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Molecular phylogeny of *Acremonium* and its taxonomic implications

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Abstract: *Acremonium* is generally considered to be a highly polyphyletic form genus containing distantly related fungi. Sectional divisions within *Acremonium* distinguish the clavicipitaceous grass endophytes of sect. *Albolanosa* from the generally saprobic species of sections *Acremonium, Chaetomioides, Gliomastix,* and *Nectrioidea*. In an effort to assess the possible number of lineages currently placed within *Acremonium* and to determine which groups of sexual ascomycetes are phylogenetically affiliated with *Acremonium* species, maximum parsimony and neighbor-joining analyses were performed using partial sequences of the nuclear small subunit ribosomal DNA (18S rDNA). *Acremonium* was shown to be a polyphyletic taxon with affiliations to at least three ascomycetous orders: 1) most of the examined species from the sections *Acremonium, Gliomastix,* and *Nectrioidea* showed a relationship to the Hypocreaceae even though many of these species have never been associated with any teleomorph; 2) the grass endophytes of sect. *Albolanosa* and other taxa from the Clavicipitaceae formed a monophyletic group derived from within the Hypocreales; 3) the thermophilic *A. alabamense* of sect. *Chaetomioides* was derived from within the Sordariales. *Acremonium alternatum,* the type species of the genus, was one of the species showing affiliation to the Hypocreaceae. In order to eliminate some of the heterogeneity within *Acremonium* while also emphasizing the unique biological, morphological, and ecological characteristics of the grass endo-

phytes, we are proposing that the anamorphs of *Epichloë* and closely related asexual grass endophytes be reclassified into the new form genus *Neotyphodium*. Phylogenetic and taxonomic considerations are also presented for other taxa.

Key Words: Clavicipitaceae, endophytes, *Epichloë*, Hypocreales, *Neotyphodium*, rDNA

INTRODUCTION

Members of the family Clavicipitaceae (Hypocreales; Ascomycotina) are well known pathogens of a diverse assemblage of hosts including grasses, sedges, other ascomycetes, and insects. These fungi are found throughout the tropical and temperate regions of the world (Diehl, 1950; White, 1994b). Rogerson (1970) listed 31 genera in the family, but eight of these are considered to be synonyms. The family is divided into three subfamilies with the insect and fungal pathogens variously placed in the Oomycetoideae and Cordycipitoideae (Diehl, 1950). The plant pathogens are by far the most widely studied and economically important members of the family and are classified as the subfamily Clavicipitoideae (Diehl, 1950). Diehl (1950) further divides the taxa of the Clavicipitoideae into the three tribes Clavicipiteae, Balansieae, and Ustilaginoideae. The fungus-host interactions of these taxa can be broadly categorized as being epibiotic or endophytic, systemic or localized, and heavily pathogenic, moderately pathogenic, or mutualistic.

Most of the grass pathogens are in the tribe Balansieae (Diehl, 1950) and are either localized to epibiotic reproductive stromata on leaves or inflorescences or form systemic endophytic infections intercellularly within their hosts in addition to forming external reproductive stromata. The Balansieae include the teleomorphic taxa *Atkinsonella, Balansia, Balansiopsis, Epichloë,* and *Myriogenospora* (Diehl, 1950; Luttrell and Bacon, 1977; Rykard et al., 1984). *Myriogenospora* is completely superficial without any penetration of the host epidermis (Luttrell and Bacon, 1977; White and Glenn, 1994), but *Atkinsonella* has some localized intercellular hyphae associated with the stroma (Leuchtmann and Clay, 1988; Morgan-Jones and White, 1989). *Balansia* contains a few epibiotic species (Leuchtmann and Clay, 1988; Clay and Frentz, 1993), but most species have a well de-

veloped endophytic habit (White et al., 1995). All species of the *Epichloë typhina* (Pers. : Fr.) Tul. group, including the apparent asexual derivatives of *Epichloë,* are intercellular grass endophytes (White, 1994b). Asexual grass endophytes were largely overlooked as being related to *Epichloë* until Bacon et al. (1977) found intercellular hyphae growing within tall fescue. They referred to the fescue endophyte as a biotype of *E. typhina* based on concurrent *in vitro* examinations and the earlier report by Sampson (1933). The obscurity of these asexual endophytes was a result of the host grasses never showing any symptoms or signs of a fungal infection. For the anamorph of the fungus, Bacon et al. (1977) applied the name *Sphacelia typhina* Sacc. which was the binomial designated by Saccardo (1881) for the anamorph of *E. typhina*. However, *S. typhina* was later considered a *nomen dubium* by Morgan-Jones and Gams (1982) because of insufficient type material. *Typhodium* (Link, 1826) is another genus of uncertain application to the anamorph of *E. typhina* and is now considered a synonym of *Epichloë* (Hawksworth et al., 1983). However, as a "convention of convenience," Diehl (1950) applied *Typhodium* as a form genus and used the term "typhodial" when referring to the anamorph of *Epichloë.*

Based on *in vitro* similarities, Morgan-Jones and Gams (1982) placed the anamorphs of both the fescue endophyte and *E. typhina* in the form genus *Acremonium*. The authors erected the new sect. *Albolanosa* for these endophytic fungi with the *in vitro* characteristics of slow-growing white to yellow colonies, solitary phialides narrower at the base than the subtending hyphae, hyaline and smooth-walled conidia, and teleomorphs in the Clavicipitaceae. They stated that the exclusively solitary phialides of the endophytes preclude them from classification in *Verticillium* sect. *Prostrata* which was restricted to those taxa, such as *Cordyceps* spp., that possess verticillate as well as solitary phialides (see also Gams, 1971). Morgan-Jones and Gams (1982) reported slight *in vitro* morphological differences between the anamorph of *E. typhina* and those of *Holcus mollis* L., *Dactylis glomerata* L., and *Sphenopholis obtusata* (Michx.) Scribn., which they treated as *A. typhinum* Morgan-Jones & W. Gams, and the tall fescue endophyte, which they described as the separate species *A. coenophialum* Morgan-Jones & W. Gams.

Acremonium is a cosmopolitan, morphologically simple genus (see Gams, 1971). *Acremonium alternatum* Link : Fr. is the lectotype and produces conidia in chains or heads. The genus *Cephalosporium* has often been distinguished from *Acremonium* because of the accumulation of conidia in slimy heads. However, Gams (1971) concluded heads versus chains was

untenable as a generic character and merged the two under the older *Acremonium*. The form genus *Gliomastix* and monophialidic species of *Paecilomyces* were also merged into *Acremonium* (Gams, 1971), thereby expanding its definition to comprise generally slow-growing species with hyaline or pigmented conidia that are one-celled or exceptionally two-celled, in chains or slimy heads, and produced from orthophialides or basitonously branched conidiophores.

