Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences

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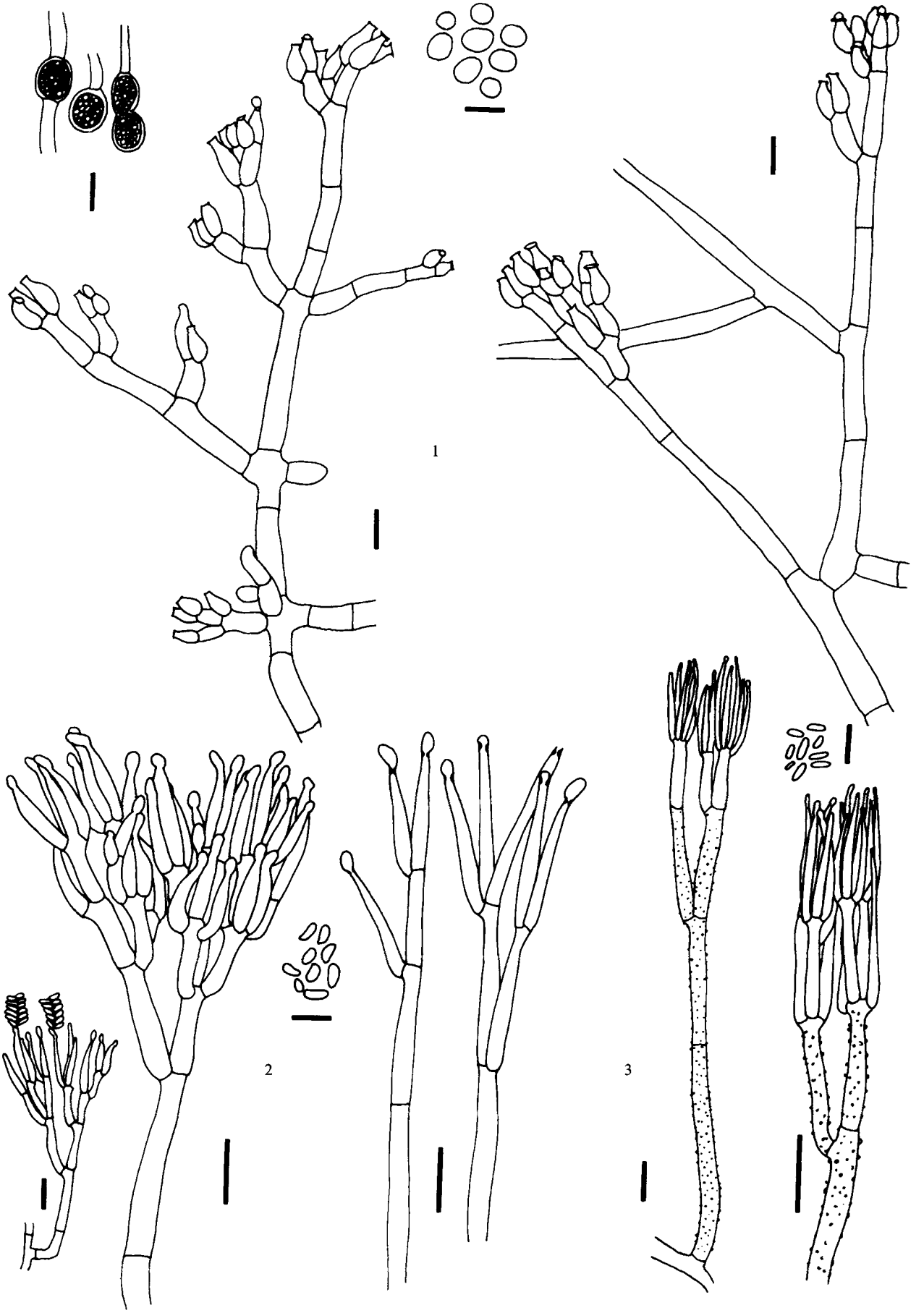
The phylogenetic distribution of *Gliocladium* within the Hypocreales was investigated by parsimony analysis of partial sequences from the nuclear large subunit ribosomal DNA (28S rDNA). Two principal monophyletic groups were resolved that included species with anamorphs classified in *Gliocladium*. The first clade includes elements of the genera *Hypocrea* (*H. gelatinosa, H. lutea*) plus *Trichoderma, Hypocrea pallida, Hypomyces* and *Sphaerostilbella*, which are each shown to be monophyletic but whose sister group relationships are unresolved with the present data set. *Gliocladium* anamorphs in this clade include *Gliocladium penicillioides*, the type species of *Gliocladium* and anamorph of *Sphaerostilbella aureonitens*, and *Trichoderma virens* (= *G. virens*) which is a member of the clade containing *Hypocrea* and *Trichoderma*. A second clade, consisting of species with pallid perithecia, is grouped around *Nectria ochroleuca*, whose anamorph is *Gliocladium roseum*. Other species in this clade having *Gliocladium*-like anamorphs are *Nectriopsis sporangiicola* and *Roumegueriella rufula*. The species of *Nectria* represented in this study are polyphyletic and resolved as four separate groups: (1) *N. cinnabarina* the type species, (2) three species with *Fusarium* anamorphs including *N. albosuccinea, N. haematococca* and *Gibberella fujikoroi*, (3) *N. purtonii* a species whose anamorph is classified in *Fusarium* sect. *Eupionnotes*, and (4) *N. ochroleuca*, which is representative of species with pallid perithecia. The results indicate that *Gliocladium* is polyphyletic and that *G. penicillioides*, *G. roseum*, and *Trichoderma virens* (= *G. virens*), are generically distinct.

