J. Parasitol., 91(6), 2005, pp. 1352–1356

DIFFERENTIAL IDENTIFICATION OF *ASCOGREGARINA* SPECIES (APICOMPLEXA: LECUDINIDAE) IN *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE) BY POLYMERASE CHAIN REACTION

Maria E. Morales, Clara B. Ocampo*, Horacio Cadena*, Claudia S. Copeland, Michael Termini†, and Dawn M. Wesson†‡
Interdisciplinary Program in Molecular and Cellular Biology, School of Public Health and Tropical Medicine, Tulane University Health Sciences Center, New Orleans, Louisiana, 70112. *email: wesson@tulane.edu*

ABSTRACT: We report 2 polymerase chain reaction (PCR)-based methods for distinguishing morphologically similar gregarine species based on amplification of variable regions of the internal transcribed spacer region of ribosomal DNA. The gregarines we investigated were *Ascogregarina barretti* (Vavra), *A. culicis* (Ross), and *A. taiwanensis* (Lien and Levine), parasites of the mosquitoes *Ochlerotatus triseriatus* (Say), *Aedes aegypti* (Linnaeus), and *Ae. albopictus* (Skuse), respectively. These 3 important vector mosquitoes often utilize the same container habitats, where larval development and infection by the parasite occurs, leaving ample opportunity for cross-species gregarine infection. Because previous studies have shown that the parasites *A. culicis* and *A. taiwanensis* variably affect fitness in both normal and abnormal mosquito hosts, distinguishing parasite infection and species is important. The task is complicated by the fact that these 2 parasite species are virtually identical in morphology, whereas *A. barretti* is morphologically distinct. Of the 2 PCR-based assays reported here, the first provides a rapid, sensitive, and straightforward means of general ascogregarine detection based on a single PCR amplification. The second method provides a means of differentiation between *A. culicis* and *A. taiwanensis* based on a species-specific PCR assay. Together, these assays allow whole mosquitoes to be tested for the presence of *Ascogregarina* species as well as identification of both *A. culicis* and *A. taiwanensis* singly or in dual infections.

Ascogregarina (Apicomplexa: Lecudinidae) contains a group of protozoan parasites that naturally infect certain insects. In mosquitoes (Diptera: Culicidae), infections have only been described from some container-breeding species (Beier and Craig, 1985). Three such species, *Aedes aegypti* (Linnaeus), *Ae. albopictus* (Skuse), and *Ochlerotatus triseriatus* (Say), are known or potential vectors of a number of arboviruses (Calisher, 1994). Each of the 3 mosquito species can naturally harbor a specific gregarine parasite that allows the host mosquito to carry out life functions relatively unaffected by its presence. The parasite is transmitted to other mosquitoes in the aquatic environment when feeding larvae ingest oocysts released by defecating or dead adult mosquitoes. Gregarine trophozoites feed intracellularly in the midgut epithelium and increase in size during mosquito larval development; gametogony and sporogony in the Malphigian tubules during the pupal and early adult stages result in new oocysts that are released back into the larval environment.

In the southeastern United States, larval *Oc. triseriatus*, *Ae. aegypti*, and *Ae. albopictus* and their ascogregarine parasites, *Ascogregarina barretti* (Vavra), *A. culicis* (Ross), and *A. taiwanensis* (Lien and Levine), respectively, co-occur in containers in some urban and suburban areas, including New Orleans, Louisiana (Comiskey et al., 1999). Although *A. barretti* trophozoites can be visually distinguished from the other 2 parasite species in mosquito midgut infections, currently there is no simple and accurate method to distinguish between *A. taiwanensis* and *A. culicis* because they have similar size and morphology (Lien and Levine, 1980). Reyes-Villanueva et al. (2001) reported differences in the shape and pigmentation between the gamonts of *A. culicis* and *A. taiwanensis* when viewed with phase-contrast microscopy. However, these morphological differences may be strain-specific and not seen in all *A. taiwanensis* isolates (Reyes-Villanueva et al., 2001). Morphological differentiation of gamonts involves a process of dissection, requiring considerable technical skill and careful timing of dissections to reliably view extracellular gamonts. This may not always be reliable when dealing with field-collected mosquito larvae, which could have consumed oocysts of both gregarine species at different times during larval development, resulting in trophozoites and gamonts of varying ages in an individual larva. Although isoenzyme electrophoresis has been used to separate *A. barretti* and *A. geniculati*, which occur naturally in *Oc. triseriatus* and *Oc. geniculatus*, respectively, this method has not been optimized for other members of the genus (Rowton and Munstermann, 1984). These obstacles significantly hinder field studies to estimate prevalence of dual infections in competition studies between *Ae. aegypti* and *Ae. albopictus* (Blackmore et al., 1995). Therefore, a reliable and specific method is needed for accurate identification of infection with both parasite species.

