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Molecular and phenotypic descriptions of *Stachybotrys chlorohalonata* sp. nov. and two chemotypes of *Stachybotrys chartarum* found in water-damaged buildings

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Abstract: Twenty-five *Stachybotrys* isolates from two previous studies have been examined and compared, using morphological, chemical and phylogenetic methods. The results show that *S. chartarum sensu lato* can be segregated into two chemotypes and one new species. The new species, *S. chlorohalonata*, differs morphologically from *S. chartarum* by having smooth conidia, being more restricted in growth and producing a green extracellular pigment on the medium CYA. *S. chlorohalonata* and *S. chartarum* also have different *tri5, chs1* and *tub1* gene fragment sequences. The two chemotypes of *S. chartarum*, chemotype S and chemotype A, have similar morphology but differ in production of metabolites. Chemotype S produces macrocyclic trichothecenes, satratoxins and roridins, while chemotype A produces atranones and dolabellanes. There is no difference between the two chemotypes in the *tub1* gene fragment, but there is a one nucleotide difference in each of the *tri5* and the *chs1* gene fragments.

Key words: atranones, beta-tubulin, chemotypes, chitin synthase, metabolite profiles, morphological species, phylogenetic species, roridins, satratoxins, sick-building syndrome, trichodiene synthase

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INTRODUCTION

Most *Stachybotrys* isolates found in water-damaged buildings in recent years have been recorded in the literature as *S. chartarum* (Ehrenb.) Hughes, or as its synonym *S. atra* Corda (Jarvis et al 1986; Johanning et al 1993; Nikulin et al 1997; Jarvis et al 1998; Vesper et al 1999, 2000b). However, different *S. chartarum* isolates from water-damaged buildings are reported in the literature to have different toxic, inflammatory and/or immunological effects (Jarvis et al 1998; Fung et al 1998; Routsalainen et al 1998; Vesper et al 1999, 2000a), which have resulted in taxonomic and medical confusion.

Jong and Davis (1976) reviewed *Stachybotrys* and treated 15 species primarily based on examination of living cultures. Their work has been the starting point for modern *Stachybotrys* systematics. Since then at least 25 additional *Stachybotrys* species have been described. Although there are now more than 40 described *Stachybotrys* species, only a few are reported frequently in literature.

Results of a study by Andersen et al (2002) of *Stachybotrys* isolates from water-damaged buildings in Northern Europe and the United States showed that isolates segregated into two distinct groups based on morphology, physiology and chemistry. Cruse et al (2002) independently showed a similar segregation into two distinct groups of another set of *Stachybotrys* isolates from the U.S.A., based on DNA sequence analyses. A collaboration was initiated to determine the relationship between these groups of isolates and their taxonomic placement. This paper reports the results of a combined phenotypic and phylogenetic study and the description of a new species of *Stachybotrys.*

MATERIALS AND METHODS

Fungal description.—*Stachybotrys* isolates were inoculated in three points (as described by Singh et al 1991) on cornmeal agar (CMA, DIFCO 1969) and Czapek yeast autolysate agar (CYA, Samson et al 2002) media. The unsealed, vented plates were put in perforated plastic bags and incubated for 7 d at 25 C in the dark. Micromorphological observations were made from CMA cultures, whereas cultural descriptions were based on CYA cultures. The isolates first were examined directly on the CMA plate and then mounted in a drop of lactophenol using tape preparations (Butler and

Mann 1959) and examined at 400× and 1000× magnification. All isolates are held in the IBT culture collection at BioCentrum-DTU, Denmark, and maintained as dried soil cultures.

Physiological characterization.—The isolates were three-point inoculated on alkaloid-forming agar (ALK) (Reshetilova et al 1992), CYA, potato-sucrose agar (PSA) (Samson et al 2002), Sigma yeast-extract sucrose agar (SYES) (Filtenborg et al 1990), and V8 juice agar (V8) (Simmons 1992) media. The inoculated, unsealed plates were put in perforated plastic bags and incubated in the dark at 25 C. After 7 d the colony diameter and pigment production were recorded as described by Andersen at al (2002). PSA cultures were reincubated for an additional 7 d before they were used for chemical characterization.

Chemical characterization.—Cultures on PSA were extracted after 14 d of growth at 25 C in the dark. Five to six agar plugs, approx. 1.5 cm^2 of colony and agar, were extracted with methanol as described in Andersen et al (2002) but without the polyethylene imine (PEI) clean-up step.

Methanol extracts (5 μL) were analyzed by liquid chromatography—photo diode array detection—positive electro spray high-resolution mass spectrometry (Nielsen and Smedsgaard 2003). Samples were separated by reversed-phase chromatography on a C_{18} column by a water-acetonitrile gradient system on an Agilent HP 1100 Liquid Chromatograph (Waldbronn, Germany) interfaced with a Micromass LCT (Manchester, United Kingdom) Time of Flight mass spectrometer (MS). The MS was tuned to a resolution of 6000 and collected as centroid data from m/z 100 to 900, with a scan time of 1 s. Potential difference between the two skimmers was set to 6 V, to minimize fragmentation of the labile trichothecenes.

The two atranone precursors, 3,4-epoxy-6-hydroxy-dolabella-7,12-diene-one and 6-hydroxydolaballa-3,7,12-trien-14-one, and the atranones A, B and F were detected as their protonated molecular ions, $[M+H]^+$ by plotting m/z 319.23, 303.23, 417.23, 447.24, 433.22, respectively. The simple trichothecenes, trichodermol and trichodermin, were detected as $[M+H]^+$ by plotting m/z 251.16 and 293.18, respectively. The macrocyclic trichothecenes, roridin E and epi-roridin E, were detected as their $[M+NH_4]^+$ ion m/z 532.29, and satratoxins G, H, and iso-F, as well as roridin L-2 and hydroxyroridin E, as their $[M+H]^+$ ions by plotting m/z 545.20, 529.24, 543.22, 531.26, 531.26, respectively. Specificity was achieved by using a window of m/z ± 0.04 of each of the mentioned ions.

Cluster analysis.—A data matrix of 25 objects (*Stachybotrys* isolates) and 10 variables (colony diameters on the five media and five metabolite families) was constructed. The matrix was standardized (the mean of each variable was subtracted and then divided by the standard deviation of each variable) and analyzed using the Manhattan coefficient and unweighted pair-group method, arithmetic average (UPGMA) in NTSYS 2.02j (Applied Biostatistics Inc., New York).

