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First report of *Leptolegnia chapmanii* (Peronosporomycetes: Saprolegniales) affecting mosquitoes in central Brazil

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A B S T R A C T

Numerous isolates of an oomycete 'fungus', *Leptolegnia chapmanii*, are reported from Brazil for the first time. This aquatic pathogen was baited with *Aedes aegypti* sentinel larvae from stagnant, temporary bodies of water in selected locations under secondary tropical forest in and near the central Brazilian city of Goiânia and from more distant sites in the western and northern regions of the state of Goiás. Isolates were identified based on their morphological and developmental characters, comparative sequence data for the ITS and TEF loci, as well as their rapid activity against *A. aegypti* larvae. Taxonomic issues affecting the application of the name *L. chapmanii* and its typification are rectified. This study contributes to a better understanding of the presence and distribution of this oomycete in Brazil, its sequence-based identification, and of its potential as a biological agent against mosquito vectors.

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1. Introduction

Leptolegnia chapmanii Seymour (1984; Straminipila: Peronosporomycetes: Saprolegniales) is notable for its rapid activity against mosquito larvae and its ability to infect these hosts either by its encysted zoospore germtubes' abilities to penetrate directly through the cuticle or through the gut after ingestion of cysts (Zattau and McInnis, 1987). This entomopathogen has interest as an agent for biological control because it is rapidly lethal for important culicine mosquito vectors of diseases in humans and animals (McInnis and Zattau, 1982; Lord and Fukuda, 1990). This fungus (this term is used here for convenience in its generalized, traditional sense) was first isolated from mosquito larvae in southern USA in the 1970s and later described as a new species (Seymour, 1984).

Our current findings in Brazil suggest that *L. chapmanii* may be relatively widely distributed–and possibly even comparatively common–throughout central Brazil and provide simplified means to detect this pathogen elsewhere in the world. One of the few certainties about this mosquito pathogen is the need to collect and to evaluate more isolates to extend our knowledge about both the actual distribution and the taxonomy of mosquito-pathogenic *Leptolegnia* species. The present study reports on the recovery of several *L. chapmanii* isolates in central Brazil and their pathogenicity for *Aedes aegypti* larvae.

L. chapmanii is well known for the characteristic formation of zoospores in a single file inside comparatively narrow zoosporangia, but the taxonomic character that most definitively distinguishes this species is its distinctive oogonia decorated by spiked or finger-like projections. Nonetheless, there is a more complex underlying taxonomic issue involving this species' typification and lack of authentic material (either specimens or cultures identified by the species' author) to allow verifications of identifications. The problem with this species, then, is an unresolved ambiguity about exactly how this species name should be applied. These issues are treated in some detail here.

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2. Materials and methods

2.1. Sampling for natural occurrences of fungal pathogens from collecting sites

Entomopathogens were surveyed by using *A. aegypti* larvae as bait insects between May 2014 and May 2015 in and around the city of Goiânia, Goiás state, in central Brazil. Additional collections were made during surveys of more remote sites near the city of Aruanã on the Araguaia River which divides the states of Goiás and Mato Grosso, as well as in the mountainous northern part of Goiás at sites in and near the city of Cavalcante (13°48′05.59″ S 47°27′36.39″ W–16°35′45.99″ S 49°16′57.33″ W).

The search of pathogens of mosquito larvae involved placements of sentinel larvae of *A. aegypti* in sheltered sites with still water at all of the collecting sites surveyed. In each instance, groups of 20–25 laboratory-reared second or third instar larvae (L2 or L3) of *A. aegypti* were transferred into a transparent plastic container (85 × 60 mm) with its bottom and a part of the lid replaced with a fine (150–400 μm) mesh, and to which several styrofoam blocks and a length of string were glued to float on the surface and to secure the container to adjacent vegetation (Fig. 1). All plastic cups and tubes used for field collections were thoroughly cleaned and sterilized by a 20 min exposure to ultraviolet light in a vertical flow sterile hood before being used in the field. Cups with larvae were generally set in the morning (8–10 am) and submerged in stagnant, shallow (10–25 cm depth) transitory bodies of water in randomly selected locations in or adjacent to secondary tropical forest (Fig. 2). Overhanging vegetation protected the collecting sites against continuous sunlight. Ambient temperature and relative humidity were measured when placing the cups with the bait larvae.

