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## Molecular variation within and among species of Harpellales

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**Abstract:** Intra- and interspecific variation of the nuclear ribosomal internal transcribed spacers (ITS) of 77 *Smittium* isolates and related genera was studied using RFLP. Due to the sequence and length variation encountered, the ITS is unsuitable for comparison at the species level within *Smittium*, as presently delimited. Intra- and interspecific variation of 18S rDNA was also analyzed. Cladistic analysis of 32 new 18S rDNA sequences resolved at least five lineages and suggests that *Smittium* is not monophyletic. In addition, 17 18S rDNA sequences from 5 orders of Zygomycota were included to assess phylogenetic relationships with the Trichomycetes. The kickxellid origin of Trichomycetes was not strongly supported.

**Key Words:** 18S rDNA, ITS, phylogeny, RFLP, *Smittium*, Trichomycetes, Zygomycota

### INTRODUCTION

The class Trichomycetes (Zygomycota) consists of a worldwide group of fungi that have various relationships with their arthropod hosts (Horn and Lichtwardt 1981, Lichtwardt 1986, 1996, Misra 1998). Known associations range from commensalistic to deleterious and possibly mutualistic in some cases (Lichtwardt 1986). The class consists of three orders, Harpellales, Asellariales and Eccrinales, of which only Harpellales contains culturable species. Traditionally, the order Amoebidiales has been placed in the same class. Although Amoebidiales and Harpellales share some similarities that perhaps reflect convergence attributable to having evolved under similar habitat constraints, they are not related phylogenetically (Lichtwardt 1986). There is evidence that zygomycetes belonging to the order Kickxellales and some Harpellales share common ancestry. They have some serological affinities (Sangar et al 1972, Peterson and Lichtwardt 1987) and similarities in septal and cell

wall structure, and both orders produce basipetal monosporous sporangia (Moss and Young 1978). In particular, a bifurcate type of septum has been reported in Harpellales (Farr and Lichtwardt 1967), Asellariales (Manier 1973, Saikawa et al 1997, Moss 1975), Kickxellales (Young 1969), Dimargaritales (Saikawa 1977, Jeffries and Young 1979) and Mersitaciaceae (Entomophthorales) (Saikawa 1989). However, Porter and Smiley (1979) concluded that the Harpellales, Kickxellales and Mucorales were not closely related based on disparate RNA molecular weights. Walker's (1984) 5S rRNA sequence comparison indicated that Harpellales were more related to Mucorales than to Kickxellales, but the differences were too small to be convincing. Recently, O'Donnell et al (1998) showed certain phylogenetic relatedness in the sequences of the 18S ribosomal DNA of some Kickxellales and four representatives of the Harpellales.

Emphasis in this study is on the trichomycete genus *Smittium* Poisson (Harpellales), with related fungal taxa included to give a broader perspective. The Harpellales currently includes 33 genera and 141 species (Misra and Lichtwardt 2000). At present only 7 genera are available in axenic culture. The genus *Smittium* has 55 described morphological species, all characterized by ellipsoidal to almost cylindrical trichospores (unisporous sporangia) with a collar and a single appendage; zygospores, when present, are biconical to fusiform and obliquely attached to the zygosporophore and also have a collar and a single appendage (Lichtwardt 1986). The number of appendages is considered a generic character (Lichtwardt 1996). Trichospore appendages of Harpellales and sporangiospore appendages of one representative of Eccrinales [*Astreptonema gammari* (Léger & Duboscq) Manier] are different in structure and ontogeny, presumably a result of convergent evolution in species subjected to similar environmental pressures (Moss 1979). Physiological studies of some *Smittium* cultures were conducted by Starr et al (1979), Williams (1983a, b) and Horn (1989a), among others. More recently, Grigg and Lichtwardt (1996) studied the isozyme variation of a worldwide sample of culturable trichomycetes.

Hosts of the Harpellales are larval stages of primitive orders of aquatic insects, namely Ephemero-

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tera, Plecoptera and nematoceran Diptera. Patterns of Trichomyces infestation of chironomids (Chironominae, Podonominae, Orthoclaadiinae, Diamesinae) at the subfamily and generic levels were suggestive of a high degree of co-evolution among trichomyces and their hosts (Slaymaker et al 1998). Some species of *Smittium* have a very wide distribution, while other species may be restricted geographically due to high host specificity or to poor dispersion. Worldwide distribution may be an expression of antiquity of the class, rather than the dispersal ability of individual species (Lichtwardt 1994a).

Analysis of nuclear ribosomal DNA (rDNA) regions has been applied to many fungal systematic questions (Bruns and Szaro 1992, Bruns et al 1992, Feibelman et al 1994, Dick et al 1999). Several studies have shown that the internal transcribed spacer (ITS) regions are often variable among fungal species (Gardes et al 1991, Hibbett and Vilgalys 1991, Feibelman et al 1994, Lobuglio et al 1993, Lloyd-MacGilp et al 1996). However, intraspecific heterogeneity also has been reported (Bunyard et al 1996). Nucleotide sequences of the small and large ribosomal subunit genes (18S and 28S) are more conserved, and thus have been generally used for comparison at higher taxonomic levels (Bruns and Szaro 1992, Bruns et al 1992, Berbee et al 1995, Gargas and Taylor 1995, Hibbett et al 1997, Harrington et al 1999). These molecules have divergent domains that form stem loops of relaxed secondary structure that are known to be variable in shape and in size among phylogenetically distant taxa (Bruns and Szaro 1992, Hillis and Dixon 1991). The existence of among-site rate variation within 18S rDNA allows the same region to be used for both higher and lower relationships (Abouheif et al 1998). Highly conserved sites could be used for deep divergence but contribute little to phylogenetic information about recent divergences, while highly variable sites can recover recent divergences (Abouheif et al 1998).

The first objective of this study was to determine the suitability of the ITS region as a genetic marker by assessing inter- and intraspecific variation in several species of *Smittium* by means of PCR-RFLP and DNA sequencing. The second objective was to use cladistic analysis of 18S rDNA sequences to elucidate phylogenetic relationships and to determine whether morphologically defined species are supported by the phylogenetic data. We tested the hypothesis that the genus *Smittium* is monophyletic and analyzed the phylogenetic relationships among 23 species.

#### MATERIALS AND METHODS

**Cultures.**—The organisms used in this study (TABLE I) were selected to obtain the maximal range in geographical and

host diversity. When available, multiple isolates of several species of *Smittium* were examined. Mycelia were grown in liquid tryptone-glucose-salts medium TGv (Lichtwardt 1986) in a rotary shaker (200 rpm) at 22 C. After 1–2 wk, mycelia were either lyophilized or harvested by vacuum filtration.

**DNA extraction.**—CTAB buffer-suspended samples (2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl pH 8.0, 0.25 mM EDTA) were frozen and thawed at least twice by submerging the tubes into liquid nitrogen and incubating at 65–70 C in a heat block. After the final thaw, mycelia were crushed with a sterile glass pestle and incubated for 30 min at 65 C. One volume of chloroform was added to each sample and mixed by vortexing for 10–15 s. Samples were centrifuged at 12 000 *g* for 10 min at room temperature. DNA was precipitated from the aqueous supernatant with two volumes of 100% ethanol (–20 C) and pelleted with a 5 min centrifugation at 12 000 *g*. After a 70% ethanol (ice-cold) wash, the genomic DNA was redissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The DNA extracts were treated with RNase ONE (Promega Corp., Madison, Wisconsin) for 30 min at 37 C and then extracted with chloroform, precipitated with ethanol, and resuspended in 50–100  $\mu$ L of TE buffer.

