RIBOSOMAL RNA GENE-BASED AND MULTIGENE PHYLOGENIES OF *SMITTIUM* (HARPELLALES) AND ALLIES—TOWARD UNRAVELING RELATIONSHIPS AMONG EARLY-DIVERGING FUNGI

by

Yan Wang

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Biology

Boise State University

May 2012

BOISE STATE UNIVERSITY GRADUATE COLLEGE

DEFENSE COMMITTEE AND FINAL READING APPROVALS

of the thesis submitted by

Yan Wang

Thesis Title: Ribosomal RNA gene-based and multigene phylogenies of Smittium (Harpellales) and allies—toward unraveling relationships among early-diverging fungi

Date of Final Oral Examination: 20 March 2012

The following individuals read and discussed the thesis submitted by student Yan Wang, and they evaluated his presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

Merlin M. White, Ph.D.	Chair, Supervisory Committee
Stephen J. Novak, Ph.D.	Member, Supervisory Committee
James F. Smith, Ph.D.	Member, Supervisory Committee

The final reading approval of the thesis was granted by Merlin M. White, Ph.D., Chair of the Supervisory Committee, and Stephen J. Novak, Ph.D., and James F. Smith, Ph.D., Members of the Supervisory Committee. The thesis was approved for the Graduate College by John R. Pelton, Ph.D., Dean of the Graduate College.

ACKNOWLEDGEMENTS

I would like to first thank Dr. Merlin M. White for serving as my thesis advisor and mentor. Dr. White was always willing to offer suggestions for experimental design, protocol improvement, and thesis writing. His support for creative ideas and tools used in this thesis is greatly appreciated. He did a great job to share with me the reality of science, not only with the early-diverging fungal groups we have been studying, but also many other interesting organisms in the kingdom of Fungi, let alone the molecular systematic tools. The annual meetings of the Mycological Society of America (MSA) were a great experience; Dr. White showed and introduced me there, which really helped engage my scientific career. Those were my golden opportunities to present pieces of my thesis work and get extra training as a young scientist. Without him, I would never have been this familiar and enjoyed so much with the community of Mycologists.

I also wish to give my sincere thanks to my two other committee advisors, Dr. Stephen J. Novak and Dr. James F. Smith, for their helpful suggestions with project design, progress reviews and suggestions for this research and thesis writing, especially from a broader, molecular phylogenetic point of view. Thanks to your wise and timely suggestions, my academic horizon was extended even broader to fields of Molecular Ecology, Evolution, Phylogeny, and Phylogeography. Without your advice and support, this work would not be accomplished.

Dr. Robert W. Lichtwardt, University of Kansas, also provided a lot of suggestions from a morphological point of view, as well as many vouchers used for molecular phylogenies or photographs used for morphological identification/confirmation. Samples, whether dissected or accessioned as isolates in our culture collection, were gratefully received by our laboratory from colleagues and friends working in the field, particularly Dr. CE Beard, Dr. MJ Cafaro, Dr. LC Ferrington Jr., Dr. CC López Lastra, Dr. WK Reeves, Dr. A Rizzo, Dr. D Strongman, Dr. T Sweeney, Dr. LG Valle, Dr. R Willey, and Dr. G Mazzucchelli, as well as PV Clarke.

Eric D. Tretter, my laboratory mate and peer graduate student colleague, helped with protocol designs and testing, especially with his bioinformatic resources. He also introduced me to several softwares, which improved the process of data analysis. Two undergraduate students from our laboratory, Prasanna Kandel and Eric M. Johnson, helped with some of the data collection. Emma R. Wilson, another graduate student of Dr. White, has always been helpful and supportive, in so many ways. She also provided several *Smittium* photographs from local collections in Boise, Idaho. Thanks also are given to other lab members, both old and new, especially Molly Bench, Justin Gause, Sarah Oman, Nicole Reynolds, Lance Steele, and Luke Sugden.

This is presented in memory of the earlier researchers in France, from Poisson through Manier, having recently celebrated the 75th anniversary since the description or "birth" of *Smittium.* Financial support from NSF REVSYS Award DEB-0918182 (to Merlin M. White, Ph.D.) and a Teaching Assistantship from Department of Biological Sciences, Boise State University is gratefully acknowledged. Consistent support and help from Ms. Judy E. Lonsdale (my teaching mentor and the coordinator of BIOL 100

Concepts of Biology Laboratory, of which I worked as instructor) made me a better skilled biologist and instructor. Other support included travel funds from the Department of Biological Sciences, Boise State University and residence funds from Graduate Residential Scholars Program (GRSP), Graduate College, at Boise State University.

Lastly, I would like to express my thanks to my family. Thanks to them, my life is good. I want my parents, grandparents, uncles, aunts, cousins, and friends to know that I love them very much, as always.

ABSTRACT

Smittium is one of the oldest members of the Harpellales, a group commonly referred to as the "gut fungi". Gut fungi are endosymbiotic microorganisms that live in the digestive tracts of various Arthropods, worldwide. During the 75 years since the first species, *Smittium arvernense*, was described, *Smittium* has increased in number and now includes 81 species. Research on this genus has also helped to advance our understanding of the gut fungi, by serving as a "model" for laboratory studies of the fungal trichomycetes. Many isolates of *Smittium* have been used for ultrastructural, physiological, host feeding, serological, as well as isozyme, and now ongoing molecular systematic studies. Previous and current 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* species. Morphological (zygospore and trichospore shape), molecular (18S and 28S rRNA genes), immunological, and isozyme evidence are used to establish a new genus *Zancudomyces*, and to accommodate *Smittium culisetae*. A multigene dataset (consisting of 18S and 28S rRNA genes, with RPB1, RPB2, and MCM7 translated protein sequences) for *Smittium* and related Harpellales (*Austrosmittium, Coleopteromyces, Furculomyces, Pseudoharpella, Stachylina* and *Trichozygospora*) was used for phylogenetic analyses and provided strong support at multiple levels in the trees generated. The clades and branches of the consensus tree are assessed relative to morphological traits, including

holdfast shape, thallus branching type, trichospore or zygospore characters as an aid to inform the current taxonomy and eventual systematic revisions and reclassification. Some patterned separation was found within the “Smittium” clade, including the separation of “True *Smittium*” clade and “Parasmittium” clade, which was supported also by thallus branching types and trichospore shapes, and perhaps lending support to an earlier narrower definition of the genus. Suggestions are offered for future morphological- and molecular-based studies, as ongoing efforts are unfolding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The Trichomycetes was established, as a formally recognized rank, 44 years ago by Manier and Lichtwardt (1968) with four orders: Amoebidiales, Asellariales, Eccrinales, and Harpellales. All members of the Trichomycetes are associated with arthropods, almost entirely as gut endosymbionts, living in the digestive tract of their hosts. Significant changes in our evolutionary understanding of the group have been made with molecular phylogenetic approaches and tools. Cafaro (2005) demonstrated that the "fungal-like" Eccrinales was actually a sister order to the Amoebidiales, both protozoans related to the Mesomycetozoa (based on 18S and 28S rRNA genes). Thus, putatively, the only fungal orders of "Trichomycetes" remaining are the Asellariales and Harpellales. Based on published multigene phylogenies, significant changes were made to the higher level classification of many fungal groups, including the suggested deconstruction of Trichomycetes (Hibbett et al. 2007). In fact, the early-diverging fungal tree is now considered to be a loose aggregation of fragmented clades in need of revision. White (2006) made the last attempt to infer relationships among the Harpellales, but no published molecular systematic data exists for the Asellariales to date (Hibbett et al. 2007).

In the Harpellales, the most species-rich genus, *Smittium*, includes species that live in the hindguts of lower Diptera worldwide. They typically occur in the larval aquatic stages of black flies (Simuliidae), bloodworms (Chironomidae), mosquitoes

(Culicidae), and solitary (Thaumaleidae) and biting (Ceratopogonidae) midges from varied habitats (Ferrington et al. 2005, Lichtwardt et al. 1999, Valle et al. 2011). These microfungi have evolved with various morphological and physiological adaptations that allowed them to live in association with their hosts for millions of years. Some species of *Smittium* have a wide distribution, while other species may be restricted geographically due to high host specificity, poor dispersal, or lack of surveying. Although they are generally considered to be commensals, their relationships range from lethal or parasitic to mutualistic for insects that are experiencing nutritional stresses (Horn and Lichtwardt 1981).

Within *Smittium*, several questions await further study or improved resolution, particularly from a phylogenetic and molecular systematic perspective. One species, *Smittium culisetae,* is widespread and culturable. Genomic DNA from one or more isolates of this species has been used in phylogenetic studies (Gottlieb & Lichtwardt 2001, James et al 2006, Jones et al 2011, Liu et al 2006, O'Donnell et al 1998, Seifert et al 2007, Tanabe et al 2000, Walker 1984, White 2006). *Smittium culisetae* has been recognized as a distinct clade with "Non-*Smittium*" Harpellales based on both 18S and 28S rRNA gene trees (White 2006). Other *Smittium* species have formed a polyphyletic clade and been included with other Harpellales (allies such as *Austrosmittium*, *Furculomyces*, *Pseudoharpella*, and *Stachylina*) based on separate, single gene (18S and 28S rRNA) phylogenetic analyses (Gottlieb and Lichtwardt 2001, White 2006).

The main objective of this dissertation study was to establish combined and multigene phylogenies of *Smittium* species and taxa putatively associated with the "Smittium" clade to test the monophyly of *Smittium*. It was believed that with a focus on

improved gene and taxon sampling, inferred (strongly supported) reconstruction of evolutionary relationships would permit an overall assessment of the morphology-based taxonomy of the group. *Smittium culisetae* has been suspected of not being a member of *Smittium*, pending the results of a multigene analysis. Would *Smittium culisetae* remain as a distinct lineage or cluster with the larger "Smittium" clade as more data were added for a more complete phylogenetic assessment*?* For the other Smittium species, might they too deserve other generic designations? Is this a large group of microfungi with diversity that is masked by convergent morphology, or might it be that some of the other taxa—*Austrosmittium*, *Furculomyces*, *Pseudoharpella*, *Stachylina*—were unwarranted and may require revision.

The thesis is comprised of two complementary studies in separate chapters. In the first, 75 years of research on *Smittium* is reviewed and a new genus, *Zancudomyces*, is proposed to accommodate *Smittium culisetae* based on a combined 2-gene (18S and 28S rRNA) analyses and other molecular and morphological support. That effort encompasses 137 taxa, including 127 Harpellales. The second chapter uses a 5-gene, combined analysis (18S and 28S rRNA again, but also with RPB1, RPB2 and MCM7 genes), to estimate phylogenetic relationships among fungal lineages. The inclusion of more variable domain regions with this study addresses natural relationships at lower levels as exemplified by other studies as well (Cafaro 2005, Gottlieb & Lichtwardt 2001, Hibbett 2007, James et al 2006, Liu et al 2006, McLaughlin et al 2009, O'Donnell et al 1998, Walker 1984, White 2006). Ultimately, the aim is to provide strong molecular-based phylogenetic support to begin to assess and eventually further reorganize the large "Smittium" clade.

References

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.

Ferrington LC, Lichtwardt RW, Hayford B, Williams MC. 2005. Symbiotic Harpellales (Trichomycetes) in Tasmanian aquatic insects. *Mycologia* 97:254–262.

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

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.

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

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.

Jones MDM, Forn I, Gadelha C, Egan MJ, Bass D, Massana R, Richards TA. 2011. Discovery of novel intermediate forms redefines the fungal tree of life. *Nature* 474:200–205.

Lichtwardt RW, Ferrington LC Jr., López Lastra C. 1999. Trichomycetes in Argentinean aquatic insect larvae. *Mycologia* 91:1060–1082.

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.

Manier JF, Lichtwardt RW. 1968. Révision de la systématique des Trichomycètes. *Ann Sci Nat Bot Biol* 12(9):519–532.

McLaughlin DJ, Hibbett DS, Lutzoni F, Spatafora JW, Vilgalys R. 2009. The search for the fungal tree of life. *Trends Microbiol* 17(11):488–497.

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

Seifert KA, Samson RA, deWaard JR, Houbraken J, Lévesque CA, Moncalvo JM, Louis-Seize G, Hebert PDN. 2007. Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *P Natl Acad Sci USA* 104(10):3901–3906.

Tanabe Y, O'Donnell K, Saikawa M, Sugiyama J. 2000. Molecular phylogeny of parasitic Zygomycota (Dimargaritales, Zoopagales) based on nuclear small subunit ribosomal DNA sequences. *Mol Phylogenet Evol* 16:253–262.

Valle LG, White MM, Cafaro MJ. 2011. Dipteran-associated Harpellales from lowland and submontane tropical rain forests of Veracruz (Mexico). *Mycologia* 103:656–673.

Walker WF. 1984. 5S ribosomal RNA sequences from Zygomycotina and evolutionary implications. *System Appl Microbiol* 5:448–456.

White MM. 2006. Evolutionary implications of a RNA-based phylogeny of Harpellales. *Mycol Res* 110:1011–1024.

CHAPTER ONE: OVERVIEW OF 75 YEARS OF *SMITTIUM* RESEARCH, ESTABLISHING A NEW GENUS FOR *SMITTIUM CULISETAE*, AND PROSPECTS FOR FUTURE REVISIONS OF THE "SMITTIUM" CLADE

Abstract

The Harpellales includes 38 genera of endosymbiotic microfungi associated with various Arthropods. *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 years 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. Previous and current 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* species. 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.

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 earlier studies of "entophytes" by American naturalist Joseph Leidy (1849a, 1849b, 1850a, 1850b, 1853). Several decades later, the foundation of the field of trichomycetology was taking form with the efforts of protozoologists in France. This began with Léger and Duboscq (1903, 1905a, 1905b), 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, 1935b, 1937) continued the tradition until just before the 2nd World War. Their active research period overlapped with the fungal studies of Poisson (1927, 1936). Gauthier (1936, 1960, 1961) published individually as well, but infrequently, over another 3-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, 1955b), Tuzet and Manier (1950) also revised "Les Trichomycètes". This was a significant publication, although some of the included taxa

were later 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 the USA and with Moss (1972) from England. Lichtwardt and Moss also published (Lichtwardt and Moss 1981, 1984a, 1984b; 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 & Lichtwardt (1968) with four orders of "hair-like" endosymbionts (Harpellales, Asellariales Manier ex Manier & Lichtw., Amoebidiales L. Léger & Duboscq, and Eccrinales), all associated with various members of 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 even been obtained in pure cultured by the 1960's (Clark et al. 1963; Lichtwardt 1964; Whisler 1962, 1966, 1968). Since that time, 8 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 that time, the trichomycetes (in non-taxonomic, lower case form) have been recognized by some as an ecological group with two fungal orders—the Asellariales and Harpellales (Cafaro 2005, Lichtwardt 1978, Moss and Young 1978). Though not included in this study, the Asellariales, with 3 genera and 14 species, is one of the key missing lineages amongst 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 lifestyle 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 just over 75 years 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 (Ferrington et al. 2005, Lichtwardt and Williams 1999, 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 commensalistic, but actually ranges from mutualistic for insects (mosquitoes) that are experiencing nutritional stress (Horn and Lichtwardt 1981), to lethal or parasitic, as with *Smittium morbosum* A.W. Sweeney, which kills mosquito larvae by preventing molting (Lichtwardt 2004, Sweeney 1981). 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, non-motile 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 years 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. 1.1), with six Smittiums described in 1969, six more in the 1970's, fifteen in the 1980's, 23 in the 1990's and with 25 since the new millennium. Although *Smittium culisetae* has been commonly recovered, reported, and even cultured from different places during this time (Farr and Lichtwardt 1967; Horn 1989b; Lichtwardt 1964; López Lastra et al. 2005; Manier 1969b; McCreadie and Beard 2003; Starr et al. 1979; Strongman and White 2008; Valle et al. 2010, 2011; White et al. 2006a; Williams 1983a, 1983b; Williams and Lichtwardt 1972b), 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 the USA, Canada, and Norway (Lichtwardt and White 2011, Lichtwardt and Williams 1999, Strongman and White 2008, White and Lichtwardt 2004). *Smittium mucronatum,* also culturable, is recognizable on the basis of a small nipple-like protruberance on the tip of the trichospore (Lichtwardt and White 2011). Despite being well studied and the second oldest species, *S. culisetae* was not considered as an epitype because it is now recognized to be quite 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. 1.2–1.5) and molecular evidence (FIGS. 1.6–1.11). We start to resolve some of the relationships between *Smittium* and its allies for what previously have 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, amongst these and other early-diverging lineages.

Materials and Methods

Host Collection and Specimen Preparation

Methods for collecting larval aquatic insects followed those described by White et al. (2001). Fungal vouchers consisted of living clumps of thalli placed in 500 ml of 2× Hexadecyltrimethyl ammonium bromide (CTAB) buffer (2% CTAB, 1.4 M Tris-HCl pH 8.0, 0.25 mM EDTA) (Gottlieb & Lichtwardt 2001) immediately after dissection and identification. Invariably, specimens of gut fungi included host tissue or other microscopic organisms associated with or passing through the host gut. The digestive tract, once removed from the host, was dissected with fine needles or forceps, and gut fungi were identified in wet mounts based on the morphological features noted (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 was then 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 the earlier study of Gottlieb and Lichtwardt (2001). Sample selection attempted to maximize the number of *Smittium* species 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 (Gottlieb and Lichtwardt 2001, O'Donnell et al. 1997, White 2006). In some cases, specimens were repeatedly frozen, 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 using glass milk or glass bead columns following the protocols of the GENECLEAN II Kit (Bio 101, Vista, CA) or the GENECLEAN Turbo Kit (Quantum Biotechnologies, Carlsbad, CA), 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 used included: 11 µL Promega Go-Taq green master mix, 0.66 µL of both the forward and reverse primer (0.3 pM/µL), 0.86 µL of 25 mM $MgCl_2$, 6.8 µL of molecular biology

grade H_2O, and 2 µL of 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 of 2.5 µM dNTP mix, 0.44 µL of 25 mM $MgCl_2$, 0.50 µL of 50 mg/mL BSA, 4.40 µL of 5M Betaine, 0.66 µL of each primer (0.3 pM/µL), 9.42 µL H_2O, and 0.11 µL TaKaRa EX Taq. For both amplification reaction kits, the final concentration of $MgCl_2$ used was 2.5 mM.

Thermal cycling protocols used 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 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 used 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 a 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

Gel electrophoresis was performed with a 1% gel (1× TAE buffer, modified to 1/10 concentration of EDTA) using 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 using

pipet tips (cut to increase bore accordingly), and then purified using a freeze and squeeze method. Microcentrifuge tubes (1.5 ml) containing the tips with cut gel were frozen at –20°C and then spun for 10 min in a microcentrifuge at 10,000 RPM. Tubes were refrozen at –20°C for 60 min and then spun again. The remaining gel in the pipet tips was expelled from the tubes, and the liquid with buffered PCR product squeezed from the cut gel was used as template for direct sequencing.

Direct Sequencing

Sanger sequencing was performed using 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 of sequencing premix, 3.75 µL of 5× sequencing buffer, 0.32 µL of each primer (0.16 pM/uL), 10.43 µL of H_2O, and 5 µL of template (squeezed gel solution). The thermal cycling regime used was adapted from the manufacturer's instructions (Applied Biosystems, Gene Amp PCR System 2700). The protocol used 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 capillary 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 utilized the nearly complete 18S and

part of the 28S rRNA gene. Data for the 18S were provided for all taxa in the study, while data on the 28S were available for 108 of them (TABLE 1.1).

Alignment and Model Determination

Data for the 18S and 28S ribosomal coding regions were first automatically aligned using the MUSCLE v3.8.31 (Edgar 2004) and then manually adjusted using MESQUITE v2.73 (Maddison and Maddison 2010). Ambiguously aligned regions (exsets) were excluded from analysis using 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 18S was GTR+G and for 28S was GTR+G+I; however, because the results for GTR+G and GTR+G+I were similar, the latter was used for both to simplify analysis. Alignments have been deposited in TreeBASE, under study number S12212.

Phylogenetic Tree Inference

Phylogenetic trees were estimated with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Five independent runs were conducted, each with four chains for $1x10^7$ generations, in which trees were sampled every 1000 generations. Stationarity of Markov chain Monte Carlo (MCMC) sampling and the appropriate burn-in values were assessed using AWTY (Wilgenbusch et al. 2004). Support for clades was also determined by a maximum likelihood analysis. One hundred bootstrap replicates were performed in GARLI v2.0 (Zwickl 2006), with the best three out of five taken for each replicate. Branch support given above and below were Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap proportions (MLBP) separately, with those considered to

be strongly supported (above 95% and 0.70 for each respectively) indicated with a bold line (FIGS. 1.7–1.11, SUPP. FIG. 1.1).

Results

We are establishing a new genus for *Smittium culisetae* based on both morphological and molecular data, as summarized below. We also highlight phylogenetic relationships among the remaining *Smittium* taxa sequenced for ribosomal RNA gene data.

Taxonomy

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

MycoBank: MB 563343

Thalli commonly verticillately branched, attached to the larval insect hindgut cuticle by a simple holdfast, producing trichospores that are wider below the midregion, 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. 1.2–1.5

MycoBank: MB 563846

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) x (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) x (5.5–)6(–8) µm, with a collar (6–)7(–8) x (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 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 morphological 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. 1.2–1.4) 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, 1983b) 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 may have contained more than one hindgut species (not unusual in some Harpellales hosts), Lichtwardt (University of Kansas) has studied one of Williams' voucher slides, and according to which, 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. 1.5).

Prior Immunological and Isozymic Evidence

Sanger et al. (1972) used serological methods, by obtaining antisera from rabbits against selected cultures from amongst 21 *Smittium* and 7 non-Harpellales isolates, to assess affinities among the fungal taxa. Phenograms and 3-dimensional projections of cluster and principal component analyses of immunoelectrophoretic data separated the 28 isolates into 5 groups. The Smittiums were in 4 different groups but with all 7 *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. 1.6) revealed a distinct and separate cluster of *Z. culisetae* (as *Smittium culisetae*) for 32 isolates, varying geographically from Australia, Japan, and seven states of the USA, including Hawaii.

Current Molecular Phylogenetic Results

For this and a number of other points, we present an overview tree (FIG. 1.7) of the major portions of a larger phylogenetic tree inferred from combined 18S and 28S rRNA gene (see Supp. FIG. 1.1 for the complete version). The 129 taxa include 126 exemplars of Harpellales and 3 members of Kickxellales as the outgroup (TABLE 1.1), 19 "Non-*Smittium*" genera of Harpellales and 3 genera of Kickxellales to anchor *Smittium* subclades, particularly included for placement of the *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 (Hibbett et al. 2007, James et al. 2006, White et al. 2006a). Of 226 sequences used herein, 142 are new. This includes 65 isolates representing 27 identified and three previously unidentified *Smittium* morpho-species.

Guide Tree and Node Description

Both the complete (Supp. FIG. 1.1) and the guide or overview tree (FIG. 1.7) indicate major, well-supported clades or subclades labeled as 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 have been 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 since 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, as that will be the focus of a future paper.

Subtending Clades

Node A of the guide tree (FIG. 1.7) represents the ordinal separation, specifically most of the Harpellales (except for *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. 1.8) includes *Zancudomyces*, with representatives that were accessioned, either as cultures or micro-dissected 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 non-molecular corroboration (see TABLE 1.1). 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. 1.7) circumscribes the greatest number of *Smittium* exemplars, whether from isolates or non-cultured representatives, yet analyzed (TABLE 1.1). Three major subclades (1–3) of "Smittium" (FIGS. 1.7, 1.9–1.11) 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.* cf. *morbosum*, amongst other *Smittium* species. Throughout the *Smittium* subclades there are terminal branchlets that are both strongly (bold lines) and less well-supported. Molecular data suggest that some species may have been misidentified at time of collecting, and others may 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 (bp). For *Z. culisetae*, nine sequences for eight isolates with 1776 bp of the 18S rRNA gene data, as well as 10 sequences for nine isolates across 971 bp for the 28S region, showed no variation. For *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 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 (El-Buni and Lichtwardt 1976a, 1976b; Farr and Lichtwardt 1967; Gottlieb and Lichtwardt 2001; Horn 1989a, 1989b, 1990; Horn and Lichtwardt 1981; Koontz 2006; White 2006; White et al. 2006a; Williams 1983a; Williams and Lichtwardt 1972a, 1972b). 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* (James et al. 2006, Liu et al. 2006, O'Donnell et al. 1998, Walker 1984).

Walker (1984) constructed the first phylogenetic tree based on 5S rRNA gene 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 sequence diversity to be great 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) and18S rRNA gene phylogeny was mapped with morphological as well as physiological characters and lifestyles. Compared to the earlier 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 various 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 5 lineages, though still lacking resolution with the single 18S

rRNA gene data, 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 at the species level within *Smittium*. This undoubtedly highlights the diversity within the genus itself, but perhaps it does not necessarily preclude the possible future utility of this region at the bar coding level once all the major subclades and lineages are resolved (Bellemain et al. 2010).

These phylogenetic studies have disproportionally included culturable taxa, understandably since they provide pure and higher concentrations of genomic DNA. However, PCR has also 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 permits us to define and refine relationships among taxa within nodes (A–D) and/or as subclades (in FIGS. 1.9–1.13).

"Non-*Smittium*" clade

Zancudomyces culisetae forms a strongly supported cluster of 10 different representatives from 6 geographic areas and reinforces earlier notions (Grigg and Lichtwardt 1996, Lichtwardt and White 2011, Sangar et al. 1972, White 2006) 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 only been observed with sexual spores on two occasions at one site in Nebraska [FIGS. 1.2–1.4; 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.

Earlier 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 bold, TABLE 1.1). For example, *Coleopteromyces* Ferrington, Lichtw. & López Lastra*, Graminella, Lancisporomyces* Santam.*, Spartiella,* and *Trichozygospora*, are all 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 also to note that *Z. culisetae* has been recorded once from a mayfly host (Lichtwardt et al. 2001) and is clustered with these and other mayfly gut fungi (*Zygopolaris* and *Bojamyces*). There are exceptions to this host specificity notion, which expands to include gut fungi from stonefly and caddis worm hosts (with the unnamed Harpellales from CA) as well, although with slightly less support. Stronger branch support might permit further discussion of possible host switching events, 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 was first 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 with one other southern hemisphere isolate (ARG-GM-2) from Argentina (TABLE 1.1). 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, and 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 even 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 size ranges 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 (*Smittium* subclade 3, FIG. 1.11) 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 earlier that this isolate did not prevent molting of larvae that were infected with it *in vitro*. Since 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. 1.9) carries some significance since it includes the epitype *Smittium mucronatum* (Lichtwardt and White 2011) and will in some way carry the name *Smittium*, pending future 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 morpho-species 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, amongst 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 is also worthy of finer scrutiny for lineage sorting and possible cryptic species.

Subclade #2

Smittium subclade 2 (FIG. 1.10) 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 the symbiotic lifestyle 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 or not taxa morphologically similar to *Smittium morbosum* exist that are also parasitic or whether such a lifestyle 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 recently 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. 1.11), which includes the largest number of *Smittium* and allies, split with strong support from the subclade 2 (FIG. 1.7). *Smittium simulii* was notably dispersed amongst 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 amongst 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* + *orthocladii* + *perforatum*) 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 or not 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 on this pending further analysis and better resolution with our ongoing efforts to build a multigene data set that will hopefully help resolve some of these issues.

"Non-*Smittium*" Allies amongst *Smittium* Subclades 1–3

Finally, several "Non-*Smittium*" genera, referred to as allies above, warrant further commentary (Supp. FIG. 1.1). 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, are also 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 with 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 (see TABLE 1.1) as either a *Stachylina* sp. or *Smittium* sp., emerged in subclade 3, and we now conservatively refer to this as a *Smittium* sp. indet. 3 (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 amongst the *Austrosmittium* samples in hand has even presented some challenges with the primers and cycling profiles that are otherwise 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 over this timeframe. 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 quite 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 for this study, either qualitatively or quantitatively, we have conducted a rather cursory examination of the morphology of the trichospore. Amongst 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. Subclade3 tends to have members with longer and narrower trichospores (see SUPP. TABLE 1.1). Specifically members of subclade 3 maintain a ratio of length to width from 3.75 to 9.76, whereas subclade 1 ranges from 2.67 to 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 length to width 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.

References

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 Microbiology* 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.

Clark TB, Kellen WR, Lindegren JE. 1963. Axenic culture of two Trichomycetes from Californian mosquitoes. *Nature* 197:208–209.

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.

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

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

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

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

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

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.

Grigg R, Lichtwardt RW. 1996. Isozyme patterns in cultured Harpellales. *Mycologia* 88:219–229

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.

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.

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

———. 1990. Physiological changes associated with sporangiospore extrusion from trichospores of *Smittium culisetae*. *Exp Mycol* 14:113–123.

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

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.

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.

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 Nat Sci Phila* 4:225–233.

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

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

———. 1850b. Descriptions of new Entophyta growing within animals. *Proc Acad Nat 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]. University of Illinois. 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. *Amer J Bot* 51:836–842.