Cups were retrieved after a 24–36 h exposure. Each batch of larvae, whether alive or dead, was transferred to a plastic tube (50 ml; Falcon Plastics) for transportation to the laboratory where they were transferred to plastic cups (35 × 50 mm) with 20 ml sterile tap water (Fig. 3), fed on alternating days with small amounts of ground pellets of cat food (Black Jack; Alisul Alimentos S.A., São Leopoldo, Rio Grande do Sul, Brazil) and checked for mortality daily for 15 days.

2.2. Isolation, preservation and characterization of cultures

Attempts to isolate the pathogen were made by placing infected individual mosquito larvae into a small pit or a wedge (with a vertical wall and the agar removed from the side) cut with a scalpel into but, importantly, not through a thick layer of Peptone-Yeast extract-Glucose (PYG) agar supplemented with streptomycin (0.06%), neomycin (0.06%), and Supertrin (sodium sulphadiazine and trimethoprim at 0.06%; Europharma Laboratórios Ldta, São Paulo, Brazil) and chloramphenicol (0.004%) in small Petri dishes (45 × 15 mm). A sterilized coverslip was sometimes placed over this cut in the medium to force hyphal growth into the medium and while minimizing or containing aerial growth by either the desired, rapidly growing pathogen or by rapidly growing saprobic fungal contaminants. Mycotized larvae were then incubated at 24 °C and 12 h photophase (Fig. 4). After 48–72 h the plates were examined on a stereo microscope with illumination through the base, and small blocks of hyphal tips growing into the medium without any obvious contamination were removed and transferred on PYG medium without antibiotics in a new Petri dish. The axenic nature of new *Leptolegnia* isolates was confirmed by transferring the isolate to PYG broth to check for any rapid growth by bacterial contaminants. Cultures were grown for 10 days as mentioned and then preserved both at 4 °C and in darkness both on PYG medium and in water stasis. For water stasis (Humber, 2012), cultures on small medium blocks (about 15 mm^3) were stored submerged in 5 ml of sterile deionized water in plastic tubes (150 × 15 mm) with screw caps and maintained at 4 °C; the fungus has been found to remain viable in these tubes for at least one year.

The Brazilian isolates were co-deposited in water stasis in the culture collection of the Laboratory of Invertebrate Pathology at

Figs. 1–4. Collection and isolation of *Leptolegnia chapmanii*. 1. Detail of plastic cup construction used in field sites to place and to recover sentinel larvae of *Aedes aegypti*. 2. Floating cup with larvae at collecting site. 3. Cup with recovered sentinel larvae of *A. aegypti* from field in laboratory to check the mortality. 4. Mycotized larva set into a small pit cut with a scalpel into but not through Peptone-Yeast extract-Glucose medium supplemented with antibiotics.

IPTSP and cryogenically at −196 °C in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, New York, USA).

Zoospores were produced by submerging blocks (about 15 mm^3) of PYG medium with mycelium (10 days old) into 30 ml sterile deionized water for 48 h. The pathogenicity for mosquitoes of each isolate was confirmed by placing 10 L2 or L3 into suspensions of actively swimming zoospores and incubated for up to 96 h at 24 °C and 12 h photophase (Pelizza et al., 2007). Larval mortality was checked daily, and the development of the pathogen on dead larvae examined by brightfield or phase contrast microscopy. Specific structures such as hyphae (as grown on PYG medium), zoosporangia, encysted zoospores and oogonia (grown *in vitro* on dead larvae) of at least two series were stained with cotton blue, measured microscopically (Nova 180i-T; Toupview), and documented with a digital camera (Leica UCMOS01300KPA). The measurements were based on up to 50 objects per isolate and series. For each series a mean value was calculated, and the final value of each object consisted of the mean and its standard error of the mean of both series. Values of measurements were compared with those reported for *L. chapmanii* in the protolog of this taxon (Seymour, 1984) as well as from strains from the southern USA (ARSEF 2681) and Argentina (ARSEF 5499; López Lastra et al., 1999).

The need to resolve the taxonomic issues involving *L. chapmanii* Seymour (1984) included trying to locate the holotype collection of this species, to discover whether this type specimen does still exists, and to determine whether any cultures of *L. chapmanii* noted by Seymour (1978, 1987) might still be available from any source.

