Characterization of a coelomic gregarine parasite from *Thitarodes pui* (Lepidoptera: Hepialidae) in the Tibetan Plateau

Zixuan Sun a,b, Qingyun Peng c, Wenjing Wu b, Guren Zhang b,*

a School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang 212013, China
b State Key Laboratory for Biological Control, Sun Yat-sen University, Guangzhou 510275, China
c Food and Health Engineering Research Center of State Education Ministry, Sun Yat-sen University, Guangzhou 510275, China

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**A B S T R A C T**

*Thitarodes pui,* one of the host species of entomopathogenic fungus *Ophiocordyceps sinensis,* has great economic importance in the Tibetan Plateau. We report here, for the first time, a gregarine parasite found in the coelom of 7th instar and adults of *T. pui.* Gregarine gamonts (ovoid, ~15 × 8 μm) underwent syzygy to produce reproductive gametocysts in *T. pui* larval hemolymph. All infected *T. pui* carried 2–17 mature gametocysts filled with numerous oocysts (lemon-shaped, 17.17 ± 0.73 × 6.49 ± 0.4 μm). Transmission electron microscopy showed that these oocysts contained vacuoles of various sizes and amylopectin granules in the cytoplasm; scanning electron microscopy revealed a number of small bumps all over the surface of these oocysts. Small subunit ribosomal DNA sequence analysis showed a close relationship between the gregarine and the species of *Ascogregarina* (Eugregarinorida: Lecudinidae). Internal transcribed spacers and 5.8S ribosomal DNA from this gregarine exhibited 76% highest sequence identity with that from *Ascogregarina culicis* Ross.

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1. Introduction

Gregarines are a group of apicomplexans that parasitize the body cavity of invertebrates (Perkins et al., 2000). They are traditionally divided into three categories, archigregarines, eugregarines and neogregarines, based on their habitats, host range and trophozoite morphological features (Vivier and Desportes, 1990). Approximately 250 genera and 1650 species of gregarines have now been identified, but a more diverse gregarine fauna may remain to be discovered within the huge invertebrate world (Clopton et al., 1992; Rueckert et al., 2010). Many genera are restricted to certain groups of invertebrate hosts. The occurrence of most gregarines has so far been recorded for insects, including a wide variety of aquatic insects, many coleopterans and dipterans (Votý pka et al., 2009). Only a small percentage of gregarines have been reported as parasites of Lepidoptera.

*Thitarodes pui* (Zhang et al., 2007) is a beneficial lepidopteran (Lepidoptera: Hepialidae). Internal transcribed spacers and 5.8S ribosomal DNA from this gregarine exhibited 76% highest sequence identity with that from *Ascogregarina culicis* Ross.

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characters by electron microscopy; and revealed its phylogenetic position through analyses of SSU rDNA and internal transcribed spacers and 5.8S ribosomal DNA (ITS-5.8S rDNA) sequences.

2. Materials and methods

2.1. Field sampling

*T. pui* were collected around the Tibetan Plateau Peculiar Bio-resources Research Station of Sun Yat-sen University at Nyingchi in Tibet Autonomous Region (4156 m altitude, 29°36’N, 94°36’E) from June to August in 2011. Because of the plateau climate, this region has almost 7 months of snow each year, below 5°C of annual mean temperature, and above 650 mm of mean annual rainfall (Zhang et al., 2007). All *T. pui* individuals (Table 1) were collected from the fields and transported alive to the station. The larvae were grouped into different instars mainly by the width of head capsule, and were singly reared on carrots in plastic boxes (5.0 cm dia. and 3.5 cm height) filled with soil from their habitat. All captured *T. pui* insects were kept at 12–15°C with 41–72% relative humidity in a dark climate chamber until being tested.

2.2. Parasites examination

All the specimens (Table 1) were examined for gregarine parasites. The insects were surface-sterilized with 75% ethanol, punctured to collect the hemolymph into 50 μL of ice-cold N-Phenylthiourea-saturated phosphate-buffered saline (PBS, pH 7.4), and dissected in 0.85% NaCl physiological solution. Hemolymph and fat body samples were searched for gregarine parasites under microscope (Eclipse 80i & Eclipse Ti-U, Nikon Corp., Tokyo, Japan). Gregarines were identified as previously described (Perkins et al., 2000). Gregarine gametocysts were carefully isolated from the host coelom and washed in PBS. Fresh oocysts within the gametocysts were fixed in glutaraldehyde fixative (2.5% glutaraldehyde, 2% NaH₂PO₄·2H₂O, 0.33% NaOH, 0.54% glucose, pH 7.2) were prepared for electron microscope examination. Gametocysts kept in Sample Protector (TaKaRa, Tokyo, Japan) were prepared for DNA extraction. All samples were stored at −40°C until being tested.

