Gene Genealogies Reveal High Nucleotide Diversity and Admixture Haplotypes Within Three *Alternaria* Species Associated with Tomato and Potato

Tika B. Adhikari,^{1,†} Thomas Ingram,¹ Dennis Halterman,² and Frank J. Louws^{1,3,†}

¹ Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695

² United States Department of Agriculture-Agricultural Research Service, Vegetable Crops Research Unit, Madison, WI 53706

³ Department of Horticultural Science, North Carolina State University, Raleigh, NC 27695

Accepted for publication 22 March 2020.

ABSTRACT

Early blight (EB) and leaf blight are two destructive diseases of tomato in North Carolina (NC), caused by *Alternaria linariae* and *A. alternata*, respectively. During the last decade, EB caused by *A. solani* has increased in potato-producing areas in Wisconsin (WI). We collected 152 isolates of three *Alternaria* spp. associated with tomato and potato in NC and WI and used the gene genealogical approach to compare the genetic relationships among them. Two nuclear genes: the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*), RNA polymerase second largest subunit (*RPB2*), and the rDNA internal transcribed spacer (*ITS*) region of these isolates were sequenced. Besides, sequences of the *GPDH* locus from international isolates described in previous studies were included for comparison purposes. A set of single nucleotide polymorphisms was assembled to identify locus-specific and species-specific haplotypes. Nucleotide diversity varied among gene sequences and species analyzed. For example, the estimates of nucleotide diversity and Watterson's theta were higher in *A. alternata* than in *A. linariae* and *A. solani*. There was little or no

polymorphisms in the *ITS* sequences and thus restricted haplotype placement. The *RPB2* sequences were less informative to detect haplotype diversity in *A. linariae* and *A. solani*, yet six haplotypes were detected in *A. alternata*. The *GPDH* sequences enabled strongly supported phylogenetic inferences with the highest haplotype diversity and belonged to five haplotypes (AaH1 to AaH5), which consisted of only *A. alternata* from NC. However, 13 haplotypes were identified within and among *A. linariae* and *A. solani* sequences. Among them, six (AsAIH1 to AsAIH6) were identical to previously reported haplotypes in global samples and the remaining were new haplotypes. The most divergent haplotypes were AaH1, AsAIH2/AsAIH3, and AsAIH4 and consisted exclusively of *A. alternata*, *A. linariae*, and *A. solani*, respectively. Neutrality tests suggested an excess of mutations and population expansion, and selection may play an important role in nucleotide diversity of *Alternaria* spp.

Keywords: mycology, population biology

Tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) represent important vegetables in North Carolina (NC) and Wisconsin (WI), respectively. Most of the tomato production is in western NC and sporadic in eastern NC and the Piedmont region. On the other hand, potato is a major vegetable in WI, which is the third largest potato-producing state in the country (USDA-NASS 2018). Commercial potato cultivars grown in WI are Russet, White, Red, Yellow, Blue, and Fingerling. Different *Alternaria* species (spp.) were found to be associated with early blight (EB) of tomato and potato (Rotem 1994; Simmons 2000). Based on morphology, *Alternaria* specimens from Solanaceae were revised into several new species including *A. grandis* (Simmons 2000, 2007). Besides *A. solani* sensu stricto (Ell. and Mart.) Jones and Grout and *A. grandis* from potato, a new species called *A. tomatophila* was identified to cause EB on tomato (Simmons 2007). Later, this species was named *A. linariae* (Neerg.) Simmons infecting Solanaceae, Cucurbitaceae, and Scrophulariaceae (Woudenberg et al. 2014). *A. alternata* sensu lato (Fr.) Keissler has been repeatedly isolated from Solanaceae with symptoms resembling that of EB. Although *A. alternata* causes brown leaf spot on potato (Ding et al. 2019; Droby et al. 1984) and leaf blight and stem canker on tomato (Grogan et al. 1975), both

diseases pose a serious challenge for tomato and potato growers due to the lack of effective resistant cultivars (Adhikari et al. 2017).

DNA sequencing approaches have provided data for both gene genealogies and phylogenetic analyses of genetic variations in different fungi (Brewer and Milgroom 2010; O'Donnell et al. 2000; Peever et al. 2002; Zaffarano et al. 2009). Gene genealogies have provided the evolutionary history and genetic relationships among and within populations in some fungi (Brewer and Milgroom 2010; O'Donnell et al. 2000; Zaffarano et al. 2009) including *A. alternata* from citrus (Peever et al. 2002). Multigene phylogenies using ribosomal DNA and the nuclear genes such as glyceraldehyde-3-phosphate dehydrogenase (*GPDH*), internal transcribed spacer (*ITS*), translation elongation factor 1 (*EF-1 α*), and the gene encoding the allergenic protein alt a 1 (*Alt a 1*) have been used to determine phylogenetic relationships among isolates of *A. solani* and *A. alternata* from tomato and potato from Brazil and WI (Ding et al. 2019; Lourenço et al. 2009). Leaf blight and EB, caused by *A. alternata* and *A. linariae* (syn. *A. tomatophila*), respectively, are destructive diseases of tomato in the United States (Jones et al. 2014). *A. linariae* is considered a primary pathogen causing EB on tomato (Woudenberg et al. 2014), but it can also infect potato (Ayad et al. 2017). Similarly, EB is a major disease of potato and is spread worldwide including in the United States (Pryor and Gilbertson 2000).

Tomato is an important vegetable and widely cultivated in western NC. *A. linariae* is a serious threat to tomato production, resulting in severe economic losses in the Southeastern United States including NC (Kuhar et al. 2019). Unlike *A. linariae*, *A. alternata* on tomato has been a neglected pathogen and is poorly understood in NC. Although these pathogens were common in NC, the currently available knowledge on molecular diversity of

[†]Corresponding authors: T. B. Adhikari; tbadhika@ncsu.edu, and F. J. Louws; fjlouws@ncsu.edu

*The e-Xtra logo stands for "electronic extra" and indicates that supplementary tables are published online.

The author(s) declare no conflict of interest.

TABLE 1. Identity, host plant, geographic location of origin, accession number, and isolates of *Alternaria* species used in this study^a

Serial number	Species	Isolate ^b	Haplotype ^c	Host plant of origin	Country of origin	County	State	Year	Accession number
1	<i>A. alternata</i>	GA4	AaH1	Asian ginseng	China			2019	MK451978
2		GSJP6	AaH1	Asian ginseng	China			2019	MK451976
3		GR1	AaH1	Asian ginseng	China			2019	MK451975
4		MRY1	AaH1	Ashitaba	China			2019	MK210169
5		MFLUCC:18-1586	AaH1	Cherry	China			2018	MH853658
6		JZB380002	AaH1	Cherry	China			2018	MH853645
7		HMCH-9	AaH1	Italian ryegrass	China			2018	MH567107
8		A01	AaH1	Chinese rose	China			2018	MH248152
9		YMAa1/IRAN2643C	AaH1	Sea squill	Iran			2016	KY231161
10		CCTU 211	AaH1	Common sunflower	Iran			2013	KC835770
11		CCTU 212	AaH1	Common sunflower	Iran			2014	KC835771
12		AE1	AaH1	Cast iron	Italy			2017	KY676195
13		211	AaH1	Peace lily	Serbia			2015	KP851761
14		181	AaH1	Peace lily	Serbia			2016	KP851756
15		986/2	AaH1	Horseradish	Serbia			2017	MF741818
16		RG3	AaH1	Radish	South Korea			2017	MG250620
17		RGW8	AaH1	Radish	South Korea			2018	MG250622
18		MUTITA:856	AaH1	Sea cucumber	Tunisia			2018	MG832208
19		T-5	AaH1	Tomato	U.S.A.	Henderson	NC	2012	MK928664
20		T-49	AaH5	Tomato	U.S.A.	Henderson	NC	2012	MK928686
21		T-51	AaH5	Tomato	U.S.A.	Henderson	NC	2012	MK928687
22		T-52	AaH1	Tomato	U.S.A.	Henderson	NC	2012	MK928688
23		T-53	AaH1	Tomato	U.S.A.	Henderson	NC	2012	MK928689
24		T-54	AaH1	Tomato	U.S.A.	Henderson	NC	2012	MK928690
25		T-16	AaH2	Tomato	U.S.A.	Pender	NC	2012	MK928670
26		T-36	AaH3	Tomato	U.S.A.	Pender	NC	2012	MK928678
27		T-66	AaH1	Tomato	U.S.A.	Pender	NC	2012	MK928699
28		T-8	AaH1	Tomato	U.S.A.	Sampson	NC	2012	MK928666
29		T-9	AaH1	Tomato	U.S.A.	Sampson	NC	2012	MK928667
30		T-56	AaH1	Tomato	U.S.A.	Sampson	NC	2012	MK928691
31		T-57	AaH1	Tomato	U.S.A.	Sampson	NC	2012	MK928692
32		T-7	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928665
33		T-11	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928668
34		T-12	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928669
35		T-17	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928671
36		T-18	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928672
37		T-23	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928673
38		T-28	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928674
39		T-30	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928675
40		T-32	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928676
41		T-35	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928677
42		T-37	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928679
43		T-38	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928680
44		T-39	AaH4	Tomato	U.S.A.	Stokes	NC	2012	MK928681
45		T-40	AaH5	Tomato	U.S.A.	Stokes	NC	2012	MK928682
46		T-41	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928683
47		T-43	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928684
48		T-46	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928685
49		T-59	AaH9	Tomato	U.S.A.	Stokes	NC	2012	MK928693
50		T-60	AaH5	Tomato	U.S.A.	Stokes	NC	2012	MK928694
51		T-61	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928695
52		T-62	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928696
53		T-63	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928697
54		T-65	AaH1	Tomato	U.S.A.	Stokes	NC	2012	MK928698
55		T-67	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928700
56		T-68	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928701
57		T-69	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928702
58		T-71	AaH3	Tomato	U.S.A.	Stokes	NC	2014	MK928703
59		T-72	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928704
60		T-73	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928705
61		T-74	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928706
62		T-76	AaH1	Tomato	U.S.A.	Stokes	NC	2014	MK928707
63		T-77	AaH3	Tomato	U.S.A.	Stokes	NC	2014	MK928708
64		T-79	AaH10	Tomato	U.S.A.	Stokes	NC	2014	MK928709
65		SAa1	AaH1	Potato	U.S.A.		WI	2019	MG525459
66		SAa2	AaH1	Potato	U.S.A.		WI	2019	MG525460

(Continued on next page)

^a The sequences of the global isolates of *Alternaria* spp. were included for comparison purposes.^b In all, 152 isolates of *Alternaria* spp. collected from tomato and potato from North Carolina (NC) and Wisconsin (WI) were analyzed. Among these, *A. alternata* isolates ($N = 46$) were collected from heirloom tomatoes and hybrids in the northeast and central NC. *A. linariae* ($N = 59$) were collected from commercial hybrid tomatoes in the western NC. *A. solani* isolates ($N = 47$) were collected from potato cultivars and lines in Waushara County, WI. Sequences of the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) gene of the isolates from different countries were downloaded (<https://www.ncbi.nlm.nih.gov/genbank/>) for comparison purposes.^c Haplotypes were assigned arbitrarily based on sequences of the *GPDH* gene sequences. For *A. linariae* and *A. solani* sequences, haplotypes were identical to those described previously (Lourenço et al. 2009), and – indicates isolate sequenced but not assigned to a designated haplotype.

