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ORIGINAL ARTICLE

Ulocladium systematics revisited: phylogeny and taxonomic status

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Abstract The genus *Ulocladium* represents phaeodictyosporic Hyphomycetes that produce conidia that are essentially obovoid in shape. Previous molecular studies that included *Ulocladium* and related taxa in *Alternaria*, *Embellisia*, and *Stemphylium* revealed a conflict between morphology and phylogeny, and *Ulocladium* was supported as polyphyletic with a paraphyletic core group. Moreover, the genus consistently resolved within a larger *Alternaria*/*Ulocladium* clade, resulting in paraphyly of *Alternaria* and questions as to the taxonomic status of *Ulocladium*. In the present study, 13 *Ulocladium* species and three genetic loci were included for a more comprehensive systematic analysis of the genus than had previously been conducted. Total genomic DNA was extracted from representative taxa and sequences were determined for the nuclear internal transcribed spacer region, including the 5.8S rDNA gene, and the protein-coding genes glyceraldehyde-3-phosphate dehydrogenase and Alt a1. Subsequent phylogenetic analyses based on maximum parsimony and Bayesian methods included related *Alternaria*, *Embellisia*, and *Stemphylium* spp. Results supported previous findings of polyphyletic and paraphyletic relationships of *Ulocladium* among other taxa. Ten *Ulocladium* species clustered into a core *Ulocladium* clade and all taxa possessed the key diagnostic feature of *Ulocladium*, namely, conidia essentially obovoid in shape. However, *A. cheiranthi* and *E. indefessa* also clustered within this group with high bootstrap support but did not posses this diagnostic feature. This paraphyletic clade resolved basal to the core *Alternaria* clade with high bootstrap support, unlike previous studies in which its position was imbedded within the primary *Alternaria* clade. Thus, the status of the genus as an independent lineage and a unique taxon is strongly supported. As previously reported, *U. alternariae* and *U. oudemansii*, which posses the key conidium characteristics of *Ulocladium*, clustered as a separate clade sister to the core *Ulocladium* clade. Further studies are necessary to determine if these taxa represent an independent lineage or share a common ancestor with other *Ulocladium* species. Obovoid conidia were poorly represented in the isolate of *U. lanuginosum* that was included in these analyses (the only *U. lanuginosum* isolate currently available), and the isolate resolved as *A. radicina* based upon all three loci sequenced. Based upon these data and the origin of the isolate, which was originally deposited as *A. malvae*, a reassessment of its identity is supported.

Keywords *Ulocladium* · ITS · *gpd* · Alt a1 · Parsimony · Bayesian inference

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Introduction

The genus *Ulocladium* represents anamorphic fungi in the Pleosporaceae, mostly found as common saprobes on plant material and in soil. Species can also be recovered from diverse substrates, such as plant debris, lumber, paper, and water-damaged building materials (Gravesen et al. 1999; Simmons 1967; Andersen and Hollensted 2008). *Ulocladium* spores are primarily dispersed by air and are known to

be common airway allergens (Al-Suwaine et al. 1999). In addition, some species have been reported as opportunistic human pathogens (de Hoog and Horre 2002).

The genus *Ulocladium* was first established by Preuss (1851) with *U. botrytis* as the type. Species are represented by dematiaceous phaedictyosporic Hyphomycetes with conidia essentially obovoid in shape. Related genera such as *Alternaria* and *Stemphylium* are also been characterized by the production of dematiaceous phaedictyospores (Nees 1816; Elliot 1917; Joly 1964; Neegard 1945; Simmons 1967; and Wiltshire 1933) and early taxonomy for this group of fungi was complicated because of gross similarities in conidium morphology among genera. As a consequence, many species have been placed into more than one of these genera since their initial description and the taxonomy of these fungi, especially *Ulocladium*, evolved with considerable historical controversy. For example, before a formal description of the genus *Ulocladium*, the type, *Ulocladium botrytis*, had been known by the synonyms *Stemphylium botryosum* Wallr. var. *Ulocladium* Sacc., v.s. and *Stemphylium botryosum* Wallr. var. *botrytis* (Pr.) Lindau, v.s. Following circumscription of the genus by Preuss with two described species, few additional taxa were added in the years following and the genus was infrequently mentioned. In 1967, the genus *Ulocladium* was reviewed by Simmons in his seminal paper "Typification of *Alternaria, Stemphylium* and *Ulocladium*" (Simmons 1967). Species of these three genera were reviewed based upon morphological characteristics and compared with the type for each genus. It was concluded that several atypical *Alternaria* and *Stemphylium* species should actually be classified as *Ulocladium* and seven additional species were included in the genus, the first additions in over 100 years. Simmons highlighted that the fundamentally obovoid and nonbeaked conidia (i.e., do not taper distally) are the principal morphological characteristics that distinguishes *Ulocladium* from *Alternaria* and other related genera (Simmons 1967). However, this distinguishing character is often best viewed only in immature conidia and may subsequently be lost as the conidia mature. Moreover, ellipsoidal and spherical forms may also be common in some species, particularly among mature conidia. In regard to *Stemphylium*, species could be readily differentiated from *Ulocladium* on the basis of the conidiophores apex and the percurrently proliferating conidiophore, which are the major morphological characteristic that distinguishes *Stemphylium* from other phaedictyosporic Hyphomycetes (Simmons 1969).

Another genus with ties to *Ulocladium, Alternaria* and *Stemphylium* is *Embellisia* Simmons. These phaeosporic Hyphomycetes have conidia that are morphologically reminiscent of both phragmosporic *Helminthosporium* species and dictyosporic *Alternaria* species (Simmons 1971). Description of the diagnostic characteristics of *Embellisia* revealed by Simmons (1992) proposed a close relationship to *Alternaria* and other allied genera in the Pleosporaceae. However, the synonomies required during the erection of *Embellisia* suggested taxonomic placement may not be without some taxonomic uncertainty.

In most previous studies involving *Ulocladium*, the phylogenetic status of the genus has only peripherally been discussed as such studies were focused primarily on allied taxa of *Alternaria, Embellisia*, or *Stemphylium*. Based on the analysis of the nuclear internal-transcriber spacer (ITS), mitochondrial small subunit (mt SSU), glyceraldehydes-3-phosphate dehydrogenase (*gpd*) sequences and the *Alternaria* allergen gene Alt a1 (Pryor and Gilbertson 2000; Chou and Wu 2002; de Hoog and Horre 2002; Pryor and Bigelow 2003; Hong and Pryor 2005), a large monophyletic clade comprising *Alternaria*, *Ulocladium*, and *Embellisia* was supported with *Stemphylium* as the sister taxa. *Ulocladium* was revealed as polyphyletic within the paraphyletic clade of *Alternaria, Ulocladium,* and *Embellisia*. A core *Ulocladium* clade was revealed that included four species of *Ulocladium*, which possess the key taxonomic character of obovoid conidia, but also two other species, *Alternaria cherianthi* and *Embellisia indefessa,* which do not possess the key taxonomic character. In addition, *U. alternariae*, which does possess the key taxonomic character, did not cluster within the core *Ulocladium* clade. Moreover, the primary *Ulocladium* clade appeared to be imbedded within a larger *Alternaria* clade resulting in paraphyly of *Alternaria* and uncertainty as to the phylogenetic status of a distinct *Ulocladium* genus. Indeed, in a recent study by Xue and Zhang (2007), which was focused primarily on *Ulocladium* and was perhaps the most comprehensive review of the genus to date (seven previously described species and one newly described species), molecular data based on the nuclear ITS rDNA region and the glyceraldehyde-3-phospate dehydrogenase (*gpd*) gene continued to support a polyphyletic and paraphyletic *Ulocladium* lineage which did not resolve independent from the genus *Alternaria*. Thus, as continued conflict was revealed between morphology and phylogeny of *Ulocladium* species, it became important to further examine the phylogenetic status of *Ulocladium* and its relationship with allied taxa and provide additional perspectives on the disposition of the genus, especially as it relates to *Alternaria*.

