Identification and Enumeration of Small-Spored *Alternaria* Species Associated with Potato in the U.S. Northwest

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Abstract

Tymon, L. S., Peever, T. L., and Johnson, D. A. 2016. Identification and enumeration of small-spored *Alternaria* species associated with potato in the U.S. Northwest. Plant Dis. 100:465-472.

A number of *Alternaria* spp. have been isolated from potato worldwide but only *Alternaria solani* and *A. alternata* have been described as pathogenic to this host in the United States. These taxa are easily differentiated based on conidial morphology but species delimitation among the small-spored *Alternaria* spp. associated with potato are much more challenging. Accurate identification methods for small-spored *Alternaria* spp. are necessary so that a more thorough understanding of *Alternaria* epidemiology can be obtained. Isolations of *Alternaria* fungi from lesions on potato leaves collected in the U.S. Northwest were made between 2008 and 2011. Large-spored taxa (putatively *A. solani*), were isolated less frequently than small-spored taxa (putatively *A. alternata* sensu lato), except in 2010. *Colletotrichum coccodes* was isolated from necrotic lesions in 2008 to 2010 but not in 2011. Frequency of isolation ranged from 0.05 (5%) to 0.11 (11%) during the 3 years the fungus was detected. Anonymous genomic region

OPA1-3, previously used for *Alternaria* systematics, allowed for the discrimination of phylogenetic lineages among 210 small-spored isolates. When OPA1-3 was restricted using enzyme *ApaI*, 65 isolates (31%) displayed a restriction banding pattern consistent with previously characterized morphospecies *A. alternata* and *A. tenuissima* and 145 (69%) displayed a restriction banding pattern consistent with the previously characterized morphospecies *A. arborescens*. Morphological characterization of a subsample of 59 small-spored *Alternaria* isolates randomly selected with each restriction pattern was compared with phylogenetic lineage. In all, 54 (92%) isolates were consistently assigned to the same group by both methods. Three isolates exhibited conidial morphologies that were inconsistent with any described morphospecies. A small number of isolates were identified as *A. arbusi* (infectoria group) via sequencing of the glyceraldehyde-3-phosphate-dehydrogenase locus and BLAST searches.

A number of *Alternaria* spp. have been isolated from potato worldwide (Ardestani et al. 2010; Rodrigues et al. 2010; van der Waals et al. 2011) but only *Alternaria solani* and *A. alternata* have been described as pathogenic to this host in the United States. Early blight, caused by *A. solani* Sorauer 1896, is a major disease of potato in the U.S. Northwest that results in tan lesions with distinctive concentric rings on the foliage (Rotem 1994). Infection can reduce overall yields, tuber size, and tuber quality, and disease control practices can account for up to 10% of production costs (Stevenson et al. 2007). *A. solani* is recognized as the dominant pathogen infecting potato, although *A. alternata* (Fr.) Keissl. 1912 is increasingly affecting American potato production (Nolte 2008).

Infection by *A. alternata*, which causes brown spot, is characterized by small, irregular to circular lesions on leaves that can expand to approximately 3 mm². These lesions can coalesce, resulting in significant leaf damage (Kirk and Wharton 2012). Brown spot can be difficult to diagnose in the field because symptoms overlap with those of early blight (Kirk and Wharton 2012) and recovery of *A. alternata* from lesions does not necessarily confirm brown spot disease. *A. alternata* can behave as a saprophyte (Rotem 1994) and *A. alternata* was not considered a pathogen of potato until 1984, when *A. alternata* was isolated from potato leaf lesions and tubers in Israel (Droby et al. 1984a,b). Inoculum concentration and disease incidence were positively correlated and infection of potato tubers by *A. alternata* resulted in sunken, black-pitted lesions (Droby et al. 1984b). Young lesions caused by *A. alternata* can also be confused

with lesions caused by *Colletotrichum coccodes* (Wallr.) S. Hughes 1958, which causes black dot (Mohan et al. 1992).

Species delimitation in *Alternaria* is challenging due to a paucity of morphological characteristics and considerable morphological plasticity (Rotem 1994; Simmons 2007). Simmons arranged the genus *Alternaria* into 14 morphospecies based on morphological features, primarily the three-dimensional sporulation apparatus (Simmons 1992). In general, morphospecies classifications have been concordant with clades defined through molecular systematic analyses (Hong et al. 2005; Peever et al. 2004; Pryor and Bigelow 2003; Pryor and Gilbertson 2000). However, some morphospecies have been associated with more than one clade or multiple morphospecies associated with the same clade (Andrew et al. 2009; Lawrence et al. 2013; Peever et al. 2004; Pryor and Michailides 2002). Although molecular sequence data have made species identification more accurate and facilitated the determination of evolutionary relationships among *Alternaria* spp., phylogenetic resolution is highly dependent upon loci used for analyses (Hong et al. 2005; Peever et al. 2004; Pryor and Bigelow 2003; Pryor and Gilbertson 2000). Species designation within the small-spored *Alternaria* taxa is further confounded by the inability of sequence data to distinguish between two morphologically distinct fungi, *A. alternata* and *A. tenuissima* (Andrew et al. 2009; Hong et al. 2005; Pryor and Michailides 2002). A comprehensive survey using both morphological and molecular criteria is required to assess the significance of different small-spored *Alternaria* taxa associated with potato in the United States.