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

———. 1986. The Trichomycetes: Fungal Associates of Arthropods. Springer-Verlag, New York, 343 p.

———. 2004. Trichomycetes: Fungi in relationship with insects and other arthropods. *Symbiosis* 4:575–588

———, Arenas JM. 1996. Trichomycetes in aquatic insects from southern Chile. *Mycologia* 88:844–857.

———, Cafaro MJ, White MM. 2001. The Trichomycetes, fungal associates of arthropods. Revised edition. 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.

———, Moss ST. 1981. Vegetative propagation in a new species of Harpellales, *Graminella microspora*. *Trans Br Mycol Soc* 76:311–316.

———, ———. 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.

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

———, ———, White MM. 2011. *Klastostachys*, a new genus of Harpellales in Chironomidae larvae. *Mycologia* 103:915–917.

———, White MM. 2011. Typification of *Smittium*, an important genus in the taxonomy of Harpellales. *Mycologia* 103:915–917.

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.

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.

Maddison WP, Maddison DR. 2010. Mesquite: A modular system for evolutionary analysis. Version 2.73 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.

Moss ST. 1972. Occurrence, cell Structure and taxonomy of the Trichomycetes, with special reference to electron microscope studies of *Stachylina* [Ph. D. dissertation]. University of Reading. 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.

———, ———. 1977. Zygospores of the Harpellales: An ultrastructural study. *Can J Bot* 55:3099–3110.

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

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

———, Young TWK. 1978. Phyletic Considerations of the Harpellales and Asellariales (Trichomycetes, Zygomycotina) and the Kickxellales (Zygomycetes, Zygomycotina). *Mycologia* 70:944–963.

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

———, ———, 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.

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 Endomycète nouveau: *Smittium arvernense* n. g., n. sp., parasite intestinal delarves de *Smittia* sp. (Diptères Chironomides) et description d'une nouvelle espèce du genre *Stachylina* Léger et Gauth. 1932. *Bull Soc Sci Bretagne* 14(18):20–31.

Porter D, Smiley R. 1979. Ribosomal RNA molecular weights of Trichomycetes and Zygomycetes. *Exp Mycol* 3:188–193.

Posada D. 2008. jModelTest: Phylogenetic Model Averaging. *Mol Biol Evol* 25:1253–1256.

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.

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

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

Strongman DB, White MM. 2008. Trichomycetes from lentic and lotic aquatic habitats in Ontario, Canada. *Botany* 86:1449–1466.

Sweeney AW. 1981. An undescribed species of *Smittium* (Trichomycetes) pathogenic to mosquito larvae in Australia. *Trans Br Mycol Soc* 77:55–60.

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(15):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(17):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(17):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.

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

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.

Whisler HC. 1961. Cultural studies of the Trichomycetes [Ph. D. dissertation]. University of California, Berkeley. 141 p.

———. 1962. Culture and nutrition of *Amoebidium parasiticum*. *Am J Bot* 49:193–199.

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

———. 1968. Developmental control of *Amoebidium parasiticum*. *Dev Biol* 17:562–570.

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

———. 2006. Evolutionary implications of a RNA-based phylogeny of Harpellales. *Mycol Res* 110:1011–1024.

———, 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: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.

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

———,———, 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.

———, Siri A, Lichtwardt RW. 2006c. Trichomycete insect symbionts in Great Smoky Mountains National Park and vicinity. *Mycologia* 98:333–352.

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 Inc: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.

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

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

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

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [Ph.D. dissertation]. The University of Texas at Austin. 125 p.

TABLE 1.1. List of taxa used in this study, with species isolate or strain codes, whether it was from culture, with collector information. Also 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[1] or Source	Host	Origin	Bench Code (18S, 28S)	GenBank Numbers[2]	
							18S	28S
Coemansi areversa	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[3]	ARG-GM-4	yes	GM/CLL	Diptera	Argentina	754	**JQ302880**	**JQ302954**
Zancudomyces culisetae	ARG-LL-6	yes	CLL	*Aedesal bopictus*	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[4]	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	*Aedesaegypti*	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[4]	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	-
Harpella meridianalis[5]	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[5]	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**	-
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**
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**
Smittiume longatum	AUS-59-5L	yes	RWL	*Cardiocladius australiensis*	Australia	326	10442589	-

Smittium sp. indet. 1[6]	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[6]	AS-22-15	yes	AS	*Cricotopus sp.*	New Zealand	367	**JQ302858**	**JQ302936**
*Smittium*sp. indet. 2[6]	LCF-27-15	n	LCF	Orthocladiinae	New Zealand	368	**JQ302859**	**JQ302937**
*Smittium*sp. indet. 2[6]	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	*Dixafluvica*	United States	193	89033406	-
Stachylina grandispora	KS-70-W11&18	n	MMW	*Chironomus riparius*	United States	290	**JQ302846**	**JQ302924**
Smittium sp. indet. 3[6]	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[6]	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**	-

Footnotes:

[1.] 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.

[2.] Accession numbers in bold were generated for this study.

[3.] Isolates of "Non-*Smittium*" taxa in bold are presented for the first time in this study.

[4.] The 18S rRNA gene was obtained from GenBank, and the 28S rRNA gene was sequenced from this study.

[5.] 18S and 28S for two samples from the same region were combined for the 18S and 28S analysis.

[6.] Supplemental information on these samples: *Smittium* sp. indet. 1 ("stenosporum" is an epithet that has been considered); *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 being 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).

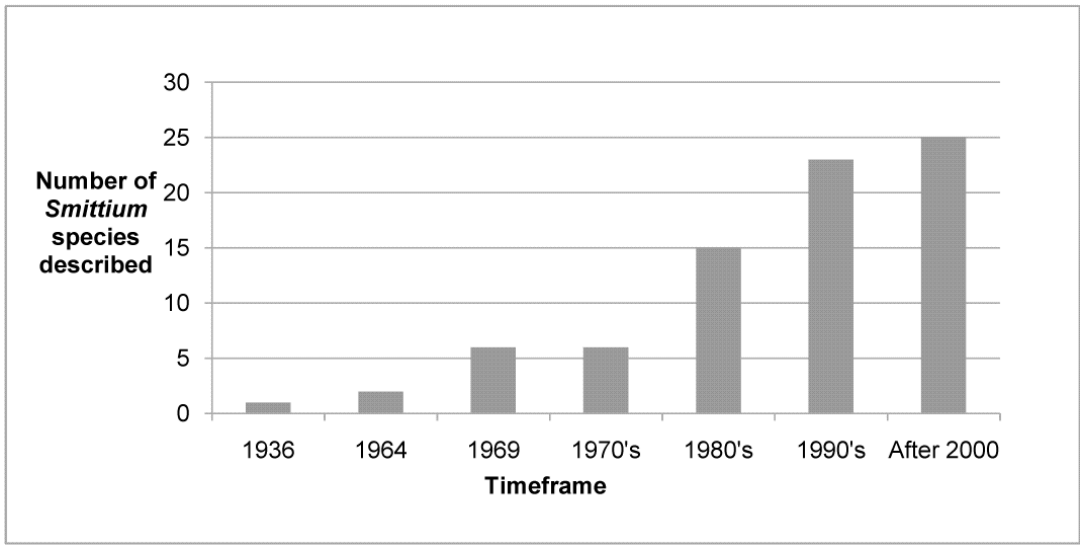
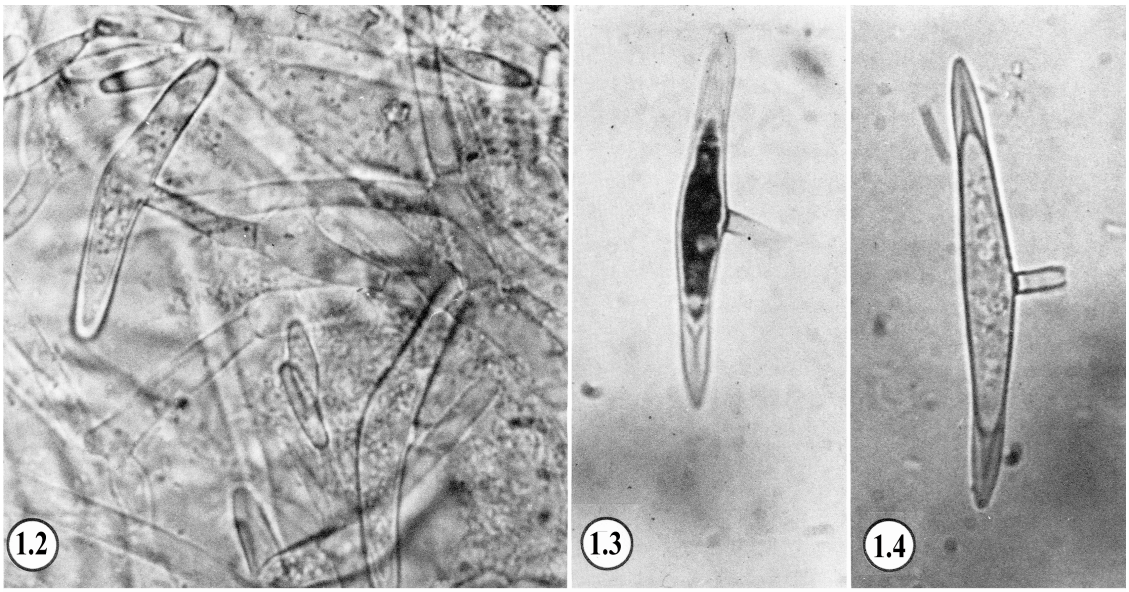


FIG. 1.1. Number of new species of *Smittium* described per indicated timeframe after the first type species, *Smittium arvernense*, was described by Poisson (1936). The trend presented by the numbers has been increasing continuously from 1969 to date. *Smittium culisetae* (now *Zancudomyces culisetae*) described by Lichtwardt (1964) is included in this representation.



FIGS. 1.2–1.4. *Zancudomyces culisetae* zygospores. 1.2. Immature zygospores in a mass of *Z. culisetae* hyphae and some trichospores, x 800. 1.3–1.4. Mature, loose zygospores, x 1000. [From Williams (1983b)].

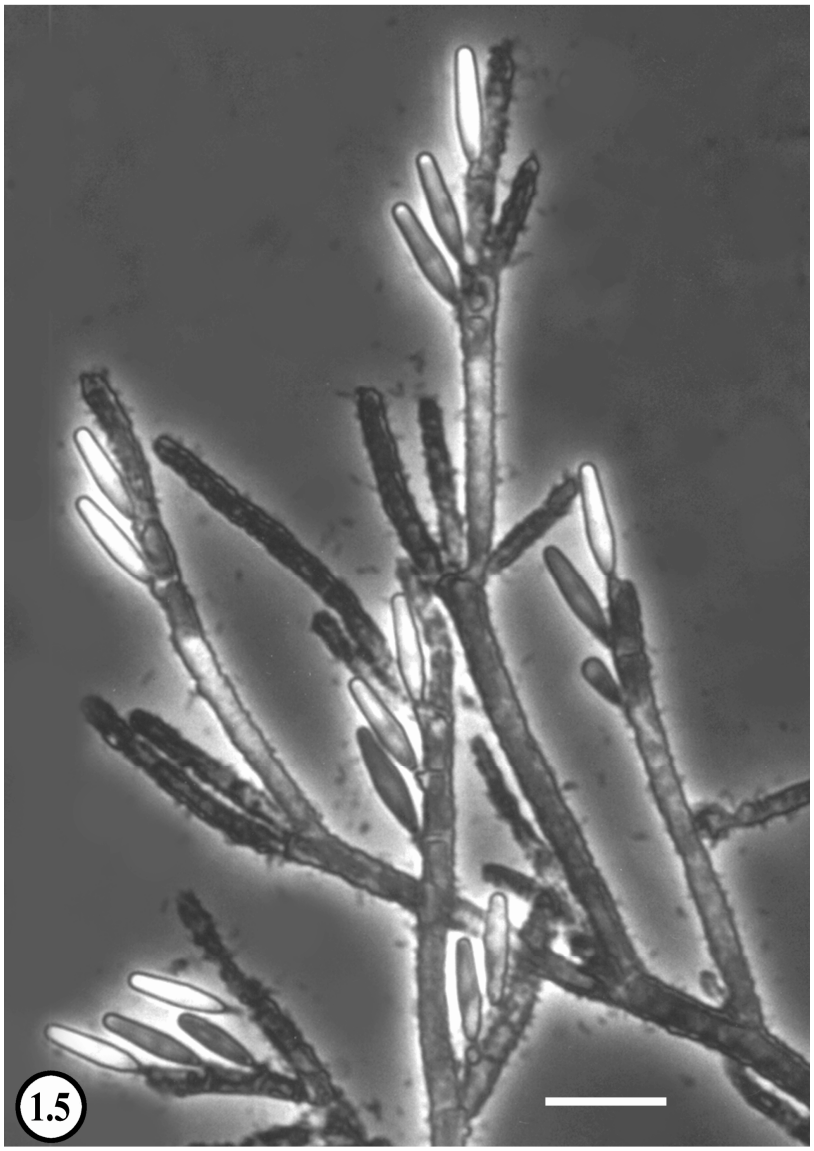


FIG. 1.5. *Zancudomyces culisetae* with attached trichospores and some verticillate branching. Dissected from a mosquito larva (microscope slide TN-46-7, photomicrograph TN-S-1) and collected from the Great Smoky Mountains National Park, USA. Scale bar = 20 µm.

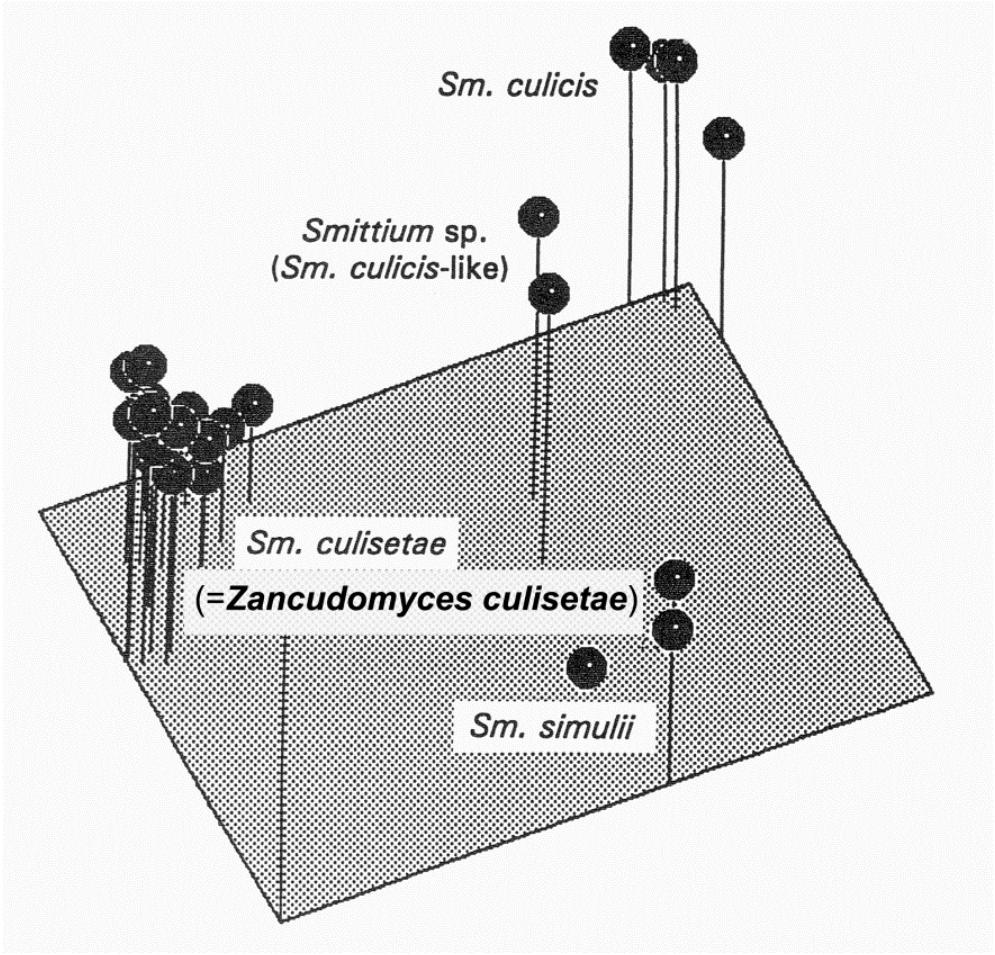


FIG. 1.6. Three dimensional model constructed from the three principal coordinates of enzyme variation similarity in 11 enzyme systems with 13 loci for 41 isolates of *Smittium* representing four species. Thirty-two isolates of *Z. culisetae* from different geographical regions are not apparent in the cluster because of many identical isozyme patterns. [Modified, from Grigg and Lichtwardt (1996)].

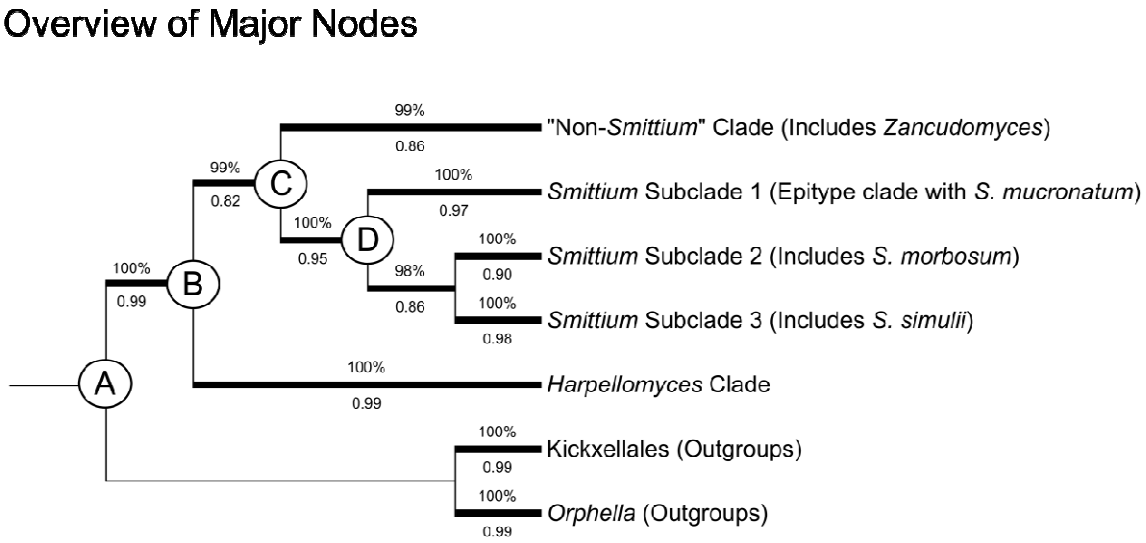


FIG. 1.7. Overview tree of major clades and nodes from complete phylogenetic tree including representative Harpellales and some Kickxellales. Subclades are collapsed for clarity. For this and all further trees, supports above the branches are Bayesian posterior probabilities (BPP) and below are maximum-likelihood bootstrap proportions (MLBP). Branches in bold are considered to be with strong support (with BPP> 95% and MLBP>.70).

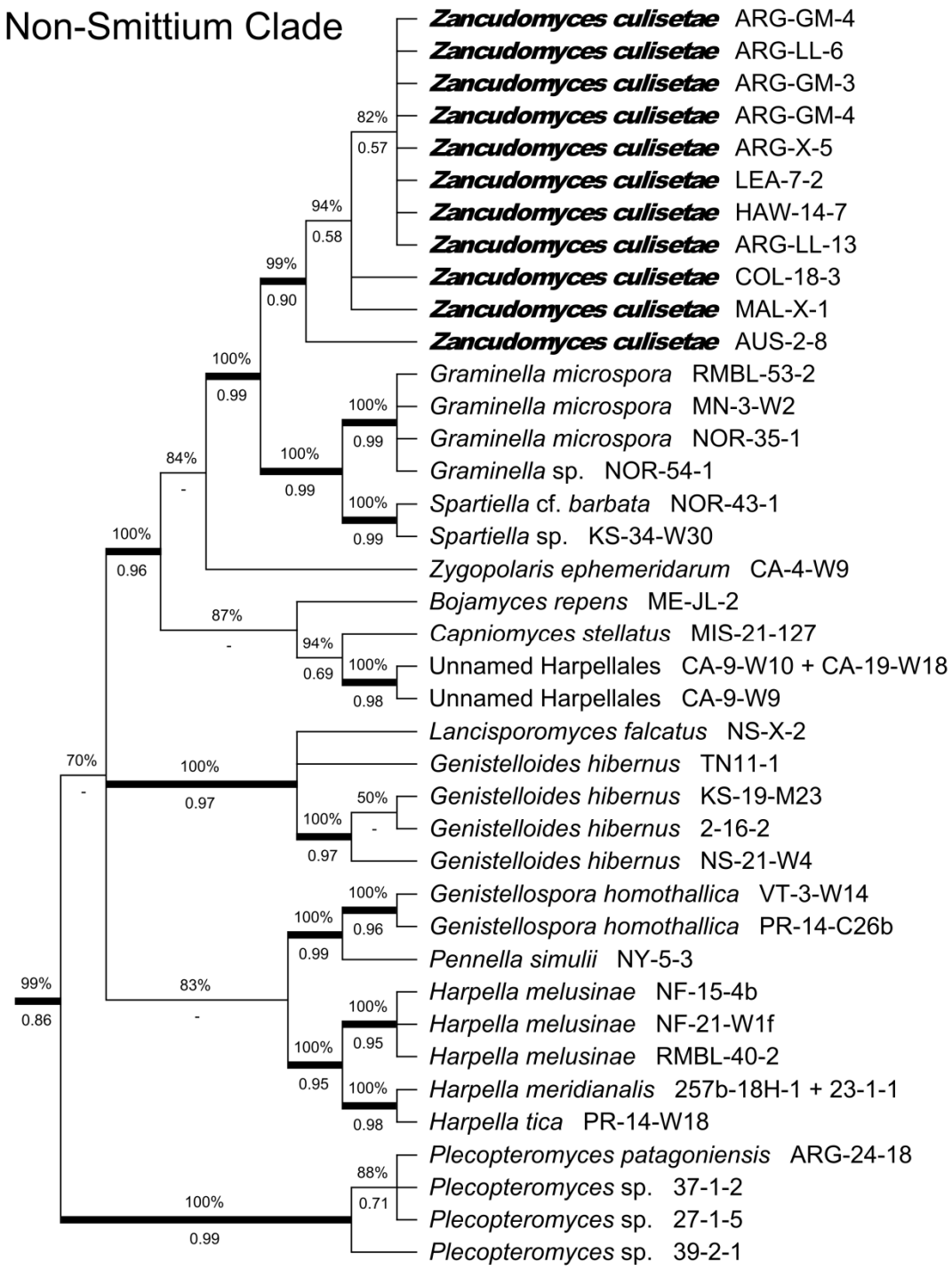


FIG. 1.8. "Non-*Smittium*" clade from the complete phylogenetic tree, including *Zancudomyces culisetae* (previously known as *Smittium culisetae*). This clade includes species from both the Harpellaceae and Legeriomycetaceae.

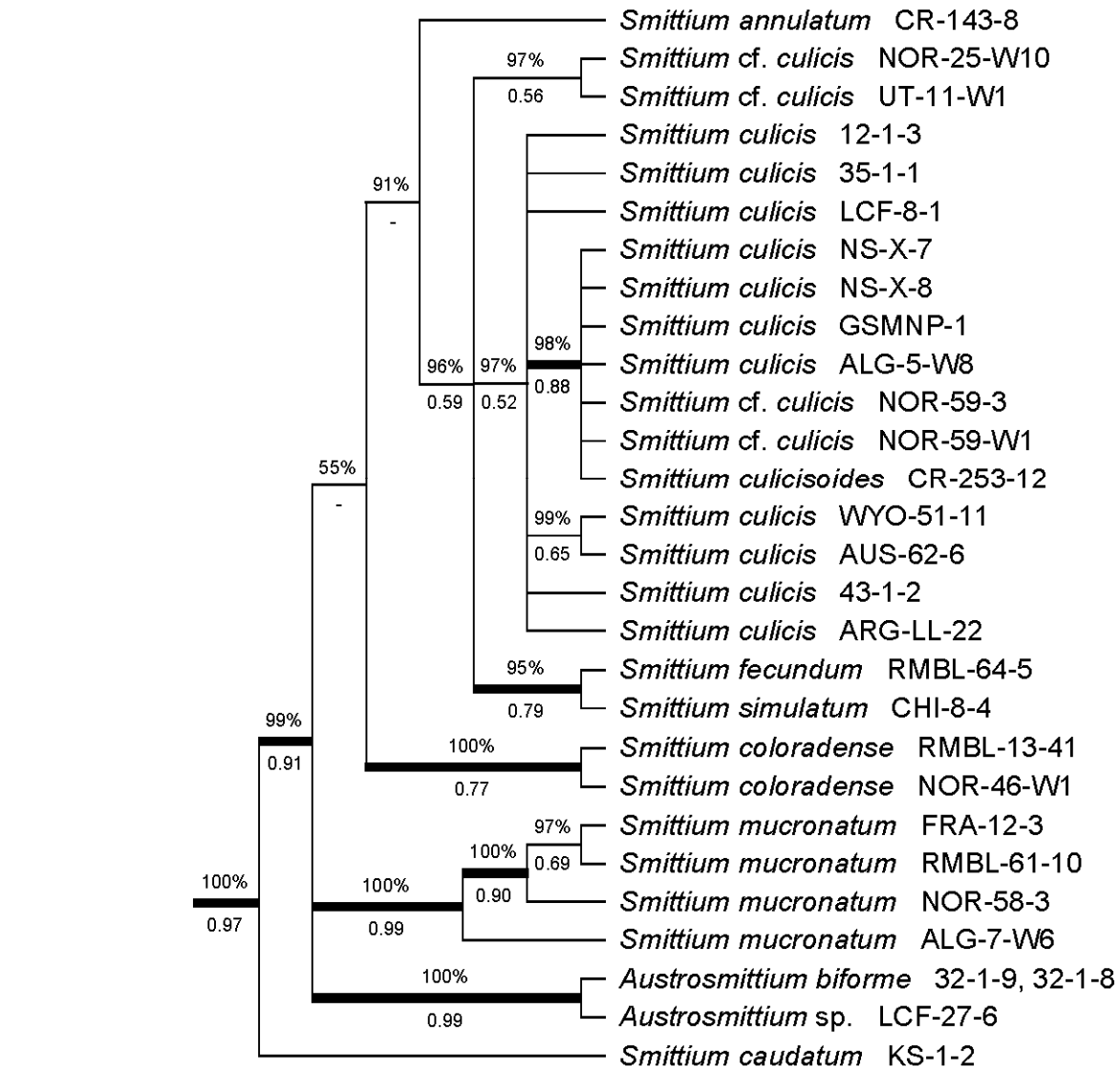


FIG. 1.9. *Smittium* subclade 1, including the epitype *Smittium mucronatum* amongst other Smittiums, as well as the well-studied and wide spread *S. culicis* and *Austrosmittium*.

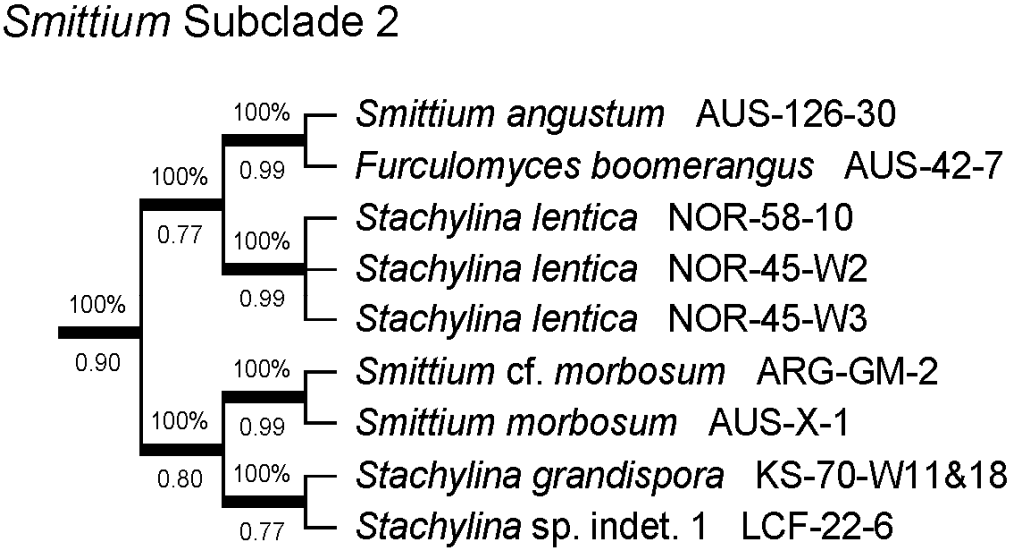


FIG. 1.10. *Smittium* subclade 2, including the true *Smittium morbosum* (AUS-X-1), the only recognized parasitic *Smittium* as well as all sequenced members of the genera *Furculomyces* and *Stachylina*. Isolate AUS-X-1 is the authentic culture of *Smittium morbosum* solidifying its true position in the tree. *Smittium angustum* may actually 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.