The objective of the present study was to continue to explore phylogeny and the taxonomic status of *Ulocladium* species based on additional taxa and additional genetic loci for phylogenetic analyses. Relationships among most described *Ulocladium* spp. and a number of related *Alternaria, Embellisia* and *Stemphylium* spp. were examined based on sequences from nuclear rDNA internal transcriber spacer (ITS) region and two protein coding

genes, *gpd* and the Alt a1 allergen gene. Morphological characteristics were re-examined for further assessment of taxonomic relationships.

Materials and methods

Isolates included in this study

Thirteen species of *Ulocladium,* twenty-five species of *Alternaria,* five species of *Embellisia*, and two species of *Stemphylium* were included in this study. The *Ulocladium* species represent most of the previously described taxa (15 total species), but not the five most recently described taxa as recorded in Index Fungorum (http://www.indexfungorum.org). *Alternaria* species include representatives of the five most common species-groups described to date: the asexual clades of the alternata species-group, the porri species-group, the radicina species-group, and the brassicicola species-group, and the sexual clade of the infectoria species-group (teleomorph = *Lewia*), which has been shown in previous studies to be distantly related to asexual *Alternaria* (Pryor and Bigelow 2003; Hong and Pryor 2005; Xue and Zhang 2007). Isolate identity and source are included in Table 1.

DNA extraction, PCR amplification and sequencing

For DNA extraction and purification, previously described protocols (Pryor and Gilbertson 2000) were followed. DNA was adjusted to a final concentration of 10 ng/μL for each PCR reaction. Primers ITS4 and ITS5 (White et al. 1990) were used to amplify rDNA from the ITS region (ITS1, 5.8s, ITS2). Primers *gpd1* and *gp2* (Berbee et al. 1999) were used to amplify DNA from the *gpd* locus. Primers Alt-for and Alt-rev (Hong et al. 2005) were used to amplify DNA from the Alt a1 locus. PCR products were sequenced in both the forward and reverse directions for confirmation of nucleotide with FS Dye Terminator reactions (Applied Biosystems, Foster City, CA) and ABI automated DNA sequencer. The resulting nucleotide sequences were aligned by the BioEdit Program version 7 (Hall 2004). For all amplified regions, manual adjustments of sequence alignments were performed with the data editor program MacClade Phylogenetic software (version 4.0, Sinauer Associates Inc., Sunderland, Massachusetts). For some species ITS, *gpd*, and Alt a1 sequences included in the alignments were determined in previous studies. (Table 1).

Phylogenetic analysis of nucleotide sequences

Phylogenetic analyses were performed using methods of maximum parsimony and Bayesian inference. Maximum parsimony (MP) was performed using heuristic searches with random stepwise addition sequences of 1,000 replicates and branch swapping by tree-bisection-reconnection (TBR) with the "MulTree" option in effect and Max Tree was set to 1000 [PAUP 4.0 10b (Swofford 2002)]. Gaps were treated as missing. For statistical analyses of resolved trees, 1000 non-parametric bootstrap replicates were performed under the MP criteria. MrBayes 3.0b4 Software (Huelsenbeck and Ronquist 2001) was used for Bayesian analyses with four Markov chains, 2,000,000 numbers of generations, 1000 sample frequency, and 10,000 burn-in value. The model GTR+I+G obtained from Modeltest version 3.7 was used to construct Bayesian trees. PAUP 4.0b10 was used to reconstruct consensus tree with maximum posterior probability among 2000 sampled trees. Concordance between datasets was evaluated with the partition–homogeneity test (PHT). For concordant data, data sets were combined and maximum parsimony and Bayesian analyses were run as previously described.

Morphological characterization of Ulocladium spp

For examination of morphological characters, conidial suspensions were prepared from 7-day-old cultures on PCA and all suspensions were adjusted to an equal concentration (2.5×10^6 spores/ml). Slides were prepared from spore suspensions and images acquired at 100× using a Fuji S2 digital camera (FujiFilm USA, Inc., Edison, NJ) attached to an Olympus BX51 compound microscope (Olympus America, Inc., Center Valley, PA). Five pictures of relatively full fields of view (100×) were taken and conidia (30–50) with different septa numbers (1, 2, 3 and 4) were selected and length and width were measured using Olympus Microsuite software. In addition, 50 conidia were randomly selected and transepta number determined to calculate the average transepta number per species. One way ANOVA (Sigmastat software package; Systat Software Inc., San Jose, CA) was used to determine significant differences (≤0.05) in conidium length, width, and l/w ratio for each conidium category and in average transepta/conidium, and means separation was performed using Tukey's test.

Results

PCR amplification, sequencing and alignment

PCR resulted in the amplification of 555–610 bp fragments using primers ITS4 and ITS5, 585–600 bp fragments using primers *gpd1* and *gpd2*, and 416–486 bp fragments using primers Alt-for and Alt-rev, from all *Ulocladium* species. The sequences determined in this study have been submitted to GenBank, and these and other sequences used in this study are listed in Table 1.

Table 1 Isolates used in this study, their source, and GenBank accession numbers for sequences used in phylogenetic analyses

