Phylogenetic, Morphological, and Pathogenic Characterization of *Alternaria* Species Associated with Fruit Rot of Blueberry in California

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ABSTRACT

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Fruit rot caused by *Alternaria* spp. is one of the most important factors affecting the postharvest quality and shelf life of blueberry fruit. The aims of this study were to characterize *Alternaria* isolates using morphological and molecular approaches and test their pathogenicity to blueberry fruit. *Alternaria* spp. isolates were collected from decayed blueberry fruit in the Central Valley of California during 2012 and 2013. In total, 283 isolates were obtained and five species of *Alternaria*, including *Alternaria alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. rosae*, were identified based on DNA sequences of the plasma membrane ATPase, *Alt a1* and Calmodulin gene regions in combination with morphological characters of the culture and sporulation. Of the 283 isolates, 61.5% were

identified as *A. alternata*, 32.9% were *A. arborescens*, 5.0% were *A. tenuissima*, and only one isolate of *A. infectoria* and one isolate of *A. rosae* were found. These fungi were able to grow at temperatures from 0 to 35°C, and mycelial growth was arrested at 40°C. Optimal radial growth occurred between 20 to 30°C. Pathogenicity tests showed that all five *Alternaria* spp. were pathogenic on blueberry fruit at 0, 4, and 20°C, with *A. alternata*, *A. arborescens*, and *A. tenuissima* being the most virulent species, followed by *A. infectoria* and *A. rosae*. Previously *A. tenuissima* has been reported to be the primary cause of *Alternaria* fruit rot of blueberry worldwide. Our results indicated that the species composition of *Alternaria* responsible for *Alternaria* fruit rot in blueberry can be dependent on geographical region. *A. alternata*, *A. arborescens*, *A. infectoria*, and *A. rosae* are reported for the first time on blueberry in California. This is also the first report of *A. infectoria* and *A. rosae* infecting blueberry fruit.

Highbush blueberry (*Vaccinium corymbosum* L.) fruit are known for their rich antioxidants, vitamins, and fiber and are becoming popular around the world, and production has steadily increased, with 87% blueberry production occurring in North America (Anonymous 2011; Caruso and Ramsdell 2007). Historically, the Central Valley of California is not known for production of blueberries because of mild winter weather in the region. However, recent establishment of low-chill southern highbush blueberry cultivars in California's warm climate has significantly increased the acreage of blueberry production in Central California, which is now a major southern highbush blueberry production region in the United States (Jimenez et al. 2005). The vast majority of blueberries grown in California are destined for the fresh market, with a 2013 production value of \$127 million (USDA-National Agricultural Statistics Service 2014). As the production continues to grow, the blueberry industry has a need to prolong the storage life of blueberry in order to extend marketing period and increase exports. However, the postharvest life of blueberry fruit is limited by fruit rots caused by fungal pathogens (Cappellini et al. 1982; Mehra et al. 2012).

The major fungi responsible for postharvest fruit rots of blueberries are *Alternaria* spp., *Botrytis cinerea*, *Colletotrichum acutatum*, *C. gloeosporioides*, and *Gloeosporium minus* (Cline 1996; Lambert 1990; Mehra et al. 2012; Milholland and Jones 1972). Previous research indicated that *A. tenuissima* is the primary causal agent of *Alternaria* fruit rot of blueberry (Cline 1996; Greco et al. 2012; Mehra et al. 2012; Milholland and Jones 1972; You et al.

2013). However, most *Alternaria* spp., including *A. tenuissima*, exhibit considerable morphological similarities. Moreover, there are several small-spored *Alternaria* spp. with morphological characteristics that overlap those of *A. tenuissima*; the most commonly known is *A. alternata*. During a survey for postharvest diseases of blueberries grown in the Central Valley of California conducted during 2012 to 2013, we observed that *Alternaria* rot is a significant problem and there is considerable variation among the small-spored *Alternaria* spp. isolates recovered from decayed blueberry fruit (C. L. Xiao, unpublished data). Thus, we initiated a study to characterize the *Alternaria* species profile in blueberry in California. Understanding species composition is important to the development of relevant disease control practices as different species of *Alternaria* may respond to fungicides differently (Ma et al. 2003; Rosenzweig et al. 2008).

In addition to the use of morphological characters to differentiate *Alternaria* species, with the advancement of molecular techniques and DNA sequencing, research on taxonomy of *Alternaria* was conducted using multigene phylogenetic analyses based on sequences of internal transcribed spacer (ITS), the second largest subunit of RNA polymerase II (*RPB2*), chitin synthase, *Tsr1*, translation elongation factor (*TEF*), plasma membrane ATPase, *Alternaria* major allergen (*Alt a1*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*), beta-tubulin, actin, Calmodulin, and partial coding sequence of histone 3 gene, etc. (Andrew et al. 2009; Deng et al. 2012; Greco et al. 2012; Hong et al. 2005; Jasnic et al. 2011; Lawrence et al. 2013; Luan et al. 2007; Wang et al. 2014; Woudenberg et al. 2013; You et al. 2013). Among those genes or regions, *Alt a1* and *gpd* are commonly used. In this study, we downloaded sequences of some small-spored *Alternaria* species, including *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. infectoria* from GenBank and compared them, and we found that the sequences of the plasma membrane ATPase are informative in

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differentiating those *Alternaria* species. Thus, in this study we selected plasma membrane ATPase, *Alt a1*, and Calmodulin genes or regions to differentiate our *Alternaria* spp. isolates from blueberries.

The objectives of this study were to (i) determine the species profiles of *Alternaria* associated with decayed blueberry fruit using molecular phylogenetic and morphological approaches and (ii) test pathogenicity of isolates representative for several *Alternaria* spp. on blueberry fruit.

MATERIALS AND METHODS

Collection of isolates. In 2012 and 2013, harvested blueberry fruit were obtained from commercial blueberry fruit packing operations. The fruit were stored at 0°C for 5 weeks, and decayed fruit were selected for isolation of fungal pathogens. Decayed fruit were sprayed with 70% ethanol until runoff and allowed to dry. The symptomatic tissue just behind the advancing lesion margin was excised using a sterile scalpel and placed in petri dishes containing potato dextrose agar (PDA) (Difco Laboratories, Detroit) acidified with lactic acid (4 ml of 25% [vol/vol] per liter of medium) to minimize bacterial growth. Isolation plates were incubated at room

temperature (20 to 21°C) for 7 to 14 days, and *Alternaria*-like colonies were subcultured to new acidified PDA for 3 to 4 days. Pure cultures were then cultured on PDA. In total, 283 *Alternaria* spp. isolates were obtained in this study.

To obtain single-spored cultures, the method described by Zhu et al. (2011) was used with some modifications. Specifically, conidia were taken from sporulating colonies cultured for 7 to 14 days using an inoculation loop and then streaked onto 2% water agar plates. The plates were incubated for 18 to 20 h at 22°C to allow for conidial germination, and germinating conidia were transferred under a dissecting microscope to a new PDA plate. After 7 days of incubation, mycelial plugs of those isolates were cut and stored in 15% glycerol at -80°C. Eight isolates of *Alternaria* spp. that were isolated from pistachio in the same region and provided by T. Michailides at the University of California, Kearney Agricultural Research and Extension Center (Pryor and Michailides 2002), including *A. alternata* (03.I95, 03.J32), *A. tenuissima* (03.J11, 03.J12), *A. arborescens* (03.J24, 03.J29), and *A. infectoria* (03.J08, 03.J14), were used for comparison (Table 1).

