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Development of a Multilocus Sequence Typing System for Medically Relevant *Bipolaris* Species

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Multilocus sequence typing (MLST) is the gold standard genotyping technique for many microorganisms. This classification approach satisfies the requirements for a high-resolution, standardized, and archivable taxonomic system. Here, we describe the development of a novel MLST system to assist with the investigation of an unusual cluster of surgical site infections caused by *Bipolaris* spp. in postoperative cardiothoracic surgery (POCS) patients during January 2008 to December 2013 in the southeastern United States. We also used the same MLST system to perform a retrospective analysis on isolates from a 2012 *Bipolaris* endophthalmitis outbreak caused by a contaminated product. This MLST system showed high intraspecies discriminatory power for *Bipolaris spicifera*, *B. hawaiiensis*, and *B. australiensis*. Based on the relatedness of the isolates, the MLST data supported the hypothesis that infections in the POCS cluster were from different environmental sources while confirming that the endophthalmitis outbreak resulted from a point source, which was a contaminated medication.

Dematiaceous fungi belonging to the genus *Bipolaris* are abundant in the environment. Many species in this genus are known to cause devastating disease in plants and staple crops around the world (1). In humans, they are common etiological agents of fungal sinusitis. Surgical site infections (SSIs) and deep tissue and invasive infections by members of this genus do occur, but they are extremely rare and typically associated with immunocompromised patients (2–7). The common disease-causing *Bipolaris* species in humans are *Bipolaris spicifera*, *B. hawaiiensis*, and, occasionally, *B. australiensis* (3). A recent proposal transfers these species to the genus *Curvularia* (1), but this new name has not yet been widely accepted.

In the clinical setting, *Bipolaris* infections are usually diagnosed using microscopy to distinguish the morphological characteristics of the fungus following culture. In recent years, the sequence comprising the internal transcribed spacer (ITS) regions and the 5.8S nuclear rRNA genes of the ribosomal cistron has been exploited for species assignment of dematiaceous fungi (8, 9). In most cases, ITS-based sequence identification is sufficient for determining species. However, ITS-based sequencing is limited by its dependence on the completeness and accuracy of available DNA sequence databases. A DNA typing system employing two or more loci, such as multilocus sequence typing (MLST), can be used when it is necessary to distinguish between isolates within a species (10, 11). MLST has been successfully implemented for species identification within a species complex as well as strain differentiation within a species for various fungal pathogens of humans such as *Aspergillus fumigatus*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Cryptococcus gattii*, *Cryptococcus neoformans*, and *Fusarium* sp. (12–18). The recent investigations of infections caused by dematiaceous fungi (5, 19) have highlighted the importance of high-resolution, standardized, and archivable typing system(s) for this group of fungi.

In November 2013, the CDC was contacted by the Texas Department of State Health Services about a cluster of SSIs caused by *Bipolaris* spp. among patients who had recently undergone cardiothoracic surgery. Upon further investigation, 21 cases in postoperative cardiothoracic surgery (POCS) patients were identified from 10 different hospitals in three states (Texas, Arkansas, and Florida) during 2008-2013. Three compelling epidemiological features emerged during the investigation: (i) all patients had a recent history of cardiothoracic surgery and had SSIs caused by *Bipolaris* spp., (ii) we could not find cases among patients who had undergone other invasive surgery procedures, and (iii) the cases were clustered together temporally (more than half of the cases occurred in 2013) and geographically (cases were found in the southeastern United States). Exposure to a common contaminated product used during surgery was initially suspected, but common medical products or devices unique to the case patients were not identified in the epidemiologic investigation (A. Purfield, S. S. Vallabhaneni, K. Benedict, U. Luvsansharav, S. R. Lockhart, C. D. Pham, A. Laufer, N. Pascoe, G. Heseltine, W. Chung, E. Hall, K. B. Brust, C. F. Wheeler, S. Chideya, and B. J. Park, unpublished data). The investigation concluded that patients likely acquired the infections from separate environmental sources. In such cases, investigators often rely on laboratory-based techniques to understand the relatedness of patient isolates for validating the conclusions of the epidemiologic investigation. Although 10 patient isolates were available for analysis, a reliable method for strain typing did not exist at the time of the outbreak.