To address this need, we developed an identification method for *A. taiwanensis* and *A. culicis* using the polymerase chain reaction (PCR). We selected the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) as our target for several reasons. As Beckingham (1982) suggested, eukaryotic ribosomal RNA (rRNA) genes are typically present in hundreds to thousands of copies, and are arranged as repeating units linked in 1 or a few tandem arrays. Each single unit consists of a conserved transcribed region, within which are the genes for the 18S, 5.8S, and 28S subunits, as well as 2 internal transcribed spacers (ITS1 and ITS2) and a variable intergenic spacer (IGS) (Beckingham, 1982). With the help of PCR, and as a result of the high rRNA copy number, even small amounts of genomic DNA produce reliable rDNA target fragments. Within the rDNA, the ITS segments have been used by a number of researchers to develop PCR-based species- or taxon-specific diagnostic assays. This is possible because although the ITS regions are not highly conserved, the flanking coding regions (18S, 5.8S, and 28S) are highly conserved. Thus, development

Received 29 July 2004; revised 5 March 2005; accepted 8 March 2005.
* Centro Internacional de Entrenamiento e Investigaciones Médicas, Cali, Colombia.
† Department of Tropical Medicine, Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, Louisiana, 70112.
‡ To whom correspondence should be addressed.

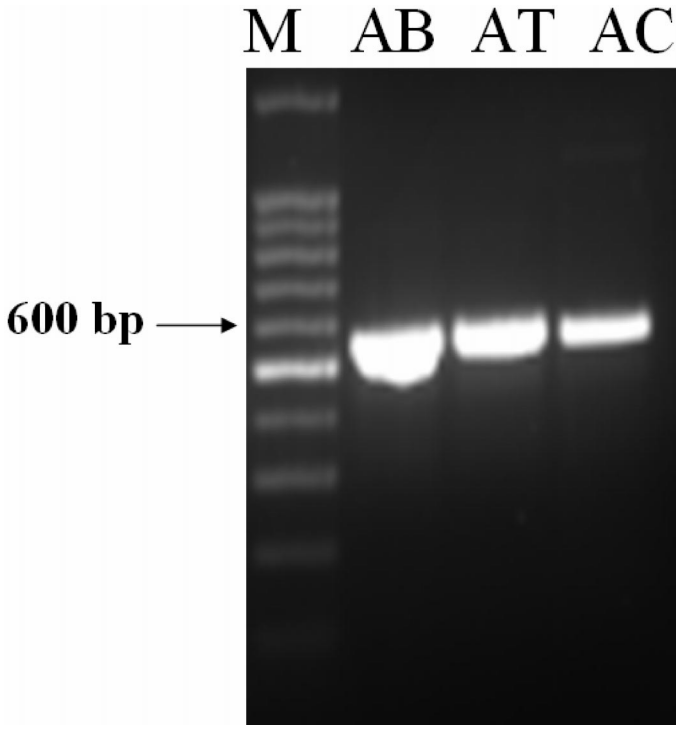

FIGURE 1. An ethidium bromide-stained 2% agarose gel under UV light showing *Ascogregarina*-specific PCR products spanning the ITS region. Lane M, 100-bp ladder; lane AB, *A. barretti*; lane AC, *A. culicis*; lane AT, *A. taiwanensis*.

of PCR primers within the coding regions, designed to span the less conserved ITS segments, allows differentiation of closely related species or species groups. For example, this tactic has been successful with insects (Porter and Collins, 1991; Gallego and Galian, 2001), protozoans (Cupolillo et al., 1995; Beltrame-Botelho et al., 2005), and nematodes (Gasser et al., 1996; Zhu et al., 2000), as well as a variety of other organisms. Here, we report a PCR-based assay which utilizes ITS variation between morphologically similar species of *Ascogregarina*.

