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A re-examination of the phylogenetic relationship between the causal agents of carrot black rot, *Alternaria radicina* and *A. carotiincultae*

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Abstract: The phylogenetic relationship between *Alternaria radicina* and *A. carotiincultae* was re-examined based on morphology, sequence analysis of rDNA (ITS and mitochondrial small subunit [mtSSU]), protein coding genes (actin [ACT], β-tubulin, chitin synthase [CHS], translation elongation factor [EF-1a], *Alternaria* allergen a1 [Alt a1], and glyceraldehyde-3-phosphate dehydrogenase [*gpd*]), and RAPD and ISSR analysis of total genomic DNA. Although some morphological characters overlapped to a degree, with *A. radicina* isolates expressing moderate variation and *A. carotiincultae* isolates being highly uniform, *A. carotiincultae* could be differentiated from *A. radicina* based on significantly greater growth rate on potato dextrose agar (PDA) or acidified PDA (APDA) and average number of transverse septa per conidium. Sequence of rDNA and two protein coding genes, ACT and CHS, were invariant between species. However polymorphism with the EF-1a, β-tubulin, and Alt a1 gene strictly separated the population of *A. radicina* and *A. carotiincultae* as distinct lineages, as did RAPD and ISSR analysis. The polymorphic *gpd* gene did not strictly separate the two species. However isolates of *A. radicina* encompassed several haplotypes, one of which was the exclusive haplotype possessed by *A. carotiincultae* isolates, suggesting evidence of incomplete lineage sorting. The results suggest that *A. carotiincultae* is closely related to *A. radicina* but is a recently divergent and distinct lineage, which supports its status as a separate species.

Key words: *A. carotiincultae*, *A. radicina*, lineage sorting, phylogenetic relationship, protein coding genes, rDNA

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INTRODUCTION

Alternaria radicina Meier, Drechsler and Eddy is an economically important fungal pathogen causing black rot disease of carrot (Meier et al 1922, Grogan and Snyder 1952, Maude 1966). The fungus can be found wherever carrots are commercially produced and infects carrot roots and foliage in the field and carrot roots in cold storage causing significant crop losses worldwide (Lauritzen 1926, Maude 1966). In 1995 a similar fungus (EGS 26-010) was recovered from necrotic foliar lesions on wild carrot in Ohio and could be distinguished from *A. radicina* based on spore morphology and sporulation pattern. This taxon was described as a new species, *A. carotiincultae* (Simmons 1995). Similar to *A. radicina*, *A. carotiincultae* has been reported to be strongly pathogenic on carrot, infecting crown, root and leaf tissue and can be recovered from commercial lots of carrot seed (Pryor and Gilbertson 2002). In addition *A. carotiincultae* was found to be weakly pathogenic or non-pathogenic on other apiaceous plants (parsley, celery, cilantro and fennel), as was *A. radicina* (Pryor and Gilbertson 2002).

Although *A. radicina* and *A. carotiincultae* originally were described as separate species, both morphological and molecular analyses have revealed that these species are closely related. In early studies of *A. radicina*, Neergaard (1945) reported three distinct types of *A. radicina*, which he termed types a, b and c. Types a and b, isolated from carrot, were strongly pathogenic on carrot and weakly pathogenic on celery, whereas type c, isolated from celery, was weakly pathogenic on carrot but was strongly pathogenic on celery. Subsequent pathogenicity tests with contemporary isolates of *A. radicina*, *A. carotiincultae* and the morphologically similar and related fungus *A. petroselini*, revealed that *A. radicina* and *A. carotiincultae* were strongly pathogenic on carrot but weakly pathogenic on celery, parsley, cilantro and fennel, whereas *A. petroselini* exhibited the converse in pathogenicity (Pryor and Gilbertson 2002). This suggested that Neergaard's type c isolates were likely *A. petroselini*. Similarly a contemporary *Alternaria* isolated from celery, ATCC 58405 (Wearing 1980), which originally was described as *A. radicina*, subsequently was shown to be *A. petroselini* based on morphological, host range and molecular analyses (Pryor and Gilbertson 2002). Furthermore Neergaard's *A. radicina* types a and b could be differen-

tiated by growth rate, colony margin, pigment and crystal production, and spore size, which are precisely the criteria that separate contemporary isolates of *A. radicina* and *A. carotiincultae* (Pryor and Gilbertson 2002). Thus it was suspected that in his original work Neergaard was working with both carrot pathogens as well.

In recent years various molecular tools have been used for delimitation of fungal taxa that were described previously based on morphological and host range criteria. Regarding *Alternaria*, DNA fingerprinting techniques such as RAPD, PCR-RFLP, amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR) and restriction mapping (Weir et al 1998, Roberts et al 2000, Pryor and Michailides 2002, Peever et al 2002, Hong et al 2005, 2006) and sequence analysis of rDNA such as the nuclear internal transcribed spacer (ITS) and mitochondrial small subunit (mtSSU) rDNA, and protein-coding genes such as glyceraldenyde-3-phosphate dehydrogenase (*gpd*), endopolygalacturonase (*endo*-PG), β-tubulin, and Alt a1 (an allergen) protein-coding genes (de Hoog and Horre 2002, Kang et al 2002, Peever et al 2002, Pryor and Michailides 2002, Serdani et al 2002, Pryor and Bigelow 2003, Peever et al 2004, Hong et al 2005) have been used for studies on relationship at the population and species level. In the first study of molecular differences between *A. radicina* and *A. carotiincultae* the species could be differentiated based on RAPD analysis but generally showed high genetic similarity and a close phylogenetic relationship (Pryor and Gilbertson 2002). Sequence analysis revealed no differences at the mtSSU locus and one nucleotide difference (a deletion) at the ITS locus (Pryor and Gilbertson 2002). Furthermore the neotype of *A. radicina*, ATCC 6503, which is morphologically atypical of both *A. radicina* and *A. carotiincultae*, was revealed to be genetically *A. carotiincultae* and not *A. radicina* by both RAPD and sequence analysis. Thus, because the two fungi are similar morphologically and genetically, both are carrot pathogens causing similar symptoms, and the neotype of *A. radicina* is genetically *A. carotiincultae*. Pryor and Gilbertson (2002) proposed that two species be considered conspecific.

However subsequent studies on the relationship between these two fungi have not corroborated the proposed synonymy, nor have they strongly suggested the converse. Studies based on IGS restriction analysis revealed that *A. radicina* and *A. carotiincultae* could be differentiated from each other by size differences of IGS PCR product (3.9 kb and 2.8 kb respectively). However the two fungi had the same restriction sites based on the mapping of eight restriction enzymes, revealing a close relationship (Hong et al 2005). Subsequent sequence analysis revealed that *A. radicina* and *A. carotiincultae* differed at the *gpd* locus by 5 bps, suggesting distinct taxa (Pryor and Bigelow 2003). However, at the Alt a1 locus, *A. radicina* and *A. carotiincultae* had identical sequences that, considering the variability at the Alt a1 locus, suggested a single taxon (Hong et al 2005). Thus taken together morphological and molecular characteristics of *A. radicina* and *A. carotiincultae* revealed to date have not lead to a clear demarcation of species. The failure of previous work to clearly reconcile these sister taxa as either synonymous or distinct might be due in part to low representation of each taxon in these previous studies.

The objective of this study was to re-evaluate the relationship between *A. radicina* and *A. carotiincultae* based on examination of a larger number of isolates from more divergent geographic sources than that used in previous work. Characteristics of morphology, sequence analysis of rDNA and protein coding genes and DNA fingerprinting were determined in an effort to reveal synapomorphies within taxa that could separate these fungi into distinct species or confirm the previously proposed synonymy.

MATERIALS AND METHODS

Strains, cultural and morphological characterization.—Twenty isolates of *Alternaria radicina*, 10 isolates of *A. carotiincultae* and one isolate each of *A. petroselini*, *A, selini*, and *A. smyrnii* were used in this study (TABLE I). Isolates of *A. radicina* and *A. carotiincultae* represent taxa from diverse geographic origins and substrates. Included in this study were the *A. radicina* neotype, the designated *A. radicina* representative strain, and the type of *A. carotiincultae* (ATCC 6503, ATCC 96831 and EGS 26-010 respectively). All isolates were maintained on potato dextrose agar (PDA; Difco, Plymouth, Minnesota) plate at 22 C, or on PDA slants at 10 C.

Data for conidia and cultural characteristics were obtained with the procedures described by Pryor and Gilbertson (2002). All isolates initially were incubated on PDA at 22 C. When colonies were 5–7 d old, a 3 mm diam plug was taken from the colony edge and transferred to three replicate plates each of PDA, acidified PDA (APDA, pH 5.0) and V8 agar (Simmons 1992). Cultures were incubated in clear plastic boxes with the surface of each dish 40 cm beneath fluorescent lights (Sylvania cool-white, 10/14 light/dark) at 22 C for 8 d.