DNA extraction, PCR amplification, and sequencing. In this study, all 283 *Alternaria* spp. isolates were initially identified

TABLE 1. Sources and GenBank accession numbers of sequences of three genes (plasma membrane ATPase, *Alt a1*, and Calmodulin) of *Alternaria* spp. isolated from blueberry used for phylogenetic analysis, pathogenicity tests and temperature response study

Name in GenBank	Isolate	Location	Collection date (mm/dd/yyyy)	Accession number		
				Plasma membrane ATPase	<i>Alt a1</i>	Calmodulin
<i>Alternaria alternata</i>	X1020 ^{x,y}	Delano, CA	5/16/2013	KJ908221	KJ921010	KJ920963
<i>A. alternata</i>	X1048 ^y	Delano, CA	4/10/2013	KJ908230	KJ920997	KJ920957
<i>A. alternata</i>	X1432 ^y	Delano, CA	6/7/2013	KJ908223	KJ921014	KJ920966
<i>A. alternata</i>	X1431	Stockton, CA	6/7/2013	KJ908255	KJ920985	KJ920965
<i>A. alternata</i>	X1280	Tulare, CA	6/6/2013	KJ908252	KJ920986	KJ920971
<i>A. alternata</i>	X1040	Kingsburg, CA	5/16/2013	KJ908251	KJ920983	KJ920972
<i>A. alternata</i>	X1092	Kingsburg, CA	5/16/2013	KJ908256	KJ920988	KJ920968
<i>A. alternata</i>	X1104	Kingsburg, CA	5/16/2013	KJ908254	KJ920984	KJ920969
<i>A. alternata</i>	X1111	Kingsburg, CA	5/16/2013	KJ908233	KJ920999	KJ920958
<i>A. alternata</i>	X1122	Kingsburg, CA	5/16/2013	KJ908253	KJ920989	KJ920970
<i>A. alternata</i>	X1191	Kingsburg, CA	5/31/2013	KJ908234	KJ920991	KJ920956
<i>A. alternata</i>	X1265 ^{x,y}	Kingsburg, CA	6/6/2013	KJ908237	KJ921004	KJ920960
<i>A. alternata</i>	X1269 ^y	Kingsburg, CA	6/6/2013	KJ908238	KJ921005	KJ920961
<i>A. alternata</i>	X1283	Kingsburg, CA	6/6/2013	KJ908222	KJ921015	KJ920938
<i>A. arborescens</i>	X1312	Richgrove, CA	8/2/2013	KJ908242	KJ921008	KJ920948
<i>A. arborescens</i>	X1033 ^{x,y}	Delano, CA	5/16/2013	KJ908248	KJ921009	KJ920941
<i>A. arborescens</i>	X1205	Delano, CA	5/28/2013	KJ908225	KJ921002	KJ920939
<i>A. arborescens</i>	X1263 ^y	Delano, CA	6/7/2013	KJ908226	KJ921003	KJ920947
<i>A. arborescens</i>	X945 ^y	Delano, CA	3/18/2013	KJ908228	KJ920995	KJ920943
<i>A. arborescens</i>	X1276 ^y	Stockton, CA	6/6/2013	KJ921006	KJ921006	KJ920945
<i>A. arborescens</i>	X100 ^{x,y}	Kingsburg, CA	8/10/2012	KJ908224	KJ921013	KJ920950
<i>A. arborescens</i>	X1013	Kingsburg, CA	5/16/2013	KJ908247	KJ920987	KJ920949
<i>A. arborescens</i>	X1126	Kingsburg, CA	5/16/2013	KJ908245	KJ920990	KJ920940
<i>A. arborescens</i>	X1392	Kingsburg, CA	5/16/2013	KJ908246	KJ920992	KJ920946
<i>A. infectoria</i>	X1273 ^{x,y}	Tulare, CA	6/6/2013	KJ908218	KJ920982	KJ920953
<i>A. rosae</i>	X1272 ^{x,y}	Tulare, CA	6/6/2013	KJ908217	KJ920981	KJ920952
<i>A. tenuissima</i>	X1003 ^y	Delano, CA	4/4/2013	KJ908229	KJ920996	KJ920954
<i>A. tenuissima</i>	X1026 ^{x,y}	Delano, CA	5/16/2013	KJ908240	KJ921011	KJ920944
<i>A. tenuissima</i>	X1068 ^y	Delano, CA	5/1/2013	KJ908231	KJ920998	KJ920962
<i>A. tenuissima</i>	X1309	Delano, CA	6/7/2013	KJ908241	KJ921007	KJ920942
<i>A. tenuissima</i>	X1085	Kingsburg, CA	6/5/2013	KJ908232	KJ920993	KJ920967
<i>A. tenuissima</i>	X1099	Kingsburg, CA	5/16/2013	KJ908227	KJ920994	KJ920951
<i>A. tenuissima</i>	X1125 ^y	Kingsburg, CA	5/16/2013	KJ908235	KJ921000	KJ920959
<i>A. tenuissima</i>	X1147	Kingsburg, CA	5/23/2013	KJ908236	KJ921001	KJ920955
<i>A. tenuissima</i>	X1259 ^{x,y}	Kingsburg, CA	6/7/2013	KJ908216	KJ921012	KJ920964
<i>A. alternata</i>	03.I95 ^z	CA	6/6/1990	KJ908249	KJ921021	KJ920976
<i>A. alternata</i>	03.J32 ^z	CA	9/9/1998	KJ908250	KJ921020	KJ920975
<i>A. arborescens</i>	03.J29 ^z	CA	9/9/1998	KJ908243	KJ921022	KJ920980
<i>A. arborescens</i>	03.J24 ^z	CA	9/9/1998	KJ908244	KJ921023	KJ920979
<i>A. infectoria</i>	03.J14 ^z	CA	9/9/1998	KJ908219	KJ921017	KJ920977
<i>A. infectoria</i>	03.J08 ^z	CA	9/9/1998	KJ908220	KJ921016	KJ920978
<i>A. tenuissima</i>	03.J11 ^z	CA	9/9/1998	KJ908258	KJ921019	KJ920974
<i>A. tenuissima</i>	03.J12 ^z	CA	9/9/1998	KJ908257	KJ921018	KJ920973

^x Isolates used for pathogenicity tests on blueberry fruit and temperature response.

^y Isolates used for the test of mycelial growth rate and measurement of conidia size.

^z Reference isolates from pistachio provided by T. J. Michailides at the University of California, Davis.

by amplifying the plasma membrane ATPase gene and by comparing results to sequences in GenBank. Based on plasma membrane ATPase gene sequences (see below for the detail), a subset of 27 isolates, representing different species of *Alternaria*, the range of genetic diversity and geographic origin (Table 1) were further sequenced for *Alt al* gene and Calmodulin gene.

Mycelia and spores from 7- to 10-day-old PDA cultures incubated at 20°C were used for DNA extraction. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and FastPrep-24 Instrument following the manufacturer's instructions (MP Biomedicals, Santa Ana, CA). DNA concentration was quantified using NanoDrop 2000c (Thermo Scientific, Wilmington, DE).