Species	Source[a]	GenBank accession		
		ITS	*gpd*	Alt a1
Alternaria alternata	EGS 34–016	AF347031	AY278808	AY563301
A. arborescens	EGS 39–128	AF347033	AY278810	AY563303
A. brassicicola	EEB 2232	AF229462	AY278813	AY563311
A. carotiincultae	EGS 26–010	AF229465	AY278798	AY563287
A. cheiranthi	EGS 41–188	AF229457	AY278802	AY563290
A. conjuncta	EGS 37–139	FJ266475	AY562401	AY563281
A. dauci	ATTCC 36613	AF229466	AY278803	AY563292
A. ethzedia	EGS 37–143	AY278833	AY278795	AY563284
A. infectoria	EGS 27–193	AY347034	AY278793	FJ266502
A. limoniasperae	EGS 45–100	FJ266476	AY562411	AY563306
A. longipes	EGS 30–033	AY278835	AY278811	AY563304
A. macrospora	DGG Ams1	AF229469	AY278805	AY563294
A. mimicula	EGS 01–056	FJ266477	AY562415	AY563310
A. petroselini	EGS 09–159	AF229454	AY278799	AY563288
A. porri	ATCC 58175	AF229470	AY278806	AY563296
A. oregonensis	EGS 29–194	FJ266478	FJ266491	FJ266503
A. radicina	ATCC 96831	AF229472	AY278797	AY563286
A. selini	EGS 25–198	AF229455	AY278800	FJ266504
A. smyrnii	EGS 37–093	AF229456	AY278801	AY563289
A. solani	ATCC 58177	AF229475	AY278807	AY563299
A. targetica	EGS 44–044	FJ266479	AY562407	AY563297
A. tenuissima	EGS 34–015	AF347032	AY278809	AY563202
Embellisia conoidea	CBS 132.89	FJ348226	FJ348227	FJ348228
E. indefessa	EGS 30–195	AY278841	AY278828	AY563323
E. novae-zelandiae	EGS 39–099	AY278844	AY278831	AY563324
E. proteae	EGS 39–031	AY278842	AY278829	FJ266505
E. hyacinthi	EGS 49–062	AY278843	AY278830	FJ266506
E. planifunda	CBS 537.83	FJ266480	FJ266492	FJ266507
E. tumida	CBS 589.83	FJ266481	FJ266493	FJ266508
Stemphylium botryosum	ATCC 42170	AF229481	AY278820	AY563274
S. vesicarium	ATCC 18521	AF229484	AY278821	AY563275
Ulocladium alternariae	BMP 0352	AF229485	AY278815	AY563316
U. atrum	ATCC 18040	AF229486	AY278818	AY563318
U. botrytis	ATCC 18043	AF229487	AY278817	AY563317
U. chartarum	ATCC 18044	AF229488	AY278819	AY563319
U. consortiale	CBS 201–67	FJ266482	FJ266494	FJ266509
U. cucurbitae	EGS 31–021	FJ266483	AY562418	AY563315
U. dauci	CBS 102062	FJ266484	FJ266495	FJ266510
U. lanuginosum[b]	CBS 102.26	FJ266485	FJ266496	FJ266511
U. multiforme	CBS 102060	FJ266486	FJ266497	FJ266512
U. obovoideum	CBS 101229	FJ266487	FJ266498	FJ266513
U. oudemansii	CBS 114.07	FJ266488	FJ266499	FJ266514
U. septosporum	CBS 109.38	FJ266489	FJ266500	FJ266515
U. tuberculatum	CBS 202.67	FJ266490	FJ266501	FJ266516

Sequences that were determined in the course of this study appeared in bold

[a] Abbreviations for source are as follows: ATCC, American Type culture collection, Manassas, VA 20108; BMP, B. M. Pryor, Division of Plant Pathology, Department of Plant Science, The University of Arizona, Tucson, AZ 85721;; DGG, D. G. Gilchrist, Department of Plant Pathology, University of California, Davis, CA 956161; EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA 95616; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933; CBS, Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

[b] No ex-type or representative cultures of *U. lanuginosum* are currently available. The isolate used in this study, CBS 102.26, is of questionable identity as revealed in this manuscript

Alignment of ITS sequences resulted in a 604 character dataset of which 104 characters (17.2%) were variable and 86 characters (14.2%) were parsimony informative. In the aligned data set several variable regions were apparent. A notable indel spanned characters 64–98 and was present in the sequences from *U. alternariae*, *U. oudemansii*, and the members of the infectoria and brassicicola species-groups. Alignment of the gpd sequences resulted in a 589 character dataset of which 202 (34.3%) characters were variable and 165 (28.0%) were parsimony informative. In the aligned data set two introns were present and spanned characters 23–79 and 144–267. Alignment of the Alt a1 sequence resulted in a 492 character dataset, of which 261 characters (53.0%) were variable and 219 characters (44.5%) were parsimony informative. In the aligned data set, one intron was present and spanned characters 328–404. Alignment of each data set has been submitted to Tree BASE for review (S2185).

Phylogenetic analyses

Maximum parsimony analysis of the ITS dataset resulted in 12 most-parsimonious trees (total length=180, CI=0.739, RI=0.915). All trees revealed *Alternaria* clades described in previous studies, namely the alternata species-group, the porri species-group, the radicina species-group, the brassicicola species-group, and the infectoria species-group, as well as the *Ulocladium* clade, the *Embellisia* clade, and the *Stemphylium* clade (Fig. 1a-k). The 12 MP trees differed only in an exchange in the position of the porri-species group and the radicina species-group, and minor changes in the relationship among members within the porri species-group and the alternata species-group (Fig. 2). Among nine principle clades, four *Alternaria* clades (alternata species-group, porri species-group, brassicicola species-group, infectoria species-group) and the *Stemphylium* group were strongly supported by high bootstrap values of >95%. The radicina species-group was moderately supported by a bootstrap value of 76% and the *Embellisia* group was weakly supported by a bootstrap value of 62%. Ten species of *Ulocladium*, *A. cherianthi*, and *E. indefessa* were clustered as a core *Ulocladium* clade, *Ulocladium* I, which was moderately supported by a bootstrap value of 72%. Two other *Ulocladium* species, *U. alternariae and U. oudemansii*, were clustered together in a separate clade, *Ulocladium* II, which was strongly supported by a bootstrap value of 99% and was immediately basal to the primary *Ulocladium* clade. The position of the brassicicola species-group was directly basal to the two *Ulocladium* clades, as was the infectoria species-group. Of the deeper nodes, only the basal ones received strong bootstrap support. Interestingly, the isolate of *U. lanuginosum* used in this study, CBS 102.26, clustered with the radicina species-group, specifically with *A. radicina* and *A. carotiincultae*, and this association was strongly supported by high bootstrap value (84%). Bayesian analyses resulted in a tree with near-identical topology as shown in Fig. 2 with minor changes in the position of radicina species-group relative to the position of porri species-group (data not shown). However support as measured by Bayesian posterior probabilities was higher and provided significant support (>95%) for most of the clades (Fig. 2).

Maximum-parsimony analysis of the *gpd* data set yielded 16 equally (Fig. 3) most-parsimonious trees (total length=413, CI=0.663, RI=0.869), which varied only in position of the brassicicola species-group, and minor changes in the relationships among the members of the radicina species-group, the *Ulocladium* group and the *Embellisia* group (Fig. 4). The topology of the *gpd* trees were nearly identical to those from the ITS data set with the exception of the placement of the brassicicola species-group, which was generally distal to the primary *Ulocladium* clade and basal to the radicina species-group. All primary clades received high bootstrap support (>95%) with the exception of the radicina species-group and the *Ulocladium* group, both weakly supported by a bootstrap value of 62%. As with the ITS data set, *U. alternariae and U. oudemansii*, clustered together in a separate clade, *Ulocladium* II, which was strongly supported by a bootstrap value of 99% and basal to the primary *Ulocladium* clade. Similarly, CBS 102.26 clustered with *A. radicina* with strong bootstrap support (98%). Bayesian analysis revealed a nearly identical tree as displayed in Fig. 4 with minor differences in the relationship among the members of *Embellisia* group. Supports measured by Bayesian posterior probabilities were higher and provided significant support for most clades (>95%) (Fig. 4).

