Mycologia, 105(1), 2013, pp. 90–111. DOI: 10.3852/11-311

Overview of 75 years of *Smittium* research, establishing a new genus for *Smittium culisetae*, and prospects for future revisions of the 'Smittium' clade[1]

Yan Wang
Eric D. Tretter
Boise State University, Department of Biological Sciences, Boise, Idaho 83725-1515

Robert W. Lichtwardt
University of Kansas, Department of Ecology & Evolutionary Biology, Lawrence, Kansas 66045-7534

Merlin M. White[2]
Boise State University, Department of Biological Sciences, Boise, Idaho 83725-1515

Abstract: The Harpellales includes 38 genera of endosymbiotic microfungi associated with various Arthropoda. *Smittium*, the second genus to be described, is now also the most species rich of the order. Species of *Smittium* inhabit the digestive tracts of larval aquatic insects, especially lower Diptera, worldwide. During the 75 y since the type, *Smittium arvernense*, was described a number of advances in our understanding of the gut fungi have unfolded, in whole or in part, with *Smittium* as a model for the fungal trichomycetes. This in part relates to the high number of successful isolation attempts, with about 40% of known species having been cultured, a total number that far exceeds any other genus of gut fungus. Many isolates of *Smittium* have been used in laboratory studies for ultrastructural, physiological, host feeding, serological, as well as isozyme, and now ongoing molecular systematic studies. Molecular studies have shown that *Smittium* is polyphyletic but with consistent separation of *Smittium culisetae*, one of the most common and widespread species, from the remainder of *Smittium*. A brief overview of *Smittium* research is provided. Zygospore and trichospore morphology and molecular evidence (immunological, isozyme, DNA sequences and phylogenetic analyses) are used to establish *Zancudomyces* and to accommodate *Smittium culisetae*. For the latter evidence, we include the first two-gene phylogenetic analysis, using combined 18S and 28S rRNA gene sequence data to show a cluster of *Zancudomyces culisetae* separate from *Smittium*. As the broadest taxon sampling of *Smittium* to date, this also serves a molecular systematic update toward revisionary syntheses of this and other Harpellales taxa.

Key words: Diptera, early-diverging fungi, Insecta, Kickxellomycotina, symbiosis, Zygomycota

Introduction

Early researchers, studies of gut fungi and timeline.— The history of research on what would become known as the Trichomycetes Manier & Lichtw., a group of obligate endosymbionts associated with Arthropoda, began with the studies of ''entophytes'' by American naturalist Joseph Leidy (1849a, b, 1850a, b, 1853). Several decades later, the foundation of the field of trichomycetology was developed by protozoologists in France. This began with Léger and Duboscq (1903, 1905a, b), whose studies spanned three decades, first on the Eccrinales L. Léger & Duboscq and later with fungal trichomycetes (Léger and Duboscq 1929). Léger and Gauthier (1931, 1932, 1935a, b, 1937) continued the tradition until just before World War II. Their active research overlapped with the fungal studies of Poisson (1927, 1936). Gauthier (1936, 1960, 1961) published individually as well, but infrequently, over another three-decade span.

The monograph of Duboscq et al. (1948) was advanced posthumously by Tuzet and Léger. Although it included Trichomycètes in the title, it did not include the Harpellales Lichtw. & Manier. While carrying on the tradition of studies in France (Tuzet and Manier 1947, 1953, 1954, 1955a, b), Tuzet and Manier (1950) also revised ''Les Trichomycètes''. This was a significant study, although some of the taxa were validated by Manier (1968). Not only did she publish with her students in France, but also she collaborated with early-career mycologists who obtained their doctoral degrees from abroad, specifically with Lichtwardt (1951) and Whisler (1961) from USA and with Moss (1972) from England. Lichtwardt and Moss also published (Lichtwardt and Moss 1981, 1984a, b; Moss and Lichtwardt 1976, 1977, 1980), both field and laboratory investigations on the Trichomycetes, and ultimately mentored a number of trichomycetologists.

The class Trichomycetes was established by Manier and Lichtwardt (1968) with four orders of hair-like endosymbionts (Harpellales, Asellariales Manier ex

Submitted 21 Sep 2011; accepted for publication 31 May 2012.

[1] This paper is dedicated to Dr Marvin Williams and his former students for their contributions to *Smittium* and other Harpellales and also to a former student of both Drs Williams and Lichtwardt, the late Dr Roger Grigg, whose isozyme studies, among others, have helped us unravel some of these stories.

[2] Corresponding author. E-mail: merlinwhite@boisestate.edu

Manier & Lichtw., Amoebidiales L. Léger & Duboscq and Eccrinales), all associated with various Arthropoda (Lichtwardt 1986, Lichtwardt et al. 2001). Lichtwardt's (1951, 1954) early work was on the Eccrinales, but later his focus was on the Harpellales. Taxonomically the Harpellales offered a relatively more reasonable group for morphological study and some species had been obtained in pure culture by the 1960s (Whisler 1962, 1966, 1968; Clark et al. 1963; Lichtwardt 1964). Since then, eight of the 38 genera of Harpellales have been established in pure culture. However, about 80% of all axenic isolates are species of *Smittium* R.A. Poiss., which accounts for about 40% of the species of this genus (Lichtwardt et al. 2001). Many of those isolates have proven to be fruitful for in vitro studies (see below).

Molecular versus morphological data and nature of the symbiosis.—Hibbett et al. (2007) published a phylogeny-based revision of the Fungi, which prompted significant changes in the higher level classification of many fungal groups. It was suggested that the Trichomycetes be deconstructed until molecular-based data more fully substantiated the lineages that comprise the gut fungi. Since then, the trichomycetes (in non-taxonomic, lowercase form) have been recognized by some as an ecological group with two fungal orders—the Asellariales and Harpellales (Lichtwardt 1978, Moss and Young 1978, Cafaro 2005). Although not included in this study, the Asellariales, with three genera and 14 species, is one of the key missing lineages among phylogenetic studies of early-diverging fungi (Lichtwardt et al. 2001). Hereafter, the focus is within the Harpellales, with all but one genus (White 1999) that live nearly exclusively in the digestive tracts of immature aquatic insects, worldwide.

Without question, the intimacy of the relationship and symbiotic lives of these fungi have prompted adaptations over evolutionary time. This is true whether considering the various morphological and physiological adaptations that accommodate the day to day challenges of maintaining a gut-dwelling residence or the obvious success they have had in evolving, with some degree of host specificity, for millions of years (Lichtwardt et al. 2001).

History of the Harpellales.—*Harpella melusinae* was the first Harpellales to be described (Léger and Duboscq 1929) and is now known to be widespread in the midguts of black flies in the northern and southern hemispheres. The first *Smittium*, *Smittium arvernense* R.A. Poiss, was named over 75 y ago by Poisson (1936) after the host midge *Smittia*. *Smittium* now has 81 species and is the most species rich of the Harpellales.

Species of *Smittium* exhibit varying degrees of specificity but typically inhabit the hindguts of lower Diptera, including not only black flies (Simuliidae) but also bloodworms (Chironomidae) and mosquitoes (Culicidae) as well as solitary (Thaumaleidae) and biting (Ceratopogonidae) midges from varied habitats (Lichtwardt 1999, Ferrington et al. 2005, Valle et al. 2011). Some species of *Smittium* are cosmopolitan and widespread, while others have narrower geographic distributions. The relationship is generally considered to be commensal, but actually ranges from mutualistic for insects (mosquitoes) that are under nutritional stress (Horn and Lichtwardt 1981), to lethal or parasitic, as with *Smittium morbosum* A.W. Sweeney, which kills mosquito larvae by preventing molting (Sweeney 1981, Lichtwardt 2004). Aside from *S. morbosum*, parasitism is rare, at least among immature stages of their dipteran hosts, but members of the Harpellales also are known to invoke a parasitic, ovarian cyst stage for dispersal via the flying adult female (White et al. 2006b).

Morphologically all species of *Smittium* are branched, septate fungi that attach to the chitinous hindgut linings of their hosts. Asexual spores or trichospores (= monosporous sporangia) are variable in shape (ranging from ellipsoidal to cylindrical) and upon detachment have a collar and a single, nonmotile appendage. The sexual spore or zygospore is biconical to fusiform and attached obliquely and submedially to the subtending zygosporophore. Detached zygospores, where known, also have a collar and a single appendage (Lichtwardt et al. 2001). Other, putatively closely related taxa from Diptera hindguts are known, but differ either in the nature of the conjugation (*Furculomyces* Lichtw. & M.C. Williams), shape of the zygospore (*Austrosmittium* Lichtw. & M.C. Williams and *Furculomyces*) or in appendage number for the trichospores and/or zygospores (*Trichozygospora* Lichtw. and *Sinotrichium* J. Wang, S.Q. Xu & Strongman).

Considering that *Smittium* is now the most species-rich genus of the Harpellales by a wide margin, it is remarkable that it would take nearly 30 y for the second two species, *Smittium culisetae* Lichtw. and *Smittium simulii* Lichtw., to be described (Lichtwardt 1964). After those three species the number increased rapidly and substantially (FIG. 1), with six Smittiums described in 1969, six more in the 1970s, 15 in the 1980s, 23 in the 1990s and 25 in the new millennium. Although *Smittium culisetae* has been commonly recovered, reported and even cultured from different places during this time (Lichtwardt 1964; Farr et al. 1967; Manier 1969b; Williams et al. 1972b; Starr et al. 1979; Williams 1983a, b; Horn 1989b; McCreadie et al. 2003; López Lastra et al. 2005; White et al. 2006a;

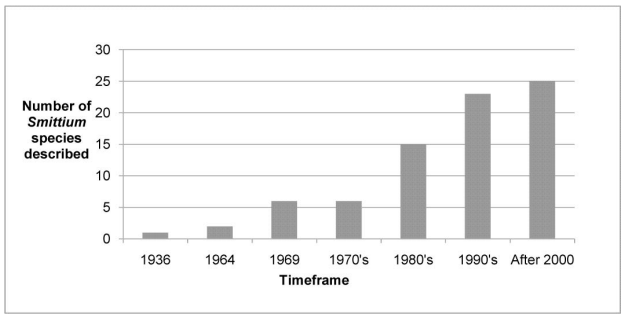


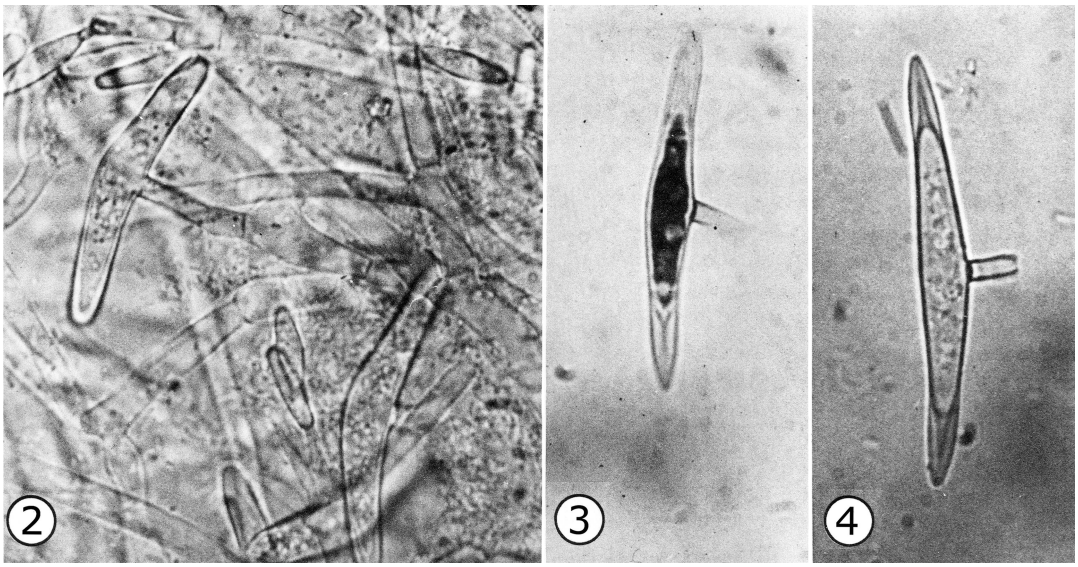
FIG. 1. Number of new species of *Smittium* described per indicated timeframe after the first type species, *Smittium arvernense*, was described by Poisson (1936). The trend presented by the numbers has been increasing continuously since 1969. *Smittium culisetae* (now *Zancudomyces culisetae*) described by Lichtwardt (1964) is included in this representation.

Strongman et al. 2008; Valle et al. 2010, 2011), the type species, *Smittium arvernense*, has yet to be found again. This and ongoing revisionary systematic studies prompted the establishment of an epitype, namely *Smittium mucronatum* Manier & Mathiez ex Manier, a species originally recorded in France (Manier 1969a) and subsequently found in USA, Canada and Norway (Lichtwardt and Williams 1999, White and Lichtwardt 2004, Strongman and White 2008, Lichtwardt and White 2011). *Smittium mucronatum*, also culturable, is recognizable on the basis of a small nipple-like protuberance on the tip of the trichospore (Lichtwardt and White 2011). Despite being well studied and the second oldest species, *S. culisetae* was not considered an epitype because it now is recognized to be unlike the other Smittiums and perhaps did not belong in the genus (White 2006).

Our overall goal is to contribute the first combined rRNA gene-based phylogenetic analyses for the largest number of *Smittium* species to test relationships among *Smittium* and closely related Harpellales genera (allies). One specific objective is to assess the monophyly of *Smittium* with a combined analysis and expanded taxon sampling. We consider this to be the first step in the revision of this genus. Herein we establish a new genus for *Smittium culisetae*, based on both morphological (FIGS. 2–5) and molecular (FIGS. 6–11) evidence. We start to resolve some of the relationships between *Smittium* and its allies for what had been regarded as the polyphyletic ''Smittium'' and ''non-Smittium'' clades (White 2006). One species is relocated, whereas others are being included in these clades for the first time, but lineages are beginning to be better resolved with ongoing efforts to generate sequence data both for more taxa and genes, among these and other early-diverging lineages.

