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First report of *Coelomomyces santabrancae* sp. nov. (Blastocladiomycetes: Blastocladiales) infecting mosquito larvae (Diptera: Culicidae) in central Brazil

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ABSTRACT

A project from 2013 to 2017 sought to discover pathogenic fungi and oomycetes from dipteran species that are vectors of major diseases of humans and animals in central Brazil and to begin evaluating the potential of these pathogens as potential biological control agents concentrated on mosquito larvae. Some collecting sites proved to be especially productive for pathogens of naturally occurring mosquito species and for placements of healthy sentinel larvae of *Aedes aegypti* in various sorts of containers in a gallery forest in the Santa Branca Ecoturismo Private Reserve of Natural Patrimony (RPPN) near Terezópolis de Goiás (GO). Collections during May-April of 2016 and February 2017 yielded a few dead mosquito larvae of an undetermined *Onirion* sp. (Culicidae: Sabethini) whose hemocoels contained many ovoid, thick-walled, yellow-golden to golden-brown, ovoid thick-walled resistant sporangia, 38.3 ± 4 × 22.8 ± 2.3 µm, decorated by numerous, closely and randomly spaced punctations of variable size and shape. These were the first indisputable collections from Brazil of any *Coelomomyces* species. Comparisons of the morphology of these sporangia with those of other species of *Coelomomyces*, confirmed that this Brazilian fungus represented a new species that is described here as *Coelomomyces santabrancae*.

1. Introduction

Comparatively little effort has been expended in Brazil or many other tropical countries to survey the diversity of fungal pathogens affecting mosquito larvae despite the significant impact of these insects as vectors of such serious diseases affecting humans as malaria, dengue, yellow fever and other emerging arboviroses – e.g., those causing Chikungunya, Zika and Mayaro fevers. As part of a three-year research project on the fungal pathogens affecting dipteran vectors of human and animal diseases in two states of central Brazil (from sites throughout Goiás and with more limited collections in southern Tocantins), a major effort has placed on discovering and cataloging the biodiversity of those fungi that are active against mosquitoes (Montalva et al., 2016a, 2016b, and several other publications in preparation).

The climate of central Brazil usually has a rainy period from October to March and a dry season from April to September. The richly diverse habitats in this region vary from open savannah to many different types of woodlands and forests that support innumerable habitats suitable for the growth and distribution of mosquito populations throughout the year. The incidence and diversity of mosquito species and their populations are usually greatest in the rainy periods that create a myriad of temporary breeding sites distributed both horizontally and vertically in forested sites (Silva et al., 2010), and raise the risks for the seasonal increases and spread of the disease agents so well known to be vectored by mosquitoes (Lira-Vieira et al., 2013).

Species of the genus *Coelomomyces* (Blastocladiomycetes: Blastocladiales) are historically best known from their life history's diploid phase as pathogens of mosquito larvae, but for the last several decades it has been known that the haploid phase of these fungi occurs as an obligatory pathogen affecting aquatic microcrustacean–copepod or ostracod–hosts (Couch and Bland, 1985; Gleason et al., 2010). Aquatic culicine and anopheline mosquito stages are the hosts most frequently attacked by these pathogens (Gleason et al., 2010). The maintenance and manipulation in the laboratory of these fungi are

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hampered by their complex life cycles, with an obligatory alternation of diploid and haploid generations between mosquito and microcrustacean hosts, respectively (Whisler et al., 1974, 1999, 2009; Whisler, 1985; Couch and Bland, 1985). There are no *Coelomomyces* studies to date that support any speculation whether this genus includes any alternative (truncated) life histories similar to those known for the closely related genus *Allomyces* (Whisler, 1985), and neither does any evidence published after Whisler's (1985) chapter support such hypothetical possibilities. It should not be surprising that these life history complications also effectively terminated the hopes in the last century that *Coelomomyces* species might become useful biological control agents against mosquitoes.

