Gregarines (Apicomplexa, Gregarinasina) in psocids (Insecta, Psocoptera) including a new species description and their potential use as pest control agents.

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Abstract:

Gregarine apicomplexans are unicellular organisms that infect invertebrate hosts in marine, freshwater and terrestrial habitats. The largest group of invertebrates infested on land is the insects. The insect order Psocoptera (booklice) has recently gained wider interest due to specimens occurring in stored food products and therefore being considered pest organisms. Biological control agents are often used to eliminate pest organisms. In this study we examined the psocid *Dorypteryx domestica*, an invasive psocid species that is spreading all over the world. We were able to isolate and describe a new gregarine species (*Enterocystis dorypterygis* sp. n.) infecting *D. domestica*. The trophozoites are panduri- or pyriform and their association/syzygy is caudo-frontal. The surface is inscribed by longitudinal epicytic folds covering the complete cell. Phylogenetic analyses of the SSU rDNA gene revealed an only weakly supported relationship with two *Gregarina* species *G. ormieri* and *G. basiconstrictonea*, both from tenebrionid beetles. Gregarines have been proposed to have some potential as biological control agents for several insects. Identifying the gregarine species infecting pest organisms like psocids is a first step and prerequisite for the probable utilization of these parasites as biological control agents in the future.

Keywords

Apicomplexan parasites, Enterocystidae, phylogeny, biological control agent

Introduction

Psocoptera is an order of small soft-bodied hemimetabolous insects commonly called psocids, barklice or booklice. There are around 2,000 species described in the world. The geographical origin of many domestic species remains unknown, as psocids have been transported by humans, via the holds of ships, in packing materials and trade goods (New, 1987). *Dorypteryx domestica* (Smithers, 1958) is an interesting psocid species that was originally described from human habitations in Zimbabwe, Africa and is an invasive species spreading all over the world since 1973 (Lienhard, 1977). Since then it has been detected from at least 16 European countries.

A considerable number of psocids occur in buildings, such as human dwellings, food stores, warehouses and granaries (Baz and Monserrat, 1999; Mockford, 2003). In general, Psocoptera feed on algae, fungi, lichens, particles of organic debris, small eggs and dead bodies of insects (Mockford, 2003). Some species occur typically in domestic environments such as humid rooms, basements, damp walls where they feed on fungal hyphae and spores of moulds (Baz and Monserrat, 1999). A few psocid species occurring in buildings feed on the paste and bindings of old books as well as on the fungal spores and hyphae, which invade the pages (Mockford, 2003). In human dwellings, the main effect due to psocopteran presence in/on buildings is lowering property values (New, 1987). In Spain for example, three psocid species – one of them was *Dorypteryx domestica* – have been described as one cause for the propagation of the alga *Pleurococcus* on recently constructed buildings (Baz and Monserrat, 1999), which is at least disfiguring, but might also have effects on the decay of the building material.

This insect order has only recently become of greater interest, when a large number of specimens have been found in stored products. Even though the infestation of food products by psocids rarely causes health problems in humans such as asthmatic reactions and allergies from house dust (Spieksma & Smits, 1975; Mockford, 2003) or skin diseases (Conci and Franceschi, 1953; Agostini et al., 1982), it is still unhygienic and certain psocid species can even serve as intermediate hosts of some ruminant infecting cestodes (Svadzhian, 1963; Kuznetsov, 1966).

Some psocids, like *Dorypteryx domestica*, may occasionally become a nuisance in habitations, or play a role as pest insects in stored food products. Parasites and predators are known be important natural regulators of pest population densities in some pest insects. So far, endoparasites (gregarines, cestodes, nematodes and fungi), ectoparasites (acari) and parasitoids (Hymenoptera) have been reported for psocids. As parasites can shape the community structure of their host organisms, it is important to gain knowledge on these parasites. Records on the occurrence of for example gregarines in psocids are sparse (e. g. Geus, 1969; Sarkar and Haldar, 1980; Devetak et al., 2013) and there is no literature about protozoan pathogens found in natural populations of *Dorypteryx domestica*. In general gregarine apicomplexans infect marine, freshwater and terrestrial invertebrates. Due to the sheer number of insects in terrestrial habitats most eugregarines are described from terrestrial hosts. However, gregarines have been reported from less than 1% of all invertebrate species, leaving the gregarine fauna of 99% of potential invertebrate hosts to be discovered (Clopton, 2000). Even though most gregarine species are described from insects, there are only five gregarine species described from around 12 psocopteran species (two only identified to genus level) belonging to 10 genera in Germany, Switzerland and India. There are four species of septate gregarines (*Hyalospora psocorum*, *H. stenopoci*, *Liposcelius coronata* and *Ancyrophora similis*) belonging to two families (Hirmocystidae Grassé, 1953; Actinocephalidae Léger, 1892) and three genera (*Hyalospora* Schneider, 1875; *Liposcelius* Sarkar & Haldar, 1980; *Ancyrophora* Léger, 1892). There is also one aseptate gregarine

(*Enterocystis bengalensis*) of the family Enterocystidae Codreanu, 1940 and the genus *Enterocystis* Zwetkow, 1926, known to infect psocids (Sarkar, 1983; Desportes and Schrével, 2013). All available descriptions of these species are based on line drawings only (von Siebold 1839, Geus, 1969; Sarkar and Haldar, 1980; Sarkar, 1983). There are no ultrastructural or molecular data available for the species infecting Psocoptera hosts (Desportes and Schrével, 2013).