TABLE 1. (Continued from previous page)

Serial number	Species	Isolate ^b	Haplotype ^c	Host plant of origin	Country of origin	County	State	Year	Accession number
67	<i>A. linariae</i>	SAA3	AaH1	Potato	U.S.A.		WI	2019	MG525461
68		SAA4	AaH1	Potato	U.S.A.		WI	2019	MG525462
69		SAA5	AaH1	Potato	U.S.A.		WI	2019	MG525463
70		SAA6	AaH1	Potato	U.S.A.		WI	2019	MG525464
71		SAA7	AaH1	Potato	U.S.A.		WI	2019	MG525465
72		SAA8	AaH1	Potato	U.S.A.		WI	2019	MG525466
73		SAA9	AaH1	Potato	U.S.A.		WI	2019	MG525467
74		SAA10	AaH1	Potato	U.S.A.		WI	2019	MG525468
75		Aa35	AaH1	Potato	U.S.A.		WI	2019	MF279611
76		Aa79	AaH1	Potato	U.S.A.		WI	2019	MF279640
77		Aa60	AaH1	Potato	U.S.A.		WI	2019	MF279624
78		Aa52	AaH2	Potato	U.S.A.		WI	2019	MF279619
79		Aa70	AaH2	Potato	U.S.A.		WI	2019	MF279631
80		NB264	—	Tomato	Algeria			2016	KR911760
81		NB242	AsAIH2/H3	Tomato	Algeria			2016	KR911762
82		NB272	AsAIH4	Tomato	Algeria			2016	KR911758
83		NB248	AsAIH1	Tomato	Algeria			2016	KR911748
84		1	—	Tomato	U.S.A.	Haywood	NC	2014	MK928757
85		2	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928758
86		4	AsAIH9	Tomato	U.S.A.	Haywood	NC	2014	MK928759
87		7	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928760
88		8	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928761
89		9	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928762
90		10	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928763
91		14	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928764
92		17	AsAIH7	Tomato	U.S.A.	Haywood	NC	2014	MK928765
93		111	AsAIH11	Tomato	U.S.A.	Haywood	NC	2014	MK928811
94		113	AsAIH11	Tomato	U.S.A.	Haywood	NC	2014	MK928812
95		114	AsAIH11	Tomato	U.S.A.	Haywood	NC	2014	MK928813
96		115	AsAIH2/H3	Tomato	U.S.A.	Haywood	NC	2014	MK928814
97		19	—	Tomato	U.S.A.	Swain	NC	2014	MK928766
98		20	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928767
99		22	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928768
100		23	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928769
101		30	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928770
102		33	AsAIH8	Tomato	U.S.A.	Swain	NC	2014	MK928771
103		34	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928772
104		36	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928773
105		37	AsAIH8	Tomato	U.S.A.	Swain	NC	2014	MK928774
106		40	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928775
107		41	AsAIH7	Tomato	U.S.A.	Swain	NC	2014	MK928776
108		45	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928777
109		46	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928778
110		47	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928779
111		48	AsAIH10	Tomato	U.S.A.	Macon	NC	2014	MK928780
112		49	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928781
113		50	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928782
114		51	AsAIH1	Tomato	U.S.A.	Macon	NC	2014	MK928783
115		52	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928784
116		54	AsAIH10	Tomato	U.S.A.	Macon	NC	2014	MK928785
117		55	AsAIH11	Tomato	U.S.A.	Macon	NC	2014	MK928786
118		56	AsAIH9	Tomato	U.S.A.	Macon	NC	2014	MK928787
119		57	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928788
120		59	—	Tomato	U.S.A.	Macon	NC	2014	MK928789
121		60	AsAIH9	Tomato	U.S.A.	Macon	NC	2014	MK928790
122		62	AsAIH10	Tomato	U.S.A.	Macon	NC	2014	MK928791
123		64	—	Tomato	U.S.A.	Macon	NC	2014	MK928792
124		65	AsAIH10	Tomato	U.S.A.	Macon	NC	2014	MK928793
125		67	AsAIH2/H3	Tomato	U.S.A.	Macon	NC	2014	MK928794
126		73	AsAIH10	Tomato	U.S.A.	Macon	NC	2014	MK928795
127		75	AsAIH10	Tomato	U.S.A.	Macon	NC	2014	MK928796
128		81	AsAIH10	Tomato	U.S.A.	Madison	NC	2014	MK928797
129		83	AsAIH8	Tomato	U.S.A.	Madison	NC	2014	MK928798
130		84	AsAIH2/H3	Tomato	U.S.A.	Madison	NC	2014	MK928799
131		85	AsAIH2/H3	Tomato	U.S.A.	Madison	NC	2014	MK928800
132		87	AsAIH11	Tomato	U.S.A.	Madison	NC	2014	MK928801
133		88	AsAIH11	Tomato	U.S.A.	Madison	NC	2014	MK928802
134		89	AsAIH10	Tomato	U.S.A.	Madison	NC	2014	MK928803
135		92	AsAIH10	Tomato	U.S.A.	Madison	NC	2014	MK928804
136		93	AsAIH2/H3	Tomato	U.S.A.	Madison	NC	2014	MK928805
137		94	AsAIH9	Tomato	U.S.A.	Madison	NC	2014	MK928806
138		95	AsAIH11	Tomato	U.S.A.	Madison	NC	2014	MK928807
139		96	AsAIH2/H3	Tomato	U.S.A.	Madison	NC	2014	MK928808

(Continued on next page)

TABLE 1. (Continued from previous page)

Serial number	Species	Isolate ^b	Haplotype ^c	Host plant of origin	Country of origin	County	State	Year	Accession number
140	<i>A. solani</i>	97	AsAIH7	Tomato	U.S.A.	Madison	NC	2014	MK928809
141		98	AsAIH9	Tomato	U.S.A.	Madison	NC	2014	MK928810
142		T22	—	Tomato	U.S.A.	Stokes	NC	2012	MK928815
143		CBS 107.61	AsAIH4	Tomato	Belgium			2014	KJ718026
144		AS069_T	AsAIH2/H3	Tomato	Brazil			2008	EU617508
145		AS063_T	AsAIH5	Tomato	Brazil			2008	EU617542
146		AS307_P	AsAIH6	Potato	Brazil			2008	EU617539
147		AS300_T	AsAIH2	Tomato	Brazil			2008	EU617537-H2
148		AS084_P	AsAIH4	Potato	Brazil			2008	EU617510-H4
149		AS252_T	AsAIH5	Tomato	Brazil			2008	EU617532-H5
150		LGBA14	—	Greater burdock	China			2018	MH754543
151		BA 1946	AsAIH4	Poroporo	Denmark			2012	HE796751
152		A151	AsAIH4	Potato	Iran			2014	KP276233
153		NL03003	AsAIH4	Potato	Netherland			2018	CP022029
154		SEH-As 1999	AsAIH4	Potato	Pakistan			2016	LT714702
155		CBS 116651	AsAIH4	Potato	U.S.A.			2014	KC584139
156		BMP 181	AsAIH4	Potato	U.S.A.			2014	HE796747
157		BMP 183	AsAIH4	Unknown	U.S.A.			2012	HE796743
158		BMP 185	AsAIH4	Potato	U.S.A.			2012	HE796749
159		BMP 186	AsAIH4	Black night shade	U.S.A.			2012	HE796748
160		ATCC 58177	AsAIH2/H3	Potato	U.S.A.			2003	AY278807
161		SAs11	AsAIH4	Potato	U.S.A.		WI	2017	MG525469
162		3-579	AsAIH2/H3	Potato	U.S.A.		AZ	2003	AY278807
163		AS012_P	AsAIH1	Potato	U.S.A.			2008	EU617505-H1
164		As38	AsAIH4	Potato	U.S.A.			2019	MF279642
165		P-1	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928710
166		P-2	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928711
167		P-7	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928712
168		P-11	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928713
169		P-12	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928714
170		P-15	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928715
171		P-16	AsAIH12	Potato	U.S.A.	Waushara	WI	2008	MK928716
172		P-17	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928717
173		P-18	—	Potato	U.S.A.	Waushara	WI	2008	MK928718
174		P-20	—	Potato	U.S.A.	Waushara	WI	2008	MK928719
175		P-21	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928720
176		P-23	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928721
177		P-27	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928722
178		P-28	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928723
179		P-30	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928724
180		P-31	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928725
181		P-32	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928726
182		P-34	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928727
183		P-37	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928728
184		P-39	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928729
185		P-44	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928730
186		P-47	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928731
187		P-50	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928732
188		P-51	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928733
189		P-53	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928734
190		P-55	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928735
191		P-56	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928736
192		P-58	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928737
193		P-59	AsAIH12	Potato	U.S.A.	Waushara	WI	2008	MK928738
194		P-62	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928739
195		P-64	AsAIH4	Potato	U.S.A.	Waushara	WI	2008	MK928740
196		P-205	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928741
197		P-208	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928742
198		P-211	—	Potato	U.S.A.	Waushara	WI	2009	MK928743
199		P-214	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928744
200		P-221	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928745
201		P-225	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928746
202		P-226	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928747
203		P-234	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928748
204		P-239	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928749
205		P-240	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928750
206		P-254	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928751
207		P-256	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928752
208		P-257	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928753
209		P-258	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928754
210		P-270	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928755
211		P-277	AsAIH4	Potato	U.S.A.	Waushara	WI	2009	MK928756

(Continued on next page)

TABLE 1. (Continued from previous page)

Serial number	Species	Isolate ^b	Haplotype ^c	Host plant of origin	Country of origin	County	State	Year	Accession number
212	<i>A. porri</i>	CBS 116698	–	Onion	U.S.A.			2014	KC584132
213	<i>A. grandis</i>	CBS 116695	AsAlH1	Potato	U.S.A.			2014	KJ718070
214	<i>A. tomatophila</i> (= <i>A. linariae</i>)	BA 1523	AsAlH2/H3	Tomato	Australia			2012	HE79673
215		HaAt4	AsAlH4	Sunflower	China			2019	MK411013
216		BA 1526	AsAlH2/H3	Tomato	New Zealand			2012	HE796740
217		MF-P585011	AsAlH4	Tomato	Russia			2014	KJ397987
218		MF-P138061	AsAlH2/H3	Tomato	Russia			2014	KJ397985
219		BA 1443	AsAlH2/H3	Tomato	U.S.A.		IN	2012	HE796739
220		BA 1528	AsAlH2/H3	Tomato	U.S.A.		PA	2012	HE796736
221		BA 1527	AsAlH2/H3	Tomato	Venezuela			2012	HE796737

Alternaria spp. from tomato is limited. The outbreaks of brown spot, caused by *A. alternata* and EB caused by *A. solani* on potato have increased recently in WI (Ding et al. 2019; Weber and Halterman 2012). However, it is unknown whether recurrent disease epidemics in tomato and potato in NC and WI are caused by the same or different haplotypes within each species of *Alternaria*. To address this question, we used coalescent genealogical approaches to obtain phylogenetically informative data derived from DNA sequences of *Alternaria* spp. using two nuclear loci: *GPDH*, RNA polymerase second largest subunit (*RPB2*) (Liu and Hall 2004), and the *ITS* region.

