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## Description of three novel *Lagenidium* (Oomycota) species causing infection in mammals

Leonel Mendoza<sup>a,b,\*</sup>, John W. Taylor<sup>c</sup>, Edward D. Walker<sup>a</sup>, and Raquel Vilela<sup>a,d</sup>

<sup>a</sup>Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, United States

<sup>b</sup>Biomedical Laboratory Diagnostics, Michigan State University, East Lansing, MI 48824, United States

<sup>c</sup>Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, United States

<sup>d</sup>Faculty of Pharmacy, Universidade Federal do Minas Gerais, Belo Horizonte, Brazil

### Abstract

**Background**—Recent molecular phylogenetic analysis of *Lagenidium* strains recovered from subcutaneous lesions in cats, dogs, and a human with lagenidiosis resolved into four clades, one of them was *Lagenidium giganteum*, but three others were novel.

**Aims**—Due to the recent increase in *L. giganteum* infections from mammals, we studied 21 *Lagenidium* strains isolated from dogs and a human available in our collection.

**Methods**—Molecular phylogenetic studies and phenotypic characteristics were used to characterize strains.

**Results**—We report the finding of three novel species, herein designated as: *Lagenidium ajelloi*, sp. nov., *Lagenidium albertoi* sp. nov., and *Lagenidium vilelae* sp. nov. Their morphological and growth features are also presented.

**Conclusions**—Our study revealed the presence of three novel *Lagenidium* species infecting mammals.

### Keywords

Lagenidium; Lagenidiosis; Oomycota; Oomycetes; *Pythium insidiosum*; Peronosporomycetes; Straminipila

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For more than a century *Pythium insidiosum* was considered the only species in the Oomycota commonly causing infection in mammalian hosts.<sup>10,13</sup> This paradigm was

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\*Corresponding author. mendoza9@msu.edu (L. Mendoza).

#### Conflict of interest

The authors declare no conflict of interest.

#### Uncited references

7,26.

challenged by reports in the past decade indicating that strains of *Lagenidium* spp. also infected apparently healthy mammals, including humans, causing cutaneous and systemic infections.<sup>16,17,23,28</sup> Recent phylogenetic analyses of several *Lagenidium giganteum* isolates recovered from mammals with lagenidiosis revealed that some isolates formed a monophyletic clade with other *L. giganteum* strains approved by the Environmental Protection Agency (EPA) as a mosquito control agent.<sup>34</sup> Three other clades of *Lagenidium* isolates found during these investigations, and also associated with mammalian infection, displayed distinctive taxonomic and phylogenetic characteristics.<sup>34</sup> The unique phenotypic attributes and phylogenetic affinities of these three novel *Lagenidium* clades represent previously undescribed species and are herein proposed as new.<sup>34</sup>

## Materials and methods

### Living cultures

The complete list of the strains and genomic DNA samples used in this study is shown in Table 1. The following isolates were studied: two thermo-sensitive *Lagenidium giganteum* ATCC 36492 and ATCC 48336 (mosquito larvae pathogen, USA) strains; *Lagenidium humanum* = ATCC 76726 (dead skin of humans, USA); *Lagenidium albertoi* = MTLA13 (ATCCMYA-4932); *Lagenidium ajelloi* = MTLA06 (ATCCMYA-4936), MTLA07 (ATCCMYA-4937), and MTLA19 to 23; 12 thermo-tolerant *L. giganteum* = MTLA01 (ATCCMYA-4933), MTLA03, MTLA-04 (ATCCMYA-4934), MTLA-05 (ATCCMYA-4935), MTLA10, and MTLA12–18 recovered from dogs and a cat; and *Lagenidium vilelae* = MTLA24. *L. albertoi* was cultured by Dr. A. Chindamporn from a Thai man with keratitis<sup>28</sup>; *L. ajelloi* was first isolated from two dogs (MTLA06 and 07) with protracted subcutaneous swellings mimicking skin pythiosis in Alabama, USA by Dr. J. Newton. The other five isolates (MTLA19 to 23) also were recovered from dogs with protracted subcutaneous lesions (4) and with cutaneous and systemic infections involving the intestinal tract and arteries (1) by Florida, Georgia and North Carolina veterinarians in the USA. The 12 thermo-tolerant *L. giganteum* strains were isolated from dogs and a cat with acute cases of subcutaneous granulomas mimicking skin pythiosis as per Vilela et al.<sup>34</sup> The only *L. vilelae* strain (MTLA24) in this study was isolated by Dr. J. Lewis from a North Carolina cat with a protracted mass at the base of the tail.<sup>23</sup> The MTLA25 of *L. vilelae* was formalin fixed tissue from a Virginia cat with a protracted abdominal mass containing *P. insidiosum*-like hyphae and thus, studied only using its extracted genomic DNA.

### Media and culture conditions

The purity of the strains was determined by the induction of zoospores using Mendoza and Prendas<sup>24</sup> protocol in culture and then selecting a single colony. The pure isolates obtained by this method were kept in our collection and later used in these analyses. With the exception of MTLA25 (genomic DNA sample extracted from biopsied tissue),<sup>30</sup> genomic DNAs were extracted from cultures of isolates grown on 2% Sabouraud dextrose broth (SDB). Temperature studies at 25 and 37 °C and the production of sexual and asexual structures were evaluated on brain heart infusion (BHI) (DIFCO, Detroit, MI USA), corn meal agar (CMA) (BBL, Sparks, MD, USA), and 2% Sabouraud dextrose agar (SDA). The different stages of sporangium formation, zoospore cleavage and release were assessed on

colonized grass leaves in water cultures containing  $\text{Ca}^{2+}$  and other ions as described by Mendoza and Prendas.<sup>24</sup> Briefly, the strains in Table 1 were subcultured on SDA plates at 37 °C for 24 h. After incubation 5 mm × 5 mm blocks were cut from the advancing edges and placed on 2% water agar plates. Sterile 4 mm × 10 mm grass blades were laid on top of the agar blocks and to parasitize the grass blades. The grass blades were then placed in beakers containing 50 ml of sporulation mix. The sporulation mix was made of two solutions: solution number one consists of  $(\text{NH}_4)_2\text{HPO}_4$  (66.04 g),  $\text{KH}_2\text{PO}_4$  (68.05 g), and  $\text{K}_2\text{HPO}_4$  (87.09 g) in 500 ml of  $\text{H}_2\text{O}$ ; and the other consists of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (18.38 g), and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (25.42 g) in 250 ml of  $\text{H}_2\text{O}$ . The sporulation mix was obtained by mixing 0.5 ml of the first solution plus 0.1 ml of the second solution in 1.0 L of sterile distilled water. Beakers containing 50 ml sporulation mix plus parasitized grass blades were incubated at 37 °C and the development of sporangia and zoospores was evaluated microscopically at 30 min intervals for 6 or more hours. Some strains required additional overnight incubation at 25 °C to develop zoospores.

