides, 1955.

This gregarine exhibits phenotypic dimorphism in *S. punctatus*, in the small and large form. Standard morphologic measurements for the two phenotypes observed are shown in Tables 2 and 3.

Trophozoite (Fig. 1e–g): epimerite globular, diameter: 8.75 ± 1.41 μm; range 6–11, $n = 20$. There are no differences in shape or size between trophozoite stages of *G. ormierei* in both genotypes. Moreover, trophozoites are identical in morphology irrespective of the host considered, either

Table 1 Results of the microscopic survey for gregarines conducted in 560 arthropods in Spain

Arthropod order/family	Arthropod species	Number of specimens studied	Gregarine species found	Infected/prevalence %
Coleoptera/Tenebrionidae	*Scaurus punctatus*	142	*Gregarina ormierei* (1FS + 1FH)	Small phenotype 47/33.1 Large phenotype 42/29.6 Mixed phenotypes 22/15.5 Total infected 111/78.1
Coleoptera/Tenebrionidae	*Blaps lusitanica*	16	*Stylocephalus gigas* (1FS)	9/56.2
Coleoptera/Tenebrionidae	*Akis acuminata*	15	*Oocephalus hispanus* (1FH)	2/13.3
Coleoptera/Tenebrionidae	*Tenebrio molitor*	25	–	0
Coleoptera/Carabidae	*Carabus* sp.	5	*Actinocephalus permagnus* (1FS)	1/NA
Coleoptera/Silphidae	*Silpha tristis*	10	–	0
Coleoptera/Cetonidae	*Cetonia aurata*	1	*G. ormierei* (1FS + 1FH)	small phenotype 1/NA
Coleoptera/Staphylinidae	*Ocypus olens*	6	–	0
Dermaptera/Forficulidae	*Forficula auricularia*	20	*G. ovata* (1FS) *G. ovata* and *G. chelidurellae* (1FS)	*G. ovata* only 6/30 mixed infection 1/5 total infected 7/35
–	–	240 (insect subtotal)	NA	131/54.5
Decapoda/Atyidae	*Atyaephyra desmaresti*	320	*Gregarina* sp.-AD (US)	245/76.5
–	–	560 (arthropods total)	NA	376/67.1

Prevalence has not been calculated for species with less than ten individuals sampled

1FS first finding in Spain, *1FH* first finding in this host species, *US* Undescribed species, *NA* not applicable

S. punctatus or *Cetonia aurata*. They present a spherical epimerite, jug-shaped deutomerite, no sarcocyte layer in deutomerite, shallow ovoid protomerite and relatively clear cytoplasmic granulation. The nucleus is oval, compact and variable in position.

Gamonts and associations (Figs. 1h, 2 and 3a–c): gamonts of the small phenotype are <160 μm long. They look similar to trophozoites but are slightly bigger. Association interface is a truncated surface in the satellite protomerite apex. In contrast, gamonts of the large phenotype are much bigger in size (mature trophozoites > 290 μm). In the latter, the protomerite is variable in shape: either irregular, orbicular, sphero-conical, broadly oblong or broadly obpanduriform. The nucleus is slightly oval and variable in position; in contrast with that of the small phenotype, it is always vesicular, with a single oval to spherical nucleolus. Association of the large phenotype shows a crescentic or bowl-like acetabulum, with shallow wrinkles visible only when examined by SEM.

Gametocysts (Figs. 1i, j and 3d, e): in the small phenotype, gametocysts are whitish, spherical, less than 60 μm in diameter and with a clear mucilaginous outer layer. They are usually embedded in insect's peritrophic membrane. Dehiscence occurs through a single sporoduct, and oocysts are emitted in masses. In the large phenotype, gametocysts are spherical and always larger than 210 μm in diameter. They are whitish and covered with a multilayered external mucilaginous coat. Gametocysts are normally released free in feces and dehiscence occurs through 10–20 sporoducts. The gametocyst sporulation mechanism for each phenotype is identical irrespective of environmental conditions either in saline solution or in air.

Oocysts (Figs. 1k, 3f, and 4): in the small phenotype, oocysts are dolioform, without polar plates and measure 5.77 ± 0.40 μm (range 4.9–6.5). Oocysts of the large gregarine are emitted in linear chains with almost identical shape and size (5.92 ± 0.52 μm; range 4.7 – 6.8) as those of the small form, but they are clearly distinct, as in this case, the resistant stage presents two polar plates. Ultrastructurally, the oocyst surface is relatively smooth and shows only a fine circumferential striation when observed at high magnification.

Comments

With regard to the phenotypic dimorphism, statistically, there was a significant difference between most of the morphological parameters obtained for both phenotypes in all stages (Tables 2 and 3); nevertheless, with five exceptions, PriPL/SatPL, PriPWM/SatPWM, OL, OW and OL/OW, which indicates that oocyst shape was almost identical between the small and large gregarine forms, not considering the presence of polar plates in the latter.