2.3. Genomic evaluations of cultures

Fungal isolates were grown in 150 ml SDY/4 broth (2.5 g/L yeast extract) for 7 days in a shaker at 125 rpm and 25 °C. DNA was extracted from mycelium using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The internal transcribed spacer (ITS) was amplified by PCR using the primers ITS1 and ITS4 and the PCR protocol conditions reported by White et al. (1990). The primers and PCR protocol used to amplify TEF loci for the Brazilian and ex-epitype isolates–IP 392 and ARSEF 2681, respectively–were 983F and 2218R (Rehner and Buckley, 2005). PCR products were visualized using agarose gel electrophoresis, and sent for purification and sequencing to Helixxa Genomic Services (Paulínia, SP, Brazil). Both strands of the PCR products were sequenced using the Applied Biosystems Big Dye v.3.1 kit and the same primers described above with an ABI 3500 automatic sequencer. Contigs of sequence data were assembled using Chromas Pro (vers. 1.5; Technelysium Pty Ltd) and aligned using the ClustalX (vers. 2.0.11). The ITS sequences were deposited in the GenBank database and accession numbers of KU896915 (for IP 392), KU896916 (for ARSEF 2681), and KU896917 (for ARSEF 5499); the TEF sequences were deposited in GenBank as KU937148 (for IP 392) and KU937149 (for ARSEF 2681).

3. Results

3.1. Sampling for occurrence of fungal pathogens from collecting sites

During the survey of entomopathogenic fungi affecting mosquitoes, moribund larvae, dead and obviously mycotized larvae bearing external hyphal growth were removed from the collections of sentinel larvae brought into the laboratory from field sites. Microscopic observations of the cadavers frequently revealed infections caused by the saprolegnialen genus *Leptolegnia*; this determination was based on the production of zoospores in a single file in narrow zoosporangia, and by subsequent laboratory tests confirming the rapid larvicidal activity of these fungi against *A. aegypti* larvae. The first finding of mycosed larvae in Campus Samambaia, UFG, Goiânia, in August 2014 was followed by similar finds from eight different localities in the state of Goiás during the rainy and dry seasons between September 2014 and May 2015 (Fig. 5). A total of eight axenic cultures of *L. chapmanii* were obtained and are being stored by serial transfer and refrigerated storage in the Laboratory of Invertebrate Pathology (IPTSP-UFG, Goiânia, Brazil). Further, these Brazilian isolates of *L. chapmanii* are being preserved cryogenically by the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA) as ARSEF 12817 (=IP 394), 12819 (=IP 390), 12829 (=IP 392), 12831 (=IP 397), 12835 (=IP 401), 12840 (=IP 406), 12845 (=IP 411), and 12847 (=IP 413).

Morphological measurements of major characters for three representative *L. chapmanii* isolates from different distantly separated localities in the state of Goiás, the *L. chapmanii* holotype described by Seymour (1984), the *L. chapmanii* isolate from South Carolina (USA) that we designate here as the epitype for *L. chapmanii*, and the only isolate of this fungus from Argentina are presented in Table 1.

The morphology of all *Leptolegnia* isolates from *A. aegypti* in Brazil was completely consistent with that of *L. chapmanii*. Characteristic slender, sparingly branched hyphae that produce lateral swellings were routinely observed to emerge from infected larvae about 24 h after death (Figs. 6 and 7). Zoospores were formed mostly in a single row in filamentous zoosporangia that are not broader than the vegetative hyphae (Figs. 8 and 9). Zoospores were released by extrusion from the zoosporangia (often with a more or less worm-like shape that quickly rounds up to produce the swimming zoospores that may encyst either immediately or after a period of swimming) (Fig. 10). Oogonia were only very rarely observed to be produced by the Brazilian isolates, but when they were formed, these sexual structures were borne on short lateral branches and showed prominent finger-like ornamentations (Figs. 11–13); oogonia produced mostly one but up to three oospores.

3.2. Confirmation of fungal pathogenicity against *A. aegypti* larvae

In the laboratory, the rapid development of the disease was observed on larvae in small plastic cups (3.5 × 5 cm) exposed to zoospores released from two cubes (ca. 15 × 15 × 15 mm) of axenic cultures of four *L. chapmanii* isolates (ARSEF: 12829, 12831, 12835, 12847). All of these isolates caused 100% mortality within 36 h of exposure to the zoospores. The larvicidal activity of the other Brazilian isolates was lower (<50% cumulative mortality) during the same period; no mortality at all was observed in any control containers that did not include the fungus.