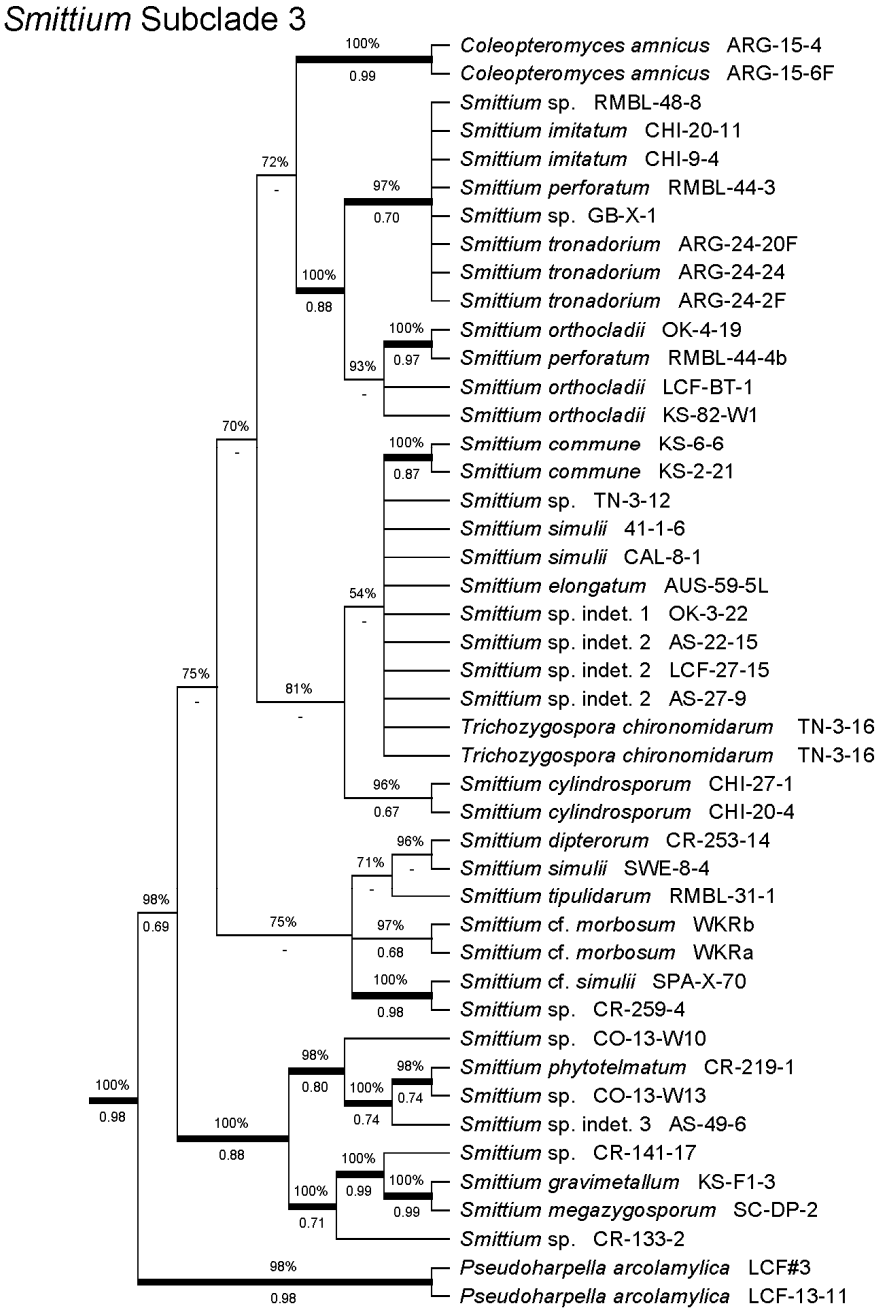
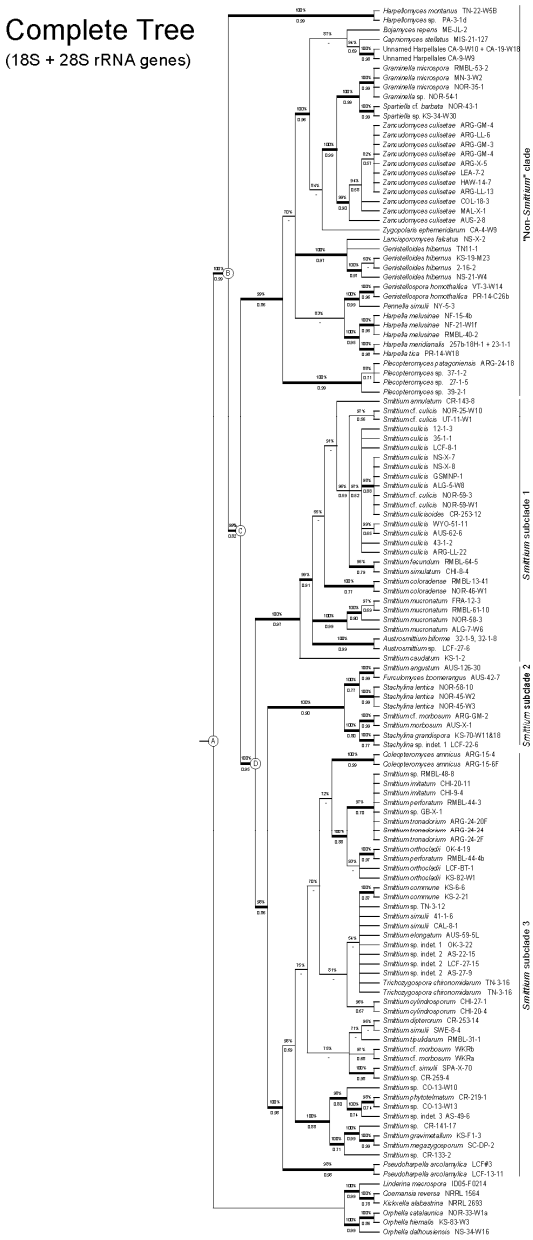


FIG. 1.11. *Smittium* subclade 3. A diverse group with numerous *Smittium* species, including *Smittium simulii.* Also included are *Coleopteromyces, Pseudoharpella* and *Trichozygospora*. Conspicuously, two isololates (WKRa and WKRb) originally thought to be *Smittium morbosum* did not cluster with the type culture for this species (AUS-X-1) and represent misidentifications. Some morpho-species (such as exemplars of *Smittium commune* and *Smittium cylindrosporum*) are well-supported, based on their earlier identifications, but clusters of others may represent cryptic species, although poor resolution hinders a more complete assessment of many of these, pending future study.

SUPP. TABLE 1.1. Comparison of trichospore length, width, and collar length, within and between members of *Smittium* subclades 1 and 3.

	Species	Average Trichospore Length	Average Trichospore Width	Trichospore Length/Width	Average Collar Length	Trichospore Length/Collar Length
Smittium subclade 1	*Smittium annulatum*	20	5	4.00	4.5	4.44
	Smittium caudatum	16	6	2.67	12.5	1.28
	Smittium coloradense	26	8	3.25	12.5	2.08
	Smittium culicis	20	6	3.33	7	2.86
	Smittium culicisoides	22.5	8	2.81	7.5	3.00
	Smittium fecundum	18.5	6.5	2.85	7.5	2.47
	Smittium simulatum	21	7	3.00	5	4.20
	Smittium mucronatum	35	6.75	5.19	8.25	4.24
	Subclade-1 Average:	22.38	6.66	3.39	8.09	3.07
Smittium subclade 3	*Smittium commune*	15	4	3.75	2	7.50
	Smittium cylindrosporum	29.5	5	5.90	5	5.90
	Smittium dipterorum	15	2.5	6.00	2	7.50
	Smittium elongatum	34	4.5	7.56	3	11.33
	Smittium gravimetallum	28.5	3	9.50	1.5	19.00
	Smittium imitatum	19	5	3.80	2	9.50
	Smittium megazygosporum	41.5	4.25	9.76	3.75	11.07
	Smittium orthocladii	30	7	4.29	7.5	4.00
	Smittium perforatum	38	7.9	4.81	7	5.43
	Smittium phytotelmatum	21	2.5	8.40	2.5	8.40
	Smittium simulii	23	5	4.60	2.5	9.20
	Smittium tipulidarum	17.5	4.5	3.89	2.6	6.73
	Smittium tronadorium	23	4	5.75	2	11.50
	Subclade-3 Average:	25.77	4.55	6.00	3.33	9.00



SUPP. FIG. 1.1. Complete phylogenetic tree with combined 18S and 28S rRNA genes. Supports above the branches are Bayesian posterior probability, and below the branches are based on the maximum-likelihood bootstrap proportions. Branches in bold indicate high support (BPP> 95%, MLBP> .70). This tree is summarized with the guide tree (FIG. 1.7).

CHAPTER TWO: TESTING MORPHOLOGY-BASED HYPOTHESES OF PHYLOGENETIC RELATIONSHIPS OF THE MAJOR "SMITTIUM" CLADE (HARPELLALES) USING FIVE-GENE PHYLOGENY

Abstract

Smittium, one of the first described genera of gut fungi, is part of a larger group of endosymbiotic microorganisms (Harpellales) that live predominantly, in the digestive tracts of aquatic insects. As a diverse and species-rich taxon, *Smittium* has helped to advance our understanding of the gut fungi, in part, due to its high culturability rate (approximately 40%) amongst the 81 known species. From those isolates, earlier studies have ranged from those relating to host specificity, growth parameters, thallus development, ultrastructure, serological, and isozyme variability as well as ongoing molecular phylogenetic and systematic efforts. *Smittium* is polyphyletic based on previous molecular-based phylogenetic analyses using single and combined ribosomal RNA genes. Species of *Smittium* and related taxa have clustered loosely and generally been regarded as the "Smittium" clade. A multigene dataset consisting of 18S and 28S rRNA genes, as well as RPB1, RPB2, and MCM7 translated protein sequences was

constructed for *Smittium* and related taxa of Harpellales (including *Austrosmittium, Coleopteromyces, Furculomyces, Pseudoharpella, Stachylina* and *Trichozygospora*). The supermatrix was used for phylogenetic analyses and provided strong support for inferred relationships at multiple levels, based on Bayesian and maximum likelihood assessments. Strongly supported clades and branches of the consensus tree were assessed relative to morphological traits for the taxa of interest. Features including holdfast shape, thallus branching type, trichospore and zygospore characters are assessed as an aid to inform the current morphologically-based taxonomy and to move toward eventual molecular systematic-based revisions and reclassification. Some patterned separation was found within the "Smittium" clade, including the separation of "True *Smittium*" clade and "Parasmittium" clade, which was supported also by morphological features including thallus branching types, trichospore shapes, and perhaps lending support to an earlier narrower definition of the genus. Parasmittium subclades near and sister to the "True *Smittium*" clade are similarly compared. Suggestions for future collection, description, and studies are also provided as ongoing efforts are unfolding.

Introduction

From a modern point of view, adaptation and evolution are critically important for diversity at every level of organismal biology, from DNA molecules to individuals, populations and species (Hall and Hallgrímsson 2008). Coevolution is the reciprocal response by individuals of two populations to invoke evolutionary changes in a trait (Janzen 1980). Symbiosis, a lifestyle presented across organismal types, should not be underestimated especially when accentuated via coevolution, which has been a driver of some remarkable relationships and patterns (Blackwell 2010, Clark et al. 2000, Currie et al. 2003, Little and Currie 2007, Moran and Jarvik 2010, Scarborough et al. 2005, Slaymaker et al. 1998).

One group that has received less attention for its potential to eventually demonstrate coevolutionary patterns is the gut fungi or Trichomycetes. Trichomycetes, as a class, was established by Manier and Lichtwardt (1968). With one genus (*Amoebidium*) as an exception, they are all obligately endosymbiotic within the digestive tracts of arthropods. Traditionally, Trichomycetes included not only the Amoebidiales (Léger and Duboscq 1929) but also the Asellariales (Manier ex Manier and Lichtwardt 1978, in Lichtwardt and Manier 1978), Eccrinales (Léger and Duboscq 1929), and Harpellales (Lichtwardt and Manier 1978). Molecular-based phylogenies have revolutionized our

understanding of fungal taxonomy and systematics (Hibbett et al. 2007, James et al. 2006). This is also true for the Trichomycetes, where the Amoebidiales (Benny and O'Donnell 2000) and Eccrinales (Cafaro 2005) have both been reclassified as Protists.

Members of Harpellales are commonly associated with immature stages of various non-predaceous insects, or rarely Isopoda (White 1999). *Smittium* R.A. Poiss., the most species-rich genus of the Harpellales, was described from the gut of, and named after, the host midge *Smittia* (Poisson 1936). *Smittium* is one of the oldest genera of the harpellids, currently loosely included within the Kickxellomycotina (Hibbett et al. 2007). They all live in the hindgut of larval Nematocera (Diptera) (Lichtwardt et al. 2001). Owing to the culturability of some species, *Smittium* has been used as a "model harpellid" to assess the nature of the symbiosis, from growth studies to spore germination and host feeding assessments (El-Buni and Lichtwardt 1976a, 1976b; Lichtwardt 2008; Lichtwardt et al. 2001; Sweeney 1981; White et al. 2006a; Williams 1983a, 1983b). Now consisting of 81 species, the generic description for *Smittium* has expanded to include members with branched thalli, ellipsoidal (or sub-ellipsoidal) to almost cylindrical trichospores (asexual spores) having a short or long collar and a single appendage (when detached), and biconical to fusiform zygospores (sexual spores), attached to the zygosporophore obliquely and submedially, upon detachment having a collar and single appendage (Lichtwardt et al. 2001).

Molecular-based phylogenies helped to prompt and permit the reclassification of Kingdom Fungi (Hibbett et al. 2007). Among the most dramatic shifts in the classification was deconstruction of the phylum Zygomycota. Orders were variously distributed and several subphyla listed as *incertae sedis*, including not only the Kickxellomycotina but also the Mucoromycotina, Entomophthoromycotina and Zoopagomycotina. In fact, the early-diverging section of the fungal tree of life remained as a loose aggregation of clades. Some of this relates to a lack of morphological characters and/or states, as much as any misapplication of them (Wang et al. 2012, White 2006), but the effort highlighted the importance of robust and well-supported molecular phylogenies to better understand the evolutionary patterns among the early-diverging fungi (Hibbett et al. 2007).

Phylogenetically, *Smittium* is polyphyletic based on single and combined 18S rRNA and 28S rRNA gene analyses (Wang et al. 2012, White 2006). Smittiums have phylogenetically associated with "Non-*Smittium*" Harpellales, including species of *Austrosmittium, Coleopteromyces, Furculomyces, Pseudoharpella, Stachylina,* and *Trichozygospora*, though not always with strong support (Gottlieb and Lichtwardt 2001, Wang et al. 2012, White 2006). *Zancudomyces culisetae* Y. Wang, Tretter, Lichtw. & M.M. White (previously known as *Smittium culisetae* Lichtw.), the newly established type for this monotypic genus, has been proved distinct from *Smittium*, based on

combined 18S and 28S rRNA gene phylogenies, as well as the different zygospore type, trichospore morphology, isozyme patterns and immunological evidence from earlier studies (Grigg and Lichtwardt 1996, Sanger et al. 1972, Wang et al. 2012, Williams 1983b). However, even with the establishment of *Zancudomyces*, *Smittium* still requires further study.

Among the allied (=putatively closely related) genera, *Austrosmittium* is distinguished morphologically based on its medially-expanded biconical zygospores, although other features are similar to *Smittium* (Lichtwardt and Williams 1992a). Despite having a beetle host (rather than a lower dipteran), the trichospore of *Coleopteromyces amnicus* is very similar to *Smittium*, although the isthma, a structure between the collar and trichospore was considered in distinguishing *C. amnicus* from *Smittium* (Lichtwardt et al. 1999). *Furculomyces boomerangus* is distinguished by its boomerang-shaped (bent) zygospores borne on a furculum (=wishbone-like conjugation apparatus), formed by the thallus (Lichtwardt and Williams 1992b). *Pseudoharpella arcolamylica* has a long and coiled trichospore as well as three broad appendages when detached (Ferrington et al. 2003), and both features are different from *Smittium*. *Stachylina* are all unbranched and midgut dwelling; therefore, they are members of the other family, Harpellaceae (Lichtwardt et al. 2001). *Trichozygospora chironomidarum* might otherwise be considered a *Smittium,* except for its multiple (>10) appendages on both trichospores and

zygospores (Lichtwardt 1972). With the exception of *Stachylina*, all of these are branched hindgut dwelling members traditionally included in the Legeriomycetaceae. Based on both morphological and molecular assessment (single and combined 18S and 28S rRNA genes phylogenies), they are all considered to be *Smittium* allies (Gottlieb and Lichtwardt 2001, Wang et al. 2012, White 2006).

The resolving power and stability offered by a multigene phylogenetic approach provides a powerful tool for molecular systematics and has revolutionized our understanding of various parts of the tree of life. For example, the loss of the flagellum has been tracked during fungal evolution from the oceans to terrestrial environments (James et al. 2006), and the evolution of hyphal septa features have been revealed in the Kingdom of Fungi (Lutzoni et al. 2004). Findings in other kingdoms of life, such as the origin of animals (Shalchian-Tabrizi et al 2008), confirmation of Coleochaetales as the closest relative of land plants (Finet et al. 2010), and the evolutionary position of "primitive" eukaryotes, the jakobids, within excavate protists (Simpson et al. 2006), have been aided by multigene phylogenies. This is also true for other examples related to the longer-term interactions of fungi with other organisms (Blackwell 2010, Clark et al. 2000, Currie et al. 2003, Little and Currie 2007, Moran and Jarvik 2010, Scarborough et al. 2005, Slaymaker et al. 1998).

The challenge of molecular phylogenetics is to match maximal taxon sampling with sufficient and informative data for the level of questioning and hypothesis testing. Gene selection is critical for the analysis. It must be conservative enough for reliable sequence alignment and sufficiently variable to offer informative evolving characters (Schmitt et al. 2009). Nuclear rRNA genes, both the small and large subunits, have been used previously with the Trichomycetes (Ogawa et al. 2005; Porter and Smiley 1979; Tehler et al. 2000; Walker1984; White 2006; White et al. 2006a, 2006b) although the ITS region was found not to be suitable for comparison at the species level within *Smittium*, due to the sequence and length variation encountered (Gottlieb and Lichtwardt 2001). During the last decade, the single copy protein-coding genes RPB1 and RPB2 have provided well-resolved and highly supported fungal phylogenies (Frøslev et al. 2005; James et al. 2006; Liu et al. 2006; Matheny 2005; Matheny et al. 2002, 2007). More recently, MCM7 and TSR1, two newly developed markers, have shown great resolving power and have outperformed many other single-copy protein-coding genes (not only RPB1, RPB2, β-tubulin, but also EF-1α, and γ-actin) according to bioinformatic assessments of gene performance in phylogenetic analysis (Aguileta et al. 2008, Schmitt et al. 2009).

Although the number of multigene phylogenetic analyses of fungi has increased over the past decades, the proportion of such studies in Harpellales (gut fungi) is still rare

(James et al. 2006, Matheny et al. 2007). In this study we used a multigene approach including the traditional 18S and 28S rRNA genes and the previously used protein-coding genes RPB1, RPB2, and MCM7 in an attempt to resolve the evolutionary relationship within *Smittium*. One of the main objectives of this research was to test the monophyly of this species-rich genus of Harpellales and map morphological characters, where possible, to assess their taxonomic significance against a molecular-based phylogeny. To help legitimize the assessment of evolutionary relationships, as many allied genera as possible (*Austrosmittium*, *Coleopteromyces*, *Furculomyces*, *Pseudoharpella*, *Stachylina*, and *Trichozygospora*) were targeted for a combined five-gene phylogenetic analysis and morphological comparison across taxa. The morphological characters assessed here include holdfast shape, thallus branching type, trichospore and zygospore shapes. The overarching goal is toward a more solid phylogenetic-based framework for *Smittium*, incorporating a morphological perspective.

Materials and Methods

Host Collection and Specimen Preparation

Collection of larval aquatic insects and preparation of fungal thalli for DNA extraction were as described by Wang et al. (2012). Representative exemplars (vouchers of morpho-species) of *Smittium* were selected based on availability, with efforts to include as much morphological variability as possible, including holdfast shape, thallus

branching type, trichospore shapes, and zygospore plasticity within *Smittium*; but this approach also extended to the selection of allied genera. The results of the combined rRNA genes analysis of Wang et al. (2012) also helped inform taxon sampling with the current knowledge of relationships within the Harpellales. Some specimens were prepared by placing colonies of axenic cultures into 500 µl CTAB buffer. Several samples were from genomic DNA preparations used earlier by Gottlieb and Lichtwardt (2001). In total this study included 99 taxa, 60 of which represented 25 *Smittium* species, with the rest being 13 *Smittium* allies, 23 "Non-*Smittium*" Harpellales, and 3 Kickxellales—*Coemansia reversa*, *Kickxella alabastrina*, and *Linderina pennispora*—as the outgroup (TABLE 2.1).

DNA Extraction, PCR Amplification, and Direct Sequencing

DNA was extracted from samples in CTAB buffer according to earlier protocols (Gottlieb and Lichtwardt 2001, O'Donnell et al. 1998, Wang et al. 2012, White 2006). General procedures for PCR amplification of 18S and 28S rRNA genes and direct sequencing method were described in Wang et al. (2012). Primers NS1AA and NS8AA (a new primer combination that is Harpellales/*Smittium* specific and developed to minimize host amplification) as well as NL1AA and LR7AA (similarly specific) were used to obtain amplified PCR products as well as new sequences of 18S and 28S.

Amplifications for the RPB1 and RPB2 were attempted with primer pairs RPB1 (Afl–Drl) and RPB2 (5F–7cR) (modified from Ben Hall unpubl., Liu et al. 1999). For MCM7, we used the primer pair 8bf–16r (modified from Schmitt et al. 2009). For the list of primers and codes used for various amplification types in this study see TABLE 2.2.

The Promega green hot master mix kit was used for RPB1, RPB2 and MCM7. The reaction cocktail contained: 11 µL Promega Go-Taq green master mix, 2.20 µL (or 1.76 µL for RPB2) of both forward and reverse primers at a concentration of 10.0 pM/uL, 0.44 µL (0.66 µL for RPB2) of 25 mM $MgCl_2$ (to a total concentration of 2.5 mM for RPB1 and MCM7; 2.75 mM for RPB2), 4.16 µL (4.82 µL for RPB2) of molecular biology grade H_2O, and 2 µL of diluted DNA template.

Thermal cycling protocols for the primer combinations of NS1AA / NS8AA and NL1AA / LR7AA were modified from Wang et al. (2012) with the annealing temperature being changed to 62°C for the 18S rRNA gene and 56°C (no touch-down) for the 28S. For RPB1 and RPB2, cycling conditions included an initial denaturation step of 95°C for 2 min, 50 cycles of denaturation at 95°C for 1 min, used with a touch-down annealing section of the profile programmed to step down from 57°C to 47°C (reduced a tenth of a degree every cycle) except for RPB2 where it stepped from 53°C to 43°C for 75 s, and with an extension at 72°C for 165 s, followed by a final extension step at 72°C for 10

min, with a final hold at 4°C. For the MCM7 gene, we included an initial denaturation step of 95°C for 2 min, 45 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 90 s, followed by a final extension step at 72°C for 10 min and then a final hold at 4°C.

Sequencher (v5.0) was used to assemble sequences. In a few instances, we used PeakTrace Basecaller (http://www.nucleics.com/peaktrace-sequencing/) to obtain slightly longer, usable sequencing reads before assembling.

Sequence Alignment and Model Determination

Assembled sequences of 99 taxa consisting of various *Smittium* species as well as other members of the Harpellales and some outgroups from the Kickxellales were combined into a single data set with previously published or submitted sequences (Gottlieb and Lichtwardt 2001, James et al. 2006, Liu et al. 2006, O'Donnell et al. 1998, Wang et al. 2012, White 2006). This study utilized five genes with 18S and 28S as nucleotides, and RPB1, RPB2, and MCM7 translated into amino acids. Most of the 18S rRNA gene and approximately the first 1500 bp of the 28S, as well as partial single-copy protein-coding genes for RPB1, RPB2, and MCM7 were used in single and combined phylogenetic analyses. The number of 18S sequences was 98 and for 28S there were 99. For protein-coding genes, we included 75 RPB1, 80 RPB2, and 85 MCM7 sequences

(TABLE 2.1). We attempted to generate data for all of the target sequences. However, secondary structures, homopolymer repeats, and "contamination" of genomic DNA with host DNA prevented us from successfully obtaining some of the protein-coding sequences.

Sequences were first aligned automatically with MUSCLE v3.8.31 (Edgar 2004) and then manually adjusted, aligned, and ambiguous regions excluded using Mesquite v2.75 (Maddison and Maddison 2011). For the protein-coding genes RPB1, RPB2, and MCM7, reading frames were set, introns were removed, and nucleotide sequences were translated into amino acids in Mesquite v2.75 (Maddison and Maddison 2011), after which they were re-aligned with MUSCLE v3.8.31 (Edgar 2004) and adjusted manually.

JModelTest v0.1.1 (Posada 2008) and ProtTest (Abascal et al. 2005) were used to estimate the most appropriate models of gene and protein evolution. The favored models were the general-time-reversible model with gamma distributed rates and a proportion of invariant sites (GTR+G+I; for 18S rRNA gene), GTR+G (for the 28S), and LG+G+I (for RPB1, RPB2, and MCM7 translated protein sequences).

Phylogenetic Tree Inference

The 18S and 28S rRNA genes as well as RPB1, RPB2, and MCM7 protein sequences were concatenated as a single file (gaps were scored as missing) and

partitioned for analysis in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and GARLI v2.0 (Zwickl 2006). Five independent runs were conducted in the Bayesian analysis, each with four chains for $1x10^7$ generations ($2x10^7$ generations for the five-gene phylogenetic tree), in which trees were sampled every 1000 generations. Stationarity of Markov chain Monte Carlo (MCMC) sampling and the appropriate burn-in (50%) values were assessed using AWTY (Wilgenbusch et al. 2004). One hundred bootstrap replicates were performed in maximum likelihood analyses, with the best tree out of three taken for each replicate. Branch support given above and below were Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap proportions (MLBP) respectively. Branches considered to be strongly supported (above 95% and 0.70 for BPP and MLBP, respectively) are indicated with a bold line (FIGS. 2.1–2.5, SUPP. FIGS. 2.1–2.7). All five single gene trees were compared for congruency of topology (SUPP. FIGS. 2.3–2.7). Consensus trees were produced using the SumTrees program from the DendroPy package v3.10.1 (Sukumaran and Holder 2010). Trees were edited and produced by Mesquite v2.75 (Maddison and Maddison 2011), TreeGraph 2 v2.0.47-206 beta (Stöver and Müller 2010), and Adobe Illustrator.

Ancestral character state reconstructions of morphological features including holdfast shapes, thallus branching pattern, trichospore and zygospore shapes were conducted using maximum likelihood model Mk1 in Mesquite v2.75 (Maddison and

Maddison 2011). Taxa were assigned character states on the basis of published literature (Lichtwardt et al. 2001).

Results

Phylogenetic Analyses and Overview of Tree

An overview tree highlights the clade labels or specific taxa for the main sections of the complete tree with strength of branch support (FIG. 2.1) from the full set of taxa (SUPP. FIG. 2.1). All five single gene trees were congruent (SUPP. FIGS. 2.3–2.7) with the five-gene consensus tree, with a burn-in of 50% [suggested by AWTY (Wilgenbusch et al. 2004)]. Among 60 of the *Smittium* samples included, 25 were species that were known or previously identified, and six were unidentified but thought to belong to the genus, based on morphological features of the voucher specimens and information from collections.

We incorporate the clade terminology of Wang et al. (2012), itself extending from that of White (2006). Thus, we present a main paraphyletic "Non-*Smittium*" clade of Harpellales including eight genera, which in this case also has two, *Harpellomyces* and *Caudomyces*, as part of a grade leading to the two clades of interest. Specifically outside these "Non-*Smittium*" taxa, two main clades encompass *Smittium* and putative allies

included in this analysis, which we refer to as the "True *Smittium*" and "Parasmittium" subclades (FIGS. 2.2–2.5 and SUPP. FIG. 2.1).

The "True *Smittium*" clade (FIG. 2.2) is so named based on the inclusion of the epitype, *Smittium mucronatum* (Lichtwardt and White 2011). The term "Parasmittium" is used for the first time here, for the clades "nearest" the "True *Smittium*" clade. No formal rank designation is implied or declared for Parasmittium at this time, pending further taxon sampling and specific subclade analysis. The Parasmittium group is presented as subclades 1–3 (FIGS. 2.3–2.5), based on strength of support. Within the clades or subclades of interest, we highlight relationships and clustering of taxa, with particular interest toward scrutinizing morphological features of taxonomic interest (FIGS. 2.1–2.5). Despite some nuances, the resolution among *Smittium* and its allies in this representation is the best to date.

