A geographically diverse set of isolates indicates two phylogenetic lineages within *Stachybotrys chartarum*

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Abstract: *Stachybotrys chartarum* is a black mitosporic fungus capable of dense colonization of cellulose-based building materials such as drywall. The presence of *S. chartarum* in indoor environments has been reported as linked to a variety of alleged environment-related illnesses including infant acute idiopathic pulmonary hemorrhage, although there continues to be insufficient (especially exposure) data to support such associations. We investigated genetic variation among 52 morphologically and geographically diverse, indoor and outdoor isolates of *S. chartarum* sensu lato using molecular markers based on β-tubulin, calmodulin, elongation factor-1 alpha, and trichodiene synthase genes, as well as the internal transcribed spacer region of nuclear ribosomal DNA. Gene genealogies proved concordant in dividing all isolates into two strongly supported clades. The majority of the variable sites separating these lineages were fixed within each of these clades, and there was no evidence of recombination between genotypes. The results of this study therefore provide strong support for the recognition of two phylogenetic species within *S. chartarum* sensu lato, and further demonstrate the limitations of morphological characters in delineating monophyletic taxa among morphologically simple fungi.

Key words: Stachybotrys atra, cryptic speciation, calmodulin, elongation factor-1 alpha, trichodiene synthase.

Résumé : Le *Stachybotris chartarum* est un champignon mitosporal noir, capable de coloniser densément les matériaux des édifices à base de cellulose, tel que les cloisons sèches. On rapporte fréquemment que, dans les environnements intérieurs, la présence du *S. chartarum* serait possiblement impliqué dans une variété de maladies reliées à l'environnement, incluant l'hémorragie pulmonaire idiopathique aiguë, bien que les données supportant ces associations demeurent insuffisantes (surtout l'exposition). À l'aide de marqueurs moléculaires basés sur la β-tubuline et la calmoduline, sur les gènes du facteur d'élongation 1-alpha et de la trichodiène synthase, ainsi que la région ITS de l'ADN ribosomal transcrit, les auteurs ont examiné la variation génétique au sein de cinquante deux isolats du *S. chartarum* sensu lato géographiquement et morphologiquement différents, et provenant de l'intérieur et de l'extérieur. Les généalogies des gènes concordent quant à la division de l'ensemble des isolats en deux clades fortement supportés. La majorité des sites variables permettant de séparer ces lignées sont fixes dans chacun de ces clades, et il n'y a pas de preuve de recombinaison entre ces génotypes. Par conséquent, les résultats de cette étude fournissent un solide support permettant de reconnaître deux espèces phylogénétiques à l'intérieur du *S. chartarum* sensu lato et démontrent une fois de plus les limitations des caractères morphologiques pour délimiter les taxons monophylétiques parmi des champignons à morphologie simple.

Mots clés : Stachybotris atra, spéciation cryptique, calmoduline, facteur d'élongation 1-alpha, trichodiène synthase.

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Introduction

The black mould *Stachybotrys chartarum* has recently gained notoriety as a potential etiologic agent of building-related illness. *Stachybotrys chartarum* (Ehrenb. ex Link) Hughes is a saprotrophic mitosporic fungus of purportedly worldwide distribution (Samson et al. 1996), tentatively allied with the ascomycete order Hypocreales (Rossman et al. 1999). A sexual state of *S. chartarum* is not known. To date, a diverse assortment of environment-related illnesses including chronic fatigue, allergy, impaired lung function, as well as central nervous system disorders have been ascribed to putative environmental exposures to this fungus, resulting from contaminated indoor air and construction materials (Croft et al. 1986; Johanning et al. 1996; Etzel et al. 1998; Hodgson et al. 1998). Historically, however, *S. chartarum* has been known for both its ability to degrade natural and manufactured cellulose-based products (Sui 1951; Jermyn 1965) and act as agents of mycotoxic syndromes (e.g., stachybotryotoxicosis) in livestock (Forgacs 1972; Ueno 1983).

Much of the current interest in *Stachybotrys* is the result of the work of Etzel et al. (1998), which linked a cluster of

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10 infants with acute idiopathic pulmonary hemorrhage to the presence of indoor moulds including *S. chartarum* (reported as *S. atra*). However, reviews conducted by the Centers for Disease Control and Prevention concluded that, while the study's findings merited further investigation, the epidemiological evidence did not sufficiently support an association between exposure to *S. chartarum* and acute idiopathic pulmonary hemorrhage (Centers for Disease Control and Prevention 1999*a*, 1999*b*, 2000). Work in this area however continues to be hampered by the lack of clinically meaningful, standardized methods of assessing exposure to either the fungus or to specific mycotoxins (Centers for Disease Control and Prevention 1999*b*; Burge 2001; Page and Trout 2001).

Despite current interest in applied fields such as environmental hygiene, medicine, and toxicology, the evolutionary relationships of *S. chartarum* and closely related taxa remain inadequately explored. Past and current taxonomic treatments of the genus have been based on a limited number of morphological characters (Bisby 1943; Jong and Davis 1976). Recent work by Anderson et al. (2002) however demonstrated variable toxin production among isolates identified as *S. chartarum* (sensu Jong and Davis 1976), while molecular characterization of *S. chartarum* isolates suggested that this morphotype may harbor at least two distinct genetic lineages (Haugland and Heckman 1998; Cruse et al. 2002). These findings are consistent with accumulating evidence demonstrating the inability of morphological markers to delimit species-level taxa among many groups of mitosporic fungi and other morphologically simple organisms (see Koufopanou et al. 1997; Geiser et al. 1998; Jousson et al. 2000; O'Donnell et al. 2000; Shaw 2000; Hoare et al. 2001). As noted by Anderson et al. (2002), the ability to identify monophyletic species is a crucial first step in applied investigations of substrate susceptibility and mycotoxin biosynthesis. More fundamentally however, limited understanding of the evolutionary relationships within this group of fungi represents a significant impediment to research addressing basic biological questions such as those relating to habitat, substrate utilization, reproductive biology, as well as population structure and distribution.