Gliocladium Corda is a genus of hyphomycetes that includes two fungi commonly used in experimental biological control of soil-borne plant diseases, *G. virens* J. H. Mill., Giddens & A. A. Foster (1957) and *G. roseum* Bainier (Papavizas, 1985; Chet, 1987, 1990). While these two species broadly conform to the concept of *Gliocladium*, the differences that separate them from each other and from the type species of the genus, *G. penicillioides* Corda, indicates that their interrelationship is not as close as their current generic placement implies and that they may not be congeneric.

One way of assessing the taxonomic and natural relationships of anamorphic fungi is to compare them with the proven anamorphs of ascomycetes. Although most species of *Gliocladium*, including *G. virens*, do not have proven teleomorphs, culture studies have demonstrated that many are similar or identical to the anamorphs produced by species of at least seven hypocrealean genera (*sensu* Rogerson, 1970) including *Hypocrea* (Webster, 1964; Webster & Rifai, 1968; Doi, 1972; Doi & Yamatoya, 1989), *Hypomyces* (Petch, 1938), *Nectria* (e.g. Booth, 1959; Samuels, 1976), *Nectriopsis* (Samuels, 1973, 1988), *Roumegueriella* (Bainier, 1910), *Sarawakus* (Samuels & Rossman, 1992), and *Sphaerostilbella* (Seifert, 1985). The linkage of *Gliocladium* anamorphs with such a diverse array of taxonomically distinct teleomorph genera clearly indicates that *Gliocladium*, as currently defined, is polyphyletic.

Another means of establishing connections between anamorphs and teleomorphs is through analysis of genotypic (DNA) data. Comparison of shared, unique polymorphisms can help link closely related or conspecific anamorphs and teleomorphs, but the full potential of this approach is best realized when coupled with phylogenetic analysis. Gene phylogenies can be considered to be hypotheses of organismal relationships, and provide information on both contemporary and historical anamorph and teleomorph connections. Gene phylogenies may ultimately lead to the integration of anamorphic and teleomorphic fungi into a unified system of classification (Reynolds & Taylor, 1993). Within the short time that molecular methods have been available, there have been many demonstrations of the usefulness of this approach to fungal systematics (reviewed by Bruns, White & Taylor, 1991), which include investigations of anamorph and teleomorph relationships (Berbee & Taylor, 1992*a*, 1992*b*; Gaudet *et al.*, 1989).

The purpose of this study is to evaluate the phylogenetic relationships within *Gliocladium*, focusing particularly on the biocontrol fungi *G. virens* and *G. roseum*, and the type species of *Gliocladium*, *G. penicillioides* (Figs 1–3). We have constructed a gene phylogeny for the 5′ region of the nuclear 28S large subunit rRNA based on sequences determined from conidial and ascospore isolates of hypocrealean anamorphs that can be identified as *Gliocladium*, and other hypocrealean taxa which do not have *Gliocladium*-like anamorphs. The gene phylogeny, interpreted as a species phylogeny, enables us to make inferences about the phylogenetic position of *G. penicillioides*,



Figs 1–3. For captions see facing page.

Table 1. Source of isolates

	Taxon[a]	Strain no.[b]	Source	GenBank accession no.
1	*Hypocrea* cf. *gelatinosa*	GJS 89-114	Maryland, U.S.A.	U00737
2	*Hypocrea gelatinosa*	GJS 88-80	Yunnan Prov., China	U00738
3	*Trichoderma virens*	GL18	California, U.S.A.	U00760
4	*Trichoderma virens*	GL20	Maryland, U.S.A.	U00759
5	*Hypocrea lutea*	GJS 89-129	North Carolina, U.S.A.	U00739
6	*Hypocrea pallida*	GJS 89-105	French Guiana	U00741
7	*Hypocrea pallida*	GJS 89-83	New Hampshire, U.S.A.	U00740
8	*Hypomyces berkeleyanus*	GJS 82-274	New Zealand	U00756
9	*Sphaerostilbella* sp.	GJS 82-40	New Zealand	U00758
10	*Sphaerostilbella aureonitens*	GJS 83-286	New Zealand	U00755
11	*Sphaerostilbella lutea*	GJS 83-277	Gabon	U00757
12	*Hypomyces aurantius*	GJS 74-86	New Zealand	U00742
13	*Hypomyces australis*	CTR 76-163	Venezuela	U00743
14	*Hypomyces rosellus*	CTR 78-121	New York, U.S.A.	U00744
15	*Roumegueriella rufula*	GJS 91-169	CBS 346-85	U00754
16	*Nectriopsis sporangiicola*	CTR 67-135	New Jersey, U.S.A.	U00753
17	*Nectria ochroleuca*	GJS 90-167	Venezuela	U00750
18	*Nectria ochroleuca*	GJS 90-179	Venezuela	U00751
19	*Gliocladium roseum*	Ns-30	New York, U.S.A.	U00736
20	*Nectria ochroleuca*	GJS 90-227	Venezuela	U00752
21	*Nectria aureofulva*	GJS 91-52	Brazil	U00746
22	*Nectria aureofulva*	CTR 71-328	Venezuela	U00747
23	*Mycoarachis inversa*	ATCC 22107	Uganda	U00745
24	*Nectria haematococca*[c]	NRRL 13425	NA	—
25	*Nectria albosuccinea*[c]	NRRL 20459	Venezuela	—
26	*Gibberella fujikuro*[c]	NRRL 13563	North Carolina, U.S.A.	—
27	*Nectria purtonii*[c]	NRRL 13625	NA	—
28	*Nectria cinnabarina*	GJS 89-107	New York, U.S.A.	U00748
29	*Nectria cinnabarina*	GJS 89-108	New York, U.S.A.	U00749

[a] Strains listed as teleomorphs were isolated from single ascospores, anamorph strains were obtained from conidial soil isolates.

