Research

Automated Ribosomal Intergenic Spacer Analysis to Detect Pathogenic Fungi in Dark-Colored Spots on Apple Fruits

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Abstract

Dark-colored spots (DCSs) found on apples at harvest time worldwide significantly reduce the commercial value of the fruits. In this study, we conducted an automated ribosomal intergenic spacer analysis (ARISA) to reliably detect fungal pathogens in these lesions. The fragment sizes of peaks for eight species and two species complexes that might cause DCSs on apples in northern Honshu Island, Japan, were determined on the basis of the ARISA. The fragment sizes for six species (*Botryosphaeria kuwatsukai*, *Diplocarpon mali*, *Mycosphaerella pomi*, *Neonectria galligena*, *Phlyctema vagabunda*, and *Venturia inaequalis*) were unique, enabling their detection in a single ARISA trial. However, two other species and two species complexes had similar fragment sizes. To identify these fungi, PCR amplifications using species- or genus-specific primers were needed. Using this method to examine the fresh tissue of DCSs (i.e., after removing the epidermis) on commercially produced apples resulted in the detection of six fungal species and two *Colletotrichum* species complexes. Because the pathogenic fungi in the tissue underlying lesions are most likely the causal agents, these fungi could be responsible for the DCSs on apple fruits. An automated analysis of the PCR products amplified for the ARISA enables the rapid examination of many samples. These results indicate that the ARISA-based technique developed in the present study is useful for the accurate, reliable, and comprehensive detection of the pathogens causing DCSs on apple fruits.

Keywords: apple, automated ribosomal intergenic spacer analysis, dark-colored spots (DCSs), detection, fungal pathogen, postharvest diseases

H. Furuya and Y. Sato contributed equally to this work.

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Many airborne pathogenic fungi of apple (*Malus × domestica*) infect fruits as well as leaves, branches, and trunks. Small lesions at early developmental stages caused by these pathogens are often found at harvest time (Giraud and Bompeix 2012; Sutton et al. 2014). These lesions not only significantly decrease the commercial value of apples but also serve as inocula for postharvest diseases during storage and transport if they are overlooked (Wenneker 2019). The lesions, called dark-colored spots (DCSs), are

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circular or subcircular, dark brown to black, and most are less than 5 mm in diameter (Fig. 1).

Apple fruits with DCSs at harvest time are found in many orchards worldwide (Giraud and Bompeix 2012; Moggia and Yuri 2003; Sutton et al. 2014; Wenneker 2019). According to a survey conducted by the Department of Agriculture and Forestry of Akita Prefecture, Japan, in 2011, apple fruits with DCSs were found in virtually every orchard in southern Akita Prefecture. This major apple-growing region in Japan has 1,000.5 ha of apple orchards, including 600.5 ha used for the production of Fuji apples; this information was obtained from the Horticulture Promotion Division, Department of Agriculture, Forestry and Fisheries, Akita Prefectural Government. The damaged fruits, which were sorted as a lower-grade product, were not sold as fresh apples but were used for food processing (Narita 2005) or discarded if they were badly damaged. To prevent such damage, DCS development must be controlled following an accurate diagnosis before apples are harvested, but this is not currently done.

The infectious diseases that might cause DCS development in northern Honshu Island, Japan, include bitter rot caused by the *Colletotrichum gloeosporioides* species complex or the *Colletotrichum acutatum* species complex, Botryosphaeria rot caused by *Botryosphaeria dothidea* (Moug.) Ces. & De Not. and *Botryosphaeria kuwatsukai* (Hara) G.Y. Sun & E. Tanaka, Marssonina blotch caused by *Diplocarpon mali* Y. Harada and Sawamura [ana. *Marssonina coronaria* (Ellis & Davis) Davis, syn. *Ma. mali* (Henn.) Ito], apple scab caused by *Venturia inaequalis* (Cooke) G. Winter, Brooks fruit spot caused by *Mycosphaerella pomii* (Pass.) Lindau, and Alternaria blotch caused by *Alternaria mali* Roberts. Additionally, Sato et al. (2021) recently reported that *Phlyctema vagabunda* Desm (syn. *Neofabraea alba* E.J. Guthrie.), a causal fungus of bull's-eye rot (Chen et al. 2016), causes DCSs. Other studies proved that DCSs

are also caused by physiological disorders, such as lenticel blotch pit (Casero et al. 2009), lenticel spot (Brook 1968), and lenticel breakdown (Singh et al. 2016), as well as foliar disease-associated pathogens.