Acremonium is perceived to be a heterogeneous taxon because several morphologically distinct teleomorphic genera have *Acremonium*-like anamorphs. Most of the known teleomorphs of *Acremonium* are species of *Nectria* (Gams, 1971; Samuels, 1973, 1976a,b; Lowen, 1995), but teleomorphic genera such as *Hypocrea, Hypomyces, Thielavia, Pronectria, Nectriopsis, Epichloë, Emericellopsis, Mycoarachis,* and *Nigrosabulum* also have anamorphs classified in *Acremonium* (Malloch and Cain, 1970; Gams, 1971; Morgan-Jones and Gams, 1982; Samuels, 1988; Lowen, 1995). Gams (1971, 1975) divided *Acremonium* into three sections: *Acremonium, Nectrioidea,* and *Gliomastix*. The majority of the species in these sections are saprobic in a wide variety of habitats, but some are plant parasites. Some species of sect. *Nectrioidea* have teleomorphs in the genus *Nectria,* but most of the species in the three sections do not have any known association to teleomorphs. Along with the establishment of sect. *Albolanosa,* Morgan-Jones and Gams (1982) also erected the new sect. *Chaetomioides* for anamorphs of ascomycetes within the family Chaetomiaceae that have, among other characteristics, short-aculeate to lageniform phialides. *Acremonium alabamense* Morgan-Jones, the type species of sect. *Chaetomioides,* is a thermophilic fungus originally isolated from fallen pine needles (Morgan-Jones, 1974), and it is the anamorph of *Thielavia terrestris* (Apinis) Malloch & Cain (Morgan-Jones and Gams, 1982). Other species of this section are anamorphs of several *Chaetomium* species and cannot be distinguished without their teleomorphs (Morgan-Jones and Gams, 1982). Most recently, sect. *Lichenoidea* was erected for lichenicolous species (Lowen, 1995), and some of these species have hypocreaceous teleomorphs while others are not associated with any teleomorph.

Because of the possible heterogeneity of *Acremonium,* the placement of the grass endophytes in sect. *Albolanosa* was done with no claim of phylogenetic relationship to the nonendophytic species in the other sections (Morgan-Jones and Gams, 1982; White and Morgan-Jones, 1987). This classification scheme was based strictly on *in vitro* morphological similarities. Some disagreement with this classification of the endophytes was initially expressed, and it continues

TABLE I. *Acremonium* taxa included in the analyses and the various sections in which they are classified[a]

Sect. Acremonium[b,c]	Sect. Nectrioidae[b]	Sect. Gliomastix[b]	Sect. Albo-lanosa[d]	Sect. Chaetomioides[d]
A. alternatum	A. rutilum	A. murorum	A. coenophialum	A. alabamense (= Thielavia terrestris)
A. kiliense	A. chrysogenum		A. uncinatum Epichloë festucae (= A. typhinum)	
A. strictum	A. furcatum			
Emericellopsis terricola (= Acremonium sp.)	Nectria vilior[e] (= A. berkeleyanum)		Epichloë amarillians (= A. t. var. typhinum)	
Emericellopsis minima (= Acremonium sp.)				

[a] Teleomorphs are indicated if they are known, and precedence in the listing is given to the teleomorphs except for the ease of *A. alabamense* which was obtained from ATCC (#26796) as *A. alabamense* and not as *T. terrestris*.

[b] Gams (1971).

[c] Gams (1975).

[d] Morgan-Jones and Gams (1982).

[e] See Samuels et al. (1991) and Gams and Van Zaayen (1982).

to be debated (Latch et al., 1984; Rykard et al., 1984; Morgan-Jones et al., 1992; Gams, 1995). The simple morphology of the anamorphs may have been derived multiple times in the evolution of fungi. However, the connection of so many *Acremonium* species to *Nectria* and other genera of the Hypocreaceae suggests that while there may be some heterogeneity created by sect. *Albolanosa* and sect. *Chaetomioides*, *Acremonium* may be more homogeneous than postulated.

The application of strict monophyly has been suggested for fungal systematics and taxonomy so as to reflect the phylogenetic history of a group of organisms (Vilgalys et al., 1993). If a monophyletic classification scheme is to be the ultimate goal for any group of fungi, then the current delimitation of *Acremonium* is open to question. Molecular phylogenetic data are particularly helpful in resolving relationships of morphologically simple organisms such as those in *Acremonium*. Therefore, using sequences of the nuclear encoded small subunit ribosomal DNA (18S rDNA), this project was undertaken to 1) assess the minimum number of separate lineages currently placed within the form genus *Acremonium*, 2) determine which groups of sexual ascomycetes are closely affiliated with *Acremonium* species in the current classification, 3) determine if *Acremonium* sect. *Albolanosa* is appropriate for classification of the grass endophytes, and if not, 4) propose a new taxon for this group.

MATERIALS AND METHODS

Fungal isolates.—A total of fifteen taxa having anamorphs currently classified in *Acremonium* were selected from sections *Acremonium, Albolanosa, Chae-*

tomioides, Gliomastix, and *Nectrioidea* for inclusion in the rDNA comparisons (TABLES I, II). Isolates from sect. *Lichenoidea* were not sampled because of the recent establishment of this newest section. All isolates of *Acremonium* were either type or authenticated cultures, and additional taxa were either personally collected or obtained from other researchers (TABLE II). The fungi were maintained on cornmeal-malt agar (CMM) (Rykard et al., 1982) at room temperature.

Nucleic acid extraction.—For extraction of total genomic DNA from mycelium, all isolates were grown in M102 liquid medium (Rykard et al., 1982) on a rotary shaker (200 rpm) at room temperature until adequate growth occurred (usually 1–2 wk). The mycelium was collected by centrifugation, and the pelleted tissue was washed once with sterile distilled water to remove excess medium. The tissue was then ground in liquid nitrogen and stored at -80 C until ready for nucleic acid extraction. Extractions were made from 0.2–0.5 g (wet weight) of ground mycelium.

The nucleic acid extraction procedure was a modification of Lee and Taylor (1990) in which the chloroform:phenol step was repeated at least once so as to remove as much cellular debris as possible. The extracted nucleic acid samples were diluted to provide solutions with a DNA concentration range of 0.1 to 1.0 ng μL^{-1}.

Polymerase chain reaction and sequencing.—Fifty μL of each diluted sample was used as template for polymerase chain reactions (PCR) (Mullis and Fallona, 1987; Saiki et al., 1988). For each amplification, a total reaction volume of 100 μL was made containing diluted template, 10 mM Tris-HCl (pH 8.3), 50 mM

TABLE II. Taxa included in the analyses, source of isolates, and accession numbers for 18S rDNA sequences

Taxon	Isolate source[a]	GenBank accession #
Acremonium alabamense	ATCC 26796[b]	U43969
Acremonium alternatum	CBS 223.70 & 406.66	U43970
Acremonium chrysogenum	ATCC 14615[b]	U43971
Acremonium coenophialum	ATCC 52274[b]; *Festuca arundinacea*	U45942
Acremonium furcatum	CBS 122.42[b] & 508.65	U43972
Acremonium kiliense	ATCC 34716[b]	U43973
Acremonium murorum	CBS 214.69	U43966
Acremonium rutilum	CBS 225.70 & 394.66	U43967
Acremonium strictum	ATCC 34717[b]	U43968
Acremonium uncinatum	J. F. White; *Festuca pratensis*	U45943
Ascosphaera apis	UCB 78-018	M83264
Atkinsonella hypoxylon	A. E. Glenn; *Danthonia spicata*	U44034
Balansia aristidae	J. F. White; *Aristida* sp.	U44035
Balansia henningsiana	A. E. Glenn; *Andropogon* sp.	U44036
Balansia obtecta	J. F. White; *Cenchrus echinatus*	U44037
Balansia sclerotica	ATCC 16582	U32399
Balansia strangulans	J. F. White; *Panicum* sp.	U44038
Candida tropicalis	MUCL 30002	M55527
Ceratocystis fimbriata	T. C. Harrington C89	U32418
Chaetomium globosum	A. E. Glenn	U44039
Claviceps purpurea	R. T. Hanlin; *Dactylis glomerata*	U44040
Cordyceps capitata	A. E. Glenn; *Elaphomyces* sp.	U44041
Cryhonectria parasitica	R. T. Hanlin 5806	Denise Silva, unpubl.
Daldinia concentrica	ATCC 36659	U32402
Diaporthe phaseolorum	F. A. Uecker 458	L36985
Echinodothis tuberiformis	J. F. White; *Arundinaria tecta*	U44042
Emericellopsis minima	ATCC 16216	U44043
Emericellopsis terricola	CBS 120.40[b]	U44112
Epichloë amarillians	J. F. White; *Agrostis hiemalis* (Ah3)	U35034
Epichloë festucae	J. F. White; *Festuca rubra rubra*	U44113
Eurotium rubrum	UCB 88-016	U00970
Hypocrea pallida	G. J. Samuels 89–83	U32408
Hypomyces polyporinus	ATCC 46844	U32410
Microascus trigonosporus	RSA 1942	L36987
Monascus purpureus	ATCC 16365	M83260
Myriogenospora atramentosa	A. E. Glenn; *Andropogon* sp.	U44114
Myriogenospora atramentosa	A. E. Glenn *Erianthus brevibarbis*	U44115
Nectria cinnabarina	G. J. Samuels 89–107	U32412
Nectria vilior	ATCC 16217	U44116
Neocosmospora vasinfecta	ATCC 28867	U44117
Saccharomyces cerevisiae	—	M27607
Taphrina deformans	ATCC 34556	U00971
Xylaria hypoxylon	ATCC 42768	U20378