In the current study we set out to investigate *Dorypteryx domestica* from Slovenia for the presence of gregarines. We studied the general morphology and phylogenetic position of the gregarine found to parasitize *D. domestica* and we discuss the possibility of employing gregarines as biological control agents.

Material and Methods

Collection and isolation of organisms

Specimens of *Dorypteryx domestica* were collected by the second author from a basement of a house in Maribor, Slovenia (46°33'58.5"N 015°39'15.2"E). The gut content was released in 0.9% saline solution by teasing apart the intestines of the psocid with fine-tipped forceps under a dissecting microscope (Zeiss Stemi 2000). The gut material was examined under an inverted microscope (Zeiss Axiovert A1) and parasites were isolated with a handdrawn glass pipette and washed three times in 0.9% saline solution, before being examined and photographed under the inverted microscope or prepared for DNA extraction.

Light and scanning electron microscopy

Light micrographs of specimens were taken with a digital camera Nikon DN100 attached to a microscope (Nikon E 800). Differential interference contrast (DIC) light micrographs were taken with a 5 megapixel CMOS camera AxioCam Erc 5s, attached to an inverted microscope (Zeiss Axiovert1).

Between 10 and 20 specimens of the isolated gregarine species were prepared for scanning electron microscopy (SEM). Individuals were deposited directly into the threaded hole of a Swinnex filter holder, containing a 10 µm polycarbonate membrane filter (Millipore Corp., Billerica, MA), that was submerged in 10 ml of 0.9% saline solution within a small canister (2 cm diam. and 3.5 cm tall). A piece of Whatman No. 1 filter paper was mounted on the inside base of a beaker (4 cm diam. and 5 cm tall) that was slightly larger than the canister. The Whatman filter paper was saturated with 4% (w/v) OsO_4 and the beaker was turned over the canister. The parasites were fixed by OsO_4 vapors for 30 min. Ten drops of 4% (w/v) OsO_4 were added directly to the saline solution and the parasites were fixed for an additional 30 min. A 10-ml syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. Filters were washed with water and dehydrated with a graded series of ethyl alcohol. They were critical point dried with CO_2. Filters were mounted on stubs, sputter coated with 5 nm of gold, and viewed under a scanning electron microscope (Hitachi). Some SEM data were presented on a black background using Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA).

DNA isolation, PCR, cloning, and sequencing

Two individual trophozoites were isolated from the dissected hosts, washed three times in saline solution, and deposited into a 1.5-ml microcentrifuge tube. DNA was extracted using the MasterPureTM Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Small subunit rDNA (SSU rDNA) sequences were PCR-amplified using a total volume of 25µl containing 2 µl of primer, 2.5 µl of DNA template, 20.5 µl of dH2O and one PuReTaq Ready-to-go PCR Bead (GE Healthcare, Quebec, Canada). The SSU rDNA sequences from these species were amplified in one fragment (~1800 basepairs) using universal eukaryotic PCR primers F1 5´-GCGCTACCTGGTTGATCCTGCC-3´ and R1 5´-GATCCTTCTGCAGGTTCACCTAC-3´. PCR was performed using the following protocol: After 4 cycles of initial denaturation at 94 °C for 4.30 min, 45 °C for 1 min and 72 °C for 1.45 min, 34 cycles of 94 °C for 30 sec (denaturation), 50 °C for 1 min (annealing), 72 °C for 1.45 min (extension), followed by a final extension period at 72 °C for 10 min. PCR products corresponding to the expected size were gel isolated using the UltraCleanTM 15 DNA Purification kit (MO Bio, Carlsbad, California) and cloned into the pSC-A-amp/kan vector using the StrataClone PCR Cloning Kit (Stratagene, AgilentTechnologies, California). Eight cloned plasmids were digested with EcoRI and screened for size. Two clones were sequenced with ABI big dye reaction mix using vector primers and internal primers oriented in both directions using the cycle sequencing technology on an ABI 3730XL sequencing machine (eurofins Genomics, Germany).

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The new SSU rDNA sequences were initially identified by BLAST analysis and subsequently verified with molecular phylogenetic analyses (GenBank Accession number for *Enterocystis dorypterygis* sp. n.: KY697695).

