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Phylogenetic relationships of *Memnoniella* and *Stachybotrys* species and evaluation of morphological features for *Memnoniella* species identification

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Abstract: Members of the anamorphic fungal genus *Memnoniella* demonstrate morphological and biological similarities to species within the genus *Stachybotrys,* and the taxonomic distinctions between the genera have been the subject of controversy in the past. Sixteen strains representing described species of *Memnoniella* were examined for morphology using light and scanning electron microscopy and for phylogenetic relationships using comparative sequence analysis of a segment of the nuclear ribosomal RNA gene operon (rDNA) including the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2) and 5.8S gene. These analyses resolved the *Memnoniella* strains into two highly divergent phylogenetic clades with morphologies generally consistent with the current descriptions of *M. echinata* and *M. subsimplex.* One strain, showing morphological features more similar to *M. subsimplex,* was placed in the *M. echinata* clade in the phylogenetic analysis and probably represents a new species. A second strain, showing a typical *M. echinata* DNA sequence, showed morphological features that were similar to *Stachybotrys* species when grown on certain culture media. The evolutionary relationships between the genera were evaluated by phylogenetic analyses of sequence data from the 18S, 28S, 5.8S rDNA genes and ITS1 and ITS2 regions. Results of several different analyses were in agreement in indicating that *Memnoniella* is paraphyletic to *Stachybotrys.*

Key Words: morphology, phylogenetic analysis, rDNA, taxonomy

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INTRODUCTION

The anamorphic genus *Memnoniella* Höhnel currently comprises four species: *M. echinata* (Rivolta) Galloway, *M. subsimplex* (Cooke) Deighton, *M. levispora* Subramanian and *M. zingiberis* Rao. In the most recent analytical treatment of the genus, Jong and Davis (1976) examined only *M. echinata* and *M. subsimplex. M. levispora* and *M. zingiberis,* were mentioned but neither was available in culture at the time; however the latter species was suggested, based on the description and illustration of Rao (1962), to be synonymous with *M. echinata.* Morphologically, *Memnoniella* species resemble members of the related genus *Stachybotrys* Corda in the production of macronematous conidiophores with an apical cluster of several unicellular conidiogenous cells bearing unicellular enteroblastic-phialidic conidia (Barron 1968, Kendrick and Carmichael 1973). They are distinguished principally by the maintenance of their phialoconidia in long persistent chains rather than conidia in slime-covered aggregates (Ellis 1971, Campbell 1975) and by their more globose, rather than typically ellipsoid shaped, conidia. The species differ from one another primarily in conidium size with reported diameter ranges of 3–6 μm for *M. echinata* and 6–9 μm for *M. subsimplex* (Jong and Davis 1976) and 4.4–6.8 μm for *M. zingiberis,* (Rao 1962). *M. levispora* was described as having smooth walled conidia with a diameter range of 3–7 μm (Subramanian 1954).

While widely accepted at present, the recognition of *Memnoniella* and *Stachybotrys* as separate genera has been controversial. Several investigators have considered them to be synonymous (Smith 1962, Kendrick and Carmichel 1973, Carmichael et al 1980). Species of both genera appear to occupy similar natural habitats as saprobes in soil or on litter (Jong and Davis 1976). The two most extensively studied species, *M. echinata* and *S. chartarum,* are both widely distributed geographically and share many physiological and biochemical characteristics (Marsh and Bollenbacher 1946, Perlman 1948, Jermyn 1953).These include the ability to degrade cellulose and use it as a carbon source for growth. Both species have received attention for their roles in the deterioration of fabrics made from plant fibers (White et al 1949). Another similarity is their pro-

duction of an overlapping spectrum of biologically active secondary metabolites and mycotoxins. *Stachybotrys chartarum* produces both simple and macrocyclic trichothecenes, cyclosporins and phenylspirodrimanes whereas *M. echinata* produces simple trichothecenes, phenylspirodrimanes and griseofulvins (Jarvis et al 1995, Jarvis et al 1998). Several other species of *Stachybotrys* have been found to also produce trichothecene mycotoxins (El-Maghraby et al 1991, Ayer and Miao 1993).

Interest in *Stachybotrys* and *Memnoniella* has increased greatly in recent years with the recognition that their habitats can include water-damaged building interiors. Numerous adverse health effects among building occupants have been reported to be associated with indoor occurrences of *S. chartarum* (Croft et al 1986, Johanning et al 1993, Johanning et al 1996, Cooley et al 1998, Etzel et al 1998). These effects have been hypothesized to result from the inhalation of toxin-laden conidia of this organism (Sorenson et al 1987). *M. echinata* has also been found in buildings, and has been suggested to also present a health risk to occupants (Sorenson et al 1996, Jarvis et al 1998). Other species of *Stachybotrys* and *Memnoniella* have been found less commonly in general and have not been reported in human dwellings.

Recent comparative analyses of nuclear ribosomal DNA sequences have shown that purported strains of *M. echinata* and *M. subsimplex* exhibit sequence variability with respect both to one another and to various *Stachybotrys* species (Haugland and Heckman 1998). The sequences examined in that study included 361 bases at the 3′ terminus of the 18S rRNA gene, the complete 5.8S rRNA gene and internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and 81 bases at the 5′ terminus of the 28S rRNA gene. Phylogenetic analyses using these sequences indicated that the two *Memnoniella* species occurred within a clade of *Stachybotrys* species that included *S. chartarum, S. kampalensis, S. dichroa, S. oenanthes, S. cylindrospora, S. microspora* and *S. nephrospora*. Although the evolutionary relationships between most of the species within this clade were poorly resolved, the results did suggest that the two *Memnoniella* species were monophyletic. These results, however, were based on nucleotide sequence data from only one strain each of *M. echinata* and *M. subsimplex*.

In the present investigation we have extended our ribosomal DNA (rDNA) sequence based analysis of the genus *Memnoniella* by examining 14 additional strains, mainly representing *M. echinata* and *M. subsimplex* and one isolate identified originally as *M. zingiberis*. The main objectives were to assess the degree of sequence variability both within and between these species and to further investigate their evolutionary relationships with members of the genus *Stachybotrys*. For the latter purpose, an additional ca 542 bases of 28S rDNA from representative strains and species of both *Memnoniella* and *Stachybotrys* were sequenced and incorporated, together with the previous data, into phylogenetic reconstructions. It was found that the species assignments previously given to several of the *Memnoniella* strains were inconsistent with their sequence derived relationships. For this reason, a second objective became to reassess whether currently used morphological features are reliable for the accurate identification of *Memnoniella* species.