Amplifications of fragments of plasma membrane ATPase and Calmodulin genes were performed using primer pairs ATPDF1/ATPDR1 and CALDF1/CALDR1, respectively (Lawrence et al. 2014). Amplification of the *Alt al* gene was performed using primer pairs Alt-for/Alt-rev described in Hong et al. (2005). PCR amplification was conducted in a 25- μ l reaction volume containing 12.5 μ l of GoTaq Mix (Promega, Madison, WI), 1 μ l of each primer (10 μ M), and 1 μ l (20 to 30 ng) of template DNA. PCR reactions were carried out in a thermal cycler S1000 (Bio-Rad Laboratory, Forster City, CA). The thermocycling parameters for the plasma membrane ATPase gene were the same as described by Lawrence et al. (2013): an initial heating step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at temperature gradient of 59°C for 30s and extension at 72°C for 1 min; and final extension at 72°C for 5 min. The thermocycling parameters for *Alt al* and Calmodulin genes were the same as described by Hong et al. (2005): an initial heating step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at temperature gradient of 57°C for 40s and extension at 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 1% agarose gels in 1.0 $\times$ Tris borate EDTA (TBE) buffer and photographed. PCR products were then purified using GeneJet purification kit (Thermo Fisher Scientific, Pittsburgh, PA) following the manufacturer's instructions. PCR products were quantified using Nanodrop 2000c (Thermo Scientific) and diluted to a range between 5 and 15 ng/ μ l for sequencing, depending on the length of the PCR products.

The PCR products were sequenced in both directions using the PCR primers and ABI Prism BigDye Terminator v. 3.1 cycle Sequencing Kit in a 3130 $\times$ L Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The nucleotide sequences were read with an ABI Prism 3130 $\times$ L sequencer (Applied Biosystems) and software Bioedit 7.0.4.1 (Hall 1999). Sequences obtained in this study were deposited in GenBank (Table 1).

Phylogenetic analyses. To obtain preliminary identification for the isolates in this study, the plasma membrane ATPase, Calmodulin and *Alt al* sequences were subjected to BLAST search using the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>) nucleotide database. Sequences of the type species specimen strains for closely related *Alternaria* species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) to compile datasets for phylogenetic analyses (Table 2). Sequences for each of the three gene regions were aligned using DNAMAN version 7 (Lynnon Corporation). Sequence alignments for each of the three gene regions were manually edited in DNAMAN 7.0 and deposited in TreeBase (<http://www.treebase.org>).

Maximum parsimony (MP) phylogenetic analyses were performed for each of the plasma membrane ATPase, Calmodulin, and *Alt al* datasets as well as for the combined datasets of the three genes in PAUP* 4.0b10 (Swofford 2000). Gaps were treated as missing data. Trees were inferred using the heuristic search with TBR branch swapping and 1,000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple parsimonious trees were saved. Branch stability

was evaluated in a bootstrap analysis with 1,000 replicates, each with 100 replicates of random stepwise addition of taxa. A second phylogenetic analysis using Markov Chain Monte Carlo (MCMC) algorithm was conducted to generate trees with Bayesian posterior probabilities in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Nucleotide substitution models were determined using Modeltest v 3.7 (Posada and Crandall 1998) for each gene region and included in the analyses. Two analyses of four MCMC chains were run from random trees for 10 million generations and sampled every 1,000 generations. The first 25% of trees were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining tree. The phylogenetic trees were viewed and printed using Treeview 1.6.6 (Page 1996).

Morphological observations. In total, 17 isolates representing different *Alternaria* species in the phylogenetic analysis were studied for morphological characteristics (Table 1). To observe the colony morphology, selected isolates were cultured on PDA for 7 days and then transferred to petri dishes containing PDA (Pryor and Michailides 2002) or potato carrot agar (PCA) (Simmons 2007). For each isolate, three replicate plates were used. These cultures were incubated at 22°C under cool white fluorescent light with an 8 h photoperiod and 16 h darkness. Colony diameters were measured at two perpendicular directions 3 and 5 days after inoculation. For each of the tested isolates, the average colony growth per day between 3 to 5 days was then calculated. After 7 days, cultures were examined for colony color, colony margin, colony texture, and the development of pigments or crystals in the agar medium. Colony color was determined based on color standards of Ridgway (1912). The experiment was performed twice.

To observe sporulation patterns, isolates were cultured on PDA for 7 days and then transferred to petri dishes containing PCA, 0.05 $\times$ PDA (Pryor and Michailides 2002), or synthetic nutrient agar (SNA) (Woudenberg et al. 2013). Dishes were incubated for 7 days under the same conditions as described above. After incubation, cultures were examined under a dissecting microscope for characteristics of the sporulation apparatus, including length of conidial chains, presence of elongated secondary conidiophores, and manner by which branching of conidial chains occurred. The sellotape technique was used for making slide preparations (Woudenberg et al. 2013) with Shear's medium as mounting fluid. Photographs of characteristic structures such as conidial chains and conidia were made under a compound microscope. For each isolate, the dimensions (length and width) of 50 conidia were measured. Average (mean), standard error, and minimum and maximum measurements were calculated for each isolate. Conidial shape and color and septation were also recorded.

On the basis of preliminary results, colony color of *Alternaria* spp. was more evident on PDA than on PCA. Sporulation of *Alternaria* spp. on PCA was more abundant than that on PDA and SNA. Thus, we selected PDA for observation of colony color development and PCA as the medium for further study of sporulation in order to compare the sporulation characteristics with published results from other studies.

Effect of temperature on mycelial growth. Effects of temperature on mycelial growth were evaluated on PDA (Pryor and Michailides 2002). Two isolates for each species were used in this study. Isolates were grown on PDA at 20°C in the dark for 7 days. Mycelial plugs (4 mm diameter) were removed with a sterile cork borer (4 mm in diameter) from the leading edges of colonies, and one such plug was placed in the center of each 90 mm Petri plate containing the medium. Inoculated plates were placed in plastic boxes and incubated at 0, 5, 10, 15, 20, 25, 30, 35, or 40°C in the dark. There were three replicate plates for each isolate at each temperature. Colony diameter in each plate was measured at two perpendicular directions after 3 and 5 days of inoculation at 10, 15, 20, 25, 30, and 35°C, 10 and 20 days after inoculation at 0 and 5°C; the two measurements taken at each time were averaged. Cultures that did not grow after 10 days at 40°C were moved to 22°C, and

colony diameters were measured after 4 days to determine whether the fungus would resume growth. Daily radial growth rates were calculated to give a final value. The colony morphology also was noted. The experiment was conducted twice.

Pathogenicity tests. Eight isolates of *Alternaria* spp. (*A. alternata*: X1020, X1265; *A. arborescens*: X100, X1033; *A. tenuissima*: X1026, X1259; *A. infectoria*: X1273; and *A. rosae*: X1272) recovered from decayed blueberry fruit were used in the pathogenicity tests. Conidia of tested isolates produced on 7-day-old PCA cultures incubated at 22°C under 8 h fluorescent light /16 h dark were used to make conidial suspensions. Conidia were collected by scraping them off the surface of the medium with a sterile scalpel and placing them in a test tube with sterile water to

make the conidial suspensions, which were then filtered through four layers of gauze. Concentrations of the spore suspensions were estimated using a hemocytometer and adjusted to 1×10^6 conidia per ml.