As part of an ongoing study of fungi from indoor environments we examined several thousand samples of contaminated building materials from various regions across Southern Ontario, Canada. Direct microscopic examination of adhesive-tape lifts derived from wallboard proved members of the genus *Stachybotrys* (consistent with *S. chartarum* sensu Jong and Davis (1976)) to be among the most dominant fungi on this substratum. Our examinations of cultures derived from these materials as well as from air samples showed these isolates to be highly variable both with respect to microscopic and colony morphology. The objectives of this study were the following: (*i*) to develop and evaluate a set of molecular genetic markers for use in systematic studies of *S. chartarum* sensu lato; (*ii*) to investigate the genetic variation encompassed by a morphologically diverse set of isolates identified as *S. chartarum* (s. str. Jong and Davis 1976); and (*iii*) to compare the genotypic diversity of Ontario representatives to that found across a geographically diverse set of both indoor and outdoor isolates.

Materials and methods

Source and culturing of *Stachybotrys* isolates

To obtain isolates, conidial aggregates were looped from the surface of contaminated materials and streaked on Rose Bengal agar (Malloch 1981) amended with chloramphenicol at 100 mg/L. Single representative colonies were selected and transferred to Modified Leonian's agar (Malloch 1981) and were identified based on descriptions by Jong and Davis (1976). Thirty-six isolates, identified as *Stachybotrys chartarum*, were chosen to represent the range of both morphological variation and substrate origin, across our larger set of 150 Ontario isolates (Table 1). We also obtained 16 isolates representing novel geographical regions and habitats, and an isolate of *S. kampalensis* (UAMH 7746), which was used as the outgroup.

DNA extraction and PCR conditions

Conidia from Modified Leonian's agar plates were inoculated in Modified Leonian's broth and incubated at room temperature with vigorous agitation. Undifferentiated mycelium was harvested after 3 days and stored at –20°C. DNA was extracted from mycelia using a small-scale method described by Scott et al. (2000) using diatomaceous earth (Dicalite 1400, Grefco Inc., Torrance, Calif.) as a grinding medium. We employed five DNA-based primer sets in total, based on the protein-encoding genes beta-tubulin (*benA*), calmodulin (*cmd*), elongation factor-1 alpha (*tef1*), and trichodiene synthase (*tri5*) as well as the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA). The 10 primer sequences are shown in Table 2. Oligonucleotide primers for loci *cmdS* and *tef1S* were designed from conserved protein-encoding regions of aligned sequences of calmodulin and elongation factor-1 alpha genes available in GenBank (GenBank U14100, Z23012 and AF072882, J05545, D44468, U15993, L02964, AF089808, respectively). Primers for the *tri5S* locus were designed from *S. chartarum* trichodiene synthase gene sequence (GenBank AF053926). Target loci were amplified in a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, Mass.). Each 50-μL amplification reaction consisted of 1.5 units of *Taq* DNA polymerase (Roche Molecular Biochemicals, Laval, Que.), 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.2 mM of each primer, and approximately 50 ng high molecular weight template DNA. PCR consisted of 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at annealing temperature (5°C below the theoretical T_M (Innis and Gelfand 1990)), and 1–1.5 min at 72°C with a final extension of 3–4 min. A template-free control was included with each set of reactions. Product size and yield was roughly quantified employing lambda phage DNA co-digested with *Hin*dIII and *Eco*RI.

Screening for sequence variability

We used a heteroduplex mobility assay (HMA) (Delwart et al. 1993) with the overlapping pairs comparison method described by Scott et al. (2000). Products of the *cmdS* locus were also heteroduplexed against isolate J1 (as a standard) to confirm small shifts in mobility seen among overlapping pairs. Heteroduplexed products were separated by poly-

Table 1. Isolates, source, and genotype.