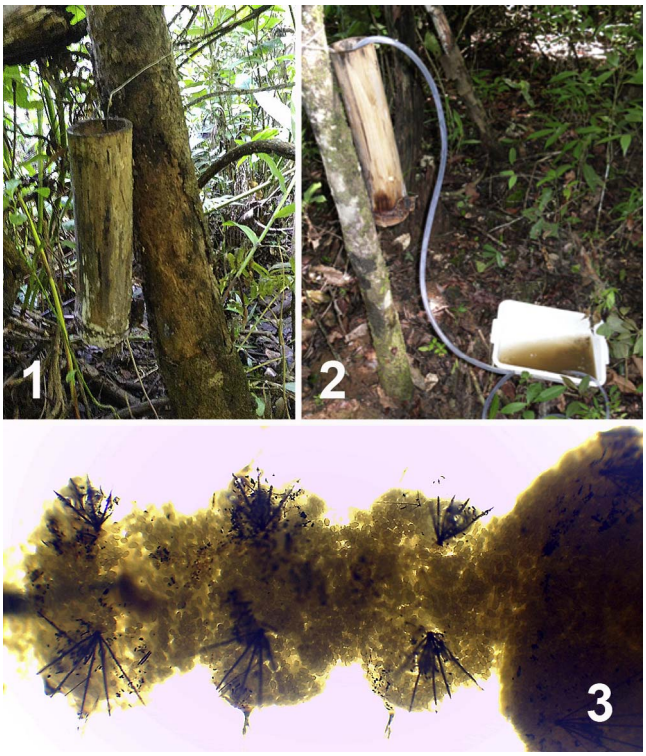
Coelomomyces includes nearly 70 species and varieties known from around the world (except from Antarctica). In the New World *Coelomomyces* spp. have been reported from the USA, Costa Rica, Panama, Colombia and Argentina (MacNitt and Couch, 1977; Couch and Bland, 1985; Sosa-Gómez et al., 2010). Taxonomy of *Coelomomyces* has been based virtually entirely on morphological characters as the size, shape, dehiscence slit and specific ornamentation of the resistant sporangia (RS), geographical origins, and the identification of their mosquito hosts (Bland and Couch, 1973; Tampieri et al., 1997). Too little continues to be known about the identities of the copepod, ostracod or other microcrustacean hosts of the haploid phase of the *Coelomomyces* life histories (Whisler, 1985; Bland and Couch, 1985) to have allowed these alternative hosts to play any significant role in the taxonomy of *Coelomomyces* species. Despite the currently expected use of gene sequences for the taxonomy of nearly all organisms, there are significant reasons discussed below why gene-based insights into the taxonomy of *Coelomomyces* still remain nearly nonexistent.

We here report the first *Coelomomyces* species found in Brazil as a pathogen of mosquito larvae (albeit with a very low incidence) of the culicine genus *Onirion*, and with a demonstrated capacity to persist on a single specific site to cause new infections at a later time. All evidence suggests that this fungus is a new species that we describe here as *C. santabrancae*.

2. Materials and methods

During a survey of entomopathogenic fungi affecting mosquitoes in Central Brazil between years 2015 and 2017 up to 30 bamboo containers (approx. 500 ml volume; Figs. 1 and 2) were set at a 1–1.5 m height in a tropical gallery forest inside the privately owned Santa Branca Ecoturismo RPPN (Private Research of Natural Patrimony) located close to the municipality of Terezópolis de Goiás, Brazil. Containers had open tops that allowed natural rainfall to replenish the water inside as well as for the unrestrained entry and development of local mosquitoes. Once a month containers were checked for aquatic mosquito stages; all living and dead mosquito larvae in the containers were retrieved using large-mouthed pipettes sterilized by rinses in 70% ethanol between each use to collect larvae, quantified and transferred to the laboratory where they were assessed with a Leica CM/LS microscope for any indications of fungal infections. On occasion, the total contents of any trap were siphoned into a white plastic pan (Fig. 2). Living larvae and pupae were placed in small cups (50 ml) with 25 ml of field water until emergence of adults; survival of larvae, pupae and adults was checked up to 15 days after collection. Larval or adult mosquitoes were identified morphologically (Harbach and Peyton, 2000).

Individuals containing visible fungal structures (Fig. 3) were kept in 70% ethanol and then dissected with insect needles under a Leica EZ4 stereoscopic microscope. Samples with fungal structures retrieved from the coelomic cavity were preserved overnight in a sodium phosphate solution (0.1 M, pH 7.2). Fungal structures were then put onto a glass slide and carefully rinsed with sterile distilled water (Figs. 4–7). These samples of resistant sporangia were dried in a desiccating chamber with silica gel for two weeks at room temperature, and then sputter-coated with gold for observation in a Jeol JSM-6610 scanning electron microscope.