Taxonomic summary

Type of hosts: *Gonocephalum rusticum* (Theodorides 1955a), *S. punctatus* (Forster 1771), *Cetonia aurata* (Linnaeus 1758).

Other host records: *Tentyria taurica* (Theodorides 1958b), *Gonocephalus prolixum*, *G. controversum*, *Mesomorphus*

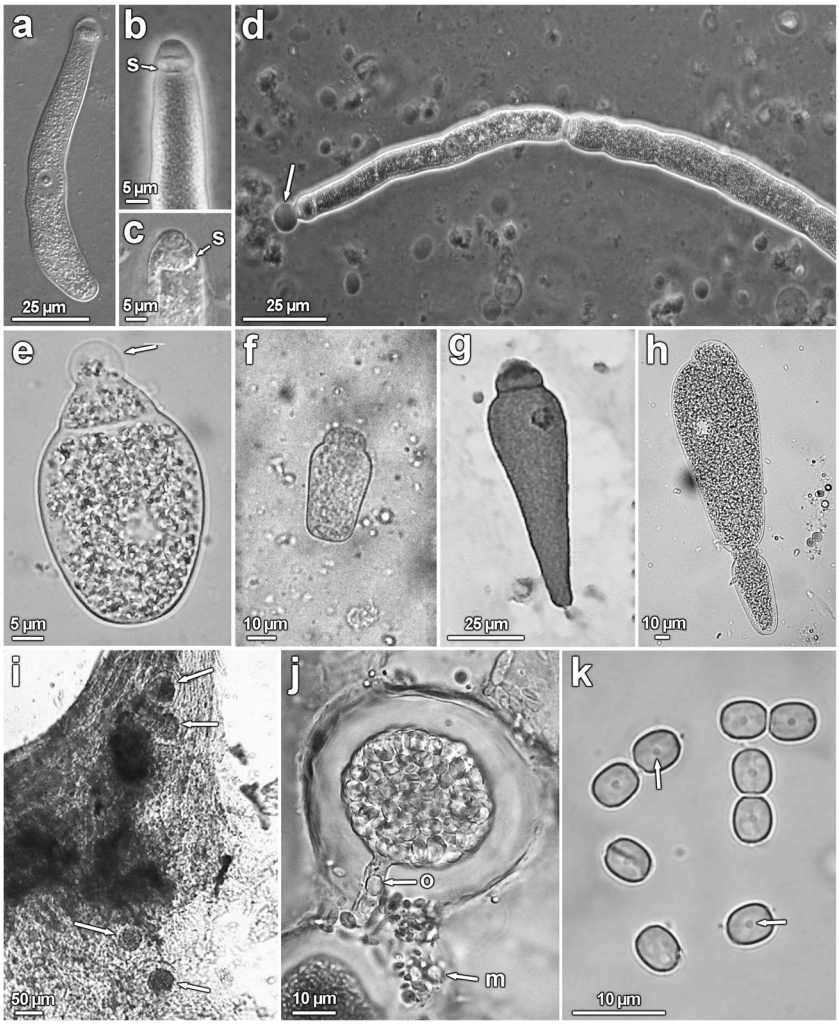

Fig. 1 Micrographs showing different stages of *Gregarina* sp.-AD from *Atyaephyra desmaresti* (phase contrast microscopy) and *G. ormierei* (small phenotype) from coleopterans (light microscopy). **a** Individual gamont of *Gregarina* sp.-AD from *Atyaephyra desmaresti*. The nucleus is oval, placed in a central rear position in the deutomerite and has a nucleolus. **b** Gamont of *Gregarina* sp.-AD: lateral view of the anterior region showing a septum that separates protomerite and deutomerite (*arrow with* "*s*"). **c** Gamont of Gregarina sp.-AD: frontolateral view of the anterior region showing the septum (*arrow with* "*s*") separating a granular region (located in the basal zone of the protomerite) from a smooth area in the anterior part of the deutomerite. **d** Association of *Gregarina* sp.-AD. The primite shows a globular epimerite-like structure (*arrow*). **e** Trophozoite of *G. ormierei* (small phenotype) showing an orbicular epimerite (*arrow*), as observed in *Scaurus punctatus*. **f** *G. ormierei* trophozoite (small phenotype) as observed in *Cetonia aurata*. **g** Trophozoite of *G. ormierei* (small phenotype) with compact nucleus (carmine stain). **h** Mature gamonts of *G. ormierei* (small phenotype) in association with no acetabulum. **i** Undehiscent gametocysts (*arrows*) of *G. ormierei* (small phenotype) attached to the beetle peritrophic membrane (PM), as observed in wet mounts of insect feces. **j** Dehiscent gametocyst of *G. ormierei* (small phenotype) in PM showing a single sporoduct containing oocysts not arranged in chains (*arrow with* "*o*"). Oocyst masses, retained by PM, surround the gametocyst in some spots (*arrow with* "*m*"). **k** Oocysts of *G. ormierei* (small phenotype). *Arrows* point to residual bodies

setosus (Theodorides et al. 1964), *G. simplex* (Theodorides et al. 1965) and *M. villiger* (Theodorides et al. 1976).

