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Botrytis fragariae, a New Species Causing Gray Mold on Strawberries, Shows High Frequencies of Specific and Efflux-Based Fungicide Resistance

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ABSTRACT *Botrytis cinerea* causes pre- and postharvest decay of many fruit and vegetable crops. A survey of German strawberry fields revealed *Botrytis* strains that differed from *B. cinerea* in diagnostic PCR markers and growth appearance. Phylogenetic analyses showed that these strains belong to an undescribed species in *Botrytis* clade 2, named *Botrytis fragariae* sp. nov. Isolates of *B. fragariae* were detected in strawberry fields throughout Germany, sometimes at frequencies similar to those of *B. cinerea*, and in the southeastern United States. *B. fragariae* was isolated from overwintering strawberry tissue but not from freshly infected fruit. *B. fragariae* invaded strawberry tissues with an efficiency similar to or lower than that of *B. cinerea* but showed poor colonization of inoculated nonhost plant tissues. These data and the exclusive occurrence of this fungus on strawberry plants indicate that *B. fragariae* is host specific and has a tissue preference different from that of *B. cinerea*. Various fungicide resistance patterns were observed in *B. fragariae* populations. Many *B. fragariae* strains showed resistance to one or several chemical classes of fungicides and an efflux-based multidrug resistance (MDR1) phenotype previously described in *B. cinerea*. Resistance-related mutations in *B. fragariae* were identical or similar to those of *B. cinerea* for carbendazim (E198A mutation in *tubA*), azoxystrobin (G143A in *cytB*), iprodione (G367A+V368F in *bos1*), and MDR1 (gain-of-function mutations in the transcription factor *mrr1* gene and overexpression of the drug efflux transporter gene *atrB*). The widespread occurrence of *B. fragariae* indicates that this species is adapted to fungicide-treated strawberry fields and may be of local importance as a gray mold pathogen alongside *B. cinerea*.

IMPORTANCE Gray mold is the most important fruit rot on strawberries worldwide and requires fungicide treatments for control. For a long time, it was believed to be caused only by *Botrytis cinerea*, a ubiquitous pathogen with a broad host range that quickly develops fungicide resistance. We report the discovery and description of a new species, named *Botrytis fragariae*, that is widely distributed in commercial strawberry fields in Germany and the southeastern United States. It was observed on overwintering tissue but not on freshly infected fruit and seems host specific on the basis of its occurrence and artificial infection tests. *B. fragariae* has also developed resistance to several fungicides that is caused by mutations similar to those known in *B. cinerea*, including an efflux-based multidrug resistance. Our data indicate that *B. fragariae* could be of practical importance as a strawberry pathogen in some regions where its abundance is similar to that of *B. cinerea*.

KEYWORDS fungicide resistance, phylogeny

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The gray mold fungus *Botrytis cinerea* Pers. ex Fr. is one of the most important plant pathogens worldwide. It attacks about 1,000 known plant species and causes pre- and postharvest losses of cultivated fruits, vegetables, and ornamental flowers (1). It is the major representative of the genus *Botrytis*, which currently includes about 30 described species that are mainly necrotrophs (2). DNA sequence-based studies revealed *Botrytis* to be divided into two phylogenetic clades (3). Clade 1 includes *B. cinerea* and *B. pseudocinerea* which infect mostly or exclusively dicotyledonous plants, as well as host-specific species such as *B. fabae*, *B. calthae*, *B. sinoviticola*, and *B. californica*. Clade 2 is phylogenetically more diverse and consists of host-specific members that infect predominantly monocots but also several dicots (2). DNA sequencing and the use of PCR-based genetic markers have greatly improved genetic studies of gray mold field isolates and contributed to the identification of new *Botrytis* species such as the recently described species *B. californica*, *B. deweyae*, *B. prunorum*, *B. pyriformis*, and *B. sinoviticola* (4–8).

Gray mold is a major disease of strawberries and other soft fruits worldwide. Disease control involves several fungicide applications during bloom and sometimes fruit maturation, imposing strong selection pressure on the fungal population. In recent years, high frequencies of fungicide resistance that threaten the efficacy of chemical control of gray mold have been observed in *B. cinerea* populations from strawberry fields in Germany, Greece, Italy, and the United States (9–12). In addition to the prevailing target site resistances, drug efflux-mediated multidrug resistance (MDR) phenotypes are widespread in *B. cinerea* populations (13, 14). The MDR1 phenotype and a more resistant MDR1 variant called MDR1h are caused by overexpression of the ABC-type drug efflux transporter AtrB. The MDR1 and MDR1h phenotypes are likely to reduce fungicide field efficacy because they confer partial resistance to two important botryticides, fludioxonil and cyprodinil (10, 14, 15). Genetic studies revealed a diverse composition of *Botrytis* populations in German strawberry fields, comprising several genotypes of *B. cinerea* and minor frequencies of *B. pseudocinerea* (10, 16; M. Hahn and S. Rupp, unpublished data). In striking contrast to *B. cinerea*, *B. pseudocinerea* has almost no documented fungicide resistance, the exception being a low-level intrinsic resistance to fenhexamid (16).

During large-scale monitoring of gray mold populations in German strawberry fields, we observed *Botrytis* isolates with a growth morphology distinct from that of *B. cinerea* and *B. pseudocinerea*, which gave unclear results with PCR primers for these two species. Sequence analysis of five phylogenetically informative genes characterized these isolates as members of a previously undescribed species, designated *Botrytis fragariae* sp. nov., on account of its main host plant. Further analyses were conducted to perform a detailed morphological description of the new species, to analyze its occurrence in strawberry fields and on wild strawberries, to determine its host and possibly tissue specificity, and to identify fungicide resistances and the underlying mutations.

RESULTS

Discovery, phylogenetic placement, and molecular identification of a new *Botrytis* species. *Botrytis* strains with growth behavior on MYA agar plates that differed from that of *B. cinerea* and *B. pseudocinerea* strains were observed in several strawberry fields. DNA from these *Botrytis* strains was poorly amplified with *mrr1* primers Mrr1-spez-F and Mrr1-spez-R. For three of these strains, a PCR with primers flanking a *B. pseudocinerea*-specific deletion (see Table S1 in the supplemental material) (16) resulted in a product with a size (ca. 145 bp) larger than that of the product obtained with either *B. cinerea* (136 bp) or *B. pseudocinerea* (112 bp; see Fig. S1 in the supplemental material). For phylogenetic placement, four of these unusual strains and three similar strains isolated from strawberry flowers in South Carolina (see below) were selected for sequencing of the *hsp60*, *g3pdh*, *rpb2*, *nep1*, and *nep2* genes (Table 1). A combined tree placed all of the strains into a clade that was clearly separated from all other *Botrytis* spp., the closest relative being *B. paeoniae* (Fig. 1). Trees based on each of the five

TABLE 1 GenBank accession numbers of *B. fragariae* sequences determined in this study

Strain[d]	GenBank accession no. of:								
	hsp60	*g3pdh*	*rpb2*	*nep1*	*nep2*	*bos1*	*mrr1*	*cytB*	*tubA*
U14_P1	KX429692	KX429699	KX429706	KX429713	KX429720	ND[a,b]	ND	ND	ND
U14_G2	KX429693	KX429700	KX429707	KX429714	KX429721	ND	ND	ND	ND
U14_H3	KX429694	KX429701	KX429708	KX429715	KX429722	ND	ND	ND	ND
D11_H_R4	KX429695	KX429702	KX429709	KX429716	KX429723	KX429730	KX429733	KX429739	KX429741
D13_F_Me3	KX429696	KX429703	KX429710	KX429717	KX429724	ND	KX429734	KX429740	ND
D13_F_Ju10	KX429697	KX429704	KX429711	KX429718	KX429725	ND	ND	ND	ND
D13_H_J2-34	KX429698	KX429705	KX429712	KX429719	KX429726	KX429727	ND	ND	KX429742
D13_F_Nba1	ND	ND	ND	ND	ND	KX429728	KX429735	ND	ND
D13_F_Nba10	ND	ND	ND	ND	ND	KX429729	ND	ND	ND
D13_F_Ju21	ND	ND	ND	ND	ND	KX429732	KX429736	ND	ND
D13_F_Ju26	ND	ND	ND	ND	ND	ND[b]	KX429737[c]	ND	ND
D14_F_Ju20	ND	ND	ND	ND	ND	ND[b]	KX429738	ND	ND
D14_Bl.427-22	ND	ND	ND	ND	ND	ND[b]	ND[b,c]	ND	ND
D14_Bl.427-23	ND	ND	ND	ND	ND	ND[b]	ND[b,c]	ND	ND
D15_F_WR3	ND	ND	ND	ND	ND	KX429731	ND[b]	ND	ND

[a]ND, sequence not determined.
[b]Resistance mutation identified.
[c]Mutation identical to MDR1-related mutation in *B. cinerea* (22).
[d]D13_F_Ju10 is listed in GenBank as D13_D_F_Ju10, D13_H_J2-34 is listed in GenBank as D13_B_F_HJ_2-34, D13_F_Nba1 is listed in GenBank as D13_T_F_Nba1, and D13_F_Ju26 is listed in GenBank as D13-D-F-Ju26.

individual gene sequences also separated the strains from all other *Botrytis* species with high bootstrap values (see Fig. S2).