The main objective of this study was to compare the relative level of DNA polymorphisms of *GPDH*, *ITS*, and *RPB2* within each species and determine haplotype diversity and infer phylogenetic relationships of these isolates with publicly available sequences of the *GPDH* gene of *Alternaria* spp. from different countries. *GPDH* sequences were selected because this nuclear gene has been used previously to analyze the phylogenetic analysis of *A. alternata* and *A. solani*, respectively (Ding et al. 2019; Lourenço et al. 2009). A haplotype herein is defined as a set of DNA variations or single nucleotide polymorphisms (SNPs) found on the gene regions (Rebbeck et al. 2004). The findings of this study are important for understanding the phylogenetic relationships among isolates of *Alternaria* spp. recovered from tomato and potato and for devising disease resistance breeding programs.

MATERIALS AND METHODS

Sample locations and collection. Tomato is mainly grown in NC, and leaf blight symptoms caused by *A. alternata* and *A. linariae* in tomato were found at late and early stages of plant growth, respectively. The northeast and central NC (~100 m above sea level) regions have a warm humid and coastal climate and mild winters. *A. alternata* was found to be sporadic in these regions in NC, and we intentionally sampled this species from the northeast and central region between 2012 and 2014 (Table 1). In the case of *A. alternata*, samples from Stokes County were mainly collected from heirloom tomatoes grown on an organic farm while samples from Pender and Sampson counties were collected from commercial hybrids. The western NC represents high elevations (>600 m above sea level) and has a temperate climate with cold winters and rainy summers. *A. linariae* was endemic in western NC and leaf samples were intentionally collected from major tomato-growing counties during the early season in 2014. The EB symptoms showing circular lesions with concentric rings surrounded by a yellow hollow caused by *A. linariae* were commonly found in hybrid tomato and sampled from five counties: Haywood, Henderson, Macon, Madison, and Swain using a stratified random sampling approach (Cochran 1977). Up to four young leaflets with EB lesions were sampled from each plant and ranged from 20 to 60 samples per county. The procedures for isolation of *A. alternata* and *A. linariae* from tomato have been described previously (T. B. Adhikari, S. Timilsina, K. Bhattarai, I. Meadows, D. Halterman, and F. J. Louws, unpublished data).

Weather conditions in WI typically include extreme cold winters and warm summers and tomato are rarely grown in WI. *A. alternata* isolates were isolated from potato in WI but they only considered *A. alternata* as an opportunistic pathogen (Weber and Halterman 2012). EB caused by *A. solani* was an endemic disease mainly in Waushara County, WI. Isolation and purification of *A. solani* isolates were performed previously (Weber and Halterman 2012). In all, 152 single-spore isolates of *Alternaria* spp. were collected and used in this study (Table 1). Population herein is referred to as samples or isolates collected from a location or county.

Morphological characterization. Each isolate was transferred to acidified potato dextrose agar (A-PDA) medium (4 g of potato starch, 20 g of dextrose, and 15 g of agar/liter of distilled water and amended with two antibiotics: ampicillin [0.06 g/liter] and rifampicin [0.024 g/liter]). Morphological characters (e.g., conidia type, septate, presence and number of beaks) of the isolates were examined under a binocular stereomicroscope (Leica Microsystems, Wetzlar, Germany). Species identification based on morphology was performed as described previously (Simmons 2007; Woudenberg et al. 2014).

Molecular characterization. Each culture was transferred to 50 ml of Difco potato sucrose broth medium in Erlenmeyer flasks that were continuously agitated at 110 rpm at room temperature. After 7 days, mycelium was transferred to 2-ml centrifuge tubes and lyophilized. To obtain the fine powder, the lyophilized-mycelium tissues were ground using a microtube homogenizer (Model D1030-E, Beadbug, Benchmark Scientific Inc., Edison, NJ) at 400 rpm for 3 min. Genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The DNA concentration was quantified using a spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE) and adjusted to a concentration of DNA at 10 ng/μl for each isolate. In the previous study (Gannibal et al. 2014), OAsF7 and OAsR6 primers were used to differentiate morphologically similar large-spores *Alternaria* spp. such as *A. solani* and *A. linariae*. We used *A. solani*-specific primers OAsF7 (5'-CGAC GAGTAAGTTGCCCTCA-3') and OAsR6 (5'-TGTAGGCGTCA GAGACACCATT-3'), which amplified the *Alt a1* genomic region only in *A. solani*, and another primer set, OAtF4 (5'-TGCGGCT TGCTGGCTAAGGT-3') and OAtR2 (5'-CAGTCGATGCGGCC GTCA-3'), which amplified DNA fragment from the calmodulin encoding gene of *A. linariae* and some other large-spored *Alternaria* species excluding *A. solani* (Gannibal et al. 2014).

PCR assays and sequencing. Three nuclear loci: *GPDH*, *RPB2*, and the *ITS* region were selected because some of these genes were used previously to compare patterns of genetic variation of *A. solani* from tomato and potato in Brazil (Lourenço et al. 2009) and *A. solani* and *A. alternata* from potato in WI (Ding et al. 2019). Partial sequences of *GPDH*, *RPB2*, and the *ITS* region were amplified using primer sets GPD-F/GPD-R (Berbee et al. 1999), RPB2-F/RPB2-R (Woudenberg et al. 2015), and *ITS4* and *ITS5* (White et al. 1990), respectively. A 25-μl mixture was used for the amplification and each PCR contained 8.5 μl of sterile distilled water, 2 μl of 10 ng/μl genomic DNA, 1 μl each of 10 μM reverse and

forward primer, and 12.5 µl of GoTaq Green PCR mix (Promega Inc., Madison, WI). The amplification with *GPDH* and *ITS* primer sets was performed individually using the same program: 95°C for 30 s, followed by 44 cycles: 94°C for 15 s, 50°C for 30 s, and 68°C for 3 min; and final elongation of 72°C for 15 min. The amplification of *RPB2* was the following: 95°C for 30 s; 30 cycles: 94°C for 15 s, 60°C for 30 s, 68°C for 3 min and final extension 72°C for 7 min. The quality of the PCR product of each sample and fragment of the expected size was confirmed by electrophoresis on a 1.5% agarose gel (wt/vol). PCR product purification was done using ExoSAP-IT PCR product cleanup reagent (Affymetrix, Inc., Santa Clara, CA). Six microliters of PCR product and 1 µl of 10 µM reverse or forward primer were added to each reaction and sent for sequencing at Genomic Sciences Laboratory, North Carolina State University, Raleigh, NC.

Sequence analysis and alignments. Raw sequence data were visually evaluated for quality using Geneious ver. 11.1.4 (1 May 2018; Biomatters Ltd., Auckland, New Zealand). Consensus sequences were generated and aligned using eight iterations of MUSCLE (Edgar 2004). Base substitutions were grouped as phylogenetically informative or uninformative and each polymorphic site received a specific site number on the consensus sequence.

Nucleotide diversity and haplotype assignment within species. The relative degree of DNA polymorphisms for each gene sequence data set was examined among isolates of each species. Nucleotide diversity at the three loci: *GPDH*, *ITS*, and *RPB2* were determined using DNA Sequence Polymorphism software (DNASP) ver.6 (Rozas et al. 2017). To arbitrarily assign locus-specific and species-specific haplotype, sequence data sets were generated for each locus separately. Three data sets were mined to identify haplotype within species in this study: (i) AaH1 to AaH6 haplotype for *RPB2* sequences for all 46 isolates of *A. alternata*; (ii) AaH1 to AaH5 haplotype for *GPDH* sequences for all 48 isolates of *A. alternata*; and (iii) AsAIH1 to AsAIH12 haplotype for *GPDH* sequences for all 106 isolates of *A. solani* and *A. linariae*. For comparisons, *A. solani* and *A. linariae* haplotypes AsAIH1 to AsAIH6 were like previously reported haplotype nomenclature

(Lourengo et al. 2009). For each gene sequence data set, the SNAP Map program (Price and Carbone 2005) was used to collapse nucleotide sequences into haplotypes (Aylor et al. 2006). The SNAP Workbench Java program package was used to analyze gene genealogies and population parameters (Price and Carbone 2005). Compatibility matrices were developed for haplotypes that share a common ancestry to examine the overall or conflict among variable sites in DNA sequence alignments as described previously (Jakobsen et al. 1997). The population mutation parameter per nucleotide site Θ using Watterson's (1975) Θ_w , based on the number of segregating sites (*S*), and the average pairwise nucleotide diversity (π) (Tajima 1989) were estimated using DNASP ver.5 (Librado and Rozas 2009) for each locus across isolates of each species.

Tests of neutrality. Neutrality test statistics were estimated with Tajima's *D* (TD) (Tajima 1989), Fu and Li's *D* (FLD), Fu and Li's *F* (FLF) (Fu and Li 1993), and Fu's *F* (Fu 1997) using 5,000 permutations (Kimura 1980). Based on these tests, if only TD, FLD, and FLF were significant and Fu's *F* was nonsignificant, this indicated background selection (Fu 1997). If only Fu's *F* was significant, this suggested population growth. Likewise, when TD and Fu's *F* test values were negative and nonsignificant, it indicated an excess of mutations. Positive values suggested a population division or background selection (Carbone et al. 2007). These four neutrality tests were calculated for all isolates of each species for each locus and the combined data of *A. linariae* and *A. solani* (*N* = 106).