[b] Collectors/culture collections: ATCC, American Type Culture Collection; GJS, Gary Samuels; CTR, Clark Rogerson; GL, Robert Lumsden; NS, Systematic Mycology Laboratory, U.S. Dept. Agriculture, Beltsville, MD; NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research, U.S. Dept. Agriculture, Peoria, IL.

[c] Sequences for these isolates were obtained from Kerry O'Donnell, NCAUR, ARS, USDA, Peoria, IL.

NA, locality information not available.

G. roseum, and *G. virens* within the Hypocreales, which supports the hypothesis that *Gliocladium* is polyphyletic.

MATERIALS AND METHODS

Strains and culture methods

Strains used in this study were obtained either from single ascospores or conidia and are listed in Table 1. Cultures, which are maintained at BPI, were stored on Difco cornmeal agar slants at 4°C. Mycelia for DNA extractions were grown for 2–5 d in 15 ml liquid CYM (Raper & Raper, 1972) in darkness at room temperature.

DNA extraction

DNA was extracted by a method modified from Raeder & Broda (1985). Cultured mycelia were transferred to filter paper and blotted dry, then frozen at −80° and lyophilized. The lyophilized mycelia were ground in a 1·5 ml microcentrifuge tube with a sterile applicator stick under liquid nitrogen, then suspended in 500 µl lysis buffer (200 mM Tris/HCl (pH 8·3), 250 mM NaCl, 50 mM Na_2EDTA, 1% SDS) and extracted successively with equal volumes of phenol:chloroform and chloroform. DNA was precipitated with 0·54 volumes of isopropanol and pelleted by spinning 10–30 s at 12000 ***g*** in a microcentrifuge. The supernatant was discarded and the tubes were drained of residual supernatant by inverting on a paper towel. The pellets were resuspended in 100 µl TE (10 mM Tris/HCl (pH 8·0), 1 mM Na_2EDTA) containing 100 mM NaCl and incubated at 55° for 1 h. RNAase A was added to the samples to 50 µg ml^{-1} and incubated at 37° for 30 min. Samples were extracted with an equal volume of chloroform, adjusted to 2·5 M ammonium acetate and precipitated by the addition of 2·5 volumes of 95% ethanol and pelleted by spinning for 30 s in a microcentrifuge. The

Figs 1–3. Fig. 1. *Gliocladium virens* with chlamydospores, conidiophores from *Trichoderma*-like aggregates (left) and from aerial hyphae (right), and conidia. **Fig. 2.** *Nectria ochroleuca* (anam. = *Gliocladium roseum*) showing conidia in imbricate chains from a penicillately branched conidiophore (left), and two verticillately branched conidiophores (right), and conidia. **Fig. 3.** *Sphaerostilbella aureonitens* (anam. *Gliocladium penicillioides*) showing two conidiophores and conidia. Fig. 1 drawn from the 'type' isolate (ATCC 13213), Fig. 2 drawn from GJS 86-236, Fig. 3 drawn from GJS 83-286. Scale bars: 10 µm.

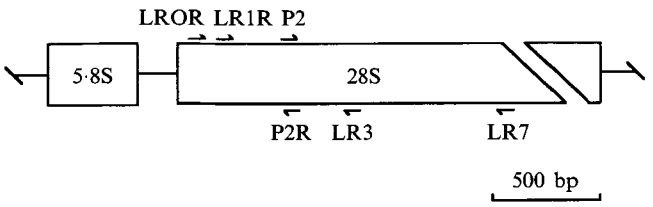


Fig. 4. Diagram of the 5′ end of the 28S rDNA showing location of amplification and sequencing primers.

pellets were rinsed in 70% ethanol, vacuum dried and resuspended in 100 µl TE. The DNA concentrations were estimated visually on 1% agarose gels (Bethesda Research Laboratories, Gaithersburg, MD) by comparison with lambda phage and pUC119 DNA standards. DNA was diluted to a final concentration of 0·1–1·0 ng μl^{-1} by adding 1–10 µl of the genomic DNA stock solutions to 500 µl TTE (1 mM Tris (pH 8·0), 0·1 mM Na_2EDTA).

Polymerase chain reaction

A fragment of DNA, spanning approximately 1400 bases of the 5′ end of the nuclear 28S ribosomal gene, was symmetrically amplified using AmpliTag DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Primer positions used for amplification and sequencing are shown in Fig. 4. DNA samples were amplified in 50 µl reaction volumes using 0·5–5·0 ng genomic DNA as template, 25 pmol of primers LROR (5′-GTACCCGCTGAACTTAAGC; Vilgalys & Hester, 1990) and LR7 (5′-TACTACCACCAAGATCT; Vilgalys & Hester, 1990), 1·25 U Taq polymerase, 100 mM total dNTPs in 10 mM Tris/HCl (pH 8·3), 50 mM KCl, and 1·5 mM $MgCl_2$. Samples were amplified by 35 repeated cycles on a temperature cycler (MJ Research, Inc., Watertown, MA) using the following parameters: DNA denaturation, 1 min at 94°; primer annealing, 1 min at 50°; primer extension, 2 min at 72°. Negative control reactions omitting DNA were included in all sets of amplifications to monitor for potential contamination by exogenous DNAs. Following amplification the samples were extracted once with an equal volume of chloroform to remove the mineral oil overlay. Residual nucleotides and primers were removed from the reaction mixtures by binding the PCR product to glass powder and eluting in water as described by Vogelstein & Gillespie (1979). The samples were adjusted to 2 M ammonium acetate and precipitated with an equal volume of isopropanol, pelleted by centrifugation for 10 min, washed with 70% ethanol, dried and resuspended in 20 µl TTE.