On the basis of the sequencing data, their association with strawberries, and several unique phenotypic characters (see below), the seven strains were identified as members of a new species, which was named *Botrytis fragariae* sp. nov. For rapid preliminary identification of *B. fragariae*, a PCR-restriction fragment length polymorphism (RFLP) analysis method based on a species-specific sequence polymorphism was developed.

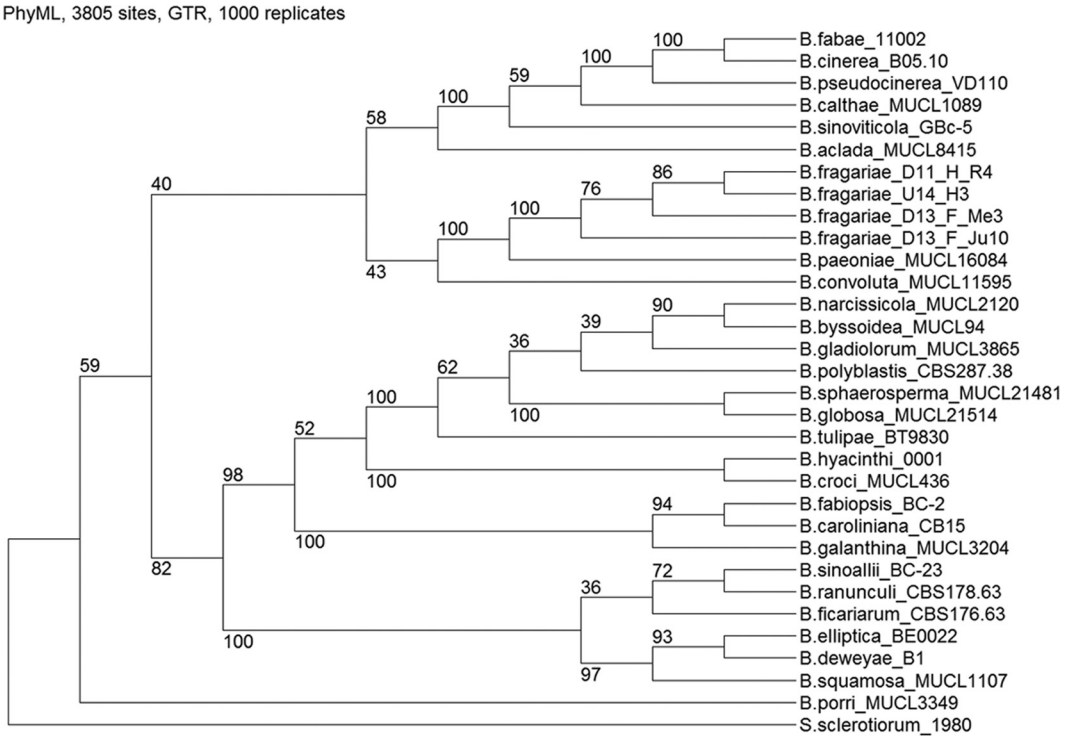

FIG 1 Phylogenetic tree of *Botrytis* spp., including three strains of *B. fragariae* from Germany (D11_H_R4, D13_F_Me3, and D13_F_Ju10) and one from South Carolina (U14_H3), based on combined *hsp60*, *g3pdh*, *rpb2*, *nep1*, and *nep2* sequences. *Sclerotinia sclerotiorum* was used as the outgroup.

A PCR fragment covering *g3pdh* was digested with the restriction enzyme BsaJI in all of the *B. fragariae* strains tested, but the BsaJI site was missing from the *g3pdh* genes of all of the other 29 *Botrytis* spp. described, including *B. cinerea* and the closely related species *B. paeoniae*, on the basis of the available sequence data in the NCBI database (see Fig. S3).

Distribution and host and tissue preference of *B. fragariae*. By applying the diagnostic PCR-RFLP for identification of *B. fragariae* and the indel-based differentiation of *B. cinerea* and *B. pseudocinerea* (16), we estimated the relative abundance of the three *Botrytis* species by analyzing a total of 1,425 isolates from 28 fields (several fields were sampled up to three times) in Germany in different seasons between 2011 and 2015. In nine of the fields, *B. fragariae* was detected. In four of these fields, 25 to 65% of the isolates recovered belonged to *B. fragariae*, indicating that the species can be locally abundant (see Fig. S4). Overall, *B. cinerea* was clearly dominant (90.3%), followed by *B. pseudocinerea* (7.2%) and *B. fragariae* (2.5%). Curiously, all of the *B. fragariae* isolates obtained so far were isolated from overwintering, partly rotten vegetative strawberry tissues and fruit mummies collected before the fungicide treatments but none were from freshly harvested fruit. Among the *Botrytis* isolates collected before the fungicide treatments ($n = 689$; 22 fields), 87.1% of the isolates were *B. cinerea*, followed by *B. pseudocinerea* (8.6%) and *B. fragariae* (4.4%). Because of the prevalence of *B. fragariae* in spring 2013 in Gernsheim (13 of 20 isolates), samplings in that field were repeated. *B. fragariae* was not found among 20 isolates from moldy fruit in summer 2013 after the treatments but was detected again on leaves (8 of 20 isolates) in spring 2014 (Table 2). In contrast, in 2014, no isolate of *B. fragariae* was obtained from a total of 88 isolates obtained from three raspberry fields within a 2-km radius of the location of the type strain in Nottensdorf (Table 2). To determine whether *B. fragariae* occurs on wild strawberries, green and rotten petioles and leaves of *Fragaria vesca* were collected in November 2016 at four sites in a forest in western Germany. Of the 43 *Botrytis* isolates recovered, 32 were classified as *B. cinerea* and 11 were classified as *B. pseudocinerea* but none were classified as *B. fragariae* (see Table S3).

Genetic variability of *B. fragariae*. The *B. fragariae* isolates were analyzed for the occurrence of the two mating type loci, the presence of the DNA transposon flipper (17), and the restriction patterns of PCR fragments generated with primers based on the intergenic region of the ribosomal DNA (IGS) (18). In contrast to *B. cinerea*, no variability was observed in the IGS-PCR patterns of the *B. fragariae* strains (not shown). Both mating type loci were observed, with a predominance of *MAT1-1* (25 isolates) over *MAT1-2* (6 isolates). Nineteen of the 33 strains tested contained flipper DNA. The sequences of two strains were 99 to 100% identical to the *B. cinerea* flipper DNA sequence (data not shown). When fungicide resistance was scored as equivalent to a genetic marker, 18 of the 38 isolates tested represented different haplotypes. Sequencing of the five genes used for taxonomic classification (Fig. 1; Table 1) revealed that all seven strains were genetically different. No significant genetic differentiation was possible between German and U.S. strains.

Phenotypic characterization of *B. fragariae*. Growth parameters were tested for selected *B. fragariae* strains and compared to those of *B. cinerea*. On solid minimal or rich medium, the appearance of colonies was similar for the two species except that the growth of *B. fragariae* was significantly slower than that of *B. cinerea* (Fig. 2A; Table 3). When cultures started to sporulate, they turned gray to brown (Fig. 2A). The sporulation yield of *B. fragariae* on minimal medium was only about half of that of *B. cinerea*, and the macroconidia were slightly smaller than those of *B. cinerea* (Table 3). After 4 weeks of incubation in the dark, black sclerotia were produced; their number and size varied considerably between the strains of the two species (Fig. 2A). On minimal and rich agar media, the sporulating mycelium of all *B. fragariae* strains was flatter and more compact than that of *B. cinerea* (Fig. 2B). This difference was also evident on infected strawberry tissues (Fig. 2C).