MATERIALS AND METHODS

Fungal cultures and DNA manipulations.—The *Memnoniella* and *Stachybotrys* species and strains analyzed are listed in TABLE I. Type cultures were unavailable in most cases; however, at least one strain representing each species in this study was also examined in the previous analytical evaluation of the two genera by Jong and Davis (1976). These strains are identified with asterisks in TABLE I. Procedures for the growth and harvesting of mycelia and extraction and gel electrophoretic analysis of genomic DNA from some of the strains were previously described (Haugland and Heckman 1998). A slight modification of the previous procedure was used for the preparation of genomic DNA from the newly acquired *Memnoniella* strains. Mycelia were obtained from 5-d-old cultures grown at 21–22 C on potato dextrose agar (Difco Laboratories, Detroit, Michigan). DNA were prepared by grinding 10–30 mg of frozen-thawed mycelia for ~2 min in 1.5-mL polypropylene tubes with a Teflon pestle (Kimble/Kontes, Vineland, New Jersey). The pestle was driven at ~300 rpm by a variable speed homogenizer (Glas-Col, Terre Haute, Indiana). Approximately 50 μL of 3% sodium dodecyl sulfate lysis buffer was added to the tissue prior to grinding. After grinding, an additional 200 μL of the same lysis buffer was mixed with the samples which were then further processed using a modification of the method of Lee and Taylor (1990) as previously described (Haugland and Heckman 1998).

Polymerase chain reaction and DNA sequencing.—Ribosomal DNA fragments from the different strains were amplified with the primers NS91 and IT60 (Haugland et al 1999) and/or with the primers NS70 or NS71 and NL21 (Haugland and Heckman 1998). The former primers amplify 164 and 81 bases of the 18S and 28S rRNA genes, respectively, together with the intervening ITS1–5.8S-ITS2 regions. The latter primers amplify the same regions plus an additional ca 197 and 542 bases of 18S and 28S rDNA. Polymerase chain reaction (PCR) reagents, thermal cycling conditions and amplicon purification procedures have been previously described (Haugland and Heckman 1998); however, for some reactions a model PTC-200 thermal cycler (M J Research, Watertown, Massachusetts) with a heated lid was used. In these instances, no mineral oil was used in the reactions and the previously used hot start procedure (i.e.,

TABLE 1. Fungal cultures and rDNA sequences

Species Strain[a], name as received	Origin; substrate	GenBank sequence accession No.	Sequence boundaries[b]	Sequence group[c]
Stachybotrys echinata (Rivolta) Smith				
UAMH 6594, *Memnoniella echinata*	Alberta, Canada; Indoor air	AF081470	NS70, NL21	1
NRRL 1982, *Memnoniella echinata*	S. Pacific Is.; Military equip.	AF205443	NS91, IT60	2
NRRL 2181, *Memnoniella echinata*	Unknown; Unknown	AF205444	NS91, IT60	2
*NRRL 2373, *Memnoniella echinata*	New Guinea; Canvas	AF205445	NS91, IT60	2
NRRL 1694, *Memnoniella echinata*	Unknown; Unknown	AF205446	NS91, IT60	3
ATCC 200581, *Memnoniella echinata*	Ohio; Home interior	AF205447	NS91, IT60	3
JS63-09[d], *Memnoniella echinata*	Ohio; Home interior	AF205448	NS91, IT60	3
NRRL 1884, *Memnoniella echinata*	Unknown; Unknown	AF205449	NS91, IT60	4
ATCC 20513, *Memnoniella echinata*	Japan; Soil	AF205450	NS91, IT60	4
ATCC 34173, *Memnoniella echinata*	Alabama; *Bambusa* sp.	AF205451	NS91, IT60	5
*ATCC 22697, *Memnoniella echinata*	New Guinea; forest soil	AF205452	NS91, IT60	6
Stachybotrys subsimplex Cooke				
ATCC 32888, *Memnoniella echinata*	Florida; Water hyacinth	AF205439	NS70, NL21	7
ATCC 32334[e], *Memnoniella echinata*	Sri Lanka, *Zingiber* sp.	AF205442	NS91, IT60	8
*ATCC 22700, *Memnoniella subsimplex*	New Guinea; Forest soil	AF205440	NS91, IT60	9
*ATCC 18838, *Memnoniella subsimplex*	Japan; Unknown	AF205441	NS91, IT60	10
Stachybotrys sp.				
*ATCC 22699, *Memnoniella subsimplex*	Japan; Forest soil	AF081471	NS70, NL21	11
Stachybotrys chartarum (Ehrenberg: Link) Hughes				
*ATCC 9182, *Stachbotrys chartarum*	Unknown; Unknown	AF081468	NS70, NL21	—
UAMH 6417, *Stachybotrys chartarum*	Namibia; Desert sand	AF206273	NS91, IT60	—
Stachybotrys albipes (Berkeley & Broome) Jong & Davis				
*ATCC 18873, *Stacybotrys albipes*	England: *Ulmus* sp.	AF081478	NS71, NL21	—
Stachybotrys bisbyi (Srinivasan) Barron				
*ATCC 22173, *Stachybotrys bisbyi*	Uknown; *Zea mays*	AF081480	NS70, NL21	—
Stachybotrys cylindrospora Jensen				
*ATCC 18851, *Stachybotrys cylindrospora*	Canada; Peat soil	AF081474	NS70, NL21	—
Stacybotrys dichroa Grove				
*ATCC 18913, *Stachybotrys dichroa*	England; *Senecio jacobaca*	AF081472	NS70, NL21	—
Stachybotrys kampalensis Hansford				
*ATCC 22705, *Stachybotrys kampalensis*	Forest soil; New Guinea	AF081477	NS70, NL21	—
Stachybotrys microspora (Mathur & Sankhla) Jong & Davis				
*ATCC 18852, *Stachybotrys microspora*	Nigeria; *Arachis hypogaea*	AF081475	NS70, NL21	—
Stachybotrys nephrospora Hansford				
*ATCC 18839, *Stachybotrys nephrospora*	Japan; Herb stem	AF081476	NS70, NL21	—
Stachybotrys parvispora Hughes				
*ATCC 18877, *Stachybotrys parvispora*	Congo; Soil	AF081483	NS70, NL21	—
Fusarium sambucinum Fuckel				
NRRL 13708, *Fusarium sambucinum*	Unknown; Unknown	Multiple[f]	NS70, NL21	—