Several species were monophyletic across broad ranges, including *S. mucronatum* as well as *S. coloradense*, whereas other morpho-species were monophyletic but not always strongly so (i.e. *S. orthocladii*). Conversely, *S. culicis* was paraphyletic, clustered also with exemplars of *S. culicisoides*, *S. fecundum*, and *S. simulatum* (FIG. 2.2).

"True *Smittium*" Clade

Molecular-based phylogenies supported a smaller group of *Smittium*, including the epitype of *Smittium*—*Smittium mucronatum* (Lichtwardt and White 2011). Clustering the epitype were *S. annulatum*, *S. caudatum*, *S. coloradense*, *S. culicis*, *S. culicisoides*, *S. fecundum*, *S. simulatum*, one *Smittium* sp. as well as *Austrosmittium biforme.* Thus, this well-resolved "True *Smittium*" clade included *Austrosmittium* as well (FIG. 2.2).

"Parasmittium" Clade

Eighteen other identified *Smittium* species (*Smittium angustum, S. commune, S. cylindrosporum, S. dipterorum, S. gravimetallum, S. hecatei, S. imitatum, S. lentaquaticum, S. megazygosporum, S. morbosum, S. orthocladii, S. perforatum, S. phytotelmatum, S. simulii, S. tipulidarum,* and *S. tronadorium,* as well as *Smittium* sp. indet. 1 and *Smittium* sp. indet. 2), five unidentified Smittiums, as well as eight *Smittium* allies—*Coleopteromyces amnicus, Furculomyces boomerangus, Pseudoharpella arcolamylica, Stachylina grandispora, St. lentica, Trichozygospora chironomidarum,*and *Stachylina* sp., as well as *Stachylina* sp. indet. 1—are also included in this large clade of 49 vouchers total.

Within the Parasmittium clade, we resolved three supported subclades (FIGS. 2.3–2.5). Parasmittium subclade 1 (FIG. 2.3) mostly includes *Smittium* allies—*Furculomyces*

boomerangus, St. grandispora, St. lentica, Stachylina sp., and *Stachylina* sp. indet.1 with a specimen accessioned as *Smittium angustum* and the only *Smittium* known to kill mosquitoes, *S. morbosum*. Parasmittium subclade 1 has slightly weaker support, limiting some of our confidence in the species relationships. Parasmittium subclade 2 (FIG. 2.4) comprises only *Smittium* species, including *S. dipterorum, S. gravimetallum, S. lentaquaticum, S. megazygosporum, S. phytotelmatum*, and 1 unidentified *Smittium* species. Parasmittium subclade 3 (FIG. 2.5) includes 12 *Smittium* species (*S. commune, S. cylindrosporum, S. dipterorum, S. hecatei, S. imitatum, S. lentaquaticum, S. morbosum, S. orthocladii, S. perforatum, S. simulii, S. tipulidarum, S. tronadorium,* and two likely new but unnamed species, specifically *Smittium* sp. indet.1 and *Smittium* sp. indet. 2). Four others were listed more loosely as *Smittium* sp. as well as allies, *Coleopteromyces amnicus, Pseudoharpella arcolamylica*, and *Trichozygospora chironomidarum*.

Discussion

Wang et al. (2012) used a combined nuclear rRNA gene analysis to assess *Smittium* and its allies, most notably with the establishment of *Zancudomyces* to accommodate *Z. culisetae*. This five-gene analysis added three additional protein-coding genes (RPB1, RPB2, and MCM7), and offered increased support for the inferred and

distinct subclades (FIGS. 2.1–2.5, see SUPP. FIG. 2.1 for full tree). It is clear that certain "Non-*Smittium*" allies—*Austrosmittium biforme, Coleopteromyces amnicus, Furculomyces boomerangus, Pseudoharpella arcolamylica, Stachylina* spp., and *Trichozygospora chironomidarum*—are still clustered with *Smittium* species, though scattered. They do present some patterns with mapped characters (see below and FIGS. 2.2–2.5).

Broad Morphological Patterns across *Smittium* and Allies

We assess not only the trichospore and zygospore as diagnostic characters, but also the nature of the thallus branching type, holdfast shape, and lifestyle characteristics between and among the "True *Smittium*" clade and Parasmittium subclades presented in the tree (FIGS. 2.1–2.5, TABLE 2.3). The combined rRNA genes analysis of Wang et al. (2012) suggested that the length/width ratio of the trichospore as well as ratio of the lengths of trichospore/collar between some of the taxa (which are distributed here between the "True *Smittium*" clade and Parasmittium subclades 2 and 3) may possess some phylogenetic signal. As an extension of that, trichospore shape also seems to be diagnostic for the "True *Smittium*" clade and Parasmittium subclades (FIG. 2.1, SUPP. FIG. 2.10). In the "True *Smittium*" clade, the epitype *Smittium mucronatum* has a trichospore

that is generally longer than the others in the clade. However, all other members also have a more compact ovoid trichospore shape, except for dimorphic *Austrosmittium biforme*, which includes not only the ovoid but a second, cylindrical spore type. Similarly, almost all members of the Parasmittium clade possess longer and narrower to cylindrical trichospores, except for another dimorphic species, *Smittium orthocladii,* which has not only a cylindrical but also an ovoid spore type. More problematic is the inclusion of *Trichozygospora chironomidarum*, which has not only an ovoid trichospore but also multiple appendages on both it and the zygospore.

It is perhaps not surprising that the original generic description of *Smittium* (Poisson 1936) has changed (expanded qualitatively and quantitatively for the trichospore) over the last three quarters of a century (Lichtwardt et al. 2001). Poisson (1936) referred to the asexual spore (=trichospore) as an “ovoid azygospore”. The modern concept (Lichtwardt et al. 2001) describes trichospores as “ellipsoidal (or subellipsoidal) to almost cylindrical”. *Smittium* has perhaps become the default genus for any hindgut dwelling, branched fungus in lower Diptera, provided they have a trichospore within this basic range of shapes with a collar and single appendage upon detachment. It is undoubtedly true that as the number of *Smittium* and Smittium-like

species grew, so did the description of *Smittium*, which now also includes species with cylindrical-shaped trichospores, in the Parasmittium clade herein (FIGS. 2.3–2.5). It is possible that the members of the Parasmittium clade are not *Smittium* and may warrant the consideration of new genera to accommodate them. We refer to the "True *Smittium*" clade because the epitype is there and all members loosely possess the original ovoid asexual spores, as documented in the original genus diagnosis by Poisson (1936).

For the morphological taxonomist (and trichomycetologists in particular) a challenge is presented; when in a single collection or across repeated collections, not all life history stages of a species are available for study. For example, many species of *Smittium* have been described without zygospores (Lichtwardt et al. 2001). Only seven of the 25 *Smittium* species included here have been recorded with the zygospores (specifically *S. coloradense, S. culicis, S. cylindrosporum, S. dipterorum, S. megazygosporum, S. mucronatum,* and *S. orthocladii*), which limits the extent to which comparisons can be made and conclusions drawn.

However, even with limited characters in hand, we found another morphological character supporting the separation of the "True *Smittium*" clade from the Parasmittium clade. Specifically, the shape of holdfast (the base of the thalli) for many members of the

"True *Smittium*" clade [i.e. *S. culicis, S. culicisoides, and S. coloradense* and *Austrosmittium* (Lichtwardt 1997, Lichtwardt and Williams 1992a, Manier 1969b, Williams and Lichtwardt 1987)] is tapered, except for the ring-like holdfast of *S. annulatum* (Lichtwardt 1997) (FIG. 2.2, SUPP. FIG. 2.8). Taxa with some form of horseshoe-shaped (or enlarged) holdfast [such as *S. angustum, S. lentaquaticum, S. simulii, Furculomyces boomerangus, Trichozygospora chironomidarum* and *Pseudoharpella arcolamylica* (although the latter might be somewhat knotted as well) (Ferrington et al. 2003; Lichtwardt 1964, 1972; Lichtwardt and Williams 1992b, 1992c; White et al. 2006c)] were scattered across the Parasmittium subclades (FIGS. 2.3–2.5, SUPP. FIG. 2.8).

Historically, much taxonomic weight has been given to the asexual and sexual spores, with other aspects of the thallus and developmental features included in some but not all *Smittium* species descriptions. For example, holdfasts and, to some extent branching patterns or even information regarding conjugations have been included (Lichtwardt 1997, Strongman and Xu 2006, White et al. 2006c). However, many species of *Smittium* have been described with emphasis on just those spore types, first qualitatively but also with a morphometric overlay. Typically a range and average are

given for spore size variation within a collection (Lichtwardt 1997, Strongman and Xu 2006, White et al. 2006c).

In this study, we have attempted to collate the morphological information as inclusively as possible, either from original publications or vouchers, photographs, images, etc. that are available. The morphological information and characters for *Smittium* species and allied taxa were mapped onto the consensus tree from the 5-gene phylogeny (FIGS. 2.1–2.5, SUPP. FIGS. 2.8–2.11). In the preliminary mapping, we physically placed features including holdfast shapes, thalli branching types, trichospore shapes, and zygospore shapes on the trees (FIGS. 2.1–2.5). The four characters were also analyzed and mapped in Mesquite v2.75 (Maddison and Maddison 2011) using a consensus maximum likelihood tree to show the probabilities of ancestral states for the characters of interest (SUPP. FIGS. 2.8–2.11).

The type of branching pattern, although it can be a bit ambiguous depending on thallus maturation, may carry some phylogenetic signal. The branching pattern of *Smittium* species has been recorded as a morphological character for some species (Ferrington et al. 2000, Lichtwardt 1994, Lichtwardt 1997, Lichtwardt and Arenas 1996, White et al. 2006c), but it has not been consistently recognized, rigorously categorized, or

explicitly examined in a phylogenetic context. With the phylogenetic tree at hand (FIGS. 2.1–2.5, SUPP. FIGS. 2.1, 2.8–2.11), we suggest that thallus branching pattern may reflect evolutionary significance and be considered for its possible taxonomic value. The entire "True S*mittium*" clade has non-verticillate branching. Parasmittium subclade 1 is also non-verticillate, whereas all members of Parasmittium subclade 2 have verticillate branching. Parasmittium subclade 3 includes a mix of examples with either one or the other of these branching patterns (FIGS. 2.1–2.5, SUPP. FIG. 2.9, TABLE 2.3).

Clade-by-clade Commentary

True *Smittium* Clade

The three isolates of *S. mucronatum* from different countries (France, Canada, USA), with one representing the epitype (ALG-7-W6), clustered tightly with strong support (FIG. 2.2). This "True *Smittium*" clade would be monophyletic, except for the inclusion of *Austrosmittium biforme*. *Austrosmittium* species are distinguished by their medially swollen zygospores. However *A. biforme* is the only one of the six *Austrosmittium* species so far described, where zygospores are not known. *Austrosmittium biforme* was described primarily on the basis of its trichospore morphology, although at the time it was placed confidently in that genus (Lichtwardt and Williams 1992a). Since *A. biforme* is the only *Austrosmittium* species that we

successfully amplified sequences using current Harpellales/*Smittium* specific protocols, there remains the question of whether *A. biforme* is a misidentified *Smittium* species. On the other hand, inspection of phylograms that include branch lengths (SUPP. FIG. 2.2) revealed that *Austrosmittium* is on a substantially longer branch and manual examination of sequence data (18S and 28S rRNA genes, RPB2 and MCM7 genes) suggesting that this is justifiable and real based on sequence divergence. Internally, weaker clade support for the exact placement of that lineage leaves it vulnerable to collapse or movement within the clade, possibly with long branch attraction tendencies as well. Future placement of exemplars of other species of *Austrosmittium*, confirmed with zygospores, would help to inform any possible taxonomic suggestions or revisions, either for the possible misidentification of *A. biforme* or whether the distinct zygospore shape of *Austrosmittium* is autapomorphic within the "True *Smittium*".

With our emphasis on branching pattern and thallus features with this five-gene phylogeny, we add that all members of this clade possess a non-verticillate branching type plus a tapered or simple holdfast shape, including also for *A. biforme* (FIG. 2.2, SUPP. FIG. 2.8–2.9). Two other features may be worthy of future consideration in this clade, in terms of clarifying the position of *A. biforme*. First, there is a tendency for some members of this clade to present a campanulate collar (i.e. *S. mucronatum* and *S. coloradense*). The shape of the trichospore collar has not been of great significance

taxonomically. *Smittium caudatum*, with a cylindrical collar offers an exception here, but it also subtends as a grade from the *S. mucronatum* cluster. Secondly, we note that *A. biforme* possesses small tuberculate projections near the base, not unlike what was reported for *Smittium fecundum* (Lichtwardt and Williams 1992a, Lichtwardt and Williams 1999). These kinds of projections are neither always easily resolved nor are they always noted in descriptions or commentaries across genera of Harpellales. Therefore, we are reluctant to place too much emphasis on the latter character at this point, but we do not suggest that it is beyond future consideration.

Possession of multiple trichospore forms is known not only in the clade discussed here, but also in some members of other clades of Harpellales. The dimorphic nature of *A. biforme* and its placement in the tree prompted a search for and comparison with dimorphic species of *Smittium* in other parts of the tree, such as *S. orthocladii* in Parasmittium subclade 3 (FIG. 2.5). The published plates of *A. biforme* (Lichtwardt and Williams 1992a) and *Smittium biforme* (White and Lichtwardt 2004), the latter from Norway, showed trichospore and collar shapes that were strikingly similar, although *S. biforme*'s long trichospore (34–42 x 9–12 µm) is longer and wider than that of *A. biforme* (18–29 x 7.2–8 µm). *Smittium biforme* was described with zygospores, which do not appear to possess any spherical expansion of the zygospore medially (as is characteristic of *Austrosmittium*). It is certainly worth sequencing *S. biforme* to place it on the tree in

the future. Alternatively, one could attempt to inoculate candidate midge hosts with an *A. biforme* culture for hopeful recovery of the zygospore, a strategy that is recommended because zygospores are not typically produced *in vitro* with axenic cultures.

The "True *Smittium*" clade also contains multiple isolates of *Smittium culicis*. The species is well-known for its broad distribution and morphological plasticity. Its placement on the tree (FIG. 2.2) indicates that it might represent a cluster with cryptic species, suggesting a possible species complex. Representatives are well separated with strong support on the tree, with a couple exceptions. Two *S. culicis* vouchers (ALG-5-W8 and GSMNP-1) clustered with *S. culicisoides* and *S. fecundum*, both of which were distinctly similar for their short generative cells (Lichtwardt 1997, Lichtwardt and Williams 1999) and differing from the original description of *S. culicis* (Manier 1969b). The other eight *S. culicis* representatives from three different countries (Australia, New Zealand, United States–Utah, Wyoming, Colorado) were joined by *S. simulatum*, from Chile (Lichtwardt and Arenas 1996). The original description of *S. simulatum* indicated that *S. simulatum* cannot be distinguished in culture from *S. culicis* based on trichospore shape and size, but it did have a distinct isozyme pattern when compared with five *Smittium* species and 16 total isolates (Lichtwardt and Arenas 1996). However, within the scope of this five-gene analysis, the placement of *S. simulatum* again suggests it

similarity with *S. culicis* (FIG. 2.2). The *S. culicis* section of this clade does have a distinct pattern (FIG. 2.2) that separates them.

One unidentified *Smittium* sp. (NOR-11-W21) from Norway (White and Lichtwardt 2004) clustered within the "True S*mittium*" clade. This *Smittium* sp. is morphologically similar to *S. coloradense* and is from the same host species as well as from a similar habitat (seepy cliffs in Norway), but based on analyses of sequence data, it was not as closely matched as the specimen (RMBL-13-41) collected in North America (White and Lichtwardt 2004, Williams and Lichtwardt 1987). It should be studied further and compared morphologically with specimens of *S. coloradense*, as a candidate species match.

Parasmittium Subclade 1

Parasmittium subclade 1 includes the mosquito killing gut fungus, *Smittium morbosum*, as well as *Furculomyces boomerangus* and all of the *Stachylina* spp. that were sequenced. *Smittium morbosum* is unusual among the Harpellales, in terms of its destructive lifestyle. It was first 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, phylogenetically was a close match with an isolate (ARG-GM-2) from Argentina, which was selected for inclusion based on an earlier two-gene study (Wang et al. 2012).

Stachylina was earlier thought to be polyphyletic based on separate phylogenetic analyses with 18S and 28S rRNA genes (White 2006). With marginally increased taxon sampling, combined analyses and three more genes (RPB1, RPB2, and MCM7) for *Stachylina* in this study, we were surprised to find them nearly all together and within one subclade. We believe that an effort and focus toward adding exemplars of *Stachylina*, the second largest Harpellales genus, will serve as the next critical step to help resolve the actual relationships not only within *Stachylina* but also between *Smittium* and allies, especially in this subclade. With only one provisionally identified *Stachylina* outside this otherwise fairly well-supported cluster of Stachylinas, it is possible that this genus, as a group of midgut dwelling fungi, will not be so severely dispersed across the "Smittium" clade, as earlier anticipated (White 2006).

Wang et al. (2012) discussed the possibility that *Smittium angustum* (AUS-126-30) is really a *Furculomyces* (FIG. 2.3). *Smittium angustum* is an axenic culture from an earlier study (Lichtwardt and Williams 1992c), and it possess narrow and subcylindrical trichospores, which on average had a trichospore length/width ratio of 8.43 (Lichtwardt and Williams 1992c). This ratio is similar to that of *F. boomerangus* (with a ratio of 7). Additionally, the trichospore of *F. boomerangus* was described with a medial swelling (Lichtwardt and Williams 1992b). This feature was not defined for *S. angustum*, but it seems to be apparent in the original plate (Lichtwardt and Williams 1992c). Thus it is

possible that *S. angustum* and *F. boomerangus* are indeed synonymous, and addition of other *Furculomyces* species would help clarify this possibility.

Furculomyces boomerangus represents the only genus in this clade for which zygospores are known, specifically boomerang shaped and borne on a wishbone-like conjugation apparatus. Since sexual spore features have never been observed for *Stachylina* or *Smittium morbosum*, whether they have similarly bent zygsopores will have to await further collecting and documentation.

Parasmittium Subclade 2

This clade consists of *S. dipterorum, S. gravimetallum, S. lentaquaticum, S. megazygosporum*, *S. phytotelmatum*, and *Smittium* sp. indet. 3 (AS-49-6). For all of those for which we have data, they all have verticillate branching (FIG. 2.4, SUPP. FIG. 2.9). This is the only subclade in which this pattern is so distinct. Thus, the verticillate branching type is a character shared among members of this subclade with possible evolutionary signal.

Only *S. megazygosporum* in the Parasmittium subclade 2 had a known zygospores type, which has a long and fusiform shape. The long and thin zygospore is variably bent near one end, where it attaches to the zygosporophore. Considering this aspect of the zygospore, it is the most extreme of all Smittiums, with a length/width ratio of 11.8, attached as it is to the zygosporophore approximately $1/6^{th}$ from the end. More data is

required before these zygospores characters can be properly evaluated; however, the bend itself and the orientation and presentation of the zygospore on the zygosporophore are features that undoubtedly deserve further morphological analysis.

Parasmittium Subclade 3

Pseudoharpella arcolamylica is sister to all other members of Parasmittium subclade 3 (FIG. 2.5). The unusual coiled nature of the trichospore of *P. arcolamylica*, with three broad appendages when detached (Ferrington et al. 2003), are both features of the asexual spore that are distinct from other members of subclade 3. Additionally, the zygospore of *P. arcolamylica* can be somewhat bent. Considering that none of the other members of Parasmittium subclade 3 possess this bent type of zygospore, this character state may have been lost over evolutionary time in this subclade.

The topology of Parasmittium subclade 3 is not strongly supported. Future clade based analyses (and/or analyses with a reduced number of taxa) could help inform some of the relationship in this subclade. *Coleopteromyces amnicus*, with somewhat cylindrical trichospores, is morphologically similar to other *Smittium* species here, even though its beetle host makes it unique compared to hosts of *Smittium* (Lichtwardt et al. 1999). It is possible that a host switching event occurred in this instance. Additionally, the isthma, a

structure between collar and trichospore that was considered a unique feature to help distinguish *Coleopteromyces* from *Smittium* (Lichtwardt et al. 1999), may need to be reconsidered for its taxonomic value.

Unlike the true *S. morbosum* (isolate AUS-X-1), *Smittium* cf. *morbosum* (isolates WKRa and WKRb) from the southeastern USA were earlier determined not to be pathogenic to mosquitoes (Wang et al. 2012). It is likely that these two isolates were misidentified. Another *Smittium* ally, *Trichozygospora chironomidarum*, has similar morphology with *Smittium* (Parasmittium) and has a Dipteran host, but has multiple (>10) appendages on both the trichospore and zygospore. Phylogenetically, multiple appendages could be a true autapomorphy in this subclade, or it may not be taxonomically informative. Future efforts to collect and place other representatives of this rare species (Lichtwardt 1972) should be undertaken. Additionally, increased efforts to incubate wet mounts of freshly dissected zygospores in moist chambers to promote spore release and appendage counting and documentation would be valuable.

Future Investigations

With the new Harpellales/*Smittium* specific primer sets used here, amplification of the DNA of insect hosts can be avoided, allowing the direct sequencing of the trichomycete from the PCR product as template. Comparatively, this direct sequencing

approach returned consistently, high quality sequence read, as judged by assembled sequences and individual chromatograms. Besides error reduction, costs are reduced if labor-intensive cloning step are avoided.

We also suggest that future collections or investigations record morphological characters as completely as possible, not only for new species descriptions but also for unnamed species sometimes included in publications. Molecular-based phylogenetic analyses can serve as a powerful tool to guide taxonomy and species discrimination (or higher taxonomic levels). From a molecular systematics perspective, as phylogenetic trees began to delineate closely related taxa in sometimes surprising ways, the pursuit and assessment of sometimes sparse morphological data becomes a concern. It would be valuable to have morphological characters not just presented in descriptions, but also augmented with images of the holdfast, thallus branching pattern, generative cells, trichospore shape (and variation) with length/width ratio, collar shape (attached and detached), zygospore shape with nature of conjugation and zygosporophore features. Additionally, to the extent possible, information on the host taxa, collecting site location, and other site information (such as water temperature, pH etc.) should all be obtained. Molecular phylogenetics is a tool for reconstrcting evolutionary relationships at various levels, but these phylogenies also allow morphological characters to be mapped onto

phylogenetic trees. Ultimately, this combined approach will enable us to more precisely estimate the true evolutionary tree for *Smittium* (and allies).

Much of our morphologically-based taxonomy of gut fungi is taken from the level of light microscope. Ultrastructural studies of *Smittium* are few (Manier and Coste-Mathiez 1968, Moss and Lichtwardt 1976, Valle and Santamaria 2004) and have lagged behind the progress made with other fungal groups. However, concentric, electron-dense rings were found in cross sections of appendages of *S. culicis* and *S. mucronatum* according to transmission electron microscopic (TEM) studies (Manier and Coste-Mathiez 1968, Moss and Lichtwardt 1976). Both of these species are in the "True *Smittium*" clade. It would be worth testing whether this is a feature possessed only by "True *Smittium*" members and whether this feature is found in members of the Parasmittium subclades.

Valle and Santamaria (2004) used scanning electron microscopy (SEM) to show ultrastructural variation in the trichospore appendage, describing it as either ribbon-like (in *S. heterosporum*) or cylindrically shaped (in *S. hecatei*). *Smittium hecatei* occurs in Parasmittium subclade 3, with a cylindrical trichospore typical of that clade. Whereas we did not succeed in sequencing *S. heterosporum*, it does possess an ovoid trichospore. Thus, with additional molecular data, combined with ultrastructure analyses, appendage form and function could be another feature. Members of "True *Smittium*" clade should be

included in future electron microscopic studies (TEM and SEM) especially considering what is known regarding entire and cross-sectioned appendages. Coincident with this, efforts should be maintained to obtain axenic cultures of species across these clades to aid such efforts. Overall scrutiny of the "whole fungus" and assessing its ultrastructure could be critical for finer detailed analysis and mapping of such features.

We consider these analyses to be a first step and some subsets of these data could be analyzed less broadly to better resolve relationships within subclades, such as for the *Smittium* allies and Smittiums in Parasmittium clade 3. Subclade analyses might recover synonymous and/or cryptic species. These kinds of analyses could also be used to examine the ecological interactions between the host and the fungus, over the shorter or longer term, to better understand the nature of this symbiotic relationship, which has undoubtedly shaped a multitude of adaptive responses over evolutionary time.

References

Abascal F, Zardoya R, Posada D. 2005. ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics* 21(9):2104–2105.

Aguileta G, Marthey S, Chiapello H, Lebrun MH, Rodolphe F, Fournier E, Gendrault-Jacquemard A, Giraud T. 2008. Assessing the performance of single-copy genes for recovering robust phylogenies. *Syst Biol* 57:613–627.

Benny GL, O'Donnell K. 2000. *Amoebidium parasiticum* is a protozoan, not a Trichomycete. *Mycologia* 92:1133–1137.

Blackwell M. 2010. Fungal evolution and taxonomy. *Biol Control* 55:7–16.

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.

Clark MA, Moran NA, Baumann P, Wernegreen JJ. 2000. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution* 54(2):517–525.

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung GH, Spatafora JW, Straus NA. 2003. Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science* 299:386–388.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32(5):1792–1797.

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

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

Ferrington LC, Lichtwardt RW, Hayford B. 2000. *Smittium gravimetallum* (Trichomycetes: Harpellales), a new species of gut fungus from *Dicrotendipes fumidus* (Johannsen) (Diptera: Chironomidae) in a metal-polluted stream. In: Hoffrichter O, ed. Late 20th. Century Research on Chironomidae: An Anthology from the 13th International Symposium on Chironomidae. Shaker Verlag, Aachen:253–257.

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

Frøslev TG, Matheny PB, Hibbett DS. 2005. Lower level relationships in the mushroom genus *Cortinarius* (Basidiomycota, Agaricales): A comparison of RPB1, RPB2 and ITS phylogenies. *Mol Phylogen Evol* 37:602–618.

Finet C, Timme RE, Delwiche CF, Marlétaz F. 2010. Multigene phylogeny of the green lineage reveals the origin and diversification of land plants. *Curr Biol* 20:2217–2222.

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

Grigg R, Lichtwardt RW. 1996. Isozyme patterns in cultured Harpellales. *Mycologia* 88:219–229.

Hall BK, Hallgrímsson B. 2008. Strickberger's Evolution (4th edition). Jones & Bartlett. 762 p.

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.

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.

Janzen DH. 1980. When is it coevolution? *Evolution* 34:611–612.

Léger L, Duboscq O. 1929. *Eccrinoïdes henneguyi* n. g. n. sp. et la systématique des Eccrinides. *Arch Anat Microsc* 25:309–324.

Lichtwardt RW. 1964. Axenic culture of two new species of branched Trichomycetes. *Amer J Bot* 51:836–842.

———. 1972. Undescribed genera and species of Harpellales (Trichomycetes) from the guts of aquatic insects. *Mycologia* 64:167–197.

———. 1994. Trichomycete fungi living in the guts of Costa Rican phytotelm larvae and other lentic dipterans. *Rev Biol Trop* 42:31–48.

———. 1997. Costa Rican gut fungi (Trichomycetes) infecting lotic insect larvae. *Rev Biol Trop* 45:1349–1383.

———. 2008. Trichomycetes and the arthropod gut. In: Brakhage AA, Zipfel PF, eds. Human and Animal Relations (2nd Edition). Berlin, Germany: Springer-Verlag: The Mycota VI:3–19.

———, Arenas J. 1996. Trichomycetes in aquatic insects from southern Chile. *Mycologia* 88:844–857.

———, Cafaro MJ, White MM. 2001. The Trichomycetes, fungal associates of arthropods. Revised edition. Published on the Internet www.nhm.ku.edu/~fungi. (last accessed 4th Mar. 2012)

———, Ferrington LC Jr., López Lastra C. 1999. Trichomycetes in Argentinean aquatic insect larvae. *Mycologia* 91:1060–1082.

———, Manier J-F. 1978. Validation of the Harpellales and Asellariales. *Mycotaxon* 7:441–442.

———, White MM. 2011. Typification of *Smittium*, an important genus in the taxonomy of Harpellales. *Mycologia* 103:915–917.

———, Williams MC. 1992a. Tasmanian Trichomycete gut fungi in aquatic insect larvae. *Mycologia* 84:384–391.

———, Williams MC. 1992b. *Furculomyces*, a new homothallic genus of Harpellales (Trichomycetes) from Australian midge larvae. *Can J Bot* 70:1196–1198.

———, Williams MC. 1992c. Western Australian species of *Smittium* and other Trichomycetes in aquatic insect larvae. *Mycologia* 84:392–398.