MATERIALS AND METHODS

Ascogregarina collection and genomic DNA extraction

Ascogregarina culicis and *A. taiwanensis* oocysts were obtained from laboratory stocks maintained in their known host mosquito species as described previously (Beier and Craig, 1985). The *A. taiwanensis* stock originated from infected *Ae. albopictus* collected in New Orleans, Louisiana, from an area with no *Ae. aegypti* and isolated as described previously (Munstermann and Wesson, 1990). The *A. culicis* stock was obtained in 2000 from naturally infected *Ae. aegypti* collected by workers with the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Cali, Colombia. At the time, there were no *Ae. albopictus* present in Cali, and so no potential for dual infections. A local strain of *Oc. triseriatus* infected with *A. barretti* was collected from New Orleans tree holes in 2002. To verify infection and parasite morphology, *Oc. triseriatus* mosquito larvae were dissected in saline solution (pH 7.0); the extracted midgut was then placed under a glass coverslip and a dissecting microscope was used to observe trophozoites. For *A. culicis* and *A. taiwanensis*, genomic DNA was extracted from oocyst suspensions from which most, but not all, mosquito DNA was excluded. For *A. barretti*, genomic DNA was extracted from the midgut, which was visibly infected with gregarines matching the known morphology of *A. barretti*. For all 3 species, genomic DNA was extracted using a 'salting-out' protocol (Collins et al., 1987). In brief, the whole larva was ground in 100 μl of grinding buffer (NaCl, sucrose, Tris, EDTA, and SDS; see Collins et al., 1987) followed by the addition of 8M potassium acetate. The reaction was then centrifuged at 11,000 *g* in a microcentrifuge at 4 C for 10 min. The supernatant was transferred into a new microfuge tube containing cold (−20 C) 100% ethanol. The precipitated DNA was centrifuged for 10 min and the DNA pellet was washed with 70 % ethanol. All pellets were resuspended in 50 μl sterile deionized water. DNA concentrations were determined spectrophotometrically. The concentrated stock was kept at −80 C and a working stock, diluted 1:10 with sterile water, was used in the PCR reactions.

TABLE I. Primers used in the initial amplification of the ascogregarine ITS region. ASCO-18S-F is located approximately 140 bp from the 3′ end of the 18S rRNA region. ASCO-28S-R is located approximately 20 bp from the 5′ end of the 28S rRNA region. TM = melting temperature.

Primer name	Primer sequence	Tm (C)
ASCO-18S-F	5′ CGA CTG GAT GAT CCG G 3′	52.0
ASCO-28S-R	5′ CAG TGG GTA GCC TTG TC 3′	48.0

PCR amplification and sequencing of ITS

The first part of the ITS targeted for sequencing was the ITS1. A degenerate primer, ASCO-IT1 Reverse (5′ GCT GCG MCC TTC ATC G 3′), located in the 5.8S region and oriented toward the 18S region, was designed based on similarity among GenBank sequences in the following apicomplexan taxa: *Neospora caninum* (GenBank L49389), *Toxoplasma gondii* (GenBank L49390), *Perkinsus atlanticus* (GenBank U07697), *Cryptosporidium parvum* (GenBank AF040725), *Eimeria maxima* (GenBank AF027726), and *Plasmodium falciparum* (GenBank U21939). A second primer, SP-18S Forward (5′ GTA AGC TTC CTT TGT ACA CAC CGC CCG T 3′), (Wesson et al., 1992), based on the 18S region and oriented toward the 5.8S region, was paired with ASCO-IT1 Reverse to amplify the ITS1. That set of primers, SP-18S Forward and ASCO-IT1 Reverse, successfully amplified the ITS1 region not only from *A. culicis* and *A. taiwanensis*, but also from their mosquito hosts. The ascogregarine fragments (~296 bp) were separated from the mosquito fragments (510–1,020 bp) on an ethidium bromide-stained 2% agarose gel and visualized under a UV lamp. The parasite fragments were excised from the gel, purified (Bioclean Kit, U.S. Biochemicals, San Diego, California), and ligated into a pGEM-T plasmid vector (Promega Corp., Madison, Wisconsin). The ascogregarine fragments were sequenced by the dideoxy termination method (Sanger et al., 1977) using Sequenase (U.S. Biochemicals) according to the manufacturer's instructions. Based on these sequence data, *Ascogregarina* spp.-specific primers were designed in the 18S region. The new 18S primer, ASCO-18S-F, based on the *A. culicis* and *A. taiwanensis* sequences, is the primer listed in Table I. PCR amplifications using this primer and ASCO-IT1-R amplified *Ascogregarina* spp. ITS1, but did not amplify mosquito DNA (data not shown). To amplify a fragment encompassing both the ITS1 region and the ITS2 region, a degenerate primer was designed based on the conserved 28S region and oriented toward the 5.8S region (ASCO-28S-Reverse, Table I) based on the following GenBank sequences: *Cryptosporidium parvum* (GenBank AF040725), *Isospera felis* (GenBank U85705), *Neospora caninum* (GenBank L49389), *Plasmodium falciparum* (GenBank U21939), and *Sarcocystis muris* (GenBank AF012883).

