Life Cycle Completion of Parasite *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae) in Non-Native Host *Ochlerotatus japonicus* (Diptera: Culicidae)

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ABSTRACT *Ascogregarina taiwanensis* (Lien and Levine), a protist gut parasite of *Aedes albopictus* (Skuse), is not known to complete its life cycle within the potential competitor species, *Ochlerotatus japonicus* (Theobald). In a laboratory cross infection study we demonstrated that *A. taiwanensis* completed its life cycle within *Oc. japonicus* and remained infectious. We also sampled cohabitating mosquito larvae in Mercer County, NJ, and based on ribosomal DNA sequence data, we determined that *Oc. japonicus* cohabitating with *Ae. albopictus* can become infected with *A. taiwanensis*.

KEY WORDS *Ochlerotatus japonicus*, *Ascogregarina taiwanensis*, mosquito, parasite

Ascogregarina (Syn. *Lankesteria; Ascocystis*) is a genus of protist gut endosymbionts of container-dwelling mosquitoes (Ward et al. 1982, Votýpka et al. 2009). *Ascogregarina* can have significant fitness consequences, particularly under deficient nutrient conditions (Comiskey et al. 1999) or high exposure levels (Sulaiman 1992). Although some earlier research suggests that *Ascogregarina* have little effect on the mortality of their natural hosts (McCray et al. 1970, Jacques and Beier 1982, Mourya and Soman 1985, Reyes–Villanueva et al. 2003), a more recent study of infection by *Ascogregarina* showed alteration of the competitive interactions between mosquito species (Aliabadi and Juliano 2002), suggesting that the impact of *Ascogregarina* parasites requires further investigation. We address here the potential for an introduced *Ascogregarina* species to infect non-native hosts in situations involving two invasive host species.

Species of *Ascogregarina* have been characterized as host-specific, meaning that each species completes its life cycle in a single host species (Lien and Levine 1980, Beier and Craig 1985, Chen 1999). However, more recent studies have demonstrated that some *Ascogregarina*, while more reproductively efficient in one particular species, are capable of life cycle completion in multiple host species (Munstermann and Wesson 1990, Garcia et al. 1994). Frequently, these cross infections in non-native species result in high-host mortality (Walsh and Olson 1976, Munstermann and Wesson 1990, Garcia et al. 1994, Comiskey and Wesson 1997).

Ascogregarina taiwanensis (Lien and Levine) is a well-characterized gregarine that primarily infects *Aedes albopictus* (Skuse). It can also infect several species across multiple Culicidae genera, but the majority of these infections occur without successful life cycle completion of the parasite (Munstermann and Wesson 1990, Garcia et al. 1994, Reeves and McCullough 2002). In non-native hosts *Ochlerotatus epactius* (Dyar and Knab = *Aedes epactius;* see Reinert 2000), *Ochlerotatus atropalpus* (Coquillett) (Munstermann and Wesson 1990), and *Ochlerotatus taeniorhynchus* (Wiedemann) (Garcia et al. 1994) it has been demonstrated to complete its life cycle and produce infectious oocysts (Fig. 1). In field collection studies, *A. taiwanensis* gamonts have been found in *Aedes aegypti* (L.) (Fukuda et al. 1997) and *Oc. epactius* larvae (Munstermann and Wesson 1990). Because of similarities in morphology between species of *Ascogregarina*, a polymerase chain reaction (PCR)-based assay has been developed by Morales et al. (2005) to distinguish specifically *A. taiwanensis* from similar gregarines *Ascogregarina culicis* (Ross) and *Ascogregarina barretti* (Vavra). Using PCR primers targeting the ITS region of ribosomal DNA they distinguish *A. taiwanensis* as a 450 bp amplicon on an electrophoretic gel (Morales et al. 2005).

Previous research has delineated the life cycle of *A. taiwanensis* within *Ae. albopictus* (Chen et al. 1997, Roychoudhury and Kobayashi 2006). *Ae. albopictus* larvae become infected upon ingesting *A. taiwanensis* oocysts from the container habitat and are vulnerable to gregarine infection at all larval instars (Roychoudhury and Kobayashi 2006). Approximately 1 hr after ingestion, *A. taiwanensis* oocysts release sporozoites into the larval gut (Roychoudhury and Kobayashi 2006). These sporozoites enter midgut epithelial cells and use host cell mitochondria to supply the energy required to mature into a trophozoite (Chen et al. 1997). Trophozoites generally mature concurrently