2.3. Electron microscopy

For scanning electron microscopy (SEM) examination, the fixed suspensions of oocysts were placed on coverslips and rinsed 20 min × 6 times with 0.1 M phosphate buffer (2% NaH₂PO₄·2H₂O, 0.33% NaOH, 0.54% glucose, pH 7.2). After ethanol serial dehydration (30% and 50% 10 min × 2 times; 70% and 90% once; and 100% 15 min × 3 times), the samples were treated with tert-butyl alcohol for 15 min twice to replace the ethanol before being frozen at −20°C for 5 min and dried by Freeze Drying Device (JFD-320, JEOL Ltd., Tokyo, Japan). Finally, the oocysts were mounted on stubs, coated with gold, and examined by SEM (Quanta 400, Philips FEI Co., Amsterdam, Netherlands; JSM-6330F, Electronics Co., Ltd., Takasaki, Japan). The sizes of the oocysts (*n* = 17) were measured using the scanning micrographs.

For transmission electron microscopy (TEM) examination, oocysts were fixed overnight at 4°C in 5% (v/v) glutaraldehyde and 3% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Rinsed with this buffer for 15 min × 3 times; post-fixed in 2% (w/v) OsO₄ overnight; dehydrated in ascending ethanol series, followed by three washes with acetone (20 min for each); and then embedded with Spurr’s resin. Ultrasectioning was performed with LKB ultramicrotome. Sections were stained with uranyl acetate and lead citrate consecutively, and examined by TEM (JEM-100CX, JEOL, Japan).

2.4. DNA extraction, PCR amplification, and sequencing

Genomic DNA was isolated from the gregarine gametocyts using the AxyPrep™ Multisource Genomic DNA Kit (Axygen, CA, USA). DNA concentration was determined by spectrophotometry.

Table 1

<table>
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<th>L4</th>
<th>L5</th>
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<td>31</td>
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</tr>
<tr>
<td>Number positive</td>
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<td>-</td>
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<tr>
<td>Infection rate (%)</td>
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<td>-</td>
<td>-</td>
<td>3.23</td>
<td>5.26</td>
<td>23.53</td>
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</table>

Surveys were conducted in the Tibetan Plateau (4156 m altitude, 29°36’N, 94°36’E) from June to August in 2011. Ln, nth instar larvae (*n* = 4–7); A♀, female adults; A♂, male adults. ‘-’ indicates no gregarines found in *T. pui* coelom.
2.5. Phylogenetic analysis

Sequences with high homology to ITS-5.8S rDNA and SSU rDNA from the gregarine parasite of T. pui were searched using the nucleotide BLAST at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The percentage identity of the related sequences was calculated using DNAMAN software (Version 6.0.40, Lynnon Corp., Quebec, Canada). Multiple alignments of the related sequences were performed with Clustal X (Thompson et al., 1997). Published SSU rDNA sequences from gregarines and Cryptosporidium spp. (outgroup) were used for phylogenetic analysis. Molecular phylogenetic trees inferred from the aligned sequences were constructed using maximum likelihood (ML) and Bayesian methods. Kimura 2-Parameter Distance model and 1000 bootstrap repetitions were applied for ML analysis in PHYML package version 3.6 (Felsenstein, 2002). Bayesian analysis using GTR + Γ + I model was performed with MrBayes 3.1.2. Four MCMC chains were executed for 300,000 generations, sampling every 100 generations and discarding the first 750 samples as burn-in. Bayesian posterior probabilities were obtained from the consensus tree (Ronquist and Huelsenbeck, 2003).

3. Results

3.1. Gregarine parasites in T. pui coelom

Table 1 shows the percent prevalence of T. pui infected with coelomic gregarines. No gregarine parasites were found in the coelom of 4th to 6th instars. In one 7th instar larval hemolymph, gregarine gamonts were observed (Fig. 1A). They were ovoid, ~15 × 8 μm. Two gamonts associated with cell membrane fusion at the time of syzygy. Gametocysts were ~25 μm in diameter (Fig. 1B). Large gametocysts visible to naked eyes were found in the coelom of the 7th instar larva, as well as some adults. The prevalence was approximately four times greater in males than in females (Table 1). All T. pui infected were active, without any symptoms. Most adult hosts carried two or three gametocysts, accumulating in the posterior segment of host body cavity (Fig. 2).