**PCR amplification.**—ITS amplification was carried out by using primers ITS4 and ITS5 (White et al 1990) and 5.8S and 5.8SR (Vilgalys and Hester 1990). Primer pairs ITS5–5.8S and ITS4–5.8SR were used to amplify ITS 1 and ITS 2 regions, respectively. Amplification of the 18S rDNA was done either using universal primers NS1–NS8 (White et al 1990) or using universal and zygomycete-specific primers (O'Donnell et al 1998) NS1-Z411G and Z31G-NS8. Amplifications were performed in 0.225 mM of each dNTP (Promega Corp., Madison, Wisconsin), 0.25 mM of each primer, 10% of 10X buffer (0.5 M KCl, 0.1 M Tris-HCl pH 8.4, 25 mM MgCl<sub>2</sub>, 1 mg/mL gelatin) and sterile double-distilled water. Taq DNA polymerase (Promega Corp., Madison, Wisconsin) was added at 2.5 units per 100  $\mu$ L of reaction mix. Total volume was adjusted to 20, 50 or 100  $\mu$ L as needed. One  $\mu$ L of genomic DNA template was used in each 20  $\mu$ L reaction. Conditions for PCR amplification were those of Vilgalys and Hester (1990). Amplifications were carried out in a Perkin-Elmer Gene Amp PCR System 2400 Thermal Cycler. Control samples without DNA template were included in each PCR run. PCR products were checked by electrophoresing 1  $\mu$ L aliquots in 1% or 2% agarose gels (Fisher Sci., Pittsburgh, Pennsylvania) in 1X TAE buffer (0.04 M Tris, 1 mM EDTA pH 8.0, 0.114% glacial acetic). Gels were stained with ethidium bromide for 20–30 min and photographed under UV light. Each PCR amplification was repeated at least twice.

**Restriction endonuclease digestions.**—Single restriction endonuclease digests of amplified products were performed after chloroform extraction and ethanol precipitation, or directly on an aliquot of the PCR reaction. Digestions were done using 0.5–0.7 units of each endonuclease per  $\mu$ g of amplified DNA following the manufacturer's protocol. PCR products were digested with *Cfo*I, *Dra*I, *Eco*RI, *Hae*II,

TABLE I. Trichomyces used in this study

Species	Isolate	Host	Locality	ITS1 (bp)	ITS2 (bp)	GenBank 18S; ITS1, ITS2.
<i>Capniomyces stellatus</i> Peterson & Lichtwardt	MIS-10-108	Capniidae	Missouri, USA	—	—	AF007531
<i>Furculomyces boomerangus</i> (Williams & Lichtw.) Williams & Lichtw.	AUS-42-7	<i>Procladius</i> ? <i>paludicola</i>	Victoria, Australia	250	400	AF007535; AF277009; AF277010
	AUS-77-4	<i>Tanytarsus</i> nr. <i>inextentus</i>	Tasmania, Australia	250	400	AF277013
<i>Genistelloides hibernus</i> Peterson, Lichtw. & Horn	AL-7-10	<i>Allocaptnia</i> sp.	Alabama, USA	350	400	
	TN-11-1	<i>Allocaptnia</i> sp.	Tennessee, USA	1000	850	AF007536
<i>Simuliomyces spica</i> Peterson & Lichtw.	TN-1-11	<i>Allocaptnia</i> sp.	Tennessee, USA	350	400	
<i>Smittium angustum</i> Williams & Lichtw.	AUS-126-30	<i>Tanytarsus</i> sp.?	West Australia, Australia	250	400	AF277005; AF277006
<i>Smittium annulatum</i> Lichtwardt	CR-143-8	Simuliidae	Costa Rica	700	900	AF277024
<i>Smittium caudatum</i> Lichtw. & Grigg	KS-1-2	Chironomidae	Kansas, USA	500	1100	AF277031; AF277032; AF277033
<i>Smittium coloradense</i> Lichtw. & Williams	RMBL-13-41	<i>Cricotopus</i> sp.?	Colorado, USA	700	1000	AF277041; AF277042
	RMBL-13-49	<i>Orthocladius</i> sp.?	Colorado, USA	700	1000	
<i>Smittium commune</i> Lichtw. & Grigg	DGK-2-3	Chironomidae	Kansas, USA	700	900	
	KS-12-13 L	<i>Orthocladius</i> sp.	Kansas, USA	700	900	
	KS-2-21	Chironomidae	Kansas, USA	700	900	AF277034
	KS-5-2	Chironomidae	Kansas, USA	700	900	
	KS-6-6	Chironomidae	Kansas, USA	700	900	AF277035; AF277036
	OK-8-4	Chironomidae	Oklahoma, USA	700	900	
<i>Smittium culicis</i> Manier	ARG-X-3	<i>Culex renatoi</i>	Argentina	900	800	
	ARG-X-4	<i>Aedes albifasciatus</i>	Argentina	900	800	
	AUS-62-17	<i>Limonia</i> sp.	Australia	900	800	
	AUS-62-6	<i>Austrothaumalea</i> sp.	Victoria, Australia	900	800	AF277012
	AUS-77-5	<i>Tanytarsus</i> nr. <i>inextentus</i>	Tasmania, Australia	900	800	
	CAL-X-1	<i>Aedes melaninom</i>	California, USA	900	800	
	CAN-X-1	<i>Prosimulium</i> sp.	Canada	900	800	
	FRA-6-16	Chironomidae	France	900	800	
	WYO-51-11	<i>Aedes sticticus</i>	Wyoming, USA	900	800	AF277047; AF277048
	ZEA-6-3	<i>Culex pervigilans</i>	North Island, New Zealand	900	800	
<i>Smittium culicisoides</i> Lichtwardt	CR-253-12	Chironomidae	Costa Rica	850–900	800	AF277028



TABLE I. Continued

Species	Isolate	Host	Locality	ITS1 (bp)	ITS2 (bp)	GenBank 18S; ITS1, ITS2.
<i>Smittium culisetae</i> Lichtwardt	ARG-X-1	<i>Aedes crinifer</i>	Argentina	450	650	
	ARG-X-2	<i>Culex dolosus</i>	Argentina	450	650	
	ARG-X-5	<i>Aedes crinifer</i>	Argentina	450	650	
	AUS-2-10	<i>Chironomus alternans</i>	New South Wales, Australia	450	650	
	AUS-2-8	<i>Chironomus alternans</i>	New South Wales, Australia	450	650	AF277007; AF277008
	CAL-X-2	<i>Culiseta incidens</i>	California, USA	450	650	
	COL-18-3	<i>Culiseta impatiens</i>	Colorado, USA	450	650	AF007540, D29950, AF277029
	FL-X-1	<i>Aedes albopictus</i>	Florida, USA	450	650	
	FRA-7-1	<i>Dasyhelia</i> sp.	France	450	650	
	HAW-5-8	<i>Aedes albopictus</i>	Hawaii, USA	450	650	
	HAW-E-2	Culicidae	Hawaii, USA	450	650	
	JAP-30-3	<i>Culex</i> sp.	Japan	450	650	
	JAP-77-8	Culicidae	Japan	450	650	
	LEA-7-2	<i>Simulium vittatum</i>	Kansas, USA	450	650	
<i>Smittium cylindrosporum</i> Lichtw. & Arenas	CHI-19-3	<i>Cricotopus</i> sp.	Chile	500	1100	
	CHI-20-4	<i>Cricotopus</i> sp.	Chile	500	1100	AF277017
	CHI-27-1	<i>Cricotopus</i> sp.	Chile	500	1100	AF277018
<i>Smittium dipterorum</i> Lichtwardt	CR-253-14	<i>Simulium</i> sp.	Costa Rica	800	1100	AF277026; AF277027
<i>Smittium dipterorum?</i>	CR-141-17	<i>Simulium</i> sp.	Costa Rica	900	1000	AF277023
<i>Smittium fecundum</i> Lichtw. & Williams	RMBL-61-13	<i>Psectrocladius</i> sp.	Colorado, USA	800	800	
	RMBL-64-5	<i>Psectrocladius</i> sp.	Colorado, USA	800	800	AF277044
<i>Smittium gravimetallum</i> Lichtw., Ferrington & Hayford, nom. prov.	KS-F1-3	<i>Dicrotendipes</i> sp.	Kansas, USA	800	800	AF277037; AF277038
<i>Smittium imitatum</i> Lichtw. & Arenas	CHI-20-11	<i>Simulium</i> sp.	Chile	800	800	AF277016
	CHI-9-4	<i>Simulium</i> sp.	Chile	800	800	AF277021
<i>Smittium megazygosporum</i> Manier & Coste	SC-DP-2	<i>Simulium vittatum</i>	South Carolina, USA	800	800	AF277045
<i>Smittium morbosum</i> Sweeney	AUS-X-1	<i>Anopheles hilli</i>	New South Wales, Australia	550	800	AF277014
<i>Smittium mucronatum</i> Manier & Mathiez ex Manier	FRA-12-3	<i>Psectrocladius sordidellus</i>	France	700	1100	AF277030
<i>Smittium orthocladii</i> Manier emend. Lichtw.	OK-4-19	Chironomidae	Oklahoma, USA	700	800	AF277040
	TN-3-12	Chironomidae	Tennessee, USA.	700	900	
<i>Smittium perforatum?</i> Williams & Lichtw.	RMBL-44-3	<i>Diamesa</i> sp.	Colorado, USA	700	800	
<i>Smittium phytotelmatum</i> Lichtw.	CR-219-1	<i>Chironomus</i> sp.	Costa Rica	700	1100	AF277025
<i>Smittium phytotelmatum?</i>	CR-133-2	<i>Chironomus</i> sp.	Costa Rica	700	900	AF277022
<i>Smittium simulatum</i> Lichtw. & Arenas	CHI-8-3	<i>Aphrophila bidentata</i>	Chile	900	800	
	CHI-8-4	<i>Aphrophila bidentata</i>	Chile	800	800	AF277019; AF277020
	CHI-8-7	<i>Aphrophila bidentata</i>	Chile	900	800	

