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An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex

M. Andrew
T.L. Peever[1]
Department of Plant Pathology, Washington State University, Pullman, Washington 99164-6430

B.M. Pryor
Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721-0036

Abstract: Small-spored *Alternaria* species are a taxonomically challenging group of fungi with few morphological or molecular characters that allow unambiguous discrimination among taxa. The protein-coding genes most commonly employed in fungal systematics are invariant among these taxa, so noncoding, anonymous regions of the genome were developed to assess evolutionary relationships among these organisms. Nineteen sequence-characterized amplified regions (SCAR) were screened for phylogenetic utility by comparing sequences among reference isolates of small-spored *Alternaria* species. Five of nineteen loci were consistently amplifiable and had sufficient phylogenetic signal. Phylogenetic analyses were performed with 150 small-spored *Alternaria* isolates using sequence data from an endopolygalacturonase gene and two anonymous loci. Associations among phylogenetic lineage, morphological classification, geography and host were evaluated for use as practical taxonomic characters. Samples included isolates from citrus in Florida, pistachio in California, desert plants in Arizona, walnuts in France/Italy and apples in South Africa. No associations were found between host or geographic associations and phylogenetic lineage, indicating that these characters were not useful for cladistic classification of small-spored *Alternaria.* Similarly strict congruence between morphology and phylogenetic lineage was not found among isolates grouped morphologically with *A. alternata* or *A. tenuissima.* In contrast 34 isolates grouped morphologically with *A. arborescens* fell into discrete clades for all datasets. Although 5–9 well supported clades were evident among isolates, it is currently unclear if these clades should be considered phylogenetic species or emerging evolutionary lineages within the phylogenetically defined alternata species-group.

Key words: population-level systematics, small-spored *Alternaria* species, taxonomy

INTRODUCTION

Genus *Alternaria* is ubiquitous, with species found worldwide in association with a wide variety of substrates. Many species are saprophytes isolated from unusual substrates such as sewage or jet fuel (Rotem 1994), but most are known for their impact as animal and plant pathogens (de Hoog and Horre 2002, Hong and Pryor 2004). *Alternaria* have a wide host range as plant pathogens, ranking 10th in terms of total number of plant hosts (Farr et al 1989). As postharvest pathogens, *Alternaria* species contribute to extensive losses of our agricultural output due to spoilage (Wilson and Wisniewski 1994). In addition, they are one of the most common airborne allergens, as well as being one causative agent of phaeohyphomycosis in immunocompromised patients (de Hoog and Horre 2002).

As a genus, *Alternaria* encompasses considerable morphological diversity and there have been a number of attempts to organize taxa into subgeneric groupings based on shared morphological characters (Elliot 1917, Neergaard 1945, Joly 1964). Most recently Simmons proposed a series of 14 morphological groups to describe discrete clusters of morphospecies (Simmons 1992). Subsequent molecular studies have supported many of these groups as monophyletic lineages, termed ''species-groups'', an epitaph herein used to describe a phylogenetically based subgrouping within genus *Alternaria* (Pryor and Gilbertson 2000, Pryor and Bigelow 2003, Hong et al 2006). For example, molecular phylogenies have supported the division between the alternata and porri species-groups, morphologically distinguished by small and large conidial sizes, respectively. Conidia produced by fungi in the alternata species-group are 20–50 μm long, while conidia produced by the porri species-group are generally more than 100 μm long (Simmons 1995, 1999a, b). Within species-groups, there is considerable controversy as to which variants represent distinct species and which represent population variation within species. Because many *Alternaria* species are prominent pathogens of important crops a precise taxonomy is needed that allows

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[1] Corresponding author. E-mail: tpeever@wsu.edu

predictions of biological characteristics useful to the scientific community. Thus all stable molecular and phenotypic characters should be evaluated in developing a more predictive classification system.

Small-spored species within the alternata species-group are particularly challenging because few morphological characters are able to clearly differentiate taxa and these characters are strongly influenced by the environment. Peever et al (2004) has suggested that all citrus-associated small-spored species be given the species epithet *A. alternata* until further stable genetic or physiological data can be produced to differentiate them. Other scientists similarly consider the small-spored, plant pathogenic *Alternaria* species to be variants of *A. alternata* but differentiate them in terms of host specificity by labeling them ''pathotypes'' (Otani and Kohmoto 1992, Scheffer 1992, Isshiki et al 1997, Johnson et al 2001, Masunaka et al 2005). Other workers differentiate species within this group on the basis of metabolite profiling, colony morphology on standardized media and conidial chain branching patterns (Simmons 1967–2002, Simmons and Roberts 1993, Andersen and Thrane 1996, Andersen et al 2001, 2002, Andersen and Frisvad 2002, Roberts 2005). For example, *A. alternata* (Fr.:Fr) Keissler produces a mass of secondary and tertiary branched conidial chains on a short primary conidiophore whereas *A. tenuissima* (Nees & T. Ness:Fr.) Wiltshire produces conidial chains in reduced to nonbranching patterns (Simmons 1999b). *Alternaria arborescens* Simmons produces conidial chains that branch in a distinctive sympodial pattern atop a long primary conidiophore, giving the sporulation apparatus a characteristic ''arborescent'' appearance (Simmons 1995). Moreover numerous isolates, both saprobes and plant pathogens, share characteristics with these three representative morphospecies or have features that are intermediate among them. The morphological diversity observed within the alternata species-group creates a continuum of morphological features among taxa, making it difficult to definitively demarcate species.

To date broad morphological categories within *Alternaria* have been supported by phylogenetic analyses (Pryor and Gilbertson 2000, Peever et al 2004). Phylogenetic studies have demonstrated a clear distinction between large and small-spored *Alternaria* species (Pryor and Bigelow 2003, Peever et al 2004), however it is among the small-spored taxa within the alternata species-group that there is still much debate. Previous molecular phylogenies of this group have revealed little to no variation in the genetic loci commonly employed in fungal systematics. Sequence data of the nuclear ribosomal internal transcribed spacer (ITS) and the mitochondrial small subunit (mtSSU) provided no resolution among these taxa (Pryor and Gilbertson 2000, Chou and Wu 2002, Kang et al 2002, Pryor and Bigelow 2003). The mitochondrial large subunit (mtLSU) ribosomal DNA, beta-tubulin, actin, calmodulin, chitin synthase, translation elongation factor alpha and 1,3,8-trihydroxynaphthalene reductase also revealed no differentiation among members of this group (Peever et al 2004).

To date, only an endopolygalacturonase (*endoPG*) gene, and two anonymous loci have proven sufficiently variable to differentiate members of the alternata species-group (Peever et al 2004). The resultant molecular phylogeny was compared to the morphological classification of Simmons (1999a), in which 10 new morphospecies were described from citrus hosts. At this level of resolution there was general agreement, but not strict congruence, between morphological classification and the phylogeny. Many morphospecies were paraphyletic and *A. citrimacularis* was polyphyletic (Peever et al 2004), indicating that there are more morphospecies than can be supported within a phylogenetic framework. The present study expands this research by adding an additional highly variable locus to increase resolution within this group, and by evaluating morphology under standardized environmental conditions to address whether the lack of association between morphological and molecular research could a result of the lack of variation in the previously used loci.

The major goal of the current research was to determine the relationships among morphology, phylogeny, host association and geographic origin of a diverse sample of fungi representing the alternata species-group and to establish which characters reliably can predict phylogenetic lineage. Limited sampling introduces bias into phylogenetic analyses, so we used the largest sample of *Alternaria* isolates employed in a phylogenetic analysis to date to better estimate population-level variation. The specific objectives of this research were: (i) to infer a phylogeny of the small-spored *Alternaria* species (primarily those morphologically grouped with *A. alternata*, *A. tenuissima* or *A. arborescens*) from sequence data from an endopolygalacturonase gene and two anonymous regions of the genome; (ii) to compare phylogenetic lineages defined above within the small-spored *Alternaria* species complex to prior and ongoing morphological classification of these taxa; and (iii) to identify associations among phylogenetic lineage, morphology and geography/host associations that are useful as taxonomic characters. These objectives were addressed through extensive phylogenetic and morphological analyses of small-spored *Alternaria* isolates from distinct host/geographic associations.