Molecular phylogenetic analysis

The new SSU rDNA sequence was aligned with 116 other SSU rDNA sequences, representing the major lineages of gregarines (with an emphasis on terrestrial gregarines and environmental sequences) and dinozoans as relevant outgroup. The 117-sequence alignment was subsequently edited and fine-tuned using MacClade 4.08 (Maddison and Maddison, 2005). The program PhyML (Guindon and Gascuel 2003) was used to analyze the 117-

sequence alignment (774 unambiguously aligned sites; gaps excluded) with maximum-likelihood (ML). Smart Model Selection selected a general-time reversible (GTR) model of nucleotide substitutions (Posada and Crandall, 1998) that incorporated invariable sites and a discrete gamma distribution (six categories) (GTR + G+ I + F model: $\alpha = 0.725$ and fraction of invariable sites = 0.187) under the Akaike Information Criterion (AIC) (Guindon et al., 2010). ML bootstrap analyses were performed on 500 pseudoreplicates, with one heuristic search per pseudo-replicate (Zwickl, 2006), using the same program set to the GTR model +G + I + F. Bayesian analysis of the 117-sequence dataset was performed using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The programme was set to operate using the following parameters: nst=6, ngammacat=5, rates=invgamma. Parameters of Metropolis Coupling Markov Chains Monte Carlo (mcmc) were set to: nchains=4, nruns=4, temp=0.2, ngen=7000000, samplefreq=100, burninfrac= 0.5 (the first 50% of 70000 sampled trees, i.e. the first 35000, were discarded in each run). The computation was performed on the CIPRES Science Gateway V 3.3 (Miller et al., 2010).

Results

Of 31 investigated psocopteran specimens, 21 were infected with gregarines, giving an infection prevalence of 68%. The mean intensity of infection was 13 gregarines per host with a range of 6-24 gregarines per host.

Morphology of *Enterocystis dorypterygis* sp. n.

Enterocystis dorypterygis sp. n.: Trophozoites were isolated from the psocid *Dorypteryx domestica* (Smithers, 1958) (Psocoptera; Psyllipsocidae). The cell morphology showed general similarities with *Enterocystis bengalensis* from a psocid species (*Psocathropos* sp.)

described by Sarkar (1983). The cells were elongated and panduriform with a little indentation at the anterior end of the cell, but no septum (Fig. 1). Trophozoites were 59.3 ± 6.9 µm (45.6– 69.0 µm, n = 35) long (mean ± SD; min–max; number) and 25.4±2.5 µm (21.5–30.7 µm, n = 35) wide at their widest part. The anterior end was mostly a bit globular and rounded, while the posterior end was more blunt (Fig. 1a-c). Some of the cells were more pyriform with a long anterior neck-like region (Fig. 1a). The round nucleus [7.8 (5.6-10) µm x 8.7 (6.5-11), n = 21] was situated in the posterior half of the cell (Fig. 1a-b), but sometimes shifted to the anterior half (Fig. 1c-d). Gametocysts were spherical and the diameter was 30 µm (25.5-39.2 µm; n=4). Associations between mature trophozoites (or gamonts) appear to be caudo-frontal (Fig. 1c-d). The SEM micrographs demonstrated that the whole cell surface was inscribed by longitudinal epicytic folds (~200) (Fig. 1e). Neither the anterior nor the posterior end was free of folds (Fig. 1e-f). The epicytic folds appeared to be arranged in waves along the longitudinal axis (Fig. 1e, g). In the middle of the cell, the density of folds was 6-8 folds/micron (Fig. 1g). Trophozoites were stiff and capable of gliding movements.

Molecular phylogeny of *Enterocystis dorypterygis* sp. n.

Molecular phylogenetic analyses of the 117-sequence dataset produced a tree topology with a moderately supported clade of dinoflagellates (outgroup) and a moderately supported clade of apicomplexan sequences (Fig. 2). The deeper branches within the tree were all poorly resolved. Within the apicomplexans three clades were formed, consisting of (1) piroplasmids, coccidians, cryptosporidians, rhytidocystids, monocystids, neogregarines and mainly terrestrial eugregarines, (2) archi- and eugregarines from mainly polychaete hosts and (3) mainly eugregarines from ascidian, polychaete, nemertean and crustacean hosts. The new sequence of *Enterocystis dorypterygis* sp. n. clustered within the strongly supported clade of mainly terrestrial eugregarines comprised of the genera *Amoebogregarina*, *Gregarina*, *Leidyana*, *Protomagalhaensia* and the as archigregarine considered *Caliculium*. The new sequence formed a low supported clade with two *Gregarina* species *G. ormieri* and *G. basiconstrictonea* both from tenebrionid beetles. Those three sequences formed a sistergroup to the strongly supported clade of *Leidyana* and *Protomagalhaensia* species.