A single stranded DNA template was synthesized in the 5′–3′ direction by the asymmetric-primer-ratio method (Gyllensten & Erlich, 1988). Asymmetric amplifications were performed in 50 µl volumes using the same amplification profile described above and altering the following reaction components: 25 µl of a 1:1000 dilution of the LROR-LR7 symmetric amplification product was used as the template; an internal primer, LR1R (5′-AGGAAAAGAAACCAACC; Vilgalys & Hester, 1990), was substituted in place of LROR using a LR1R/LR7 primer ratio of 50:2 pmoles. Following amplification the mineral oil overlay was removed by a single extraction with chloroform and the reaction product was examined on a 1% agarose gel (Bethesda Research Laboratories, Gaithersburg, MD). Unincorporated primers and nucleotides were removed by centrifugal filtration with Centricon-100 filter units (Amicon Div., W. R. Grace & Co., Beverly, MA) according to the manufacturer's directions. The samples were lyophilized then resuspended in 15 µl sterile distilled water.

Sequencing

Nucleotide sequences of the PCR fragments were obtained for both strands using two direct sequencing strategies; double strand sequencing without dimethyl sulfoxide for the 5′ to 3′ direction (Winship, 1989), and direct sequencing of the asymmetrically amplified templates for sequences in the 3′ to 5′ direction (White *et al.*, 1990). All sequencing reactions were performed with Sequenase 2·0 (US Biochemical, Cleveland, OH) and ^{35}S-labelled dATP (Amersham, Arlington Hts, IL) following the manufacturers recommendations except that the labelling reactions were performed on ice and the termination reactions at 45°. Two sequencing primers were used in each direction; LR0R and P2 (5′-AGATGAAAAGAACTTTGAAAAGAGAG; Guadet *et al.*, 1989) for sequences in the 5′ to 3′ direction, and LR-3 (5′-CCGTGTTTCAAGACGGG; Vilgalys & Hester, 1990) and P2R (5′-CTCTCTTTTCAAAGTTCTTTTCATCT, the reverse complement of P2) for sequences in the 3′ to 5′ direction. Sequencing reactions were run on wedge or standard 5% acrylamide gels in 1·35X TBE buffer (135 mM Tris, 45 mM boric acid, 2·5 mM Na_2EDTA) at 40 w for approximately 3·5 and 6·5 h, respectively. Gels were fixed in 10% methanol/5% acetic acid, vacuum dried, and exposed to Kodak XAR-5 film for 12–24 h.

Data entry and analysis

Sequence data entry and editing were performed on an IBM PC using the computer program MICROGENIE (Beckman Instruments, Palo Alto, CA). Sequences were initially aligned with the computer program Align Plus (Scientific & Educational Software, State Line, PA) using the program default parameters, then edited visually where necessary.

Synapomorphic nucleotide substitutions (shared by two or more but not all taxa) from unambiguously aligned regions were used as characters for cladistic analyses with the computer program PAUP 3.0r (Swofford, 1991). Analyses were performed on a Macintosh IIci. Due to the large size of the data set, parsimony trees were constructed using heuristic methods. Heuristic searches were implemented using the random addition sequence option and the tree bisection-reconnection branch swapping algorithm. Branch robustness was examined by bootstrapping (Felsenstein, 1985) and from the decay index (Mishler, Donoghue & Albert, 1991; Donoghue, *et al.* 1992). The bootstrap analysis, which is a resampling of the data with replacement with randomly assigned characters, was performed with 1000 replications using the same heuristic search options described above. The