Isolate	Source	Habitat	Location	Genotype‡				
				Bt2	*cmdS*	*tef*S	*tri5*S	ITS
J1*	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B2	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B3	Wall paper	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B4	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B5	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B6*	Air sample	Indoor	Ont., Canada	1_1	1_1	1_1	*1*	*1*
B7	Air sample	Indoor	Ont., Canada	*2*	*2*	*2*	*2*	*1*
B8	Air sample	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B9*	Drywall	Indoor	Ont., Canada	*2*	2_1	*2*	*2*	1_1
B10	Insulation	Indoor	Ont., Canada	*2*	*2*	*2*	*2*	*1*
B11	Wood	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B12	Cork board	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
D13*	Leaf	Outdoor	Belgium	*2*	2_2	*2*	*2*	1_1
B14*	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	1_1	*1*
B15	Air sample	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B16	Ceiling tile	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B17	Sheetrock	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B18*	Ceiling tile	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B19	Ceiling tile	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B20	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B21	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B22	Drywall	Indoor	Ont., Canada	*2*	*2*	*2*	*2*	*1*
B23	Wood	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B24	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B25	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B26	Filter	Indoor	Ont., Canada	*2*	*2*	*2*	*2*	*1*
B27*	Air sample	Indoor	Ont., Canada	*2*	*2*	*2*	*2*	1_1
B28	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B29	Paper	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B30	Sheetrock	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B31	Drywall	Indoor	Ont., Canada	*2*	*2*	*2*	*2*	*1*
B32	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B33	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B34	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B35	Ceiling tile	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B36	Drywall	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
B37	Air sample	Indoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
ATCC 9182*	na	na	Wash. D.C. U.S.A.	*2*	2_2	*2*	*2*	1_1
ATCC 16026*	na	na	U.K.	*1*	*1*	1_1	*1*	*1*
ATCC 26303*	Cardboard	na	Finland	*1*	1_2	1_1	*1*	*1*
ATCC 48994	Wheat straw	na	Egypt	*2*	*2*	*2*	*2*	*1*
CBS 177.42	Soil	Outdoor	France	*1*	*1*	*1*	*1*	*1*
CBS 329.37	Paper	na	Italy	*2*	*2*	*2*	*2*	*1*
CBS 330.37	Paper	na	Italy	*2*	*2*	*2*	*2*	*1*
CBS 492.96	Leaf litter	Outdoor	Cuba	*1*	*1*	*1*	*1*	*1*
CBS 109285*	na	na	Denmark	2_1	2_3	*2*	*2*	1_1
CCF 1094	Air sample	Indoor	Czech Republic	*2*	*2*	*2*	*2*	*1*
JS 135	Dung	Outdoor	Ont., Canada	*1*	*1*	*1*	*1*	*1*
UAMH 3228*	na	Outdoor	Calif., U.S.A.	2_1	2_1	*2*	2_1	1_1
UAMH 6715*	Air sample	Indoor	Sask., Canada	*1*	1_2	1_1	*1*	*1*
UAMH 7568	Cardboard	indoor	B.C., Canada	*1*	*1*	*1*	*1*	*1*
UAMH 7720	Air sample†	Indoor†	Alta., Canada	*2*	*2*	*2*	*2*	*1*

Note: na, information not available or ambiguous; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; CCF, The Culture Collection of Fungi; JS, James Gloer Collection (housed at the University of Toronto, Botany Department); UAMH, University of Alberta Microfungus Collection and Herbarium.

*Proxy isolates.

†Sampled from *Apis mellifera* (honeybee) overwintering facility.

‡Major allele types are designated *1* and *2*. Subscripts indicate minor allelic variation (1–3 bp) as evidenced by sequence data.

Table 2. Primer sequence and source.

Target gene	Locus	Primer	Sequence (5′ → 3′)	Source
Beta-tubulin (*benA*)*	Bt2	Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC	Glass and Donaldson 1995
		Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	
Calmodulin (*cmd*)†	*cmdS*	cmdS2	TCC/T GAA/G TA/TC AAG GAG GCC TTC TC	This study
		cmdS5	TCC/T TCA/C/G GAR TCG GTA/G TCC TTC AT	
Translation elongation factor-1 alpha (*tef1*)‡	*tef1S*	tefS1	GTA AGG ARG ACA AGA CTC AC	This study
		tefS6	GAR GTA CCA GTG ATC ATG TTC	
Trichodiene synthase (*tri5*)§	*tri5S*	tri5S1	CCT CAC CCT CAG ATG TTG ACA TAC	This study
		tri5S2	TCC TTG TAG AAG GAC ATG AGG TCG	
Ribosomal DNA (rDNA)	ITS	ITS5	GGA AGT AAA AGT CTT AAC AAG G	White et al. 1990
		ITS4	TCC TCC GCT TAT TGA TAT GC	

*After Orbach et al. 1986.
†After Yasui et al. 1995.
‡After Ichi-ishi and Inoue 1995.
§After Trapp et al. 1998.

acrylamide gel electrophoresis using a variation of the gel formula described by Xing et al. (1996). Gels consisting of 12% acrylamide, 0.15% bis-acrylamide, 10% glycerol, and 0.5% agarose in distilled deionized water were run on a vertical electrophoresis apparatus (Protean II, BioRad, La Jolla, Calif.) at 10 V/cm, 8°C for approximately 24 h. Following electrophoresis, gels were stained with 1 μg/mL ethidium bromide in dH_2O and visualized under ultraviolet light. Isolates with like alleles were assigned to HMA groups, and proxy isolates were chosen from within these groups for sequencing. All proxy target loci were re-amplified and purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, Calif.). Forward and reverse sequences were obtained from selected proxy isolates for all loci using an ABI prism automated sequencer (model 377) at the York University, Core Molecular Biology Facility, Toronto, Ont., Canada. Products were amplified and sequenced as above for the outgroup *S. kampalensis* (UAMH 7746) for all loci except *tri5*.

Sequence analysis

Sequences were aligned using Clustal X Multiple Sequence Alignment Program version 1.81 (Higgins and Sharp 1988; Jeanmougin et al. 1998). All phylogenetic analyses were conducted using PAUP* version 4.0b8. (Swofford 1998). Phylogenetic trees were generated from noncoding (intron regions) plus ITS or coding (exon) regions plus ITS sequence data for nonrooted and rooted trees, respectively, with gaps coded as missing. Maximum parsimony (MP) trees were generated using the branch-and-bound algorithm. Maximum likelihood (ML) trees were generated using the heuristic search algorithm with 10 replicates and random sequence addition under the Hasegawa–Kishino–Yano (Hasegawa et al. 1985) distance model. Congruence between loci was assessed for the combined tree using the parsimony method of Koufopanou et al. (1997). Support for MP trees was assessed using bootstrap analysis (Felsenstein 1985) of 1000 resampled replicate data sets. Graphical representations of trees were generated using TreeView version 1.5 (Page 1996).

