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Evolutionary implications of a rRNA-based phylogeny of *Harpellales*†

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ABSTRACT

The *Harpellales* (*Trichomycetes*) are endosymbiotic microfungi, mostly unculturable and predominantly associated with larval aquatic insects worldwide. Molecular phylogenies including 'gut fungi' have included at most only four axenic isolates of the 38 genera of *Harpellales*. Cladistic analyses were used to infer the phylogeny of the *Harpellales* using partial 18S or 28S nu-rRNA sequences generated for 16 genera of *Harpellales*, with 64 of 72 sequences generated from unculturable samples. Both analyses placed *Orphella* outside an otherwise monophyletic group of *Harpellales*, more closely allied to the *Kickxellales*. The current classification recognizing two families is not corroborated and continued use of the family *Legeriomycetaceae* may not be supportable. The largest genera of *Harpellales*, *Smittium* and *Stachylina*, were polyphyletic and the 28S rRNA sequences separate *Smittium culisetae* from the remainder of its genus. The cladograms did not support the consistent mapping of important morphological taxonomic characters, including trichospore shape and zygospore type or appendage numbers for both. This study demonstrates the use of microscopic thalli from host guts for molecular phylogenies and suggests the need for more data from the remaining *Harpellales*, especially with the future inclusion of protein-coding genes.

Introduction

Just two decades ago the class *Trichomycetes* (*Zygomycota*) was thought to include three true fungal orders, all obligate endosymbionts of various *Arthropoda* (Lichtwardt 1986). The gut fungi occur worldwide in all habitats where appropriate hosts have been sought (Misra 1998; White *et al.* 2000, 2001). The *Harpellales* attach to the midgut or hindgut linings of larval aquatic insects or, exceptionally, in freshwater isopods (White 1999). The *Asellariales* includes species that inhabit terrestrial, freshwater and marine *Isopoda* or *Collembola* (springtails). The *Eccrinales* occur in the foregut or hindgut of *Diplopoda*, *Crustacea* or *Insecta*. A fourth order, the *Amoebidiales*, long regarded as an unnatural member of the *Trichomycetes*, was the first to be recognized as protistan based on molecular data (Benny & O'Donnell 2000; Ustinova *et al.* 2000). Recently, phyogenetic studies using rRNA sequence data have revealed *Eccrinales* are also protists (Cafaro 2003, 2005), and the classification of the *Trichomycetes*, as other *Zygomycota*, is in a state of flux (White *et al.* unpubl.).

The *Harpellales* (herein gut fungi) includes two families: the *Harpellaceae* (Léger & Duboscq 1929) for all unbranched species attached to midgut linings of lower dipteran (*Nematocera*) hosts, and the *Legeriomycetaceae* (Pouzar 1972) for all branched species commonly associated with lower *Diptera*, mayflies (*Ephemeroptera*), stoneflies (*Plecoptera*), beetles (*Coleoptera*) and caddisflies (*Trichoptera*) (Lichtwardt 1986; Lichtwardt *et al.* 1999, 2001a; White 1999). *Harpellales* have unique asexual trichospores and four kinds of sexual biconical zygospores (Lichtwardt 1986; Moss *et al.* 1975) [but see Discussion on sexual spores of *Orphella*]. Zygospores are designated according to their arrangement on the zygosporophore: (1) perpendicular and medially attached to the zygosporophore (type I); (2)

† This paper is dedicated to the memory of Stephen T. Moss for his significant contributions to our understanding of the gut fungi.

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oblique and submedial (type II); (3) parallel and medial (type III); (4) coaxial and polar (type IV) (Lichtwardt 1986 and see current monograph by Lichtwardt *et al.* 2001a; Moss *et al.* 1975). Other morphological taxonomic characters include the size and shape of the spores, number of appendages, spore number per thallus branch, shape of the holdfast, nature of the attachment (e.g., presence of adhesive exudate or mucilage, etc.), and kind of host.

Several morphologically based hypotheses have been proposed regarding the natural affinities of the orders of *Trichomycetes* (Cavalier-Smith 1998; Lichtwardt 1986; Moss 1979; Moss & Young 1978). Studies have shown that the *Harpellales* may be closely related to the *Kickxellales* (class *Zygomycetes*) (Benjamin 1979) based on serological affinities and even at the gross morphological or ultrastructural level (Benny & White 2001; Moss 1979, 1998; Moss & Young 1978; Sangar *et al.* 1972). The lack of a clear consensus on the ordinal relationships is in part due to the paucity of taxonomic characters and the unculturability of many of these fungi.

Molecular systematic approaches have led to the reconsideration of evolutionary histories among many organisms across many fields, and mycology is no exception. Cladistic analyses of sequence data helped establish the *Glomeromycota* (Schuessler *et al.* 2001) and changed our understanding of major evolutionary lineages across fungal phyla. Comparatively slower progress has been made with the *Chytridiomycota* and *Zygomycota* with phylogenetic analyses highlighting the polyphyletic or paraphyletic nature of these basal fungi, and with a recognized need for further contributions to various understudied taxa (see reviews pending by James *et al.* unpubl. and White *et al.* unpubl.).

To date, nu-rRNA phylogenies including gut fungi have only considered culturable representatives. Walker (1984) included *Harpellales* and *Kickxellales*, but the 5S rRNA sequences lacked resolving power to determine the sister group relationships that O'Donnell *et al.* (1998) demonstrated using 18S rRNA sequences (and morphological characters) for four genera of *Harpellales*. Gottlieb & Lichtwardt (2001) illustrated a similar pattern using more taxa of culturable *Harpellales*, but also demonstrated that *Smittium* was polyphyletic, with at least five lineages.

The *Harpellales* is the only culturable fungal order of *Trichomycetes*, but only eight of the 38 genera have been isolated axenically, and about 80% of those (in The University of Kansas Mycology Culture Collection) are *Smittium* species. Despite the successful isolation of nearly half of all known species of *Smittium*, the inability to culture the majority of gut fungi has hindered phylogenetic studies due to the omission of unculturable taxa (most *Harpellales* and all *Asellariales*). The goal of this study was to infer an expanded phylogeny of *Harpellales* by including unculturable taxa with sequences from fungal specimens taken directly from the host gut.

Materials and methods

Host collection and specimen preparation

Methods for collecting larval aquatic insects followed White *et al.* (2001). Fungal specimens consisted of living clumps of thalli placed in 500 µl of 2× Hexadecyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1.4 M Tris–HCl pH 8.0, 0.25 m M EDTA) (Gottlieb & Lichtwardt 2001) immediately after dissection and identification. Invariably, vouchers 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. Thalli of a separated fungal species (multiple taxa of gut fungi can be found in a single gut) were kept in CTAB buffer at −20 °C (up to 4 y) before DNA extraction. Exemplars were selected to maximize the number of genera of *Harpellales* for phylogenetic analysis. Other samples were colonies of axenic cultures similarly placed in CTAB buffer. A few dilutions of genomic DNA were donations from the earlier study of Gottlieb & Lichtwardt (2001).

DNA extraction

Standard procedures for CTAB extraction were followed (Gottlieb & Lichtwardt 2001; O'Donnell *et al.* 1997). In some cases, specimens were repeatedly frozen, by submerging in liquid nitrogen and thawing at 65 °C in a heat block (but no attempt was made to crush the microscopic amounts of thalli). After two chloroform extractions, DNA was precipitated, eluted in TE buffer (10 mM Tris–HCl pH 8.0, 1 m M EDTA pH 8.0) and either used directly or after dilution in sterile double distilled water (ddH_2O) for PCR amplification. Some genomic DNA extracts were cleaned using glassmilk 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 of rRNA

Several modifications of published protocols were introduced to amplify the regions between the 5′ end of the 18S and 3′ end (near the second domain) of the 28S nuclear rRNA gene. Initial attempts followed the protocol and cycling conditions of O'Donnell *et al.* (1997) and Gottlieb & Lichtwardt (2001). Occasionally, buffer conditions were altered or PCR products were reamplied to generate sufficient product for sequencing reactions. Various primer combinations were tested using the universal primers of White *et al.* (1990) or modifications of them (Gottlieb & Lichtwardt 2001; O'Donnell *et al.* 1998) in combinations that spanned various regions of the rRNA operon. Owing to mixed genomic DNA templates typically obtained from the unculturable specimens taken directly from guts, often multiple (sized) PCR products were recovered. Therefore, several new primers were designed in an attempt to enhance specificity of the reactions: HL2, 5′ GCTTGGCTAC CAACTGGTTCCCTTTC 3′; HR7, 5′ GCTTGGCTACCAACTGGTTC CCTTTC 3′; IS6, 5′ CGTTCGTTATCGGAATTAACCAGA 3′; HS1, 5′ TTGTCTCAAAGATTAAGCCATG 3′); HS8, 5′ GTTCACCTACG GAAACCTTGTT 3′, and HS6, 5′ CCGTTAGTTAACCACTAA CAGTCC 3′ (for a complete list of primers used see Table 1). The (now discontinued) program Primerfinder (http://eatworms.swmed.edu/%7Etim/primerfinder/) was used to help minimize hairpins and primer dimers. Table 1 lists of all primers used in this study and is cross-referenced with Table 2 (see clone no. details). Concurrent with primer design and