After incubation growth rate was determined from measurements of colony diameter on PDA and APDA across two axes. Cultural and micromorphological characteristics including colony margin and the production of pigment and crystals on PDA and APDA were determined according to the protocols of Pryor and Gilbertson (2000). In addition the number of transepta per conidium was

TABLE I. Isolates used in this study and their sources

Species	Isolate No.[a]	Host/substrate	Tissue[b]	Location of origin
A. radicina	BMP 0047	*Daucus carota* L.	Root	California
	BMP 0055	Soil	—	California
	BMP 0062	*Daucus carota* L.	Seed	Japan
	BMP 0065	*Daucus carota* L.	Seed	Japan
	BMP 0066	*Daucus carota* L.	Crown	California
	BMP 0073	*Daucus carota* L.	Seed	USA
	BMP 0074	*Daucus carota* L.	Seed	The Netherlands
	BMP 0079 (ATCC 96831)	*Daucus carota* L.	Seed	France
	BMP 0089	Soil	—	California
	BMP 0090	Soil	—	California
	BMP 0093	Soil	—	California
	BMP 0094	*Daucus carota* L.	Crown	Australia
	BMP 0096	*Daucus carota* L.	Leaf	California
	BMP 0097	Soil	—	California
	BMP 0098	Soil	—	California
	BMP 0099	Soil	—	California
	BMP 0104	Soil	—	California
	BMP 0105	*Daucus carota* L.	Seed	USA
	BMP 0108	Soil	—	California
	BMP 0111	*Daucus carota* L.	Seed	USA
A. carotiincultae	BMP 0064	*Daucus carota* L.	Seed	Japan
	BMP 0075	*Daucus carota* L.	Seed	The Netherlands
	BMP 0076	*Daucus carota* L.	Seed	The Netherlands
	BMP 0078	*Daucus carota* L.	Seed	USA
	BMP 0095	*Daucus carota* L.	Crown	Australia
	BMP 0129 (EGS 26-010)	*Daucus carota*	Foliage	Ohio
	BMP 0130	Soil	—	California
	BMP 0131	*Daucus carota* L.	Seed	Japan
	BMP 0132	*Daucus carota* L.	Seed	Japan
	BMP 0133 (ATCC 6503)	*Daucus carota* L	Root	Washington, DC
A. petroselini	BMP 0144 (EGS 09-159)	*Petroselinum crispum* (Mill.) Nym.	Seed	California
A. selini	BMP 0145 (EGS 25-198)	*Petroselinum crispum* (Mill.) Nym.	Foliage	Saudi Arabia
A. symrnii	BMP 0147 (EGS 37-093)	*Smrynium olus-atrum* L.	Stem	England
A. alternata	BMP 0269 (EGS 34-016)	*Arachis hypogaea* L.	Foliage	India

[a] Abbreviations for isolate number: ATCC, American Type Culture Collection, Manassas, VA 20108; BMP, B.M. Pryor, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; EGS, E.G. Simmons, Mycological Services, Crawfordsville, IN 47933.

[b] For Seed sources, the location of Seed producer is given.

determined for 50 randomly selected conidia in each of four fields of view (200×), and a mean number of septa per conidium was calculated (Pryor and Gilbertson 2000). All experiments were repeated three times. For growth rate and number of transepta, a Student's *t*-test (SigmaStat 3.5; Systat Software Inc, Point Richmond, California) was performed to determine significant difference ($P = 0.05$) between taxa.

DNA extraction and PCR amplification.—DNA extraction and purification were conducted according to the protocol of Pryor and Gilbertson (2002). Amplification of ITS and mtSSU rDNA was conducted according to previously described protocols using respectively primer pairs ITS5/ITS4 (White et al 1990) and NMS1/NMS2 (Li et al 1994) (Pryor and Gilbertson 2002). Amplification of protein coding genes actin (ACT), Alt a 1, β-tubulin, chitin synthase (CHS), EF-1α, and *gpd* was accomplished with primers pairs ACT-512F/ACT-783R (Carbone and Kohn 1999), Alt-for/Alt-rev (Hong et al 2005), Bt1a/Bt1b (Glass and Donaldson 1995), CHS-79F/CHS-354R (Carbone and Kohn 1999), EF1-728F/EF1-986R (Carbone 1999), and gpd1/gpd2 (Berbee et al 1999) respectively. Each PCR mixture contained 5 pmol of each primers, 200 μM dNTP, 1× *Taq* reation buffer, 2 unit of Ampli*Taq*-DNA polymerase, 2.5 mM $MgCl_2$ and 10 ng of template DNA in a final volume of 25 μL. PCR conditions for ACT, β-tubulin, EF-1α,and *gpd* were 35 cycles of 94 C for 40 s denaturing, 55 C for 40 s annealing and 72 C for 1 min extension. For Alt al, PCR conditions were 35 cycles of 94 C for 40 s denaturing, 57 C for 40 s annealing and 72 C for 1 min extension. Initial denaturing at 94 C was extended to 5 min and the final extension was at 72 C for 15 min.

Sequencing, alignment and phylogenetic analysis.—The

sequences of PCR products were determined with FS Dye Terminator reactions (Applied Biosystems, Foster City, California) and ABI automated DNA sequencer. Sequences were determined for both DNA strands of PCR products for sequence confirmation. The sequences were proofread, edited and merged into composite sequences with the PHYDIT program version 3.1 (Chun 1995 available at http://plaza.snu.ac.kr/jchun/phydit/). Nucleotide sequences were aligned manually where necessary.

Phylogenetic analyses were performed in PAUP 4.0 10b (Swofford 2002). Ambiguously aligned regions were excluded from analyses. Sequence gaps were treated as missing data. Maximum parsimony (MP) analyses were estimated by heuristic searches consisting of 100 stepwise random addition replicates and branch swapping by tree bisection-reconnection (TBR). Branch stability was assessed by 1000 bootstrap replications using a heuristic search with simple sequence addition. Maximum likelihood (ML) analyses were performed on heuristic searches with the best-fit model, which was deduced as the best fit for the data by the likelihood ratio test using Modeltest ver3.06 (Posada and Crandall 1998). Branch stability was assessed by 500 bootstrap replications using rearrangements limited to 1000 per replications to reduce computational time. Sequences of *A. alternata* were used as outgroup based on results from Hong et al (2005), Pryor and Bigelow (2003), Pryor and Gilbertson (2000). The statistical concordance between sequence datasets was evaluated with the partition homogeneity test (incongruence length difference [ILD] test [Farris et al 1995]) option of PAUP 4.0 10b (Swofford 2002).

RAPD analysis.—(RAPD analyses were conducted with isolates listed in TABLE I.) Four random primers (OPA-04, OPA-08, OPA-09 and OPA-10) (Operon Kit A, Operon Technologies, Alameda, California) were chosen to perform random amplification of genomic DNA for all selected isolates because they produced the greatest number of polymorphic bands and reproductive profiles. RAPD analyses were carried out as described by Pryor and Gilbertson (2002). PCR amplification was conducted with a PTC-100 thermal cycler (MJ Research Inc., Watertown, Massachusetts) programmed for these parameters: 45 cycles of 94 C for 1 min denaturing, 36 C for 1.5 min annealing and 72 C for 2 min extension. Initial denaturing at 94 C was extended to 5 min and the final extension was at 72 C for 10 min. Six μL of PCR products were analyzed by electrophoresis in 1.4% agarose gels in 0.5% Tris-borate-EDTA (TBE) buffer and viewed by UV illumination after staining in ethidium bromide. RAPD analyses were conducted at least twice to confirm reproducibility of amplification products. Gels were photographed on a UV-transilluminator with a UVP BioImaging GDS-8000 system (UVP Inc., Upland, California), and all RAPD fragments between 250 and 2500 bp were recorded with UVP Bioimaging software. Each isolate was scored for the presence or absence of a given fragment, and a binary matrix was constructed. Cluster analysis of the data matrix was performed with the unweighted pair group method with arithmetic mean (UPGMA) with Jaccard's similarity coefficient (Sneath and Sokal 1973), and the goodness of fit of the clustering to the data matrix was measured by cophenetic correlation (r) analysis with the software NTSYSpc ver. 2.1 (Exeter Software, Setauket, New York). The unbiased genetic identity and genetic distance of Nei (1978) was calculated with POPGENE version 1.31 (Yeh et al 1999).

ISSR analysis.—One microsatellite primer ($[ACA]_5$) and two minisatellite primers (M13 and T3B, 5′-GAGGGTGGCGGTTCT-3′ and 5′-AGGTCGCGGGTTCGAATCC-3′ respectively) were chosen based on production of high polymorphism and reproductive profiles. The PCR amplification were conducted in 25 μL volume containing 20 ng of genomic DNA, 1× PCR buffer (Promega, Madison, Wisconsin), 2.5 mM $MgCl_2$ (Promega, Madison, Wisconsin), 0.5 mM primer, 0.2 mM each dNTP, and 1.0 unit of of Taq DNA Polymerase (Promega, Madison, Wisconsin). PCR was performed in a PTC-100 thermal cycler (MJ Research Inc., Watertown, Massachusetts) under these conditions: 45 cycles of 94 C for 1 min denaturing, 55 C for 1.5 min annealing and 72 C for 2 min extension. Initial denaturing at 94 C was extended to 5 min, and the final extension was at 72 C for 10 min. Six μL of PCR products were viewed on 1.4% agarose gels in 0.5% Tris-borate-EDTA (TBE) buffer. Gels were stained with ethidium bromide and photographed on a UV-transilluminator with a UVP BioImaging GDS-8000 system (UVP Inc., Upland, California). ISSR analyses were conducted at least twice to confirm profiles. Isolates were scored for the presence or absence of bands between 300 bp and 3000 bp, and a binary matrix was constructed. Cluster analysis of the data matrix was conducted as for RAPD analysis.

RESULTS

Cultural and micromorphological characteristics.—Cultural and micromorphological characteristics including growth rate and the mean number of transepta per conidium were determined to be useful characteristics for delimitation of *A. radicina* and *A. carotiincultae* (TABLES III and IV). Quantitative data was not normally distributed, thus significant differences were determined based on the Mann-Whitney rank sum test (SigmaStat 3.5). Growth of *A. radicina* was significantly less from that of *A. carotiincultae* on PDA ($P < 0.001$) and APDA ($P < 0.001$; FIG. 1, TABLE IV). Conidia of *A. carotiincultae* had a significantly greater ($P < 0.001$) mean number of transepta than those of *A. radicina* (5.99 vs. 4.23 respectively, TABLE IV).