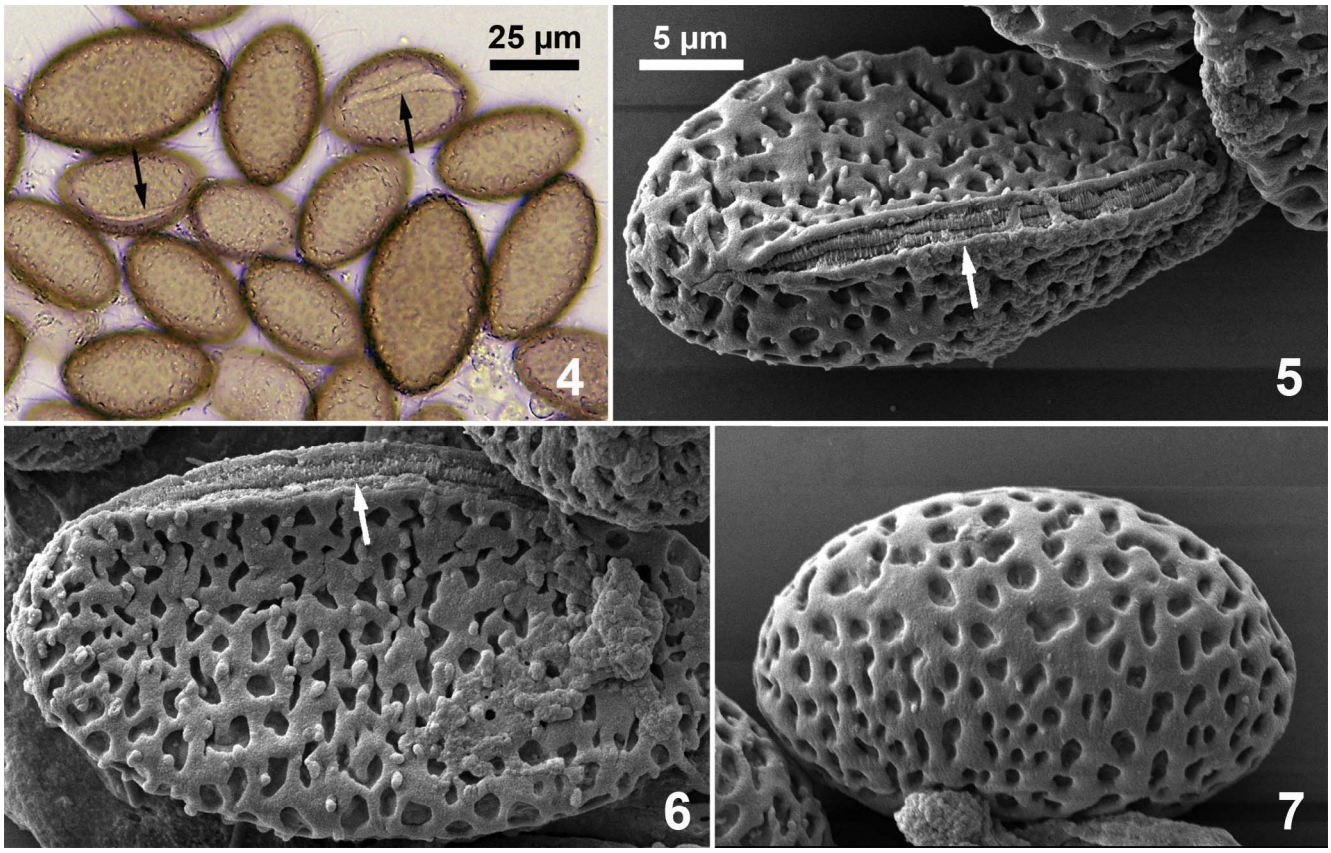
Figs. 1–3. Collection site and appearance of infected larva. 1–2. Bamboo containers open to colonization by local arthropods and their pathogens; 2. Siphoning contents of trap to white plastic tray for initial *in situ* evaluation of contents. 3. Resistant sporangia of *Coelomomyces santabrancae* filling the hemocoel of an *Onirion* sp. larva.