Phylogenetic inference. To determine genetic relationships among isolates within species, both ends of each gene sequences were trimmed to the same length before concatenating the sequences. The concatenation of the three gene sequences of each isolate was used for phylogenetic analysis. Phylogenetic relationships were analyzed using maximum likelihood (ML). For ML analysis, PAUP* ver. 4.b10 (Swofford 2004) was used with resampling estimated log-likelihood of 1,000 bootstrap replicates. Multilocus tree building was performed using the Tamura-Nei genetic distance model (Tamura and Nei 1993). Metadata were analyzed with the Tamura-Nei model using the Tree-Based

TABLE 2. Variable bases in two nuclear genes, glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) and RNA polymerase second largest subunit (*RPB2*), and the internal transcribed spacer (*ITS*) rDNA region were analyzed on the 152 isolates of three *Alternaria* spp. from tomato in North Carolina (NC) and potato in Wisconsin (WI)^a

Species	<i>GPDH</i> (483 nt) polymorphic sites															
	14	19	86	91	99	100	109	110	111	113	118	120	121	150	151	152
<i>A. linariae</i>	Y	S	T	A	A	T	G	C	A	C	C	T	T	A	C	A
<i>A. solani</i>	C	G	T	A	A	T	G	C	A	C	C	T	T	A	C	A
<i>A. alternata</i>	C	G	C	C	G	C	A	A	G	T	A	A	C	G	T	G
Species	<i>ITS</i> (474 nt) polymorphic sites															
	5	6	7	26	32	66	78	84	87	88	97	103	107	128	129	136
<i>A. linariae</i>	G	G	C	C	G	C	A	C	C	C	T	C	G	T	G	T
<i>A. solani</i>	G	G	C	C	G	C	A	C	C	C	T	C	G	T	G	T
<i>A. alternata</i>	T	A	–	–	T	T	T	A	–	–	–	T	T	A	T	–
Species	<i>RPB2</i> (699 nt) polymorphic sites															
	3	18	21	27	30	39	51	66	75	78	87	90	108	129	132	138
<i>A. linariae</i>	C	A	C	C	C	C	C	T	T	C	T	T	C	C	C	T
<i>A. solani</i>	C	A	C	C	C	C	T	T	C	T	C	C	C	C	C	T
<i>A. alternata</i>	M	G	T	T	T	T	C	C	T	T	T	C	Y	G	T	C
	369	429	441	447	456	498	519	537	540	546	552	558	562	564	567	570
<i>A. linariae</i>	G	G	C	A	C	T	A	A	G	A	T	C	A	T	T	G
<i>A. solani</i>	A	G	C	A	C	C	A	G	A	A	T	T	A	T	T	A
<i>A. alternata</i>	G	A	T	G	T	C	R	A	A	T	C	T	G	C	A	A

(Continued on next page)

^a Data refer to polymorphic sites from each locus and nucleotide consensus based on majority agreement at each variable location. In all, 152 isolates of *Alternaria* spp. were collected and analyzed in this study (Table 1). Among these, *A. alternata* isolates (*N* = 46) were collected from mainly heirloom tomato in the northeast and central NC. *A. linariae* (*N* = 59) were collected from hybrid tomato in the western NC. *A. solani* isolates (*N* = 47) were collected from potato cultivars and lines in Waushara County, WI. Y = C/T, S = G/C, W = A/T, M = A/C, and R = A/G.

Alignment Selector T-BAS v 2.0 (Carbone et al. 2017). Bootstrap analysis was used to determine the statistical support for each branch of trees generated with 1,000 replications. Phylogenetics was displayed in the circle tree format and the results were visualized as the outer rings of multigene ML phylogeny.

Comparison of *GPDH* sequences between the current isolates of *A. alternata* and the global isolates. The *GPDH* gene sequences of 46 isolates of *A. alternata* from tomato in NC (Table 1) were compared with publicly available *GPDH* gene sequences of international isolates downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). For haplotype comparisons, sequences of 15 representative isolates of *A. alternata* from potato from the previous study of Ding et al. (2019) were retrieved. The isolates included were SAa1 (accession no. MG525459), SAa2 (MG525460), SAa3 (MG525461), SAa4 (MG525462), SAa5 (MG525463), SAa6 (MG525464), SAa7 (MG525465), SAa8 (MG525466), SAa9 (MG525467), and SAa10 (MG525468) collected from Grand Marsh, Hancock, and Plover counties from WI in 2017, and Aa35 (MF279611), Aa52 (MF279619), Aa60 (MF279624), Aa70 (MF279631), and Aa79 (MF279640) collected from Hancock and Plover counties from WI in 2012. Also, sequences of the 17 international isolates of *A. alternata* collected from various host plants were downloaded from GenBank and included for comparisons (Table 1). These isolate sequences were from China ($N = 8$), Iran ($N = 3$), Italy ($N = 1$), Serbia ($N = 3$), and South Korea ($N = 2$). The results were visualized in T-BAS v.2.0 (Carbone et al. 2017).

Comparison of *GPDH* sequences between the current isolates of *A. linariae* and *A. solani* and the global isolates. *GPDH* gene sequences of the 59 isolates of *A. linariae* sampled from tomato in NC and 47 isolates of *A. solani* from potato were analyzed (Table 1). Additionally, six isolate sequences AS063_T (accession no. EU617542), AS307_P (EU61539), AS084_P (EU617510), AS069_T (EU17508), AS300_T (EU617537), and AS252_T (EU617532) of *A. solani* from Brazil were included for haplotype comparisons (Lourenço et al. 2009). For *A. linariae*, *GPDH* sequences downloaded from GenBank were from Algeria ($N = 4$) and Belgium ($N = 1$). For *A. solani*, *GPDH* sequences were from China ($N = 1$), Denmark ($N = 1$), Iran ($N = 1$), Netherland

($N = 1$), Pakistan ($N = 1$), and the United States ($N = 10$). Also, *A. tomatophila* (syn. *A. linariae*) isolate sequences used were one each from Australia, China, New Zealand, and Venezuela and two each from Russia and the United States. We also included sequences of *A. porri* and *A. grandis* from the United States (Table 1). FASTA format of each isolate was trimmed and aligned using Geneious ver. 11.1.4. Tree outputs were assembled for each isolate using the T-BAS v.2.0 as described previously (Carbone et al. 2017).

Comparison of the *RPB2* and *GPDH* locus-based haplotypes of *Alternaria* spp. identified in the current study with publicly available *Alternaria* whole-genome sequences. We retrieved publicly available whole-genome sequence data of three isolates: BMP 0185 (*A. solani*) (Dang et al. 2015), HWC-128 (*A. solani*; accession no. JRWV000000000), and SRC1lrK2f (*A. alternata*; accession no. GCA_001642055) were retrieved from GenBank and compared with the haplotypes identified in the current study. Sequence data were evaluated using Geneious ver. 11.1.4 (1 May 2018; Biomatters Ltd., Auckland, New Zealand) and aligned using MUSCLE (Edgar 2004). Base substitutions were between the *RPB2* and *GPDH* locus-based haplotypes and whole-genome isolates were compared and grouped at a specific site on the consensus sequences.

RESULTS

Morphological characterization. Three *Alternaria* spp. can cause leaf blight symptoms with the same appearance of lesions in both tomato and potato in NC and WI. In total, 152 isolates were collected and identified as *Alternaria* spp. based on the microscopic examination of morphological traits. For purified isolates grown on A-PDA, colonies with aerial mycelium were dense and black in reverse. Dark brown conidia were small and large, and separated with or without a long beak. Based on the size of the conidia and the presence of beak, the isolates of *Alternaria* spp. were classified into two groups: small-spore and large-spore. All samples collected from the north and central NC were small-spored without beak and were identified as *A. alternata*. Similarly, all samples collected from the western NC were large-spored with long multiple beaks and

TABLE 2. (Continued from previous page)

GPDH (483 nt) polymorphic sites														
154	155	177	179	186	324	339	400	409	424	436	448	454	478	
C	T	C	W	C	T	T	T	T	T	T	C	C	C	
C	T	C	A	C	T	T	T	T	T	T	C	C	C	
T	C	T	A	Y	C	C	C	C	C	C	T	T	T	
ITS (474 nt) polymorphic sites														
332	334	335	339	340	342	347	366	410	417	418	422	423	441	442
T	T	G	C	C	C	G	C	G	T	C	C	C	C	C
T	T	G	C	C	C	G	C	G	T	C	C/–	C/–	C	C
G	C	–	A	G	T	T	A	A	A	T	–	–	T	T
RPB2 (699 nt) polymorphic sites														
139	156	159	183	204	219	222	231	234	261	267	273	318	351	357
T	C	C	C	G	T	G	C	C	A	C	C	C	G	C
T	C	C	T	G	T	G	C	C	A	C	C	C	G	C
C	T	T	C	A	Y	A	T	T	G	Y	Y	A	A	T
576	579	594	603	609	621	627	660	684	693	694	699			
T	T	C	T	T	A	C	T	G	G	A	C			
T	T	T	T	C	G	C	T	G	G	A	C			
C	C	Y	C	T	W	T	C	A	R	T	Y			

were assigned to *A. linariae*, while all large-spored conidia with a single long beak collected from potato in WI were *A. solani*.

Molecular characterization. As expected, the primer set OAsF7/OAsR6 amplified a 164 bp fragment from *A. solani* only, while OAtF4 and OAtR2 primers amplified a 438-bp DNA fragment from *A. linariae* (Gannibal et al. 2014). PCR assay based on DNA samples from tomato and potato confirmed the isolates to be *A. linariae* and *A. solani*, respectively.

Tests of neutrality. Nucleotide polymorphisms analyzed through gene-by-gene comparisons in each species included 483 nucleotides for *GPDH*, 474 for *ITS*, and 669 for *RPB2* and concatenated data for the three genes was 1,626 bp in length (Table 2). Intraspecific variability was detected in each species across the isolates analyzed. By comparisons across three species, 30, 31, and 59 nucleotide polymorphisms were identified in the *GPDH*, *ITS*, and *RPB2* genes, respectively (Table 2). Nucleotide diversity (π) estimates in three species ranged from 0.00088 to 0.00264 for the *GPDH* gene; from 0 to 0.00019 for the *ITS* region, and 0 to 0.00674 for the *RPB2* gene (Table 3). For the combined *A. linariae* and *A. solani* populations, π values for the *GPDH*, *ITS*, and *RPB2* genes were 0.00278, 0.00004, and 0.01079, respectively. The *GPDH* and *RPB2* genes had a higher number of segregating sites (*S*) and mean mutation parameters per site (Watterson's Θ_w) than the *ITS* region (Table 3).