Freshly harvested organic ‘Snow Chaser’ blueberry fruit were obtained from a local blueberry farm and used for pathogenicity tests. The fruit were organically produced avoiding potential interference by fungicide residues on/in the fruit. The fruit were surface-disinfested in 0.5% NaOCl (10% bleach) for 2 min, rinsed three times with sterile distilled water and then air-dried. Fruit were wounded with a sterile needle to a depth of 2 mm. Each fruit was inoculated by placing 1 µl of conidial suspension with a pipette into the wound of each fruit. Inoculated fruit were placed on

TABLE 2. Accession numbers for sequences of three genes (*Alt al*, plasma membrane ATPase, and Calmodulin) of *Alternaria* species and other taxa obtained from the GenBank used for comparison in the phylogenetic analysis

Name in GenBank	Isolate number ^y	Accession number		
		<i>Alt al</i>	Plasma membrane ATPase	Calmodulin
<i>Alternaria alternata</i>	EGS 34-016 ^{tab}	AY563301	JQ671874	JQ646208
<i>A. alternata</i>	CBS 117130	FN689396	N/A ^z	N/A
<i>A. alternata</i>	CBS 117143	FN689397	N/A	N/A
<i>A. angustiovoidea</i>	EGS 36-172 ^{ta}	JQ646398	JQ671869	JQ646203
<i>A. arborescens</i>	EGS 39-128 ^{tab}	AY563303	JQ671880	JQ646214
<i>A. arborescens</i>	MAP 05	FN689414	N/A	N/A
<i>A. arborescens</i>	CBS 109730	FN689398	N/A	N/A
<i>A. arborescens</i>	BMP 0602	N/A	JQ811991	N/A
<i>A. arborescens</i>	BMP 0462	N/A	JQ811990	N/A
<i>A. argyroxiphii</i>	EGS 35-122 ^{ta}	JQ646434	JQ671926	JQ646260
<i>A. bursii</i>	CBS 107.38 ^a	JQ646388	JQ671860	N/A
<i>A. cerealis</i>	EGS 43-072 ^{ta}	JQ646405	JQ671883	JQ646217
<i>A. citri</i>	CBS 192.81	FN689399	N/A	N/A
<i>A. citriarbasti</i>	SH-MIL-8s ^a	JQ646406	JQ671884	JQ646218
<i>A. citrimaculata</i>	BC2-RLR-17s ^a	JQ646407	JQ671885	JQ646219
<i>A. colombiana</i>	BMP 2337 ^a	JQ646409	JQ671887	JQ646221
<i>A. destruens</i>	EGS 46-069 ^{ta}	JQ646402	JQ671873	JQ646207
<i>A. dumosa</i>	EGS 45-007 ^{ta}	N/A	JQ671877	JQ646211
<i>A. gaisen</i>	CBS 632.93	FN689401	N/A	N/A
<i>A. gaisen</i>	BMP 0243 ^a	JQ646400	JQ671871	JQ646205
<i>A. herbiphorbicola</i>	EGS 40-140 ^{ta}	JQ646410	JQ671888	JQ646222
<i>A. hordeicola</i>	EGS 50-184 ^{ta}	JQ646372	JQ671812	JQ646146
<i>A. infectoria</i>	EGS 27-193 ^{tab}	FJ266502	JQ671804	JQ646138
<i>A. infectoria</i>	MAP 03	FN689415	N/A	N/A
<i>A. infectoria</i>	CBS 210.86	FN689402	N/A	N/A
<i>A. limoniasperae</i>	EGS 45-100 ^{tab}	N/A	JQ671879	JQ646213
<i>A. longipes</i>	EGS 30-033 ^{ab}	AY563304	JQ671864	JQ646198
<i>A. longipes</i>	CBS 917.96	FN689403	N/A	N/A
<i>A. macrospora</i>	DGG Ams1 ^a	N/A	JQ671909	JQ646243
<i>A. marlvae</i>	CBS 447.86 ^a	JQ646397	JQ671878	JQ646212
<i>A. rosae</i>	EGS 41-130 ^{ta}	JQ646370	JQ671803	JQ646137
<i>A. septorioides (A. resedae)</i>	CBS 175.80 ^a	JQ646408	JQ671886	JQ646220
<i>A. solani</i>	ATCC 58177 ^a	AY563299	JQ671898	JQ646232
<i>A. steviae</i>	CBS 632.88 ^a	JQ646423	JQ671906	JQ646240
<i>A. tangelonis</i>	EV-MIL-2s ^a	JQ646392	JQ671865	JQ646199
<i>A. tenuissima</i>	BMP 0517	N/A	JQ811982	N/A
<i>A. tenuissima</i>	BMP 1153	N/A	JQ811985	N/A
<i>A. tenuissima</i>	BMP 0238	N/A	JQ811984	N/A
<i>A. tenuissima</i>	BMP 0508	N/A	JQ811987	N/A
<i>A. tenuissima</i>	BMP 1098	N/A	JQ811986	N/A
<i>A. tenuissima</i>	BMP 1254	N/A	JQ811988	N/A
<i>A. tenuissima</i>	MAP 01	FN689408	N/A	N/A
<i>A. tenuissima</i>	CBS 880.95	FN689407	N/A	N/A
<i>A. tenuissima</i>	EGS 34-015 ^{tab}	AY563302	JQ671875	JQ646209
<i>A. tenuissima</i>	BRIP 48081	JQ282277	N/A	N/A
<i>A. tomatophila</i>	CBS 109156 ^a	JQ646431	JQ671923	JQ646257
<i>A. toxicogenica</i>	PR 320 ^a	JQ646411	JQ671889	JQ646223
<i>A. tropica</i>	CBS 631.93 ^a	JQ646438	JQ671930	JQ646264
<i>A. turkisafria</i>	EGS 44-159 ^a	JQ646404	EF504051	JQ646216
<i>A. perangusta</i>	BMP 2336 ^a	JQ646403	JQ671881	JQ646215
<i>Embellisia proteae</i>	EGS 39-031 ^a	FJ266505	JQ671777	JQ646111
<i>Ulocladium chartarum</i>	ATCC 18044 ^a	AY563319	JQ671828	JQ646162
<i>U. cucurbitae</i>	EGS 31-021 ^a	AY563315	JQ671836	JQ646170
<i>U. dauci</i>	CBS 102062 ^a	FJ266510	JQ671832	JQ646166

^y t = ex-type isolate, a = isolate also included in Lawrence et al. (2013), and b = isolate also included in Woudenberg et al. (2013).

^z N/A = not available.

double-sided sticky tapes in the petri dishes and plates were kept in plastic boxes with a shallow depth of sterile water (about 0.5 cm) on the bottom to keep high humidity. The plastic boxes were placed in incubators with designated temperatures at 0, 4 or 20°C (commercially blueberries are commonly stored at 0 to 1°C). Three 10-blueberry fruit replicates were inoculated with each isolate of the fungus for each temperature. For a control treatment, fruit were wounded as described above and treated with 1 µl of sterile water instead of the conidial suspension. Fruit rot development was evaluated after incubation for 6 days at 20°C, 5 weeks at 4°C, and 3 additional days at room temperature after removal from 6-week storage at 0°C. As blueberry fruit are small and difficult to measure the size of decay lesion on the fruit, for ease of assessment, severity of decay, expressed as percentage of the fruit surface decayed, was estimated for each fruit using the following scale: 0 = no decay; 1 = ≤1/8 of the fruit surface decayed; 2 = 1/8 to 1/4 of the fruit surface decayed; 3 = 1/4 to 1/2 of the fruit surface decayed; 4 = 1/2 to 3/4 of the fruit surface decayed; and 5 = ≥3/4 of the fruit surface decayed. The incidence of decay expressed as the percentage of the inoculated fruit that developed decay in each replicate was calculated. Reisolation of fungi from the decayed fruit was attempted. The experiment was conducted twice. Decay incidence and severity were analyzed by analysis of variance (ANOVA) performed using PROC ANOVA in SAS (version 9.3, SAS Institute, Cary, NC) with mean separation using Fisher's protected least significance test with $P = 0.05$. Decay incidence data in percentage were arcsine-square-root-transformed prior to analysis.

