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Molecular characterization of *Embellisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium* and *Stemphylium*

Barry M. Pryor[1]
Donna M. Bigelow

Department of Plant Pathology, Forbes 204, University of Arizona, Tucson, Arizona 85721-0036

Abstract: DNA sequences from rDNA and protein-coding regions were determined for six *Embellisia* and two *Nimbya* spp. and were compared to those from *Alternaria*, *Ulocladium* and *Stemphylium* spp. Sequences determined included rDNA from the nuclear internal transcribed-spacer region (ITS1/5.8S/ITS2) and the mitochondrial small-subunit (mt SSU) and a portion of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene. Phylogenetic analyses were performed on each dataset separately and then combined for total evidence analysis using methods of maximum parsimony and maximum likelihood. Results revealed that *Embellisia* and *Nimbya* clustered within a large monophyletic *Alternaria-Nimbya-Embellisia-Ulocladium* clade with *Stemphylium* as the sister taxon. Members of the *infectoria* species-group were the most basal group in this large polygeneric clade. *Embellisia* and *Nimbya* were sister taxa of the remaining *Alternaria* and *Ulocladium* spp. and were related more closely to *Alternaria* than was *Stemphylium*. Four *Embellisia* spp. formed a monophyletic clade. However, *E. allii* clustered with the two *Nimbya* spp. and *E. indefessa* clustered with *Alternaria* and *Ulocladium* spp., revealing that *Embellisia*, as currently circumscribed, is polyphyletic. Potential revisions of taxonomy for all genera are discussed.

Key words: *gpd*, ITS, maximum likelihood, mitochondrial SSU, parsimony, phylogeny

INTRODUCTION

Embellisia Simmons and *Nimbya* Simmons are anamorphic-based genera of phaeosporic Hyphomycetes described as *genera nova* in 1971 and 1989, respectively (Simmons 1971, 1989). Species in both genera possess characteristics of conidia that are morphologically reminiscent of both phragmosporic *Helminthosporium* species and dictyosporic *Alternaria* species. Moreover, numerous species in both genera are synonyms of earlier described species of *Helminthosporium*, *Alternaria* or other morphologically similar genera, which reveals a level of taxonomic ambiguity that historically has shadowed these and related taxa. Additional review using modern systematic methods might be useful in resolving some of the uncertainty regarding true evolutionary relationships.

The genus *Embellisia* originally was erected to accommodate the atypical *Helminthosporium* spp., *H. allii* Campanile, causal agent of garlic bulb canker (Simmons 1971). Simmons recognized that the production of conidiophores as successive sympodial proliferations by *H. allii* was inconsistent with the erect growth of *Helminthosporium* conidiophores and was more similar to those produced by *Curvularia*, *Bipolaris*, *Drechslera*, *Alternaria* and *Ulocladium* (Shoemaker 1959, 1962; Simmons 1967; Alcorn 1988, 1991). Like *Curvularia* spp., conidia of *H. allii* variously can be curved and branched (Campanile 1924). But unlike *Curvularia*, the curvature is not generated by the inequilateral growth of a single central cell (Simmons 1971). Moreover, the production of significant numbers of dictyoconidia among a majority of phragmoconidia by *H. allii* was inconsistent with the strict production of phragmoconidia by *Curvularia, Bipolaris* and *Drechslera* and more resembled those produced by *Alternaria* and *Ulocladium*. Simmons further noticed that conidium septation was particularly thick, dark and rigid in contrast to the external wall of the conidium. Thus, based on a combination of several characters, the placement of *H. allii* into pre-existing genera was deemed unsuitable and the new genus *Embellisia* was erected with *E. allii* (Campanile) Simmons as the type (Simmons 1971).

Since the inception of *Embellisia*, 19 additional species have been described or transferred from other genera (David et al 2000; de Hoog and Muller 1973; de Hoog et al 1985; Muntanola-Cvetkovic and Ristanovic 1976; Simmons 1971, 1983, 1990). The combination of characters that are diagnostic for *Embellisia* include: 1) a low percentage of dictyoconidia among the dominant phragmoconidia; 2) variously swollen, smoothly curved or sigmoid conidia as exceptions to the usually straight-elliptical or oblong-elliptical population; 3) umbilicate sites (''pores'') of conidium production at conidiophore geniculations; 4) intra-

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[1] Corresponding author. E-mail: bmpryor@u.arizona.edu

hyphal proliferating chlamydospores and hyphal coils in culture (Simmons 1971, 1983). Conidial septation distinctly contrasts with the external wall of the conidium and is a key differentiating character. For most species, conidia generally are borne singly, although the production of secondary conidia is documented for several species. A notable exception to solitary conidia is *E. indefessa*, in which conidia often are borne in chains up to 10–12 conidia in length (Simmons 1983). In addition, several members have recognized teleomorphic states in the genus *Allewia* Simmons (Simmons 1990). Characteristics of *Allewia* ascostroma are indistinguishable from those of *Lewia* Simmons & Barr, the teleomorphic state of several *Alternaria* spp. (Simmons 1986), the only difference between the genera being the anamorphic state.

The designation of *Nimbya* as a new genus followed a similar course as the designation of *Embellisia*. The genus *Nimbya* was erected to accommodate the atypical characteristics of *Sporidesmium scirpicola* Fuckel, a pathogen of river bulrush (Simmons 1989). The taxonomy of this fungus has been controversial and different authors previously have described the species as *Sporidesmium*, *Clasterosporium*, *Cercospora* or *Alternaria* (Fuckel 1863, Saccardo 1886, Zinderen Bakker 1940, Sivanesan 1984). However, Simmons said, ''*Sporidesmium scirpicola* was the earliest of a few taxa described with a combination of conidium characters suggestive of both *Alternaria* and *Drechslera* to modern students of the group. I found the combination of characters too miscellaneous for *Drechslera* and unacceptable of *Alternaria*'' (Simmons 1989). Isolates of *Sporidesmium scirpicola* produce solitary conidia, initially long obclavate with a tapered apical region that matures into a long filiform beak. Internal cell-like lumina are delimited by distosepta, which become rarely, partially or completely euphragmoseptate on maturity. No longisepta are present. Based on these characters, Simmons found the placement of this taxon in *Sporidesmium* unacceptable and genus *Nimbya* was erected with *N. scirpicola* (Fuckel) Simmons as the type (Simmons 1989).

Since its inception, 13 additional taxa have been added to *Nimbya*, several of which formerly were recognized in other genera (Chen et al 1997; Johnson et al 2002; Simmons 1989, 1995, 1997, 2000). Species of *Nimbya* produce one to several conidia on closely geniculate conidiophores. Conidia generally are solitary or rarely catenate, long obclavate or short to long rostrate. Most conidia are porogenic; however, in some species conidia are holoblastic. Initially the conidia are distophragmoseptate becoming rarely, partially or completely euphragmoseptate, which is a key taxonomic character and clearly distinguishes *Nimbya* from *Drechslera*, *Alternaria* and other related genera (Simmons 1989). Conidia often are distinctly constricted at eusepta. Longitudinal or oblique septa occasionally are produced, similar to *Alternaria* and *Ulocladium*. It is interesting to note that the teleomorph of *Sporidesmium scirpicola* has been recognized as a species of *Macrospora* Fuckel, a genus morphologically distinct from the *Embellisia* teleomorph *Allewia* Simmons or the *Alternaria* teleomorph *Lewia* Barr & Simmons (Simmons 1986, 1990). However, the taxonomy of *Macrospora scirpicola* is not without controversy because some mycologists have treated the fungus as a species of *Pleospora* (Crivelli 1983), which morphologically is quite similar to *Lewia* (Simmons 1986).