Here, we describe the development and implementation of a novel MLST system using six different DNA loci to differentiate *B. spicifera*, *B. hawaiiensis*, and *B. australiensis* isolates from case pa-

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TABLE 1 Isolate collection information and MLST-based allelic and sequence-type assignment

Isolate collection information			MLST locus allele no.						MLST typing	
Name	Source	Year	*BRN1*	*EF1α*	*GPDH*	*RPB1*	*RPB2*	*SAL1*	ST	Species
B10583[a]	CS[b] patient	2013	4	1	1	1	3	3	1	*B. spicifera*
B10588[a]	Environment	2013	2	1	4	3	4	5	2	*B. spicifera*
B10582[a]	CS patient	2013	3	1	3	3	8	2	3	*B. spicifera*
CBS125738	Sinus	Unknown	1	1	8	4	2	3	4	*B. spicifera*
B10680[a]	Sinus	2014	3	1	5	2	4	5	5	*B. spicifera*
B4388	Sinus	1987	3	1	7	4	4	1	6	*B. spicifera*
B10575[a]	CS patient	2013	3	1	4	3	6	5	7	*B. spicifera*
B10586[a]	Environment	2013	3	1	7	3	3	5	8	*B. spicifera*
B10730[a]	Sinus	2014	3	1	7	3	4	5	9	*B. spicifera*
B6138	Environment	2001	3	1	5	3	4	5	10	*B. spicifera*
B10681[a]	Sputum	2014	3	1	5	2	7	5	11	*B. spicifera*
B10578[a]	CS Patient	2013	5	1	6	4	4	5	12	*B. spicifera*
B10574[a]	CS patient	2013	4	3	5	6	3	5	13	*B. spicifera*
B10585[a]	Environment	2013	3	1	6	3	8	5	14	*B. spicifera*
B10581[a]	Environment	2012	3	1	6	3	8	5	14	*B. spicifera*
B10590[a]	Environment	2013	3	1	4	6	3	7	15	*B. spicifera*
B10788[a]	Nose	2014	6	1	4	6	4	5	16	*B. spicifera*
B10677[a]	Sinus	2014	5	1	7	4	4	5	17	*B. spicifera*
ATCC28335	Alfalfa seed	1973	7	3	3	6	1	5	18	*B. spicifera*
CBS274.52[c]	Mandarin orange	Unknown	7	3	3	6	3	5	19	*B. spicifera*
B10584[a]	Environment	2013	3	1	6	4	8	5	20	*B. spicifera*
B10621	Breast	2013	3	1	4	3	9	5	21	*B. spicifera*
B10791[a]	Sputum	2014	6	1	4	3	4	7	22	*B. spicifera*
B10794[a]	Sinus	2014	5	1	7	4	3	6	23	*B. spicifera*
B10580[a]	CS patient	2012	3	1	4	4	9	5	24	*B. spicifera*
B10589[a]	Environment	2013	3	1	5	6	8	5	25	*B. spicifera*
B10576[a]	CS patient	2013	5	1	6	3	8	6	26	*B. spicifera*
B10618[a]	Environment	2013	6	1	6	3	8	5	27	*B. spicifera*
B10619[a]	Environment	2013	6	1	6	3	8	5	27	*B. spicifera*
B10617[a]	CS patient	2013	3	2	7	3	4	8	28	*B. spicifera*
B10793[a]	Tracheal aspirate	2014	3	1	4	3	8	8	29	*B. spicifera*
B10579[a]	CS patient	2011	5	1	6	2	8	8	30	*B. spicifera*
B10682[a]	Sinus	2013	8	1	5	5	5	5	31	*B. spicifera*
B11060	Calcium gluconate	2013	3	1	8	5	8	5	32	*B. spicifera*
B10594[a]	Sinus	2013	6	1	4	4	8	7	33	*B. spicifera*
B10587[a]	Environment	2013	9	3	2	5	8	5	34	*B. spicifera*
MSG06003	Human, source unknown	2014	6	1	6	5	8	5	35	*B. spicifera*
B10592[a]	Environment	2013	5	1	6	6	8	8	36	*B. spicifera*
B3515	Human, source unknown	1981	7	1	8	5	9	5	37	*B. spicifera*
B4432	Corneal scraping	1986	1	4	1	1	2	1	1	*B. hawaiiensis*
B10790[a]	Bronchial wash	2014	2	1	2	2	1	2	2	*B. hawaiiensis*
B10620[a]	CS patient	2013	4	2	3	5	3	3	3	*B. hawaiiensis*
B10336	Environment	2013	3	3	3	6	4	4	4	*B. hawaiiensis*
B10795[a]	Sinus	2014	5	3	3	4	3	5	5	*B. hawaiiensis*
B9526[d]	Endophthalmitis	2012	3	3	3	6	4	9	6	*B. hawaiiensis*
B9573[d]	Endophthalmitis	2012	3	3	3	6	4	9	6	*B. hawaiiensis*
B9574[d]	Endophthalmitis	2012	3	3	3	6	4	9	6	*B. hawaiiensis*
B9575[d]	Endophthalmitis	2012	3	3	3	6	4	9	6	*B. hawaiiensis*
B9525[d]	Endophthalmitis	2012	3	3	3	6	4	9	6	*B. hawaiiensis*
B9528[#]	Endophthalmitis	2012	3	3	3	6	4	9	6	*B. hawaiiensis*
B10789[a]	Bronchial wash	2014	7	5	4	3	5	6	7	*B. hawaiiensis*
B10679[a]	Sputum	2013	6	5	4	3	5	7	8	*B. hawaiiensis*
B10678[a]	Sinus	2013	6	5	4	3	5	7	8	*B. hawaiiensis*
CBS127091	Unknown	Unknown	6	5	4	3	5	8	9	*B. hawaiiensis*
CBS173.57[c]	Asian rice	1957	6	5	4	3	5	8	9	*B. hawaiiensis*
CBS126975	Unknown	Unknown	1	2	1	1	1	3	1	*B. australiensis*
B10792[a]	Leg abrasion	2014	2	1	3	2	3	1	2	*B. australiensis*
CBS172.57[c]	Asian rice	1957	3	3	2	3	2	2	3	*B. australiensis*
B4361	Cat granuloma	1986	1	1	1	1	1	1	1	*B. ellisii*
B10591[a]	Environment	2013	-	-	-	-	-	-	-	*B. cynodontis*