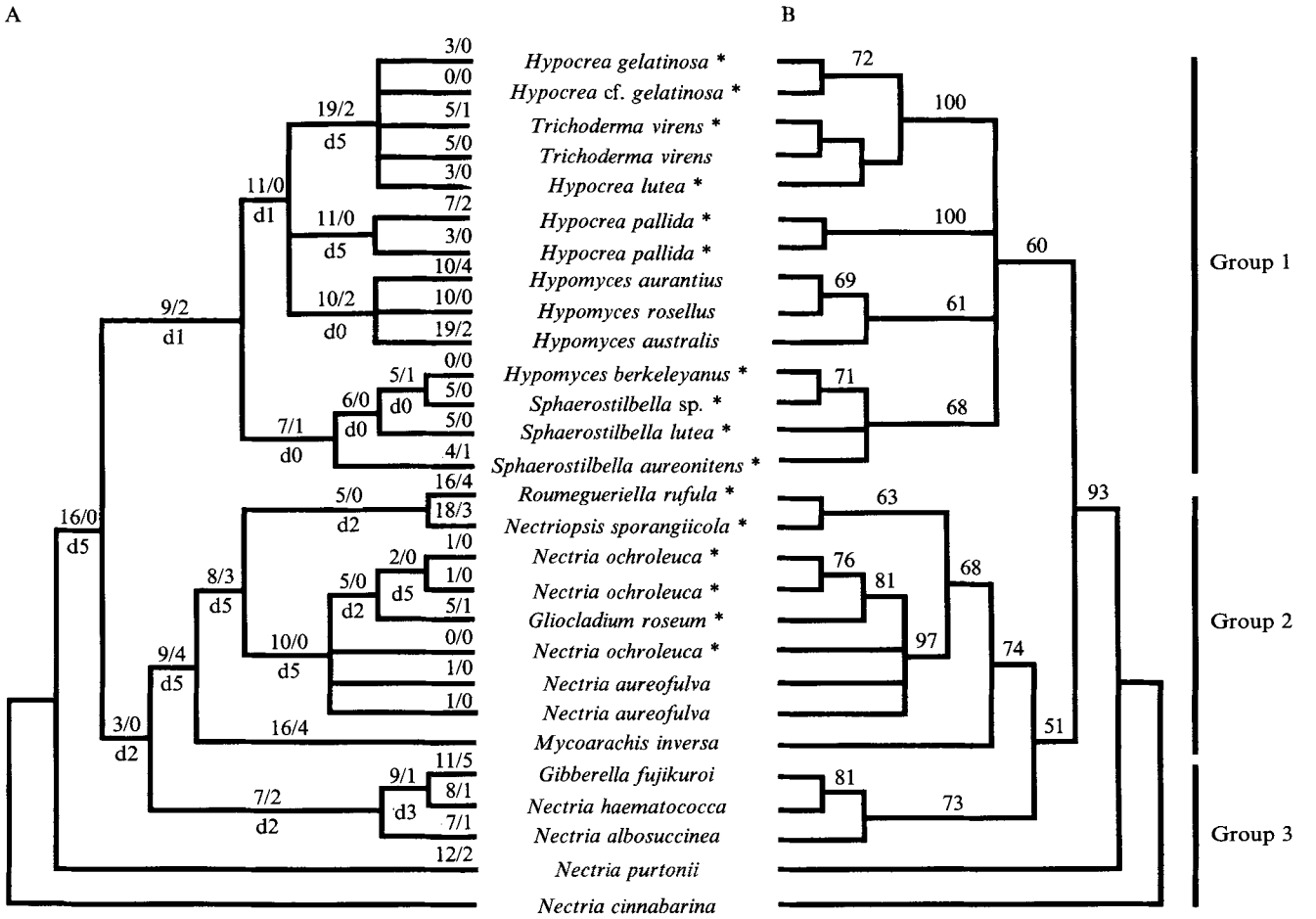


Fig. 5. Cladograms inferred by maximum parsimony analysis. A, strict consensus of 56 shortest trees of 320 steps, consistency index = 0·469. Branch lengths and the number of characters unequivocally supporting each branch are indicated above branches and separated by a '/', respectively. Decay indices (d) are indicated below branches. B, bootstrap parsimony analysis with bootstrap intervals from 1000 replications; nodes supported in > 50% of bootstrap replicates are indicated. Asterisks indicate species whose anamorphs have a *Gliocladium*-like morphology.

decay index is the number of extra steps in tree length necessary to lose resolution of individual branches resolved in the strict consensus tree of the most parsimonious tree(s). Decay indices were computed by performing a heuristic search and saving all trees that were up to five steps longer than the most parsimonious trees. The trees output from this analysis were segregated into incremental length classes by using the filter trees option of PAUP from which strict consensus trees were computed.

RESULTS

A 563 base region of the 5′ end of the 28S rDNA, spanning the eukaryote specific divergent regions 1 and 2 (Hassouna, Michot & Bachellerie, 1984), was completely sequenced on both strands for the strains listed in Table 1. The region analysed corresponds to positions 79-658 of *Saccharomyces* (Gutell & Fox, 1988). These sequences may be obtained individually from GenBank (accession numbers are given in Table 1) or collectively in an aligned format from the first author.

In preliminary parsimony analyses, both *Saccharomyces cerevisiae* and *Neurospora crassa* were used separately and jointly as outgroups to determine the basal hypocrealean taxon(a) or clade included in this study. In these analyses (results not shown), *Nectria cinnabarina* was placed as the sister group to the other hypocrealean taxa and was used as the outgroup for rooting all subsequent analyses.

The heuristic search for minimal length trees yielded fifty-six equally parsimonious trees with 320 steps and a consistency index = 0·469, excluding uninformative characters. Separate heuristic searches implementing different addition sequences and swapping strategies yielded identical results. Approximate searches have the potential to get trapped in local optima, and the shortest trees may not be found (Swofford, 1991). To increase the probability that the shortest trees were identified in the heuristic search, the trees output from this analysis were used as starting trees for mounting 1000 additional heuristic searches. When setting up this analysis a high value for the random seed was specified in the heuristic search menu of PAUP. Selecting a high random seed value randomizes the point at which the data set is entered and the tree search initiated. This increases the likelihood of discovering shorter

trees if such exist. All the shortest trees found in the additional searches were identical to the trees identified in the original search, which suggests that the results of the heuristic analyses are most likely the shortest trees for this data set.

A strict consensus cladogram of the 56 trees is presented in Fig. 5A. In the following discussion of the tree outputs, we divide the taxa contained therein into three groups. Group 1 is monophyletic and includes *Hypocrea* spp. (*H. gelatinosa* and *H. lutea*) plus *Trichoderma virens*, *Hypocrea pallida* plus *Hypomyces*, with *Sphaerostilbella* spp. as the sister group to the preceding taxa. Group 2, also monophyletic, is centred around *Nectria ochroleuca* but also includes *Nectriopsis* and *Roumegueriella*, which are collectively referred to here as 'pallid nectrias'. Group 3, a non-monophyletic assemblage, incorporates representatives of three distinct groups within *Nectria*, including: *N. cinnabarina* the type species; three species with *Fusarium* anamorphs including *N. albosuccinea* with an unnamed *Fusarium* anamorph (*Fusarium* sect. *Spicarioides*), *N. haematococca* with *F. solani* anamorph (*Fusarium* sect. *Martiella*), and *Gibberella fujikuro* with *F. moniliforme* anamorph (*Fusarium* sect. *Liseola*), which are the sister group to Group 2; and *N. purtonii* with a *F. aquaeductuum* anamorph (*Fusarium* sect. *Eupionnotes*), which is basal to the ingroup.

The results from the bootstrap (Fig. 5B) are largely congruent with the strict consensus cladogram of the heuristic analysis, except for two areas within Group 1. In Group 1, *Sphaerostilbella* spp. is the sister group to *Hypocrea* spp. plus *Trichoderma*, *Hypocrea pallida*, and *Hypomyces* spp., whereas these four groups form an unresolved polytomy in the bootstrap analysis. Also, the relationship of *Trichoderma virens* and *Hypocrea lutea* is unresolved in the heuristic analysis but are placed as sister groups in the bootstrap analysis.