While we now have reliable and relatively easy techniques to isolate new cultures of *L. chapmanii* and to confirm their pathogenicity for mosquitoes, two other aspects of this fungus have proven to be more challenging: Cultures of *L. chapmanii* tend to lose their infectivity to mosquitoes and/or their ability to produce zoospores after a comparatively few serial transfers *in vitro*. We have not confirmed whether serial *in vivo* passage of isolates (from infected to healthy larvae) is able to retard or to stop this loss. Continued later attempts to expose susceptible mosquitoes to *in vitro* cultures of the fungus does not seem to augment the activity of this fungus. The other problem, which might perhaps be linked to the loss of pathogenicity, is that standard cryopreservation protocols commonly used for the vast majority of fungi (Humber, 2012) are neither adequate nor reliable for *L. chapmanii* but remain under study to improve the protocols to preserve this and related oomycete pathogens affecting invertebrates.

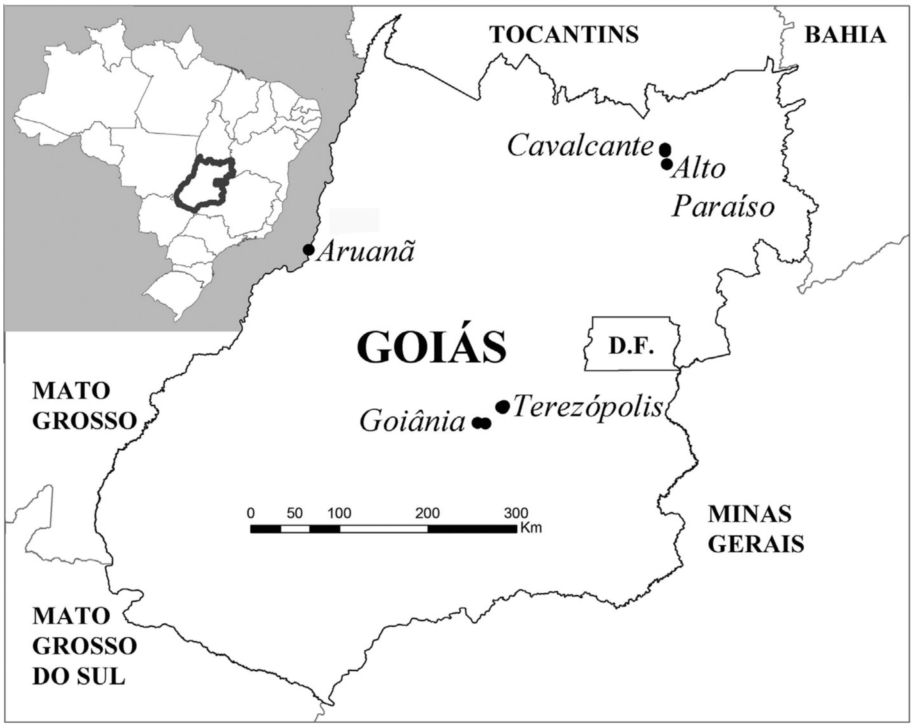


Fig. 5. Map indicating the locations of the major collecting regions within the central Brazilian state of Goiás from which *Leptolegnia chapmanii* has been recovered.

Table 1
Major morphological features of *Leptolegnia chapmanii* as originally described (Seymour, 1984), selected isolates from Brazil and Argentina. All dimensions [in µm] except for those given in the original description represent 50 measurements and are presented as mean ± standard error of the mean; overall ranges are given in square brackets.

Isolate	Source	Hypha diameter	Zoosporangium		Encysted zoospore diameter	Oogonium diameter[c]	Oospore diameter
			Length	Width			
Holotype[a]	Ohio, USA	[d]	[d] [70–240]	[d] [15–40]	[d]	[d] [26–63]	[d] [18–52]
ARSEF[b] 12829	Goiânia, Brazil	7.4 ± 0.2 [4.5–10.5]	1056.1 ± 120.8 [746.1–1319.2]	11.7 ± 0.5 [6.4–28.2]	11.1 ± 0.1 [9.0–14.6]	31.8 ± 0.4 [26.2–43.5]	11.4 ± 0.2 [8.9–13.6]
ARSEF[b] 12847	Alto Paraíso, Brazil	9 ± 0.3 [5.4–13.2]	736.1 ± 89.1 [260–2049]	11.1 ± 0.3 [7.8–16.4]	8.8 ± 0.2 [6.1–11.9]	29.5 ± 0.5 [22.4–39.3]	15.3 ± 0.3 [11.3–19.8]
ARSEF[b] 12831	Aruanã, Brazil	9.0 ± 0.2 [6.0–12.5]	[d]	[d]	8.6 ± 0.1 [6.4–11.6]	[d]	[d]
ARSEF[b] 5499	La Plata, Argentina	9.4 ± 0.3 [5.2–15.7]	[d]	[d]	[d]	42 ± 0.6 [34.7–51]	[d]
ARSEF[b] 2681 (epitype)	Clemson, USA	6.4 ± 0.2 [3.3–10.7]	668.3 ± 41.6 [412.2–1098.4]	10.6 ± 0.3 [7.4–15.2]	[d]	[d]	[d]