Maximum-parsimony analysis of the Alt a1 data set yielded 20 most-parsimonious trees (total length=618, CI=0.607, RI=0.834). The trees differed only in minor changes in the relationship among members of the porri species-group (Fig. 5). Topography of Alt a1 trees were nearly identical to those of the gpd trees in terms of species clusters; however, the primary *Ulocladium* clade was further resolved as two distinct clades, *Ulocladium* Ia and Ib, with the second clade composed of *U. chartarum*, *U. septosporum*, *A. cheiranthi*, and *E. indefessa*. As with the ITS and gpd data sets, *U. alternariae and U. oudemansii*, clustered together in a separate clade, *Ulocladium* II, which was strongly supported by a bootstrap value of 99% and immediately basal to the primary *Ulocladium* clade. Similarly, CBS 102.26 clustered with *A. radicina* with strong bootstrap support (98%). The principle clades were all strongly supported by high bootstrap values (>95%) with the exception of the second clade in the primary *Ulocladium* group, which was moderately supported (77%).

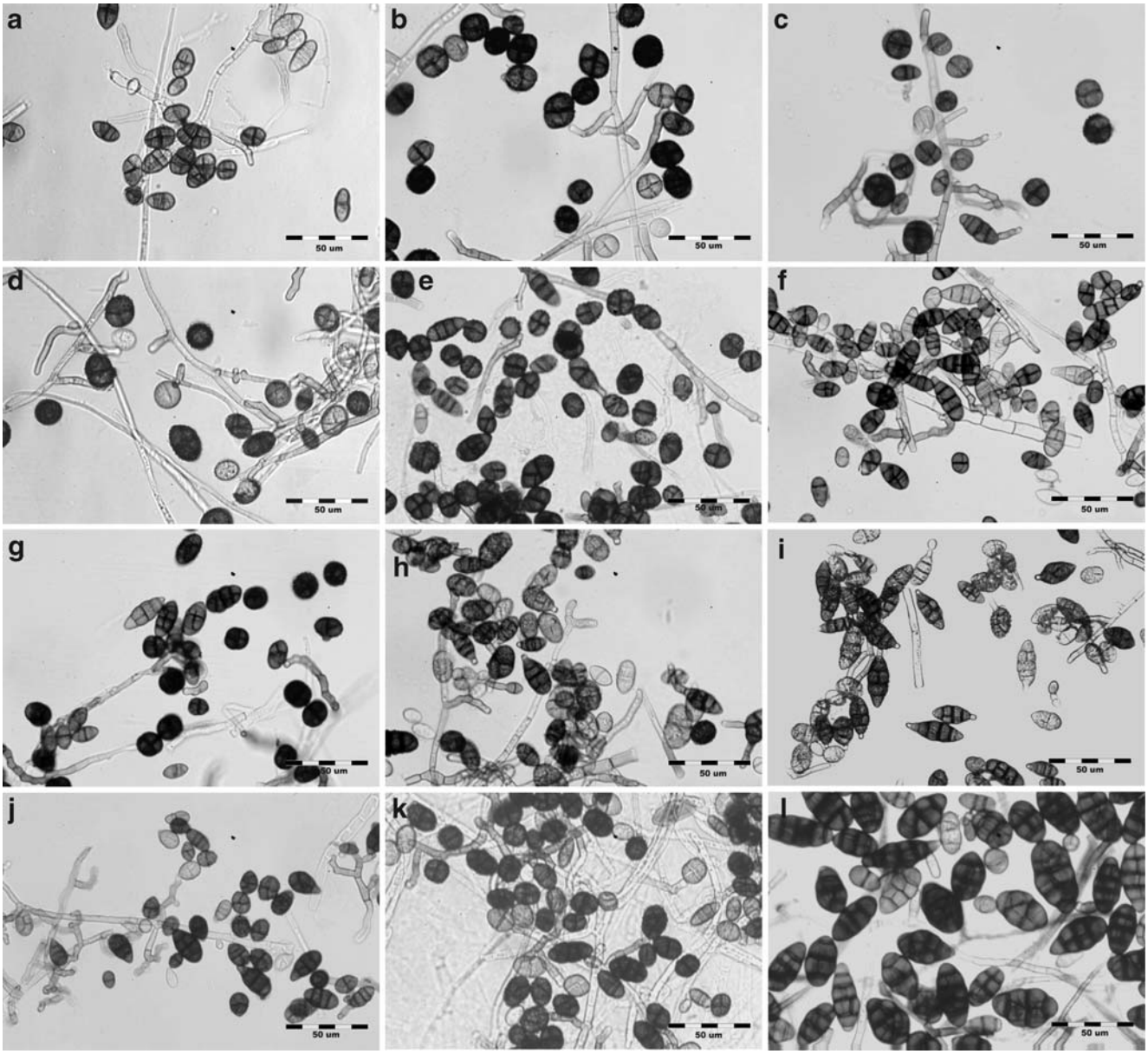

Fig. 1 Conidia of (**a**) U. botrytis, (**b**1) U. atrum, (**c**) U. cucurbitae, (**d**) U. dauci, (**e**) U. multiforme, (**f**) U. consortiale, (**g**) U. obovoideum, (**h**) U. chartarum, (**i**) U. septosporum, (**j**) U. alternariae, (**k**) U. oudemansii, (**l**) CBS 102.26, on PDA after 2 weeks. Scale bar equal 100 μm

Bayesian analyses of Alt a1 data set resulted a tree with near identical topology as that revealed in Fig. 4 and most groups were strongly supported by Bayesian posterior probabilities >95% (Fig. 4).

The three datasets were combined pairwise for the partition homogeneity tests. The tests revealed that the datasets were not significantly inconcordant ($P \geq 0.010$, based upon criteria proposed by Cunningham 1997) with each other (ITS:gpd, $P=0.057$; ITS:Alt a1, $P=0.194$; gpd: Alt a1, $P=0.448$). When combined in a 3-way comparison, the PHT also revealed that the sets were not significantly inconcordant ($P=0.042$). Maximum parsimony analysis of the combined dataset yielded eight equally most-parsimonious trees (total length=1,230, CI=0.636, RI=0.854). The trees differed primarily in the relationship among the members of porri species-group and the *Ulocladium* group within their respective clades. All clades revealed in the analysis of the combined dataset were revealed in analysis of individual datasets and the topologies were most similar to those revealed in the Alt a1 trees. The one exception being that the *Ulocladium* Ia and Ib clades in the Alt a1 trees resolved as a single clade in the combined data set. Bootstrap support of 100% was revealed for all primary clades with the exception of the *Ulocladium* group, which