Table 1 – List of oligonucleotide primers (with original citation) used in this study

Primer	Oligonucleotide sequence	Reference
NL1	5′ GCATATCAATAAGCGGAGGAAAAG 3′	O'Donnell (1993)
NL4	5′ GGTCCGTGTTTCAAGACGG 3′	O'Donnell (1993)
HL2	5′ GCTTGGCTACCAACTGGTTCCCTTTC 3′	This study
HR7	5′ TCGGATTTACTACCACCAAGA 3′	This study[a]
NS1	5′ GTAGTCATATGCTTGTCTC 3′	White *et al.* (1990)
PNS1	5′ GAATTCGTAGTCATATGCTTGTCTC 3′	O'Donnell (pers. comm.)[b]
PNS1b	5′ TTGAATTCGTAGTCATATGCTTGTCTC 3′	O'Donnell (pers. comm.)[b]
HS1	5′ TTGTCTCAAAGATTAAGCCATG 3′	This study[b]
HS8	5′ GTTCACCTACGGAAACCTTGTT 3′	This study[c]
HS6	5′ CCGTTAGTTAACCACTAACAGTCC 3′	This study
IS6	5′ CGTTCGTTATCGGAATTAACCAGA 3′	This study
NS51	5′ GGGGGAGTATGGTCGCAAGGC 3′	O'Donnell *et al.* (1997) (from T. Bruns)
NS4	5′ CTTCCGTCAATTCCTTTAAG 3′	White *et al.* (1990)
NS8	5′ TCCGCAGGTTCACCTACGGA 3′	White *et al.* (1990)
T1	5′ AACCAATGGGGGCAACCTCTTACTT 3′	Gottlieb and Lichtwardt (2001)
T8	5′ CATCGTGCTGGGGATAGTCCAT 3′	Gottlieb and Lichtwardt (2001)
5.8S	5′ CGCTGCGTTCTTCATCG 3′	Vilgalys and Hester (1990)
5.8SR	5′ TCGATGAAGAACGCAGC 3′	Vilgalys and Hester (1990)

a Modification of LR7 from Vilgalys & Hester (1990).
b Modification of NS1 from White *et al.* (1990).
c Modification of NS8 from White *et al.* (1990).

testing, adjustments to the thermal cycling regime were also undertaken to optimize cycling parameters, including touchdown approaches with extended numbers of cycles per trial and/or several successive drops in annealing temperature per cycle per regime and increased total number of cycles.

Amplifications were performed in a mixture with a total of 0.25 mM of each primer, 0.225 mM of each dNTP and a 10% solution of 10× Buffer (0.1 M Tris–HCl pH 8.4, 0.5 M KCl, 25 mM $MgCl_2$, 1 mg ml^{-1} gelatin) that was adjusted to final volume with filtered (0.2 μm) sterile ddH_2O. Some trials used the 10× Buffer and magnesium chloride, adjusted according to instructions, supplied with the *Taq* DNA polymerase (Cat. no. M1661, Promega, Madison, WI), added at 2.5 units per 100 μl of final reaction mix. Reaction volumes were 10–50 μl with 0.5–2 μl of genomic DNA template (except for the negative control) with thermal cycling in a Perkin-Elmer 2400 or a MJ Research PTC-100. After electrophoresis on 1–2% agarose gels, ethidium bromide staining and photographing, PCR products were scored by comparison with a 1 kb ladder (cat. no. G2101, Promega, Madison, WI).

Product cleaning, cloning, and sequencing

For cloning, PCR products purified directly were prepared with either the Wizard PCR Preps Kit (Promega, Madison, WI), the Microcon PCR device (Millipore, Bedford, MA), or QIAquick PCR purification kit (QIAGEN, Valencia, CA). Otherwise, PCR products were first separated in sterilized 1–2% agarose gels (1× TAE buffer) and purified using a QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). Cleaned PCR products were cloned using either the Prime PCR Cloner Cloning System (5′–3′ Prime, Boulder, CO) or the pGEM T-Easy Vector System II cloning kit (Promega, Madison, WI). Clones were screened by direct PCR of bacterial cells or by adapting a modification of a gel lysis protocol of Sekar (1987). The latter permitted both the determination of positive clones and the scoring (sizing) of clone inserts. [The molecular methods used here are described more fully in White (2002).]

Selected positive bacterial colonies were grown in 3 ml Luria-Bertani Broth, Miller + ampicillin (100 μg ml^{-1}), plasmids were harvested and purified using a QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA) and then quantified using a Beckman DU® 640B spectrophotometer (Beckman Instruments, Fullerton, CA) for use as sequencing reaction template. Sequencing reactions were prepared with a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's protocol, using universal Infrared-labelled sequencing primers (T7, M13Rev or SP6) and a simultaneous bi-directional sequencing approach (SBS™; LI-COR, Lincoln, Nebraska). Reactions were run on acrylamide gels, and data were captured using a LI-COR 4000L sequencer apparatus equipped with BaseImagIR Sequencing Software, all at the Biochemical Research Service Laboratory, University of Kansas.

Analyses of sequence data

Raw sequence data from each clone were BLASTed first against the GenBank nucleotide database (at http://www.ncbi.nlm.nih.gov/BLAST) to determine sequence similarity before it was completely edited (leading vector sequence removed, remaining sequences correctly oriented and joined) in SEQUENCE NAVIGATOR v. 1.01 (Perkin-Elmer, Foster City, CA). Sequences from obvious contaminating genomic DNA (i.e., associated host group, algae, nematodes, etc.) were culled from further analyses. Ambiguous residues received the appropriate IUPAC code and consensus sequences were exported as separate text files. Sequences generated (deposited under GenBank accession numbers DQ367440–DQ367512) were appended in a larger text file with sequences

Table 2 – List of taxa used in this study (by species name, clone code, collector, host associate, origin, and GenBank accession number)