### **Morphological description of hyphal structures, sporangia, zoospores, and other propagules**

The morphological features were evaluated after subculture on BHI, CMA, and SDA following 24–72 h of incubation at both 25 and 37 °C. Briefly, 4 mm × 4 mm agar blocks were cut from the inoculated plates and mixed with one drop of lactophenol cotton blue (phenol 20 ml, lactic acid 20 ml, glycerol 40 ml, and distilled water, 20 ml) to assess the microscopic features of the hyphae and other structures. The development of sporangia and zoospores were evaluated in sporulation medium (see above). After incubation at the induction temperatures, beakers containing 50 ml of sporulation medium and parasitized grass blades were inspected on inverted microscope. Grass blades with more than 10 vesicles that contained zoospores were removed and placed on glass slides containing 5 µl of merthiolate (0.02%) to kill the zoospores and facilitate their measurements.

### **Molecular analysis**

The strains (Table 1) were inoculated into 250 ml flasks containing 100 ml of SDB and incubated for 72 h at 37 °C on a shaker rotating at 150 rpm as per Vilela et al.,<sup>32</sup> After incubation the cultures were killed with merthiolate (0.02%, wt/vol) and then filtrated to recover the hyphal cell mass. The hyphal cell mass was then transferred to a mortar and ground in the presence of liquid nitrogen. The DNA from the disrupted hyphae was treated with sodium dodecyl sulfate and proteinase K and then incubated at 60 °C for 1 h and their genomic DNA extracted with phenol, chloroform, isoamyl alcohol following Sambrook and Russell protocol.<sup>30</sup> PCR was by hot start amplification of the complete *ITS* sequences and the partial coding DNA sequences of *cytochrome oxidase II (COXII)*; *heat shock protein 90 (HSP90)*; *cell division cycle:42 (CDC42)*; and *tubulin (TUB)*, as per Vilela et al.<sup>34</sup> (Table 1). Briefly, the PCR temperature cycling parameters were as follows: initial DNA denaturation for 10 min at 95 °C followed by 40 cycles of DNA denaturation for 1 min at 95 °C, annealing for 1.0 min at 60 °C, and elongation at 72 °C for 2 min, concluded by a final elongation of 2 min at 72 °C. The amplicons were ligated into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), purified and then sequenced using BigDye Terminator chemistry in an ABI Prim 310 genetic analyzer (Perkin-Elmer, Foster City, CA, USA).

## Phylogenetic analysis

Each of the sequenced genes was analyzed independently in combination with sequences of *Lagenidium* spp. and *Pythium* spp. available at the National Center for Biotechnology Information (NCBI). Concatenated genomic DNA sequences from the studied strains containing the ITS sequences and the partial gene sequences of the selected four exons (*CDC42*, *COXII*, *HSP90*, and *TUB*) were aligned with *Lagenidium* and *Pythium* spp. groups a–c, e–g, i and j<sup>22</sup> DNA sequences from NCBI using CLUSTAL W, v. 1.81 with default settings followed by a visual inspection (Table 1). Dashes (–) were used to annotate missing nucleotide data especially in *Pythium* spp. *CDC42*, *COXII*, *HSP90*, and *TUB* exons. The aligned sequences were exported for parsimony analysis using heuristic search with TBR branch swapping and distance analysis by Neighbor-Joining (MEGA-6, Tamura, Stecher, Peterson, Filipski, and Kumar). Large insertions were coded as one event by excluding all but one nucleotide per insertion and the resulting gaps were treated as missing data. Neighbor-Joining analysis used one of four approaches: uncorrected distances, maximum-likelihood estimates of distance with a time reversible model (6ST), empirical base frequencies with no rate variation among sites, or a gamma distribution with four rate categories and a shape parameter of 0.5. Branch support was estimated as the percentage of trees from resampled data sets containing the branch, using either parsimony (1000 resampling, heuristic, nni branch swapping) or Neighbor-Joining trees (1000 resampling, maximum-likelihood distances). Concatenated DNA sequences of the five investigated loci were used also in Bayesian analysis as per Vilela et al.<sup>34</sup> Briefly, the test was conducted in MrBayes v3.2.1 ×64 using the GTR + I + gamma model, with two chains (one heated), two runs, sampling every 100th generation for  $1 \times 10^6$  generations, and exclusion of the first  $2.5 \times 10^5$  samples (representing the burn-in) prior to analysis. Support for branches was estimated as the percentage of parsimony trees (1000 resampling, heuristic, nni branch swapping) or Neighbor-Joining trees (1000 resampling, maximum likelihood distances) containing the branch as well as by determining the Bayesian probability estimated as the percentage of Bayesian trees possessing the branch after discarding the burn-in sample.

## Results

Based on the above studies, three novel *Lagenidium* clades are herein described as containing new species.

### ***Lagenidium ajelloi*, Newton, Vilela, Taylor, Walker, Mendoza sp. nov**

Holotype = ATCCMYA 4936 (MTLA-07) isolated from a dog with lagenidiosis by Dr. J. Newton, Alabama, USA.=*Lagenidium karlingii*, sensu. Grooters, Proceedings, 25th Annual American College of Veterinary Internal Medicine Forum, 2007.=*Paralagenidium karlingii*, Grooters, Grooters, Spies, Chen, Glockling, Lévesque, de Cock. Nomenclatural novelties. Index Fungorum No. 34, 2013. Nomen nudum (<http://www.bgbm.org/iapt/nomenclature/code/saintlouis/0001ICSLContents.htm>; article 36)Etymology = In recognition of the numerous medical and veterinary mycological contributions of our friend and mentor (LM) Dr. Libero Ajello.