Descriptions of *Nimbya* and *Embellisia* spp. reveal some characters unique and some similar to those of *Alternaria* and other genera in the Pleosporaceae. Following the description of *Embellisia* and *Nimbya*, Simmons (1992) proposed a close relationship of these genera to *Alternaria*, based on common conidium characteristics. *Alternaria*, together with *Ulocladium* and *Stemphylium*, comprise a large group of closely related phaeodictyosporic Hyphomyetes whose members are plant pathogens (predominantly) or saprobes. Recent phylogenetic analyses, based on nuclear internal-transcribed spacer (ITS), mitochondrial small-subunit (mt SSU), and 18S rDNA sequences, revealed that typical members of these genera comprised a monophyletic group with *Stemphylium* as the sister group of a large monophyletic clade of *Alternaria* and *Ulocladium* spp. (Pryor and Gilbertson 2000). Within the large *Alternaria/Ulocladium* clade, species were clustered into distinct species-groups, which corresponded fairly well with previously described species-groups based on morphology (Simmons 1992, 1995). *Ulocladium*, it is interesting to note, was polyphyletic within this clade. Subsequent studies, based on ITS sequences analysis, have supported these findings (de Hoog and Horre 2002, Chou and Wu 2002, Konstantinova et al 2001). Further studies examining relationships based on other genetic regions, such as protein coding genes, would contribute considerably to our confidence in phylogenetic relationships developed thus far. However, such studies are lacking. One potential gene for this purpose is glyceraldehyde-3-phosphate dehydrogenase (*gpd*), which has been used extensively in the resolution of phylogenetic relationships among *Cochliobolus/Bipolaris*, *Pyrenophora/Drechslera*, *Setosphaeria/Exserohilum* spp. (Zhang and Berbee 2001, Berbee et al 1999), genera established as sister taxa of *Pleospora/Stemphylium* and *Lewia/Alternaria* spp. (Pryor and Gilbertson 2000, Berbee 1996, Berbee et al 1999). This region has been found to have a similar level of genetic variation as that found in the ITS

region. To date, only a few *gpd* sequences of *Alternaria* spp. have been determined. No phylogenetic assessment of the suggested relationship among *Embellisia*, *Nimbya* and *Alternaria* spp., based on any gene, has been performed. The objective of this study is to examine the phylogenetic relationships among *Embellisia* and *Nimbya* spp. and the genera *Alternaria*, *Ulocladium* and *Stemphylium*, based on nuclear and mitochondrial rDNA sequences and those of a single-copy protein-coding gene, *gpd*.

MATERIALS AND METHODS

Isolates used in this study.—All isolates of *Embellisia* and *Nimbya* were obtained from the collection of E. G. Simmons (TABLE I). Other isolates in this work were obtained from various sources, and many of the isolates have been included in previous phylogenetic work (Pryor and Gilbertson 2000; TABLE I). All isolates were cultured on 0.05× PDA and PCA, under strictly defined incubation conditions (Pryor and Michailides 2002) and examined for characteristics of the sporulation apparatus and conidium morphology to confirm species identity and compare morphological characters.

DNA extraction, PCR amplification and sequencing.—DNA extraction and purifications were conducted according to previously established protocol (Pryor and Gilbertson 2000). The DNA concentrations were diluted to a final concentration of 10 ng/μL for PCR reactions. rDNA from the ITS region (ITS1, 5.8S, ITS2) was amplified via PCR with primers ITS5 and ITS4 (White et al 1990). rDNA from the mt SSU was amplified via PCR with primers NMS1 and NMS2 (Li et al 1994). DNA from the *gpd* gene was amplified via PCR with primers *gpd*1 and *gpd*2 (Berbee et al 1999). Amplification of the *gpd* region was carried out for all isolates in this study. Amplification of the ITS and mt SSU region was carried out for all *Nimbya* and *Embellisia* species and for several of the *Alternaria* and *Ulocladium* species for which ITS and mt SSU sequences had not been determined in previous work (Pryor and Gilbertson 2000). PCR annealing temperature for ITS and mt SSU amplification was 60 C, and annealing temperature for *gpd* amplification was 50 C. PCR product purifications were carried out as previously described (Pryor and Gilbertson 2000). The sequences of PCR products were determined with FS DyeTerminator reactions and ABI automated DNA sequencers. Sequences were determined for both DNA strands of the PCR products for sequence confirmation.

Phylogenetic analysis.—DNA sequences from each isolate were combined with sequences from previously established monophyletic species-groups contained within the *Alternaria/Ulocladium* clade and the *Stemphylium* clade (TABLE I) and were aligned with the PILEUP program of the GCG Sequence Analysis Software Package (version 10.2, Accelrys Inc., San Diego, California). For all amplified regions, manual adjustments of sequence alignments were performed with the data editor program of MacClade Phylogenetic Software (version 4.0, Sinauer Associates Inc., Sunderland, Massachusetts). Phylogenetic analyses were performed with programs contained in PAUP Phylogenetic Software (version 4.0 β, Sinauer Associates Inc., Sunderland, Massachusetts). Sequences of *Exserohilum pedicillatum* were included as outgroup in all analyses. Parsimony analysis heuristic searches for the most-parsimonious trees were conducted with random stepwise addition and branch swapping by tree bisection-reconnection (TBR). Sequence gaps were treated as missing data. To assess the effect of ambiguous regions in the alignment, analysis was repeated after removing ambiguous sections (ambiguous insertion/deletions regions are eliminated to the point where a conserved site is present in all taxa). For maximum-likelihood analysis, parameters of ti/tv ratio, base frequencies, proportion of invariable sites and gamma distribution were estimated from the most-parsimonious tree with the best-likelihood score. For each analysis, 500 bootstrap replicates were performed to assess the statistical support for each tree topology. During maximum-likelihood bootstrap analysis, re-arrangements were limited to 1000 per bootstrap replicate to reduce computational time.

Total evidence analysis.—Concordance between datasets was evaluated with the Partition-Homogeneity Test implemented in PAUP. For concordant data, datasets were combined and parsimony and maximum-likelihood analyses were run as previously described.

RESULTS

Isolates used in this study.—For all isolates, morphological characteristics of conidia produced in cultures grown on 0.05× PDA and PCA generally were consistent with published descriptions of isolates cultured on PCA (Simmons 1967, 1983, 1986, 1989, 1990, 1995; FIG. 1). However, characteristics of *E. indefessa* conidia were more consistent with those of many *Alternaria* spp., e.g., conidia shape typically ovoid to short obclavate rather than ovoid to elliptical as previously reported on PCA (FIG. 1F).

DNA extraction, PCR amplification and sequencing.—PCR resulted in the successful amplification of 591–626 bp fragments using primers ITS5 and ITS4, 639–765 bp fragments using primers NMS1 and NMS2 and 545–606 bp fragments using primers *gpd*1 and *gpd*2 for all isolates. All sequences determined in this study have been submitted to GenBank, and accession numbers are listed in TABLE I.

Phylogenetic analysis.—Alignment of the ITS sequences with those of other *Alternaria*, *Ulocladium*, *Stemphylium* and *Exserohilum* spp. resulted in a 632-character dataset, of which 135 characters (21.4%) were variable and 88 characters (13.9%) were parsimony informative. In the aligned dataset, four regions of numerous indels were apparent and the alignment of characters within these regions was variable. Variable Region 1 (ITS-VR1) spanned characters 55–123.