RESULTS

Phylogenetic analysis. The initial analysis of the partial sequences of the plasma membrane ATPase gene of the 283 *Alternaria* spp. isolates obtained from blueberry revealed a total of five groups. Compared with the sequences of the reference isolates and the ex-type or authentic representative isolates from the NCBI nucleotide database by the BLAST search, analysis of these five groups suggested that they belonged to five species: *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. rosae*, respectively. Of the isolates sequenced for the plasma membrane ATPase gene, 35 representing the five groups (species) and covering all the sampled counties, along with eight reference *Alternaria* isolates from pistachio and some sequences of *Alternaria* spp. downloaded from GenBank (Table 2), were selected for further multilocus analysis comprising of plasma membrane ATPase, Calmodulin, and *Alt a1* genes. Sequences of *Alternaria* spp. used in this study were deposited in GenBank (Table 1). Sequences of the plasma membrane ATPase gene of the *Alternaria* species ranged from 1,195 to 1,225 bp in which there were eight base pair differences between *A. alternata* and *A. tenuissima* and nine base pair differences between *A. arborescens* and *A. alternata/A. tenuissima*. Sequences of the Calmodulin gene ranged from 676 to 796 bp in which there were six base pair differences between *A. arborescens* and *A. alternata/A. tenuissima*. Sequences of the *Alt a1* gene ranged from 431 to 473 bp. The aligned sequences of each dataset of plasma membrane ATPase (85 taxa, 1,201 characters), Calmodulin (76 taxa, 756 characters), and *Alt a1* (86 taxa, 353 characters) gene regions were deposited in TreeBASE (<http://www.treebase.org>).

In the parsimony analysis, statistical data for the plasma membrane ATPase gene were as follows: tree length for MP = 493, consistency index (CI) = 0.7525, retention index (RI) = 0.9196, rescaled consistency index (RC) = 0.6920, homoplasy index (HI) = 0.3020; for Calmodulin: tree length = 447, CI = 0.7673, RI = 0.9404, RC = 0.7216, HI = 0.2613; for *Alt a1*: tree length = 303, CI = 0.7228, RI = 0.9190, RC = 0.6642, HI = 0.3387; and for the three-gene combined sequences: tree length = 1,247, CI = 0.7193, RI = 0.9098, RC = 0.6544, HI = 0.3265. For Bayesian analysis, model test analysis recommended appropriate models as follows: GTR+G model (Lset base = [0.2162 0.3089 0.2523 0.2226]; Nst = 6;

Rmat = [1.8069 3.3351 1.9043 1.8198 7.7586]; rates = gamma; shape = 0.2163; pinvar = 0) for the plasma membrane ATPase gene region, a TIM+G model (Lset base = [0.2381 0.3539 0.2339 0.1741]; Nst = 6; Rmat = [1.0000 4.3033 1.6394 1.6394 7.2626]; rates = gamma; shape = 0.3751; pinvar = 0) for the Calmodulin gene region, a HKY+G model (Lset base = [0.2348 0.2951 0.2225 0.2476]; Nst = 2; Tratio = 1.7759; rates = gamma; shape = 0.6253; pinvar = 0) for the *Alt a1* gene region, and a TrN+I+G model (Lset base = [0.2294 0.3225 0.2432 0.2050]; Nst = 6; Rmat = [1.0000 2.6464 1.0000 1.0000 5.3196]; rates = gamma; shape = 1.0929; pinvar = 0.4612) for the combined sequences of the three gene regions. For all the three gene regions and their combination, topologies of their trees were basically similar in the maximum parsimony and Bayesian inference. Four phylogenetic trees reconstructed from parsimony bootstrap values and posterior probabilities are presented in Figures 1 to 4.

The phylogenetic analysis for the plasma membrane ATPase gene showed that the 35 isolates from blueberry could be divided into five groups: 14 isolates were clustered in the same group with the reference isolates of *A. alternata* by high bootstrap value of 97/1.00, nine isolates were clustered in the same group with the reference isolates of *A. tenuissima* by high bootstrap value of 95/1.00, 10 isolates were clustered in the same group with the reference isolates of *A. arborescens* by high bootstrap value of 100/1.00, one isolate X1273 was clustered in the same group with the reference isolates of *A. infectoria* by high bootstrap value of 99/0.98, and one isolate X1272 was clustered in the same group with the reference sequence of *A. rosae* from GenBank, which were supported by high MP bootstrap support of 80/0.99 (Fig. 1).

Compared with the reference isolates, the phylogenetic analysis of Calmodulin gene differentiated *A. arborescens*, *A. infectoria*, and *A. rosae* from other *Alternaria* spp. supported by a high bootstrap value; however, *A. alternata* and *A. tenuissima* were grouped in the same cluster (Fig. 2). The phylogenetic analysis of *Alt a1* gene differentiated *A. infectoria* and *A. rosae* from others (Fig. 3). Although reference isolates 03.J24 and 03.J29 of *A. arborescens* were grouped in one cluster, the MP bootstrap support was lower than 70, which was believed not to be reliable (Fig. 3). Similarly *A. alternata* and *A. tenuissima* were clustered in the same groups (Fig. 3). In the phylogenetic analysis of combined sequences of plasma membrane ATPase, Calmodulin and *Alt a1* genes, *A. infectoria* and *A. rosae* could be differentiated by high MP bootstrap supports. *A. arborescens* isolates were grouped in one cluster, but both *A. alternata* and *A. tenuissima* isolates were clustered into the same groups (Fig. 4).

Morphological characteristics of cultures and sporulation.

In total, 17 isolates representing five species of *Alternaria* identified based on the sequences of the three gene regions were used for morphological observations (Table 1). Based on the colony morphology, colony growth, and sporulation patterns, these *Alternaria* isolates were grouped in five distinct groups as described below.

The isolates in group A and group B developed colonies that were lettuce green to olive green and usually had a prominent white margin. Colony texture was felty to woolly, and 46% of the isolates in these two groups produced whitish crystals in the agar medium underneath the mycelial mat (Fig. 5). Isolates in group A developed conidial chains of 6 to 15 conidia in length and the secondary chains of 1 to 6 conidia in length. Conidia chain branched in a sympodial manner by the elongation of secondary conidiophores from distal terminal conidial cells or through the lateral growth of secondary conidiophores from median or basal conidial cells and subsequently formed conidia. Conidia were typically ovate to obclavate in shape. Those isolates were identified as *A. alternata*, which grew at 9.1 mm per day at 22°C and the conidia size was 14.6 to 36.2 × 5.2 to 15.0 µm (Table 3). The isolates in group B developed conidial chains of 6 to 18 conidia in length and the uncommon secondary chains of 1 to 4 conidia in length. Conidial chains typically branched by the lateral growth of secondary conidiophores from

distal terminal conidial cells and subsequently formed conidia. Conidia were typically ovate to obclavate in shape. These isolates were identified as *A. tenuissima*, which grew 9.8 mm per day at 22°C (Table 3). The conidia size of *A. tenuissima* (13.9 to 43.0 × 5.8 to 13.7 µm) is similar to that of *A. alternata*.