*L. ajelloi* (MTLA07) developed similar colonies on all media and showed slower growth rate at 37 °C (~2 to 4 mm/day, diameter of the entire colony) and 25 °C (0–1 mm/day) compared to that of *L. albertoi* or *L. vilelae*). Macroscopic and microscopic features were based on *L. ajelloi* strain MTLA07 subcultured on SDA. On this medium *L. ajelloi* developed yellowish, submerged, radiate colonies with few aerial hyphae. Microscopically, *L. ajelloi* developed a filamentous thallus strongly constricted at septa, resulting in the formation of long, branched chains of 60–600 µm, with chains of cylindrical to ellipsoid detached segments (Fig. 1B,C). However, this feature was lost after several subcultures. In addition, *L. ajelloi* isolates displayed the presence of hyaline sparsely septate mycelioid hyphae (6–16 µm) resembling fungal mycelia (Fig. 1C arrow). The strain did not form oogonia.

Zoospore formation of *L. ajelloi* in induction medium at 37 °C took more than 24 h. The development of one or more exit tubes from branched or detached ~40 µm × 120 µm in diameter sporangia was the first step (Fig. 2). First, the nuclei and cytoplasm flowed from the sporangia (Fig. 2, arrows) through exit tubes (Fig. 2, arrow heads) to form vesicles 35–65 µm in diameter (Fig. 3A–C). Vesicles which formed on the grass blades harbored 30–45 reniform (11–19 µm × 8–10 µm) biflagellate zoospores (Fig. 3A–C arrow). After vesicle formation, zoospores developed fully within 20 min. The detachment of the entire vesicle from the tip of the hyphae was sometimes observed (Fig. 3B,C). The zoospores were released after disruption of the vesicle's cell wall by active zoospores. After release they swam for the next 25 min before the formation of spherical cysts (11–16 µm in diameter), which germinated by forming hyphal tubes (Fig. 3D,E) (Table 2).

#### ***L. albertoi*, Chindamporn, Vilela, Taylor, Walker, Mendoza sp. nov**

Holotype = ATCCMYA-4932 (MTLA13) strain isolated from a Thai man with ocular lagenidiosis by Dr. Ariya Chindamporn. Reinprayoon U, Permpalung N, Kasetsuwan N, Plongla R, Mendoza L, Chindamporn A, *Lagenidium* sp. ocular infection mimicking ocular pythiosis. J. Clin. Microbiol. 2013;51:2778–2780. = *Lagenidium* sp. sensu Reinprayoon U, Permpalung N, Kasetsuwan N, Plongla R, Mendoza L, Chindamporn A. *Lagenidium* sp. ocular infection mimicking ocular pythiosis. J Clin Microbiol. 51:2778–2780, 2013. Etymology: Dedicated to an author's relative (LM).

*L. albertoi* (MTLA13) developed similar colonies on all media and grew comparatively faster at 37 °C (8 mm/day) and 25 °C (3 mm/day) (Table 2) than *L. ajelloi* but similar to *L. vilelae*. On SDA at 37 °C *L. albertoi* developed white to yellowish submerged colonies with few aerial hyphae and folds radiating from the center of the colony (Fig. 1D). The main microscopic features were hyaline, 10–18 µm filamentous, branched mycelioid structures with few septa (Fig. 1D–F). In liquid medium (SDB), the mycelioid structures developed constrictions at the septa with the occasional formation of long segments. Coiled hyphae were also detected on CMA and SDA. Oogonia were not found.

In induction medium *L. albertoi* developed 45–95 µm detached segments (sporangia) with exit tubes very similar to *L. ajelloi* (Fig. 2). Vesicles 25–65 µm in diameter contained biflagellated zoospores after 6 h of incubation (37 °C) near the end of the exit tubes. The number of vesicles harboring zoospores on grass blades was greater than those found using

the same methodology in *L. ajelloi* or *L. vilelae*. Some vesicles detached from the opening of the exit tube just before zoospore release as described in *L. ajelloi* (Fig. 3C). The zoospores were similar in shape and size (12–18  $\mu\text{m} \times 8\text{--}10\ \mu\text{m}$ ) to *L. ajelloi* and *L. vilelae* (see below). Upon release, zoospores swam 20–40 min and then formed spherical cysts (11–18  $\mu\text{m}$  in diameter) and developed coenocytic germ tubes as described in *L. ajelloi* (Fig. 3).

### **Lagenidium vilelae, Lewis, Vilela, Taylor, Walker, Mendoza sp. nov**

Holotype = MTLA24 isolated from a cat with lagenidiosis, North Carolina, USA submitted to us by Dr. John Lewis. = *L. deciduum*, Chi Y. Chen, Grooters, Spies, de Cock, Lévesque. Nomenclatural novelties. Index Fungorum No34, 2013. Nomen nudum (<http://www.bgbm.org/iapt/nomenclature/code/saintlouis/0001ICSLContents.htm>; article 36) Etymology: Dedicated to an author's family member (RV).

*L. vilelae* strain MTLA24 had a moderate growth rate at 37 °C (9 mm/day) and more slowly at 25 °C (4 mm/day) on all media. This strain developed submerged colonies with a white to yellowish radiate pattern with few aerial hyphae (Fig. 1G). In a rich medium such as BHI the colony morphology showed similar patterns, but the presence of aerial hyphae was more apparent. Microscopically, *L. vilelae* showed hyaline hyphae (7–18  $\mu\text{m}$ ) strongly constricted near septa (Fig 1H,I arrows) with few lateral branches and the presence of occasional cylindrical, smooth (17–300  $\mu\text{m}$  long) hyphal segments on fresh samples. The finding of short- and long-coiled-hyphae was also detected in subcultured strains, especially on CMA and occasionally on the other media (Fig. 1E arrows, lower section). When *L. vilelae* cultures on grass blades were placed in induction medium the hyphae developed detached or connected sporangial segments containing one or more exit tubes from which 35–65  $\mu\text{m}$  in diameter vesicles were formed. The vesicles contained numerous reniform, 12–17  $\mu\text{m} \times 8\text{--}10\ \mu\text{m}$  biflagellate zoospores, similar to that depicted in Fig. 2. Oogonia were not found.