3.2. Morphology and ultrastructure of gregarine oocysts

Gregarine gametocysts in T. pui host coelom were oval, translucent or white, 0.44 × 0.38–1.26 × 1.18 mm, full of numerous oocysts that were enveloped by a gametocyst wall. Oocysts were formed via internal budding from the cytoplasmic mass within young gametocysts (Fig. 3A). Each bud produced one oocyst. Newly formed oocysts were spherical or ovoid, and subsequently transformed into lemon-shaped oocysts (Fig. 3B). Oocysts were colorless, with two truncate round ends, and contained large vacuoles inside. Gametocysts dehisced by simple rupture and released oocysts (Fig. 3C).

SEM examination showed that oocyst main body was rough with a number of small bumps (Fig. 3D), while both truncate ends were smooth in texture (Fig. 3E). Moreover, oocysts exhibited an equatorial line as shown in Fig. 3D. In micrographs, the length of oocysts (n = 17) was 17.17 ± 0.73 μm (15.53–18.45 μm), the width of midsection was 6.49 ± 0.4 μm (5.93–7.24 μm), and the diameter of the truncate ends was 1.98 ± 0.16 μm (1.67–2.48 μm).

Fig. 3. Gregarine oocysts from T. pui coelom. Light micrographs showing preoocysts (arrow) budding from the cytoplasmic mass within a young gametocyst (A), oocysts within a mature gametocyst (B), and oocysts released via simple rupture of a gametocyst wall (C), scale bar = 20 μm. SEM micrographs showing the oocyst was characterized with an equatorial line (arrow) and a number of small bumps all over the major surface (D), and its truncate ends were round and smooth in texture (E). A TEM micrograph (F) showing the oocyst ultrastructure: ow, oocyst wall; V, vacuole; AP, amylopectin granule. Scale bar = 200 nm.
The TEM micrograph of the oocyst showed a thick oocyst wall (Ow). Vacuoles (V) of various sizes were distributed in the cytoplasm. A few amylopectin granules (AP) also appeared (Fig. 3F).

3.3. Molecular identification of the gregarine parasite from T. pui coelom

Amplified fragments of SSU rDNA and ITS-5.8S rDNA of the gregarine parasite were 1796 bp and 494 bp, respectively. The sequence data sets were deposited into the GenBank nucleotide sequence database under accession numbers HQ619958 for ITS-5.8S rDNA and HQ619959 for SSU rDNA. Nucleotide BLAST results revealed the highest similarity between these sequences and those of Ascogregarina species.

The percentage identity of the SSU rDNA sequences was 96% between the gregarine and Ascogregarina taiwanensis Lien and Levine (1980). ML and Bayesian methods yielded almost identical topologies. Both phylogenetic trees indicated that the newly described gregarine was more related to Ascogregarina species and Paraschneideria metamorphosa Nowlin (1922) than to other gregarines including species with lemon-shaped oocysts (Lantová et al., 2010; Schrével and Philippe, 1993; Valigurová and Koudela, 2006) (Fig. 4). Percentage pairwise identities of published SSU rDNA from lepidopteran gregarine genera are shown in Table 2.

All published ITS-5.8S rDNA sequences from gregarines showed low similarity to that of the gregarine from T. pui. Multiple alignments of the related ITS-5.8S rDNA sequences were shown in Fig. 5. The highest percentage identity of the ITS-5.8S rDNA sequences (excluding the primer sequences) was 76% between the gregarine and Ascogregarina culcis Ross (Table 3). Ascogregarina

![Fig. 4. ML tree inferred from SSU rDNA sequences showing phylogenetic position of the gregarine from T. pui. Underlines denote some genera in which some species had been isolated from Lepidoptera. Some species in the genera with lemon-shaped oocysts are highlighted in grey boxes. Cryptosporidium spp. served as an outgroup. Codes in parentheses represent GenBank accession numbers of SSU rDNA sequences. Bootstrap values (1000 replicates) are shown above the branches.](image-url)

<table>
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<th>Gregarine species (SSU rDNA GenBank ID)</th>
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<th>2</th>
<th>3</th>
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<td>1. Gregarine parasite of <em>Thitarodes pui</em></td>
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<td></td>
<td></td>
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<tr>
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<tr>
<td>3. <em>Mattesia</em> sp. (AY334569)</td>
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<td>84.53</td>
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<tr>
<td>4. <em>Mattesia geminata</em> (AY334568)</td>
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<td>82.61</td>
<td>89.56</td>
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</table>

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species differed from the gregarine from *T. pui* in the ITS1 region by a 22 bp long gap.