Molecular characterization.—Methods of growing mycelium for DNA extraction, DNA extraction, PCR amplification, DNA sequencing, sequence alignment and phylogenetic analysis are as described by Cruse et al (2002).

DNA sequencing.—Mycelium was grown in yeast broth for 3–4 d, lyophilized and stored. Lyophilized mycelium was broken in a bead beater and DNA extracted using a CTAB protocol followed by the use of solvents and a Qiagen Dneasy kit. The trichodiene synthase 5 fragment (*tri5*), the beta-tubulin 1 fragment (*tub1*) and the chitin synthase 1 fragment (*chs1*) were PCR amplified using primers and conditions described in Cruse et al (2002). PCR products were prepared for sequencing with Qiagen's QIAquick PCR purification kit or with an isopropanol precipitation. Purified PCR product was sequenced with an ABI model 3100 Sequencer and ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California). Sequences were analyzed and aligned with Sequencing Analysis 3.0 and Sequence Navigator 1.01 (Applied Biosystems, Foster City, California).

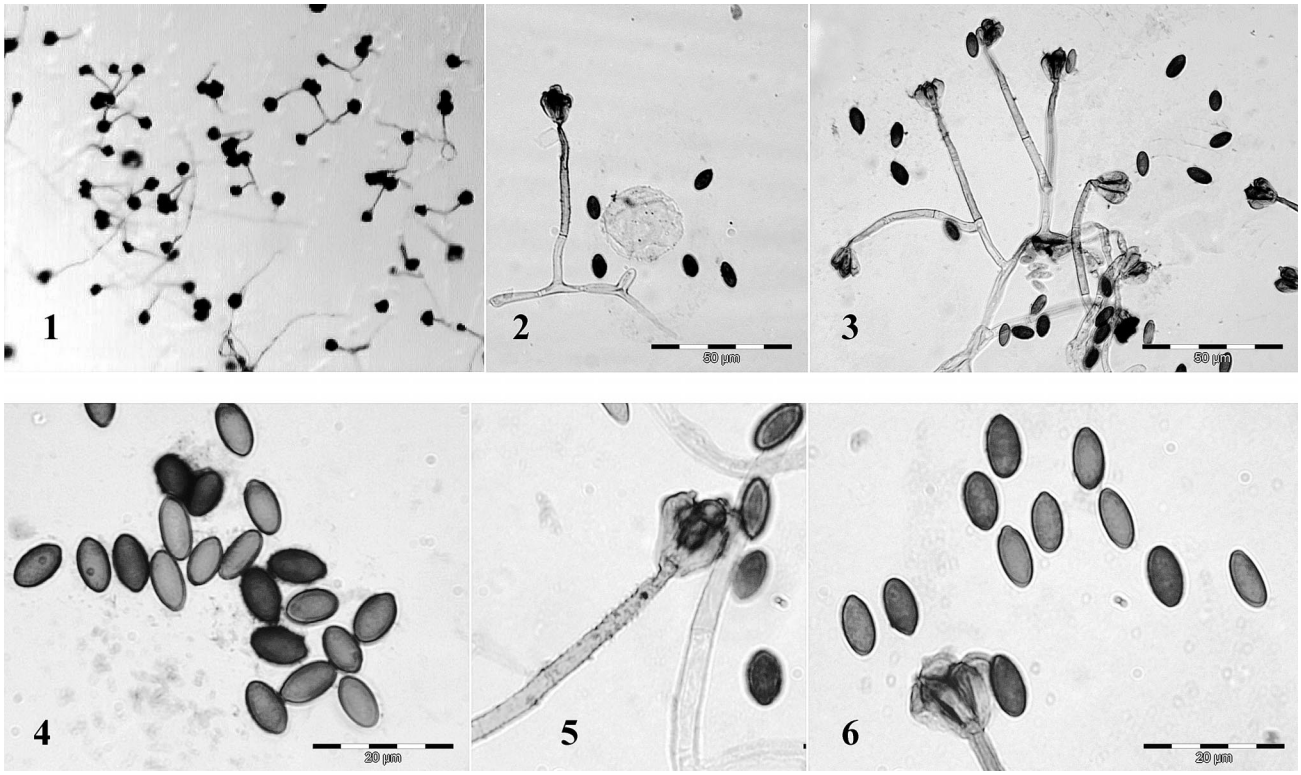
Phylogenetic analysis.—Sequences obtained from *tri5, tub1* and *chs1* were aligned and checked visually with Sequence Navigator 1.01. There were no gaps in the alignments. The aligned sequences were exported to a NEXUS file and analyzed with PAUP 4.0b8 (Swofford 2001). All sequences were placed in a single NEXUS file and partitioned by each locus to create the complete dataset. These data then were analyzed by maximum parsimony using settings described in Cruse et al (2002). Analysis was done individually on each locus and on all loci combined. Heuristic searches were carried out with tree-bisection reconnection and 1000 random sequence additions. Support for internal branches was assessed with a heuristic parsimony search of 1000 bootstrapped datasets. The trees shown are rooted at the midpoint.

TAXONOMY

Stachybotrys chlorohalonata Andersen et Thrane, sp. nov. FIGS. 1–6

In agaris CMA et CYA descripta. Coloniae in CMA 35 mm diam a 7d, 25 EC, luce excluso; mycelium superficiale, hyalinum, arachnoideum, capitulis atris, mucosis manifestis; pigmenta extracellulosa nulla. Coloniae in CYA a 7 d, 25 EC, luce excluso, 14 mm diam, glaucae, pubescentibus, margine angusto, albido; pigmento extracelluloso atroviridente, in agaro trans marginem coloniae 4–5 mm diffuso. Conidiophora in CMA erecta, stricta, vulgo ramosa, (0–)1–2(–3) septata, cellulis basalibus tumidis exortis, basi plerumque laevibus, hyalinis, saepe apice versus fuscatis et irregulariter verruculosis; vulgo 44–69 μm, basi ad 5 μm, apice deminutis. Phialides apicales, 3–5 fasciculatae, clavatae vel obovoideae, basi hyalinae, apice fuscatae, laevibus, eseptatae, monophialidicae, vulgo 8–11 × 4–6 μm. Conidia juvenilia ellipsoidea, citrino-viridia; matura late ellipsoidea vel obovoidea, basi saepe inconspicue papillata, eseptata, atroviridia, laevia, plerumque 8–10.5 × 4–5.5 μm. Teleomorphosis ignota.