For the *Ascogregarina* genus-specific PCR, amplifications were performed in 50.0 μl reaction volumes containing 100 ng of genomic DNA from the species *A. culicis*, *A. taiwanensis*, and *A. barretti*, 5.0 μl of 10× reaction buffer, 1,200 μM of each dNTP, 2 mM $MgCl_2$, and 1 U of Taq polymerase (Promega). The samples were heated initially at 94 C for 1 min, followed by 30 cycles of amplification at 94 C for 1min, at 50 C for 1 min, at 72 C for 2 min, and a final extension step at 72 C for 10 min.

The amplified products were purified on columns (Promega) and sequenced using PCR primers by the DNA Sequencing Core at Tulane's Center for Gene Therapy using BigDye™ Terminator chemistry (Applied Biosystems [ABI], Foster City, California) and Prism 3100 se-

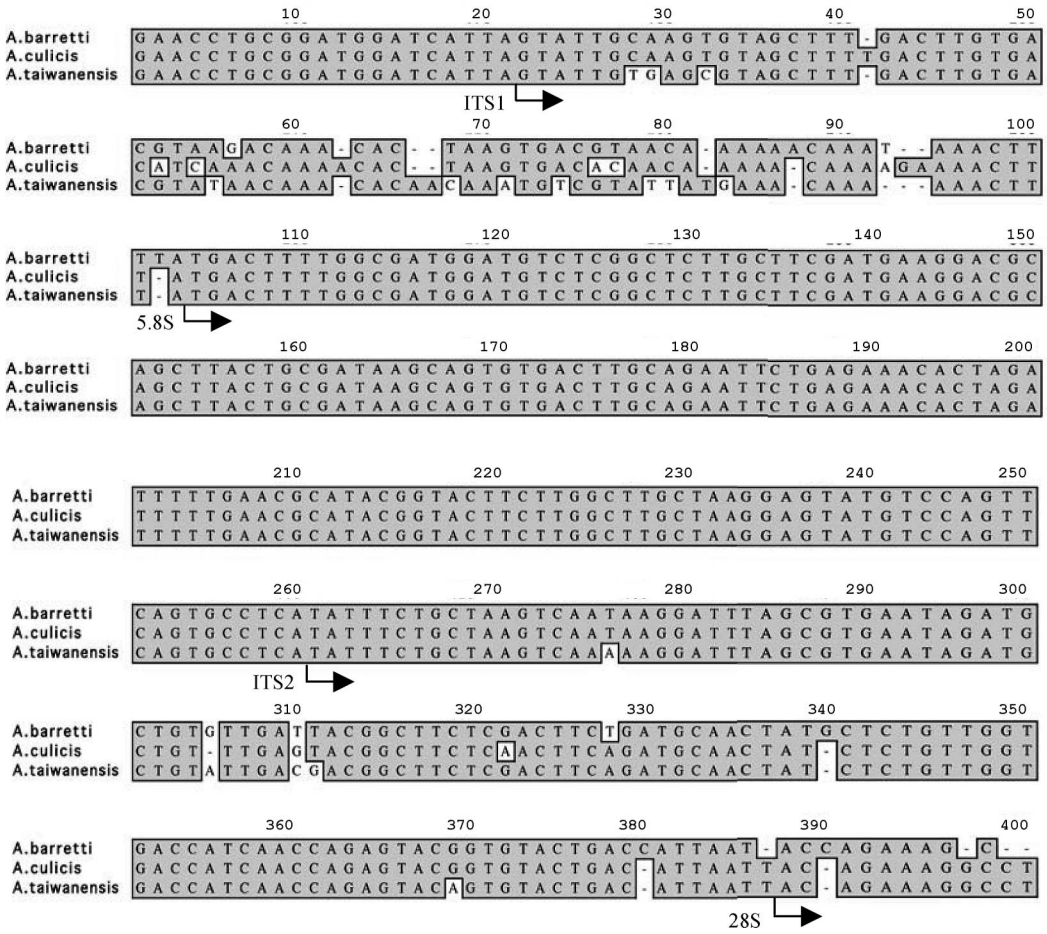

FIGURE 2. Alignment of the ITS1, 5.8S, ITS2, and flanking 18S and 28S sequences of the 3 *Ascogregarina* species. Shaded areas indicate where at least 2 of the sequences are in consensus; dashes represent gaps introduced to maximize overall sequence similarity. Arrows represent the start of the ITS1, 5.8S, ITS2, and 28S. The length of the ITS1 ranges from 75 to 77 bp; the length of 5.8S in all species is 157 bp; ITS2 length ranges from 124 to 126 bp. Specific primer AC (from Table 2) for *A. culicis* is located from 49 to 73, while primer AT for *A. taiwanensis* is located from 300 to 321; universal primer AU is not shown on this figure.

quencers (Applied Biosystems [ABI]). The sequences were aligned using the Jellyfish software package (LabVelocity, San Francisco, California). GenBank accession numbers for the rDNA sequences are AY327258 (*A. culicis*), AY327259 (*A. barretti*), and AY326461 (*A. taiwanensis*).

Ascogregarina species-specific PCR

For the *Ascogregarina* species-specific PCR, the PCR mixture consisted of 5.0 μl of 10× reaction buffer, 1.2 mM of each dNTP, 2 mM $MgCl_2$, 1 U of Taq polymerase (Promega), and 100 ng of template DNA in 50.0 μl final reaction volume. The amount of each primer used in the PCR assay was 10 μM. PCR conditions included an initial denaturation step at 94 C for 2 min, followed by 30 cycles of 1 min at 94 C, 30 sec at 53 C, and 1 min at 72 C, with a final extension step of 10 min at 72 C. The amplified fragments were separated by electrophoresis on an ethidium bromide-stained 2% agarose gel and visualized under a UV lamp.