MATERIALS AND METHODS

Isolate sampling.—Isolates of small-spored, catenulate *Alternaria* species were sampled from five distinct hosts and geographic locations worldwide (TABLE I). Isolates were selected primarily from the alternata species-group, although we also included some members of the infectoria species-group causing core-rot of apples (TABLE I) as outgroup taxa (Pryor and Michailides 2002, Pryor and Bigelow 2003, Hong et al 2006). Most of the isolates were employed in population genetic and systematic studies of *Alternaria* spp. from citrus in Florida (Peever et al 1999, 2004, 2005), walnut and hazelnut in Italy and France (Belisario et al 2004, Hong et al 2006), pistachio in California (Pryor and Michailides 2002) and apple in South Africa (Kang et al 2002, Serdani et al 2002) and had been characterized morphologically. Also included in this study were morphologically and genetically uncharacterized small-spored isolates obtained from soil and leaf litter from desert environments in southern Arizona. Our sampling strategy represented a wide variety of hosts, a diverse range of associations with plants, and sampling was performed worldwide. Many of the citrus pathogens cause brown spot and produce host-specific toxins; the apple pathogens cause core rot, a postharvest disease, and the isolates associated with the Arizonian desert plants were presumed saprophytes. Citrus isolates were selected based on previous population genetic and phylogenetic studies (Peever et al 1999, 2000, 2002, 2004, 2005) and were sampled from a variety of hosts including white and red grapefruit, rough lemon, sweet orange, tangerines and tangelos. In addition several citrus-associated morphospecies were included that were classified by E.G. Simmons (1999a) to allow comparison with Peever et al (2002, 2004, 2005). All isolates employed in this study were initiated from single conidia and stored at −20 C on sterile filter papers (Peever et al 1999). Sequences have been deposited in GenBank under accession numbers EF503727–EF504220.

Morphological classification.—Isolates were grown on standard potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan) and on 5% PDA with standardized light, humidity and temperature conditions (Simmons and Roberts 1993, Pryor and Michailides 2002). Isolates were placed into one of four previously defined morphological groups of small-spored taxa, the *A. alternata* group, the *A. tenuissima* group, the *A. arborescens* group or the *A. infectoria* group, by comparing colony morphology and sporulation branching patters to ex-type (or representative) cultures of defined morphospecies (Pryor and Michailides 2002).

Fungal cultures and DNA extraction.—Isolates were grown in 2-YEG medium (10 g dextrose and 2 g yeast extract per liter) on an orbital shaker at 200 rpm for 4–6 d. Cultures were vacuum filtered, frozen at −20 C, lyophilized and ground to a fine powder using a metal rod. Lyophilized mycelium was used for genomic DNA extraction as described by Peever et al (1999) with the following modifications. Phenol/chloroform extraction was performed with a 1:1 ratio, followed by a second chloroform extraction. Quantity of DNA was estimated on 0.8% ethidium bromide-stained agarose gels, with known quantities of phage lambda DNA as standards. DNA extractions were diluted to 10–20 ng/μL in elution buffer (10 mM Tris-HCl, pH 8.0) for use in PCR.

Screening of sequence characterized amplified regions (SCAR) markers.—Nineteen primer sets amplifying anonymous regions of the genome (Peever et al 2004) were screened for variation and suitability for phylogenetic analyses by comparing sequence variation among four representative isolates from clades 1, 2, 4 and 7 (FIG. 4) in Peever et al (2004). When greater than 2% variation was evident among the screening isolates, these loci were chosen for further study by examining additional isolates from each of the clades and comparing relative sequence diversity among the larger sample of isolates. Five of 19 loci were chosen for further study, and of these one did not consistently amplify from all *Alternaria* isolates and consequently was removed from the test group. Sequences of regions OPA10-2, OPA19-650, AA-SCAR-11, AA-SCAR-9, OPB 15-2 were translated in six frames and BLAST X analysis performed to determine sequence similarity to known proteins. The most variable locus, OPA10-2, was selected for the present study and used in conjunction with the previously employed *endoPG* and OPA1-3 (Peever et al 2004, 2005).

PCR and sequencing.—Polymerase chain reactions were performed with a combination PCR/loading buffer containing 2.0 μM $MgCl_2$, BSA, Ficoll and tartrazine (Buffer 1779, Idaho Technology, Salt Lake City, Utah); 10 μM deoxyribonucleotide triphosphates (New England BioLabs, Ipswich, Massachusetts); 0.2 μM of each primer; 1 unit of Taq polymerase (New England BioLabs); 10–20 ng of DNA template to a total volume of 25 μL per tube. *EndoPG* and OPA1-3 were amplified and sequenced as described in Peever et al (2004, 2005) with modifications. A 485 bp segment of an endopolygalacturonase (*endoPG*) gene, first characterized by Isshiki et al (1997, 2001), was amplified with primers PG3 (5′-TACCATGGTTCTTTCCGA-3′) and PG2b (5′-GAGAATTCRCARTCRTCYTGRTT-3′). An approximately 800 bp segment of the anonymous noncoding region, OPA10-2, was amplified with primers OPA 10-2R (5′-GATTCGCAGCAGGGAAACTA-3′) and OPA 10-2L (5′-TCGCAGTAAGACACA TTCTACG-3′). Cycling conditions consisted of 1 min initial denaturation at 95 C, followed by 35 cycles of 95 C melt for 30 s, 62 C annealing for 30 s, 72 C extension for 30 s and a final 7 min elongation cycle at 72 C.

Amplicons were purified through QIAquick Columns (QIAGEN, Valencia, California), or with ExoSAP IT (USB Corp., Cleveland, Ohio), following the manufacturer's directions. DNA concentrations were estimated visually in 1.5% ethidium-bromide stained agarose gels, by comparing band intensity to known quantities of lambda DNA. Cycle sequencing reactions were carried out with a standard mixture that contained 20 ng DNA template, 480 nM primer, 4 μL of Big Dye Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City,

TABLE I. Geographical/host association and morphological classification of *Alternaria* species used in this study