Discussion

The majority of eugregarine species are reported from insects. Most of these belong to the septate gregarines (trophozoite devided into protomerite and deutomerite by a septum) and only a few belong to the aseptate gregarines (trophozoite composed of single compartment lacking the septum). As they have been reported from less than 1% of the known insect species the gregarine fauna of over 99% is still to be discovered (compare Clopton, 2000). A good example here are the Psocoptera, of the ~2000 described psocid species, only 10 have been reported to be infected with gregarine apicomplexans. In this study we were able to describe a new gregarine species (*Enterocystis dorypterygis* sp. n.) from a new psocid host species (*Dorypteryx domestica*).

Enterocystis dorypterygis sp. n.

The new gregarine species isolated from the psocopteran *D. domestica* is an aseptate gregarine, and a first record of a gregarine infection in this psocid. The characteristic of a missing septum distinguishes the new species from all described septate gregarines (*Hyalospora psocorum*, *H. stenopoci*, *Liposcelius coronata* and *Ancyrophora similis*) infecting Psocoptera (nine species including: *Peripsocus alboguttatus*, *Amphigerontia bifasciata*, *Psocus longicornis*, *P. quadripunctatus*, *Caecilius flavidus*, *Lachesilla quercus*, *Mesopsocus unipunctatus*, *Graphopsocus cruciatus*, *Stenopsocus immaculatus*). All nine Psocoptera species are actually infected by *H. psocorum* (Geus, 1969), whereas all other sepatate gregarine species are reported from just a single psocid host species (Desportes and Schrével, 2013). There is only one known aseptate gregarine species (*Enterocystis bengalensis*), which belongs to the family Enterocystidae Codreanu, 1940, and was described by Sarkar (1983) from *Psocathropos (syn. Psocatropos)* sp. in India. Desportes (2013) questions this identification and new species description as all other species belonging to the family Enterocystidae infect the aquatic larvae of Ephemoptera and not any Psocopteridae. The psocid genus infected with *E. bengalensis* is falsly named as *Psocoptrips* sp. in Desportes (2013). The families/genera/species of aseptate gregarines known to infect terrestrial and freshwater invertebrates, are all recorded from other invertebrates than Psocoptera. The newly described gregarine species is most similar to *Enterocystis bengalensis* than any other *Enterocystis* species, based on the morphology of the trophozoites and associations. Due to its smaller size and the different host species the described gregarine is considered a new *Enterocystis* species.

Molecular phylogeny of *Enterocystis*

Up to this date there is no reference sequence of any *Enterocystis* species available in any of the public databases such as GenBank. The sequence of our newly described species *E. dorypterygis* sp. n. clustered within the highly supported clade of mainly terrestrial gregarines (Fig. 2). The closest relatives were species of the genus *Gregarina* infecting Tenebrionidae, *Leidyana* and *Protomagalhaensia* both infecting Blattaria, all of which are septate eugregarines. This is an example of another aseptate eugregarine clustering within a clade of septate eugregarines, all infecting arthropods and in this case insects. It has been questioned before, if the separation of septate and aseptate gregarines, estblished by Chakravarty (1959) reflects the actual phylogenetic relationships of eugregarines (e.g. Rueckert et al., 2011). The current study and the study by Rueckert et al. (2011) clearly show that the septate eugregarines do not form a monophyletic clade. Therefore, the taxonomic separation of sepatate and aseptate eugregarines based on a morphological feature (the septum) should be deemed invalid. What still remains uncertain is the evolutionary history of certain septate and aspetate gregarines infecting insects. At the moment there are two possible scenarios: i) according to Leander (2006, 2008) a lecudinid stem group gave rise to all other eugregarine lineages and the eugregarines of insects became comparmentalized by forming a transverse septum between cell regions, so there might be some lineages that remained without a septum or ii) it could be a loss of the septum giving rise to secondary aseptate gregarines as was suggested by Grassé (1953). The latter one also indicating that the septum is not a reliable character in gregarine taxonomy. One example of such an aseptate gregarine in an insect is the genus *Gamocystis*, which only presents a septum in a very early trophozoite stage (Clopton, 2000; Desportes and Schrével, 2013). No septum was detected in any of the observed trophozoit stages of *E. dorypterygis* sp. n. in this study.

Based on our current knowledge we have decided to assign the gregarine species infecting the psocopteran *Dorypteryx domestica* to the genus *Enterocystis.* The two species *Enterocystis dorypterygis* sp. n. and *E. bengalensis* will be validated, as soon as molecular sequence data of the type species of *Enterocystis* or any other species belonging to this genus become available.