[a] ATCC = American Type Culture Collection, USA; CBS = Centraalbureau voor Schimmelcultures, Netherlands; MUCL = Mycothèque de l'Université Catholique, Louvain-la-Neuve, Belgium; RSA = Rancho Santo Ana, USA; UCB = University of California, Berkeley Microgarden, USA.

[b] Type culture.

KCl, 2.5 mM MgCl$_2$, 0.5 μM of each primer, 200 μM of each dNTP, and 2.5 units of AmpliTaq® DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ). Each reaction mixture was topped off with a thin layer of mineral oil and amplified using a Perkin-Elmer DNA Thermal Cycler 480 (Norwalk, CT).

A segment of the small subunit ribosomal RNA gene (18S rDNA) was amplified using primers NS1 and NS6 (White et al., 1990). Thermal cycling parameters for amplification consisted of one initial cycle with denaturation at 95 C for 5 min, annealing at 54 C for 30 s, and extension at 72 C for 45 s. This cycle was followed by 38 cycles with denaturation at 95 C for 30 s, annealing at 54 C for 30 s, and exten-

sion at 72 C for 45 s. (plus 4 sec addition to extension segment per cycle). A final cycle was performed with an extension segment of 72 C for 10 min.

Amplified products were separated from unincorporated nucleotides and primers using either mini-columns (Wizard™ PCR Preps, Promega Corp., Madison, WI) or microconcentrators (Microcon™ 100, Amicon, Inc., Beverly, MA) following each manufacturer's protocol. Purified samples were sequenced by the Molecular Genetics Facility of the University of Georgia (Athens, GA) using an Applied Biosystems automated sequencer (model 373A, version 1.2.1). Primers NS1, NS2, and NS3 (White et al., 1990) were used for sequencing the 18S rDNA. Primers NS1 and NS2 provide complementary sequences, but the complementary sequence to NS3 was not determined. The rDNA sequences were easily aligned by direct examination.

Data analysis.—Maximum parsimony analysis of the aligned sequences was conducted using PAUP v. 3.1.1 (Swofford, 1993) on a Macintosh Performa 6115CD. Alignment gaps were treated as missing data (GAP-MODE=MISSING). However, one gap was included as an additional character in the data matrix (absence and presence of gap were coded as 0 and 1, respectively). In total, 43 isolates were included in the analysis (TABLE II). As a result of the large data set, only heuristic searches were performed with the following options in effect: tree-bisection-reconnection (TBR) swapping algorithm, collapsing zero length branches, and saving all minimal length trees (MULPARS). Ten replications with random addition of taxa were performed for each heuristic search in order to find any additional islands of minimum length trees (Maddison, 1991). To measure the relative support and stability of the resulting clades, bootstrap values (Efron, 1982; Felsenstein, 1985) and decay indices (Bremer, 1988; Donoghue et al., 1992) were calculated using PAUP v. 3.1.1. The computational intensity of these two values is directly dependent on the number of taxa and characters. Executing these computations on all of the taxa included was beyond the capability of the computer. Therefore, selected taxa were excluded, and the bootstrap values and decay indices were calculated on the smaller representative selection. Bootstrapping was performed with 250 replications. Decay indices up to 4 steps longer than the most parsimonious trees were determined. *Saccharomyces cerevisiae* E. C. Hansen, *Taphrina deformans* (Berk.) Tul., and *Candida tropicalis* (Castellani) Berkhout were used as outgroup taxa based on the results of previous analyses at a broader taxonomic scale by Berbee and Taylor

(1992), Bruns et al. (1992), and Spatafora and Blackwell (1993).

Phenetic analyses were also performed on the aligned 18S rDNA sequences using MEGA v. 1.01 (Kumar et al., 1993). Since MEGA isn't capable of evaluating binary code, the alignment gap was not included as an additional character in the data set. For neighbor-joining analyses (Saitou and Nei, 1987), the gamma (Kimura 2-parameter) distance method was used with complete deletion of all sites containing gaps or missing information. Bootstrapping of the neighbor-joining tree was performed with 500 replications. Again, *S. cerevisiae*, *T. deformans*, and *C. tropicalis* were used as outgroup taxa.

RESULTS

Segments of the ssrRNA gene (18S rDNA) encompassing 937 bp were analyzed. Alignment of sequences was easily accomplished by direct examination due to the gene's conserved nature. An alignment gap of one base pair, common to the outgroup taxa and the Clavicipitaceae, was added to the data set as an additional character. Of the 43 taxa included in this study (TABLE II), sequences from 16 of these were obtained from either GenBank or other researchers. The remaining 27 were sequenced for this study. Our sequencing efforts focused primarily on the form genus *Acremonium* and the ascomycete family Clavicipitaceae. Aligned sequences are available from the authors upon request.

A maximum parsimony analysis with all taxa included yielded eight equally parsimonious trees of 656 steps. For each of these trees, the consistency index (CI) was 0.585 with autapomorphies included, the retention index (RI) was 0.712, and the rescaled consistency index (RC) was 0.417. The strict consensus of the eight trees is presented in FIG. 1. In order to determine bootstrap and decay values for the broad range of taxa included in this study, a smaller subset of selected taxa was chosen to represent the various clades, and the support values were computed on the smaller collection of taxa. The maximum parsimony strict consensus cladogram of this subset is presented in FIG. 2 along with the bootstrap and decay indices. This same subset of taxa was also analyzed using the neighbor-joining method, and the results are presented in FIG. 3.