Typus: pars ex cultura IBT 9467 ex tabula gypsea, lecta Kristian F. Nielsen, X-1997, Selandia, Dania, desiccata et in



FIGS. 1–6. *Stachybotrys chlorohalonata*. 1. Sporulation pattern directly on CMA (no scale bar). 2–3. Simple and branched conidiophores and phialides on CMA (scale bar = 50 µm). 4–6. Conidiophore, phialides and conidia on CMA (scale bar = 20 µm).

C 60160 (Holotypus) conservanda. Culturae ex typo IBT 9467, CBS 109285.

Colonies on CMA attain 35 mm diam after 7 d of growth at 25 C in the dark. Mycelium is superficial, hyaline and cobweb-like with visible black slime heads (FIG. 1) thinning toward the edge. No extracellular pigment is produced on CMA. Colonies on CYA are 14 mm diam after 7 d at 25 C in the dark. They are grayish green to dull green (color plates 28 E 4–5 in Methuen Handbook of Color, Kornerup and Wanscher 1978) with narrow white edges and a downy texture. A dark green to blackish green (color plates 27–28 F 6, Kornerup and Wanscher 1978) extracellular pigment is produced on CYA, which extends 4–5 mm beyond the edge of the colonies. On CMA, conidiophores are erect, straight or slightly flexuous and mostly branched once or twice with (0–)1–2(–3) septa (FIGS. 2–3). Solitary conidiophores usually arise from swollen basal cells. Conidiophores are mostly smooth and hyaline at the base, often darker toward the apex, and the upper portion is sometimes irregularly verrucose (FIG. 5).

The whole conidiophore apparatus may be up to 100 µm long. Most conidiophores are 44–69 µm long from first point of branching to apex, up to 5 µm at the base and tapering toward the apex. Phialides, which are produced in groups of 3–5, are clavate to obovoid. They are smooth, aseptate and monophialidic. The phialides are hyaline at the base and darker toward the apex. Most phialides are 8–11 × 4–6 µm (FIGS. 5–6). Immature conidia initially are ellipsoid and yellowish green, becoming broadly ellipsoidal to obovoid, often inconspicuously papillate at the base. Mature conidia are aseptate, blackish green, opaque and smooth. Most conidia are 8–10.5 × 4–5.5 µm (FIGS. 4–6). Teleomorph unknown.

Habitat: Wet cellulose-containing material such as fabric, hay, seaweed, grain, paper and soil. Known distribution: Belgium, Denmark, Finland, Iraq, New Guinea, Spain and U.S.A.

Etymology: *chlorohalonata*, refers to green halo of extracellular pigment around the colonies that can be seen on CYA medium.

Type specimen. DENMARK: Sjælland, cardboard on gypsum board, OCT 1997, *Kristian F. Nielsen*. (HOLOTYPE: C 60160; Living cultures EX-TYPE: IBT 9467 (ALK pl. 2.), CBS 109285).

Additional cultures examined. Stachybotrys chlorohalonata (IBT 9225, IBT 9226, IBT 9293, IBT 9294, IBT 9299 [= CBS 109284], IBT 9714 [= HT-016], IBT 9755, IBT 9756, IBT 9757, IBT 9824, IBT 9826, IBT 9827, IBT 10219, IBT 40285, IBT 40287, IBT 40290, IBT 40292, IBT 40294, IBT

TABLE I. Isolate number, species, substratum, origin and identity of the 25 *Stachybotrys* isolates examined

Isolate #	Species	Substratum[a]/origin	Identity # and collection[b]
005[c]	*S. chartarum*	Plant/California, USA	IBT 40289
007[c]	*S. chartarum*	Build/California, USA	IBT 40288
011[c]	*S. chartarum*	Build/California, USA	
201[c]	*S. chartarum*	Build/California, USA	IBT 40293
205[c]	*S. chartarum*	Build/California, USA	IBT 40286
206[c]	*S. chartarum*	Build/California, USA	IBT 40291
7711[d]	*S. chartarum*	Build/Denmark	IBT 7711
9290[d]	*S. chartarum*	Build/Denmark	IBT 9290
9460[d]	*S. chartarum*	-/Finland	CBS 414.95
9466[d]	*S. chartarum*	Build/Denmark	IBT 9466
9631[d]	*S. chartarum*	Build/Cleveland, USA	JS58-03
9633[d]	*S. chartarum*	Build/Cleveland, USA	BBJ-22
9807[d]	*S. chartarum*	Build/Cleveland, USA	JS58-01
14915[d]	*S. chartarum*	Build/Denmark	IBT 14915
14916[d]	*S. chartarum*	Build/Denmark	IBT 14916
006[c]	*S. chlorohalonata*	Plant/California, USA	IBT 40295
102[c]	*S. chlorohalonata*	-/Texas, USA	IBT 40290
103[c]	*S. chlorohalonata*	-/New Mexico, USA	IBT 40292
203[c]	*S. chlorohalonata*	Build/California, USA	IBT 40287
204[c]	*S. chlorohalonata*	Build/California, USA	IBT 40285
211[c]	*S. chlorohalonata*	Build/California, USA	IBT 40294
9299[d]	*S. chlorohalonata*	Build/Denmark	CBS 109284
9467[d] Type	*S. chlorohalonata*	Build/Denmark	CBS 109285
9754[d]	*S. chlorohalonata*	Water/Belgium	IHEM 9905[e]
9825[d]	*S. chlorohalonata*	Build/USA	ATCC 20I863 = JS58-06[f]

[a] Build: Building material; Plant: Leaf litter and other plant material.

[b] IBT: Culture collection at BioCentrum-DTU, Denmark; CBS: Culture collection in Utrecht, the Netherlands; IHEM: Culture collection (BCCM™) in Brussels, Belgium; JS and BBJ: Culture collection at Jarvis's lab in Maryland; ATCC: American Type Culture Collection.

[c] Isolates used in Cruse et al (2002).

[d] Isolates used in Andersen et al (2002).

[e] Isolate used in Peltola et al (2002).

[f] Isolate used in Jarvis et al (1998) and Vesper et al (1999, 2000a).

40295, IHEM 2248 [= ba 173], IHEM 9905 [= IBT 9754], ATCC 201860 [= JS51–08 = IBT 9823], ATCC 201863 [= JS58-06 = IBT 9825] and NRRL 29940 [= QM 94d = IBT 9767]).