[a] Strains designated with asterisk (*) symbols were examined for morphology in the most recent extensive treatment of the genera *Strachybotrys* and Memnoniella (Jong and Davis 1976) and were concluded to be representative of the assigned species. Culture collection sources of strains: University of Alberta Microfungus Collection and Herbarium (UAMH), Devonian Botanic Garden, Edmonton, Alberta, Canada, T6G 2E1; Northern Regional Research Laboratory (NRRL), U. S. Department of Agriculture, Agricultural Research Science Culture Collection, Northern Regional Research Laboratory, 1815 University Street, Peoria, Illinois, 61604; American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110.

addition of polymerase enzymes to the reactions after a preliminary DNA melting step) was omitted.

DNA sequencing reactions were performed using double-stranded PCR amplicons as templates and ABI PRISM™ Dye Terminator Cycle Sequencing Kit reagents including AmpliTaq FS (PE Biosystems, Foster City, California) according to the manufacturer's instructions. Sequencing strategies and sequences of the majority of primers used in this study have been reported earlier (Haugland and Heckman 1998, Summerbell et al 1999). Additional primers used for the sequencing of 28S rDNA were NL11 (5′-AAGCATATCAATAAGCGGAGGA, base positions 895–916 of the strain UAMH 6594 sequence), NL10 (5′-GAGACCGATAGCGCACAAGTA, base positions 1184–1204) and NL20 (reverse complement of NL10). Purification of the extension products was performed by precipitation with 74 μL of 70% ethanol containing 0.5 mM MgCl2 for 30–60 min at room temperature followed by centrifugation at 20 800 *g* in a microcentrifuge for 20 min. The pellets were washed with 0.4 mL 70% ethanol, dried for 15–30 min in a vacuum centrifuge (Savant Industries, Holbrook, New York) and redissolved with subsequent heating for 2 min at 90 C in 5:1 formamide/25 mM EDTA loading buffer according to guidelines provided by the sequencing kit manufacturer. Electrophoresis and automated analyses of extension products were performed using a model 373A DNA sequencer (PE Biosystems). The SeqMan™ software program (PE Biosystems) was used for compilation and editing of the multiple sequences generated from each template as well as for the identification of redundant final sequences from different strains. GenBank accession numbers of the sequences for each strain are listed in TABLE I.

Sequence alignments and phylogenetic analyses.—Sequence alignments were obtained from the Clustal V software program (Higgins et al 1992) using default parameters. The positions of some gaps in the outputs from this program were manually changed with a word processing program to improve the alignments. When required for subsequent analyses, exclusion of positions containing gaps as well as those appearing to contain ambiguous alignments was performed in MacClade version 3.0 (Madison and Madison 1992). Searches for most parsimonious trees were performed in PAUP version 3.0 (Swofford 1993) using either branch and bound or heuristic methods with characters defined as unordered and with equal weights. Bootstrap results were obtained from 1000 heuristic searches with groups occurring at 50% or greater frequencies being retained in consensus trees. Unless otherwise specified, all heuristic searches were performed by the tree-bisection-reconstruction branch swapping method with simple sequence addition and MULPARS options in effect. Searches for trees based on maximum likelihood and distance criteria were performed in PAUP* version.4.0b2 (Swofford 1998) using program default settings. Congruence between sequence data sets was measured using the partition-homogeneity test (Farris et al 1995) in PAUP* v.4.0b2 based on 1000 heuristic searches. Statistical comparisons of the relative likelihoods of different tree topologies were obtained from maximum likelihood analyses using the Kishino-Hasagawa test (Kishino and Hasagawa 1989), also in PAUP* v.4.0b2. Phylograms were generated using the TreeView software program (Page 1996). The sequence alignments are available from TreeBASE S527.

Morphological studies.—Cultures were grown for 2–6 wk at 21–22 C on corn meal agar (CMA) (Difco Laboratories, Detroit, Michigan), cellulose agar (CA) (ATCC 1991) or malt extract agar (MEA) (Raper and Thom 1949) plates overlaid with nylon filters (Micron Separations, Westboro, Massachusetts). Conidial structures from the colonies were examined in 85% lactic acid on microscope slides under oil immersion at ×1000 in a Zeiss Axiophot microscope. Conidia and phialide size measurements were determined from photomicrographs using a calibrated 9.9-μm objective scale bar imprinted on the photographs. For scanning electron microscopy (SEM), 10-mm^2 sections of the filters containing fungal colonies were fixed for 2 h with a 3% glutaraldehyde solution, prepared from 25% glutaric dialdehyde stock reagent diluted in phosphate Millonig buffer, pH 7.3 (JEOL 1993, Millonig 1976). The specimens were washed twice for 10 min with phosphate Millonig buffer and dehydrated by 5-min exposures to a series of increasing concentrations of ethanol in water from 30 to 100%, in gradations of 10% (Hayat 1989). Critical-point drying was performed with the specimens initially submerged in absolute ethanol in a Autosamdri 814B automatic critical point drying apparatus (Tousimis, Rockville, Maryland) operated in the manual operation mode. The dried specimens were mounted on 12-mm-diam aluminum posts with 12-mm carbon-conducting adhesive tabs and gold coated at an applied current of 45 mA for 160 s in a Large Desk II Cold Sputter Etch Argon Purged Coater (Denton Vacuum, Inc., Cherry Hill, New Jersey), with the vacuum adjusted to 80 mT. SEM was performed with a JSM 5800LV microscope (JEOL Ltd., Tokyo, Japan) under high vacuum using secondary emission imaging. Polaroid type 55 positive/negative film was used to record the images.

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[b] Positions adjacent to the 3′ and 5′ ends of the indicated forward and reverse primers, respectively.

[c] Denotes different sequences among *Memnoniella* strains only, based upon comparisons of the rDNA region bounded by primers NS91 and IT60.