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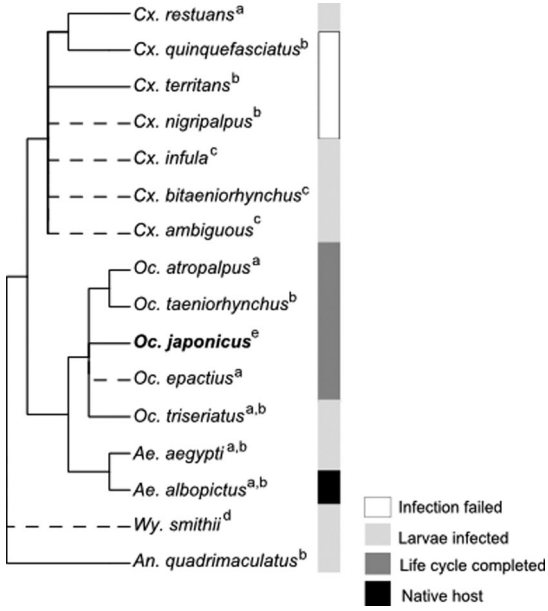

Fig. 1. Cladogram of *Culicidae* mosquito hosts and summarized reports of infectivity of *A. taiwanensis* in those hosts. The relationships shown between species are taken from the Maximum Likelihood tree displayed by Shepard et al. (2006). Species that were not included in Shepard et al.'s study have been placed at the genus level based on current classification and indicated with dotted lines. (a) Munstermann and Wesson (1990), (b) Garcia et al. (1994), (c) Mourya and Soman (2000), (d) Reeves and McCullough (2002), (e) this study.

with their host and upon the release of host molting hormone 20-hydroxyecdysone, trophozoites exit midgut epithelial cells, travel down the digestive tract, and enter the Malpighian tubules (Chen 1999). Once in the Malpighian tubules, the trophozoite becomes segmented and divides into gametes (Chen et al. 1997). Gametes combine via syzygy, creating gametocysts that in turn bud oocysts (Chen et al. 1997). When *Ae. albopictus* pupae eclose, or when adults defecate or oviposit in container habitats, they release these *A. taiwanensis* oocysts and expose the next generation of *Ae. albopictus* larvae to infection (Roychoudhury and Kobayashi 2006).

The invasive species *Ae. albopictus* was introduced from Japan via the international used tire trade (Hawley et al. 1987, Reiter and Sprenger 1987) and sustaining populations were first detected in the United States in 1985 near Houston, TX (Sprenger and Wuithiranyagool 1986). *A. taiwanensis* was not detected in the United States until 1988 (Munstermann and Wesson 1990). It has been conjectured that this delay between the arrival of the host and parasite occurs because hosts with rapid range expansion may temporarily outrun and escape their parasite in newly founded populations (Blackmore et al. 1995). *Ae. albopictus* has been an efficient colonizer in the United States and is a successful competitor with previously established mosquito species (Ho et al. 1989, Livdahl and Willey 1991, O'Meara et al. 1995, Juliano and Lounibos 2005). In the past 26 yr, *Ae. albopictus* has expanded its range to 36 states and has invaded regions of the Middle East, Europe, Africa, and Central and South America (Enserink 2008).

Like *Ae. albopictus*, *Ochlerotatus* (*Finlaya*) *japonicus japonicus* (Theobald) is a container-dwelling mosquito native to Japan. First detected in the United States in 1998 in Connecticut, New York, and New Jersey (Peyton et al. 1999, Andreadis et al. 2001), the range of *Oc. japonicus* includes Japan, Korea, South China, Taiwan, Russia, Canada, and at least 22 states on both the east and west coasts of the United States (Williges et al. 2008). Like *Ae. albopictus*, *Oc. japonicus* appears to have been transported to the United States via international tire shipping (Peyton et al. 1999) and is also a successful competitor with resident mosquito species (Burger and Davis 2008, Andreadis and Wolfe 2010).

The natural parasite of *Oc. japonicus*, *Ascogregarina japonicus* (Roychoudhury, Isawa, Hoshino, Sasaki & Kobayashi) was recently described in Japan (Roychoudhury et al. 2007b), but has not been reported in the United States. However, because *Oc. japonicus* and *Ae. albopictus* have overlapping ranges and occupy the same container habitats, it seems likely that *Oc. japonicus* would be exposed to *A. taiwanensis*. Susceptibility of *Oc. japonicus* to cross infection by the parasites of its potentially competing container-dwelling mosquitoes has not yet been established. If *Oc. japonicus* could be infected by *A. taiwanensis*, the parasite could influence the competitive capacity and range expansion of both mosquito species (Munstermann and Wesson 1990, Garcia et al. 1994, Blackmore et al. 1995). In this study, we infected *Oc. japonicus* with *A. taiwanensis* in the laboratory to investigate whether *A. taiwanensis* can complete its life cycle in *Oc. japonicus*. We also collected *Oc. japonicus* cohabitating with *Ae. albopictus* to test for natural infections of *Oc. japonicus* by *A. taiwanensis*.