TABLE I. Continued

Species	Isolate	Host	Locality	ITS1 (bp)	ITS2 (bp)	GenBank 18S; ITS1, ITS2.
<i>Smittium simulii</i> Lichtwardt	CAL-8-1	<i>Simulium argus</i>	California, USA	700	900	AF277015
	JAP-31-7	<i>Simulium uchidai</i>	Japan	700	900	
	JAP-51-5	<i>Simulium</i> sp.	Japan	700	900	
	SWE-8-4	<i>Diamesa</i> sp.	Sweden	700	900	AF277046
<i>Smittium tipulidarum</i> Williams & Lichtw.	RMBL-31-1	Tipulidae	Colorado, USA	800	900	AF277043
	RMBL-31-2	Tipulidae	Colorado, USA	800	900	
<i>Smittium tronadorium</i> Lichtw., Ferrington & López Lastra	ARG-24-2F	Diamesinae	Argentina	800	800	AF277004
	ARG-24-20F	Diamesinae	Argentina	800	800	
<i>Smittium</i> sp.	AR-19-4L	Chironomidae	Arkansas, USA	700	800	
	AUS-59-L	<i>Cardiocladius australiensis</i>	Victoria, Australia	700	900	AF277011
	AUS-59-5S	<i>C. australiensis</i>	Victoria, Australia	350	400	
	CR-259-4	Chironomidae	Costa Rica	1000	1000	
	MO-3-15	Chironomidae	Missouri, USA	700	900	
	MO-3-16	Chironomidae	Missouri, USA	700	900	
	OK-3-22	Chironomidae	Oklahoma, USA	800	900	AF277039
	RMBL-48-8	Chironomidae	Colorado, USA	750	800	
	SWE-3-5	Chironomidae	Sweden	700	900	
	SWI-4-13	Chironomidae	Switzerland	700	900	
	SWI-7-6	Chironomidae	Switzerland	700	900	
TN-3-16	Chironomidae	Tennessee, USA	700	900		
<i>Entomophaga aulicae</i> (Reichardt) Humber	—	—	—	—	—	U35394
<i>Entomophthora muscae</i> (Cohn) Fresenius	—	—	—	—	—	D29948
<i>Conidiobolus coronatus</i> (Constantin) Batko	—	—	—	—	—	D29937
<i>Zoophthora radicans</i> (Brefeld) Batko	—	—	—	—	—	D29949
<i>Dipsacomyces acuminosporus</i> Benjamin	—	—	—	—	—	AF007534
<i>Linderina pennispora</i> Raper & Fennell	—	—	—	—	—	AF007538
<i>Kickxella alabastrina</i> Coemans	—	—	—	—	—	AF007537
<i>Coemansia reversa</i> van Tiegh. & Le Monn.	—	—	—	—	—	AF007533
<i>Spirodactylon aureum</i> Benjamin	—	—	—	—	—	AF007541
<i>Spiromyces aspiralis</i> Benny & Benjamin	—	—	—	—	—	AF007543
<i>Spiromyces minutus</i> Benjamin	—	—	—	—	—	AF007542
<i>Mucor racemosus</i> Fresenius	—	—	—	—	—	X54863
<i>Syncephalastrum racemosum</i> Cohn	—	—	—	—	—	X89437
<i>Endogone pisiformis</i> Berk.	—	—	—	—	—	X58724
<i>Glomus intraradices</i> Schenck & G. S. Smith	—	—	—	—	—	X58725
<i>Acaulospora spinosa</i> Walker & Trappe	—	—	—	—	—	Z14004
<i>Gigaspora gigantea</i> (Nicol. & Gerd.) Gerde- mann & Trappe	—	—	—	—	—	Z14010
<i>Blastocladiella emersonii</i> Cantino & Hyatt	—	—	—	—	—	M54937

*HindIII*, *HinfI*, *RsaI* and *Sau3AI* (Promega Corp., Madison, Wisconsin). Incubation was held in a water bath at 37 C for 3–4 h. Restriction products were electrophoresed in 2% agarose gels in 1X TAE buffer. Size of restriction products was estimated by comparison with a molecular size marker (100 bp DNA Ladder, Promega Corp., Madison, Wisconsin), which was included in duplicate on all gels.

**Cloning and sequencing.**—PCR products were cut from the gel and purified using the QIAquick Gel Extraction kit (QIAGEN Inc., Valencia, California). Cleaned PCR products were cloned using either the Prime PCR Cloner Cloning System (5 Prime-3 Prime Inc., Boulder, Colorado) or the pGEM-T Easy Vector System (Promega Corp., Madison, Wisconsin) cloning kit. Putative positive colonies were screened for the insert by PCR amplification. Recombinant bacterial colonies were extracted with QIAprep Spin Miniprep kit (QIAGEN Inc., Valencia, California) and were sequenced in a LI-COR 4000L DNA Sequencer apparatus using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey). Direct sequencing was performed in an ABI PRISM 310 DNA sequencing apparatus (Perkin-Elmer Corp., Foster City, California) at the Biochemical Research Service Laboratory, University of Kansas. Both strands of each rDNA fragment were sequenced. Overlapping sequencing reactions for the 18S rDNA were done using primers Z411G, Z31G (O'Donnell et al 1998), NS41, NS51 (Simon et al 1992) and two primers for Harpellales, T1: 5'-AACCAATGGGGGCAACCTCTTACTT-3' (position 223–244 in *Saccharomyces cerevisiae* 18S rDNA) and T8: 5'-CATCGTGCTGGGGATAGTCCAT-3' (position 1630–1650 in *S. cerevisiae* 18S rDNA). Gene regions for the 18S, 5.8S and 28S rRNA genes were identified according to those of *S. cerevisiae* (GenBank M27607, J01353) and to those of some Harpellales already published (O'Donnell et al 1998). Sequences have been deposited in the GenBank Database and are identified by the accession numbers listed in TABLE I.

**Data analyses.**—Restriction digest banding patterns were coded. The absence of a target site and the length of amplified ITS1 and 2 were considered as additional phenotypes. The similarity matrix was obtained by using Simple Matching Coefficient as specified in the NTSYS-PC program (Rohlf 1993). Dendrograms were constructed by SAHN clustering method using UPGMA (unweighted pair-group method using arithmetic averages) cluster analysis to visualize relationships. The cophenetic correlation index was assessed by MXCOMP.