Decay indices are loosely correlated with bootstrap values; branches with high bootstrap values tend also to have a high decay index whereas branches with low bootstrap intervals have low decay indices. Five of seven branches with a decay index of five have bootstrap values above 90%, but two branches have significantly lower bootstrap values; the branch below *Mycoarachis inversa* and the branch below *Roumegueriella rufula* plus *Nectriopsis sporangiicola* and *Nectria aureofulva* plus *N. ochroleuca*, have bootstrap values of 74% and 68%, respectively. Wide variation among bootstrap values was observed among branches with decay indices ranging from zero to two: branches with decay indices of zero had bootstrap values ranging from 61 to 71%, a branch with a decay index of one had a bootstrap value of 60%, and branches with decay indices of two had bootstrap values from 62 to 81% (Fig. 5A, B).

DISCUSSION

Terminal groupings of taxa in the 28S rDNA phylogeny are consistent with current taxonomic concepts of teleomorph genera in the Hypocreales, including those with *Gliocladium* anamorphs (i.e. *Hypocrea* with *Trichoderma* anamorphs, *Sphaerostilbella*, and species of *Nectria* that have pallid perithecia), and those without (*Hypomyces*, *Mycoarachis*). *Nectria* is polyphyletic in this analysis and the species treated here are divided into four groups consistent with current taxonomic concepts based on teleomorph and anamorph characters. The internal branching structure of the 28S cladogram yields insights into the phylogenetic relationships among morphologically diverse genera of the Hypocreales. Taxonomic interpretations of these results are discussed in the context of Groups 1–3 (Fig. 5).

Group 1

The 28S rDNA sequence data support the monophyly of Group 1 at a low level. Although the 28S data are not particularly robust for this branch, a separate study, based on sequence data from the nuclear small subunit found strong support for the monophyly of Group I (Spatafora & Blackwell, 1992). Topological congruence among gene phylogenies increases confidence in phylogenetic hypotheses because of the likelihood that sequence evolution at different loci occurs independently (Doyle, 1992). However, evolution of the 28S and 18S are probably not strictly independent as these genes are linked in tandem arrays in the nuclear genomes of most fungi, plants and animals (Rogers & Bendich, 1987). Nonetheless, given the different rates at which these two genes are known to evolve, we view the congruence of the 18S and 28S phylogenies as further support for the monophyly of these genera.

Neither the 28S phylogeny presented here nor the 18S phylogeny of Spatafora & Blackwell (1991) resolved relationships among the genera within Group I. In the 18S data, the short terminal branches characterizing each of these genera suggests a radiation that outpaced the rate of sequence evolution of the 18S gene. In contrast, the internal and terminal branch lengths of the 28S gene tree within Group I are evidence of nucleotide substitutions accumulating in the 28S gene between cladogenic events. However, most substitutions along this branch are homoplasious, thus obscuring phylogenetic signal. Additional data from other genes will perhaps resolve these relationships. In the following we discuss the terminal taxa comprising Group I.

Hypocrea gelatinosa, *H. lutea*, and *Trichoderma virens* (= *G. virens*) form a well supported monophyletic group. This result is significant for its implications for the taxonomy of *T. virens*, which has been placed in both *Gliocladium* and *Trichoderma* by different workers (eg. von Arx, 1987; Bissett, 1991). On purely morphological grounds, there are justifications for including *G. virens* in either *Gliocladium* or *Trichoderma*. Characteristic of *Gliocladium*, the conidia are held in drops of liquid on a penicillus of phialides that form at the tips of branches of more or less discrete phialides. However, the green conidia, compact phialides, essentially indeterminate conidiophores that branch at right angles and that are often joined in hemispherical tufts on agar media, production of chlamydospores in culture, and the rapid growth rate and overall colony characters, are indicative of *Trichoderma*. Thus, taxonomic conclusions based upon traditional morphological characters alone have not been wholly convincing for the placement *T. virens* in either *Trichoderma* or *Gliocladium*.

References in the literature of *Gliocladium virens* reflect the taxonomic confusion surrounding the identification of this taxon. *Gliocladium virens* first received attention for its ability

to produce the antifungal toxins, gliotoxin (Weindling, 1937, 1941; Weindling & Emerson, 1936) and viridin (Brian & McGowan, 1945), and has since been referred to as *G. fimbriatum*, *G. virens*, *Trichoderma lignorum*, and *T. viride*. Weindling (1941) first referred to the species as *G. fimbriatum*, a name which is now known to be *Myrothecium verrucosa* (Domsch, Gams & Anderson, 1980). Webster & Lomas (1964) identified Weindling's original gliotoxin-producing isolate and Brian & McGowan's two gliotoxin- and viridin-producing isolates as *G. virens*. Webster & Lomas (1964) and Dennis & Webster (1971) were unable to demonstrate the production of gliotoxin or viridin by any representatives of the nine species aggregates of *Trichoderma* defined by Rifai (1969), including *T. viride*. Thus, *Gliocladium virens* emerged as the name in common usage in the biocontrol literature for the past 25 yr (see review in Papavisas, 1985).

von Arx (1987), without providing justification, transferred *G. virens* to *Trichoderma*. This placement was followed more recently by Bissett (1991*c*) in his monograph of *Trichoderma*. The results of the rDNA analysis in the present study support the taxonomic usage of von Arx and of Bissett.