Isolate code	Alternate code[a]	Location	Host	Haplotype			Morphospecies group[b]
				endoPG	OPA1-3	OPA10-2	
APP1	CR1	South Africa	*Malus domestica*	8	1	6	*A. arborescens* (*A. arborescens*)
APP2	CR2	South Africa	*Malus domestica*	8	1	6	*A. arborescens* (*A. arborescens*)
APP3	CR3	South Africa	*Malus domestica*	7	3	7	*A. arborescens* (*A. arborescens*)
APP4	CR4	South Africa	*Malus domestica*	7	3	6	*A. arborescens* (*A. arborescens*)
APP5	CR5	South Africa	*Malus domestica*	6	3	6	*A. arborescens* (*A. arborescens*)
APP6	CR6	South Africa	*Malus domestica*	7	25	9	*A. arborescens* (*A. arborescens*)
APP7	CR7	South Africa	*Malus domestica*	18	15	12	nc (*A. tenuissima*)
APP8	CR8	South Africa	*Malus domestica*	7	3	14	*A. arborescens* (*A. arborescens*)
APP9	CR9	South Africa	*Malus domestica*	2	—	—	nc (*A. infectoria*)
APP10	CR10	South Africa	*Malus domestica*	7	3	6	*A. arborescens* (*A. arborescens*)
APP11	CR11	South Africa	*Malus domestica*	7	3	7	*A. arborescens* (*A. arborescens*)
APP13	CR13	South Africa	*Malus domestica*	16	15	12	nc (*A. tenuissima*)
APP14	CR14	South Africa	*Malus domestica*	7	3	7	*A. arborescens* (*A. arborescens*)
APP16	CR16	South Africa	*Malus domestica*	16	21	12	nc (*A. tenuissima*)
APP17	CR17	South Africa	*Malus domestica*	19	22	27	*A. tenuissima* (*A. tenuissima*)
APP18	CR18	South Africa	*Malus domestica*	23	8	28	*A. alternata* (*A. arborescens*)
APP19	CR19	South Africa	*Malus domestica*	19	13	12	*A. tenuissima* (*A. tenuissima*)
APP20	CR20	South Africa	*Malus domestica*	16	20	3	*A. tenuissima* (*A. tenuissima*)
APP21	CR21	South Africa	*Malus domestica*	12	7	28	Aa/At intermediate (*A. tenuissima*)
APP22	CR22	South Africa	*Malus domestica*	16	7	27	Aa/At intermediate (*A. tenuissima*)
APP23	CR23	South Africa	*Malus domestica*	7	4	6	Aa/Aarb intermediate (*A. arborescens*)
APP24	CR24	South Africa	*Malus domestica*	12	7	28	*A. tenuissima* (*A. tenuissima*)
APP25	CR25	South Africa	*Malus domestica*	16	20	12	Aa/At intermediate (*A. tenuissima*)
APP26	CR26	South Africa	*Malus domestica*	19	20	12	Aa/At intermediate (*A. tenuissima*)
APP27	CR27	South Africa	*Malus domestica*	16	—	3	*A. tenuissima* (*A. tenuissima*)
APP29	CR29	South Africa	*Malus domestica*	2	—	—	*A. infectoria* (*A. infectoria*)
APP30	CR30	South Africa	*Malus domestica*	2	—	—	*A. infectoria* (*A. infectoria*)
APP31	CR31 (EGS-27-193)	—	*Triticum* spp.	3	—	—	*A. infectoria* (*A. infectoria*)
APP32	CR32 (EGS 34-016)	India	*Arachis hypogaea*	1	14	12	*A. alternata* (*A. alternata*)
DES02002	BMP1068	Arizona	Desert soil/mixed leaf litter	12	7	1	*A. tenuissima*
DES02003	BMP1069	Arizona	Desert soil/mixed leaf litter	12	22	1	*A. tenuissima*
DES02004	BMP1070	Arizona	Desert soil/mixed leaf litter	12	7	1	*A. tenuissima*
DES02005	BMP1071	Arizona	Desert soil/mixed leaf litter	12	7	1	*A. tenuissima*
DES009	BMP1079	Arizona	Desert soil/mixed leaf litter	5	2	—	*A. tenuissima*
DES030	BMP1098	Arizona	Desert soil/mixed leaf litter	12	13	5	*A. tenuissima*
DES031	BMP1099	Arizona	Desert soil/mixed leaf litter	24	15	27	Aa/At intermediate
DES032	BMP1100	Arizona	Desert soil/mixed leaf litter	12	7	1	Aa/At intermediate
DES501	BMP1756	Arizona	Desert soil/mixed leaf litter	16	18	12	*A. alternata*

TABLE I. Continued

Isolate code	Alternate code[a]	Location	Host	Haplotype			Morphospecies group[b]
				endoPG	OPA1-3	OPA10-2	
DES502	BMP1757	Arizona	Desert soil/mixed leaf litter	16	14	—	*A. tenuissima*
DES503	BMP1758	Arizona	Desert soil/mixed leaf litter	16	7	28	Aa/At intermediate
DES504	BMP1759	Arizona	Desert soil/mixed leaf litter	12	14	12	*A. alternata*
DES506	BMP1761	Arizona	Desert soil/mixed leaf litter	12	7	1	*A. alternata*
DES509	BMP1764	Arizona	Desert soil/mixed leaf litter	12	7	5	*A. alternata*
DES510	BMP1765	Arizona	Desert soil/mixed leaf litter	15	13	1	Aa/At intermediate
DES512	BMP1767	Arizona	Desert soil/mixed leaf litter	12	7	24	*A. tenuissima*
DES513	BMP1768	Arizona	Desert soil/mixed leaf litter	16	14	24	Aa/At intermediate
DES515	BMP1770	Arizona	Desert soil/mixed leaf litter	12	13	1	*A. tenuissima*
DES516	BMP1771	Arizona	Desert soil/mixed leaf litter	12	7	5	*A. tenuissima*
DES518	BMP1773	Arizona	Desert soil/mixed leaf litter	1	14	12	Aa/At intermediate
DES523	BMP1778	Arizona	Desert soil/mixed leaf litter	12	11	5	Aa/At intermediate
DES525	BMP1780	Arizona	Desert soil/mixed leaf litter	—	14	1	Aa/At intermediate
DES528	BMP1783	Arizona	Desert soil/mixed leaf litter	—	7	—	Aa/At intermediate
DES531	BMP1786	Arizona	Desert soil/mixed leaf litter	—	13	1	Aa/At intermediate
DES532	BMP1787	Arizona	Desert soil/mixed leaf litter	—	13	21	*A. tenuissima*
DES535	BMP1790	Arizona	Desert soil/mixed leaf litter	—	7	24	*A. tenuissima*
I1	BMP0910	Italy	*Juglans regia*	7	5	13	*A. arborescens*
I3	BMP0908	Italy	*Juglans regia*	—	7	12	*A. alternata*
I4	BMP0923	Italy	*Juglans regia*	16	7	12	*A. tenuissima*
I6	BMP0906	Italy	*Juglans regia*	7	1	9	*A. arborescens*
I7	BMP0905	Italy	*Juglans regia*	19	—	28	*A. tenuissima*
I8	BMP0903	Italy	*Juglans regia*	7	3	9	*A. arborescens*
I0902	BMP0902	Italy	*Juglans regia*	9	1	14	*A. arborescens*
I00	BMP0901	Italy	*Juglans regia*	6	3	15	*A. arborescens*
I11	BMP0943	Italy	*Juglans regia*	6	9	9	*A. arborescens*
I0900	BMP0900	France	*Juglans regia*	10	7	28	*A. alternata*
I13	BMP0899	France	*Juglans regia*	8	9	9	*A. arborescens*
I14	BMP0922	Italy	*Juglans regia*	16	7	28	*A. alternata*
I0942	BMP0942	France	*Juglans regia*	—	20	3	*A. alternata*
I0921	BMP0921	France	*Juglans regia*	16	7	28	*A. tenuissima*
I17	BMP0920	France	*Juglans regia*	7	3	6	*A. arborescens*
I18	BMP0919	Italy	*Corylus avellana*	16	20	24	*A. tenuissima*
I20	BMP0916	Italy	*Corylus avellana*	16	—	12	*A. tenuissima*
I22	BMP0914	Italy	*Corylus avellana*	16	—	12	*A. tenuissima*
I23	BMP0913	Italy	*Corylus avellana*	7	3	9	*A. arborescens*
I0912	BMP0912	Italy	*Corylus avellana*	11	7	28	*A. tenuissima*
I25	BMP0911	Italy	*Corylus avellana*	7	3	14	*A. arborescens*

