Kappamyces, a new genus in the Chytridiales (Chytridiomycota)

by

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With 18 figures and 1 table

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Abstract: A new genus for a chytrid member of the Rhizophydium clade is described. Many Rhizophydium clade members exhibit simple and variable thallus morphology, and thus some species may be identical, while others may be members of species complexes and represent distinct genera. A minute chytrid found colonizing pollen grains had zoospore ultrastructure and a sequence of the large subunit ribosomal rRNA gene different from the molecular constitution of 22 isolates in the core Rhizophydium clade. Unlike typical Rhizophydium zoospores, the zoospore of Kappamyces laurelensis gen. et sp. nov. lacked a rumposome, kinetosome-associated electron-opaque spur, and kinetosome-associated microtubular root. Distinct from the typical Rhizophydium zoospore, the kinetosome abutted a single mitochondrion, both the kinetosome and non-flagellated centriole had an electron-opaque core composed of dense material, and a ring of distinct vesicles surrounded the kinetosome. In a parsimony analysis of large subunit rRNA gene sequences, a grouping of K. laurelensis and two other isolates were sister to the core Rhizophydium clade, and both the K. laurelensis group and the core Rhizophydium clade had 100% bootstrap support. The zoospore of K. laurelensis may be the simplest type of zoospore in the Chytridiales.

Key words: chytrid, large subunit rRNA gene, Rhizophydium, ultrastructure, zoospore

Introduction

The phylum Chytridiomycota is composed of five orders (Blastocladiales, Chytridiales, Monoblepharidales, Neocallimastigales, and Spizellomycetales). These fungi exhibit morphological diversity, and although they are commonly considered to be aquatic, they are in fact ubiquitous in soil (Powell 1993, Letcher & Powell 2001, 2002). Much of the current chytrid classification is based not only on thallus morphology (Sparrow 1943, 1960) and development (Karling 1932, Whiffen 1944, Roane &
Paterson 1974, Karling 1977) but also on zoospore ultrastructure (Barr 1980, 1990, 2001). In the Chytridiomycota, orders are delineated on the basis of zoospore ultrastructure, while circumscription of families (especially in Spizellomycetales and Chytridiales) often relies on thallus development. Genera and sub-family groups are defined primarily on zoospore ultrastructure (Barr 1980). However, several studies have suggested that, on the basis of comparison of ultrastructural features, many families and genera are polyphyletic (Beakes et al. 1988, Longcore 1992, 1995). Most of the approximately 1000 species of Chytridiomycota have not been grown in culture. Many chytrids are of relatively simple thallus morphology, and thallus morphology has been demonstrated repeatedly and in many taxa to be significantly variable (Paterson 1963, Miller 1976, Powell & Koch 1977, Miller et al. 1978).

The order Chytridiales is the most diverse of the five orders. *Rhizophydium* is the largest genus in the Chytridiales, with more than 220 described species (Sparrow 1960, Longcore 1996). Members of the genus are characterized (sensu Sparrow 1960) as eucarpic and monocentric, with an epibiotic, inoperculate, uniporous or multiporous sporangium, an endobiotic rhizoidal system, and an epibiotic resting spore. Some species in *Rhizophydium* may be identical, while other taxa may be members of species complexes in which character states of specific morphological features intergrade with other genera such as *Rhizidium*, *Phlyctochytrium*, and *Rhizophlyctis*, confounding species determination.

**Molecular data**

Molecular data are contributing to our understanding of chytrid phylogeny. An analysis of nuclear small-subunit (SSU) ribosomal RNA data (James et al. 2000) suggested that the members of the order Chytridiales are not a monophyletic group, but that there are well-supported monophyletic clades within the Chytridiales: the *Chytromyces* (*Chytridium*), *Lacustromyces*, *Nowakowskiiella*, and *Rhizophydium* clades. Chambers (2003), using secondary structure alignment of the nuclear large subunit (LSU) rRNA gene, showed the gene to have sufficient variation among related chytrids to resolve close relationships. In seeking the generic boundaries of *Rhizophydium*, Letcher (2003) demonstrated that, while several isolates of provisional *Rhizophydium* species formed a core of the *Rhizophydium* clade, other isolates provisionally placed in the genus were clearly delineated from the core taxa. This investigation reports the circumscription of three isolates in the *Rhizophydium* clade that exhibit significant differences in zoospore morphology, ultrastructure, and molecular constitution from the core *Rhizophydium* clade isolates.