Type of locality: Sête (France).

Other locations: Kolabey-Dûzlugu (Turkey), Khartoum (Sudan), Grid-Wonji (Ethiopia), Sintaien (South Korea) and Alcalá de Henares (Spain).

Site of infection: Midgut.

Specimens: the voucher slide series (neotypes) for this redescription is deposited in the Museo de Ciencias Naturales de Madrid (José Gutiérrez Abascal 2, 28006 Madrid—Spain) and comprises of 12 hapantotype slides containing multiple trophozoites, gamonts, gametocysts and oocysts: MNCN 35/64 (large phenotype), MCN 35/65 (large phenotype), MCN 35/66 (large phenotype), MCN 35/67 (large phenotype), MCN 35/68 (large phenotype), MCN 35/69 (large phenotype), MCN 35/70 (large phenotype), MCN 35/71 (small phenotype), MCN 35/72 (small phenotype), MCN 35/73 (small phenotype), MCN 35/74 (small phenotype) and MNCN 35/75 (small phenotype).

Table 2 Standard indices/measurements for *G. ormierei* (associations)

Character	Small phenotype—primite	Small phenotype—satellite	Large phenotype—primite	Large phenotype—satellite
TL	119.80 ± 22.35* (90–158)	77.23 ± 17.75* (47–113)	599.1 ± 185.3 (320–990)	485.5 ± 173.26 (290–940)
PL	16.6 ± 3.12* (9–22)	12.20 ± 3.05* (6–20)	100.4 ± 19.35 (75–130)	61.66 ± 14.40 (40–100)
PWS	24.56 ± 3.39* (15–31)	21.83 ± 4.67* (12–31)	70.73 ± 14.73 (45–115)	59.56 ± 15.12 (35–110)
PWMax	25.13 ± 3.24* (16–31)	22.26 ± 4.35* (12–31)	75.7 ± 19.17 (45–110)	67 ± 15.33 (40–120)
DL	103.56 ± 20.75* (74–143)	65.0 ± 15.41* (37–96)	514.2 ± 171.8 (260–940)	426.16 ± 166.22 (250–870)
DWEq	34.53 ± 7.94* (19–50)	29.0 ± 7.73* (12–42)	75.7 ± 24.8 (40–150)	59.56 ± 15.12 (35–110)
DWMax	41.43 ± 9.39* (25–60)	31.36 ± 9.14* (14–48)	87.16 + 20.41 (50–130)	80 ± 20.04 (45–145)
NL	16.66 ± 2.94* (10–21)	13.8 ± 2.97* (5–18)	25.2 ± 2.86 (15–36)	23.23 ± 4.4 (14–32)
NW	10.30 + 2.11* (7–15)	9.03 ± 2.29* (5–14)	21.93 + 5.31 (11–33)	20.43 + 3.84 (14–32)
KD	NA	NA	9.63 ± 2.02 (6–13)	9.86 ± 2.08 (5–15)
Distance nucleus- septum	19.56 ± 14.07* (1–65)	13.9 ± 9.36* (1–45)	353 ± 222.16 (50–750)	352.25 ± 265.26 (60–820)
TL/PL	7.46 ± 1.80* (5.09–12.44)	6.40 ± 1.19* (4.2–10)	6.1 ± 1.45 (3.85–10)	8.07 ± 2.3 (5–13.42)
DL/PL	6.52 ± 1.79* (4.09–11.44)	5.27 ± 1.42* (1.2–9)	5.16 ± 1.47 (2.14–9)	7.13 ± 2.33 (4–12.42)
TL/DL	1.15 ± 0.04* (1.02–1.24)	1.19 ± 0.05* (1.07–1.31)	1,23 ± 0.2 (0.86–2)	1.15 ± 0.07 (0.89–1.38)
DWM/PWM	1.37 ± 0.22* (1.08–2.08)	1.36 ± 0.25* (0.88–1.71)	1.16 ± 0.23 (0.52–1.54)	1.06 ± 0.22 (0.66–1.53)
NL/NW	1.65 ± 0.27* (1.25–2.42)	1.57 ± 0.30* (1–2.14)	1.14 ± 0.13 (0.75–1.46)	1.16 ± 0.15 (1–1.47)

Nomenclature/abbreviations follow terminology proposed by Clopton (2004) and Janovy et al. (2007)

NA not applicable

*$P < 0.005$ or lower (Student's *t* test)