Isolates in group C were *A. arborescens*. The colonies were typically dark olive gray, iron gray to castor gray in color. The colony margin was generally wavy or torn (Fig. 5) and 98% of the isolates in this group produced a diffusible pale orange pigment and

abundant crystals in the agar medium underneath the mycelial mat. Colony grew slower than other species (4.7 mm per day between 3 to 5 days incubation at 22°C) (Fig. 5, Table 3). The isolates in this group formed conidial chains of two to six (occasionally 8) conidia in length and produced numerous secondary, tertiary, and quaternary chains of two to four conidia in length. Chain branching typically occurred in a sympodial manner by the elongation of secondary conidiophores from distal terminal conidial cells and subsequently formed conidia. Some isolates generally formed long

Fig. 1. Phylogenetic trees inferred from the plasma membrane ATPase gene sequences from maximum parsimony (MP) and Bayesian analysis for *Alternaria* spp. from blueberry and reference isolates of *Alternaria* spp. *Embellisia proteae* (EGS 39-031) was selected as outgroup. Isolates beginning with X were collected from blueberry in California and all other isolates used in the phylogenetic analyses are listed in Tables 1 and 2. Bootstrap support values (1,000 replicates) above 70% for MP and above 0.95 for Bayesian analysis are shown. Species are followed by strain numbers.

primary conidiophores and then had the initiation of conidial chains. Conidia were typically ovate in shape (11.7 to 35.3 × 6.3 to 15.0 μm) (Table 3).

The isolates in group D belonged to *A. infectoria*. The colony was apricot orange in color. The colony generally had a cottony texture and tufts of sterile white hyphae were present at the center of the colony (Fig. 5). It produced an orange pigmentation without crystals. Colony growth rate was 10.2 mm per day at 22°C (Table 3). Isolates in this group formed conidial chains of two to eight conidia

in length and developed numerous secondary, tertiary, and quaternary chains of two to four conidia in length. Chain branched typically in sympodial manner. Secondary conidiophores were generally one to three times the length of conidia. Conidia (12.9 to 25.5 × 5.3 to 10.9 μm) were typically ovate to obclavate in shape (Fig. 5, Table 3). Some conidia developed distinctive apical extensions in no less than the length of the conidium body.

In group E, isolates produced colonies lettuce green in color and had a prominent white margin with about 15 to 20 mm in width

Fig. 2. Phylogenetic trees inferred from maximum parsimony (MP) and Bayesian analysis of Calmodulin gene sequences for *Alternaria* spp. from blueberry and reference isolates of *Alternaria* spp. *Embellisia proteae* (EGS 39-031) was selected as outgroup. Isolates beginning with X were collected from blueberry in California and all other isolates used in the phylogenetic analyses are listed in Tables 1 and 2. Bootstrap support values (1,000 replicates) above 70% for MP and above 0.95 for Bayesian analysis are shown. Species are followed by strain numbers.

(Fig. 5, Table 3). The colony growth of this fungus was about 11.1 mm per day at 22°C (Table 3). Primary conidiophores in granular clumps under the dissecting microscope, simple or branched, arose in close proximity from hyphae and with a short conidial chain of one to seven conidia in length formed on the conidiogenous site at each geniculate extension or tip of the conidiophores. Conidia (10.1 to 24.6 × 4.7 to 8.9 µm) were ovoid or elliptical (Table 3, Fig. 5). One isolate X1272 with those characteristics was identified as *A. rosae*.

Species distribution of *Alternaria* spp. Of the 283 *Alternaria* isolates obtained from blueberries, 61.5% were identified as *A. alternata*, 32.9% were *A. arborescens*, 5.0% were *A. tenuissima*, and only one isolate of *A. infectoria* and one isolate of *A. rosae* were found. *A. alternata* and *A. arborescens* were present at all five sampled locations. *A. tenuissima* was present at two locations (Kingsburg and Delano). *A. infectoria* and *A. rosae* were found only in Tulare (Table 1).

Fig. 3. Phylogenetic trees inferred from maximum parsimony (MP) and Bayesian analysis of *Alt a1* gene sequences for *Alternaria* spp. from blueberry and reference isolates of *Alternaria* spp. *Embellisia proteae* (EGS 39-031) was selected as outgroup. Isolates beginning with X were collected from blueberry in California and all other isolates used in the phylogenetic analyses are listed in Tables 1 and 2. Bootstrap support values (1,000 replicates) above 70% for MP and above 0.95 for Bayesian analysis are shown. Species are followed by strain numbers.

Temperature response. The *Alternaria* isolates were able to grow at temperature from 0 to 35°C (Fig. 6). Radial growth rate increased with temperature up to 25 or 30°C, and then decreased rapidly as temperature increased. Optimal radial growth occurred between 20 and 30°C. The average radial growth of *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. rosae* was 8.7, 9.7, 9.0, 11.9, and 10.1 mm per day at 25°C, respectively. At 40°C, mycelial growth was arrested, but growth resumed when culture of *A. infectoria* and *A. rosae* isolates were incubated at 22°C. However,

after 10 days of incubation at 40°C, all *A. alternata*, *A. tenuissima*, and *A. arborescens* isolates failed to resume growth after an additional 10 days of incubation at 22°C and were presumably dead.

Pathogenicity and virulence. ANOVA indicated that there were no significant differences between the results of the two experiments; therefore, the data were combined for further analyses. All inoculated fruit that were incubated at 4 and 20°C developed *Alternaria* rot symptoms, except 98% of the fruit inoculated with *A. rosae* and incubated at 20°C were decayed

Fig. 4. Phylogenetic trees inferred maximum parsimony (MP) and Bayesian analysis of combined sequences of plasma membrane ATPase, Calmodulin and *Alt al* genes for *Alternaria* spp. from blueberry and reference isolates of *Alternaria* spp. *Embellisia proteae* (EGS 39-031) was selected as outgroup. Isolates beginning with X were collected from blueberry in California and all other isolates used in the phylogenetic analyses are listed in Tables 1 and 2. Bootstrap support values (1,000 replicates) above 70% for MP and above 0.95 for Bayesian analysis are shown. Species are followed by strain numbers.

(Table 4). Decay developed slowly during the 6-week incubation at 0°C, but the majority of the fruit developed decay symptoms after 3 additional days at 20°C after removal from 0°C (Table 4). No decay developed on fruit in the control treatment, except 2% decay was observed in the control at 4°C (Table 4). The original fungi were reisolated from decayed fruit that were inoculated with respective isolates. Under the same incubation conditions, differences in lesion sizes (disease severity) on the fruit were not significantly different on the fruit inoculated with isolates among the *alternata*, *tenuissima*, or *arborescens* species-group (Table 4). In contrast, average disease severity for the isolates of *A. infectoria* and *A. rosae* was 1.7 and 1.8, respectively, which were significantly lower than those of isolates of *A. alternata*, *A. tenuissima*, and *A. arborescens* (Table 4).