[a] Isolate from the POCS investigation.
[b] CS, cardiothoracic surgery.
[c] Isolate from the endophthalmitis.
[d] Type strain from CBS.

TABLE 2 MLST loci information

Locus	GenBank accession no.[a]	Primer type[b]	Primer sequence (5′ to 3′)	No. of bp analyzed	Analyzed sequence fragment start and end points
β*TUB*	NA	F	AAATCGGTGCTGCTTTCTGGC	287−296	5′-CCAT(T/C)TC(C/T).....TGGGCCAA-3′
		R	GTAGTGACCCTTGGCCCAGTT		
BRN1	AB011638.1	F	TATTGGAAAGGCTATGGCCA	485	5′-TACGCCAA.....AGAAG(G/A)TC-3′
		R	GGCAGACAGCGTGGTACAT		
*EF1*a	JN601010.1	F	TCGGTGTCAAGCAGCTCAT	569	5′-GAGGAGCG.....CAGGTCGG-3′
		R	AATCTTCTCGAGGAGCTCGG		
GPDH	JN601036.1	F	GTCTCGCATGCGTAGGTGT	448–453	5′-TCCATTGA .....CGTCATGG-3′
		R	AGTGGTTGTGCAGGAGGC		
RPB1	JQ965141.1	F	TACAACCTGGCTACCCCG	556	5′-TTCTCC(T/A)G.....C(G/A)TT(C/T)TTC-3′
		R	CAAGAGCCTGCTTCTTCTTGTT		
RPB2	JQ585695.1	F	GTGGAAAACAACCAAGACTTCAA	437	5′-CGGC(C/T)TGA.....(A/G)GT(C/T)GTGC-3′
		R	ATATCACGAATCAAACTCATCTCGT		
SAL1	AB587805.1	F	CATGGCTAACTAGTTTGCAGATGT	275	5′-G(G/T)CGGC(A/G/C)T.....TCA(C/T)CA (G/A)C-3′
		R	CCGCGACTCATCCGTGTAT		

[a] Accession number of gene sequence from which the primers were derived.
[b] F, forward; R, reverse.

tients, controls, and the environment, collected during the investigation of the cluster of fungal SSIs among POCS patients as well as from a preexisting culture collection at the CDC, including *Bipolaris* isolates from a previous outbreak of endophthalmitis (5).