Species	Sample[a]	Clone no.[b]	Collector[c]	Host	Origin[d]	18S[e]	28S[e]
Genistellospora homothallica	RMBL-82-7	164-1-1*	RWL	*Diptera*, *Simuliidae*	CO		DQ367490
G. homothallica	VT-3-W14	185-1H-9	MMW	*Diptera*, *Simuliidae*	VT		DQ367495
Pennella simulii	NF-19-8	274-11H-2	MMW/RWL	*Diptera*, *Simuliidae*	NF		DQ367502
P. simulii	NF-1-W44	276-11-6	MMW/RWL	*Diptera*, *Simuliidae*	NF		DQ367503
Genistelloides hibernus	NS-21-W4	118-1-2	MMW	*Plecoptera*, *Capniidae*, *Allocapnia* sp.	NS		DQ367480
G. hibernus	2-16-2	117-1-2	AS	*Plecoptera*, *Capniidae*, *Allocapnia vivapara*	KS		DQ367479
G. hibernus	TN-11-1	GenBank	KUMYCOL	*Plecoptera*, *Capniidae*, *Allocapnia* sp.	TN		AF031062
Zygopolaris ephemeridarum	MN-3-W1/LCF-MN3-3	294-12M-1	LCF/MMW	*Ephemeroptera*	MN		DQ367506
Z. ephemeridarum	MN-3-W4	83-1-5	LCF/MMW	*Ephemeroptera*	MN		DQ367475
Z. ephemeridarum	CA-4-W9	346-12H-1	MMW/PVC	*Ephemeroptera*	CA		DQ367508
Legeriomyces sp. (new?)	CA-10-W16	359-12H-2	MMW/PVC	*Ephemeroptera*	CA		DQ367510
Stachylina sp.	NS-7-W35	50-1-12	MMW	*Diptera*	NS		DQ367470
Capniomyces stellatus	MIS-10-108	GenBank	KUMYCOL	*Plecoptera*, *Capniidae*, *Allocapnia* sp.	MO		AF031073
C. stellatus	MIS-21-127	167-1-1*	KUMYCOL	*Plecoptera*, *Capniidae*, *Allocapnia* sp.	MO		DQ367491
Bojamyces repens	ME-JL-2	113-1H-1	JL	*Ephemeroptera*, *Leptophlebiidae*, *Leptophlebia intermedia*	ME		DQ367478
Trichoptera gut fungus (new?)	CA-19-W18	356-12H-1	MMW/PVC	*Trichoptera*	CA		DQ367509
Harpella meridianalis	ARG-46a-14	24-1-4	RWL	*Diptera*, *Simuliidae*	ARG		DQ367468
H. tica	PR-14-W18	26-1-2	MMW/RWL/MJC	*Diptera*, *Simuliidae*, *Simulium bipunctatum*	PR		DQ367469
H. meridianalis	ARG-25-5	23-1-1	RWL	*Diptera*, *Simuliidae*	ARG		DQ367467
Harpella sp.	ARG-20-3	259-11H-12	RWL	*Diptera*, *Simuliidae*	ARG		DQ367499
Gripopterygidae gut fungus	27-1-7	229b-12M-1	LCF/BH	*Plecoptera*, *Gripopterygidae*	TAS		DQ367498
Plecopteromyces sp.	37-1-2	106-1-20	LCF/BH	*Plecoptera*, *Gripopterygidae*	TAS		DQ367476
Plecopteromyces sp.	39-2-1	227b-11H-1	LCF/BH	*Plecoptera*, *Gripopterygidae*	TAS		DQ367497
Smittium culisetae	COL-18-3	GenBank	KUMYCOL	*Diptera*, *Culicidae*, *Culiseta impatiens*	CO		AF031072
S. culisetae	LEA-7-2	168-1-1*	KUMYCOL	*Diptera*, *Simuliidae*, *Simulium vittatum*	KS		DQ367492
S. culisetae	HAW-14-7	169-1-1*	KUMYCOL	*Diptera*, *Culicidae*, *Aedes albopictus*	HI		DQ367493
S. tronadorium	ARG-24-7	139-1-2	RWL	*Diptera*, *Diamesinae*, *Limaya?*	ARG		DQ367486
S. tronadorium	ARG-24-18F	138-1-1	LCF	*Diptera*, *Diamesinae*, *Paraheptagyia* sp.	ARG		DQ367485
S. tronadorium	ARG-24-24	288-11-8	RWL	*Diptera*, *Diamesinae*, *Limaya?*	ARG		DQ367505
S. orthocladii?	KS-82-W4/LCF-BT-6	131-1-1	LCF/MMW	*Diptera*, *Orthocladiinae*, *Hydrobaenus* sp.	KS		DQ367484
S. orthocladii?	KS-82-W1/ LCF-BT-4	130-1-3	LCF/MMW	*Diptera*, *Orthocladiinae*, *Orthocladius* sp.	KS		DQ367483
S. orthocladii?	LCF-BT-1	108-1-2	LCF/MMW	*Diptera*, *Orthocladiinae*, *Corynoneura*	KS		DQ367477
Smittium sp. (blackfly)	ARG-47-3	140-1-1	RWL	*Diptera*, *Simuliidae*, *Simulium bonaerense*	ARG		DQ367487
S. phytotelmatum	CR-219-1	61-1-4	KUMYCOL	*Diptera*, *Chironominae*, *Chironomus* sp.	CR		DQ367471
Smittium? (hindgut fungus)	LCF-21-2	226b-11-5	LCF	*Diptera*, *Chironominae*, *Chironomus* sp.	NZ		DQ367496
Furculomyces boomerangus	AUS-42-7	GenBank	KUMYCOL	*Diptera*, *Tanypodinae*, *Procladius paludicola?*	AUS		AF031074
S. culicis	35-1-1	361-12H-1	LCF/BH	*Diptera*, *Thaumaleidae*	TAS		DQ367511
S. culicis	43-1-2	362-12H-4	LCF/BH	*Diptera*, *Chironominae*, *Chironomus* sp.	TAS		DQ367512
S. culicis	WYO-51-11	63-1-2	KUMYCOL	*Diptera*, *Culicidae*, *Aedes sticticus*	WY		DQ367472
S. culicisoides	CR-253-12	64-1-1	KUMYCOL	*Diptera*, *Chironomidae*	CR		DQ367473
S. mucronatum	FRA-12-3	68-1-2	KUMYCOL	*Diptera Orthocladiinae*, *Psectrocladius simulans*	FRA		DQ367474
S. mucronatum	RMBL-61-10	142-1-1	RWL	*Diptera*, *Orthocladiinae*, *Psectrocladius* sp.	CO		DQ367488
Austrosmittium biforme	32-1-8	170-1-1*	KUMYCOL	*Diptera*, *Orthocladiinae*	TAS		DQ367494
Stachylina penetralis	CO-3MJC-C4	152-1-3	MJC	*Diptera*	CO		DQ367489
Smittium longisporum	SD-2-W15	283-11-6	MMW/PVC	*Diptera*	SD		DQ367504

Orphella avalonensis	NF-26-W8	272-11H-3	MMW/RWL	*Plecoptera, Leuctridae, Leuctra ferruginea*	NF	**DQ367501**
O. avalonensis	NF-26-W8	272-11H-2	MMW/RWL	*Plecoptera, Leuctridae, Leuctra ferruginea*	NF	**DQ367500**
Orphella nr. *hiemalis*	KS-83-W3	125-1-3	MMW/LCF	*Plecoptera, Leuctridae, Zealeuctra claassenii?*	KS	**DQ367482**
Orphella nr. *haysii*[g]	NS-18-W6	124-1-4	MMW	*Plecoptera, Capniidae, Paracapnia* sp.	NS	**DQ367481**
Orphella nr. *haysii*[g]	NS-34-W18	302-11H-3	MMW	*Plecoptera, Capniidae, Paracapnia* sp.	NS	**DQ367507**
Linderina pennispora	-	GenBank	-	-	-	AF031063
Kickxella alabastrina	-	GenBank	-	-	-	AF031064
Dipsacomyces acuminosporus	-	GenBank	-	-	-	AF031065
Martensiomyces pterosporus	-	GenBank	-	-	-	AF031066
Spiromyces minutus	-	GenBank	-	-	-	AF031070
S. aspiralis	-	GenBank	-	-	-	AF031071
Syncephalastrum monosporum var. *pluriproliferum*	NRRL22812	GenBank	-	-	-	AF157215
Mucor ramosissimus	NRRL3042	GenBank	-	-	-	AF113472
Basidiobolus ranarum[f]	NRRL20525	GenBank	-	-	-	AF113452
B. haptosporus[f]	NRRL28635	GenBank	-	-	-	
Conidiobolus coronatus[f]	NRRL28638	GenBank	-	-	-	AF113456
C. coronatus[f]	NRRL1912	GenBank	-	-	-	AF113455
Blastocladiella emersonii	ATCC22665	GenBank	-	-	-	X90411
Smittium orthocladii?	LCF-BT-1	108-18H-1	LCF/MMW	*Diptera, Orthocladiinae, Corynoneura*	KS	**DQ367446**
S. commune	KS-2-21	GenBank	KUMYCOL	*Diptera, Chironomida,*	KS	AF277034
S. vulgare nom. prov.	AS-27-9	366-18H-1	AS/LCF	*Diptera, Orthocladiinae*	NZ	**DQ367466**
S. tipulidarum	RMBL-31-1	GenBank	KUMYCOL	*Diptera, Tipulidae*	CO	AF277043
S. dipterorum	CR-253-14	GenBank	KUMYCOL	*Diptera, Simuliidae, Simulium* sp.	CR	AF277026
Pseudoharpella arcolamylica nom. prov.	LCF-13-11	193-9-3 194-9-2 194-6-2	LCF	*Diptera, Dixidae, Dixa fluvica*	MN	**DQ367457**
S. phytotelmatum	CR-219-1	GenBank	KUMYCOL	*Diptera, Chironominae, Chironomus* sp.	CR	AF277025
S. megazygosporum	SC-DP-2	GenBank	KUMYCOL	*Diptera, Simuliidae, Simulium vittatum*	SC	AF277045
Furculomyces boomerangus	AUS-77-4	GenBank	KUMYCOL	*Diptera, Chironominae, Tanytarsus nr. Inextentus*	AUS	AF277013
F. boomerangus	AUS-42-7	GenBank	KUMYCOL	*Diptera, Tanypodinae, Procladius ?paludicola*	AUS	AF007535
Stachylina rivularia nom. prov.	LCF-22-6(AFR no. 14)	200-6-1	LCF	*Diptera, Chironominae, Tanytarsus* sp.	AF	**DQ367458**
Smittium morbosum	AUS-X-1	GenBank	KUMYCOL	*Diptera, Culicidae, Anopheles hilli*	AUS	AF277014
Austrosmittium biforme	32-1-9	338-18H-1	LCF/BH	*Diptera, Orthocladiinae*	TAS	**DQ367462**
Austrosmittium sp.?	LCF-27-6	98-4-1	LCF/AS	*Diptera, Orthocladiinae, Cricotopus* sp.	NZ	**DQ367443**
S. mucronatum	FRA-12-3	GenBank	KUMYCOL	*Diptera, Orthocladiinae, Psectrocladius sordidellus*	FRA	AF277030
S. simulatum	CHI-8-4	GenBank	KUMYCOL	*Diptera, Simuliidae, Aphrophila bidentata*	CHI	AF277019
S. caudatum	KS-1-2	GenBank	KUMYCOL	*Diptera, Chironomidae*	KS	AF277031
Harpellomyces nr. *abruptus*	PA-3-1d	81b-18H-4	LCF/MMW	*Diptera, Thaumaleidae, Thaumalea veralli*	PA	**DQ367442**
Harpella melusinae[f]	RMBL-40-2	181-5-16	RWL	*Diptera, Simuliidae*	CO	**DQ367452**
H. meridianalis	ARG-46a-15	257b-18H-1	RWL	*Diptera, Simuliidae*	ARG	**DQ367460**
H. tica	PR-14-W18	26-3-1	MMW/RWL/MJC	*Diptera, Simuliidae, Simulium bipunctatum*	PR	**DQ367441**
Genistellospora homothallica	VT-3-W14	185-5-2	MMW	*Diptera, Simuliidae*	VT	**DQ367454**
G. homothallica	PR-14-C26b	184-5-7	MJC/RWL/MMW	*Diptera, Simuliidae, Simulium bipunctatum*	PR	**DQ367453**
Genistelloides hibernus	KS-19-M23	192-5-3	JKM	*Plecoptera, Capniidae*	KS	**DQ367456**
G. hibernus	2-16-2	117-4-1	AS	*Plecoptera, Capniidae, Allocapnia vivapara*	KS	**DQ367448**
G. hibernus	NS-21-W4	118-5L-2	MMW	*Plecoptera, Capniidae, Allocapnia* sp.	NS	**DQ367449**