Slide preparations of resistant sporangia were also observed and photographed digitally at 400× magnifications using a Leica DM 750 microscope fitted with a Leica ICC50HD digital camera (in Goiânia, BR) and an Olympus BX51 microscope fitted with a JenOptik ProgRes CF^{scan} digital camera (in Ithaca, NY).

3. Results

During collections in April and May of 2016 a single dead culicid larva, identified as *Onirion* sp. (Diptera, Culicidae, Sabethini) was detected in each of two bamboo containers hanging from local vegetation growing adjacent to the João Leite River, and separated by a horizontal distance of 1.5 km. These containers were intended to serve as artificial tree hole habitats capable of attracting and sustaining the development of a diverse group of mosquito species and their possible pathogens.

These bamboo containers were left in place and allowed to remain empty along the dry season (April–September), but again filling naturally with ambient rainwater from the beginning of the rainy season in October 2016. During a collection made on 11 February 2017, three dead mosquito larvae were found inside one single bamboo container. The accumulated sediment inside that container was collected the next day (after discovering the presence of fungus-infected larvae) and transported to the laboratory in addition to all remaining mosquito larvae from the container. Many resistant sporangia were found in the sediment in this trap, thus suggesting that an undetected but ongoing infection event had occurred. The exuviae of mosquito larvae showing no indications of any internal fungal infective structures, and cadavers of microcrustaceans that might or might not have been the hosts for the haploid phase of the life history of this new *Coelomomyces* species were also confirmed to be present (Fig. 8). No vegetative growth or sporulation by the haplophase of this fungus were observed.



Figs. 4–7. *Coelomomyces santabrancae* resistant sporangia. 4. Light micrograph showing characteristic decoration of resistant sporangia and dehiscence slits partially opened by pressure of coverslip on the sporangia. 5–7. Scanning electron micrographs showing dehiscence slits (arrows) and characteristic pattern of randomly shaped and sized punctations through the outer wall layer of the resistant sporangia; possible bacterial contaminants of the spore surfaces and punctae are visible on 5 and 6 but are absent from 7. White bar shows measurements for Figs. 5–7.

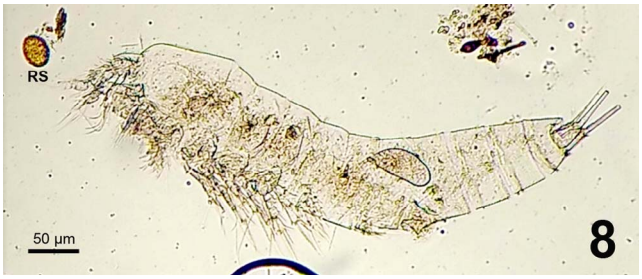


Fig. 8. Sediments from the bottom of a bamboo container recovered from the field in February 2017 (containing three mosquito larvae infected by *C. santabrancae*) included individual resistant sporangia (RS) freed from one or more infected mosquito larvae as well as an unidentified microcrustacean (shown here) and copepod cadavers (not shown) that might have been served as hosts for the gamete-producing haploid phase of the fungus.

4. Taxonomy

4.1. Coelomomyces santabrancae Rueda-Páramo, Montalva, Luz & Humber, sp. nov. Figs. 3–7

Registration # IF 553240

The coelomic cavities of infected larvae included numerous ovoid yellow-orange to gold-brown resistant sporangia (RS), 38.3 ± 4 µm × 22.8 ± 2.3 µm (n = 22; ranges 32.6–43.5 × 18.5–26.4 µm), with walls 2.5 ± 0.5 µm thick (n = 22; range 1.6–3.4 µm), and showing many closely spaced, irregularly shaped punctations into the otherwise smooth surface of the sporangium. (Figs. 3–7). A straight, pre-formed dehiscence slit was visible on the more broadly curved side of the asymmetrically shaped sporangia (Figs. 4–6). No vegetative hyphae were observed in any living or dead mosquito larvae from these collections.

Holotype: An infected mosquito larva containing numerous resistant sporangia of the new fungus, preserved in 70% ethanol, sealed in a cryovial, and deposited as UFG 50749 in the Herbário da Universidade Federal de Goiás (Goiânia, BR).
Type locality: Adjacent to the River João Leite, in Santa Branca Ecoturismo (Terezópolis de Goiás, Brazil), 16°25′04.83″ S 49°05′45.90″ W, 798 m altitude above sea level.
Type host: An undetermined species of *Onirion* (Diptera: Culicidae: Culicinae: Sabethini).
Etymology: The specific epithet recognizes the collection of this new species in the Santa Branca Ecoturismo RPPN (Private Reserve of Natural Patrimony) several kilometers to the northwest of Terezópolis de Goiás (state of Goiás), a site that has been an exceptionally productive, reliable location for collecting a wide range of entomopathogenic fungi.