Materials and Methods

Collection and Culturing of *A. taiwanensis* and *Ae. albopictus*. Water samples containing *Ae. albopictus* were collected from container habitats (e.g., buckets, vases, tarpaulins, and tires) in St. Georges' and Hamilton, Bermuda by the Department of Vector Control of the Bermuda Ministry of Health. We dissected *Ae. albopictus* to determine the presence of *Ascogregarina* and confirmed the identity of these gregarines as *A. taiwanensis* via parasite morphology (Lien and Levine 1980) and PCR assays identical to those described below. We amplified *A. taiwanensis* stocks by infecting multiple generations of *Ae. albopictus* with oocysts collected from *Ae. albopictus* in Bermuda. The oocyst homogenate was stored at 4°C until needed for experimental use or further amplification of the parasite. Oocyst concentration was determined using a hemacytometer. Since 2005, *Ae. albopictus* has been the only *Aedes* mosquito species on Bermuda (Kaplan et al. 2010), preventing any chance of cross infection with any other gregarine.

The *Ae. albopictus* colony used in this study to amplify *A. taiwanensis* and determine the viability of oocysts passed through *Oc. japonicus* (see below) was established from egg batches obtained in Bermuda ovitraps. Because the field populations of these mosquitoes were infected with *A. taiwanensis*, we rinsed the egg slats with distilled water before hatching them in 1 g/L of nutrient broth (Difco Laboratories, Detroit, MI) and removed then after 20 h, placing them in distilled water. Larvae dissected from this initial hatching were uninfected (data not shown) and dissections of the next generation also suggested that the colony was uninfected with *Ascogregarina*. The colony was maintained at 24°C with 80% RH and a photoperiod of 16:8 (L:D) h.

Collection of *Oc. japonicus*. *Oc. japonicus* adults were captured in Hadwen Arboretum of Clark University, Worcester, MA (42 15′2″ −71 49′58″), bloodfed, and kept in cages with oviposition containers in 80% humidity. We collected egg sheets collected from these adults and immersed them 1 g/L of nutrient broth for 24 h to hatch larvae.

Experimental Design. We disbursed 10 first instars into 14, 10 cm petri dishes. each containing a 30 ml solution of *A. taiwanensis* oocysts in distilled water containing 1,000 oocysts per ml (3,000 oocysts per larva). Larvae were not fed for the first 20 h after hatching to encourage oocyst ingestion after which they were fed 0.05 g powdered yeast (Kal, Neutraceutical Corp., Park City, UT) every other day. Larvae were reared at 24°C.

From each dish, we sampled and dissected *Oc. japonicus* larvae, as pupae, and as adults. On days 4, 7, and 8, one larva from each dish was dissected, with the final two larvae being dissected on day 12 as they were the only remaining mosquitoes in of the dishes. Dissections of pupae occurred on days 8, 9, 10, and 11; no more than one pupa per dish was dissected per day, if there was a pupa available. Infection status of larvae and pupae was confirmed through mid-gut dissection and visual confirmation of *A. taiwanensis* gamonts in the mid-gut lining. Before dissecting larvae and pupae, we rinsed them in distilled water, after which we placed specimens on a microscope slide in 0.05 ml distilled water, removed the head with a dissecting needle, and inserted one dissecting needle into the thorax while pulling posteriorly with another dissecting needle inserted into the terminal abdominal segment. This process separated the terminal segment from the body, drawing out the Malpighian tubules and gut along with it. Gut contents flooded into the surrounding medium. We covered gut and Malpighian tubules with a cover slip and examined at 400× under a compound microscope.

In specimens reared to adulthood, pupae were removed individually from the oocyst solution, rinsed in distilled water and placed in 1.5 ml centrifuge tubes with 0.5 ml distilled water which were plugged with cotton. When the adults emerged they were transferred to a second 1.5 ml centrifuge tube in which they were frozen. Adults were then homogenized within the centrifuge tube using a motorized pestle and positive *A. taiwanensis* infection was confirmed and quantified using a hemacytometer. Homogenized pupal exuviae of emerged adults were also analyzed because *Ascogregarina* oocysts are frequently shed during eclosion (Fellous and Koella 2009).