**The Rhizophydium zoospore**

Compared with morphological features, the ultrastructural arrangement of organelles in the chytrid zoospore is quite conserved, but variation also exists at that level. Taxa in the genus *Rhizophydium* exhibit variable ultrastructure morphology (Barr & Hadland-Hartmann 1978, Barr 1980, Letcher 2003), including presence or absence of a rumposome, electron-opaque kinetosome-associated spur, and microtubular root.
In hypothesizing a taxonomic and phylogenetic scheme among the chytrids, Barr (1978) suggested that thallus development was of primary importance in chytrid evolution, and that increasing complexity in zoospore ultrastructural organization occurred simultaneously. In the evolution of eucarpic chytrids, Barr (1978) proposed that Rhizophydium types of morphology, thallus development, and ultrastructural organization were primitive. Among the less complex taxa was Rhizophydium patellarium E. Scholz (Scholz 1958). In R. patellarium, encysted zoospore development was endogenous, a small, thin-walled sporangium dissolved away during zoospore release, and the ultrastructural features of the zoospore were less complex in comparison with more advanced Rhizophydium taxa exhibiting the Rhizophydium Group III-type zoospore (Barr 1980). Specifically, the zoospore of R. patellarium possessed a single lipid globule, a microbody and simple cisterna intimately associated with the lipid globule, ribosomes aggregated in a core region of the zoospore, and a kinetosome and non-flagellated centriole that were parallel (Chong & Barr 1974, Barr & Hadland-Hartmann 1978). Barr’s evolutionary precept was that more complex zoospores, such as those characteristic of several other Rhizophydium isolates, evolved from a simpler R. patellarium-type of thallus and zoospore. Molecular data were not available when Barr developed his hypothesis.

In culturing soils from various locations in the eastern US, a chytrid was isolated that superficially resembled R. patellarium, on the basis of morphology and ultrastructural features. A more detailed examination of this chytrid has revealed features inconsistent with the concept of R. patellarium. The objective of this paper is to describe this chytrid as the type of a new genus, Kappamyces, illustrate its morphology and zoospore ultrastructure, and evaluate its position in a nuclear 28S LSU rRNA gene inferred phylogeny.

Materials and methods

Taxonomic sampling

Pure cultures of 28 isolates in the order Chytridiales were obtained from chytrid culture collections maintained at The University of Alabama and University of Maine (Table I). In the phylogenetic analysis, ingroup taxa were 25 isolates in the Rhizophydium clade. The outgroup taxa were Diplophlyctis sarcopoides (H.E. Petersen) Dogma and Obelidium mucronatum L. Nowakowski of the Chytriomyces clade, and Phlyctochytrium planicorne G.F. Atk.

Sample preparation

Cultures were grown on the following media: PmTG, mPmTG, ½ CM+, ¼ YpSs and ½ YpSs (Barr 1987, Longcore 1995). Cells were fractured by freezing with liquid nitrogen and grinding with 0.1 mm and 0.5 mm silica beads (Biospec Products, Inc., Bartlesville, OK, USA) in a sterile 1.5 mL microcentrifuge tube with a sterile plastic pestle. Nine parts CTAB lysis buffer (1.0 M Tris base pH 8.0, 0.05 M EDTA, 0.2 M NaCl, and 2% CTAB) and one part 20% SDS were added to the ground tissue. The mixture was vortexed, incubated at 68°C for 2 h in a water bath, and then centrifuged for 10 min at 16 000 × g. The supernatant was transferred to a sterile 1.5 ml microcentrifuge tube and extracted twice with an equal volume of chloroform. DNA was precipitated by the addition of 0.6 volumes of isopropanol, incubated at room temperature overnight, and then centrifuged for 10 min at 16 000 × g. The DNA pellet was washed with 70% ethanol once, 100% ethanol once, and air dried in a 37°C incubator. The DNA pellet was resuspended in 50 µL sterile water.
DNA amplification and sequencing