4. Discussion

We report here a coelomic gregarine parasite in *T. pui*, a lepidopteran insect from the Tibetan Plateau. So far, only a very limited number of gregarines have been described in Lepidoptera, such as *Ophriocystis elektroscirrha* McLaughlin and Myers (1970) from the monarch butterfly *Danaus plexippus* L., *Mattesia dispora* Naville, 1930 and *Leidyana ephestiae* Daviault, 1929 from the flour moth *Ephestia kuehniella* Zeller. Some gregarine parasites are potential disease agents for the biological pest control because of their pathogenicity, e.g. the pathogenicity of *Ascogregarina taiwanensis* to the mosquito *Aedes aegypti* L. (Beier and Craig, 1985; Reyes-Villanueva et al., 2003). In the present study, *T. pui*, the host of our newly characterized gregarine, is an important economic lepidopteran in the Tibetan Plateau (Zhang et al., 2007). Caterpillar Fungus including the mummified *T. pui* larvae infected by *Ophiocordyceps sinensis* has always been the main source of income for the natives, so understanding the occurrence and pathogenicity of the gregarine parasite in *T. pui* may help sustain Caterpillar Fungus.

Gregarine oocyst morphology may serve as a species characteristic (Schrével and Philippe, 1993). Oocysts of several genera, such as *Ascogregarina*, *Psychodictyiella*, *Mattesia*, *Farinocystis* Weiser, *Monocystis*, are all lemon-shaped with other specific characters (Lantová et al., 2010; Roychoudhury et al., 2007; Schrével and Philippe, 1993; Valigurová and Koudela, 2006; Weiser, 1953). The gregarine from *T. pui* was characterized by lemon-shaped oocysts with apparent truncate round ends, which were more similar to those of the genus *Ascogregarina*. However, their oocyst sizes were different from each other. Furthermore, significantly different surface ultrastructures were observed among their oocysts. The oocyst surfaces of *Ascogregarina taiwanensis*, *Ascogregarina armigerei* and *Ascogregarina* sp. from *O. j. japonicas* were smooth; that of *Ascogreg- arina Culicis* contained numerous dense spots (Roychoudhury et al., 2007) whereas that of the gregarine parasite from *T. pui* was full of small bumps.

DNA sequences have been used to identify the species of gregarines and study their phylogenetic positions (Leander et al., 2003; Morales et al., 2005). The molecular sequences from gregarines are now mainly restricted to a small number of SSU rDNA sequences derived from GenBank. In the present study, the results from SSU rDNA and ITS-5.8S rDNA sequence analyses revealed the relatively close relationship between the gregarine from *T. pui* and the species of *Ascogregarina*. Meanwhile, the newly characterized gregarine markedly differed from *Ascogregarina* species in the ITS rDNA sequence, which suggest maybe it does not belong to the genus *Ascogregarina*. Moreover, with the fact that the taxon *Ascogregarina* was more related to *Ophriocystis elektroscirrha* (order...
Neogregarinorida) than to many gregarines at the same order in the phylogenetic tree, the current grouping of many asperate gregarines within eugregarines is untenable based on SSU rDNA (Votýpka et al., 2009). More definite phylogenetic positions of gregarines might require further studies in the future.

The life cycle of the newly characterized gregarine parasite of *T. pui* might be different from that of most gregarines including the well-studied *Ascogregarina* species. *Ascogregarina* oocysts could infect the digestive tract of any instar of their dipteran hosts and develop into gametocysts in the Malpighian tubule by the time of pupation (Chen, 1999; Roychoudhury and Kobayashi, 2006). Hundreds of new oocysts were then formed through internal budding from a large cytoplasmic mass within the gametocyst (Chen, 1999). In *T. pui* hosts, the gregarine gametocysts with oocysts already appeared in the coelom at the late larval stage. Further studies will be required to determine the parasitism mechanism of the gregarine parasite. We are also currently collecting more tissue samples from *T. pui* larvae and pursuing the earlier developmental stages of this gregarine parasite which could occur in the host digestive tract, intestine and/or coelom.

A number of gregarines may cause disease in their invertebrate hosts, especially some coelomic gregarines. In our study, *T. pui* hosts infected with the coelomic gregarine parasite showed no symptoms. Moreover, a few oocysts were also found in the coelom of one female adult that had already oviposited. These oocysts might be the residual ones from the open gametocysts that had been expelled by the female adults during oviposition. Previous studies had reported that the dissemination of gregarine parasites could depend on the host oviposition behavior (Beier and Craig, 1985). Many non-pathogenic gregarine parasites have been demonstrated to reduce the fecundity and longevity of their hosts (Comiskey et al., 1999; Villanueva, 2004). We are currently pursuing the non-lethal effects of this newly characterized gregarine parasite on *T. pui* hosts. More detailed studies on this gregarine's life cycle will be conducted to further understand its parasitism mechanisms.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2012.07.024.

References