TABLE 2 Phenotypic and genetic characterization of *B. fragariae* strains isolated from strawberry fields in Germany and the United States[a]

Field site (time of isolation)	Plant tissue	Strain	Fungicide resistance							*MAT1* locus	Flip	Haplotype
			Flu[b]	Fen	Cyp	Carb	Ipr	Bos	Azo			
Nottensdorf (autumn 2011)	Fruit mummies	D11_H_R3	r	S	S	S	R	S	S	*1*	Yes	1
		D11_H_R4[c]	r	S	S	S	R	S	S	*2*	No	2
		D11_H_R5	r	S	S	S	R	S	R	*1*	Yes	3
		D11_H_R8	r	S	S	R	R	S	R	*1*	Yes	4
		D11_H_R11	r	S	S	S	R	S	S	*2*	Yes	5
Iffezheim (spring 2013)	Flower	D13_HJ2-34	r	S	S	R	S	S	R	*2*	No	6
Weiterstadt (spring 2013)	Flower	D13_F_Me3[c]	r	S	S	S	S	S	R	*2*	Yes	7
Grafschaft (spring 2012)	Leaf	D12_K_Mai_MU10	R	S	S	S	R	S	S	NA[e]	NA	
Wittlich-A (spring 2013)	Leaves and stems	D13_F_Nba1	R	S	S	R	S	S	S	*1*	No	8
		D13_F_Nba9	R	S	S	S	S	S	S	*1*	No	9
		D13_F_Nba10	r	S	S	R	S	S	S	*1*	No	10
		D13_F_Nba12	r	S	S	R	S	S	S	*1*	No	10
		D13_F_Nba13	R	S	S	S	S	S	S	*1*	No	9
Gernsheim-1 (Spring 2013)	Leaves and stems	D13_F_Ju1	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju4	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju10[c]	r	S	S	S	S	S	S	*1*	No	12
		D13_F_Ju11	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju13	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju15	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju18	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju19	r	S	S	S	S	S	S	*1*	No	12
		D13_F_Ju21	R	S	R	S	R	S	S	*1*	No	13
		D13_F_Ju23	r	S	S	S	S	S	S	*1*	No	12
		D13_F_Ju26	R	S	S	S	R	S	S	*2*	No	14
		D13_F_Ju28	r	S	S	S	S	S	S	*1*	Yes	11
		D13_F_Ju29	R	S	R	S	R	S	S	*1*	Yes	15
Gernsheim-2 (Spring 2014)	Leaves and stems	D14_F_Ju12	R	S	R	S	R	S	S	*1*	Yes	15
		D14_F_Ju13	R	S	R	S	R	S	S	*1*	Yes	15
		D14_F_Ju14	r	S	S	S	S	S	S	*1*	Yes	11
		D14_F_Ju16	R	S	R	NA	NA	S	R	NA	NA	
		D14_F_Ju17	R	S	R	S	R	S	S	*1*	No	13
		D14_F_Ju18	r	S	S	NA	NA	S	R	*1*	No	
		D14_F_Ju19	R	S	R	NA	NA	S	S	NA	NA	
		D14_F_Ju20	R	S	R	S	R	S	S	*2*	Yes	16
Wittlich-B (Spring 2014)	Leaf	D14_F_Nb3	r	S	S	R	S	S	R	*2*	Yes	17
Wennigsen-Deister (Spring 2014)	Leaves and stems	D14_Bl.427-22	R	S	R	S	R	S	S	NA	NA	
		D14_Bl.427-23	R	S	R	S	R	S	S	NA	NA	
Wagshurst (Spring 2015)	Leaf	D15_W_R3	r	S	R	R	R	S	R	NA	Yes	18
Pelion, SC, USA (spring 2015)	Flower	U14_P1	r	S	S	R	S	S	S	NA	NA	
Gilbert SC, USA (spring 2015)	Flower	U14_G2	r	S	S	R	S	S	R	NA	NA	
Holly Hill, SC, USA (spring 2015)	Flower	U14_H3	r	S	S	R	S	S	S	NA	NA	

[a]The fungicide treatment histories of the fields are shown in Table S4. R, resistant; S, sensitive; NA, not analyzed. Flip, flipper DNA.
[b]r, growth on 0.2 μg/ml fludioxonil; R, growth on 1 μg/ml fludioxonil.
[c]Deposited in the CBS culture collection.

Infection behavior of *B. fragariae* and *B. cinerea* on different tissues. The virulence of *B. fragariae* was studied by artificial inoculation of several known host plants of *B. cinerea*. On strawberry fruit, *B. fragariae* showed a somewhat lower infection rate than *B. cinerea* and both the mycelium development and sporulation of *B. fragariae* occurred later and were less profuse than those of *B. cinerea* (Fig. 3; see Fig. S3A). On wounded strawberry leaves inoculated with agar discs containing germinated spores, both species produced expanding lesions but the lesions due to *B. fragariae* were smaller ($P < 0.001$) than those due to *B. cinerea* (see Fig. S3B). On strawberry flower petals, the two species formed necrotic lesions at similar rates (Fig. 3 and 4). On nonstrawberry tissues, *B. fragariae* strains were much less aggressive than *B. cinerea*, except for the infections of dog rose petals, in which the two species were comparable. *B. cinerea* rapidly colonized and formed spreading lesions on tomato leaves, *Gerbera* and blackberry flower petals, and apple fruit. *B. fragariae* formed only small primary lesions or sometimes no lesions at all (Fig. 3 and 4). The competitiveness of *B. fragariae* and *B. cinerea* was evaluated by mixed-inoculation experiments. On strawberry leaves,

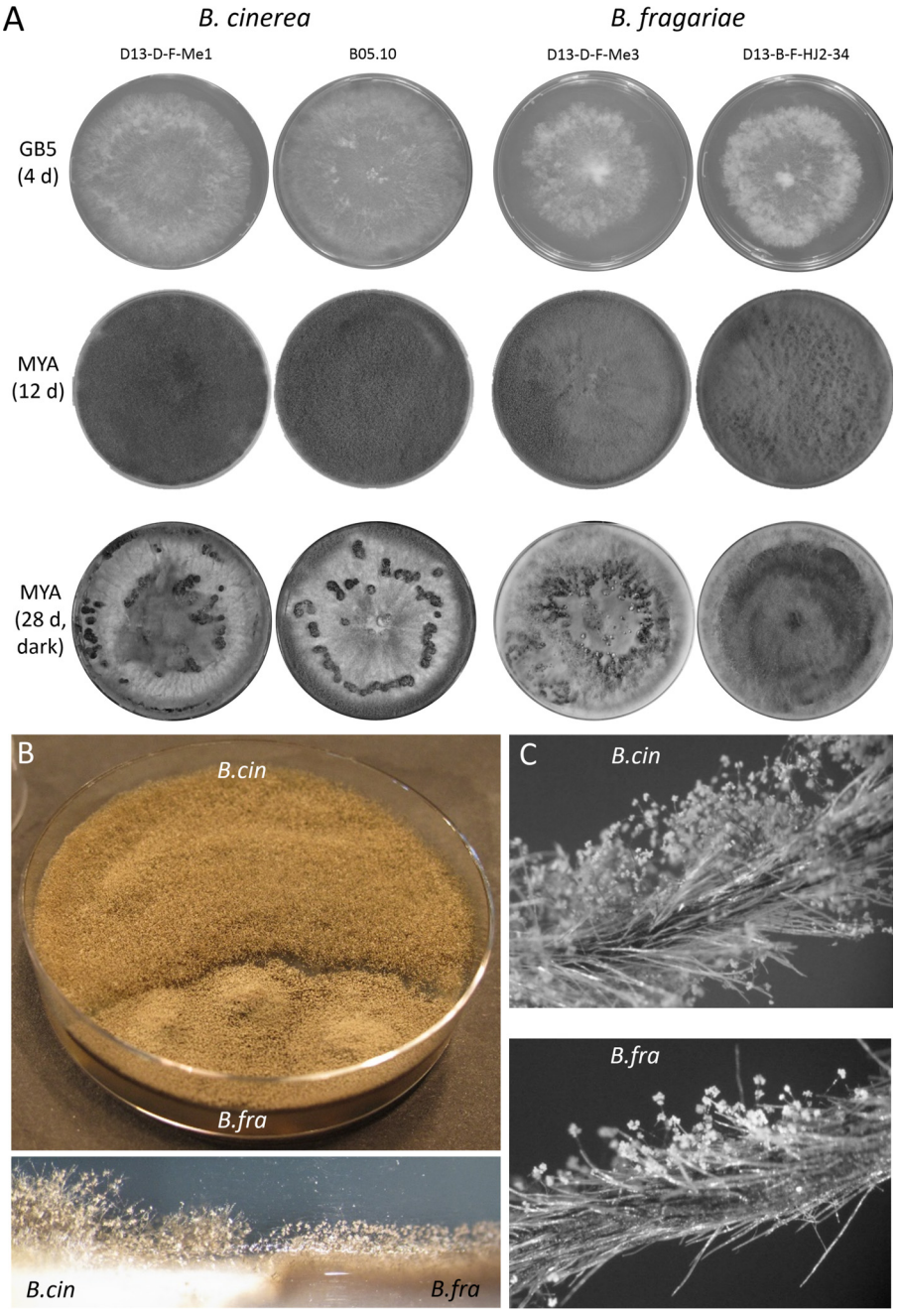

FIG 2 Growth behavior and morphology of *B. fragariae* and *B. cinerea*. (A) Mycelium growth and sclerotium formation. (B) Different heights of aerial mycelia of *B. cinerea* (*B.cin*) and *B. fragariae* (*B.fra*). (C) Aerial hyphae with conidiophores bearing clusters of macroconidia on strawberry petioles.

B. cinerea strains outcompeted *B. fragariae* strains in six out of eight experiments in the ability to form mycelium in the spreading lesions. In all experiments with strawberry fruit, *B. cinerea* dominated over *B. fragariae* (Table 4).