Other species examined. *Stachybotrys albipes* (CBS 100343); *S. bisbyi* (CBS 142.97); *S. chartarum* (CBS 414.95); *S. cylindrospora* (IHEM 17451); *S. dichroa* (CBS 526.50); *S. microspora* (CBS 186.79); *S. nephrospora* (CBS 796.95); *S. nilagirica* (IHEM 17453); *S. parvispora* (CBS 173.97) and *S. theobromae* (IHEM 17456).

RESULTS

Morphology.—In this study, a subset of 12 *Stachybotrys* isolates from Cruse et al (2002) and a subset of 13 isolates from Andersen et al (2002) were examined (TABLE I) and compared with isolates of 10 other *Stachybotrys* species from CBS and IHEM culture collections (see Taxonomy section). These cultures that arrived as *S. albipes*, *S. bisbyi*, *S. chartarum*, *S. cylindrospora*, *S. dichroa*, *S. microspora*, *S. nephrospora*, *S. parvispora* and *S. theobromae* all fit the descriptions of those species in Jong and Davis (1976), and *S. nilagirica* fit the description by Subramanian (1957). The culture from CBS culture collection, CBS 414.95 (= IBT 9460) and 14 other *Stachybotrys* isolates from Denmark and the U.S.A. (see TABLE I), were similar morphologically on CMA and fit the description of *S. chartarum* in Jong and Davis (1976).

Nine isolates from Denmark and the U.S.A. and one culture from IHEM culture collection, IHEM 9905 (= IBT 9754) (see TABLE I), were similar morphologically on CMA. They originally had been identified as *S. chartarum* but did not fit any of the *S. chartarum* (or *S. atra*) descriptions by Bisby (1943), Subramanian (1957), Barron (1961), Ellis (1971, 1976), Jong and Davis (1976) or Domsch et al (1980). Nor did they fit the descriptions of *S. bambusicola* (Rifai 1964), *S. breviusculus*, *S. freycinetiae*, *S.*

TABLE II. Comparison of *S. chlorohalonata* to other *Stachybotrys* species examined (this study) and *Stachybotrys* species with smooth conidia (literature data). Morphology and colony characteristics are based on 7-day-old cultures

	Phialides on CMA		Conidia on CMA			Colonies on CYA	
Species	Shape	Max. size (μm)	Texture	Max. size (μm)	Shape	Pigment	Size (mm)
S. albipes	Cylindrical	12 × 4	Smooth	8 × 6	Broadly ellipsoidal	NR[c]	NR
S. bambusicola[a]	Cylindrical	13 × 7	Smooth	15 × 8	Obovoid	—[d]	—
S. bisbyi	Cylindrical	16 × 4	Smooth	16 × 9	Fusiform	NR	NR
S. cannae[a]	—	39 × 7	Smooth	17 × 11	Clavate	—	—
S. chartarum	Obovoid	13 × 6	Rough	12 × 6	Ellipsoidal	None	23
S. chlorohalonata	Obovoid	11 × 6	Smooth	11 × 6	Ellipsoidal	Green	14
S. cylindrospora[b]	Subclavate	11 × 5	Rough	16 × 5	Cylindrical	None	15
S. dichroa[b]	Subclavate	10 × 6	Rough	10 × 7	Ovoid	None	20
S. guttulispora[a]	Cylindrical	15 × 4	Smooth	12 × 5	Ellipsoidal	—	—
S. indica[a]	Ellipsoidal	11 × 3	Smooth	7 × 3	Clavate	—	—
S. microspora	Obovoid	10 × 5	Rough	7 × 5	Broadly ellipsoidal	Yellow	22
S. nephrospora	Obovoid	10 × 6	Rough	12 × 7	Reniform	None	22
S. nilagirica[a]	Clavate	15 × 8	Rough	25	Globose	Yellow	12
S. parvispora	Ellipsoidal	11 × 4	Rough	6 × 3	Ellipsoidal	None	16
S. ramosa[a]	—	5 × 3	Smooth	9 × 7	Subspherical	—	—
S. renispora[a]	Obovoid	9 × 4	Smooth	7 × 5	Reniform	—	—
S. theobromae	Cylindrical	25 × 8	Rough	33 × 14	Fusiform	None	15

[a] Morphological data from original description (see references in Results).
[b] Morphological data from Jong & Davis (1976).
[c] NR: Not recorded.
[d] Not available.

nephrodes (McKenzie 1991), *S. cannae* (Batista and Vital 1957), *S. clitoriae* (Batista et al 1960), *S. globosa*, *S. mangiferae* (Misra and Srivastava 1982), *S. guttulispora* (Muhsin and Al-Helfi 1981), *S. havanensis*, *S. xanthosomae* (Mercado-Sierra and Mena-Portales 1988), *S. humilis*, *S. virgata* (Krzemieniewska and Badura 1954), *S. indica* (Misra 1975), *S. kapiti*, *S. reniverrucosa*, *S. waitakere* (Whitton et al 2001), *S. queenslandica* (Matsushima 1989), *S. ramosa* (Dorai and Vittal 1986), *S. renispora* (Misra 1976), *S. ruwenzoriensis*, *S. verrucispora* (Matsushima 1985), *S. sphaerospora* (Morgan-Jones and Sinclair 1980), *S. stilboidea* (Munjal and Kapoor 1969), *S. yunnanensis* (Kong 1997), *S. zeae* (Morgan-Jones and Karr 1976), or *S. zuckii* (Matsushima and Matsushima 1995).

The morphological appearance of these 10 questionably identified isolates, however, was consistent with a description of a "*Stachybotrys* sp." isolated from water in Iraq by Muhsin and Al-Helfi (1981). Unfortunately, it was not possible to obtain this isolate. A new *Stachybotrys* species, *S. chlorohalonata* Andersen & Thrane, therefore, has been described to accommodate these isolates. The *S. chlorohalonata* isolates in TABLE I and cultures cited in earlier works ("*Stachybotrys* sp. Group A" isolates in Andersen et al [2002] and "small clade" isolates in Cruse et al [2002]) are morphologically identical to *S. chlorohalonata* and have the same distinctive colony appearance on CYA. We have encountered more than of 25 *Stachybotrys* isolates that belong to *S. chlorohalonata* (See Additional cultures examined).