The morphological characters of colony margin, pigment and crystal production were consistent in most cases with previously descriptions for each species (Pryor and Gilbertson 2002). Most isolates of *A. radicina* formed irregular or torn colony margins and produced a yellow diffusible pigment and crystals within 20 d (TABLE III). However two isolates identi-

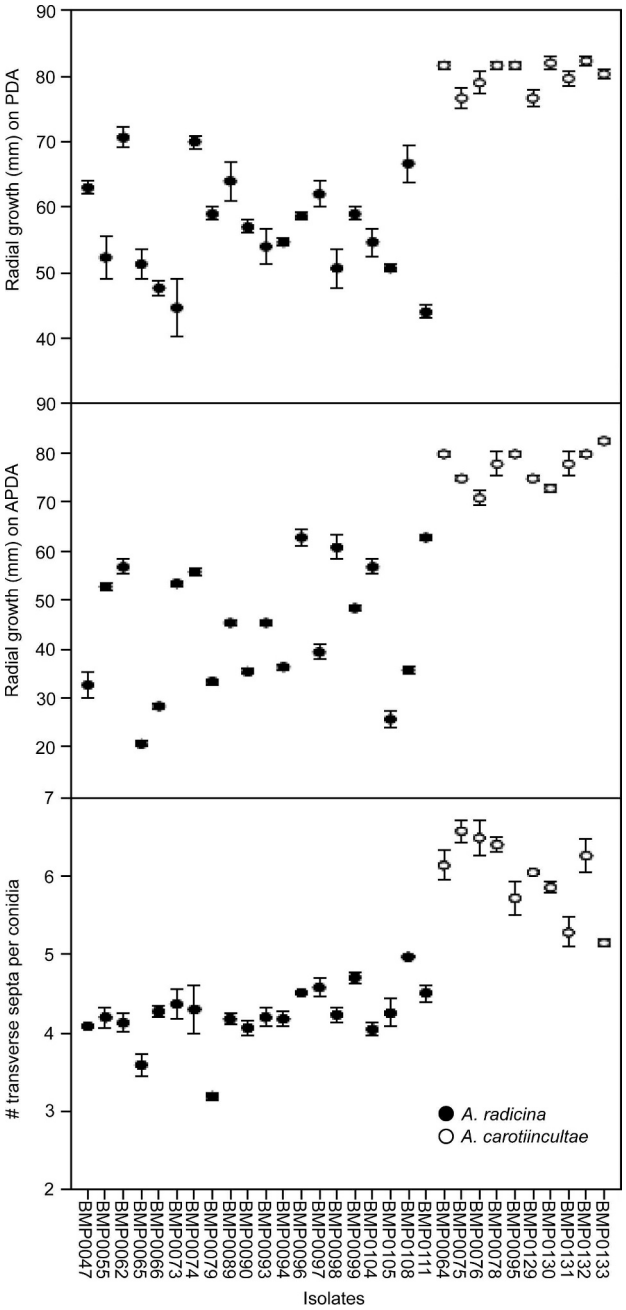

FIG. 1. Morphological characteristics including radial growth on PDA and APDA and the mean number of transepta per conidium on V8A. For each species, values indicate mean of three replications.

fied as *A. radicina* based on growth rate and average number of transepta (BMP 0097 and BMP 0099) formed smooth colony margins and did not produce yellow pigment or crystals. Other isolates identified as *A. radicina* based on growth rate and average number of transepta (BMP 0062, BMP 0066, BMP 0073, BMP 0074, BMP 0094, BMP 0097 and BMP 0098) did not produce crystals on either medium. All isolates identified as *A. carotiincultae* had smooth colony margins and covered the surface of the plate within 10–15 d. Yellow pigment and crystal production was never observed for these isolates on either medium. Although *A. petroselini*, *A. selini* and *A. smyrnii* grew rapidly and produced yellow pigment to varying degree they did not produce crystals in the medium.

Phylogenetic analysis.—Sequence analysis of the ITS, mtSSU, ACT and CHS revealed data that could not differentiate between *A. radicina* and *A. carotiincultae*. PCR amplification of the ITS region for all isolates generated 594–595 bp fragments. Alignment of ITS sequences revealed that the isolates of *A. radicina* and *A. carotiincultae* were both polymorphic (C/T) at position 483 of ITS2 region. All other positions were identical between the isolates of *A. radicina* and *A. carotiincultae* (results not shown). PCR amplification of the mtSSU region for all isolates generated 719 bp fragments. Sequence analysis of mtSSU rDNA showed 100% sequence identify among all isolates. PCR amplification of the ACT and CHS genes for all isolates generated 243 bp fragments and 219 bp fragments respectively. Sequence analysis of ACT and CHS revealed few polymorphic characters (1 nt and 3 nt respectively) and that the polymorphisms were shared among isolates of *A. radicina* and *A. carotiincultae* including the representative isolates (results not shown).

PCR amplification of the EF-1α gene for all isolates generated 279–280 bp fragments and alignment (minus primer sequences) resulted in a 243 characters dataset (12.8% variable and 5.8% parsimony informative). Parsimony analysis based yielded one most parsimonious tree (steps = 32, CI = 1.000, RI = 1.000) with two clades (FIG. 2). All isolates of *A. radicina* and *A. carotiincultae* fell within one well supported clade with the *A. carotiinculae* isolates comprising a monophyletic subclade. *A. petroselini*, *A. selini* and *A. smyrnii* resolved in a monophyletic clade basal to *A. radicina* and *A. carotiincultae*. All *A. radicina* isolates including the representative strain (BMP0079) had identical sequences. Similarly all *A. carotiincultae* including the ex-type of *A. carotiincultae* (BMP0129) and the neotype of *A. radicina* (BMP0133) had identical sequences and differed from those of *A. radicina* by 2 nt.

PCR amplification of the β-tubulin gene for all isolates generated 480 bp fragments and alignment (minus primer sequences) resulted in a 416 characters dataset (3.4% variable and 1.9% parsimony informative). Parsimony analysis yielded one most parsimonious tree (steps = 14, CI = 1.0000, RI = 1.0000) with two clades (FIG. 3). The β-tubulin trees had similar topology as that revealed in analysis of EF-

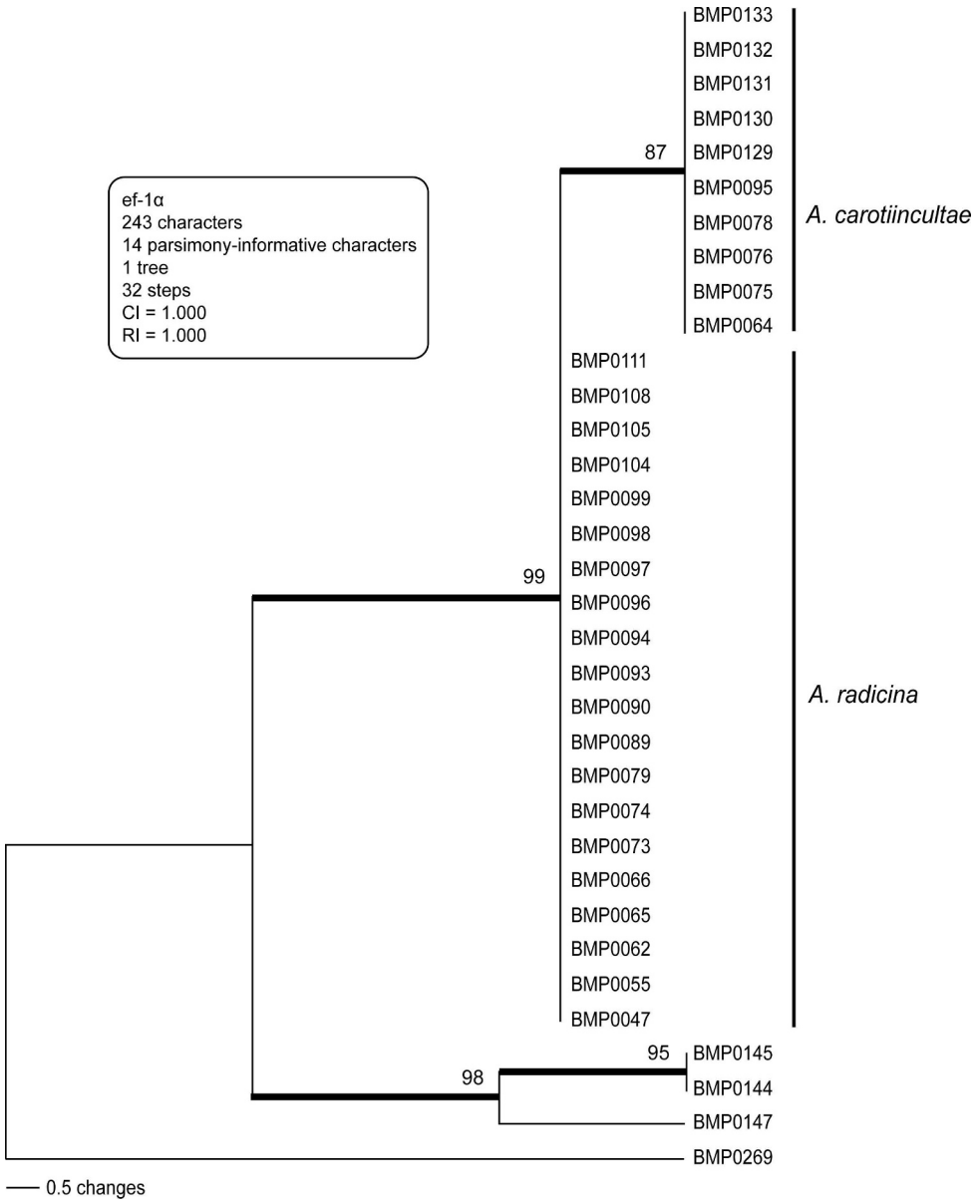

FIG. 2. Single parsimonious trees inferred from sequence of EF-1α gene. Bold branches indicate branch conserved in analyses using distance, parsimony and ML methods. Numbers represent parsimony bootstrap values obtained after a bootstrap test with 1000 replications. Bar indicates the number of nucleotide substitutions.