The LSU rRNA gene was amplified by PCR in 50 µL reactions (25.4 µL sterile deionized water, 5 µL 10x buffer, 15 mM MgCl₂, [Sigma-Aldrich, St. Louis, MO, USA], 4 µL dNTP mix [2.5 µM each dNTP], 2.5 µL each primer [10 pM/µL LROR and LR5, Vilgalys & Sun 1994], 0.6 µL Taq DNA polymerase [Sigma-Aldrich], 2 µL unquantified DNA, and 8 µL Gene Releaser [BioVentures, Inc., Murphreesboro, TN, USA]) using an MJ research PTC-200 thermal cycler. Thermal cycling conditions for PCR amplification were: 94°C denaturation step (29 s), 55°C annealing step (29 s), and 72°C extension (90 s) for 10 cycles, followed by 94°C denaturation step (29 s), 50°C annealing step (29 s), and 72°C extension (90 s) for 29 cycles, with a final 4°C hold. PCR products were purified by gel electrophoresis on 1.5% low melt agarose (NuSieve, Cambrex Corp, East Rutherford, NJ, USA), then extracted from the gel by the manufacturer’s protocol. Cycle sequencing reactions were carried out using dye-labeled dideoxy terminators and primers LROR and LR5, and read by capillary electrophoresis on an ABI 3100 automated sequencer.

Table I. Taxon sampling for nuclear LSU rDNA phylogenetic analysis of 28 isolates in the order Chytridiales.

<table>
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<tr>
<th>Taxon</th>
<th>Culture number</th>
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<tr>
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<td></td>
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<td>NSW, AUS</td>
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<td>AY439043</td>
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*New South Wales, Australia
bTasmania, Australia
Molecular data analysis

Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI, USA) was used to assemble contiguous sequences. Partial LSU rRNA sequences (721-995 bases from the 5’ end) were aligned automatically using ClustalX 1.81 (Thompson et al. 1997), and manually using BioEdit (Hall 1999) based on the secondary structure model of Saccharomyces cerevisiae (Gutell et al. 1994, Van de Peer et al. 1997), GenBank U53879, available on-line at http://www.rna.icmb.utexas.edu. Equally weighted maximum-parsimony (MP) analysis was performed using PAUP* 4.0b10 (Swofford 2001) on a Power Macintosh computer. Starting trees were obtained by 100 heuristic searches employing random sequence addition and tree-bisection-reconnection (TBR) branch swapping. Support using MP was assessed with 1000 bootstrap replicates, with uninformative characters deleted. Neighbor-joining analysis (NJ) was performed on the same dataset using the Hasegawa-Kishino-Yano 85 (HKY85) substitution model.

Fixation for electron microscopy

The zoospores from all isolates in the ingroup were examined by transmission electron microscopy. For each culture, an initial zoospore suspension was prepared by flooding a culture plate with sterile P/3 water (1 part pond water, 2 parts deionized water). The water was allowed to stand and moisten the surface of the inoculant plate for 24 hours, during which the plate was periodically agitated gently to distribute discharged zoospores over the agar surface. After draining the water from the plate, the culture plate was sealed with Parafilm and incubated at 22°C for 2-6 d, during which time a lawn of sporangia formed on the agar surface. Upon observation of zoospore discharge, zoospores were harvested to serve as inoculum for 10 agar plates, which were treated in the same manner as the single plate. Synchronous zoospore discharge from mature sporangia was initiated 2-6 d after inoculation by flooding each plate with 0.75 ml of P/3 water. Discharge commenced between 0.25 h and 12 h after the plates were flooded. Zoospores were fixed in 2.5% glutaraldehyde in 0.1 M s-collidine buffer, pH 7.4, for 2 h at 21°C followed by 22 h at 5°C. Fixed zoospores were pelleted at low speed (setting = 2, IEC Clinical Centrifuge) for 1 h, washed three times without resuspension in 0.05 M s-collidine buffer, and post-fixed in 1% osmium tetroxide for 1 h at 21°C in the dark. The zoospore pellet was washed once in 0.05 M s-collidine buffer, three times in distilled water, then embedded in 2% agar. The solidified agar containing zoospores was cut into 1 mm³ pieces and en bloc stained in 2% aqueous uranyl acetate for 18 h. Blocks were dehydrated in a graded acetone series (10%, 30%, 50%, 70%, 75%, and 90% for 15 min per step, and 95% once and 100% twice for 20 min per step), and embedded in EPOX 812 (Ernest F. Fullam, Inc., Latham, NY, USA). Random sections were cut with a diamond knife on a Sorvall MT5000 ultramicrotome and collected on nickel grids. Sections were cleaned in 1% periodic acid for 5 min (Barr & Désaulniers 1986) and then post-stained for 5 min in saturated uranyl acetate in 70% ethanol (Lewis & Knight 1977), followed by 10 min in lead citrate (Pease 1967). Sections were examined on a Zeiss 10A transmission electron microscope at 60 kV.