Morphologically on CMA, *S. chlorohalonata* most closely resembles *S. chartarum sensu lato*. The conidiophore apparatus of *S. chlorohalonata* are similar in appearance to that of *S. chartarum*, but individual conidiophores are shorter (up to 70 μm and 90 μm, respectively). The phialides of *S. chlorohalonata* also resemble those of *S. chartarum* but are shorter (see TABLE II). The conidia of *S. chlorohalonata* are ellipsoidal to broadly ellipsoidal with a smooth surface and papillate at the base, in contrast to those of *S. chartarum*, which are slightly longer, ellipsoidal and have a rough surface. Several *Stachybotrys* species are reported to produce smooth conidia, but *S. chlorohalonata* can be distinguished from these species either by its conidial shape and size and/or the shape and size of its phialides (see TABLE II).

Metabolite production and colony characters.—Result of the cluster analysis based on five colony diameter measurements and five metabolite families is shown in FIG. 7. The analysis produced only one tree (no ties) and had a cophenetic correlation (r) of 0.88227. The dendrogram shows three clusters: one with iso-

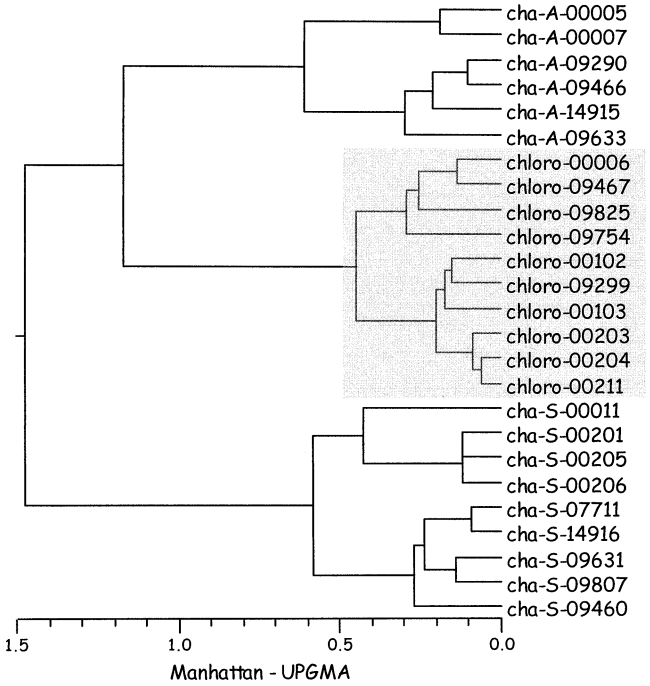


FIG. 7. Dendrogram produced by Manhattan coefficient and UPGMA cluster analysis based on diameters on five media and five metabolite families. The top cluster contains *S. chartarum* chemotype A isolates (cha-A), the hatched cluster contains *S. chlorohalonata* isolates (chloro) and the bottom cluster *S. chartarum* chemotype S isolates (cha-S).

lates identified as *S. chlorohalonata* (gray-hatched one marked as chloro) and two clusters with isolates morphologically identified as *S. chartarum* (marked as cha-A and cha-S, respectively).

The main division between *S. chlorohalonata* (chloro) and *S. chartarum* (cha-A) on one hand and *S. chartarum* (cha-S) on the other (FIG. 7) is a result of differences in metabolite production. The *S. chlorohalonata* (chloro) isolates were atranone and dolabellane-producers. *S. chartarum* (cha-A) isolates also were atranone and dolabellane-producers, while *S. chartarum* (cha-S) isolates produced satratoxins and roridins. The atranone and dolabellane-producing cha-A isolates basically showed the same metabolite profile as *S. chlorohalonata*, although *S. chlorohalonata* generally produced fewer spirocyclic drimanes than the cha-A isolates. It was possible to differentiate between *S. chlorohalonata* and *S. chartarum* (cha-S) based on metabolite production alone, but not between *S. chlorohalonata* and *S. chartarum* (cha-A).

The metabolite production, recorded as metabolite families, of all 25 *Stachybotrys* isolates is shown in TABLE III. Spirocyclic drimanes (stachybotrys-lactams, lactones and dialdehydes and Mer5003 terpeneoids), produced by all 25 *Stachybotrys* isolates, were the dominant compounds in all metabolite profiles. All *S. chlorohalonata* and all *S. chartarum* (cha-A) isolates, except IBT 9754, produced atranones (A, B, F and E) and their precursors (3,4-epoxy-6-hydroxy-dolabella-7,12-diene-one and 6-hydroxy-dolaballa-3,7,12-trien-14-one), but the quantities of these varied by nearly three orders of magnitude. The LC-MS method used in this study showed that the three main trichothecenes produced by *S. chartarum* (cha-S) were satratoxin H, roridin E and L-2, followed by lower quantities of satratoxin G, iso-F and occasionally isosatratoxin H, verrucarins J and B, epiroridin E. A new metabolite, hydroxyroridin E, was found in all the extracts of *S. chartarum* (cha-S). Hydroxyroridin E tentatively was identified by accurate mass by LC-MS (m/z 531.2594 $[M+H]^+$ and m/z 548.2860 $[M+NH_4]^+$), retention time (4 min before roridin E, Nielsen and Smedsgaard 2003) and its UV-spectrum (almost identical to roridin E).

The production of extracellular pigment on CYA of all 25 *Stachybotrys* isolates also is shown in TABLE III. The *S. chlorohalonata* (chloro) isolates produced a green extracellular pigment on CYA, while *S. chartarum* (cha-A) produced no pigment. *S. chartarum* (cha-S) either produced a yellow pigment or no pigment at all on CYA.

Differences in colony diameters distinguished *S. chlorohalonata* (chloro) from isolates of *S. chartarum* (cha-A), as seen in the dendrogram (FIG. 7). Growth of *S. chlorohalonata* isolates was more restricted on all five media than *S. chartarum* (cha-A and cha-S) isolates. The mean diameter (± twice the standard deviation) of *S. chlorohalonata* on CYA was 15 mm (± 4 mm) compared to the mean diameters of the cha-A isolates and the cha-S isolates of 23 mm (± 3 mm) and 20 mm (± 3 mm), respectively. It was not possible to differentiate between *S. chartarum* (cha-A) and *S. chartarum* (cha-S) on the basis of colony diameter alone, although the cha-A isolates appeared to grow slightly faster on all media than the cha-S isolates.