TABLE II. Primers used for identification of *A. culicis* and *A. taiwanensis*. AU is the ascogregarine universal primer that binds to the 18S DNA for both species. AT is the *A. taiwanensis* primer that binds to the ITS2 rDNA region, while AC is the *A. culicis* primer that binds to the ITS1 rDNA region.

Primer name	Primer sequence	Tm (C)
AU	5′ ACC GCC CGT CCG TTC AAT CG 3′	63.5
AT	5′ GAG AAG CCG TCG TCA ATA CAG C 3′	59.1
AC	5′ CAC TTA GTG TTT TGT TTG ATG TC 3′	52.0

RESULTS

Analysis of ITS sequence

The rDNA ITS was successfully amplified from the 3 ascogregarine species using the ASCO-18S-Forward and ASCO-28S-Reverse primers (Table I). A single band of approximately 600 bp was revealed by electrophoresis (Fig. 1). This set of primers could, therefore, be used as a diagnostic PCR for the presence of *Ascogregarina* spp. infection in mosquitoes. Presence of infection (in the form of trophozoites in larvae or gametocysts or oocysts in adults) is not difficult to verify if one is well versed in midgut dissection, but misidentification in the course of extensive population surveys in the field may occur. This method, therefore, can serve as an additional checkpoint that can be applied after a specimen has been morphologically destroyed and can also serve to improve time efficiency when processing multiple potentially infected specimens.

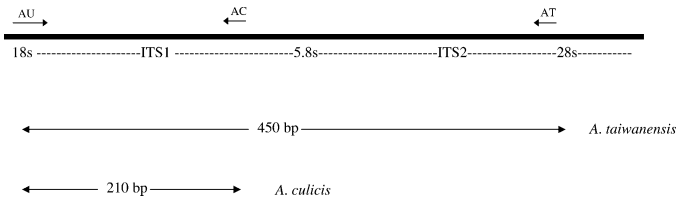

FIGURE 3. Schematic diagram of the design of the species-specific primers. Primers are represented by single headed arrows, shown above a diagram of a 630-bp fragment of gregarine rDNA. Primer names are abbreviated as follows: AC, *A. culicis* reverse; AT, *A. taiwanensis* reverse; and UN, universal forward. Approximate sizes of the bands resulting from PCR using these primers and template DNA from *A. taiwanensis* or *A. culicis* are shown in bp below the rDNA diagram.