Accurate identification of fungi is critical for disease management. For example, populations of *A. solani* and *A. alternata* differed in resistance to specific fungicides in the Columbia Basin (Tymon and Johnson 2014), which necessitates different choices of fungicides and rotations of fungicides. Three small-spored *Alternaria* spp. that are difficult to distinguish from *A. alternata* based on morphology are *A. tenuissima*, *A. arborescens*, and *A. infectoria* (Andersen and Thrane 1996; Taralova et al. 2011). However, *A. tenuissima* and *A. infectoria* can be distinguished from *A. alternata* and *A. arborescens* based on key morphological features (Simmons 2007). *A. tenuissima*

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Accepted for publication 17 August 2015.

<http://dx.doi.org/10.1094/PDIS-03-15-0263-RE>
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Table 1. Species designation of small-spored *Alternaria* isolates from potato in the Pacific Northwest as *Alternaria alternata* or *A. arborescens* based on morphology and a restriction digest assay

Isolate	Location ^a	Species designation		GenBank accession number
		Morphology	Restriction digest ^b	
aa10_216	CB	<i>alternata</i>	<i>alternata/tenuissima</i>	...
aa10_351	SRV	<i>alternata</i>	<i>alternata/tenuissima</i>	...
aa08_21	CB	<i>arborescens</i>	<i>alternata/tenuissima*</i>	...
aa10_253	BF	<i>arborescens</i>	<i>alternata/tenuissima*</i>	...
aa11_300	SRV	<i>arborescens</i>	<i>alternata/tenuissima*</i>	...
aa08_38	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa09_12	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa09_172	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa09_60	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa09_182	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa09_225	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_14	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_20	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_30	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_32	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_91	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_105	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_121	SRV	<i>arborescens</i>	<i>arborescens</i>	...
aa10_174	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_207	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_212	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa10_280	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa10_281	SRV	<i>arborescens</i>	<i>arborescens</i>	...
aa10_284	SRV	<i>arborescens</i>	<i>arborescens</i>	...
aa10_304	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa10_310	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa10_312	SRV	<i>arborescens</i>	<i>arborescens</i>	...
aa11_14	OR	<i>arborescens</i>	<i>arborescens</i>	...
aa11_20	OR	<i>arborescens</i>	<i>arborescens</i>	...
aa11_31	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_41	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_43	CB	<i>arborescens</i>	<i>arborescens</i>	KM589065.1
aa11_45	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_110	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_115	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_206	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa11_234	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_238	CB	<i>arborescens</i>	<i>arborescens</i>	...
aa11_253	SRV	<i>arborescens</i>	<i>arborescens</i>	...
aa11_257	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa11_232	BF	<i>arborescens</i>	<i>arborescens</i>	...
aa11_352	OR	<i>arborescens</i>	<i>arborescens</i>	...
aa10_214	BF	<i>tenuissima</i>	<i>arborescens*</i>	...
aa11_290	BF	<i>tenuissima</i>	<i>arborescens*</i>	...
aa09_211	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_21	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_28	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	KM5890661.1
aa10_170	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_180	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_195	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_198	BF	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_224	BF	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_231	BF	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_263	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_273	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa10_317	BF	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa11_09	OR	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa11_72	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa11_229	CB	<i>tenuissima</i>	<i>alternata/tenuissima</i>	...
aa08_33	CB	...	<i>alternata/tenuissima</i>	...
aa08_37	CB	...	<i>alternata/tenuissima</i>	...

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^a Location of isolate collection: CB = Columbia Basin, WA; BF = Bonner's Ferry, ID; SRV = Snake River Valley, ID; OR = LaGrande, OR; MI = Three Rivers, MI; and CO = Alamosa, CO.

^b An asterisk (*) indicates that species designation based upon morphology and restriction digest were inconsistent. DNA was reextracted from colonies used in the morphological assay and restriction digest was performed a second time. Results from the two restriction digest assays were consistent.