1α sequences except with greater genetic diversity among isolates of *A. radicina* and *A. carotiincultae*. Isolates of *A. radicina* and *A. carotiincultae* included two distinct β-tubulin haplotypes each. All *A. radicina* isolates fell into two distinct groups and included the representative strain (BMP0079) and all fell into one clade. All *A. carotiincultae* isolates fell into two distinct groups and included the ex-type of *A. carotiincultae* (BMP0129) and the neotype of *A. radicina* (BMP0133).

PCR amplification of the Alt a1 gene for all isolates generated 512–513 bp fragments and alignment (minus primer sequences) resulted in a 474 characters dataset (22.3% variable and 6.8% parsimony informative). Parsimony analysis yielded six most parsimonious tree (steps = 108, CI = 0.9444, RI = 0.9627) with near identical topology (FIG. 4). The Alt a1 trees had similar topology as that revealed in analysis of EF-1α and β-tubulin sequences except with greater genetic diversity among isolates of *A. radicina* and *A. carotiincultae*. Isolates of *A. radicina* included three Alt a1 haplotypes and formed monophyletic clade supported by moderate bootstrap values (75%), whereas isolates of *A. carotiincultae* encompassed two Alt a1 haplotypes and formed monophyletic clade supported by high bootstrap values (98%). As with

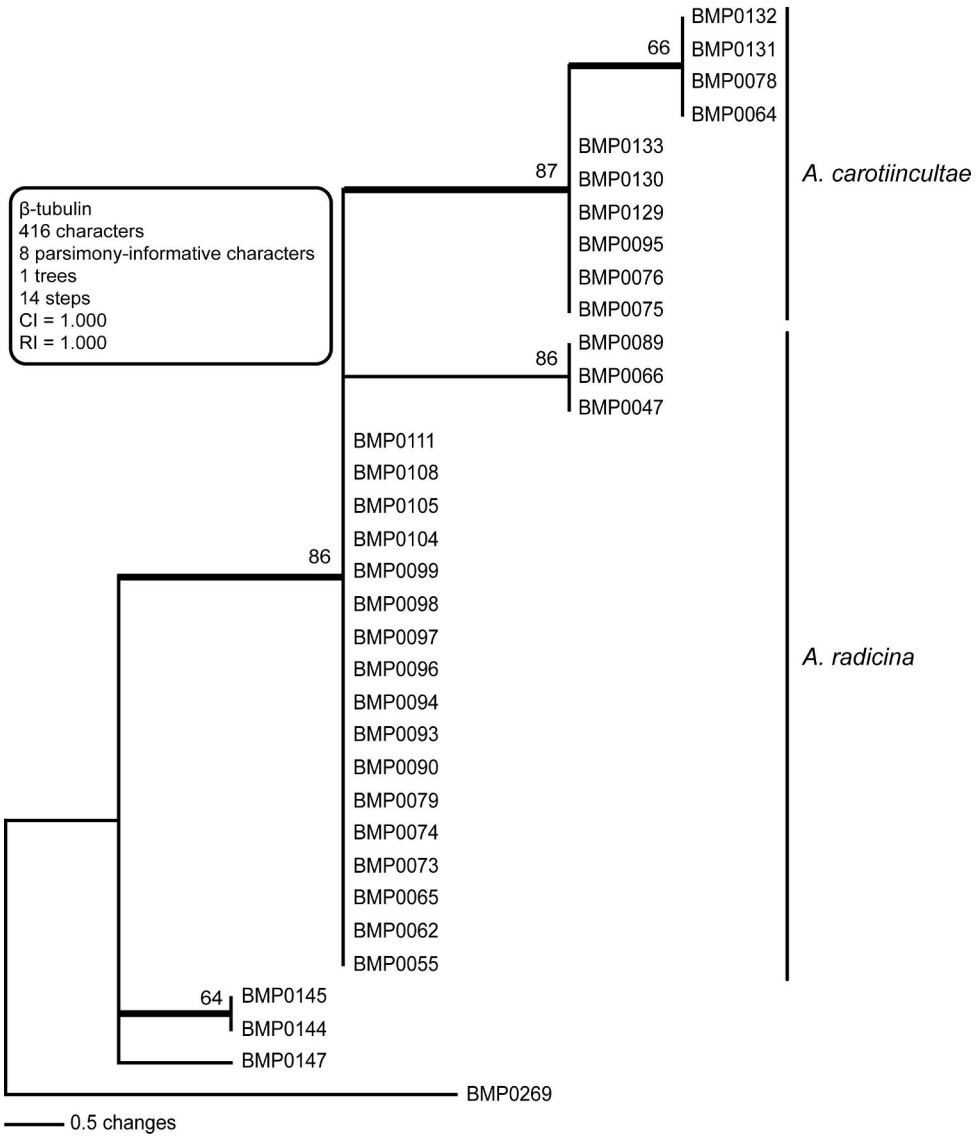

FIG. 3. Single parsimonious trees inferred from sequence of β-tubulin gene. Bold branches indicate branch conserved in analyses using distance, parsimony and ML methods. Numbers represent parsimony bootstrap values obtained after a bootstrap test with 1000 replications. Bar indicates the number of nucleotide substitutions.

the EF-1α and β-tubulin sequences, the *A. radicina* grouping included the representative strain (BMP0079) and the *A. carotiincultae* grouping included the ex-type of *A. carotiincultae* (BMP0129) and the neotype of *A. radicina* (BMP0133).

PCR amplification of the *gpd* gene for all isolates generated 612–618 bp fragments and alignment (minus primer sequences) resulted in a 577 characters dataset (7.8% variable and 3.6% parsimony informative). Parsimony analysis yielded one most parsimonious tree (steps = 46, CI = 1.000, RI = 1.000) with two clades (FIG. 5). The first clade included isolates of *A. radicina* and the representative strain (BMP0079) of *A. radicina* with strong bootstrap support (88%). The second clade included all isolates of *A. carotiincultae*, the ex-type of *A. carotiincultae* (BMP0129), the neotype of *A. radicina* (BMP0133) and two isolates (BMP0096 and BMP0108) referred to as *A. radicina* based on morphological characters and was well supported by 86% bootstrap value. Isolates of *A. radicina* were included in three *gpd* haplotypes. Two of them formed the monophyletic clade of *A. radicina*, whereas the other haplotype was included in the *A. carotiincultae* clade. *A. petroselini*, *A. selini* and *A. smyrnii* formed a well supported clade with *A. radicina* and *A. carotiincultae* as sister taxa. ML analysis of EF-1α, β-tubulin, Alt a1 and *gpd* sequences revealed trees with identical or near identical topology as those obtained in parsimony analysis (data not shown).