TABLE I. Isolates used in this study, their sources, and GenBank accession numbers for sequences used in phylogenetic analyses. Sequences that were determined in the course of this study appear in bold

Species	Source	GenBank accession		
		ITS	mt SSU	*gpd*
Alternaria alternata	EGS 34-016	AF347031	**AY278849**	**AY278808**
A. arborescens	EGS 39-128	AF347033	**AY278851**	**AY278810**
A. brassicicola	EEB 2232	AF229462	AF229652	**AY278813**
A. carotiincultae (syn. *A. radicina*)	EGS 26-010	AF229465	AF229654	**AY278798**
A. cheiranthi	EGS 41-188	AF229457	AF229655	**AY278802**
A. crassa	DGG Acr 1	AF229464	AF229656	**AY278804**
A. dauci	ATCC 36613	AF229466	AF229657	**AY278803**
A. destruens	EGS 46-069	**AY278836**	**AY278853**	**AY278812**
A. ethzedia	EGS 37-143	**AY278833**	**AY278847**	**AY278795**
A. infectoria	EGS 27-193	AF347034	**AY278846**	**AY278793**
A. japonica	ATCC13618	AF229474	AF229661	**AY278814**
A. longipes	EGS 30-033	**AY278835**	**AY278852**	**AY278811**
A. macrospora	DGG Ams1	AF229469	AF229663	**AY278805**
A. petroselini	EGS 09-159	AF229454	AF229664	**AY278799**
A. porri	ATCC 58175	AF229470	AF229667	**AY278806**
A. radicina	ATCC 96831	AF229472	AF229668	**AY278797**
A. selini	EGS 25-198	AF229455	AF229673	**AY278800**
A. smyrnii	EGS 37-093	AF229456	AF229674	**AY278801**
A. solani	ATCC 58177	AF229475	AF229675	**AY278807**
A. tenuissima	EGS 34-015	AF347032	**AY278850**	**AY278809**
A. triticina	EGS 41-050	**AY278834**	**AY278848**	**AY278796**
Embellisia allii	EGS 38-073	**AY278840**	**AY278857**	**AY278827**
E. leptinellae	EGS 40-187	**AY278845**	**AY278862**	**AY278832**
E. indefessa	EGS 30-195	**AY278841**	**AY278858**	**AY278828**
E. novae-zelandiae	EGS 39-099	**AY278844**	**AY278861**	**AY278831**
E. proteae	EGS 39-031	**AY278842**	**AY278859**	**AY278829**
E. hyacinthi	EGS 49-062	**AY278843**	**AY278860**	**AY278830**
Exserohilum pedicillatum	EEB 1336	AF229478	AF229660	**AY278824**
Lewia infectoria	ATCC 12054	AF229480	AF229666	**AY278794**
Nimbya caricis	EGS 13-094	**AY278839**	**AY278856**	**AY278826**
N. scirpicola	EGS 19-016	**AY278838**	**AY278855**	**AY278825**
Pleospora herbarum	ATCC 11681	AF229479	AF229665	**AY278823**
Stemphylium botryosum	ATCC 42170	AF229481	AF229671	**AY278820**
S. callistephi	EEB 1055	AF229482	AF229672	**AY278822**
S. vesicarium	ATCC 18521	AF229484	AF229677	**AY278821**
Ulocladium alternariae	BMP 3141005	AF229485	AF229679	**AY278815**
U. atrum	ATCC 18040	AF229486	AF229680	**AY278818**
U. botrytis	ATCC 18043	AF229487	AF229681	**AY278817**
U. chartarum	ATCC 18044	AF229488	AF229682	**AY278819**
U. consortiale	BMP 3151001	**AY278837**	**AY278854**	**AY278816**

Abbreviations for sources are as follows: ATCC, American Type Culture Collection, Manassas, VA 20108; BMP, B. M. Pryor, Department of Plant Pathology, University of Arizona, Tucson, AZ 85721; DGG, D. G. Gilchrist, Department of Plant Pathology, University of California, Davis, CA 95616; EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA 95616; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933.

Within this region, a notable indel (characters 88–113) was present in sequences from *N. caricis*, *N. scirpicola* and *E. allii*, members of the infectoria species-group, and members of the brassicicola species-group but not in sequences from any other isolate. Variable regions 2, 3 and 4 (ITS-VR2, ITS-VR3 and ITS-VR4, respectively) spanned characters 236–244, 447–469 and 540–590, respectively.

Alignment of the mt SSU dataset with those of other *Alternaria*, *Ulocladium*, *Stemphylium* and *Exserohilum* spp. resulted in a 769-character dataset, of which 82 characters (10.7%) were variable and 50 charac-

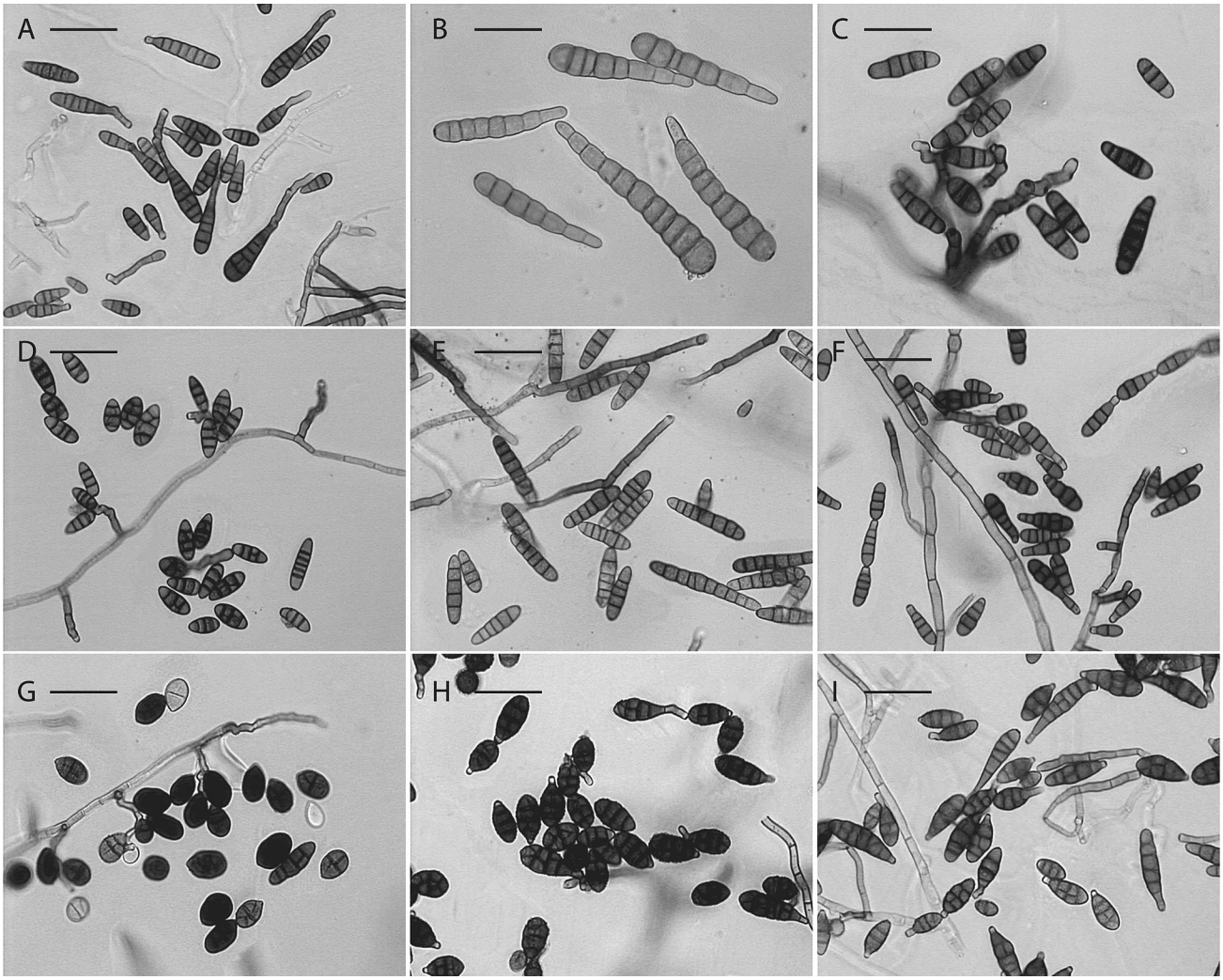

FIG. 1. Conidia of (A) *Alternaria infectoria*, (B) *Nimbya caricis*, (C) *Embellisia allii*, (D) *E. proteae*, (E) *E. leptinellae*, (F) *E. indefessa*, (G) *Ulocladium alternariae*, (H) *U. chartarum* and (I) *A. alternata*. Scale bars equal 35 μm.

ters (6.5%) were parsimony informative. In the aligned dataset, one very large region of indels was present and spanned characters 365–559. Within this region, numerous indels were present, ranging in size from 3 to 38 bp, and alignment of these indels was variable. However, the presence of specific indels among members of previously established species-groups generally was conserved and sequence identity among members of species-groups was usually >98%.