Gregarines as possible biological control agents

Insects encompass a great number of pest species and so far several have been recorded as hosts of gregarine apicomplexans. The present knowledge on the interactions between pest insects and their pathogens is still insufficient. Gregarines infecting mosquitos have been mentioned in the literature as potential biological control agents with opposing views for a few decades (Lantova and Volf, 2014). Whereas, the possibility of utilizing gregarines as pest control agents in cockroaches, grasshoppers, fleas, beetles and flies has only recently entered any form of discussion.

Lopes and Alves (2005) for example tested the effect of gregarines on the susceptibility of cockroaches towards control measures. *Blatella germanica* adults infected with gregarines were more susceptible to the treatment with the fungus *Metarhizium anisopliae* and triflumuron than healthy cockroaches. Studies have shown that gregarines have an impact on fecundity, feeding and mortality rates in economically important grasshopper species, justifying the utilization of gregarine apicomplexans in biological control monitoring of these pest species (Pushkala and Muralirangan, 1997; Johny et al., 2000). Cat fleas, *Ctenocephalides felis*, are infected with the gregarine *Steinina ctenocephali* and Alarcon et al. (2011) were able to confirm its potential as biological control agent for this cat parasite. A few studies have also been carried out on beetles. Due to their high infection rate in the grey corn weevil, *Tanymecus dilaticollis*, gregarines play a role as natural regulators of the beetle’s population density (Takov et al., 2013). A few gregarines species have been reported in bark beetles (Curculionidae: Scolytinae) to date (Takov et al., 2011; Pernek et al., 2009; Yaman and Baki, 2010). Consequently, they could play a role as pest control agents against bark beetles in the future.

Among Diptera or flies, phlebotomine sand flies (Psychodidae) and mosquitos (Culicidae) are important vectors of human pathogens. Gregarines infecting phlebotomine flies of the genera *Lutzomyia* and *Phlebotomus* were studied (Lantova et al., 2011; McCarthy et al., 2011) and the results suggested that they could possibly be an efficient control method of phlebotomine populations. Despite the fact that gregarines increased the mortality of immature stages in *Phlebotomus sergenti* and negatively affected the survival of adult flies, their potential for use in pest control is questionable as a result of several factors, including this pathogen’s strict host specificity (Lantova et al., 2011). While the study of gregarines in *Lutzomyia longipalpis*, the vector of visceral leishmaniasis, suggested that they are a possible efficient control agents under natural conditions (McCarthy et al., 2011).

In mosquitoes, the susceptibility of *Culex bitaeniorhynchus* to two species of *Ascogregarina* parasites naturally infecting *Aedes* mosquitoes was determined (Mourya and Soman, 2000). The gregarines caused high mortality of *Culex* mosquitoes, but were not able to complete their life cycle in the unnatural hosts. The survival of infected mosquitoes was significantly reduced.

Gregarines do infect numerous invertebrates that are classified as pest organisms due to their negative impact on for example crops and human health. Understanding the gregarine-host interactions is crucial to make any progress in the possible utilization of gregarines to control aforementioned pests. The identification of gregarine species infecting these pest organisms examplified in this study by *E. dorypterygis* sp. n. infesting *Dorypterix domestica* is a first and essential step in that direction.

Taxonomic Summary

Superphylum Alveolata Cavalier-Smith, 1991

Phylum Apicomplexa Levine, 1980, emend. Adl et al., 2005

Class Conoidasida Levine, 1988

Subclass Gregarinasina Dufour, 1828

Order Eugregarinorida Léger, 1900

Family Enterocystidae Codreanu, 1940

Genus *Enterocystis* Zwetkow, 1926

Enterocystis dorypterygis sp. n. Devetak and Rueckert, 2017

Species diagnosis: Trophozoites elongated and panduriform, little indentation at anterior end, but no septum. Trophozoites on average 59.3 µm long and 25.4 µm wide. Anterior end globular and rounded, posterior end more blunt. Some cells pyriform with a long anterior neck-like region. Round nucleus situated in the posterior half of the cell, sometimes shifted to the anterior half. Gametocysts spherical, diameter 30 µm. Associations caudo-frontal. Cell surface inscribed by longitudinal epicytic folds (~200), including anterior and posterior end, arranged in waves along longitudinal axis. Density of folds 6-8 folds/micron. Trophozoites stiff, capable of gliding movements.

Type host: *Dorypteryx domestica* (Smithers, 1958) (Psocodea: Psyllipsocidae).

Site: Intestine.

Type locality: Maribor, Slovenia (46°33'58.5"N 015°39'15.2"E).

Type micrographs: Figs. 1a, e.

DNA sequence: Small subunit rDNA (Genbank Accession number: xxx).

Etymology: Species-group name *dorypterygis* is is a noun in the genitive case (Article 11.9.1.3 of the ICZN 1999) derived from the genus-group name *Dorypteryx*, referring to the host of the new *Enterocystis* species.