MATERIALS AND METHODS

Host collection and specimen preparation.—Methods for collecting larval aquatic insects followed those of White et al. (2001). Fungal vouchers consisted of living clumps of thalli placed in 500 mL 2× Hexadecyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1.4 M Tris–HCl pH 8.0, 0.25 mM EDTA) (Gottlieb and Lichtwardt 2001) immediately after dissection and identification. Specimens of gut fungi invariably included host tissue or other microscopic organisms associated with or passing through the host gut. The digestive tract, once removed from the host, was



FIGS. 2–4. *Zancudomyces culisetae* zygospores. 2. Immature zygospores in a mass of *Z. culisetae* hyphae and some trichospores, 800×. 3, 4. Mature, loose zygospores, 1000×. (From Williams 1983b.)

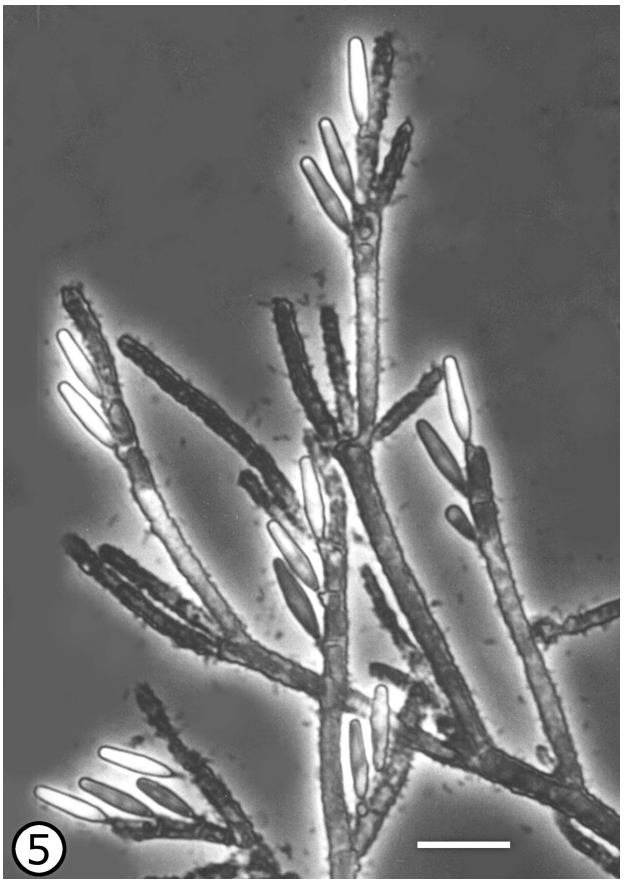


FIG. 5. *Zancudomyces culisetae* with attached trichospores and some verticillate branching, as dissected from a mosquito larva (microscope slide TN-46-7, photomicrograph TN-S-1) sampled from Great Smoky Mountains National Park, USA. Bar = 20 μm.

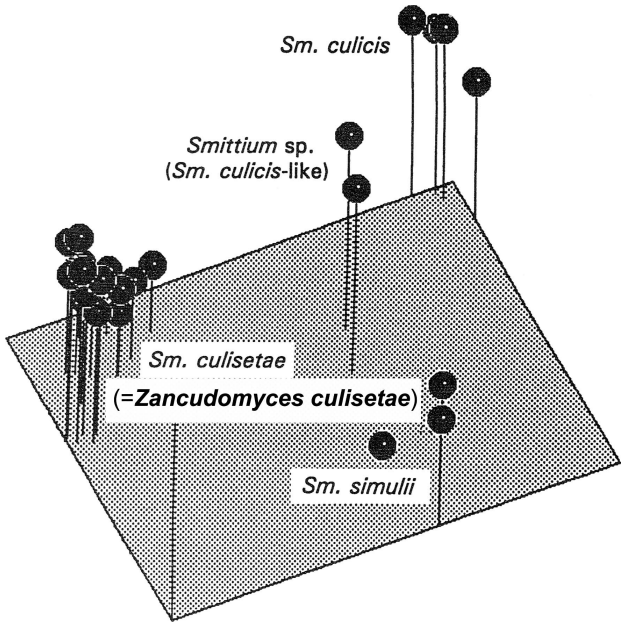


FIG. 6. Three-dimensional model constructed from the three principal coordinates of enzyme variation similarity in 11 enzyme systems with 13 loci for 41 isolates of *Smittium* representing four species. Thirty-two isolates of *Z. culisetae* from different geographical regions are not apparent in the cluster because of many identical isozyme patterns. (Modified, from Grigg and Lichtwardt 1996.)

dissected with fine needles or forceps, and gut fungi were identified in wet mounts based on the morphological features noted by Lichtwardt et al. (2001). Every attempt was made to place thalli of a single fungal species (multiple taxa of gut fungi can be found in a single gut) in the CTAB buffer, which then was placed at −20 C (up to 4 y) before DNA extraction. Other samples were a few colonies from axenic cultures similarly placed in CTAB buffer. Additional samples were obtained as genomic DNA preparations from Gottlieb and Lichtwardt (2001). Sample selection attempted to maximize the number of species of *Smittium* and broadly sample some of the other genera of Harpellales for phylogenetic analysis.

DNA extraction.—Standard procedures for DNA extraction from samples in 2× CTAB buffer were followed (O'Donnell et al. 1997, Gottlieb and Lichtwardt 2001, White 2006). In some cases specimens were frozen repeatedly by submerging in liquid nitrogen and thawing at 65 C in a heat block (no attempt was made to crush microscopic amounts of thalli). After two chloroform extractions, DNA was precipitated, eluted in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0) and either used directly or after dilution in sterile double-distilled water (ddH_2O) in PCR amplification. Some genomic DNA extracts were cleaned with glass-milk or glass-bead columns following the protocols of the GENECLEAN II Kit (Bio 101, Vista, California) or the GENECLEAN Turbo Kit (Quantum Biotechnologies, Carlsbad, California) respectively.

PCR amplification.—For amplification of the nuclear small subunit, rRNA gene, or 18S, we used the primers SR1R (Vilgalys and Hester 1990) and NS8 (White et al. 1990). For the portion of the 28S we amplified, we used the primers ITS3 (White et al. 1990) and LR5 (Vilgalys and Hester 1990). The Promega Green Master Mix kit (Cat. No. M7122) was used for the 18S sequences and some of the 28S sequences. For these amplifications, the cocktail included 11 μL Promega Go-Taq Green Master mix, 0.66 μL both the forward and reverse primer (0.3 pM/uL), 0.86 μL 25 mM $MgCl_2$, 6.8 μL molecular biology-grade H_2O and 2 μL diluted DNA template. For some 28S reactions, a TaKaRa EX Taq-based kit was used. The TaKaRa amplification cocktail included: 2.2 μL EX Taq buffer, 1.76 μL 2.5 μM dNTP mix, 0.44 μL 25 mM $MgCl_2$, 0.50 μL 50 mg/mL BSA, 4.40 μL 5M Betaine, 0.66 μL of each primer (0.3 pM/uL), 9.42 μL H_2O, and 0.11 μL TaKaRa EX Taq. For both amplification reaction kits, the final concentration of $MgCl_2$ was 2.5 mM.

Thermal-cycling protocols were adapted from the instructions included with the Promega Go-Taq Green Master Mix kit. The protocol for the 18S region consisted of an initial denaturation of 95 C for 2 min; 45 cycles consisting of 95 C

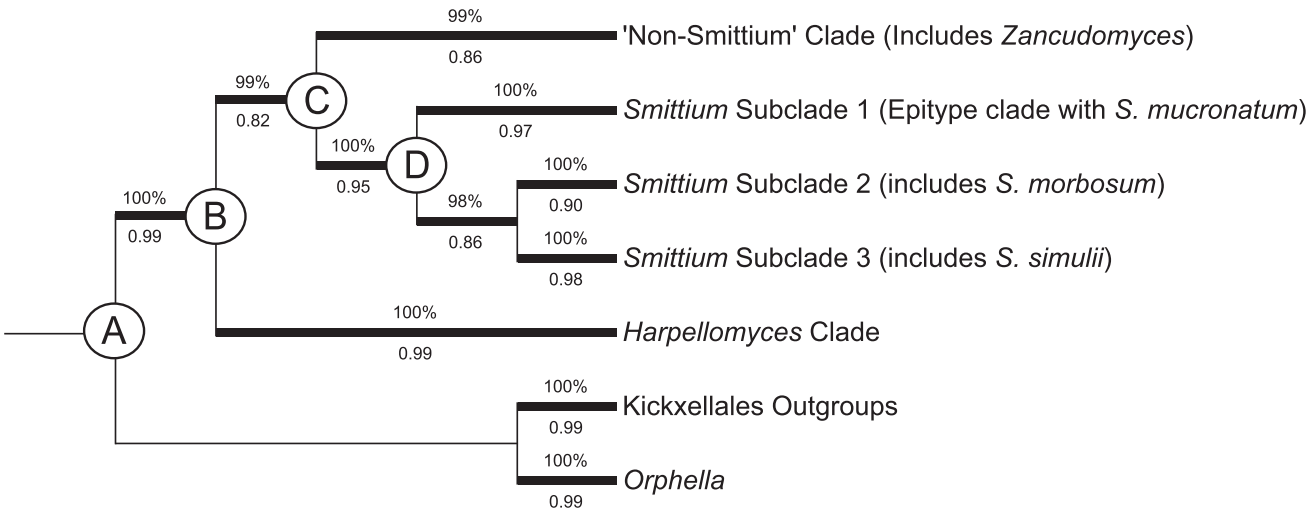


FIG. 7. Overview tree of major clades and nodes from complete phylogenetic tree including representative Harpellales and some Kickxellales. Subclades are collapsed for clarity. For this and all further trees, supports above the branches are Bayesian posterior probabilities (BPP) and below are maximum likelihood bootstrap proportions (MLB). Branches in bold represent strong support (with BPP > 95% and MLB > 0.70).

for 30 s, annealing at 52 C for 45 s and an extension at 72 C for 3 min; a final extension of 72 C for 10 min was followed by a final hold at 4 C. The cycling protocol for the 28S gene consisted of an initial denaturation of 95 C for 2 min; 45 cycles consisting of a denaturation at 95 C for 30 s, with annealing for 45 s starting at 52 C (but being reduced by one-tenth of a degree every cycle) and an extension at 72 C for 4 min; a final extension of 72 C for 10 min, was followed with a final hold at 4 C.

Gel electrophoresis.—It was performed with a 1% gel (1× TAE buffer, modified to 1/10 concentration of EDTA) with a high quality agarose (SeaPlaque GTG, Lonza USA, Cat. No. 50110) for ease of DNA handing and downstream processing. Amplified products were visualized by adding Gelstar stain (Lonza USA, Cat. No. 50535) to molten solution (4 μL/100 mL) before pouring the gel and then illuminating, after electrophoresis, with a dark reader (Clare Chemical Research DR-45M). Bands of interest were sized by comparison with 1000 bp ladder (5 Prime Ref No. 2500360), cored from the gel with pipet tips (cut to increase bore accordingly), and purified with a freeze and squeeze method. Microcentrifuge tubes (1.5 mL) containing the tips with cut gel were frozen at −20 C and spun 10 min in a microcentrifuge at 10 000 RPM. Tubes were refrozen at −20 C for 60 min and spun again. The remaining gel in the pipet tips was expelled into the tubes, and the buffered PCR product squeezed from the cut gel was used as template for direct sequencing.

Direct sequencing.—Sanger sequencing was performed with the Applied Biosystems BigDye Terminator 3.1 cycle sequencing kit. The most successful reaction cocktail, which was used for the majority of our results, was 0.5 μL sequencing premix, 3.75 μL 5× sequencing buffer, 0.32 μL each primer (0.16 pM/uL), 10.43 μL H_2O, and 5 μL template (squeezed gel solution). The thermal-cycling regime was adapted from the manufacturer's instructions (Applied Biosystems, Gene Amp PCR System 2700). The protocol included an initial denaturation of 96 C for 1 min; 80 cycles consisting of a denaturation at 96 C for 10 s, annealing at 50 C for 10 s, an extension at 60 C for 4 min; with a final hold at 4 C. Reactions were shipped overnight in strip tubes (of eight) to the University of Wisconsin Biotechnology Center (UWBC) for cleanup and electrophoresis.

Gene regions sampled.—Sequences of 129 taxa, consisting of representatives from the genus *Smittium* as well as other members of the Harpellales and some outgroups from the Kickxellales and *Orphella*, were assembled. Other sequences were taken from the GenBank (http://www.ncbi.nlm.nih.gov/) database. This study used the nearly complete 18S and part of the 28S rRNA gene. Data for the 18S are provided for all taxa in the study while data on the 28S are available for 108 of them (TABLE I).

Alignment and model determination.—Data for the 18S and 28S ribosomal coding regions first were aligned automatically with the MUSCLE algorithm (Edgar 2004) and then manually adjusted with MESQUITE (Maddison and Maddison 2010). Ambiguously aligned regions (exsets) were excluded from analysis with MESQUITE, and the two genes combined into a matrix consisting of 2666 characters. We used jModeltest (Posada 2008) to determine the most appropriate model of evolution for use. The method suggested for the 28S was GTR + G + I and for 18S was GTR + G; however, because the results for GTR + G + I and GTR + G were similar, the former was used for both to simplify analysis. Alignments were deposited in TreeBASE under study number S12212.