Isolation and culture of isolates P.L. 74, 75, and 98

The three isolates (P.L. 74, P.L. 75, and P.L. 98) were isolated by P. M. Letcher, and the number for each isolate refers to the collection of P. M. Letcher, Department of Biological Sciences, The University of Alabama. The fungi were isolated by standard methods (Barr 1987) using sweet gum pollen (Liquidambar styraciflua L.) and pine pollen (Pinus strobus L.) as bait, and grown on ½ CM+ agar (8.5 g corn meal agar, 1.0 g glucose, 0.50 g yeast extract, 8.5 g agar, 1 L deionized water) or ½ YpSs (20 g Emerson’s YpSs agar, 1 L deionized water). Cultures were grown and maintained at ambient laboratory temperatures (~21-23°C) and transferred at 2-month intervals. Isolates examined in this study were recovered from soils collected from under moss from Laurel Run, Rockbridge Co., VA, USA (isolates P.L. 74 and P.L. 75), and Cloudland Canyon State Park, Walker Co., GA, USA (isolate P.L. 98).

Results

All Rhizophydium clade isolates in this study were obtained from soils from a wide variety of habitats in North America and Australia. Of the 25 isolates examined,
three isolates (P.L. 74, P.L. 75, and P.L. 98) from deciduous forests of the eastern United States exhibited zoospore ultrastructure and molecular constitution different from the 22 isolates composing the core *Rhizophydium* clade (Letcher 2003). Isolate P.L. 98 was critically examined via serial sections by transmission electron microscopy and designated as the type for a new genus.

**Development**

Figs 1-8

Zoospores observed in the aquatic environment were spherical, with a single lipid globule (Fig. 1). Germlings initially produced a long slender rhizoidal axis (Fig. 2), from the tip of which developed a compact mass of branched rhizoids (Figs 3-5). The spherical sporangium was minute, 6-12 µm diam. (Fig. 6), and on pollen the sporangia were either solitary or crowded. Zoospores swarmed in the sporangium prior to discharge, and were discharged in a mass (although not in an exogenous vesicle) through a single, wide apical discharge pore (Fig. 7). Upon discharge, many of the zoospores remained clustered in the vicinity of the exit orifice, and either germinated *in situ*, or attached directly to the same pollen grain. Other zoospores were observed swimming freely in the aqueous medium. Empty sporangia (Fig. 7) did not collapse subsequent to discharge, and a clear indentation in the sporangial wall was visible where the wide discharge pore was located. Spherical resting spores (Fig. 8) developed on pollen grains in older cultures, were either sessile or stipitate, and contained a single large, eccentrically located oil globule. Germination of resting spores was not observed.

**Zoospore ultrastructure**

Figs 9-15

The zoospores of the three isolates P.L. 74, P.L. 75, and P.L. 98 lacked a rumposome, kinetosome-associated electron-opaque spur, and kinetosome-associated microtubular root. Distinct from the typical *Rhizophydium* zoospore, the kinetosome abutted a single mitochondrion, both the kinetosome and non-flagellated centriole had an electron-opaque core, and several vesicles surrounded the kinetosome.

The zoospore was spherical to broadly elliptical in longitudinal section (Fig. 9) and circular in transverse section (Fig. 10). A central core region, surrounded by a network of endoplasmic reticulum, contained aggregated ribosomes, a lipid globule, a


Abbreviations used in Figs 9-16: DM, dense material; ER, endoplasmic reticulum; F, flagellum; FB, fibrillar bridge; K, kinetosome; L, lipid globule; M, mitochondrion; Mb, microbody; N, nucleus; NFC, non-flagellated centriole; P, flagellar prop; R, ribosomes; SC, simple cisterna; Sp, spiral; TP, terminal plate; Vac, vacuole; LVe, large electron-opaque vesicle adjacent to kinetosome; Sve, small electron-opaque vesicle in peripheral cytoplasm.
microbody, a nucleus, and a mitochondrion (Figs 9, 10). The lipid globule was centrally located in the ribosome core. The microbody was appressed to the lipid globule, and a lobe of the microbody extended into a pocket of the single mitochondrion (Figs 9, 10). The mitochondrion also contacted the nucleus and was intimately in contact with the kinetosome.

The microbody-lipid globule complex (MLC) was type 5A (Powell & Roychoudhury 1992) in which a simple cisterna surrounded the surface of the lipid globule and extended or branched around the microbody and mitochondrion. No microtubular root was observed, and the lipid globule appeared to be fixed in its central position by the mass of the ribosome core (Figs 9, 10).