[d] Strain provided by William Sorenson, National Institutes of Occupational Safety and Health, 1095 Willowdale Road, Morgantown, West Virginia. Referenced in Jarvis et al 1998.

[e] Strain also maintained as *Memnoniella zingiberis* (strain number 696.73) at the Centraal Bureau voor Schimmelcultures (CBS) Culture Collection, Oosterstraat 1, 3740 A G Baarn, The Netherlands.

[f] Merged sequences from GenBank: AF081467, X65480 and X65474.

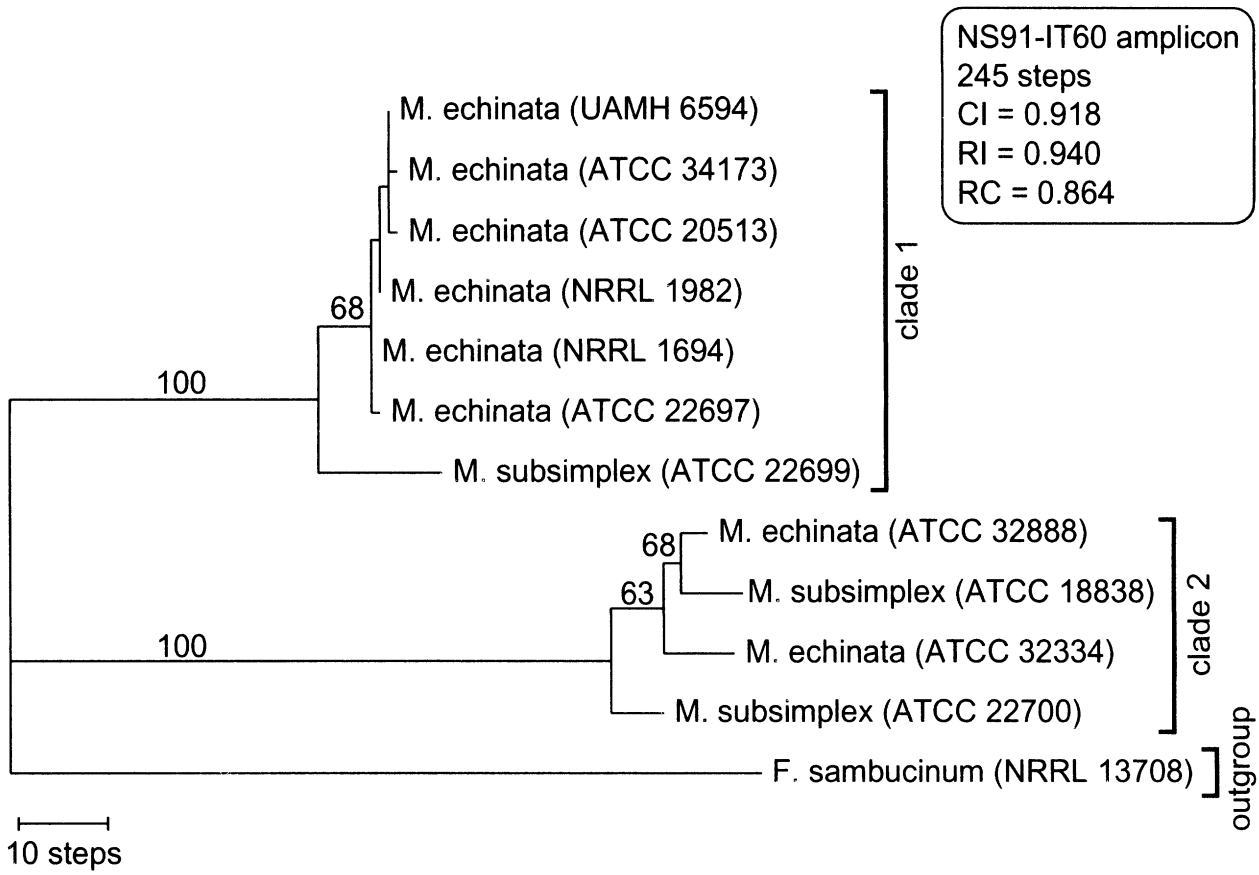

FIG. 1. One of 44 most parsimonius trees obtained from a branch and bound search of aligned NS91–IT60 sequences (Treebase matrix accession number M772) of *Memnoniella* strains. The other 43 most parsimonius trees differed only in the topology of the *M. echinata* strains in clade 1. Species names listed are those given by the sources of the strains. The sequence alignment contained 756 positions. All positions were included in the analysis and gaps were treated as a fifth character state to allow resolution of highly similar sequences. A total of 109 positions was parsimony informative. This is an unrooted tree. Bootstrap values greater than 50%, calculated from 1000 heuristic search replications, are indicated above the branches.

RESULTS

Phylogenetic and morphological analyses of Memnoniella *strains.*—Eleven different sequences were identified among the 16 *Memnoniella* strains examined (TABLE I) for the rDNA region amplified by the primers NS91 and IT60. Parsimony analysis based on an alignment of these 11 unique sequences indicated that the organisms were grouped into two highly divergent and well supported clades (FIG. 1). Clade 1 predominantly comprised strains identified by their sources as *M. echinata* (TABLE I), but also contained strain ATCC 22699 which was the *M. subsimplex* representative used in our previous phylogenetic analyses (Haugland and Heckman 1998). Clade 2 contained two strains on deposit in ATCC as *M. subsimplex* (ATCC 22700, ATCC 18838) and one as *M. echinata* (ATCC 32888). A fourth strain in clade 2 (ATCC 32334) is listed as *M. echinata* by ATCC but held under the name *M. zingiberis* at CBS (TABLE I).