Trichoderma virens has never been unequivocally linked to a teleomorph, but it is morphologically similar to the anamorph of *Hypocrea gelatinosa* (Webster, 1964; Bissett, 1991). *Hypocrea gelatinosa* was included by Doi (1972) in *Hypocrea* sect. *Hypocrea* subsect. *Creopus* with species such as *H. aureoviridis* and *H. flavovirens* that have *Gliocladium* or *Trichoderma* anamorphs. The teleomorphs typically occur on decaying, decorticated wood. While *Gliocladium virens* appears to be morphologically distinct from *Trichoderma* because the conidia are held in slime, there is a continuous variation into more typical *Trichoderma*, as judged by *Hypocrea* species similar to *Hypocrea gelatinosa* that have more or less *Trichoderma* or *Gliocladium* anamorphs (see Doi, 1972; Bissett, 1991). *Hypocrea gelatinosa* (GJS 88-80) and a morphologically and anatomically similar but distinct, and as yet unnamed *Hypocrea* were included in this study. The anamorph of isolate GJS 88-80 is similar morphologically and culturally to *T. virens* (= *G. virens*) strains GL18 and GL20 used in biocontrol research whereas the anamorph of *H.* cf. *gelatinosa* GJS 89-114 is *T. strictipilis* Bissett. Isolates of *Hypocrea gelatinosa* were included in the study to determine whether rDNA sequence data could help to establish its relationship to *T. virens*. Sequence divergence within the alignable regions among other isolates of *H. gelatinosa* and additional species of *Trichoderma* (data not shown) not thought to be closely related to *H. gelatinosa* was less than 1·5%, and most of these substitutions were autapomorphies or homoplasious. Investigation of more rapidly evolving sequences such as the nuclear ribosomal internal transcribed spacers (ITS) or intergenic regions (IGS) may provide a greater number of informative characters for delineating relationships with *Hypocrea* and *Trichoderma*.

Hypocrea lutea, although anatomically close to *H. gelatinosa*, differs in having colourless (not green), ascospores and a *Gliocladium*-like anamorph known as *G. viride*. Despite the morphological dissimilarity between the anamorph of *H. lutea* to *Trichoderma* species, its placement within the clade containing *H. gelatinosa* and *G.* (*Trichoderma*) *virens*, and its removal from *Gliocladium sensu stricto* is supported.

Species of *Sphaerostilbella* were monophyletic in both the heuristic and bootstrap analyses, although support for this association was low. However, our results support Seifert's (1985) hypothesis that *Sphaerostilbella* is monophyletic, being characterized by a unique suite of morphological characters. The type species of *Gliocladium*, *G. penicillioides* (Fig. 3), has been known for a long time to be the anamorph of *Sphaerostilbella aureonitens* (Petch, 1938). Species of *Sphaerostilbella* occur on tough basidiocarps of the Aphyllophorales. The anamorph of the type and other species of *Sphaerostilbella* have synnematous anamorphs that Seifert (1985) included in *Gliocladium*. The anamorph of *Hypomyces berkeleyanus* is a *Gliocladium*-like species that is morphologically similar to the anamorph of *S. aureonitens* and this analysis indicates that *H. berkeleyanus* should be placed in *Sphaerostilbella*. These results confirm that *Sphaerostilbella* is monophyletic and the genus *Gliocladium sensu stricto* should be restricted to the anamorphs of *Sphaerostilbella*.

Hypocrea pallida has been difficult to place taxonomically because it is morphologically intermediate between *Hypocrea* and *Sphaerostilbella*. Doi & Yamatoya (1989) placed this species in *Hypocrea* because its ascospores disarticulate at the median septum during spore maturation. Also, they noted that the perithecia of *H. pallida* do not form in groups within a stroma as in *Hypocrea*, but have a two-layered wall structure, the distinctive outer layer forming a more or less pseudoparenchymatous layer that appears similar to the stromal tissues of *Hypocrea*. However, as noted by Seifert (1985), *H. pallida* possesses characteristics that ally it with *Sphaerostilbella*. Like *Sphaerostilbella*, *H. pallida* occurs on tough basidiocarps and its *Gliocladium* anamorph is morphologically very similar to *G. penicillioides*. In this analysis the two isolates of *Hypocrea pallida* form a monophyletic group within Group 1 but the rDNA sequence data did not resolve their phylogenetic position to either *Hypocrea*, *Sphaerostilbella* or *Hypomyces*.

The three species of *Hypomyces* examined here, *H. aurantius*, *H. australis* and *H. rosellus*, formed a monophyletic group within Group 1, with *H. australis* basal to *H. aurantius* plus *H. rosellus*. *Hypomyces* includes species that are parasites on the basidiomata of agarics, boletes and polypores although a few species occur on ascomycetes (Rogerson & Samuels, 1985, 1989, 1993). *Hypomyces* is characterized morphologically by its perithecia, which are situated on or within a subiculum and by fusiform, usually bicellular ascospores, that are usually warted and bear polar apiculi. The species considered here are thought to be related; they all occur on polypores and have *Cladobotryum* anamorphs (Rogerson & Samuels, 1993), a grouping resolved in both the heuristic and bootstrap analyses.

Group 2

The species in Group 2 are biologically and morphologically heterogeneous and include saprobes and fungal parasites with either perithecial or cleistothecial ascocarps.

Of primary interest in Group 2 is the clade containing *Nectria ochroleuca*, which has *Gliocladium roseum* as its anamorph. This *Gliocladium*, which has been used in biocontrol research (see Papavizas, 1985), is isolated from soil and wood (e.g. Park, Stack & Kenerley, 1992), and is clearly distinct both

from the type species of *Gliocladium* and from *T. virens*. *Gliocladium roseum* (Fig. 2) is also easily distinguished from other *Gliocladium* species by the asymmetric conidia, the way that the conidia are held in opaque slime in imbricate chains, and in colony characters. Domsch *et al.* (1980) have proposed that *Clonostachys* is the appropriate genus for this anamorph. *Gliocladium roseum* is further distinguished by the formation of two distinct types of conidiophores, one *Gliocladium*-like and producing chains of conidia, and the other *Verticillium*-like and producing conidia in colorless drops of liquid (Domsch *et al.*, 1980). It should be mentioned that the production of a *Verticillium*-like synanamorph is not unique to *G. roseum*. *Sphaerostilbella lutea* also produces a *Verticillium*-like state in addition to its typical synnematous anamorph. The *Verticillium*-like synanamorphs produced by *G. roseum* and *S. lutea* are not strictly equivalent and the similarity is most likely due to convergence.