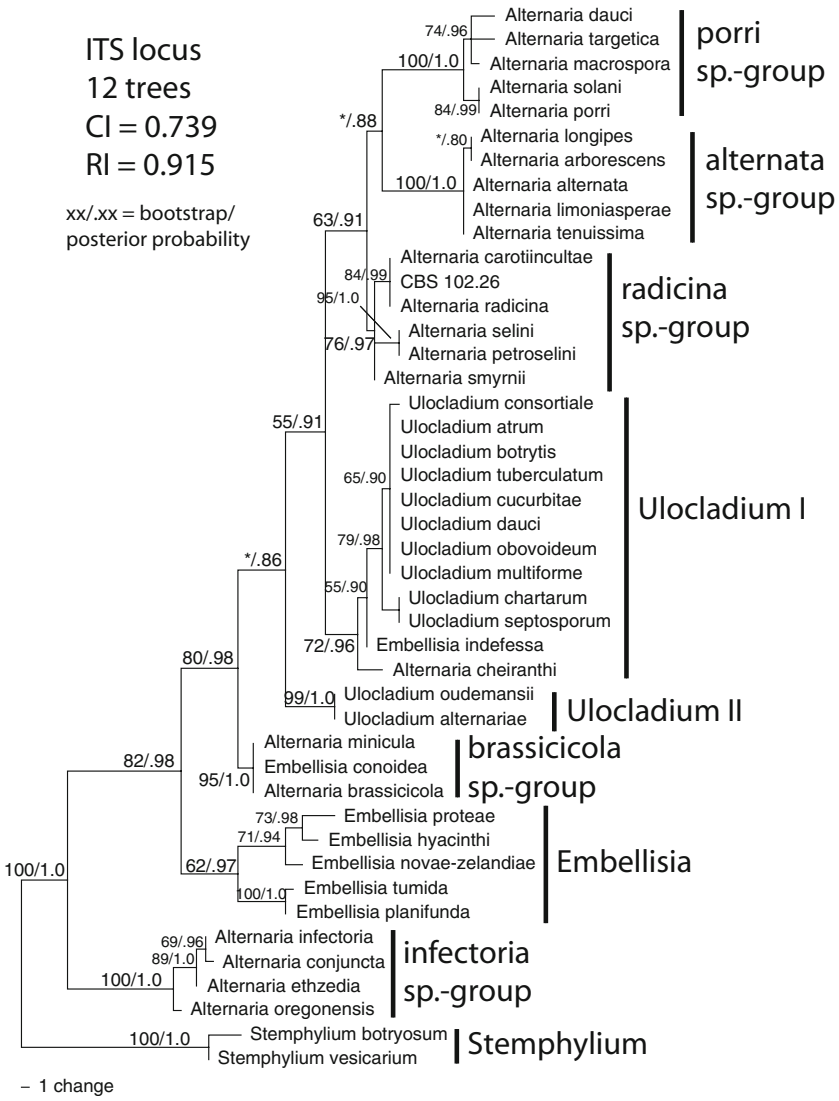

Fig. 2 One of 12 most-parsimonious trees generated from analysis of ITS1/5.8S/ITS2 sequences from select *Ulocladium*, *Alternaria*, *Embellisia*, and *Stemphylium* species. Number in the front of "/" represents parsimony bootstrap values from 1,000 replicates and number after the "/" represents Bayesian posterior probabilities. Values represented by an "*" were less than 50%. The scale bar indicates the number of nucleotides substitution; vertical distances have no significance

was strongly supported by a bootstrap value of 86%. Moreover, most intermediate clades were strongly supported by bootstrap values of >85% with one exception of moderate support (72%) immediately basal to the brassicicola species-group. Thus, with the combined data sets, nearly all clades were resolved with strong support. As with the individual data sets, the position of CBS 102.26 was sister to *A. radicina* and strongly supported (100%). Bayesian analysis revealed an identical tree to that displayed in Fig. 5, and posterior probabilities for nearly all clades were significant (>95%) (Fig. 5).

Morphological characterization of Ulocladium species

The morphological characteristics for all *Ulocladium* isolates were consistent with published descriptions for each species (Simmons 1967, 1983, 1986, 1990a, b, 1994, 1995, 1996, 1998; David et al. 2000), and conidium measurements for each taxon grown on PCA are presented in Table 2. Most species presented the morphological character of obovoid conidia, at least in immature conidia, which is diagnostic for *Ulocladium*. In summary, conidium morphology was as follows: *U. botrytis*, mostly obovoid to broadly obovoid; *U. atrum*, mostly sub-spherical and uncommonly obovoid; *U. cucurbitae*, mostly broad obovoid to sub-spherical; *U. dauci*, mostly sub-spherical and uncommonly obovoid; *U. multiforme*, a mixture of sub-spherical and broad obovoid conidia; *U. consortiale*, obovoid to long ellipsoidal; *U. obovoideum*, obovoid to broadly obovoid; *U. tuberculatum*, grossly tuberculate and uncommonly obovoid; *U. chartarum*, obovoid to ovoid to short ellipsoidal, with most conidia in concatenate chains of six or more; *U. septosporum*, obovoid to ellipsoidal conidia with broadly conical to rounded base; *U. alternariae*, obovoid to broadly ellipsoidal; *U. oudemansii*, broadly obovoid or ellipsoidal . Conidia of CBS 102.26 were ovoid

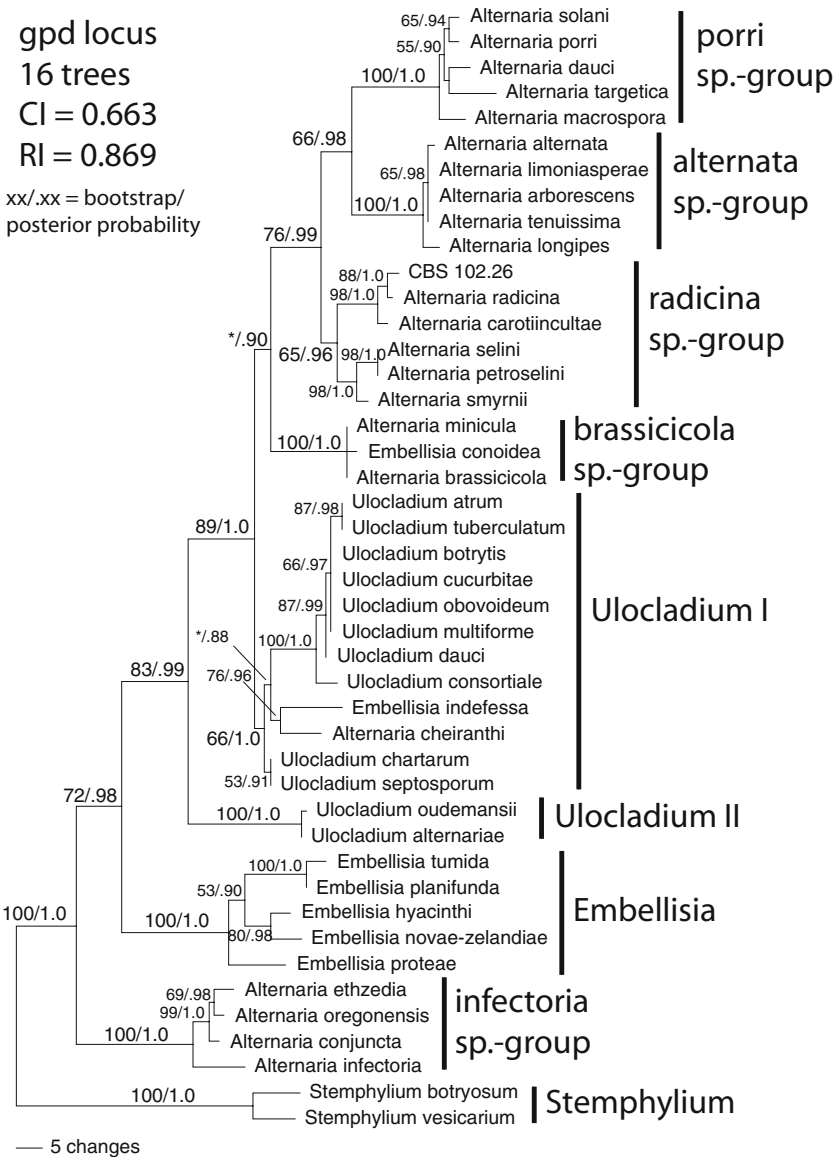

Fig. 3 One of 16 most-parsimonious trees generated from maximum-parsimony analysis of *gpd* sequences from select *Ulocladium*, *Alternaria*, *Embellisia* and *Stemphylium* species. The number in the front of "/" represents parsimony bootstrap values from 1,000 replicates and the number after the "/" represents Bayesian posterior probabilities. Values represented by an "*" were less than 50% for bootstrap. The scale bar indicates the number of nucleotides substitution; vertical distances have no significance