Phylogeny.—The results of maximum-parsimony analysis based on the trichodiene synthase 5 gene (*tri5*), beta-tubulin 1 gene (*tub1*) and chitin synthase 1 gene (*chs1*) are shown in FIGS. 8–10, respectively. The differentiation between *S. chlorohalonata* and *S. chartarum* was strongly supported by all three gene fragments. There were 28 fixed nucleotide substitutions between isolates in the two species for *tri5,* 27 for *tub1* and 9 for *chs1,* for a total of 64 in the combined analysis (FIG. 11). In all single-gene genealogies, the branch separating the two species was supported in 100% of bootstrap resampled datasets, and the same result was found in parsimony trees based on the data from all three gene fragments. In *S. chartarum*, the

TABLE III. Production of different metabolite families by *Stachybotrys* isolates on PSA detected by LC-MS and color of extracellular pigment produced on CYA

Isolate #	Species/ chemotype	Atranones	Dolabellanes	Tricho-dermin	Satratoxins	Roridins	Hydroxy roridin E	Pigmen-tation
011	*chartarum*/S	ND[a]	ND	+	+	+	+	None
7711*	*chartarum*/S	ND	ND	+	+	+	+	None
9460	*chartarum*/S	ND	ND	+	+	+	+	Yellow
9631	*chartarum*/S	ND	ND	+	+	+	+	Yellow
9807	*chartarum*/S	ND	ND	+	+	+	+	Yellow
14916	*chartarum*/S	ND	ND	+	+	+	+	None
201*	*chartarum*/S	ND	ND	ND	+	+	+	None
205	*chartarum*/S	ND	ND	ND	+	+	+	None
206*	*chartarum*/S	ND	ND	ND	+	+	+	None
005	*chartarum*/A	+	+	+	ND	ND	ND	None
007*	*chartarum*/A	+	+	+	ND	ND	ND	None
9290*	*chartarum*/A	+	+	+	ND	ND	ND	None
9466	*chartarum*/A	+	+	+	ND	ND	ND	None
9633	*chartarum*/A	+	+	+	ND	ND	ND	None
14915*	*chartarum*/A	+	+	+	ND	ND	ND	None
006	*chlorohalonata*	+	+	+	ND	ND	ND	Green
9467*	*chlorohalonata*	+	+	+	ND	ND	ND	Green
9825*	*chlorohalonata*	+	+	+	ND	ND	ND	Green
102	*chlorohalonata*	+	+	ND	ND	ND	ND	Green
103*	*chlorohalonata*	+	+	ND	ND	ND	ND	Green
203	*chlorohalonata*	+	+	ND	ND	ND	ND	Green
204	*chlorohalonata*	+	+	ND	ND	ND	ND	Green
211	*chlorohalonata*	+	+	ND	ND	ND	ND	Green
9299	*chlorohalonata*	+	+	ND	ND	ND	ND	Green
9754	*chlorohalonata*	ND	ND	ND	ND	ND	ND	Green

[a] ND: not detected.

* Good representative isolate of the taxon.

cha-A and cha-S isolates were separated by a single nucleotide substitution in the *tri5* gene fragment. With *chs1,* all cha-A isolates except one (IBT 9466) could be distinguished from the cha-S isolates by a single nucleotide substitution. With *tub1,* there were no consistent differences between cha-A and cha-S isolates. In the combined analysis, both the *tri5* and *chs1* single nucleotide substitutions supported a clade of cha-A isolates emerging from a nonmonophyletic assemblage of cha-S isolates, although the latter nucleotide substitution showed a reversal in isolate IBT 9466.

DISCUSSION

The results of morphological, chemical and molecular analyses concurrently show that the *Stachybotrys* isolates that grouped separately from *S. chartarum* in Andersen et al (2002) (*Stachybotrys* sp. Group A) and in Cruse et al (2002) (smaller bottom clade) are conspecific and described here as *S. chlorohalonata.* Furthermore, our results show that two chemotypes exist within *S. chartarum*: *S. chartarum* chemotype S, producing the macrocyclic trichothecenes, satratoxins and roridins, and S. *chartarum* chemotype A, producing atranones and dolabellanes. *S. chlorohalonata* easily can be distinguished from *S. chartarum sensu lato* by a combination morphology and growth characteristics: smooth conidia and smaller colonies on CYA with green extracellular pigment. *S. chlorohalonata* can be distinguished from *S. chartarum* (cha-S) by its morphology, phylogeny, growth characteristics, pigmentation and its metabolite profile, while *S. chlorohalonata* can be distinguished from *S. chartarum* (cha-A) by its morphology, phylogeny, pigmentation and growth characteristics. *S. chartarum* (cha-S) and *S. chartarum* (cha-A) can be separated by their metabolite profiles and their phylogeny.

Both phenotypic and phylogenetic methods have proven useful for recognizing fungal species because they have demonstrated cryptic species within a single morphological species or a species complex (Taylor et al 2000, Larsen et al 2001). These cryptic species often have distinctive and important phenotypes; for example, phylogenetic species found within the human pathogenic fungus *Histoplasma capsulatum*

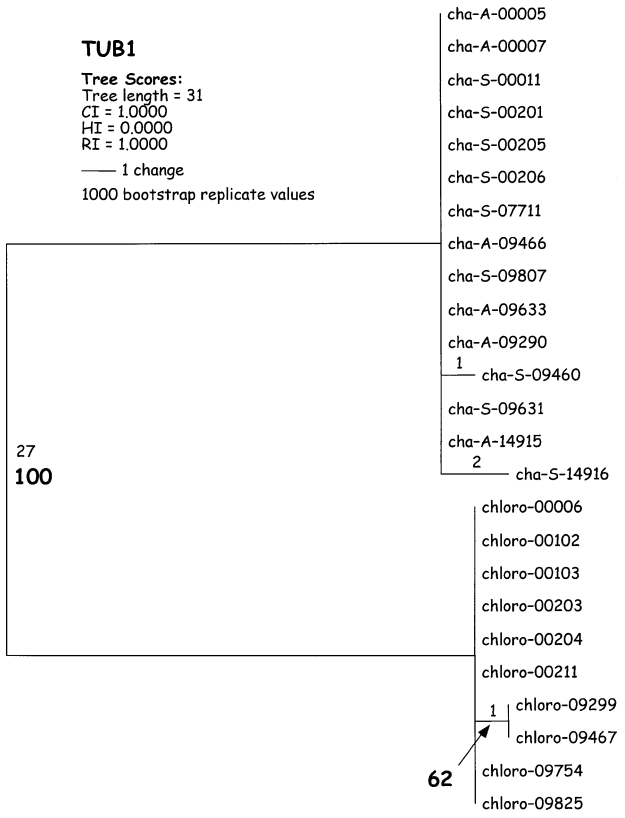


FIG. 8. The single most-parsimonious tree for *tub1*. The combined clade of cha-A plus cha-S and the chloro clade are monophyletic, however, neither cha-A nor cha-S, alone, are monophyletic.