Sequence data for the proxy isolates as well as the outgroup were deposited in GenBank under accession numbers; Bt2 AY095953–AY095959, AY180246–AY180252; cmdS AY095960–AY095966, AY180253–AY180259; *tef1S* AY095967–AY095973, AY180264–AY180270, *tri5S* AY095981–AY095987, AY180271–AY180276; ITS AY095974–AY095980, AY180260–AY180263, AY185565–AY185567. Alignment matrices for individual loci are available from TreeBASE under accession numbers S916, M1516, and M1517. Cultures of proxy isolates are available from University of Alberta Microfungus Collection and Herbarium (UAMH) under accession numbers UAMH 10150–10156.

Results

Genetic screening using heteroduplex mobility assays

HMA analysis of *S. chartarum* revealed two alleles at the Bt2, *tef1S*, and *tri5S* loci, consistently partitioning our 52 isolates into two groups. Isolates J1, B6, B14, B18, ATCC 16026 and B9, D13, B27, ATCC 9182 were chosen as proxy isolates, representing each of these two HMA groups, respectively. HMA analysis of the *cmdS* locus revealed five alleles. Isolates ATCC 26303 and UAMH 6715, CBS 109285, and UAMH 3228 served as additional proxy isolates representing the three additional HMA groups. HMA analysis of amplification products for ITS revealed only a single allele.

Summary of sequence alignments

Sequencing of PCR products for proxy isolates resulted in five alignments of 13 sequences each. Out of a total of 1042 noncoding sites (combined intron regions and ITS), 82 were variable among proxy isolates. Sixty-nine of these sites were fixed within each of two groups as defined by HMA analysis (see above). Of the 13 polymorphic sites, seven represented singleton sites (i.e., autapomorphies in parsimony analyses); three occurring in isolate B6; two in isolate CBS 109285; and one each in isolates B14 and UAMH 3228. There were no shared polymorphisms between the two groups.

The combined alignment, which included the *S. kampalensis* outgroup, had a total 665 sites (combined exon regions of Bt2, *cmdS* and *tef1S*) and an additional 347 from ITS1 and ITS2. Amplification products of the Bt2, *cmds* and *tef1s* and ITS loci for *S. kampalensis* were comparable to those for *S. chartarum* isolates with the exception that the former species possessed an additional 100 base pair (bp)

intron within the *tef1S* locus located within exon no. 2 (compared with *Trichoderma reesei*, GenBank Z23012). Repeated attempts to obtain amplification product from *S. kampalensis* using *tri5S* primers (Table 2), as well as other primer sets (not shown), failed. Of the 90 variable coding sites in the combined alignment, 82 were singleton sites. Eighty-one of these singleton sites occurred within the outgroup. Alignments of intron regions of individual loci that included the outgroup *S. kampalensis* (UAMH 7746) were ambiguous, as they contained multiple insertions or deletions (indels) and were highly variable. Further summary of the aligned sequence data is presented in Table 3.

Phylogenetic analysis

Unrooted trees

Phylogenetic analysis of noncoding sequence alignment data for the 13 proxy isolates produced a single unrooted ML tree for each of the five loci (trees not shown). As expected from inspection of alignment data, all gene trees possessed a single long internal branch separating isolates J1, B6, B14, B18, ATCC 16026, ATCC 26303, and UAMH 6714 from isolates B9, D13, B27, ATCC 9182, CBS 109285, and UAMH 3228. Comparison of the topologies of ML trees across the five loci showed them to differ only with respect to the extent of exterior branching as dictated by the presence of uniquely derived character states (singleton sites) occurring in 0–2 isolates per locus. All ML trees were topologically consistent with those produced by MP analysis. Bootstrap analyses of Bt2, *cmdS*, *tef1S*, and *tri5S* MP trees subsequently resulted in very strong support (98–100%) for the two clades. These clades were not supported by bootstrap analysis of the ITS MP tree. MP analyses revealed no evidence for homoplasy within individual loci (i.e., all gene trees were of minimum length (see Table 3), consistency index (CI) = 1).

Analysis of the combined data set resulted in one unrooted ML tree with two very divergent clades (Fig. 1). Parsimony analysis resulted in three MP trees (each with CI = 0.988) that each confirmed the topology of the ML tree with respect to the major partition. MP trees differed with each other only with respect to the degree and pattern of branching within clade 2. Bootstrap analysis of the MP tree resulted in 100% support for the two major clades. MP trees generated from the combined data set were one step longer than the additive lengths of the five MP trees derived from individual loci. Both visual inspection of the alignments and a comparison of the resulting gene trees confirmed incongruency between the Bt2 and *cmdS* loci with respect to the relative placement of isolate UAMH 3228 within clade 2. As this did not affect the major partition, sequence data from both Bt2 and *cmdS* were maintained in the combined tree.

Rooted trees

Analysis of sequence data derived from amplified coding regions of Bt2, *cmdS*, *tef1S*, as well as ITS resulted in a single rooted ML tree for each of these four loci. The single most likely tree produced from analysis of the combined data set is presented in Fig. 2. The combined tree again partitioned isolates J1, B6, B14, B18, ATCC 16026, ATCC 26303, and UAMH from isolates B9, D13, B27, ATCC 9182, CBS 109285, and UAMH 3228 (i.e., into clades 1 and 2 as defined above) with a very long branch separating *S. chartarum* sensu lato isolates from the outgroup *S. kampalensis*. MP analysis of the combined data set confirmed the topology of the ML tree. Bootstrap analysis of the single most parsimonious tree (CI = 1) resulted in strong support for nodes defining clades 1 and 2 (98% and 94%, respectively). Comparison of MP gene trees with the combined tree revealed no evidence for homoplasy (i.e., the total combined branch length of the trees generated from the combined data were equal to the sum of the lengths of all gene trees). The lack of novel homoplasy in the combined tree confirmed that the contributing data sets were highly compatible and should be combined (Koufopanou et al. 1997).