[a] Seymour (1984).
[b] USDA-ARS Collection of Entomopathogenic Fungal Cultures, Robert W. Holley Center for Agriculture and Health, Ithaca, NY [USA].
[c] Excluding the ornamentations projecting from the oogonial wall.
[d] No data.

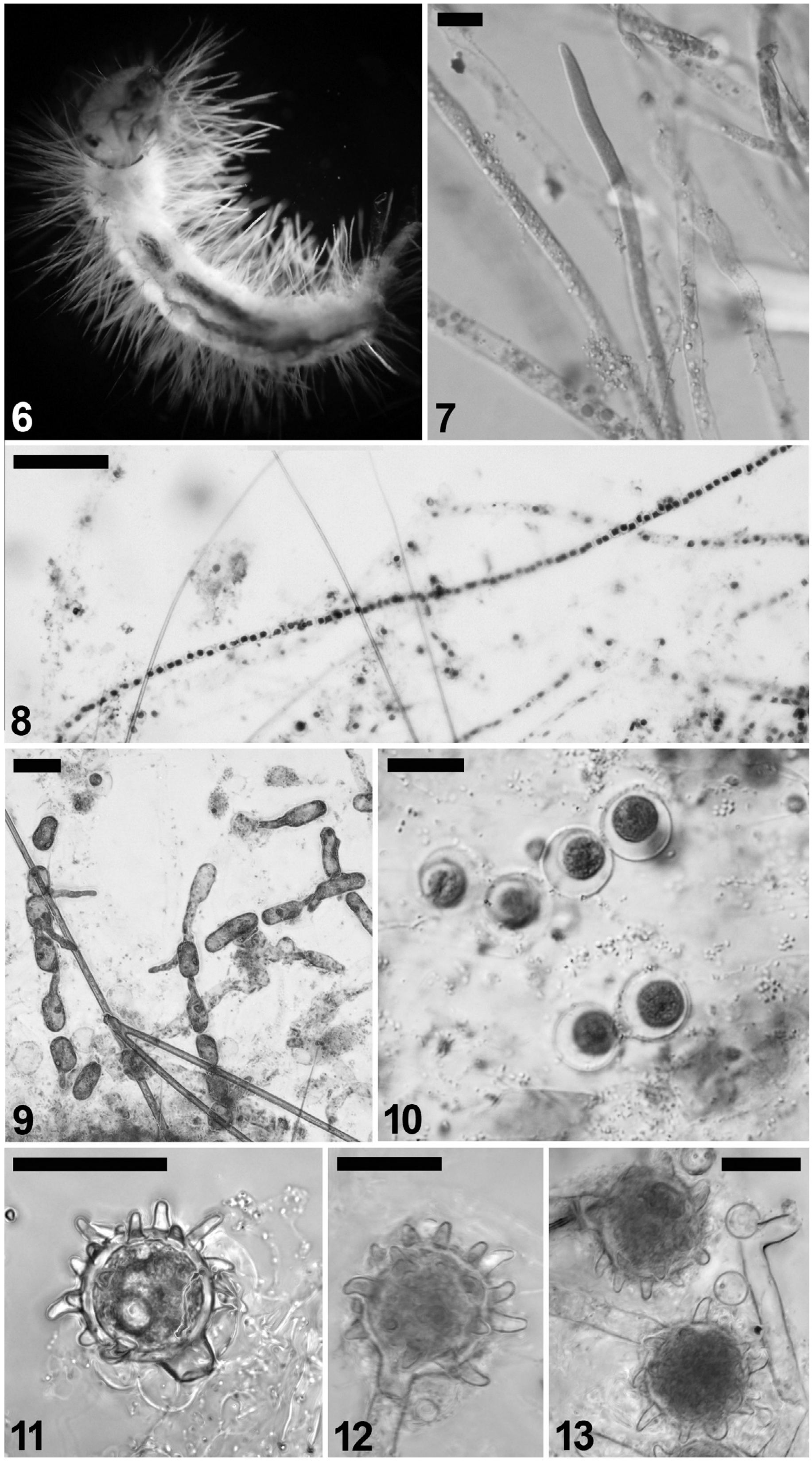
3.3. *Taxonomic considerations involving* L. chapmanii