Traditional diagnostic techniques for DCSs, such as visual and microscopic examinations, are not effective because there is a lack of distinct characteristics associated with each disease, including spores on lesions, which are seldom detected during the early infection stages (Sutton et al. 2014; Wenneker 2019). It is therefore common to wait for several weeks, or even a few months, for typical symptoms to appear. If fast and reliable techniques for identifying pathogens are available, then the causal agent of DCSs on apples in each orchard or region can be determined, thereby enabling targeted control measures to be implemented in the following season. Moreover, accurate detection of the causal agent of DCSs is crucial for international export and import because several apple-associated pathogenic fungi are subjected to quarantine regulations in many countries.

In the last two decades, many molecular techniques have been developed to detect the causal fungi of these diseases (Garipey et al. 2003; Michalecka et al. 2016; Skidar et al. 2014; Wenneker et al. 2017). These techniques can specifically detect a single species or the species of a single genus (Garipey et al. 2003; Michalecka et al. 2016). To detect pathogens in lesions such as DCSs, which have a range of potential causal agents, a comprehensive technique is required. In the present study, on the basis of the automated ribosomal intergenic spacer analysis (ARISA), we developed a fast, reliable, and comprehensive technique to detect the pathogenic fungi causing DCSs on apple fruits.

The ARISA was first introduced by Fisher and Triplett (1999) to investigate freshwater bacterial diversity and community compositions. Since then, it has been extensively used for bacterial

FIGURE 1

Examples of dark-colored spots (DCSs) found on Fuji apple fruits during several weeks after harvest. *Botryosphaeria dothidea*, *Phlyctema vagabunda*, and *Diplocarpon mali* were detected in lesions A and B; E, F, and G; and D, respectively. C was not examined. Bar indicates 5 mm.

community analyses of a variety of habitats, such as soils (Lerner et al. 2006; Ranjard et al. 2001), decomposing organic materials (Ciesielski et al. 2013), aquatic environments (Danovaro et al. 2006), and the mammalian gut (Jami et al. 2014). Ranjard et al. (2001) adopted the method to characterize fungal communities, using length polymorphisms of the nuclear ribosomal DNA region, which contains two internal transcribed spacer regions and the 5.8S rRNA gene. This method involves the PCR amplification of the rRNA intergenic spacer region, which may encode tRNAs. The fluorescently labeled and amplified DNA fragments can be separated and sized using a capillary electrophoresis system. These amplified fragments are denatured with formamide, and the capillary is maintained at a consistent temperature so that highly reproducible results are generated by the genetic analyzer (Fisher and Triplett 1999; Ranjard et al. 2001). The program output is a series of peaks (i.e., an electropherogram). The peaks represent fragments of varying sizes. The size of each peak is determined according to a relative comparison of the migration speeds during electrophoresis involving a size-standard peak. Fisher and Triplett (1999) observed that peaks differing in size by one base can be easily separated; this was subsequently confirmed by Ranjard et al. (2001). The peak height reflects the relative proportion of the fragments in the total product. The heterogeneity of the amplified fragment lengths and nucleotide sequences allow bacterial and fungal species to be distinguished. Each taxon, mostly at the species level, has a unique fragment size. Thus, the taxon, often at the species level, can be assumed according to the fragment size. Ikeda et al. (2007) successfully used an ARISA to efficiently detect plant pathogenic fungi at a high resolution and sensitivity in infected milled rice grain tissues. Furthermore, Pancher et al. (2012) used an ARISA to detect pathogenic fungi and to elucidate fungal endophytic communities in grapevine stems.