### **Phylogenetic features of the mammalian-pathogenic Lagenidium species**

Phylogenetic analysis by either parsimony or neighbor-joining of the *ITS* sequences or each the four exons showed no topological conflicts. Bayesian analysis of the *ITS* sequences and the four exons consistently placed the *Lagenidium* strains into four clades as described by Vilela et al.<sup>34</sup> In Maximum Parsimony analyses, the thermo-tolerant *L. giganteum* (mammals) and thermo-sensitive *L. giganteum* (mosquito) form a monophyletic clade located at the crown of the genus (Fig. 4). These taxa, plus *L. humanum* (Lh1), and two unnamed entomophagous clades (Lspa and Lspb) were the sister taxon to *L. vilelae* (Lv). These clades plus *L. vilelae* form a larger strongly supported clade, sister to the cluster formed by *L. albertoi* (Lal1), *L. callinectes* (Lc 1 to 3), *L. caudatum* (Lca), *L. myophilum* (Lm) and three entomophagous *Lagenidium* isolates (Lspc1-3). *L. ajelloi* (La) formed a strongly supported taxon sister to the described clades (Fig. 4). An entomophagous *Lagenidium* sp. strain accession number HQ111455 = CBS 127042 (Lv1, Table 1) clustered with strong bootstrap support with the two DNA sequences of *L. vilelae* (Fig. 4). In Bayesian and Parsimony analyses *L. albertoi* (Lal1) always grouped on a single clade (data not shown, Fig. 4).

## Discussion

Our phylogenetic analyses and that of Vilela et al.<sup>34</sup> support the placement of the three new mammalian *Lagenidium* species basal to *L. giganteum* types recovered from mammals and mosquitoes. Thus, we propose three novel *Lagenidium* species based on the following: (a) except for the mammalian pathogen *L. giganteum*,<sup>34</sup> there is no a formal description of other *Lagenidium* species pathogenic for mammals and (b) our phylogenetic data placed these unique *Lagenidium* species in three well supported taxonomic clades. Thus, they represent previously undescribed species. Concatenate Bayesian and Maximum Parsimony analyses of the 25 *Lagenidium* gene sequences available at the NCBI and the 21 gene sequences from the strains in this study placed the pathogen of blue crabs *L. callinectes* (Lc) (placed by Dick<sup>11</sup> in the genus *Salilagenidium*), the saprotrophic *L. humanum* (Lh1) (placed by Dick<sup>11</sup> in the genus *Myzocytiopsis*), and eight yet to be name entomopathogenic *Lagenidium* spp. (Lspa and Lspb) from Taiwan basal to *L. giganteum* types (Lgm and Lgp). The mammalian pathogen *L. vilelae* (Lv) (including an entomophagous strain from Taiwan,<sup>15</sup> (Lv1) formed a strongly supported taxon basal to the Lspa and Lspb entomophagous clades. *L. albertoi* (Lal1) was strongly supported in its own clade, suggesting this strain is unique among mammalian *Lagenidium* species. Similar results were recently reported using Bayesian analysis.<sup>34</sup> The clade formed by three entomopathogenic *Lagenidium* spp. from Taiwan (Lspc1-3), the pathogens of nematodes *L. caudatum* (Lca) (placed by Dick<sup>11</sup> in the genus *Pythium*), and shrimps *L. myophilum* (Lm) (placed by Dick<sup>11</sup> in the genus *Salilagenidium*) were sister to the seven *L. ajelloi* (La) species recovered from mammals. The finding of *Lagenidium* spp. forming a monophyletic cluster to *Pythium* spp. challenges Dick<sup>11</sup> decision to remove a number of *Lagenidium* species to other straminipilan groups. Surprisingly, of the 46 *Lagenidium* spp. in the study, only one has not yet been reported as an animal pathogen, indicating that the capacity to infect animals, including mammals, is deeply rooted in the genus.<sup>14,34</sup> This and other studies suggest members of the genus *Lagenidium* are well equipped with virulence factors to infect animals including mammalian hosts.

Recently Grooters et al.<sup>15</sup> proposed the genus *Paralagenidium* based on a single strain isolated from an USA dog. We had used their ITS DNA sequence in phylogenetic analysis (data not shown) and found that their KF915306 is analogous to the DNA sequences of *L. ajelloi* in this study. Grooters et al.<sup>15</sup> proposal of the genus *Paralagenidium* was based on a single strain and two genes (*COXI*, *ITS*). But, the finding in this study and that of Vilela et al.<sup>34</sup> indicate that the genus *Lagenidium* is monophyletic, thus of the proposed genus *Paralagenidium* is not supported in these analyses and thus, considered a nomen nudum. Likewise, the Taiwan nematode strain *L. deciduum* (HQ111455) described by the same authors<sup>15</sup> clustered with the two of our *L. vilelae* DNA sequences from mammals (Lv2, Lv3). Vilela et al.<sup>34</sup> called the attention on two populations of *L. giganteum*: a thermo-sensitive (mosquito pathogen) and thermo-tolerant (mammalian pathogen). Although *L. deciduum* (HQ111455) clustered with *L. vilelae* in our analysis (data not shown), and the fact this strain was recovered from nematodes suggests that our *L. vilelae* strain from mammals may be a different type as is the case with *L. giganteum* and therefore, its true relationship with the mammalian *L. vilelae* strains are dubious at best. Thus, the legitimacy of these epithets is challenged under “The International Code of Botanical Nomenclature”

Chapter V article 52 and 58.1. <http://www.bgbm.org/iapt/nomenclature/code/saintlouis/0001ICSLContents.htm>; and by the “International Code of Nomenclature for algae, fungi, and plants (Melbourne Code)” Chapter V article 36 <http://www.iapt-taxon.org/nomen/main.php?page=title>.