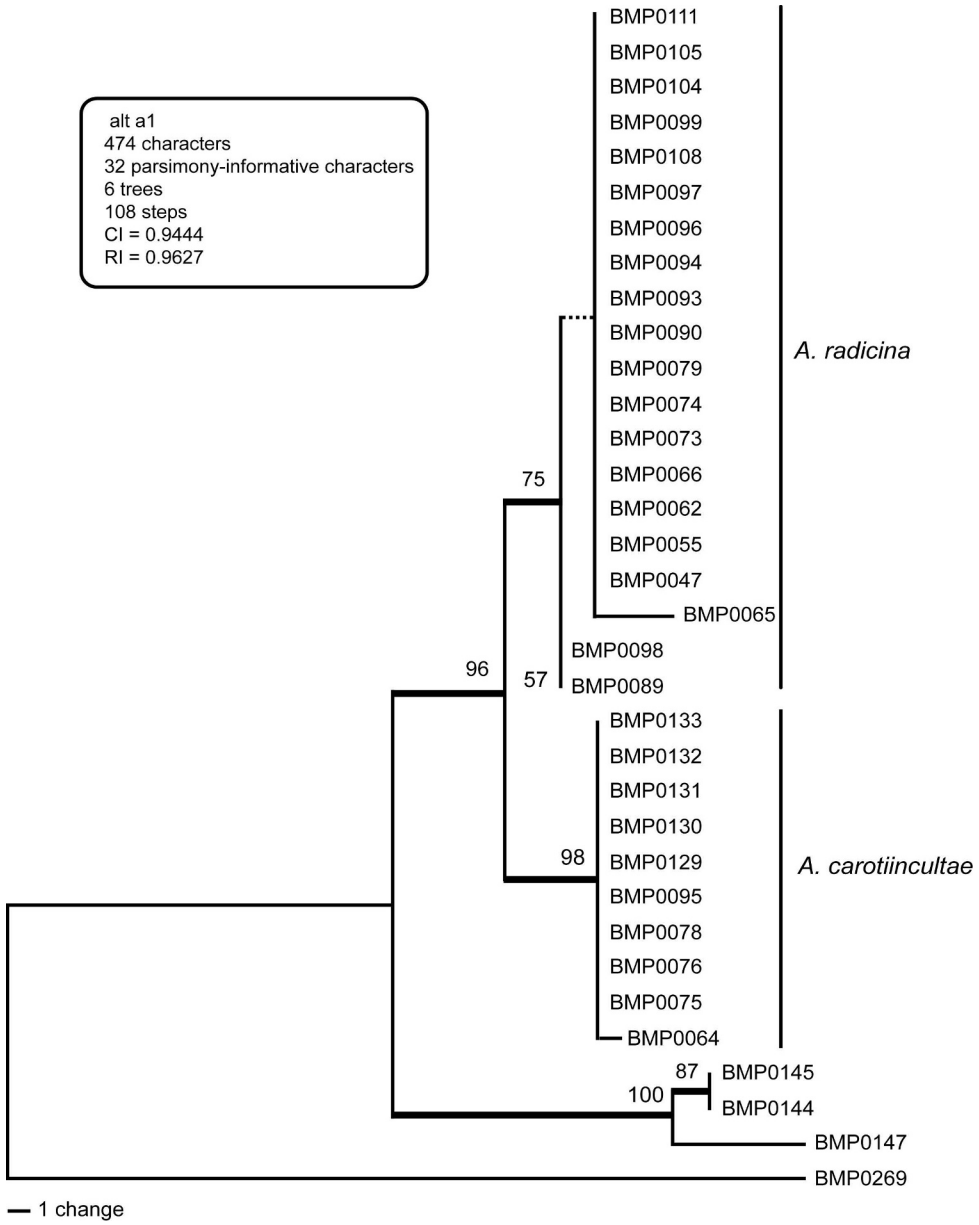

FIG. 4. One of six parsimonious trees inferred from sequence of alt a1 gene. Broken line indicated branches that were not conserved in the strict consensus tree of six parsimonious trees and bold branches indicate branch conserved in analyses using distance, parsimony and ML methods. Numbers represent parsimony bootstrap values obtained after a bootstrap test with 1000 replications. Bar indicates the number of nucleotide substitutions.

Phylogenetic analysis based on combined sequence of EF-1α, β-tubulin, Alt a1 and *gpd* yielded eight most parsimonious tree (steps = 206, CI = 0.9417, RI = 0.9633), which differed primarily in the position of two isolates (BMP0096 and BMP0108) of *A. radicina* (FIG. 6). Partition homogeneity tests revealed the four individual data sets were not significantly inconcordant (P = 0.095). Parsimony analysis of the combined dataset revealed a tree with similar topology as that revealed in analysis of with the EF-1α, β-tubulin, and Alt al data, whereas the combined dataset differed with *gpd* data in the positions of two isolates (BMP0096 and BMP0108) of *A. radicina*, which were ambiguous in combined data. ML analysis of the combined dataset revealed a tree with near identical topology as that obtained in parsimony analysis with exception in the position of isolates BMP0096 and BMP0108, which were placed as sister taxa of *A. carotiincultae* clade. Most *A. radicina* isolates formed monophyletic clade supported with a 64% bootstrap values, with the isolates BMP0096 and BMP0108 as a poorly supported sister clade. If isolates BMP0096 and BMP0108 were

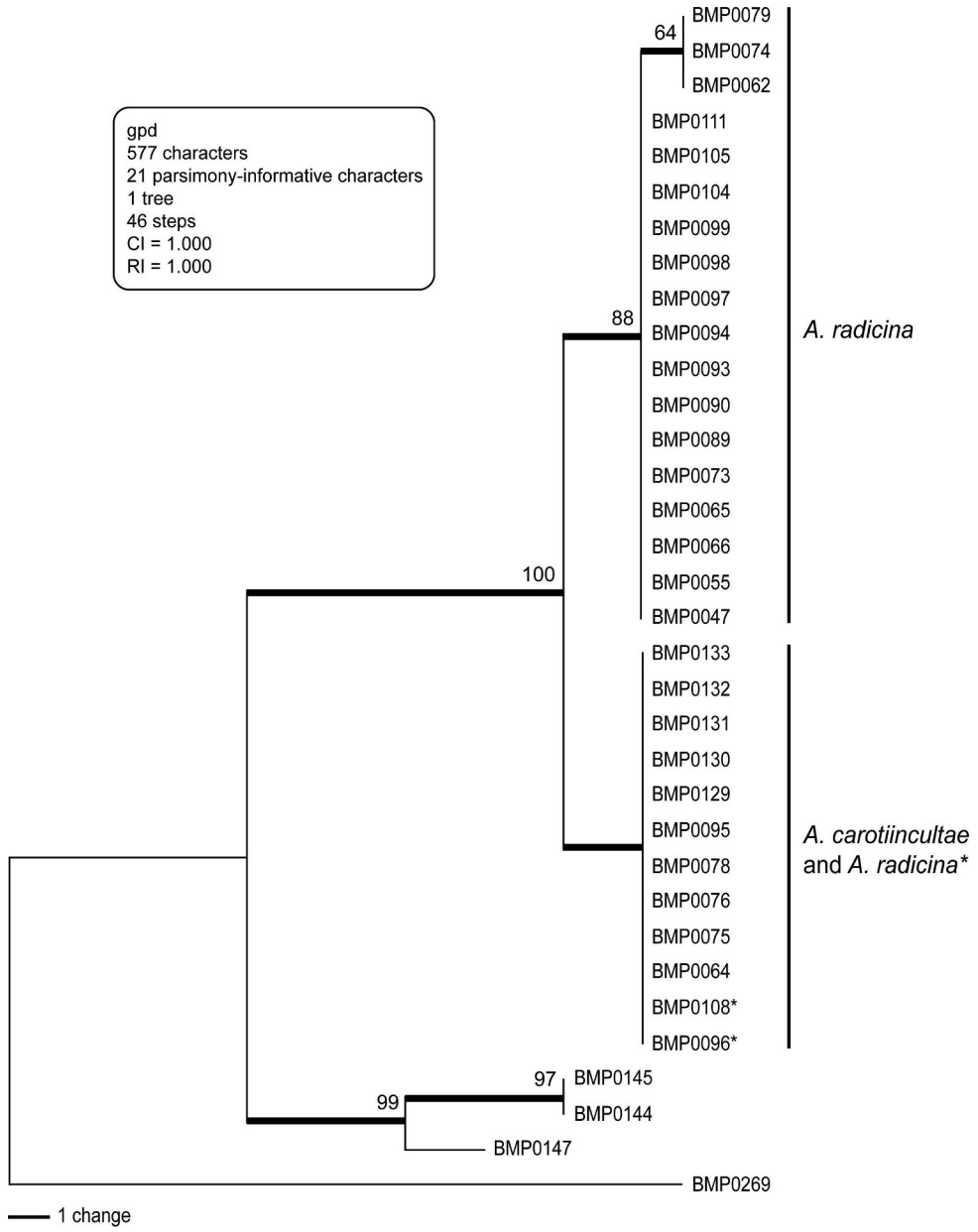

FIG. 5. Single parsimonious trees inferred from sequence of *gpd* gene. Bold branches indicate branch conserved in analyses using distance, parsimony, and ML methods. Numbers represent parsimony bootstrap values obtained after a bootstrap test with 1000 replications. Bar indicates the number of nucleotide substitutions.

excluded from the analyses, the *A. radicina* clade was supported by a bootstrap value of 97%. All isolates of *A. carotiincultae* formed a monophyletic clade supported by strong bootstrap values (100%).

Summarizing results of sequence analysis, genetic diversity within *A. radicina* was high compared to *A. carotiincultae*. Isolates of *A. radicina* and *A. carotiincultae* encompassed seven and three haplotypes, respectively (TABLE II). Within *A. radicina* 10 isolates of *A. radicina* sharing identical EF-1α, β-tubulin, Alt a1 and *gpd* haplotypes formed the basal haplotype of *A. radicina*. Other haplotypes differed in by single substitutions in *gpd* sequences (three haplotypes), four substitutions in *gpd* sequences (two haplotypes), two substitutions in β-tubulin sequences (two haplotypes), two substitutions in each of the β-tubulin sequences and alt a1 sequences (one haplotype), two substitution in the alt a1 sequences (one haplotypes) and four substitutions in the alt a1 sequences (one haplotype). Within *A. carotiincultae* six isolates of *A. carotiincultae* sharing identical EF-1α, β-tubulin, Alt a1, and *gpd* haplotype formed the basal haplotype of *A. carotiincultae*. Other haplotypes differed in by a single substitution in the β-tubulin sequences (three