or broadly ellipsoidal and consistent with those of *A. radicina* (Pryor and Gilbertson 2002) (Fig. 1l). Obovoid conidia diagnostic for *Ulocladium* were not apparent for this taxon. In addition, the production of yellow pigment was observed within 14 days growth of CBS 102.26 on PDA, and was consistent with that of *A. radicina* (Pryor and Gilbertson 2002).

Measurements of length, width, and l/w ratio were also consistent with those previously reported. Although there were significant differences in length and/or width among specific taxa, in general, most species represented spores that were 12–24 μl in length (depending on age) and 10–12 μ in width and no clear pattern was evident that separated the three primary *Ulocladium* clades that were evident from molecular analyses (*Ulocladium* Ia, Ib, and II) (Table 2). The notable exceptions were conidium measurements from CBS 102.26, which averaged 30–40 μl in length and 15–18 μ in width. Measurement of length, width, l/w ratio, and average number of transverse septa for CBS 102.26 also reflected the previous findings of conidium characteristics of *A. radicina.*

Among most of the *Ulocladium* species examined, average number of transepta were similar and not significantly different. Most of the species were predominantly 1-transeptate on PCA except *U. consortiale* and CBS 102.26. Most of the species also possessed additional representative conidia that were 2- or 3-transeptate except *U. atrum* and *U. dauci*, which only produced conidia 1- or 2-transeptate, and *U. tuberculatum,* which only produced 1-transeptate conidia. Four-transeptate conidia were observed for *U. consortiale* and CBS 102.26. The average number of transepta per conidium was significantly higher for CBS 102.26 than that of the other *Ulocladium* species. Maximum number of transepta for most species of *Ulocladium* was less

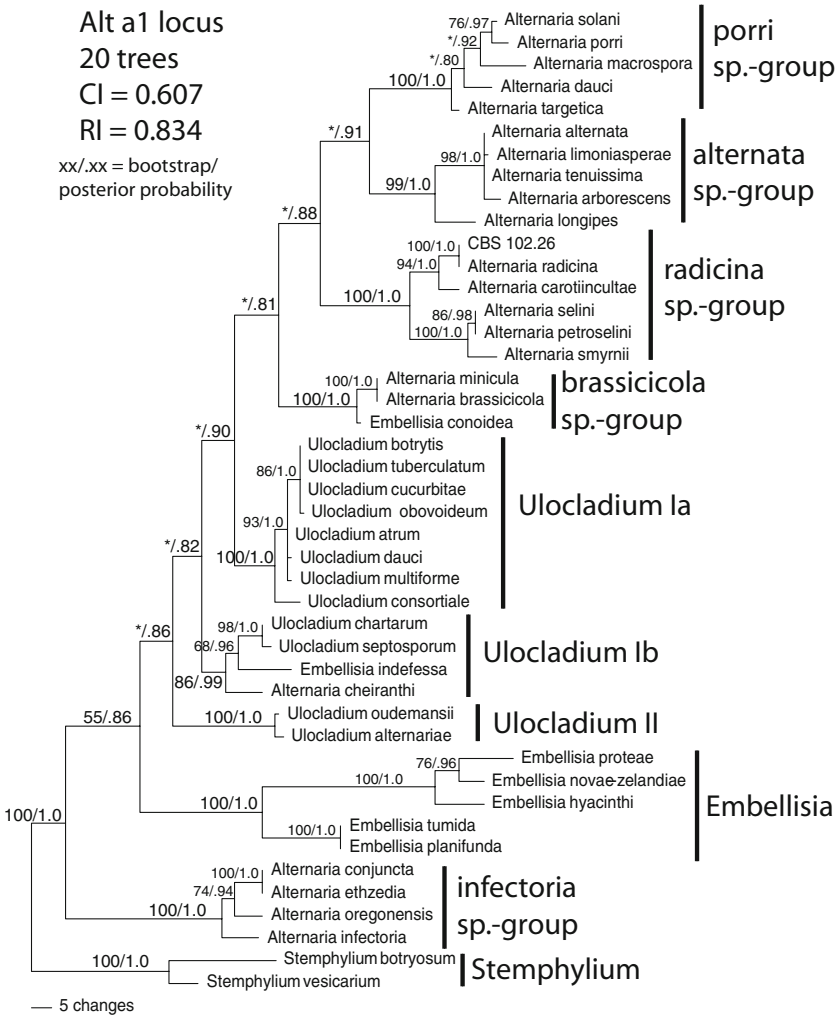

Fig. 4 One of 20 most-parsimonious trees generated from maximum-parsimony analysis of Alt a1 sequences from select *Ulocladium*, *Alternaria*, *Embellisia* and *Stemphylium* species. Number in the front of "/" represents parsimony bootstrap values from 1,000 replicates and number after the "/" represents Bayesian posterior probabilities. Values represented by an "*" were less than 50% for bootstrap. The scale bar indicates the number of nucleotides substitution; vertical distances have no significance

than five. For CBS 102.26, the maximum number of transepta was six.

Discussion

The present study describes the phylogenetic relationship of 13 species of *Ulocladium* with other related species of *Alternaria, Embellisia*, and *Stemphylium* based on sequences from three different genetic regions, nuclear ITS rDNA and the protein coding genes *gpd* and Alt a1. The molecular data sets used for phylogenetic study were not significantly inconcordant and results from the combined data set revealed the same conclusion with greater bootstrap and posterior probability supports than results from analysis of the independent loci. This study revealed that ten species of *Ulocladium* clustered into a core *Ulocladium* group, which also included *A. cheiranthi* and *E. indefessa*. *Ulocladium alternariae* and *U oudemansii* clustered together in a second clade sister to and immediately basal to the primary *Ulocladium* clade. Thus, these results supported previous finding of polyphyletic and paraphyletic relationship of *Ulocladium* among other related taxa of *Alternaria*, *Embellisia*, and *Stemphylium* (Pryor and Gilbertson 2000, Chou and Wu 2002, de Hoog and Horre 2002, Pryor and Bigelow 2003, Hong and Pryor 2005).