The kinetosome and non-flagellated centriole (NFC) lay side by side (Fig. 13), were parallel or orientated at a slight angle, and were connected by a narrow fibrillar bridge between the number 6 triplet of the kinetosome and the NFC (Fig. 13). Both the kinetosome and NFC had an electron-opaque core (Figs 11, 13, 14).

Adjacent to the kinetosome, and rarely in the cytoplasm peripheral to the ribosomal mass, were vesicles of distinct morphology with electron-opaque content (Figs 9, 13-15). These single-membrane bound vesicles had a fenestrated inner shell of electron-opaque material and an electron-opaque core that appeared to be continuous with a portion of the inner shell (Fig. 15). The electron-transparent region of these vesicles occupied about half of the interior of the vesicle and was often reniform, while the contrasting electron-opaque content was papillate (Figs 14, 15). In longitudinal sections through the zoospore and across the kinetosome and NFC, a vesicle often appeared on the side of the kinetosome opposite the NFC. In longitudinal sections 90 degrees from the kinetosome-NFC plane, vesicles were often present on both sides of the kinetosome (Fig. 9). In transverse section, a ring of up to five vesicles encircled the kinetosome (Fig. 14). Other vesicles within the cytoplasm included a type with uniformly electron-opaque contents. Within the cytoplasm peripheral to the ribosomal mass were one or more vacuoles with granular contents (Figs 9, 10). At the proximal end of the flagellum was a terminal plate, and props supported the base of the flagellum (Fig. 11). Immediately distal to the terminal plate was a spiral structure that turned approximately nine times in which the coils were evenly spaced (Fig. 12). Ultrastructural features of the zoospore are schematically presented in Fig. 16.

Figs 9-15. Ultrastructural features of the zoospore of Kappamyces laurelensis. 9. Longitudinal section. 10. Transverse section. 11. Longitudinal section through kinetosome, illustrating dense material in core of kinetosome. 12. Longitudinal section through kinetosome and base of flagellum, illustrating electron-opaque spiral. 13. Transverse section through kinetosome and NFC, illustrating dense material in the core of the structures, and the fibrillar bridge between the kinetosome and NFC. 14. Transverse section through kinetosome, illustrating five vesicles surrounding the kinetosome, with the electron-opaque portion of the vesicles orientated toward the kinetosome. 15. Kinetosome-associated vesicle. Scale bars in Fig. 9 = 0.5 µm (for Figs 9, 10), in Fig. 11 = 0.25 µm (for Figs 11, 13, 14), in Fig. 12 = 0.25 µm, in Fig. 15 = 0.25 µm.
A strict consensus, most parsimonious tree (Fig. 17) was constructed, and was 319 steps in length, CI = 0.718, and RI = 0.788. A grouping of the three isolates P.L. 74, P.L. 75, and P.L. 98 was sister to the core *Rhizophydium* clade of 22 isolates, and had 100% bootstrap support. The core *Rhizophydium* clade also had 100% bootstrap support. The strong bootstrap support indicated a high confidence level for the derived topology. The CI suggested that homoplasy levels for the molecular characters were acceptable; the RI indicated that the informative characters fit the tree well.

In the three isolates P.L. 74, P.L. 75, and P.L. 98, sequence lengths (757, 721, and 771 bases respectively), agreement of paired sequences (0.86-0.87), and G+C content (46.37%, 47.16%, and 47.47% respectively) were similar.

A Neighbor Joining tree (Fig. 18) showed distances among and between isolates in the core *Rhizophydium* clade and the grouping of isolates P.L. 74, P.L. 75, and P.L. 98, sequence lengths (757, 721, and 771 bases respectively), agreement of paired sequences (0.86-0.87), and G+C content (46.37%, 47.16%, and 47.47% respectively) were similar.
P.L. 98. Substitutions per site indicated greater divergence within the grouping of three isolates than among most isolates in the core *Rhizophydium* clade.

**The genus**

*Kappamyces* Letcher & M.J. Powell, gen. nov.

Thallus monocentricus, eucarpicus, e sporangio sessili, globoso, epibiotico atque spora remanente et systemate endobiotico rhizoidarum capillarium compositus. Sporae remanentes crassitunicatae, globosae, hyalinae, sessiles vel stipitatae, unico magno globulo repletae. Zoosporae sphaericae, molem ribosomalae, unicum globulum lipoideum, corpusculum parvum (‘microbody’), nucleum, atque unicum mitochondrium, omnia duplicibus membranis circumdatae; vesiculae membranis crassis circumdatae, 0.2-0.3 µm diam., opacae, seu kinetosoma et centriolum circumdantes seu in cytoplastmate peripherico dispositae; kinetosoma et centriolum flagelli carens consimilia, parallela vel fere parallela.