MATERIALS AND METHODS

Fungal collection, growth conditions, and DNA purification. Sixty *Bipolaris* species isolates were included in this study (Table 1). Thirty-eight isolates were collected as part of the investigation into a cluster of fungal

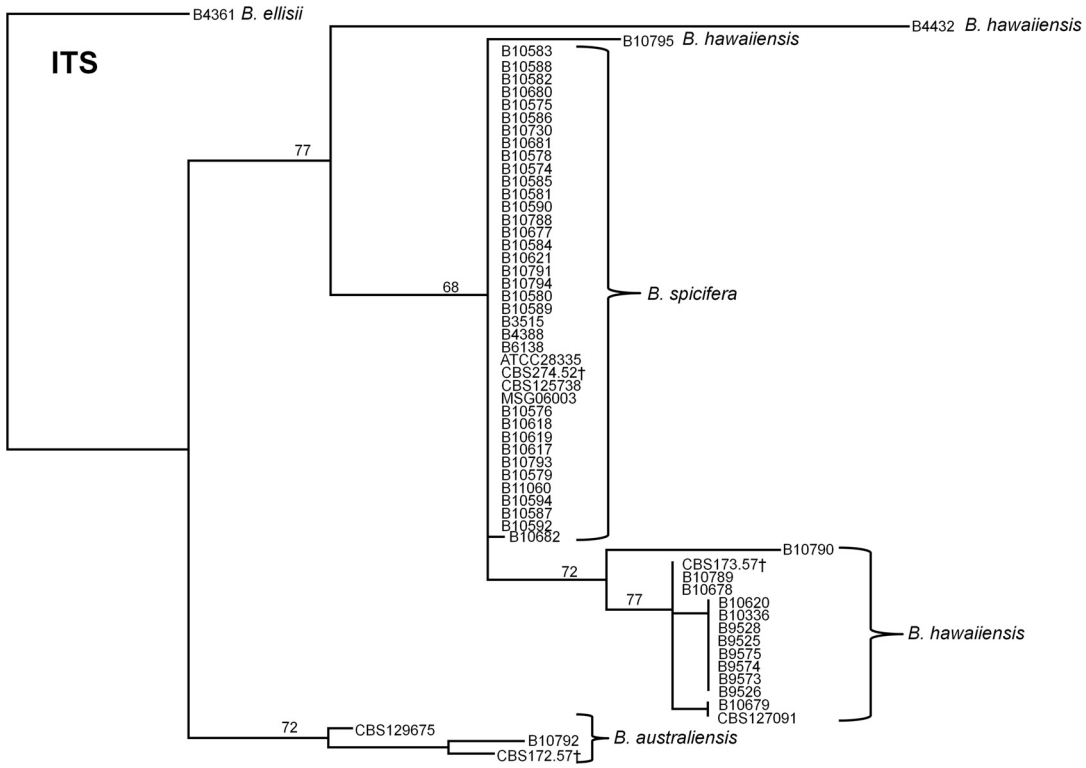

FIG 1 Dendrogram showing the discrimination of *B. australiensis*, *B. hawaiiensis*, and *B. spicifera* isolates inferred from ITS sequence data. The dendrogram was constructed with the neighbor-joining method using CLUSTALW alignment and rooted at *B. ellisii*. The genetic distance and topology reliability were calculated using the Tamura-Nei model and 1,000 bootstrap replicates, respectively. †Type strain from CBS.

TABLE 3 Allelic information at each MLST locus[a]

Species	Allele no.	*BRN1*	*EF1α*	*GPDH*	*RPB1*	*RPB2*	*SAL1*	ITS
B. spicifera								
No. of isolates per allele	1	1	34	1	1	1	1	38
	2	1	1	1	3	1	1	1
	3	18	4	3	15	6	1	0
	4	2	0	9	8	10	1	0
	5	6	0	6	5	1	26	0
	6	6	0	10	7	1	2	0
	7	3	0	6	0	1	3	0
	8	1	0	3	0	15	4	0
	9	1	0	0	0	3	0	0
SI		0.75	0.23	0.84	0.77	0.77	0.55	0.05
B. hawaiiensis								
No. of isolates per allele	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1
	3	7	8	9	5	2	1	1
	4	1	1	5	1	7	1	5
	5	1	5	0	1	5	1	8
	6	4	0	0	7	0	1	0
	7	1	0	0	0	0	2	0
	8	0	0	0	0	0	2	0
	9	0	0	0	0	0	6	0
SI		0.78	0.68	0.62	0.77	0.73	0.86	0.68
B. australiensis								
No. of isolates per allele	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1
	3	1	1	1	1	1	1	1
SI		1	1	1	1	1	1	1
Total no. of isolates		58	58	58	58	58	58	58
Composite SI		0.87	0.79	0.90	0.88	0.87	0.79	0.55

[a] Allele information will be stored at http://mlst.mycologylab.org/DefaultSlideUD.aspx?Page=Home.