In previous studies, *U. alternariae* was resolved as distinct from the other *Ulocladium* spp. and described as a problematic taxon do to its positional instability when included in phylogenetic data sets. (Pryor and Bigelow 2003, Pryor and Gilbertson 2000). However, with the inclusion of *U. oudemansii* in the present data set, the position of *U. alternariae* stabilized and resolved with high statistical support. This stability was also evident in the recent study of Xue and Zhang (2007). Both *U. alternariae* and *U. oudemansii* have the characteristic *Ulocladium* conidium shape as well as other common *Ulocladium* features, e.g., geniculate conidiophores and little to no catenation of spores. But based upon each genetic locus used in this study, they do not appear to be share the

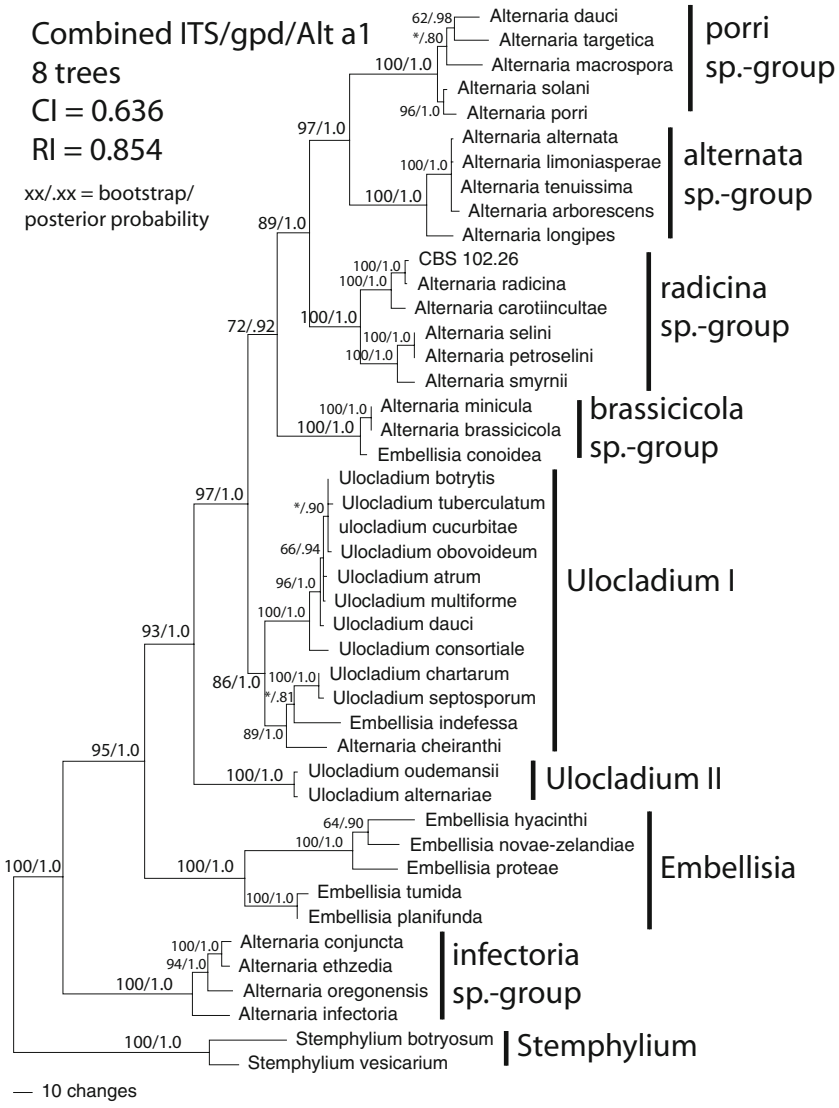

Fig. 5 One of eight most-parsimonious trees generated from maximum-parsimony analysis of a combined dataset containing ITS, *gpd*, and Alt a1 sequences from select *Ulocladium*, *Alternaria*, *Embellisia* and *Stemphylium* species. The number in the front of "/" represents parsimony bootstrap values from 1,000 replicates and number after the "/" represents Bayesian posterior probabilities. The scale bar indicates the number of nucleotides substitution; vertical distances have no significance

immediate common ancestor with the primary *Ulocladium* clade, suggesting polyphyly. However, the position of this second *Ulocladium* clade was sister to and immediately basal to the primary *Ulocladium* clade, and this position resolved with high statistical support. Thus, a close phylogenetic relationship is evident. The inclusion of additional taxa in the present study provided increased resolution and support for *Ulocladium* phylogeny previously resolved using smaller data sets (Pryor and Bigelow 2003, Hong and Pryor 2005, Xue and Zhang 2007). Considering the very strong morphological correlations between the *Ulocladium* I and *Ulocladium* II clades, and their close topological proximities in robustly resolved trees as evident it this study, it is believed that further expansion of the data sets in terms of taxa and phylogenetically informative loci will ultimately resolve both clades with a common ancestor and unifying them as a monophyletic clade.

Where this study differentiates from previous studies is in the final resolution of the *Ulocladium* I clade in relation to other asexual *Alternaria* clades. In most previous studies, the relationship between the primary *Ulocladium* clade and the asexual *Alternaria* clade of the brassicicola species-group remained unresolved with weak statistical support. In most previous studies, the asexual brassicicola species-group resolve basal to the *Ulocladium* group, resulting in paraphyly of *Alternaria* and uncertainty as to the taxonomic status of *Ulocladium*. The primary problem resided with the ITS data set, in which a unique indel is shared by members of the sexual infectoria species-group, the asexual brassicicola species-group, and *U. alternariae* and *U. oudemansii*, thus pulling these taxa together in subsequent phylogenetic analyses. In this study, sequences of the Alt a1 locus were included for a larger number of *Ulocladium* species, and subsequent phylogenetic analyses clearly support a distinct *Ulocladium* lineage that is basal to all asexual *Alternaria*

Table 2 Conidium measurements for *Ulocladium* spp. on potato carrot agar

Species	Conidium measurements[a]												# Transverse septa/conidium[b]	
	1-septa			2-septa			3-septa			4-septa				
	l	w	l/w	l	w	l/w	l	w	l/w	l	w	l/w	Max	Avg
Ulocladium alternariae	15.6bcd	11.1b	0.93b	18.4b	11.0bc	1.70b	21.7bc	11.5b	1.94bc				3	2.2bc
Ulocladium atrum	17.0a	12.4a	1.39b	19.8b	12.3b	1.63bc							3[c]	1.2bcd
Ulocladium botrytis	17.8a	10.1bc	2.41a	20.2b	10.5bc	1.96a	24.7b	10.6b	2.37a				3	2.2bc
Ulocladium chartarum	16.2b	10.2bc	1.64a	18.9b	10.6bc	1.81b	24.3b	10.6b	2.31a				4	2.5bc
Ulocladium consortiale				18.0bc	10.0bcd	1.85b	22.2b	10.6b	2.44a	29.9b	12.8b	2.50a	5[d]	3.1b
Ulocladium cucurbitae	18.3a	14.4a	1.55a	20.4b	11.9b	1.78b	24.9b	10.8b	2.37a				4	1.9bcd
Ulocladium dauci	18.4a	13.7a	1.35b	20.0b	13.0b	1.60bc							3[c]	1.5bcd
Ulocladium lanuginosum[e]				29.4a	16.5a	1.835b	34.1a	17.4a	2.06bc	40.0a	17.4a	2.40a	6[d]	3.7a
Ulocladium multiforme	17.2a	11.2b	1.56a	19.8b	11.0bc	1.865b	24.2b	11.5b	2.18a				3	2.1bc
Ulocladium obovoideum	17.6a	10.4bc	1.71a	20.3b	9.6bcd	2.15a	23.6b	9.4bc	2.60a				3	2.2bc
Ulocladium oudemansii	16.3b	12.1b	1.39b	20.2b	11.5b	1.75b	24.7b	11.5b	2.16a				3	2.2bc
Ulocladium septosporum	15.8bc	10.0bc	1.60a	17.3bcd	9.7bcd	1.81b	21.0bcd	10.7b	1.97bc				4	2.3bc
Ulocladium tuberculatum	11.8bcd	9.5bcd	1.20b										1	1.0bcd