Table 1. (continued from preceding page)

Isolate	Location ^a	Species designation		GenBank accession number
		Morphology	Restriction digest ^b	
aa09_63	CB	...	<i>alternata/tenuissima</i>	...
aa10_36	CB	...	<i>alternata/tenuissima</i>	...
aa10_43	CB	...	<i>alternata/tenuissima</i>	...
aa10_49	CB	...	<i>alternata/tenuissima</i>	...
aa10_50	CB	...	<i>alternata/tenuissima</i>	...
aa10_88	CB	...	<i>alternata/tenuissima</i>	...
aa10_103	CB	...	<i>alternata/tenuissima</i>	...
aa10_104	CB	...	<i>alternata/tenuissima</i>	...
aa10_160	CB	...	<i>alternata/tenuissima</i>	...
aa10_184	CB	...	<i>alternata/tenuissima</i>	...
aa10_193	CB	...	<i>alternata/tenuissima</i>	...
aa10_252	BF	...	<i>alternata/tenuissima</i>	...
aa10_270	BF	...	<i>alternata/tenuissima</i>	...
aa10_313	BF	...	<i>alternata/tenuissima</i>	...
aa10_327	BF	...	<i>alternata/tenuissima</i>	KM5 89063.1
aa10_345	BF	...	<i>alternata/tenuissima</i>	...
aa11_13	CB	...	<i>alternata/tenuissima</i>	...
aa11_21	OR	...	<i>alternata/tenuissima</i>	...
aa11_28	CB	...	<i>alternata/tenuissima</i>	...
aa11_33	CB	...	<i>alternata/tenuissima</i>	...
aa11_37	CB	...	<i>alternata/tenuissima</i>	...
aa11_42	CB	...	<i>alternata/tenuissima</i>	...
aa11_47	CB	...	<i>alternata/tenuissima</i>	...
aa11_114	CB	...	<i>alternata/tenuissima</i>	...
aa11_188	BF	...	<i>alternata/tenuissima</i>	...
aa11_245	BF	...	<i>alternata/tenuissima</i>	...
aa11_251	BF	...	<i>alternata/tenuissima</i>	...
aa11_282	CB	...	<i>alternata/tenuissima</i>	...
aa11_298	CB	...	<i>alternata/tenuissima</i>	...
aa11_299	CB	...	<i>alternata/tenuissima</i>	...
aa11_301	BF	...	<i>alternata/tenuissima</i>	...
aa11_317	SRV	...	<i>alternata/tenuissima</i>	...
aa11_329	MI	...	<i>alternata/tenuissima</i>	...
aa11_332	MI	...	<i>alternata/tenuissima</i>	...
aa11_337	OR	...	<i>alternata/tenuissima</i>	...
aa11_342	OR	...	<i>alternata/tenuissima</i>	...
aa11_348	MI	...	<i>alternata/tenuissima</i>	...
Othello_2011	CB	...	<i>alternata/tenuissima</i>	...
r011_r25e	OR	...	<i>alternata/tenuissima</i>	...
ro11_r19a	CB	...	<i>alternata/tenuissima</i>	...
ro11_r38d	OR	...	<i>alternata/tenuissima</i>	...
ro11_r4_R	OR	...	<i>alternata/tenuissima</i>	...
ro11_r4B	OR	...	<i>alternata/tenuissima</i>	...
aa09_04	CB	...	<i>arborescens</i>	...
aa09_28	BF	...	<i>arborescens</i>	...
aa09_50	CB	...	<i>arborescens</i>	...
aa09_54	CB	...	<i>arborescens</i>	...
aa09_61	CB	...	<i>arborescens</i>	...
aa09_62	CB	...	<i>arborescens</i>	...
aa09_63	CB	...	<i>arborescens</i>	...
aa09_64	CB	...	<i>arborescens</i>	...
aa09_75	CB	...	<i>arborescens</i>	...
aa09_76	CB	...	<i>arborescens</i>	...
aa09_131	CB	...	<i>arborescens</i>	...
aa09_148	CB	...	<i>arborescens</i>	...
aa09_167	CB	...	<i>arborescens</i>	...
aa09_171	BF	...	<i>arborescens</i>	...
aa09_192	BF	...	<i>arborescens</i>	...
aa09_220	CB	...	<i>arborescens</i>	...
aa10_05	CB	...	<i>arborescens</i>	...
aa10_06	CB	...	<i>arborescens</i>	...
aa10_07	CB	...	<i>arborescens</i>	...
aa10_08	CB	...	<i>arborescens</i>	...
aa10_10	CB	...	<i>arborescens</i>	...
aa10_12	CB	...	<i>arborescens</i>	...