infections among POCS patients: 10 isolates obtained from surgical sites from POCS case patients, 15 isolates from respiratory tract or skin from patients who presented with *Bipolaris* infections that were not SSIs to hospitals where *Bipolaris* SSI cases were identified, and 12 isolates obtained through environmental sampling in hospitals with *Bipolaris* SSI cases. Twenty-two isolates used as unrelated controls were obtained from the CDC collection ($n = 16$) or the Centraalbureau voor Schimmelcultures (CBS) (http://www.cbs.knaw.nl/Collections/) collection ($n = 6$, including the type strain for each species). The CDC collection isolates included six *B. hawaiiensis* isolates which were collected as part of an investigation into a 2012 *Bipolaris* endophthalmitis outbreak linked to contaminated medication (11).

Fungal isolates were propagated on Sabouraud dextrose agar. All isolates were maintained at 25°C. Total genomic DNA was purified using bead agitation and reagents in the DNeasy blood and tissue kit (Qiagen; Valencia, CA, USA) as previously described (20).

Molecular investigations of the ribosomal DNA and MLST loci. PCR and DNA sequencing of both strands with BigDye Terminator (Life Technologies, Grand Island, NY, USA) were performed as described previously (21). The same primer sets were employed for both PCR and DNA sequencing at each locus. Locus-specific primers were designed as described previously (22) (Table 2). The sequences for melanin reductase (*BRN1*) (23), glyceraldehyde-3-phosphate dehydrogenase (*GPDH*), transcription elongation factor-1 alpha (*EF1α*), RNA polymerase II subunit 1-like (*RPB1*), RNA polymerase II subunit 1-like (*RPB2*), and scytalone dehydratase (*SAL1*) (24) were from the National Center for Biotechnology Information (Table 1). The β-tubulin primers were designed using the β-tubulin gene sequences from *B. spicifera* ($n = 3$) and *Curvularia* sp. ($n = 1$). PCR programs were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 2 min.

MLST marker stability and reproducibility were determined by performing sequential DNA analysis on two unrelated *B. hawaiiensis* isolates and one *B. spicifera* isolate. These isolates were subcultured every 2 weeks over a period of 6 weeks, followed by DNA extraction and sequencing of the MLST loci to monitor for genetic instability.

Molecular computational phylogenetic analysis. Geneious (Biomatters Limited, San Francisco, CA, USA) was employed for visualizing and annotating DNA sequences and for phylogenetic analysis. The species assignment of fungal isolates was accomplished by using the ITS DNA sequence and the Basic Local Alignment Search Tool (BLAST) on the CBS fungal database website (http://www.cbs.knaw.nl/Collections/BiolomICSSequences.aspx?file=all). For phylogenetic analysis, single-locus sequences or concatenated multilocus sequences of all *Bipolaris* isolates were pool aligned using MUSCLE (or otherwise specified) prior to the construction of a phylogram. All phylograms were generated using the neighbor-joining model and rooted on *Bipolaris ellisii*. The genetic distance and reproducibility of the phylograms were calculated according to Tamura-Nei parameters and 1,000 bootstrap replicates, respectively. Simpson's index (SI) of diversity was calculated as previously described by Hunter and Gaston (25).

Database hosting. The typing systems for *B. spicifera*, *B. hawaiiensis*, and *B. australiensis* with allele number and sequence type assignment are

