Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences

Barry M. PRYOR and Robert L. GILBERTSON

Department of Plant Pathology, University of California, One Shields Ave., Davis, CA 95616, USA.
E-mail: bmpryor@ucdavis.edu

Received 29 June 1999; accepted 14 February 2000.

To elucidate relationships among *Alternaria*, *Ulocladium*, and *Stemphylium* species, nuclear internal transcribed spacer (ITS) and mitochondrial small subunit (SSU) ribosomal DNA (rDNA) sequences from 18 *Alternaria*, four *Ulocladium* and four *Stemphylium* spp. were determined and compared. Phylogenetic analysis of the ITS and SSU rDNA sequences, performed by the neighbour joining and maximum parsimony methods, revealed that the *Stemphylium* spp. were phylogenetically distinct from the *Alternaria* and *Ulocladium* spp. Most *Alternaria* spp. and the *Ulocladium* spp. were placed together in a large *Alternaria*/*Ulocladium* clade. Within this large clade, the *Alternaria* spp. clustered into several distinct species-clades, most of which correlated with species-groups previously established based upon morphological characteristics. The *Ulocladium* spp. were placed into two species-clades, each of which also included *Alternaria* spp. *A. longissima* was distantly related to the other *Alternaria* spp., as well as the *Ulocladium* and *Stemphylium* spp. Based upon ITS and nuclear 18S rDNA sequence identities, *A. longissima* was most closely related to *Leptosphaeria*.

INTRODUCTION

The genus *Alternaria* includes nearly 100 species of dematiaceous hyphomycetes that occur worldwide in a variety of habitats (Ellis 1971, 1976, Joly, 1964, Simmons 1992). Several species are saprobes commonly found in soil and/or on dead or dying plant tissue. The majority, however, are plant pathogens that, collectively, cause a range of economically important diseases on a variety of crops worldwide (Rotem 1994, Strandberg 1992).

The genus *Alternaria* was originally described by Nees (1816) with *A. tenuis* as the type. Characteristics of the genus included the production of chains of dark-coloured multicelled conidia with longitudinal and transverse septa (phaeodictyospores) and a beak or tapering apical cells. Subsequent identification of additional *Alternaria* species, many of which did not produce conidia in chains and/or have a conidium beak, prompted several re-descriptions of the genus in order to include these new species (Elliot 1917, Joly 1964, Neergaard 1945, Simmons 1967, Wiltshire 1933). However, the production of dark-coloured conidia with longitudinal and transverse septa has remained a key taxonomic characteristic of the genus.

Within the genus *Alternaria*, species are defined primarily upon conidium characteristics including size, septation, presence and/or size of a beak, and pattern of catenation (Ellis 1971, 1976, Joly 1964, Simmons 1992). Because of the diversity of *Alternaria* species, several subgeneric groups have been proposed, either formally or informally, that include species with similar conidium characteristics (Elliot 1917, Ellis 1971, 1976, Joly 1964, Neergaard 1945). Most recently, Simmons (1992) has organized the genus into a number of species-groups, each of which is typified by a representative species. For example, the alternata species-group includes species with small catenate spores such as *A. alternata* and the porri species-group includes species with large, long-beaked, non-catenate spores such as *A. porri*. Other species-groups include the brassicicola group, the cheiranthi group, the infectoria group, and the tenuissima group (Simmons 1995).

Other fungal genera, such as *Stemphylium*, *Ulocladium*, and *Macrosporium*, also possess phaeodictyosporic conidia (Elliot 1917, Simmons 1967, Wiltshire 1933), and the morphology of these conidia is similar to that of some *Alternaria* species. Similarities in conidium size and shape among members of these genera have complicated the classification of certain species, as well as the practical identification of some of the plant pathogenic members of these genera (e.g. many species of *Alternaria*, *Ulocladium* and *Stemphylium* can be found on carrot seed, but only *A. radicina* and *A. dauci* are plant pathogens). The confusion surrounding the taxonomy of these fungi is revealed by the fact that some atypical species have been placed into more than one of these genera since their initial identification (e.g. *U. consortiale* (Thüm.) Simmons 1967 with the synonyms *M. consortiale* Thümen 1876, *S. consortiale* (Thüm.) Groves & Skolko 1944, and *A. consortiale* (Thüm.) Groves & S. Hughes 1953).

Wiltshire (1933, 1938) reviewed this taxonomic problem,

and proposed revised criteria for the genera *Alternaria* and *Stemphylium*. *Macrosporium* was placed on a list of *nomina ambigua*, and many of the atypical species were placed in a subgenus of *Stemphylium*, *Pseudostemphylium*. Joly (1964) reviewed the genus *Alternaria* and related species, and proposed that most of these atypical species be classified as *Alternaria* rather than *Pseudostemphylium*. However, Simmons reviewed *Alternaria*, *Stemphylium*, and the long-neglected genus *Ulocladium*, and concluded that many of the atypical *Alternaria* and *Stemphylium* species should be classified as *Ulocladium*. Based upon the characteristics defined by Simmons, *Stemphylium* species are readily identified on the basis of the conidiophore apex, whereas differentiation of certain *Alternaria* and *Ulocladium* species requires an examination of the basal end of immature conidia.

In our laboratory, we have been conducting research on the biology and ecology of *A. radicina*, causal agent of carrot black rot disease (Meier, Drechsler & Eddy 1922). *A. radicina* is a member of the cheiranthi species-group, all of which have large dark-coloured conidia that lack prominent beaks and are produced singly or in chains of 2 (or rarely 3) conidia (Simmons 1995). In addition to *A. radicina*, a number of other pathogens of carrot and/or other umbelliferous crops in this group, including *A. petroselini* and *A. smyrnii*, and two recently described species, *A. carotiincultae* and *A. selini* (Simmons 1995). This group also includes *A. cheiranthi* and *A. japonica* which are pathogenic on wallflower and *Brassica* species, respectively; and *A. instipitata* and *A. atrocariis*, which were described from herbarium specimens on conifer wood and carrot, respectively (Simmons 1995). Some of the morphological characteristics of *A. radicina* and the other umbelliferous pathogens of the cheiranthi species-group are very similar, making identification of these species difficult (Neergaard 1945, Simmons 1995). Thus, we have been investigating molecular-based approaches for the rapid and reliable detection and differentiation of these fungi.