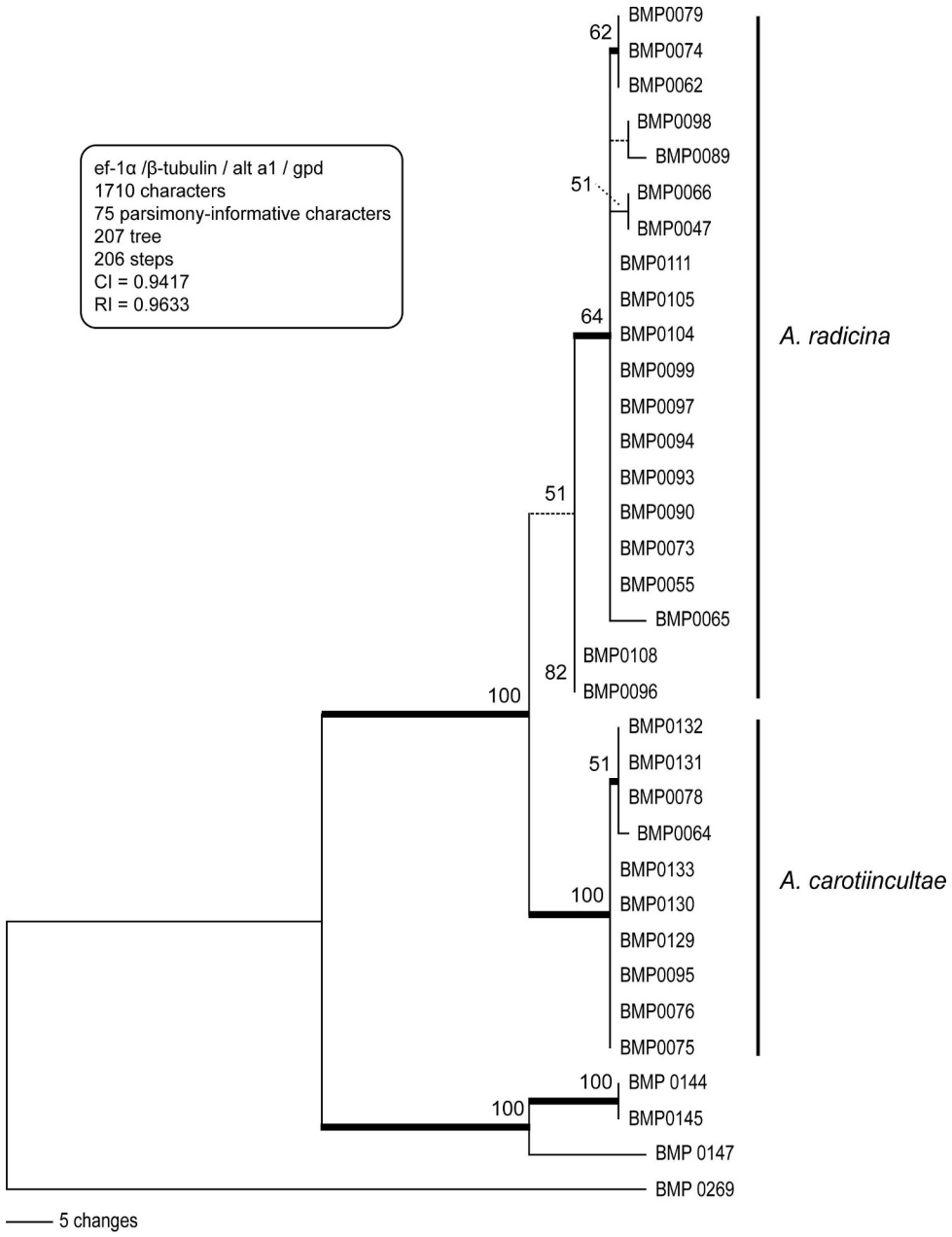

FIG. 6. One of 207 parsimonious trees inferred from combined dataset of EF-1α, β-tubulin, Alt a 1 and *gpd* genes sequence. Broken line indicated branches that were not conserved in the strict consensus tree of eight parsimonious trees and bold branches indicate branch conserved in analyses using distance, parsimony and ML methods. Numbers represent parsimony bootstrap values obtained after a bootstrap test with 1000 replications. Bar indicates the number of nucleotide substitutions.

haplotypes) and one substitution each in the β-tubulin sequences and Alt a1 sequences (one haplotype). All sequences have been deposited in GenBank (TABLE II) and EF-1α, β-tubulin, Alt a1, and *gpd* alignments have been deposited in TreeBase (SN3622) for review.

RAPD analysis.—Four random primers (OPA-04, OPA-08, OPA-09 and OPA-10) produced polymorphic and reproducible fragment and were used for RAPD analysis of all isolates. A total of 73 polymorphic bands were scored. The UPGMA tree obtained from the data matrix constructed with the four combined RAPD primers is provided (FIG. 7) (r = 0.991). In this tree three distinct clusters were evident: one cluster containing isolates of *A. radicina*; a second cluster containing isolates of *A. carotiincultae*; and a third cluster containing *A. petroselini*, *A, selini* and *A. smyrnii*. The isolates of *A. radicina* formed a single cluster with the representative isolate of *A. radicina* (BMP 0079) at

TABLE II. Sequences (GenBank accession numbers) used in the phylogenetic analyses. Only representative sequences for each haplotype are shown. Those in boldface were generated in this study

Species/isolate	ITS	mtSSU	ACT	Alt a1	β-tubulin	CHS	EF1a	*gpd*
A. radicina BMP 0047	**EU136661**		**EU141973**		**EU139383**	**EU141979**	**EU139417**	**EU142009**
A. radicina BMP 0065				**EU139346**				
A. radicina BMP 0079	**EU136654**	AF229668	**EU141971**	**EU139342**	**EU139376**	**EU141977**	**EU139410**	AY278797
A. radicina BMP 0089				**EU139341**				
A. radicina BMP 0098								**EU141995**
A. carotiincultae BMP 0064				**EU139329**	**EU139363**		**EU139397**	
A. carotiincultae BMP 0075	**EU136640**							
A. carotiincultae BMP 0129	**EU136636**	AF229654	**EU141969**	**EU139324**	**EU139358**	**EU141975**	**EU139392**	AY278798
A. petroselini BMP 0144				AY563288	**EU139353**		**EU139387**	AY278799
A. selini BMP 0145				**EU139319**	**EU139352**		**EU139386**	**EU141980**
A. smyrnii BMP 0147				AY563289	**EU139351**		**EU139385**	AY278801
A. alternata BMP 0269				AY563301	**EU139350**		**EU139384**	AY278808

74% similarity level and included four haplotypes. The isolates of *A. carotiincultae* also constitute a single cluster with the ex-type isolate of *A. carotiincultae* (BMP 0129) and the neotype isolate of *A. radicina* (BMP0133) at 74% similarity level and included two haplotypes. The genetic distance between *A. radicina* population and *A. carotiincultae* population was 0.0317 and between *A. radicina* population and others was 0.5429 (TABLE IV). The isolates of *A. radicina* were genetically different from those of *A. carotiincultae* but were more similar to those of *A. carotiincultae* than to *A. petroselini*, *A*, *selini* and *A. smyrnii*.

ISSR analysis.—Three ISSR primers produced polymorphic and reproducible fragments and were used for ISSR analysis of all isolates. The three primer combinations revealed a total of 49 polymorphic bands between 300 and 3000 bp and these bands were scored as present and absent for each isolate. The UPGMA tree generated with the Jaccard similarity coefficients based on these ISSR bands revealed the same three distinct groups that were recognized in RAPD analysis ($r = 0.992$) (FIG. 8). However the haplotype of isolates was not identical to that from RAPD data. The isolates of *A. radicina* formed a single cluster at ca. 85% similarity and encompassed three haplotypes. The isolates of *A. carotiincultae* also constitute a single cluster at ca. 90% similarity and encompassed a single haplotype. The isolates of *A. radicina* were different genetically not only from those of *A. carotiincultae* with genetic distance of 0.1438 but also from *A. petroselini*, *A*, *selini*, and *A. smyrnii* with genetic distance of 0.6186 (TABLE V).

DISCUSSION

Carrot black rot disease is one of the most economically important diseases in carrot, however comprehensive studies on the taxonomic relationship between the causal agents, *A. radicina* and *A. carotiincultae*, have been limited. In the primary study of *A. carotiincultae*, spore size, spore morphology and sporulation pattern were the sole characters used to distinguish *A. carotiincultae* from *A. radicina* (Simmons 1995). A subsequent study included additional characters to differentiate the taxa such as growth rate and the presence of pigments and crystals in culture, hallmarks of the production of radicinin, a secondary metabolite with phytotoxic and antifungal activity (Aldridge and grove 1964, Grove 1964, 1970, Pryor and Gilbertson 2002). In addition the later study included the use of molecular characters to provide a robust means to differentiate these taxa, which revealed that the neotype of *A. radicina* actually was similar genetically to *A. carotiincultae*. At that time the close morphological, molecular and ecological similarities between the two taxa and the systematic status of the *A. radicina* neotype required the two species to be synonymized (Pryor and Gilbertson 2002). The current study reported herein was substantially different from previous studies in that it included a larger number of taxa with a broader geographic representation to more fully reveal variation in populations. Moreover it used a more robust polyphasic approach with morphological analysis and various molecular analyses, which included sequence analysis of rDNA and protein coding genes and RAPD and ISSR analysis of total genomic DNA. Thus the conclusions of this study may be viewed as a much more comprehensive review of the systematic status of the taxa in question.