Discussion

Summary of phylogenetic analyses

Five loci, four representing protein-encoding genes in addition to the ITS region of rDNA, were used to investigate genetic variation among a diverse set of 52 isolates identified as *Stachybotrys chartarum* sensu lato. Combined analyses of sequence data divided all isolates into two strongly supported clades. Concordance of gene trees with respect to this partition was confirmed through comparative parsimony analysis of individual, relative to the combined, data sets. Under the principle of genealogical concordance (see Avise and Ball 1990), the results of our phylogenetic analyses strongly support the recognition of two distinct evolutionary lineages among our set of isolates. Further, the two major genotypes were found to be persistent over a broad geographical area and were recovered from various indoor and outdoor niches, with no evidence of recombination between them. Given the diversity of our sample set it is highly improbable that we have repeatedly sampled two groups (of a single species) that by chance do not share a single polymorphism out of a total of 82 variable sites. As such, our results concur with those of Cruse et al. (2002) insofar as recognizing two species-level taxa within the current broad concept of *S. chartarum*.

Our macroscopic and microscopic observations of these fungi suggest that these two lineages may be morphologically differentiable. Examination of colonies growing on Modified Leonian's agar (data not included) showed that clade 1 isolates were relatively faster growing and generally produced more conidia and less extracellular, media-soluble pigments than did clade 2 isolates. Microscopically, clade 1 isolates had rough, darkly pigmented conidia that were ellipsoidal in shape and had broadly rounded apices. In contrast, the relatively lightly pigmented conidia of clade 2 isolates were ellipsoidal to broadly obovoid and acute at one or both apices (Fig. 3). These characters, however, were highly variable within and between isolates and depended on both the age of the culture as well as the number of times an isolate had been subcultured.

As noted by Cruse et al. (2002), despite being very similar morphologically, the degree of evolutionary divergence between these two species is consistent with a long period of genetic isolation. Evidence however suggests that the two species are likely more closely related to each other than ei-

Table 3. Summary of sequence alignments for individual loci.

Locus	Length of alignment (bp)*	Introns (or ITS)				Exons			TI/TV
		No. of sites†	No. of variable sites‡	No. of MP info sites§	No. of indels	No. of sites†	No. of variable sites‡	No. of MP info. sites§	
Characterization of alignments without outgroup									
Bt2	294	60	7	5	0	—	3	—	6.29
cmdS	461	293	31	28	1	—	2	—	2.69
tef1S	542	278	30	30	1	—	9	—	1.59
tri5S	409	75	13	11	1	—	21	—	0.89
ITS	546	336	1	1	0	—	0	—	5.92e+36¶
Characterization of alignments including *S. kampalensis* outgroup									
Bt2	295	—	(~59%)	—	2	234	21	3	4.60
cmdS	472	—	(~40%)	—	11	168	15	1	6.90
tef1S	643	—	(~45%)	—	13	263	10	3	9.19
ITS	556	347	44	1	10	—	—	—	1.07

Note: Coding and noncoding regions were submitted to separate phylogenetic analyses (see Methods). Dashes (—) indicate that these regions (combined intron or conversely combined exon sites) were not included in the corresponding analyses. The number of variable sites in these regions are included for comparative purposes. Approximate values indicate ambiguities in corresponding alignment. TI/TV, transition/transversion ratio for regions used in phylogenetic analysis as indicated (values calculated from maximum parsimony tree using maximum likelihood method (see Methods section)).

*Total length of the alignment.

†Total number of noncoding (intron or ITS) or coding (exon) sites for each locus.

‡Total number of variable sites in noncoding (intron or ITS) or coding (exon) regions for each locus.

§Number of parsimony informative sites in the region.

¶Value based on a single substitution.

ther is to other species of *Stachybotrys* or *Memnoniella* accepted by Jong and Davis (1976). For instance, Haugland and Heckman (1998) demonstrated considerable differences in ITS sequence between isolates identified as *S. chartarum* (sensu Jong and Davis 1976) and other members of the genus *Stachybotrys* (compare with results for ITS within *S. chartarum* sensu lato, Table 3.) Outgroup comparison further supported this interpretation, as we found a considerable number of substitutions in both individual and combined analyses between *S. kampalensis* and *S. chartarum* sensu lato as compared with that found between lineages of the latter. It should be noted that, while we tried all five of our primer sets across a number of other species of *Stachybotrys* including *S. albipes* (UAMH 7750), *S. bisbyi* (UAMH 1526), *S. microspora* (UAMH 7747), *S. parvispora* (UAMH 7749), as well as our own isolates identified as *S. elegans* and *Memnoniella echinata*, only *S. kampalensis* yielded product for the majority of our loci (data not shown).