The apparent discrepancies between the sequence based relationships of some of the strains and their original identifications led us to reexamine the morphological characteristics of the different strains using light microscopy and SEM. These observations

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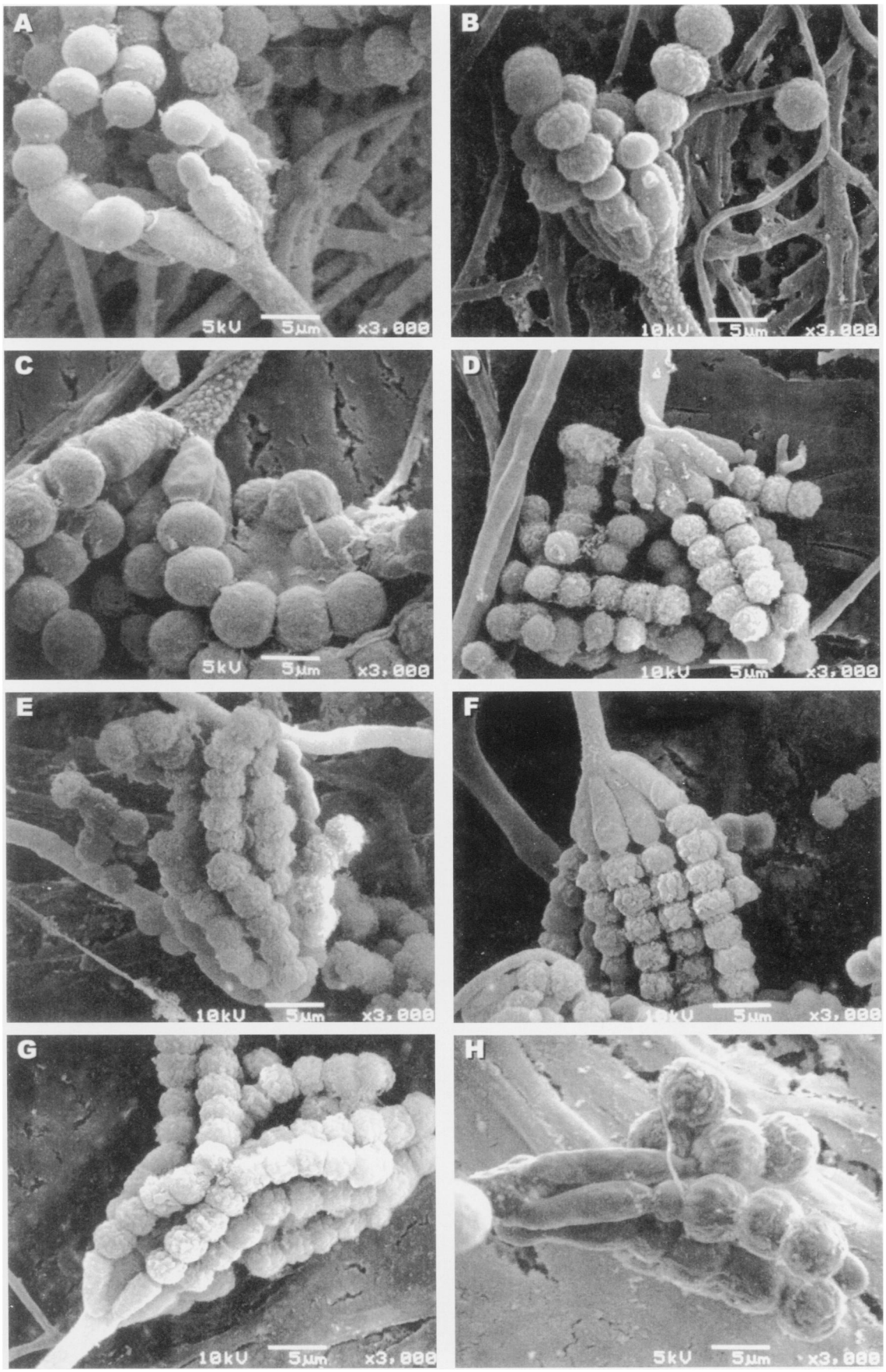
FIG. 2. SEM SEI (secondary emission imaging) micrographs showing examples of conidiophore, phialide and phialoconidia morphologies of representative *Memnoniella* strains grown on CMA medium. A: *M. subsimplex* strain ATCC 32888 (received as *M. echinata*). B: *M. subsimplex* strain ATCC 22700. C: *M. subsimplex* strain ATCC 18838. D: *M. echinata* strain UAMH 6594. E: *M. echinata* strain ATCC 20513. F: *M. echinata* strain ATCC 34173. G: *M. echinata* strain NRRL 1982, H: *M. sp.* strain ATCC 22699 (received as *M. subsimplex*).