———, Williams MC. 1999. Three Harpellales that live in one species of aquatic chironomid larva. *Mycologia* 91:396–399.

Little AEF, Currie CR. 2007. Symbiont complexity: Discovery of a fifth symbiont in the attine ant-microbe symbiosis. *Biol Lett* 3:501–504.

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.

Liu YL, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Mol Biol Evol* 16:1799–1808.

Lutzoni F, Kauff F, Cox C, McLaughlin D, Celio G, Dentinger B, Padamsee M, Hibbett D, James T, Baloch E, Grube M, Reeb V, Hofstetter V, Schoch C, Arnold AE, Miadlikowska J, Spatafora J, Johnson D, Hambleton S, Crockett M, Shoemaker R, Sung GH, Lücking R, Lumbsch T, O'Donnell K, Binder M, Diederich P, Ertz D, Gueidan C, Hansen K, Harris RC, Hosaka K, Lim YW, Matheny B, Nishida H, Pfister D, Rogers J, Rossman A, Schmitt I, Sipman H, Stone J, Sugiyama J, Yahr R, Vilgalys R. 2004. Assembling the fungal tree of life: Progress, classification, and evolution of subcellular traits. *Am J Bot* 91:1446–1480.

Maddison WP, Maddison DR. 2011. Mesquite: A modular system for evolutionary analysis. Version 2.75 http://mesquiteproject.org (last accessed 28 Feb. 2012)

Manier JF. 1969b. Trichomycètes de France. *Ann Sci Nat Bot Biol* 12(10):565–672.

———, Coste-Mathiez F. 1968. L'ultrastructure du filament de la spore de *Smittium mucronatum* Manier, Mathiez 1965 (Trichomycète, Harpellales). *C R Acad Sci Hebd Seances Acad Sci D* 266:341–342.

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

Matheny PB. 2005. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*, Agaricales). *Mol Phylogenet and Evol* 35:1–20.

———, Liu YJ, Ammirati JF, Hall BD. 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). *Am J Bot* 89:688–698.

———, Wang Z, Binder M, Curtis JM, Lim YW, Nilsson RH, Hughes KW, Hofstetter V, Ammirati JF, Schoch CL, Langer E, Langer G, McLaughlin DJ, Wilson AW, Frøslev T, Ge Z-W, Kerrigan RW, Slot JC, Yang Z-L, Baroni TJ, Fischer M, Hosaka K, Matsuura K, Seidl MT, Vauras J, Hibbett DS. 2007. Contributions of rpb2 and tef1 to the phylogeny of mushrooms and allies (Basidiomycota, Fungi). *Mol Phylogen Evol* 43:430–451.

Moran NA and Jarvik T. 2010. Lateral transfer of genes from fungi underlies carotenoid production in aphids. *Science* 328:624–627.

Moss ST, 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.

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

Ogawa Y, Kurihara Y, Suda A, Kusama-Eguchi K, Watanabe K, Tokimasu S. 2005. Taxonomic position of the genus *Ramacandelaber,* Kickxellales, inferred from 18S rDNA. *Nippon Kingakukai Kaiho* 46:13–17.

Poisson R. 1936 (1937). Sur un Endomycète nouveau: *Smittium arvernense* n. g., n. sp., parasite intestinal de larves de *Smittia* sp. (Diptères Chironomides) et description d'une nouvelle espèce du genre *Stachylina* Léger et Gauth. 1932. *Bull Soc Sci Bretagne* 14(18):20–31.

Porter D, Smiley R. 1979. Ribosomal RNA molecular weights of Trichomycetes and Zygomycetes. *Exp Mycol* 3:188–193.

Posada D. 2008. jModelTest: Phylogenetic Model Averaging. *Mol Biol Evol* 25:1253–1256.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.

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

Scarborough CL, Ferrari J, Godfray HCJ. 2005. Aphid protected from pathogen by endosymbiont. *Science* 310:1781.

Schmitt I, Crespo A, Divakar PK, Fankhauser JD, Herman-Sackett E, Kalb K, Nelsen MP, Nelson NA, Rivas Plata E, Shimp AD, Widhelm T, Lumbsch HT. 2009. New

primers for promising single-copy genes in fungal phylogenetics and systematics. *Persoonia* 23:35–40.

Shalchian-Tabrizi K, Minge MA, Espelund M, Orr R, Ruden T, Jakobsen KS, Cavalier-Smith T. 2008. Multigene phylogeny of choanozoa and the origin of animals. *PLoS ONE* 3(5):e2098.

Simpson AG, Inagaki Y, Roger AJ. 2006. Comprehensive multigene phylogenies of excavate protists reveal the evolutionary positions of "primitive" eukaryotes. *Mol Biol Evol* 23(3):615–625.

Slaymaker AK, Ferrington LC, Jr., Lichtwardt RW. 1998. Chironomidae-Trichomycete associations: A literature review. *J Kansas Ent Soc* 7:490–500.

Stöver BC, Müller KF. 2010. TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics* 11:7.

Strongman DB, Xu S. 2006. Trichomycetes from China and the description of three new *Smittium* species. *Mycologia* 98:479–487.

Sukumaran J, Holder MT. 2010. DendroPy: A Python library for phylogenetic computing. *Bioinformatics* 26:1569–1571.

Sweeney AW. 1981. An undescribed species of *Smittium* (Trichomycetes) pathogenic to mosquito larvae in Australia. *Trans Br Mycol Soc* 77:55–60.

Tehler A, Farris JS, Lipscomb DL, Källersjö M. 2000. Phylogenetic analyses of the fungi based on large rDNA data sets. *Mycologia* 92:459–474.

Walker WF. 1984. 5S ribosomal RNA sequences from Zygomycotina and evolutionary implications. *System Appl Microbiol* 5:448–456.

Wang Y, Tretter ED, Lichtwardt RW, White MM. 2012. Overview of 75 years of *Smittium* research, establishing a new genus for *Smittium culisetae*, and prospects for future revisions of the "Smittium" clade. *Mycologia* (in review).

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

———. 2006. Evolutionary implications of a RNA-based phylogeny of Harpellales. *Mycol Res* 110:1011–1024.

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

———, 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.

———, Lichtwardt RW, 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.

———, Siri A, Lichtwardt RW. 2006c. Trichomycete insect symbionts in Great Smoky Mountains National Park and vicinity. *Mycologia* 98:333–352.

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 Inc: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 4 Mar. 2012)

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

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

Williams MC, Lichtwardt RW. 1987. Three new species of *Smittium* (Trichomycetes) with notes on range extensions. *Mycologia* 79:832–838.

Valle LG, Santamaria S. 2004. The genus *Smittium* (Trichomycetes, Harpellales) in the Iberian Peninsula. *Mycologia* 96:682–701.

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *J Bacteriol* 172:4239–4246.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [Ph.D. dissertation]. The University of Texas at Austin. 125 p.

TABLE 2.1. List of 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 name	Strain No.	DNA Bench Code	Collected by[1] or Source	Host	Origin	18S rRNA	28S rRNA	RPB1	RPB2	MCM7
Smittium angustum	AUS-126-30	314	RWL	*Tanytarsus* sp.	Australia	10442583	JQ302822	314-62	314-82	314-310
Smittium annulatum	CR-143-8	66	RWL	Simuliidae	Costa Rica	10442602	JQ302832	66-62	66-82	66-310
Smittium caudatum	KS-1-2	69	KUMYCOL/RWL	Chironomidae	United States	10442609	JQ302948	69-602	69-82	69-310
Smittium coloradense	RMBL-13-41	67	RWL	Cricotopus sp.	United States	10442619	JQ302912	67-602	67-82-1	67-310
Smittium coloradense	NOR-46-W1	679	MMW	Chironomidae	Norway	JQ302869	679-183	679-602	679-82	679-310H
Smittium commune	KS-6-6	57	RWL	Chironomidae	United States	10442613	57-183	57-602	57-82	57-310
Smittium commune	KS-2-21	315	KUMYCOL/RWL	Chironomidae	United States	10442612	JQ302901	315-62	315-82	315-310
Smittium culicis	ALG-5-W8	925	MMW	*Bactylolabis montana*	Canada	JQ302899	JQ302915	925-602	925-82	925-310
Smittium culicis	WYO-51-11	63	KUMYCOL/RWL	*Aedes sticticus*	United States	10442625	JQ302830	63-62	63-82	63-310
Smittium culicis	12-1-3	373	LCF/BH	Culicidae	Australia	JQ302860	JQ302938	373-602	373-82	-
Smittium culicis	GSMNP-1	879	RWL	Culicidae	United States	JQ302885	JQ302959	879-602	879-82	879-310
Smittium culicis	43-1-2	362	LCF/BH	*Chironomus* sp.	Australia	JQ302893	89033461	362-62	362-82	362-310
Smittium culicis	AS-42-1	364	AS	*Corynoneura* sp.	New Zealand	364-177	364-183	-	364-82	364-310
Smittium culicis	35-1-1	361	LCF/BH	Thaumaleidae	Australia	JQ302855	JQ302933	361-602	361-82	361-310
Smittium culicis	LCF-8-1	365	LCF	Thaumaleidae	New Zealand	JQ302856	JQ302934	365-602	365-82	365-310
Smittium culicis	AUS-62-6	316	RWL	*Austrothaumalea* sp.	Australia	10442590	JQ302902	316-62	316-82-1	316-310

Smittium cf. *culicis*	UT-11-W1	761	MMW	Diptera	United States	JQ302881	JQ302955	761-602	761-82	761-310
Smittium culicisoides	CR-253-12	64	KUMYCOL	Chironomidae	Costa Rica	10442606	JQ302831	64-602	64-82-1	64-310
Smittium cylindrosporum	CHI-27-1	56	RWL	*Cricotopus* sp.	Chile	10442596	JQ302828	-	-	56-310
Smittium dipterorum	CR-253-14	59	KUMYCOL	*Simulium* sp.	Costa Rica	10442604	JQ302909	59-62	59-82-1	59-310
Smittium dipterorum	CR-141-17	319	RWL	*Simulium* sp.	Costa Rica	10442601	JQ302928	319-602	319-82	319-310
Smittium fecundum	RMBL-64-5	65	RWL	*Psectrocladius* sp.	United States	10442622	JQ302911	65-602H	65-82	65-310
Smittium fecundum	SPA-X-67	856	LGV	Diptera	Spain	856-177	856-183	-	856-82	-
Smittium gravimetallum	KS-F1-3	60	LCF	*Dicrotendipes fumidus*	United States	10442615	60-183	60-602	60-82	60-310
Smittium hecatai	SPA-X-63	854	LGV	Diptera	Spain	854-177	854-183	-	-	-
Smittium imitatum	CHI-20-11	54	RWL	*Simulium* sp.	Chile	10442594	JQ302907	54-62	54-82	54-310
Smittium imitatum	CHI-9-4	320	RWL	*Simulium* sp.	Chile	10442599	JQ302903	320-602	320-82	320-310
Smittium lentaquaticum	TN-27-A4	906	Siri/MMW/RWL	*Chironomus* sp.	United States	906-177	906-183	906-602	906-82	906-310
Smittium lentaquaticum	TN-27-A5	911	Siri/MMW/RWL	*Chironomus* sp.	United States	911-177	911-183	911-602	911-82	911-310
Smittium megazygosporum	SC-DP-2	321	KUMYCOL/CEB	*Simulium vittatum*	United States	10442623	JQ302823	321-601	321-82-1	321-310
Smittium morbosum	AUS-X-1	70	KUMYCOL/RWL	*Anopheles hilli*	Australia	10442592	JQ302913	70-62	70-82	70-310
Smittium cf. *morbosum*	WKRa	881	WKR/CEB	*Ochlerotatus triseriatus*	United States	JQ302886	JQ302960	881-602	881-82	881-310
Smittium cf. *morbosum*	WKRb	883	WKR/CEB	*Ochlerotatus triseriatus*	United States	JQ302895	JQ302834	883-62	883-82	883-310H
Smittium cf. *morbosum*	ARG-GM-2	307	GM/CLL	Diptera	Argentina	JQ302849	JQ302927	307-602	307-82	307-310
Smittium mucronatum	ALG-7-W6	916	MMW	Chironomidae	Canada	JQ302898	JQ302914	916-602	916-82	916-310
Smittium mucronatum	RMBL-61-10	142	RWL	*Psectrocladius* sp.	United States	JQ302840	89033437	-	-	-

Smittium mucronatum	FRA-12-3	68	KUMYCOL/RWL	*Psectrocladius sordidellus*	France	10442608	JQ302833	68-602	68-82-1	68-310
Smittium orthocladii	OK-4-19	55	RWL	Chironomidae	United States	10442618	JQ302827	55-62M-1	55-82	55-310
Smittium orthocladii	LCF-BT-1	108	LCF/MMW	*Corynoneura* sp.	United States	89033395	JQ302900	108-602	108-82	108b-310
Smittium orthocladii	KS-82-W1	130	LCF/MMW	*Orthocladius abiskoensis*	United States	JQ302838	JQ302917	-	-	-
Smittium perforatum	RMBL-44-3	332	RWL	*Diamesa* sp.	United States	JQ302851	JQ302930	332-602	332-82	332-310
Smittium phytotelmatum	CR-219-1	61	KUMYCOL/RWL	*Chironomus* sp.	Costa Rica	10442603	JQ302910	61-602L	61-82-2	61-310
Smittium simulatum	CHI-8-4	323	KUMYCOL/RWL	*Aphophila bidentata*	Chile	10442597	JQ302824	323-602	323-82	323-310
Smittium simulii	SWE-8-4	58	RWL	*Diamesa* sp.	Sweden	10442624	JQ302908	58-602	58-82H-1	58-310
Smittium simulii	CAL-8-1	324	RWL	*Simulium argus*	United States	10442593	JQ302825	324-62-1	324-82-1	324-310
Smittium simulii	41-1-6	374	LCF/BH	*Orthocladius* sp.	Australia	JQ302861	JQ302939	374-602	374-82	374-310
Smittium cf. *simulii*	SPA-X-70	858	LGV	Culicidae	Spain	JQ302883	JQ302957	858-602	858-82	858-310
Smittium sp. indet. 1[2]	OK-3-22	327	RWL	Chironomidae	United States	10442617	327-183	327-602	327-82	327-310
Smittium tipulidarum	RMBL-31-1	52	KUMYCOL/RWL	*Elliptera astigmatica*	United States	10442621	JQ302836	52-62	52-82-1	52-310
Smittium tronadorium	ARG-24-20F	53	LCF	*Limaya* sp.	Argentina	JQ302894	JQ302906	53-602	53-82	53-310
Smittium tronadorium	ARG-24-2F	325	LCF	*Paraheptagyia* sp.	Argentina	10442582	JQ302904	325-62	325-82-1	325-310
Smittium sp. indet. 2[2]	AS-22-15	367	AS	*Cricotopus* sp.	New Zealand	JQ302858	JQ302936	367-602	367-82	367-310
Smittium sp. indet. 2[2]	LCF-27-15	368	LCF	Orthocladiinae	New Zealand	JQ302859	JQ302937	368-602	368-82	368-310
Smittium sp. indet. 2[2]	AS-27-9	366	AS/LCF	Orthocladiinae	New Zealand	JQ302857	JQ302935	366-602	366-82	366-310
Smittium sp.	TN-3-12	331	RWL	Chironomidae	United States	JQ302850	JQ302929	331-602	331-82	363543787
Smittium sp.	CR-259-4	329	RWL	*Simulium* sp.	Costa Rica	JQ302891	JQ302826	329-62	329-82	329-310

Smittium sp.	RMBL-48-8	330	RWL	*Prosimulium* sp.	United States	JQ302892	JQ302905	330-62	330-82	330-310
Smittium sp.	GB-X-1	885	AR/SM	*Simulium ornatum*	United Kingdom	JQ302896	885-97	885-62	885-82-1	885-310
Smittium sp.	NOR-11-W21	785	MMW/RWL	Chironomidae	Norway	785-177	785-183	-	-	785-310
Smittium sp. indet. 3[2]	AS-49-6	210	AS	Chironomidae (*Paratanytarsus* sp.?)	New Zealand	JQ302844	210-183	210-602	210-82	210-310
Smittium allies										
Austrosmittium biforme	32-1-9	338	LCF/BH	Orthocladiinae	Australia	338-177	338-183	-	338-82	-
Austrosmittium biforme	32-1-8	345	KUMYCOL	Cricotopus sp./Orthocladiinae	Australia	-	89033443	-	-	345-310
Coleopteromyces amnicus	ARG-15-3	239	RWL	Scirtidae	Argentina	239-177	239-183	-	-	239-310H
Coleopteromyces amnicus	ARG-15-6F	339	LCF	Scirtidae	Argentina	JQ302853	JQ302931	-	-	339-310
Furculomyces boomerangus	AUS-77-4	1031	RWL	*Tanytarsus* nr. *inextentus*	Australia	10442591	1031-183	120561214	120561246	1031-310
Furculomyces boomerangus	AUS-42-7	1030	RWL	*Procladius? paludicola*	Australia	2226385	82398545	-	1030-82	-
Pseudoharpella arcolamylica	LCF-3	766	LCF	Dixidae	United States	JQ302882	JQ302956	-	-	766-310
Stachylina grandispora	KS-70-W11&18	290	MMW	*Chironomus riparius*	United States	JQ302846	JQ302924	-	-	-
Stachylina lentica	NOR-58-10	701	RWL	*Chironomus* sp.	Norway	JQ302874	701-183	701-602	701-82	701-310
Stachylina lentica	NOR-45-W3	686	MMW	Chironomidae	Norway	JQ302871	JQ302947	-	-	-
Stachylina sp. indet. 1[2]	LCF-22-6	200	LCF	*Tanytarsus* sp.	South Africa	89033407	JQ302922	-	-	-
Stachylina sp.	NS-X-10	723	DBS	Chironomidae	Canada	723-177	723-183	-	-	723-310
Trichozygospora chironomidarum	TN-3-16	166	RWL	Chironomidae	United States	JQ302842	JQ302919	-	-	166a-310

Non-*Smittium* taxa

***Bojamyces* sp.**	**CA-18-W17**	**767**	**MMW**	**Ephemeroptera**	**United States**	**767-177**	**767-183**	**-**	**-**	**767-310**
Capniomyces stellatus	**MIS-21-127**	**167**	**GenBank/RWL**	***Allocapnia* sp.**	**United States**	**89033400**	**125747107**	**120561212**	**120561244**	**167-310**
***Caudomyces* sp.**	**UT-1-W16a**	**763**	**MMW**	**Diptera**	**United States**	**763-177**	**763-183**	**-**	**-**	**763-310**
Genistelloides hibernus	**KS-19-M23**	**192**	**GenBank/JKM**	**Capniidae**	**United States**	**89033405**	**JQ302921**	**192-602**	**192-82**	**192-310**
Harpella melusinae	**NF-15-5A**	**244**	**MMW**	**Simuliidae**	**Canada**	**244-177&170**	**244-183**	**244-602**	**244-82**	**244-304**
Harpellomyces montanus	**TN-22-W5B**	**954**	**MMW**	**Thaumaleidae**	**United States**	**JQ302887**	**JQ302961**	**-**	**-**	**954G-310H**
Lancisporomyces falcatus	**NS-X-2**	**520**	**DBS**	***Paracapnia angulata***	**Canada**	**JQ302865**	**JQ302943**	**520-602**	**520-82**	**520-310**
Legeriomyces minae	**PEI-X-6**	**930**	**DBS**	***Ephemerella invaria***	**Canada**	**930-177**	**930-183**	**930-602**	**930-82**	**930-310**
***Pteromaktron* sp.**	**OR-11-W8**	**983**	**MMW**	**Ephemeroptera**	**United States**	**983-177**	**983-183**	**-**	**-**	**983G-310**
Unnamed Harpellales	**ALG-10-W3**	**913**	**MMW**	**Trichoptera**	**Canada**	**913-177**	**913-183**	**-**	**913-82**	**913-310**
Unnamed Harpellales	**ALG-13-W1**	**918**	**MMW**	**Trichoptera**	**Canada**	**918-177**	**918-183**	**--**	**918-82**	**918-310**
Zancudomyces culisetae	**LEA-7-2**	**176**	**RWL**	***Simulium vittatum***	**United States**	**176-177**	**176-183**	**176-602**	**176-82**	**176-310**
Zancudomyces culisetae	**ARG-X-5**	**375**	**CLL**	**Culicidae**	**Argentina**	**JQ302862**	**JQ302940**	**375-602**	**375-82**	**375-310**
Zancudomyces culisetae	**ARG-LL-13**	**734**	**CLL**	***Aedes aegypti***	**Argentina**	**JQ302879**	**JQ302953**	**734-602**	**-**	**734-310**
Zancudomyces culisetae	**AUS-2-8**	**62**	**KUMYCOL/RWL**	***Chironomus alternans***	**Australia**	**10442585**	**JQ302829**	**62-62**	**62-82**	**62-310**
Zancudomyces culisetae	**LEA-7-2**	**168**	**KUMYCOL/RWL**	***Simulium vittatum***	**United States**	**JQ302888**	**JQ302820**	**168-62-1**	**168-82-1**	**168-310**
Zancudomyces culisetae	**HAW-14-7**	**169**	**KUMYCOL/RWL**	***Aedes alpopictus***	**United States**	**JQ302889**	**JQ302821**	**169-62**	**169-82-1**	**169-310**
Zancudomyces culisetae	**COL-18-3**	**317**	**GenBank/RWL**	***Culiseta impatiens***	**United States**	**296035099**	**311235631**	**120561210**	**120561242**	**317-310**
Zancudomyces culisetae	**KS-108-02**	**927**	**JAK**	***Aedes vexans***	**United States**	**927-177**	**927-97**	**927-602**	**927-82**	**927-310**

Zancudomyces culisetae	**ARG-GM-4**	**754**	**GM/CLL**	**Diptera**	**Argentina**	**JQ302880**	**JQ302954**	**754-602**	**754-82**	**-**
Zancudomyces culisetae	**ARG-GM-3**	**306**	**GM/CLL**	**Diptera**	**Argentina**	**JQ302848**	**JQ302926**	**-**	**306-82**	**-**
Zancudomyces culisetae	**ARG-GM-4**	**305**	**GM/CLL**	**Diptera**	**Argentina**	**JQ302847**	**JQ302925**	**305-602**	**305-82**	**-**
Zancudomyces culisetae	**MAL-X-1**	**889**	**CLL**	***Aedes crinifer***	**Malaysia**	**JQ302897**	**JQ302835**	**889-62**	**889-82**	**889-301**
Outgroups										
Coemansia reversa	**NRRL 1564**	**415**	**GenBank**	**None, free-living**	**N/A**	**44936090**	**44936641**	**83320443**	**83415480**	**jgi: e_gw1.81.36.1**
Kickxella alabastrina	**NRRL 2693**	**420**	**GenBank**	**None, free-living**	**N/A**	**2226387**	**3786354**	**420-62L**	**420-82**	**420-310**
Linderina pennispora	**NRRL 3781**	**418**	**GenBank**	**None, free-living**	**N/A**	**2226388**	**3786353**	**418-602**	**418-82**	**418-310**

Footnote:

[1.] AS, Amy Slaymaker; AR, Alen Rizzo; BH, Barb Hayford; CEB, Charles “Eddie” Beard; CLL, Claudia Lopez Lastra; DBS, Douglas B. Strongman; GM, Maria Gabriela Mazzucchelli; JAK, Jason Koontz; JKM, JK Misra; LCF, Leonard C. Ferrington, Jr.; LGV, Laia Guàrdia Valle; MMW, Merlin White; RWL, Robert W. Lichtwardt; SM, Steve Moss; Siri, Augusto Siri; WKR, Will K. Reeves. Some of the sequences were generated from culturable isolates from the University of Kansas Mycological Culture Collection, represented as KUMYCOL.

[2.] Supplemental information on these samples: *Smittium* sp. indet. 1 (“stenosporum” is an epithet that has been considered); *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 being 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).

TABLE 2.2. List of primers and bench codes for primer combinations used to amplify 18S and 28S rRNA genes, as well as RPB1, RPB2, and MCM7 protein-coding genes.

Gene	Bench code	Primer name	Sequences	Note
18S rRNA	170	SR1R	5' TACCTGGTTGATYCTGCCAGT 3'	Vilgalys and Hester 1990
		NS8	5' TCCGCAGGTTCACCTACGGA 3'	White et al. 1990
	177	NS1AA	5' AAGCCATGCATGTCTAAGTATAA 3'	Novel
		NS8AA	5' TACTTCCTCTAAATGACCAAGTTTG 3'	Novel
28S rRNA	97	ITS3	5' GCATCGATGAAGAACGCAGC 3'	White et al. 1990
		LR5	5' TCCTGAGGGAAACTTCG 3'	Vilgalys and Hester 1990
	183	NL1AA	5' GAGTGAAGCGGGAAIAGCTCAAG 3'	Novel
		LR7AA	5' CCACCAAGATCTGCACTAGA 3'	Novel
RPB1	62	RPB1-Af	5' GARTGYCCDGGDCAYTTYGG 3'	Hall (unpubl.).
		RPB1-Dr	5' TTCATYTCRTCDCCRTCRAARTC 3'	Hall (unpubl.).
	601	smRPB1-Afor	5' GARTGYCCBGGHCAYTTYGGWC 3'	Modified RPB1-Af
		kxRPB1-D3r	5' CCRTCRAARTCNGCRTTGTAMG 3'	Modified RPB1-Dr
	602	RPB1-AfL	5' GARTGYCCDGGDCAYTTYGGICA 3'	Modified RPB1-Af
		RPB1-DrL	5' TTCATYTCRTCDCCRTCRAARTCIGC 3'	Modified RPB1-Dr
RPB2	82	fRPB2-5f	5' GAYGAYMGWGATCAYTTYGG 3'	Liu et al. 1999
		fRPB2-7cR	5' CCCATRGCTTGYTTRCCCAT 3'	Liu et al. 1999
MCM7	310	MCM7-8bf	5' GTIGCIGCITAYYTITGYGAY 3'	Modified from Schmitt et al. 2009
		MCM7-16r	5' GTYTGYTGYTCCATIACYTCRTG 3'	Modified from Schmitt et al. 2009

TABLE 2.3. List of morphological characters for taxa presented in "True *Smittium*" clade and Parasmittium subclades. Details of morphology and status per specimen, including trichospore shape, branching pattern, holdfast shape, zygospore shape, host, and origin, were offered and for the sketches mapped onto cladograms.