Analysis of ribosomal DNA (rDNA) sequences has become a common tool in modern systematics, and has been used to establish molecular phylogenetic relationships within many groups of fungi (Miyamota & Cracraft 1991, Moritz & Hillis 1990, White *et al*. 1990). For example, analysis of the relatively conserved nuclear 18S sequence has been used to describe family-genus relationships within the *Pleosporales*, and revealed a close phylogenetic relationship between *Pleospora* (anamorph *Stemphylium*) and *Alternaria* (Berbee 1996, Morales *et al*. 1995). For analysis of fungal taxa at or below the species level, the more variable internal transcribed spacer (ITS) region is commonly used. This region has been used to examine phylogenetic relationships among *Alternaria* species that produce host-specific toxins (Kubaba & Tsuge 1995) or that are pathogenic on crucifers (Jasalavich *et al*. 1995), as well as among other species of the *Pleosporales* (Khashnobish & Shearer 1996, Morales, Pelcher & Taylor 1992, Morales *et al*. 1995). The mitochondrial small subunit (SSU) rDNA sequence has also been used to investigate relationships among closely related fungus species (Kretzer *et al*. 1996, Li, Rouse & German 1994). Compared with nuclear rDNA, mitochondrial rDNA generally has higher rates of nucleotide substitution and, thus, may provide additional insight into relationships among closely related fungi (Bruns & Szaro 1992, Wolstenholme 1992).

The objective of this study was to examine phylogenetic relationships among *A. radicina* and other members of the cheiranthi species-group, with an emphasis on umbelliferous pathogens, based upon nuclear ITS and mitochondrial SSU rDNA sequences. We also examined relationships among selected *Alternaria*, *Stemphylium*, and *Ulocladium* species, particularly those with conidia morphologically similar to members of the cheiranthi group (i.e. large non-catenate non-beaked conidia).

MATERIALS AND METHODS

Fungal cultures

Sources of the isolates used are listed in Table 1. All cultures were maintained on potato dextrose agar (PDA, Difco, Plymouth, MN) or cornmeal agar (CMA, Difco) plates at 22 °C, as well as on PDA slants stored at 5 °. The identification of each isolate was determined or confirmed based upon established morphological criteria (Ellis 1971, 1976, Simmons 1967, 1992). The identity of selected isolates was confirmed by Mycological Services (Crawfordsville, IN 47933; Table 1).

DNA isolation

Fungi were grown in liquid culture for DNA extraction. To prepare liquid cultures, agar plates (PDA or CMA) with 10-day-old cultures of test fungi were flooded with 10 ml of sterile water, and mycelia and conidia were dislodged with a plastic rod. Two ml of this suspension were then added into 100 ml of sterile liquid growth medium (20.7 g D-glucose, 1.2 g DL-asparagine, 1.2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g yeast extract, 0.1 g NaCl l^{-1}) in a 250 ml Erlenmeyer flask. Flasks were agitated on a rotary shaker at 120 rpm for 3–5 days at 22 °, and mycelia was harvested by filtration through Miracloth (Calbiochem, San Diego, CA). Mycelia was lyophilized and stored at −20 ° under desiccation.

For extraction of fungal total genomic DNA, 100 mg of lyophilized mycelia was ground into a powder in liquid N_2. Two ml of lysis buffer (10 mM Tris–HCl, pH 8.0; 10 mM EDTA; 10% SDS; 0.5 M NaCl) was immediately added to the powdered mycelia, and the sample was ground for an additional 10–15 s. Each sample was transferred to a 15 ml centrifuge tube and three ml of phenol-chloroform (1:1) was added to each tube. The tubes were shaken vigourously for 30 s, and then centrifuged at 12000 ***g*** for 20 min at 4 °. The aqueous phase was transferred to a new tube, and a second extraction was performed with three ml of chloroform. The aqueous phase was transferred to a new tube, and the DNA was precipitated by the addition of 0.6 volumes of isopropanol. The tubes were kept at −20 ° for 1 h, and then centrifuged at 12000 ***g*** for 5 min at 4 °. The nucleic acid pellet was washed with 5 ml of 70% ethanol and then vacuum-dried. The nucleic acids were re-suspended in 200 µl of TE buffer pH 8.0 (Sambrook, Fritsch & Maniatis 1989). The DNA concentration of each preparation was determined with a DNA fluorometer (model TKO 100, Hoefer Scientific Instruments, San Francisco,

Table 1. Fungal isolates and the GenBank accession numbers for the rDNA sequences.

			GenBank accession number		
			ITS	SSU	18S
Species	Isolate	Source[a]	ITS5–ITS4	NMS1–NMS2	NS1–NS2/NS3–NS8
Alternaria alternata	28329	ATCC	AF229459	AF229648	AF229490/AF229504
	214107	BMP	AF229460	AF229649	
	214110	BMP	AF229461	AF229650	
A. dauci	36613	ATCC	AF229466	AF229657	AF229495/AF229509
	213109	BMP	AF229467	AF229658	
	213116	BMP	AF229468	AF229659	
A. radicina	96831	ATCC*	AF229471	AF229668	AF229500/AF229514
	212107[b]	BMP	AF229472	AF229669	
	212114[b]	BMP	AF229473	AF229670	
A. brassicae	216102	BMP	AF229463	AF229651	AF229491/AF229505
A. brassicicola	2232	EEB	AF229462	AF229652	AF229492/AF229506
A. carotiincultae	26-010	EGS	AF229465	AF229654	AF229493/AF229507
A. cheiranthi	41-188	EGS	AF229457	AF229655	AF229494/AF229508
A. crassa	Acr1	DGG	AF229464	AF229656	
A. infectoria	211115[b]	BMP	AF229458	AF229647	
A. japonica	13618	ATCC*	AF229474	AF229661	AF229496/AF229510
A. longissima	18552[b]	ATCC*	AF229489	AF229662	AF229497/AF229511
A. macrospora	Ams1	DGG	AF229469	AF229663	
A. petroselini	09-159	EGS	AF229454	AF229664	AF229498/AF229512
A. porri	58175	ATCC	AF229470	AF229667	
A. selini	25-198	EGS	AF229455	AF229673	AF229501/AF229515
A. smyrnii	37-093	EGS	AF229456	AF229674	
A. solani	58177	ATCC	AF229475	AF229675	
A. tenuissima	16423	ATCC*	AF229476	AF229678	
Ulocladium alternariae	314105[b]	BMP	AF229485	AF229679	AF229502/AF229516
U. atrum	18040[b]	ATCC	AF229486	AF229680	AF229503/AF229517
U. botrytis	18043[b]	ATCC	AF229487	AF229681	
U. chartarum	18044[b]	ATCC	AF229488	AF229682	
Stemphylium botryosum	42170	ATCC	AF229481	AF229671	
S. callistephi	1055	EEB	AF229482	AF229672	
S. sarcinaeforme	1072	EEB	AF229483	AF229676	
S. vesicarium	18521	ATCC*	Af229484	AF229677	
Pleospora herbarum	11681	ATCC	AF229479	AF229665	AF229449/AF229513
P. infectoria	12054[b]	ATCC	AF229480	AF229666	
Bipolaris tetramera	513101	BMP	AF229477	AF229653	
Exserohilum pedicellatum	1336	EEB	AF229478	AF229660	

[a] Abbreviations for sources are as follows: ATCC, American Type Culture Collection, Manassas, VA 20110-2209; BMP, B. M. Pryor, Department of Plant Pathology, University of California, Davis, CA 95616; DGG, D. G. Gilchrist, Dept. of Plant Pathology, University of California, Davis, CA 95616; EEB, E. E. Butler, Dept. of Plant Pathology, University of California, Davis, CA 95616; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933. ATCC isolates marked with an asterisk (*) originated from EGS.