(continued on next page)

Table 1. (continued from preceding page)

Isolate	Location ^a	Species designation		GenBank accession number
		Morphology	Restriction digest ^b	
aa10_19	CB	...	<i>arborescens</i>	...
aa10_27	CB	...	<i>arborescens</i>	...
aa10_36	CB	...	<i>arborescens</i>	...
aa10_37	CB	...	<i>arborescens</i>	...
aa10_44	CB	...	<i>arborescens</i>	...
aa10_45	CB	...	<i>arborescens</i>	...
aa10_46	CB	...	<i>arborescens</i>	...
aa10_48	CB	...	<i>arborescens</i>	...
aa10_69	CB	...	<i>arborescens</i>	...
aa10_107	CB	...	<i>arborescens</i>	...
aa10_146	CB	...	<i>arborescens</i>	...
aa10_157	SRV	...	<i>arborescens</i>	...
aa10_160	CB	...	<i>arborescens</i>	...
aa10_172	CB	...	<i>arborescens</i>	...
aa10_199	BF	...	<i>arborescens</i>	...
aa10_203	CB	...	<i>arborescens</i>	...
aa10_205	CB	...	<i>arborescens</i>	...
aa10_206	BF	...	<i>arborescens</i>	...
aa10_209	BF	...	<i>arborescens</i>	...
aa10_210	BF	...	<i>arborescens</i>	...
aa10_22	CB	...	<i>arborescens</i>	...
aa10_223	SRB	...	<i>arborescens</i>	...
aa10_225	BF	...	<i>arborescens</i>	...
aa10_228	BF	...	<i>arborescens</i>	...
aa10_235	BF	...	<i>arborescens</i>	...
aa10_236	CB	...	<i>arborescens</i>	...
aa10_239	CB	...	<i>arborescens</i>	...
aa10_243	CB	...	<i>arborescens</i>	...
aa10_300	BF	...	<i>arborescens</i>	...
aa10_305	BF	...	<i>arborescens</i>	...
aa10_306	BF	...	<i>arborescens</i>	...
aa10_307	BF	...	<i>arborescens</i>	KM589064.1
aa10_314	BF	...	<i>arborescens</i>	...
aa10_328	BF	...	<i>arborescens</i>	...
aa10_335	SRV	...	<i>arborescens</i>	...
aa10_344	BF	...	<i>arborescens</i>	...
aa10_347	BF	...	<i>arborescens</i>	...
aa10_348	BF	...	<i>arborescens</i>	...
aa10_350	BF	...	<i>arborescens</i>	...
aa10_352	CB	...	<i>arborescens</i>	...
aa11_16	CB	...	<i>arborescens</i>	...
aa11_32	CB	...	<i>arborescens</i>	...
aa11_34	CB	...	<i>arborescens</i>	...
aa11_39	CB	...	<i>arborescens</i>	...
aa11_40	CB	...	<i>arborescens</i>	...
aa11_44	CB	...	<i>arborescens</i>	...
aa11_49	CB	...	<i>arborescens</i>	...
aa11_59	CB	...	<i>arborescens</i>	...
aa11_77	CB	...	<i>arborescens</i>	...
aa11_80	CB	...	<i>arborescens</i>	...
aa11_109	CB	...	<i>arborescens</i>	...
aa11_113	CB	...	<i>arborescens</i>	...
aa11_152	CB	...	<i>arborescens</i>	...
aa11_154	CB	...	<i>arborescens</i>	...
aa11_161	CB	...	<i>arborescens</i>	KM589067.1
aa11_183	SRV	...	<i>arborescens</i>	...
aa11_188	BF	...	<i>arborescens</i>	...
aa11_205	BF	...	<i>arborescens</i>	...
aa11_225	CB	...	<i>arborescens</i>	...
aa11_229	CB	...	<i>arborescens</i>	...
aa11_244	BF	...	<i>arborescens</i>	...
aa11_246	BF	...	<i>arborescens</i>	...
aa11_251	BF	...	<i>arborescens</i>	...
aa11_259	BF	...	<i>arborescens</i>	...
aa11_29	CB	...	<i>arborescens</i>	...