Phylogenetic tree inference.—Phylogenetic trees were estimated with MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Five independent runs were conducted, each with four chains for 1×10^7 generations, in which trees were sampled every 1000 generations. Stationarity of MCMC sampling and the

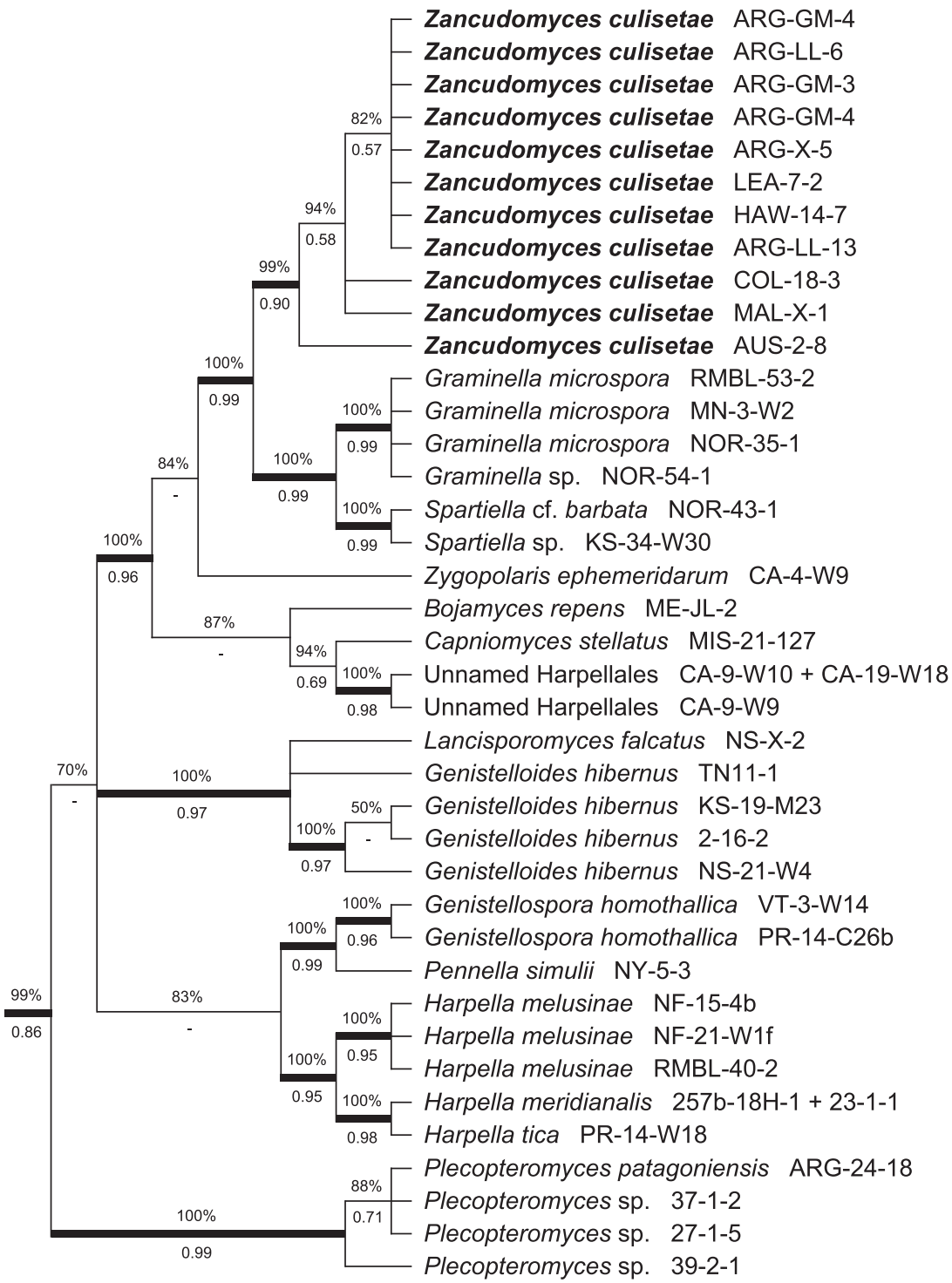


FIG. 8. ''Non-Smittium'' clade from the complete phylogenetic tree, including *Zancudomyces culisetae* (previously known as *Smittium culisetae*). This clade includes species from both the Harpellaceae and Legeriomycetaceae.

appropriate burn-in values were assessed with AWTY (Wilgenbusch et al. 2004). Support for clades was determined with a maximum likelihood analysis. One hundred bootstrap replicates were performed in GARLI (Zwickl 2006), with the best tree out of five taken for each replicate.

RESULTS

We are establishing a new genus for *Smittium culisetae* based on both morphological and molecular data, as summarized below. We also highlight phylogenetic

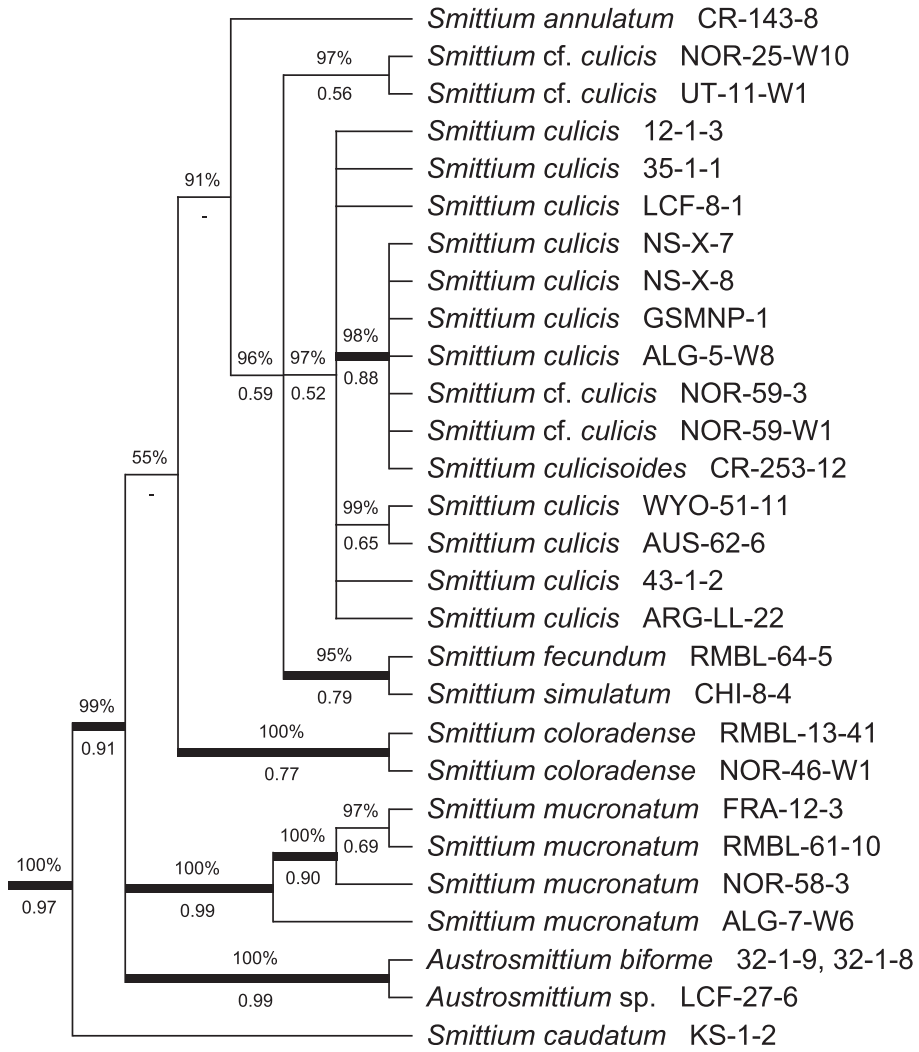


FIG. 9. *Smittium* subclade 1, including the epitype *Smittium mucronatum* among other Smittiums, as well as the well studied and widespread *S. culicis* and *Austrosmittium*.

relationships among the remaining *Smittium* taxa sequenced for ribosomal RNA gene data.

TAXONOMY

Zancudomyces gen. nov. Y. Wang, Tretter, Lichtw. & M.M. White
MycoBank MB563343

Thalli commonly verticillately branched, attached to the larval insect hindgut cuticle by a simple holdfast, producing trichospores that are wider below the mid-region, with a collar and single appendage. Biconical zygospores attached medially and perpendicularly to the zygosporophore.

Etymology: Zancudos, which literally means having long, thin legs, was used by Hispanic Americans for mosquitoes, a common and widespread host of this fungus. In its adjectival form, one also could imagine it referring to the long, thin branches of the cladogram that, at this time, distance this new taxon from its former *Smittium* clade.

Type species: **Zancudomyces culisetae** comb. nov. Y. Wang, Tretter, Lichtw. & M.M. White FIGS. 2–5
MycoBank MB563846

Thalli attached to host cuticle by an inconspicuous holdfast, often verticillately branched, sporulating prolifically. Trichospores usually 4–10 per fertile branchlet, long-ovoid, (11–)16(–30) × (3–)4(–7) µm, with a short collar 1–2.5 µm long often flared outward; single appendage fine and relatively short. Zygospores rare, biconical, (46–)52(–58) × (5.5–)6(–8) µm, with a collar (6–)7(–8) × (3.5–)3.8(–4.5) µm attached medially and perpendicularly to the zygosporophore.

Basionym: Smittium culisetae Lichtw. 1964 Amer. J. Bot. 51:837. HOLOTYPE: culture COL-18-3 isolated

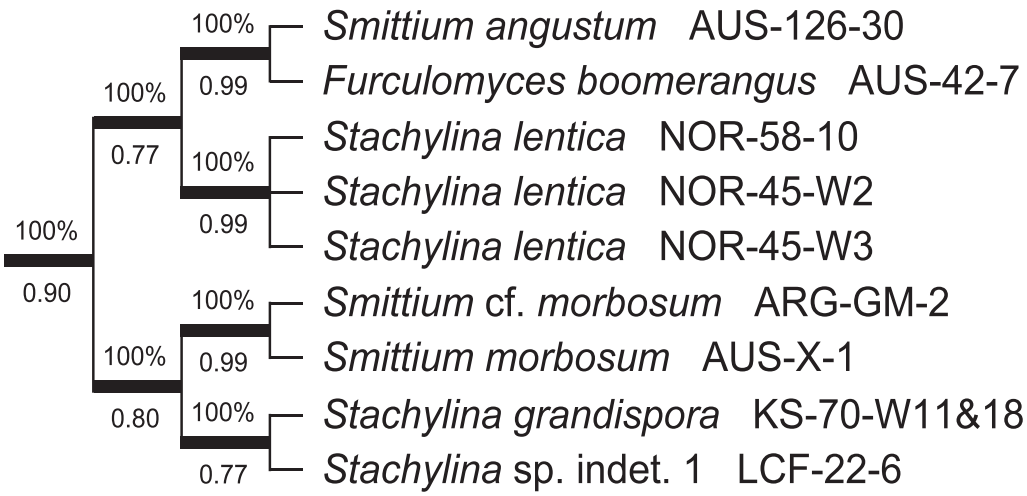


FIG. 10. *Smittium* subclade 2, including the true *Smittium morbosum* (AUS-X-1), the only recognized parasitic *Smittium* as well as all sequenced members of the genera *Furculomyces* and *Stachylina*. Isolate AUS-X-1 is the authentic culture of *Smittium morbosum* and solidifying its true position in the tree. *Smittium angustum* actually may represent a species of *Furculomyces*. Three species of *Stachylina*, a large and unculturable genus with numerous and diverse species, form a paraphyletic grouping in this subclade.

from the hindgut of a *Culiseta impatiens* (Wlk.) larva, Gunnison County, Colorado, USA, deposited with the University of Kansas Mycological Culture Collection, as well as accessioned in the American Type Culture Collection (as 16244), and the ARSEF Collection of Entomopathogenic Fungal Cultures (as 9012), Ithaca, New York, USA.

Basis for establishment of Zancudomyces.—*Prior morphological evidence*. The first evidence that *Smittium culisetae*, hereafter *Zancudomyces culisetae*, did not belong to *Smittium* was the discovery of zygospores by Williams (1983b) in two larvae of *Aedes vexans*. The zygospores (reproduced as FIGS. 2–4) were attached medially and at right angles to the zygosporophore, also known as type I (Moss et al. 1975), whereas the biconical zygospores of *Smittium* (Lichtwardt and White 2011) and for that matter *Austrosmittium*, *Furculomyces*, *Sinotrichium*, *Trichozygospora* as well are attached obliquely (or type II). Williams (1983a, b) dissected mosquito larvae from the same locality and other sites in Nebraska, USA. In his laboratory, larvae were fed simultaneously with several different isolates of the fungus on the chance that sexual reproduction might be heterothallic but found no additional zygospores. Regarding any question that field-collected larvae with zygospores actually might have contained more than one hindgut species (not unusual in some Harpellales hosts), one of us (RWL) studied one of Williams' voucher slides, and we can confirm that no other fungus was present. In addition to the different zygospore type, *Z. culisetae* differs from *Smittium* species in that its trichospores are widest just below the midregion (FIG. 5).

Prior immunological and isozymic evidence. Sanger et al. (1972) used serological methods by obtaining antisera from rabbits against selected cultures from among 21 *Smittium* and seven non-Harpellales isolates, to assess affinities among the fungal taxa. Phenograms and three-dimensional projections of cluster and principal component analyses of immunoelectrophoretic data separated the 28 isolates into five groups. The Smittiums were in four groups but with all seven *Z. culisetae* isolates distinctly separated from three other groups of *Smittium* spp. and the non-Harpellales group. Curiously enough, two Kickxellales did show some positive immunodiffusion reactions with Smittiums and the nature of their relationship was suggested as topic for further investigation.

The third indication that *Z. culisetae* might not be a *Smittium* came from a study of isozyme patterns in 108 cultures representing 18 species in six genera of Harpellales (Grigg and Lichtwardt 1996). Their phenogram (see Grigg and Lichtwardt 1996, modified here as FIG. 6) revealed a distinct and separate cluster of *Z. culisetae* (as *Smittium culisetae*) for 32 isolates, varying geographically from Australia, Japan and seven states of USA, including Hawaii.