[b] Isolates that were examined by E. G. Simmons. The identity of each isolate was confirmed to the species level, except for isolates of *A. infectoria*, *P. infectoria*, *U. botrytis*, which were confirmed to the species-group level.

CA), and then adjusted to a final concentration of 10 ng μl^{-1} with TE buffer.

DNA amplification and sequencing

The nuclear rDNA ITS region, including ITS 1 and 2 and the 5.8S ribosomal gene, was amplified using PCR and primers ITS5 and ITS4 (White *et al*. 1990). An internal region of the mitochondrial rDNA SSU gene was amplified using PCR and primers NMS1 and NMS2 (Li *et al*. 1994). The nuclear 18S region was amplified as three separate fragments using PCR and primer pairs NS 1 and 2, 3 and 6, and 5 and 8, respectively (White *et al*. 1990). For PCR, a 50 µl reaction mixture containing 10 ng DNA, 0.5 mM of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, and 1.0 U Amplitaq DNA polymerase in 1X Amplitaq PCR buffer II (PE Applied Biosystems, Foster City, CA) was used. PCR was carried out in a thermal cycler (Model 480, PE Applied Biosystems) programmed for the following parameters: 94° for 1 min, 60° for 1.5 min, and 72° for 2 min, for 35 cycles. PCR-amplified DNA fragments were fractionated in 1.0% agarose gels in 0.5 × TBE buffer (Sambrook *et al*. 1989), and DNA was visualized by ethidium bromide staining and uv illumination. DNA was recovered from agarose using a silica matrix (GeneClean; Bio 101, Vista, CA) according to the manufacturer's recommendations.

DNA sequences of PCR-amplified fragments were determined with an ABI PRISM 377 DNA Sequencer using either dRhodamine terminator or Big Dye Terminator chemistry (Perkin–Elmer/ABI, Foster City, CA). Primers used for sequencing were the same as those used to direct the amplification of the given DNA fragment. The sequence of

both strands of each fragment were determined for sequence confirmation.

DNA sequence analysis

DNA sequences were aligned with the PILEUP program of the GCG Sequence Analysis Software Package (version 9.1; Genetics Computer Group, Madison, WI). In some cases, manual adjustments of sequence alignments were performed using the data editor program of MacClade Phylogenetic Software (version 3.05; Sinauer Associates, Sunderland, MA). Phylogenetic analyses were performed using programs contained in PAUP Phylogenetic Software (version 4.0-beta; Sinauer Associates, Inc., Sunderland, MA). For each set of rDNA sequences, phylogenetic trees were constructed using distance and maximum parsimony methods. For distance analysis, the neighbour-joining method was performed using Kimura 2-parameter distances. For parsimony analysis, heuristic searches for the most parsimonious trees were conducted using closest step-wise addition and branch swapping by tree bisection-reconnection. Sequence gaps were treated as missing data. For each analysis, 1000 bootstrap replicates were performed to assess the statistical support for each tree.

RESULTS

For nuclear ITS and mitochondrial SSU rDNA sequence analysis, 18 species of *Alternaria* were examined, including 8 members of the cheiranthi species-group (Table 1). Four species each of *Stemphylium* and *Ulocladium* were also included, as well as 2 species of *Pleospora*: *P. herbarum*, which has a *Stemphylium* anamorph, and *P. infectoria*, which has an *Alternaria* anamorph (Simmons 1986). *Exserohilum pedicellatum* and *Bipolaris tetramera* were included as outgroups. Most isolates used in this study had morphological characteristics that were consistent with type descriptions, with the exception of the *P. infectoria*, *A. infectoria* and *U. botrytis* isolates. Examination of these isolates by Mycological Services concluded that the *P. infectoria* and *A. infectoria* isolates were members of the infectoria species-group, and that the *U. botrytis* isolate was a member of the *U. botrytis* species-group.

Nuclear ITS

The ITS 4 and ITS 5 primers directed the amplification of an approximately 600 bp ITS rDNA fragment from all isolates. DNA sequencing revealed that these fragments ranged in size from 591 to 623 bp (including primer sequences). The GenBank accession numbers for all ITS sequences determined in this study are provided in Table 1.

The alignment of the ITS sequences was deposited in TreeBASE (http://herbaria.harvard.edu). In addition to the sequences determined in this study, the alignment included 17 ITS sequences obtained from GenBank. Alignment of these sequences revealed variability in the ITS 1 (first 250 nucleotides [nt]) and ITS 2 (last 200 nt) regions, and little or no variability in the 5.8S ribosomal gene (centre 150 nt). Several small indels ($\leqslant$ 8 nt) were present in the ITS 1 and 2 regions of some species, but these indels did not affect the local sequence alignment. A large indel in the ITS 1 region (alignment position 60–83) in *A. brassicicola*, *A. japonica*, *U. alternariae*, *A. infectoria*, and *P. infectoria*, did influence the local alignments depending on which gap and gap width penalties were used in the alignment procedure. Therefore, analyses were performed with and without this indel included.

The sequence alignment revealed that the outgroups, *B. tetramera* and *E. pedicellatum*, were distinct from the other species, and the ITS sequences of these two species were 84–88% identical to those of *Stemphylium*, *Ulocladium* and most of the *Alternaria* species. Unexpectedly, the ITS sequence of *A. longissima* was less similar (79% identity) to those of *Stemphylium*, *Ulocladium* and the other *Alternaria* species than were those of *B. tetramera* or *E. pedicellatum*. A GenBank search conducted with the ITS sequence of *A. longissima* revealed that the similar sequence was the ITS sequence of *Leptosphaeria maculans* (98% identity). Consistent with these findings, in phylogenetic analyses that included the *A. longissima* ITS sequence, this species was placed outside of the clades established for the *Alternaria*, *Stemphylium*, and *Ulocladium* species (data not shown). Therefore, to use *E. pedicellatum* and *B. tetramera* as outgroups, and to more accurately resolve taxa within the main *Alternaria*/*Stemphylium*/*Ulocladium* group, this highly divergent *Alternaria* species was not included in subsequent ITS analyses.