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Table 1. (continued from preceding page)

Isolate	Location ^a	Species designation		GenBank accession number
		Morphology	Restriction digest ^b	
aa11_296	MT	...	<i>arborescens</i>	...
aa11_297	CB	...	<i>arborescens</i>	...
aa11_302	BF	...	<i>arborescens</i>	...
aa11_303	SRV	...	<i>arborescens</i>	...
aa11_305	SRV	...	<i>arborescens</i>	...
aa11_307	SRV	...	<i>arborescens</i>	...
aa11_311	SRV	...	<i>arborescens</i>	...
aa11_313	MI	...	<i>arborescens</i>	...
aa11_314	SRV	...	<i>arborescens</i>	...
aa11_316	SRV	...	<i>arborescens</i>	...
aa11_318	CB	...	<i>arborescens</i>	...
aa11_320	BF	...	<i>arborescens</i>	...
aa11_323	CB	...	<i>arborescens</i>	...
aa11_330	CO	...	<i>arborescens</i>	...
aa11_338	ND	...	<i>arborescens</i>	...
aa11_343	CB	...	<i>arborescens</i>	...
r011_r35a	OR	...	<i>arborescens</i>	...
ro11_r17	OR	...	<i>arborescens</i>	...
ro11_r46B	OR	...	<i>arborescens</i>	...

produces conidia in long, unbranched chains whereas *A. infectoria* produces distinct secondary conidiophores (Simmons 2007). It is important to differentiate among these fungi because some small-spored species produce toxins that are involved in pathogenicity, while *A. infectoria* does not. AAL-toxin, produced by *A. arborescens*, affects mitochondria and rough endoplasmic reticulum of tomato (Rotem 1994), resulting in the formation of stem lesions as well as necrosis in leaf tissue (Gilchrist et al. 1995; Thomma 2003).

Small-spored *Alternaria* spp. associated with potato in the U.S. Northwest have been poorly studied and, therefore, are the primary focus of this study. Due to the number of small-spored *Alternaria* spp. that potentially can cause foliar lesions on potato, the objectives of this research were to (i) determine the frequency of different *Alternaria* taxa and *C. coccodes* associated with necrotic lesions on potato foliage in the U.S. Northwest and (ii) distinguish between *A. alternata* and the potential host-specific, toxin-producing *A. arborescens*. Discriminating among *Alternaria* spp. on potato in the U.S. Northwest will allow for future research on targeted management with fungicides and cultural tactics as well as providing further understanding of potential pathogen epidemiology.

Materials and Methods

Sampling and isolation. Potato fields in Washington and Idaho are usually managed with fungicides, which reduce the incidence of infection by *Alternaria* spp. When potato leaves with characteristic early blight and brown spot lesions were observed, leaves were collected. Leaves were collected by lab members, field managers, and Washington State extension agents from potato fields in the Columbia Basin of Washington and Oregon, the Skagit Valley of Washington, the Snake River Valley in Idaho, and near Bonner's Ferry, ID between 2008 and 2011 (Table 1). Foliage was placed in plastic bags, sealed, and transported to a laboratory in Pullman, WA. Leaves were stored at 4°C until processed. Potato leaflets with lesions were surface disinfested by dipping in 70% ethanol for 3 to 5 s. Leaflets were blotted with paper towel and air dried. One to three lesions per leaf (one lesion per leaflet) were sampled. Lesion margins were excised and plated on modified potato dextrose agar (mPDA; Difco Bactoagar at 20 g/liter, Difco PDA at 1 g/liter, and 95% ethanol at 6 ml/liter). Isolates were examined under a stereomicroscope and were assigned to *A. solani*, small-spored *Alternaria*, or *C. coccodes* based on morphological characteristics. Single conidial isolates were obtained by streaking a conidial suspension on mPDA. A single conidium was selected after 24 h. Colonies were grown on mPDA at 21 to 23°C under continuous light. Isolates were grown

on mPDA for 7 to 10 days and stored on sterile filter paper, as previously described (Peever et al. 1999), for future inoculation experiments. Filter paper was placed inside sterile coin envelopes and maintained at room temperature.

DNA extraction. *Alternaria* isolates were grown in Difco potato dextrose broth (Becton, Dickinson and Company, Franklin Lakes, NJ) for 1 week and vacuum filtered, and mycelia were lyophilized for 48 h. DNA was extracted using a modified phenol chloroform extraction (Dobinson 1995; Lee et al. 1990). Mycelia were vortexed in 2.0-ml microfuge tubes (Fisherbrand Premium; Fisher Scientific, Inc., Hampton, New Hampshire) with one ceramic bead (MP Biomedicals, LLC, Santa Ana, CA) until mycelia were ground to a fine powder. Lysis buffer (200 µl of 0.5 M NaCl, 0.2 M Tris-HCl [pH 8.0], 0.01 M EDTA, and 1% sodium dodecyl sulfate) were added to the mixture. Powdered mycelia were then vigorously vortexed for 3 min at 6- to 30-s intervals, where cells were placed on ice every 30 s. An additional 200 µl of lysis buffer was added to the mycelia and briefly vortexed. Phenol-chloroform (300 µl, 1:1) was added and the sample was vortexed for 30 s. The sample was centrifuged at 12,000 rpm for 5 min to separate the phases. Phenol-chloroform (300 µl) was added to the aqueous phase and the sample was vortexed for 30 s. The aqueous phase was separated once more by centrifugation and a nucleic acid pellet was precipitated with 200 µl of 95% ethanol and placed on ice for 5 min. This step was repeated without placing the sample on ice. The pellet was then washed with 200 µl of 70% ethanol. RNA was digested by 2 µl of RNase (1 mg/ml) to the DNA and incubated at 37°C overnight.