Alignment of the *gpd* sequences resulted in a 613-character dataset, of which 237 characters (38.7%) were variable and 168 characters (27.4%) were phylogenetically informative. In the aligned dataset, two regions of indels were present and alignment of sequences within these regions was variable. Variable Region 1 (*gpd*-VR1) spanned characters 26–93, and Variable Region 2 (*gpd*-VR2) spanned characters 169–282. Alignments of each dataset have been submitted to TreeBASE for review (SN1412).

Maximum-parsimony analysis of the ITS dataset yielded nine equally most-parsimonious trees (steps = 266, CI = 0.654, RI = 0.834), which differed primarily in minor changes in the relationship among members of the porri species-group and in minor changes in the relationships among members of the *Ulocladium* group (FIG. 2). Nine principle clades were evident, seven of which corresponded to previously established monophyletic groups: *Stemphylium*, infectoria species-group, brassicicola species-group, porri species-group, radicina species-group, alternata species-group and *Ulocladium* group (Pryor and Gilbertson 2000). Most of these groups were supported strongly by bootstrap values of >83%, with the exception of the brassicicola species-group, which was weakly supported by bootstrap values of 56%. Two

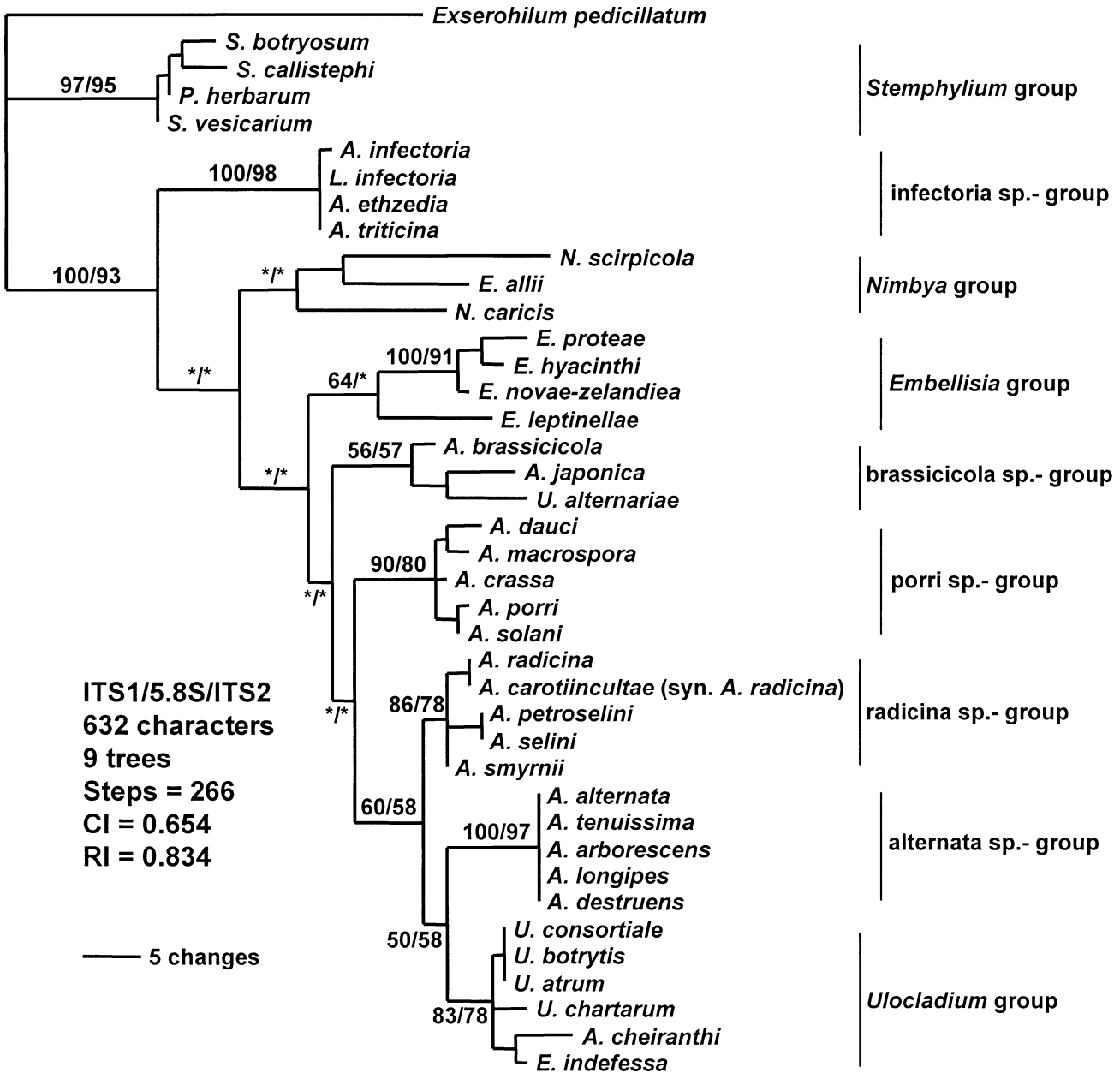

FIG. 2. One of nine most-parsimonious trees generated from maximum-parsimony analysis of ITS1/5.8S/ITS2 sequences from select *Alternaria*, *Embellisia*, *Nimbya*, *Ulocladium* and related species. Number in the front of "/" represents parsimony bootstrap values from 500 replicates, and number after the "/" represents maximum-likelihood bootstrap values from 500 replicates. Values represented by an "*" were less than 50%. Bootstrap values for terminal nodes are not presented. The scale bar indicates the number of nucleotide substitutions; vertical distances have no significance.

additional clades were evident, referred to herein as the *Nimbya* group and the *Embellisia* group. The *Nimbya* group comprised *N. caricis*, *N. scirpicola* and *E. allii*. The *Embellisia* group comprised *E. hyacinthi*, *E. leptinellae*, *E. novae-zelandiae* and *E. proteae*. These groups were weakly supported by bootstrap values of <50% and 64%, respectively. Within *Embellisia*, three *Embellisia* species clustered together and were strongly supported with a bootstrap value of 100%. The inclusion of *E. leptinellae* as a fourth species within this group was supported only weakly in bootstrap analysis. It is interesting to note that *E. indefessa* clustered with the *Ulocladium* species and *A. cheiranthi*, previously established as the *Ulocladium* group. This association was strongly supported by a bootstrap value of 83% (FIG. 2). Removal of ITS-VR2, ITS-VR3 and ITS-VR4 had no significant effect on tree topology or tree support (data not shown). Removal of ITS-VR1

resulted in 563-character dataset, of which 96 (17.1%) characters were variable and 60 (10.7%) were parsimony informative. A heuristic search of this dataset resulted in more than 5000 most-parsimonious trees (steps = 178, CI = 0.674, RI = 0.847), which varied primarily in the relationships among *Alternaria* species-groups but not in the composition of the species-groups (data not shown). Bootstrap analysis of this dataset had only minor effects on the resulting topology of the bootstrap consensus tree compared to that resulting from analysis of the complete ITS dataset, but it resulted in significantly lower bootstrap values (<50%) for most clades previously established, with the exception of the *Stemphylium* group and alternata species-group (data not shown).