(continued on next page)

Table 2 (*continued*)

Species	Sample[a]	Clone no.[b]	Collector[c]	Host	Origin[d]	18S[e]	28S[e]
Plecopteromyces patagoniensis	ARG-24-18	18-4-1	RWL	*Plecoptera, Gripopteryigidae*	ARG	**DQ367440**	
Plecopteromyces sp.	37-1-2	106-4-2	LCF/BH	*Plecoptera, Gripopteryigidae*	TAS	**DQ367445**	
Gripopteryigidae gut fungus	27-1-5	105-5-2	LCF/BH	*Plecoptera, Gripopteryigidae*	TAS	**DQ367444**	
Plecopteromyces sp.	39-2-1	227b-17-4	LCF/BH	*Plecoptera, Gripopteryigidae*	TAS	**DQ367459**	
Capniomyces stellatus	MIS-21-127	167-1-1*	KUMYCOL	*Plecoptera, Capniidae, Allocapnia* sp.	MO	**DQ367451**	
C. stellatus	MIS-10-108	GenBank	KUMYCOL	*Plecoptera, Capniidae, Allocapnia* sp.	MO	AF007531	
Trichoptera gut fungus	CA-9-W9	353-18H-4	MMW/PVC	*Trichoptera*	CA	**DQ367464**	
Trichoptera gut fungus	CA-9-W10	354-18H-3	MMW/PVC	*Trichoptera*	CA	**DQ367465**	
Smittium culisetae	COL-18-3	GenBank	KUMYCOL	*Diptera, Culicidae, Culiseta impatiens*	CO	AF007540	
S. culisetae	AUS-2-8	GenBank	KUMYCOL	*Diptera, Chironominae, Chironomus alternans*	AUS	AF277007	
Zygopolaris ephemeridarum	CA-4-W9	346-18H-1	MMW/PVC	*Ephemeroptera*	CA	**DQ367463**	
Bojamyces repens	ME-JL-2	113-4-1	JL	*Ephemeroptera, Leptophlebiidae, Leptophlebia intermedia*	ME	**DQ367447**	
Orphella nr. *haysii*[g]	NS-34-W16	191-5-3 191-5-7	MMW	*Plecoptera, Capniidae, Paracapnia* sp.	NS	**DQ367455**	
Orphella nr. *haysii*[g]	NS-34-W18	302b-18-2	MMW	*Plecoptera, Capniidae, Paracapnia* sp.	NS	**DQ367461**	
Orphella nr. *Hiemalis*	KS-83-W3	125-3-3 125-3-8 125-5-2 125-5-22 125-5H-1	MMW	*Plecoptera, Leuctridae, Zealeuctra claassenii?*	KS	**DQ367450**	
Spiromyces minutes	NRRL 3067	GenBank	-	-	-	AF007542	
S. aspiralis	NRRL 22631	GenBank	-	-	-	AF007543	
Coemansia reversa	NRRL 1564	GenBank	-	-	-	AF007533	
Spirodactylon aureum	NRRL 2810	GenBank	-	-	-	AF007541	
Kickxella alabastrina	NRRL 2693	GenBank	-	-	-	AF007537	
Linderina pennispora	NRRL 3781	GenBank	-	-	-	AF007538	
Dipsacomyces acuminosporus	NRRL 2925	GenBank	-	-	-	AFF007534	
Martensiomyces pterosporus	NRRL 2642	GenBank	-	-	-	AF007539	
Mucor racemosus[f]	NRRL3640	GenBank				AF113430	
Blastocladiella emersonii	L17	GenBank	-	-	-	M54937	

a Generally, the sample number is the dissection number of the investigator, which is also the code given the axenic isolates.

b Clone number is [(numeric sample code)-(primer combination for amplicon)-(picked colony number)]. Primer combinations are: -1- (NL1-NL4); -3- (PNS1-IS6); -4- (PNS1-HS6); -5- (PNS1-T8); -6- (T1-T8); -9- (NS51-NS8); -11- (5.8SR-NL1-NL4); -12- (5.8SR-NL4); -17- (PNS1/PNS1b–NS8-5.8S); -18- (HS1-HS8). Primer combinations ending in a letter are as follows: H, Heavy; M, Medium; L, Light. These are used to designate bands of different size (length) that were cut from stained gels, prior to gel extraction and amplification. Clone numbers ending in an asterisk (*) were sequenced with Kerry O'Donnell. Where more than one clone number is given, a consensus sequence of these clones was used in the alignment. If designated as GenBank, sequences were downloaded directly from the NCBI data base, specified by the accession code in either the 18S or 28S column.

c AS, Amy Weis (née Slaymaker); BH, Barb Hayford; JKM, JK Misra; JL, Joyce Longcore; LCF, Leonard Ferrington, Jr.; MJC, Matías Cafaro; MMW, Merlin White; PVC, Paula Clarke; RWL, Robert Lichtwardt. Some of the sequences were generated from culturable isolates from the University of Kansas Mycological Culture Collection, represented as KUMYCOL.

d Country Codes: AF, Africa; ARG, Argentina; AUS, Australia; CHI, Chile; CR, Costa Rica; FRA, France; NZ, New Zealand; TAS, Tasmania, Australia. Canadian Provinces: NF, Newfoundland and NS, Nova Scotia. USA states: CA, California; CO, Colorado; HI, Hawaii; KS, Kansas; ME, Maine; MN, Minnesota; MO, Missouri; PA, Pennsylvania; SC, South Carolina; SD, South Dakota; TN, Tennessee; VT, Vermont; WY, Wyoming. PR, Puerto Rico.

e Entries in bold were generated during this study.

f These taxa appear in cladograms of White *et al.* (2006a), but were not included in the alignments for this paper.

g This specimen was later recognized as a new species now being described under a different name by D Strongman & MM White.

downloaded from GenBank for certain *Harpellales*, *Kickxellales* and other fungi as well as outgroup taxa from the *Chytridiomycota* (Table 2).