Polymerase chain reaction amplification and restriction digest assay. In all, 210 isolates were subsampled from 940 putative *A. alternata* and *A. arborescens* isolates for the restriction digest assay. Isolates with distinct secondary conidiophores were eliminated from this study because this is a characteristic of morphospecies *A. infectoria* (Simmons 2007) and beyond the scope of this assay. An anonymous 900-bp region, OPA1-3, was amplified using polymerase chain reaction (PCR) and digested with a restriction enzyme to identify *A. arborescens* (Peever et al. 2004). At this locus, *A. alternata* has two restriction sites, resulting in three bands, whereas isolates of *A. arborescens* have only one restriction site. PCR was performed using 25-µl reaction volumes in thin-walled 0.2-ml PCR strip tubes. Reaction mixes comprised 2.0 µl of 10× PCR buffer (NEB, Waltham, MA), 0.48 µM MgCl₂, 2.4 µM dNTP (Thermo Fisher Scientific, Waltham, MA), 0.1 µM each primer (Invitrogen Life Technologies, Carlsbad, CA), 1 U of *Taq* polymerase (NEB), and 20 ng of DNA. Reactions were run using a

touchdown PCR protocol on a Bio-Rad MyCycler Personal Thermal Cycler (Hercules, California). Thermal cycler conditions were 4 min at 94°C (denaturation) followed by 18 cycles of 45 s at 94°C, 45 s at 61°C (with the temperature dropping 0.5°C each cycle), and 2 min at 72°C; followed by 20 cycles of 45 s at 94°C, 45 s at 53°C, and 2 min at 72°C; with a final extension cycle of 10 min at 72°C. Amplicons were run on a 1% agarose gel to confirm amplification. Amplicons were restricted with *Apal* (Invitrogen Life Technologies) in 15- μ l reaction volumes using 1 μ l of enzyme and 2 μ l of buffer per reaction. Samples were incubated at 37°C overnight and digested DNA was visualized on 1% agarose gels. Because a restriction digest of OPA1-3 cannot distinguish *A. alternata* from *A. tenuissima*, isolates were designated as either *A. arborescens* or *A. alternata/tenuissima* (Table 1). The OPA1-3 region of five representative isolates was amplified and sequenced to confirm identity of the isolates and integrity of the restriction digest. Sequences were aligned using Clustal W in Geneious 8.0 with reference sequences of *A. alternata* (GenBank accession number JQ859819.1), *A. tenuissima* (GenBank accession number JQ859820.1), and *A. arborescens* (GenBank accession number JQ859821.1). All reference sequences selected were from isolates identified by E. G. Simmons.

Sequencing. PCR cleanup was performed by adding 1.5 μ l of Exosap-It (Affymetrix, Santa Clara, CA) to 6 μ l of PCR product and incubating at 37°C for 15 min followed by an incubation at 80°C for 15 min. DNA sequencing was performed by Elim Biopharm (Hayward, CA). BLAST searches were performed to the National Center for Biotechnology Information nucleotide sequence databases to identify the closest sequence matches.

Glyceraldehyde-3-phosphate-dehydrogenase amplification and sequencing. Three isolates exhibited conidial morphology that was inconsistent with *A. alternata*, *A. tenuissima*, or *A. arborescens*. Amplification of glyceraldehyde-3-phosphate-dehydrogenase (*G3PD*) was performed using *gpd1* and 2 primers (Berbee et al. 1999) because the *G3PD* locus is sufficient at distinguishing among different small-spored *Alternaria* groups (Pryor and Bigelow 2003). PCR

was performed using 25- μ l reaction volumes in thin-walled 0.2-ml PCR strip tubes. Reaction mixes comprised 2.0 μ l of 10 $\times$ PCR buffer (NEB), 0.48 μ M MgCl₂, 2.4 μ M dNTPs (Thermo Fisher Scientific), 0.1 μ M each primer (Invitrogen Life Technologies), 1 unit of *Taq* polymerase (NEB), and 20 ng of DNA. Cycling was performed in a Techne TC-3000X Thermal Cycler (Burlington, NJ). For amplification of *G3PD*, cycles consisted of 4 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at 57°C, and 2 min at 72°C, with a final extension cycle of 10 min at 72°C.