The aim of this study was to develop an ARISA-based technique to detect fungal pathogens in apple fruit DCSs. To the best of our knowledge, this is the first report of an ARISA being used to comprehensively detect pathogenic fungi in plant lesions.

MATERIALS AND METHODS

Collection of DCS samples

In this study, the DCSs used for the ARISA were obtained from Fuji apple fruits. The fruits with DCSs were collected from trees in an orchard at the Akita Fruit-Tree Experiment Station (latitude: 39.242475; longitude: 140.530043) that had not been sprayed with either fungicides or insecticides for many years and at regional fruit-sorting facilities in the Sakae, Masuda, and Daigo districts of Yokote City. Samples were collected from the second half of November, which is the normal harvest time in these regions, to March from 2017 to 2021. One to seven DCSs were obtained from each fruit. The whole tissue of each DCS, including the epidermis and the underlying fresh tissue, mostly less than 5 mm in diameter and 3 mm deep, respectively, and the fresh tissue that was obtained after removing the epidermis were used. The epidermis and fresh tissue from a DCS were excised soon after the fruit was collected. Tissues were excised using a sterilized knife, placed into 2-ml plastic tubes, and stored at 4°C in a refrigerator. Genomic DNA was extracted from these tissues within 1 week of storage.

DNA extraction from the fresh tissue or whole tissue of DCSs or fungal mycelia

Total genomic DNA was extracted from apple fruit tissues with DCSs using the DNeasy Plant Mini Kit (Qiagen, Hilden,

Germany). Tissue segments (50 to 100 mg) derived from each DCS were placed in 2-ml tubes. After adding a metal cone, the tubes were placed in liquid nitrogen. The frozen tissue segments were ground in a Multi-beads shocker (Yasui Kikai Corporation, Osaka, Japan) before the DNA extraction.

Genomic DNA was also extracted from the fungal isolates obtained from DCSs using the commercial kit. Isolates were grown in 6-cm-diameter Petri dishes containing 5 ml of potato dextrose broth at 25°C for 2 to 3 days in the dark. Mycelial mats were harvested by filtration, rinsed with sterile distilled water, blotted dry, placed in 2-ml tubes containing zirconia beads (Sarstedt K.K., Tokyo, Japan), ground using a BugCrasher GM-01 (Taitech, Koshigaya-shi, Saitama, Japan), and used for the DNA extraction. When storage was necessary, the mycelial mats were transferred to 2-ml tubes and stored at −30°C until used.