After running the single-round infectivity trial, oocysts produced from *Oc. japonicus* adult and pupal exuviae samples were collected for use in confirming the viability of these oocysts through infection of *Ae. albopictus.* Thirty newly hatched *Ae. albopictus* were divided into two petri dishes, with one petri dish containing 20 ml of 406 oocysts (produced by *Oc. japonicus*) per ml and the other containing 20 ml of distilled water. Four larvae from each petri dish were dissected at the third instar to assess parasite success. The remaining mosquitoes were reared to adulthood and handled as described above to determine if the oocysts from *Oc. japonicus* were able to complete their life cycle in *Ae. albopictus.*

Confirmation of Cross Infection via PCR Amplification. We confirmed the accuracy of hemacytometer results by extracting DNA from samples of 12 homogenized adult samples and 12 homogenized pupal exuviae, including both negative and positive infection status. DNA was extracted using the E.Z.N.A Forensic DNA Kit (Omega Biotek, Norcross, GA). We amplified a sequence of rDNA encompassing the partial 18s, complete ITS1, complete 5.8s, and partial ITS2 regions using PCR with primers and a PCR cycle described by Morales et al. (2005). We added a 10 μM primer specific for the genus *Ascogregarina* AU (5′-ACC GCC CGT CCG TTC AAT CG-3′) and a 10 μM primer specific for *A. taiwanensis* AT (5′-GAG AAG CCG TCG TCA ATA CAG C-3′) to a reaction mix containing 12.5 μl of GoTaq Master Mix (Promega Corporation, Madison, WI) and 7.5 μl of sterile, distilled water. We analyzed PCR products on a 1% agarose gel using standard electrophoretic procedures (Sambrook et al. 1989), scoring samples as positive or negative based the presence or absence of an appropriately sized amplicon (≈450 bp) as determined by comparison with PCR Markers DNA ladder (Promega Corporation, Madison, WI). For DNA samples that were negative for the presence of *A. taiwanensis*, we then amplified using PCR with primers specific to *Oc. japonicus* ITS2 and 28S regions of the rDNA, using 10 μM of forward primer specific for the start of the ITS2 region (5′-GCG TGC GCG TTT CAC TTC GG-3′) and 10 μM of reverse primer specific for the start of the 28S region (5′-GCC TAC TGG AGT GTT ATA TGT GGG C-3′) in a reaction mix containing 12.5 μl of GoTaq Master Mix (Promega Corporation) and 7.5 μl of sterile, distilled water. We analyzed PCR products on a 1% agarose gel using standard electrophoretic procedures (Sambrook et al. 1989), scoring samples as positive or negative based the presence of or absence of an appropriately sized amplicon (≈267 bp) in comparison with PCR Markers DNA ladder (Promega Corporation). After running the PCR products on the agarose gel we detected the appropriate 267 bp *Oc. japonicus* amplicon in all samples scored as negative for *A. taiwanensis* and confirmed that the absence of

Table 1. Parasite species, host, origin, ungapped sequence length in our alignment, and accession number for the rDNA sequences used in this study

Parasite species	Host collected in	Origin	Sequence length	Accession no.
Ascogregarina armigerei	*Armigeres subalbatus* (Coquillett)	New Jersey	1733	DQ462459
Ascogregarina barretti	*Oc. triseriatus*	Massachusetts	1735	JX131296
Ascogregarina culicis	*Ae. aegypti*	Columbia	1733	DQ462457
Ascogregarina japonicus	*Oc. japonicus*	Japan	1733	DQ462458
Ascogregarina **sp.**	*Oc. japonicus* (J1)	New Jersey	1733	JX131300
Ascogregarina **sp.**	*Oc. japonicus* (J6)	New Jersey	1733	JX131299
Ascogregarina **sp.**	*Ae. albopictus* (J3)	New Jersey	1733	JX131297
Ascogregarina taiwanensis	*Ae. albopictus*	Bermuda	1733	JX131298
Ascogregarina taiwanensis	*Ae. albopictus*	Japan	1733	DQ462454

Samples in bold were sequenced in this study.

an *A. taiwanensis* amplicon was not because of irregularities with the DNA samples.

Field Collection and DNA Extraction. We collected larvae from sampling sites in Trenton and Hopewell, NJ (40.295655, −74.782884) with the assistance of the Mercer County Department of Mosquito Control. We selected this area because at this latitude, *Oc. japonicus* and *Ae. albopictus* ranges overlap and both species have been observed occupying the same containers at the same time, providing a natural opportunity for cross infection. We collected samples from habitats where *Oc. japonicus* and *Ae. albopictus* were cohabitating (children's pools, cemetery vases, etc.). Samples were washed in distilled water and dissected under a dissecting microscope at 40× magnification. We extracted DNA from these *Oc. japonicus* samples (and one *Ae. albopictus* sample) using the E.Z.N.A Forensic DNA Kit (Omega Bio-Tek Inc., Norcross, GA) and performed PCR as described above to test for presence of parasite DNA.