TABLE I. Continued

Isolate code	Alternate code[a]	Location	Host	Haplotype			Morphospecies group[b]
				endoPG	OPA1-3	OPA10-2	
I26	BMP0934	Italy	*Corylus avellana*	6	3	9	*A. arborescens*
I27	BMP0933	Italy	*Corylus avellana*	7	9	15	*A. arborescens*
I28	BMP0932	Italy	*Corylus avellana*	12	7	24	*A. alternata*
I29	BMP0944	Italy	*Corylus avellana*	7	3	23	*A. arborescens*
PIST0453	BMP0453	California	*Pistacia vera*	16	—	19	*A. alternata*
PIST0454	BMP0454	California	*Pistacia vera*	16	14	—	*A. alternata*
PIST0462	BMP0462	California	*Pistacia vera*	6	3	9	*A. arborescens*
PIST0463	BMP0463	California	*Pistacia vera*	17	14	12	*A. tenuissima*
PIST0466	BMP0466	California	*Pistacia vera*	16	20	29	*A. alternata*
PIST0472	BMP0472	California	*Pistacia vera*	16	7	9	*A. alternata*
PIST0474	BMP0474	California	*Pistacia vera*	16	14	12	*A. alternata*
PIST0480	BMP0480	California	*Pistacia vera*	17	13	—	*A. tenuissima*
PIST0497	BMP0497	California	*Pistacia vera*	16	14	11	*A. alternata*
PIST0508	BMP0508	California	*Pistacia vera*	16	14	12	*A. tenuissima*
PIST0510	BMP0510	California	*Pistacia vera*	6	1	6	*A. arborescens*
PIST0517	BMP0517	California	*Pistacia vera*	14	20	3	*A. tenuissima*
PIST0523	BMP0523	California	*Pistacia vera*	6	9	6	*A. arborescens*
PIST0549	BMP0549	California	*Pistacia vera*	16	7	12	*A. alternata*
PIST0561	BMP0561	California	*Pistacia vera*	16	14	—	*A. alternata*
PIST0582	BMP0582	California	*Pistacia vera*	6	3	16	*A. arborescens*
PIST0583	BMP0583	California	*Pistacia vera*	16	26	12	*A. tenuissima*
PIST0591	BMP0591	California	*Pistacia vera*	16	13	3	*A. alternata*
PIST0592	BMP0592	California	*Pistacia vera*	7	3	9	*A. arborescens*
PIST0596	BMP0596	California	*Pistacia vera*	7	1	22	*A. arborescens*
PIST0599	BMP0599	California	*Pistacia vera*	16	—	12	*A. alternata*
PIST0600	BMP0600	California	*Pistacia vera*	6	1	8	*A. arborescens*
PIST0602	BMP0602	California	*Pistacia vera*	6	3	9	*A. arborescens*
PIST0610	BMP0610	California	*Pistacia vera*	16	7	10	*A. tenuissima*
PIST0612	BMP0612	California	*Pistacia vera*	16	7	12	*A. alternata*
PIST0627	BMP0627	California	*Pistacia vera*	16	—	6	*A. alternata*
PIST0630	BMP0630	California	*Pistacia vera*	6	3	8	*A. arborescens*
PIST0635	BMP0635	California	*Pistacia vera*	7	25	9	*A. arborescens*
PIST0638	BMP0638	California	*Pistacia vera*	6	3	8	*A. arborescens*
PIST0651	BMP0651	California	*Pistacia vera*	6	9	9	*A. arborescens*
PIST0653	BMP0653	California	*Pistacia vera*	16	14	—	*A. alternata*
PIST0660	BMP0660	California	*Pistacia vera*	14	—	4	*A. tenuissima*
EV17	EV-17	Florida	*Citrus reticulata* x *C. paradisi*	12	7	28	Aa/At intermediate
EV18	EV-18	Florida	*Citrus reticulata* x *C. paradisi*	20	6	25	*A. alternata*
EV19	EV-19	Florida	*Citrus reticulata* x *C. paradisi*	12	7	28	nc
EV21	EV-21	Florida	*Citrus reticulata* x *C. paradisi*	12	7	28	Aa/At intermediate
EV26	EV-26	Florida	*Citrus reticulata* x *C. paradisi*	12	19	28	nc
EV33	EV-33	Florida	*Citrus reticulata* x *C. paradisi*	12	7	28	*A. alternata*
WGF1	VB-WGF-1	Florida	*Citrus paradisi*	20	14	25	Aa/At intermediate
WGF6	VB-WGF-6	Florida	*Citrus paradisi*	12	23	2	Aa/At intermediate
WGF8	VB-WGF-8	Florida	*Citrus paradisi*	12	7	27	*A. alternata*
WGF9	VB-WGF-9	Florida	*Citrus paradisi*	20	23	25	Aa/At intermediate
WGF11	VB-WGF-11	Florida	*Citrus paradisi*	20	6	25	Aa/At intermediate
WGF16	VB-WGF-16	Florida	*Citrus paradisi*	12	—	27	*A. alternata*
WGF22	VB-WGF-22	Florida	*Citrus paradisi*	16	20	12	*A. tenuissima*

TABLE I. Continued

Isolate code	Alternate code[a]	Location	Host	Haplotype			Morphospecies group[b]
				endoPG	OPA1-3	OPA10-2	
RGF5	VB-RGF-5	Florida	*Citrus paradisi*	20	23	25	Aa/At intermediate
RGF8	VB-RGF-8	Florida	*Citrus paradisi*	12	7	27	*A. alternata*
RGF10	VB-RGF-10	Florida	*Citrus paradisi*	12	16	27	nc
RGF15	VB-RGF-15	Florida	*Citrus paradisi*	25	—	27	Aa/At intermediate
RGF18	VB-RGF-18	Florida	*Citrus paradisi*	24	10	2	*A. tenuissima*
alternata1	EGS 34-016	India	*Arachis hypogaea*	1	14	12	*A. alternata* [Rep]
alternata2	EGS 34-039	India	*Datura metel*	1	14	20	*A. alternata* [Rep]
alternata3	EGS 39-192	California	*Citrus paradisi*	1	14	18	nd
arborescens	EGS 39-128	California	*Lycopersicon esculentum*	8	1	14	*A. arborescens* [Ex]
citri1	A.citri-1	California	*Citrus sinensis*	6	1	8	nd
citri4	EGS 39-190	Florida	*Citrus sinensis*	12	17	—	nd
citri6	UC-7s	California	*Citrus limon*	16	14	—	nd
citriarbusti1	SH-MIL-8	Florida	*Citrus reticulata x C. paradisi*	—	7	27	*A. citriarbusti* [Auth]
citriarbusti2	SH-MIL-15	Florida	*Citrus reticulata x C. paradisi*	24	7	27	*A. citriarbusti* [Ex]
citrimac1	BC2-RLR-17s	Florida	*Citrus jambhiri*	—	7	27	*A. citrimacularis* [Auth]
citrimac2	BC2-RLR-32s	Florida	*Citrus jambhiri*	—	7	28	*A. citrimacularis* [Ex]
colombiana	EGS 45-017	Colombia	*Citrus reticulata x C. paradisi*	—	—	25	*A. columbiana* [Ex]
gaisen	EGS 90-0512	Japan	*Pyrus serotina*	21	8	26	*A. gaisen* [Rep]
gaisen15A	EGS 37-1321	Japan	*Pyrus serotina*		24	12	*A. gaisen* [Rep]
limonias1	BC2-RLR-1s	Florida	*Citrus jambhiri*	16	13	17	*A. limoniasperae* [Auth]
limonias2	PR325	Florida	*Citrus jambhiri*	16	13	17	*A. limoniasperae* [Auth]
longipes	EGS 30-033	North Carolina	*Nicotiana tabacum*	20	6	—	*A. longipes*
perangusta	EGS 44-160	Turkey	*Citrus reticulata x C. paradisi*	13	20	12	*A. perangusta* [Ex]
tangelon1	EV-MIL-2s	Florida	*Citrus reticulata x C. paradisi*	20	6	25	*A. tangelonis* [Ex]
tangelon2	SH-MIL-4	Florida	*Citrus reticulata x C. paradisi*	22	23	25	*A. tangelonis* [Auth]
tenuissima	EGS 34-015	United Kingdom	*Dianthus caryophyllus*	16	13	—	*A. tenuissima* [Rep]
toxicogenica	PR320	Florida	*Citrus reticulata*	12	12	1	*A. toxicogenica* [Ex]
turkisafria	EGS 44-159	Turkey	*Citrus reticulata x C. paradisi*	4	20	12	*A. turkisafria* [Auth]

[a] As defined in previous studies (apple, Serdani *et al.* 2002; walnut/hazelnut, Belisario et al 2004; citrus, Peever et al 1999; pistachio and desert, B.M. Pryor. All other isolates are representative morphospecies designated by E.G. Simmons and referred to in Peever et al 2004, 2005).

[b] Morphologically identified by B.M. Pryor (morphological designations in parentheses from Serdani et al 2002); nc = not classifiable into a discrete morphological group; Aa/At intermediate = an isolate possessing an intermediate phenotype between the *A. alternata* and *A. tenuissima* groups; Aa/Aarb intermediate = an isolate possessing an intermediate phenotype between the *A. alternata* and *A. arborescens* groups; nd = not determined; [Ex] denotes ex-type isolates described by E.G. Simmons; [Auth] denotes authentic isolates described by E.G. Simmons; [Rep] denotes representative isolates described by E.G. Simmons.

California) and sterile distilled water in a total volume of 15 μL. All sequencing reactions were carried out in a BioRad MyCycler thermal cycler (BioRad, Hercules, California) with 35 cycles of 96 C for 15 s, 50 C for 15 s and 60 C for 4 min. Sequencing products were purified through gel filtration cartridges (Edge BioSystems, Gaithersburg, Maryland), following the manufacturer's directions and dried at 60 C in a vacufuge. Sequence reads were performed on an Applied Biosystems Model 373A Automated DNA Sequencing System in the Laboratory for Biotechnology

and Bioanalysis, School of Molecular Biosciences, Washington State University.