DISCUSSION

This research presented the first study on the characterization of *Alternaria* spp. responsible for fruit rot of blueberry in California. Based on multiple DNA sequence comparisons and fungal morphological

characteristics, we documented that five *Alternaria* species, including *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. rosae*, are present in blueberry in California, and that *A. alternata* and *A. arborescens* are the dominant species present in all five sampling locations in the major blueberry production region in central California.

The species *A. tenuissima* has been reported to be the primary causal agent of *Alternaria* leaf spot and fruit rot in blueberry worldwide (Cline 1996; Greco et al. 2012; Milholland and Jones 1972; You et al. 2013), though *A. alternata* has also been reported to be associated with fruit rot of blueberry (Mehra et al. 2012). In our study, only 5% of the isolates were *A. tenuissima*, whereas 61.5 and 32.9% were *A. alternata* and *A. arborescens*, respectively. With the exception of *A. tenuissima*, *A. alternata*, *A. arborescens*, *A. infectoria*, and *A. rosae* are newly reported on blueberry in California. This is also the first report of *A. infectoria* and *A. rosae* causing *Alternaria* fruit rot on blueberry worldwide. Our results indicated that the species composition of *Alternaria* responsible for *Alternaria* fruit rot in blueberry can be dependent on geographical region. In the Central Valley of California which is the primary

TABLE 3. Conidia size and mycelial growth rate of *Alternaria* spp. from blueberry^z

Species	Isolate	Length of conidia (μm)	Width of conidia (μm)	Length/width	Growth rate at 22°C under 8 h light/16 h darkness (mm/day)
<i>Alternaria alternata</i>	X1020	22.62 ± 0.62 (14.60–31.70)	7.92 ± 0.17 (5.20–10.50)	2.88 ± 0.07 (2.00–4.00)	9.7 ± 0.20 (9.40–10.70) bc
	X1269	20.69 ± 0.47 (15.20–28.5)	7.62 ± 0.14 (5.80–)	2.74 ± 0.07 (1.80–4.50)	7.80 ± 0.64 (6.70–8.50) de
	X1048	23.02 ± 0.66 (16.10–36.20)	9.58 ± 0.22 (7.20–14.40)	2.46 ± 0.09 (1.60–4.10)	9.70 ± 0.91 (8.60–10.90) bc
	X1265	22.89 ± 0.53 (16.80–30.60)	9.39 ± 0.28 (6.40–15.00)	2.54 ± 0.10 (1.30–4.60)	7.90 ± 0.37 (6.70–9.00) de
	X1432	20.98 ± 0.53 (16.10–27.80)	8.99 ± 0.25 (6.70–14.60)	2.40 ± 0.07 (1.50–3.60)	10.20 ± 0.20 (9.70–10.70) b
	Mean	22.04 a	8.70 ab	2.60 a	9.06 B
<i>A. tenuissima</i>	X1125	22.02 ± 0.32 (13.90–36.60)	9.62 ± 0.12 (7.00–13.70)	2.35 ± 0.04 (1.30–3.60)	9.50 ± 0.22 (8.80–10.10) c
	X1068	20.20 ± 0.18 (15.90–26.7)	9.31 ± 0.12 (5.80–13.2)	2.25 ± 0.03 (1.40–3.60)	9.90 ± 0.21 (9.50–10.90) bc
	X1003	21.74 ± 0.24 (15.00–30.30)	10.15 ± 0.13 (6.50–4.20)	2.19 ± 0.03 (1.60–3.50)	11.10 ± 0.38 (10.00–12.30) a
	X1259	24.23 ± 0.42 (16.8–43.00)	8.35 ± 0.08 (5.80–10.40)	2.94 ± 0.05 (1.80–4.90)	10.20 ± 0.21 (9.70–10.90) b
	X1026	22.82 ± 0.23 (15.30–32.20)	8.30 ± 0.07 (6.00–10.60)	2.78 ± 0.03 (1.90–4.40)	8.30 ± 0.15(8.00–8.90) d
	Mean	22.18 a	9.14 a	2.50 ab	9.80 B
<i>A. arborescens</i>	X1276	21.23 ± 0.21 (16.20–28.50)	9.84 ± 0.13 (6.60–14.40)	2.16 ± 0.03 (1.2–4.00)	3.40 ± 0.36 (2.00–4.40) g
	X1263	19.43 ± 0.23 (13.50–28.50)	9.15 ± 0.11 (6.90–15.00)	2.16 ± 0.03 (1.50–3.10)	5.00 ± 0.45 (4.30–5.90) f
	X945	16.21 ± 0.19 (11.70–27.70)	7.84 ± 0.05 (6.40–9.70)	2.08 ± 0.03 (1.40–4.30)	2.7 ± 0.47 (1.20–4.50) h
	X1033	24.31 ± 0.30 (15.90–35.30)	9.00 ± 0.09 (6.60–12.20)	2.75 ± 0.04 (1.70–5.10)	7.50 ± 0.41 (6.30–9.10) e
	X100	23.74 ± 0.31 (14.90–33.40)	8.92 ± 0.09 (6.30–12.10)	2.72 ± 0.04 (1.60–4.50)	5.00 ± 0.29 (4.10–6.00) f
	Mean	20.96 ab	8.92 a	2.40 ab	4.72 C
<i>A. infectoria</i>	X1273	19.12 ± 1.33 (12.90–25.50)	7.76 ± 0.61 (5.30–10.90)	2.54 ± 0.27 (1.60–3.90)	10.20 ± 0.28 (9.30–11.00) b
	Mean	19.12 b	7.76 bc	2.54 a	10.20 AB
<i>A. rosae</i>	X1272	13.94 ± 1.41 (10.10–24.60)	6.82 ± 0.41 (4.70–8.90)	2.07 ± 0.22 (1.40–3.60)	11.10 ± 0.31 (10.10–11.90) a
	Mean	13.94 c	6.82 c	2.07 b	11.10 A

^z Values are average ± standard error (minimum–maximum), and values followed by the same letter within the same column are not significantly different ($P = 0.05$). Only means are compared for length, width, and length/width ratio among the species groups.

TABLE 4. Incidence (%) and severity of *Alternaria* rot on blueberry fruit inoculated with different *Alternaria* spp.^z

Species	Isolates	20°C for 6 days		4°C for 5 weeks		0°C for 6 weeks		3 days after removal from 0°C	
		Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity
<i>Alternaria alternata</i>	X1020	100.0 a	3.7 ± 0.2 c	100.0 a	4.0 ± 0.2 a	48.3 ± 10.1 bcd	0.5 ± 0.1 bc	98.3 ± 1.7 a	3.3 ± 0.3 a
	X1269	100.0 a	3.8 ± 0.1 bc	100.0 a	3.8 ± 0.1 a	71.7 ± 13.0 ab	1.2 ± 0.3 a	100.0 a	3.6 ± 0.2 a
<i>A. tenuissima</i>	X1026	100.0 a	4.0 ± 0.1 ab	100.0 a	3.9 ± 0.2 a	80.0 ± 12.9 a	1.1 ± 0.2 a	100.0 a	3.5 ± 0.3 a
	X1259	100.0 a	3.9 ± 0.2 bc	100.0 a	4.1 ± 0.1 a	28.3 ± 7.5 de	0.3 ± 0.1 cd	100.0 a	3.1 ± 0.1 a
<i>A. arborescens</i>	X100	100.0 a	3.9 ± 0.2 bc	100.0 a	2.8 ± 0.3 b	31.7 ± 10.5 de	0.4 ± 0.1 bcd	100.0 a	3.2 ± 0.3 a
	X1033	100.0 a	4.2 ± 0.2 a	100.0 a	3.9 ± 0.2 a	60.0 ± 10.6 bc	0.9 ± 0.2 ab	100.0 a	3.5 ± 0.1 a
<i>A. infectoria</i>	X1273	100.0 a	1.7 ± 0.1 d	100.0 a	2.1 ± 0.2 c	33.3 ± 8.5 cde	0.5 ± 0.1 bcd	100.0 a	1.7 ± 0.2 b
<i>A. rosae</i>	X1272	98.3 ± 1.7 b	1.8 ± 0.2 d	100.0 a	2.0 ± 0.2 c	21.7 ± 8.7 e	0.3 ± 0.1 cd	86.7±7.1 b	1.1 ± 0.1 c
Control		0 c	0 e	1.7 ± 1.7 b	0.1 ± 0.1 d	0 f	0 d	0 c	0 d