The file was opened as DNA and multiple alignments performed using either version 1.64 or 1.81 of Clustal X (Thompson *et al.* 1994, 1997). Multiple alignments were edited further by eye, using Se-Al v2.0a8 [the Sequence Alignment Editor (Se-Al) program by Andrew Rambaut, Department of Zoology, University of Oxford; http://www.evolve.zoo.ox.ac.uk/software/Se-Al/main.html]. Ambiguous regions were removed from the analysis (earlier alignments included various *Ascomycota* and *Basidiomycota* to ensure that homologous characters were aligned across conserved domains and to incorporate secondary structure). Both alignments have been deposited in TreeBASE.

Phylogenetic analyses were performed by MP using PAUP 4.0b4 (Swofford 1999). Each data set was analysed via the heuristic search option using stepwise addition (both simple and random sequence addition of 100 replicates) and tree bisection–reconnection (TBR) branch swapping. All characters were treated as unordered (weighted equally), and gaps were treated as missing data. Search settings were as follows: steepest descent option not in effect, MulTrees option in effect, zero length branches were set to collapse to yield polytomies, and multistate taxa were considered as uncertainty. Support for internal branches was evaluated by BS analyses from 500 heuristic searches (with MAXTREES not to exceed 500) and by decay indices (Bremer 1988) calculated using AutoDecay version 4.0 (available at http://www.bergianska.se/personal/TorstenE/) (Eriksson 1999).

Results

Technical commentary

The generation of multiple bands on check gels of PCR reaction products obtained using universal primers (Berbee & Taylor 2001; White *et al.* 1990) with mixed genomic templates was minimized by using specific primers to screen for the amplicons of interest. The 5.8SR-NL4 and 5.8SR-HR7 primer combinations used, in addition to NL1–NL4, produced a greater proportion of gut fungal amplicons, all of which were larger in size. This not only enhanced specificity and sequence read lengths, but also has initiated an ITS sequence database that may be useful for developing highly specific primers and addressing other evolutionary questions. The next step, to amplify and to clone nearly the entire repeat region (over 5 kb), was achieved using various combinations of primers listed here for culturable isolates (White, unpublished). This ensures that the 18S and 28S sequences are from the same genome (in samples of mixed DNA) and permits size fractionation and discrimination of PCR products (obtained from mixed template DNA samples), owing to the known length variability of the two ITS regions in *Harpellales* (Gottlieb & Lichtwardt 2001).

18S and 28S rRNA sequencing

The nuSSU (18S) rRNA sequences generated for this study varied in length depending on the primers used to amplify them. Therefore, the data set used in the analysis was reduced to 1169 characters, of which 746 were constant and 296 were parsimony informative. Cladistic analysis produced 312 equally most parsimonious cladograms (trees) of 1035 steps with CI, homoplasy index (HI), RI and rescaled consistency (RC) index of 0.537, 0.462, 0.768 and 0.462, respectively. The strict consensus 18S rRNA tree (Fig 1) suggests that the *Harpellales* are polyphyletic if *Orphella* spp. are included. With the exception of *Orphella*, the remaining taxa of *Harpellales* sampled form a well-supported monophyletic clade (BS = 100%, hereafter BSs are given as percentages only). Several of the nodes for terminal branches within the larger *Harpellales* clade received moderate to high BS values, although this analysis was unable to resolve many of the relationships among genera within that clade. A distinct lineage of *Orphella* species received moderate support (70%) as a sister clade to members of *Kickxellales*. The 18S tree groups the larger *Harpellales* cluster within a slightly more inclusive grade that includes a distinct *Spiromyces* lineage, although with less overall support by bootstrapping (66%). It should be noted that in earlier analyses the *Spiromyces* lineage also formed a sister group to the *Orphella–Kickxellales* clade, depending on the ingroup taxa and number of aligned characters.

The nuLSU (28S) rRNA sequences varied in length depending on the primers used to amplify them. The data set included 429 characters, with 145 constant and 232 parsimony informative. Cladistic analysis produced two equally most parsimonious cladograms of 1415 steps with CI, HI, RI and RC index of 0.391, 0.608, 0.709 and 0.277, respectively. The 28S rRNA strict consensus tree (Fig 2) suggests that the *Harpellales* are paraphyletic. The *Orphella* species form a well-supported lineage (100%) as a sister group (65%) with a *Kickxellales* + *Spiromyces* clade (67%). Aside from *Orphella* the remaining *Harpellales* formed a well-supported clade (94%). Overall, there was strong support (90%) for the *Harpellales* (minus *Orphella*) as sister to the *Kickxellales*/*Spiromyces* + *Orphella* clade. Within the *Harpellales* clade, two groups were distinguished with moderate support (61 and 67%) with several terminal branches having moderate to high BS values. The analysis was unable to fully resolve all of the relationships among the taxa at the generic level.

Although several branches collapsed (those <50% are designated with asterisks), both strict consensus trees indicated a distinct *Orphella* lineage outside an otherwise well-supported monophyletic *Harpellales* clade but more closely allied with the *Kickxellales*. The 28S tree provides better resolution at the generic level, but data sets were not been combined because not all of the taxa are represented equally. Nonetheless, as the most inclusive phylogenies of the Harpellales to date, the tree congruence permits significant discussion of the evolutionary relationships of the gut fungi. These findings are presented in consideration of the morphologically based classification of the *Harpellales*.

Discussion

Evolutionary relationships within the Zygomycota

The dilemma presented by trying to tease apart the relationships of two similar orders, *Harpellales* and *Kickxellales*,

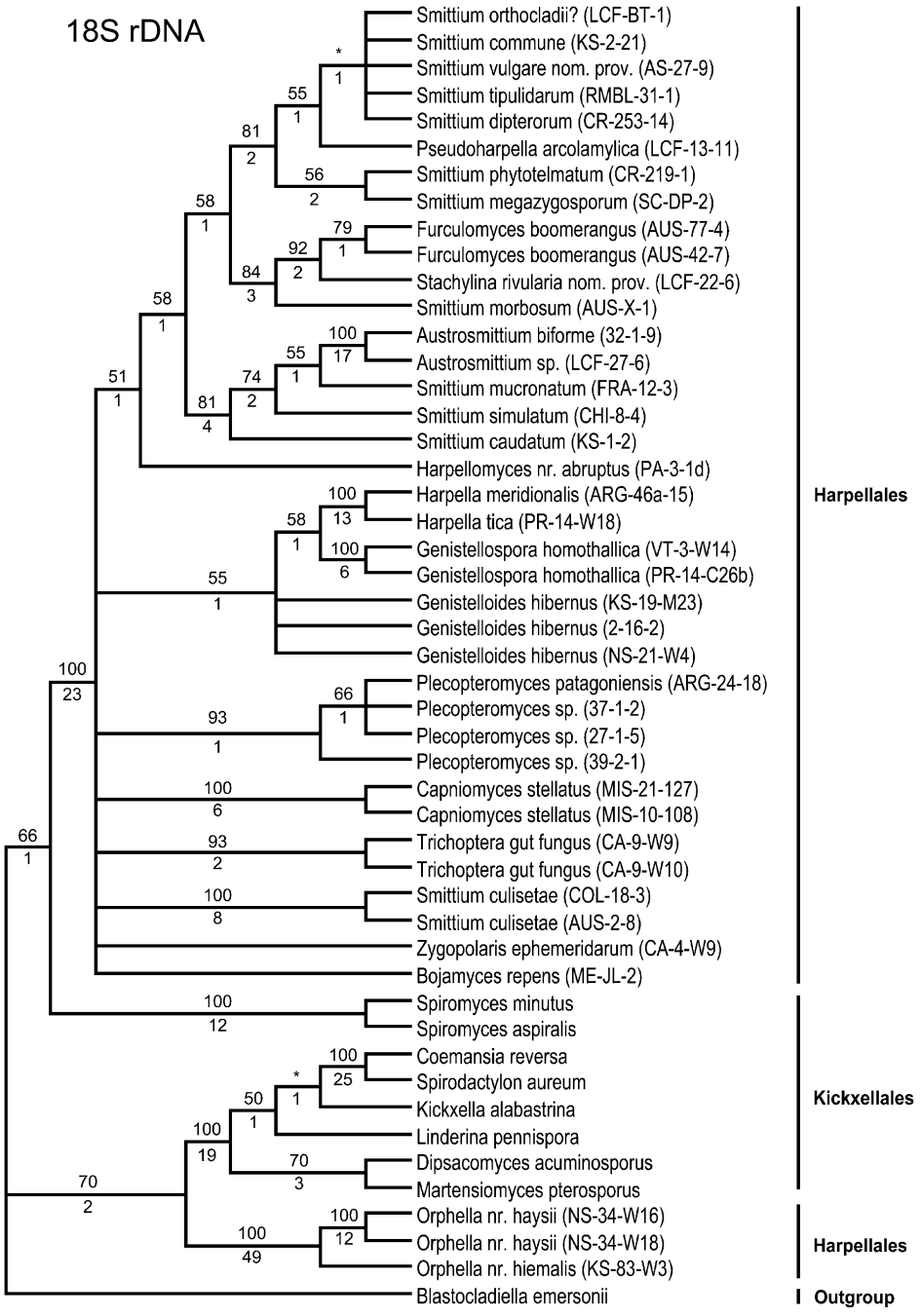

Fig 1 – Strict consensus of 312 equally most parsimonious trees (1035 steps, CI = 0.537, RI = 0.768) inferred from the 18S rRNA data set. Numbers above branches indicate BS values (above 50% from 500 replicates; asterisks (*) indicate nodes that collapse in the BS tree), numbers below are decay indices.