The closest relative of *Nectria ochroleuca* included in this study is *N. aureofulva* which has a sporodochial anamorph that is currently referred to as *Dendrodochium*. Morphologically, *G. roseum* is similar to *Sesquicillium* species (not treated here), which are anamorphs of species of *Nectria* also with pallid perithecia (Samuels, 1989). A unifying feature of all these anamorphs is a tendency for the conidia to be held in salmon or orange slime and for the conidia to be asymmetric. Both anamorphs and teleomorphs, including *N. ochroleuca*, most often occur on small branches of recently dead trees. Our data suggest no relationship between these *Nectria* species and either *H. gelatinosa* or *Sphaerostilbella* species. Taxonomic and nomenclatural studies of *G. roseum* and the genus *Clonostachys* are continuing.

The basal group within Group 2 is *Mycoarachis inversa*. This species and *Roumegueriella rufula* (discussed later) are unusual because of their cleistothecial ascocarps. Although Malloch & Cain (1970) placed *M. inversa* in the Pseudoeurotiaceae, the *Acremonium*-like anamorph of *M. inversa* implies that its affinity is within the Hypocreales. Malloch (1970) has described many examples where a loss of taxonomically salient characters such as the arrangement of asci in a hymenium, sterile filaments in the centrum, ascomatal opening, and ascal discharge mechanism has lead to the derivation of cleistothecia in unrelated pyrenomycetes. Additional examples of independent loss of forcible ascospore discharge or derivation of cleistothecial ascocarps have been inferred from molecular based phylogenies within other groups of Pyrenomycetes (Spatafora & Blackwell, 1991; Berbee & Taylor, 1992*a*).

Nectriopsis sporangiicola and *Roumegueriella rufula* are united on a branch that places them as the sister group to *Nectria ochroleuca* and *N. aureofulva*. Both species have *Gliocladium*-like anamorphs that are morphologically similar to *G. penicillioides*, however, the rDNA phylogeny does not support their close relationship to other species with *Gliocladium*-like anamorphs. *Nectriopsis* has about 75 species, all but one of which have pallid perithecia and according to Samuels (1988) may be heterogeneous. The *Gliocladium*-like anamorph of *N. sporangiicola* is unlike the relatively few proven anamorphs of other species of *Nectriopsis* which have morphologically simpler conidiophores. Species of *Nectriopsis* have few characters that can be used to indicate their relationships within the Hypocreales. While our analysis places *N. sporangiicola* basal to the clade containing the 'pallid nectrias', we cannot comment on the broader relationships of *Nectriopsis* spp. until sequences of more species in this genus are available.

Roumegueriella rufula has bright yellow cleistothecia and globose, spinulose ascospores held in globose asci that are apparently produced without order within the cleistothecium (Bainier, 1910; Malloch, 1970). Like *Nectriopsis*, the teleomorph morphology of *R. rufula* provides little indication of its position within the Hypocreales, but the rDNA phylogeny clearly shows it to be derived from within Group 2.

Group 3

Nectria sensu lato was found to be both paraphyletic and polyphyletic, and was resolved into four distinct groups in this analysis. Teleomorphs of *Fusarium* sections *Liseola*, *Martiella*, and *Spicarioides*, currently placed in *Nectria* and *Gibberella* form a monophyletic group basal to Group 2, which together form a monophyletic group that is the sister group to Group I. However, support for this clade is weak and requires further confirmation. Guadet *et al.* (1989), in an analysis based on 28S sequence data, have shown that *Gibberella* and its *Fusarium* anamorphs are derived from within a clade also containing *Nectria* with *Fusarium* anamorphs, demonstrating that *Nectria* is paraphyletic. O'Donnell (1993), in a more comprehensive survey of 28S sequences from fusaria, confirmed the findings of Gaudet *et al.* and showed that elements of *Nectria* and *Fusarium* are polyphyletic.

Our results suggest that the fungicolous species *Nectria purtonii*, which is currently placed in *Nectria* subg. *Dialonectria* (Samuels *et al.*, 1991) and the anamorph of which is classified in *Fusarium* sect. *Eupionnotes*, is less related to both *Nectria* and *Gibberella* than these groups are from each other. We conclude from this analysis that species centred around *N. cinnabarina*, *N. purtonii* and the species of *Nectria* with pallid perithecia each represent distinct groups worthy of formal taxonomic recognition.

General conclusions

Parsimony analyses of large subunit ribosomal RNA gene sequences permitted us to examine phylogenetic relationships of ascomycetes in the Hypocreales. The inferred tree topologies are consistent with the current generic concepts of *Sphaerostilbella*, *Hypomyces*, *Hypocrea*, and several groups recognized within the large genus *Nectria*. This has allowed the phylogenetic placement of soil isolates of *Trichoderma virens* and *Gliocladium roseum* that have not been directly derived from teleomorphs. The results of this research clearly indicate that *Trichoderma virens* (= *G. virens*) is phylogenetically distinct from the type species of *Gliocladium*, *G. penicillioides*, and is derived from within *Hypocrea*. *Gliocladium roseum* is phylogenetically distant from *G. penicillioides* and *T. virens* and forms a group with species of *Nectria* that have pallid perithecia. These results confirm our hypothesis that *G. penicillioides*, *G. roseum* and *T. virens* are generically distinct.

The data presented here illustrate that gene phylogenies

can provide credible phylogenetic hypotheses of organismal relationships. A more inclusive molecular analysis of the Hypocreales should help to define better the natural limits of this order as well as improve the delimitation of its families and genera. However, given the different levels of phylogenetic support provided by the 28S data it is evident that well-supported molecular phylogenies will require data sets derived from several or more genes.

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