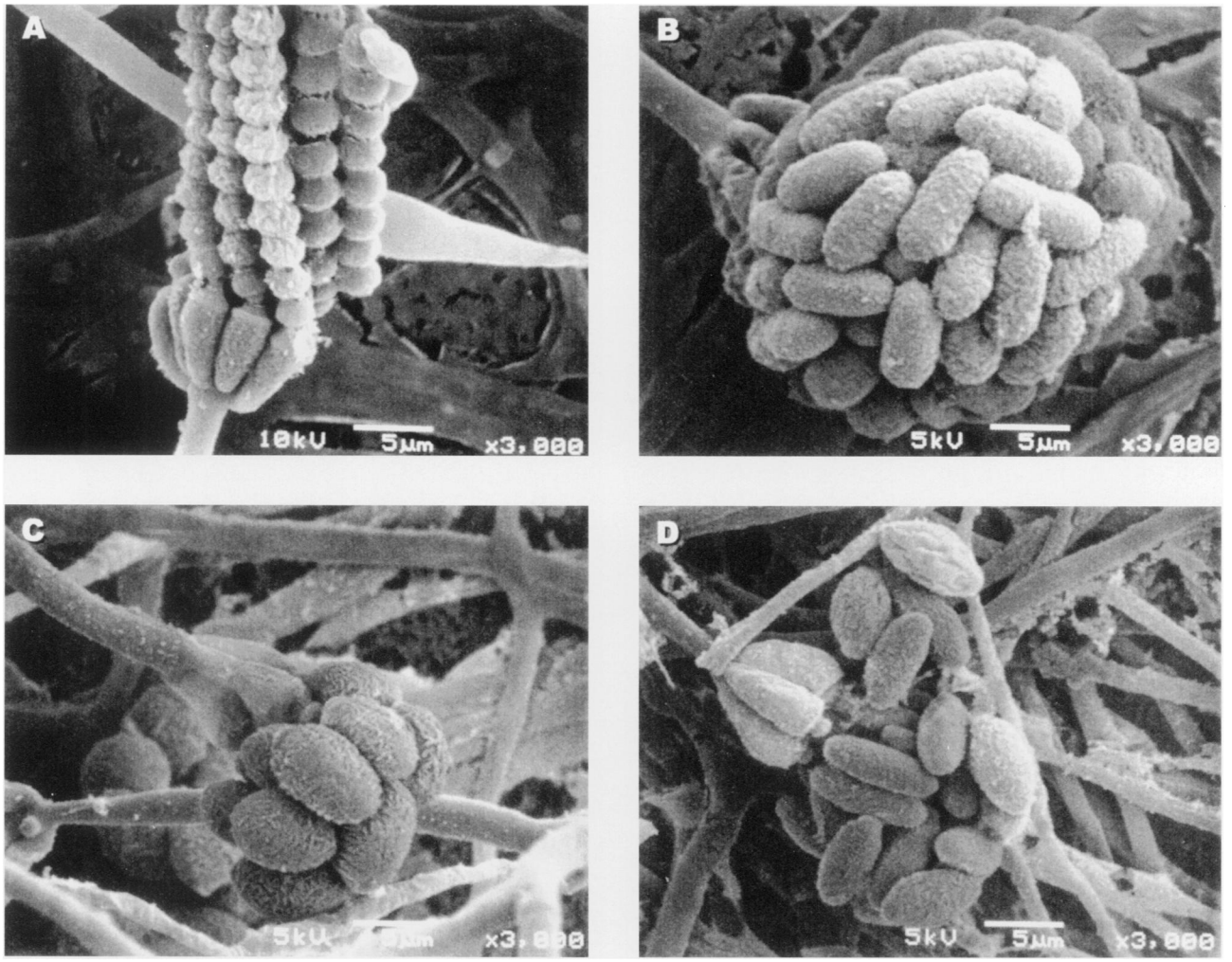


FIG. 3. SEM SEI micrographs showing examples of conidiophore, phialide and phialoconidia morphologies of *Memnoniella echinata* strain ATCC 22697 and *Stachybotrys chartarum* strains grown on CA medium. A: Strain ATCC 22697 with typical *M. echinata* morphological characters. B: Strain ATCC 22697 with *Stachybotrys*-like characters from the same colony as shown in panel A. C: *S. chartarum* strain UAMH 6417. D: *S. chartarum* strain ATCC 9182.

revealed that the strains in clade 2, with the exception of strain ATCC 32334 which failed to sporulate in our laboratory, showed similar morphologies (FIG. 2, panels A, B, and C) that were most consistent with the current description of *M. subsimplex* (Jong and Davis 1976). Mature conidia were globose, approx 5–7 µm diam, slightly to only moderately roughened and consistently present in chains. Phialides were approx 9 × 5 µm. Conidiophores were often extensively warted.

The majority of strains in clade 1 were also morphologically similar to one another and were consistent with the current description of *M. echinata* (cf. FIG. 2, panels D, E, F and G). Mature conidia were globose but often flattened along their vertical axis, approx 3–5 µm diam, and usually coarsely roughened and present in long chains. Phialides were approximately 7 × 3 µm. Conidiophores were usually either smooth or only slightly roughened.

Two strains in clade 1 exhibited morphologies and/or conidial arrangements that were distinct. Mature conidia from strain ATCC 22699 (FIG. 2, panel H) were globose but larger, approx 7–8 µm diam, not usually as deeply roughened and formed on larger phialides of approximately 12 × 4 µm. Conidiophores of this strain were often warted in a similar manner as the strains in clade 2.

Strain ATCC 22697, grown on either CMA or CA, assumed two distinctly different forms. One was typified by extended chains of small, nearly globose conidia (FIG. 3, panel A), similar to the other *M. echinata* strains in clade 1. Conidia of the second form were predominantly cylindrical but occasionally ellipsoidal, approx 7–9 × 3–4 µm, moderately roughened

and present in aggregates rather than chains (FIG. 3, panel B). Conidia of *Stachybotrys chartarum* strains, grown on the same media, were similar to the latter form (FIG. 3, panels C and D) but were usually more ellipsoidal, slightly larger (8–11 × 3–6 μm) and were smooth or irregularly ridged rather than uniformly roughened. Both strain ATCC 22697 and *S. chartarum* strains exhibited much looser aggregates of conidia on CMA than on CA medium (results not shown). This appeared to correspond to a higher water content of the slime associated with these aggregates on the former medium. The relative abundance of the two different morphological forms in strain ATCC 22697 was also observed to be medium-dependent. On CMA, the *Stachybotrys*-like form was predominant; on CA the two forms were present in roughly equal abundance (varying somewhat from culture to culture), and on MEA nearly all structures were of the typical *M. echinata* form. These respective forms and relative abundances were maintained on each of the media types during repeated subculturing.