Neighbour-joining analysis of ITS sequences placed *E. pedicellatum*, *B. tetramera*, and a distinct clade comprised of the *Stemphylium* spp. outside of a large clade that contained the *Alternaria* and *Ulocladium* species (Fig. 1). This *Alternaria*/*Ulocladium* clade was supported with a bootstrap value of 100%. Within this large *Alternaria*/*Ulocladium* clade, the following distinct species-clades were revealed: the porri clade with *A. porri*, *A. solani*, *A. dauci*, *A. crassa*, and *A. macrospora*; the brassicicola clade with *A. brassicicola*, *A. japonica*, and *U. alternariae*; the *Ulocladium* clade with *U. atrum*, *U. botrytis*, *U. chartarum*, and *A. cheiranthi*; the alternata clade with *A. alternata* and *A. tenuissima*; the radicina clade with *A. radicina*, *A. carotiincultae*, *A. petroselini*, *A. selini* and *A. smyrnii*; and the infectoria clade with *A. infectoria* and *P. infectoria* (Fig. 1). The establishment of these clades was supported by bootstrap values of $\geqslant$ 65%. With a few exceptions, the clades revealed by this ITS sequence analysis were similar to several *Alternaria* species-groups previously established based upon morphological characters (Simmons 1995). One notable exception was the cheiranthi species-group. In the ITS analysis, members of this species-group were distributed among the radicina, brassicicola, and the *Ulocladium* clades (Fig. 1). In addition, the inclusion of *A. japonica* and *U. alternariae* in the brassicicola clade is not consistent with previous groupings based upon morphological criteria. Finally, although *A. brassicae* was not placed into any of the previously mentioned species-clades (Fig. 1), it did have a high level of sequence identity ($>$ 90%) with the other *Alternaria* species.

ITS sequence identities (entire fragment) within species-clades were usually $>$ 98%, whereas identities between species from different clades ranged from 90–98%. No intra-species ITS sequence variability was detected among the three isolates each of *A. alternata*, *A. dauci*, and *A. radicina* for which sequences were determined in this study.

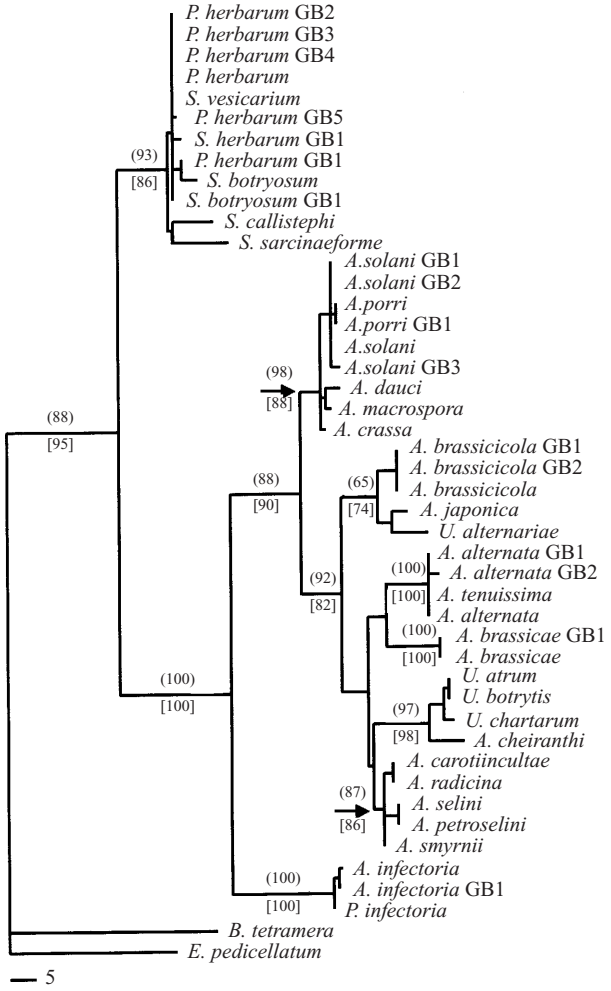


Fig. 1. Phylogenetic tree generated from neighbour-joining and parsimony analyses of nuclear ITS/5.8S rDNA sequences of selected *Alternaria* and related species. In addition to fungal sequences determined in this study, sequences from the following GenBank accessions were also included: *A. alternata* GB1, U05195; *A. alternata* GB2, AF071346; *A. brassicae* GB1, U05253; *A. brassicicola* GB1, U05198; *A. brassicicola* GB2, AF201964; *A. infectoria* GB1, Y17066; *A. porri* GB1, AB026159; *A. solani* GB1, Y17070; *A. solani* GB2, Y17069; *A. solani* GB3, U08203; *P. herbarum* GB1, AF071345; *P. herbarum* GB2, AB02165; *P. herbarum* GB3, AB026164; *P. herbarum* GB4, AB026163; *P. herbarum* GB5, U05202; *S. botryosum* GB1, Y17068; and *S. herbarum* GB1, AF071344. The scale bar indicates the number of base changes per 1000 nucleotide positions in the neighbour-joining analysis, and the number of nucleotide substitutions in the parsimony analysis; vertical distances have no significance. Bootstrap confidence measures from 1000 bootstrap replicates are indicated in parentheses for the neighbour-joining analysis and brackets for the parsimony analysis.

Parsimony analysis of ITS data set revealed 63 most parsimonious trees that differed mainly in the position of *A. brassicae* and the alternata and *Ulocladium* clades relative to the remaining clades. One of these trees (length = 302, CI = 0.76, RI = 0.92) had near identical topology to the tree generated with neighbour-joining analysis (Fig. 1). The large *Alternaria*/*Ulocladium* clade was again supported by a bootstrap value of 100%, and the establishment of the species-clades was supported by bootstrap values $\geqslant$ 74%. Among the *Alternaria*, *Stemphylium*, and *Ulocladium* species, 595 total characters were examined of which 171 were variable (29%), and 108 (18%) were phylogenetically informative.