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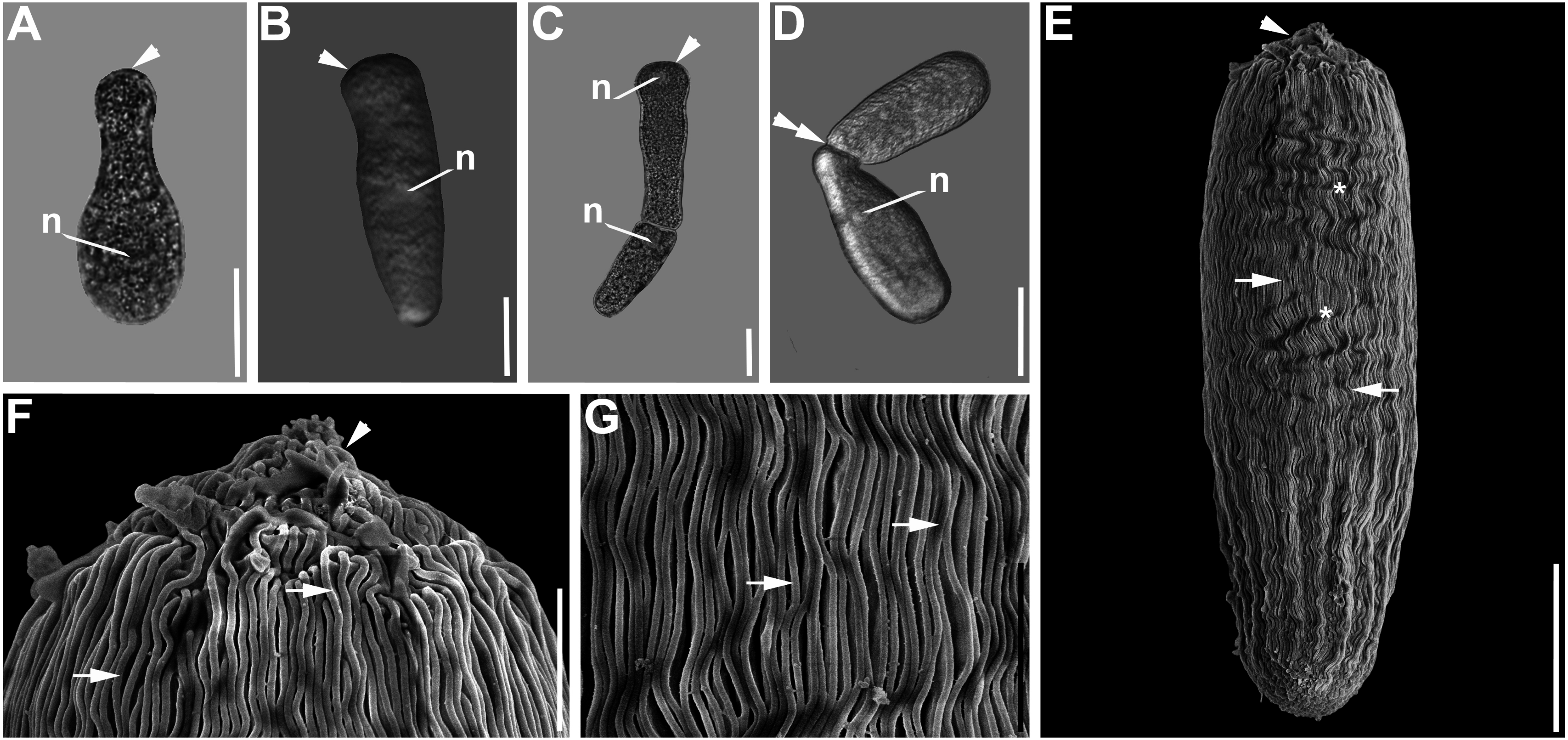
Figure legends

Fig. 1: Differential interference contrast (DIC) light micrographs and scanning electron micrographs (SEM) of *Enterocystis dorypterygis* sp. n. from psocopteran *Dorypteryx domestica*. A-B) Differently shaped trophozoite cells with a flattened or rounded anterior end (mucron, arrowhead). The nucelus (n) is visible in the posterior half of the cell. C-D) Two

gamonts in caudo-frontal syzygy. The nucleus (n) here lies in the anterior part of the cell. The attachment zone is marked with a double arrowhead. E) SEM of a trophozoite cell showing epicytic folds (arrow) running longitudinallyand undulating (asterisk) along the whole cell including the mucron area (arrowhead). F) Higher magnification SEM of the anterior end with epicytic folds covering the mucron area. G) Higher magnification SEM of the longitudinal epicytic folds (arrows). Scale bars: A – 25µm; B – 15µm; C – 25µm; D – 35µm; E – 10µm; F – 2.5µm; G – 2.5µm.