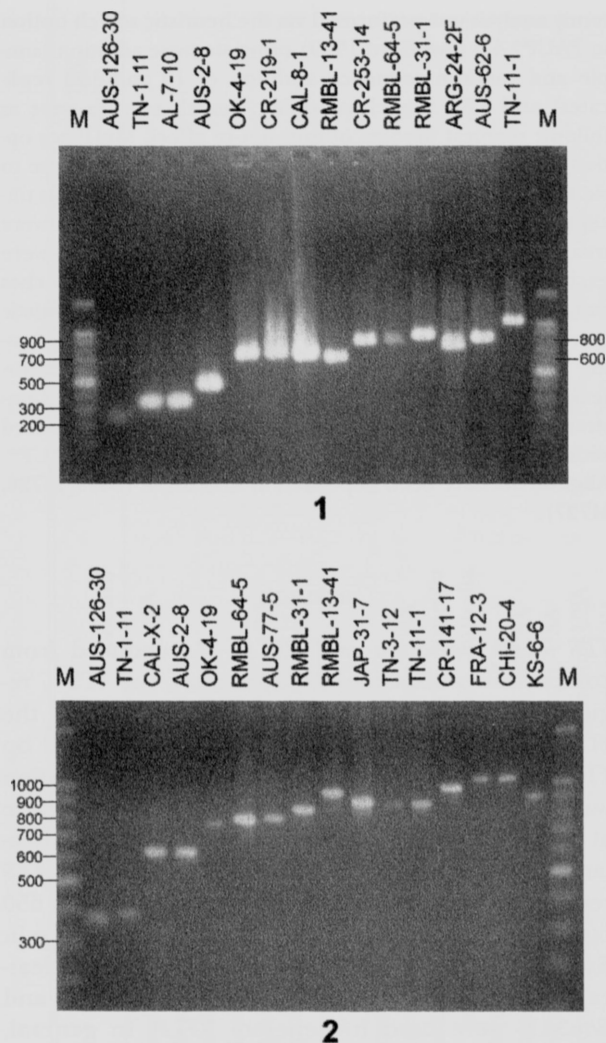
Alignment of sequences was done with CLUSTAL W (Thompson et al 1994); default settings for GOP and GEP were used (10 and 0.05, respectively). Alignments were visually inspected and edited to maximize for homology. Two sequences from Kickxellales and from Entomophthorales (TABLE I) were used to root the cladograms. Fourteen additional sequences downloaded from GenBank for comparison with ingroup sequences are listed in TABLE I. Consensus sequences were obtained for those species with more than one sequence using SEQUENCE NAVIGATOR v. 1.01 (Perkin-Elmer Corp., Foster City, California). The IUPAC code was used for ambiguous residues. Maximum parsimony

analysis was performed via the heuristic search option in PAUP\* 4.0 (Swofford 1999) using stepwise addition (simple and random sequence addition of 10 and 100 replicates) and TBR branch swapping. 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. Characters were treated as unordered. Gaps were treated as missing and all ambiguously aligned regions were excluded from the analyses. Support for internal branches was evaluated by bootstrap analyses from 500 heuristic searches and by decay indices (Bremer 1988) calculated using TREEROT (Sorenson 1996). Pairwise distance (uncorrected "p") between sequences was done with PAUP\*. Constraint and unconstraint tree topologies were compared with the Kishino-Hasegawa test implemented in PAUP\*. Alignments have been deposited in TreeBASE (S499, M726, M727).

## RESULTS

**ITS length variation and RFLP.**—As deduced from comparison with a molecular marker, the ITS 1 region ranged from 250 to about 1000 bp, while the ITS2 region ranged from 400 bp to about 1100 bp (TABLE I, FIGS. 1, 2). The greatest length variation was determined to be in the amplified ITS 1, where at least 10 different lengths were recorded. Particularly within *Smittium*, the size of the complete ITS region, including the 5.8S gene, ranged between 650 bp in *Smittium angustum* AUS-126-30 and 2000 bp in *Smittium* sp. CR-259-4 (TABLE I). Eight endonucleases, *EcoRI*, *CfoI*, *DraI*, *HaeIII*, *HindIII*, *HinfI*, *RsaI* and *Sau3AI*, were used to generate RFLP. In general, there was good agreement between size estimates for the undigested PCR products and the sums of fragment sizes, but some anomalies were observed: the sum of fragment sizes obtained with *Sau3AI* in CR-259-4 and CR-253-14 were higher than the estimated size. Coded ITS-RFLP patterns rendered the dendrogram depicted in FIG. 3 with a high cophenetic correlation coefficient ( $r = 0.963$ ).

Some taxa for which multiple isolates were scored provided the basis for intraspecific comparison of ITS-RFLP (FIG. 3). With the exception of two Argentinian samples ARG-X-1 and ARG-X-2, the remaining 12 *Smittium culisetae* isolates shared identical restriction patterns, despite their occurrence in different countries and continents (e.g., USA, France, Australia, or Japan). However, isolate JAP-77-8 showed an additional 400 bp band for ITS2. Three *RsaI* profiles were encountered in the ITS1 of *Smittium culicis*; isolates FRA-6-16 and ZEA-6-3 exhibited unique patterns. Particularly for isolate FRA-6-16, the sum of fragment sizes did not agree with the estimated size of undigested product. The ITS2 of *S. culicis* isolates



FIGS. 1, 2. Internal transcribed spacer (ITS) length polymorphism in some harpelllean fungi. 1. ITS1 length variation. 2. ITS2 length variation. M stands for molecular marker. Band sizes (base pairs) of the marker are indicated. Isolate numbers are indicated for each lane. For complete reference, see TABLE I.

was also variable; for instance, isolates WYO-51-11, CAN-X-1 and ZEA-6-3 lacked *Hin*I target sites. Excluding isolate FRA-6-16, remaining *S. culicis* isolates formed a cluster with 87% similarity (FIG. 3). In *S. simulii*, the ITS1 was more homogeneous than the ITS2, where sequence variation was detected with *Hae*III and *Dra*I, and resulted in isolates that did not group together (FIG. 3). Similarly, *S. cylindrosporium* isolates showed variability in ITS2 profiles obtained with *Dra*I, *Sau*3AI and *Rsa*I, while the ITS1 restriction profiles were identical. Though only two strains each of *Smittium orthocladii*, *S. phytotelmatum* and *S. dipterorum* were studied, high intraspecific variation was observed in one or both spacers. Consequently,

they failed to cluster co-specifically in the dendrogram (FIG. 3). In contrast, *S. coloradense* and *S. tipulidarum* isolates formed separate groups within which no variation was detected. Some species shared identical ITS-RFLP or had a high level of similarity. For example, the two isolates of *S. tronadorium* and one isolate of *S. imitatum* CHI-9-4 exhibited identical patterns, while *S. culicisoides* and *S. fecundum* shared 91% similarity. Likewise, *S. gravimetallum* KS-F1-3 and *S. megazygosporum* SC-DP-2 showed 89% similarity. The Australian *Smittium* isolate AUS-59-5S had 94% similarity with two isolates belonging to two different genera, *Simulium* *spica* and *Genistelloides hibernus* (FIG. 3). Significant variation in length and RFLP was observed between strains of *G. hibernus* included in this analysis, TN-11-1 and AL-7-10 (36% similarity). On the other hand, *Furculomyces boomerangus* isolates AUS-42-7 and AUS-77-4 and *Smittium angustum* AUS-126-30 had 100% similarity. Six unidentified *Smittium*s showed high similarity with members of the *S. commune* cluster.