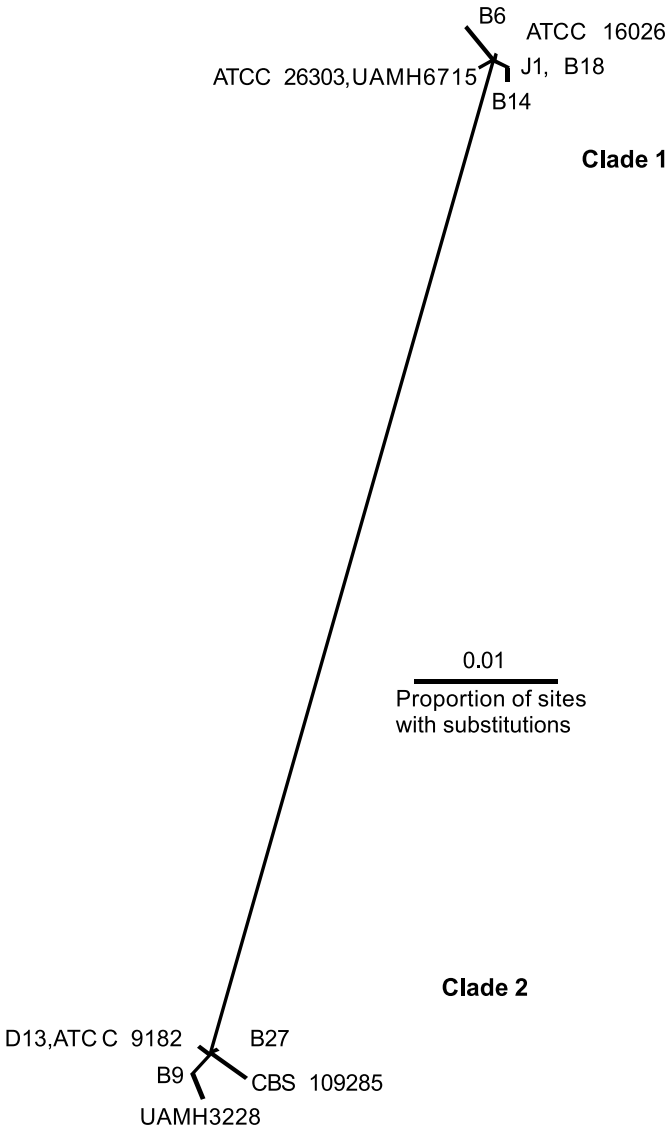
Evaluation of marker set

Protein-encoding loci Bt2, *tef1S*, and *tri5S* showed comparable resolution of our set of *S. chartarum* isolates. These loci possessed two major allele types with one to three subtypes. While we found five HMA groups with the *cmdS* locus, sequencing showed this locus to be comparable to others in having two major allele types. In alignments restricted to isolates of *S. chartarum* sensu lato, the Bt2, *cmdS*, and *tef1S* loci were comparable with respect to the overall level of intron variability, with variable sites representing 11–12% of the total number of noncoding sites. Variability within the *tri5* intron was relatively higher (17%) among these isolates. In contrast, all alignments, which included the outgroup *S. kampalensis*, demonstrated considerable variability within noncoding sequence and included a number of indels. As a result alignments across intron regions of all protein-encoding loci were ambiguous and could not be used in phylogenetic analyses. However the combined coding regions of all four protein-encoding loci in addition to ITS contained sufficient variability to differentiate clade 1 and 2 isolates while allowing comparison to this outgroup.

The amplified portion of the *tef1* gene was similar to the sequence for *Neurospora crassa* (GenBank D45837) with respect to both number and relative length of intron regions, including introns no. 2 and 3, exon no. 3, and partial sequence for exons no. 2 and 4. The *tef1S* locus had the greatest number of noncoding sites and contributed a number of variable characters to our data set. This finding is consistent with recent work in both insect and fungal systematics demonstrating the utility of intron regions of the *tef1* gene in elucidation of subgeneric and species-level relationships (O'Donnell et al. 1998; Cruickshank et al. 2001; Danforth and Ji 2001; Skovgaard et al. 2001). Our results and those of other studies suggest that coding regions of this gene may also prove useful in resolving higher-level relationships. For example, Voigt and Wostemeyer (2001) employed *tef1* to study ordinal and subordinal relationships of Mucorales and related taxa of zygomycetous fungi, while Yang et al. (2000) used this gene to investigate relationships among genera of stilleto flies.

To date, comparatively few systematic studies of fungi and other organisms have employed the *cmd* gene. Recent exceptions among the fungi include the work of Feibelman et al. (1998) and Peterson et al. (2001), who used a segment of this gene to support morphology-based species delineation within intrageneric groupings of the Aspergilli. The alignment of *cmdS* included introns no. 2, 3, and 4, exons no. 3 and 4, and 30 bp of exon no. 5 (based on *N. crassa* complete sequence for calmodulin GenBank L02964). Intron position and relative length were comparable to those observed for homologous regions in *N. crassa* (GenBank L02964), *Emericella nidulans* (GenBank J05545), and *Fusarium oxysporum* (GenBank AF158365). Our experience

Fig. 1. Unrooted single most likely tree from combined data (noncoding sequence of Bt2, cmdS, tef1S, and tri5S with ITS) for 13 proxy isolates. Bootstrap support for the two major clades was 100%.



with the *cmdS* locus suggests significant sequence variability at primer sites, and primer designs is likely to be hampered by the fact that exon no. 3 is short (16 bp) and thus allows little opportunity for modification based on even nominal positional shifts. Despite these difficulties the *cmd* gene remains an attractive target for studies of *S. chartarum* and closely related species, as this locus contributed several infraclade polymorphisms to our data set.