Fig. 2: Phylogenetic tree of gregarine apicomplexans using dinoflagellate species as outgroup. This gamma-corrected maximum likelihood tree (-ln L = 17107.63238, α = 0.725, fraction of invariable sites = 0.187, 6 rate categories) inferred using the GTR model of substitution on an alignment of 117 small subunit (SSU) rDNA sequences and 774 unambiguously aligned sites. Numbers at the branches denote bootstrap percentage (top) and Bayesian posterior probabilities (bottom). When both values were below 50% or 0.50 numbers were not reported. Black dots on branches denote Bayesian posterior probabilities and bootstrap percentages of 0.95/95% or higher.


A
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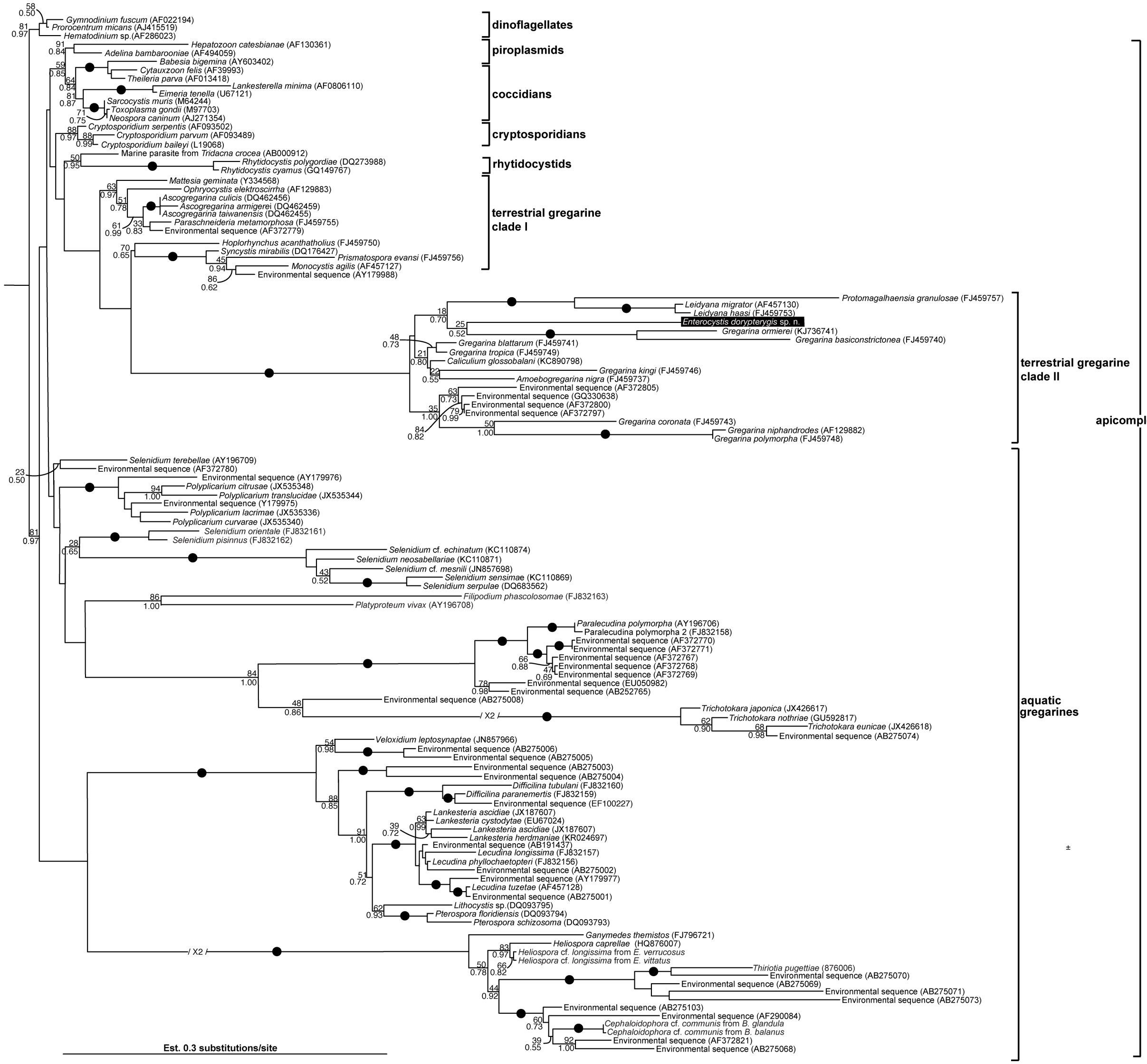
dinoflagellates
piroplasmids
coccidians
cryptosporidians
rhytidocystids
terrestrial gregarine clade I
terrestrial gregarine clade II
aquatic gregarines
apicomplexans
Gymnodinium fuscum (AF022194)
Prorocentrum micans (AJ415519)
Hematodinium sp.(AF286023)
Hepatozoon catesbianae (AF130361)
Adelina bambarooniae (AF494059)
Babesia bigemina (AY603402)
Cytauxzoon felis (AF39993)
Theileria parva (AF013418)
Lankesterella minima (AF0806110)
Eimeria tenella (U67121)
Sarcocystis muris (M64244)
Toxoplasma gondii (M97703)
Neospora caninum (AJ271354)
Cryptosporidium serpentis (AF093502)
Cryptosporidium parvum (AF093489)
Cryptosporidium baileyi (L19068)
Marine parasite from Tridacna crocea (AB000912)
Rhytidocystis polygordiae (DQ273988)
Rhytidocystis cyamus (GQ149767)
Mattesia geminata (Y334568)
Ophryocystis elektroscirrha (AF129883)
Ascogregarina culicis (DQ462456)
Ascogregarina armigerei (DQ462459)
Ascogregarina taiwanensis (DQ462455)
Paraschneideria metamorphosa (FJ459755)
Environmental sequence (AF372779)
Hoplorhynchus acanthatholius (FJ459750)
Syncystis mirabilis (DQ176427)
Prismatospora evansi (FJ459756)
Monocystis agilis (AF457127)
Environmental sequence (AY179988)
Protomagalhaensia granulosae (FJ459757)
Leidyana migrator (AF457130)
Leidyana haasi (FJ459753)
Enterocystis dorypterygis sp. n.
Gregarina ormierei (KJ736741)
Gregarina basiconstrictonea (FJ459740)
Gregarina blattarum (FJ459741)
Gregarina tropica (FJ459749)
Caliculium glossobalani (KC890798)
Gregarina kingi (FJ459746)
Amoebogregarina nigra (FJ459737)
Environmental sequence (AF372805)
Environmental sequence (GQ330638)
Environmental sequence (AF372800)
Environmental sequence (AF372797)
Gregarina coronata (FJ459743)