Fungicide resistance frequencies. Fungicide sensitivity assays were performed on agar plates containing discriminatory fungicide concentrations to detect resistance to the seven main site-specific fungicides used against *Botrytis* (10). Of the 38 German and three North American *B. fragariae* isolates analyzed (Table 2), 30 were resistant to at least one fungicide (excluding the low intrinsic resistance to fludioxonil) and 1 was resistant to four fungicides (see Fig. S4). The fungicide resistance frequencies in nine fields in which all three species were isolated were compared (Fig. 5). While the

TABLE 3 Growth parameters of selected *B. cinerea* and *B. fragariae* strains[a]

Species and strain	Mean radial growth on agar after 72 h (mm) ± SD		Mean sporulation on agar (10^5 conidia cm^{-2}) after 9 days ± SD		Mean conidial length, width (μm) ± SD
	GB5	MA	GB5	MA	
B. cinerea					
B05.10	8.8 ± 1.3	9.9 ± 0.7	11.8 ± 3.6	12.7 ± 2.6	9.9 ± 1.3, 7.0 ± 0.9
D13_F_Ju3	6.1 ± 1.9	5.0 ± 1.5	8.2 ± 6.8	7.6 ± 1.4	11.9 ± 1.8, 8.5 ± 1.2
D13_F_Me1	5.8 ± 0.7	8.4 ± 2.2	7.1 ± 3.4	4.8 ± 1.1	9.3 ± 1.2, 7.2 ± 0.9
All	6.9 ± 2.0	7.7 ± 2.6	9.0 ± 2.8	8.3 ± 5.8	10.4 ± 2,2, 7.5 ± 1.2
B. fragariae					
D11_H_R4H-R4	2.6 ± 0.8	3.0 ± 0.8	3.2 ± 1.2	2.4 ± 2.4	8.7 ± 1.5, 6.2 ± 0.7
D13_HJ2-34	3.5 ± 1.7	5.2 ± 2.0	3.7 ± 3.5	7.0 ± 2.3	10.0 ± 2.2, 7.1 ± 1.2
D13_F_Me3	3.6 ± 1.6	5.5 ± 1.7	4.5 ± 1.2	1.9 ± 0.9	10.2 ± 1.9, 7.2 ± 0.8
D13_F_Ju10	2.1 ± 0.5	4.4 ± 1.7	5.1 ± 2.1	7.5 ± 0.3	9.3 ± 1.8, 6.0 ± 0.5
All	3.0 ± 1.4	4.5 ± 1.9	4.1 ± 1.7	5.3 ± 3.3	9.6 ± 1.9, 6.6 ± 1.0

[a]Growth and sporulation data are the mean values of five and three biological replicates, respectively. Conidial sizes were determined from 100 conidia of each strain. Mean values of growth and sporulation of *B. cinerea* and *B. fragariae* are significantly different ($P < 0.0001$), except for sporulation on MYA. Mean conidial sizes were also different (length, $P = 0.013$; width, $P < 0.001$ [two-sided *t* test]).

dominant species, *B. cinerea*, showed high frequencies of resistance to all five fungicides registered for gray mold control in Germany, all *B. pseudocinerea* isolates were sensitive, except for one carbendazim-resistant isolate. Some *B. fragariae* strains were resistant to cyprodinil, azoxystrobin, fludioxonil, iprodione, and carbendazim, but none was resistant to boscalid or fenhexamid.

Quantitative evaluation of the sensitivity and resistance of *B. fragariae* strains to the fungicides fludioxonil, cyprodinil, and iprodione was performed. High levels of resistance of *B. cinerea* to fludioxonil have not been found in most field environments, but intermediate resistance caused by efflux-mediated MDR type 1 (MDR1) occurs frequently in gray mold populations in fungicide-treated fields in Europe and at lower frequencies in the United States (10, 19). All of the *B. fragariae* strains showed a sensitivity to fludioxonil approximately 10-fold lower than that of sensitive *B. cinerea*

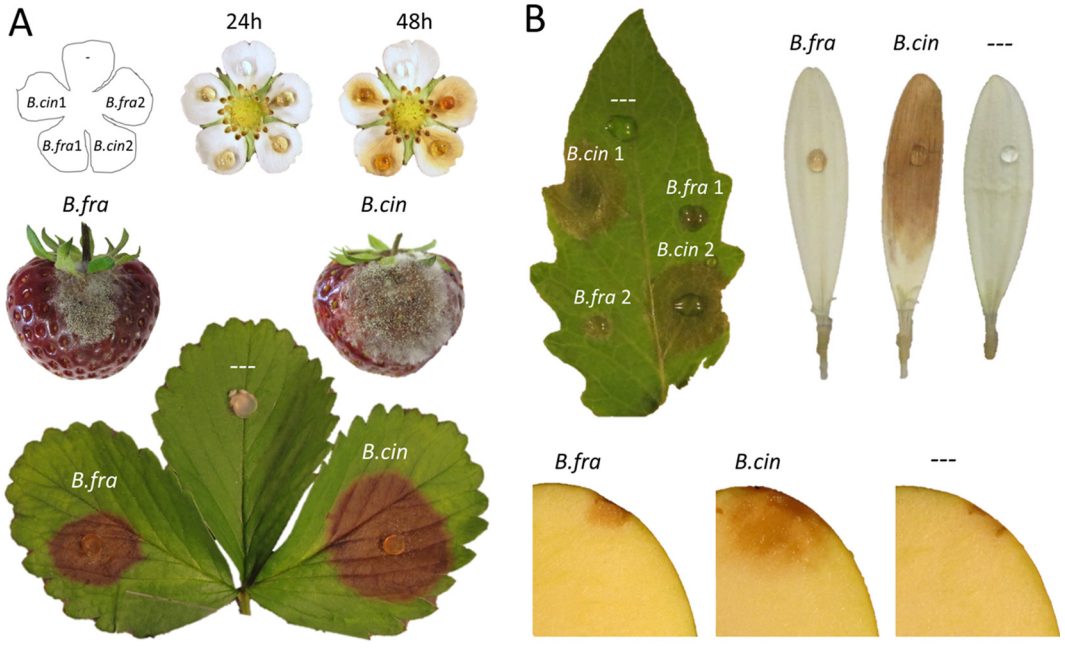

FIG 3 Symptoms induced by *B. cinerea* (*B.cin*) and *B. fragariae* (*B.fra*) on strawberries (A) and the nonhost tissues tomato leaf, *Gerbera* petals, and apple fruit (B).

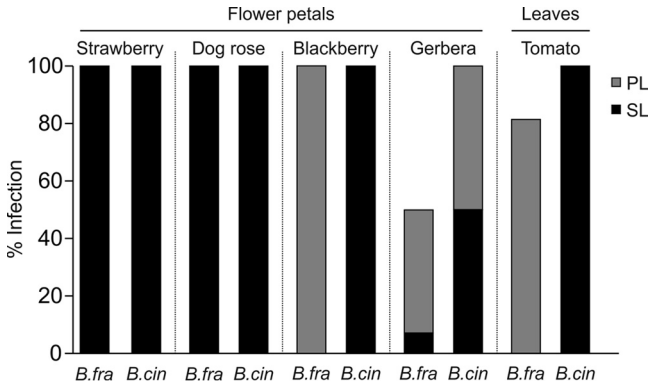

FIG 4 Virulence of *B. cinerea* (*B.cin*) and *B. fragariae* (*B.fra*) on different plant tissues 72 h after inoculation. On tomato leaves, strawberry petals, and *Gerbera* petals, five *B. cinerea* strains and seven *B. fragariae* strains were analyzed. On dog rose and blackberry petals, two *B. cinerea* strains and two *B. fragariae* strains were analyzed. PL, primary localized lesions; SL, secondary expanding lesions. Tests were performed three times with duplicates.

strains (Fig. 6A) and were able to grow on media containing 0.2 mg of fludioxonil liter^{-1}, a concentration that inhibits the growth of sensitive but not MDR1 *B. cinerea* strains. Because these strains were sensitive to cyprodinil, the reduced fludioxonil sensitivity of *B. fragariae* was probably independent of drug efflux-related MDR. However, several *B. fragariae* strains were able to grow on plates containing 1 mg of fludioxonil liter^{-1}. All of these strains had medium or high resistance to cyprodinil (Fig. 6B) and high resistance to tolnaftate (not shown), strongly indicating an MDR1-like phenotype (10). The fludioxonil resistance levels of these *B. fragariae* strains were similar to those of *B. cinerea* strains with the MDR1h phenotype, a more resistant variant of MDR1 (Fig. 6A) (10). The baseline sensitivity of *B. fragariae* strains to cyprodinil was similar to that of *B. cinerea* (Fig. 6B). The cyprodinil resistance levels of *B. fragariae* MDR1 strains were higher than those of *B. cinerea* MDR1 or MDR1h strains (Fig. 6B). The baseline sensitivities of *B. fragariae* and *B. cinerea* to iprodione were similar. Resistant *B. fragariae* strains could be grouped into medium- and high-resistance strains (Fig. 6C).