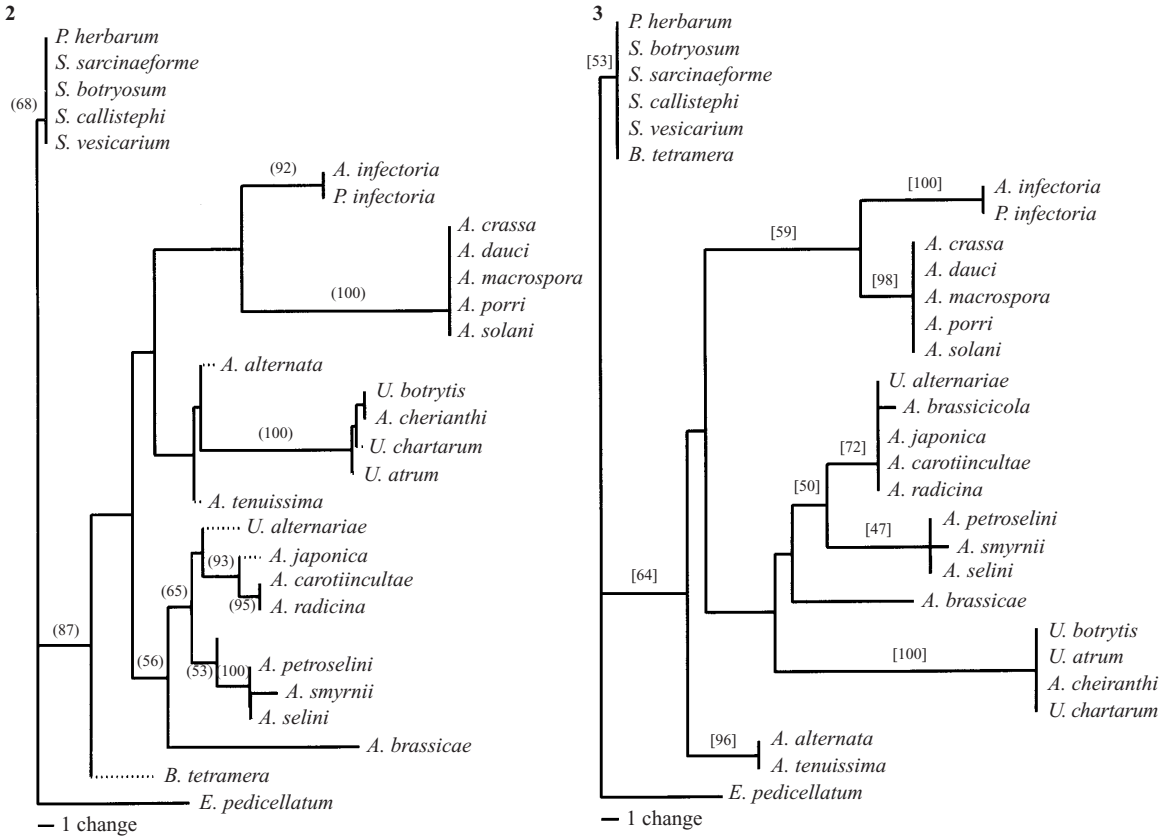
Additional parsimony analyses, performed with the ITS data set after removal of the large indel present in the ITS 1 region of some species, did not affect the overall tree topology, but did slightly increase bootstrap values for several clades (data not shown). However, the most parsimonious trees obtained during heuristic searches had similar confidence statistics (length = 274, CI = 0.76, RI = 0.92).

Mitochondrial SSU

The NMS2 and NMS3 primers directed the amplification of an approximately 700 bp fragment from all isolates. For the *S. callistephi*, *A. petroselini*, *A. selini*, and *A. smyrnii* isolates, one or two small secondary fragments (less intensely stained with ethidium bromide) also were amplified. In these cases, the primary SSU fragment was excised from an agarose gel and purified prior to sequencing. DNA sequencing revealed that the SSU fragments were more variable in size than were the ITS fragments, with SSU fragments ranging in size from 633 to 750 bp (including primer sequences). The GenBank accession numbers for all SSU sequences determined in this study are provided in Table 1.

The alignment of the SSU sequences was deposited in TreeBASE (http://herbaria.harvard.edu). No SSU sequences for any of the fungal species used in this study were found in GenBank. Alignment of the SSU sequences revealed two conserved regions flanking a highly variable central region of approximately 180 nt. The variation in the size of the SSU fragments was due to numerous indels that ranged in size from 2 to 38 nt and often contained poly-A or poly-T sequences. The largest indel was a 38 nt palindrome (alignment position 388–425), which was present in the SSU sequences of certain members of the cheiranthi species-group: *A. petroselini*, *A. selini*, and *A. smyrnii*.

Alignment of the SSU sequences also revealed species groups that were similar to those identified in the ITS analysis. However, there was greater sequence identity between/among members of each group for SSU compared with ITS sequences. For example, there was no variability in SSU sequences for species within the porri, alternata, and infectoria species-groups, whereas ITS sequences of these same species had differences ranging from 2 to 18 nt. There was also no intra-species SSU sequence variability among the three isolates each of *A. alternata*, *A. dauci*, or *A. radicina* for which sequences were determined in this study. The level of sequence variability between species of different groups was similar for SSU (93–98%) and ITS (90–98%) sequences. For the SSU sequences, most of this variability was due to the indels, which were particularly numerous in members of the radicina, porri, and *Ulocladium* groups. As with the ITS region, sequence identities between the *A. longissima* SSU and those of the other fungal species, including *E. pedicellatum* and *B. tetramera*, were the lowest of all pairwise comparisons (93–94%). Therefore, this species was not included in the SSU phylogenetic analyses. A GenBank search conducted with the *A. longissima* SSU



Figs 2–3 Phylogenetic trees generated from analyses of mitochondrial SSU rDNA sequences of selected *Alternaria* and related species. **Fig. 2.** Neighbour-joining analysis. **Fig. 3.** Parsimony analysis. The bar in Fig. 2 indicates the number of base changes per 1000 nucleotide positions, and the bar in Fig. 3 represents the number of nucleotide substitutions; vertical distances have no significance. Bootstrap confidence measures (> 50%) from 1000 bootstrap replicates are indicated in parenthesis for the neighbour-joining analysis and brackets for the parsimony analysis.

sequence failed to reveal sequences with higher identity, but no *Leptosphaeria* SSU sequences were available for comparison.

Neighbour-joining analysis of the SSU sequences revealed relationships among these fungi that were similar to those found in the ITS analyses. *E. pedicellatum*, *B. tetramera*, and the *Stemphylium* spp. were placed outside of a large *Alternaria*/*Ulocladium* clade (Fig. 2). The bootstrap value for this *Alternaria*/*Ulocladium* clade was < 50%. However, if the *B. tetramera* sequence was omitted, the bootstrap value for this *Alternaria*/*Ulocladium* clade increased to 85% (data not shown). Most of the species-clades revealed in the SSU analysis corresponded to those identified in the ITS analyses. One exception was the brassicicola clade, which was a distinct clade in the ITS analysis but was grouped with the radicina clade in the SSU analysis (Fig. 2). In general, bootstrap values for the SSU clades were lower than those for equivalent clades in the ITS analysis, although values for the porri and *Ulocladium* species-clades were higher in the SSU analysis.