separated into both classes of the *Zygomycota* is not new. Molecular data build on earlier morphologically based phyletic discussions of the relationships of these two orders (Moss & Young 1978). Moss (1979) considered the *Harpellales* to be members of the *Zygomycota*, but with noteworthy similarities to the *Kickxellales* based on immunology, sterol formation, cell wall analysis, septal pore structure, asexual spores, and especially zygospores. Asexually the *Harpellales*, with trichospores having aseptate collars produced laterally from generative cells, and *Kickxellales*, with spores on pseudophialides (except *Spiromyces*) borne on sporocladia, have been suggested to share a basic, 'coemansioid pattern' (Benjamin 1966; Moss & Young 1978). Zygospores, although different in the two orders (biconical in *Harpellales* versus globose in the *Kickxellales*), have been suggestive of a closer relationship (Benjamin 1979) among various shared similarities (Benny & White 2001). O'Donnell *et al.* (1998) inferred a phylogeny with both orders, using 18S rRNA sequence data with morphological and physiological characters. In that analysis the *Harpellales* were sister to a monophyletic clade with *Kickxellales* and *Spiromyces*

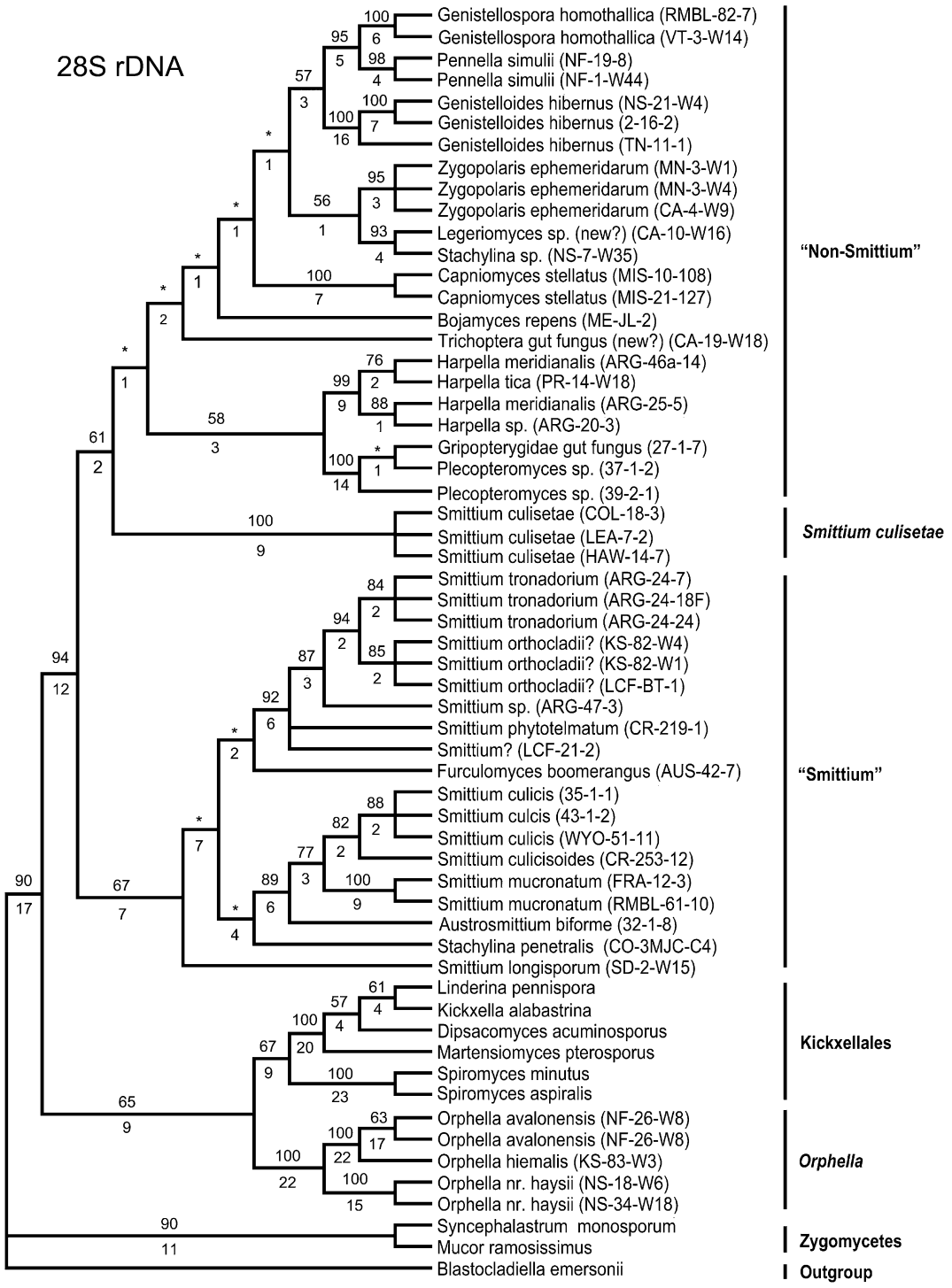

Fig 2 – Strict consensus of two equally most parsimonious trees (1415 steps, CI = 0.391, RI = 0.277) inferred from the 28S rRNA data set. Numbers above branches indicate BS values (above 50% from 500 replicates; asterisks (*) indicate nodes that collapse, < 50%, in the BS tree), numbers below are decay indices.

(although overall support was poor). Other molecular phylogenetic studies have demonstrated that the *Harpellales* and *Kickxellales* share a common ancestor but have also highlighted the significance of a broader sampling of the gut fungi (see review pending by White *et al.* unpubl.). All previous molecular phylogenies have included, at most, four genera of the *Harpellales*.

This report is taxonomically the most comprehensive molecular phylogenetic analysis of the gut fungi to date. A total of 72 rRNA sequences were generated (twenty-six 18S plus forty-six 28S rRNA) with 64 from uncultured samples of *Harpellales* (see Table 2). The analyses support earlier suggestions that the most parsimonious explanation for the origin of the *Harpellales* and *Kickxellales* is a polytomous radiation from

their most common ancestor (Gottlieb & Lichtwardt 2001; O'Donnell *et al.* 1998), but the unculturable taxa of gut fungi also present some novel findings of considerable impact on our understanding of their evolutionary history.

Placement of Orphella

These are the first analyses to indicate that the *Harpellales* are not monophyletic. Resolution of an *Orphella* lineage with *Kickxellales* (*Zygomycetes*) falling outside an otherwise well-supported *Harpellales* clade for both data sets was unexpected. Morphologically, *Orphella* was believed to be one of the most derived of the *Harpellales* (Lichtwardt 1986). It is the only harpellid with dissemination units as the asexual propagules, and a number of specialized cells have been defined to distinguish species and to describe development (Santamaria & Girbal 1998). All species of *Orphella* are associated with non-predaceous stoneflies in North America and Europe (Lichtwardt *et al.* 2001a). The thalli attach to the hindgut and extend posteriorly (within the lumen of the gut) via axial extension of the thallus. At maturity, sporulating heads protrude beyond the anus with dissemination units attached in clusters. The 'basal' position of species of *Orphella* in the trees, as symbionts of *Plecoptera*, (among the oldest of aquatic *Insecta*), supports the notion that they have been coevolving with their hosts for an extremely long time (Lichtwardt 1995).