Current molecular phylogenetic results. For this and a number of other points we present an overview tree (FIG. 7) of the major portions of a larger phylogenetic tree inferred from combined 18S and 28S rRNA gene (see SUPPLEMENTARY FIG. 1 for the complete version). The 129 taxa include 126 exemplars of Harpellales and three members of Kickxellales as outgroup (TABLE I). The 19 ''non-Smittium'' genera of Harpellales and three genera of Kickxellales anchor *Smittium*

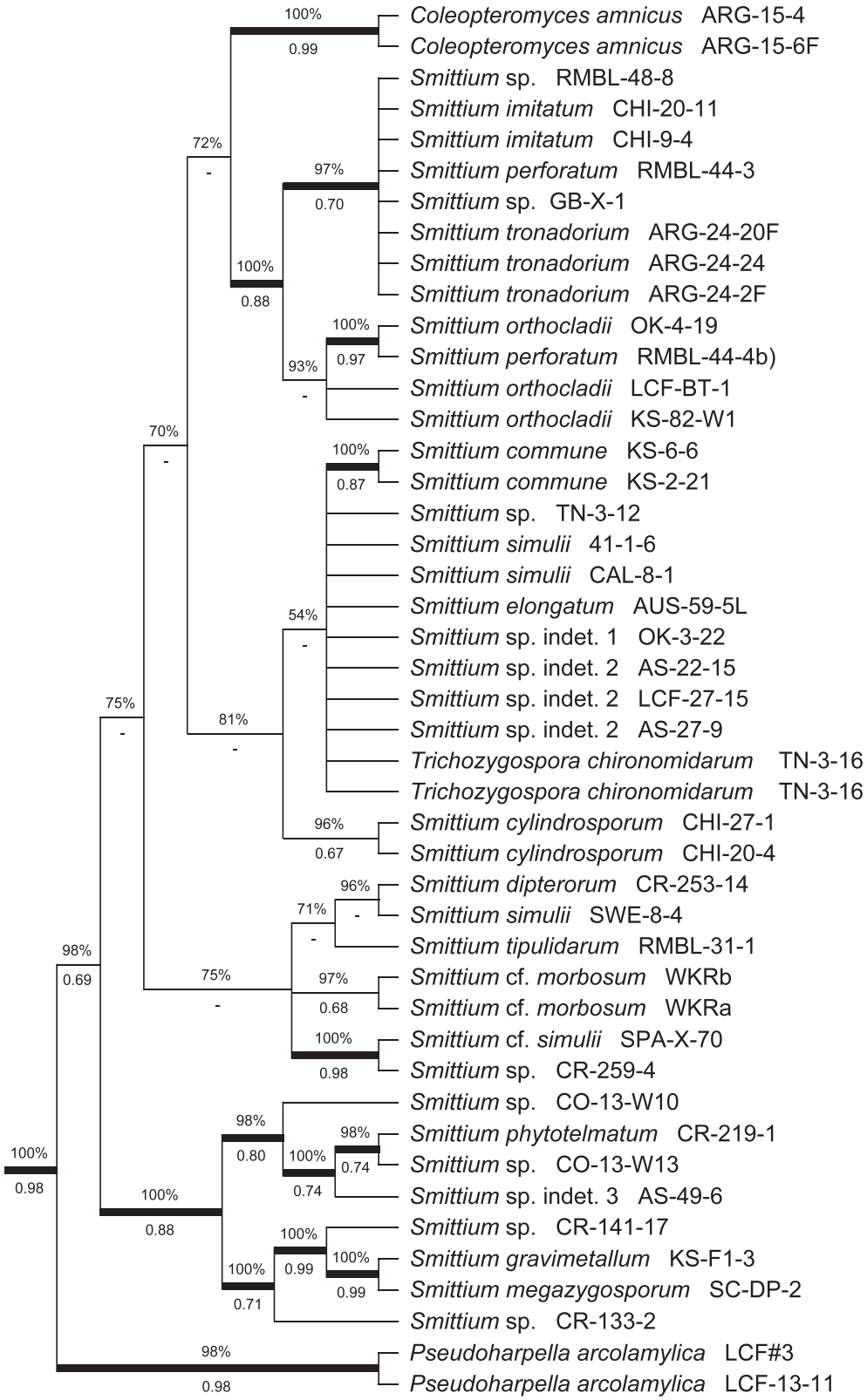


FIG. 11. *Smittium* subclade 3. A diverse group with numerous *Smittium* species, including *Smittium simulii*. Also included are *Coleopteromyces*, *Pseudoharpella* and *Trichozygospora*. Conspicuously, two isololates (WKRa and WKRb) originally thought to be *Smittium morbosum* did not cluster with the type culture for this species (AUS-X-1) and represent misidentifications. Some morphospecies, such as exemplars of *Smittium commune* and *Smittium cylindrosporum*, are well supported, based on their

subclades, and were particularly included for placement of *Zancudomyces culisetae*. We are using Kickxellales and *Orphella* L. Léger & Gauthier as outgroups based on our current understanding of the relationships among the closest relatives (James et al. 2006, White et al. 2006a, Hibbett et al. 2007). Of 226 sequences used herein, 142 are new. This includes 65 isolates representing 27 identified and three previously unidentified *Smittium* morphospecies.

Guide tree and node description. Both the complete (SUPPLEMENTARY FIG. 1) and the guide or overview tree (FIG. 7) indicate major, well supported clades or subclades labeled nodes A–D. We refer to nodes when speaking broadly or as clades/subclades especially with reference to *Smittium* species. With this first combined two-gene analysis of *Smittium* and its allies, we wish to highlight the distinct separation that exists between *Zancudomyces culisetae* (in the ''non-Smittium'' clade) and the *Smittium* subclades. The ''non-Smittium'' and ''Smittium'' clades, at node C, cluster with strong support (99% and 0.82). Much can be gleaned from the two-gene analyses, but our intention is to use it to assess the relationships among two major portions that were referred to as the ''Smittium'' and ''non-Smittium'' clades by White (2006), a labeling system we also use here, for continuity. The three *Smittium* subclades are the lowest level we will discuss because the finer branches do not have complete support. Whereas we detail some of the other lineages with *Zancudomyces culisetae* we refrain from detailed discussion of ''non-Smittium'' taxa because that will be the focus of a future paper.

Subtending clades. Node A of the guide tree (FIG. 7) represents the ordinal separation, specifically most of the Harpellales (except *Orphella*) and the Kickxellales. These outgroup taxa are split from the subclades of interest and subtended at node B with *Harpellomyces* Lichtw. & S.T. Moss, forming a lineage on a long branch and in a relatively novel position. Sister to the *Harpellomyces* lineage are 126 representatives of Harpellales. Again node C forms a split between ''non-Smittium'' and ''Smittium'' clades (subclades 1–3).

''*Non-Smittium*'' *clade.* The ''non-Smittium'' clade (FIG. 8) includes *Zancudomyces*, with representatives that were accessioned, either as cultures or microdissected samples in our DNA repositories, as *Smittium culisetae*. Some were not identified as such, but we identify them here as *Z. culisetae* with sequences generated for this study and with retrospective morphological reassessment and/or non-molecular corroboration (TABLE I). Replicate samples of *Z. culisetae* have been sequenced for this analysis to emphasize the stability of its position and to help justify the description of *Zancudomyces*, with *Z. culisetae* as the type species of this widespread genus of gut fungus in mosquitoes and other Diptera. This monotypic genus is deeply nested within the ''non-Smittium'' clade with *Graminella* L. Léger & Gauthier ex Manier and *Spartiella* Tuzet & Manier ex Manier as well supported sister taxa.

Smittium subclades. Node D (FIG. 7) circumscribes the greatest number of *Smittium* exemplars, whether from isolates or non-cultured representatives, yet analyzed (TABLE I). Three major subclades (1–3) of ''Smittium'' (FIGS. 7, 9–11) are recognized. Of note: Subclade 1 includes *S. culicis* Manier, *S. mucronatum* and relatives; subclade 2 includes *Smittium morbosum*, *Smittium angustum* M.C. Williams & Lichtw. and two other *Smittium* allies, *Stachylina lentica* M.M. White & Lichtw. and *Furculomyces boomerangus* M.C. Williams & Lichtw; subclade 3 includes *S. simulii* and *S. morbosum*, among other *Smittium* species. Throughout *Smittium* subclades there are terminal branchlets that are both strongly (bold lines) and less well supported. Molecular data suggest that some species might have been misidentified at time of collecting and others might actually require reconsideration and restudy, but overall the analysis presents an improved phylogeny and permits further commentary on *Smittium* lineages.

Variation among Zancudomyces culisetae *and* Smittium culicis.—We examined the sequences of *Z. culisetae* and *S. culicis*, the species for which we had the greatest number of representatives, and that varied widely in a geographic context. Bases were trimmed closest to the priming regions (approx. 20 for each end) and compared across all base pairs. For *Z. culisetae*, nine sequences for eight isolates with 1776 bases of the 18S rRNA gene data, as well as 10 sequences for nine isolates, across 971 bases for the 28S region, showed no variation. Concerning *S. culicis* representatives, 1790 bp of the 18S were the same, but within 954 bp for the 28S gene region 34 variable characters were found.

DISCUSSION

Prior studies with Z. culisetae.—One objective is to establish the new genus *Zancudomyces*, based on the

←

earlier identifications, but clusters of others may represent cryptic species, although poor resolution hinders a more complete assessment of many, pending futhur study.

TABLE I. Taxa used in this study, with species isolate or strain codes, whether it was from culture, with collector information. The host is given, where known and appropriate, with origin, our molecular bench code and GenBank accession/GI number

Species	Isolate/strain or collection code	Culture?	Collected by[a] or source	Host	Origin	Bench code (18S, 28S)	GenBank nos.[b]	
							18S	28S
Coemansia reversa	NRRL 1564	−	GenBank	None, free-living	N/A	415	44936090	44936641
Kickxella alabastrina	NRRL 2693	−	GenBank	None, free-living	N/A	419	2226387	3786354
Linderina macrospora	ID05-F0214	−	GenBank	None, free-living	N/A	−	166788502	166788502
Orphella catalaunica	NOR-33-W1a	−	GenBank/MMW	Leuctridae	Norway	576	125747106	125747109
Orphella dalhousiensis	NS-34-W16	−	GenBank/MMW	*Paracapnia* sp.	Canada	191	84039757	82398589
Orphella hiemalis	KS-83-W3	−	GenBank/MMW	*Zealeuctra classenii*	United States	125	89033399	89033431
Zancudomyces culisetae[c]	ARG-GM-4	Yes	GM/CLL	Diptera	Argentina	754	**JQ302880**	**JQ302954**
Zancudomyces culisetae	ARG-LL-6	Yes	CLL	*Aedes albopictus*	Argentina	285	**JQ302845**	**JQ302923**
Zancudomyces culisetae	ARG-GM-3	Yes	GM/CLL	Diptera	Argentina	306	**JQ302848**	**JQ302926**
Zancudomyces culisetae	ARG-GM-4	Yes	GM/CLL	Diptera	Argentina	305	**JQ302847**	**JQ302925**
Zancudomyces culisetae	ARG-X-5	Yes	CLL	Culicidae	Argentina	375	**JQ302862**	**JQ302940**
Zancudomyces culisetae	COL-18-3	Yes	GenBank/RWL	*Culiseta impatiens*	United States	317	296035099	311235631
Zancudomyces culisetae[d]	AUS-2-8	Yes	KUMYCOL/RWL	*Chironomus alternans*	Australia	62	10442585	**JQ302829**
Zancudomyces culisetae	LEA-7-2	Yes	KUMYCOL/RWL	*Simulium vittatum*	United States	168	**JQ302888**	**JQ302820**
Zancudomyces culisetae	HAW-14-7	Yes	KUMYCOL/RWL	*Aedes alpopictus*	United States	169(a)	**JQ302889**	**JQ302821**
Zancudomyces culisetae	ARG-LL-13	N	CLL	*Aedes aegypti*	Argentina	734	**JQ302879**	**JQ302953**
Zancudomyces culisetae	MAL-X-1	Yes	CLL	*Aedes crinifer*	Malaysia	889	**JQ302897**	**JQ302835**
Bojamyces repens	ME-JL-2	N	GenBank/JL	*Leptophlebia intermedia*	United States	113	89033396	89033427
Capniomyces stellatus	MIS-21-127	Yes	GenBank/RWL	*Allocapnia* sp.	United States	167	89033400	125747107
Coleopteromyces amnicus	ARG-15-4	N	RWL	Scirtidae	Argentina	341	**JQ302854**	**JQ302932**
Coleopteromyces amnicus	ARG-15-6F	N	LCF	Scirtidae	Argentina	339	**JQ302853**	**JQ302931**
Lancisporomyces falcatus	NS-X-2	N	DBS	*Paracapnia angulata*	Canada	520	**JQ302865**	**JQ302943**
Genistelloides hibernus	TN-11-1	−	GenBank/RWL	*Allocapnia* sp.	United States	−	2226386	3786352
Genistelloides hibernus[d]	KS-19-M23	N	GenBank/JKM	Capniidae	United States	192	89033405	**JQ302921**
Genistelloides hibernus	NS-21-W4	−	GenBank/MMW	*Allocapnia* sp.	Canada	118	89033398	89033429
Genistelloides hibernus	2-16-2	−	GenBank/AS	*Allocapnia vivipara*	United States	117	89033397	89033428
Genistellospora homothallica	VT-3-W14	−	MMW	Simuliidae	United States	185	89033403	89033444
Genistellospora homothallica	PR-14-C26b	−	MJC/RWL/MMW	*Simulium bipunctatum*	Puerto Rico	184	89033402	−
Graminella microspora	RMBL-53-2	N	RWL	*Baetis tricaudatus*	United States	172	**JQ302843**	**JQ302920**
Graminella microspora	MN-3-W2	N	LCF/MMW	Mayfly	United States	119	**JQ302837**	**JQ302916**
Graminella microspora	NOR-35-1	N	RWL	*Baetis rhodani*	Norway	662	**JQ302867**	**JQ302945**
***Graminella* sp.**	NOR-54-1	N	RWL	*Baetis rhodani*	Norway	687	**JQ302872**	−
Harpella melusinae	NF-15-4b	−	GenBank/RWL	*Prosimulium mixtum*	Canada	13	89033463	89033467
Harpella melusinae	NF-21-W1f	−	GenBank/MMW	*Prosimulium mixtum*	Canada	11	89033462	89033466
Harpella melusinae	RMBL-40-2	−	GenBank/RWL	Simuliidae	United States	181	89033401	−