We used four neutrality tests to determine whether the data departed from an equilibrium model of neutral evolution and provided insight into an indication of recent population expansion. In *A. alternata*, most of the neutrality test values were negative for the *GPDH* locus and the *ITS* region (Table 3). Although FLD and FLF estimates were negative, these two values were significant ($P \leq 0.05$) for the *GPDH* gene, suggesting an excess of recent mutations in the *A. alternata* population. In the case of *A. linariae*, TD, FLD, and Fu's *F* values were negative for the *RPB2* gene, and only the FLF estimate was positive. For *A. solani*, all four estimates were negative. However, FLF values were significant for both *GPDH* and *ITS* within selective species (Table 3). For the combined *A. linariae* and *A. solani*, TD and FLF statistics were positive and significant for the *RPB2* gene. In contrast, all four neutrality test statistics (TD, FLD, FLF, and Fu's *F*) were negative for both *GPDH* and *ITS* genes, but TD, FLD, and FLF values for the *GPDH* gene were significant (Table 3).

Haplotype identification within species. For the *ITS* gene sequences, low nucleotide polymorphism was observed. Thus, it was less informative to identify haplotypes in all species tested. For *A. alternata*, the *GPDH* gene sequence data set yielded five haplotypes (AaH1, AaH2, AaH3, and AaH4, and one unique haplotype AaH5) of *A. alternata* with seven SNPs (Fig. 1; Table 4). Likewise, the *GPDH* gene data set yielded 12 haplotypes of *A. solani* and *A. linariae* with seven SNPs (Table 5; Fig. 1). Among them, six haplotypes (AsAIH7, AsAIH8, AsAIH9, AsAIH10, AsAIH11, and AsAIH12) were unique and new (Fig. 1). Intriguingly, a single SNP at position 20 led to an amino acid change from valine (Val) to leucine (Leu) in the isolates belonging to three haplotypes of *A. linariae* and *A. solani*: H7, H8, and H9 (Table 5). The *RPB2* gene sequences had a lower number of polymorphic sites than the *GPDH* locus. Therefore, at least six haplotypes (H1 to H6) were detected with 15 SNPs in *A. alternata* for the *RPB2* sequences (Table 6). Although 15 SNPs at the *RPB2* sequences were found between *A. linariae* and *A. solani*, the identified nucleotide polymorphisms were not variable enough to allow for resolution to differentiate haplotypes within *A. linariae* or *A. solani* (Table 2).

For the *GPDH* gene sequences, AaH1 to AaH5 were more common in *A. alternata* than other haplotypes in all counties in NC sampled. However, AaH1 ($N = 25$) was the most frequent haplotype in Stokes County followed by Henderson and Sampson counties (Supplementary Table S1; Fig. 1). The number of isolates detected in each haplotype in the other counties was low (between 0 and 3). One unique haplotype was detected in Henderson and Stokes counties ($N = 4$). Based on the *RPB2* gene, six haplotypes (AaH1 to AaH6) and one unique haplotype were found in *A. alternata* from NC (Supplementary Table S2; Fig. 1). In Stokes County, haplotype AaH3 was common ($N = 10$) followed by AaH5 ($N = 6$) and AaH4 ($N = 5$). In other counties, the distribution of isolates in each haplotype was low ranging from 0 to 3.

In *A. solani*, AsAIH4 ($N = 42$) was exclusively found in Waushara County, WI (Supplementary Table S3; Fig. 1) followed by AsAIH12 ($N = 2$). Only one unique haplotype ($N = 3$) was found. However, no other haplotypes were detected in Waushara County, WI. In *A. linariae*, haplotypes AsAIH2/H3 were found in Haywood ($N = 7$), Macon ($N = 8$), and Madison ($N = 4$) counties (Supplementary Table S3; Fig. 1). Similarly, haplotype AsAIH7 was detected in Haywood

TABLE 3. Estimates for neutrality tests and genetic diversity for three closely related *Alternaria* spp. collected from tomato in North Carolina (NC) and from potato in Wisconsin (WI)

Species	Host	N ^a	Locus ^b	S ^c	TD ^d	FLD ^d	FLF ^d	Fu's F ^d	π ^e	Θ_w ^f
<i>A. solani</i>	Potato	47	<i>RPB2</i>	0	NA ^g	NA	NA	NA	0.0000	NA
			<i>ITS</i>	1	-1.1	-2.35* ^h	-3.19*	-4.08	0.00009	0.00047
			<i>GPDH</i>	9	-2.24	-3.84	-3.68*	-2.53	0.00088	0.00422
<i>A. linariae</i>	Tomato	59	<i>RPB2</i>	2	-1.44	-2.6	2.5	-1.01	0.0001	0.00062
			<i>ITS</i>	0	NA	NA	NA	NA	0.0000	NA
			<i>GPDH</i>	4	1.04	0.99	1.15	-1.81	0.00264	0.00179
<i>A. alternata</i>	Tomato	46	<i>RPB2</i>	18	-0.47	0.85	0.8	-0.67	0.00674	0.00588
			<i>ITS</i>	2	-1.47	-2.49	-2.4	-2.91	0.00019	0.00097
			<i>GPDH</i>	9	-1.9	-2.52**	-2.57**	-4.93	0.0014	0.00425
<i>A. linariae</i> and <i>A. solani</i> combined	Tomato and potato	106	<i>RPB2</i>	17	3.68***	0.64	2.04***	21.99	0.01079	0.00466
			<i>ITS</i>	1	-1.02	-2.05	-1.95	-2.27	0.00004	0.00040
			<i>GPDH</i>	13	-1.24*	-3.3*	-2.94**	-4.32	0.00278	0.0038

^a *N* = sample size analyzed. In all, 152 isolates of *Alternaria* spp. were collected and analyzed in this study (Table 1). Among these, *A. alternata* isolates ($N = 46$) were collected from mainly heirloom tomato in the northeast and central NC. *A. linariae* ($N = 59$) were collected from hybrid tomato in the western NC. *A. solani* isolates ($N = 47$) were collected from potato cultivars and lines in Waushara County, WI.

^b Multilocus DNA sequences from the three nuclear genes, the internal transcribed spacer (*ITS*) rDNA region, glyceraldehyde-3-phosphate dehydrogenase (*GPDH*), and RNA polymerase second largest subunit (*RPB2*), were sequenced and analyzed. Unique sequence determined by nucleotide polymorphism(s) present.

^c *S* = number of segregating sites calculated according to Watterson (1975).

^d TD = Tajima's *D* (Tajima 1989), FLD = Fu and Li's *D*, and FLF = (Fu and Li 1993) and Fu (Fu 1997) statistics were calculated according to statistical methods described previously.

^e π = nucleotide diversity was calculated as described previously (Tajima 1989).

^f For each analysis, Θ_w is the population mutation parameter per nucleotide estimated according to Watterson (1975).

^g NA = not applicable.

^h *, **, and *** indicate significance at $P < 0.10$, $P < 0.05$, and $P > 0.01$, respectively.

($N = 1$), Madison ($N = 1$), and Swain ($N = 8$) counties. Haplotype AsAIH8 was rare but it was found only in Madison and Swain counties. Likewise, haplotypes H9 and H10 were detected only in Madison and Macon counties. Haplotype AsAIH11 was present in Haywood and Madison counties. Interestingly, haplotypes AsAIH4 and AsAIH12 were absent in *A. linariae* in NC. One unique

haplotype was detected in Haywood ($N = 2$), Macon ($N = 3$), and Swain ($N = 1$) counties (Supplementary Table S3; Fig. 1). At the *RPB2* locus, 15, 43, and 44 nucleotide variations occurred between *A. linariae* and *A. solani*, *A. linariae* and *A. alternata*, and *A. solani* and *A. alternata*, respectively (Supplementary Table S4). Interestingly, all isolates of *A. linariae* analyzed had similar SNPs at

Fig. 1. Identification and distribution of haplotypes (H) of *Alternaria* spp. analyzed using the single-copy two nuclear genes: the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) and RNA polymerase second largest subunit (*RPB2*). In all, 152 isolates of three closely related *Alternaria* spp. collected from the sampled counties in tomato- and potato-producing area (red spots on the map) in North Carolina (NC) and Wisconsin (WI). Numbers in pie charts indicate the frequency of isolates from each haplotype from each species of *Alternaria*.

the *RPB2* region and had a poor resolution to distinguish isolates within this species. Similarly, for *A. solani* isolate sequences were too conserved and did not distinguish isolates within *A. solani*. However, there was an apparent separation of *A. linariae* isolates and those of *A. solani* isolates based on the metadata profile of the sequences mined from the *RPB2* locus (Table 2; Supplementary Table S4).

Gene genealogy. The rooted trees generated from the *RPB2* gene sequences revealed that haplotype networks showed mutations along with clades specific to *A. alternata*; however, these gene

sequences were not variable enough to allow for resolution among the isolates within *A. solani* and *A. linariae* to form discrete subbranches (Fig. 2A). Based on the *RPB2* locus sequence, high bootstrap score (97%) supported haplotypes AaH4, AaH5, and AaH6 network and appeared to be independent; however, the remaining haplotypes (bootstrap score >58%) AaH1, AaH2, and AaH3 were likely divergent from other haplotypes. Sequences of the two representative isolates of *A. linariae* (isolate 1) and *A. solani* (P-2) were located in two branches but had no discernible bootstrap score (100%) between these two isolates. The patterns of haplotype branches based on ML analysis for the *GPDH* gene sequences broadly separated three species into two clades (Fig. 2B). The top clade contained the *A. alternata* haplotypes: (AaH1 to AaH4 [bootstrap score >74%]) while another clade consisted of the two haplotypes (AsAl12 and AsAlH4 [score 100%]) of *A. solani* and six new haplotypes (AsAl H7 to AsAl H11 located at the bottom belonging to *A. linariae*) (Fig. 2B).

Phylogenetic analysis. In the previous studies (Ding et al. 2019; Woudenberg et al. 2015), single-gene phylogenetic analyses could not separate isolates of small-spored *Alternaria* into groups. In this study, phylogenetic analyses based on the concatenation of three nuclear loci (*GPDH*, *ITS*, and *RPB2*) sequences showed evolutionary diversity within each species (Fig. 3). ML analysis of *A. alternata* isolates placed them into few clades. Although the tree topologies identified eight haplotypes (Fig. 2B), combined sequence data within isolates of *A. linariae* and *A. solani* appeared to represent monophyletic species with 100% bootstrap value (Fig. 3). No association between haplotype or species and host geographic locations was observed. Using the multigene phylogeny analyses, there was no cluster detected by location in *A. alternata* isolates collected from three locations: Grand Marsh, Hancock, and Plover from potato in WI (Ding et al. 2019). *A. alternata* genotype 1A was predominantly found in most locations sampled from potato in WI.