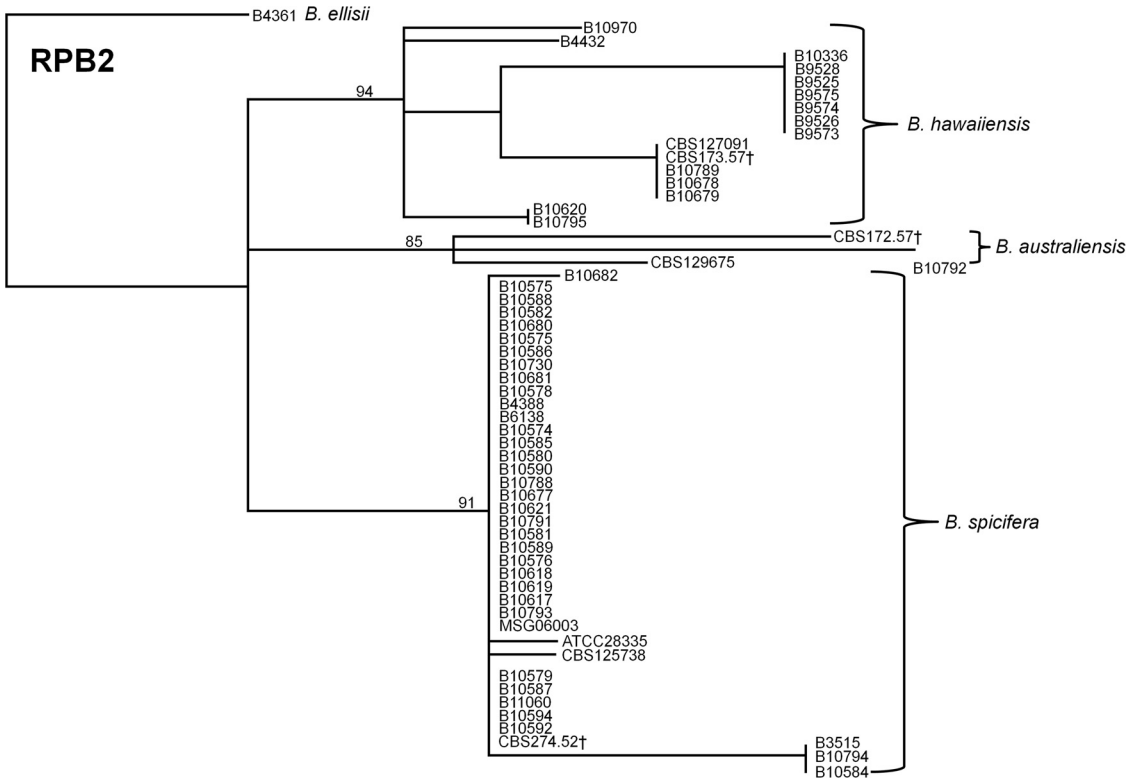

FIG 2 Dendrogram showing discrimination of *B. australiensis*, *B. hawaiiensis*, and *B. spicifera* isolates by the *RPB2* sequence, which was constructed with the neighbor-joining method and rooted at *B. ellisii*. The genetic distance and topology reliability were calculated using the Tamura-Nei model and 1,000 bootstrap replicates, respectively. †Type strain from CBS.

publicly available and hosted at the Fungal MLST Database site (http://mlst.mycologylab.org/DefaultInfo.aspx?Page=Home).

RESULTS

Morphological and ribosomal DNA sequence characteristics of mold isolates. As part of the *Bipolaris* SSI cluster investigation, 38 *Bipolaris* isolates were received. By BLAST analysis of the ITS sequence, the *Bipolaris* isolates were further classified into *B. spicifera* ($n = 30$), *B. hawaiiensis* ($n = 6$), *B. australiensis* ($n = 1$), and *B. cynodontis* ($n = 1$). ITS sequences of the 22 isolates of *Bipolaris* used as unrelated controls for the development of the MLST system confirmed them as *B. australiensis* ($n = 2$), *B. ellisii* ($n = 1$), *B. hawaiiensis* ($n = 10$), and *B. spicifera* ($n = 9$). The *B. spicifera* isolates exhibited high ITS sequence identity; 38 of 39 shared identical ITS sequences (Fig. 1). The ITS sequences of *B. australiensis* and *B. hawaiiensis*, including their respective type strains, showed higher intraspecies diversity with Simpson's index (SI) values of 1 and 0.68, respectively.

Validation of genetic stability and amplification reproducibility for the genetic markers employed to differentiate *Bipolaris* species. We investigated seven different loci (β*TUB*, *BRN1*, *GPDH*, *EF1*α, *RPB1*, *RPB2*, and *SAL1*) for the development of the MLST system. All primer sets generated robust bands for *B. australiensis*, *B. ellisii*, *B. hawaiiensis*, and *B. spicifera*. Not all of the loci were amplified for the *B. cynodontis* isolate, so it was dropped from the analysis. DNA sequences for all seven loci were stable in isolates that were subcultured three times over a period of 6 weeks. Analysis of the DNA sequences revealed a highly variable intron in the β*TUB* marker which is similar to introns observed in the β*TUB* gene of various other fungal species (26). The β*TUB* intron is approximately 46 to 55 bp long and contains 36 single nucleotide polymorphisms (SNPs). Upon further analysis of the β*TUB* locus, it was determined that the β*TUB* primers amplified two tubulin paralogs. As a result, β*TUB* was not included in the final MLST system.