Node names	Trichospore shape	Branching pattern	Holdfast shape	Zygospore (LW=Length/Width ratio)	Host	Origin
Smittium caudatum KS-1-2	Ovoid	Non-verticillate	N/A	N/A	Chironomidae	United States
Smittium fecundum SPA-X-67	Ovoid	Non-verticillate	N/A	N/A		
Smittium mucronatum ALG-7-W6	Ovoid	Non-verticillate	N/A	LW: 4.2, zygosporephore attached at 1/3 end	Chironomidae	United States
Smittium mucronatum RMBL-61-10	Ovoid	Non-verticillate	N/A	LW: 4.2, zygosporephore attached at 1/3 end	*Psectrocladius* sp.	United States
Smittium mucronatum FRA-12-3	Ovoid	Non-verticillate	N/A	LW: 4.2, zygosporephore attached at 1/3 end	*Psectrocladius sordidellus*	France
Smittium coloradense RMBL-13-41	Ovoid	Non-verticillate	tapering	LW: 5.1, zygosporephore attached at 1/4 end	*Cricotopus* sp.	United States
Smittium coloradense NOR-46-W1	Ovoid	Non-verticillate	tapering	LW: 5.1, zygosporephore attached at 1/4 end	Chironomidae	United States
Smittium sp. NOR-11-W21	Ovoid	Non-verticillate	N/A	N/A	Chironomidae	United States
Austrosmittium biforme 32-1-9	Dimorphic	Non-verticillate	tapering	N/A	Orthocladiinae	New Zealand
Austrosmittium biforme 32-1-8	Dimorphic	Non-verticillate	tapering	N/A	*Cricotopus* sp./Orthocladiinae	Australia
Smittium annulatum CR-143-8	Ovoid	Non-verticillate	ring-like	N/A	Simuliidae	Costa Rica
Smittium culicis ALG-5-W8	Ovoid	Non-verticillate	tapering	LW: 4.4, zygosporephore attached at 1/3 end	*Bactylolabis montana*	Canada
Smittium culicis GSMNP-1	Ovoid	Non-verticillate	tapering	LW: 4.4, zygosporephore attached at 1/3 end	Culicidae	Australia
Smittium culicisoides CR-253-12	Ovoid	Non-verticillate	tapering	N/A	Chironomidae	United States
Smittium fecundum RMBL-64-5	Ovoid	Non-verticillate	simple	N/A	*Psectrocladius* sp.	United States

***Smittium culicis* WYO-51-11**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	***Aedes sticticus***	**United States**
***Smittium culicis* AUS-62-6**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	***Austrothaumalea* sp.**	**Australia**
***Smittium culicis* 12-1-3**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	**Culicidae**	**Australia**
***Smittium culicis* 43-1-2**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	***Chironomus* sp.**	**Australia**
***Smittium culicis* LCF-8-1**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	**Thaumaleidae**	**Australia**
***Smittium culicis* AS-42-1**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	***Corynoneura* sp.**	
***Smittium culicis* 35-1-1**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	**Thaumaleidae**	**Australia**
***Smittium* cf. *culicis* UT-11-W1**	**Ovoid**	**Non-verticillate**	**tapering**	**LW: 4.4, zygosporephore attached at 1/3 end**	**Dipteran**	**United States**
***Smittium simulatum* CHI-8-4**	**Ovoid**	**Non-verticillate**	**simple**	**N/A**	***Aphophila bidentata***	**Chile**
***Stachylina* sp. indet. 1 LCF-22-6[1]**	**N/A**	**Non-verticillate**	**N/A**	**N/A**	***Tanytarsus* sp.**	**Australia**
***Furculomyces boomerangus* AUS-42-7**	**Cylindrical**	**Non-verticillate**	**horseshoe shaped**	**Bend (like boomerangus), LW: 7.9, zygosporephore attached at 1/2 end**	***Procladius ?paludicola***	**Australia**
***Smittium angustum* AUS-126-30**	**Cylindrical**	**Non-verticillate**	**horseshoe shaped**	**N/A**	***Tanytarsus* sp.**	**Australia**
***Furculomyces boomerangus* AUS-77-4**	**Cylindrical**	**Non-verticillate**	**horseshoe shaped**	**Bend (like boomerangus), LW: 7.9, zygosporephore attached at 1/2 end**	***Tanytarsus* nr. *inextentus***	**Australia**
***Smittium morbosum* AUS-X-1**	**Short and thin with median swollen**	**Non-verticillate**	**N/A**	**N/A**	***Anopheles hilli***	**Australia**
***Smittium* cf. *morbosum* ARG-GM-2**	**Short and thin with median swollen**	**Non-verticillate**	**N/A**	**N/A**	**Diptera**	**Argentina**
***Stachylina* sp. NS-X-10**	**N/A**	**Non-verticillate**	**N/A**	**N/A**	**Chironomidae**	**United States**
***Stachylina grandispora* KS-70-W11&18**	**Cylindrical**	**Non-verticillate**	**small and round**	**N/A**	***Chironomus riparius***	**United States**
***Stachylina lentica* NOR-58-10**	**Cylindrical**	**Non-verticillate**	**small and round**	**N/A**	***Chironomus* sp.**	**Australia**
***Stachylina lentica* NOR-45-W3**	**Cylindrical**	**Non-verticillate**	**small and round**	**N/A**	**Chironomidae**	**United States**

***Smittium dipterorum* CR-141-17**	**Cylindrical**	**Verticillate**	**N/A**	**LW: 5.8, zygosporephore attached at 1/3 end**	***Simulium* sp.**	**Costa Rica**
***Smittium gravimetallum* KS-F1-3**	**Cylindrical**	**Verticillate**	**disk-like**	**N/A**	***Dicrotendipes fumidus***	**United States**
***Smittium megazygosporum* SC-DP-2**	**Cylindrical**	**Verticillate**	**N/A**	**LW: 11.8, zygosporephore attached at 1/6 end**	***Simulium vittatum***	**United States**
***Smittium lentaquaticum* TN-27-A5**	**Short and thin**	**Verticillate**	**horseshoe shaped**	**N/A**	***Chironomus* sp.**	**Australia**
***Smittium phytotelmatum* CR-219-1**	**Cylindrical**	**Verticillate**	**N/A**	**N/A**	***Chironomus* sp.**	**Australia**
***Smittium* sp. indet. 3 AS-49-6**[1]	**N/A**	**N/A**	**N/A**	**N/A**	**Chironomidae (*Paratanytarsus* sp.?)**	**New Zealand**
***Pseudoharpella arcolamylica* LCF-3**	**Cylindrical but coiled**	**N/A**	**horseshoe shaped at beginning**	**Bend (sometimes), LW: 8.4, zygosporephore attached at 4/9 end**	**Dixidae**	**United States**
***Smittium dipterorum* CR-253-14**	**Cylindrical**	**Verticillate**	**N/A**	**LW: 5.8, zygosporephore attached at 1/3 end**	***Simulium* sp.**	**Costa Rica**
***Coleopteromyces amnicus* ARG-15-3**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	**Scirtidae**	**Argentina**
***Coleopteromyces amnicus* ARG-15-6F**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	**Scirtidae**	**Argentina**
***Smittium tipulidarum* RMBL-31-1**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	***Elliptera astigmatica***	**United States**
***Smittium lentaquaticum* TN-27-A4**	**Short and thin with median swollen**	**Non-verticillate**	**N/A**	**N/A**	***Chironomus* sp.**	**Australia**
***Smittium* cf. *morbosum* WKRa**	**N/A**	**N/A**	**N/A**	**N/A**	***Ochlerotatus triseriatus***	**United States**
***Smittium* cf. *morbosum* WKRb**	**N/A**	**N/A**	**N/A**	**N/A**	***Ochlerotatus triseriatus***	**United States**
***Smittium* cf. *simulii* SPA-X-70**	**N/A**	**N/A**	**N/A**	**N/A**	**Culicidae**	**Australia**
***Smittium* sp. CR-259-4**	**Cylindrical**	**N/A**	**N/A**	**N/A**	***Simulium* sp.**	**Costa Rica**
***Smittium orthocladii* OK-4-19**	**Dimorphic**	**Non-verticillate**	**N/A**	**LW: 8.7, zygosporephore attached at 1/4 end**	**Chironomidae**	**United States**
***Smittium orthocladii* LCF-BT-1**	**Dimorphic**	**Non-verticillate**	**N/A**	**LW: 8.7, zygosporephore attached at 1/4 end**	***Corynoneura* sp.**	
***Smittium orthocladii* KS-82-W1**	**Dimorphic**	**Non-verticillate**	**N/A**	**LW: 8.7, zygosporephore attached at 1/4 end**	***Orthocladius abiskoensis***	**United States**

***Smittium perforatum* RMBL-44-3**	**N/A**	**Non-verticillate**	**N/A**	**N/A**	***Diamesa* sp.**	**United States**
***Smittium* sp. RMBL-48-8**	**N/A**	**N/A**	**N/A**	**N/A**	***Prosimulium* sp.**	**United States**
***Smittium* sp. GB-X-1**	**N/A**	**N/A**	**N/A**	**N/A**	***Simulium ornatum***	**United Kingdom**
***Smittium tronadorium* ARG-24-20F**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	***Limaya* sp.**	**Argentina**
***Smittium tronadorium* ARG-24-2F**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	***Paraheptagyia* sp.**	**Argentina**
***Smittium imitatum* CHI-20-11**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	***Simulium* sp.**	**Costa Rica**
***Smittium imitatum* CHI-9-4**	**Cylindrical**	**Non-verticillate**	**N/A**	**N/A**	***Simulium* sp.**	**Costa Rica**
***Smittium cylindrosporum* CHI-27-1**	**Cylindrical**	**Verticillate**	**N/A**	**LW: 4.9, zygosporephore attached at 1/3 end**	***Cricotopus* sp.**	**United States**
***Smittium simulii* SWE-8-4**	**Subcylindrical**	**Non-verticillate**	**horseshoe shaped**	**N/A**	***Diamesa* sp.**	**United States**
***Smittium simulii* CAL-8-1**	**Subcylindrical**	**Non-verticillate**	**horseshoe shaped**	**N/A**	***Simulium argus***	**United States**
***Smittium* sp. TN-3-12**	**N/A**	**N/A**	**N/A**	**N/A**	**Chironomidae**	**United States**
***Smittium* sp. indet. 1 OK-3-22[1]**	**N/A**	**N/A**	**N/A**	**N/A**	**Chironomidae**	**United States**
***Trichozygospora chironomidarum* TN-3-16**	**Ovoid**	**N/A**	**N/A**	**Multiple appendages (>10), LW: 4.1, zygosporephore attached at 1/4 end**	**Chironomidae**	**United States**
***Smittium commune* KS-6-6**	**Cylindrical**	**Verticillate**	**N/A**	**N/A**	**Chironomidae**	**United States**
***Smittium commune* KS-2-21**	**Cylindrical**	**Verticillate**	**N/A**	**N/A**	**Chironomidae**	**United States**
***Smittium simulii* 41-1-6**	**Subcylindrical**	**Non-verticillate**	**horseshoe shaped**	**N/A**	***Orthocladius* sp.**	**Australia**
***Smittium hecatai* SPA-X-63**	**N/A**	**Verticillate**	**N/A**	**N/A**		
***Smittium* sp. indet. 2 AS-22-15[1]**	**N/A**	**Non-verticillate**	**small secreted**	**N/A**	***Cricotopus* sp.**	**United States**
***Smittium* sp. indet. 2 LCF-27-15[1]**	**N/A**	**Non-verticillate**	**small secreted**	**N/A**	**Orthocladiinae**	**New Zealand**

***Smittium* sp. indet. 2 AS-27-9**[1]	**N/A**	**Non-verticillate**	**small secreted**	**N/A**	**Orthocladiinae**	**New Zealand**

[1.] Supplemental information on these samples: *Smittium* sp. indet. 1 ("stenosporum" is an epithet that has been considered); *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 being 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).

Overview tree

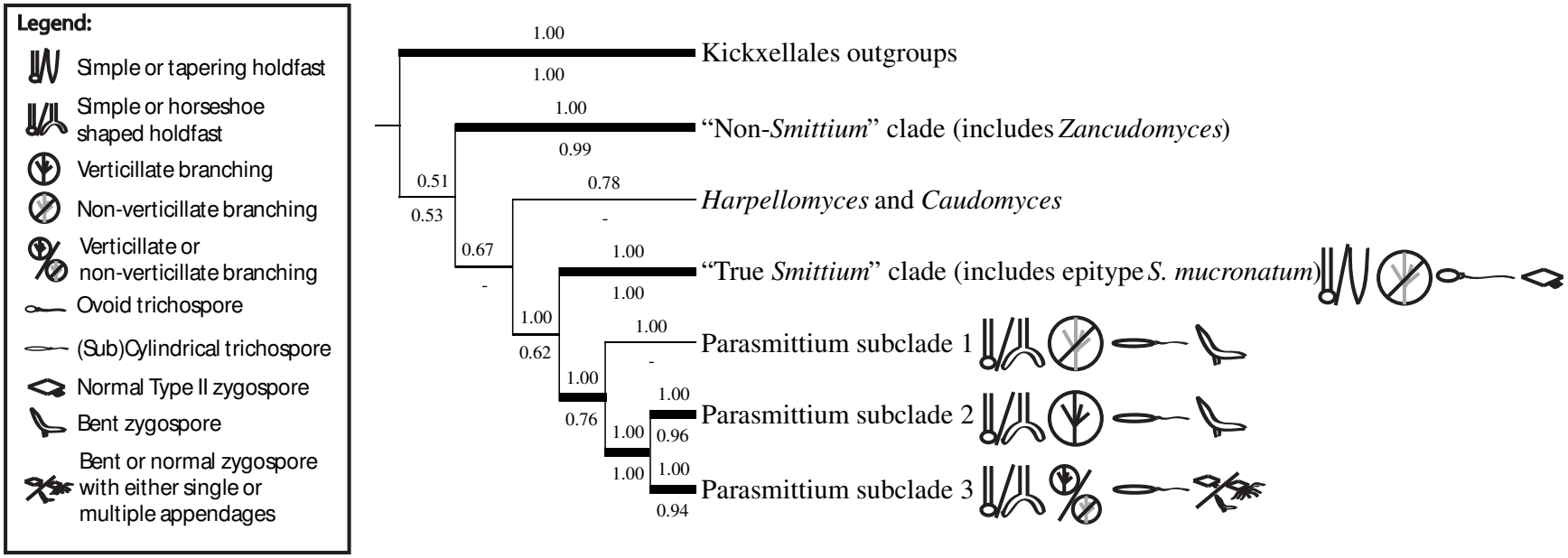


FIG. 2.1. Overview tree summarized from complete combined multigene tree. It includes representative species of *Smittium*, a broad sampling of other Harpellales and some Kickxellales as outgroups. Subclades are collapsed for clarity. For this and all further trees, supports given above the branches are Bayesian posterior probabilities (BPP), and below are maximum-likelihood bootstrap proportions (MLBP). Branches in bold are considered to have high support (BPP > 95%, MLBP > .70). The term "Parasmittium" is used here for the first time, but does not carry, nor is it implied any rank designation. Sketches of morphological characters, particularly those either in use or as candidates for taxonomic consideration are also mapped here, as well as in the subclade figures (FIGS. 2.2–2.5).

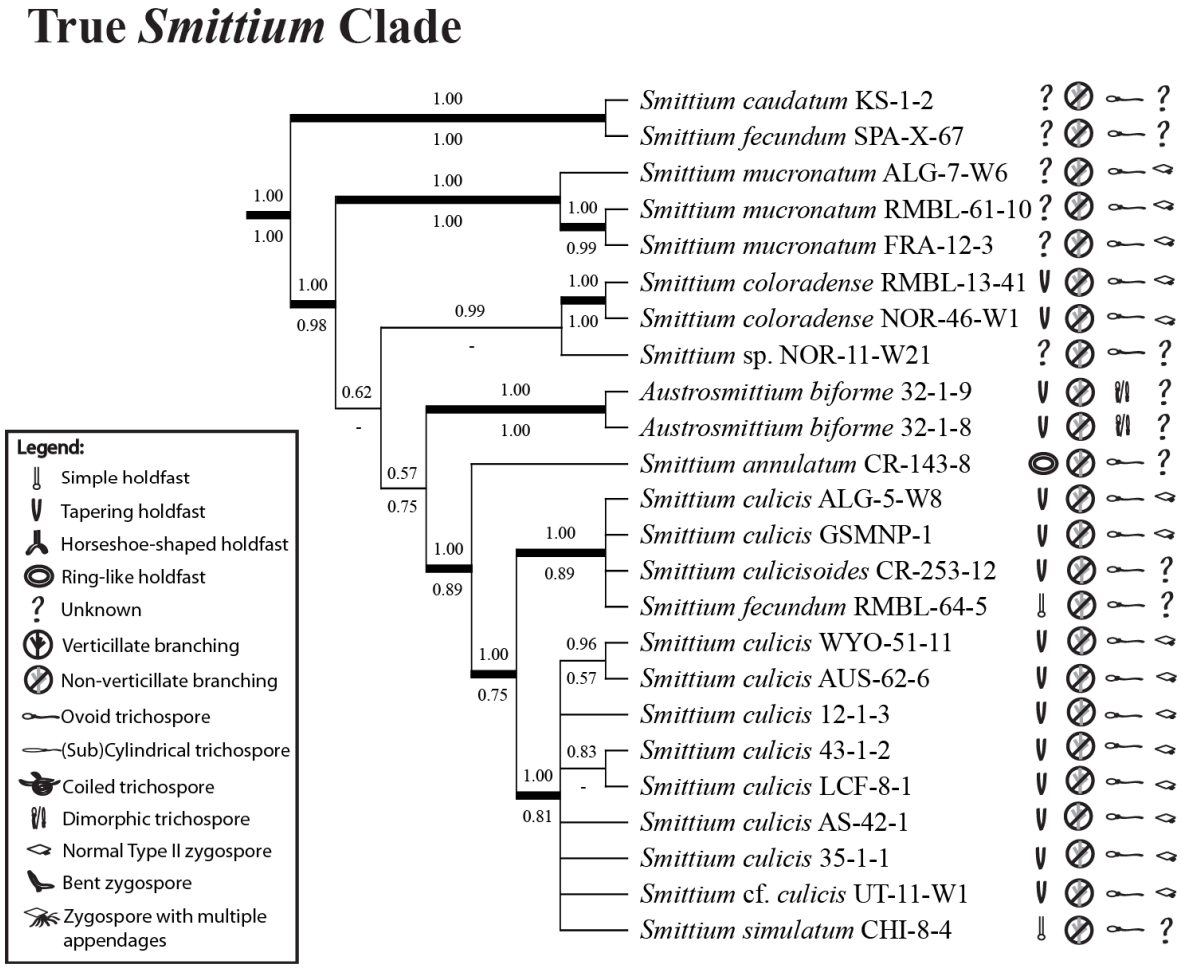


FIG. 2.2. "True *Smittium*" clade, from the complete phylogenetic tree. It includes the epitype *Smittium mucronatum* among other Smittiums, as well as the widespread taxon *S. culicis*. *Austrosmittium biforme* is the only "Non-*Smittium*" included.

Parasmittium subclade 1

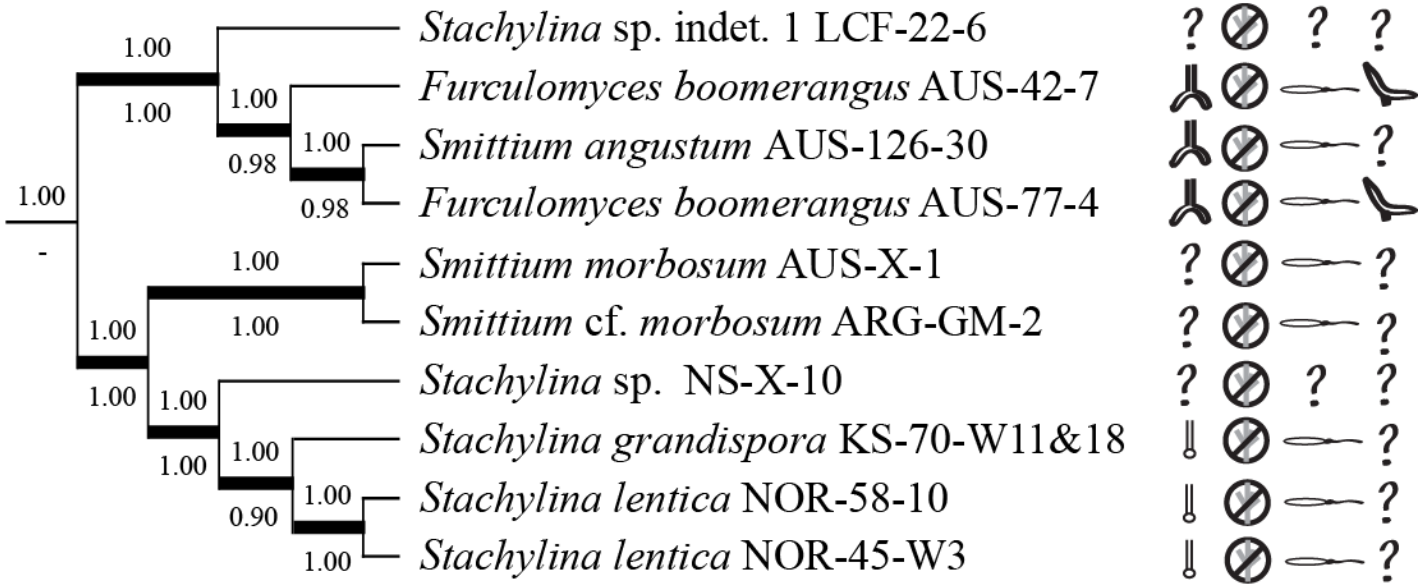


FIG. 2.3. Parasmittium subclade 1, from the complete tree. It includes *Smittium morbosum* (AUS-X-1), the only parasitic *Smittium*, as well as representatives of *Furculomyces* and *Stachylina*. Isolate AUS-X-1 is considered to be the authentic *Smittium morbosum*, anchored as it is in this subclade of the tree.

Parasmittium subclade 2

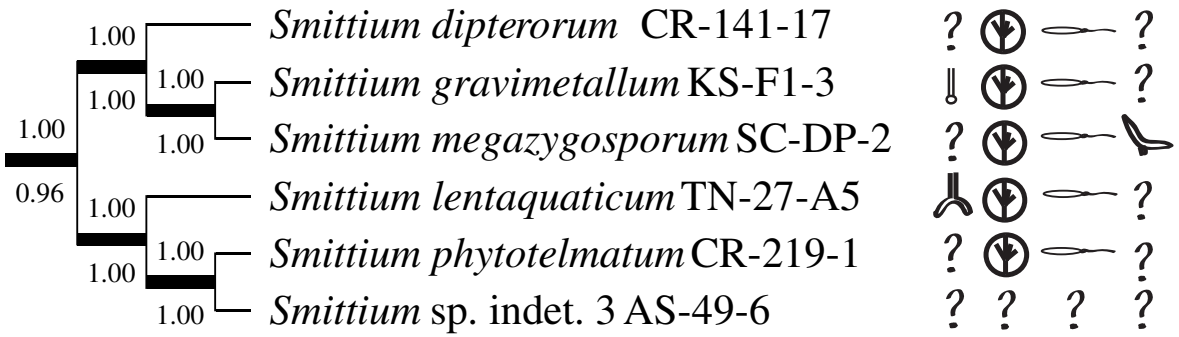


FIG. 2.4. Parasmittium subclade 2, from the complete tree. It represents a small clade of six *Smittium* species, all with verticillate branching type where known, making it a distinguishing feature among the three Parasmittium subclades.

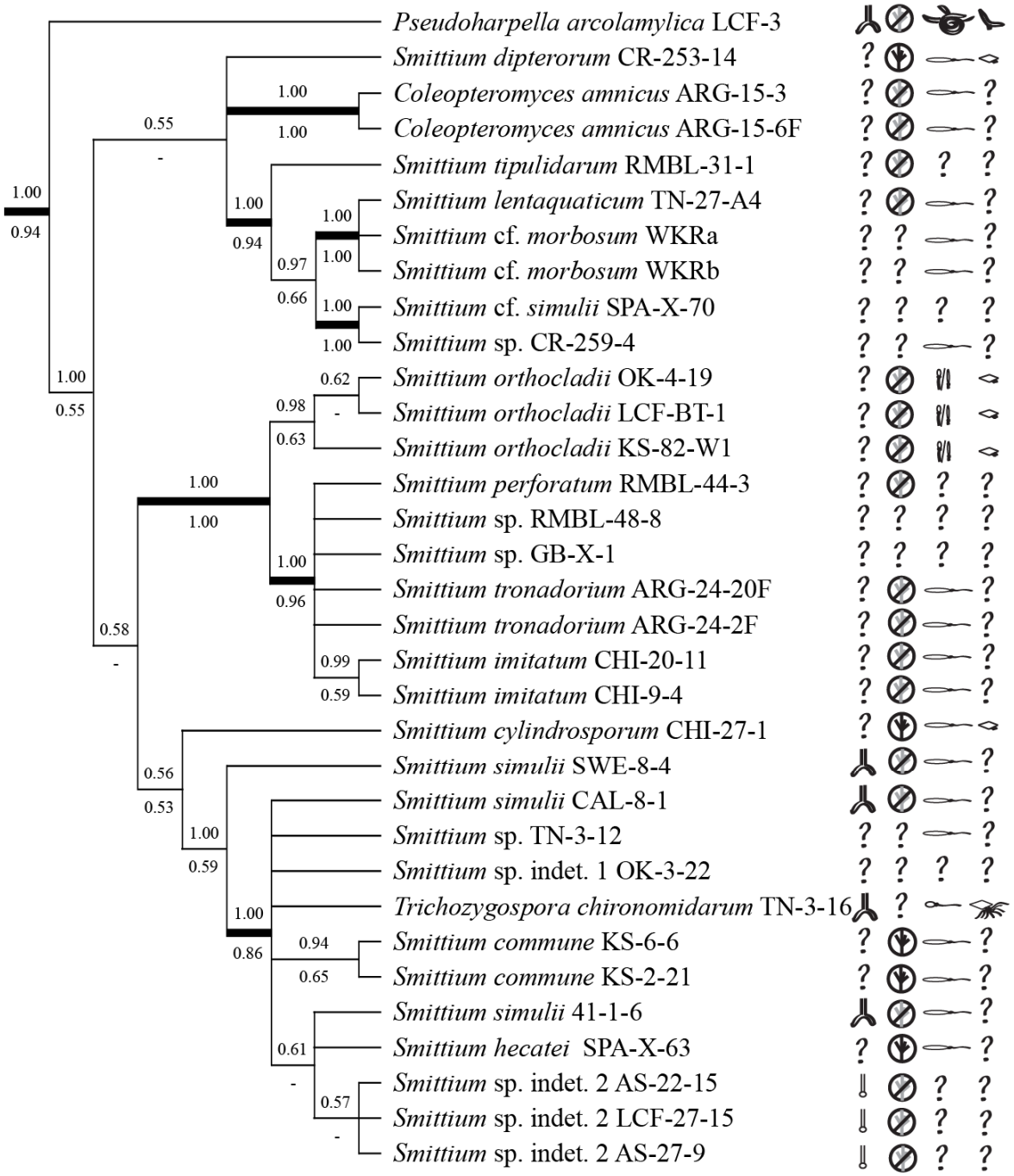
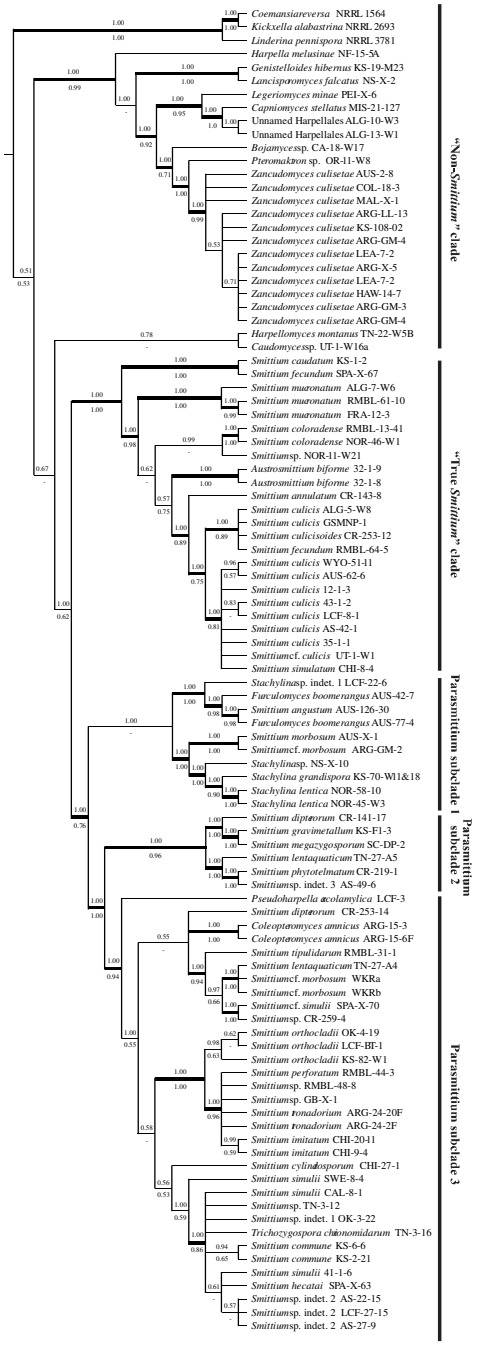
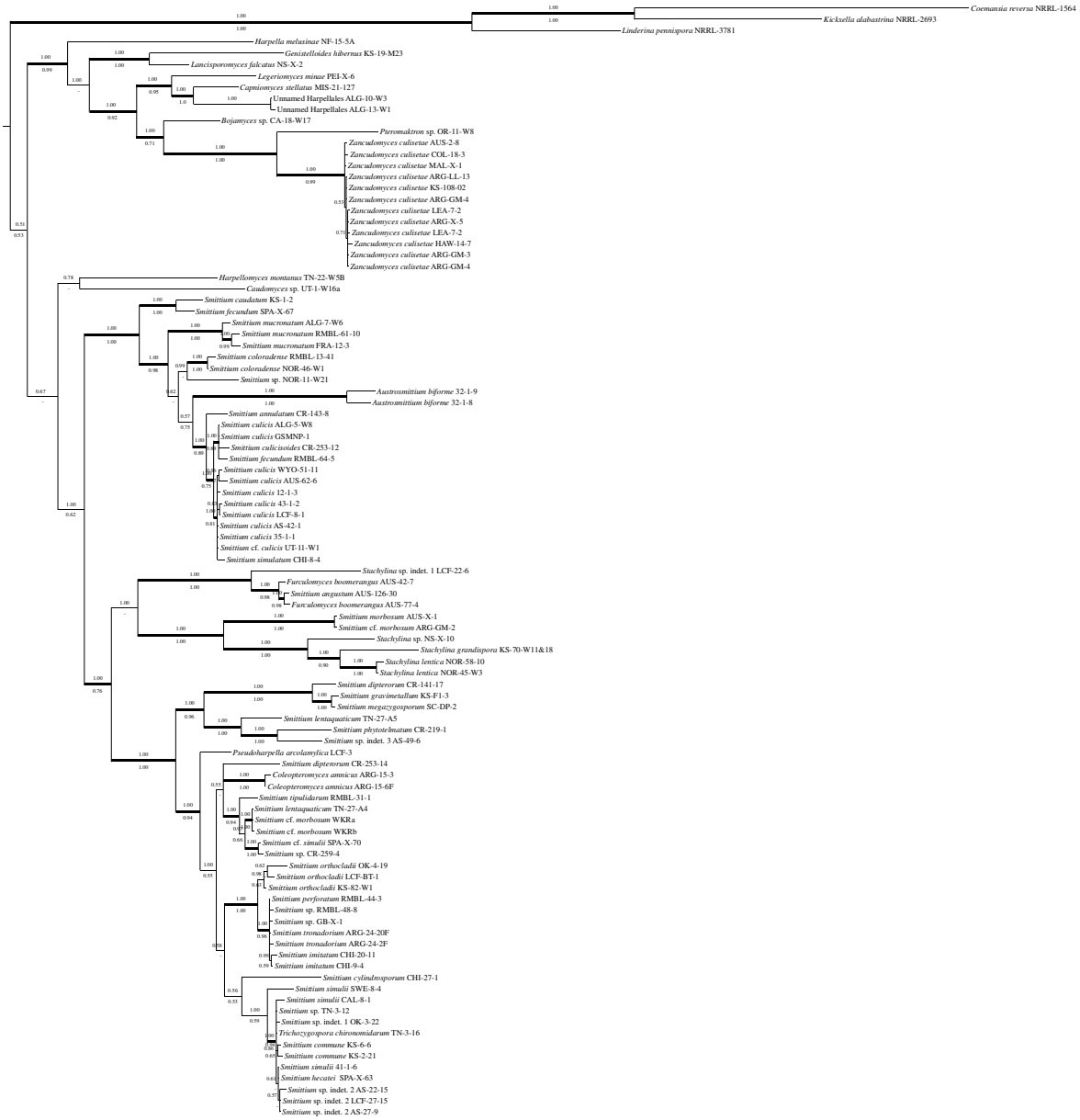


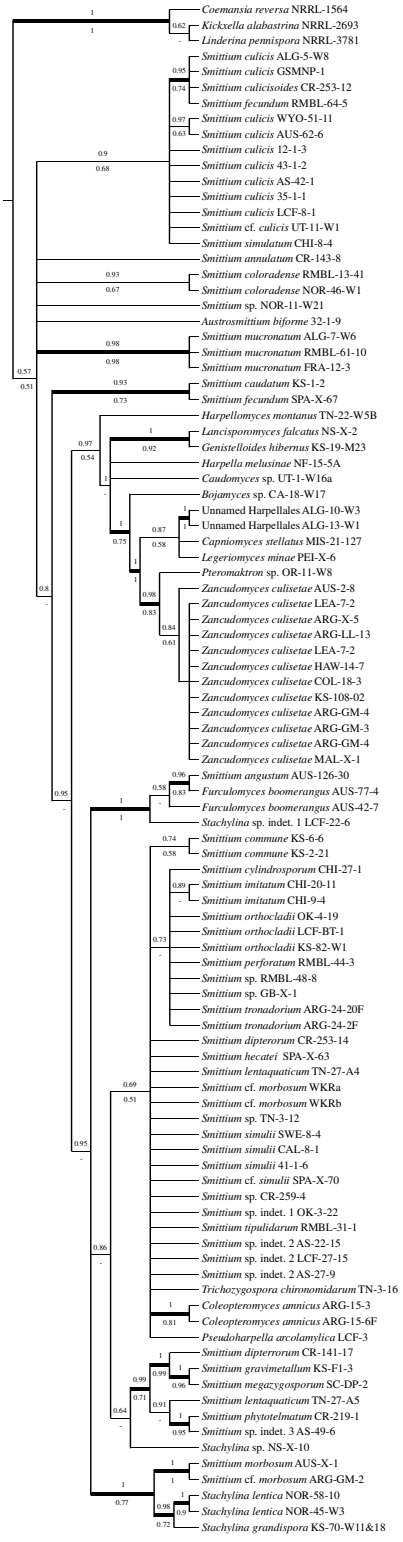
FIG. 2.5. Parasmittium subclade 3, from the complete tree. It is the largest and most diverse subclade with numerous *Smittium* species, including *Smittium simulii*. This subclade also includes representatives of *Coleopteromyces*, *Pseudoharpella*, and *Trichozygospora*.