^z Severity was based on the lesion development on inoculated blueberry fruit using the following scale: 0, no decay; 1, lesion ≤ 12.5%; 2, 12.5% < lesion ≤ 25%; 3, 25% < lesion ≤ 50%; 4, 50% < lesion ≤ 75%; and 5, 75% < lesion ≤ 100%. Values followed by the same letter within the same column are not significantly different ($P = 0.05$).

region for production of a wide range of fruit and nut crops such as almond, citrus, and pistachio, blueberries are commonly grown in fields in close proximity to those crops. *A. alternata* is the cause of Alternaria leaf spot of almond and Alternaria late blight of pistachio, and *A. arborescens* and *A. tenuissima* have also been

reported on these crops in the region (Pryor and Michailides 2002; Teviotdale and Adaskaveg 2002). The similarity in *Alternaria* species composition between blueberry and the nut crops (pistachio and almond) in the region may suggest that these nut crops may be an important source of *Alternaria* inoculum contributing to the

Fig. 5. Morphological features of *Alternaria* spp. isolated from blueberry. **a to e**, Colony morphology on potato dextrose agar after 7 days under cool white fluorescent light with an 8 h photoperiod and 16 h darkness; **f to o**, sporulation pattern on potato carrot agar after 7 days under cool white fluorescent light with an 8 h photoperiod and 16 h darkness; **a, f, and k**, *A. alternata* isolate X1020; **b, g, and i**, *A. tenuissima* isolate X1259; **c, h, and m**, *A. arborescens* isolate X1033; **d, i, and n**, *A. infectoria* isolate X1273; and **e, j, and o**, *A. rosae* isolate X1272. Scale bar of f to j = 0.05 mm; scale bar of k to o = 10 μ m.

Fig. 6. Effect of temperature on radial mycelial growth rate (mm/day) of *Alternaria alternata* (X1269, X1020), *A. arborescens* (X100, X1033), *A. tenuissima* (X1026, X1259), *A. infectoria* (X1273), and *A. rosae* (X1272) on potato dextrose agar in the dark. Values are the means of pooled data from the two runs of the experiment.

establishment of *Alternaria* spp. population in blueberry in California as blueberry is a relatively new crop in the region compared with these nut crops (Jimenez et al. 2005).

Previously, identification of *Alternaria* spp. associated with blueberry, pistachio, and other crops has been largely based on morphological characters such as colony and sporulation morphology (Cline 1996; Greco et al. 2012; Milholland and Jones 1972; Pryor and Michailides 2002; You et al. 2013). Recently, DNA sequence-based analysis offers an increasingly important tool for speciation of *Alternaria*, particularly small-spored species (Andrew et al. 2009; Deng et al. 2012; Greco et al. 2012; Hong et al. 2005; Jasnic et al. 2011; Lawrence et al. 2013; Luan et al. 2007; Woudenberg et al. 2013; You et al. 2013). Plasma membrane ATPase, Calmodulin, and *Alt a1* genes were selected to identify *Alternaria* spp. in this study. All the isolates from blueberry in this study could be clearly distinguished from each other by analyzing the sequences of the plasma membrane ATPase gene. *A. arborescens*, *A. infectoria*, and *A. rosae* could also be differentiated by the sequences of *Alt a1* or Calmodulin, except that *A. alternata* and *A. tenuissima* were still in the same group. Although small-spored *Alternaria* spp. in this study can be largely differentiated by DNA sequence, morphological characters are still important in differentiating some species. The phylogenetic analysis from the plasma membrane ATPase gene indicated that some species including *A. resedae*, *A. turkisafrina*, *A. perangusta*, and *A. destruens* were clustered into the same group with *A. alternata* isolates from blueberry and the reference isolates of *A. alternata*, and those species could be differentiated from *A. alternata* by morphological characteristics. In addition, two isolates of *A. tenuissima* (BMP 0238 and BMP 0508) from GenBank were divided into the same group with *A. alternata* in this study and the reference isolates of *A. alternata*. Thus, the two isolates in the GenBank could be re-examined to verify their identity by morphological characters described by Simmons (2007). Of the several small-spored *Alternaria* spp. with morphological characteristics that overlap those of *A. alternata*, the most commonly known are *A. tenuissima* and *A. infectoria*, and the intermediate characteristics make them frequently misidentified as *A. alternata* (Roberts et al. 2000; Simmons and Roberts 1993). In the present study, colonies of *A. alternata* and *A. tenuissima* were similar, though the sporulation was usually different. Moreover, for those isolates without typical characteristics of *A. alternata*, *A. tenuissima*, and *A. arborescens*, they could not be distinguished easily. *A. arborescens* generally grew more slowly than other species, the margin of the colony often wavy and torn, the colors of colony were usually dark olive gray to castor gray. Moreover, sporulation of *A. arborescens* was distinct with numerous secondary to quaternary branching. Morphology and sporulation of *A. infectoria* and *A. rosae* were significantly different from those of the other species. Our results suggest that DNA sequence comparisons of multiple gene regions combined with morphological characteristics are important in speciation of *Alternaria* associated with blueberry.

Fruit inoculation studies showed that all five *Alternaria* species caused fruit rot on detached blueberry fruit stored at 0, 4, or 20°C, indicating that these species were pathogenic to blueberry fruit and can cause fruit rot at the storage temperature of 0°C. Although all five *Alternaria* spp. were pathogenic to blueberry fruit, the decay lesion sizes on the fruit inoculated with *A. infectoria* and *A. rosae* were significantly smaller than those on the fruit inoculated with *A. alternata*, *A. tenuissima*, or *A. arborescens*, suggesting that *A. infectoria* and *A. rosae* were less virulent to blueberry fruit than the other *Alternaria* species tested in this study. Similar observations have also been reported by Pryor and Michailides (2002) who documented that *A. infectoria* was less virulent on pistachio than other *Alternaria* species.

In summary, we documented in this study that *Alternaria* fruit rot in blueberry in California is caused by a species complex of

Alternaria, including *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. rosae*, with *A. alternata* and *A. arborescens* being the dominant species. *A. infectoria* and *A. rosae* were less virulent to blueberry fruit than other *Alternaria* species. As different species of *Alternaria* may respond to fungicides differently, the findings of this study will provide the basis for the development of chemical control practices targeting specific pathogens.

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