Morphological characters including growth rate, the mean number of transepta per conidium, colony margin and pigment and crystal production on PDA and APDA were compared as in previous studies. Although the characters of growth rate and transepta

TABLE III. Cultural and molecular characteristics for *A. radicina*, *A. carotiincultae*, *A. petroselini*, *A, selini*, and *A. smyrnii*

		Cultural characteristics					Molecular characteristics						
			Pigment production[b]		Crystal production[b]		Sequence analysis					DNA fingerprinting	
Species	Isolates	Colony sharp [a]	P[c]	AP	P	AP	EF-1α	β-tubulin	Alt a 1	*gpd*	Combined data	RAPD	ISSR
A. radicina	BMP 0047	I	+[c]	+	−	+	R1	R2	R1	R1	R3	R1	R1
	BMP 0055	I	+	+	+	+	R1	R1	R1	R1	R1	R2	R3
	BMP 0062	I	+	+	−	−	R1	R1	R1	R2	R2	R2	R2
	BMP 0065	I	+	+	+	+	R1	R1	R2	R1	R5	R2	R2
	BMP 0066	I	+	+	−	−	R1	R2	R1	R1	R3	R2	R2
	BMP 0073	I	+	+	−	−	R1	R1	R1	R1	R1	R1	R2
	BMP 0074	I	+	+	−	−	R1	R1	R1	R2	R2	R2	R2
	BMP 0079	I	+	+	+	+	R1	R1	R1	R2	R2	R2	R2
	BMP 0089	I	+	+	−	+	R1	R2	R3	R1	R6	R1	R2
	BMP 0090	I	+	+	+	+	R1	R1	R1	R1	R1	R2	R2
	BMP 0093	I	+	+	+	+	R1	R1	R1	R1	R1	R2	R2
	BMP 0094	I	+	+	−	−	R1	R1	R1	R1	R1	R2	R1
	BMP 0096	I	+	+	+	+	R1	R1	R1	C1	R4	R3	R2
	BMP 0097	S	−	−	−	−	R1	R1	R1	R1	R1	R3	R3
	BMP 0098	I	+	+	−	−	R1	R1	R3	R1	R7	R2	R2
	BMP 0099	S	−	−	−	−	R1	R1	R1	R1	R1	R3	R3
	BMP 0104	I	+	+	+	+	R1	R1	R1	R1	R1	R2	R2
	BMP 0105	I	+	+	−	+	R1	R1	R1	R1	R1	R1	R2
	BMP 0108	I	+	+	−	+	R1	R1	R1	C1	R4	R4	R2
	BMP 0111	I	+	+	+	+	R1	R1	R1	R1	R1	R4	R3
A. carotiincultae	BMP 0064	S	−	−	−	−	C1	C2	C2	C1	C3	C1	C2
	BMP 0075	S	−	−	−	−	C1	C1	C1	C1	C1	C2	C1
	BMP 0076	S	−	−	−	−	C1	C1	C1	C1	C1	C2	C1
	BMP 0078	S	−	−	−	−	C1	C2	C1	C1	C2	C1	C2
	BMP 0095	S	−	−	−	−	C1	C1	C1	C1	C1	C1	C2
	BMP 0129	S	−	−	−	−	C1	C1	C1	C1	C1	C1	C2
	BMP 0130	S	−	−	−	−	C1	C1	C1	C1	C1	C2	C2
	BMP 0131	S	−	−	−	−	C1	C2	C1	C1	C2	C1	C2
	BMP 0132	S	−	−	−	−	C1	C2	C1	C1	C2	C1	C2
	BMP 0133	S	−	−	−	−	C1	C1	C1	C1	C1	C1	C2
A. petroselini	BMP 0144	S	+	+	−	−	P	P	P	P	P	P	P
A. selini	BMP 0145	S	+	+	−	−	S	S	S	S	S	S	S
A. symrnii	BMP 0147	S	+	+	−	−	Sm	Sm	Sm	Sm	Sm	Sm	Sm

[a] Colony sharp within 14 d on APDA was recorded as irregular margins (I) and smooth margin (S).
[b] Production of pigment and crystals within 14 d was recorded as present (+) or absent (−).
[c] P, PDA (potato dextrose agar); AP, APDA (acidified PDA).

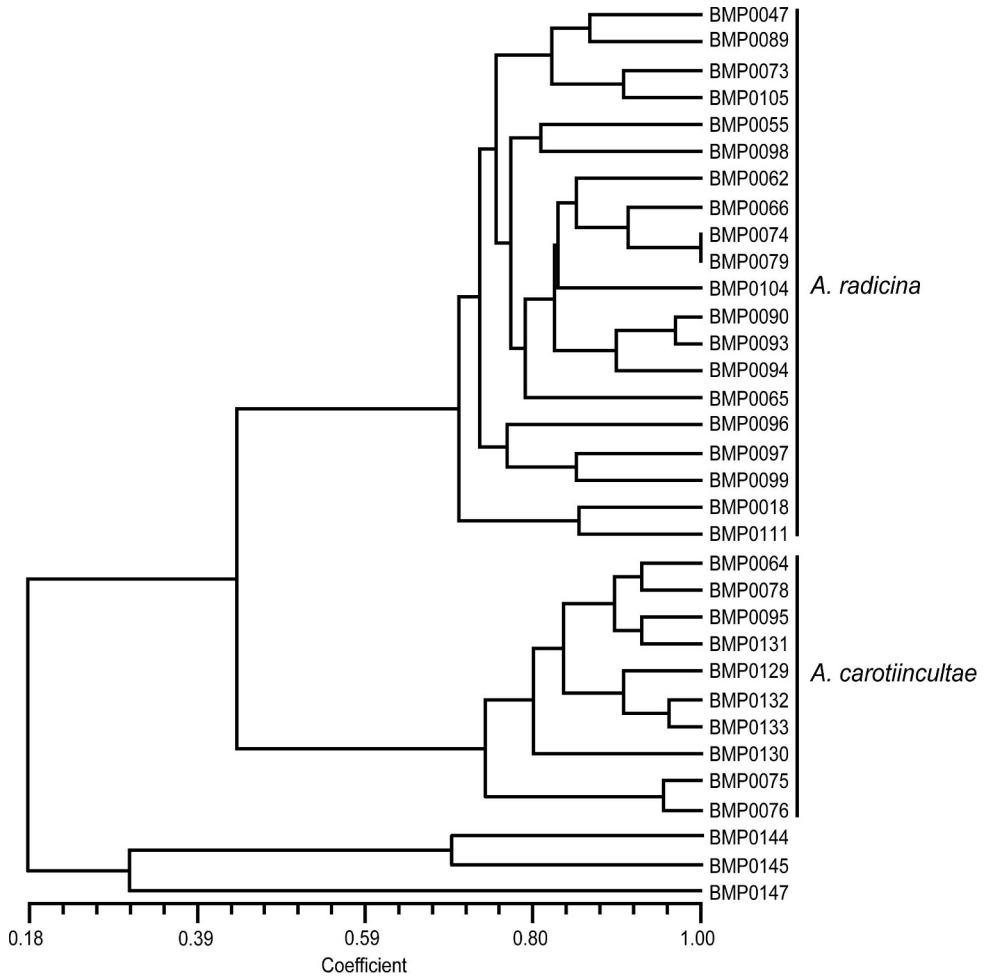

FIG. 7. UPGMA dendrogram from RAPD analysis of isolates of *A. radicina*, *A. carotiincultae*, *A. petroselini*, *A. selini* and *A. smyrnii* based on a combined dataset using four RAPD primers.

number could be used alone for differentiation between *A. radicina* and *A. carotiincultae*, the other characters could not due to the extent of their morphological variability. In most cases isolates of *A. radicina* grew slowly, produced yellow pigments and crystals on PDA and APDA and had irregular colony margins, whereas *carotiincultae* grew more rapidly, did not produce yellow pigments or crystals on PDA and APDA and had smooth colony margins. In addition conidia of *A. radicina* had significantly fewer transepta than those of *A. carotiincultae*. However isolates BMP 0097 and BMP 0099 had characteristics intermediate between *A. radicina* and *A. carotiincultae*. Thus species delimitation based on morphology was

TABLE IV. Morphological characters for *A. radicina* and *A. carotiincultae*

Species	Number of isolates	Radial growth[a]		No. transverse septa/conidium[b]
		PDA	APDA	
A. radicina	20	56.7a	44.4a	4.23a
A. carotiincultae	10	80.2b	76.9b	5.99b

[a] Mean colony diameter for each isolate was calculated from three replicate plates after 8 d incubation on potato dextrose agar (PDA) or acidified PDA (APDA) at 22 C. Values presented are the mean for each species (n = 20, n = 10). Values in each column followed by the different letter are significantly different based upon Mann-Whitney rank sum test, $P = 0.05$.

[b] Mean transepta per conidium for each isolate was calculated from 50 conidia randomly observed in each of four fields of view (200×) after 8 d incubation on V8 agar at 22 C. Values presented are the mean of each species (n = 20, n = 10). Values in each column followed by the different letter are significantly different based upon Mann-Whitney rank sum test, $P = 0.05$.