Maximum-likelihood analysis of the ITS dataset revealed a most-likely tree that had near-identical topology as displayed in FIG. 2 (minor differences in branch lengths), with the exception of the position of *E. leptinellae*, which did not cluster with any previously described clade and its position was basal to the *Embellisia* clade (data not shown). Bootstrap support for maximum-likelihood topology was similar to that for parsimony topology (FIG. 2).

Maximum-parsimony analysis of the mt SSU dataset yielded more than 20 000 most-parsimonious trees (steps = 122, CI = 0.869, RI = 0.936), due to the number of large indels and identical or nearly identical sequences within the dataset. Most of these trees had similar topography in terms of species clusters as those revealed in analysis of the ITS dataset (FIG. 3). However, branch lengths often were significantly longer due to the presence of species-group-specific indels of varying lengths (3–38 characters). Similarities included strong bootstrap support (>81%) for infectoria and porri species-groups and the *Ulocladium* group and moderate support (>70%) for the *Stemphylium* group. *Nimbya* and *Embellisia* groups and the radicina species-group were weakly supported. Moreover, the relationships among the *brassicicola*, *porri*, *alternata* and *radicina* species-groups and the *Ulocladium* group were unresolved as a polytomy. The removal of the large variable section resulted in a 574-character dataset, of which 26 (4.5%) of the characters were variable and 13 (2.3%) were parsimony informative. A heuristic search of this dataset resulted in 126 most-parsimonious trees (steps = 33, CI = 0.879, RI = 0.920), most of which were unresolved as polytomies (data not shown). Bootstrap analysis of this dataset also placed most species in unresolved polytomies (data not shown).

Maximum-likelihood analysis of the mt SSU dataset revealed a nearly identical tree as displayed in FIG. 3 (minor differences in branch lengths), with the exception of a reversal in positions of the porri species-group and *Ulocladium* group relative to the positions of the remaining clades (data not shown). Bootstrap support for maximum-likelihood topology was similar to that for parsimony topology (FIG. 3).

Maximum-parsimony analysis of the *gpd* dataset yielded 12 equally most-parsimonious trees (steps = 560, CI = 0.579, RI = 0.788), which differed primarily in the relationships among members of the porri species-group and in the position of the radicina and alternata species-groups relative to the porri species-group (FIG. 4). Similar clades were revealed in analysis of the *gpd* dataset as were revealed in analysis of the ITS and SSU datasets. Excluding minor differences in branch lengths, the only significant differences in results from parsimony analysis of the *gpd* dataset compared to the ITS dataset was that *E. leptinellae* and *U. alternariae* did not cluster in the *Embellisia* clade and the brassicicola clade, respectively, or in any of the other previously described clades. Bootstrap analysis revealed strong support (>81%) for all previously described clades, with the exception of the *Nimbya* clade and the brassicicola clade (*A. brassicicola* and *A. japonica*). Removal of *gpd*-VR1 and *gpd*-VR2 independently had only minor effects on tree topology but generally reduced bootstrap support for most clades (data not shown). However, removal of both variable regions resulted in a 431-character dataset, of which 93 (21.6%) characters were variable and 33 (7.7%) were parsimony informative. A heuristic search of this dataset resulted in 12 most-parsimonious trees (steps = 187, CI = 0.561, RI = 0.754), which differed from trees derived from the complete dataset in a reversal of polarity (basal versus distal) of nearly all clades in the large *Alternaria/Nimbya/Embellisia/Ulocladium* clade (i.e., the radicina and *Ulocladium* clades were most basal and the infectoria and *Nimbya* clades were most distal; data not shown). The bootstrap consensus tree derived from this dataset did not reflect this reversal in clade position, but bootstrap support was poor (<50%) for most clades that were resolved, with the exception of the *Stemphylium* group and infectoria species-group (data not shown).

Maximum-likelihood analysis revealed a nearly identical tree as displayed in FIG. 4 (minor differences in branch lengths), with the exception of a reversal in positions of the porri and radicina clades relative to the positions of the remaining clades. In addition, *A. brassicicola* and *A. japonica* did not cluster together or with any other previously described group. Bootstrap support for maximum-likelihood topology was similar to that for parsimony topology (FIG. 4).

Total evidence analysis.—Datasets were combined and partitioned by gene in this analysis. Test for concor-

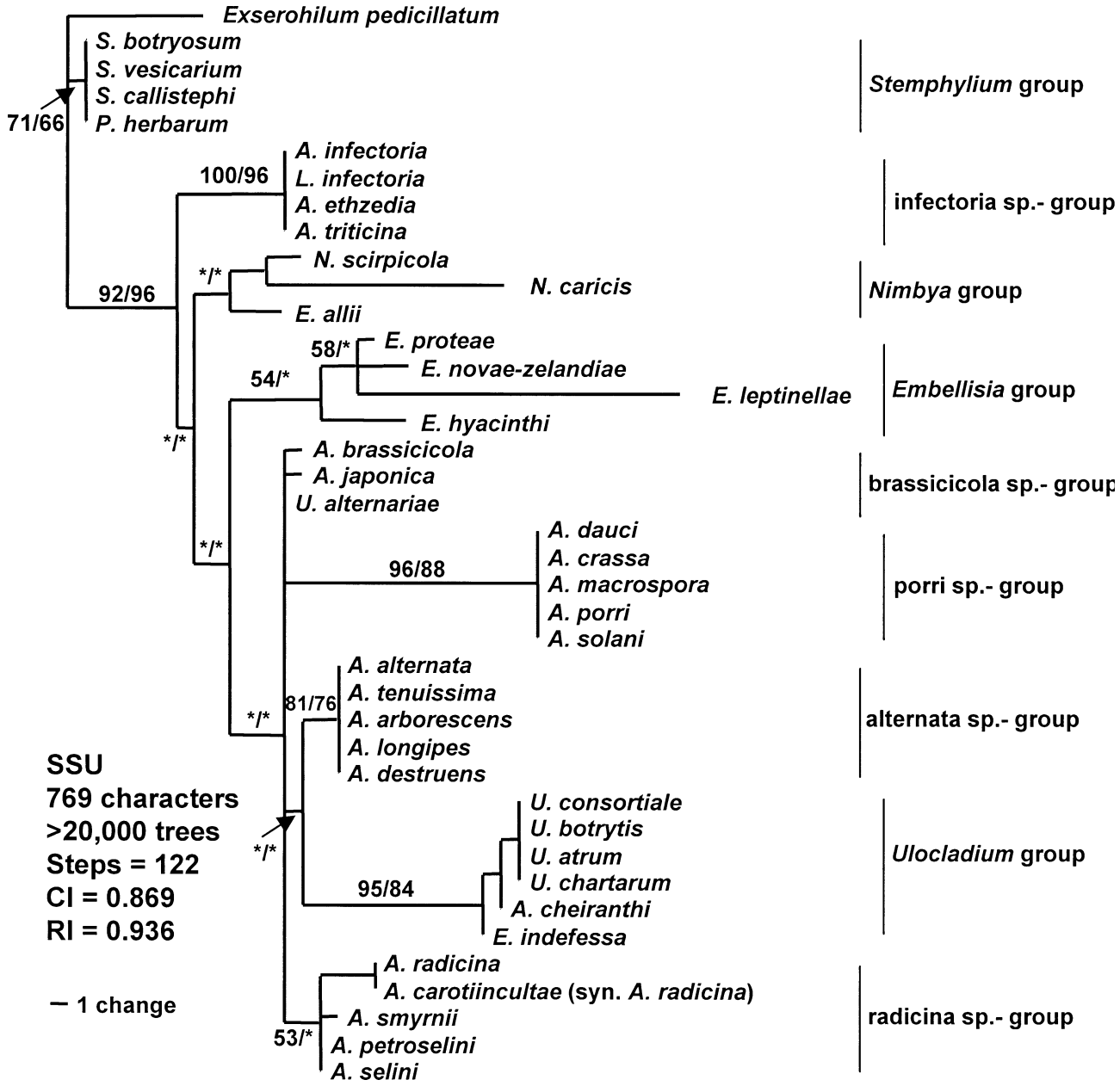

FIG. 3. One of more than 20 000 most-parsimonious trees generated from maximum-parsimony analysis of mitochondrial SSU sequences from select *Alternaria*, *Embellisia*, *Nimbya*, *Ulocladium* and related species. Number in the front of "/" represents parsimony bootstrap values from 500 replicates, and number after the "/" represents maximum-likelihood bootstrap values from 500 replicates. Values represented by an "*" were less than 50%. Bootstrap values for terminal nodes are not presented. The scale bar indicates the number of nucleotide substitutions; vertical distances have no significance.