DNA sequence alignment and phylogenetic analyses.—Sequences were aligned in Clustal X v. 1.81 (Thompson et al 1997) and further edited manually. Maximum parsimony and maximum likelihood analyses were performed in PAUP* 4.0b10 (Swofford 2003) with heuristic searches. Characters were treated as unordered and gaps as missing data. Bootstrap support for internal branches was estimated from 1000 pseudoreplicates for both parsimony and maximum likelihood analyses. Models of sequence evolution were estimated for each locus with ModelTest 3.7 (Posada and Crandall 1998, Posada and Buckley 2004). When the hierarchical likelihood ratio tests (hLRT) and Akaike Information Criterion (AIC) selected different evolutionary models, the more complex model was chosen. An equal-frequency Tamura-Nei model with gamma-distributed sites was selected for *endoPG* (base frequencies = equal; rate matrix = [1.0000, 2.8595, 1.0000, 1.0000, 10.4236]; shape parameter for gamma distribution = 0.3502; proportion of invariant sites = 0). A transversion model with gamma-distributed sites was selected for OPA1-3 (base frequencies = [0.2906 0.2491 0.1906 0.2697]; rate matrix = [2.0014, 9.4219, 1.7741, 4.1739, 9.4219]; shape parameter for gamma distribution = 0.4880; proportion of invariant sites = 0). An equal-frequency transversion model with gamma distribution with invariant sites was selected for OPA10-2 (base frequencies = equal; rate matrix = [0.3381, 2.9409, 0.2995, 0.3749, 2.9409]; shape parameter for gamma distribution = 0.8114; proportion of invariant sites = 0.6320).

Bayesian analyses were performed independently for each genetic locus in MrBayes v.3.0b4 (Huelsenbeck and Ronquist 2001) to estimate the posterior probabilities of tree topologies with Metropolis-coupled Markov chain Monte Carlo (MCMCMC) searches. All analyses employed one cold chain and three incrementally heated chains, where the heat of the *i*th chain is $b = 1/[1 + (I - 1)T]$ and $t = 0.2$; when $I = 1$, $B = 1$, corresponding to the cold chain. Independent analyses of each of the three loci were conducted with 3 000 000 generations each, with a sampling frequency of one tree every 100 generations. The average standard deviation of split frequencies stabilized (to a difference of less than 1%) after 10 000 generations in all analyses. Therefore the initial 10 000 generations from each run were discarded as burn-in when summarizing tree parameters and topology. Flat Dirichlet probability densities were used as priors for the substitution rate parameters and stationary nucleotide frequencies and uniform priors were used for the shape and topology parameters and an exponential unconstrained prior was used for the branch lengths parameter. Maximum likelihood bootstrap values greater than 70% combined with posterior probabilities greater than 0.95 were used to infer clades.

Tests for neutrality and combinability among datasets.—Haplotype diversity was estimated for each locus with DnaSP version 4.10 (Rozas et al 2003). Tajima's *D* (Tajima 1989), Fu's and Li's *D** and *F** statistics (Fu and Li 1993) were estimated with DnaSP (Rozas et al 2003). Tajima's *D*, and Fu's and Li's *D** and *F** statistics measure departure from the null hypothesis of neutral evolution where significant values may indicate change in population sizes and/or purifying or balancing selection (Tajima 1989, Fu and Li 1993). Thirty-nine isolates could not be amplified across all loci (TABLE I) despite PCR optimization, possibly due to point mutations in priming sites. Individual tree topologies were compared with the nonparametric Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) implemented in PAUP* 4.0b10. Distributions of differences in log-likelihood values were generated with 1000 bootstrapped replicates generated using RELL sampling. To ensure that only well supported nodes were being compared, individual tree topologies were compared with a cutoff criterion of 70% bootstrap support for all nodes. To further explore the potential causes of topological incongruence among loci site compatibility matrices were estimated for each aligned dataset with the clade and matrix methods implemented in the SNAP workbench (Price and Carbone 2004). Phylogenetically incompatible sites were manually pruned from the datasets and topological incongruence tests were repeated with each pruned dataset as described above to determine the effect of phylogenetically incompatible sites on incongruence among and combinability of datasets.

Hypothesis testing.—The hypothesis that isolates assigned morphologically to the *A. arborescens* group were monophyletic was tested with nonparametric Shimodaira-Hasegawa tests (Shimodaira and Hasegawa 1999) performed in PAUP* 4.0b10. Topologies estimated from each locus were compared to a constraint tree that enforced the monophyly of isolates assigned morphologically to the *A. arborescens* group. Constrained and unconstrained ML trees with the highest log-likelihoods were compared in PAUP* 4.0b10 with 1000 bootstrap replicates generated using RELL sampling.

RESULTS

Phylogenetic analyses.—Consensus tree topologies for the most parsimonious trees for each locus were similar to those estimated with maximum likelihood and Bayesian methods. Bayesian phylogenies are shown (FIGS. 1–3) with clades inferred from joint ML bootstrap and posterior probabilities as described above. Amplification of the partial endopolygalacturonase gene (*endoPG*) yielded amplicons that varied in length 484–1104 bp. This size discrepancy was due primarily to isolates classified morphologically in the *A. infectoria* group that shared a 61 bp indel, as well as 500 bp of additional flanking DNA compared to other taxa. Isolates classified as *A. infectoria* were used to root the *endoPG* phylogeny. Aligned sequence length was 521 characters, of which 70 were parsimony-informative. Three equally parsimonious trees were recovered and tree scores were TL = 151; CI = 0.757; RI = 0.745; RC = 0.564; HI = 0.243. The