TABLE I. Continued

Species	Isolate/strain or collection code	Culture?	Collected by[a] or source	Host	Origin	Bench code (18S, 28S)	GenBank nos.[b]	
							18S	28S
Harpella meridianalis[c]	ARG-46a-15	–	GenBank/RWL	Simuliidae	Argentina	257b	89033409	–
	ARG-25-5	–	GenBank/RWL	Simuliidae	Argentina	23	–	89033416
Harpella tica	PR-14-W18	–	GenBank/ MMW/RWL/MJC	*Simulium bipunctatum*	Puerto Rico (US)	26	89033390	89033418
Harpellomyces montanus	TN-22-W5B	N	MMW	Thaumaleidae	United States	954	**JQ302887**	**JQ302961**
Harpellomyces sp.	PA-3-1d	–	GenBank/LCF/ MMW	Thaumaleidae	United States	81b	125747105	125747108
Pennella simulii	NY-5-3	–	GenBank/RWL/ MMW	Simuliidae adult	United States	186	89033464	–
Plecopteromyces patagoniensis	ARG-24-18	–	GenBank/RWL	Gripopterygidae	Argentina	18	89033389	–
Plecopteromyces sp.	39-2-1	–	GenBank/LCF/BH	Gripopterygidae	Australia	227b	89033408	89033446
Plecopteromyces sp.	37-1-2	–	GenBank/LCF/BH	Gripopterygidae	Australia	106	89033394	89033425
Plecopteromyces sp.	27-1-5	–	GenBank/LCF/BH	Gripopterygidae	Australia	229b	89033393	89033447
Spartiella* cf. *barbata	NOR-43-1	N	RWL	*Baetis rhodani*	Norway	675	**JQ302868**	**JQ302946**
***Spartiella* sp.**	KS-34-W30	N	MMW	Baetid	United States	49	**JQ302864**	**JQ302942**
Unnamed Harpellales[c]	CA-9-W10	–	MMW/PVC	Trichoptera	United States	354	89033414	–
	CA-19-W18	–	MMW/PVC	Trichoptera	Puerto Rico (US)	356	–	89033458
Unnamed Harpellales	CA-9-W9	–	MMW/PVC	Trichoptera	United States	353	89033413	–
Zygopolaris ephemeridarum	CA-4-W9	–	MMW/PVC	Ephemeroptera	United States	346	89033412	89033457
Smittium angustum	AUS-126-30	Yes	RWL	*Tanytarsus* sp.	Australia	314	10442583	**JQ302822**
Smittium annulatum	CR-143-8	Yes	RWL	Simuliidae	Costa Rica	66	10442602	**JQ302832**
Smittium caudatum	KS-1-2	Yes	KUMYCOL/RWL	Chironomidae	United States	69	10442609	**JQ302948**
Smittium sp.	CR-141-17	Yes	RWL	*Simulium* sp.	Costa Rica	319	10442601	**JQ302928**
Smittium cf. *morbosum*	ARG-GM-2	Yes	GM/LL	Diptera	Argentina	307	**JQ302849**	**JQ302927**
Smittium sp.	CR-133-2	Yes	RWL	*Chironomus* sp.	N/A	322	10442600	–
Smittium coloradense	RMBL-13-41	Yes	RWL	*Cricotopus* sp.	United States	67	10442619	**JQ302912**
Smittium commune	KS-6-6	Yes	RWL	Chironomidae	United States	57	10442613	–
Smittium commune	KS-2-21	Yes	KUMYCOL/RWL	Chironomidae	United States	315	10442612	**JQ302901**
Smittium cf. *culicis*	NOR-25-W10	N	MMW	Mosquito	Norway	574	**JQ302866**	**JQ302944**
Smittium cf. *culicis*	UT-11-W1	Yes	MMW	Dipteran	United States	761	**JQ302881**	**JQ302955**
Smittium culicis	12-1-3	Yes	LCF/BH	Culicidae	Australia	373	**JQ302860**	**JQ302938**
Smittium culicis	35-1-1	Yes	LCF/BH	Thaumaleidae	Australia	361	**JQ302855**	**JQ302933**
Smittium culicis	LCF-8-1	Yes	LCF	Thaumaleidae	New Zealand	365	**JQ302856**	**JQ302934**
Smittium culicis	NS-X-7	N	DBS	Mosquito	Canada	720	**JQ302877**	**JQ302951**
Smittium culicis	WYO-51-11	Yes	KUMYCOL/RWL	*Aedes sticticus*	United States	63	10442625	**JQ302830**
Smittium culicis	AUS-62-6	Yes	RWL	*Austrothaumalea* sp.	Australia	316	10442590	**JQ302902**
Smittium culicis	43-1-2	Yes	LCF/BH	*Chironomus* sp.	Australia	362	**JQ302893**	89033461
Smittium coloradense	NOR-46-W1	N	MMW	Chironomidae	Norway	679	**JQ302869**	–

TABLE I. Continued

Species	Isolate/strain or collection code	Culture?	Collected by[a] or source	Host	Origin	Bench code (18S, 28S)	GenBank nos.[b]	
							18S	28S
Smittium culicis	NS-X-8	N	DBS	Mosquito	Canada	721	**JQ302878**	**JQ302952**
Smittium culicis	GSMNP-1	Yes	RWL	Culicidae	United States	879	**JQ302885**	**JQ302959**
Smittium culicis	ALG-5-W8	Yes	MMW	*Bactylolabis montana*	Canada	925	**JQ302899**	**JQ302915**
Smittium culicis	ARG-LL-22	N	CLL	Mosquito	Argentina	866	**JQ302884**	**JQ302958**
Smittium cf. *culicis*	NOR-59-3	N	RWL	*Psectrocladius (Psectrocladius) limbellatus*	Norway	707	**JQ302875**	**JQ302950**
Smittium cf. *culicis*	NOR-59-W1	N	MMW	*Psectrocladius (Psectrocladius) limbellatus*	Norway	712	**JQ302876**	–
Smittium culicisoides	CR-253-12	Yes	KUMYCOL	Chironomidae	Costa Rica	64	10442606	**JQ302831**
Smittium cylindrosporum	CHI-27-1	Yes	RWL	*Cricotopus* sp.	Chile	56	10442596	**JQ302828**
Smittium cylindrosporum	CHI-20-4	–	RWL	*Cricotopus* sp.	Chile	318	10442595	–
Smittium dipterorum	CR-253-14	Yes	KUMYCOL	*Simulium* sp.	Costa Rica	59	10442604	**JQ302909**
Smittium sp.	RMBL-48-8	Yes	RWL	*Prosimulium* sp.	United States	330	**JQ302892**	**JQ302905**
Smittium fecundum	RMBL-64-5	Yes	RWL	*Psectrocladius* sp.	United States	65	10442622	**JQ302911**
Smittium gravimetallum	KS-F1-3	Yes	LCF	*Dicrotendipes fumidus*	United States	60	10442615	–
Smittium imitatum	CHI-20-11	Yes	RWL	*Simulium* sp.	Chile	54	10442594	**JQ302907**
Smittium imitatum	CHI-9-4	Yes	RWL	*Simulium* sp.	Chile	320	10442599	**JQ302903**
Smittium megazygosporum	SC-DP-2	Yes	KUMYCOL/CEB	*Simulium vittatum*	United States	321	10442623	**JQ302823**
Smittium morbosum	AUS-X-1	Yes	KUMYCOL/RWL	*Anopheles hilli*	Australia	70	10442592	**JQ302913**
Smittium cf. *morbosum*	WKRb	Yes	WKR/CEB	*Ochlerotatus triseriatus*	United States	883	**JQ302895**	**JQ302834**
Smittium cf. *morbosum*	WKRa	Yes	WKR/CEB	*Ochlerotatus triseriatus*	United States	881	**JQ302886**	**JQ302960**
Smittium mucronatum	FRA-12-3	Yes	KUMYCOL/RWL	*Psectrocladius sordidellus*	France	68	10442608	**JQ302833**
Smittium mucronatum	ALG-7-W6	Yes	MMW	Chironomidae	Canada	916	**JQ302898**	**JQ302914**
Smittium mucronatum	RMBL-61-10	N	RWL	*Psectrocladius* sp.	United States	142	**JQ302840**	89033437
Smittium mucronatum	NOR-58-3	N	RWL	*Psectrocladius (Psectrocladius) limbellatus*	Norway	696	**JQ302873**	**JQ302949**
Smittium orthocladii	OK-4-19	Yes	RWL	Chironomidae	United States	55	10442618	**JQ302827**
Smittium orthocladii	LCF-BT-1	Yes	LCF/MMW	*Corynoneura* sp.	United States	108	89033395	**JQ302900**
Smittium orthocladii	KS-82-W1	N	LCF/MMW	*Orthocladius abiskoensis*	United States	130	**JQ302838**	**JQ302917**
Smittium sp.	TN-3-12	Yes	RWL	Chironomidae	United States	331	**JQ302850**	**JQ302929**
Smittium perforatum	RMBL-44-3	Yes	RWL	*Diamesa* sp.	United States	332	**JQ302851**	**JQ302930**
Smittium perforatum	RMBL-44-4b)	N	RWL	*Diamesa* sp.	United States	132	**JQ302839**	**JQ302918**
Smittium phytotelmatum	CR-219-1	Yes	KUMYCOL/RWL	*Chironomus* sp.	Costa Rica	61	10442603	**JQ302910**
Smittium simulatum	CHI-8-4	Yes	KUMYCOL/RWL	*Aphophila bidentata*	Chile	323	10442597	**JQ302824**
Smittium simulii	41-1-6	Yes	LCF/BH	*Orthocladius* sp.	Australia	374	**JQ302861**	**JQ302939**
Smittium simulii	SWE-8-4	Yes	RWL	*Diamesa* sp.	Sweden	58	10442624	**JQ302908**

TABLE I. Continued

Species	Isolate/strain or collection code	Culture?	Collected by[a] or source	Host	Origin	Bench code (18S, 28S)	GenBank nos.[b] 18S	GenBank nos.[b] 28S
Smittium simulii	CAL-8-1	Yes	RWL	*Simulium argus*	United States	324	10442593	**JQ302825**
Smittium cf. *simulii*	SPA-X-70	Yes	LGV	Culicidae	Spain	858	**JQ302883**	**JQ302957**
Smittium elongatum	AUS-59-5L	Yes	RWL	*Cardiocladius australiensis*	Australia	326	10442589	–
Smittium sp. indet. 1[f]	OK-3-22	Yes	RWL	Chironomidae	United States	327	10442617	–
Smittium sp.	CR-259-4	Yes	RWL	*Simulium sp.*	Costa Rica	329	**JQ302891**	**JQ302826**
Smittium sp.	GB-X-1	Yes	AR/SM	*Simulium ornatum*	United Kingdom	885	**JQ302896**	–
Smittium sp.	CO-13-W10	N	MMW	Chironomidae	United States	433	**JQ302863**	**JQ302941**
Smittium tipulidarum	RMBL-31-1	Yes	KUMYCOL/RWL	*Elliptera astigmatica*	United States	52	10442621	**JQ302836**
Smittium tronadorium	ARG-24-20F	Yes	LCF	*Limaya sp.*	Argentina	53	**JQ302894**	**JQ302906**
Smittium tronadorium	ARG-24-24	N	RWL	Diamesinae	Argentina	288	**JQ302890**	89033454
Smittium tronadorium	ARG-24-2F	Yes	LCF	*Paraheptagyia sp.*	Argentina	325	10442582	**JQ302904**
Smittium sp. indet. 2[f]	AS-22-15	Yes	AS	*Cricotopus sp.*	New Zealand	367	**JQ302858**	**JQ302936**
Smittium sp. indet. 2[f]	LCF-27-15	N	LCF	Orthocladiinae	New Zealand	368	**JQ302859**	**JQ302937**
Smittium sp. indet. 2[f]	AS-27-9	Yes	AS/LCF	Orthocladiinae	New Zealand	366	**JQ302857**	**JQ302935**
Austrosmittium biforme	32-1-8	–	KUMYCOL	Orthocladiinae	Australia	170	–	89033443
	32-1-9	–	LCF/BH	Orthocladiinae	Australia	170	89033411	–
Austrosmittium sp.	LCF-27-6	–	LCF/AS	*Cricotopus* sp.	New Zealand	98	89033392	–
Furculomyces boomerangus	AUS-42-7	–	KUMYCOL	*Psectrocladius paludicola*	Australia	–	2226385	82398545
Smittium sp.	CO-13-W13	N	MMW	Chironomus	United States	334	**JQ302852**	–
Pseudoharpella arcolamylica	LCF#3	N	LCF	Dixidae	United States	766	**JQ302882**	**JQ302956**
Pseudoharpella arcolamylica	LCF-13-11	N	LCF	*Dixa fluvica*	United States	193	89033406	–
Stachylina grandispora	KS-70-W11&18	N	MMW	*Chironomus riparius*	United States	290	**JQ302846**	**JQ302924**
Smittium sp. indet. 3[f]	AS-49-6	N	AS	Chironomidae (*Paratanytarsus* sp.?)	New Zealand	210	**JQ302844**	–
Stachylina lentica	NOR-58-10	N	RWL	*Chironomus* sp.	Norway	701	**JQ302874**	–
Stachylina sp. indet. 1[f]	LCF-22-6	N	LCF	*Tanytarsus sp.*	South Africa	200	89033407	**JQ302922**
Stachylina lentica	NOR-45-W2	N	MMW	Chironomidae	Norway	685	**JQ302870**	–
Stachylina lentica	NOR-45-W3	N	MMW	Chironomidae	Norway	686	**JQ302871**	**JQ302947**
Trichozygospora chironomidarum	TN-3-16	Yes	RWL	Chironomidae	United States	166 b	**JQ302842**	**JQ302919**
Trichozygospora chironomidarum	TN-3-16	Yes	RWL	Chironomidae	United States	166 a	**JQ302841**	–

[a] AS, Amy Slaymaker; AR, Alan Rizzo; BH, Barb Hayford; CEB, Charles ''Eddie'' Beard; CLL, Claudia López Lastra; DBS, Douglas B. Strongman; GM, Maria Gabriela Mazzucchelli; JKM, JK Misra; JL, Joyce Longcore; LCF, Leonard C. Ferrington, Jr.; LGV, Laia Guàrdia Valle; MJC, Matías J. Cafaro; MMW, Merlin White; PVC, Paula Clarke; RWL, Robert W. Lichtwardt; SM, Steve Moss; WKR, Will K. Reeves. Some of the sequences were generated from samples prepared from isolates in the University of Kansas Mycological Culture Collection, represented as KUMYCOL.