The punctate decorations of the *C. santabrancae* resistant sporangia (RS) resemble those on *C. punctatus* Couch & H.R. Dodge (1962), a species known only from the United States (Couch and Bland, 1985). However, the RS of *C. punctatus* (42–75 × 32–41 µm) are much larger, and the punctations of this species are more numerous, comparatively smaller, and show a more obviously linear arrangement than in the Brazilian collections. The sizes, shapes, and distribution of the punctae on the outer surface of the Brazilian RS (Figs. 4–7) are clearly of more or less random sizes, shapes, and distribution. Some scanning electron

micrographs sometimes showed the presence of more or less regularly shaped forms (Fig. 5; less prominently in Fig. 6), on the RS surface and/or in the depressed areas of the punctae, but it seems most likely that these are probably bacteria adhering to the sporangial surface rather than any structural feature of the RS.

5. Discussion

5.1. Taxonomic issues involving the recognition of C. santabrancae

That *Coelomomyces* species appear to be very rare from sites in South America simplified the question of whether the fungus described here was morphologically distinct from all of those previous South American reports. It is also notable that the distributions of most *Coelomomyces* species tend to be restricted and endemic rather than global (Couch and Bland, 1985). *Coelomomyces reticulatus* var. *parvus* Couch, Farr & Mora in Couch and Bland (1985) from *Aedomyia squamipennis* collected in Acacias (Meta, Colombia) has resistant sporangia 22–35 × 33.5–45 μm; while these sporangia are similar in size to those of *C. santabrancae* the surfaces of the Colombian sporangia are decorated by prominent pentagonal to hexagonal, reticulate ridging. The Argentinean collection from Buenos Aires province by López Lastra and Garcia (1997; also see López Lastra, 1999) affected *Culex dolosus* larvae and was identified as *Coelomomyces iliensis* var. *indus* Couch & Iyengar in Couch and Bland (1985). These Argentinean resistant sporangia were 47.4–71.1 × 26.1–37.9 μm and decorated by long, raised (and occasionally branching), ribbon-like bands separated by raised, regularly striate regions in the scanning electron micrograph. Both Argentinean and Colombian collections of *Coelomomyces* differed unmistakably in both their sizes and patterns of decoration of their resistant sporangia from *C. santabrancae*.

In the first reports of any *Coelomomyces* from Brazil, Arêa Leão and Pedroso (1964, 1965) twice named this collection–invalidly, both times, without typification or any (required) Latin diagnosis–as the new species, *Coelomomyces ciferrii*, affecting the eggs of a *Phlebotomus* species (Diptera: Psychodidae) from Belo Horizonte (Minas Gerais, BR). The globose, (dark) brown resistant sporangia of *C. ciferri* had spiculate (minutely spiny) surfaces on a wall 4 μm thick; no pre-formed dehiscence slit was mentioned or illustrated in either characterization. Nothing about the shape, color, decoration, lack of a dehiscence slit of the thick-walled sporangia; its ovoparasitic habit; or its phlebotomine host suggested that *C. ciferri* was characteristic of any *Coelomomyces*. This species was appropriately rejected from this genus by Couch and Bland (1985). Whether *C. ciferrii* might represent a blastocladiomycotan (or chytridiomycotan) fungus in the current systematics cannot be confirmed. Neither is it possible to reject or to confirm the speculation by Dedet and Laird (1981) that *C. ciferri* represents the resting spores of a fungus in the Entomophthorales.