White (2002) predicted that the future documentation of zygospores in *Orphella* would be of great interest considering its phylogenetic position near the *Kickxellales* (Figs 1–2). The recent discovery of zygospores that are neither biconical nor globose in the three species of *Orphella* from Spain (Valle & Santamaria 2005) was as unexpected as the placement of *Orphella* in the rRNA trees. The sexual spores of *Orphella* have varying degrees of coiling (Valle & Santamaria 2005). Thus, it is apparent that *Orphella* is the most unusual genus of *Harpellales* from both a morphological and molecular aspect and deserves reclassification pending further collections and analyses. Retrospectively, the morphological characters thought to be derived for *Orphella* may actually be more ancestral. If subsequent phylogenies confirm its ancestral placement amongst gut fungi, it may be one of the most significant extant taxa for reconstructing evolutionary relationships amongst the *Harpellales*.

The intuitive phylogeny of the *Harpellales* and *Kickxellales* by Moss and Young (1978), left *Orphella* out of their schematic (listing it as a footnote) because of insufficient morphological information (see their Fig 1). They suggested that *Pteromaktron protrudens* (Whisler 1963) was basal because of the similarity between its subsidiary cell and the pseudophialide of *Kickxella* (Moss & Young 1978). Future efforts to generate sequence data for this putatively basal taxon, *Pteromaktron*, and other possibly significant *Harpellales* are worthy [see White *et al.* (2006b) for an unusual new genus].

The 28S tree revealed one clade (61% support) that includes symbiont associates from the most primitive hosts of the *Harpellales* (stoneflies and mayflies, but also with a few from lower dipterans). Relationships between the fungi are unresolved, but the sequestering of these taxa suggests that they have coevolved with their hosts. The instability of the *Spiromyces* lineage between the *Harpellales* and *Kickxellales* has been noted in previous analyses (O'Donnell *et al.* 1998; Tanabe *et al.* 2000). More sequence data and more taxa may stabilize its placement, but it may also be a case of long branch attraction (Bergsten 2005). Long branches leading to *Zygomycetes* in cladograms, suggesting accelerated rates of sequence evolution of the 18S rRNA gene, have been recognized as a concern for inferring phylogenies (Berbee & Taylor 2001; Bruns *et al.* 1992; James *et al.* 2000; Tanabe *et al.* 2000). Future studies including protein coding genes hold promise, compared with rRNA, to overcome long branch attraction problems (White *et al.* unpubl.). A few long branches were apparent with the phylograms for this study but the analyses will serve as a benchmark toward understanding their evolutionary relationships.

Circumscription of the Harpellales *(excluding* Orphella*)*

The remainder of the discussion pertains to the remaining *Harpellales* within well supported monophyletic clades for each data set. Neither analysis supports the two family system of the *Harpellales*. Species belonging to two (*Harpella* and *Stachylina*) of the five genera of *Harpellaceae* have been included (Figs 1–2) without recovering a monophyletic arrangement in any of the previous analyses (not all shown). The unbranched *Harpellaceae* include the smallest gut fungi (just a few trichospores per thallus), often with only a few thalli per midgut, and these have also been the most difficult unculturable taxa to amplify and sequence. It is unclear whether this is because of the small amounts of genomic template, poor primer annealing or other reaction conditions. More taxa from the *Harpellaceae* should be added to sequence data sets before any reclassification, but if the apparent polyphyly of the *Harpellaceae* is confirmed, then the *Legeriomycetaceae* may need to be removed as a formal rank. Again, these data are only suggestive of such a removal at this time, pending further sequence data, and preferably including at least one single-copy gene.

Polyphyly of Smittium *and* Stachylina

The two largest genera of *Harpellales*, *Smittium* and *Stachylina* are polyphyletic (Figs 1 and 2). Gottlieb & Lichtwardt (2001) also demonstrated, using 18S rRNA, that *Smittium* was polyphyletic and comprises five lineages but with no discernible pattern correlated with either geographic origin or host order among the culturable isolates in that study. A few exemplars of the lineages delimited by Gottlieb & Lichtwardt (2001) have been sequenced for the 28S data set with some other specimens of *Smittium* taken directly from guts. Compared with 18S, the 28S rRNA data allows better overall resolution but still supports the earlier conclusion that *Smittium* is polyphyletic (Gottlieb & Lichtwardt 2001). The partial LSU gene should be useful to resolve relationships among 'Smittiums' and between closely related taxa, with a smaller subsampling of taxa that permits more of the gene to be included in the analysis. Preliminary analyses (not shown) indicate several subclades of *Smittium*, possibly multiple genera, may be masked by convergent morphology. An investigation to resolve the 'Smittium' clade, perhaps including a protein coding gene, should be considered to resolve this polyphyletic assemblage. There is no discernible pattern in the cladogram that

corresponds to the adaptive radiation of lower dipteran hosts, and any hypothesized coevolution of the symbionts (Slaymaker *et al.* 1997, 1999) will require more data and subsequent analyses. Similarly, the polyphyly of *Stachylina* is based on three specimens scattered across the trees for each data set and clearly needs future efforts as well. Curiously, the polyphyly of both *Stachylina* and *Smittium* is offset by the stable placement of *Smittium culisetae*.

S. culisetae is cosmopolitan and usually associated with various mosquitoes, but it may also infest blackflies, solitary midges, or rarely, mayflies (Grigg & Lichtwardt 1996; Lichtwardt 1986; Lichtwardt & Williams 1990). Morphologically, it has a distinct trichospore shape and demonstrated homogeneity regarding immunological (Peterson & Lichtwardt 1987), isozymic (Grigg & Lichtwardt 1996) as well as 18S-RFLP and ITS-RFLP data (Gottlieb & Lichtwardt 2001). *S. culisetae* was a strongly supported terminal branch in the 18S and 28S rRNA trees. In the 28S tree, *S. culisetae* split from the other *Smittium* species in the 'Smittium' clade, which are all associated with lower dipteran families. In all phylogenetic analyses, *S. culisetae* was either separate from the other groups, or most closely allied with the 'non-Smittium' clade. As a distinct lineage it may warrant future generic reconsideration, pending further sequence data.

Resolution of non-*Smittium* taxa, *Furculomyces*, *Austrosmittium*, *Pseudoharpella*, *Harpellomyces* and *Stachylina* with *Smittium* species (other than *S. culisetae*) in the 18S and 28S cladograms (Figs 1–2), presents a clade of lower dipteran endobionts. *Furculomyces* has a unique 'wish bone' (furculum-like) conjugation apparatus and bent zygospores that distinguish it both morphologically (Misra *et al.* 1999) and molecularly (Figs 1–2). Similarly, in each tree *Austrosmittium* is a distinct lineage near a clade that included *S. mucronatum*. Mature trichospores of *S. mucronatum* bear a distinctive small, distal nipple-like projection. In the 28S tree (Fig 2) an axenic culture (strain FRA-12-3) of *S. mucronatum*, isolated from near the (now destroyed) type locality in France, paired with a gut-removed specimen (RMBL-61-10) from the Colorado Rockies, tentatively identified as *S. mucronatum*-like (R. Lichtwardt, pers. comm.). *S. mucronatum* was also reported from Norway (White & Lichtwardt 2004) and in each case was associated with same midge genus, *Psectrocladius*. This illustrates the usefulness of sequences for identification and represents a significant range extension for *S. mucronatum* considering the loss of the type locality.

Ferrington *et al.* (2003) described a new genus, *Pseudoharpella*, from meniscus midges (*Dixidae*). *Pseudoharpella* is an unusual harpellid because it possesses a coiled trichospore with three broad trichospore appendages and a type II zygospore. The separation of *Pseudoharpella* as a lineage in the 'Smittium' clade in the 18S tree (Fig 1) was unexpected because the other taxa in the 'Smittium' clade have only one trichospore appendage (but they do share the same zygospore type). It remains to be seen how much significance trichospore shape and appendage number have as taxonomic characters for *Harpellales* associated with midges. *Harpellomyces* is restricted to solitary midges (*Thaumaleidae*), a host family with low vagility and specific to restricted habitats. It separated as a weakly supported, more inclusive grade that encompasses the 'Smittium' cluster in the 18S tree. *Harpellomyces* also has type II zygospores, but trichospores have two to five appendages. It had been regarded as unbranched until recent investigations noted its branching base (Lichtwardt *et al.* 2001b). Based on its unusual trichospore shape and placement on the tree, further collections of *Harpellomyces* species in solitary midges are warranted (see White *et al.* 2006b for a new species).