The newly-described *Lagenidium* species can be definitively separated from other closely related Oomycota genera. For instance, with the exception of *P. insidiosum* and *P. aphanidermatum*,<sup>13</sup> none of the other *Pythium*, *Phytophthora*, and *Phytophthium* species have been found to cause infections in mammals.<sup>11,12,19</sup> Also, no other species in the Oomycota have been found to infect mammals except *P. insidiosum*, *P. aphanidermatum*,<sup>13</sup> the thermo-tolerant species of *L. giganteum*,<sup>32</sup> and the three novel species in this study. Furthermore, besides its pathogenic capabilities, our mammalian *Lagenidium* species in liquid cultures develop mycelioid structures strongly constricted at septa that soon became sporangia. Although *Pythium* and some other species can also develop this feature, in *Lagenidium* spp. these segmented structures become sporangia. The genera *Myzocytiium* and *Myzocytiopsis* differ from our strains in that they have not been reported to cause disease in mammals.<sup>5,11,12,14,19</sup> In addition, these species develop very short, endobiotic, and articulate or sometimes disarticulate mycelioid bodies (unicellular sporangia) with sexual oogonia within their infected hosts.<sup>5,20,25</sup> None of our strains developed oogonia in their infected hosts. Likewise, our strains can be also differentiated from the genus *Lagenocystis* (*Lagena*) (*Lagena* nomen nudum, Foraminifera; see Williamson<sup>35</sup>) by their lack of sexual reproduction in the infected hosts and in culture.<sup>6</sup> Additionally, the unique endobiotic holocarpic thalli of *Pythiella* (placed in the genus *Pleocystidium* by Dick<sup>11</sup>) set this genus apart from the three *Lagenidium* species in this study.<sup>6,18,25</sup>

*Lagenidium* species are well known pathogens of animals such as insects, crab, fish, nematodes and others.<sup>1,8,9,11,12,19,29</sup> With the exception of *L. humanum* recovered from dead skin of humans and snakes,<sup>19</sup> the other members of the genus typically parasitize invertebrates and, as recently discovered, mammals.<sup>16,17,23,28,34</sup> Did the species pathogenic for mammals evolve from zoopathogenic ancestors infecting invertebrates to become mammalian pathogens? This kind of host switching across kingdoms has been postulated for the Oomycota and indeed there is strong scientific evidence supporting these views.<sup>9,18</sup> *P. insidiosum* for instance, was recently recovered from an infected mosquito in India.<sup>31</sup> Otherwise, *P. insidiosum* infection in mosquitoes has rarely been observed, compared with the increasing numbers of new cases of pythiosis diagnosed every year in mammals inhabiting tropical and subtropical areas of the world.<sup>13</sup> It is then quite possible that the mammalian pathogenic *Lagenidium* species, like other Oomycota species, evolved from marine ancestors, to later acquire traits to infect plants, and those capacities were adapted later to infect lower animals and more recently evolved to affect mammals including humans.<sup>3,4,32</sup> Vilela et al.<sup>34</sup>, based on the phylogenetic finding of several *Lagenidium* clades, concluded that the “trait of infecting mammals has arisen several times, independently, in the genus *Lagenidium*”, a finding supported also by our phylogenetic data.

Gene transfer has been implicated in the gaining of new virulence factors and several mechanisms of disease in the Oomycota.<sup>2,33</sup> A recent study of *L. giganteum* transcriptome showed the presence of virulence factors from plant pathogenic Oomycota species that were



found in other peronosporans such as *Phytophthora* and *Pythium* species.<sup>21,27</sup> However, the source of virulence factors related to mammalian infectivity from within the Oomycota, but outside of the genus *Pythium*, is unknown. Based on our data and that of Vilela et al.<sup>34</sup> two putative scenarios to explain the sudden emergence of thermo-tolerant *L. giganteum* as a pathogen of mammals can be postulated: (1) the release of thermo-sensitive *L. giganteum* during field studies increased the population of this organism in the target ecological niches. The elevated numbers of the biocontrol organisms increased the likelihood of interacting with mammals, and perhaps allowed the mosquito pathogen also interacts with other Oomycota species and fungi, thus facilitating the acquisition of novel traits by horizontal gene transfer, or (2) the thermo-tolerant (mammals) and the thermo-sensitive (mosquito-larvae) strains are simple individuals with different phenotypes in one or more populations of *L. giganteum* in nature. It is then quite possible that *L. giganteum* (thermo-tolerant) wild-type strains may have been accidentally selected and used for field studies by mosquito control investigators. Although these hypotheses could explain in part the emergence of this species as a pathogen of mammals, the true ecological pressures leading the ancestor of the mammalian pathogenic *Lagenidium* species to gain new features infecting mammals have yet to be investigated.