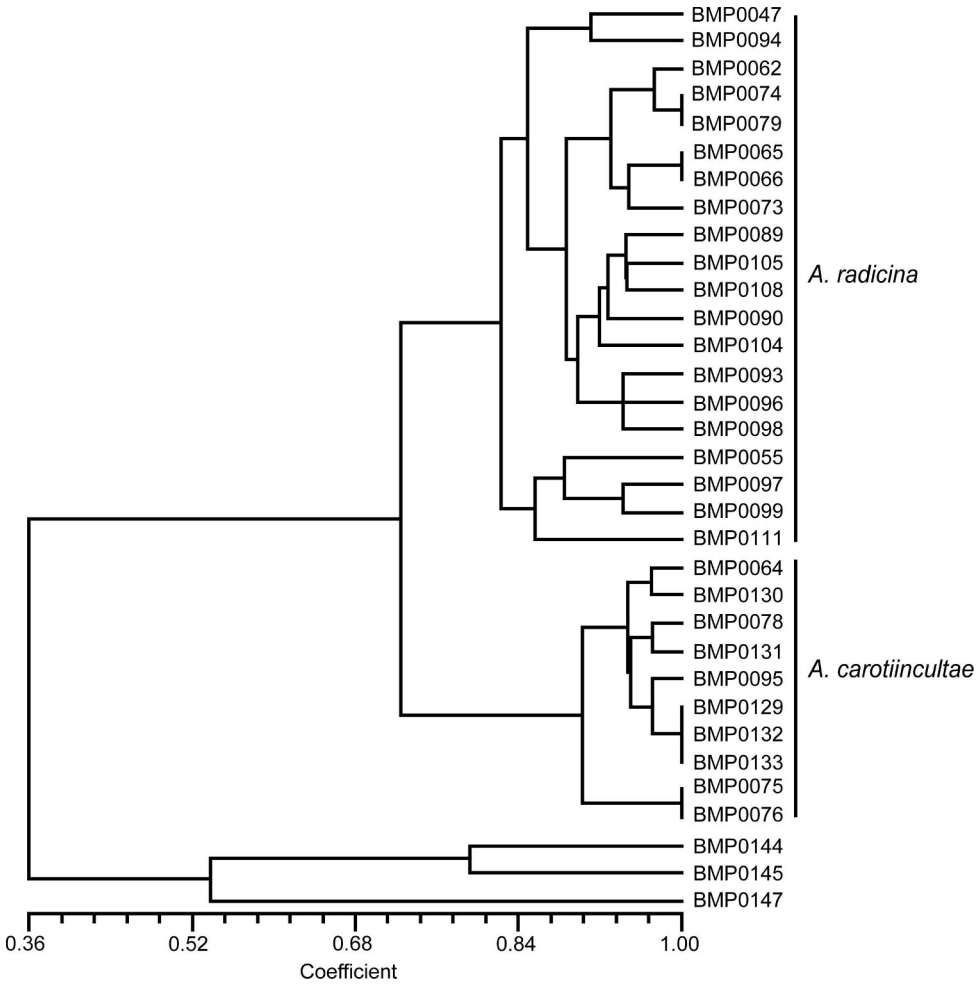

FIG. 8. UPGMA dendrogram from ISSR analysis of *A. radicina*, *A. carotiincultae*, *A. petroselini*, *A. selini* and *A. smyrnii* based on a combined dataset using three ISSR primer.

not absolute and misidentification might be possible using only these criteria.

Pryor and Gilbertson (2002) suggest that the morphological differences between *A. radicina* and *A. carotiincultae*, such as slow growth, irregular colony margin, production of pigments and crystals and reduce number of transepta, might be associated with the production of radicinin and are useful diagnostic characters. However this study revealed that some isolates of *A. radicina* are intermediate in these characters. For example isolates BMP 0097 and BMP 0099 displayed relatively slow growth and fewer transepta per conidium, which appeared to be taxonomically useful characteristics for taxon differentiation but lacked the ability to produce yellow pigments and crystals, which have been reported as hallmarks of radicinin production (Aldridge and Grove 1964, Pryor and Gilbertson 2002). Although the presence of radicinin was shown to affect certain morphological characteristics of *A. radicina* and *A. carotiincultae* (Pryor and Gilbertson 2002) this study suggested that the ability of isolates to produce radicinin might be variable or might not critically affect morphological characters. Further studies on the secondary metabolite profiles of all isolates may be necessary to fully answer this question.

TABLE V. Estimated values of Nei's (1978) unbiased genetic identity (above diagonal) and genetic distance (below diagonal) of *A. radicina*, *A. carotiincultae* and others (*A. petroselini*, *A. selini*, and *A. smyrnii*)

Species	*A. radician*	*A. carotiincultae*	Others
RAPD			
A. radicina	—	0.9688	0.5811
A. carotiincultae	0.0317	—	0.6212
Others	0.5429	0.4760	—
ISSR			
A. radicina	—	0.8661	0.5387
A. carotiincultae	0.1438	—	0.6065
Others	0.6186	0.5000	—

Morphological characteristics of fungi, and of *Alternaria* in particular, are subject to environmental influences and vary substantially from culture to culture (Simmons 1992, Pryor and Michailides 2002, Steaby 1996). Thus taxonomy, based solely on phenotype, may be subject to ambiguities caused by environmental conditions. Moreover identification of *Alternaria* is often problematic due to interspecific similarities and intraspecific polymorphism of morphological characters (Simmons 1990, Simmons and Roberts 1993, Roberts et al 2000, Pryor and Michailides 2002, Serdani et al 2002, Belisario et al 2004, Hong et al 2006). In this study *A. radicina* reveals considerable phenotypic variability among isolates and some characters of *A. radicina* were varied and overlapped with those of *A. carotiincultae*. In contrast *A. carotiincultae* has relatively uniform phenotypic characteristics.

In previous studies sequences of 18S, ITS, and mtSSU rDNA and of the protein-coding genes *gpd* and alt a1 were used to infer phylogenetic relationship among members of the radicina species-group of *Alternaria* (Pryor and Gilbertson 2000, Pryor and Michailides 2002, Pryor and Bigelow 2003, Hong et al 2005). However sequence analysis of rDNA (18S, ITS, and mtSSU) was not sufficient for delimitation between *A. radicina* and *A. carotiincultae*. The two species had identical 18S and mtSSU rDNA sequences and one base difference in the ITS sequence (1-nt deletion of *A. carotiincultae*). This study based on a much larger sample size also confirmed that the mtSSU rDNA sequences were identical but could not confirm the one base difference in the ITS sequence. However *A. radicina* and *A. carotiincultae* did possess one polymorphic ITS site but this was shared by isolates of both species.

Phylogenetic analysis of EF-1α, β-tubulin and the Alt a1 gene in this study did support recognition of *A. radicina* and *A. carotiincultae* as two distinct lineages, whereas analyses of other protein coding genes (ACT and CHS) did not provided characters for delimitation into distinct lineages. However for almost all of these genes polymorphisms still exist but are shared by the two species. Similarly sequence analysis of EF-1α, CAL, CHS and 1, 3, 8-trihydroxynaphthalene (THN) reducatase among small-spored catenulate *Alternaria* in the alternata species-group also revealed genetic regions insufficient for estimating phylogenies among closely related taxa (Peever et al 2004). In previous studies based on smaller sample sizes analyses of the *gpd* gene distinguished *A. radicina* from *A. carotiincultae* and also separated these taxa from other taxa in the radicina species-group (Pryor and Bigelow 2003, Hong et al 2005). However in this study based on a larger sample size analyses of the *gpd* gene revealed shared polymorphisms and did not strictly separate these species. This study revealed that *gpd* analysis separated isolates of *A. radicina* into two distinct clades, with most isolates of *A. radicina* and the representative strain (BMP0079) in one clade and *A. radicina* isolates BMP0096 and BMP0108, all isolates of *A. carotiincultae* and the neotype isolate of *A. radicina* (BMP0133) in a second distinct clade. However isolates BMP0096 and BMP0108 had slow growth and a fewer number of transepta, which are taxonomically useful characteristics for differentiating *A. radicina* from *A. carotiincultae*, as well as possessing irregular margins and pigment and crystals production, which are common to most other *A. radicina* isolates. In addition analysis of EF-1α, β-tubulin and alt a1 gene place these two isolates with other isolates of *A. radicina* and distinct from isolates of *A. carotiincultae*. Although phylogenetic analysis of combined dataset (EF-1α, β-tubulin, alt a1, and *gpd* gene) did support recognition of *A. radicina* and *A. carotiincultae* as two distinct lineages, the positions of isolates BMP0096 and BMP0108 of *A. radicina* were ambiguous due to shared polymorphisms in the *gpd* phylogeny. Other recent studies also have revealed incongruence between phylogeny based on single specific loci and actual species phylogeny (Buckley et al 2006, Crews and Hedin 2006, Ford et al 2006). Such incongruence could be the result of incomplete lineage sorting, gene duplication or horizontal transfer. In this study no signs of gene duplication and horizontal transfer were noted. However incomplete lineage sorting appears to be evident in the *gpd* locus as revealed in the sequences of BMP0096 and BMP0108, which clustered with isolates of *A. carotiincultae* in the *gpd* phylogeny.

The occurrence and systematic challenges associated with incomplete lineage sorting is more evident for recently divergent species (Rosenberg 2003, Maddison and Knowles 2006, Carstens and Knowles 2007). For example sequence analysis of the *gpd*, the triose phosphate isomerase (TPI), and the malate synthase (MS) genes among species of *Rosa* sect. *Cinnamomeae* in North America revealed low genetic distance between species and incomplete lineage sorting, particularly at the *gpd* locus, which supported a recent divergence among taxa (Joly and Bruneau 2006). All previous studies have revealed a relatively low genetic distance between *A. radicina* and *A. carotiincultae* as well, which suggests a close relationship and recent divergence between the two species. However fingerprint analysis of total DNA in this study and in previous work have always revealed that isolates of *A. radicina* are distinct from isolates of *A. carotiincultae* and formed a distinct monophyletic clade. Of note, this study based on a larger number of

isolates from diverse locations revealed higher genetic diversity among isolates of *A. radicina* than observed among isolates of *A. carotiincultae*. Moreover when polymorphisms exist more often those observed in *A. radicina* were shared in *A. carotiincultae* instead of the reverse, revealing both synapomorphic and autapomorphic characters in *A. carotiincultae* and suggesting a recently derived lineage.

The results of this study based on morphological and molecular characteristics confirmed the relationship of *A. radicina* and *A. carotiincultae* as sister taxa and distinct species. Some isolates of *A. radicina* have morphological and molecular polymorphism shared by *A. radicina* and *A. carotiincultae*, whereas isolates of *A. carotiincultae* generally were fixed for alleles that characterized *A. carotiincultae*. Our results revealed that the neotype of *A. radicina*, ATCC 6503 (BMP0133) and *A. carotiincultae* are conspecific as suggested by Pryor and Gilbertson (2002). Because the neotype of *A. radicina* was confirmed as *A. carotiincultae* this study concludes that in light of subsequent data the assignment of ATCC 6503 as the *A. radicina* neotype is no longer appropriate. Future studies that include *A. radicina* should use the designated representative strain, ATCC 96831, as an appropriate reference for the species.

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