Thallus monocentric, eucarpic, with an epibiotic sporangium and resting spore and a compact endobiotic mass of thread-like rhizoids. Resting spores thick-walled, spherical,
hyaline, sessile or stalked, with a single large globule. Zoospores spherical, containing a ribosomal mass, a single lipid globule, a microbody, a nucleus, and a single mitochondrion, all enveloped in a double-membrane system; thick-membraned vesicles, 0.2-0.3 µm diam., with electron-opaque contents, either surrounding the kinetosome and non-flagellated centriole or occurring in the peripheral cytoplasm; kinetosome and non-flagellated centriole parallel, or proximal end of non-flagellated centriole slightly angled toward the kinetosome. Both the kinetosome and the non-flagellated centriole have an electron-opaque core.

Fig. 18. Neighbor-joining tree from LSU rRNA gene sequence analysis of 28 isolates in the Chytridiales (three Kappamyces isolates, 22 isolates in the core Rhizophydium clade, and three outgroup taxa).
Etymology: The genus is named for the Greek letter Kappa, for the kinetosome-associated vesicles.

Type species: *Kappamyces laurelensis*.

**The species**

*Kappamyces laurelensis* Letcher & M.J. Powell, sp. nov.

Sporangium globosum, 6-12 µm diam., poro evacuationis unico lato praeditum; zoosporae liberatae vel stipitatae, unico magno globulo repletae. Zoosporae globosae, 2.5-3 µm diam., massam ribosomalem, globulum lipoidem, corpusculum parvum nucleum, atque mitochondrium, duplici membrana circumdatae continentis. Kinetosoma atque centriolum flagello carens plus minusve parallela, utraque uno ad quinque vesiculis lata membrana circumdatae, intus opacis cincta. Pars translucida reniformis, pars opaca papillata. Ex solo musco tecto, in Cloudland Canyon State Park, prope Chickamauga, GA, USA, GPS locus 34° 49.514' N, 65° 29.573' W. 28S rRNA GenBank #AY439034.

Typus: Figurae 1-15. Diagnosis cultura P.L. 98 ex pollonis esca posita in aquacultura solo musco tecti composita iuxta amnem in Cloudland Canyon State Park, Chickamauga, in Georgia, USA.

Thallus monocentric, eucarpic, with an epibiotic spherical sporangium 6-12 µm diam., with a single wide discharge pore; zoospores released as a mass, but not in an exogenous vesicle; sporangial remains inflated following zoospore discharge. Rhizoidal system composed of a single rhizoidal axis and compact, finely-branched, thread-like rhizoids.

Resting spores epibiotic or interbiotic, spherical, thick-walled, hyaline, 6-10 µm diam., with a single large lipid globule. Zoospores spherical, 2.5-3 µm diam., containing a ribosomal mass, a single lipid globule, a microbody, a nucleus, and a single mitochondrion, all enclosed in a double-membrane system. The kinetosome and non-flagellated centriole are parallel or nearly parallel and are surrounded by one to five thick-membraned, spherical vesicles with electron-opaque contents; the electron-transparent content usually reniform, the electron-opaque content usually papillate; vesicles also occur singly in the peripheral cytoplasm. From moss-covered soil, Cloudland Canyon State Park, near Chickamauga, GA, USA; GPS coordinates 34° 49.514’ N, 65° 29.573’ W; saprotrophic on pine pollen. 28S rRNA gene GenBank #AY439034.

Type: Figs 1-15. Diagnosis based on isolate P.L. 98, obtained from pollen bait placed with water cultures of moss-covered soil from along a stream in Cloudland Canyon State Park, Chickamauga, GA, USA. A culture of the type isolate has been deposited with Centraalbureau voor Schimmelcultures.

Etymology: The species is named for Laurel Run, a creek in Rockbridge County, VA, USA, along which the initial isolates (P.L. 74 and P.L. 75) of this chytrid were found.