Sequencing of 18s rDNA. While the morphology of our samples from *Oc. japonicus* resembled that of *A. taiwanensis*, we could not conclude definitively from morphology alone the identity of our samples because there has been no published morphology on the trophozoite life stage of *A. japonicus*. Instead, we sought to compare the DNA sequences from our samples to those of *A. japonicus*, *A. taiwanensis*, and other species of *Ascogregarina* parasites in the eastern United States. The only available nucleotide sequence for *A. japonicus* on GenBank was an 1,800 bp fragment from the 18s region, so we amplified and sequenced this region of rDNA from our samples collected from New Jersey, as well as a sample of *A. barretti* from *Ochlerotatus triseriatus* (Say) collected in Worcester, MA, and a sample of *A. taiwanensis* from *Ae. albopictus* collected in Bermuda.

To amplify only rDNA from *Ascogregarina*, as our DNA extractions contained mosquito DNA as well, we used a universal 18s forward primer (5′-CGAATTCAACCTGGTTG ATCCTGCCAGT-3′) from Roychoudhury et al. (2007a) and an *Ascogregarina*-specific reverse primer Asco28sR(5′-CAG TGG GTA GCC TTG TC-3′) that binds to the beginning of the 28s region from Morales et al. (2005). These primers amplified roughly 1,800 nucleotides of the 18s region along with the complete ITS1, 5.8s, ITS2, and partial 28s rDNA regions. The PCR conditions included an initial denaturing step at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 3 min, with a final extension step of 10 min at 72°C. PCR products were sequenced directly using the universal 18s forward and universal 18s reverse (5′-CCG GAT CCT GAT CCT TCT GCA GGT TCA CCT AC-3)′ primers, two 18s internal primers (5′-GGA GAG GGA GCC TGA GAA-3′ and 5′CTC TAA GAA GCG ACG CCA-3′) described by Roychoudhury et al. (2007a), the *Ascogregarina*-specific reverse primer, and an *Ascogregarina*-specific forward primer Asco18sF (5′-CGA CTG GAT GAT CCG G-3′) from Morales et al. (2005) that binds near the end of the 18s region.

We cleaned the amplified PCR products via ethanol precipitation and sequenced in both directions with the above primers using BigDye 3.1 Terminator chemistry and manufacturer's protocols, and the sequencing reaction was cleaned using Agencourt CleanSeq magnetic plate (Beckman Coulter Inc., Brea, CA). We used a 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA) to obtain DNA sequences.

We assembled the six reads for each sample in Geneious (Drummond et al. 2012) and trimmed low quality regions at the start and end of the reads resulting in a 2070 bp sequence of rDNA from *Ascogregarina* collected in New Jersey, Massachusetts, and Bermuda. We aligned resulting rDNA sequences in Geneious using MAFFT (Katoh et al. 2002) and trimmed to just the 18s region, resulting in 1733 bp (or 1735 bp in the case of our sample from *A. barretti*). We aligned the trimmed sequences again in Geneious using MAFFT with four 18s Ascogregarina sequences from GenBank (Table 1), trimming sequences from GenBank to the length of our sequences. We examined the alignment visually to ensure that trimming did not cause any alignment errors.

Data Analyses. We regressed infection status in *Oc. japonicus* against time since hatch using a nominal logistic model. We included larval and pupal dissection data, as well as adult and exuviae life cycle detection. After finding significant differences in prevalence of *A. taiwanensis* among life stages of experimentally infected *Oc. japonicus* by χ^2 square contingency analysis, we tested for specific differences among larvae, pupae and adults using pairwise

Table 2. Prevalence of *A. taiwanensis* in three life stages of *Oc. japonicus*

Stage	Number examined	Prevalence
Larva	44	95%a
Pupa	28	39%b
Adult	53	30%b

Prevalence in different stages that are significantly different ($P < 0.01$) are marked by different letters.

Table 3. Presence of *A. taiwanensis* oocysts in adult and pupal exuviae of *Oc. japonicus* by sex

Sex	Total individuals	IP[a]	IA[b]	O[E][c]
Female	17	24%	18%	24%
Male	36	25%	14%	33%
Combined	53	25%	15%	30%

Differences between sexes not significant (Fisher's exact tests, $P > 0.05$).

[a] I^P Infected pupal exuviae.

[b] I^A Infected homogenized adult tissue.

[c] O^E Oocysts present in either pupal exuviae or homogenized adult tissue.

comparisons described by Zar (1999). We used Fisher's exact test to compare the prevalence of *A. taiwanensis* in dissections of *Ae. albopictus* larvae exposed to oocysts from *Oc. japonicus* with those unexposed to oocysts, frequency of life cycle completion by *A. taiwanensis* in experimentally infected *Oc. japonicus* with experimentally infected *Ae. albopictus*, as well as prevalence of *A. taiwanensis* in field collected *Ae. albopictus* versus *Oc. japonicus* from New Jersey. We used JMP v.9 (SAS Institute 2010) for contingency analysis, logistic regression, and Fisher's exact tests.