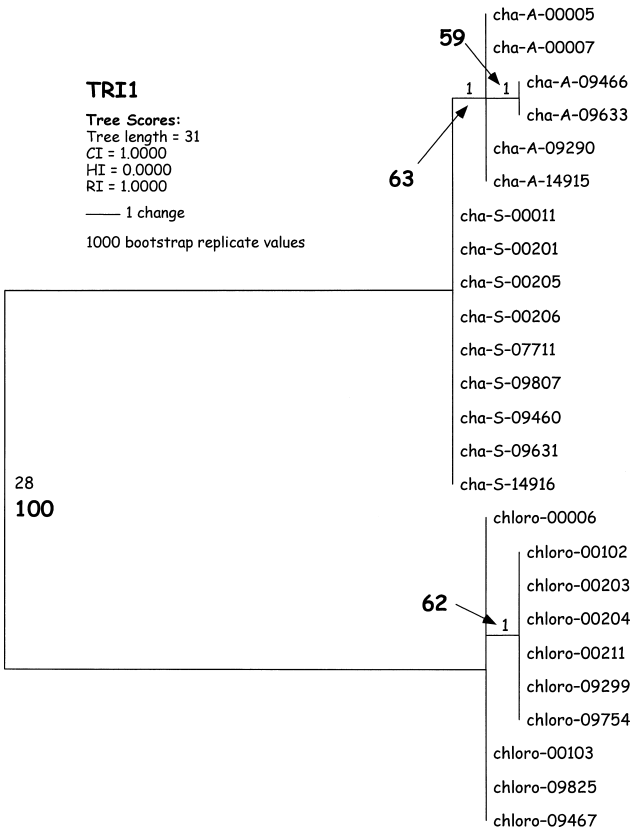


FIG. 9. The single most-parsimonious tree for *tri5*. The combined clade of cha-A plus cha-S, and the cha-A clade and the chloro clade are monophyletic. The clade cha-S is paraphyletic.

correlate in their ability to cause systemic or superficial disease and in their ability to cause disease in immuno-competent or immuno-compromised hosts (Kasuga et al 1999). Similarly, phylogenetic species within the toxigenic fungus *Aspergillus flavus* correlate in the size of their reproductive propagules (sclerotia) and in the production of mycotoxins (aflatoxins B and G) (Geiser et al 1998, 2000).

There are several types of phylogenetic species recognition (Mayden 1997), and the type applied in these studies and in previous work on *S. chartarum* (Cruse et al 2002) is by congruence of multiple gene genealogies. In recombining organisms, genealogies for different genes will conflict within species because different genes are inherited from different parents. However, as emerging species become genetically isolated, drift will reduce the ancestral variation in most genes to one ancestral allele, so that all newly developed alleles will coalesce to one ancestral allele. The result is that branches between species will be congruent for most gene genealogies and the shift from conflict to congruence in gene genealogies can be used to identify the limits of phylogenetic species (Avise and Ball 1990, Taylor et al 2000). The phenotypic approach used for *Stachybotrys* in this and previous studies (Andersen et al 2002) is based on the presence or absence of different metabolite families rather than quantities of individual metabolites and the use of growth characteristics under standardized growth conditions. These measures have been taken to minimize the influence of environmental factors, which always has been a major argument against the phenotypic species concept (Lumbsch 1998). Combinations of different and independent phenotypic characters in multivariate analyses can reveal cryptic species, often determined to be new species based on subsequent morphological and phylogenetic re-examination.

The separation of *S. chlorohalonata* from *S. chartarum sensu lato,* suggested by Andersen et al (2002) based on secondary metabolite production, colony diameter and morphology, is shown here to agree exactly with the separation suggested by Cruse et al (2002) based on three gene genealogies. In this case, morphological species recognition and phylogenetic species recognition arrived at the same conclusion and any of 64 fixed nucleotide substitutions in the three gene fragments could be used to distinguish

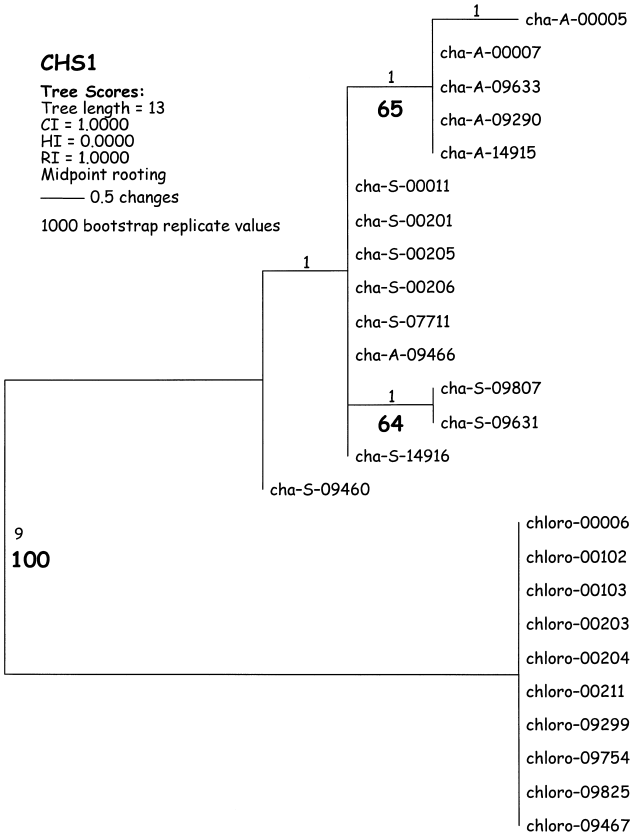


FIG. 10. One of two most-parsimonious trees for *chs1,* both of which support the monophyly of the combined clade cha-S plus cha-A and the monophyly of the clade chloro. The difference between the two topologies involves the position of cha-S-07711, which groups with cha-A-09460 in the other most-parsimonious tree. Note that neither cha-A nor cha-S are monophyletic, due to the placement of cha-A-09466 among cha-S isolates.