## Acknowledgments

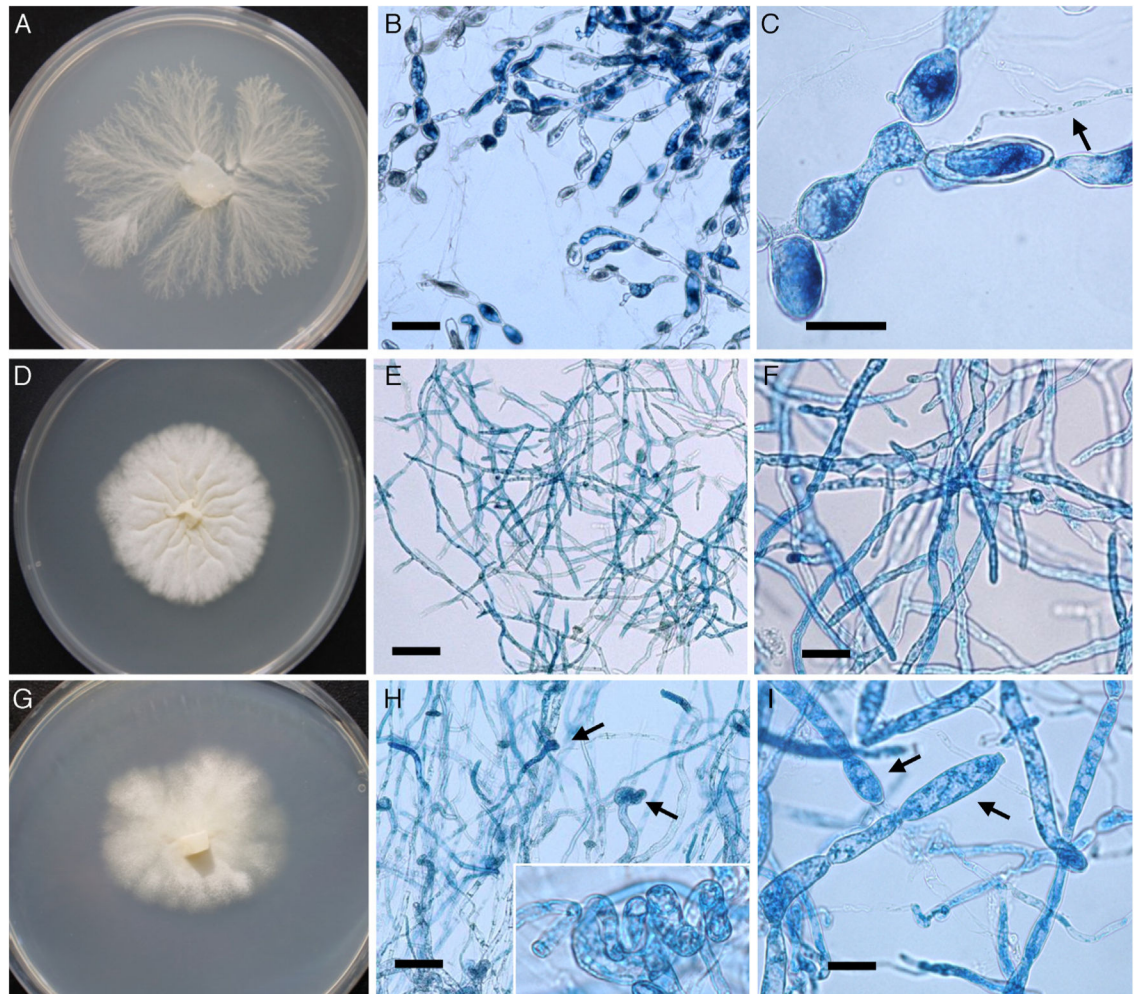
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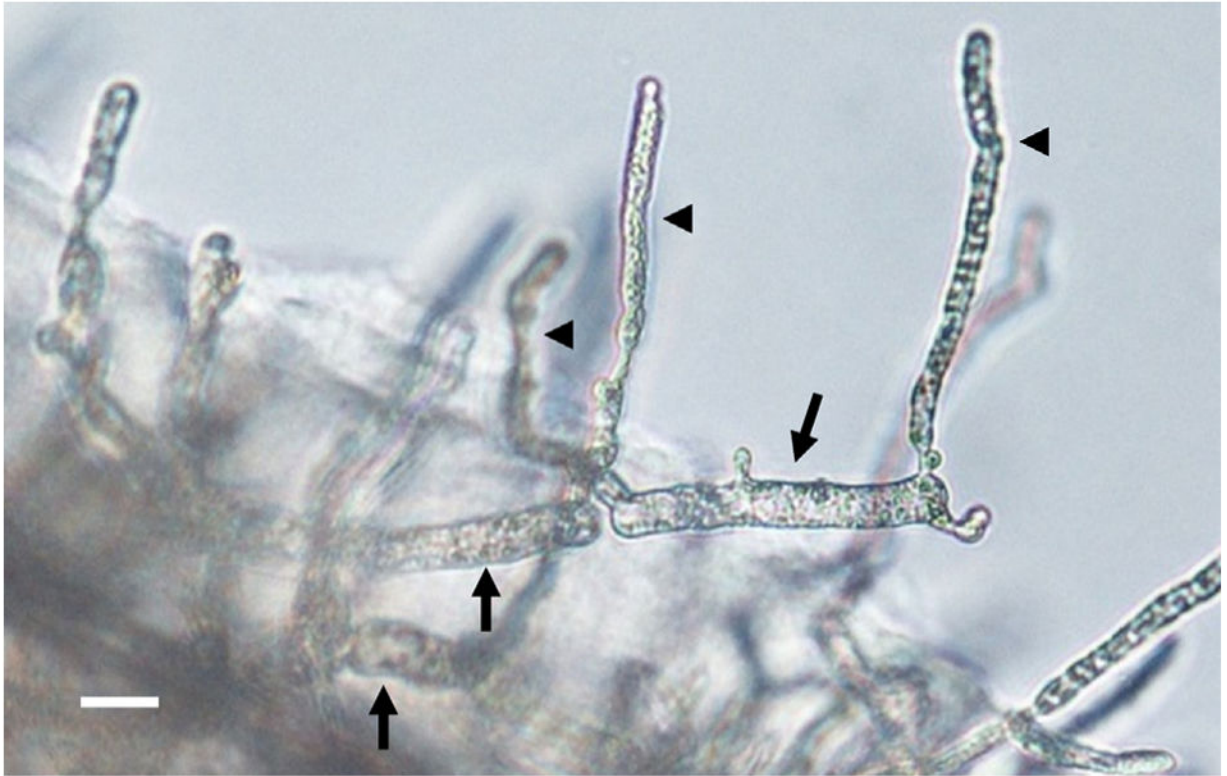
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**Fig. 1.**

The figure depicts the macroscopic and microscopic features of the three novel mammalian *Lagenidium* species. *Lagenidium ajelloi* (Panels A–C) is characterized by the formation of submerged colonies with a branching radiate pattern on SDA (A). Microscopically *L. ajelloi* displays branching mycelioid structures strongly constricted at septa resulting in the formation of elongated smooth and ovoid segments (Panel B, Bar = 80  $\mu\text{m}$  and Panel C, Bar = 100  $\mu\text{m}$ ). The arrow in Panel C shows a coenocytic hypha of *L. ajelloi* commonly found along with ovoid segments. The *L. albertoi* colony macroscopic features are shown in Panel D. Microscopically, this species developed mostly hyaline branching hyphae (E, Bar = 60  $\mu\text{m}$ , F, Bar = 20). The culture macroscopic feature of *L. vilelae* is shown in Panel G. Panels H and I depict the development of branched hyphae strongly constricted at the septa sometimes forming disarticulate elongate segments. The presence of numerous coiled hyphae was also found (Panel H arrows). Lower Panel H shows a close-up of the coiled hyphal structures. Long elongate hypha-like structures constricted at septa, as those depicted in Panel I were more common in liquid cultures (Panel I, arrows) (H, Bar = 13  $\mu\text{m}$  and Panel I, Bar = 15  $\mu\text{m}$ ).