dance in datasets following combination revealed the data were not significantly inconcordant ($P = 0.360$), therefore, parsimony and maximum-likelihood analysis proceeded on the combined dataset. Maximum-parsimony analysis of the combined dataset yielded two equally most-parsimonious trees (steps = 964, CI = 0.627, RI = 0.810), which differed primarily in the relationship among members of the porri species-group (FIG. 5). Most clades revealed in the analysis of the combined dataset were revealed in analysis of the individual datasets with the exception of the brassicicola group, in which *U. alternariae* was not included and was resolved separately. Most resolved groups and species-groups previously discussed were strongly supported (>91%) in bootstrap analysis. The *Nimbya* clade was moderately supported (74%) and brassicicola clade (*A. brassicicola* and *A. japonica*) was weakly supported (51%). Removal of all variable regions re-

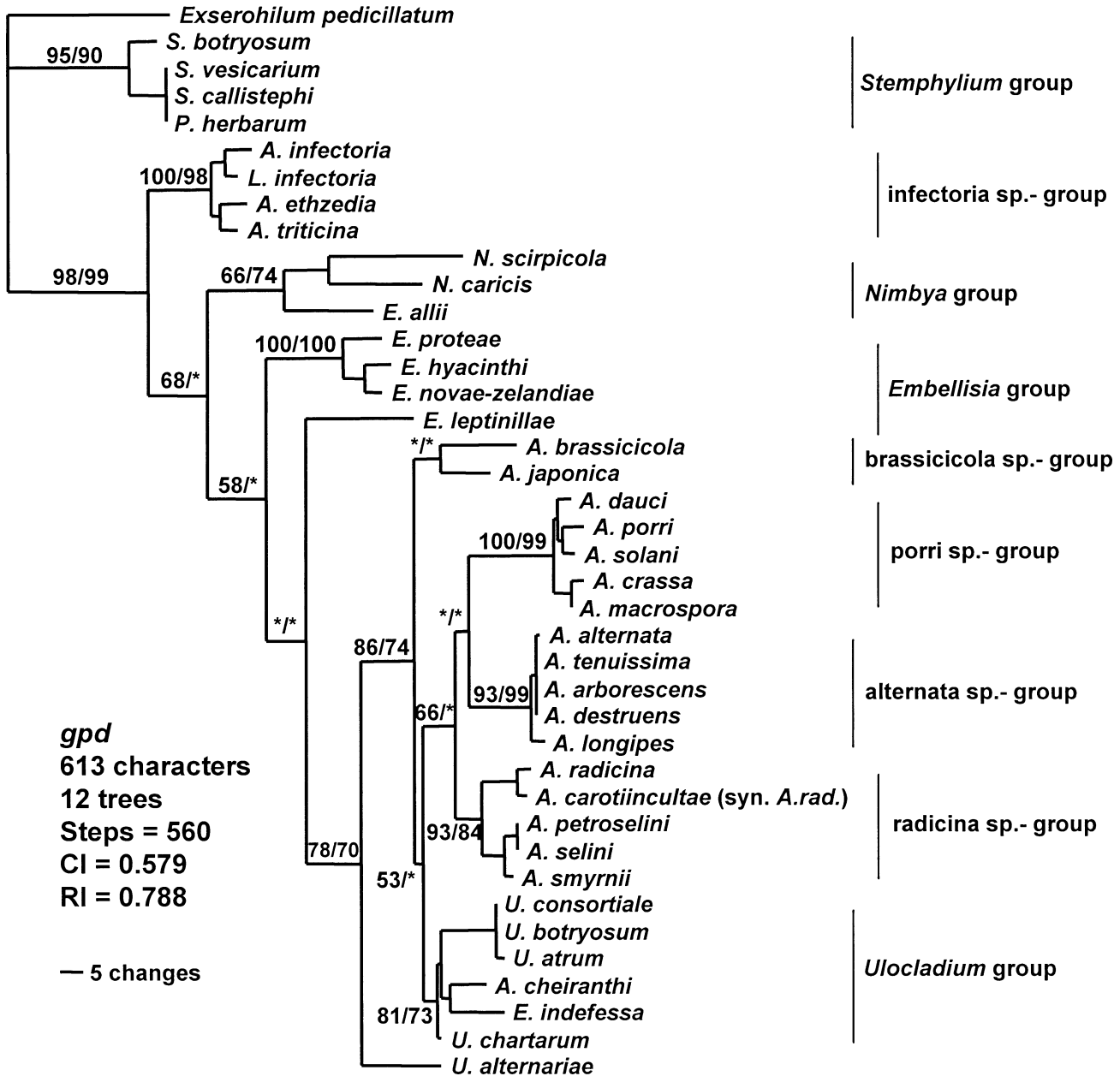

FIG. 4. One of 12 most-parsimonious trees generated from maximum-parsimony analysis of *gpd* sequences from select *Alternaria*, *Embellisia*, *Nimbya*, *Ulocladium* and related species. Number in the front of "/" represents parsimony bootstrap values from 500 replicates, and number after the "/" represents maximum-likelihood bootstrap values from 500 replicates. Values represented by an "*" were less than 50%. Bootstrap values for terminal nodes are not presented. The scale bar indicates the number of nucleotide substitutions; vertical distances have no significance.

sulted in a 1493-character dataset, of which 183 (12.3%) characters were variable and 113 (7.6%) were parsimony informative. A heuristic search of this dataset resulted in 18 most-parsimonious trees (steps = 346, CI = 0.613, RI = 0.789), which differed from trees derived from the complete dataset in a reversal in positions of the porri species-group and the *Ulocladium* group, relative to the positions of the remaining clades (data not shown). In addition, the infectoria species-group and *Nimbya* group clustered in a single clade, as did the brassicicola species-group and *Ulocladium* group. Bootstrap analysis of these data resulted in a consensus tree with similar topology as the most-parsimonious trees. Bootstrap support for most clades was somewhat lower, particularly the radicina clade (<50%). In addition, bootstrap