Based on these results, *Acremonium* is a polyphyletic taxon having species associated with at least three or four currently recognized ascomycete orders (FIG. 1). First, *A. furcatum* F. & V. Moreau : W. Gams is potentially associated with *Microascus* and *Ceratocystis* of the order Microascales. The bootstrap values for this connection are 67% using maximum parsi-
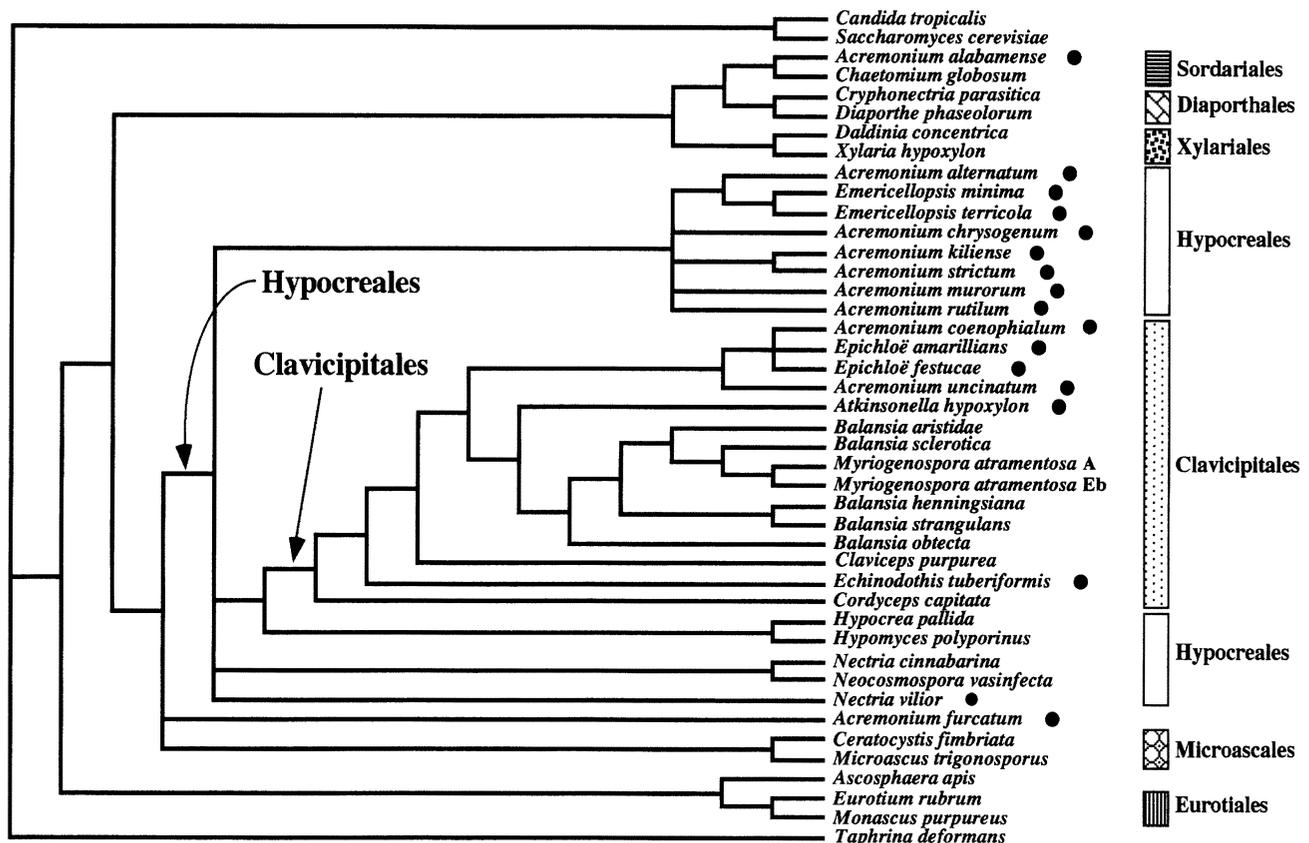
FIG. 1. Strict consensus of eight cladograms (each of 656 steps, CI = 0.585, RI = 0.712, RC = 0.417) resulting from maximum parsimony analysis of 18S rDNA sequences of all taxa included in the study. Orders of the Euascomycetes are indicated. Black dots indicate species having *Acremonium*-like anamorphs.

mony (FIG. 2) and 83% with the neighbor joining analysis (FIG. 3). Second, *A. alabamense* is the known anamorph of *T. terrestris* of the Sordariales (see TABLE I), and our results definitively show this anamorph is strongly associated with *Chaetomium* from the Sordariales. Both maximum parsimony and neighbor-joining analyses resulted in a 100% bootstrap value for the *A. alabamense-Chaetomium* clade (FIGS. 2 and 3, respectively). Third, *Epichloë* and related asexual endophytes are well known members of the order Clavicipitales (FIG. 1), and their anamorphs are currently classified in *Acremonium* sect. *Albolanosa* (see TABLE I). Fourth, a large number of *Acremonium* species show affiliation to the Hypocreales (FIG. 1). Six conidial fungi classified in *Acremonium,* two *Emericellopsis* species which have *Acremonium* anamorphs, and *Nectria vilior* Starbäck, which also has an *Acremonium* anamorph (see TABLE I), are all placed within the moderately well supported clade comprising the Hypocreales. Based on maximum parsimony analysis, the Hypocreales had a 76% bootstrap value and a decay index of +2 (FIG. 2). MEGA's neighbor-joining analysis gave a bootstrap value of 87% for the comparable clade (FIG. 3).

Among the *Acremonium* species having affiliation to the Hypocreales is the type species of the genus, *A. alternatum.* It was weakly associated with other taxa such as *A. kiliense* Grütz, *A. strictum* W. Gams, *Emericellopsis minima* Stolk, and *E. terricola* J. F. H. Beyma. The parsimony clade of these five taxa had a bootstrap of only 59% and a decay index of +1 (FIG. 2). The phylogenetic placement of the cleistothecial *Emericellopsis* in the Hypocreales is supported here by the 18S rDNA sequences of both *E. terricola* and *E. minima.*

The monophyletic Clavicipitales appears to have been derived from within the Hypocreales (FIGS. 1–3). Monophyly of the Clavicipitaceae, the only family in the order Clavicipitales, was supported by neighbor-joining analysis with an 89% bootstrap value (FIG. 3), and support from maximum parsimony analysis was only slightly weaker with a bootstrap of 85% and a decay index of +3 (FIG. 2).