Products for the Bt2 locus included intron no. 5, exons no. 4 and 5, and partial sequence for exons no. 3 and 6 as based on a comparison with the complete sequence of the *benA* gene of *N. crassa* (GenBank M13630). *Stachybotrys chartarum* is therefore similar to *F. sporotrichioides* (GenBank AF006367) in having two fewer introns than the comparable region of both *N. crassa* and *E. nidulans* (GenBank M17519). The potential utility of the *benA* gene sequence for infrageneric systematic studies has been demonstrated within a number of morphologically inscrutable taxa (Koufopanou et al. 1997; Geiser et al. 1998; de Jong et al. 2001; Schoch et al. 2001). The Bt2 locus typically includes multiple introns, and the primer sites appear to be highly conserved (Glass and Donaldson 1995). However an increasing number of studies report the presence of two paralogous *benA* genes (Ayliffe et al. 2001) including among members of the Hypocreales (Goldman et al. 1993; O'Donnell 2000). Clearly the indeterminate sampling of paralogous genes represents a serious potential confounder in systematic studies (no evidence of amplification of paralogous tubulin genes was observed in our study). Despite that tubulin genes remain popular for fungal systematics, the small product size of the Bt2 product and the potential for nonspecific amplification diminishes the value of this specific locus in further studies of *Stachybotrys*.

The amplified region of *tri5* contained a single 75-bp intron. This is consistent with the *tri5* sequence reported for both *Myrothecium roridum* (GenBank AF009416) and *F. sporotrichioides* (GenBank AF364179). The *tri5* gene encodes trichodiene synthase, the first pathway-specific enzyme in the biosynthesis of nonmacrocyclic and macrocyclic trichothecenes (Sweeney and Dobson 1999). A comparison of complete sequence for this gene (data not shown) with the partial sequence deposited by Cruse et al. (2002) showed their samples 5 (GenBank AF468154) and 6 (GenBank) to be consistent with our clade 1 and 2 isolates, respectively. The intron region of this gene was highly variable, as was the amplified portion of the coding sequence as compared with amplified exon regions of *benA*, *cmd*, and *tef1*. This latter finding is intriguing given recent work by Anderson et al. (2002) that demonstrated the presence of three chemotypes within *S. chartarum* sensu lato. Investigations in both *Fusarium* and *Myrotheceium* have shown that *tri5* and other trichothecene-pathway genes are clustered (Hohn et al. 1993; Trapp et al. 1998). Among the theories proposed to explain gene clustering in fungi, one is that this arrangement may promote and thus arise from horizontal transfer of a complete set of integral metabolic genes between fungi (Keller and Hohn 1997; Rosewich and Kistler 2000; Walton 2000). However, within the *F. graminearum* species complex, Ward et al. (2002) demonstrated that incongruency in evolutionary histories seen between virulence-associated genes of the trichothecene pathway and other (nonpathway) genes was best explained by the maintenance of ancestral polymorphism through balancing selection acting on chemotype differences within species (Ward et al. 2002). The t*ri5S* locus may offer an opportunity to investigate the relative contribution of these forces in the inheritance of trichothecene pathway genes in *S. chartarum* sensu lato.

Alignment of the amplified portion of rDNA included complete sequence for ITS1, 5.8S rDNA, and ITS2 as well as partial sequence for 18S and 28S. Within *S. chartarum* sensu lato the amplified region of nuclear rDNA contained a single variable site located in ITS2. Our ITS sequence is consistent with that reported for *S. chartarum* by Haugland and Heckman (1998) and Cruse et al. (2002) including the position of the variable site. The ITS1 region was invariant across our entire set of isolates. This finding is significant because "*S. chartarum*-specific" primers and fluorescent probes used in the TaqMan™ fluorogenic probe system

Fig. 2. Rooted, single most likely tree from combined data (coding sequence of Bt2, cmdS, and tef1S with ITS) for 13 proxy isolates with *S. kampalensis* outgroup. Bootstrap support for clades 1 and 2 was 98% and 94%, respectively.