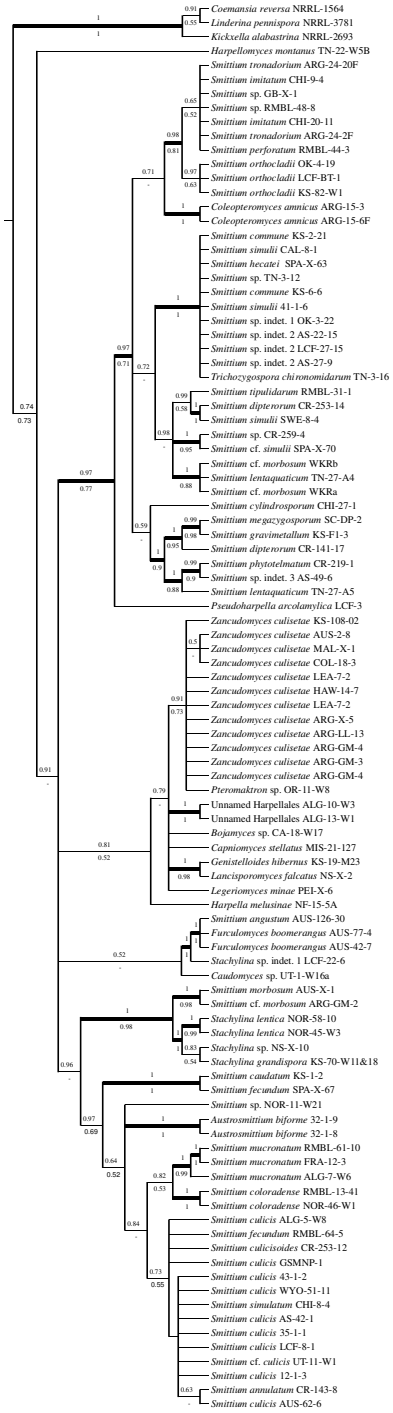
SUPP. FIG. 2.1. Complete phylogenetic tree from combined 18S, 28S rRNA gene, as well as RPB1, RPB2, and MCM7 protein sequences (translated from protein-coding genes). Support above the branches are Bayesian posterior probability (BPP), and below are maximum-likelihood bootstrap proportions (MLBP). Branches in bold indicate high support (BPP > 95%, MLBP > .70). The overview tree (FIG. 2.1) is the summarized version of this complete tree.



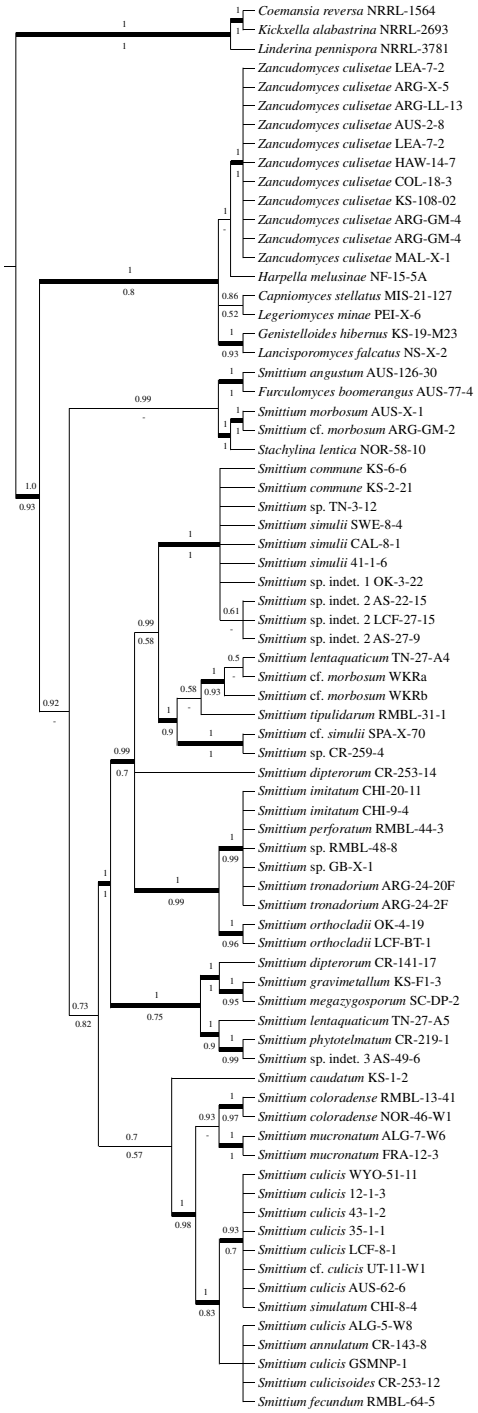
SUPP. FIG. 2.2. Complete five-gene phylogenetic tree showing branch length variation. As for SUPP. FIG. 2.1, support above the branches are Bayesian posterior probability (BPP), and below the branches are based on the maximum-likelihood bootstrap proportions (MLPP). Branches in bold indicate high support (BPP > 95%, MLBP > .70).



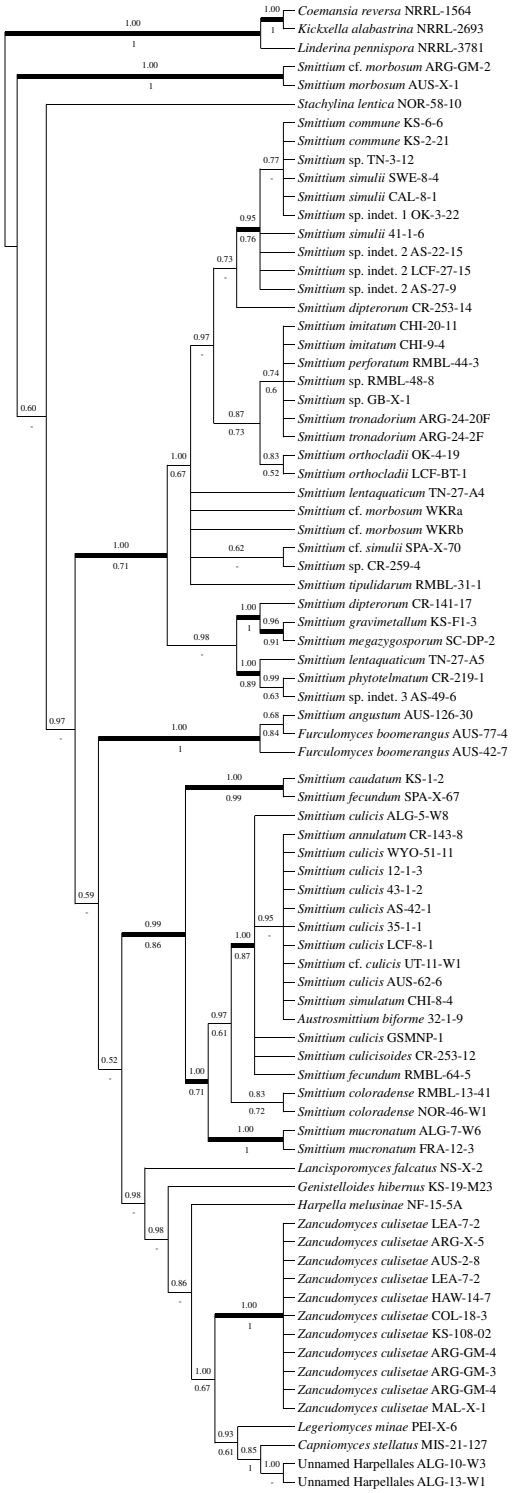
SUPP. FIG. 2.3. 18S ribosomal RNA gene phylogenetic tree, used to assess resolution and overall congruency with the other four single gene trees.



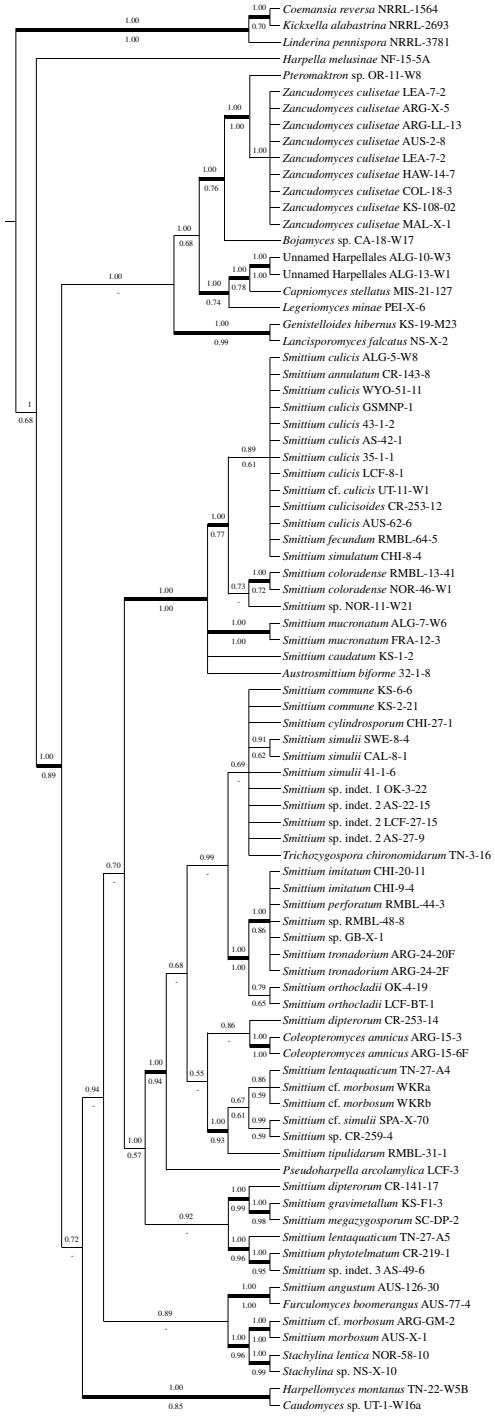
SUPP. FIG. 2.4. 28S ribosomal RNA gene phylogenetic tree, used to assess resolution and overall congruency with the other four single gene trees.



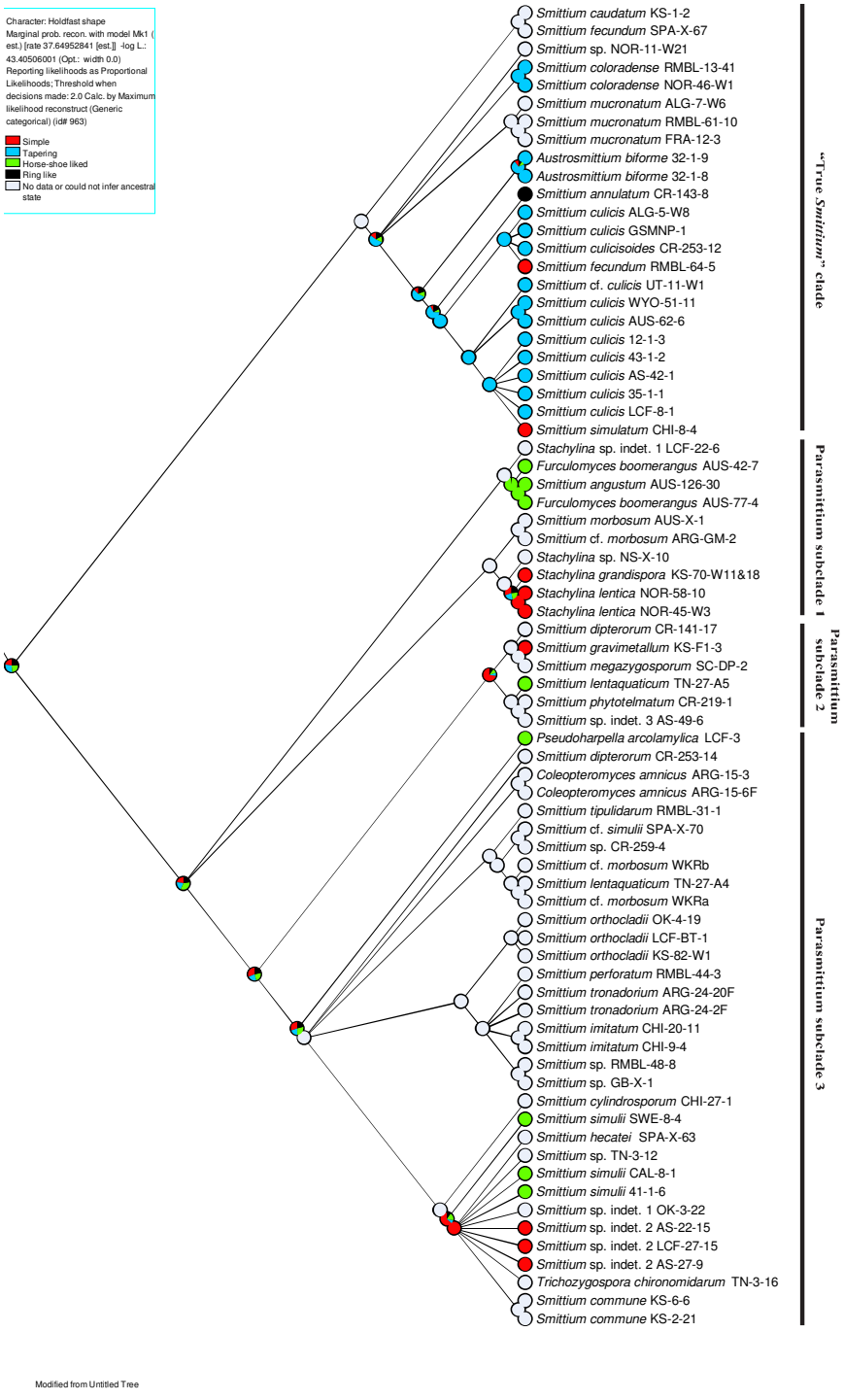
SUPP. FIG. 2.5. DNA-directed RNA polymerase II subunit 1 (RPB1) translated protein sequence-based phylogenetic tree, used to assess resolution and overall congruency with the other four single gene trees.



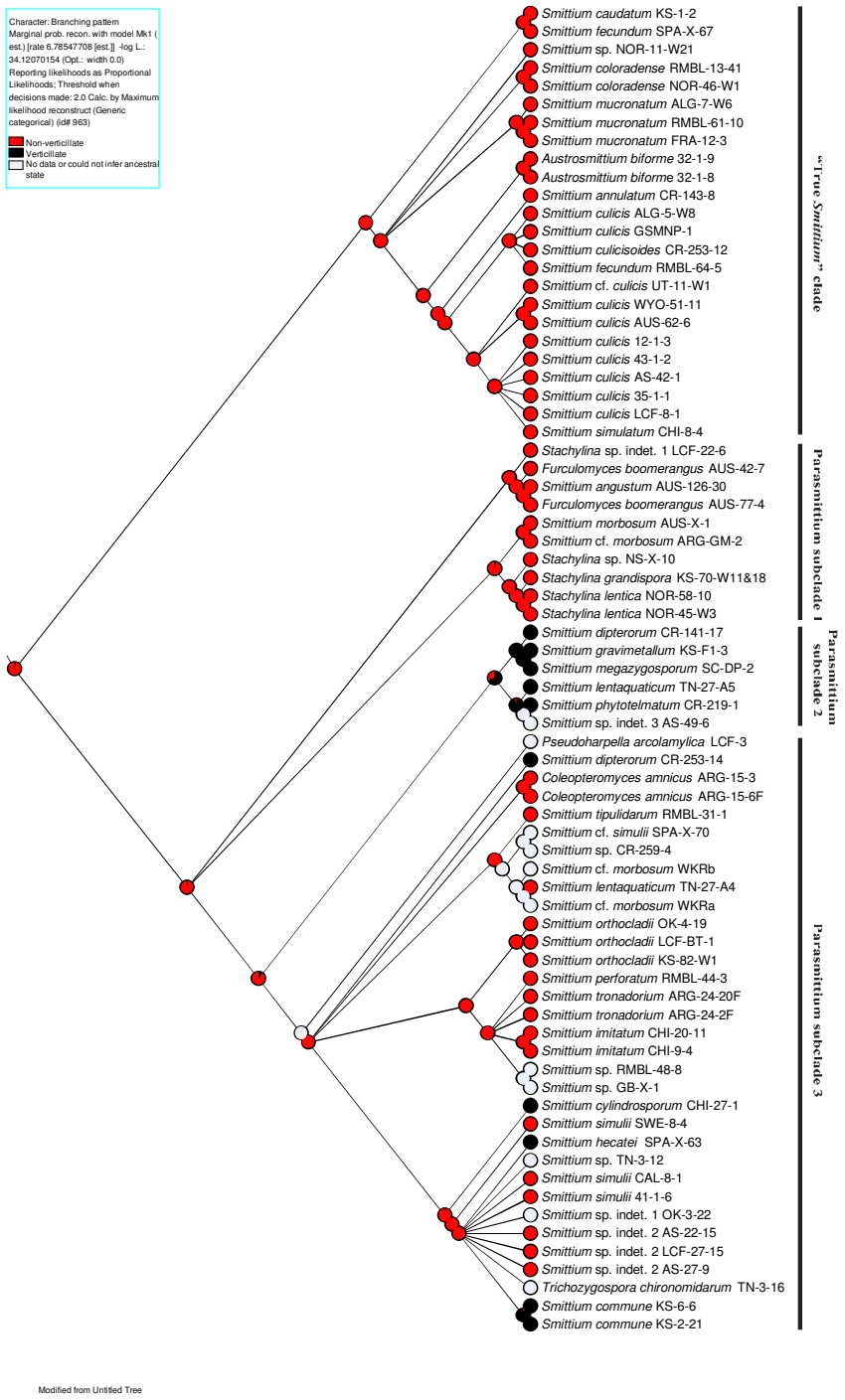
SUPP. FIG. 2.6. DNA-directed RNA polymerase II subunit 2 (RPB2) translated protein sequence-based phylogenetic tree, used to assess resolution and overall congruency with the other four single gene trees.



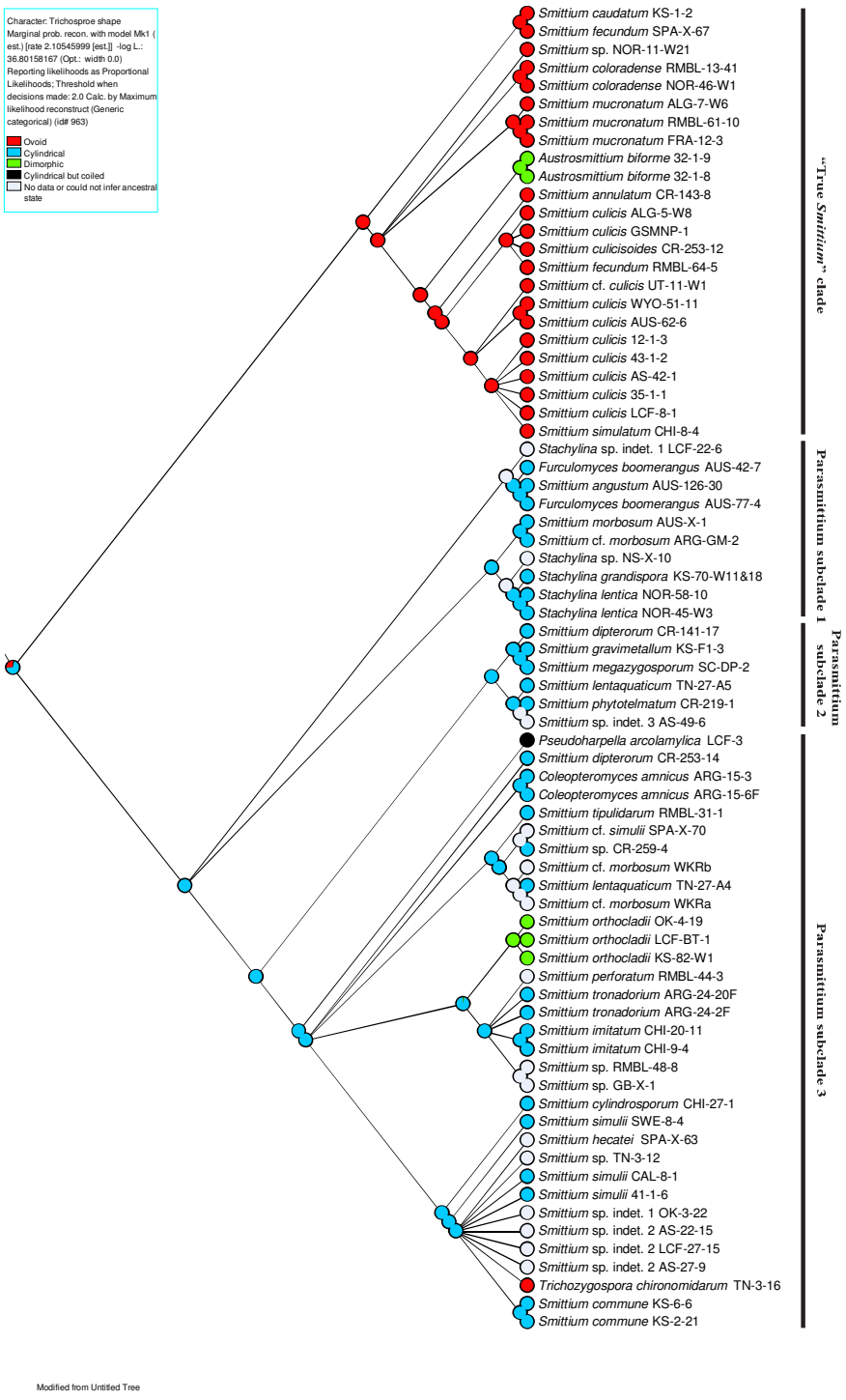
SUPP. FIG. 2.7. Mini chromosome maintenance complex component 7 (MCM7) translated protein sequence-based phylogenetic tree, used to assess resolution and overall congruency with the other four single gene trees.



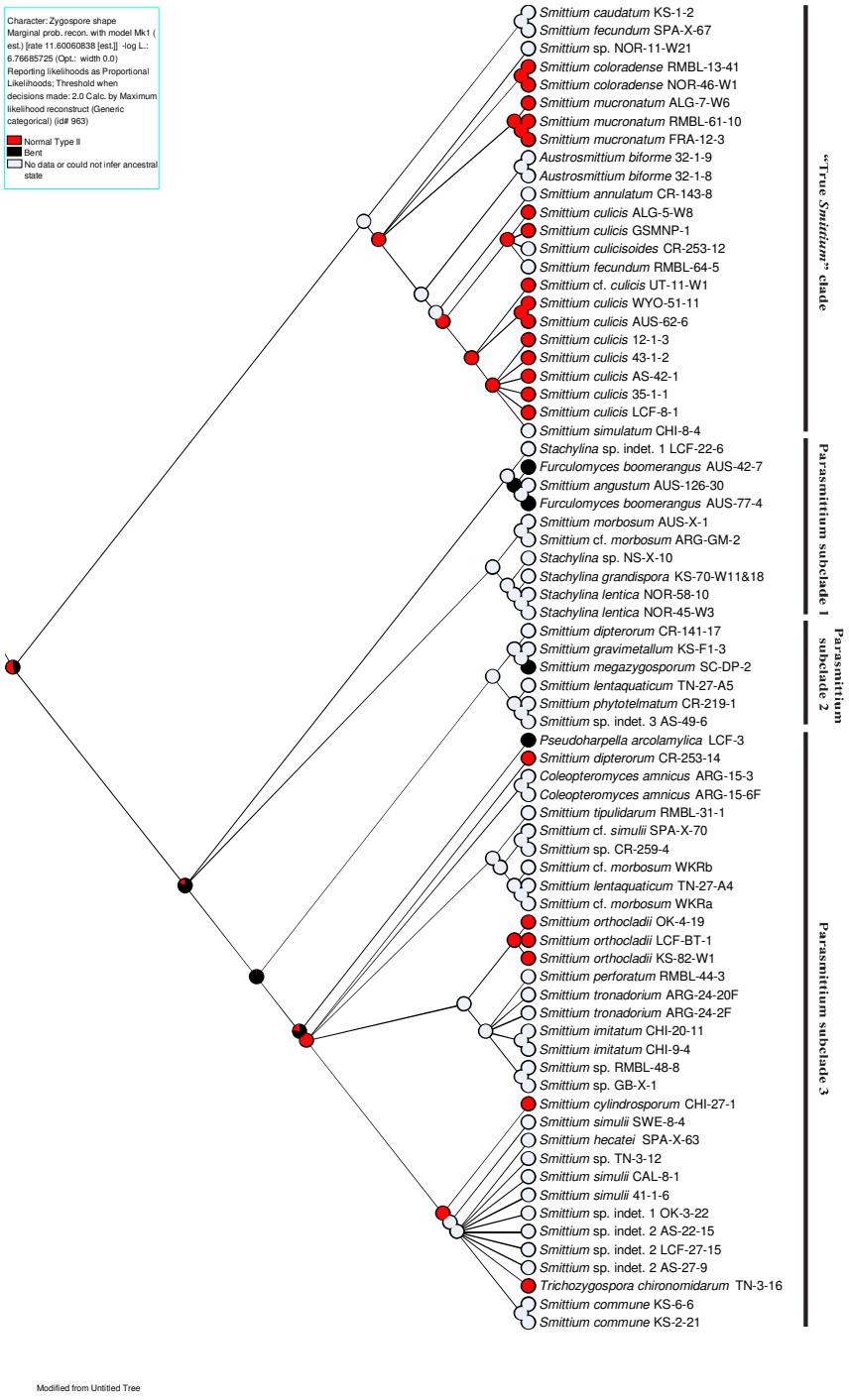
SUPP. FIG. 2.8. Likelihood morphological character mapping of holdfast shapes with *Smittium* and related Harpellales. Shown is likelihood morphological character mapping of three different holdfast shapes—simple, tapering, horseshoe-shaped, and ring-like. Tree drawn in Mesquite using consensus maximum likelihood tree; pie charts at nodes represent ancestral states probabilities calculated from the maximum likelihood reconstruction of each possible character state.