As indicated in FIGS. 2 and 3, *Epichloë* and its asexual derivatives in *Acremonium* sect. *Albolanosa* formed a well supported, monophyletic group with 84% and 96% bootstrap values, respectively. *Atkinsonella, Balansia,* and *Myriogenospora* formed a sister group to
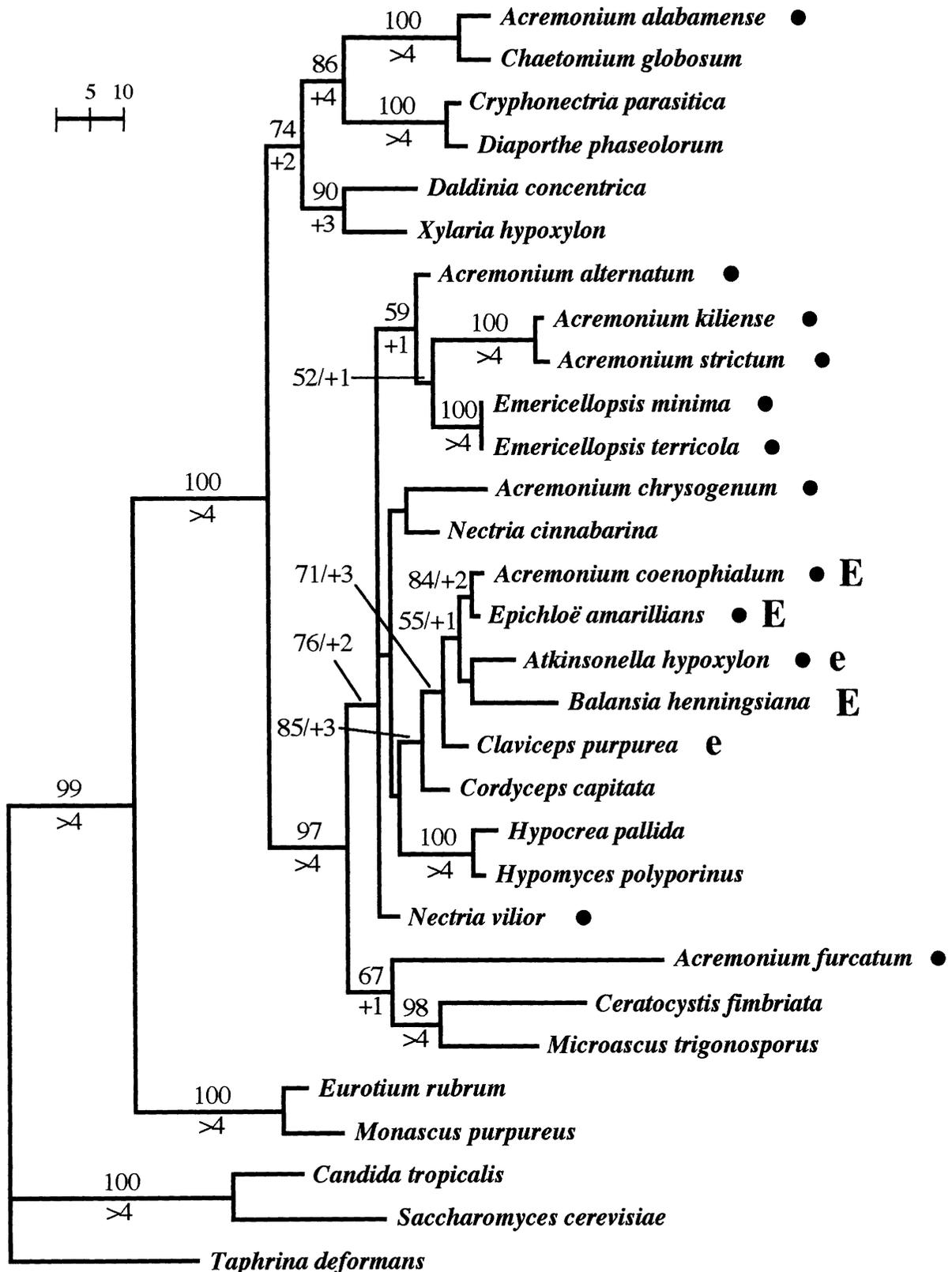
FIG. 2. Strict consensus of two cladograms (each of 527 steps, CI = 0.639, RI = 0.727, RC = 0.465) resulting from maximum parsimony analysis of 18S rDNA sequences of a selected subset of taxa. Consensus cladogram is depicted with proportional branch lengths. Scale bar indicates the number of nucleotide changes per unit of measure. Bootstrap values greater than 50% are indicated above internodes. Decay indices are indicated below internodes. Black dots indicate species having *Acremonium*-like anamorphs. E = grass endophyte; e = grass epibiont.
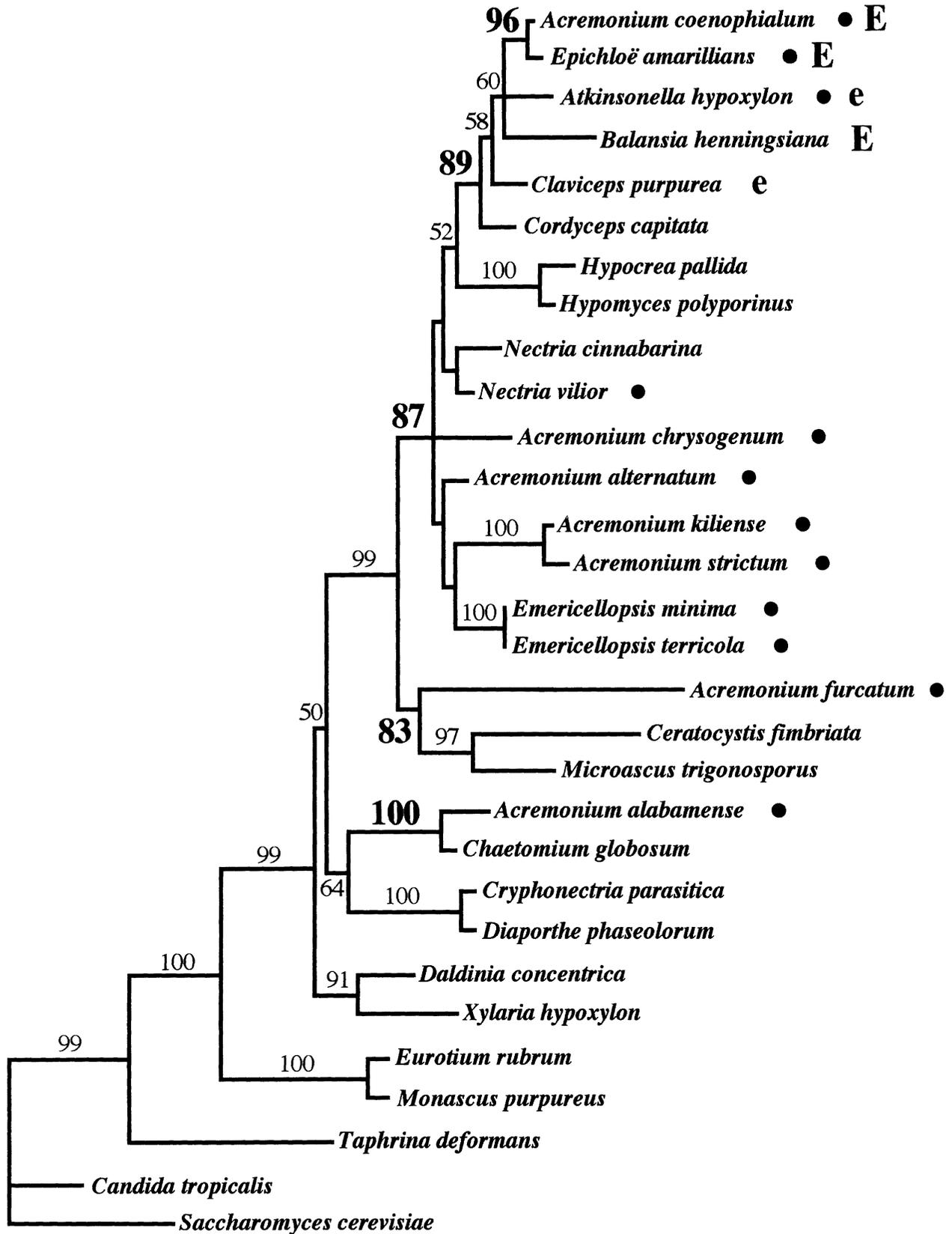
FIG. 3.   Neighbor-joining phenogram resulting from analysis of 18S rDNA sequences of a selected subset of taxa. Bootstrap confidence measures greater than 50% are indicated at internodes. Symbols are the same as in FIG. 2.

the *Epichloë* clade (FIGS. 1), but little support was available for resolving these relationships.

In general, there was a high degree of congruence between the resulting phylogenies of the cladistic (FIG. 2) and phenetic (FIG. 3) analyses. Relationships within the Hypocreales differed only in the placement of *N. vilior* and *A. chrysogenum* (Thirum. & Sukapure) W. Gams. Placement of the Xylariales also differed between the two analyses. Bootstrap values for comparable clades in the two resulting phylogenies were generally equivalent. However, three clades did show distinctly different values: the monophyletic clade comprising the Sordariales and Diaporthales, the Hypocreales clade, and the clade containing *A. furcatum* and the Microascales. These differences are probably inherent in the individual algorithms of the synapomorphy-based cladistical analysis and the similarity-based phenetic analysis and how they each affect the areas of the trees where the degree of support for the topology is limited.