ARISA

The DNA samples extracted from apple fruit tissues or fungal isolates were subjected to an ARISA. First, a PCR using the primer set 1406f (5'-TGYACACACCGCCCGT-3')/3126T (5'-ATATGCTTAAGTTCAGCGGGT-3') and 3126T (Hansgate et al. 2005) labeled with the fluorescent dye VIC (Applied Biosystems, Foster City, CA, U.S.A.) at the 5' end was conducted to amplify the region containing partial small subunits at the 3' end of the 18S ribosomal DNA sequence, two internal transcribed spacers, and the 5.8S rDNA sequence of the fungal isolates obtained from apples. The PCR mixture was prepared in 0.2-ml microtubes, each containing 20 µl of reaction mixture with 1 ng of template DNA, 200 µM of dNTP mixture, 0.2 µM of primer 1406f, 0.1 µM of primer 3126T and fluorescent 3126T, 1 × PCR buffer, and 0.05 U/µl of AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR amplification was completed using a DNA thermal cycler (TaKaRa PCR Thermal Cycler Dice Standard; TaKaRa Bio, Ohtsu, Shiga, Japan), with the following program: 1 cycle of 10 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C, followed by 1 cycle of 10 min at 72°C. The amplified products (4 ml of PCR product and 1 ml of loading dye) were analyzed by 2% agarose gel electrophoresis using Tris-acetate-EDTA buffer. The agarose gels were stained with ethidium bromide (10 mg/ml) and visualized under UV transillumination. The success of a PCR run was confirmed based on the presence or absence of fragments amplified from the DNA of *P. vagabunda* isolate AITC-KH54. After the confirmation of the amplified fragments, 1 µl of unpurified PCR product was mixed with 0.15 µl of GeneScan 1200 LIZ Size Standard (Applied Biosystems) and 9.85 µl of Hi-Di Formamide (Applied Biosystems) and then centrifuged at 140 × g for 30 s at 20°C (Hitachi CF16RX II, Koki Holdings, Hitachi-shi, Ibaraki, Japan). The fragment sizes of the fluorescent PCR products and their fluorescence intensity levels (fluorescence units) were determined using an ABI 3700 Genetic Analyzer (Applied Biosystems). The analysis was conducted by Biotechnology Center, Faculty of Bioresource Sciences, Akita Prefectural University. Peaks with fragment sizes less than 600 bases were ignored because the fragments of most fungi, including every pathogenic fungus examined in this study, are estimated to contain more than 600 bases according to the GenBank database. Peaks with fluorescence intensities of less than 50 units were also ignored because they were considered unreliable. To determine the extent of the variation in fragment sizes among genetic analyzer runs, DNA extracted from cultured *P. vagabunda* AITC-KH54 mycelia was used in each ARISA trial as a positive control for the PCR amplification and a standard fragment with a known sequence length.

PCR with specific primers

The DNA extracted from DCS tissues or fungal isolates was amplified by PCR using pathogenic fungi-specific primers. The PCR mixture was prepared in 0.2-ml microtubes, each containing 20 µl of reaction mixture with 1 ng of template DNA, 200 µM of dNTP mixture, 0.1 mg/ml of bovine serum albumin, 0.2 µM of primer pair, 1× PCR buffer, and 0.05 U/µl of AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR amplification was completed using a DNA thermal cycler (TaKaRa PCR Thermal Cycler Dice Standard), with the following program: 1 cycle of 10 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C, followed by 1 cycle of 7 min at 72°C. The following species- or genus-specific primers were used: Coll1F/3Rb (AACCWGC GGAGGGATCATTA/TCCCTBCGRRTCCCRRTGCG) for *Colletotrichum* (Mosca et al. 2014), FaF/Bt2b (CATCCGCAGCGTGGGAGAACAT/ACCCTCAGTGTAGTGACCCTTGGC) for *B. dothidea* (Xu et al. 2016), LinF1/LinR (TATCGCCTGGCCACCTACGC/TGGCCACGACAACCCACATA) for *A. alternata* (*A. mali*) (Johnson et al. 2000), and MC forward/MC reverse (GCCTACCTACCTCTGTTGC/CAGAACCAAGAGATCCGTTGTTG) for *D. mali* (Oberhänsli et al. 2014). The products of the amplification using the genus-specific primers Coll1F/Coll3Rb were diluted 100-fold with sterile distilled water, after which 1 µl of the diluted amplified products was used as the template for additional amplifications using the PCR mixtures and PCR program described above. The amplified products were analyzed by 2% agarose gel electrophoresis as previously described. The success of the PCR was confirmed based on the presence or absence of fragments amplified from the DNA of each fungal isolate for which the species- or genus-specific primer set was designed.

Sequencing of amplified products for molecular species identification

The amplified products corresponding to the ARISA peaks were sequenced. First, the amplified products were fractionated by 2% agarose gel electrophoresis, and each fragment was excised from the gel with a sterilized razor blade. These single fragments were transferred to a 1.5-ml tube and then purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited, Buckinghamshire, U.K.). Purified DNA fragments were then sequenced using primers 1406f and 3126T, the Ready Reaction Cycle Sequencing Kit (Applied Biosystems), and the ABI 3700 DNA sequencer (Applied Biosystems). The raw sequencing data were verified using 4Peaks (version 1.7) (<http://staden.sourceforge.net/>). The forward and reverse sequences were assembled using GENETYX-Mac (version 20) (Genetyx Science, Tokyo, Japan). Finally, the sequence data were used for a BLAST search of the GenBank database (National Center for Biotechnology Information) to identify the closest taxon. This analysis was conducted for peaks with high fluorescence intensity levels (ideally greater than 1,000) and target amplicons that were separated enough to be excised without contamination.