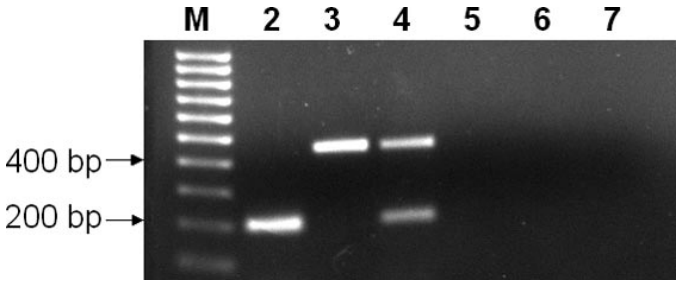

FIGURE 4. An ethidium bromide-stained 2% agarose gel under UV light showing species-specific products. Lane M is the 100-bp ladder; lane 2, *A. culicis*; lane 3, *A. taiwanensis*; lane 4, DNA of both *A. culicis* and *A. taiwanensis*; lane 5, noninfected *Ae. aegypti*; lane 6, noninfected *Ae. albopictus*; lane 7, water template control.

The entire ITS region (including ITS1 and ITS2) was sequenced from all 3 species. Sequencing was done on both strands directly from the PCR products using the PCR primers. Figure 2 shows an alignment of the consensus sequence obtained for each species, and boundaries of the 18S, 5.8S, and 28S genes. Although the sequences aligned well through most of their length, there was variation, including nucleotide insertions and deletions and base substitutions, in the ITS1 and ITS2 domains. Percent similarity among the species for the ITS segment (including the 5.8S region, but excluding gaps) was 94.6% for *A. culicis*/*A. taiwanensis*, 97.4% for *A. culicis*/*A. barretti*, and 95.2% for *A. taiwanensis*/*A. barretti*. Percent similarity for ITS1 and ITS2 only (excluding 5.8S and gaps) was 90.3% for *A. culicis*/*A. taiwanensis*, 95.4% for *A. culicis*/*A. barretti*, and 91.3% for *A. taiwanensis*/*A. barretti*.

Species-specific PCR assay

Our strategy in designing the ITS1- and ITS2-based species-specific primers followed the approach used by Scott et al. (1993) to distinguish the *Anopheles gambiae* complex. The forward primer, which binds to the 5′ end of the 18S, is universal in all 3 ascogregarine species, whereas the reverse primers are species-specific for *A. culicis* and *A. taiwanensis*, and anneal at different positions of the ITS1 or ITS2 regions (Table II, Fig. 2). Amplification products of diagnostic lengths were generated from the DNA of each species. Primer sequences were defined so that at least 3 nucleotides in their 3′ end would prevent hybridization to the alternative haplotypes and guarantee specificity of the assay (Huang et al., 1992). The resulting PCR products differed from one another by at least 100 bp, enabling them to be easily separated on agarose gels. The size of the diagnostic bands and the positions of the primer binding sites are indicated in Figure 3. The primers AU-forward, AT-reverse, and AC-reverse, therefore, successfully amplified *A. culicis*, *A. taiwanensis*, or both, depending on which gregarine species was present, and did not amplify mosquito DNA. The resulting PCR products were easily distinguished by size (Fig. 4).

An attempt was made to include a specific primer for *A. barretti*. Although amplification proceeded smoothly in a simple PCR using this primer with the universal primer and *A. barretti* DNA alone as a template, amplification of a single clean fragment from *A. barretti* was not achieved when its specific primer was used in the multiplex PCR (data not shown). In light of the fact that *A. barretti* is clearly morphologically distinct from *A. culicis* and *A. taiwanensis*, morphological identification seems to be the more reliable method for identification of *A. barretti* at this time.

DISCUSSION

The role of these intestinal parasites as potential mosquito pathogens is important because many of their hosts are vectors of human and animal diseases. Mortality in nonnative hosts due to ascogregarine infection can be significant. *Ascogregarina taiwanensis* normally infects *Ae. albopictus* and establishes a benign infection. However, when *A. taiwanensis* infects *Ae. aegypti*, mortality of up to 10% has been reported (Reyes-Villanueva et al., 2003). In addition, in native hosts, pathology such as stunted development and elevated mortality in larval *Ae. aegypti* heavily infected with *A. culicis* was reported by Barrett (1968). If larval nutrients are limited, prolonged development or reduction in adult size and longevity have been noted in *Ae. aegypti* infected with *A. culicis* and *Oc. triseriatus* infected with *A. barretti* (McCray et al., 1970; Walker et al., 1987). Comiskey et al. (1999) concluded that *Ascogregarina* spp. infections affect *Ae. albopictus* by increasing the mortality of immature stages when nutrient supplies are scarce and by decreasing the reproductive capacity of females under high nutrient conditions.