DISCUSSION

New taxon and combinations.—Based on the 18S rDNA sequence analyses presented here, the present classification of the anamorphs of *Epichloë* and related mutualists in *Acremonium* is untenable. *Acremonium*, as typified by *A. alternatum*, appears to be restricted to the family Hypocreaceae. *Epichloë* and related genera of the Clavicipitaceae form a well circumscribed, monophyletic family that appears to be derived from within the Hypocreales. The monophyly of this family is supported by the unique morphology, ecology, and obligate parasitism of the Clavicipitaceae. The anamorphs of this family are unusual in that they are associated with stromatic tissue formed on a host (Diehl, 1950; Luttrell, 1980; Rykard et al., 1984; White and Morgan-Jones, 1987; Leuchtmann and Clay, 1988; Morgan-Jones and White, 1989). Therefore, we are proposing the erection of a new genus to accommodate anamorphs of clavicipitaceous fungi that form a palisade of simple phialidic conidiogenous cells over the surface of a stroma and in culture produce simple, strictly aculeate phialides often lacking a basal septum and usually arising as lateral branches from aerial hyphae. As thus defined, this genus should include the conidial fungi currently classified in *Acremonium* sect. *Albolanosa*.

Neotyphodium Glenn, Bacon & Hanlin gen. nov.

Coloniae albae vel flavidae, lente vel modice crescentes, hyphae aeriae saepe abundantes, byssaceae, typice non fasciculatae; phialides solitariae, raro verticillatae, aculeatae, orthotropicae, exoriundae ex hyphis aeriis, basi plerumque

septo carente. Conidia oblonga, ellipsoidea vel cylindrica vel fusiformia vel lunata vel uncinata, hyalina, levia, amerospora. Fungi parasitae obligati graminum, aliqui facientes ectostroma hymenio phialidum obtectum. Genus continens anamorphoses Clavicipitacearum.

TYPUS: *Acremonium coenophialum* Morgan-Jones & W. Gams.

Colonies white to yellowish, very to moderately slow growth rate, aerial hyphae often abundant, cottony but usually not fasciculate; phialides solitary, rarely verticillate, aculeate, orthotropic, arising from aerial hyphae, bases frequently lacking a septum. Conidia oblong, ellipsoidal, cylindrical, fusiform, lunate, uncinate, hyaline, smooth-walled, amerosporous. Obligate parasites of grasses, some forming exposed ectostroma with palisade of phialides. Genus containing anamorphs of the Clavicipitaceae.

Etymology. Greek *neos* = new + *Typhodium*, a genus erected by Link (1826) that was of uncertain applicability to the anamorph of *Epichloë*.

Neotyphodium coenophialum (Morgan-Jones & W. Gams) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium coenophialum Morgan-Jones & W. Gams, *Mycotaxon* 15: 311. 1982.
No known teleomorph.

Neotyphodium typhinum (Morgan-Jones & W. Gams) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium typhinum Morgan-Jones & W. Gams, *Mycotaxon* 15: 311. 1982.
Teleomorphs are species of *Epichloë*.

Neotyphodium lolii (Latch, Christensen & Samuels) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium lolii Latch, Christensen & Samuels [as *Acremonium loliae*], *Mycotaxon* 20: 535. 1984.
No known teleomorph.

Neotyphodium chisosum (J. F. White & Morgan-Jones) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium chisosum J. F. White & Morgan-Jones, *Mycotaxon* 28: 179. 1987.
No known teleomorph.

Neotyphodium starrii (J. F. White & Morgan-Jones) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium starrii J. F. White & Morgan-Jones, *Mycotaxon* 30: 87. 1987.
No known teleomorph.

Neotyphodium huerfanum (J. F. White, G. T. Cole & Morgan-Jones) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium huerfanum J. F. White, G. T. Cole & Morgan-Jones, *Mycologia* 79: 148. 1987.
No known teleomorph.

Neotyphodium uncinatum (W. Gams, Petrini & D. Schmidt) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium uncinatum W. Gams, Petrini & D. Schmidt, *Mycotaxon* 37: 67. 1990.
No known teleomorph.

Neotyphodium chilense (Morgan-Jones, J. F. White & Piont.) Glenn, Bacon & Hanlin comb. nov.

Basionym. Acremonium chilense Morgan-Jones, J. F. White & Piont., *Mycotaxon* 39: 441. 1990.
No known teleomorph.

While not included in our analyses, *N. starrii* and *N. lolii* have been shown to be closely related to *N. coenophialum* based upon maximum parsimony analysis of the two internal transcribed spacer (ITS) regions of rDNA (Schardl et al., 1991). Unfortunately, *N. chilense*, *N. chisosum*, and *N. huerfanum* were also unavailable to us for examination, and these three have yet to be characterized by DNA-based phylogenetic analyses. We do, however, propose the transfer of these species into *Neotyphodium* based upon the examinations and personal communications of James F. White, Jr. and Walter Gams.

The conidial stromata of species of *Epichloë* are easily and logically addressed by the teleomorphic nomen even though anamorphic binomials and varieties have been erected for the various species (Morgan-Jones and Gams, 1982; White, 1992). However, the abundance of asexual endophytes that rarely or never produce teleomorphic stromata [clonal type-III endophytes of White (1988)] means that a dual system of nomenclature must be maintained whereby the type-III endophytes are classified in *Neotyphodium* and the sexually reproducing species are classified in *Epichloë*.

As defined, *Neotyphodium* is the most appropriate form genus for the typhodial stage of *Atkinsonella* (Rykard et al., 1984). However, the applicability of the nomen *Atkinsonella* to all stages of its development is empirical and conveys a holomorphic view of this fungus. Exclusively asexual species are not known. Therefore, since no binomial is currently defined for the typhodial stage of *Atkinsonella*, we do not see the necessity of defining one under *Neotyphodium*. The binomial *Ephelis borealis* Ellis & Everh. has been established for the sympodially proliferating synanamorph of *Atkinsonella* (Diehl, 1950).

The anamorph of *Echinodothis tuberiformis* (Berk. & Ravenel) G. F. Atk. presents a similar problem as found with *Atkinsonella*. The anamorph of this epibiont is similar to *Neotyphodium* in that a palisade of phialidic conidiogenous cells are produced over the surface of a host-associated stroma, but the conidia are different in being didymosporous (White, 1993b). White (1993b) suggested that *Echinodothis* and *Epichloë* were closely related and might deserve classification together in a separate tribe. While lacking statistical support, cladistical analysis suggested that *Echinodothis* may be intermediate between *Cordyceps* and *Claviceps* and is not closely related to *Epichloë* (FIG. 1). As with *Atkinsonella*, the holomorphic application of *E. tuberiformis* to its anamorph and teleomorph means there is no need to establish an anamorphic binomial.

The Clavicipitales-Hypocreales relationship.—The Clavicipitales is a morphologically well circumscribed order of mostly parasitic ascomycetes possessing morphologically distinctive characters such as deliquescent lateral paraphyses, cylindrical asci with a thickened apical cap, and filiform ascospores that are often septate and may disarticulate into part-spores (Rogerson, 1970). However, the ordinal affinity of the clavicipitaceous genera has been evaluated and interpreted differently by several researchers [see Rogerson (1970) for a thorough historical review of the taxonomic literature].