**ITS sequencing.**—ITS1 and ITS2 sequences were obtained for isolates *Smittium culisetae* AUS-2-8, *S. angustum* AUS-126-30, *S. simulatum* CHI-8-4, *S. culicis* WYO-51-11, *S. commune* KS-6-6, *S. coloradense* RMBL-13-41, *S. gravimetallum* KS-F1-3, *S. caudatum* KS-1-2 and *F. boomerangus* AUS-42-7. For isolates *S. culisetae* COL-18-3 and *S. dipterorum* CR-253-14, only the ITS1 was sequenced. In general, ITS1 and ITS2 sequences were unambiguously aligned in the regions corresponding to ribosomal genes (18S, 5.8S and 28S). For ITS1, the alignment included 11 sequences with different lengths ranging from 443 bp (*S. culisetae*) to 807 bp (*S. gravimetallum*); the G+C content varied from 18% in *S. caudatum* to 39.8% in *S. dipterorum*, suggesting that base composition may not be homogeneous among taxa. For the ITS2 data set, lengths varied from 557 bp in *S. culisetae* to 1042 bp in *S. caudatum*; the G+C content ranged from 23.5% in *S. culisetae* to 42.3% in *S. simulatum*. The alignment of seven ITS2 sequences included 1093 characters. Again, ribosomal gene sequences were unequivocally aligned. However, sequences of *S. simulatum* and *S. culicis* were completely alignable, having diverged by only 1.3%. ITS1 and ITS2 sequences of *Smittium angustum* AUS-126-30 and *Furculomyces boomerangus* AUS-42-7 were identical, having a 240 bp ITS1 and a 370 bp ITS2.

Sequences were used to confirm restriction banding patterns. Also, we looked for introns that might account for the length variation by conducting a BLAST search in GenBank, but no intron sequences were found.

**PCR-RFLP from 18S rDNA.**—No evident length variation was observed in PCR-amplified products of the 18S rRNA gene. Intraspecific variability in the 18S rDNA was analyzed by RFLP of multiple isolates of *Smittium culicis*, *S. culisetae*, *S. commune* and *S. simulii*. Although no intraspecific variability was observed using *EcoRI*, *CfoI*, *DraI*, *HaeIII*, *HindIII*, *HinI* and *RsaI*, some species could not be differentiated (FIG. 4). Isolates of *S. culisetae* were clearly distinguished and formed a highly homogeneous cluster. Isolates of *S. simulii*, *S. cylindrosporum*, *S. imitatum*, *S. orthocladii* and *S. commune* were indistinguishable from each other. Another highly homogeneous group was formed by *S. culicis*, *S. fecundum*, *S. simulatum* and *S. culicisoides*. Other species like *S. tipulidarum*, *S. annulatum*, *S. dipterorum*, *S. coloradense*, *S. mucronatum*, *S. caudatum*, *S. gravimetallum*, and *S. megazygosporum* were also differentiated, suggesting that this molecule could be useful for phylogenetic studies directed at identifying species groups.

**18S rDNA sequencing.**—Thirty-two 18S rDNA sequences were obtained for 24 morphological species of *Smittium*. Only 30–40 bp from both the 5' and 3' ends were not sequenced. No intron sequences were found in the ingroup. The harpelllean 18S rDNA sequences have an average G+C content of 43.6%, ranging from 41% in *Smittium morbosum* to 45.9% in *S. annulatum*, suggesting that the base composition for this gene is rather homogeneous. Thirty-six ingroup sequences plus 4 outgroup sequences (*Dipsacomyces acuminosporus* and *Linderina pennispora* from Kickxellales, and *Entomophaga aulicae* and *Entomophthora muscae* from Entomophthorales) were aligned at 2055 positions. From these, 1305 phylogenetically uninformative characters and 365 ambiguously aligned positions were excluded from the analysis. As a result, 385 informative sites were used to infer tree topologies.

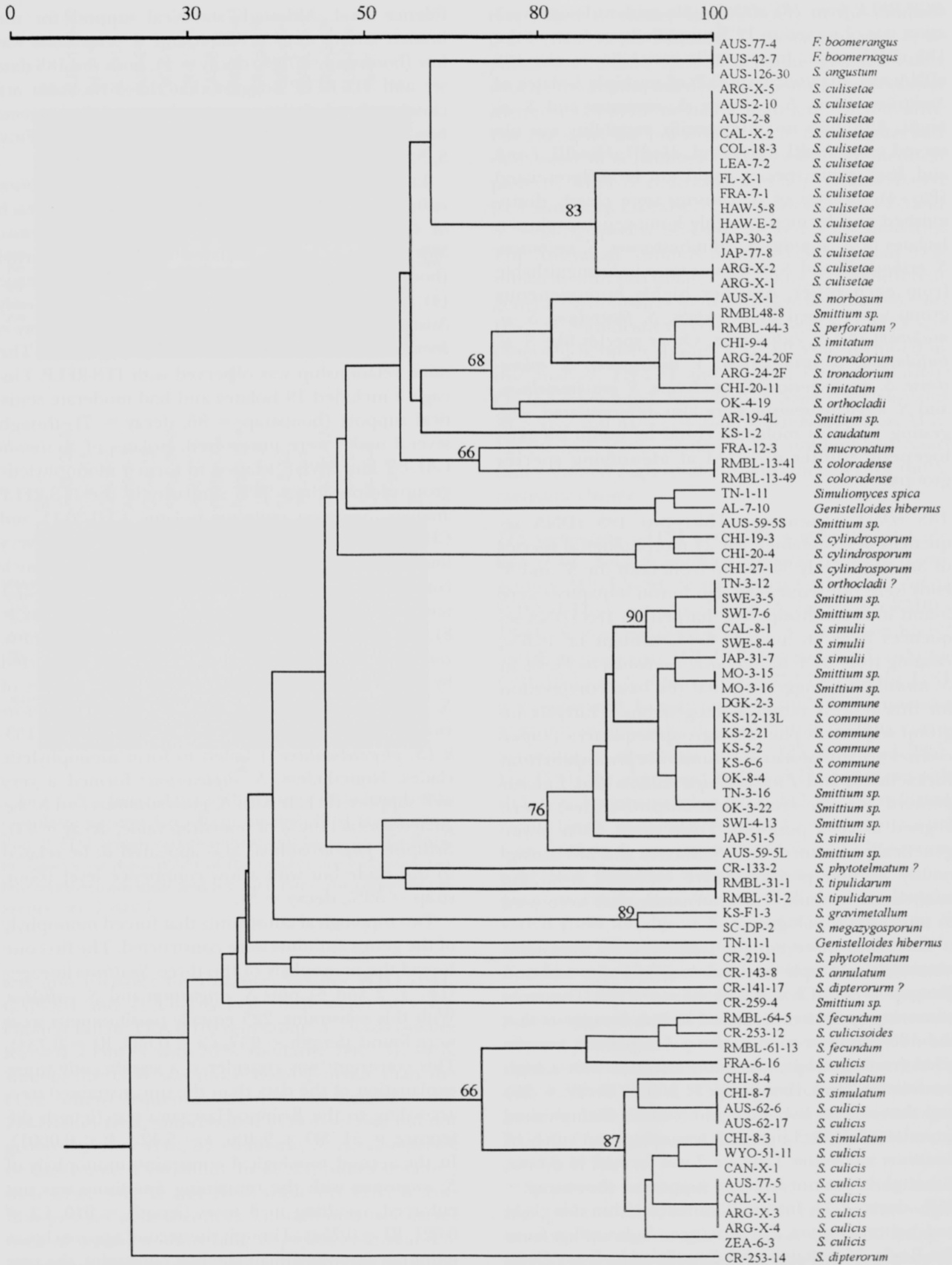
The same three equally parsimonious trees were obtained by simple and random stepwise addition (length = 886, CI = 0.638, RI = 0.771). The strict consensus gene tree depicted in FIG. 5 suggests that the 18S rDNA sequences from Harpellales are derived from a unique common ancestor with a high confidence level (bootstrap = 100%, decay = 28) and that at least five lineages could be distinguished (numbered 1–5). Lineage 1 was composed solely of *Smittium morbosum*. Lineage 2 was formed by 8 taxa, although it was not strongly supported (bootstrap = 75%, decay = 1). Internal branches within this clade had better support. For instance, *S. fecundum* from the Rocky Mountains and *S. culicisoides* from Costa Rica formed a monophyletic clade with a 96% con-

fidence level. Although statistical support for the branch connecting *S. culicis* and *S. simulatum* was low (bootstrap = 75%, decay = 1), both the 18S data set and ITS-RFLP suggest that these two taxa are closely related. *Smittium coloradense* and *S. mucronatum* were also identified as putative sister taxa (FIGS. 3, 5).