Comparison between *Alternaria* spp. *GPDH* locus-based haplotypes of the current study and global isolates. The phylogenetic relationships among global isolates of *Alternaria* spp. were inferred by analysis of *GPDH* sequences because both *ITS* and *RPB2* sequences in the data set were too invariable for accurate phylogeny. Therefore, we compared the *GPDH* sequences of 46 isolates of *A. alternata* with 22 global isolate sequences from different countries. Similarly, sequences of 59 isolates of *A. linariae* from tomato and sequences of 47 isolates of *A. solani* with 26 sequences from the previous studies (Ding et al. 2019; Lourenço et al. 2009) and GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) were downloaded. *A. alternata* metadata revealed that haplotype AaH1 exhibited the highest frequency within the *GPDH* sequences from NC and other countries and formed similar network topologies (Fig. 4). On the other hand, haplotypes AaH3, AaH5, AaH9, and AaH10 exhibited the lowest frequency among the *GPDH* sequences from tomato in NC. These haplotypes were not present

TABLE 4. Nucleotide polymorphisms in the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) among haplotypes of *Alternaria alternata* isolates (*N* = 46) collected from tomato in North Carolina

Haplotypes	<i>GPDH</i> ^a (488 nt) polymorphic sites						
	47	65	127	146	147	156	187
AaH1	C	C	C	C	C	C	C
AaH2	T	C	C	C	C	C	T
AaH3	C	C	C	C	A	T	C
AaH4	C	T	C	T	C	C	C
AaH5	C	C	T	C	C	C	C

^a Nucleotide polymorphisms at the *GPDH* gene sequences of *A. alternata*. The consensus sequence of variable nucleotides of each haplotype for the *GPDH* locus. Sequences are presented for the five haplotypes and the number indicates the base position of each variable site. *A. alternata* isolates collected from mainly heirloom tomato were from Henderson, Pender, Sampson, and Stokes counties.

TABLE 5. Nucleotide polymorphisms in the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) locus among haplotypes of *Alternaria linariae* (*N* = 59) and *A. solani* (*N* = 47) collected from tomato and potato in North Carolina and Wisconsin, respectively

Haplotypes ^b	Host	<i>GPDH</i> ^a (488 nt) consensus sequence						
		15	18	20 ^c	23	27	180	391
AsAlH2/H3	Tomato	T	–	G	G	–	A	–
AsAlH4	Potato	C	–	G	G	–	A	–
AsAlH7	Tomato	C	–	C	G	–	A	–
AsAlH8	Tomato	C	–	C	T	–	A	–
AsAlH9	Tomato	T	–	C	G	–	A	–
AsAlH10	Tomato	T	–	G	G	–	T	–
AsAlH11	Tomato	T	–	G	G	–	A	G
AsAlH12	Potato	C	C	G	G	T	A	–

^a Consensus sequence of variable nucleotides of each haplotype for the *GPDH* locus.

^b *A. linariae* isolates from hybrid tomato were collected from Haywood, Macon, Madison, and Swain counties. *A. solani* isolates (*N* = 47) were collected from potato cultivars and lines in Waushara County, WI.

^c The G to C at position 20 results in a Valine (Val) to Leucine (Leu) conversion.

TABLE 6. Nucleotide polymorphisms in the RNA polymerase second largest subunit (*RPB2*) locus among haplotypes of *Alternaria alternata* (*N* = 46) collected from tomato in North Carolina

Haplotypes ^b	<i>RPB2</i> ^a (699 nt) consensus sequence														
	3	57	108	127	219	267	273	367	483	519	594	621	651	693	699
AaH1	A	G	T	C	C	T	C	C	A	A	T	A	A	A	T
AaH2	A	A	T	C	C	T	C	C	C	A	T	A	A	A	T
AaH3	A	G	T	C	C	T	C	C	C	A	T	A	A	A	T
AaH4	C	G	T	C	T	C	T	C	C	G	T	A	A	G	C
AaH5	C	G	T	T	T	C	C	C	C	G	C	T	A	A	C
AaH6	C	G	C	C	T	C	C	T	C	G	T	A	T	A	C

^a Consensus sequence of variable nucleotides of each haplotype for the *RPB2* locus. Sequences are presented for the 15 unique haplotypes, and number indicates the base position of each variable site. Y = C/T and R = A/G.

^b *A. alternata* isolates collected from mainly heirloom tomato were from Henderson, Pender, Sampson, and Stokes counties.

in other countries. Of the *GPDH* sequences of the 15 isolates of *A. alternata* from potato in WI of the study of Ding et al. (2019) that we compared, three isolates (Aa3, Aa52, and Aa70 sequences) appeared to be identical to sequences of our five isolates (T9, T16, T36, T71, and T77) of *A. alternata* from tomato in NC. Based on the mutations, six haplotypes of *A. solani* were inferred for the *GPDH* locus in the previous study (Lourenço et al. 2009). Among these haplotypes, H2, H3, H4, and H5 were detected in tomato isolates while haplotypes H1 and H6 were found in potato isolates. Based on the frequency from where they originated, haplotype AsAIH4 described here appeared to represent the most important *GPDH* haplotype within sequences of *A. solani* isolates from WI and global isolates including Australia, Algeria, Brazil, China, New Zealand, and Russia (Table 1; Fig. 5). The most common haplotype AsAIH2/H3 was found within *A. linariae* from tomato in the current study and was also exclusively present in Algeria, Belgium, China, Demark, Iran, Netherland, Pakistan, Russia, and several other states (e.g., IN, PA, and AZ) of the United States. (Table 1). The results of this study suggest that two haplotypes (AsAIH2/H3 and AsAIH4) could be widely distributed clones in tomato- and potato-growing regions.

Comparison of the *RPB2* and *GPDH* locus-based haplotypes of *Alternaria* spp. with publicly available *Alternaria* whole-genome sequences. For both genes, the haplotypes identified in the current study using our approach were compared with publicly available *A. alternata* and *A. solani* whole-genome sequences. For comparing the *RPB2* locus sequences, we identified six haplotypes of the *A. alternata* and these haplotype sequences had little or no (0 to 8) nucleotide differences between them and their respective isolates (Supplementary Table S5). Importantly, *A. alternata* haplotype AaH3 identified from the *RPB2* gene sequence corresponded perfectly with the reference genome sequence of *A. alternata* isolate SRC11rK2f (Supplementary Table S5). For the *RPB2* locus, *A. solani* representative isolate P2 sampled from a potato field in WI was identical to the genome sequence of *A. solani* isolate BMP 0185 from Arkansas of the United States. However, there were three nucleotide differences between *A. solani* isolate P2 and another genome sequence of *A. solani* isolate HWC

168 from China (Supplementary Table S5). Our results show 47 to 50 nucleotide differences between *A. alternata* isolate SRC11rK2f versus *A. solani* and *A. linariae*, and between *A. solani* isolate BMP 0185 versus *A. alternata* haplotypes.

For the *GPDH* locus, maximum nucleotide differences resulted in haplotypes of *A. alternata* when compared with the reference genome of *A. solani* isolate BMP 0185 and isolate HWC 168 or when *A. solani* and *A. linariae* haplotypes were compared with the reference genome of *A. alternata* isolate SRC11rK2f (Supplementary Table S6). However, in each of the haplotypes corresponding to *A. solani* and *A. linariae*, there were one to two nucleotide differences. With the *GPDH* locus-based sequence dataset, *A. solani* and *A. linariae* haplotype P1-AsAIH4 was identical with the genome of *A. solani* isolate BMP 0185 and isolate HWC 168 (Supplementary Table S6). Similarly, *A. alternata* haplotype T-5AaH1 corresponded perfectly with the reference genome of *A. alternata* isolate SRC11rK2f.

Sequence data availability. The DNA sequences reported in this study have been deposited in GenBank under the accession numbers MK928512 to MK928663 (*ITS*), MK928664 to MK928815 (*GPDH*), and MK928816 to MK928967 (*RPB2*). Sequences of publicly available *Alternaria* whole-genome sequences of the three isolates used in this are available from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

DISCUSSION

One of the primary objectives of the current study was to use the gene genealogical approach to improve our understanding of the genetic diversity of economically important members of the *Alternaria* spp. Toward this end, we analyzed 152 isolates of *Alternaria* spp. sampled from major tomato and potato growing areas in NC and WI. We also included DNA sequence data per isolate used in previous studies (Ding et al. 2019; Lourenço et al. 2009; Woudenberg et al. 2014). As a result, phylogenetic analysis of a single-gene data set was informative to provide the haplotype diversity and evolutionary relationships between species of

Fig. 2. Phylogenetic relationships of *Alternaria alternata* (Aa), *A. solani* (As), and *A. linariae* (Al) of haplotypes based the sequences of the **A**, RNA polymerase second largest subunit (*RPB2*) and **B**, glyceraldehyde-3-phosphate dehydrogenase (*GPDH*). Each number indicates a representative isolate from each haplotype, and letter before isolate number refers to host: T = tomato and P = potato from which isolate was collected. All rooted maximum-likelihood trees are drawn to scale with branch lengths proportional to the substitutions per site. Bootstrap support was calculated from 1,000 replicates and values >50% are indicated above the branches.

Alternaria spp. As noted in published phylogenetic studies (Ding et al. 2019; Lourenço et al. 2009; Woudenberg et al. 2015), our results also indicate that nucleotide diversity was lower for the *ITS* sequences than the *RPB2* and *GPDH* sequences. Although the *RPB2* sequences were too conserved and did not distinguish among individuals within *A. linariae* or within *A. solani*, nucleotide substitution sites or SNPs were useful to allow delineation at the species level. The *GPDH* sequences outperformed the other two genes in providing superior resolution for haplotype placement and phylogenetic inference. For *A. linariae* or *A. solani*, sequences of haplotypes AsAIH1 to AsAIH6 were identical to previously reported haplotypes from Brazil (Lourenço et al. 2009). The remaining AsAIH7 to AsAIH12 were unique and new haplotypes. Our results suggest that *A. linariae* or *A. solani* contain new and previously reported haplotypes that are capable of causing disease epidemics in tomato- and potato-producing areas in NC and WI.