[a] Twenty conidia from each septum category were randomly observed in five fields of view (40×). Values within each column followed by different letters are significantly different ($P \leq 0.05$)

[b] Fifty conidia from each isolate were randomly observed in five fields of view (40×) and the number of transverse septa per conidium was counted and the mean number of septa per conidium is calculated. Values within the column followed by different letters are significantly different ($P \leq 0.05$)

[c] The occurrence of 3-transeptate conidia was extremely rare for these taxa and values of l/w were not recorded

[d] The occurrence of 1-transeptate conidia was uncommon for these taxa and values of l/w were not recorded

[e] No ex-type or representative cultures of *U. lanuginosum* are currently available. The isolate used in this study, CBS 102.26, is of questionable identity as revealed in this manuscript

lineages, including the brassicicola species-group. Although support for the topology resulting from independent Alt a1 analyses was only Bayesian posterior probabilities, when concatenated with sequences of the gpd locus and the ITS region, full support from bootstrap and posterior probabilities was achieved.

The polyphyly resulting from the position of CBS 102.26, listed as *U. lanuginosum*, is problematic as this study revealed that the taxon was quite unrelated to the other *Ulocladium* spp. Analyses from all three loci revealed near sequence identity between this isolate and *A. radicina*. Moreover, the morphological data also supported that CBS 102.26 did not possess the key diagnostic feature of *Ulocladium* spp., specifically the fundamentally obovoid conidium. In addition, statistically significant differences in conidium length, width, and transepta number, further support that this taxon is fundamentally different morphologically from other *Ulocladium* spp. and is not representative of the genus. However, the isolate of *U. lanuginosum* used in this study is not an ex-type (one does not exist) nor is a designated representative of the species. Until a valid isolate of *U. lanuginosum* is obtained, perhaps isolated from the same substrate as Harz's isolate, that being honeycomb from a beehive infected with foulbrood, and proper morphological, pathogenicity, and genetic tests can be conducted, the true phylogenetic status of *U. lanuginosum* will be uncertain.

Conidium morphology was also examined for comparisons with the resolved phylogeny. According to the descriptions of Simmons (Simmons 1967, 1982, 1990a, b, 1998, 2004, 2007), conidium shape and septation differ from early stage (1–3 days) to late growth stage (7 days). In this study, the suspensions of conidia prepared from PCA cultures included the conidia from the edge and center of the plate. As a result, both young and matured conidia were included for morphological measurements, and results revealed by the measurements of length, width and l/w ratio of conidia were consistent with published descriptions. Among the species of *Ulocladium* examined (not including CBS 102.26), all supported the key diagnostic feature of conidia essentially obovoid in shape. Four species of *Ulocladium*, *U. atrum*, *U. cucurbitae*, *U. multiforme*, and *U. dauci*, were mostly represented by mature conidia that were primarily subspherical in shape with few diagnostic obovoid conidia evident (Simmons 1998). However, for the most part the key taxonomic feature of *Ulocladium*, that being the presence of obovoid conidia particularly in juvenile conidia, appeared to be taxonomically consistent.

The conflict with morphology and phylogeny was revealed primarily by the presence of *A. cheiranthi* and *E.*

indefessa in the primary *Ulocladium* clade. The key diagnostic features of *Alternaria* (ovoid conidia that distinguishes *Ulocladium* from *Alternaria*) and the key diagnostic features of *Embellisia* (relatively thick, dark, rigid septation in contrast to the external wall of the conidia) were not represented in *Ulocladium* spp. but were represented in *A. cheiranthi* and *E. indefessa,* respectively. Likewise, the common morphological feature of *Ulocladium*, that being fundamentally obovoid conidia, was not represented in *A. cheiranthi* and *E. indefessa.* Thus, the presence of these taxa within a morphologically consistent *Ulocladium* clade is problematic. Perhaps these taxa represent the loss of the fundamental *Ulocladium* characteristic of obovoid conidia. Or perhaps these taxa preceded the development of this diagnostic character. Evidence for the latter hypothesis may be found in the resolution provided by the Alt a1 locus, which clustered both taxa with *U. chartarum* and *U. septosporum*. *U. chartarum*, along with *E. indefessa* and *A. cheiranthi*, demonstrate concatenation of conidia in chains, a characteristic strongly *Alternaria* and, to a lesser extent, *Embellisia*, but not notably *Ulocladium*. In particular, *U. chartarum* and *E. indefessa*, represent the most extreme examples of concatenation within their respective genera (Simmons 1967, Simmons 1983), so much so that these taxa may likely be confused with *A. alternata* or taxa within the alternata species-group. Moreover, these taxa, including *U. septosporum*, do not strongly exhibit the diagnostic *Ulocladium* characteristic of obovoid conidia with most conidia having conidia with broadly conical or rounded bases. Thus, although these four taxa cluster with other more characteristic *Ulocladium* species in terms of phylogeny, their morphology clearly has characters that are generally attributed to certain *Alternaria* lineages. Their phylogenetic placement as sister to, yet separate from, the primary *Ulocladium* clade based upon Alt a1 sequences is supportive of their morphological distinctness.

Based on the findings of this work, the taxonomic status of *Ulocladium* is firmly supported as a monophyletic clade consisting of 10 species (*Ulocladium* I) that is distinct and independent from other asexual lineages of *Alternaria*. Other recently described species not included in this work, e.g., *U. capsicuma* (Xue and Zhang 2007) have already been shown to fall within this primary clade and it is likely that additional taxa will be included in this clade in the near future as well. The current paraphyly of *Ulocladium* can be resolved with synonymy of *A. cheiranthi* and *E. indefessa* as *U. cheiranthi* and *U. indefessa*, respectively. However, such synonymy should not move forward without resolution of the morphological characters that tie these two taxa to the primary *Ulocladium* clade; something that this study was not able to accomplish. Alternatively, these two taxa, along with *U. chartarum* and *U. septosporium* may ultimately resolve as a separate lineage, as suggested by analyses of Alt a1. As the data from this locus permitted additional resolution of *Ulocladium* not possible with ITS or gpd sequences, other more informative loci may provide additional support for this resolution and a second genus may be proposed. Similarly, support for the integration of *U. alternariae* and *U. oudemansii* into the primary *Ulocladium* clade, or differentiation as a separate genus, will also likely require the inclusion of additional loci and/or taxa in future analyses to provide final resolution of this clade as well. The resolution of these remaining issues is critical to corroborate the taxonomic status of the several outlying *Ulocladium* species with the resolved phylogeny of the primary *Ulocladium* clade as presented in this work. This in turn, will support continued resolution of more basal lineages in the Pleosporaceae such as *Embellisia* and the sexual *Alternaria* clade, *Lewia*, that continue to be phylogenetically problematic.

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