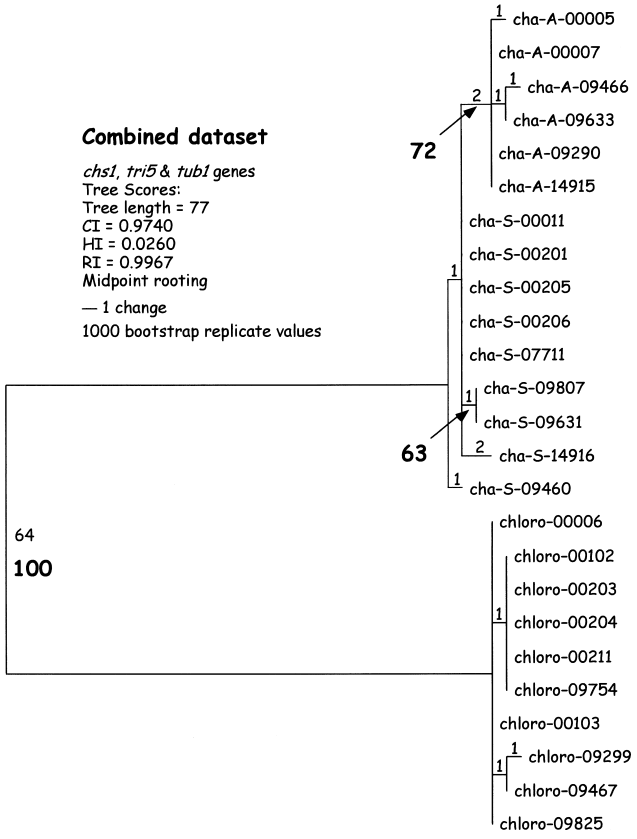


FIG. 11. One of 18 most-parsimonious trees for the combined data, all of which support the monophyly of the cha-A plus cha-S, cha-A (alone) and chloro clades. The variation in topology concerns the position of isolates cha-A-09466 and cha-A-09633 (three topologies), cha-S-07711 (two topologies), and chloro-09299 and chloro-09467 (three topologies). Note that cha-S is paraphyletic.

the two species. We also have found that the two species can be recognized by the single ITS nucleotide polymorphism reported among isolates of *S. chartarum* by Haugland and Heckman (1998); for those individuals sampled, *S. chartarum* is represented by GenBank ITS sequence AF081469 and *S. chlorohalonata* by GenBank sequence AF081468.

Within *S. chartarum*, differences in metabolite production identified the *S. chartarum* chemotype S capable of producing satratoxins and phylogenetic analysis of the *tri5* gene fragment identified the *S. chartarum* chemotype S as a monophyletic clade distinguished from *S. chartarum* chemotype A by a single nucleotide substitution in the *tri5* gene fragment. With *tub1,* no distinction was seen; with *chs1* all *S. chartarum* chemotype A isolates, except IBT 9466, had a common nucleotide substitution not seen in the *S. chartarum* chemotype S. The *tri5* and *chs1* nucleotide substitutions are important from a toxicological point of view because the two chemotypes of *S. chartarum* elicit very different toxicological responses (Nielsen et al 2001). It is interesting to note that it has been only *S. chartarum* chemotype A, the non-satratoxin producer, that has been found in both air and on material samples from case homes in which infants were diagnosed with pulmonary hemosiderosis, both in Belgium (Nielsen, 2002) and the U.S.A. (Vesper et al 2000b, Johanning, Gareis and Nielsen unpubl).

In this study we showed the presence of a *tri5* gene in all 25 *Stachybotrys* isolates examined, although the sequence in *S. chartarum* and *S. chlorohalonata* differed with 28 fixed nucleotide substitutions. In a study by Peltola et al (2002), the *tri5* gene was amplified in *S. chartarum* but not in the group of *Stachybotrys* sp. isolates identical with *Stachybotrys* Group A (= *S. chlorohalonata*). The failure of the *tri5* gene to amplify in isolates from Group A probably is due to the placement of the SCTOX5–1 primer (Peltola et al 2002) in a region now known to contain five nucleotide substitutions in the *S. chlorohalonata* se-

quence, as compared to the *S. chartarum* sequence (nucleotides 510–530 in GenBank sequences AF468155 and AF468154, respectively). None of the 10 isolates of *S. chlorohalonata* investigated in this study or the 17 isolates analyzed by Andersen et al (2002) produced satratoxins or any other macrocyclic trichothecenes, although it was possible to amplify their *tri5* gene. The eight *Stachybotrys* isolates analyzed by Peltola et al (2002) did not produce satratoxins, either. However, 30% of all *S. chlorohalonata* isolates produced detectable quantities of trichodermol and trichodermin (simple trichothecenes), a result that correlates with the presence of the *tri5* gene in *S. chlorohalonata*. The ability to produce trichodermol and trichodermin (the precursor for roridins and satratoxins), however, is not always consistent from inoculation to inoculation, a situation that was also observed with GC-MS/MS detection (Andersen et al 2002).

The improved LC-MS analysis method in this study revealed that satratoxin G and roridin L-2 co-elute in *S. chartarum* chemotype S extracts but that they can be distinguished by their different molecular masses and a difference of retention time of 0.09 min. Therefore, the peak that previously was interpreted as satratoxin G by LC-UV (Nielsen et al 2001, Andersen et al 2002) is mainly roridin L-2. Also, there has been very little focus on the spirocyclic drimanes, such as stachybotrys-lactams, lactones and di-aldehydes, Mer5003 terpenoids (Andersen et al 2002, Nielsen 2002) and the bisabosquals (Minagawa et al 2001). They are produced by *S. chlorohalonata* and both chemotypes of *S. chartarum* when growing on building materials, agar substrates and especially, on their natural habitat, hay and straw (Nielsen 2002).

This study has focused on the separation of *S. chlorohalonata* from *S. chartarum*. Further research is needed on the toxicity and phylogeny of the two chemotypes of *S. chartarum*. Because they have such different metabolite profiles, there likely will be more genes like the *tri5* gene that can distinguish between these two important chemotypes of *S. chartarum*. We recommend these isolates as best representatives for the three taxa: *S. chlorohalonata*: IBT 9467, IBT 9825 and 103 (= IBT 40292); *S. chartarum* chemotype A: IBT 9290, IBT 14915 and 007 (= IBT 40288); and *S. chartarum* chemotype S: IBT 7711, 201 (= IBT 40293) and 206 (= IBT 40291).

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