TABLE 4 Results of mixed-inoculation experiments with *B. cinerea* and *B. fragariae* strains on strawberry tissues

Substrate and *B. cinerea* strain	*B. fragariae* strain	Mean % recovery of *B. cinerea* ± SD (*n*)[a]
Leaves		
B05.10	D14_F_Ju12	90.0 ± 14.1 (5)
B05.10	D14_F_Ju12	90.7 ± 14.1 (5)
B05.10	D13_F_Ju29	94 ± 12.0 (5)
B05.10	D13_F_Ju29	95.0 ± 11.2 (5)
D09-A04	D14_F_Ju12	94 ± 12.0 (5)
D09-A04	D14_F_Ju12	50 ± 28.9 (5)
D09-A04	D13_F_Ju29	100 (5)
D09-A04	D13_F_Ju29	48 ± 40.7 (5)
All		83.3 ± 28.4
Fruit		
B05.10	D14_F_Ju12	64.7 ± 47.4 (14)
B05.10	D14_F_Ju12	93.2 ± 8.0 (20)
B05.10	D14_F_Ju12	84.5 ± 31.1 (17)
B05.10	D13_F_Me3	100 (14)
B05.10	D13_F_Me3	72.3 ± 31.5 (22)
B05.10	D13_F_Me3	66.9 ± 25.5 (13)
All		80.7 ± 30.3

[a]Mean values and standard deviations of two independent experiments are shown. Strains were identified by their resistance to carbendazim (*B. cinerea*) and to fludioxonil and cyprodinil (*B. fragariae*).

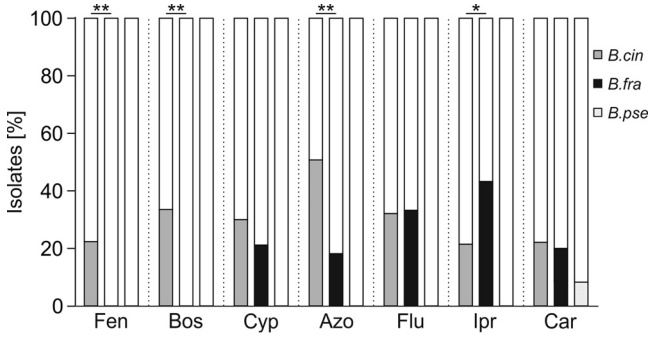

FIG 5 Fungicide resistance frequencies of *B. cinerea* (*B.cin*; $n = 134$; gray bars), *B. fragariae* (*B.fra*; $n = 33$; black bars), and *B. pseudocinerea* (*B.pse*; $n = 12$; light gray bars). Data are from isolates collected in nine German strawberry fields from which all three species were recovered. Significant differences in resistance frequencies between *B. fragariae* and *B. cinerea* are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$ [independent-sample *t* test]). Fen, fenhexamid; Bos, boscalid; Cyp, cyprodinil; Azo, azoxystrobin; Flu, fludioxonil; Ipr, iprodione; Car, carbendazim.

Fungicide resistance mutations. To identify the mutations responsible for the resistance phenotypes observed, the known target genes of carbendazim (*tubA* encoding β-tubulin), quinone outside inhibitor (QoI) fungicides (*cytB*), and iprodione (*bos1*) were sequenced. All of the carbendazim-resistant strains tested contained the E198A mutation in *tubA*, and the azoxystrobin-resistant strains contained the G143A mutation in *cytB* (Table 5). Both mutations are common in fungi resistant to carbendazim and QoI, respectively, including *B. cinerea*. The *bos1* sequences of five highly iprodione-resistant strains and one strain with medium resistance (D11_H_R4) contained two changes in adjacent codons (glycine to alanine at codon 367, valine to phenylalanine at codon 368 [G367A-V368F]). Whereas the double mutation has not been previously described in *B. cinerea*, mutations at positions 365, 368 (including V368F), and 369 are common in iprodione-resistant *B. cinerea* strains (20, 21). In medium resistance strain D13_F_Ju21, the observed changes (E62K, G748V) did not correspond to any of the known iprodione resistance-related mutations in *B. cinerea* (Table 5).

B. cinerea strains with the MDR1 phenotype harbor gain-of-function mutations in the transcriptional activator Mrr1 (13). Therefore, we sequenced most of the coding region of *mrr1* of three *B. fragariae* strains with partial fludioxonil resistance and two strains with baseline sensitivity to fludioxonil, corresponding to bp 115 to 2411 of *B. cinerea* B05.10 *mrr1*. The *mrr1* nucleotide sequences of the sensitive strains were identical to one another and encoded a protein with 87% and 84% identity to Mrr1 sequences of *B. cinerea* and *B. cinerea* group S strains, respectively (10). The *mrr1* sequences of three

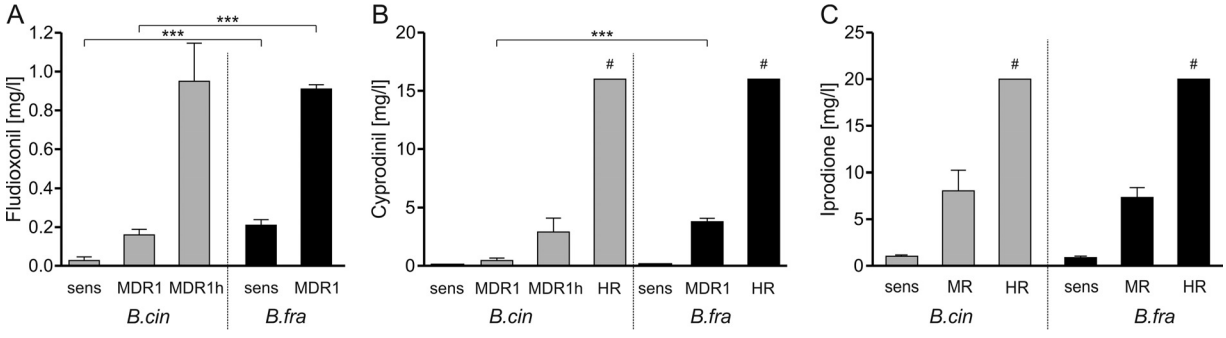

FIG 6 Different fungicide sensitivities (EC_{50}s) of *B. cinerea* (*B.cin*) and *B. fragariae* (*B.fra*) strains. (A) Fludioxonil. Mean values for sensitive (sens), MDR1, and MDR1h strains (one of each) of *B. cinerea* and three sensitive and three MDR1 strains of *B. fragariae*. (B) Cyprodinil. Mean values for sensitive, MDR1, and MDR1h strains of *B. cinerea* and three sensitive, three MDR1, and three highly resistant (HR; >16 mg liter^{-1}) strains of *B. fragariae*. (C) Iprodione. Mean values for sensitive (sens; 0.5 to 2.0 mg liter^{-1}), moderately resistant (MR; >2 to 20 mg liter^{-1}), and highly resistant (HR; >20 mg liter^{-1}) strains of *B. cinerea* (one of each) and three sensitive, three medium resistance, and three high resistance strains of *B. fragariae*. #, resistant to the highest concentration tested. The strains used are listed in Table S5. ***, significant differences between selected groups of *B. cinerea* and *B. fragariae* strains ($P < 0.001$ by two-tailed *t* test).

TABLE 5 Fungicide resistance-related mutations identified in *B. fragariae* strains and fungicide sensitivity levels of selected strains

Strain	Azoxystrobin (*cytB*)	Carbendazim (*tubA*)	Iprodione (*bos1*)		Fludioxonil/MDR1 (*mrr1*)	
			Mutation	Avg EC_{50} (mg liter^{-1}) ± SD	Mutation	Avg EC_{50} (mg liter^{-1}) ± SD
D11_H_R4	Sensitive[a]	Sensitive[a]	G367A-V368F	8.35 ± 0.2	Sensitive[a]	0.18 ± 0.04
D13_F_Me3	G143A	Sensitive	Sensitive	0.94 ± 0.2	Sensitive[a]	0.24 ± 0.06
D13_HJ2-34	G143A	E198A	Sensitive[a]	0.90 ± 0.3	Sensitive	0.20 ± 0.06
D13_F_Nba1	Sensitive	E198A	Sensitive[a]	1.95 ± 0.6	Sensitive[a]	ND[b]
D14_F_Nba3	Sensitive	E198A	Sensitive	ND	Sensitive	ND
D13_F_Nba10	Sensitive	E198A	Sensitive[a]	0.79 ± 0.3	Sensitive	ND
D13_F_Ju21	Sensitive	Sensitive	E62K, G748V	6.20 ± 2.1	C588Y	0.90 ± 0.4
D13_F_Ju26	Sensitive	Sensitive	G367A-V368F	ND	R632I	0.86 ± 0.5
D14_F_Ju18	G143A	Sensitive	Sensitive	ND	Sensitive[a]	ND
D14_F_Ju20	Sensitive	Sensitive	G367A-V368F	>32	R632I	1.01 ± 0.4
D14_Bl.427-22	Sensitive	Sensitive	G367A-V368F	>32	R632I	0.96 ± 0.2
D14_Bl.427-23	Sensitive	Sensitive	G367A-V368F	ND	R632I	ND
D15_F_WR3	G143A	E198A	G367A-V368F	>32	C588Y	ND
U14_P1	Sensitive	E198A	Sensitive	ND	Sensitive	ND
U14_G2	G143A	E198A	Sensitive	ND	Sensitive	ND
U14_H3	Sensitive	E198A	Sensitive	ND	Sensitive	ND

[a]Target gene of sensitive strain sequenced.
[b]ND, not determined.

resistant strains each possessed a single nonsynonymous mutation (Table 5). Three further resistant strains, from which *mrr1* was partially sequenced, also contained a single amino acid-changing mutation (not shown). Of these six *B. fragariae* MDR1 strains, four carried a mutation leading to an R632I exchange, which is identical to a previously described MDR1-related mutation in *B. cinerea* (19). Two strains had a C588Y exchange, which has not been described before but is located in a region in which many other MDR1-related mutations have been found in *B. cinerea* (22).