[b] Accession numbers in boldface were generated for this study.

TABLE I. Continued

[c] Isolates of ''non-Smittium'' taxa in boldface are presented for the first time in this study.
[d] The 18S rRNA gene was obtained from GenBank, and the 28S rRNA gene was sequenced from this study.
[e] 18S and 28S for two samples from the same region were combined for the 18S and 28S analysis.
[f] Supplemental information on these samples: *Smittium* sp. indet. 1 (''stenosporum'' is an epithet that has been considered in a draft manuscript); *Smittium* sp. indet. 2 (''vulgare'' is an epithet that has been considered); *Smittium* sp. indet. 3, voucher AS-49-6 was accessioned with ambiguity (with epithets being considered as either ''paratanytarsensis'' for *Stachylina* or ''corymbiatum'' for *Smittium*); *Stachylina* sp. indet. 1 (''rivularia'' is an epithet that has been considered). We do not in any way imply formal presentation of these herein and do not use them as species names, but simply loosely list them for possible continuity with future manuscripts (by Ferrington, Jr. and others).

type *Z. culisetae*, previously known as *Smittium culisetae* Lichtw. (Lichtwardt 1964), one of the most frequently encountered species of Harpellales from widespread regions of the world (Lichtwardt et al. 2001). Various dipteran larvae serve as hosts, but *Z. culisetae* is especially known from the hindguts of mosquitoes (Lichtwardt and Williams 1990). As one of the oldest and easiest of the Harpellales to isolate, axenic cultures of *Z. culisetae* have been used in numerous studies ranging from effects of temperature and pH on growth and sporulation, media preferences, utilization of various carbon and nitrogen sources, host specificity, trichospore longevity, effects on development of mosquito larvae under nutritional stress, the fine structure of trichospores and factors affecting sporangiospore extrusion from the trichospore (Farr and Lichtwardt 1967; Williams and Lichtwardt 1972a, b; El-Buni and Lichtwardt 1976a, b; Horn and Lichtwardt 1981; Williams 1983a; Horn 1989a, 1990; Gottlieb and Lichtwardt 2001; Koontz 2006; White 2006; White et al. 2006a). Certain isolates of *Z. culisetae*, including the type culture (COL-18-3), also have been used in molecular phylogenies, either as a representative of or the only species of *Smittium* (Walker 1984, O'Donnell et al. 1998, James et al. 2006, Liu et al. 2006).

Walker (1984) constructed the first phylogenetic tree based on 5S rRNA sequences, although that gene lacked the resolving power to fully determine sister-group relationships. Walker was interested in assessing the morphological features and characters that might indicate ancestral origins of various Zygomycetes. He found great sequence diversity within the small family Kickxellaceae and between sequences from supposedly derived Harpellales.

Porter and Smiley (1979) compared ribosomal RNA molecular weights of four species of *Smittium* (*S. culicis*, *S. mucronatum*, *S. simulii* and *S. culisetae* [= *Z. culisetae*]) and three species of Kickxellales. They showed that weights were highest for the *Smittium* isolates and concluded that the differences were biologically significant and that *Smittium* was not closely related to any of the Zygomycetes.

Fifteen years later, based on the shared characteristics of regularly septate hyphae with similarly plugged, flared septal pores, O'Donnell et al. (1998) assessed the relationships of the putative sister orders Harpellales and Kickxellales. Molecular and morphological trees were compared (the latter with less support), and 18S rRNA phylogeny was mapped with morphological, as well as physiological characters and living strategies. Compared to the study by Walker (1984), O'Donnell et al. (1998) resolved clades within the two orders and demonstrated monophyletic assemblages for each of the Kickxellales and

Harpellales as well as an independent *Spiromyces* clade. Whereas the trees permitted an investigation of these features, taxon sampling was limited. Only *Zancudomyces culisetae* and three other culturable genera within the Legeriomycetaceae (Harpellales) were included.

The first phylogenetic study with an emphasis on culturable *Smittium* species and the Harpellales was Gottlieb and Lichtwardt (2001), with 24 *Smittium* species. They separated *Smittium* into five lineages, although still lacking resolution with the single 18S rRNA gene data and making it difficult to assess and map morphological features. Also included was an assessment of the nuclear ribosomal internal transcribed spacers (ITS 1 and 2), for which it was concluded that they were not suitable for comparisons among species within *Smittium*. This undoubtedly highlights the diversity within the genus itself but perhaps it does not necessarily preclude the possible future use of this region in barcoding once all the major subclades and lineages are resolved (Bellemain et al. 2010).

These phylogenetic studies have disproportionally included culturable taxa, understandably because they provide pure and higher concentrations of genomic DNA. However, PCR also has allowed unculturable samples of gut fungi, micro-dissected from the guts of their hosts, to be incorporated with culturable exemplars in some analyses (White 2006). Although White's (2006) single gene (18S and 28S rRNA) trees showed S*mittium* (and the second largest genus *Stachylina* L. Léger & M. Gauthier) as a polyphyletic assemblage, it also showed *Z. culisetae* clearly offset and separated distinctly from the remainder of the ''Smittium'' clade and showed promise for further refinements using these gene regions.

Combined two-gene phylogeny.—As the most complete and the only combined analysis to date, including both culturable and unculturable species of *Smittium* and 10 different isolates of *Zancudomyces* and other putative allies, the improved resolution lets us define and refine relationships among taxa within nodes (A–D) and/or as subclades (FIGS. 9–13).

''*Non-Smittium*'' *clade. Zancudomyces culisetae* forms a strongly supported cluster of 10 representatives from six geographic areas and reinforces notions (Sangar et al. 1972, Grigg and Lichtwardt 1996, White 2006, Lichtwardt and White 2011) that the species is a distinct lineage and separate from *Smittium*. With 18S and partial 28S rRNA gene sequences that are nearly identical (see alignment file), it is interesting to recall that *Z. culisetae* has been observed only with sexual spores on two occasions at one site in Nebraska (FIGS. 2–4 from Williams 1983b) despite worldwide collections over nearly a half century. Sexual spores for certain Harpellales are extremely rare and *Z. culisetae* has almost always been identified with and based on its asexual spores alone. The concept of asexual fungi is not a new one, and this may be an example of a lineage that either maintains little sexuality or does not present this process in or associated with the digestive tract of its larval host, where most researchers would be likely to encounter it. That we observed so little variation within *Z. culisetae* supports the notion of a sustained asexual condition.

Studies that have included *Z. culisetae* did not have the benefit of the additional ''non-Smittium'' taxa, some of which we are able to present here for the first time as well (see isolates in boldface TABLE I). For example, *Coleopteromyces* Ferrington, Lichtw. & López Lastra, *Graminella*, *Lancisporomyces* Santam., *Spartiella*, and *Trichozygospora* all are newly sequenced Harpellales members that strengthen our confidence in the placement of *Z. culisetae* with its own genus outside the ''Smittium'' subclades.

Two of these, *Graminella* and *Spartiella*, appear as a well supported sister clade, both together and with *Zancudomyces culisetae* as a grade. *Graminella* and *Spartiella* possess relatively small trichospores compared to *Zancudomyces*, but qualitatively they do share the submedially swollen trichospore of *Z. culisetae*. It is interesting to note also that *Z. culisetae* has been recorded once from a mayfly host (Lichtwardt and Williams 1990) and is clustered with these and other mayfly gut fungi (*Zygopolaris* and *Bojamyces*). There are exceptions to this notion of host specificity, which expands to include gut fungi from stonefly and caddis worm hosts (with the unnamed Harpellales from California) as well, although with slightly less support. Stronger branch support might permit further discussion of possible host switching, but our data do not preclude an overall evolutionary trend for the gut fungi first associating with the much older Plecoptera or Ephemeroptera hosts and then toward certain lower Diptera hosts.

Clarification on Smittium morbosum *samples.*—*Smittium morbosum* is the only gut fungus known to kill its mosquito hosts. It first was isolated (and deposited as culture AUS-X-1) from Australia (Sweeney 1981). The Australian exemplar, which is presented as the true representative of the species, matched closely one other southern hemisphere isolate (ARG-GM-2) from Argentina (TABLE I). It clusters with representatives of *Stachylina* as well as *Furculomyces* (see Gottlieb and Lichtwardt 2001 for discussion on possible misidentification of *Furculomyces boomerangus* and *S. angustum*). Three other putatively

identified ''*S. morbosum*'' samples from Argentina (isolate numbers ARG-GM-3, ARG-GM-4, ARG-LL-6) were a match for *Z. culisetae* and have been identified as such in our files and the GenBank entry. Beyond the life habit and parasitic nature of *S. morbosum*, which can present the larval host with a melanized spot seen through the exoskeleton as a response to invasion, Sweeney (1981) also commented on potential confusion between *S. morbosum* and *Z. culisetae*. The trichospores of *S. morbosum* are usually shorter but their dimensions overlap, and, although trichospores of *S. morbosum* are widest medially, the submedial swelling of *Z. culisetae* is only subtly different. *Smittium morbosum* occupies the anterior part of the hindgut in infected larvae whereas *Z. culisetae* occupies the posterior portions of the hindgut (Sweeney 1981). The two species can be distinguished in vitro by the growing thalli, being small and dense in *S. morbosum* compared to the more floccose and more open pattern of *Z. culisetae*. However, in the absence of one or more of these features and depending on the maturity of the specimen at the time of isolation, it is not unreasonable to expect some confusion. Similarly isolates WKRa and WKRb (subclade 3) clustered with *Smittium simulii* and allies, rather than *S. morbosum*, so we have added some question to the identification of that species. Reeves (2004) noted that this isolate did not prevent molting of larvae that were infected in vitro. Because this isolate could represent a new species of *Smittium* and because it had been isolated from a host with the apparent pathology of *S. morbosum*, further laboratory studies of it with mosquitoes are warranted.

Subclade 1. Smittium subclade 1 (FIG. 9) carries some significance because it includes the epitype *Smittium mucronatum* (Lichtwardt and White 2011) and in some way will carry the name *Smittium*, pending revisions. This clade also includes *Smittium culicis*, which can exhibit morphological variation that is now matched at the molecular level as well, as demonstrated by the 28S internal variation for morphospecies included. The clade holds together fairly well, notwithstanding the inclusion of *S. culicisoides* Lichtw., *S. fecundum* Lichtw. & M.C. Williams and *S. simulatum* Lichtw. & Arenas in it. *Smittium annulatum* Lichtw. receives some support as well among the large cluster. *Smittium coloradense* Lichtw. & M.C. Williams (type RMBL-13-41) from Colorado united strongly with the same species identified from Norway (NOR-46-W1). With *S. mucronatum*, these are part of a larger grade, with two representatives of *Austrosmittium* that form a well supported lineage and finally are subtended by *Smittium caudatum* Lichtw. & Grigg. While not a feature that holds throughout this clade, many of these species possess a collar with some degree of campanulation, particularly depending on whether it is viewed while the trichospore is attached or detached—in the latter case tending to reduce the degree of curvature once the spores are released from the thallus. Weak support for some branches prevents further consideration of this as a synapomorphy, pending analyses with an expanded number of genes and/or taxa, but the collar shape and or dimensions may be worthy of mapping onto future trees. This subclade also is worthy of finer scrutiny for lineage sorting and possible cryptic species.

Subclade 2. Smittium subclade 2 (FIG. 10) is a small cluster with strong support but includes three different genera: *Smittium morbosum* (AUS-X-1) groups with *Furculomyces* and *Stachylina*. *Stachylina* is paraphyletic but that must be considered an improvement over the apparent polyphyly presented earlier (White 2006). As the second largest genus, in terms of species, *Stachylina* is undoubtedly one of the most important taxa to include in future phylogenetic analyses, but it also typically provides minimal material per dissection and low concentration DNA that are difficult to amplify, at least to date. Again, we consider this to be the true *Smittium morbosum* clade and if one considers the nature of symbiosis when analyzing relationships it will be interesting to further expand taxon sampling in this section of the tree. Might the closest relatives of *Smittium morbosum* show similar parasitic tendencies? Or might the other taxa be able to invoke such a parasitic strategy? We can only speculate at this time whether taxa morphologically similar to *Smittium morbosum* exist that are also parasitic or whether such a shift was very narrow, perhaps with only one or a few species taking on the strategy in the larval hosts. From what we have observed, there is no reason to suspect that either of the three *Stachylina* representatives in the tree or *Furculomyces boomerangus* are parasitic.