Gregarina niphandrodes (AF129882)
Gregarina polymorpha (FJ459748)
Selenidium terebellae (AY196709)
Environmental sequence (AF372780)
Environmental sequence (AY179976)
Polyplicarium citrusae (JX535348)
Polyplicarium translucidae (JX535344)
Environmental sequence (Y179975)
Polyplicarium lacrimae (JX535336)
Polyplicarium curvarae (JX535340)
Selenidium orientale (FJ832161)
Selenidium pisinnus (FJ832162)
Selenidium cf. echinatum (KC110874)
Selenidium neosabellariae (KC110871)
Selenidium cf. mesnili (JN857698)
Selenidium sensimae (KC110869)
Selenidium serpulae (DQ683562)
Filipodium phascolosomae (FJ832163)
Platyproteum vivax (AY196708)
Paralecudina polymorpha (AY196706)
Paralecudina polymorpha 2 (FJ832158)
Environmental sequence (AF372770)
Environmental sequence (AF372771)
Environmental sequence (AF372767)
Environmental sequence (AF372768)
Environmental sequence (AF372769)
Environmental sequence (EU050982)
Environmental sequence (AB252765)
Environmental sequence (AB275008)
/ X2 /
Trichotokara japonica (JX426617)
Trichotokara nothriae (GU592817)
Trichotokara eunicae (JX426618)
Environmental sequence (AB275074)
Veloxidium leptosynaptae (JN857966)
Environmental sequence (AB275006)
Environmental sequence (AB275005)
Environmental sequence (AB275003)
Environmental sequence (AB275004)
Difficilina tubulani (FJ832160)
Difficilina paranemertis (FJ832159)
Environmental sequence (EF100227)
Lankesteria ascidiae (JX187607)
Lankesteria cystodytae (EU67024)
Lankesteria ascidiae (JX187607)
Lankesteria herdmaniae (KR024697)
Environmental sequence (AB191437)
Lecudina longissima (FJ832157)
Lecudina phyllochaetopteri (FJ832156)
Environmental sequence (AB275002)
Environmental sequence (AY179977)
Lecudina tuzetae (AF457128)
Environmental sequence (AB275001)
Lithocystis sp.(DQ093795)
Pterospora floridiensis (DQ093794)
Pterospora schizosoma (DQ093793)
/ X2 /
Ganymedes themistos (FJ796721)
Heliospora caprellae (HQ876007)
Heliospora cf. longissima from E. verrucosus
Heliospora cf. longissima from E. vittatus
Thiriotia pugettiae (876006)
Environmental sequence (AB275070)
Environmental sequence (AB275069)
Environmental sequence (AB275071)
Environmental sequence (AB275073)
Environmental sequence (AB275103)
Environmental sequence (AF290084)
Cephaloidophora cf. communis from B. glandula
Cephaloidophora cf. communis from B. balanus
Environmental sequence (AF372821)
Environmental sequence (AB275068)
Est. 0.3 substitutions/site
±