Parsimony analysis of the SSU data set revealed over 10000 minimal trees due to the high number of identical or near-identical sequences in the data set. Most of these trees were very similar, and a representative tree is presented in Fig. 3 (length = 77, CI = 0.92, RI = 0.97). *E. pedicellatum*, *B. tetramera*, and the *Stemphylium* spp. were placed outside a large *Alternaria*/*Ulocladium* clade, which was supported by a bootstrap value of 64%. Most trees generated in the parsimony analysis did not differ greatly from the neighbour-joining tree except in the placement of the alternata species-clade. The bootstrap analysis showed support (⩾ 96%) for the establishment of most of the species-clades also revealed by the neighbour-joining analysis, except for the radicina and brassicicola clades. Among the *Stemphylium*, *Ulocladium*, and *Alternaria* species, 747 total characters were examined of which 64 were variable (9%), and 52 (7%) were phylogenetically informative.

Additional parsimony analyses, performed with the SSU data set after removal of various combinations of the large indels, did not affect the overall tree topology, nor increase bootstrap values (data not shown). The most parsimonious trees obtained during heuristic searches also had slightly lower confidence statistics.

Nuclear 18S

To lend support to the results of ITS and SSU sequence analyses, nuclear 18S sequences of selected isolates were determined and compared with 18S sequences of other ascomycete fungi, particularly the loculoascomycetes, obtained from the GenBank. The primer pairs NS1 and NS2, and the overlapping primer pairs NS3 and NS6, and NS5 and NS8 directed the amplification of 560, 760 and 520 bp fragments, respectively, from all the selected isolates. Assembly of the

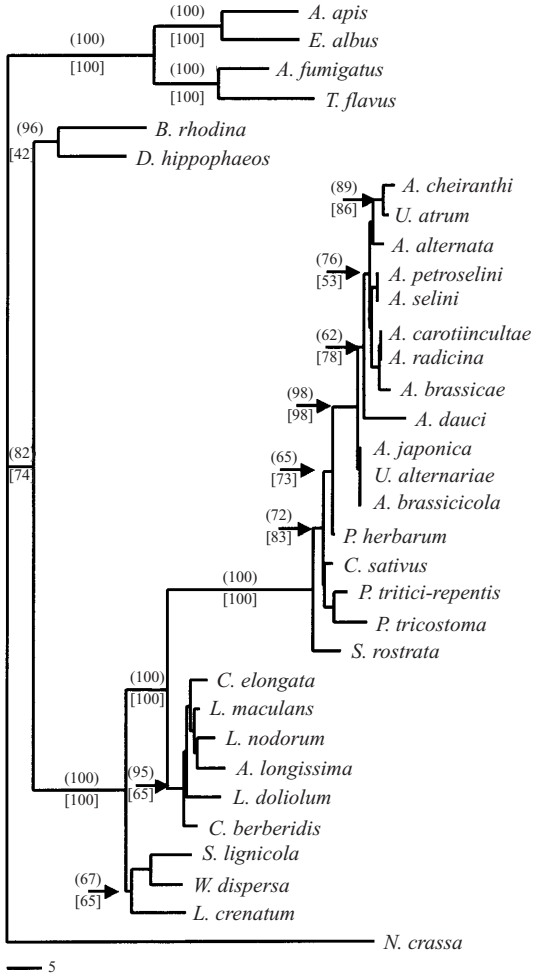


Fig. 4. Phylogenetic tree generated from neighbour-joining and parsimony analyses of nuclear 18S rDNA sequences of selected *Alternaria* and related Ascomycete species. In addition to fungal sequences determined in this study, sequences from the following GenBank accessions were also included: *Aspergillus fumigatus*, M60300; *Ascosphaera apis*, M83264; *Botryosphaeria rhodina*, U42476; *Cochliobolus sativus*, U42479; *Cucurbitaria berberidis*, U42481; *C. elongata*, U42482; *Dothidea hippophaeos*, U42475; *Eremascus albus*, M83258; *Leptosphaeria maculans*, U04238; *L. doliolum*, U04205; *L. nodorum*, U04236; *Lophiostoma crenatum*, U42485; *Neurospora crassa*, X04971; *Pyrenophora tricostoma*, U43459; *P. tritici-repentis*, U42486; *Setosphaeria rostrata*, U42487; *Sporomia lignicola*, U42478; *Talaromyces flavus*, M83262; *Westerdykella dispersa*, U42488. The scale bar indicates the number of base changes per 1000 nucleotide positions in the neighbour-joining analysis, and the number of nucleotide substitutions in the parsimony analysis; vertical distances have no significance. Bootstrap confidence measures from 1000 bootstrap replicates are indicated in parentheses for the neighbour-joining analysis and brackets for the parsimony analysis.

sequences of these fragments (including the highly conserved 21 bp NS2/NS3 priming site for which sequences were not determined) revealed that the 18S sequences of these isolates were 1687 nt, which is similar in size to the 18S sequences from GenBank. The GenBank accession numbers for all 18S sequences determined in this study are provided in Table 1.

The alignment of the 18S sequences was deposited in TreeBASE (http://herbaria.harvard.edu). The alignment did not require manual manipulation because there were few idels and those detected were $\leqslant 3$ nt. Results of neighbour-joining analyses of the 18S sequences were similar to those obtained for the ITS and SSU sequence analyses in that: (1) the *Ulocladium* and *Alternaria* spp. were closely related; (2) *Ulocladium* species were not clustered together; and (3) *A. longissima* was distantly related to the other *Alternaria* spp. and more closely related to *Leptosphaeria* spp. (Fig. 4). The establishment of the *Alternaria*/*Ulocladium* clade was strongly supported by a bootstrap value of 98%, whereas the establishment of the *Leptosphaeria*/*A. longissima* clade was supported by a bootstrap value of 95%. In addition, the 18S analysis showed that *P. herbarum* (anamorph *Stemphylium*) is related to the *Alternaria* and *Ulocladium* species, but it was placed into a distinct clade, as were species of *Cochliobolus* (anamorph *Bipolaris*), *Pyrenophora* (anamorph *Drechslera*), and *Setosphaeria* (anamorph *Exserohilum*).

A heuristic search of the 18S sequence data revealed five most parsimonious trees, one of which (length = 504, CI = 0.73, DI = 0.88) was nearly identical to the tree generated by neighbour-joining analysis (Fig. 4). The other four trees had only minor differences in terms of the positioning of *A. alternata*, and the *Cucurbitaria* species (data not shown). The establishment of the *Alternaria*/*Ulocladium* clade was strongly supported by a bootstrap value of 98%, whereas the establishment of the clade containing *Leptosphaeria* species and *A. longissima* was supported by a bootstrap value of 65%.