In addition to their contributions to pathology of their mosquito hosts, ascogregarine species may also play a role in the maintenance of arboviruses. *Ascogregarina culicis* oocysts have been implicated in the vertical transmission of Chikungunya (CHIK) virus in *Ae. aegypti* mosquitoes (Mourya et al., 2003). Adult mosquitoes infected orally with CHIK virus produced CHIK-infected *A. culicis* oocysts. Those oocysts, when ingested by *Ae. aegypti* larvae, led to CHIK-infected *Ae. aegypti* adults. This observation implicated *A. culicis* in the maintenance of CHIK virus during the interepidemic period. Further research is needed to discern whether other arboviruses can survive in this fashion.

Identification of the trophozoite stage of *Ascogregarina* spp. is done mainly on the basis of morphological characteristics. However, in addition to the labor intensive nature of dissection and identification, this method is often difficult to apply because of biological and/or technical issues, e.g., shared overlapping characters, presence of parasites of different developmental stage due to continuous filter feeding by larvae, differential sampling, and inadequate preservation of specimens. Hence, the taxonomic status of each of the species of *Ascogregarina* has relied largely on which mosquito species it preferentially in-

fects. This technique becomes much less useful when the targeted mosquito species coexist in the same aquatic container environment.

Our aim was to develop a more reliable method for *Ascogregarina* species differentiation in areas where multiple mosquito and parasite combinations may occur. With proper primer design, the PCR method is rapid, reliable, and extremely sensitive (He et al., 1994). Moreover, as is frequently highlighted as an advantage of PCR-based methods, a small quantity of biological material contains sufficient template DNA to allow amplification, which leaves the remaining body of the organism available for other studies. This method provides a rapid, reliable, inexpensive, and technically straightforward protocol for the identification and species-level differentiation of *Ascogregarina* sp. infections.

ACKNOWLEDGMENTS

We thank Lisa Pratt and Bethany Peel for their invaluable help in fieldwork. This project has been funded from the following sources: NIH U01 AI 58303-01, ASPH (CDC) S1740-21/24, and the Coypu Foundation of Louisiana. Additional external support for Clara Ocampo was provided by COLCIENCIAS and the World Health Organization.

LITERATURE CITED

BARRETT, W. L. 1968. Damage caused by *Lankesteria culicis* (Ross) to *Aedes aegypti* (L.). Mosquito News **28:** 441–444.

BECKINGHAM, K. 1982. Insect rDNA. *In* The cell nucleus, volume 10, H. Busch and L. Rothblum (eds.). Academic, New York, New York, p. 205–269.

BEIER, J. C., AND G. B. CRAIG, JR. 1985. Gregarine parasites of mosquitoes. *In* Integrated mosquito control methodologies, volume 2, M. Laird (ed.). Academic, London, U.K., p. 167–184.

BELTRAME-BOTELHO, I. T., D. GASPAR-SILVA, M. STEINDEL, A. M. DAVILA, AND E. C. GRISARD. 2005. Internal transcribed spacers (ITS) of *Trypanosoma rangeli* ribosomal DNA (rDNA): A useful marker for inter-specific differentiation. Infections, Genetics and Evolution **5:** 17–28.

BLACKMORE, M. S., G. A. SCOLES, AND G. B. CRAIG. 1995. Parasitism of *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae) by *Ascogregarina* spp. (Apicomplexa: Lecudinidae) in Florida. Journal of Medical Entomology **32:** 847–852.

CALISHER, C. H. 1994. Medically important arboviruses of the United States and Canada. Clinical Microbiology Reviews **7:** 89–116.

COLLINS, F. H., M. A. MENDEZ, M. O. RASMUSSEN, P. C. MEHAFFEY, N. J. BESANSKY, AND V. FINNERTY. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. American Journal of Tropical Medicine and Hygiene **37:** 37–41.

COMISKEY, N., R. C. LOWRIE, JR., AND D. M. WESSON. 1999. Role of habitat components on the bionomics of *Aedes albopictus* (Diptera: Culicidae) from New Orleans. Journal of Medical Entomology **36:** 313–320.