Overexpression of *atrB* in MDR1 strains. In *B. cinerea* MDR1 and MDR1h strains, *atrB* encoding the ABC-type drug efflux transporter is overexpressed in an Mrr1-dependent manner (10, 13). We therefore compared the mRNA levels of *atrB* in sensitive, MDR1, and MDR1h strains of *B. cinerea* and *B. fragariae*. *B. cinerea* MDR1 strain D06_5-16 and *B. fragariae* MDR1 strain D14_F_Ju20 both carried the same mutation in *mrr1*, R632I. For reverse transcription (RT)-PCR, a primer pair suitable for *atrB* detection in both species was designed. The basal *atrB* expression levels of sensitive *B. fragariae* strains were significantly higher than those of sensitive *B. cinerea* strains. The basal levels of *atrB* were higher in *B. fragariae* and *B. cinerea* MDR1 strains than those of the corresponding sensitive strains and even higher in a *B. cinerea* MDR1h strain (Fig. 7A). In all *B. cinerea* strains, *atrB* expression was strongly upregulated in the presence of cyprodinil, as shown previously (10). Treatment of *B. fragariae* also resulted in upregu-

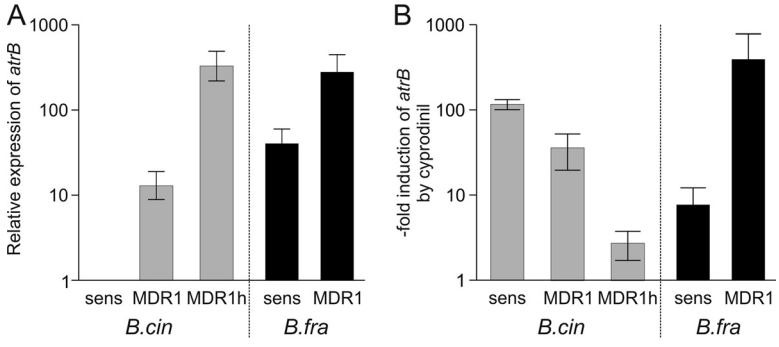

FIG 7 Expression of *atrB* in *B. cinerea* (*B.cin*) and *B. fragariae* (*B.fra*) strains with and without the MDR1 and MDR1h phenotypes. (A) Basal transcript levels. The value of the sensitive (sens) *B. cinerea* strain (B05.10) was set to 1. (B) Increased expression of *atrB* in *B. cinerea* and *B. fragariae* strains after treatment with 1 mg of cyprodinil liter^{-1} for 30 min. The strains used are listed in Table S5.

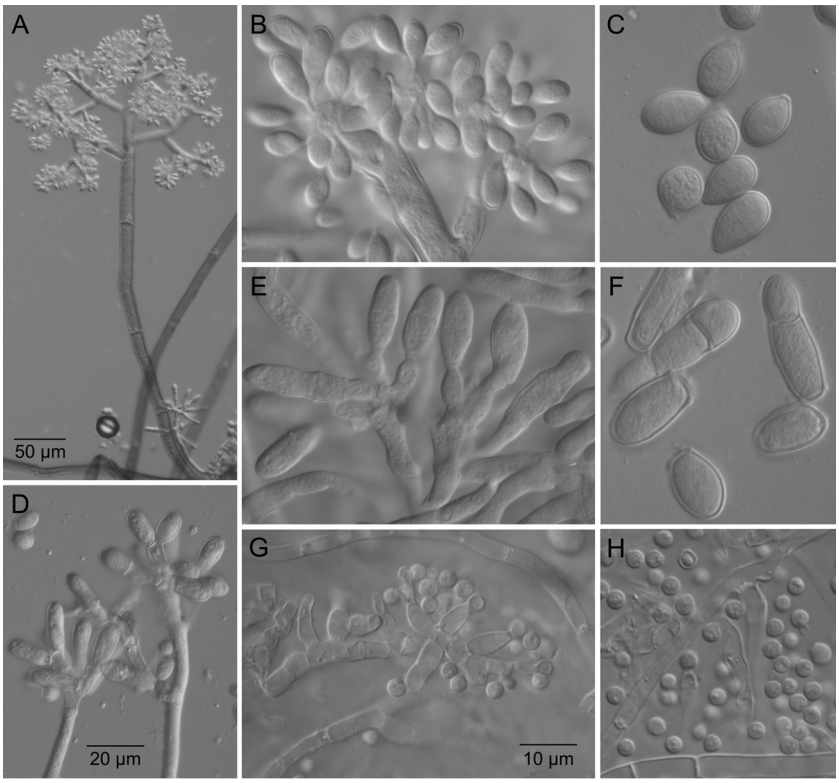

FIG 8 Microscopic features of *B. fragariae*. (A) Large and small macroconidiophores in direct comparison. (B) Details of conidiogenesis on large macroconidiophores. (C) Conidia produced from large macroconidiophores. (D and E) Small macroconidiophores and details of conidiogenesis. (F) Conidia produced from small macroconidiophores. (G) Microconidiophore. (H) Microconidia. Panels B, C, E, F, G, and H are at the same scale.

lation of *atrB*, but unexpectedly, the induction factor level was higher in the MDR1 isolate than in the sensitive isolate (Fig. 7B).

TAXONOMY

Formal description of the new species *B. fragariae*. On the basis of the molecular, morphological, and host specificity data presented here, the isolates from *Fragaria* × *ananassa* (strawberry) represent a new species of *Botrytis*, i.e., *Botrytis fragariae* C. Plesken, M. Hahn & R. W. S. Weber sp. nov. (Fig. 2 and 8).

MycoBank number 818236.

Etymology: "*fragariae*" refers to the host plant (*Fragaria* spp.).

Colonies on malt extract agar growing 2.2 to 3.0 mm in diameter per day (Table 2), colony at first white and later pale gray, developing dark gray to black sclerotia sometimes covered by mycelium, solitary or aggregated. Sclerotia on malt extract agar developing in a scattered fashion within 4 weeks of incubation at 15°C in the dark; hemispherical, convex, sometimes hollow in the center with a concave surface; black, 2- to 6-mm (average, 3-mm) diameter. Conidiophores and conidia produced on strawberry fruit and fruit mummies and on potato dextrose agar in culture. Conidiophores of two kinds: (i) large macroconidiophores determinate, erect, thick walled, smooth, repeatedly septate, brown to subhyaline from base to apex, unbranched or with only one or two branches in the pigmented region, and repeatedly branched in the fertile region near the apex, 1,000 to 1,800 (−2,500) by 10 to 13 μm, tapering only slightly in the fertile region. Conidiogenous cells arising from alternating lateral branches, hyaline, giving rise to botryose clusters of conidia through narrow pores of <1 μm. (ii) Small macroconidiophores hyaline throughout, 4 to 5 μm wide, produced from aerial hyphae, giving rise to irregularly lobed fertile cells producing small botryose clusters of conidia

through wide pores of 1.5 to 3.0 μm. Small macroconidiophores often produced earlier than large macroconidiophores. Conidia from large macroconidiophores ellipsoidal to ovoid, hyaline, aseptate, 7.5 to 13.2 by 5.9 to 9.8 μm (average, 9.6 by 6.6 μm). Conidia from small macroconidiophores ellipsoidal to oblong with a blunt base, either aseptate and 7.9 to 18.1 by 4.6 to 8.8 μm (average, 11.8 by 5.9 μm) or one-septate and 13.1 to 25.0 by 5.2 to 8.2 μm (average, 17.3 by 6.4 μm). Elongated two-septate conidia occasionally seen. All macroconidial types capable of germination on potato dextrose agar. Microconidia produced in hyaline droplets from dense clusters of phialides, spherical, smooth walled, except for abscission scar, 3.0 to 3.5 μm in diameter. Apothecia not observed.

Holotype. Germany, Lower Saxony (53.4872°N, 9.5880°E), isolated from a strawberry (*Fragaria* × *ananassa*) fruit mummy in a commercial field after harvest, 7 October 2011, collected by R. W. S. Weber. Holotype CBS 141696 is a culture of D11_H_R4 deposited in the CBS Collection of Fungi (CBS Fungal Biodiversity Centre, Utrecht, The Netherlands).

Isotypes. D13_F_Me3 (CBS 141697) and D13_F_Ju10 (CBS 141698) are isotypes.