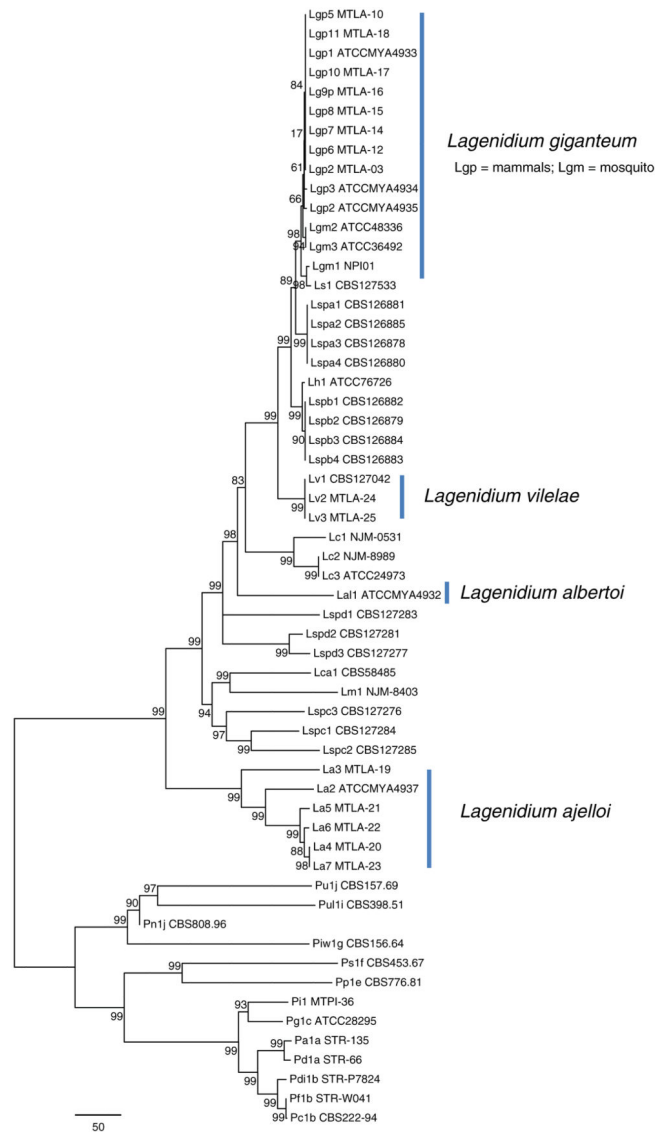


**Fig. 2.**

The figure depicts the development of articulate and disarticulated sporangia (arrows) of *Lagenidium ajelloi* on a grass blade in induction medium showing the formation of slender discharge tubes (arrow heads). The development of exit tubes from elongate sporangia (arrow heads) gives rise to vesicles at the tip of the each exit tube, as shown in Fig. 3 (Bar = 40  $\mu$ m). *L. albertoi* and *L. vilelae* developed also similar sporangia structures in induction medium before zoospore formation.



**Fig. 3.** Panels A–C show *Lagenidium ajelloi* vesicles containing zoospores on grass blades (see Fig. 2) after incubation in sporulation medium at 37 °C (Bar = 45 µm). Fully develop zoospores before release are shown in Panel C (arrows). The detachment of the whole vesicle from the tip of the exit tube (Panels B and C arrow heads) just before zoospore release was also observed in some of the strains. Note the zoospore reniform shape (Panel C, arrows) before breaking out from vesicle. Panels D and E show a group of encysted spores one of them developing long germ tubes (Bar = 15 µm). Panel E shows the encysted zoospores developing long hyaline coenocytic hyphae with some branches after several hours of encystment. *L. albertoi* and *L. vilelae* also developed their zoospores and cysts in the same fashion.



**Fig. 4.** Unrooted Maximum Parsimony phylogenetic tree based on sequence analysis of the complete rDNA ITS and 4 exons: *CDC42*, *COXII*, *HSP90*, and *TUB* concatenated data set showing the relationship among isolates of *L. ajelloi*, *L. albertoi*, *L. vilelae* with the data available in NCBI on other *Lagenidium* and *Pythium* species, as listed in Table 1. The numbers on the internal branches are percentage of trees based on 1000 bootstrapped data sets possessing the branch. The branch lengths correspond to corrected distances. In the tree, *L. ajelloi*, *L. albertoi*, and *L. vilelae* clustered in their own clades with strong bootstrap support. Several clusters of unnamed *Lagenidium* entomophagus species (*Lspa*, *Lspb*, *Lspc*, and *Lspd*) appeared also in their own clades. In addition, *L. giganteum* (*Lgm* and *Lgp*), *L. callinectes* (*Lc*), *L. humanum* (*Lh*), *L. caudatum* (*Lca*) and *L. myophilum* (*Lm*) all aligned within the *Lagenidium* monophyletic taxon with varied support. The scale bar represents number of substitutions per nucleotide.

Table 1

The table shows the strains taxonomic names, their IDs, their acronyms and their *ITS*, *CDC42*, *COXII*, *HSP90*, and *tubulin* NCBI accession numbers.

Taxonomic name	Strain ID	Acronym	<i>ITS</i>	<i>CDC42</i>	<i>COXII</i>	<i>HSP90</i>	<i>Tubulin</i>
<i>L. giganteum</i>	NPI01	Lgm1	EF016915	0	0	0	0
<i>L. giganteum</i>	ATCC48336	Lgm2	JQ745259	0	0	0	0
<i>L. giganteum</i>	ATCC36492	Lgm3	AY151183	JX985758	AF086697	JX999093	JX999124
<i>Lagenidium</i> sp.	CBS127533	Ls1	HQ395647	0	0	0	0
<i>Lagenidium</i> sp. A	CBS126881	Lspa1	HQ111460	0	0	0	0
<i>Lagenidium</i> sp. A	CBS126885	Lspa2	HQ111452	0	0	0	0
<i>Lagenidium</i> sp. A	CBS126878	Lspa3	HQ111464	0	0	0	0
<i>Lagenidium</i> sp. A	CBS126880	Lspa4	HQ111459	0	0	0	0
<i>Lagenidium</i> sp. B	CBS126882	Lspb1	HQ111461	0	0	0	0
<i>Lagenidium</i> sp. B	CBS126879	Lspb2	HQ111463	0	0	0	0
<i>Lagenidium</i> sp. B	CBS126884	Lspb3	HQ111450	0	0	0	0
<i>Lagenidium</i> sp. B	CBS126883	Lspb4	HQ111462	0	0	0	0
<i>L. callinectes</i>	NJM-0531	Lc1	AB285488	0	0	0	0
<i>L. callinectes</i>	NJM-8989	Lc2	AB285487	0	0	0	0
<i>L. callinectes</i>	ATCC24973	Lc3	AB285486	0	0	0	0
<i>L. vilelae</i>	CBS127042	Lv1	HQ111455	0	0	0	0
<i>Lagenidium</i> sp. C	CBS127284	Lspc1	HQ111472	0	0	0	0
<i>Lagenidium</i> sp. C	CBS127285	Lspc2	HQ111470	0	0	0	0
<i>Lagenidium</i> sp. C	CBS127283	Lspd1	HQ111471	0	0	0	0
<i>Lagenidium</i> sp. C	CBS127276	Lspc3	HQ111451	0	0	0	0
<i>Lagenidium</i> sp. C	CBS127281	Lspd2	HQ111473	0	0	0	0
<i>Lagenidium</i> sp. D	CBS127277	Lspd3	HQ111454	0	0	0	0
<i>L. caudatum</i>	CBS58485	Lca1	HQ643136	0	AF290309	0	0
<i>L. myophilum</i>	NJM-8403	Lm1	AB285497	0	AF290311	0	0
<i>L. humanum</i>	ATCC76726	Lh1	JX970867	JX985757	AF290310	JX999094	JX999125
<i>L. giganteum mammals</i>	ATCCMYA4933	Lgp1	JF919611	JF919613	JX970853	JX999097	JX999127
<i>L. giganteum mammals</i>	MTLA-03	Lgp2	JX999099	JF919612	JX970852	JX999099	JX999128
<i>L. giganteum mammals</i>	ATCCMYA4934	Lgp3	JX999096	JF919613	JX970854	JX999096	JX999126