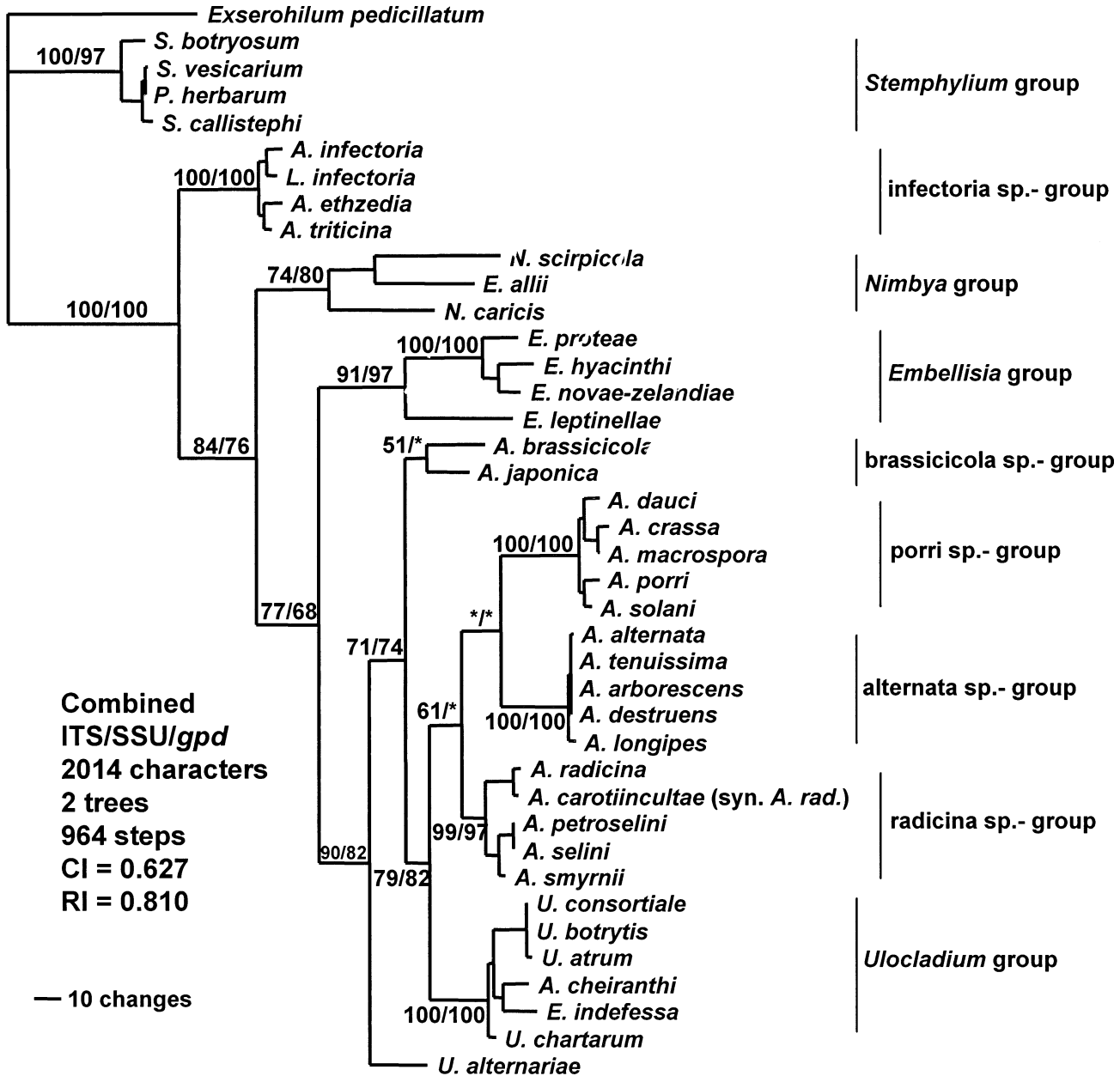

FIG. 5. One of two most-parsimonious trees generated from maximum-parsimony analysis of a combined dataset containing ITS, mt SSU and *gpd* sequences from select *Alternaria*, *Embellisia*, *Nimbya*, *Ulocladium* and related species. Number in the front of "/" represents parsimony bootstrap values from 500 replicates, and number after "/" represents maximum-likelihood bootstrap values from 500 replicates. Values represented by an "*" were less than 50%. Bootstrap values for terminal nodes are not presented. The scale bar indicates the number of nucleotide substitutions; vertical distances have no significance.

support for the infectoria/*Nimbya* and brassicicola/*Ulocladium* clusters were poor (<50%; data not shown).

Maximum-likelihood analysis revealed a tree nearly identical to that as displayed in FIG. 5, with the exception of a reversal in the positions of the porri and *Ulocladium* clades relative to the remaining clades (data not shown). Bootstrap support for maximum-likelihood topology was similar to that for parsimony topology (FIG. 5).

DISCUSSION

This work describes phylogenetic relationships among select species of *Embellisia* and *Nimbya* and species of *Stemphylium*, *Alternaria* and *Ulocladium*,

based on sequences from three different genetic regions, nuclear ITS rDNA, mitochondrial SSU rDNA, and a single-copy protein-encoding gene, *gpd*. Although the ITS sequence of *N. gomphrenae* has been included in a previous phylogenetic analysis of *Alternaria* and *Ulocladium* species (Chou and Wu 2002), to date, no further examination of molecular relationships among *Nimbya* and other genera has been explored. Moreover, no analysis of *Embellisia* phylogeny has ever been conducted. Thus, this work provides the first systematic examination of these two genera as they relate to the hypothesized related taxa of *Alternaria* (Simmons 1992).

In general, analysis of the three genetic regions independently resulted in similar phylogenetic conclusions. Although amplified fragments from all regions were roughly similar in size (545–765 bp), the *gpd* sequences contained almost twice the number of variable sites and parsimonious-informative sites as the ITS sequences, which contained approximately twice the number of variable sites and parsimonious-informative sites as the mt SSU region. The increase in the number of variable sites did not necessarily translate into improved phylogenetic resolution because results from analysis of the *gpd* sequences were very similar to those of the ITS sequences in terms of tree topology and bootstrap support. Similar results were obtained in comparisons to ITS and *gpd* phylogenies using *Cochliobolus* and *Pyrenophora* sequences as well (Berbee et al 1999, Zhang and Berbee 2002). Furthermore, results from analysis using maximum parsimony were similar to results using maximum likelihood in that clades strongly supported in parsimony were generally strongly supported in likelihood. Results from analysis of the mt SSU sequences were somewhat different from those of ITS and *gpd* sequences in that many of the intermediate nodes in the resulting tree were poorly resolved or not at all (polytomies). This low resolution was due, in part, to the low number of total variable sites within the dataset and to the paucity of variable sites outside the region of indels described previously. Although analysis of this region was variable with respect to indel alignment, the presence of specific indels was diagnostic for certain groups, such as *Ulocladium*, in which there was greater than 99% sequence identity among the six representatives of the group. This high degree of sequence identity in mt SSU sequences among members of particular groups resulted in strong support for most of the terminal clades, despite low support for more basal clades.

Perhaps more important in terms of robust phylogenetic conclusions, datasets were not significantly inconcordant and results of the combined datasets were similar in most aspects to those results obtained from independent analysis, often with greater bootstrap support. In regard to *Alternaria/Ulocladium* phylogeny, this work revealed relationships that were consistent with previous studies; specifically, that *Alternaria* and *Ulocladium* spp. composed a strongly supported monophyletic clade with *Stemphylium* as sister group of this clade (Pryor and Gilbertson 2000, de Hoog and Horre 2002). Within this clade, *Ulocladium* was polyphyletic. In addition, the primary clade containing most *Ulocladium* spp., which was strongly supported in all parsimony and maximum-likelihood analyses, included an *Alternaria* and *Embellisia* spp. and, thus, was paraphyletic. Therefore, support for *Ulocladium* as a distinct monophyletic genus was not evident, which also had been revealed in previous studies (Pryor and Gilbertson 2000, de Hoog and Horre 2002).

These findings conflict with morphology because all *Ulocladium* species examined in this study including *U. alternariae*, which did not cluster with the other *Ulocladium* spp., possessed similar characteristics. These characteristics included small, heavily pigmented conidia produced in dense clusters on closely geniculate conidiophores. Most important, all isolates produced conidia that typically were ovoid in shape, a key differentiating characteristic of the genus as described by Simmons (1967). Moreover, *E. indefessa* and *A. cheiranthi*, which clustered tightly with most *Ulocladium* spp. in all three datasets, did not possess this combination of characters. Taken together, these results suggest that the characters that circumscribe *Ulocladium* are homoplastic, derived from *Alternaria* progenitors and are not reliable criteria for determining evolutionary relationships.