RESULTS

Fragment sizes of pathogenic fungi in DCSs on apples

The fragment sizes determined on the basis of an ARISA of DNA samples derived from each representative isolate of 13 pathogenic species that could be involved in the regional DCS

incidence were examined in three separate tests (Table 1). The fragment size of each representative isolate of six species (*M. pomi*, *Neonectria galligena*, *P. vagabunda*, *D. mali*, *V. inaequalis*, and *B. kuwatsukai*) was unique among the 13 tested species and differed from the fragment sizes of other representative isolates by four or more bases. The sizes of the fragments of the remaining seven species were within three bases of the fragments from one or more other species in each trial. These species were divided into two groups, one consisting of four species with fragment sizes of 689 to 692 bases and another consisting of three species with fragment sizes of 697 to 702 bases. The former included three *Colletotrichum* species that belong to the *C. gloeosporioides* species complex as well as *A. mali*. The latter included two *Colletotrichum* species that belong to the *C. acutatum* species complex as well as *B. dothidea*. During three runs of the genetic analyzer, the fragment sizes of the peaks varied by up to two bases for the DNA samples of the authentic isolates of 11 pathogenic fungi, whereas they varied by three or four bases for the DNA samples of *V. inaequalis* and *B. kuwatsukai* (Table 1). The size of the amplified product for each pathogen was examined after sequencing the products (Table 1). The amplified products had 17 to 31 less bases than the fragments of each pathogen estimated by the ARISA.

ARISA of DCS tissues

Eight ARISA trials were conducted using DNA samples extracted from the underlying fresh tissue or from the whole tissue of 490 DCSs. The fragment size of the standard DNA sample obtained from the *P. vagabunda* isolate was between 667 and 669 bases, depending on the genetic analyzer run (Table 2). Of the tested DCSs, 452 (92%) had one or more peaks, whereas the others had no peaks. The most common peaks were those with fragments comprising 667 to 671 bases. They were detected in 68.4% of the DNA samples obtained from DCSs analyzed. More specifically, they were detected in 69.5% and 67.6% of the DNA samples from fresh and whole tissues, respectively, as well as in 74.1% of the DNA samples in which one or more peaks were detected. A BLAST search revealed that sequences of the amplified DNA products corresponding to these peaks showed high identities to *P. vagabunda* (Table 3).

ARISA of the fresh tissue from DCSs

An ARISA was conducted using DNA samples extracted from the fresh tissue of 197 DCSs obtained from fruits collected at a non-chemically sprayed orchard and three fruit-sorting facilities (Table 2). In total, 154 (78.2%) of the DNA samples obtained from the DCSs had a single peak, 10 (5.1%) had multiple peaks, and 33 (16.8%) had no peaks.

The second most common peaks among the DNA samples obtained from fresh tissues had fragments comprising 686 to 687 bases. The sequences of the amplicons corresponding to these peaks showed high identities to *D. mali* (ana. *Ma. coronaria*) (Table 3). Sequences of the amplicons corresponding to the peak with a fragment size of 1,096 bases obtained in trial No. 2 showed 99.8% identity to *Botryosphaeria* sp. sequences. The sequences of the amplicons corresponding to the peaks with fragment sizes of 702 bases in trial No. 1 and 697 and 699 bases in trial No. 3 showed 100% identity to *Aureobasidium* spp. or *Penicillium* sp., 96% to 99% identities to *B. dothidea*, and 100% identity to *Colletotrichum* spp., respectively.