**Discussion**

*Kappamyces laurelensis* differs from species of the core *Rhizophyldium* clade in morphology, zoospore ultrastructure, and molecular constitution, and these differences are the rationale for the generic delineation.
Morphology

Observations of both small and larger, epibiotic, inoperculate, spherical chytrids ("LRCs and BRCs" – little round chytrids and big round chytrids) in the genus *Rhizophydium* have often been reported in the literature (Willoughby 1962, 1965, Booth 1971, Booth & Barrett 1971, Sparrow 1975, Karling 1981, 1988, Longcore 2004). These putative taxa, normally designated as “*Rhizophydium* sp.”, often are affiliated or compared with recognized species. This situation has not been due to a lack of taxonomic expertise on the part of the observers; rather, it is a result of the fact that many monocentric chytrids of simple thallus morphology lack sufficient discriminatory morphological characters to be reliably placed within a species, especially if developmental processes are not observed. Morphological plasticity, particularly in thallus size and shape, often confuses taxonomic delineation not only at the species but also at the generic level, thus precluding accurate identification.

*Kappamyces laurelensis* was first observed on pollen bait with soil samples from temperate deciduous forests in the mountains in Virginia, eastern US (Letcher & Powell 2001, 2002) but was not named so far because, on the basis of thallus morphology, it could not be reliably assigned to a known taxon. Additional observations of single-spore axenic cultures of *K. laurelensis* indicated that it was possibly related to *Rhizophydium patellarium*. However, substantial differences in thallus morphology were observed. Most chytrids exhibit a range in sporangial size, and the range can be an important diagnostic tool. Scholz (1958) described the sporangium of *R. patellarium* as 10-30 µm diam.; the sporangia of *K. laurelensis* were 6-12 µm. Unlike *R. patellarium*, the sporangial wall of *K. laurelensis* did not deliquesce upon discharge, nor did it leave a dish- or plate-like structure (“*patella*” in Latin, and thus the etymology of the specific epithet of *R. patellarium*) as the remnant of the discharged sporangium. Rather, the sporangial wall of *K. laurelensis* remained rigid following discharge, and a single, large exit pore was visible. Differences in rhizoidal structure were also evident. *Rhizophydium patellarium* was described as having a “delicate” rhizoid (“...Rhizoid zart...”, Scholz 1958) and was illustrated as having a single rhizoidal axis with branching rhizoids sparse to non-existent. In contrast, in both pollen grains and on agar, *K. laurelensis* exhibited a single rhizoidal axis with extensive, yet compact branching rhizoids.

Zoospore ultrastructure

The zoospore of *Kappamyces* exhibits ultrastructural features not present in either *R. patellarium* or isolates in the core *Rhizophydium* clade. Those features include a ring of several morphologically distinct vesicles around the kinetosome, and both the kinetosome and non-flagellated centriole have an electron-opaque core. No other chytrid has these features.

The zoospores of the 22 isolates of the core *Rhizophydium* clade included here have been examined by transmission electron microscopy, and all have an electron-opaque structure (a spur) associated with the kinetosome (Letcher 2003). Many of the isolates
in the core *Rhizophydium* clade also have a rumposome associated with the lipid globule; many also exhibit a kinetosome-associated microtubular root. The zoospores of both *R. patellarium* and *K. laurelensis* exhibited less complexity in structure when compared with the isolates in the core *Rhizophydium* clade. The zoospores of *R. patellarium* and *K. laurelensis* have no electron-opaque spur, no rumposome, and no kinetosome-associated microtubular root.

Although the overall ultrastructural morphologies of *R. patellarium* (Chong & Barr 1974, Barr & Hadland-Hartmann 1978) and *K. laurelensis* were similar, the zoospore of *K. laurelensis* had ultrastructure features not present in *R. patellarium*. The most prominent difference between the two taxa was the presence of vesicles with distinct morphology that were adjacent to and surrounding the kinetosome in *K. laurelensis*, a feature not present in the zoospore of *R. patellarium*. Such an arrangement of vesicles has not been previously reported for any chytrid.