Overall, the 'Smittium' and 'non-Smittium' clades do not support the long-believed taxonomic value of the morphologies of trichospores or zygospores, or the number of spore appendages for the *Harpellales*. In the 18S tree, the upper 'Smittium' cluster includes species with only type II zygospores and one trichospore appendage, except that *Pseudoharpella* and *Harpellomyces* trichospores have multiple trichospore appendages. The 28S rRNA 'non-Smittium' clade, including representatives with each of the four known kinds of zygospores and with every possible number of trichospore appendages (ranging from 0 to 6), does not support the phylogenetic utility of these characters. This is not meant to deny their morphogical taxonomic value for species description. It will be illuminating, however, to determine how the Harpellalean clades respond to the addition of more unculturable taxa and other molecular markers with future sequencing efforts.

Circumscription of 'non-Smittium' taxa

The 'non-Smittium' taxa formed a weakly supported clade sister to *Smittium culisetae* on the 28S rRNA tree, but were unresolved, sister to the 'Smittium' clade on the 18S tree. Six of the 10 genera included in this clade (*Bojamyces*, *Legeriomyces*, *Zygopolaris*, *Plecopteromyces*, *Genistelloides*, and *Capniomyces*) are associated with stonefly or mayfly nymphs, two of the most basal lineages of aquatic insect hosts, and an unnamed species is from larval *Trichoptera*.

One of the most unusual gut fungi, from ecological and developmental perspectives, is *Bojamyces repens* (Longcore 1989). This genus was known only from *Leptophlebiidae* in lentic systems (ponds) in Maine, until *B. transfuga* was decribed (with a single appendaged trichospore and type I zygospore) from mayfly molts in Spain (Valle & Santamaria, 2004). Lichtwardt and Williams (1992) described, but did not name a gut fungus with striking similarity to the genus *Bojamyces*, in *Australonousia* (*Leptophlebiidae*) from Tasmania. *B. repens* trichospores have no appendages, and their formation seems to occur only in the shed exuviae of the *Leptophlebiidae* nymphs (Longcore 1989). This delay in asexual reproduction may be linked with their occurrence in non-flowing systems. All other gut fungi undergo asexual reproduction in the gut of the host during the intermolt period. The non-motile appendages of trichospores (and zygospores) are believed to aid in entanglement in the substrate to prevent their downstream movement in flowing systems. If restricted to non-flowing systems, *B. repens* may have evolved alternate strategies specific to nymph behaviour, such as reinfestation by hosts grazing on or near the shed skins following molting (Longcore 1989; White *et al.* 2006b). The position of *B. repens* on the 28S rRNA tree, as a distinct lineage, may suggest that delayed asexual reproduction was a significant alternative reproductive strategy adapted during the evolution of the *Harpellales*. Further sequences

from other species of *Bojamyces* and efforts to collect from lentic micohabitats are needed to address this possibility.

Two other mayfly-associated gut fungi fall into this non-'Smittium' clade, *Legeriomyces* and *Zygopolaris*, both widespread in lotic systems. *Legeriomyces* species occur in the hindgut, have 'bowling pin'-shaped trichospores with two appendages and type II zygospores. The trichospore shape for *Legeriomyces* species resembles those for *Legerioides* and *Legeriosimilis*, but the latter two genera are distinguished by features of sexual reproduction and number of appendages (White 1999; Williams & Lichtwardt 1999). Sequence data for *Legerioides* and *Legeriosimilis* are not available, but a new unnamed species similar to *Legeriomyces* and listed as '*Trichoptera* gut fungus' (Table 2) was obtained from caddisfly larvae collected in Yosemite National Park (California) during these studies. It has features of *Legeriomyces*, but matches no described species and appears as a distinct lineage in the 28S rRNA tree. This is yet another occurrence associated with *Trichoptera*, a new host order of gut fungi (White 1999). During the same survey, a new *Legeriomyces* species from mayfly nymphs and the lone exemplar of *Legeriomyces* studied here did not cluster with the '*Trichoptera* gut fungus' in the 28S tree. More sequence data will be needed to determine their relationships, but the occurrence of another *Legeriomyces*-like species in a non-mayfly host, i.e. *Legerioides* was described from *Isopoda*, may indicate convergent evolution. Occurrences of such morphologically 'similar' genera of gut fungi from such a wide range of hosts are unusual among harpellid gut fungi.

Zygopolaris, with *Orphella* and *Pteromaktron*, are the genera whose mature thalli protrude from the host's anus. Trichospore and zygospore appendages of *Zygopolaris* are inconsistent making it difficult to map its morphology on the trees. Trichospores may have a small 'blob' of material at their base (Lichtwardt 1986; Moss *et al.* 1975) or released trichospores of *Zygopolaris ephemeridarum* have been noted, rarely, with three short appendages (Lichtwardt & Williams 1984). Zygospores of *Zygopolaris* (type IV) usually bear no appendages, but a fibrous substance at the base of the zygosporophore (Moss & Lichtwardt 1977) may accompany the released zygosporophore that appears as an almost invisible skirt (Lichtwardt 1986; Lichtwardt & Williams 1984). The only other species with this kind of sexual spore presented herein is *Plecopteromyces* sp. *Plecopteromyces*, endobionts of southern hemisphere stoneflies (family *Gripopterygidae*), have trichospores with two thickened appendages and turbinate zygospores (type IV) (Lichtwardt *et al.* 1999). Only one other genus associated with stoneflies, *Lancisporomyces*, has type IV zygospores (Santamaria 1997) but no sequence data from this genus are yet available. Two other stonefly gut fungi genera, however, are included in the trees.

Both *Capniomyces stellatus* and *Genistelloides hibernus* have been found in winter-emerging stoneflies (*Capniidae*) from locations in eastern North America. *C. stellatus* has type II zygospores and trichospores with 1–6 broad appendages whereas *Genistelloides hibernus* has type I zygospores with two appendages. Both are culturable but difficult to maintain. *Genistelloides hibernus* appeared as a sister group in a larger clade with *Pennella simulii* and *Genistellospora homothallica*. Both *P. simulii* and *G. homothallica* are hindgut inhabitants of blackflies and have type III zygospores although their trichospores differ in having 4–6 or approximately (5–)6(–7) appendages, respectively. *Pennella* and *Genistellospora* are also separated generically by subtle features of the sexual process and the holdfast. *P. simulii* has a pointed or split holdfast, accompanied by a mucilaginous secretion (Lichtwardt *et al.* 2001a) whereas *G. homothallica* has a thumb-like extension from the supporting cell below the zygosporophore and a well-defined solid holdfast. The sequence data revealed that these gut fungi are similar though separable in the 28S rRNA tree. The clade with *Pennella* + *Genistellospora* and *Genistelloides*, aside from the similarities in the former two genera, reveal no pattern relating to host, zygospore type or trichospore appendage number. Likewise, a clade with two other genera from blackfly and stonefly hosts, *Harpella* and *Plecopteromyces*, do not reveal any morphologically informative characters that substantiate this grouping. It should be noted that both of these subclades are only weakly supported and complicated by the inclusion of *G. homothallica* with *Harpella* that is weakly supported in the 18S gene tree.

Harpella species are restricted to the peritrophic membranes of their larval blackfly hosts. *H. melusinae* is cosmopolitan but the other four species, *H. amazonica*, *H. leptosa*, *H. meridianalis* and *H. tica* apparently have much narrower geographical distributions (Lichtwardt *et al.* 2001a). The ranges of *H. meridianalis* and *H. tica* overlap geographically and a misidentified specimen may explain their grouping, particularly in the 28S rRNA tree (Fig 2). There is some indication (White, unpublished) that species of *Harpella* exhibit molecular variation that is masked by morphological convergence. Further studies of *H. melusinae* to determine possible intraspecific or population level variation are recommended and feasible as it is readily obtainable from blackflies worldwide.

Significance of this study

These results establish a working hypothesis of the phylogeny of the *Harpellales*. The tree topologies are scaffolds upon which to build a broader phylogenetic perspective of the gut fungi. There are many taxa of gut fungi to be appended to these preliminary rRNA data sets, and clearly, other kinds of sequence data, including protein genes, need to be explored and tested. The task at hand, especially with unculturable taxa, is less daunting as the molecular approaches continue to be refined and data sets expanded. Concurrently, efforts to document the biodiversity of gut fungi must be sustained because few surveys in the last 15 y have been undertaken without new taxa of harpellids being described and many more remain to be discovered. This investigation demonstrates the promise of molecular systematics for *Harpellales*, provides a provocative insight into the evolution of gut fungi, and will form the foundation of an eventual reclassification.

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