This work revealed similar findings in regard to the relationship between *Embellisia* and *Alternaria*. In the combined dataset, four *Embellisia* spp. composed a strongly supported monophyletic clade, which was basal to most *Alternaria/Ulocladium* species, except those within the infectoria species-group. However, the type species of *Embellisia*, *E. allii*, did not cluster with this group and clustered with *Nimbya* spp. with moderate bootstrap support in the combined dataset. In addition, *E. indefessa* clustered with the *Ulocladium/A. cheiranthi* clade and was strongly supported in bootstrap analysis of all individual datasets and in the combined dataset. The key character that circumscribes *Embellisia*—viz. relatively thick, dark, and rigid septation in contrast to the external wall of the conidium—was apparent in all *Embellisia* spp. examined. These data reveal that the phylogeny of *Embellisia* is similar to that of *Ulocladium* in that the genus is polyphyletic and the key differentiating characters of *Embellisia* are likely homoplastic. Thus, the results of this work questions the phylogenetic status of *Em-*

bellisia, much as it does the phylogenetic status of *Ulocladium*.

Although this work revealed that the three datasets were concordant and that results from analysis of each dataset were corroborative, several taxa were problematic in the development of strongly supported phylogenetic trees. The *Nimbya* group, comprising *N. scirpicola*, *N. caricis* and *E. allii*, was a weakly supported clade in analysis of each of the three datasets. In the phylogram generated from the combined dataset, greater bootstrap support was obtained. However, increased bootstrap values might not necessarily be confirmation of monophyly because these data might be statistically inconsistent or ''positively misleading'', i.e., estimating an incorrect clade with increasing certainty as the amount of character data increases (Felsenstein 1978, Hendy and Penny 1989). It might be that these taxa are representatives of several distinct but related clades, which might be resolved as distinct when additional related taxa are included in the dataset.

A similar situation was apparent in regard to the *Embellisia* group. Three *Embellisia* spp., *E. hyacinthi*, *E. novae-zelandiae* and *E. proteae*, clustered together with strong bootstrap support in analysis of each of the three datasets, establishing a monophyletic core for the *Embellisia* group. A fourth species, *E. leptinellae*, clustered with these three species in two of the three datasets but with low bootstrap support. However, when all three datasets were combined, support for the association of *E. leptinellae* with the remaining taxa in the *Embellisia* group was increased significantly. Again, this does not necessarily suggest a true relationship because the data might be ''positively misleading''. Additional related taxa might be needed in the dataset to resolve more firmly the true relationship of *E. leptinellae* to other *Embellisia* spp.

A different situation existed for *U. alternariae*, a particularly problematic taxon. *U. alternariae* clustered with *A. brassicicola* and *A. japonica* in analysis of the ITS region. However, this clade was weakly supported. This relationship was evident in analysis of the mt SSU dataset as well. In analysis of the *gpd* dataset, *U. alternariae* did not cluster within the brassicicola species-group, nor did it cluster in any other species-group. Bootstrap support for intermediate nodes generally was weak in analysis of each of the three datasets. So, from these data it was not possible to definitively resolve the relationship of *U. alternariae* to other clades. In the combined dataset, *U. alternariae* also did not cluster within the previously defined brassicicola species-group. However, in the combined dataset nearly all intermediate clades were moderately to strongly supported and from these data it was possible to resolve the position of *U. alternariae* within the large *Alternaria/Nimbya/Embellisia/Ulocladium* clade, as well as the positions of *A. brassicicola* and *A. japonica*, as basal to clades containing exclusively asexual taxa of *Alternaria* and *Ulocladium*. Moreover, these data clearly resolve *U. alternariae* as distinct from the other *Ulocladium* spp., which is consistent with 18S-based phylogeny of *Alternaria* and related taxa developed in previous studies (Pryor and Gilbertson 2000).

Data from this work revealed that most genera examined were not natural taxa and were in conflict with the principles of modern phylogenetic systematics (Hennig 1966, Wiley et al 1991). If *Alternaria*, *Nimbya*, *Embellisia* and *Ulocladium* are not monophyletic clades but are all closely related taxa, how might the taxonomy of all four genera be resolved so that the final taxonomic structure is consistent with contemporary concepts in systematics, i.e., each taxonomic unit be resolved into a monophyletic clade? Several possible solutions exist. The first is to collapse the four genera into one genus, *Alternaria*. If taxa within the infectoria species-group, which are phylogenetically basal to *Nimbya*, *Embellisia* and *Ulocladium*, are to be maintained within a monophyletic clade containing the remaining *Alternaria* spp., then all taxa within the clade containing both the infectoria species-group and the remaining *Alternaria* spp. necessarily would be *Alternaria*. This would require a significant revision of characters that circumscribe *Alternaria* to include those of *Nimbya*, *Embellisia* and *Ulocladium* spp., as well as synonymy of species currently described as *Nimbya*, *Embellisia* and *Ulocladium* to newly erected *Alternaria* taxa.

The second solution would be to maintain all genera as distinct. However, for this solution to be consistent with the premise of monophyly of taxonomic units, the characters that circumscribe most genera would need to be revised. For example, this work revealed that *E. allii* clustered most closely with *Nimbya* spp., *E. indefessa* and *A. cheiranthi* clustered with *Ulocladium* spp. and *U. alternariae* clustered most closely with *Alternaria* spp. Nomenclatural revision for all these taxa would be needed so that taxonomic status would be consistent with phylogenetic relationships, and all genera would need revisions of diagnostic characters to be consistent with the newly synonymized taxa. The circumscription of *Embellisia* would be problematic because the type for the genus, *E. allii*, clustered most closely with *Nimbya* spp. in a clade distinct from the *Embellisia* clade resolved in this work. The circumscription of *Ulocladium* would be equally problematic because *Alternaria* species occur both basal (e.g., *A. brassicicola* and *A. japonica*) and distal (e.g., the alternata and radicina species-groups) to the *Ulocladium* clade within the large *Al-*

ternaria/Nimbya/Embellisia/Ulocladium clade. In addition, if the phylogenetic concept of monophyly of genera is upheld, the infectoria species-group, which currently includes approximately 15 species of *Alternaria* (several of which have known teleomorphs) and is phylogenetically basal to both the *Nimbya* and *Embellisia* clade, would require genus designation distinct from *Alternaria* species-groups that contain strictly asexual taxa.

Both solutions require rather substantial changes to the taxonomic structure of this group of fungi and some significant revisions of how mycologists view these groups. However, the advancement of mycology is best served when such changes are made slowly and carefully because the consequences of erroneous conclusions based on partial data would result in unnecessary confusion in our understanding of a group of fungi already hampered by historical controversy. Results of this study do not suggest an immediate taxonomic revision of *Alternaria* and related genera. Results of this study do reveal an apparent conflict between the resolved phylogenies of several genes and contemporary taxonomic structure, and this suggests that taxonomic revision might be necessary. For this revision to proceed with confidence, the resolution of addition gene phylogenies is necessary, as well as an evaluation of concordance between those data and data presented in this work. The inclusion of more taxa would be necessary to represent the full range of diversity among species contained in *Alternaria*, *Nimbya*, *Embellisia* and *Ulocladium* and to assist in the resolution of clades that have been weakly supported to date. In addition, a comprehensive examination of morphological, biochemical and cytological characters of contemporary species would be necessary to develop the biological and ecological basis for revisionary taxonomy and to support the development of meaningful, natural taxonomic keys.

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