Sequences were aligned using the Clustal W package (EMBL-EBI, Hixton, Cambridge, UK) in Geneious 8.0 (Biomatters, Auckland, NZ) with reference sequences of *A. alternata* (GenBank accession number AY278808.1), *A. arborescens* (GenBank accession number GQ180070.1), *A. arbusti* (GenBank accession number JQ693621), *A. arbusti* (GenBank accession number FJ214806.1), *A. oregonensis* (GenBank accession number AY762957.1), and *A. metachromatica* (GenBank accession number AY762956.1). All reference sequences selected were from isolates identified by E. G. Simmons.

Morphological characterization. Morphological characterization of isolates was conducted to estimate the stability of characteristics used to separate *A. arborescens* from *A. alternata*. A randomly selected subsample of 59 isolates identified as *A. alternata* or *A. arborescens* by the restriction digest assay were morphologically characterized on potato carrot agar, as prepared according to Simmons (2007). In all, 20 g of Yukon gold potato and 20 g of carrot were autoclaved in 100 ml of distilled water for 30 min. The potato and carrot were then sieved through a number 10 USA Standard Test Sieve (WS Tyler Industrial Group, Mentor, OH). The mashed potato and carrot were added to 1 liter of distilled H₂O and 20 g of Difco BactoAgar (Becton, Dickinson and Company) and autoclaved for 20 min. Autoclaved medium was cooled and poured into petri dishes (60 by 15 mm; Greiner, Frickenhausen, Germany).

In total, 59 individual isolates were plated in the center of a petri dish and maintained under a cycle of 8 h of fluorescent light and 16 h of darkness at a mean temperature of 24°C in light conditions

Fig. 1. Frequency of isolation of *Alternaria* spp. from early blight and brown spot lesions on potato in the U.S. Northwest. Frequency of isolation is given as a proportion of the total isolates collected in one year.

and 23°C in dark conditions for 5 to 8 days. Fungi were mounted in lactic acid on slides using double-sided tape, where one side of the tape was touched gently to the fungal culture and the other side adhered to the slide. Conidia and conidiophores were examined with light microscopy on an Olympus BH2 compound microscope (Olympus Corp., Shinjuku, Tokyo) and photographed using a Nikon Coolpix 4300 (Nikon Corp., Tokyo) camera. Morphospecies assignments were based on descriptions of *Alternaria* spp. by Simmons (2007).

Results

Isolate collection and frequency of isolation. Small-spored *Alternaria* taxa were isolated more frequently than *A. solani* from leaves with lesions in all years except 2010. *C. coccodes* was isolated from necrotic lesions in 2008 to 2010 but not in 2011 (Fig. 1). Frequency of isolation ranged from 0.05 (5%) to 0.11 (11%) during the 3 years the fungus was detected. Data were not collected on coinfection of lesions with *A. alternata* and either *A. solani* or *C. coccodes* in 2008. Coinfection of lesions was observed in 2009 to 2011, although at infection frequencies of 0.33 or less.

OPA1-3 restriction digest, *G3PD* amplification, and species identification. The anonymous region of randomly selected small-spored *Alternaria* isolates was amplified and restricted with *Apa*I. In all, 65 (31%) of 210 isolates had a restriction-banding pattern that was consistent with *A. alternata/tenuissima* and 145 (69%) had a restriction-banding pattern that was consistent with *A. arborescens*. Four isolates designated as *A. alternata* or *A. arborescens* by the restriction digest were confirmed with sequence data when the OPA1-3 region of six isolates was amplified and compared with sequences in GenBank.

Three isolates exhibited conidial morphology that was inconsistent with any described morphospecies. Sequencing of *G3PD* and BLAST searches identified these isolates as *A. arbusti* (KM589057.1, KM589056.1, and KM589054.1).

Morphological characterization. *A. arbusti* isolates (Fig. 2) were distinguishable from *A. alternata* and *A. arborescens* (Fig. 3) based on morphology. *A. arbusti* isolates produced aerial hyphae that gave the colony a cottony appearance. Conidia of *A. arbusti* are produced in short chains and, with the exception of the terminal conidium,

produce apical secondary conidiophores (Simmons 2007). Of 59 isolates, 2 (3%) were putatively designated as morphospecies *A. alternata*, 40 (68%) were designated as *A. arborescens*, and 17 (29%) were designated as *A. tenuissima* based on branching pattern, conidial length and width, and conidiophore length (Table 1). However, the restriction digest assay determined that five isolate identifications were inconsistent between the morphological determination and restriction digest assay. Three isolates were designated as *A. arborescens* based on morphology but as *A. alternata/tenuissima* based on the restriction digest, and two isolates were designated as *A. tenuissima* based on the morphology but as *A. arborescens* based on the restriction digest. DNA was reextracted and the restriction digest assay was run to confirm that the inconsistencies observed were not due to isolate contamination.