From the MAFFT alignment of our sequences and the sequences for *Ascogregarina armigerei* (Lien and Levine), *A. culicis*, *A. japonicus*, and *A. taiwanensis* (Accession numbers: DQ462459, DQ462457, DQ462454, and DQ462454, respectively), we created a distance matrix of pairwise similarity to identify each field collected *Ascogregarina* sample and we constructed an unrooted maximum likelihood (ML) tree. The ML analysis was performed with 100 bootstrap replicates on the RAxML BlackBox server (http://phylogench.vital-it.ch/taxml-bb/index.php; Stamatakis et al. 2008) using *A. armigerei* (DQ462459) as an outgroup based on the previous phylogenetic analysis of Roychoudhury et al. (2007). The resulting best scoring ML tree was viewed in Dendroscope (Huson et al. 2007).

Results and Discussion

Experimental Infection. Of the 44 *Oc. japonicus* dissected as larvae, 42 (95%) were infected with *A. taiwanensis*, all dissected on days 4, 7, or 8 (Table 2). The two larvae that showed no signs of *A. taiwanensis* infection in the midgut were dissected on day 12, and as such, we expect they had ample time to consume oocysts. Therefore, we assume that all of the *Oc. japonicus* larvae were exposed to *A. taiwanensis*, even if they did not shown signs of infection at each life stage. We dissected 28 *Oc. japonicus* as pupae; 39% were infected with visible gamonts. In a sample of 53 homogenized adults, 30% had oocysts present in either the exuviae or the homogenized adult tissue (Table 3). These results demonstrate that *A. taiwanensis* is capable of completing its life cycle within *Oc. japonicus.*

Our logistic regression suggested that *Oc. japonicus* is able to clear itself of infection, as prevalence of *A. taiwanensis* decreased over time (Fig. 2; $\chi^2_1 = 31.39$, $P < 0.001$). This clearance of infection may have occurred during the pupa life stage because there was no difference between prevalence in adults compared with pupae, but parasite prevalence was significantly greater in dissected larvae (Table 2). This seems common in experimental infection of non-native hosts, as Garcia et al. (1994) found that despite a 100% prevalence of *A. taiwanensis* in experimentally infected *Ae. aegypti*, *Ae. albopictus*, and *Oc. taeniorhynchus* larvae, no oocysts were found in *Ae. aegypti* adults and only 30% of *Oc. taeniorhynchus* adults harbored oocysts, whereas all 100% of *Ae. albopictus* adults had oocysts. Further, Chen (1999) found that all of the trophozoites in *Ae. aegypti* were lysed in the pupa life stage, but despite 100% infection of pupae, roughly half of the trophozoites in *Ae. albopictus* failed to migrate to the Malpighian tubules and were lysed during the pupa life stage.

We have confirmed the viability of *A. taiwanensis* oocysts collected from parasitized *Oc. japonicus* because they produced a second-round infection in the midgut lining of all four of the dissected *Ae. albopictus*

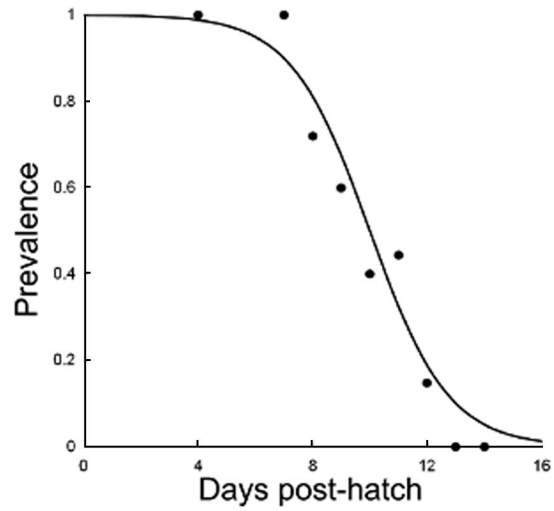

Fig. 2. Logistic regression of prevalence of *A. taiwanensis* in *Oc. japonicus* through time. Points are proportion of infected *Oc. japonicus* by *A. taiwanensis* in dissections of larvae and pupae or detection of life cycle completion in adults. Pupae were dissected starting on day 8; adults began emerging, and were checked for life cycle completion, beginning on day 10. The predictive model is shown; the coefficient for time (t), 0.73 ± 0.13, is significant ($\chi^2_1 = 31.39$; $P < 0.0001$).