DISCUSSION

The molecular phylogenetic relationships among *A. radicina*, other species of *Alternaria*, and related fungi were examined based upon rDNA sequence analysis. The two rDNA sequences chosen for analysis, the nuclear ITS/5.8S region and the mitochondrial small subunit gene, have been previously used to examine relationships among fungi at the species level (Miyamoto & Cracraft 1991, White *et al*. 1990). Furthermore, by sequencing rDNA from the independently replicating nuclear and mitochondrial genomes, two lines of evidence are provided to support conclusions derived from these analyses.

Although the results obtained from nuclear and mitochondrial sequence analyses were not identical (i.e. minor differences were found in some intermediate tree nodes, branch lengths, and relationships among some species-clades), most of the phylogenetic relationships revealed in these analyses were similar. Thus, several important conclusions can be drawn. First, our results support the current distinction of *Stemphylium* and *Alternaria* spp. as separate but related genera. Based on the ITS and SSU analyses, the four *Stemphylium* spp. and *P. herbarum* (anamorph *Stemphylium*) were always placed together in a single clade and, thus, are very closely related. The rDNA sequence analyses, including the 18S sequences, further established that the *Stemphylium* species and *P. herbarum* are related to, but phylogenetically distinct from, *Alternaria* species. Consistent with these findings, our results revealed that *P. herbarum* and *P. infectoria* (anamorph *Alternaria*) are phylogenetically distinct, with *P. infectoria* most closely related to other *Alternaria* species. Simmons (1986) examined several *Pleospora* species that have *Alternaria* anamorphs, and

placed them in *Lewia* on the basis of differences in ascostroma morphology with *Pleospora* spp. that have *Stemphylium* anamorphs. Our results support the placement of *Pleospora*-like fungi that have *Alternaria* anamorphs (e.g. *P. infectoria*) into the genus *Lewia*.

Second, *Ulocladium* and *Alternaria* species were found to be more closely related than *Stemphylium* and *Alternaria* species. The four *Ulocladium* species examined in this study were consistently placed into two separate clades, each of which also contained *Alternaria* species. Three *Ulocladium* species (*U. atrum*, *U. botrytis*, and *U. chartarum*) had nearly identical ITS and SSU sequences, and formed a distinct clade along with *A. cheiranthi*, which shared a high degree of 18S, ITS, and SSU sequence identity with these *Ulocladium* species. However, *A. cheiranthi* does not possess *Ulocladium* characteristics as defined by Simmons (1967, 1995); i.e. obovoid, non-beaked conidia. The inclusion of *A. cheiranthi* within this clade is particularly significant because of the historical debate surrounding its taxonomic status. One of the founding species of the genus *Macrosporium* Fr. 1832 was *M. cheiranthi* (syn. *A. cheiranthi*). The species was subsequently placed in *Alternaria* by Wiltshire (1933), which was one of the factors that led to the designation of *Macrosporium* as a *nomen ambiguum*. The placement of *A. cheiranthi* in the genus *Alternaria* has subsequently been supported in several studies (Joly 1964, Simmons 1995). Thus, the placement of *A. cheiranthi* into the genus *Ulocladium* on the basis of rDNA sequence identity would be inconsistent with the morphological criteria established for *Alternaria* (i.e. *A. cheiranthi* has ovoid conidia like most *Alternaria* species), and the morphological criteria that establishes *Ulocladium* as distinct from *Alternaria* (i.e. *Ulocladium* species have obovoid conidia). Furthermore, the movement of *A. cheiranthi* into *Ulocladium* would also resurrect the debate regarding the nomenclatural status of *Macrosporium*, a name which preceded *Ulocladium* by 19 years.

The 18S, ITS, and SSU rDNA sequences of the fourth *Ulocladium* species, *U. alternariae*, were significantly different from those of the other *Ulocladium* species. Consistent with this, *U. alternariae* was placed in a clade separate from that containing the other *Ulocladium* species, and it generally clustered with *A. japonica*. However, *U. alternariae* possesses the morphological characteristics of *Ulocladium* as defined by Simmons (1967), whereas *A. japonica* does not. Together, these results suggest that the diagnostic morphological characteristics defined for *Ulocladium* (obovoid, non-beaked conidia) are synapomorphic characters that may not reflect phylogenetic relationships among these fungi (Swofford & Olsen 1990).

It is also evident from a historical review of the genus *Ulocladium* that *Ulocladium sensu* Preuss is not the same as *Ulocladium sensu* Simmons. *Ulocladium* Preuss was typified by *U. botrytis* Preuss 1851, and included *U. atrum* Preuss 1852. Preuss had described *A. chartarum* in 1848 and, thus, did not consider this species to be closely related to the *Ulocladium* species he subsequently described. Saccardo (1886) also did not recognize a relationship among these three species, nor did he recognize the genus *Ulocladium*. Thus, *A. chartarum* was maintained in *Alternaria*, and *U. botrytis* and *U. atrum* were placed in the genus *Stemphylium* and named *S. botryosum* var. *ulocladium* and *S. atrum*, respectively. Simmons (1967) recognized that *S. botryosum* var. *ulocladium* and *S. atrum* shared certain morphological characteristics with *A. chartarum* and other atypical *Alternaria* and *Stemphylium* species. In order to resolve several long-standing taxonomic debates about the status of these and related fungi, the genus *Ulocladium* was brought back into use to include these species (Simmons 1967).

Our results clearly support moving *S. botryosum* var. *ulocladium* and *S. atrum* from the genus *Stemphylium* and grouping them with *A. chartarum*. However, they do not support their inclusion in a genus distinct from *Alternaria*. Rather, our results indicate that certain *Ulocladium* species, along with *A. cheiranthi*, comprise a phylogenetically distinct species-group within the genus *Alternaria*, which is as phylogenetically distinct as other *Alternaria* species-groups. Thus, in many respects, our results support the work of Joly (1964) who suggested a broader definition of the genus *Alternaria*, in which: (1) species currently in the genus *Ulocladium* were placed in the genus *Alternaria*; and (2) these species were placed in a subgeneric group that also contained *A. cheiranthi*.

Based upon these results, we propose that the four *Ulocladium* species examined in this study be placed in *Alternaria*, and that *U. botrytis*, *U. atrum*, *U. chartarum*, and *A. cheiranthi* be referred to as the ulocladium species-group. Sequence analysis of other *Ulocladium* species, as well as certain *Alternaria* species, will be necessary to further define the ulocladium species-group. By virtue of placing the type species, *U. botrytis*, into the genus *Alternaria*, the phylogenetic status of the genus *Ulocladium* is questioned.