Low level of genetic diversity was found for *Alternaria* spp. in this study. Based on the *ITS* locus, Lourenço et al. (2009) also reported a low level of diversity for isolates of *A. solani* collected from tomato and potato in Brazil. Similarly, *ITS* locus was less informative and resulted in poor resolution in *Fusarium* spp. (O'Donnell et al. 2013; Ramdial et al. 2017). In contrast, the *GPDH* gene sequences were useful and informative to compare the genetic diversity and genetic relationships of *Alternaria* spp. in previous

studies (Ding et al. 2019; Lourenço et al. 2009). In the case of *A. linariae* or *A. solani*, 13 haplotypes (including unique haplotypes) were identified, and six of them were identical to previously reported haplotypes (Lourenço et al. 2009). The remaining haplotypes were new. Our findings based on the consensus sequence across the *GPDH* locus suggest that the two most frequently diverse haplotypes, AsAIH2/AsAIH3 and AsAIH4, were associated with *A. linariae* and *A. solani* in the United States. Interestingly, all global isolates of *A. linariae* and *A. solani* included in this study share these haplotypes. Our findings support previous results showing that the majority of gene diversity may be distributed within individuals and populations are not geographically differentiated (Linde et al. 2002; McDonald et al. 1999). We found seven new haplotypes (AsAIH7 to AsAIH13) that were not present in previously reported haplotypes of *A. solani* (Lourenço et al. 2009). Among these haplotypes, AsAIH2/AsAIH3 and AsAIH4 have been mainly detected in *A. linariae* isolates from tomato in NC and in *A. solani* isolates from potato in WI, respectively, but formed a single clade in the neighbor-joining trees. These two haplotypes also displayed closer proximity to the clusters that contain haplotypes AsAIH7, AsAIH8, AsAIH9, AsAIH10, and AsAIH11 of *A. linariae* and one haplotype AsAIH12 of *A. solani*. Although population differentiation according to the host of origin in this study was not possible due

Fig. 3. The phylogenetic tree derived from a concatenated sequence made up of glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) and RNA polymerase second largest subunit (*RPB2*), and the rDNA internal transcribed spacer (*ITS*) region of the 152 isolates of *Alternaria alternata* ($N = 46$), *A. linariae* ($N = 59$), and *A. solani* ($N = 47$) isolates from North Carolina and Wisconsin. Innermost ring: host plant from which isolates were collected and each isolate designation is connected with principal component value. Letter before isolate number refers to host: T = tomato and P = potato from which collected; ring 2: fungal species *A. alternata*, *A. linariae*, and *A. solani*; and ring 3: state or county from which the isolate was sampled. Bootstrap values >50 are labeled along branches.

to limited sample size and no overlap between tomato and potato, the haplotype association with host species has been demonstrated in the previous study (Lourenço et al. 2009). For example, the *GPDH* locus-based haplotypes (H1, H2, H4, and H5) were mainly of tomato isolates, whereas two haplotypes (H6 and H7) were commonly detected in potato isolates from Brazil (Lourenço et al. 2009). Future research will need to collect additional samples from different hosts, geographic locations, and years, and a more detailed assessment involving multigene sequence analysis may provide insight into the genetic diversity of *Alternaria* spp. across the United States.

Based on combined sequences analysis of four genes (*Alt a1*, *GPDH*, *ITS*, and translation elongation factor), Ding et al. (2019) reported five genotypes of *A. alternata* from potato sampled in WI. In our study, we also included the 15 isolate sequences of *A. alternata* from potato from the previous study (Ding et al. 2019) for comparisons. Of the 15 isolate sequences analyzed, 12 isolates belonged to genotype I in the previous study and were haplotype AaH1 in this study. Similarly, one isolate was genotype III and two isolates were genotype V. Although we found the *GPDH* gene sequence similarities between the isolates of *A. alternata* from potato sampled in WI and the isolates of *A. alternata* from tomato sampled in NC, the haplotype nomenclature in the previous study was based on multigene analysis (Ding et al. 2019). However, in our analysis, it was based on single-copy *GPDH* gene sequences. Haplotype networks of *A. alternata* from tomato are considered to be a conservative exploration of understanding the phylogenetic hypothesis that described the relationships among closely related species such as those of *A. alternata* from potato between the different geographic origin (NC versus WI) and isolates infecting different host plant of origin (tomato versus potato).

We analyzed the genetic variability of *Alternaria* spp. using two diversity estimates (S and π) (Nei 1973) and assessed simple models of Watterson's Θ_w (Watterson 1975) and four neutrality tests: D , FLD , FLF , and Fu's F (Carbone et al. 2007; Fu 1997; Fu and Li 1993; Tajima 1989). Data indicate greater π and Watterson's Θ_w estimates in *A. alternata* than estimates in *A. linariae* and *A. solani* isolates. In general, a higher degree of nucleotide diversity is expected in an ancestral population (Nei 1973). Comparatively, S values for all three genes (*GPDH* = 9, *ITS* = 2, and *RPB2* = 18) were higher in *A. alternata* than *A. linariae* and *A. solani* and can explain the differences in nucleotide diversity in these populations. Four neutrality test estimates varied with *Alternaria* spp. and the nuclear genes analyzed, and most values were not significant. All negative values for the three genes indicated an excess of recent mutation and population expansion in *Alternaria* spp. The mutation is one of the principal evolutionary mechanisms, which causes a heritable change in the nucleotide sequence in several fungi (Hartl and Clark 2007). We hypothesize that new haplotypes AsAIH7 to AsAIH13 have emerged independently in each population. Our data indicate Tajima's D value was positive, but Fu's F was nonsignificant for the *GPDH* gene in *A. linariae*. This result is likely due to balancing selection where the polymorphic alleles are in a position to diversify by additional mutations (Tajima 1989). In this study, *A. alternata* and *A. linariae* were sampled from tomatoes from limited geographic areas in NC between 2012 and 2014 while *A. solani* samples were collected from a potato from a single location in WI during the 2008 season. Thus, we were unable to compare haplotypes over time and space or determine whether some historical events had favorable or unfavorable effects on estimating the number of population genomic parameters for the respective pathogen populations.

Fig. 4. The maximum-likelihood phylogenetic trees of the 46 isolates of *Alternaria alternata* sampled from tomato and potato produced from North Carolina and Wisconsin, and the representative isolates from different countries were derived using the Tamura-Nei DNA substitution model on the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) locus sequence alignment. For comparison purposes, sequences of the global isolates of *A. alternata* were obtained from the GenBank BLASTn search engine of the National Center for Biotechnology Information. Bootstrap values (>50%) are shown above the branches. *GPDH* haplotypes were determined by differences at variable sites, and dashed lines connect individual isolates. Innermost ring 1: principal component analysis, isolate designation and accession numbers from GenBank, and country of origin from which these isolates were collected. Letter before isolate numbers refers to tomato (T) isolates from which isolates were collected and sequenced; ring 2: haplotypes of *A. alternata*; and ring 3: host plants from which these isolates were sampled. The dotted lines within the ring are cosmetic, and solid lines refer to high nucleotide polymorphisms.

Morphological characterization of *Alternaria* large-spored species associated with EB of tomato and potato appeared to be insufficient to distinguish between *A. solani* and *A. linariae* (Woudenberg et al. 2014). Importantly, large-spored *A. linariae* has been isolated from Solanaceae vegetables with symptoms resembling that of EB at the early stages of infection (Ayad et al. 2017). Interestingly, *A. linariae* was not only specific to tomato but it has also been isolated from potato (Ayad et al. 2017; Bessadat et al. 2016; Rodrigues et al. 2010; Simmons 2000). Since *A. solani* and *A. linariae* are the most common species and widely distributed in tomato- and potato-producing regions, molecular markers are necessary to identify and differentiate these species. The *RPB2* gene encodes a protein with a modest rate of evolutionary change and its polypeptide sequence has been used for phylogenetic studies in green plants (Denton et al. 1998) and ascomycetes, including lichen-forming taxa (Liu and Hall 2004). In the current study, comparative genealogy analyses indicate that gene variations exist in these closely related *Alternaria* spp. might have diverged in the genome evolution. In the previous studies, both *GPDH* and *RPB2* genes have been used to compare sequence variation in agriculturally important *Fusarium* species complex (O'Donnell et al. 2000, 2013) and several *Alternaria* spp. (Woudenberg et al. 2015; 2014). In particular, we also found that the *RPB2* locus sequences provided the most conclusive and specific evidence to delineate two closely related species:

A. linariae and *A. solani*. We reasoned that both *A. linariae* and *A. solani* have identical asexual conidia morphology (Woudenberg et al. 2014) and can cause EB disease in tomato and potato (Ayad et al. 2017; Bessadat et al. 2016). Our result suggests that the development of molecular markers from the *RPB2* genomic region can be useful to differentiate *A. linariae* and *A. solani*.

All tomatoes grown in NC are for fresh market. Most of the tomato industries use conventional management practices and utilize the plasticulture system that includes the use of fumigants and conventional fungicides. However, there is a trend of growing organic tomatoes in NC. Tomato cultivars (hybrids, determinate, semideterminate, indeterminate, and heirloom) are diverse, with different combinations of fruit size, growth habit, and disease resistance (<https://plantpathology.ces.ncsu.edu/pp-vegetables/pp-vegetables-tomatoes/>). The isolates of *A. solani* and *A. linariae* were collected in the early season, while isolates of *A. alternata* were collected from tomato later of the season. EB epidemics on tomato caused by *A. linariae* in western NC are recurrent and could be due to planting susceptible tomato cultivars or hybrids, change in virulence of the pathogen, conducive weather parameters, and climate changes (Anderson et al. 2004; Stukenbrock and Bataillon 2012; Stukenbrock and McDonald 2008; Sumabat et al. 2018). In this study, *A. solani* isolates were collected from diverse potato breeding lines and sampled from only one location in WI. We found that the majority (>91%) of the isolates exhibited haplotype

Fig. 5. The maximum-likelihood phylogenetic trees of the 59 and 47 isolates of *Alternaria linariae* and *A. solani* sampled from tomato and potato production regions from North Carolina and Wisconsin, respectively, and representative isolates derived using the Tamura-Nei DNA substitution model on the glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) locus sequence alignment. For comparison purposes, sequences of the global isolates of *A. linariae*, *A. solani*, *A. grandis*, and *A. porri* were obtained from the GenBank BLASTn search engine of the National Center for Biotechnology Information. Bootstrap values (>50%) are shown above the branches. *GPDH* haplotypes were determined by differences at variable sites, and dashed lines connect individual isolates. Innermost ring 1: *Alternaria* spp., principal component analysis, and isolate designation and accession numbers from GenBank, and *A. linariae* and *A. solani*. Letter before isolate numbers refers to potato (P) isolates from which isolates were collected and sequenced. Ring 2: haplotype; ring 3: host plant of origin from which these isolates were sampled, and ring 4: country of origin from which these isolates were collected. The dotted lines within the ring are cosmetic, and solid lines represent high nucleotide polymorphisms.

AsAIH4. In the previous study, Ding et al. (2019) analyzed 33 isolates of *A. solani* sampled from Grand Marsh, Hancock, and Plover, which were different sampling sites than in our study. Based on multigene phylogeny, they also found only one genotype of *A. solani* in different potato fields in WI. It appeared that *A. solani* isolates with identical (*GPDH*, *ITS*, and *RPB2*) marker sequences were well established in these environments and can cause disease epidemics in potato in WI.

In conclusion, we used DNA sequencing at multiple loci and genealogical approaches to provide new insights into the population genetic structure of *Alternaria* spp. This study revealed the presence of previously reported haplotypes and new haplotypes in tomato- and potato-producing areas in NC and WI. Both *A. linariae* and *A. solani* can cause similar lesions on leaves and have been isolated from single symptomatic leaf tissue of tomato and potato (Ayad et al. 2017; Bessadat et al. 2016; Simmons 2007). SNP-based diagnostic molecular markers can be developed from sequences of the *RPB2* gene and used to differentiate these two species.