Taxonomic name	Strain ID	Acronym	ITS	CDC42	COXII	HSP90	Tubulin
<i>L. giganteum mammals</i>	ATCCMYA4935	Lgp4	JX999098	JF919614	JX970855	JX999098	JX999129
<i>L. giganteum mammals</i>	MTLA-10	Lgp5	KJ506111	KJ506076	KJ506083	KJ506097	KJ506111
<i>L. giganteum mammals</i>	MTLA-12	Lgp6	KJ506112	KJ506078	KJ506084	KJ506098	KJ506112
<i>L. giganteum mammals</i>	MTLA-14	Lgp7	KJ506113	KJ506077	KJ506085	KJ506099	KJ506113
<i>L. giganteum mammals</i>	MTLA-15	Lgp8	KJ506116	KJ506081	KJ506087	KJ506103	KJ506116
<i>L. giganteum mammals</i>	MTLA-16	Lgp9	KJ506115	KJ506080	KJ506086	KJ506102	KJ506115
<i>L. giganteum mammals</i>	MTLA-17	Lgp10	KJ506117	KJ506082	KJ506088	KJ506101	KJ506117
<i>L. giganteum mammals</i>	MTLA-18	Lgp11	KJ506114	JX970841	KJ506089	KJ506100	KJ506114
<i>L. ajelloi</i>	ATCCMYA4936	La1	JX970885	JX970885	JX970841	JX999112	JX999143
<i>L. ajelloi</i>	ATCCMYA4937	La2	JX970884	JX970884	JX970842	JX999113	JX999144
<i>L. ajelloi</i>	MTLA-19	La3	KJ506134	KJ506070	KJ506092	KJ506106	KJ506120
<i>L. ajelloi</i>	MTLA-20	La4	KJ506135	KJ506071	KJ506094	KJ506109	KJ506124
<i>L. ajelloi</i>	MTLA-21	La5	KJ506137	KJ506073	KJ506095	KJ506108	KJ506122
<i>L. ajelloi</i>	MTLA-22	La6	KJ506138	KJ506069	KJ506096	KJ506107	KJ506123
<i>L. ajelloi</i>	MTLA-23	La7	KJ506136	KJ506072	KJ506093	KJ506110	KJ506121
<i>L. albertoi</i>	ATCCMYA4932	Lal1	JX970870	JX970838	JX970838	JX999115	JX999142
<i>L. vilelae</i>	MTLA-24	Lv2	KJ506133	KJ506075	KJ506091	KJ506105	KJ506119
<i>L. vilelae</i>	MTLA-25-(Tissue)	Lv3	KJ506132	KJ506090	KJ506090	KJ506104	KJ506118
<i>Pythium insidiosum</i>	MTPI-36	PI1	AY486144	0	0	0	0
<i>Pythium grandisporangium</i>	ATCC28295	Pg1c	AY151182	0	0	0	0
<i>Pythium ophiandermatum</i>	STR-135	Pa1a	AY151180	0	0	0	0
<i>Pythium difforme</i>	STR-66	Pd1a	AY151181	0	0	0	0
<i>Pythium flevoense</i>	STR-W041	PH1b	EU240168	0	0	0	0
<i>Pythium diclinum</i>	STR-P7824	Pd11b	EF153675	0	0	0	0
<i>Pythium capillosum</i>	CBS222-94	Pc1b	AY598635	0	0	0	0
<i>Pythium sylvaticum</i>	CBS453.67	Ps1f	AY598657	0	0	0	0
<i>Pythium pleroticum</i>	CBS776.81	Pp1e	AY598642	0	0	0	0
<i>Pythium iwayamai</i>	CBS156.64	Pi1w1g	AY598648	0	0	0	0
<i>Pythium undulatum</i>	CBS157.69	Pul1j	AY598708	0	0	0	0
<i>Pythium ultimum</i>	CBS398.51	Pull1i	AY598642	0	0	0	0
<i>Pythium nunn</i>	CBS808.96	Ph1j	AY598709	0	0	0	0

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0 = no DNA sequences available.

MTLA and MTP1 strains.

Biomedical Laboratory Diagnostics, Michigan State.

University culture collection.

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Microscopic features of the mammalian pathogenic *Lagenidium* spp. including their appearances and structure on 2% Sabouraud dextrose agar and in induction zoospore medium.

**Table 2**

Strains	Hyphae	Structures	Vesicle	Zoospore	Encysted zoospore	Oospore
<i>L. ajellii</i> sp. nov. ATCCMYA 4936 type strain	6–12 µm	Elongate chains of oval segments 60–600 µm	Spherical/oval 35–65 µm	11–19 µm by 8–10 µm	11–16 µm	Not observed
<i>L. albertoi</i> sp. nov. ATCCMYA-4932 type strain	8–15 µm	Mainly branched hyphal structures	Spherical 27–65 µm	12–18 µm by 8–10 µm	11–18 µm	Not observed
<i>L. vitellae</i> sp. nov. (MTLA-24) type strain	7–16 µm	Few 17 to 300 µm elongate hyphal structures	Spherical 25–65 µm	12–17 µm by 8–10 µm	11–18 µm	Not observed