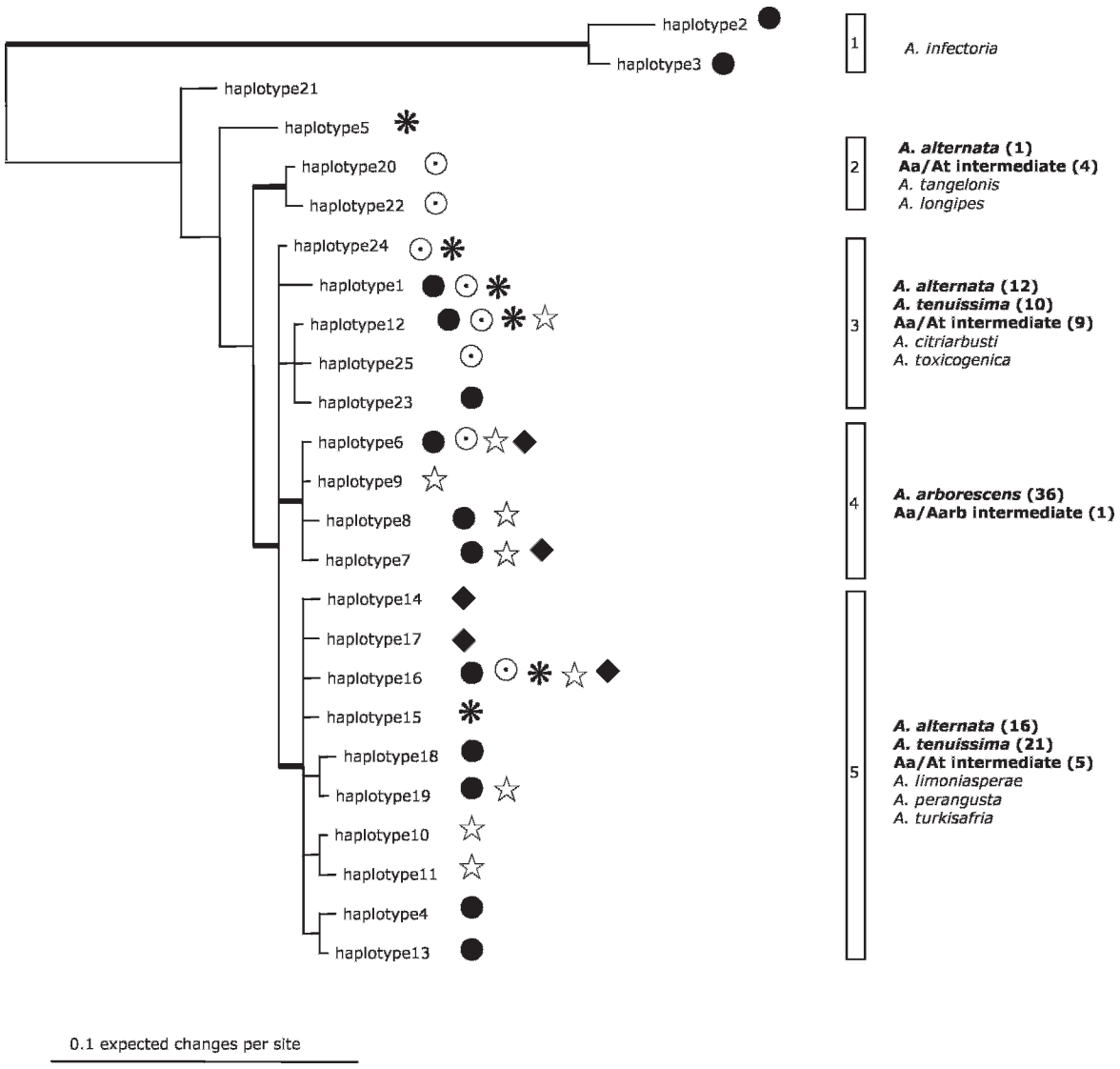

FIG. 1. Bayesian phylogeny estimated from *endoPG* sequence data and rooted by *A. infectoria*. Thick horizontal lines indicate nodes with maximum likelihood bootstrap values greater than 70% and Bayesian posterior probabilities greater than 0.95. Numbered open boxes indicate clades defined with a 70% bootstrap/0.95 posterior probability criterion. Morphological classification of isolates is shown in bold font and numbers in parentheses indicate the number of isolates of each classification. Taxa indicated in regular font are representative morphospecies defined by Simmons (1981, 1999a) and Simmons & Roberts (1993). Host/geographic associations of isolates symbolized as: isolates associated with South Africa apples are represented by the symbol ●; Florida citrus ⊙; Arizona desert grass ✻; France/Italy walnut ☆; California pistachio ◆. Scale indicates expected number of nucleotide substitutions per site.

sequences of 141 isolates were collapsed into 25 haplotypes with the SNAP workbench (Price and Carbone 2004 TABLES I, II). The resultant *endoPG* phylogeny revealed five well supported clades (FIG. 1). The OPA1-3 phylogeny was estimated with sequence data from 138 isolates that were collapsed into 26 haplotypes using the SNAP workbench (Price and Carbone 2004 TABLE I, II). Seventy-seven of 560 sites were parsimony informative. Twenty equally parsimonious trees were recovered and parsimony tree scores were TL = 202, CI = 0.782, RI = 0.866, RC = 0.677 and HI = 0.218. This locus yielded much higher levels of variation than *endoPG*, and the resultant phylogeny revealed seven well supported clades (FIG. 2). The OPA10-2 phylogeny was estimated with sequences of 29 haplotypes representing 138 isolates (TABLES I, II). Phylogenetic analyses revealed that this locus generated the most resolved phylogeny with nine well supported clades (FIG. 3). Fifty-six of 654 sites were parsimony informative. Three hundred

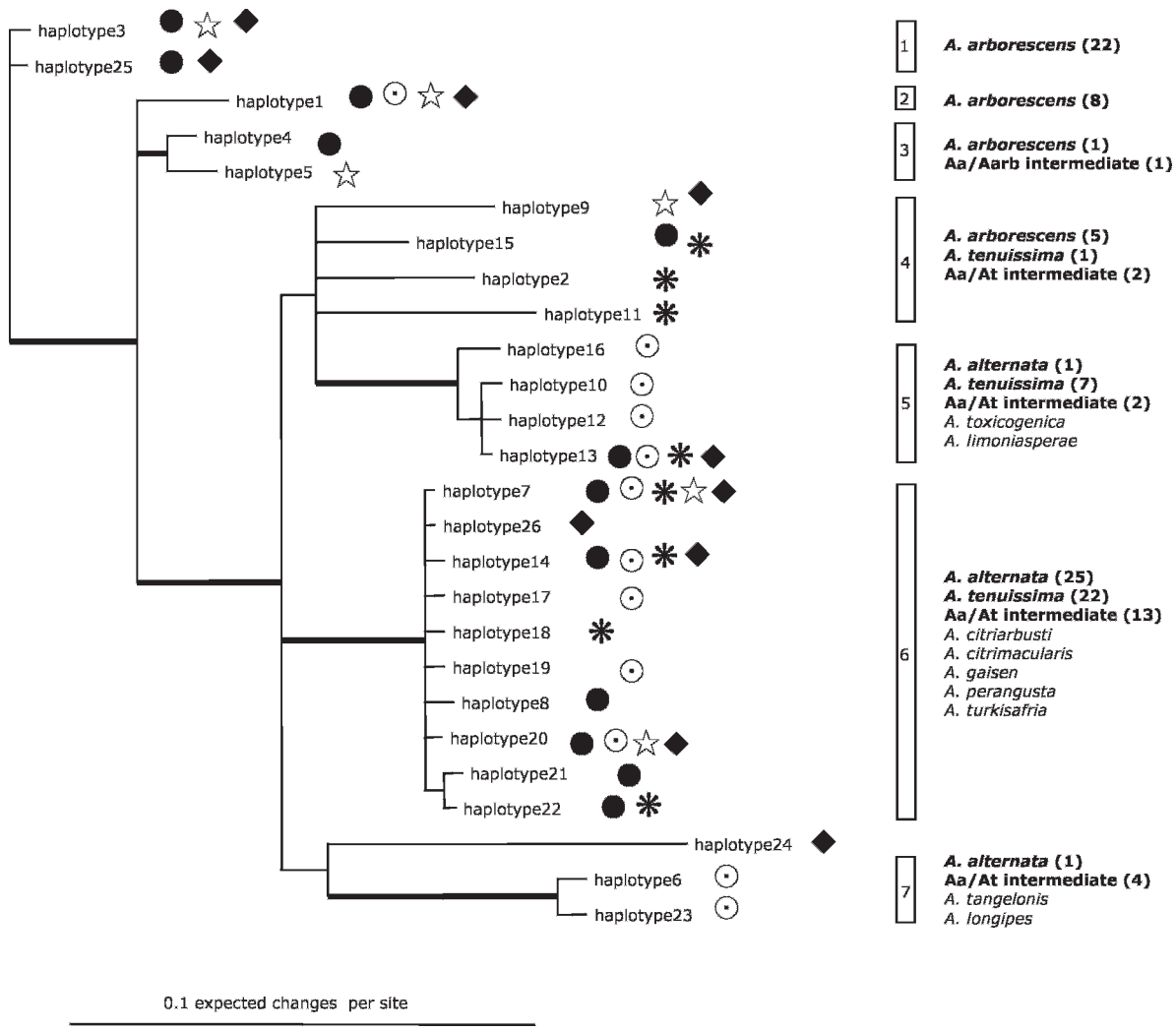

FIG. 2. Bayesian phylogeny estimated from OPA1-3 sequence data with mid-pointrooting. Thick horizontal lines indicate nodes with maximum likelihood bootstrap values greater than 70% and Bayesian posterior probabilities greater than 0.95. Numbered open boxes indicate clades defined with a 70% bootstrap/0.95 posterior probability criterion. Morphological classification of isolates is shown in bold font and numbers in parentheses indicate the number of isolates of each classification. Taxa indicated in regular font are representative morphospecies defined by Simmons (1981, 1999a) and Simmons & Roberts (1993). Host/geographic associations of isolates symbolized as: isolates associated with South Africa apples are represented by the symbol ●; Florida citrus ⊙; Arizona desert grass ✻; France/Italy walnut ☆; California pistachio ◆. Scale indicates expected number of nucleotide substitutions per site.

thirty equally parsimonious trees were recovered and parsimony tree scores were TL = 110, CI = 0.755, RI = 0.915, RC = 0.691 and HI = 0.245.