Discussion

Morphology is the traditional means of distinguishing small-spored *Alternaria* spp. However, the morphological characteristics used to identify *Alternaria* spp. are plastic and susceptible to environmental variation, making species designation difficult. For example, *A. brassicae* forms conidia in chains when grown in pure culture but not when growing on the host (Rotem 1994). Substrate, temperature, and light can affect the dimensions of the spores, which often overlap among species, preventing accurate identification on the basis of spore dimensions. Lowered humidity or temperature during conidial development can increase conidial size (Misaghi et al. 1978). Five isolate identifications were inconsistent between the morphological determination and restriction digest assay. As with other species of *Alternaria*, *A. arborescens* morphology is plastic. When isolates are grown under the recommended conditions of light and darkness (Simmons 2007), conidiophores grown in light-exposed areas are shorter, up to 200 to 300 µm, whereas conidiophores grown in the dark are between 800 µm and 1 mm or longer (Simmons 2007). This can lead to incorrectly identifying an *A. arborescens* isolate as *A. alternata*. Two isolates were designated as *A. tenuissima* based on the morphology but as *A. arborescens* based on the restriction digest. Further sequencing will be conducted to assess whether this inconsistency is due to morphological variation of the *A. arborescens* or if the isolate is a morphologically similar species to *A. tenuissima*.

The anonymous region OPA1-3 was one of three regions that has been shown to reliably distinguish among taxa of small-spored *Alternaria* spp., particularly within the *alternata* clade (Peever et al. 2004). The use of this restriction digest assay represented a robust means to distinguishing between morphospecies *A. arborescens* and *A. alternata*, particularly if key morphological features are not apparent. The assay is also rapid, allowing for many isolates to be assessed at one time, and is much more economical than sequencing. The accuracy of the assay was confirmed by sequencing of the OPA1-3 region.

Three isolates were identified as *A. arbusti*, which clusters within the infectoria clade (Lawrence et al. 2014). Along with being genetically distinct, past studies on fungal metabolite production by *A. alternata* and *A. infectoria* species show that metabolite profiles are also different

Fig. 2. *Alternaria arbusti* A, conidia and B, conidiophore. Scale bars = 34 µm.

Fig. 3. Conidia and conidiophores of A, *Alternaria alternata*, scale = 35 µm; B, *A. arborescens*, scale = 53 µm; and C, *A. tenuissima*, scale = 53 µm.

(Andersen and Thrane 1996). Species clustered within the infectoria clade are not known to produce toxins, although recent data have shown that isolates of *A. infectoria* from wheat produce more metabolites than previously observed (Oviedo et al. 2013).

The lack of stability in morphological characteristics among environments continues to make *Alternaria* taxonomy a challenge. Because of this confusion, species are often grouped together and evaluated as one. Grouping of all small-spored *Alternaria* spp. as *A. alternata* can mask subtle differences in species biology and epidemiology. For example, this will limit proper determination of host ranges for many of these species. *A. arbusi* and *A. arborescens* may be examples of fungi isolated on potato that have been previously misidentified as *A. alternata*. *A. arbusi* was first isolated from *Pyrus pyrifolia* and has also been isolated from Rainier cherry in Washington (Simmons 1993), which are grown in close proximity to potato in the Columbia Basin of Washington. In this study, *A. arborescens* was isolated more frequently than *A. alternata* from lesions on potato, which is the predominant small-spored *Alternaria* sp. isolated from potato (Boiteux and Reifschneider 1994; Drobey et al. 1984b; Hausladen 2006; van der Waals et al. 2011). *A. arborescens* has been isolated from potato in Iran (Ardestani et al. 2010); however, it has not yet been reported on potato in the United States.

More species of *Alternaria* pathogens have been shown to be associated with potato in the U.S. Northwest than previously thought. Accurate identification methods are necessary so that a more thorough understanding of *Alternaria* epidemiology can be obtained. Although morphology is capable of distinguishing among morphologically distinct small-spored *Alternaria* taxa, molecular techniques have helped to rapidly and accurately distinguish among morphologically similar species when appropriate loci are used. The results of these molecular assays for species designation will facilitate future research, such as population genetics studies, that may reveal other discrete characteristics of the species as related to disease spread or management.

Acknowledgments

We thank M. Pavsek, G. Vandemark, F. Dugan, and D. Wheeler for critical reviews of the manuscript; D. Drader, M. Hubbard, and J. Miller for providing potato leaf samples; and the Washington State Potato Commission for partial funding support.

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