CUPOLILLO, E., G. GRIMALDI, JR., H. MOMEN, AND S. M. BEVERLEY. 1995. Intergenic region typing (IRT): A rapid molecular approach to the characterization and evolution of *Leishmania*. Molecular Biochemistry and Parasitology **73:** 145–155.

GALLEGO, D., AND J. GALIAN. 2001. The internal transcribed spacers (ITS1 and ITS2) of the rDNA differentiates the bark beetle forest pests *Tomicus destruens* and *T. piniperda*. Insect Molecular Biology **10:** 415–420.

GASSER, R. B., L. E. STEWART, AND R. SPEARE. 1996. Genetic markers in ribosomal DNA for hookworm identification. Acta Tropica **62:** 15–21.

HE, Q., M. MARJAMAKI, H. SOINI, J. MERTSOLA, AND M. K. VILJANEN. 1994. Primers are decisive for sensitivity of PCR. BioTechniques **17:** 82–86.

HUANG, M.-M., N. ARNHEIM, AND M. F. GOODMAN. 1992. Extension of base mispairs by Taq DNA polymerase: Implication for single nucleotide discrimination in PCR. Nucleic Acid Research **20:** 4567–4573.

LIEN, S. M., AND N. D. LEVINE. 1980. Three new species of *Ascocystis* (Apicomplexa: Lecudinidae) from mosquitoes. Journal of Protozoology **27:** 147–151.

MCCRAY, E. M., JR., R. W. FAY, AND H. F. SCHOOF. 1970. The bionomics of *Lankesteria culicis* and *Aedes aegypti*. Journal of Invertebrate Pathology **16:** 42–53.

MOURYA, D. T., D. K. SINGH, P. YADAV, M. D. GOKHALE, P. V. BARDAE, N. B. NARAYAN, J. P. THAKARE, A. C. MISHRA, AND Y. S. SHOUCHE. 2003. Role of gregarine parasite *Ascogregarina culicis* (Apicomplexa: Lecudinidae) in the maintenance of Chikungunya virus in vector mosquito. Journal of Eukaryotic Microbiology **50:** 379–382.

MUNSTERMANN, L. E., AND D. M. WESSON. 1990. First record of *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae) in North American *Aedes albopictus*. Journal of American Mosquito Control Association **6:** 235–243.

PORTER, C. H., AND F. H. COLLINS. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). American Journal of Tropical Medicine and Hygiene **45:** 271–279.

REYES-VILLANUEVA F., J. J. BECNEL, AND J. F. BUTLER. 2001. Morphological traits for distinguishing extracellular gamonts of *Ascogregarina culicis* and *Ascogregarina taiwanensis* in *Aedes aegypti* and *Aedes albopictus*. Journal of Invertebrate Pathology **77:** 227–229.

———, ———, AND ———. 2003. Susceptibility of *Aedes aegypti* and *Aedes albopictus* larvae to *Ascogregarina culicis* and *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae) from Florida. Journal of Invertebrate Pathology **84:** 47–53.

ROWTON, E. D., AND L. E. MUNSTERMANN. 1984. Electrophoretic differences in the isoenzymes of two mosquito gregarines, *Ascogregarina barretti* and *Ascogregarina geniculati*. Journal of Parasitology **70:** 63–67.

SANGER, F., S. NICKLEN, AND A. R. COULSEN. 1977. DNA sequencing with chain terminating inhibitors. Proceedings of the National Academy of Science **74:** 5463–5468.

SCOTT, J. A., W. G. BROGDON, AND F. H. COLLINS. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. American Journal of Tropical Medicine and Hygiene **49:** 520–529.

WALKER, E. D., S. J. POIRIER, AND W. T. VELDMAN. 1987. Effects of *Ascogregarina barretti* (Eugregarinida: Lecudinidae) infection on emergence success, development time, and size of *Aedes triseriatus*. Journal of Medical Entomology **24:** 303–309.

WESSON, D. W., C. H. PORTER, AND F. H. COLLINS. 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). Molecular Phylogenetics and Evolution **1:** 253–269.

ZHU X., R. B. GASSER, D. E. JACOBS, G. C. HUNG, AND N. B. CHILTON. 2000. Relationships among some ascaridoid nematodes based on ribosomal DNA sequence data. Parasitological Research **86:** 738–744.