Stachylina can be found in the midguts of many of the same dipteran families as *Smittium*, although more rarely in black flies. *Stachylina* species have very similar trichospore features except that most have trichospores with either no collar or a reduced collar and are borne on unbranched thalli attached to the peritrophic matrix that lines this section of the digestive tract. Zygospores are not known for any current members of *Stachylina*, except *St. pedifer*, for which they were developed in vitro as wet mounts after micro-dissecting the midgut lining with attached, conjugating thalli (Beard and Adler 2003). *Stachylina reflexa* was described with zygospores, but that species was moved to a new genus (*Klastostachys*) based on other features of the thallus (Lichtwardt et al. 2011). *Stachylina* is emerging as a large group of Harpellales, still inviting further study.

Subclade 3. Smittium subclade 3 (FIG. 11), which includes the largest number of *Smittium* and allies, splits with strong support from subclade 2 (FIG. 7). *Smittium simulii* was notably dispersed among the clade and not as well resolved as one might expect given its fairly unique and substantial clamp-shaped holdfast. Morphologically the holdfast alone can suggest it as a species when noted for thalli in a collection, which is confirmed with mature trichospores for the complete morphometric assessment. Overall branch support permits only a cursory assessment of the relationships among taxa interspersed with *Smittium simulii* representatives, one of which (SPA-X-70) we have listed tentatively.

Conversely the strong support for certain branch tips are worthy of note for certain samples (i.e. *S. commune* and *S. cylindrosporum*). However, clustered groups of others (i.e. *S. imitatum + perforatum + orthocladii*) may deserve reconsideration or are cryptic species being masked by convergent morphology (perhaps also true for some of the *S. simulii* samples). *Smittium* subclade 3 is the most diverse assemblage of species we present for further consideration. The question that remains is whether some of these taxa are just simply unresolved based on the analysis of the data at hand, which is indeed possible given the breadth of our assessment, or whether they are conspecific and need to be reassessed morphologically. We decline to elaborate pending further analysis and better resolution with our ongoing efforts to build a multigene dataset that hopefully will help resolve some of these issues.

*Non-*Smittium *allies among* Smittium *subclades 1–3.* Finally several non-*Smittium* genera, referred to as allies above, warrant further commentary (SUPPLEMENTARY FIG. 1). An unexpected finding was the inclusion of *Coleopteromyces amnicus*, the only Harpellales from larval beetles, with strong terminal support deep within subclade 3. The remarkable discovery of the fungus in this host in Argentina prompted the generic description. Indeed, it is the only non-Diptera host for the entire cluster within node D. It may represent a recent host switch or fortuitous instance of growth in a non-typical host at that site. In comparing the morphology of *C. amnicus*, whereas it was described without zygospores (Lichtwardt et al. 1999), the trichospore shape, with a collar and single appendage when detached also are characters that hold for species of *Smittium*. Also in subclade 3 is the rare *Trichozygospora chironomidarum*, notable morphologically with its multiple appendages on both the trichospore and zygospore, features that are not true for Smittiums. The significance of appendage number in the *Smittium* subclades remains to be further scrutinized, pending collection of further molecular sequence data and indeed morphological data, for certain taxa.

The placement of *Pseudoharpella arcolamylica* Ferrington, Lichtw. & M.M. White and the strength of its support as a lineage at the base of subclade 3 should not be understated here. While the type II zygospore matches the other members of these subclades, where the sexual spores are known at least, *P. arcolamylica* is unique with its coiled trichospore and three broad appendages (Ferrington et al. 2003). Except for the branched growth pattern of the thallus and the Dipteran host (Dixidae), it is different morphologically and perhaps now molecularly as well, at least as it is presented on a fairly well defined and separate lineage in subclade 3.

Pseudoharpella emerges from a grade at node D that is near subclade 2 that includes both *Furculomyces* and *Stachylina* (see above). Although most *Stachylina* species have no known sexual spore (Beard and Adler 2003, Lichtwardt et al. 2011) the zygospore of *Furculomyces boomerangus* is type II but with a bent longitudinal axis reminiscent of a boomerang (and borne on a furculum or wishbone-like union of conjugating hyphae). *Pseudoharpella arcolamylica* also tends to present a variably bent zygospore (Ferrington et al. 2003). Recovery of *Stachylina* collections with zygospores would be informative in comparison with these two genera. One sample (AS-49-6) from New Zealand, which was accessioned with ambiguity (TABLE I) as either a *Stachylina* sp. or *Smittium* sp., emerged in subclade 3, and we now conservatively refer to this as a *Smittium* sp. voucher (pending publication of an earlier survey of Harpellales from that country).

Finally, *Austrosmittium* in subclade 1 is most typically recognized based on its type II zygospore that is somewhat spherically swollen at the midpoint (making it somewhat inflated in appearance) and a striking morphological feature. We adhere to this idea of uniqueness based on molecular data as well. *Austrosmittium* is notably variable for these gene regions, although this might not be obvious with it nestled in subclade 1. However, the sequence variation among the *Austrosmittium* samples in hand has presented some challenges with the primers and cycling profiles that otherwise are fairly reliable for this group of Harpellales. As the genus currently stands, *Austrosmittium* seems to be a lineage that has undergone considerable change in both regards.

As we reflect on just over 7½ decades of research, and despite the relocation of *Z. culisetae*, *Smittium* has increased on average by about one new species per year. Clearly, this is a time to both reflect upon and anticipate further the membership of this large genus. We present some clades with some remarkable

patterns. There appear to be species of Harpellales that are unique or geographically sequestered in terms of their evolutionary origins, but in other cases very similar species or even conspecific ones can be wide-ranging geographically. As growing datasets and analyses produce more trees, we also anticipate mapping key morphological features onto well supported clades, as exemplified by *Zancudomyces culisetae*.

While an in-depth morphometric critique was not undertaken in this study, either qualitatively or quantitatively, we have conducted a cursory examination of the morphology of the trichospore. Among the *Smittium* subclades, there seems to be a trend that helps to distinguish members of subclades 1 and 3, considering overall length to width ratios of asexual spores. Subclade 3 tends to have members with longer and narrower trichospores (SUPPLEMENTARY TABLE I). Specifically members of subclade 3 maintain a ratio of length to width of 3.75–9.76, whereas subclade 1 is 2.67–5.19. There is some overlap here, but this trend was surprising, even as a crude assessment. Current morphotaxonomy of *Smittium* and allies does not consider such a ratio but may be worthy of further consideration as molecular systematic efforts continue to attempt to reliably infer relationships.

We anticipate that, as we add more taxa and more genes to ongoing phylogenetic efforts, we will continue to improve tree resolution and support of various lineages and gain more confidence in offering such comparisons, perhaps unexpected. This large group of Harpellales, predominantly from lower Diptera larval hosts, represents a remarkable repertoire to be rendered for revisionary reviews.

ACKNOWLEDGMENTS

Financial support from NSF REVSYS Award DEB-0918182 (to MMW) and DEB-0918169 (to RWL) are gratefully acknowledged for this and ongoing studies toward a molecular-based reclassification of the Harpellales and Asellariales. MMW also received financial support for some of the earlier sequences from a Martin-Baker Award from the Mycological Society of America. We present this work also in memory of the earlier work in France, from Poisson through Manier, and because we recently celebrated the 75th anniversary of the establishment of *Smittium*. We are exceedingly grateful for samples, whether dissected or accessioned as isolates in our culture collection, from our colleagues and friends working in the field, particularly to Drs CE Beard, MJ Cafaro, LC Ferrington Jr, CC López Lastra, G Mazzucchelli, WK Reeves, A Rizzo, D Strongman, T Sweeney, LG Valle, as well as R Willey, while also recognizing both longer term and ongoing collaborations with some of them as well. YW also thanks his committee, Drs James Smith and Steve Novak, for their helpful suggestions with project design, progress reviews and suggestions for this research. We also thank Jeremy Niece and staff at UWBC core facility for prompt support for sequencing reactions and data handling.

LITERATURE CITED

Beard CE, Adler PH. 2003. Zygospores of selected Trichomycetes in larval Diptera of the families Chironomidae and Simuliidae. Mycologia 95:316–319.

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. 2010. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiol 10:189, doi:10.1186/1471-2180-10-189

Cafaro M. 2005. Eccrinales (Trichomycetes) are not fungi, but a clade of protists at the early divergence of animals and fungi. Mol Phylogenet Evol 35:21–34, doi:10.1016/j.ympev.2004.12.019

Clark TB, Kellen WR, Lindegren JE. 1963. Axenic culture of two Trichomycetes from Californian mosquitoes. Nature 197:208–209, doi:10.1038/197208a0

Duboscq O, Léger L, Tuzet O. 1948. Contribution à la connaissance des *Eccrinides*: les Trichomycètes. Arch Zool Exp Gen 86:29–144.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797, doi:10.1093/nar/gkh340

El-Buni AM, Lichtwardt RW. 1976a. Asexual sporulation and mycelial growth in axenic cultures of *Smittium* spp. (Trichomycetes). Mycologia 68:559–572, doi:10.2307/3758979

———, ———. 1976b. Spore germination in axenic cultures of *Smittium* spp. (Trichomycetes). Mycologia 68:573–582, doi:10.2307/3758980

Farr DF, Lichtwardt RW. 1967. Some cultural and ultrastructural aspect of *Smittium culisetae* (Trichomycetes) from mosquito larvae. Mycologia 59:172–182, doi:10.2307/3756952

Ferrington LC Jr, Lichtwardt RW, Hayford B, Williams MC. 2005. Symbiotic Harpellales (Trichomycetes) in Tasmanian aquatic insects. Mycologia 97:254–262, doi:10.3852/mycologia.97.1.254

———, White MM, Lichtwardt RW. 2003. A new genus of Trichomycetes (*Pseudoharpella arcolamylica*) from *Dixa fluvica* Peters (Diptera: Dixidae). Aquat Insects 25:85–94, doi:10.1076/aqin.25.2.85.14039

Gauthier M. 1936. Sur un nouvel Entophyte du groupe des Harpellacées Lég. et Dub., parasite des larves d'Éphémérides. Cr Hebd Acad Sci 202:1096–1098.

———. 1960. Un nouveau Trichomycète rameux parasite des larves de *Baëtis pumilus* (Burm.) Trav Lab Hydrobiol Piscic Univ. Grenoble 50–51:225–227.

———. 1961. Une nouvelle espèce de *Stachylina*: *St. minuta* n. sp., parasite des larves de Chironomides Tanytarsiens. Trav Lab Hydrobiol Piscic Univ. Grenoble 53:1–4.

Gottlieb AM, Lichtwardt RW. 2001. Molecular variation within and among species of Harpellales. Mycologia 91:66–81, doi:10.2307/3761606

Grigg R, Lichtwardt RW. 1996. Isozyme patterns in cultured Harpellales. Mycologia 88:219–229, doi:10.2307/3760926

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt RW, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N. 2007. A higher level phylogenetic classification of the Fungi. Mycol Res 122:509–547, doi:10.1016/j.mycres.2007.03.004

Horn BW. 1989a. Requirement for potassium and pH shift in host-mediated sporangiospore extrusion from trichospores of *Smittium culisetae* and other *Smittium* species. Mycol Res 93:303–313, doi:10.1016/S0953-7562(89)80157-1

———. 1989b. Ultrastructural changes in trichospores of *Smittium culisetae* and *S. culicis* during in vitro sporangiospore extrusion and holdfast formation. Mycologia 81:742–753, doi:10.2307/3759879

———. 1990. Physiological changes associated with sporangiospore extrusion from trichospores of *Smittium culisetae*. Exp Mycol 14:113–123, doi:10.1016/0147-5975(90)90070-A

———, Lichtwardt RW. 1981. Studies on the nutritional relationship of larval *Aedes aegypti* (Diptera: Culicidae) with *Smittium culisetae* (Trichomycetes). Mycologia 73: 724–740, doi:10.2307/3759499

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox C, Celio G, Gueidan C, Fraker E, Miadlikowska J, Lumbsch HT, Rauhut A, Reeb V, Arnold EA, Amtoft A, Stajich JE, Hosaka K, Sung G-H, Johnson D, O'Rourke B, Crockett M, Binder M, Curtis JM, Slot JC, Wang Z, Wilson AW, Schüßler A, Longcore JE, O'Donnell K, Mozley-Standridge S, Porter D, Letcher PM, Powell MJ, Taylor JW, White MM, Griffith GW, Davies DR, Humber RA, Morton J, Sugiyama J, Rossman AY, Rogers JD, Pfister DH, Hewitt D, Hansen K, Hambleton S, Shoemaker RA, Kohlmeyer J, Volkmann-Kohlmeyer B, Spotts RA, Serdani M, Crous PW, Hughes KW, Matsuura K, Langer E, Langer G, Untereiner WA, Lücking R, Büdel B, Geiser DM, Aptroot A, Diederich P, Schmitt I, Schultz M, Yahr R, Hibbett DS, Lutzoni F, McLaughlin D, Spatafora J, Vilgalys R. 2006. Reconstructing the early evolution of the fungi using a six gene phylogeny. Nature 443:818–822, doi:10.1038/nature05110

Koontz JA. 2006. Physiological studies on a new isolate of the gut fungus, *Smittium culisetae* (Trichomycetes: Harpellales), from wetland mosquito larvae, *Aedes vexans* (Diptera: Culicidae). *Trans Kansas Acad Sci* 109:175–183, doi:10.1660/0022-8443(2006)109[175:PSOANI]2.0.CO;2

Léger L, Duboscq O. 1903. Recherche sur les Myriapodes de Corse et leur parasites. Arch Zool Exp Gen 1:307–311.

———, ———. 1905a. Les Eccrinides, nouveau groupe de Protophytes parasites. Cr Hebd Acad Sci 141:425–427.

———, ———. 1905b. Les Eccrinides, nouveau groupe de végétaux inférieurs, parasites des Arthropodes. Assoc Franç Avanc Sci 28:331–332.

———, ———. 1929. *Harpella melusinae* n. g. n. sp. Entophyte eccriniforme parasite des larves de Simulie. Cr Hebd Acad Sci 188:951–954.