The absence of genomic data supporting our description of a new, morphologically based species is not an oversight but a practical necessity of the circumstances in which this fungus was discovered. Couch and Bland (1985) provided the indispensable taxonomic reference for *Coelomomyces* with keys, characterizations, and illustrations of 62 species and varieties; six additional taxa from diverse global locations have been described since then but none of these newer taxa resembles *C. santabrancae*. The various global genomic databases include very limited data from only four of the nearly 70 taxa in *Coelomomyces*. The most comprehensive phylogenetic review of the phylum Blastocladiomycota to date (Porter et al., 2011) included much deeper data resources in the analyses of other blastocladian taxa in the analyses but treated *Coelomomyces* using only these few available sequences. The scarcity of *Coelomomyces* genomic data reflects the absence of cultures for this genus and that field collections are usually rare events yielding very limited numbers of infected individuals with the thick-walled RS but very rarely including any vegetative hyphae. Consequently, the techniques to obtain clean DNA and usable sequence data from *Coelomomyces* remain less explored and less reliable than for most other fungi. These constraints leave mycologists with only a tiny and unrepresentative base of genomic data for *Coelomomyces* that effectively forces the taxonomy of this genus to remain dependent–at least for now–on such traditional criteria as resistant sporangial morphology, host identity, collection site, and other similar characters.

5.2. Life history and dispersal of C. santabrancae

Some persistent questions about most natural occurrences of *Coelomomyces* include how the fungus comes to be in the locations and to affect its hosts (with an apparently high degree of host specificity), and how the fungus can persist on site or be dispersed to new locations. That the *Coelomomyces* life history includes separate diploid and haploid phases affecting wholly different hosts (mosquitoes and microcrustaceans, respectively), complicates the effort to understand these fungi better.

From an ecological point of view, it is essential to acknowledge that infected female larvae can, on rare occasions, survive pupation. These infected females emerge with their ovaries invaded and 'usurped' by the fungal pathogen so that the ovaries produce RS rather than eggs. These RS can than be dispersed by new sites where *Coelomomyces* has not been present by flying infected females where the sporangia are 'oviposited' by the infected host's normal behaviors or released when an infected adult cadaver decays (Lucarotti, 1987, 1992; Laird and Sota, 1992; Lucarotti and Andreadis, 1995; Shoulkamy et al., 1997).

The capacity of *C. santabrancae* to persist in the environment was confirmed from a single bamboo container by two infection events temporally separated by a dry season during which containers remained empty and dry, as well as by the numerous loose RS in the sediment collected from the bottom of the trap during the later collection of infected mosquitoes. Some *Coelomomyces* species have been intensely studied for their high virulence for mosquitoes and for their allowing epizootic events on larval populations in the same sites over many years (Muspratt, 1963; Chapman and Glenn, 1972; Chapman, 1985; Apperson et al., 1992). *C. santabrancae*, however, is known so far from only five infected larvae as well as from the collection of loose resistant sporangia (Fig. 8) in the detritus at the bottom of the affected bamboo trap. These loose sporangia were clearly released from one or more infected (but undetected) mosquito larvae but we do not know how long they might have been present (and persistent) in this container. The only time the total contents of any trap were harvested was for the trap from which we recovered three *C. santabrancae*-infected larvae in a single collection. Microcrustaceans were observed in that trap's sediment but no evidence of vegetative stages of *C. santabrancae* were seen in any living or dead mosquito larvae or in any of living or dead microcrustaceans in this trap.

It is clear that the scarcity of collections of *C. santabrancae* (and, indeed, of most other *Coelomomyces* species wherever they occur) requires further and more intensive field searches to augment our knowledge about the biology and ecology of these pathogens. Among all entomopathogenic fungi and oomycetes, however, the genus *Coelomomyces* is unique in having an obligatory alternation of haploid and diploid generations that also alternate between two very distantly related types of arthropod hosts. Other entomopathogenic genera of the Blastocladiales such as *Myiophagus* (Sparrow, 1939; Karling, 1948) and *Coelomycidium* (Debaisieux, 1919, 1920; Weiser, 1951) have poorly understood life histories; while these fungi may or may not demonstrate alternations of haploid and diploid generations, there is no evidence suggesting the alternation of hosts that appears to be such a major feature of *Coelomomyces*.

Although the findings of this study have expanded the known geographic and host ranges of *Coelomomyces*, such limited, serendipitous collections cannot support much additional research, and they can only minimally advance the global understanding of this genus. No matter how well we might eventually understand the biotic and abiotic factors

allowing *Coelomomyces* species to cause natural infections as well as to persist and to disseminate, the inherent complexities of the biologies of these fascinating fungi probably effectively prohibit their use as practical biological control agents against mosquito populations.

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