Characteristics of the DNA markers used for differentiating *B. australiensis*, *B. hawaiiensis*, and *B. spicifera*. Partial fragments of the *BRN1*, *EF1*α, *GPDH*, *RPB1*, *RPB2*, and *SAL1* genes were combined to form the new MLST system (Table 3). No insertions or deletions were observed in any of the MLST loci for *B. australiensis*, *B. hawaiiensis*, and *B. spicifera*. The *BRN1* and the *GPDH* loci both contained introns. The *BRN1* locus contained an intron of 53 bp that contained 15 unique SNPs. The *GPDH* locus used had one full intron and part of another. The full intron was 62 bp in length and contained 29 unique SNPs. The partial intron (truncated by the end of the sequence used for analysis) was 15 bp and contained two SNPs. The predicted coding regions of *BRN1* and *GPDH* loci also contained a high number of polymorphic sites. The majority of those sites were synonymous mutations; *GPDH* only had synonymous mutations while *BRN1* also possessed five nonsynonymous mutations, an indication of possible selective pressure at this locus.

None of the three species (*B. australiensis*, *B. spicifera*, and *B. hawaiiensis*) shared an identical allele for any of the MLST markers. The ITS locus was able to differentiate both *B. australiensis* and *B. spicifera*, but suggested that *B. hawaiiensis* was polyphyletic (Fig. 1); two isolates, B4432 and B10795, did not cluster with the

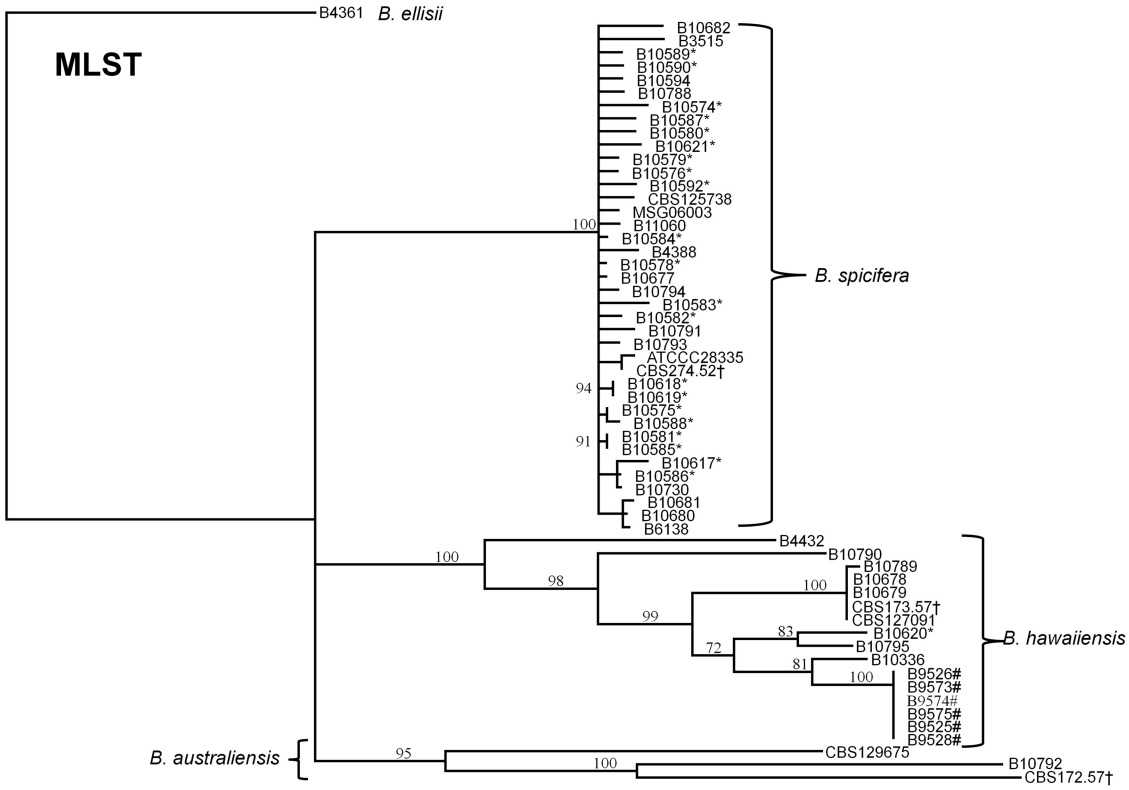

FIG 3 Dendrogram inferring the relatedness of 59 *Bipolaris* isolates using MLST. The dendrogram was constructed with the neighbor-joining method and rooted at *B. ellisii*. The genetic distance and topology reliability were calculated using the Tamura-Nei model and 1,000 bootstrap replicates, respectively. *Isolate from the POCS investigation; †type strain from CBS; #isolate from the endophthalmitis outbreak.

type isolate. Of the six markers used, *RPB2* most reliably segregated all of the isolates to specific species clusters (Fig. 2).