Tests of neutrality and combinability of datasets.— Neutrality could not be rejected ($P > 0.050$) for all loci using Fu's and Li's D^* and F^* statistics (TABLE II). Neutrality was rejected ($P < 0.05$) for *endoPG* but not for OPA1-3 and OPA10-2 using Tajima's D^* (TABLE II). Tajima's D^* was significantly negative for *endoPG*, possibly indicating purifying (negative) selection acting on this locus. Sequence length, number of polymorphic sites and number of haplotypes before and after pruning of phylogenetically incompatible sites for each alignment are provided (TABLE II). Shimodaira-Hasegawa (SH) tests revealed statistically significant incongruence ($P < 0.050$) for two of three pairwise comparisons both before and after dataset pruning. Congruence was rejected between OPA1-3 and *endoPG* and between OPA10-2 and *endoPG* before dataset pruning (TABLE III). Congruence could not be rejected between the OPA1-3 and OPA10-2 topologies. After removal of incompatible sites from each alignment two of three pairwise comparisons were significantly incongruent (TABLE III). Congruence was rejected between

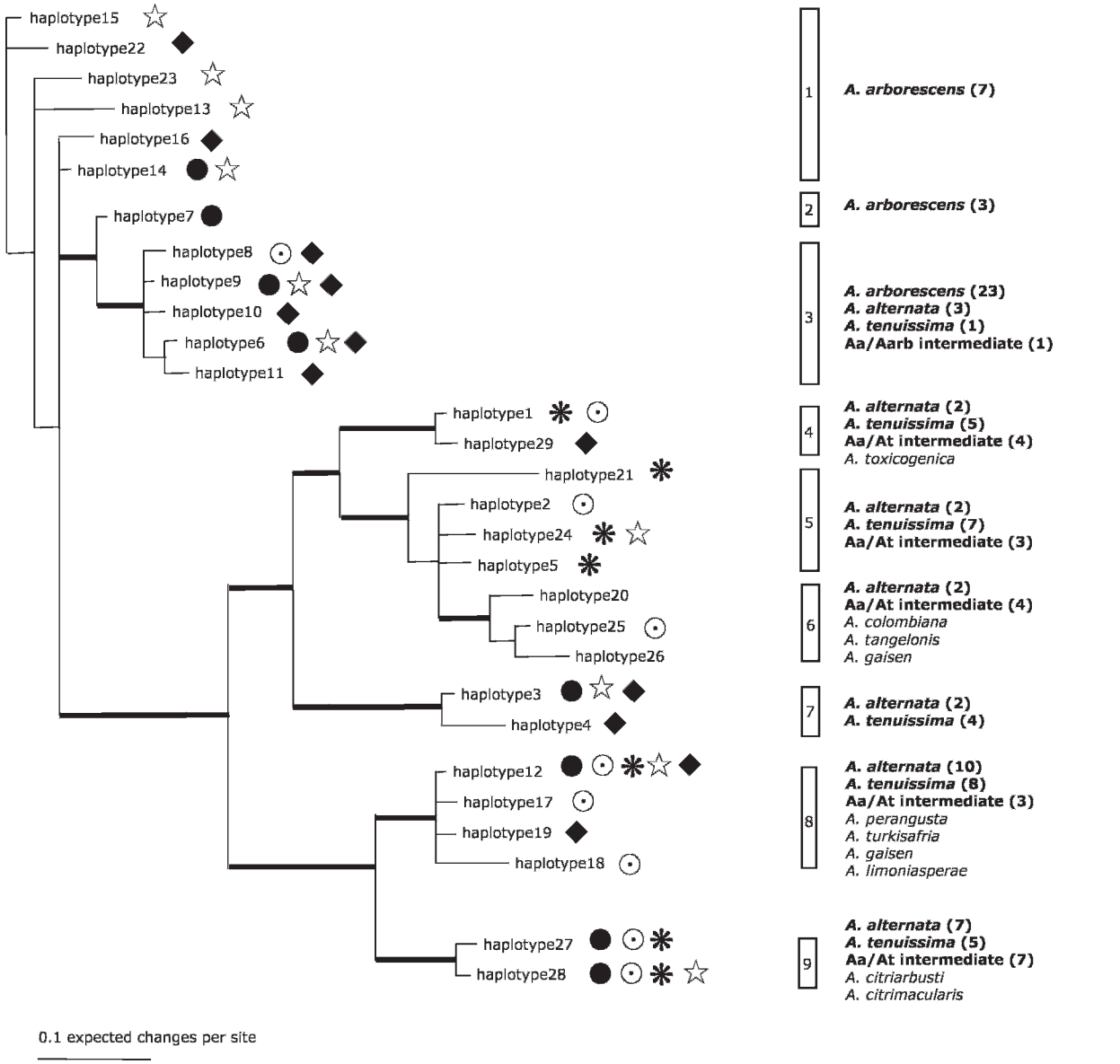

FIG. 3. Bayesian phylogeny estimated from OPA10-2 sequence data with mid-point rooting. Thick horizontal lines indicate nodes with maximum likelihood bootstrap values greater than 70% and Bayesian posterior probabilities greater than 0.95. Numbered open boxes indicate clades defined with a 70% bootstrap/0.95 posterior probability criterion. Morphological classification of isolates is shown in bold font and numbers in parentheses indicate the number of isolates of each classification. Taxa indicated in regular font are representative morphospecies defined by Simmons (1981, 1999a) and Simmons & Roberts (1993). Host/geographic associations of isolates symbolized as: isolates associated with South Africa apples are represented by the symbol ●; Florida citrus ⊙; Arizona desert grass ✻; France/Italy walnut ☆; California pistachio ◆. Scale indicates expected number of nucleotide substitutions per site.

TABLE II. Genetic diversity estimates and tests of neutrality for each locus

Locus	Analyzed sequence length in bp	Total polymorphic sites	Haplotypes	Haplotype diversity	Fu and Li's *D**	Fu and Li's *F**	Tajima's *D*
endoPG	521 (503)[a]	86 (66)[b]	25 (141)[c]	0.865	$P > 0.050$[d]	$P > 0.050$	$P < 0.050$
OPA1-3	560 (495)	144 (89)	26 (138)	0.885	$P > 0.050$	$P > 0.050$	$P > 0.050$
OPA10-2	654 (607)	78 (31)	29 (138)	0.922	$P > 0.050$	$P > 0.050$	$P > 0.050$

[a] Numbers in parentheses indicate length of the alignment after pruning of incompatible sites.
[b] Numbers in parentheses indicate number of polymorphic sites after pruning of incompatible sites.
[c] Numbers in parentheses indicate total number of isolates analyzed per locus.
[d] Probability of obtaining a more extreme value by chance in 1000 simulated coalescent datasets.

TABLE III. Pairwise Shimodaira-Hasegawa tests of topological congruence among phylogenies estimated from each genomic region before and after pruning of phylogenetically incompatible sites. Probabilities before pruning incompatible sites above the diagonal and the probabilities after pruning of incompatible sites below diagonal

	OPA1-3	*OPA10-2*	*endoPG*
OPA1-3	—	0.123	0.000
OPA10-2	0.003[a]	—	0.000
endoPG	0.000	0.043	—

[a] Probability of different topologies among 1000 bootstraps generated using RELL sampling.

OPA10-2 and OPA1-3 as well as OPA1-3 and *endoPG* but not between *endoPG* and OPA10-2 (TABLE III). Significant incongruence between the datasets before and after removal of phylogenetically incompatible sites precluded combining the datasets.

Phylogenetic associations with morphological classification.—Each phylogeny had 1–4 well supported clades composed predominantly of isolates classified in the *A. arborescens* group (FIG. 1, clade 4; FIG. 2, clades 1–4; FIG. 3, clades 1–3). Exceptions were one isolate classified as *A. tenuissima* (FIG. 2, clade 4; FIG. 3, clade 3), three isolates classified as *A. alternata* (FIG. 3, clade 3), one isolate classified as an *A. alternata*/*A. arborescens* intermediate (FIG. 1, clade 4; FIG. 3, clade 3), and two isolates classified as *A. alternata*/*A. tenuissima* intermediates (FIG. 2, clade 4), which also occurred in these clades. It seems likely that these isolates represent recombinants between members of the *A. arborescens* clades and the *A. alternata*/*A. tenuissima* clades because they did not cluster consistently with *A. arboresens* isolates for all loci. Further research to characterize these putative recombination events will be necessary to confirm this. We were unable to reject the hypothesis of monophyly of isolates classified in the *A. arborescens* group for *endoPG* and OPA1-3 datasets with *P*-values of 0.318 and 0.122 respectively (TABLE IV) but monophyly was rejected ($P < 0.05$) for the OPA10-2 dataset (TABLE IV).