3.3.1. *Typification and application of the name* L. chapmanii

A diligent search failed to locate the whereabouts of either the holotype specimen of *L. chapmanii* or of the WHO International Reference Collection where this holotype specimen was deposited. The WHO Collection was originally housed in the State of Ohio's Department of Public Health, Vector-Borne Diseases Laboratory in Columbus, Ohio. It appears, however, that all or part of this collection was discarded and/or relocated to a site now unknown to this laboratory's current staff, and the fate of the *L. chapmanii* holotype cannot now be determined. Seymour (1978, 1987) indicated that he could send cultures of *L. chapmanii* from his laboratory at the Ohio State University (Columbus, Ohio, USA), but none of these cultures was mentioned in the species description, and he never deposited any of them in any recognized culture collection. Consequently, no authentic specimens or cultures of *L. chapmanii* are available to use as the taxonomic standard to identify subsequent collections.

Further, every known extant collection or culture identified as *L. chapmanii* was obtained after the formal description of this species. All of these later collections were necessarily identified using only the developmental and morphological characters described and illustrated by Seymour (1978, 1984, 1987).

No specimens of *L. chapmanii* seem to have been deposited in major herbaria after the formal description of this species. It



Figs. 6–13. *Leptolegnia chapmanii*. 6–11 are of Brazilian isolates; 12–13 are of the epitype specimen. 6. Infected *Aedes aegypti* larva. 7. Hyphae. 8–9. Zoosporangia showing single-file arrangement of zoospores. 9. undispersed zoospores germinating in the zoosporangium. 10. Encysted zoospores. 11–13. Oogonia with finger-like decorations; 12–13 are from epitype specimen, a larva infected by ARSEF 2681. (Bar: Fig. 7 = 20 μm; Fig. 8 = 100 μm; Fig. 9 = 20 μm; Fig. 10 = 10 μm; Figs. 11–13 = 25 μm).

appears that the only cultures now identified as *L. chapmanii* are maintained in the ARSEF culture collection (Humber et al., 2014); no cultures of this species are available from such major culture collections as ATCC (Manassas, Virginia, USA), IMI-UKBRC (London, UK), CBS-KNAW (Utrecht, Netherlands), or DSMZ (Darmstadt, Germany). These ARSEF isolates come from South Carolina (ARSEF 2681) and from Florida (ARSEF 2562, 2680, 2682) in the USA, and a single isolate from Buenos Aires province, Argentina (ARSEF 5499 and later laboratory reisolations 6718, 7787), as well as the Brazilian isolates studied here.

Because Seymour's (1978, 1987) illustrations of the distinctively decorated oogonia have been the principal basis for all later identifications of *L. chapmanii*, it is appropriate to lectotypify *L. chapmanii* with Seymour's (1984) illustrations of these oogonia. A lectotype is defined as "a specimen or illustration designated from the original material as the nomenclatural type if no holotype was indicated at the time of publication, or if the holotype is missing" (McNeill et al., 2012; ICN, Article 9.2). The inability to locate the holotype collection of *L. chapmanii* makes it essential to provide this lectotypification.

Leptolegnia chapmanii Seymour, 1984, Mycologia 76, 670.

Holotype: WHO International Reference Center Accession 1518 (the continued existence or location of this collection remain unconfirmed).
Lectotype (here declared): Figures 1–14, Seymour, 1984, Mycologia 76, 671–672.
Epitype (here declared): Cornell University Plant Pathology Herbarium, CUP-068042, a single larva of *A. aegypti* infected by ARSEF 2681 bearing numerous oogonia; preserved in a sealed cryovial in 100% ethanol.
Ex-epitype isolate: *L. chapmanii* Seymour ARSEF 2681, isolated from *Culex quinquefasciatus* (as *Culex pipiens quinquefasciatus*) (Diptera: Culicidae), collected at Clemson, South Carolina, USA, by TJ McInnis; culture received by ARSEF from Jeffrey C. Lord, 6 February 1989.