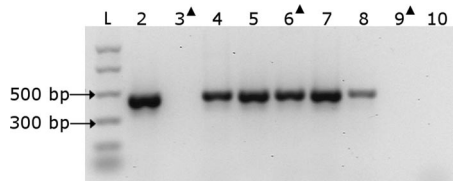

Fig. 3. An ethidium bromide-stained 1% agarose gel under ultraviolet light showing *A. taiwanensis* species-specific products. Lane L is the PCR Markers DNA ladder; lane 2, male pupal exuvia; lane 3, male adult; lane 4, male pupal exuvia; lane 5, male adult; lane 6, female pupal exuvia; lane 7, female adult; lane 8, female pupal exuvia. ▲ indicates sample was previously classified as noninfected with a hemacytometer.

larvae exposed to those oocysts, while none of the uninfected *Ae. albopictus* had any visible parasite infection (Fisher's exact test, $P < 0.03$). Furthermore, of the mosquitoes exposed to oocysts that reached adulthood, all eight had visible oocysts in the pupal exuviae, while none of the ten unexposed mosquitoes that reached adulthood did (Fisher exact test, $P < 0.0001$). Despite treatment with a much lower initial dosage of oocysts (406/ml compared with 1,000/ml), all surviving *Ae. albopictus* showed evidence of life cycle completion by *A. taiwanensis*, compared with only 30% of *Oc. japonicus* (Fisher exact test, $P = 0.002$). Although our study was not designed to thoroughly quantify the level of life cycle completion of *A. taiwanensis* in *Ae. albopictus*, these results indicate that *Ae. albopictus* may be more susceptible to *A. taiwanensis* than *Oc. japonicus.*

In previous cross infection studies, gregarine life cycle completion could only be confirmed by visually detecting oocysts or by producing second-round infections in a new generation of mosquitoes. To help resolve issues concerning the sensitivity of a visual survey, we not only scored samples for the presence of *A. taiwanensis* oocysts using a hemacytometer but also confirmed results using *A. taiwanensis* primer sequences developed by Morales et al. (2005). Of eight adult pupal exuviae samples tested with PCR amplification of parasite rDNA, six were scored as positive based on presence of a 450 bp band (Fig. 3). Lane three and lane nine were negative for infection in the hemacytometer visual survey and were also negative for infection in the PCR assay. Lane 6, however, was negative for infection in the hemacytometer visual survey but was positive for infection in the PCR assay. This shows that while a hemacytometer can be useful in detecting life cycle completion, it is not as sensitive as a PCR amplification of parasite rDNA.

Field Collection and rDNA Sequencing. From the field samples of cohabitating *Ae. albopictus* and *Oc. japonicus* larvae collected from Mercer County, NJ, we were able to detect *A. taiwanensis* infection in *Oc. japonicus* and *Ae. albopictus* both through visual identification of trophozoites in the larval midgut and through PCR amplification and sequencing of DNA obtained from dissected larvae. We observed what appeared morphologically to be *A. taiwanensis* infection in three of seventeen *Oc. japonicus* sampled and 9 of 18 *Ae. albopictus* (Fisher exact test $P = 0.075$). We were able to amplify parasite DNA from two of the infected *Oc. japonicus* samples and one *Ae. albopictus* sample. The 18s sequences from our three samples collected from New Jersey were the most similar to one another, with one to two base pairs different between them (Table 4). These sequences had a two to three base pair difference compared with the sequence from *A. taiwanensis* collected in Bermuda, but a four to five nucleotide difference in the same region compared with the sequence of *A. taiwanensis* from Japan. Because *Ae. albopictus* is thought to have been introduced to Bermuda from the United States (Kaplan et al. 2010), we expected that out of the sequences examined in this study, this sequence of *A. taiwanensis* from Bermuda would be the most similar to the sequences from New Jersey if indeed our samples of *Oc. japonicus* were infected with *A. taiwanensis.* Furthermore, the likelihood of our field collected samples being any other species of gregarine found in the eastern United States, or *A. japonicus*, seems quite low given the much larger difference in nucleotides between those four other species and our three field collected samples. This is reflected in a maximum likelihood tree comparing our New Jersey samples from *Oc. japonicus* and *Ae. albopictus* to *A. armigerei*, *A. barretti*, *A. culicis*, *A. japonicus*, and *A. taiwanensis* (Fig. 4). This analysis strongly supports the monophyly of the clade containing all three of our samples, as well as both sequences from *A. taiwanensis.*

While our own phylogenetic analysis did not resolve the relationships among *Ascogregarina* species, phylogenetic analyses of *Ascogregarina* spp. by Roychoudhury et al. (2007a) found that *A. taiwanensis* from *Ae. albopictus* and *A. japonicus* from *Oc. japonicus* were morphologically similar (in oocyst size) and their 18S rDNA sequences revealed that they were more closely related to each other than to *A. culicis* or *A. armigerei.* The similarity between these two species could be further investigated with host specificity