To summarize, on the basis of an ARISA combined with a PCR using specific primers, a single pathogenic fungus was detected in

156 of 164 DCSs (95.1%). One or more peaks were detected for the DNA samples extracted from these 164 DCSs. Saprophytic fungi were detected in only four of these samples. The other fungi were unidentified. Of the 156 samples in which pathogenic fungi were detected, 151 had a single ARISA peak. A single pathogenic fungus was detected in five samples in which multiple peaks were detected. Thus, the causal pathogen of each DCS was determined for 79.2% of the 197 examined samples.

ARISA of the whole tissue from DCSs

An ARISA was conducted using DNA samples extracted from the whole tissue of 293 DCSs obtained from 275 apple fruits (Table 2). In total, 71.7% of the DNA samples had a single peak, 26.6% had multiple peaks, and 1.7% had no peaks. A single peak was detected for 72.9% of the DNA samples that had one or more peaks. Peaks with a fragment size of 667 to 671 bases were the most common, detected in 67.6% of the examined DCSs, followed by peaks with fragment sizes of 699 to 703 (21.2%), 689 to 693 (11.9%), 685 to 686 (5.5%), and 658 to 660 (4.1%) bases. The sequences of the amplicons corresponding to the peaks with fragment sizes of 685 to 686 and 658 to 660 bases showed high identities to *D. mali* and *Neonectria* sp. sequences, respectively (Table 3).

Peaks with a fragment size of 699 to 703 bases were detected in 62 DCSs (21.2% of the examined DCSs). The sequences corresponding to these peaks showed high identities to either *Aureobasidium* spp. or species of *C. acutatum* species complexes (Table 3). Forty-five DNA samples with peaks in this range were analyzed by PCR using *B. dothidea*- or *Colletotrichum*-specific primers. The former and latter primers amplified nine and four DNA samples, respectively (Supplementary Fig. S1).

Peaks with a fragment size of 689 to 693 bases were detected in 35 DCSs (11.9% of the examined DCSs). The sequences associated with these peaks showed high identities to either *Alternaria* spp. or species of the *C. gloeosporioides* species complex (Table 3). Thirty-three DNA samples with peaks in this range were subjected to a PCR analysis using *A. alternata* (*A. mali*)- and *Colletotrichum*-specific primers. The former primers did not amplify any DNA samples, whereas the latter primers amplified six DNA samples (Supplementary Fig. S2).

All pathogenic fungi described in this section were isolated from DCSs on apple fruits in the present study.

DISCUSSION

The fragment sizes of the ARISA peaks for the authentic isolates of six fungal species (*B. kuwatsukai*, *D. mali*, *M. pomi*, *Ne. galligena*, *P. vagabunda*, and *V. inaequalis*) differed by four or more bases from the fragments of the other analyzed species (Table 1) potentially involved in the DCS development on apple fruits in northern Honshu Island, Japan. Because peaks with fragments differing in size by one base can be separated by this analysis (Fisher and Triplett 1999; Ranjard et al. 2001), the four-base differences are sufficient for detecting the peaks corresponding to specific fungi. Thus, these six species can be identified in a single ARISA trial. In fact, when the ARISA was used to detect pathogens in the DCSs on commercially produced apple fruits in the present study, peaks with fragment sizes corresponding to five of these six species were detected (i.e., *B. kuwatsukai*, *D. mali*, *Ne. galligena*, *P. vagabunda*, and *V. inaequalis*; Table 2). The identification of these peaks was confirmed by a BLAST search using the sequence data of the amplicons corresponding to the peaks (Table 3). This confirmation eliminates the need for more traditional diagnostic methods for analyzing DCSs on commercially produced apple fruits.

The fragment sizes of the ARISA peaks for the authentic isolates of four other taxa were not discriminative. However, the ARISA results indicated which primers should be used for the subsequent PCR-based species identification. More specifically, when peaks with fragment sizes of approximately 690 bases were detected, *A. mali*- or *Colletotrichum*-specific primers were used. In contrast, when peaks with fragment sizes of about 700 bases were detected, *B. dothidea*- or *Colletotrichum*-specific primers were used. This method can detect pathogens in DCSs more efficiently (i.e., in terms of time and cost) than other fingerprinting techniques, including PCR amplifications using species-specific primers without any reasonable hypothesis regarding the target fungus.