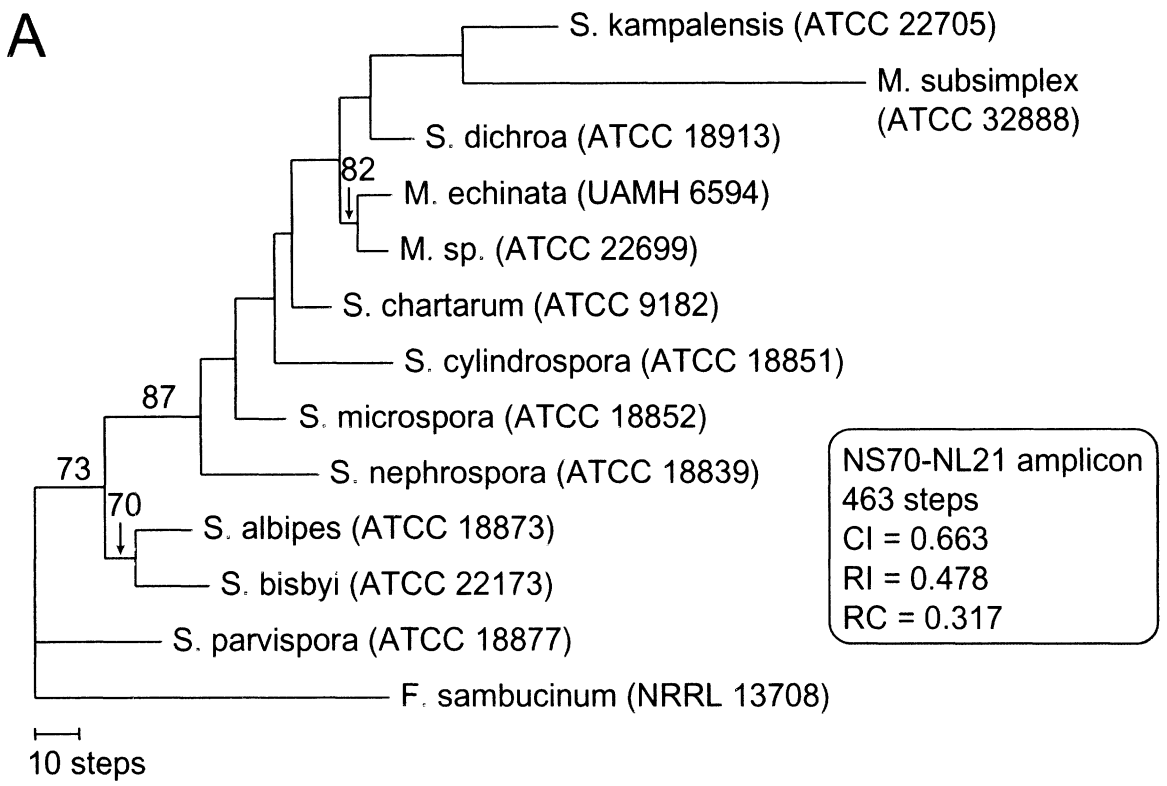
Phylogenetic relationships of Memnoniella *and* Stachybotrys *species.*—The high degree of rDNA sequence divergence exhibited by the two clades of *Memnoniella* strains described above led us to reexamine the question of whether *Memnoniella* is a monophyletic genus. To address this question, sequences of the rDNA region amplified by the primer pair NS70 (or NS71) and NL21 from representative *Memnoniella* and *Stachybotrys* strains or species, were aligned and subjected to phylogenetic analyses using maximum parsimony, neighbor joining and maximum likelihood approaches. *Memnoniella* strains incorporated into these analyses included UAMH 6594 as a representative of *M. echinata,* ATCC 32888 as a representative of *M. subsimplex* and the atypical strain ATCC 22699 from clade 1. These analyses included a total of 1141 non-excluded alignment positions (68 parsimony informative) from rRNA gene coding sequences and 275 non-excluded alignment positions (51 parsimony informative) from the internal transcribed spacer regions. Homogeneity partitioning analysis, based on 1000 heuristic searches, indicated that the coding and spacer region data sets were congruent ($P = 0.72$) and so the combined data sets were used in all analyses.

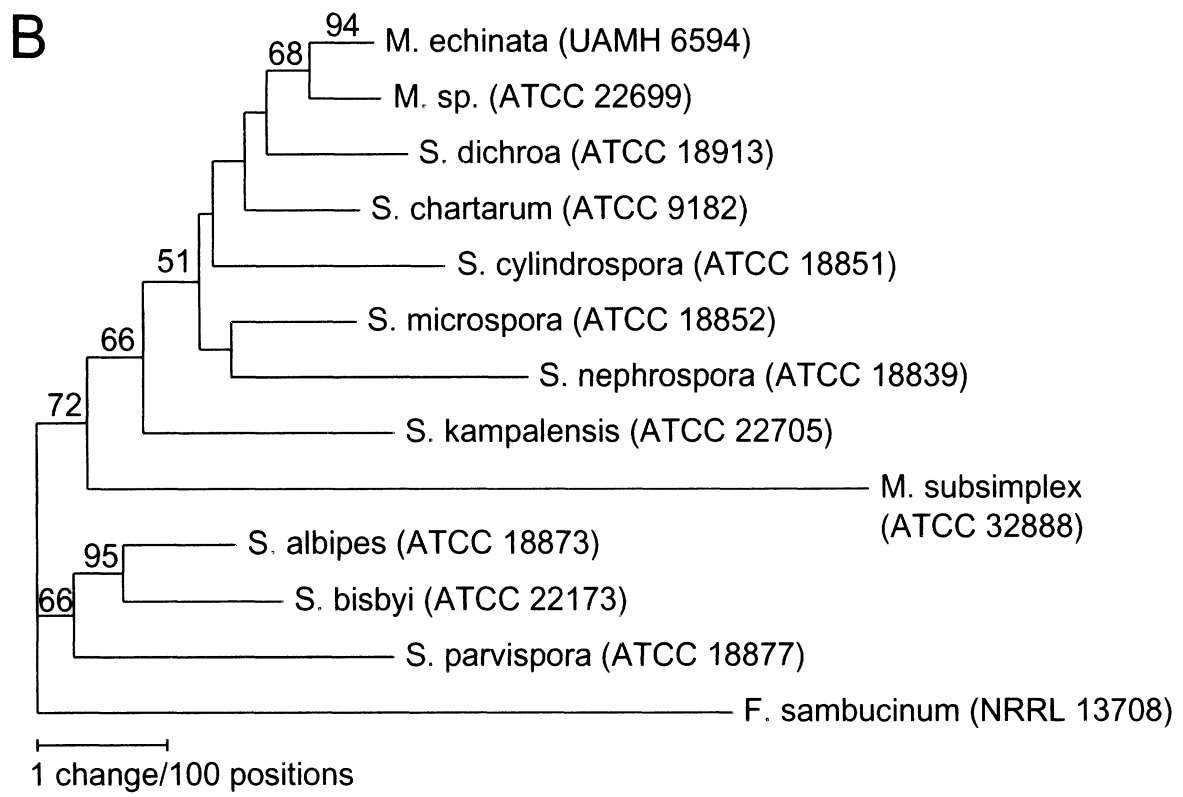
Optimal trees identified from parsimony and neighbor joining searches (FIG. 4) showed similar topologies that differed primarily in the branching positions of *Memnoniella* strain ATCC 32888 and *Stachybotrys kampalensis.* A global heuristic search using maximum likelihood as the optimality criterion resulted in a tree (−Ln = 4562) that was identical in topology to the most parsimonius tree. This tree was not found to be significantly more likely ($P = 0.54$) in the Kishino-Hasegawa test than the tree obtained from the neighbor joining search (−Ln = 4578).