ACKNOWLEDGMENTS

We thank Inga Meadows for helping with isolate collection; and Audrey Djunaedi, Stella Chang, and Virginie Rumsch for assisting in the laboratory.

LITERATURE CITED

- Adhikari, P., Oh, Y., and Panthee, D. R. 2017. Current status of early blight resistance in tomato. An update. *Int. J. Mol. Sci.* 18:E2019.
- Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., and Daszak, P. 2004. Emerging infectious diseases of plants: Pathogen pollution, climate change and agrotechnology drivers. *Trends Ecol. Evol.* 19:535-544.
- Ayad, D., Aribi, D., Hamon, B., Kedad, A., Simoeau, P., and Bouznad, Z. 2017. New data on early blight of potato and tomato caused by a complex of large-spored *Alternaria* species in Algeria. Pages 167-180 in: Sixteenth Euroblight Workshop, Special Report No. 18, Aarhus, Denmark.
- Aylor, D. L., Price, E. W., and Carbone, I. 2006. SNAP: Combine and map modules for multilocus population genetic analysis. *Bioinformatics* 22: 1399-1401.
- Berbee, M. L., Pirseyedi, M., and Hubbard, S. 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* 91:964-977.
- Bessadat, N., Berruyer, R., Hamon, B., Bataille-Simoneau, N., Benichou, S., Kihal, M., Henni, D. E., and Simoneau, P. 2016. *Alternaria* species associated with early blight epidemics on tomato and other Solanaceae crops in northwestern Algeria. *Eur. J. Plant Pathol.* 148:181-197.
- Brewer, M. T., and Milgroom, M. G. 2010. Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse *Vitis* species. *BMC Evol. Biol.* 10:268.
- Carbone, I., Jakubek, J. L., Ramirez-Prado, J. H., and Horno, B. W. 2007. Recombination, balancing selection, and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. *Mol. Ecol.* 16:4401-4417.
- Carbone, I., White, J. B., Miallikowska, J., Arnold, A. E., Miller, M. A., Kauff, F., U'Ren, J. M., May, G., and Lutzoni, F. 2017. T-BAS: Tree-Based Alignment Selector toolkit for phylogenetic-based placement, alignment downloads, and metadata visualization: An example with the Pezizomycotina tree of life. *Bioinformatics* 33:1160-1168.
- Cochran, W. G. 1977. *Sampling Techniques*. 3rd ed., Wiley & Sons, New York.
- Dang, H. X., Pryor, B., Peever, T., and Lawrence, C. B. 2015. The *Alternaria* genomes database: A comprehensive resource for a fungal genus comprised of saprophytes, plant pathogens, and allergenic species. *BMC Genomics* 16: 239.
- Denton, A. L., McConaughy, B. L., and Hall, B. D. 1998. Usefulness of RNA polymerase II coding sequences for estimation of green plant phylogeny. *Mol. Biol. Evol.* 15:1082-1085.
- Ding, S., Meinholz, K., Cleveland, K., Jordan, S. A., and Gevens, A. J. 2019. Diversity and virulence of *Alternaria* spp. causing potato early blight and brown spot in Wisconsin. *Phytopathology* 109:436-445.
- Droby, S., Dinoo, A., Prusky, D., and Barkai-Golan, R. 1984. Pathogenicity of *Alternaria alternata* on potato in Israel. *Phytopathology* 74:537-542.
- Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792-1797.
- Fu, Y. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 147:915-925.
- Fu, Y., and Li, W. 1993. Statistical tests of neutrality of mutations. *Genetics* 133:693-709.
- Gannibal, P. B., Orina, A. S., and Mironenko, N. V. 2014. Differentiation of the closely related species, *Alternaria solani* and *A. tomatophila*, by molecular and morphological features and aggressiveness. *Eur. J. Plant Pathol.* 139:609-623.
- Grogan, R. G., Kimble, K. A., and Misaghi, I. 1975. A stem canker disease caused by *Alternaria alternata* f. sp. *lycopersici*. *Phytopathology* 65:880-886.
- Hartl, D. L., and Clark, A. G. 2007. *Principles of Population Genetics*. Sinauer Associates, Sunderland, MA.
- Jakobsen, I. B., Wilson, S. R., and Easteal, S. 1997. The partition matrix: Exploring variable phylogenetic signals along nucleotide sequence alignments. *Mol. Biol. Evol.* 14:474-484.
- Jones, J. B., Zitter, T. A., Timur, M. T., and Miller, S. A. 2014. Page 176 in: *Compendium of Tomato Diseases and Pests*, 2nd ed. American Phytopathological Society, St. Paul, MN.
- Kimura, M. 1980. A simple method for estimating the evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-120.
- Kuhar, T. P., Ramon Arancibia, R., Rideout, S. L., and Reiter, M. S. 2019. Southeastern U.S. 2019 Vegetable Crop Handbook. AREC-66NP (SPES-106NP). Virginia Cooperative Extension, Virginia Tech, and Virginia State University. <https://www.pubs.ext.vt.edu/AREC/AREC-66/AREC-66.html>
- Librado, P., and Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451-1452.
- Linde, C. C., Zhan, J., and McDonald, B. A. 2002. Population structure of *Mycosphaerella graminicola*: From lesions to continents. *Phytopathology* 92:946-955.
- Liu, Y. J., and Hall, B. D. 2004. Body plan evolution of ascomycetes, as inferred from an RNA polymerase II phylogeny. *Proc. Natl. Acad. Sci. USA* 101:4507-4512.
- Lourenço, V., Jr., Moya, A., González-Candelas, F., Carbone, I., Maffia, L. A., and Mizubuti, E. S. G. 2009. Molecular diversity and evolutionary processes of *Alternaria solani* in Brazil inferred using genealogical and coalescent approaches. *Phytopathology* 99:765-774.
- McDonald, B. A., Zhan, J., Yarden, O., Hogan, K., Garton, J., and Pettway, R. E. 1999. The population genetics of *Mycosphaerella graminicola* and *Phaeosphaeria nodorum*. Pages 44-69 in: *Septoria on Cereals: A Study of Pathosystems*. H. M. Anderson, ed. CABI, Wallingford, U.K.
- Nei, M. 1973. Analysis of gene diversity in subdivided population. *Proc. Natl. Acad. Sci. USA* 70:3321-3323.
- O'Donnell, K., Kistler, H. C., Takke, B. K., and Casper, H. H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proc. Natl. Acad. Sci. USA* 97:7905-7910.
- O'Donnell, K., Rooney, A. P., Proctor, R. H., Brown, D. W., McCormick, S. P., Ward, T. J., Frandsen, R. J., Lysøe, E., Rehner, S. A., Aoki, T., Robert, V. A., Crous, P. W., Groenewald, J. Z., Kang, S., and Geiser, D. M. 2013. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genet. Biol.* 52:20-31.
- Peever, T. L., Ibañez, A., Akimitsu, K., and Timmer, L. W. 2002. Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. *Phytopathology* 92:794-802.
- Price, E. W., and Carbone, I. 2005. SNAP: Workbench management tool for evolutionary population genetic analysis. *Bioinformatics* 21:402-404.
- Pryor, B., and Gilbertson, R. 2000. Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycol. Res.* 104:1312-1321.
- Ramdiel, H., Latchoo, R. K., Hosein, F. N., and Rampersad, S. N. 2017. Phylogeny and haplotype analysis of fungi within the *Fusarium incarnatum-equiseti* species complex. *Phytopathology* 107:109-120.
- Rebbeck, T. R., Ambrosone, C. B., Bell, D. A., Chanock, S. J., Hayes, R. B., Kadlubar, F. F., and Thomas, D. C. 2004. SNPs, haplotypes, and cancer: Applications in molecular epidemiology. *Cancer Epidemiol. Biomarkers Prev.* 13.
- Rodrigues, T. T. M. S., Berbee, M. L., Simmons, E. G., Cardoso, C. R., Reis, A., Maffia, L. A., and Mizubuti, E. S. G. 2010. First report of *Alternaria tomatophila* and *A. grandis* causing early blight on tomato and potato in Brazil. *New Dis. Rep.* 22:28.
- Rotem, J. 1994. Page 48 in: *The Genus Alternaria Biology, Epidemiology, and Pathogenicity*, 1st ed. American Phytopathological Society, St. Paul, MN.
- Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., and Sánchez-Gracia, A. 2017. DnaSP 6. DNA Seq. Polymorph. Anal. Large Datasets. *Mol. Biol. Evol.* 34:3299-3302.
- Simmons, E. G. 2000. *Alternaria* themes and variations (244-286) species on Solanaceae. *Mycotaxon* 75:1-115.
- Simmons, E. G. 2007. *Alternaria: An Identification Manual*. CBS Fungal Biodiversity Centre, Utrecht, Netherlands.

- Stukenbrock, E. H., and Bataillon, T. 2012. A population genomics perspective on the emergence and adaptation of new plant pathogens in agro-ecosystems. *PLoS Pathog* 8:e1002893.
- Stukenbrock, E. H., and McDonald, B. A. 2008. The origins of plant pathogens in agro-ecosystems. *Annu. Rev. Phytopathol.* 46:75-100.
- Sumabat, L. G., Kemerait, R. C., Jr., and Brewer, M. T. 2018. Phylogenetic diversity and host specialization of *Corynespora cassiicola* responsible for emerging target spot disease of cotton and other crops in the southeastern United States. *Phytopathology* 108:892-901.
- Swofford, D. L. 2004. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4b10. Sinauer Associates, Sunderland, MA.
- Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585-595.
- Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10:512-526.
- USDA-NASS. 2018. United States Department of Agriculture (USDA)-National Agricultural Statistics Service (NASS). https://www.nass.usda.gov/Publications/Ag_Statistics/2018/index
- Watterson, G. A. 1975. On the number of segregating sites in genetical models without recombination. *Theor. Popul. Biol.* 7:256-276.
- Weber, B., and Halterman, D. A. 2012. Analysis of genetic and pathogenic variation of *Alternaria solani* from a potato production region. *Eur. J. Plant Pathol.* 134:847-858.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
- Woudenberg, J. H., Truter, M., Groenewald, J. Z., and Crous, P. W. 2014. Large spored *Alternaria* pathogens in section Porri disentangled. *Stud. Mycol.* 79:1-47.
- Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z., de Vries, M., Stielow, J. B., Thomma, B. P. H. J., and Crous, P. W. 2015. *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? *Stud. Mycol.* 82:1-21.
- Zaffarano, P. L., McDonald, B. A., and Linde, C. C. 2009. Phylogeographical analyses reveal global migration patterns of the barley scald pathogen *Rhynchosporium secalis*. *Mol. Ecol.* 18:279-293.