In recent years, the ordinal relationships among hypocreaceous and clavicipitaceous genera has again come into question as a result of phylogenetic evaluations using molecular data. Maximum parsimony analyses using the nuclear encoded 18S rDNA have indicated a monophyletic relationship exists between genera of the Clavicipitales and the Hypocreales, suggesting that all taxa may be classifiable under the single order Hypocreales with the monophyletic Clavicipitaceae being a separate family from the paraphyletic Hypocreaceae (Spatafora and Blackwell, 1993). Our results, which also utilized 18S rDNA, show the same general relationship (FIGS. 1–3). Additionally, gene phylogenies based on both the nuclear encoded large subunit ribosomal DNA (28S rDNA) and nuclear encoded orotidine-5'-monophosphate decarboxylase also support the derivation of the monophyletic Clavicipitaceae from within the Hypocreales (Rehner and Samuels, 1994b, 1995). All these gene phylogenies support the treatment by Kreisel (1969) with a single order incorporating the families Hypocreaceae (*sensu* Rogerson) and Clavicipitaceae.

The expanding Hypocreales.—Just as molecular systematics has aided in clarifying some of the taxonomic confusion concerning the ordinal relationships

among hypocreaceous and clavicipitaceous genera, the same approaches are allowing systematists to propose phylogenetically informative holomorphic connections between anamorphs and teleomorphs (Schardl et al., 1991; Rehner and Samuels, 1994a). Such phylogenetic analyses are also generating proposals of novel intraordinal teleomorphic relationships which are expanding the delimitation of the Hypocreales (Rehner and Samuels, 1994a, 1995; results reported herein).

Our approach to the form genus *Acremonium* was similar to that used to investigate other polyphyletic form genera (Rehner and Samuels, 1994a, 1995). Our results (FIGS. 1–3) suggest *Acremonium* is indeed polyphyletic as currently circumscribed. Affiliation of the type species, *A. alternatum,* and other common species such as *A. kiliense, A. strictum, A. chrysogenum, A. murorum* (Corda) W. Gams, and *A. rutilum* W. Gams to the Hypocreaceae suggests that *Acremonium* should be restricted to anamorphs of only this family. Since many *Acremonium* species, including *A. alternatum,* do not produce ascomata *in vitro,* applying the taxonomic criterion of relationship to the Hypocreaceae is currently not feasible based on morphological characteristics. In order to strictly reserve *Acremonium* as anamorphs of the Hypocreaceae, DNA sequence analyses would be needed for each of the species of *Acremonium* that do not have a known teleomorph. While perhaps justified, such an immense task is not yet practicable. However, it is possible that most of the orphaned *Acremonium* species may be phylogenetically affiliated with the Hypocreales, as suggested by FIG. 1 which shows six orphaned species of *Acremonium* from the sections *Acremonium, Gliomastix,* and *Nectrioidea* derived from within the Hypocreales. Of those sampled, only one orphaned species, *A. furcatum* of sect. *Nectrioidea,* is not derived from within the Hypocreales.

At present the best we can do to eliminate some of the heterogeneity within *Acremonium* is to remove those species which are known to be associated with teleomorphs that are not within the Hypocreaceae (Gams, 1995). Though not examined, some, if not all, species of sect. *Lichenoidea* would be expected to show affiliation to the Hypocreaceae since four of the nine species have hypocreaceous teleomorphs. Among the *Acremonium* species that were examined by us, the grass endophytes (sect. *Albolanosa*) and *A. alabamense* (sect. *Chaetomioides*) have teleomorphs that are in the Clavicipitaceae and Chaetomiaceae (Sordariales), respectively. Therefore, the generic placement of these anamorphs is in need of reevaluation. The morphological characteristics of *A. alabamense* are very similar to those of other 'true' *Acremonium* species in that it produces guttulate conidia

in chains or heads from simple phialides, but *A. alabamense* is different in that it grows much more rapidly than other species and is thermophilic (Morgan-Jones, 1974; Morgan-Jones and Gams, 1982). Before any formal generic reassignment of this anamorph is proposed, a detailed study is needed to determine if other morphological characteristics exist which distinguish it from other species of *Acremonium.* However, because of the abundance of morphological and biological information that is available concerning the grass endophytes, the generic placement of the anamorphs of *Epichloë* and related grass symbionts is now ready to be addressed. A discussion of pertinent biological and taxonomic information is presented below.

Numerous systems of higher categories for the Ascomycotina have been suggested and are summarized in Hawksworth et al. (1983). Common to a few of these systems are the categorical divisions Plectomycetes and Pyrenomycetes at varying hierarchical levels. These two divisions are based on characters of the ascomata. Plectomycetes includes those fungi having typically globose, nonostiolate cleistothecia that produce asci at varying levels throughout the centrum (Fennell, 1973; Benny and Kimbrough, 1980), and Pyrenomycetes includes those fungi having flask-shaped, mostly ostiolate perithecia that produce asci from a single basal hymenium (Müller and von Arx, 1973). Suggestions have been made that the plectomycetes are a heterogeneous assemblage of morphologically similar fungi resulting from convergent evolution, and many plectomycetous genera are suggested to be derived from within the Pyrenomycetes (Malloch, 1981; Benny and Kimbrough, 1980). Recent molecular phylogenetic studies have confirmed that such a heterogeneity indeed exists. An initial maximum parsimony analysis indicated that there was a natural sister-group relationship between selected taxa corresponding well to the traditional ascomycete classes Plectomycetes and Pyrenomycetes (Berbee and Taylor, 1992). Such traditional plectomycetous taxa as *Ascosphaera, Ajellomyces, Monascus, Talaromyces,* and *Thermoascus* were found to form a monophyletic sister group to a monophyletic group of pyrenomycetes including *Ophiostoma, Chaetomium,* and *Neurospora.*

While a true higher level division appears to naturally exist between pyrenomycetes and some plectomycetes, molecular analyses are identifying other "plectomycetes" that appear to be derived from within the pyrenomycetes. *Roumegueriella* and *Heleococcum* are cleistothecioid genera that have been previously placed in the Hypocreales (Rogerson, 1970; Malloch, 1981). This placement was supported by analysis of 28S rDNA sequences (Rehner and Sa-

muels, 1994a, 1995). *Mycoarachis* (Rehner and Samuels, 1994a) and *Emericellopsis* (FIGS. 1–3), so far classified in the cleistothecioid family Pseudeurotiaceae, also show affinity to the Hypocreales. The Pseudeurotiaceae was suggested by Malloch (1981) to be related to the Diaporthales on the basis of centrum development, but some members of this family now appear to be phylogenetically related to the Hypocreales. This relationship is directly relevant to this study in that *Mycoarachis, Emericellopsis,* and other members of the Pseudeurotiaceae possess *Acremonium* anamorphs (Malloch and Cain, 1970). *Heleococcum* also produces an *Acremonium* anamorph (Udagawa et al., 1995).