Epitypes are declared to provide an interpretation of the meaning of ambiguous taxa, and serve a particularly critical function since fungal epitype specimens can be prepared from living cultures that remain available for any and all subsequent studies of the taxon. The epitype specimen is a single *A. aegypti* larva infected on 7 June 2015 with ARSEF 2681, incubated at 16 °C, and observed on 25 August 2015 to bear numerous typically decorated oogonia (Figs. 12 and 13). These oogonia had an average diameter of 35.6 ± 3.9 μm (n = 50; range: 27.5–42.5 μm), exclusive of the decorative projections. The overall diameter of the oogonia including these finger-like decorations was 47.7 ± 5.4 μm (n = 50; range: 35.0–57.5 μm). Curiously, on another larva of *A. aegypti* infected by ARSEF 2681 but incubated at 25 °C for ca. 7 weeks, 40% of the 50 oogonia examined failed to produce the characteristic oogonial projections.

3.3.2. Morphological and genomic characters confirm identifications of L. chapmanii

The morphological and developmental assessments of the Brazilian isolates and other known collections of *L. chapmanii* agree that this one oomycete species is present in North America, Argentina, and multiple sites in Brazil. Despite the current primacy of gene-based sequence data for both identifying and distinguishing taxa, no body of meaningful sequence data exists for *L. chapmanii* isolates. Until now, the available sequences have been those of a taxonomically uniformative polyketide synthase gene (Lee et al., 2001), and for the 18S ITS gene from an Argentinean isolate (López Lastra et al., 1999) and an isolate from Florida (but with no indication of the isolate's source or provenance) that were generated as part of a taxonomic revision of the order Saprolegniales (Dick et al., 1999). As part of these studies, we present initial genomic sequence comparisons for the ITS locus confirming that isolates from Brazil, Argentina and the ex-epitype from the United States represent a single taxon (with 99% homologies). The TEF sequences for a Brazilian isolate and the ex-epitype isolate proved to be nearly identical (differing by only a single base pair) and further confirm that the Brazilian and ex-epitype isolates do represent a single widely distributed species.

4. Discussion

4.1. Natural occurrences and application of the name L. chapmanii

The genus *Leptolegnia* has been isolated as a pathogen from such diverse mosquito hosts as *Aedes albifasciatus*, *Aedes albopictus*, *Aedes triseriatus*, *Anopheles culicifacies*, *Culex quinquefasciatus* and *Mansonia titillans* (Seymour, 1976; McInnis and Zattau, 1982; Lord and Fukuda, 1990; Bisht et al., 1996; Fukuda et al., 1997; López Lastra et al., 1999). While *Leptolegnia* includes at least five accepted species as well as several uncertain, undescribed, or rejected species (Johnson et al., 2002), only *Leptolegnia caudata* de Bary and *L. chapmanii* have been isolated from insects (McInnis and Zattau, 1982; Bisht et al., 1996). Until the generation of ITS and TEF sequence data for several isolates of *L. chapmanii*, the most notable morpho-developmental difference between *L. chapmanii* and *L. caudata* is the presence or absence, respectively, of the distinctive decorations of the oogonial surface. The comparisons of the ITS sequences generated here for IP 392 (from Brazil), ARSEF 5499 (from Argentina) and ARSEF 2681 (the newly designated ex-epitype of this species) with those in NCBI for the three isolates of *Leptolegnia* from the Centraalbureau voor Schimmelcultures (CBS) (Steciow et al., 2014) provide further evidence of supporting the identifications of the Brazilian isolates as *L. chapmanii* and that *L. caudata* (whose typification may be as deficient as was that of *L. chapmanii*) appears to be genomically distinct from *L. chapmanii.* There was a ca. 96% homology of sequences between CBS 392.81 (*Leptolegnia* sp.) and the sequences generated here whereas there was only a homology of ⩽86% between these Brazilian and ex-epitype isolates of *L. chapmanii* with the CBS isolates 113431 and 680.69 that are identified as being *L. caudata.* The host for CBS 392.81 is unknown, but this isolate should probably be treated as *L. chapmanii* (and could be confirmed as such if its oogonia bear the distinctively spiky decorations of this species). The virtually 100% homology of the TEF genes in IP 392 and ARSEF 2681 (differing by a single base pair) further confirms that all of the North and South American isolates studied here represent a single taxon with a wide distribution.