Analysis of ribosomal sequences and phylogenetic analysis

The sequences of the 18S rRNA gene of *G. ormierei*, *S. gigas* and *Gregarina* sp.-AD were introduced in Genbank® with accession numbers KJ736741, KU664395 and KU664396. In *G. ormierei*, two slightly different sequences were found in samples in both phenotypes. Sequence variation consisted in just two base divergences (C → T in positions 576 and

Table 3 Standard indices/measurements obtained for associations, oocysts and gametocysts of *G. ormierei*

Character	Small gregarine phenotype	Large gregarine phenotype
PriTL/SatTL	1.09 ± 0.21 (0.8–1.8)*	1.15 ± 0.21 (0.85–1.70)
PriPL/SatPL	1.43 ± 0.47 (0.6–2.83) NS	1.56 ± 0.46 (1–3.11)
PriDL/SatDL	1.65 ± 0.37 (0.96–2.57)*	1.10 ± 0.23 (0.82–1.60)
PriPWM/SatPWM	1.13 ± 0.15 (0.74–1.44) NS	1.17 ± 0.21 (0.72–1.66)
PriDWM/SatDWM	1.37 ± 0.22 (1.08–2.08)*	1.12 ± 0.26 (0.62–2)
ASW	17.0 ± 2.47 (10–22)	NA
Acet D	NA	11.26 ± 4.23 (5–22)
Acet W	NA	55.46 ± 13.29 (35–90)
OL	5.77 ± 0.40 (4.9–6.5) NS	5.92 ± 0.52 (4.7–6.8)
OW	4.54 ± 0.44 (3.5–5.2) NS	4.67 ± 0.36 (4.2–5.6)
PPL	NA	0.27 ± 0.09 (0.1–0.4)
PPW	NA	2.07 + 0.49 (1.1–2.9)
OL/OW	1.28 ± 0.13 (1–1.52) NS	1.27 ± 0.11 (1.04–1.44)
PPL/OL	NA	0.045 ± 0.01 (0.019–0.066)
PPL/OW	NA	0.44 ± 0.11 (0.20–0.59)
Gametocyst outer diameter	47.08 ± 3.15 (45–54)*	352.08 ± 38.02 (270–470)
Gametocyst inner diameter (excluding gelatinous layer)	27.43 ± 3.51 (22–34)*	288.04 ± 32.05 (210–380)

Nomenclature/abbreviations follow terminology by Clopton (2004) and Janovy et al. (2007)

NA not applicable, *NS* non-significant

*$P < 0.005$ or lower (Student's *t* test)

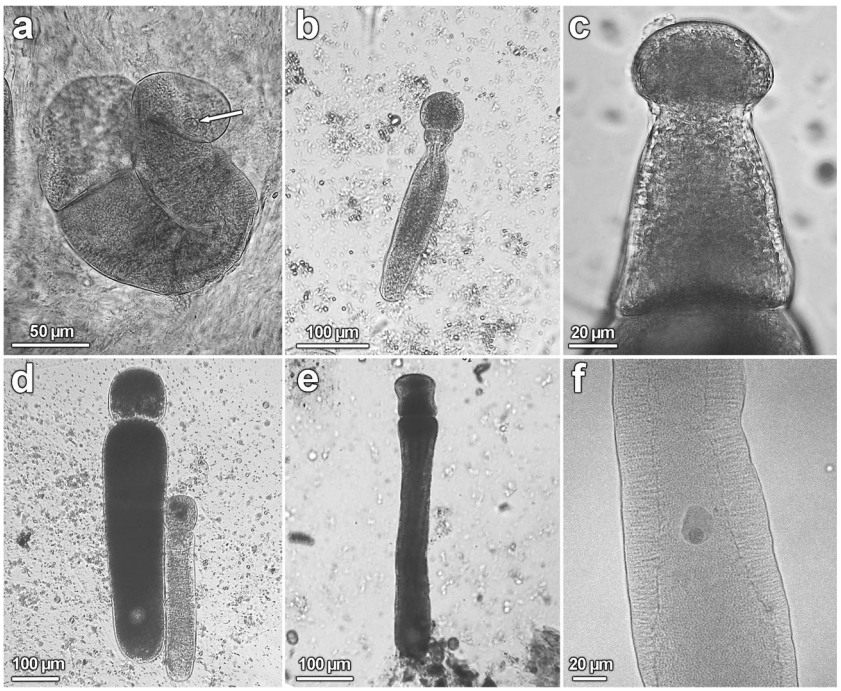

Fig. 2 Light microscopy micrographs showing different stages of the large phenotype of *G. ormierei* found in *S. punctatus* (1). **a** Young gamont in insect's intestinal tissues showing irregular shape protomerite and clear granules in cytoplasm. Such characteristic facilitates a clear view of the nucleus and nucleolus (*arrow*) in deutomerite. **b** Gamont with orbicular protomerite. **c** Conical-orbicular protomerite. **d** Gamont showing a broadly oblong protomerite. In mature gamonts, the deutomerite is narrowly oblong and filled with dark granules that make difficult a clear observation of the nucleus. **e** Broadly obpanduriform protomerite. **f** Partial view of a mature gamont stained with carmine showing the nucleus, nucleolus and a subpellicular striated sarcocyte layer