Discriminatory power of the MLST system. The MLST system displayed strong interspecies discriminatory power for the *Bipolaris* species investigated. It was able to segregate isolates of the same ITS-assigned species into distinct clades in concordance with the type strains, with bootstrapping values of >95% (Fig. 3). The MLST system also displayed good intraspecies differentiation (Table 1). For *B. spicifera*, *GPDH* displayed the highest discriminatory power with an SI of 0.84 while *EF1*α displayed the lowest discriminatory power with an SI of 0.23. Each MLST marker alone was able to divide the 39 *B. spicifera* isolates into multiple allele types, ranging between three and nine alleles per locus. For *B. hawaiiensis*, *SAL1* displayed the highest discriminatory power with an SI of 0.86, while *GPDH* displayed the lowest discriminatory power with an SI of 0.62. There were too few isolates of *B. australiensis* to adequately calculate the discriminatory power.

The MLST system divided the 39 *B. spicifera* isolates into 37 unique sequence types (STs). Isolates collected during the *Bipolaris* SSI investigation had unique STs except for two pairs of environmental isolates that belonged to the same ST; isolates B10618 and B10619 came from two different swabs of a medical instrument associated with a case-patient's care and shared an ST, and isolates B10581 and B10585 were cultured about 1 year apart from the environment of a hospital and shared an ST. The 16 *B. hawaiiensis* isolates were separated into 9 different STs. All isolates from a previous *Bipolaris* endophthalmitis outbreak (5) had identical STs, consistent with the epidemiological conclusion of a point source from a contaminated medication. The three *B. australiensis* isolates were divided into three unique STs.

DISCUSSION

In November 2013, the CDC was asked to assist in the investigation of a cluster of infections in POCS patients caused by the mold *Bipolaris*. One hypothesis was that this was a point source outbreak caused by a contaminated product. In addition to an epidemiologic investigation looking for common exposures, such a hypothesis can be investigated by DNA typing of the isolates and looking for genetic homology or heterogeneity. However, no typing system existed for *Bipolaris* at the time of the outbreak. We designed an MLST system using six distinct loci, all of which were informative. This new typing system was used to provide support against a point source in the POCS cluster and to corroborate the hypothesis of a contaminated product from an older outbreak.

This novel *Bipolaris* MLST system revealed a high degree of heterogeneity among the *B. spicifera* isolates. The 39 *B. spicifera* isolates separated into 37 unique STs. Two isolates collected a year apart at a hospital displayed identical MLST patterns. Together with the genetic stability experiments, this suggests that the MLST loci are stable. We also used this MLST system to analyze 16 *B. hawaiiensis* isolates which revealed 9 different STs. Although our isolate numbers were low, this MLST system also seemed to work for *B. australiensis* and *B. ellisii*; the MLST primers were able to amplify all six genes in both species. The typing system did not work for the species *B. cynodontis*.

Using the new MLST system, we were able to confirm that the

B. spicifera isolates from the *Bipolaris* POCS investigation were highly heterogeneous with no identical patterns among isolates from the case patients. This strongly supported the epidemiological data suggesting that the source was likely not a single-source contaminated product but rather that the likely sources of the infections were environmental. In contrast, the *B. hawaiiensis* isolates implicated in the 2012 endophthalmitis outbreak were clonal to the extent that was shown with this typing system, which confirms the epidemiological data suggesting that the mode of transmission was likely due to contaminated triamcinolone steroid injected into the eyes of patients (5).

Although fungal outbreaks are relatively uncommon, there have been a number of high-profile fungal-related outbreaks in recent years (27). Fungal typing systems were not available for the majority of these clusters, and typing was performed in most cases after the epidemiologic investigation was completed. We show here that MLST high-resolution typing systems can be developed when only a minimum number of sequences are available for the species of interest. With the increasing number of fungal whole genomes becoming available for loci identification, fungal MLST systems can be developed quickly for immediate use in public health investigations. While whole genomes or a next-generation typing system like NGMLST (28) will likely be the typing tools of the future, the comparative speed and cost of MLST make it a welcome alternative for current fungal epidemiologic investigations.

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