While ascomatal characteristics appear to vary dramatically between hypocreaceous genera, the possession of an *Acremonium* anamorph by many of these teleomorphs appears to be a phylogenetically informative character supporting their link to the Hypocreaceae. It is interesting to note that Wu and Kimbrough (1990) found much similarity in the ascogonial and ascogenous systems of *Emericellopsis, Ascosphaera,* and *Monascus* even though *Emericellopsis* is phylogenetically distinct from the more closely related *Ascosphaera* and *Monascus* (FIG. 1). Such similarity in ascomatal development despite the phylogenetic separation implies that these ascomatal characteristics may be subject to convergent evolution. Also, the anamorphs of *Ascosphaera* (= *Chrysosporium*) and *Monascus* (= *Basipetospora*) are distinctly different from the *Acremonium* anamorph of *Emericellopsis.* Some of the other *Acremonium*-producing genera of the Pseudeurotiaceae that may prove to be hypocreaceous once molecular analyses are performed include *Nigrosabulum, Hapsidospora,* and *Leucosphaerina* (Malloch and Cain, 1970; Malloch, 1989). The inclusion of cleistothecioid forms within the Hypocreales is creating a need for reevaluation and refinement of the distinguishing characters defining this order.

Taxonomic considerations.—The fact that the teleomorph of *A. alabamense* is *T. terrestris* of the family Chaetomiaceae is in opposition to the criterion of *Acremonium* being restricted to anamorphs of the Hypocreaceae. The thermophilic nature of *A. alabamense,* its rapid growth rate, and its truncated, dacryoid conidia were all considered by Morgan-Jones and Gams (1982) to be characters distinctive enough to warrant the creation of sect. *Chaetomioides* within *Acremonium.* While *A. alabamense* is typically orthophialidic like most species of *Acremonium,* perhaps its unique morphological and biological characters are more distinctive at the generic versus sectional level. Before any formal reclassification of *A. alabamense* is

considered, more detailed morphological studies are needed to determine if other tangible characters exist that could be used for its generic distinction from *Acremonium.* A comparative analysis of conidium ontogeny of *A. alternatum* and *A. alabamense* may provide additional needed characters.

The potential taxonomic problems posed by *A. furcatum* are complex. Since its affiliation to an ascomycete order is currently vague, it must be retained in the genus *Acremonium.* If further molecular or morphological analyses should indicate a phylogenetic affiliation to the Hypocreaceae, then its current status would be maintained. If additional molecular analyses should confirm an affiliation to the Microascales as suggested by our results, or if its teleomorph is discovered and classified within the Microascales, a more phylogenetically appropriate classification for *A. furcatum* may then be justified.

The gene phylogenies presented here indicate that the Clavicipitaceae is a distinctive, monophyletic family derived from within the Hypocreales. Other phylogenetic analyses have also indicated this same relationship (Spatafora and Blackwell, 1993; Rehner and Samuels, 1995). The monophyletic nature of the Clavicipitaceae is substantiated by its unique ecology and morphology. Mating compatibility studies have shown that graminicolous species of *Balansia, Atkinsonella, Echinodothis,* and *Epichloë* are heterothallic, requiring the transfer of conidia (= spermatia) from a stroma of one mating type to a stroma of the opposite mating type (White and Bultman, 1987; Leuchtmann and Clay, 1989; White, 1993a, b; White et al., 1995). Mating compatibility of *Myriogenospora* has not been examined. Conidia of *Claviceps* are infective and do not function as spermatia (Luttrell, 1980). Diehl (1950) emphasized the importance of conidial fructification for taxonomic and systematic evaluations of these fungi. *Sphacelia,* the form genus for the anamorph of *Claviceps,* is characterized by a palisade of doliiform to lageniform phialides produced along the convoluted surface of the developing sclerotium, and this palisade bears a wet mass of amerosporous conidia (Diehl, 1950; Luttrell, 1980). In contrast, the "typhodial" conidial fructifications of *Epichloë,* herein classified in the form genus *Neotyphodium,* produce dry masses of amerosporous conidia from a continuous, nonconvoluted palisade of narrowly aculeate phialides over the surface of the host-associated stroma (Diehl, 1950; White and Morgan-Jones, 1987). The anamorphs of *Balansia* and *Myriogenospora* are classified in the form genus *Ephelis* which is characterized by holoblastic, scolecosporous conidia produced successively from sympodially proliferating conidiophores in apothecioid cavities in host-associated stromata or pseudosclerotia (Diehl,

1950; Rykard et al., 1984; White et al., 1995). *Atkinsonella* is intermediate in that it initially has a *Neotyphodium*-like anamorph which is followed by an *Ephelis* anamorph (Rykard et al., 1984).

The close evolutionary connection between asexual grass endophytes and the sexually reproducing species of *Epichloë* has been examined from an ecological perspective (White, 1988), but a greater amount of attention has been given to molecular evaluations of relationships. The isozyme and molecular analyses previously performed on the asexual endophytes have mainly dealt with the variation and relationships that exist among some of the species. Leuchtmann and Clay (1990) found that isozyme profiles based on ten enzymes are virtually uniform throughout most isolates of *N. coenophialum* (= *A. coenophialum*). They also found that no distinction could be made between the *Epichloë* endophytes and the asexual endophytes, thereby suggesting that they jointly comprise a monophyletic group. Schardl et al. (1991) performed maximum parsimony analyses on sequences of the internal transcribed spacer (ITS) regions of rDNA and showed that the asexual endophytes formed a monophyletic group with *Epichloë* and that asexual species apparently arose from *Epichloë* on multiple occasions. Additionally, they found that sequence comparisons do not support some of the morphologically based species classifications of asexual endophytes. Based on maximum parsimony analyses of ITS sequences, An et al. (1992) reported that at least two distinct evolutionary origins of *Neotyphodium*-endophytes from *Epichloë* had occurred in the single host species *Festuca arizonica* Vasey. The studies by Leuchtmann and Clay (1990) and Schardl et al. (1991) are supportive of the existence of several different species of *Epichloë* (see also White, 1993a; White, 1994a; Leuchtmann et al., 1994).

The monophyletic nature of the Clavicipitaceae, the separation of *Epichloë* from *A. alternatum*, the obligate parasitism of *Epichloë* and its asexual derivatives, and the formation of a sporodochium-like palisade of phialides on an external stroma all indicate the distinct nature of *Epichloë* and support the reclassification of its anamorphs. The doubtful identity of *Sphacelia* as a form genus for these anamorphs (Diehl, 1950; Morgan-Jones and Gams, 1982) and the lack of any other valid or appropriate form genus means a new genus is needed to accommodate these fungi. *Typhodium* was erected by Link (1826), but his meager description is vague and confusing so there is uncertainty as to whether he was describing the anamorph of *Epichloë* or the teleomorph. Diehl (1950) felt that the conidial fructifications of *Epichloë* were significantly different from those of *Claviceps* and chose not to apply the form genus *Sphacelia* to

the anamorph of *Epichloë*. As an informal "convention of convenience," Diehl (1950) applied *Typhodium* as the form genus for *Epichloë* conidial fructifications. In following Diehl's concept, we have proposed the form genus *Neotyphodium* for the anamorphs of *Epichloë* and its related asexual grass endophytes.

The establishment of *Neotyphodium* helps to alleviate some of the heterogeneity of *Acremonium* while emphasizing the unique, monophyletic nature of the grass endophytes. The employment of molecular data to substantiate this reclassification indicates the utility of DNA-based phylogenetic techniques in evaluating the taxonomic affiliations of morphologically simple taxa such as *Acremonium*. However, limitations do exist. For example, the basic morphology of *A. alabamense* is essentially the same as that of *A. alternatum*, but the two are affiliated with different ascomycete orders. More detailed morphological studies are now needed to determine if informative characters exist which could be used for their generic distinction. Results of phylogenetic analyses are useful as frameworks for further, more focused morphological and molecular comparisons. Such comparisons may help to alleviate some of the remaining heterogeneity of *Acremonium*.

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