1033), which were interpreted as single nucleotide polymorphisms located in hypervariable regions of the 18S rRNA gene. *G. ormierei* and *Gregarina* sp.-AD were related (85 and 94 % identity by BLASTN, respectively) to *G. basiconstrictonea* and *G. cloptoni*, respectively, whereas *S. gigas* was closely related to *Xiphocephalus ellisi* and *S. giganteus* (98 and 97.9 % identity).

Gregarine sequences were aligned using Clustal Omega. The resultant alignment contained 2030 positions (of which 1434 were variable positions and 1221 parsimony informative positions). Figure 5 shows the phylogenetic tree obtained by the maximum likelihood method for the new gregarine sequences. A similar topology was obtained using the maximum parsimony approach. *G. ormierei* groups with

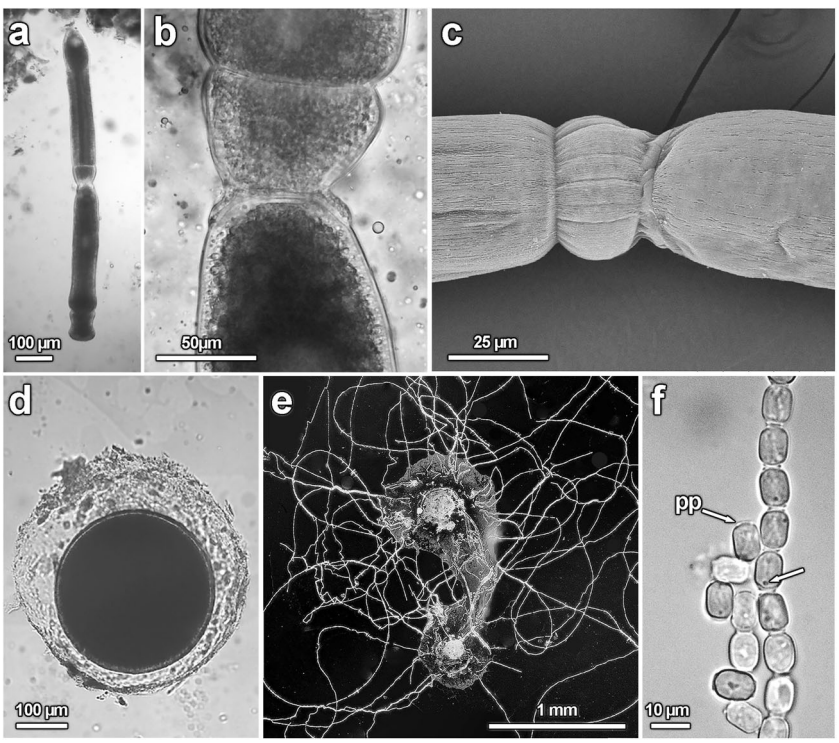

Fig. 3 Micrographs showing different stages of the large genotype of *G. ormierei* found in *S. punctatus* (2). All the images were obtained by light microscopy except **c.** **a** Mature gamonts in association. **b** Detail of the bowl-shaped association interface (acetabulum). **c** Scanning electron micrograph view of the acetabulum, which shows some shallow grooves. Such a slightly wrinkled surface is not visible under photonic microscopy. **d** Indehiscent gametocyst as observed in feces. **e** Dehiscent gametocysts with multiple sporoducts. **f** Wet mount of an oocyst chain. *Arrow* points to a residual body; *arrow with* "*pp*" denote polar plates