Lineage 3 was composed of two isolates of *Smittium culisetae*, AUS-2-8 and COL-18-3, and one isolate each of *Capniomyces stellatus* and *Genistelloides hibernus*. Monophyly of these isolates was well supported (bootstrap = 92%, decay = 5). The fourth lineage (4), had a 100% confidence level and comprised only Australian harpellids, two isolates of *Furculomyces boomerangus* and one of *Smittium angustum*. The same relationship was observed with ITS-RFLP. Lineage 5 included 19 isolates and had moderate statistical support (bootstrap = 86, decay = 7), though several nodes were unresolved. Isolates of *S. simulii* CAL-8-1 and SWE-8-4 failed to form a monophyletic group despite their 90% similarity in the ITS-RFLP analysis. *Smittium imitatum* isolates, CHI-20-11 and CHI-9-4, formed a monophyletic group with a very low bootstrap support (53%, decay = 1). The branch connecting *S. cylindrosporum* CHI-20-4 and CHI-27-1 was very well supported (bootstrap = 98%, decay = 8). On the other hand, a monophyletic origin of *S. commune* isolates KS-6-6 and KS-2-21 was supported by a bootstrap value of 91% (decay = 2). Isolates of *S. dipterorum* [CR-253-14 and CR-141-17 (*S. dipterorum?*)] and *S. phytotelmatum* [CR-219-1 and CR-133-2 (*S. phytotelmatum?*)] failed to form monophyletic clades. Nonetheless, *S. dipterorum?* formed a very well supported clade with *S. gravimetallum* and *S. megazygosporum* (100% of bootstrap value, decay = 14). *Smittium phytotelmatum?* also appeared to be related to this clade but with a low confidence level (bootstrap = 59%, decay = 3).

Two topological constraints that forced monophyly of the genus *Smittium* were constructed. The first one forced the monophyly of the three *Smittium* lineages (i.e., 1, 2 and 5) plus *S. angustum* and *S. culisetae*. With this constraint, 225 equally parsimonious trees were found (length = 937, CI = 0.603, RI = 0.734). This constraint was considered a significantly worse explanation of the data than the unconstrained tree, according to the Kishino-Hasegawa test (length difference = 51, SD = 9.406,  $t = 5.422$ ,  $P < 0.0001$ ). In the second topological constraint, monophyly of *S. angustum* with the remaining *Smittium*s was not enforced, resulting in 3 trees (length = 910, CI = 0.621, RI = 0.754). Though the second approach was more parsimonious than the first constraint, the tree length difference with respect to the unconstrained







tree was statistically significant (length difference = 24, SD = 7.253,  $t = 3.308$ ,  $P = 0.001$ ).

An additional, more inclusive analysis was conducted that included 13 sequences representing 6 orders of Zygomycota plus *Blastocladiella emersonii* (Chytridiomycetes) as outgroup. Ingroup sequences of *Smittium imitatum*, *S. commune*, *S. culicis*, *S. culisetae* and *S. cylindrosporum* were consensus between sequences used in the previous analysis. As a result, 36 sequences were aligned at 2081 positions of which 406 were phylogenetically informative. Twenty-four equally parsimonious trees were obtained and the strict consensus gene tree is depicted in FIG. 6 (length = 1374, CI = 0.476, RI = 0.693). Mucorales, Entomophthorales, Glomales and Harpellales formed strongly supported monophyletic clades (bootstrap = 99–100%). Members of the Kickxellales did not form a monophyletic clade. However, the placement of *Spiromyces* as sister taxa was poorly supported (bootstrap < 50%). The common origin of Harpellales and Kickxellales (excluding the *Spiromyces* clade) received a relatively low bootstrap interval (68%).

#### DISCUSSION

In fungi, the entire ITS region is often between 600 and 800 bp (Gardes and Bruns 1993). The Harpellales studied had a longer ITS than expected. Nonetheless, such lengths have been reported in other fungi. Farmer and Sylvia (1998) observed in a variety of ectomycorrhizal fungi complete ITS regions ranging 850–1240 bp and Feibelman et al (1994) found that the ITS1 within isolates of *Cantharellus* ranged 250–1100 bp. In the zygomycete *Entomophaga aulicae* (Entomophthorales), the complete ITS region comprises 1100 bp (GenBank U35394). The RFLP analysis of PCR-amplified fragments is an appropriate technique for screening for variation in a large number of isolates. Usually, very small restriction products are unresolvable in 1% agarose and will contribute to underestimation of RFLP. Therefore we used 2% agarose gels for sizing fragments. As judged from comparison with patterns deduced from sequence data, 2% agarose gels worked sufficiently well. In addition to length variation, some cases of within-isolate heterogeneity were suggested by the data. Heterogeneity among the rDNA repeats, in size or presence of target sites, can result in extra bands leading to

discrepancies between the actual size of the undigested PCR product and the sum of fragments. For instance, some copies of the ITS2 in isolates CR-259-4 (*Smittium* sp.) and CR-253-14 (*S. dipterorum*) may have lost one or more sites for *Sau3A1*. Only in one case was an extra band detected that may be due to heterogeneity among rDNA repeats, but no attempts were made to sequence it. Our results suggest that ITS length and sequence variation make it unsuitable for phylogenetic studies among species of the genus *Smittium* as presently circumscribed.

In our effort to infer the evolutionary history of Trichomycetes, we included 18S rDNA in the analysis. Results from this data set were also surprising. Placement of *Smittium culisetae* within a clade composed of representatives of other genera (*Capniomyces*, *Genistelloides* and *Furculomyces*) together with the split distribution of *Smittium* species suggests that the genus may not be monophyletic. A possible explanation of the phylogenetic divergence could be the convergence of similar phenotypes that have evolved independently under similar environmental constraints. Many species of the genus *Smittium* are distinguished by very subtle features that may not represent synapomorphies. In other cases, morphological characters define robust groups.

*Smittium morbosum*, which formed a solitary lineage, has been reported to penetrate the gut cuticle (Sweeney 1981, Sato et al 1989), causing 50–90% mortality in larvae of *Anopheles hilli* in Australia (Sweeney 1981). Sato et al (1989) in Japan and López Lastra (1990) in Argentina found that *S. morbosum* is able to infect many species of mosquitoes. Phylogenetic relationship with another invasive—but nonlethal—trichomycete, *S. perforatum* (Williams and Lichtwardt 1987), remains to be investigated. Isolate RMBL-44-3 was tentatively identified as *S. perforatum*, but after re-examination of morphological features, its taxonomic status is uncertain. However, RMBL-44-3 and a unidentified isolate RMBL-48-8 (*Smittium* sp.) appeared to be closely related. They showed some similarity with the Chilean species, *S. imitatum*, although there are no reports of *S. imitatum* from the USA. Lichtwardt and Arenas (1996) discussed the morphological similarity between *S. simulatum* and *S. culicis*, and they noted that they cannot be readily distinguished in culture using trichospore shape and size alone. Two taxonomically important characters, the holdfast and the branching pattern of the thalli,

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FIG. 3. UPGMA cluster analysis of 82 harpellean isolates using data from ITS-RFLP. Similarity was estimated with Simple Matching Coefficient. Isolates and their taxonomic status are indicated. Numbers above branches indicate shared percentage of similarity. Scale represents percentage of similarity.