In their ultrastructural analysis of members of the genus *Rhizophydium*, Barr & Hadland-Hartmann (1978) noted the presence of several types of vesicles in various taxa. Vesicular organelles with electron-opaque content, similar in size (but not similar in morphology) to the vesicles reported here for *K. laurelensis*, were observed in the peripheral cytoplasm of the zoospores of *R. patellarium* and *R. capillaceum* D.J.S.Barr. Those vesicles had concentric or reticulate electron-opaque contents, as opposed to the papillate shape of the electron-opaque content, and complementary reniform shape of the electron-translucent content in the vesicles of *K. laurelensis*. It was specifically noted, however (Barr & Hadland-Hartmann 1978), that in both *R. patellarium* and *R. capillaceum*, the vesicles present were not conspicuously aggregated near the flagellar apparatus. Transverse serial sections through the kinetosome region of *K. laurelensis* revealed up to five vesicles surrounding the kinetosome. Although both *R. patellarium* and *R. capillaceum* had similar vesicles in the peripheral cytoplasm, and *R. sphaerotheca* sensu Booth had similar vesicles near the flagellum, *R. capillaceum* had a rumposome and *R. sphaerotheca* sensu Booth (1971) had a kinetosome-associated spur, features which preclude their confusion with *R. patellarium*. Barr & Hadland-Hartmann (1978) also described vesicles present in the zoospores of other *Rhizophydium* taxa. In the zoospore of *R. constantineani* Sacc., electron-opaque vesicles were observed adjacent to the kinetosome; however, *R. constantineani* also had a rumposome, which distinguishes it from *R. patellarium* and our *K. laurelensis*. The zoospores of *R. subangulosum* (A. Br.) Rabenh. had vesicles morphologically similar to gamma bodies in *Blastocladiella emersonii* (see Cantino & Mills 1976), and which were observed near both the nucleus and kinetosome. McNitt (1974), in an examination of the zoospore of *Phyllyctochytrium irregularare* W.J. Koch, revealed vesicles in the peripheral cytoplasm with electron-opaque, irregularly-shaped cores. Lange & Olson (1979) commented on the presence of various types of vesicles with electron-dense contents that occurred with numerous chytrid taxa, and Beakes et al. (1988) reported the presence of “dense-cored” vesicles that occurred in the peripheral cytoplasm of *Zygophyllum affluens* Canter similar in morphology to those of *K. laurelensis*. Although the above-mentioned vesicles all occur in the peripheral cytoplasm, they are not typical of peripheral vesicles such as those observed in *Saprolegnia ferax* (Lehnen & Powell 1989), but are more similar
to encystment vesicles (extrusomes) that function in attachment of the encysting zoospore (Lehnen & Powell 1989). It is of interest that the electron-opaque, reniform content of the vesicles in *K. laurelensis* is normally oriented toward the kinetosome or flagellum base.

The attachment of and adhesion to substrata by the zoospores of lower fungi is a relatively unexplored area of research. The zoospore of *Rozella allomycis* (Chytridiales) contacts the host in the lateral region of the zoospore, encysts, and then germinates from the region of contact (Held 1973). The uniflagellate zoospore of *Catenaria anguillulae* (Blastocladiales) settles with the flagellum projecting away from the host, and attaches at the anterior region of the spore (Deacon & Saxena 1997). If the particular type of vesicle seen in *K. laurelensis* is extrusomal in function, such that the posterior of the zoospore were the site of attachment and adhesion to the substratum, such attachment would represent a departure from recognized growth polarity in the lower fungi. The biochemical constitution of the vesicles in *K. laurelensis* has not been studied, and whether they are homologous with other attachment or encystment vesicles is speculative.

A second ultrastructural feature also serves to delineate *Kappamyces*. Both the kinetosome and non-flagellated centriole in *K. laurelensis* have an electron-opaque core that is centrally positioned in both those structures, and which is apparent in both longitudinal and transverse sections. That feature alone may serve to delineate *Kappamyces* from the core *Rhizophydatum* clade, because it has not been observed previously in any other *Rhizophydatum* isolate, or other chytrid taxon.

**Molecular phylogeny**

The rationale for partitioning *Kappamyces* from the core *Rhizophydatum* clade isolates is also evident in the inferred LSU phylogeny. The *Kappamyces* isolates were sister to the *Rhizophydatum* isolates that composed the core *Rhizophydatum* clade. The strong bootstrap support for both the core *Rhizophydatum* clade and the grouping of *Kappamyces* isolates indicate their molecular differences and the divergence between *Rhizophydatum* and *Kappamyces*. The divergence is also reflected by the molecular signature of *Kappamyces* as compared with that of members of the core *Rhizophydatum* clade (Letcher 2003).

The distance between taxa, as demonstrated by the Neighbor-Joining tree (Fig. 18), indicated that not only were isolates P.L. 74 and P.L. 75 distinct from *K. laurelensis*, but were also distinct from each other. The inferred distance between P.L. 74 and P.L. 75 is the reason the two isolates are not subsumed under the same species.

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References


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