DISCUSSION

A new *Botrytis* species, *B. fragariae*, was discovered in strawberry fields in Germany and the southeastern United States. Confirmation of its status as a distinct and previously undescribed species is drawn from morphological and physiological features, its host specificity, and phylogenetic analyses of DNA sequencing data. Morphologically, *B. fragariae* isolates are distinguished from *B. cinerea* by a flatter and more compact aerial mycelium. Furthermore, growth of all *B. fragariae* strains in the presence of 0.2 mg of fludioxonil liter^{-1} can be taken as a preliminary diagnostic marker of the species. Differential sensitivities to fungicides have been useful as preliminary physiological markers to distinguish *B. pseudocinerea* (low-level HydR1 resistance to fenhexamid, hypersensitivity to fenpropidin) (16, 23) and *B. calthae* (higher tolerance to succinate dehydrogenase inhibitor fungicides) (24) from *B. cinerea* and other related species, although DNA-based methods are required for final identification. A PCR-RFLP analysis method taking advantage of a unique restriction site in *g3pdh* of *B. fragariae* was developed. On the basis of five gene sequences, four German and three North American *B. fragariae* isolates were clearly separated from the most closely related species, *B. paeoniae* (Fig. 1). On the basis of polymorphic genetic markers and fungicide resistance profiles, 18 haplotypes were distinguished among the 38 *B. fragariae* isolates tested. Genetic diversity appears to be a hallmark of *Botrytis* spp., which has been described also for *B. cinerea* (25–27) and *B. calthae* (24). The occurrence of both mating types (*MAT1-1* and *MAT1-2*) indicates that sexual reproduction is possible in *B. fragariae* populations, potentially contributing to the genetic diversity of this species.

B. fragariae was found widely distributed in strawberry fields throughout Germany. Although it was generally much less abundant than *B. cinerea*, it did contribute a major share of the *Botrytis* population within individual fields. Furthermore, it was found in North America and seems to be widely distributed along the East Coast of the United States (M. Dowling and G. Schnabel, unpublished data). The availability of a DNA-based screening method permits an analysis of the occurrence and possible origin of *B. fragariae* with higher precision. Cultivated strawberries are mostly hybrids (*Fragaria* × *ananassa*) of the American wild species *Fragaria chiloensis* and *F. virginiana*. It is unknown whether wild European strawberries (*F. vesca*, *F. moschata*, and *F. viridis*) are also natural host plants of *B. fragariae*. Despite extensive searches, we were unable to detect *B. fragariae* among several hundred isolates from other plant species sampled in the vicinity of strawberry fields with *B. fragariae* infections between 2012 and 2015, including raspberries and apples (R. W. S. Weber, unpublished data), which are also members of the family *Rosaceae*. We also failed to isolate *B. fragariae* from wild strawberries in Germany. One explanation of this could be the co-occurrence of wild strawberries with blackberries, raspberries, and blueberries in the Palatine Forest, all of which are known to be infected by *B. cinerea* and *B. pseudocinerea*. In artificial

inoculation tests, *B. fragariae* was unable or barely able to cause expanding lesions on tomato leaves, *Gerbera* petals, or apple fruit, i.e., tissues that are readily infected by *B. cinerea*. In contrast, *B. fragariae* strains caused spreading lesions on strawberry tissues. Together, these data indicate that *B. fragariae* is a host-specific species on strawberries that coexists alongside *B. cinerea* and *B. pseudocinerea*. Other plants that are susceptible to host-specific *Botrytis* spp. are also attacked by the generalists *B. cinerea* and *B. pseudocinerea*. Examples include *Caltha palustris*, the host of *B. calthae* (24); broad bean, the host of *B. fabae* and *B. fabiopsis* (16, 28); and peony, the host of *B. paeoniae* (16). All of these host-specific species need to have specific adaptations that confer a selective advantage over *B. cinerea* and *B. pseudocinerea*, which are likely to produce larger numbers of airborne conidia from a wider range of colonized host tissues. In artificial inoculation experiments, *B. fragariae* appeared to be as aggressive on strawberry flowers as *B. cinerea*, but lesion formation on leaves and fruit was retarded. Similarly, *B. fragariae* was less competitive than *B. cinerea* in mixed-inoculation experiments on strawberry leaves and fruit. Therefore, it remains unclear how *B. fragariae* is able to adapt successfully to the strawberry plant as a host. So far, *B. fragariae* has been isolated predominantly from dead tissues of overwintering strawberry plants, including fruit mummies, but never from freshly rotting fruit. This indicates that *B. fragariae* has a tissue preference different from that of *B. cinerea*, which commonly causes infections of maturing fruit. In a strawberry field in Gernsheim, *B. fragariae* was found at an elevated frequency in spring 2013, disappeared in summer 2013, and reemerged in spring 2014, indicating that *B. fragariae* is able to survive in the field by colonizing vegetative tissue. Similar observations were made in a commercial strawberry field in Mullins, SC. Isolates from the same plot collected from flowers and 2 months later from rotting berries revealed that the flowers had been colonized exclusively by *B. fragariae* and the fruit had been colonized exclusively by *B. cinerea*. A comparable host tissue preference has been observed in *B. pseudocinerea*, which was found in French vineyards predominantly on flowers in spring but was less frequent on mature grapes than was the dominant species *B. cinerea* (29). Ecological specialization by different tissue preference could thus explain the coexistence of these sympatric species on the same host plant.

Mutations in *B. fragariae* conferring resistance to carbendazim (E198A in β-tubulin) and QoI fungicides (G143A in cytochrome *b*) were identical to those that are common in resistant strains of *B. cinerea* and other fungi. Interestingly, the major mutation in iprodione-resistant *B. fragariae* strains from several fields was a G367A-V368F double mutation in *bos1*. This mutation has not been found in *B. cinerea*, which typically has other mutations in the same region of amino acids 365 to 369 (20, 21). Thus, similar but nonidentical steric constraints may determine the probability of these mutations in different *Botrytis* spp. In all six *B. fragariae* strains tested, MDR1-related mutations were located in the *mrr1* coding region. Four of the strains carried a mutation leading to an amino acid exchange (R632I) previously found in *B. cinerea* MDR1 strains (19), and two strains carried an as-yet-undescribed change (C588Y). In *B. cinerea* MDR1 strains, 15 different putative gain-of-function mutations in the transcriptional activator of *atrB*, Mrr1, have already been identified (22). MDR1 phenotypes in *B. cinerea* are correlated with the constitutive upregulation of *atrB*, which encodes an ABC-type efflux transporter. Similarly, a *B. fragariae* MDR1 strain showed 10-fold overexpression of *atrB* compared to a sensitive strain. As in *B. cinerea*, the *atrB* expression in *B. fragariae* increased in the presence of cyprodinil, confirming that *atrB* is similarly regulated in both species. In *B. cinerea*, the MDR1 phenotype is characterized by resistance factors of 5 to 20 for fludioxonil and cyprodinil (13). The effects of MDR1 on cyprodinil can only be tested in strains that have not acquired other mechanisms of resistance to cyprodinil. All *B. fragariae* MDR1 strains had medium to high levels of cyprodinil resistance, with resistance factors of at least 260, compared to non-MDR1 strains, which were all cyprodinil sensitive. It remains unclear whether these resistance levels are due only to the *mrr1* mutation or also to other types of mutations.

Overall, the resistance frequencies of *B. fragariae*, *B. cinerea*, and *B. pseudocinerea* in the same fields showed significant differences. In agreement with a previous report

(16), no resistance to a registered fungicide was found in *B. pseudocinerea*. While *B. cinerea* showed medium to high frequencies of resistance to the seven major classes of site-specific gray mold fungicides, in *B. fragariae*, somewhat lower frequencies of resistance to five fungicides were observed whereas no resistance to fenhexamid or boscalid was found. The intrinsic low-level fludioxonil resistance of *B. fragariae* and the high resistance levels of MDR1 strains, similar to those of *B. cinerea* MDR1h strains, could give *B. fragariae* a selective advantage in commercial strawberry fields when the fungicide Switch, a combination product of fludioxonil and cyprodinil and one of the most effective current botryticides, is repeatedly applied. All of the fields in which *B. fragariae* MDR1 strains were found had previously been sprayed with Switch. The resistance of *B. fragariae* to various fungicides, including the benzimidazoles that were introduced in the late 1960s but discontinued for several decades since, indicates that the species has been adapted to commercial strawberry fields for a long time. Being observed in different climatic regions of northern and southern Germany, as well as in the southeastern United States, *B. fragariae* can be expected to be discovered also in other countries and might be of local importance, together with *B. cinerea*, in causing tissue damage to strawberry plants.