Table 4. Pairwise comparison of *Ascogregarina* rDNA sequences

Species		1	2	3	4	5	6	7	8	9
A. armigerei DQ462459	1		54	58	63	55	53	55	56	56
A. culicis DQ462457	2	96.8		17	23	12	11	12	13	13
A. japonicus DQ462458	3	96.6	98.9		28	19	18	19	20	20
A. barretti US MA	4	96.3	98.6	98.3		21	22	23	24	24
A. taiwanensis DQ462454	5	96.7	99.2	98.8	98.7		4	4	5	5
A. taiwanensis BM	6	96.9	99.3	98.9	98.6	99.7		2	3	3
Ascogregarina NJ *Oc. japonicus* (1)	7	96.7	99.2	98.8	98.6	99.7	99.8		1	1
Ascogregarina NJ *Ae. albopictus*	8	96.7	99.2	98.8	98.5	99.6	99.7	99.9		2
Ascogregarina NJ *Oc. japonicus* (2)	9	96.7	99.2	98.8	98.5	99.6	99.7	99.9	99.8	

Percentage similarity (bottom) and no. of nucleotides different in the alignment of the 18S region between New Jersey collected *Ascogregarina* samples (4, 5, and 6), *A. japonicus*, and other *Ascogregarina* found in the eastern United States.

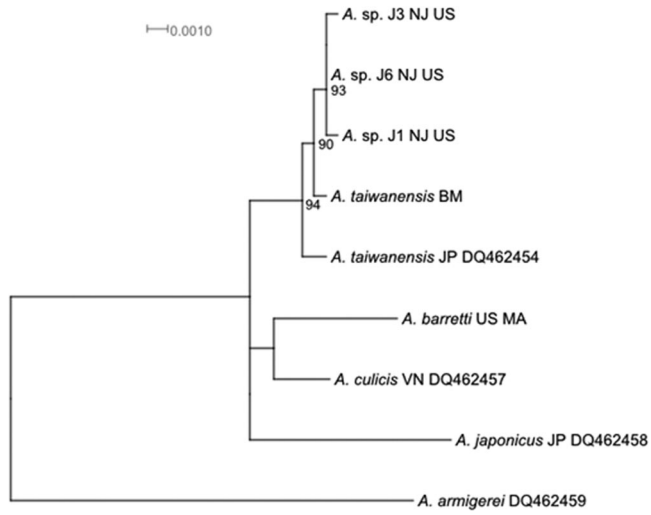

Fig. 4. The highest scoring maximum likelihood tree based on 1735 nucleotide characters in our alignment of the 18s region of the ribosomal RNA gene. Bootstrap values >50% shown. This phylogenetic analysis shows that our collected New Jersey sequences (J1, J3, J6) are more closely related to *A. taiwanensis* than other *Ascogregarina* found in the eastern United States.

studies by cross infecting *Ae. albopictus* with *A. japonicus* from *Oc. japonicus.*

Concerning implications for mosquito ecology, previous studies have suggested that *A. taiwanensis* has negligible influence on *Ae. albopictus* mortality, Aliabadi and Juliano (2002) showed that the gregarine does have the capacity to alter *Ae. albopictus* fitness because *Ae. albopictus* is a superior competitor against *Oc. triseriatus* when it is not burdened with an infection of *A. taiwanensis.* Our results suggest that *Oc. japonicus*, while a viable host for *A. taiwanensis*, may not be as competent a host as *Ae. albopictus*, though further research is needed in this area. If *Oc. japonicus* is a less competent host, the total output of oocysts from a mixed community of *Ae. albopictus* and *Oc. japonicus* may be lower than that of a community containing just *Ae. albopictus*, potentially causing successive generations of *Ae. albopictus* to be exposed to fewer parasites in those habitats and thus escape parasitism. If larger numbers of *Ae. albopictus* were able to escape parasitism, they could have an increased competitive advantage in the field. Armistead et al. (2008) established that *Ae. albopictus* is already a superior competitor relative to cohabitating *Oc. japonicus* and we suggest that this competitive advantage could be amplified in wild populations where *Ae. albopictus* can share the burden of infection with parasite-free *Oc. japonicus*, particularly if future studies can elucidate the fitness consequences of infection.

Given the ability of *A. taiwanensis* to complete its life cycle in multiple *Ochlerotatus* host species, it is possible that *A. taiwanensis* could experience a range expansion outside of the current range of its native host *Ae. albopictus.* This parasite range expansion could influence *Ochlerotatus* host species abundance and distribution, as it has been shown that several *Ochlerotatus* species experience high mortality when challenged with *A. taiwanensis* (Munstermann and Wesson 1990, Garcia et al. 1994).

Future studies that quantify the degree of infectivity of *A. taiwanensis* in *Oc. japonicus* versus the natural host *Ae. albopictus* should be performed, as well as competition studies that investigate whether *A. taiwanensis* infection has a measurable negative effect on *Oc. japonicus* fitness to assess more accurately future proliferation or range expansion for *Ochlerotatus* spp. and *Ae. albopictus.*

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