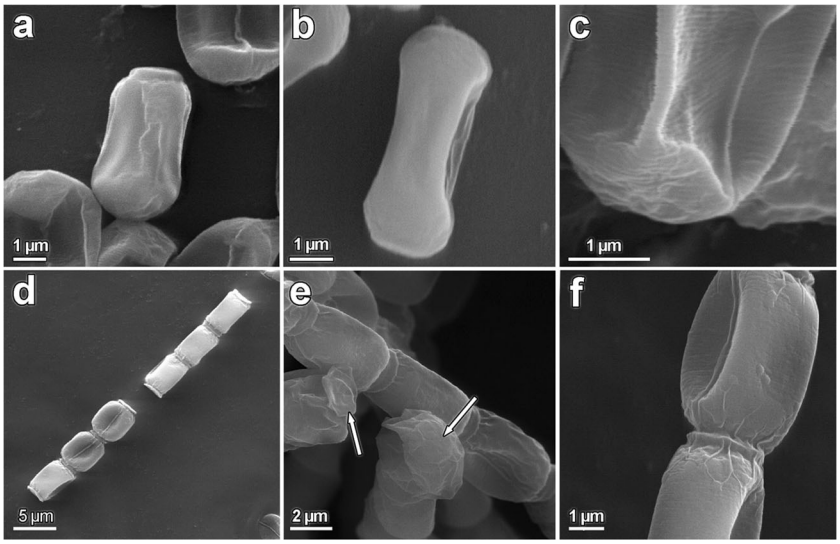

Fig. 4 Scanning electron micrographs of the oocyst stage of *G. ormierei*. **a** Dorso-ventral view of an oocyst of the small phenotype. No polar plates are evident. **b** Lateral view of a small phenotype oocyst with prismatic shape. **c** Pole of a small phenotype gregarine oocyst at high magnification. A fine circumferential striation is evident. No polar plate or pore can be observed. **d** Short broken chain of oocysts of the large phenotype gregarine. **e** Group of oocysts of the large phenotype gregarine. Two of them show polar plates in frontal or fronto-lateral view, without evidence of pore or micropile (*arrows*). **f** View of the oocyst surface (large phenotype) at high magnification. As in the small phenotype oocysts, a very fine circumferential striation is observed

G. basiconstrictonea but it is an independent species, located within the gregarine clade. On the other hand, the new *Gregarina* sp.-AD from crustaceans also groups with other species within *Gregarina*, particularly *G. cloptoni*. Finally, it is fairly evident that *Stylocephalus* and *Xiphocephalus* are paraphyletic.

Discussion

Gregarine biogeography

The present work is the first systematic survey for gregarines conducted in a significant number of arthropods in Spain. Diverse gregarines (including a possible new species) and some new hosts are being reported here for the first time for this country, thus providing a significant advancement in the current knowledge on the biogeography of these apicomplexans.

In Table 4, we present an overview of the literature concerning the gregarine species found in Spain. It can be inferred that *G. ormierei* has low parasitic specificity and wide distribution in the Old World. *S. gigas* shows a similar broad geographical distribution to that of *G. ormierei* but linked only in two coleopteran genera in Europe and North America. It is unclear whether the absence of this species in other parts of the World was due to true absence or to lack of sampling in adequate hosts.

In agreement with previous findings by other authors (Table 4), *O. hispanus* seems to be an occasional parasite of Tenebrionid beetles in the Mediterranean basin. Extensive studies on gregarines of dermapterans have been done in Europe but sparsely in other geographic locations. As pointed out by Tuzet and Ormieres (1956), in France, only earwig larvae were heavily infected but adults were not, whereas in Spain, low intensity of infection was noted in adults with moderate prevalence. With reference to literature review (Table 4), these findings agree that *G. ovata*, and to a lesser extent *G. chelidurellae*, are well represented in most earwig communities in Europe. Likewise, *A. permagnus* shows a relatively wide distribution range in Europe and North Africa (Table 4), usually associated to *Carabus* spp. and sometimes to *Cathoplius* spp. beetles.

Although gregarines recovered from *A. desmaresti* in Spain are likely to belong to a new species, a complete description was not possible due to the lack of data on gametocyst and oocyst morphology. *Gregarina* sp.-AD is smaller than *U. gammari* and *H. longissima*, yet both species showed resemblance in the gamont stage. The same is true for *Ganymedes themistos*, an aseptate gregarine with associations often bearing a ball-like bulge in the primite (Prokopowicz et al. 2010). Sprague and Couch (1971) and more recently Desportes and Schrevel (2013), in their respective reviews of gregarines of crustaceans, did not report any infections caused by *Gregarina* spp. Hence, the gregarine of *A. desmaresti* may be considered the first member of *Gregarina* ever reported in crustaceans.

In the present work, gregarines were not found in some insects (namely *S. tristis* and *O. olens*), possibly due to a small sample size. But in *T. molitor*, no gregarines were discovered when only adults were surveyed, which is in