While the *Memnoniella* strains from clades 1 and 2 were indicated to be paraphyletic to *Stachybotrys* in the optimal trees identified by each of the different search algorithms, bootstrap analyses provided either weak or moderate support for the branches resolving these organisms (FIG. 4). To further test the hypothesis that *Memnoniella* is paraphyletic to *Stachybotrys,* a parsimony search of the same data set was performed under the constraint that the *Memnoniella* strains remain monophyletic. This search identified three most parsimonius trees of 478 steps (results not shown) as compared to 463 steps for the tree previously identified in the absence of constraints. Likelihood analyses of these constraint trees gave −Ln values of 4597, 4600 and 4602 which, in each case, were significantly less likely ($P < 0.05$) than that of the unconstrained tree in the Kishino-Hasegawa test.

DISCUSSION

The results of this study support the recognition of at least three species within the genus *Memnoniella.* A fourth described species, *M. levispora,* was not examined here. Both DNA sequence and morphological data indicated that the majority of strains in clade 1 (FIG. 1) are a closely related group representing the species concept of *M. echinata* as defined by Jong and Davis (1976). The exception is strain ATCC 22699 which is indicated by these criteria to differ from all other strains and thus appears to represent a different species. The strains in clade 2 of FIG. 1 exhibited somewhat greater rDNA sequence divergence but generally agree with the species concept of *M. subsimplex.* The genetic differences could support the further taxonomic division of this group; however, since no clear differences in morphology were seen, more information about them (e.g., biochemical properties, ranges of habitat and geographic distribution and/or comparative sequence analyses of additional genes) would be useful before making any such further distinctions. Strain ATCC 32334 in clade 2 was originally identified as *M. zingiberis* by Dr. W. Gams and was isolated from *Zingiber sp.,* collected in Sri Lanka; however, it was later reassigned at the ATCC as *M. echinata* (TABLE I). The identification as *M. zingiberis* could not be confirmed because the isolate failed to sporulate in our laboratory; however, the conidial size range and description given for *M. zingiberis* (Rao 1962) is very similar to that which we observed for the other strains in clade 2. This suggests that *M. zingiberis* is synonymous with *M. subsim-*





plex and not with *M. echinata* as previously suggested (Jong and Davis 1976). Strain ATCC 32888 in clade 2 was also incorrectly assigned at *M. echinata*. The incorrect identification of *M. subsimplex* strains as *M. echinata* may be due to more widespread familiarity with the latter species (White et al 1949).

The true branching order of *M. echinata, M. subsimplex* and most *Stachybotrys* species remains uncertain, as evidenced both by the discrepancies between our neighbor joining and parsimony analysis based trees and also by the weak bootstrap support indicated for most branches in each of these trees. The representative *M. subsimplex* strain, ATCC 32888, was shown to occur on a particularly long branch that could contribute to artifacts in these trees (Swofford and Olsen 1990). Elimination of this organism from the analyses did not, however, substantially improve the overall quality measures of the resultant parsimony-derived tree (consistency index = 0.672, retention index = 0.518, rescaled consistency index = 0.348), nor did it significantly increase the bootstrap support for most of the resultant branches (results not shown). An exception was the branch setting off *S. albipes* and *S. bisbyi* as a basal sister clade which was found in 99% of all bootstrap replicates when the *M. subsimplex* sequence was excluded.

Despite the continued uncertainty in the precise relationships of many of these species, all phylogenetic analysis results support the previously proposed relegation of *Memnoniella* to synonymy with *Stachybotrys* (Smith 1962). The morphological features that are used at present to distinguish *Memnoniella* as a separate genus, i.e., production of globose phialoconidia in dry chains, are suggested by our results to be derived character states that have developed in two different terminal clades of organisms. Evidence that the alternative states, i.e., occurrence of non-globose conidia in disorganized, slimy masses, are ancestral can be seen from their presence in members of the basal sister clade represented by *S. albipes* and *S. bisbyi*. From the observations of Zuck (1946) as well as our own observations of ATCC strain 22697, the ability to exhibit these ancestral states is retained by some strains of *M. echinata*.

TAXONOMY

On the basis of the data and observations presented here, we recognize the following species.

Stachybotrys echinata (Rivolta) Smith 1962, Trans. Br. Mycol. Soc. 45: 392

Syn. *Memnoniella echinata* Rivolta 1933, Trans. Br. Mycol. Soc. 18: 165

Phialoconidia highly roughened, globose to subglobose, approximately 3–5 μm diam., in dry chains, rarely demonstrating cylindrical conidia, 7–9 by 3–5 μm, in slimy aggregates.

Represented by strains listed under this species name in TABLE I. Epitype strain: UAMH 6594.

Stachybotrys subsimplex Cooke 1883, Grevillea 12: 33

Syn. *Memnoniella subsimplex* (Cooke) Deighton 1960, CMI Mycol. Papers 78: 5

? *Memnoniella zingiberis* Rao 1962, Sydowia 16: 43

Phialoconidia slightly to moderately roughened, globose, approximately 5–7 μm diam., in dry chains. Jong & Davis (1976) reported the conidial size range as 6–9 μm diam; however, that description encompassed the strain producing larger conidia (ATCC 22699) here recognized as a separate species. Molecular results placed a representative strain (ATCC 32334) of *M. zingiberis* within the *S. subsimplex* clade. The conidial size range for *M. zingiberis* was reported as 4.4–6.8 μm (Rao 1962). Although these dimensions could not be confirmed by us because we had no authentic culture and ATCC 32334 failed to sporulate, the conidial size range agrees with our observations for *M. subsimplex*. Represented by strains listed under this species name in TABLE I. Epitype strain: ATCC 32888.

Stachybotrys sp.

Phialoconidia moderately to extensively roughened, globose, ca 7–8 μm diam. Represented by ATCC 22699. A formal diagnosis of this species will appear in a later publication.

←

FIG. 4. Phylogenetic analyses of aligned NS70(NS71)-NL21 rDNA sequences (Treebase M773) among selected *Memnoniella* and *Stachybotrys* strains and species using parsimony and distance methods. A: Single most parsimonius tree obtained from both branch and bound and heuristic searches. The sequence alignment contained 1557 positions, of which 141 were excluded due to the occurrence of gaps and/or ambiguous alignment and 119 were parsimony informative. B: Neighbor-joining tree obtained from analysis of the same sequence alignment with excluded positions. Bootstrap values greater than 50%, calculated from 1000 search replications, are indicated above the branches of both trees. *F. sambucinum* was used as an outgroup in both analyses, however, both trees are unrooted.

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