SUPP. FIG. 2.9. Likelihood morphological character mapping of thallus branching types with *Smittium* and related Harpellales. Shown is likelihood morphological character mapping of fungal thalli branching types and associated two different types—non-verticillate and verticillate branching types. Tree drawn in Mesquite using consensus maximum likelihood tree; pie charts at nodes represent ancestral states probabilities calculated from the maximum likelihood reconstruction of each possible character state.



SUPP. FIG. 2.10. Likelihood morphological character mapping of trichospore shapes with *Smittium* and related Harpellales. Shown is likelihood morphological character mapping of four different trichospore shapes—ovoid, cylindrical, dimorphic, and cylindrical but coiled. Tree drawn in Mesquite using consensus maximum likelihood tree; pie charts at nodes represent ancestral states probabilities calculated from the maximum likelihood reconstruction of each possible character state.



SUPP. FIG. 2.11. Likelihood morphological character mapping of zygospore shapes with *Smittium* and related Harpellales. Shown is likelihood morphological character mapping of different zygospore shapes—normal type II biconical shape and bent biconical or fusiform shape. Tree drawn in Mesquite using consensus maximum likelihood tree; pie charts at nodes represent ancestral states probabilities calculated from the maximum likelihood reconstruction of each possible character state.

CONCLUSIONS

Separate 2-gene and 5-gene phylogenetic analyses were used to address fundamental questions surrounding the "Smittium" clade of early-diverging fungi. A new genus, *Zancudomyces*, was established to accommodate the farthest *Smittium* outlier in the trees, *Smittium culisetae*. Total evidence for this decision also came from studies of its morphology, ecology, physiology, and immunology to help complete the molecular-based phylogeny. Chapter 1 has been peer-reviewed and revised and is in final resubmission for publication in *Mycologia* (Wang et al. 2012).

Toward resolution of the polyphyletic "*Smittium*" clade, the 5-gene phylogeny distinguished a "True *Smittium*" clade and three "Parasmittium" subclades. Morphological characters including the nature of the holdfast, branching type, trichospore and zygospore shape, were also mapped and assessed. Some misidentified *Smittium* species were identified whereas others are sequestered as unidentified (*Smittium* sp.). Some characters remain as unknown, and their recovery with future collections would be an asset. Conversely, the trees should help focus such efforts on taxa of interest. This remains a diverse and species-rich genus that warrants further analysis.

Future studies should consider the ultrastructural (electron microscopic or EM) characters of the representatives of certain species of *Smittium* and allies within these clades (i.e. for *Smittium mucronatum, S. culicis, S. simulii, S. morbosum, Austrosmittium biforme, Furculomyces boomerangus,* and *Stachylina grandispora*). That kind of data

may provide the additional support needed to confidently separate the "True *Smittium*" clade from members of the Parasmittium clade. For example, features such as the concentric electron-dense, ring-like structure, as seen in cross sections of appendages (of *S. mucronatum* and *S. culicis*) from earlier EM studies (Manier and Coste-Mathiez 1968, Moss and Lichtwardt 1976), hold promise as diagnostic morphological characters that shed light on the taxonomy and evolutionary relationship of members of *Smittium*. New generic designations may be forthcoming with such additional support. Despite the fact that this is the widest sampling of *Smittium* to date, additional taxa should be included in future analyses.

For species boundary delineation studies within metaspecies, as possibly the case for *Smittium culicis*, ITS (Schmitt et al. 2009, Schoch et al. 2012) or combined ITS and 28S rRNA genes (Schoch et al. 2012) could be used and combined with a genealogical sorting index study (Cummings et al. 2008, Sakalidis et al. 2011, Weisrock et al. 2010) to provide statistical support to uncover cryptic species and show species origins. This would be particularly exciting if it could be paired with data from the hosts to assess possible coevolutionary patterns for the group.

Whole genome sequencing projects are ongoing and will offer the next tool for molecular phylogenetics. Among the Harpellales, genome studies have been initiated for *Zancudomyces culisetae* (Liu and Voigt 2010). One can envision eventual molecular phylogenetic analyses based on the whole genomes and combined with detailed morphological data toward revised classifications. Nonetheless, multi-gene analyses are still a valuable tool to sort out relationships among taxa, especially for those species that are still unculturable.

References

Cummings MP, Neel MC, Shaw KL. 2008. A genealogical approach to quantifying lineage divergence. *Evolution* 62:2411–2422.

Liu XY, Voigt K. 2010. Molecular characters of Zygomycetous fungi. In: Gherbawy Y, Voigt K, eds. Molecular Identification of Fungi. Berlin, Heidelberg, Germany: Springer-Verlag:461–488.

Manier JF, Coste-Mathiez F. 1968. L'ultrastructure du filament de la spore de *Smittium mucronatum* Manier, Mathiez 1965 (Trichomycète, Harpellale). *C R Acad Sci Hebd Seances Acad Sci D* 266:341–342.

Moss ST, 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.

Sakalidis ML, Hardy GEStJ, Burgess TI. 2011. Use of the Genealogical Sorting Index (GSI) to delineate species boundaries in the *Neofusicoccum parvum-Neofusicoccum ribis* species complex. *Mol Phylogenet Evol* 60(320):333–344.

Schmitt I, Crespo A, Divakar PK, Fankhauser JD, Herman-Sackett E, Kalb K, Nelsen MP, Nelson NA, Rivas Plata E, Shimp AD, Widhelm T, Lumbsch HT. 2009. New

primers for promising single-copy genes in fungal phylogenetics and systematics. *Persoonia* 23:35–40.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium. 2012. The nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *P Natl Acad Sci USA* 109(16):6241–6246.

Wang Y, Tretter ED, Lichtwardt RW, White MM. 2012. Overview of 75 years of *Smittium* research, establishing a new genus for *Smittium culisetae*, and prospects for future revisions of the "Smittium" clade. *Mycologia* (in review).

Weisrock DW, Rasoloarison RM, Fiorentino I, Ralison JM, Goodman SM, Kappeler PM, Yoder AD. 2010. Delimiting species without nuclear monophyly in Madagascar's mouse lemurs. *PLoS ONE* 5(3):e9883. doi:10.1371/journal.pone.0009883.

APPENDIX

Detailed Instruction for the Molecular Phylogenetic Analysis in This Study

1. Importing Dataset to Mesquite (v2.75)

1.1. Create a nexus file in Mesquite (Master file, containing all genes). During file creation, create a taxon block with the full number of taxa in the analysis. Call this taxon block "Master taxon block".

1.2. Click the "List & Manage Taxa". Create a suitable list of taxon names, which should include some sort of numerical sample identification code (like "0001") that can be used to tie the master taxon records to their individual gene entries. Paste these taxon names into Mesquite.

1.3. Click the "Taxa &Trees" and select the "New Block of Taxa"—name this block of taxa after the gene with which it will be used (like "Taxa 18S"), and specify a number of taxa.

1.4. Click the newly created "Taxa 18S", then click the "Characters" label and select the "New Empty Matrix", choose "18S" taxa and indicate whether it will be a DNA or amino acid matrix. Now you can copy both of the FASTA Tag for the individual gene sequence and the sequence data into the Character Matrix. Make sure that your FASTA Tags contain the same numerical taxon identifier you used in the master block.

1.5. In the new version of the Mesquite (v2.75), we were able to use the MUSCLE alignment function under the "Matrix" tab to align the sequences. You may also want to click "Matrix" tab and select the "Display" button, choose "narrow columns" and "thin rows" for a better view.

1.6. Find the start and end of sequence and delete the sequences before the forward primer (as well as the primer region) and the ones after the reverse primer (also delete the primer region), using the tool—"Find sequence"—"Matching sequence" under the tab "Edit" (Be aware that, when design the primer, there could be some ambiguity code, like W, M, K et al., all of which should be counted as the "Number of allowed mismatches"; For reverse primer, do not forget to select the "Search for reverse complement" button).

1.7. Change the terminal gaps to "?": select the tab "Matrix"—"Alter/transform"—"Terminal gaps to '?'".

2. Translating DNA Sequences to Amino Acids by Mesquite (v2.75)

2.1. Use MUSCLE (embedded in Mesquite v2.75) to align the sequences, then manually check all of the gaps. If a single gap or extra base exists in only a small number of sequences, it is often helpful to check within Sequencer to make sure it is real and not simply a miscall. To translate all of the nucleotide sequences into proteins, the reading frame must be consistent, so it is vitally important to identify and fix these errors.

2.2. Attempt to remove all introns from the nucleotide alignment. Introns can usually be identified by searching for large, unalignable regions possessed by only some of the sequences in the alignment. Spliceosomal introns usually start with GT and end with AG, rarely introns may also start with AT and go to AC.

2.3. Make sure the sequences start from the real codon position 1 (the codon position "123" is stable-stable-variable) and set the codon position to "123123" in "Characters table", then change all terminal "?" to gap in Character Matrix, before translation begin, "collapse all sequences to left".

2.4. Save file (in case found some expected stop codons) and click the tab “Character”, choose “Make new matrix from”—“translate DNA to protein”.

2.5. Check the protein sequences and align them using MUSCLE (for the stop codons, we need to remember their position and revert the file to the one previously saved, then recheck the DNA sequence for any sliding issues in codon position or even mis-deleting introns; fix them and redo steps 2.4 and 2.5).

2.6. Remove gap-only characters by clicking “Matrix”—“alter/transform”—“remove gap-only characters”.

2.7. To look for conservative protein sequences, copy all rows and paste them to a word document and replace all “tabs” (^t) with “hard returns” (^p), then paste it to txt file. Upload the txt file to Jalview (http://www.jalview.org/download.html) by selecting the tab “file”—“input alignment”—“from textbox”, then present the hydrophobicity of water by clicking the tab “Colour”—“Hydrophobicity”. Then compare it with the sequences in Mesquite (to show the exclude characters, you have to click “Matrix”—“Add Characters info strip”—“Boolean Info Strip”—“Character included”). This will provide you with information about how well the sequences are aligned.

2.8. When copying the aligned sequences (both transcripted DNA and translated protein) to the excel data file, do not forget to change terminal gaps to “?” and check the length of each sequences to make sure they are correct (“V lookup” formula can help you find corresponding value to organize file).

3. Model Tests

3.1. Save the nexus file to a different name to avoid changing the master file, then simplify the taxa name by opening the taxa list to be exported, selecting “list”, then “Taxon names”—“Simplify Taxon names”.

3.2. Delete excluded characters by clicking the “List & Manage Characters”, then use the “magic wand” tool to click any one excluded character. This will select all of the excluded characters for this gene. Press backspace to delete them.

3.3. Click “file” tab and export data as “FASTA (RNA/DNA)” or “FASTA (protein)” (depends on it is gene or protein sequences) with default setting (be sure to “include gaps”).

3.4a. For DNA sequences, use jModelTest to estimate the model by analyzing the exported file with “compute likelihood scores” and default setting, then when the analysis finished, “Do AIC calculation” to show the best model;

3.4b. For protein sequences, submit the exported file to ProtTest (http://darwin.uvigo.es/software/prottest_server.html) with the setting—Build BioNJ tree, Model selection criterion using “AICc”, for the rest with default.

4. Bayesian Analyses by MrBayes (v3.1.2) through Beowulf System

4.1. Save the nexus file to a different name for export and then simplify the taxa name by clicking “Taxa ‘18S’ Taxa” and “list” then “Taxon names”—“Simplify Taxon names”.

4.2. Export the single gene data for Bayesian analysis by clicking “file”—“Export”—select “Export NEXUS for MrBayes” (for a single gene) or “Fused Matrix Export” (for a multigene supermatrix) and select the single gene matrix (for a single gene) or the master taxon block (for multiple genes). For a single gene analysis, you can input your analysis

parameters into a window that pops up; for a multigene analysis, you will have to add this information to the end of the nexus file.

For DNA sequences(“nst=6” represents “GTR”; “inv” represents “I” model; “gamma” represents “G” model):

```
begin mrbayes;
    set autoclose=yes nowarn=yes;
    lset nst=6 rates=invgamma;
    unlink statefreq=(all) revmat=(all) shape=(all) pinvar=(all);
    prset applyto=(all) ratepr=variable;
    mcmcp ngen= 10000000 relburnin=yes burninfrac=0.50 printfreq=1000
samplefreq=1000 nchains=4 savebrlens=yes;
end;
```

For protein sequences (“inv” represents “I” model; “gamma” represents “G” model; the “LG” model was listed in “aarevmatpr=dirichlet()”):

```
  begin mrbayes;
    set autoclose=yes nowarn=yes;
    lset rates=invgamma;
    unlink statefreq=(all) revmat=(all) shape=(all) pinvar=(all);
prset applyto=(all) ratepr=variable aamodelpr=fixed(gtr) aarevmatpr=dirichlet(37.4274,
24.372, 34.7904, 219.15, 91.4382, 85.3938, 181.9026, 31.5954, 13.1913, 34.8075,
47.2374, 98.9649, 22.3371, 103.6854, 416.2014, 188.3709, 15.9111, 19.278, 224.325,
```

66.1986, 10.9134, 47.0646, 32.0454, 247.2201, 34.3539, 213.6483, 11.1807, 26.5761, 556.974, 42.6249, 4.6422, 29.2779, 75.555, 50.9769, 52.2639, 27.6849, 15.0453, 446.9256, 46.5552, 47.6946, 149.301, 126.576, 397.0125, 16.8606, 6.0246, 188.8623, 32.6646, 7.8822, 14.2443, 352.9134, 176.148, 3.9951, 53.8857, 7.3683, 5.508, 461.6928, 46.0809, 74.3913, 81.6273, 0.9414, 1.3275, 24.9129, 2.2491, 1.5336, 34.7292, 109.1988, 37.4949, 2.6316, 11.8953, 3.3426, 0.3078, 7.4673, 50.1201, 56.3958, 28.2294, 52.299, 1.1682, 78.6834, 97.3107, 6.6366, 245.1573, 100.6767, 59.0013, 102.618, 172.5048, 363.4992, 30.7143, 37.3203, 3.897, 6.1344, 159.1119, 15.2964, 1.656, 36.927, 53.8803, 53.2269, 6.8544, 10.5687, 21.5739, 23.5926, 423.8019, 6.4143, 51.282, 284.7609, 147.2598, 3.1572, 54.9657, 107.7507, 95.0994, 20.7963, 22.6566, 18.5184, 27.4248, 0.7668, 3.897, 26.1171, 12.2859, 7.8876, 17.3412, 153.1962, 11.4309, 23.6394, 4.8141, 6.7527, 9.5868, 32.2524, 61.3899, 38.9574, 60.0588, 44.8011, 87.165, 51.4413, 52.5672, 467.2368, 10.4787, 364.9491, 14.0049, 376.2666, 97.9695, 6.8922, 5.6439, 91.0152, 9.8307, 20.4723, 937.593, 12.1059, 555.7671, 228.2715, 21.9285, 16.0497, 26.6715, 54.5553, 26.3826, 149.9166, 57.8106, 2.106, 34.3656, 65.9169, 100.0944, 4.3938, 11.6163, 16.3062, 158.3784, 8.7912, 30.5478, 177.8814, 61.2945, 42.3765, 167.1714, 8.3169, 31.8564, 14.5278, 216.3357, 687.0888, 57.6414, 117.8145, 50.3145, 8.3754, 7.8903, 26.1054, 569.8476, 21.9105, 35.2656, 8.6607, 12.3984, 21.645, 192.6549, 277.4997, 16.6851, 21.951) statefreqpr=dirichlet (0.079066, 0.055941, 0.041977, 0.053052, 0.012937, 0.071586, 0.040767, 0.057337, 0.022355, 0.062157, 0.099081, 0.0646, 0.022951, 0.042302, 0.04404, 0.061197, 0.053287, 0.012066, 0.034155, 0.069147)

;

mcmcp ngen= 10000000 relburnin=yes

burninfrac=0.5 printfreq=1000 samplefreq=1000 nchains=4 savebrlens=yes;

end;

4.3. Name the exported file like “smit5g-18S-1.0.nex” for Beowulf. Provide a relative short name with no special characters or spaces to avoid causing problems for the analysis software.

4.4. Connect to the Beowulf server through the SSH Secure Shell Client.

4.5. Click the “New file transfer” window on top and drag the newly exported file to the folder “mrbayes-3.1.2” on the Beowulf server.

4.6. Create a “.pbs” file (used for Beowulf system). Make sure to change the values for the MrBayes folder and for your .nex file to values appropriate for your analysis.

```
#!/bin/sh
#PBS -l nodes=4:node
#PBS -l walltime=140:00:00
#PBS -m be
#---------------------------------------------------------------------
# setup for MPICH2
MPICH2_HOME=/usr/local/mpich2
export PATH=$MPICH2_HOME/bin:$PATH
export MANPATH=$MPICH2_HOME/man:$MANPATH
unset MPI_HOST
#---------------------------------------------------------------------
cd /home/merlin/mrbayes_3.2.0
mpdboot
mpiexec -n 8 mb << END
set autoclose=yes
set nowarnings=yes
execute smit5g-18S-1.0.nex
mcmc
sump
sumt
quit
END
```

mpdallexit

4.7. Type “mb” to start MrBayes, then “execute smit5g-18S-1.0.nex” to confirm the file can be opened correctly. If the file loads correctly (as long as no error message pop up), type “quit” to close the MrBayes. Use command “qsub <filename>.pbs” to initiate the script file just created.

4.8. You can use “qstat –a” to verify your run is in the queue on the Beowulf server. You can also use “tail <filename>.nex.run1.t” to look at the end of your tree file as it forms.

4.9. Both of the tree files (“<filename>.nex.run1.t” and “<filename>.nex.run2.t”) can be downloaded and used for “Are We There Yet” (AWTY, http://ceb.csit.fsu.edu/awty/), which can help visualize which burn-in value is appropriate for convergence.

4.10. Some file type you may see and use in Beowulf:

smit5g-mcm7-1.0.pbs – original script file

smit5g-mcm7-1.0.nex – original nexus file

smit5g-mcm7-1.0.nex.con – consensus tree

smit5g-mcm7-1.0.nex.run1.t – tree files for independent run #1

smit5g-mcm7-1.0.nex.run1.p – probability files for independent run #1

smit5g-mcm7-1.0.nex.run2.t – tree files for independent run #2

smit5g-mcm7-1.0.nex.run2.p – probability files for independent run #2

smit5g-mcm7-1.0.nex.trprobs – tree probabilities

smit5g-mcm7-1.0.pb* or q* or o*- spurious files left over from run

5. Maximum Likelihood Analyses by Garli (v2.0) through Beowulf System

5.1. For single-gene or nucleotide-only analyses, you can use the same nexus file (for DNA sequences) from the Bayesian analysis. For protein sequences, we need to open the nexus file (for bayesian analysis) with notepad and replace the MrBayes code with Garli code (by adding the LG model for protein sequences):

begin garli;

[this is the LG model rate matrix, in GARLI format (upper triangle, alphabetical by single letter codes)]
[it is scaled such that the mean rate is 100, but GARLI does not require any particular scaling]

r 243.500 38.656 101.598 24.819 202.114 35.106 14.657 52.486 38.675 109.961 27.080 115.206 94.882 41.586 462.446 209.301 249.250 17.679 21.420 6.120 0.342 108.123 55.689 62.662 31.366 1.298 58.110 87.426 51.728 7.374 8.297 52.294 272.397 111.863 191.672 65.557 114.020 512.992 1.704 82.657 90.697 1.046 27.681 1.475 2.499 496.584 38.588 51.201 12.126 121.332 41.661 3.714 2.924 13.217 1.840 34.127 41.467 4.330 176.791 6.816 16.996 52.994 41.030 403.888 35.606 59.867 59.141 23.971 7.616 11.743 8.764 66.732 108.855 2.340 253.635 175.976 8.758 9.241 3.508 5.158 35.396 16.142 64.046 240.373 763.432 30.472 0.852 29.019 4.330 13.651 140.640 19.268 26.214 38.171 170.218 12.701 7.503 26.266 5.349 10.652 68.211 35.836 43.286 441.125 49.779 470.891 237.387 96.850 57.157 11.643 58.408 519.152 15.561 405.499 418.074 18.734 7.658 7.127 12.423 6.271 101.128 1041.770 10.923 22.747 13.451 64.234 209.847 38.184 316.401 618.860 73.241 111.216 18.118 4.882 12.907 617.519 6.694 24.365 56.980 29.529 17.833 29.635 166.574 60.617 29.314 36.294 9.768 163.622 47.361 33.942 197.646 185.746 68.105 47.085 15.827 165.890 73.554 392.126 195.720 8.187 4.439 59.873 61.073 32.531 130.905 55.905 29.006 9.306 8.767 274.689 119.723 105.666 20.576 23.107 25.174 83.950 56.641 16.717 58.071 30.761 633.164 9.623 24.345 39.184 214.061 13.776 24.050 18.539 24.390 308.333 ;

[these are the LG model amino acid frequencies, in GARLI order]
e 0.079066 0.012937 0.053052 0.071586 0.042302 0.057337 0.022355 0.062157 0.0646 0.099081 0.022951 0.041977 0.04404 0.040767 0.055941 0.061197 0.053287 0.069147 0.012066 0.034155
;
end;

For multigene analysis including protein data, each gene must be exported independently as a single nexus file. Copy the data blocks for each nexus file and place them end to end

in a single file. Each data block must have an entry for each taxon in the tree, even if the gene is missing for that taxon (it should be filled with "?"). Remember to add the garli block containing the LG protein model.

5.2. Create or copy a ".pbs" file and name it like "smit5g-mcm7-1.0.pbs" (all ".pbs" files are the same for Garli since the configuration data is stored within "garli.conf"):

```
#!/bin/sh
#PBS -l nodes=10:node
#PBS -l walltime=80:00:00
#PBS -m be

#---------------------------------------------------------------------
# setup for MPICH2
MPICH2_HOME=/usr/local/mpich2
export PATH=$MPICH2_HOME/bin:$PATH
export MANPATH=$MPICH2_HOME/man:$MANPATH
unset MPI_HOST
#---------------------------------------------------------------------


mpdboot
mpiexec -n 10 ../bin/Garli 10 << END
quit
END
mpdallexit
```

5.3. Copy a ".conf" file (normally it is named "garli.conf"; the program includes some basic example files) and change the data file name and prefix to current file name. The file should look like following ("br"=bootstrap): (The first model is for protein sequences LG+G+I; "model 2" is for nucleotide GTR+G+I; "model 3" is for nucleotide GTR+G. The order can be arranged according to the real concatenated sequences)

```
[general]
datafname = smit5g-mcm7-1.0.nex
constraintfile = none
streefname = stepwise
attachmentspertaxon = 50
```

```
ofprefix = smit5g-mcm7-1.0.100br
randseed = -1
availablememory = 512
logevery = 10
saveevery = 100
refinestart = 1
outputeachbettertopology = 0
outputcurrentbesttopology = 0
enforcetermconditions = 1
genthreshfortopoterm = 20000
scorethreshforterm = 0.05
significanttopochange = 0.01
outputphyliptree = 0
outputmostlyuselessfiles = 0
writecheckpoints = 0
restart = 0
outgroup = 1
outputsitelikelihoods = 0
collapsebranches = 1
searchreps = 3
linkmodels = 0
subsetspecificrates = 1

[model1]
datatype = aminoacid
ratematrix = fixed
statefrequencies = estimate
ratehetmodel = gamma
numratecats = 4
invariantsites = estimate

[model2]
datatype = nucleotide
ratematrix = 6rate
statefrequencies = estimate
ratehetmodel = gamma
numratecats = 4
invariantsites = estimate

[model3]
datatype = nucleotide
ratematrix = 6rate
statefrequencies = estimate
ratehetmodel = gamma
numratecats = 4
```

```
[master]
nindivs = 4
holdover = 1
selectionintensity = 0.5
holdoverpenalty = 0
stopgen = 5000000
stoptime = 5000000

startoptprec = 0.5
minoptprec = 0.01
numberofprecreductions = 10
treerejectionthreshold = 50.0
topoweight = 1.0
modweight = 0.05
brlenweight = 0.2
randnniweight = 0.1
randsprweight = 0.3
limsprweight =  0.6
intervallength = 100
intervalstostore = 5

limsprrange = 6
meanbrlenmuts = 5
gammashapebrlen = 1000
gammashapemodel = 1000
uniqueswapbias = 0.1
distanceswapbias = 1.0

bootstrapreps = 10
resampleproportion = 1.0
inferinternalstateprobs = 0
```

5.4. Open the Beowulf through the SSH Secure Shell Client.

5.5. Click the “New file transfer” window on top and drag the newly exported file to the folder “Garli-2.0” in Beowulf.

5.6. Use command “qsub <filename>.pbs” to initial the script file just created.

5.7. To sum up trees after a MPI Garli run: the MPI version of Garli will put out results that look like “<output file name>.100br.run00.boot.tre”. If you run the program in 10

separate instances, as we normally do, you have ten of these files (named as run00, run01, run02, etc.). To sum up the files and make a consensus tree, the syntax is:

sumtrees.py <garli output name>.100br.run0?.boot.tre --output=<consensus tree file name>.100br.con.tre

("<garli output name>" is the output file prefix specified in the "garli.conf" file (under prefix) and "<consensus tree file name>" is the name you would like the consensus tree to have. The question mark "?" allows the incorporation of all 10 files into the final product).

5.8. Check the progress: the command "tail <filename>.100br.run00.boot.tre" can be used to check the progress (since all 10 trees start and end at the same time).

6. Some Trouble-shooting for Beowulf System

6.1. If a job terminated for no reason, try to clean up the "mpds" by typing "pdsh -a mpdcleanup", then try again.

6.2. The code to show commands containing "mpd" on each processor "pdsh -a ps augx | grep mpd".

6.3. The code to kill all "mpd" runs on the processor "pdsh -a killall python2.4".

7. Maximum Likelihood Analyses by RAxML

7.1. Export ".phy" file for tree analysis by clicking "file" tab and export data as "Phylip (DNA/RNA or protein)" and with default set (be sure maximum length of taxon names to be "40").

7.2. Submit the ".phy" file to Rxaml (commands for Raxml):

For nucleotide matrix (GTR+G)—

Raxmlhpc-pthreads –f a –x 12345 –p 12345 -# 100 –m GTRGAMMA –s <filename> -n <filename without extension> -T 4

For protein matrix (LG+G)—

Raxmlhpc-pthreads –f a –x 12345 –p 12345 -# 100 –m PROTGAMMALG –s <filename> -n <filename without extension> -T 4

7.3. When you get the "RAxML_bipartitions.<filename>-raxml-1-16-2012 (date)" files, you can add an ".tre" at the end of the file to make it a tree file and open it in Mesquite.

8. Infer Ancestral States of Morphological Characters by Mesquite (v2.75)

8.1. Code your morphological characters in an excel matrix. This matrix should include the same taxa and in the same order as the tree you will use listed within Mesquite. The characters are coded pending on the model you used. We coded our characters as unordered categorical characters by giving each variation of the character an integer value, starting at zero and increasing from there. For this type of character, all states are considered equivalent and all state changes with the same distance.

8.2. Prepare the tree file. The tree file must be nexus-formatted and contain branch lengths information based on the analysis method you used.

8.3. Open the tree file in Mesquite. Create a new character matrix via the option “New empty matrix” under “Characters”. The type of matrix should be “standard categorical data”. Give it an appropriate name and a suitable number of characters (you only need one matrix for all of the morphological characters in your analysis).

8.4. Paste (or type) the coded morphological characters into the matrix heading.

8.5. To provide appropriate character names for each coded character state, click “Edit State Names” under “Matrix”. This will provide proper names for legends.

8.6. Open the tree file you are going to infer ancestral character states. Click “Trace Character History” under “Analysis” and select “Stored Characters”. Next, select the method—Parsimony, Likelihood-based calculation, or Stochastic Character mapping. We used Likelihood Ancestral States here. Next select the model. What we used here is “Current Probability Models” (Mk1), which is correct for unordered categorical characters.

8.7. When you get the ancestral states on the tree, select “Ball and Sticks” under “Tree Form” within the “Drawing” menu to change the view. You may also want to use the scissor tool to cut the unnecessary outgroups.