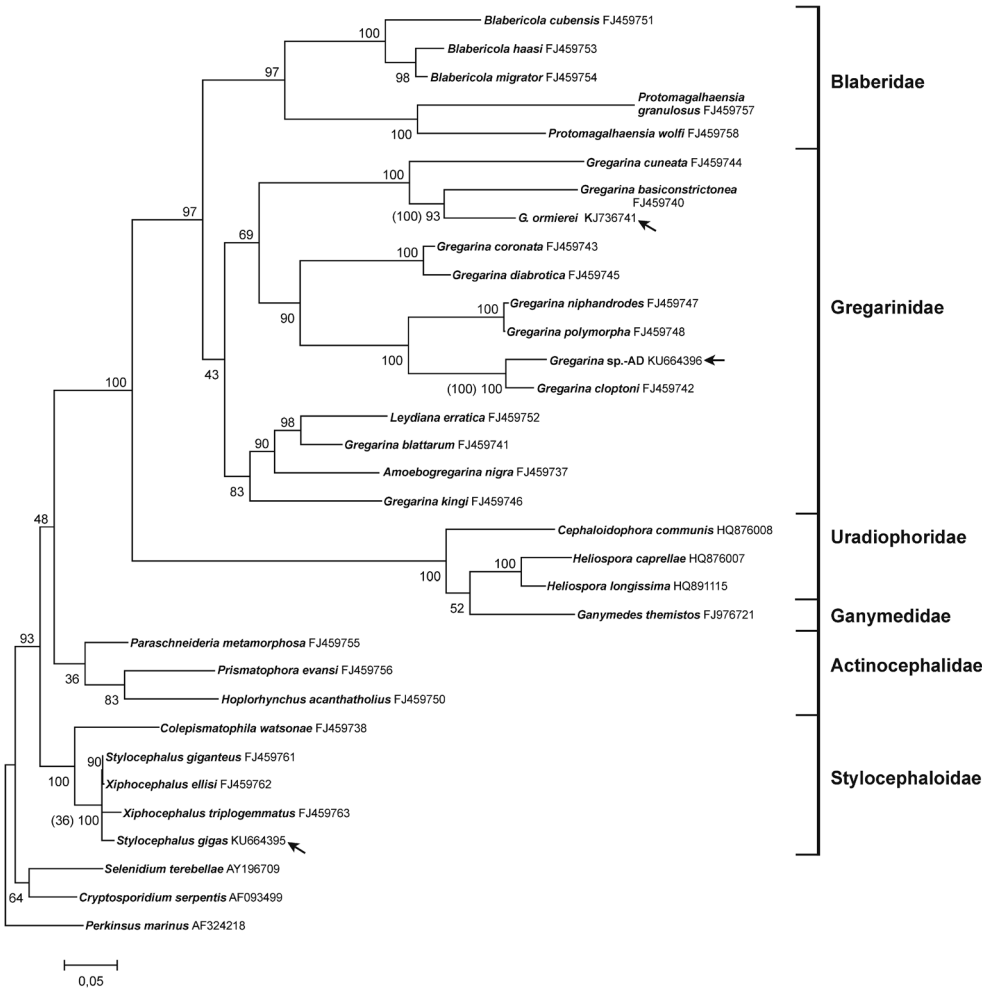

Fig. 5 Phylogenetic relationship of *Perkinsus marinus* (as outgroup), *Cryptosporidium serpentis* and 31 gregarine species based on the DNA sequence variation of the 18S rRNA gene (MEGA 6 program). GenBank® accession numbers are indicated after each name. The tree was inferred by using the maximum likelihood (with Hasegawa-Kishino-Yano substitution model and nearest neighbour interchange for tree inference). *Numbers at nodes* indicate bootstrap support with 100 replications. *Numbers in parenthesis* at the nodes of the new sequences show bootstrap support obtained with the maximum parsimony method. The gregarine isolates sequenced in Spain are indicated by *arrows*

agreement with a former report by Stein (1848). However, some studies have shown that adult beetles are sometimes infected by *G. cuneata* and *G. niphandrodes* in the USA (Clopton et al. 1991).

Redescription of *G. ormierei* Theodorides, 1955: remarks

A complete description of *G. ormierei* based on modern shape parameters (Clopton 2004) has been provided in this study. However, it is important to mention, in agreement with Rueckert et al. (2011), that taking measurements of multiple dimensions of different trophozoites is potentially uninformative due to phenotypic variability. Such variability has been reported earlier in other gregarines by Filipponi (1952). However, the present data confirm that both the feeding stages (trophozoite with epimerite) and the resistant stage (oocyst) are relatively uniform in size and shape. Hence, they should be preferentially used for morphological recognition of species, according to Filipponi (1952, 1953), Clopton et al. (1991) and Janovy et al. (2007). In contrast, dehiscence is unlikely an important trait in species definition, since *G. ormierei* shows two different sporulation mechanisms (single sporoduct or multiple sporoducts). Accordingly, similar dual dehiscence mechanisms have been reported in the gregarine *Boliviana floridensis*, depending on environmental conditions (Johny and Whitman 2005).

Theodorides (1955a) described *G. ormierei* in adults of *G. rusticum* in France; however, the morphology of gametocysts and oocysts for this species was not included in that study. Later, Theodorides (1958b) observed that young trophozoites in *T. taurica* possessed an orbicular epimerite. Theodorides (1955a) pointed out that *G. ormierei* was similar but distantly related to *G. cavalierina*. The former species (*G. ormierei*) could be recognized by the following morphological details: cytoplasm filled with clear granules, protomerite variable in shape (flattened to irregular), deutomerite more oblong than in *G. cavalierina*, association with a bowl-like acetabulum, spherical epimerite and compact nucleus in young trophozoites. In *S. punctatus*, the gregarine showed traits similar to that of (*G. ormierei*), having been found in two different coleopteran families (Tenebrionidae and Cetonidae). Furthermore, Theodorides (1955a and 1958a) and Theodorides et al. (1964, 1965 and 1976) reported the presence of this gregarine in different hosts widely distributed geographically. The slight