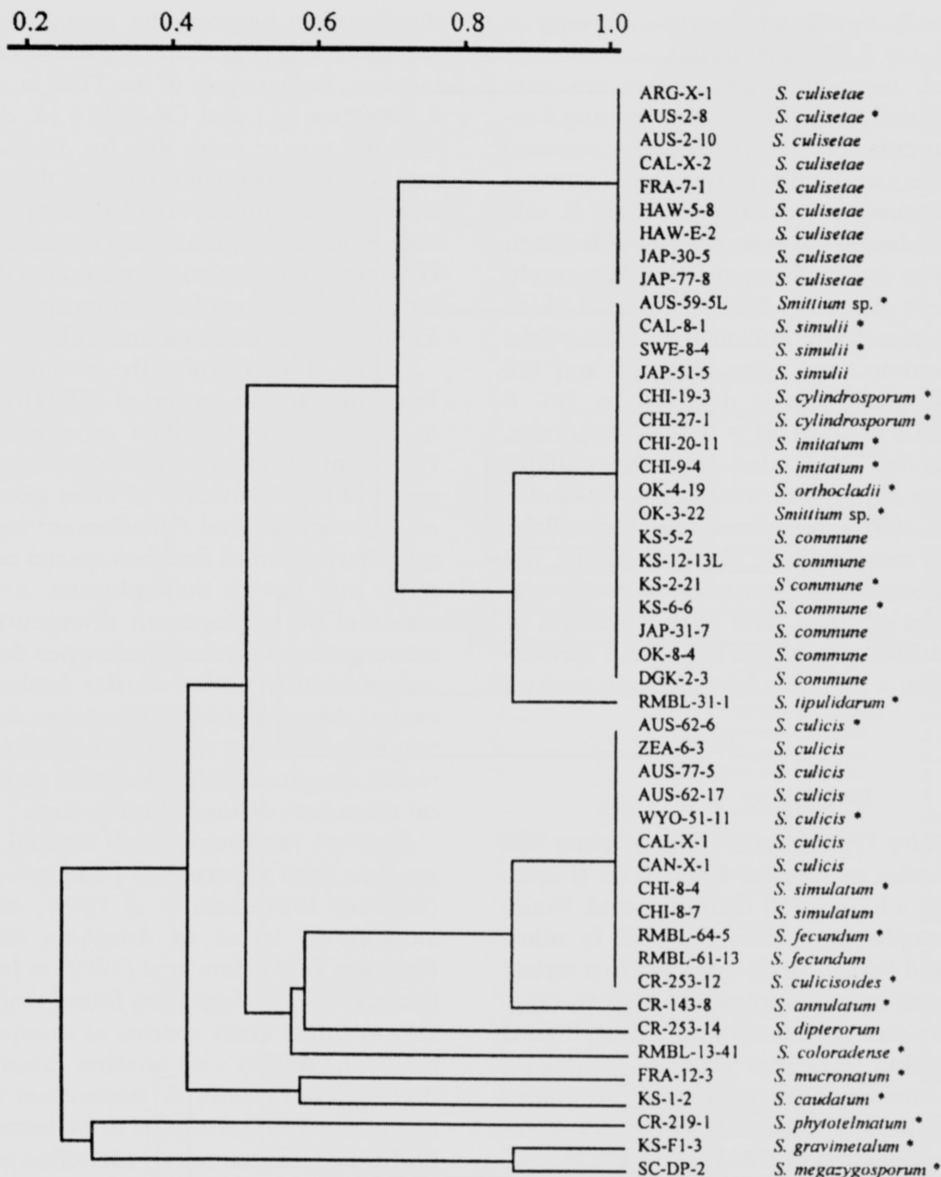


FIG. 4. Dendrogram derived from 18S-RFLP. Scale represents percentage of similarity. Isolate numbers and species names are indicated. Asterisks indicate isolates used for sequencing.

are used to distinguish them. Isozymically, these two taxa are very distinct (Grigg and Lichtwardt 1996), but our 18S rRNA gene phylogeny indicated that they are closely related. As indicated by its name, *Smittium culicisoides* is morphologically similar to *S. culicis*; trichospores of the two species vary slightly in average length to width ratio, and branching in thalli of *S. culicis* is more divergent than in *S. culicisoides*. The distinction of these two species was supported by isozyme patterns (Lichtwardt 1997). In the gene tree presented here, *S. culicisoides* appears as a sister taxon of *S. fecundum*. As a distinctive feature, *S. fecundum* produces a prolific series of short generative cells on fertile branches of mature thalli (Lichtwardt

and Williams 1999). There is some conflict between results from the isozyme analysis, ITS-RFLP and the 18S rDNA sequence analysis for *S. annulatum* CR-143-8, *S. coloradense* RMBL-13-41 and *S. caudatum* KS-1-2, such that their phylogenetic relationships are still unclear. The most recognizable feature of *S. mucronatum* is the very small nipple at the tip of the trichospore (Lichtwardt and Williams 1999). According to Moss and Young (1978), *S. mucronatum* and *S. culicis* may have lost the septum delimiting the generative cell. It would be very interesting to investigate if this constitutes a common feature among the members of that clade. *Smittium culisetae* is a very well characterized taxon for which highly homogeneous

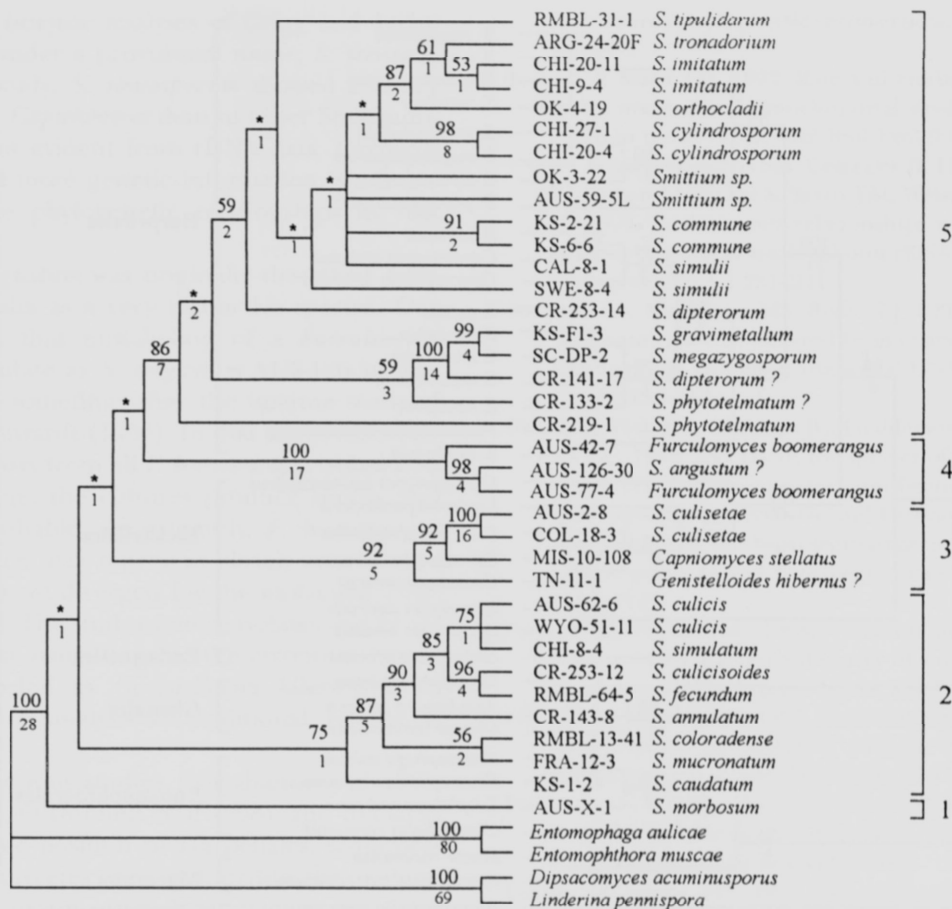


FIG. 5. Strict consensus of 3 equally parsimonious trees (886 steps, CI = 0.638, RI = 0.771) inferred from 18S rDNA data set. Numbers above branches represent bootstrap values (above 50% from 500 replicates), while those below represent decay indices. Asterisks (\*) indicate branches that collapsed in the bootstrap tree. Five lineages (numbered 1-5) are indicated next to isolate numbers and species names.

isozymic patterns (Grigg and Lichtwardt 1996) and immunological data (Peterson and Lichtwardt 1987) were obtained. In the present study, 14 isolates from various geographic origins were studied. With the exception of isolates ARG-X-1 and ARG-X-2, the remaining isolates showed high homogeneity. Surprisingly, this taxon appears not to be monophyletic with other *Smittium* species studied based on the 18S rDNA phylogeny. Horn (1989b) observed ultrastructural differences between *S. culisetae* and *S. culicis* associated with *in vitro* sporangiospore extrusion from trichospores. In *S. culisetae*, trichospores have pits in the sporangiospore wall and associated mucilage in the interwall layer only at the apical end. In contrast, *S. culicis* possesses pits and mucilage at both ends of its trichospores, and sporangiospores can be extruded from either end of the trichospore. Whether or not these traits are common within a lineage remains to be studied.