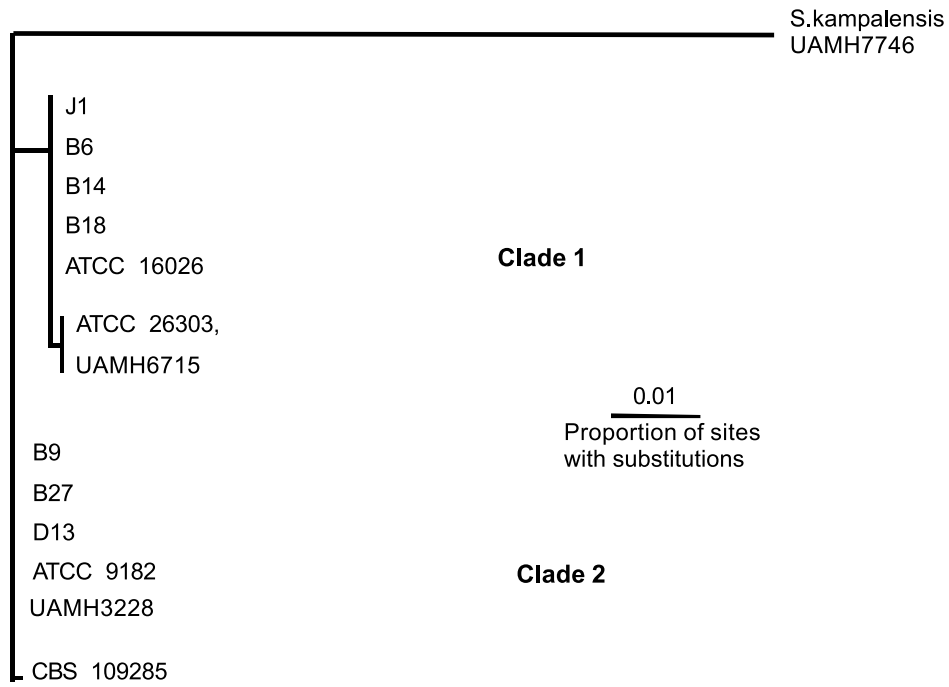
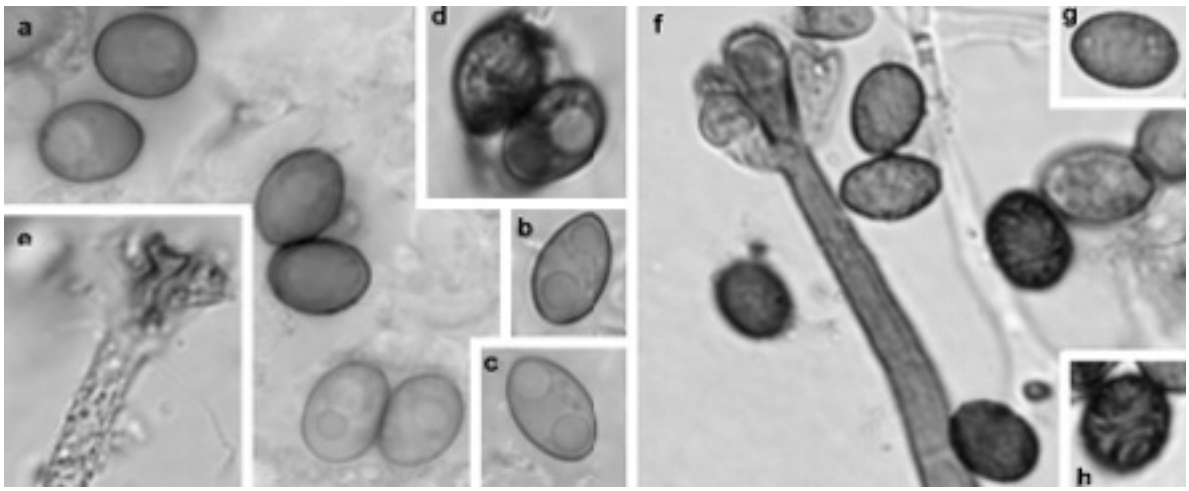


Fig. 3. Comparison of conidial morphology. Figs 3*a*–3*e*. ATCC 9182: (*a*–*c*) mature conidia; conidium in (*b*) is 9 μm long; (*d*) older conidia showing some surface roughening; (*e*) conidiophore. Figs 3*f*–3*h*. ATCC 16026: (*f*) conidiophore and mature conidia, focus emphasizes characteristic roughening of conidia; (*g*) newly formed conidium with relatively smooth surface; (*h*) older conidium.



(Haugland et al. 1999; Cruz-Perez et al. 2001) target the ITS1 region and therefore will not differentiate these two species. In contrast there was a considerable number of variable sites in the alignment of ITS, which included the outgroup *S. kampalensis*. This is consistent with results presented by Haugland and Heckman (1998) that showed ITS to be a useful marker in systematic studies of the genus as a whole.

Taxonomic and nomenclatural implications

A fundamental problem lies in our current inability to provide names for the two species represented by our clades. Ehrenberg described *Stilbospora chartarum*, now accepted as *Stachybotrys chartarum* (Hughes 1958; Jong and Davis 1976), in 1818 and no ex-type cultures (i.e., those derived from authentic material) are known to exist. Thus, the conclusion by Cruse et al. (2002) that ATCC 9182 represents the "type" collection of *S. chartarum* appears unfounded. Similarly, the assumption made by Anderson et al. (2002) that two of their chemotypes represented *S. chartarum* is speculative given that major treatments of *Stachybotrys* used to identify the isolates (i.e., Bisby 1943; Jong and Davis 1976) failed themselves to examine type material. A comparison of our isolates with those examined by Anderson et al. (2002), both with respect to conidial morphology and apparent abundance, suggests that chemotypes identified by these authors as *S. chartarum* most likely correspond to our clade 1. In contrast, the isolate identified by Cruse et al.

(2002) as the "type" of *S. chartarum* (ATCC 9182) falls within our clade 2. Although the connection between chemotype and genotype remains to be confirmed, there is clearly a need to reexamine available type material for *S. chartarum* Ehrenb. (1818) and synonyms prior to linking historic names to either lineage.

Microscopic examination alone is likely to prove insufficient as a means of verifying the descriptions of earlier authors given that certain nomenclaturally important specimens are in poor condition (S.J. Hughes, pers. commun.). Molecular characters and techniques designed to amplify "ancient" DNA (Bruns et al. 1990) may prove successful with this material. Targeting a taxonomically restricted gene may allow differential amplification of *S. chartarum* template DNA from polymicrobial specimens. Our *tri5S* primer set may be especially suitable for this type of investigation, as our experience with these primers has shown that they are specific to *S. chartarum* sensu lato (i.e., they do not amplify products from other species of *Stachybotrys* or other trichothecene producing fungi (data not shown)). If this approach fails, we then recommend that an epitype for *S. chartarum* sensu stricto be chosen such that it is morphologically (and genetically) consistent with isolates of our clade 1. Our observations suggest that, in terms of conidial morphology, this lineage may best reflect the current concept of this species. As concerns the disposition of the second lineage (i.e., that represented by clade 2), this lineage should be described as a new species only if investigations confirm that an appropriate name does not already exist.

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