Morphological characters have been traditionally used to identify *Leptolegnia* species and have been well documented (Seymour, 1984; Zattau and McInnis, 1987; Humber, 2012). It is unfortunate that there have been few molecular studies of this or other *Leptolegnia* species (Dick et al., 1999; Robertson and Tartar, 2006), but the ITS gene studied here for two Brazilian and the ex-epitype isolates of *L. chapmanii* may be the most useful single gene for the clarifying the taxonomy of oomycetes (Sandoval-Sierra et al., 2014; Sandoval-Sierra and Diéguez-Uribeondo, 2015). The morphological findings of the sexual and asexual propagules and their comparison with other reported *Leptolegnia* isolates and the ITS and TEF sequences of these fungi suggest that the Brazilian isolates are appropriately placed within the species *L. chapmanii.*

4.2. Ecological and biogeographical considerations about L. chapmanii

Much of the success of our efforts to recover *L. chapmanii* from nearly every field site sampled can be attributed to understanding the types of sheltered, still water habitats that seem to favor the presence of *L. chapmanii*, to the use of floating, mesh-bottomed containers holding mosquito larvae for the direct baiting of this pathogen from these habitats, and to the improved techniques described here for its isolation in axenic culture. The value of these combined approaches for recovering *Leptolegnia* from field sites is amply demonstrated by our recovery of this pathogen from very nearly every appropriately selected site that was sampled in the central Brazilian state of Goiás and also as a result of initial collections in adjacent state of Tocantins. We isolated *L. chapmanii* from some sites during both the dry and wet seasons, and this suggests that in the absence of suitable hosts *L. chapmanii* may survive in the unobserved oospore state.

The very obvious lack of observation of oogonial formation among the Brazilian isolates that were believed to represent *L. chapmanii* was worrisome. However, Catão et al. (2015) have shown that the Argentinean isolate of *L. chapmanii* forms oogonia after exposures of 72 h to temperatures of -10 °C to 0 °C. The Brazilian isolates treated as *L. chapmanii* may also respond to similar cold temperature stimulus in the laboratory, but the ambient temperatures during any part of the year at any of the Brazilian collecting sites for *L. chapmanii* never experience any extended period of cold. The Brazilian collections of *L. chapmanii* may, in fact, successfully complete their life histories in the field by cycling on continually augmented populations of susceptible mosquito hosts even though some of these sites also do remain without standing water in some periods of the year, and we cannot confirm in which state this larval pathogen might survive in the absence of susceptible hosts. In central Brazil, *L. chapmanii* may sustain itself without ever forming its environmentally resistant sexual structures (oogonia and oospores) that are well known from all other collecting sites in more temperate climates.

Currently, *L. chapmanii*, is reported from places with humid subtropical climate in United States of America (South Carolina and Florida) and Argentina (Humber et al., 2014), and is understood to produce its sexual structures routinely in such sites. With these new Brazilian isolates the known geographical distribution of *L. chapmanii* is expanded to areas with tropical climate in Latin America, and probably with this method to collect and to isolate might be shown to be present in other countries and continents.

A. aegypti has not been confirmed as a natural host of *L. chapmanii* in any of the collection sites probably in large part because the normal breeding sites for this species are synanthropic rather than sylvatic. At those sites where dip net collection of naturally occurring mosquito larval populations were made, none of those larvae displayed obvious signs of fungal mycoses after several days of observations. Nonetheless, several of the new Brazilian isolates studied here were shown to be active against *A. aegypti* larvae in the laboratory. *L. chapmanii* was already known to be a virulent pathogen of first and second instar larvae of *A. aegypti*, and caused 100% mortality of larvae within 24 h of exposure to zoospores (McInnis and Zattau, 1982). At least five of the Brazilian isolates of *Leptolegnia* were shown to be virulent to second and third instar larvae of *A. aegypti*. Further, the apparently narrow host specificity of this oomycete to mosquito larvae makes it a potentially valuable control agent presenting little to no threat to non-target organisms (McInnis, 1985; López Lastra et al., 2004).

The findings of this study contribute to a better vision about the geographical distribution of this important insect pathogen and suggest that the function and prevalence of *L. chapmanii* as a natural control agent of mosquitoes in South America has been underestimated until now. We are confident that the known geographical range of this fungus will continue to be expanded through further exploration activities in Brazil and, probably, in other countries throughout the world.

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