*Smittium gravimetallum* nom. prov. is attached to

the hindgut cuticle of its host by a bulbous basal cell and produces trichospores with short collars (1-2  $\mu$ m). In contrast, the original description of *S. megazygosporum* from France has no bulbous base, and trichospore collars are 3.5-4  $\mu$ m long. Trichospores of both species overlap in size. Those of *S. megazygosporum* SC-DP-2 from South Carolina, USA, are variable in size, and thalli in culture do not produce basal cells nor is the structure of the basal cell in the host known. 18S rDNA gene data suggest that *S. gravimetallum* and *S. megazygosporum* may be conspecific, but more isolates should be collected and studied before considering them to be synonymous.

Morphological characters were re-examined when molecular data showed a high intraspecific variation. Both isolates of *Smittium dipterorum* were found in simuliid guts from Costa Rica (Lichtwardt 1997). Of the two, CR-253-14 should be considered as representative of the taxon, while the taxonomic status of the other (CR-141-17) is now uncertain. *Smittium phyto-*

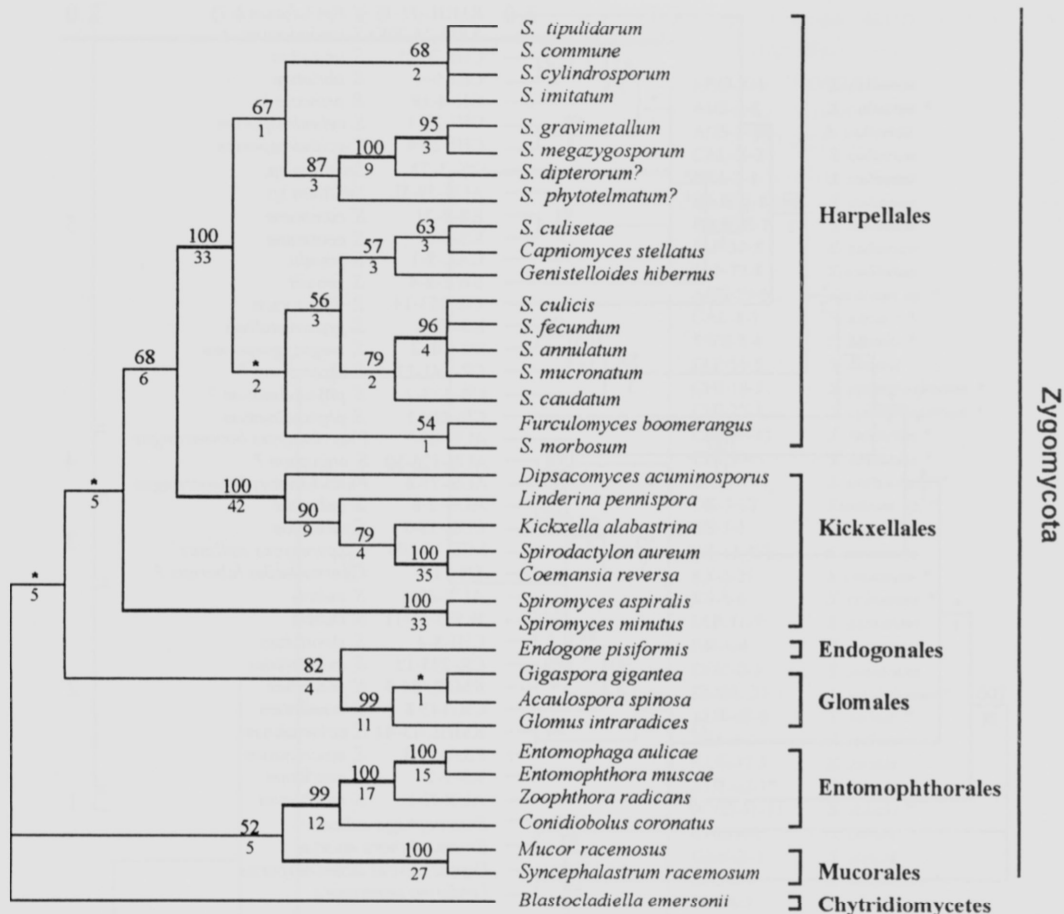


FIG. 6. Strict consensus of 24 equally parsimonious trees (1374 steps, CI = 0.476, RI = 0.693) inferred from 18S rDNA data set. Numbers above the branches represent bootstrap values (above 50% from 500 replicates), while those below represent decay indices. Asterisks (\*) indicate branches that collapsed in the bootstrap tree.

*telmatum* isolates were obtained from bloodworms (*Chironomus* sp.) living in bromeliad leaf bases in Costa Rica (Lichtwardt 1994b). Even though CR-133-2 was collected from the same bromeliad as the holotype, its branching pattern is somewhat different and the spores are longer and have a long collar. Morphological features of isolate CR-219-1 agree with the type description of *S. phytotelmatum* and is confirmed to be that species (Lichtwardt 1994b).

Trichospores of *Smittium commune* are distinct from other described species. Although *S. morbosum* has similar spore dimensions, they lack the characteristic median bulge. Also, *S. tipulidarum* has similar dimensions but the branching pattern of thalli is different. Isozymically, *S. commune* isolates formed two groupings interspersed with other taxa (Grigg and Lichtwardt 1996), but our molecular data indicate that this group is very homogeneous. *Smittium cylindrosporium* and *S. imitatum* were originally described from Chile (Lichtwardt and Arenas 1996). Based on molecular data, phylogenetic relationships were not

very well supported. A similar situation was observed for *S. imitatum* and *S. tronadorium*, both from southern South America and very similar morphologically (Lichtwardt et al 1999). Further studies using other genes might indicate whether or not they are conspecific. Isolates of *Smittium orthocladii* showed variation for molecular characters. Of the two isolates, only OK-4-19 has dimorphic trichospores, which is the distinctive feature of the taxon. Our data indicate that cultures of *Smittium simulii* may not constitute a monophyletic lineage, though more isolates should be studied.

Australian isolates AUS-59-5S and AUS-59-5L were previously considered as *S. elongatum* (Lichtwardt and Williams 1990), but this identification was only tentative. The two isolates used were obtained from a mixed culture in which two morphotypes were distinguishable. Based on ITS-RFLP, AUS-59-5S showed more similarity to a *Genistelloides* than to other *Smittium*s, and AUS-59-5L was more related to *S. commune* and *S. simulii*. Likewise, isolate OK-3-22 was in-

cluded in the isozyme analyses of Grigg and Lichtwardt (1996) under a provisional name, *S. stenosporum*. In that study, *S. stenosporum* showed a closer relationship to *Capniomyces* than to other Smittiums, but this was not evident from rDNA data. Certainly the addition of more genetic information is needed to fully resolve phylogenetic relationships in that clade.

*Smittium angustum* was originally described from Western Australia as a very distinctive species. Our results suggest that mislabeling of a *Furculomyces boomerangus* isolate as *S. angustum* AUS-126-30 may have occurred sometime after the isozyme study of Grigg and Lichtwardt (1996). In that study, AUS-126-30 clustered apart from all *F. boomerangus* isolates. At the present time, the cultures produce spores that are indistinguishable. Alternatively, *F. boomerangus* and *S. angustum* may represent closely related taxa which have not yet diverged for the molecular characters surveyed. The study of more isolates and genes might clarify the issue. Because the taxonomic status of isolates labeled as *Genistelloides hibernus* is in doubt, sequence analysis of additional isolates is needed.

Based on previous studies (Nagahama et al 1995, Sugiyama 1998, O'Donnell et al 1998), and to clarify the phylogenetic position of Harpellales, sequences of several zygomycetes were included in the analysis. The kickxellid origin of Trichomycetes as proposed by Moss and Young (1978) was not strongly supported. As suggested by O'Donnell et al (1998), a polytomous radiation from a common ancestor appears to be the most parsimonious explanation.

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