Table 4 Biogeography of the gregarine species reported in the present survey in Spain (literature review)

Gregarine species	Host genus	Geographic area	Countries	References
Gregarina ormierei	*Gonocephalum*, *Mesomorphus*	Africa	Sudan, Ethiopia	Theodorides et al. (1964) Theodorides et al. (1965)
	Tentyria	Asia	Turkey, South Korea	Theodorides (1958a) Theodorides et al. (1976)
	Gonocephalum, **Scaurus**, **Cetonia**	Europe	France, **Spain**	Theodorides (1955a) Present work
Gregarina ovata	*Forficula*	Europe	France, Germany, Poland, England, **Spain**	Dufour (1828); Tuzet and Ormieres (1956); Wellmer (1911); Lipa (1967), Ball et al. (1986) Present work
Gregarina chelidurellae	*Forficula*	Europe	Germany, Slovenia, **Spain**	Geus (1969) Devetak et al. (2013) Present work
Gregarina sp.-AD	**Atyaephyra**	Southern Europe?	**Spain**	Present work
Actinocephalus permagnus	*Carabus*	Europe	Germany, France, Poland, Slovenia, **Spain**	Wellmer (1911), Theodorides 1954, Sienkiewicz and Lipa (2009), Devetak et al. (2013) Present work
	Cathoplius	North Africa	Morocco	Theodorides (1955b)
Oocephalus hispanus	*Morica*	Mediterranean Basin	Spain, Morocco	Schneider (1886) Theodorides (1955b)
	Akis	Mediterranean Basin	Spain	Present work
Stylocephalus gigas	*Blaps*	Southern Europe	Italy, France, **Spain**	Filipponi (1949) Theodorides (1956) Present work
	Eleodes	North America	USA	Nelson (1970)

In reference to the present work, "Spain" in boldface indicates that the corresponding gregarine genus has been found in this country for the first time, whereas an arthropod genus in boldface indicates that it is the first finding in this host

morphological differences between *G. ormierei*, particularly the large phenotype in Spain and elsewhere, were indeed the result of phenotypic variability, as revealed by molecular data in individual gametocysts. Since analysis of DNA samples obtained in both phenotypes yielded identical sequences of the 18S rRNA gene, apparently one genotype is expressed as two different phenotypes. This conclusion is based on the fact that amplifications performed on individual gametocysts used DNA derived from a single cell. This strategy circumvents possible problems of mixing the trophozoites or gamonts of two distinct gregarine species when pooled together in one sample and also avoids differential PCR amplification when two organisms are present in pooled gametocyst samples. In spite of that fact, it should be noted that additional data on the sequences of the intergenic spacers (ITS1 and ITS2) of the 18S rRNA gene, plus the first domain of the 28S rRNA gene, would be indispensable to obtain conclusive evidence that both phenotypes belong to the same species. Such ribosomal sequences are very reliable for interspecific discrimination of apicomplexans (Motriuk-Smith et al. 2011).

In the unlikely event that phenotypes would be classified into distinct species, the specific name of *G. ormierei* should be retained and conserved for the large phenotype, whereas a new specific name could be proposed for the small phenotype. This idea is based on published guidelines related to genus subdivision: "when a genus is subdivided into other genera, the original name should be retained for that portion of it which exhibits in the greatest degree of essential characters as at first described" to quote Jardine (2011).

Molecular studies on the 18S rRNA gene and gregarine phylogeny

Phylogenetic analysis clearly separated *G. ormierei* from other *Gregarina* spp., in agreement with the original description of Theodorides (1955a). However, the present study has shown that *G. ormierei* is morphologically similar to *G. cavalierina*. In consequence, future research should be undertaken to further investigate and confirm if they are different taxonomic entities.

S. gigas is closely related to organisms included in two different genera. Such paraphyly between *Stylocephalus* and *Xylocephalus* has already been pointed out by Clopton (2009). Taxonomic problems in this group cannot be addressed until

more information on 18S rRNA gene sequences is gathered in other species.

The use of molecular phylogeny corroborated that the *Gregarina* sp.-AD of Spanish crustaceans is not close to *Uradiospora*, *Heliospora* or *Ganymedes*, but rather to *G. cloptoni*. Moreover, such genetic relationship is also confirmed by morphological features. From descriptions of trophozoites of *G. cloptoni* by Janovy et al. (2007), it is evident that some similarities exist in the structure of the anterior region of *Gregarina* sp.-AD and *G. cloptoni*. There is a hyaline region in the apical part of the protomerite, albeit the latter is less marked in *G. cloptoni* as compared to the Spanish gregarine.

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