———, Gauthier M. 1931. *Orphella coronata* n. g., n. sp. Entophyte parasite des larves de Némurides. Trav Lab Hydrobiol Piscic Univ. Grenoble 23:67–72.

———, ———. 1932. Endomycètes nouveaux des larves aquatiques d'Insectes. Cr Hebd Acad Sci 194:2262–2265.

———, ———. 1935a. La spore des Harpellacées (Léger et Duboscq), Champignons parasites des Insectes. Cr Hebd Acad Sci 200:1458–1460.

———, ———. 1935b. La spore des Harpellacees (Léger et Duboscq) Champignons parasites des Insectes. Trav Lab Hydrobiol Piscic Univ. Grenoble 27:3–6.

———, ———. 1937. *Graminella bulbosa* nouveau genre d'Entophyte parasite des larves d'Éphémérides du genre *Baetis*. Cr Hebd Acad Sci 202:27–29.

Leidy J. 1849a. *Enterobrus*, a new genus of Confervaceae. Proc Acad Natl Sci Phila 4:225–233.

———. 1849b. Descriptions (accompanied by drawings) of new genera and species of Entophyta. Proc Acad Natl Sci Phila 4:249–250.

———. 1850a. Observations upon an entophytic forest. Proc Acad Natl Sci Phila 5:8–9.

———. 1850b. Descriptions of new Entophyta growing within animals. Proc Acad Natl Sci Phila 5:35–36.

———. 1853. A flora and fauna within living animals. Smithson Contrib Knowl 5:1–67.

Lichtwardt RW. 1951. Studies on some species of Eccrinales inhabiting the intestinal tract of millipeds (master of science thesis). Univ. Illinois Press. 50 p.

———. 1954. Three species of Eccrinales inhabiting the hindguts of millipedes, with comments on the Eccrinids as a group. Mycologia 46:564–585.

———. 1964. Axenic culture of two new species of branched Trichomycetes. Am J Bot 51:836–842, doi:10.2307/2439889

———. 1978. Validation of the Harpellales and Asellariales. Mycotaxon 7:441–442.

———. 1986. The Trichomycetes: fungal associates of arthropods. New York: Springer-Verlag. 343 p.

———. 2004. Trichomycetes: fungi in relationship with insects and other arthropods. Symbiosis 4:575–588, doi:10.1007/0-306-48173-1_36

———, Arenas JM. 1996. Trichomycetes in aquatic insects from southern Chile. Mycologia 88:844–857, doi:10.2307/3760981

———, Cafaro MJ, White MM. 2001. The Trichomycetes, fungal associates of arthropods. Rev ed. Published on the Internet (www.nhm.ku.edu/~fungi). (last accessed 14 Nov. 2011)

———, Ferrington LC Jr, López Lastra CC. 1999. Trichomycetes in Argentinean aquatic insect larvae. Mycologia 91:1060–1082, doi:10.2307/3761637

———, Moss ST. 1981. Vegetative propagation in a new species of Harpellales, *Graminella microspora*. Trans Br Mycol Soc 76:311–316, doi:10.1016/S0007-1536(81)80151-9

———, ———. 1984a. New Asellariales (Trichomycetes) from the hindguts of aquatic isopods and springtails. Mycotaxon 20:259–274.

———, ———. 1984b. *Harpellomyces eccentricus*, an unusual Harpellales from Sweden and Wales. Mycotaxon 20: 511–517.

———, Williams MC. 1990. Trichomycete gut fungi in Australian aquatic larvae. Can J Bot 68:1057–1074, doi:10.1139/b90-133

———, ———. 1999. Three Harpellales that live in one species of aquatic chironomid larva. Mycologia 91:396–399, doi:10.2307/3761385

———, White MM. 2011. Typification of *Smittium*, an important genus in the taxonomy of Harpellales. Mycologia 103:915–917, doi:10.3852/10-414

Liu YJ, Hodson MC, Hall BD. 2006. Loss of the flagellum happened only once in the fungal lineage: phylogenetic structure of kingdom Fungi inferred from RNA polymerase II subunit genes. BMC Evol Biol 6:74–86, doi:10.1186/1471-2148-6-74

López Lastra CC, Scorsetti AC, Marti GA, Coscarón S. 2005. Trichomycetes living in the guts of aquatic insects of Missiones and Tierra del Fuego, Argentina. Mycologia 97:320–328, doi:10.3852/mycologia.97.2.320

Maddison WP, Maddison DR. 2010. Mesquite 2.73: a modular system for evolutionary analysis. (http://mesquiteproject.org) (last accessed 14 Nov. 2011)

Manier J-F. 1968. Validation de Trichomycètes par leur diagnose latine. Ann Sci Nat Bot Biol 12, 9:93–108.

———. 1969a. Changement de nom pour *Eccrina flexilis* Léger et Duboscq, 1906. Ann Sci Nat Bot Biol 12, 10: 469–471.

———. 1969b. Trichomycètes de France. Ann Sci Nat Bot Biol 12, 10:565–672.

———, Lichtwardt RW. 1968. Révision de la systématique des Trichomycètes. Ann Sci Nat Bot Biol 12, 9:519–532.

McCreadie JW, Beard CE. 2003. The microdistribution of the trichomycete *Smittium culisetae* in the hindgut of the black fly host *Simulium vittatum*. Mycologia 95:998–1003, doi:10.2307/3761907

Moss ST. 1972. Occurrence, cell structure and taxonomy of the Trichomycetes, with special reference to electron microscope studies of *Stachylina* [doctoral dissertation]. Reading, UK: Univ. Reading Press. 340 p.

———, Lichtwardt RW. 1976. Development of trichospores and their appendages in *Genistellospora homothallica* and other Harpellales and fine-structural evidence for the sporangial nature of trichospores. Can J Bot 54: 2346–2364, doi:10.1139/b76-251

———, ———. 1977. Zygospores of the Harpellales: an ultrastructural study. Can J Bot 55:3099–3110, doi:10.1139/b77-351

———, ———. 1980. *Harpella leptosa*, a new species of Trichomycetes substantiated by electron microscopy. Can J Bot 58:1035–1044, doi:10.1139/b80-128

———, ———, Manier J-F. 1975. *Zygopolaris*, a new genus of Trichomycetes producing zygospores with polar attachment. Mycologia 67:120–127, doi:10.2307/3758234

———, Young TWK. 1978. Phyletic considerations of the Harpellales and Asellariales (Trichomycetes, Zygomycotina) and the Kickxellales (Zygomycetes, Zygomycotina). Mycologia 70:944–963, doi:10.2307/3759130

O'Donnell K, Cigelnick E, Benny GL. 1998. Phylogenetic relationships among the Harpellales and Kickxellales. Mycologia 90:624–639, doi:10.2307/3761222

———, ———, Weber NS, Trappe JS. 1997. Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia 89:48–65, doi:10.2307/3761172

Poisson R. 1927. Sur une Eccrinide nouvelle: *Taeniellopsis orchestiae* nov. gen., nov. sp., Protophyte parasite du rectum de *I'Orchestia bottae* M. Edw. (Crust. Amphipode). Son cycle évolutif. Cr Hebd Acad Sci 185:1328–1329.

———. 1936 (1937). Sur un Endomyce'te nouveau: *Smittium arvernense* n. g., n. sp., parasite intestinal de larves de *Smittia* sp. (Dipte'res Chironomides) et description d'une nouvelle espe'ce du genre *Stachylina* Léger et Gauth. 1932. Bull Soc Sci Bretagne 14:20–31.

Porter D, Smiley R. 1979. Ribosomal RNA molecular weights of Trichomycetes and Zygomycetes. Exp Mycol 3:188–193, doi:10.1016/S0147-5975(79)80029-8

Posada D. 2008. jModeltest: Phylogenetic model averaging. Mol Biol Evol 25:1253–1256, doi:10.1093/molbev/msn083

Reeves WK. 2004. Oviposition by *Aedes aegypti* (Diptera: Culicidae) in relation to conspecific larvae infected with internal symbiotes. J Vector Ecol 29:159–163.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574, doi:10.1093/bioinformatics/btg180

Sangar VK, Lichtwardt RW, Kirsch JAW, Lester RN. 1972. Immunological studies on the fungal genus *Smittium* (Trichomycetes). Mycologia 44:342–358, doi:10.2307/3757837

Starr AM, Lichtwardt RW, McChesney JD, Baer TA. 1979. Sterols synthesized by cultured Trichomycetes. Arch Microbiol 120:185–189, doi:10.1007/BF00423063

Strongman DB, White MM. 2008. Trichomycetes from lentic and lotic aquatic habitats in Ontario, Canada. Botany 86:1449–1466, doi:10.1139/B08-107

Sweeney AW. 1981. An undescribed species of *Smittium* (Trichomycetes) pathogenic to mosquito larvae in Austrialia. Trans Br Mycol Soc 77:55–60, doi:10.1016/S0007-1536(81)80179-9

Tuzet O, Manier J-F. 1947. *Orphella culici* n. sp., entophyte parasite du rectum des larves de Culex hortensis Fclb. Cr Hebd Acad Sci 225:264–266.

———, ———. 1950. Les Trichomycètes. Revision de leur

diagnose. Raisons qui nous font y joindre les Asellariées. Ann Sci Nat Zool 11(12):15–23.

———, ———. 1953. Recherches sur quelques Trichomycètes rameux. *Asellaria armadillidii* n. sp. *Genistella choanifera* n. sp. *Genistella chironomi* n. sp. *Spartiella barbata* Tuzet et Manier. Ann Sci Nat Zool 11:373–391.

———, ———. 1954. Importance des cultures de Trichomycètes pour l'étude du cycle et de la classification de ces organismes. Cr Hebd Acad Sci 238:1904–1905.

———, ———. 1955a. Étude des Trichomycètes de l'intestin des larves de *Simulium eguinum* Linné récoltés aux Eyzies (Dordogne). Ann Sci Nat Zool 11: 55–62.

———, ———. 1955b. Sur deux nouvelles espèces de *Génistellales*: *Genistella rhitrogenae*, n. sp., et *Genistella mailleti* n. sp., observées dans les larves de *Rhitrogena alpestris* Eat. et *Boetis bioculatus* L. récoltés aux Eyzies (Dordogne). Ann Sci Nat Zool 11:67–71.

Valle LG, Cafaro MJ. 2010. First report of Harpellales from the Dominican Republic (Hispaniola) and the insular effect on gut fungi. Mycologia 102:363–373, doi:10.3852/09-028

———, White MM, Cafaro MJ. 2011. Dipteran—associated Harpellales from lowland and submontane tropical rain forests of Veracruz (Mexico). Mycologia 103:656–673, doi:10.3852/10-298

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246.

Walker WF. 1984. 5S ribosomal RNA sequences from Zygomycotina and evolutionary implications. System Appl Microbiol 5:448–456, doi:10.1016/S0723-2020(84)80002-8

Whisler HC. 1961. Cultural studies of the Trichomycetes [doctoral dissertation]. Berkeley: Univ. California Press. 141 p.

———. 1962. Culture and nutrition of *Amoebidium parasiticum*. Am J Bot 49:193–199, doi:10.2307/2439541

———. 1966. Host-integrated development in the Amoebidiales. J Protozool 13:183–188.

———. 1968. Developmental control of *Amoebidium parasiticum*. Dev Biol 17:562–570, doi:10.1016/0012-1606(68)90005-5

White MM. 1999. *Legerioides*, a new genus of Harpellales in isopods and other Trichomycetes from New England, USA. Mycologia 91:1021–1030, doi:10.2307/3761634

———. 2006. Evolutionary implications of a RNA-based phylogeny of Harpellales. Mycol Res 110:1011–1024, doi:10.1016/j.mycres.2006.06.006

———, Cafaro MJ, Gottlieb AM. 2001. Taxonomy and systematics of Trichomycetes—past, present and future. In: Misra JK, Horn BW, eds. Trichomycetes and other fungal groups. Enfield, New Hampshire: Science Publishers. p 27–37.

———, James TY, O'Donnell K, Cafaro MJ, Tanabe Y, Sugiyama J. 2006a. Phylogeny of the Zygomycota based on nuclear ribosomal sequence data. Mycologia 98: 872–884, doi:10.3852/mycologia.98.6.872

———, Lichtwardt RW. 2004. Fungal symbionts (Harpellales) in Norwegian aquatic insect larvae. Mycologia 96: 891–910, doi:10.2307/3762122

———, ———, Colbo MH. 2006b. Confirmation and identification of parasitic stages of obligate endobionts (Harpellales) in blackflies (Simuliidae) by means of rRNA sequence data. Mycol Res 110:1070–1079, doi:10.1016/j.mycres.2006.06.008

———, Siri A, Lichtwardt RW. 2006. Trichomycete insect symbionts in Great Smoky Mountains National Park and vicinity. Mycologia 98:333–352, doi:10.3852/mycologia.98.2.333

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. New York: Academic Press. p 315–322.

Wilgenbusch JC, Warren DL, Swofford DL. 2004. AWTY: a system for graphical exploration of MCMC convergence in Bayesian phylogenetic inference. (http://ceb.csit.fsu.edu/awty) (last accessed 14 Nov 2011)

Williams MC. 1983a. Spore longevity of *Smittium culisetae* (Harpellales, Legeriomycetaceae). Mycologia 75:171–174, doi:10.2307/3792935

———. 1983b. Zygospores in *Smittium culisetae* (Trichomycetes) and observations on trichospore germination. Mycologia 75:251–256, doi:10.2307/3792809

———, Lichtwardt RW. 1972a. Infection of *Aedes aegypti* larvae by axenic cultures of the fungal genus *Smittium* (Trichomycetes). Am J Bot 59:189–193, doi:10.2307/2441400

———, ———. 1972b. Physiological studies on the cultured Trichomycete, *Smittium culisetae*. Mycologia 64:806–815, doi:10.2307/3757935

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [doctoral dissertation]. Austin: Univ. Texas Press. 125 p.