A third interesting finding of this study was that *A. longissima* was phylogenetically distinct from the other *Alternaria* species and the *Stemphylium* and *Ulocladium* species examined. This is consistent with the fact that this species produces filiform *Cercospora*-like conidia that are somewhat atypical for *Alternaria* (Ellis 1971, 1976). In addition, *A. longissima* spores also have few longitudinal septa. Comparison of *A. longissima* 18S sequence with those of other fungi revealed a high degree of sequence identity with *L. maculans* (99.5%). This level of sequence identity is similar to that between *L. maculans* and *L. doliolum* var. *conoidea* (99.1%) or *L. nodorum* (99.5%). The genus *Leptosphaeria* includes over 500 species, many of which are similar in ecology and biology to those of *Alternaria* (Crane & Shearer 1995). Although many *Leptosphaeria* species have coelomycete anamorphs (e.g. *Phoma*, *Septoria*, *Stagonospora*), some have hyphomycete anamorphs (e.g. *Nakataea*) (Crane & Shearer 1995). Moreover, some of the coelomycete anamorphs produce phaeodictyosporic conidia (e.g. *Camarosporium*), which are morphologically similar to conidia produced by some *Alternaria* species (Crane & Shearer 1995). Moreover, for many *Leptosphaeria* species, anamorphs have not been described. Thus, our results suggest that *A. longissima* is an anamorph of a *Leptosphaeria*. Further reclassification of this fungus will be necessary as new perspectives on diversity within the genus *Leptosphaeria* emerge.

Ribosomal DNA sequence analyses revealed that the cheiranthi species-group, which was established based upon

morphological criteria (Simmons 1995), is not monophyletic. Analysis of ITS sequences placed *A. cheiranthi* and *A. japonica* in separate species-clades, and placed *A. radicina*, *A. carotiincultae*, *A. selini*, *A. petroselini*, and *A. smyrnii*, all pathogens of umbelliferous crops, in a radicina species-clade. Analysis of SSU sequences placed *A. cheiranthi* in a separate species-clade, but not *A. japonica*. Simmons (1995) has previously suggested that the cheiranthi species-group could be typified by a more representative member, such as *A. radicina*. Our results strongly support this suggestion, and we propose changing the cheiranthi species-group designation to the radicina species-group. Further analysis of molecular, morphological, and/or biological characteristics are needed to fully resolve the species that comprise the radicina species-group.

Phylogenetic analyses of the five members of the radicina species-group revealed two distinct subgroups: the radicina-carotiincultae subgroup and the petroselini-selini-smyrnii subgroup. This grouping is consistent with groupings based upon ecology and pathogenicity, as members of the radicina-carotiincultae subgroup are primarily carrot pathogens, whereas members of the petroselini-selini-smyrnii subgroup are primarily parsely pathogens. The SSU and ITS sequences of *A. petroselini* and *A. selini* were identical, and varied from those of *A. smrynii* by only a few nts. The SSU sequences of *A. radicina* and *A. carotiincultae* were identical, and there was only a 1-nt difference between ITS sequences. Studies involving other fungal genera (e.g. *Leptosphaeria*, *Suillus*, *Verticillium*) have found greater variability in ITS or SSU sequences among closely related species (Li *et al*. 1994, Morales *et al*. 1992, Kretzer *et al*. 1996), suggesting that *Alternaria* isolates with little or no ITS and SSU variation may represent infraspecific categories (i.e. subspecies, varieties, and/or strains). Additional studies are underway to further resolve the phylogenetic relationships among the fungi of the radicina species-group, particularly the relationship between *A. radicina* and *A. carotiincultae*.

The porri species-group also had a high degree of rDNA similarity among members. Of the five species examined, there was no difference in SSU sequences and only minor variation (2–18 nt) among ITS sequences. Minor ITS sequence variation within *A. solani* was also found in comparisons of GenBank ITS sequences with those determined in this study. Again, this level of variation is less than that seen among strains of other fungal species, which suggests an extremely close relationship among species in the porri species-group. It has been proposed that certain members of the porri species-group represent varieties or special forms of a single species (Joly 1964, Neergaard 1945), and our results lend support to this concept.

In general, better taxonomic resolution of *Alternaria* and related fungi was achieved by analysis of nuclear ITS sequences compared with mitochondrial SSU sequences. One important difference between these regions was the amount, type, and distribution of sequence variation among the *Alternaria* species-groups. Overall, the SSU region contained approximately 37% the number of variable sites compared with the ITS region, and almost all of the SSU variability was found within a 150 nt variable region. In contrast, variability in the ITS sequences was distributed throughout a larger region. In addition, analysis of ITS sequences revealed differences between some species that were not detected in analyses of SSU sequences (e.g. the porri species-group). Thus, the ITS region appears to be more useful for identifying/differentiating *Alternaria* isolates. In addition, the ITS region has fewer indels, which allows for a more robust phylogenetic analysis because fewer assumptions are made during sequence alignments.

However, analysis of the SSU region did reveal clear differences between members of different *Alternaria* species-clades and, thus, provides a useful criterion for placing isolates into appropriate species-groups. For example, the unique indels identified in the SSU rDNA of *A. cheiranthi* clearly revealed that it is related to the *Ulocladium* spp. In addition, the low level of SSU sequence variability among members of individual clades presented a slightly more conserved picture of species diversity than did the ITS region, and the SSU rDNA analysis provided another line of evidence for relationships among related species. Thus, analysis of SSU rDNA sequences is a useful tool for phylogenic analysis of the genus *Alternaria*.

In conclusion, the overall congruence in the phylogenies generated from sequence analysis of nuclear and mitochondrial rDNA provides strong support for the conclusions drawn from this study. The fact that analysis of the relatively conserved 18S region provided similar results as analysis of the less-conserved ITS and SSU sequences lends further support to our conclusions. These analyses revealed that most *Alternaria* and *Ulocladium* species are included within a monophyletic clade composed of several distinct species-groups. *Stemphylium* species form a distinct sister group to this clade. *A. longissima* is an atypical *Alternaria* that is not closely related to *Alternaria*, *Ulocladium*, or *Stemphylium*. Future rDNA analyses of additional typical and/or atypical *Alternaria* species will undoubtedly help resolve the taxonomic status of *Alternaria* and related genera, and will help facilitate accurate definition/differentiation of *Alternaria* species.

ACKNOWLEDGEMENTS

This work was partially supported by the California Fresh Carrot Advisory Board and the College of Agricultural and Environmental Sciences, University of California, Davis. We thank Dr E. G. Simmons for providing cultures, confirming the identity of isolates, and for useful advice during the course of this study. We also thank Dr E. E. Butler for providing cultures and for a critical review of this manuscript.

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Corresponding Editor: B. Senn-Irlet