Isolates classified morphologically in the *A. alternata* or *A. tenuissima* groups occurred throughout each phylogeny and were not associated with any specific clade for any locus (FIGS. 1–3). Isolates classified in these groups occurred in every non-*A. arborescens* clade of all three phylogenies, and many isolates shared the same multilocus haplotype. For example isolate DES02002, classified in the *A. tenuissima* group (TABLE I), shared an identical multilocus haplotype with isolate DES506, which was classified in the *A. alternata* group (TABLE I; haplotype 12, 7, 1 for *endoPG*, OPA1-3 and OPA10-2 respectively). Isolates classified as intermediates between these two groups were disbursed similarly throughout the phylogeny (FIGS. 1–3). Many of the citrus morphospecies, including *A. citriarbusti*, *A. perangusta*, *A. turkisafria*, *A. gaisen*, *A. tangelonis*, *A. toxicogenica* and *A. limoniasperae* (Simmons 1999a), were paraphyletic supporting Peever et al (2004, 2005) and the hypothesis that there are currently more morphological species among this group of fungi than can be supported in a phylogenetic framework.

Host/geographic associations across the phylogeny.—Associations between phylogenetic clade and host/geographic association was not observed (FIGS. 1–3). Most clades contained isolates from more than one host/geographic association. However in each phylogeny we observed a clade composed primarily of citrus-associated isolates (FIG. 1, clade 2; FIG. 2, clade 7; FIG. 3, clade 6). *A. longipes* (Ell. & Ev.) Mason isolate EGS 30-033 from tobacco clustered with these citrus isolates in the *endoPG* and OPA1-3 phylogenies (FIGS. 1, 2) but was not sequenced for OPA10-2 (TABLE I). EGS 30-033 did not cluster consistently with the same set of isolates in Peever et al (2004, 2005), suggesting that it might be a recombinant.

TABLE IV. Shimodaira-Hasegawa tests of monophyly of isolates classified morphologically as *A. arborescens*

Locus	Constraint	Score (-lnL)	Difference (-lnL)	*P*[a]
endoPG	None	1324.5426	—	
	A. arborescens monophyly	1328.9194	4.3768	0.318
OPA1-3	None	1954.5558	—	
	A. arborescens monophyly	1968.4831	13.9273	0.122
OPA10-2	None	1649.0717	—	
	A. arborescens monophyly	1679.1581	30.0864	0.016

[a] Probability of a larger difference in log-likelihoods by chance among 1000 bootstrapped datasets generated with RELL sampling.

DISCUSSION

Due to the economic importance of small-spored *Alternaria* species, especially within the alternata species-group, a predictive association of species names with biology is needed to allow rapid identification. This suggests that all stable characters, both molecular and morphological, should be considered in developing a more predictive classification system. No associations between host and/or geography and the resultant phylogenies were detected for any of the three loci examined. However host and geography were confounded in our sampling. For example all citrus isolates were sampled from Florida and all pistachio isolates were sampled from California, so any possible host associations might have been obscured by geography and vice versa. The current study also showed no host-specificity among clades, suggesting that host and geographic associations will likely not be useful characters for *Alternaria* classification. One possible exception was a clade identified in all three phylogenies that contained mostly citrus brown spot isolates but also included a representative isolate of *A. longipes* from tobacco. Research with *A. longipes* isolate EGS 30-033 (Peever et al 2004, Peever unpubl) have suggested that this isolate is a recombinant. More detailed analyses of this clade are required to determine whether it is composed exclusively of citrus-associated members.

Morphological classification was similarly a poor predictor of phylogenetic lineage among small-spored *Alternaria* taxa from a diverse range of habitats and geographic locations. Strict agreement between morphology and phylogenetic lineage was only observed for 22% of isolates, all of which were classified in the *A. arborescens* group. The remaining 78% of isolates had morphological classifications that could not be mapped to specific clades. Isolates classified in the *A. alternata* and *A. tenuissima* groups all were genetically undifferentiated, and many other isolates were assigned as intermediates between the two groups (TABLE I). The citrus morphospecies *A. limoniasperae*, *A. turkisafria*, *A. perangusta*, *A. citrimacularis*, *A. citriarbusti* and *A. toxicogenica* were paraphyletic, confirming the results of Peever et al (2004, 2005). Approximately 17% of the total isolates were not classifiable or were classified as intermediates between two morphological groups. *Alternaria* species are known to vary vegetatively and reproductively depending on the media used, relative humidity and light intensity (Simmons 1992). Simmons (1992) has suggested that the use of high nutrient media might result in the loss of sporulation ability after serial transfer. The morphological characters used to delineate species in the alternata species-group are phenotypically plastic and do not allow the reproducible differentiation of several morphospecies. Our classification of several isolates as *A. alternata*/*A. tenuissima* intermediates is consistent with this hypothesis. To date no research has aimed at quantifying the amount of phenotypic plasticity in any of the traits used to identify *Alternaria* species. Correct assignment relies on statistically significant differences among groups that are stable in different environments, and this has never been demonstrated experimentally for any *Alternaria* morphospecies.

The South African apple core-rot isolates originally were classified by Serdani et al (2002) and subsequently reclassified for the current study. This allowed a direct comparison of the stability of classification across laboratories and through time. Ten of 32 isolates (31%) were categorized differently between the two laboratories. Five of these isolates were not classifiable in the current study due to uncharacteristic and unstable sporulation patterns and could not be placed into any of the four morphological categories being considered. Four isolates, identified as *A. tenuissima* by Serdani et al (2002), were characterized as intermediates between two morphological groups in the present study. Isolate APP18 was described by Serdani et al (2002) as *A. arborescens*, however it was classified as a member of the *A. alternata* group in the present study and was not found in any of the clades with other members of the *A. arborescens* morphospecies-group in the phylogenetic analyses. The lack of correlation between morphospecies assignments between laboratories could have been due to either the phenotypic plasticity mentioned above and/or to different morphotaxonomic schemes between laboratories. Whatever the cause, our results clearly indicate that these characters alone will not be useful for robust identification within the alternata species-group.

Small-spored *Alternaria* isolates also have been differentiated on the basis of physiological traits, such as the production of host-specific toxins. Some scientists classify the host-specific toxin producers as pathotypes of *A. alternata* and assign pathotype names to them depending on particular host-specific toxin they produce (Otani and Kohmoto 1992, Scheffer 1992, Isshiki et al 1997, Johnson et al 2001, Hatta 2002, Masunaka et al 2005). For example an isolate of *A. mali* Roberts, the apple pathogen, was found that spontaneously lost AM-toxin production (Johnson et al 2001). This coincided with loss of pathogenicity and the loss of the 1.1 megabase chromosome on which toxin biosynthesis genes normally reside (Johnson et al 2001). Masunaka et al (2005) suggest that lateral gene transfer of toxin genes might have resulted in an *Alternaria* isolate that

was found to produce both ACT- and ACR-toxins and is pathogenic on two different hosts. The putative lateral gene transfer of toxin genes among isolates, as well as spontaneous loss of whole chromosomes carrying toxin genes, indicates that toxin production is not a stable character. Simmons and Roberts (1993) similarly reported no correlation between pathogenicity on Japanese pear and sporulation group among a sample of small-spored *Alternaria* taxa from Japanese pear. Thus a system for classifying the small-spored *Alternaria* species based on pathotype is not a practical or desirable system for *Alternaria* taxonomy.

The objective of this study was to critically test the predictive power of morphological classification of small-spored *Alternaria* taxa under defined environmental conditions and to evaluate the relationship between host/geographic association and phylogenetic lineage. We found no support for differentiation of *A. alternata* and *A. tenuissima* morphospecies in an evolutionary context. Only isolates classified in the *A. arborescens* group were phylogenetically distinct for all loci, and monophyly of these isolates could not be rejected for two of three loci. This supports the phylogenetic division of an arborescens group from other members of the alternata species-group. While we propose the phylogenetic division of an arborescens group, we suggest that all other isolates studied here be referred to as *A. alternata* until novel aspects of biology and/or biochemistry that have gone unnoticed can be assigned to each lineage to aid in species identification.

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