MATERIALS AND METHODS

Isolation and cultivation of *Botrytis* strains. *Botrytis* strains were obtained from infected strawberry tissue in different ways. Spores from sporulating lesions were directly transferred to malt yeast extract agar (MYA) plates (10 g of malt extract, 4 g of glucose, 4 g of yeast extract, and 15 g of agar per liter, pH 5.5) with sterile forceps or cotton swabs (10). Where necessary, infected tissue was incubated in a humid chamber for several days to allow new sporulation to occur. Alternatively, pieces of petioles, leaves, or inflorescences were placed onto MYA plates containing 0.5% tannic acid to suppress the growth of contaminating fungi. After sporulation, conidia were transferred to fresh MYA and the cultures were tested for the absence of contamination. Pure cultures were obtained by transfer of fresh conidia to MYA. Cultures for further processing were prepared as described previously (10). For long-term storage, cultures were kept in 30% glycerol at −80°C. Mycelial growth tests were performed at 20°C under ambient light conditions. For quantification of sporulation, MYA plates were incubated under Philips Black Light tubes (TL-D 36W, BLB) with a 16-h light, 8-h dark illumination cycle.

For isolation of *Botrytis* from wild strawberries (*F. vesca*), whole plants were collected in November 2016 at four sites ca. 0.5 to 1 km apart from each other in the Palatine Forest south of Kaiserslautern, Germany. Green petioles and leaves were cut into pieces with sterile scissors, transferred to MYA with tannic acid, and further treated as described above.

Fungicide sensitivity tests. The fungicide resistance or sensitivity of the strains was tested on agar plates as described previously (16), by using the following discriminatory fungicide concentrations (per liter): 10 mg of fenhexamid, 0.2 and 1 mg of fludioxonil, 16 mg of cyprodinil, 5 mg of carbendazim, 25 mg of iprodione, 3 mg of boscalid, and 25 mg of azoxystrobin supplemented with 100 mg of salicylhydroxamic acid. Sensitivity to fludioxonil, cyprodinil, and iprodione was quantified as described previously (10), by determining the effective concentration at which a 50% reduction of germ tube growth was achieved (EC_{50}) by using the following concentrations (mg liter^{-1}): 0,0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 for fludioxonil and cyprodinil and 1, 2, 4, 8, 16, 32, and 64 for all of the fungicides.

Plant infection tests. For inoculation with spore suspensions, freshly harvested spores were suspended in Gamborg B5 minimal medium with 10 mM sodium phosphate and 25 mM glucose, pH 5.5 (GB5) (30). Leaves from potted strawberry plants (cv. Sonata) cultivated in a phytochamber or open air were inoculated with 5-mm agar discs of GB5 inoculated with 10^3 spores in 10 μl and incubated for 16 h to allow germination. The agar discs were placed onto detached strawberry leaves wounded by a small cut with a scalpel, the aerial hyphae being in contact with the leaf surface. Strawberry and raspberry fruits from a supermarket were surface sterilized before inoculation by soaking in 1% sodium hypochlorite for 5 min, followed by three washes with sterile water. Fruits were inoculated with 10-μl droplets of GB5 containing 10^3 conidia. Leaves from tomato (cv. Moneymaker) plants raised in a phytochamber, petals from *Gerbera* flowers (purchased in a supermarket) and flowers from wild strawberry (*F. vesca*) plants grown in a private garden were inoculated with 4-μl (flowers) or 10-μl (leaves) droplets of conidia (10^5 ml^{-1}) suspended in GB5 medium. Inoculated plant tissues were incubated in a humid chamber under ambient laboratory light at 20°C for 72 h unless indicated otherwise.

For mixed-inoculation experiments with fruits, 500 conidia each of *B. cinerea* (strain B05.10 or D09-A04) and *B. fragariae* (strain D14_F_Ju12, D13_F_Ju29, or D13_F_Me3) were mixed and applied in 10-μl droplets to the intact fruit surface as described above. After 6 to 7 days of incubation, conidia that formed on the margin of the lesion were removed with wet sterile cotton swabs and transferred into a sterile tube containing a droplet of water. From the resulting suspension, 5-μl aliquots were applied to MYA plates containing discriminatory concentrations of carbendazim (5 mg liter^{-1}) as a resistance marker for *B. cinerea* strains, fludioxonil (0.2 mg liter^{-1}), or cyprodinil (16 mg liter^{-1}) as resistance markers for *B. fragariae* strains, and no fungicide as a growth control as described above. For mixed-inoculation experiments with strawberry leaves, 1,000 spores of a 1:1 mixture of a *B. cinerea* strain and a *B. fragariae* strain were applied to an agar disc. Preincubation of the agar disc and inoculation of wounded leaves

were done as described above. After 5 days of incubation, two peripheral sectors of the infected leaf tissue were transferred to an MYA plate and allowed to sporulate and the conidia were tested for resistance to carbendazim (*B. cinerea*) or fludioxonil and cyprodinil (*B. fragariae*) on fungicide-containing plates.

Genetic characterization of *Botrytis* strains. PCR analysis and DNA sequencing were performed by using standard techniques with primers shown in Table S1. Genetic differentiation of *B. cinerea* and *B. pseudocinerea* was done as described previously (16), with primers BpsID_137F and BpsID_273R. Expression of *atrB* was analyzed by quantitative RT-PCR as described previously (10), with the following modifications. A 20-ml volume of liquid MY medium was inoculated with 2.5×10^5 spores and incubated overnight under ambient laboratory light at 20°C and 200 rpm. To induce *atrB* expression, 20 μl of a cyprodinil stock solution (1 mg ml^{-1} water) was added to the culture for a further 30 min. For isolation of total RNA, the mycelium was collected by centrifugation at 4,000 rpm for 5 min at 4°C and the pellet was rinsed in 650 μl of lysis buffer RA1 containing β-mercaptoethanol (NucleoSpin Plant RNA kit; Macherey-Nagel, Düren, Germany). The mixture was transferred to a 2-ml reaction tube filled with 500 μl of glass beads (0.75 to 1 mm), and the mycelium was disrupted in a Fast-Prep-24 instrument (MP Biomedicals, Solon, OH) by using three pulses of 30 s at setting 6.5 with cooling on ice between the pulses. After centrifugation (30 s at 13,000 rpm), the supernatant was loaded to a NucleoSpin filter, and RNA isolation was performed in accordance with the user manual. RT was done with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Munich, Germany). Quantitative PCR was performed with the MyiQ Real-Time PCR detection system (Bio-Rad Laboratories) and *atrB* primers optimized for *B. fragariae* and *B. cinerea* (atrB_RT_for/atrB_RT_rev). *atrB* transcript levels were normalized against the expression levels of the reference genes encoding actin and beta-tubulin, and results are shown as relative *atrB* expression (31). Experiments were performed with three biological replicates (technical triplicates of each).

Genetic diversity of *B. fragariae* isolates was examined by analyzing the presence or absence of the flipper transposon (17) and the mating type locus *MAT1-1* or *MAT1-2* (32) with primers shown in Table S1. Differentiation of isolates from *B. cinerea* groups N and S and *B. pseudocinerea* was achieved by using the *mrr1*-specific primers BcinN-in-F/BcinN-in-R and Mrr1-spez-F/Mrr1-spez-R. *B. pseudocinerea* isolates were identified with primers g2944_137_F/g2944_273_R (16).

Detection of fungicide resistance-related mutations. Mutations related to fungicide resistance in *B. fragariae* were detected by sequencing. For detection of the E198A mutation in the *tubA* gene, which confers resistance to carbendazim, the PCR fragment obtained with primers Bc_Tub_for/Bc_Tub_rev was sequenced. The G143A mutation in *cytB* conferring resistance to the QoI azoxystrobin was detected by sequencing the PCR product generated with primers Qo13ext/Qo14ext. For detection of mutations related to iprodione resistance, the complete coding region of *bos1*, including introns, was sequenced. For detection of MDR1-related mutations, most of the *mrr1* coding region, corresponding to positions 115 to 2411 in *B. cinerea* B05.10 *mrr1*, of two *B. fragariae* strains with baseline fludioxonil sensitivity, and three strains with partial fludioxonil resistance, was sequenced. Some *B. cinerea*-specific primers were modified to allow amplification and sequencing of *B. fragariae bos1* and *mrr1* (see Table S1).

Phylogenetic analyses. Nucleotide sequences of *hsp60*, *rpb2*, *g3pdh*, *nep1*, and *nep2*, commonly used for differentiation of *Botrytis* spp., were obtained from the NCBI website (see Table S2) and aligned by using the SeaView software (33). Bootstrap frequencies were calculated with 1,000 replicates, and branches with bootstrap frequencies of >70% were considered significant.

Microscopy. Photomicrographs were taken with an Axio Scope A1 light microscope fitted with 10×, 40×, and 100× Plan-Neofluar objectives and differential interference contrast optics with an ICc 3 digital camera (all from Carl Zeiss, Jena, Germany). Conidial dimensions of *B. cinerea* (strain B05.10) and *B. fragariae* (strain D11-H-R4) were measured with the AxioVision software 4.8 by using the 100× objective. Freshly sporulating mycelium after 7 to 10 days of growth on potato dextrose agar (Carl Roth, Karlsruhe, Germany) was used for all microscopic work.

Statistics. Data were analyzed by unpaired, two-tailed *t* tests or with an independent-sample *t* test by using GraphPad Prism 5 for Windows 5.01.

Accession number(s). The nucleotide sequences generated in this study have been deposited in GenBank under the accession numbers listed in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00269-17.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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