Before 2012, apple bitter rot in Japan was considered to be caused by *C. acutatum* J. H. Simmonds and *C. gloeosporioides* (Penz.) Penz. & Sacc. (Kawai et al. 2000; Sato and Moriawaki 2009; Tsushima and Yukita 2002). On the basis of the information regarding the multigene sequence-based taxonomy of the genus, two species complexes (i.e., *C. acutatum* species complex and *C. gloeosporioides* species complex) were proposed in 2012, with many new species in each species complex (Cannon et al. 2012; Damm et al. 2012). Since then, several species have been

TABLE 1

Fragment sizes of major polymerase chain reaction products (peaks) assessed by genetic analyzer in automated ribosomal intergenic spacer analysis (ARISA) with DNA samples of pathogenic fungi that might be involved in the formation of dark-colored spots (DCSs) on apple fruits in Yokote region, Akita

Taxon	Isolate no.	Disease	No. of bp ^a	Fragment sizes in ARISA		
				Trial 1	Trial 2	Trial 3
<i>Mycosphaerella pomi</i>	MAFF 645022	Brooks fruit spot	627	652	650	650
<i>Neonectria galligena</i>	MAFF 410257	Nectria canker	635	659	658	–
<i>Phlyctema vagabunda</i>	AITC KH54	Bull's-eye rot	639	667	–	668
<i>Diplocarpon mali</i>	MAFF 645015	Marssonina blotch	659	686	685	685
<i>Colletotrichum aenigma</i>	MAFF 244310	Bitter rot	664	690	689	689
<i>C. siamense</i>	MAFF 244307	Bitter rot	664	691	691	691
<i>C. fruticola</i>	MAFF 244311	Bitter rot	665	691	692	692
<i>Alternaria mali</i>	HBA1	Alternaria blotch	661	692	–	–
<i>C. fioriniae</i>	MAFF 306630	Bitter rot	674	699	702	702
<i>Botryosphaeria dothidea</i>	25-192A	Botryosphaeria rot	673	698	700	698
<i>C. nymphaeae</i>	MAFF 306546	Bitter rot	674	701	700	701
<i>Venturia inaequalis</i>	MAFF 237305	Apple scab	1,029	1,046	1,042	1,044
<i>B. kuwatsukai</i>	YTK-1	White rot	1,072	1,095	1,096	1,097

^a The numbers were calculated based on sequence data of each pathogen.

reported as the causal agents of apple bitter rot (Misawa and Sato 2013; Phytopathological Society of Japan 2021; Yokozawa et al. 2017). Basic disease control measures, however, need not be altered depending on the species in each species complex (Akahira et al. 2016; Iwanami et al. 2003). Thus, the species complex-level identification of causal fungi by the ARISA-based technique is useful, allowing growers to make more informed decisions regarding how to control DCS development.

A single peak was detected for most fresh tissues of DCSs. Most of these peaks were for DNA samples from pathogenic fungi. Because pathogenic fungi can infect intact host plant tissues, whereas saprophytic fungi usually cannot, a pathogenic fungus detected within the underlying tissue is most likely the causal agent of the lesion. Accordingly, the pathogenic fungus detected in the fresh tissue of DCSs could be considered the causal agent. Another benefit to using the fresh tissue of DCSs for an ARISA is that it can minimize the chances of detecting other fungi unrelated to DCS development, including saprophytic and pathogenic fungi that might have appeared by chance at the lesion surface. These fungi often interfere with the detection of causal fungi by masking the causal fungi or adversely affecting detection sensitivity. Therefore, to accurately identify the causal agents, the DNA samples used for an ARISA should be obtained from the fresh tissue of DCSs (i.e., after the epidermis is removed). A pathogenic fungus was detected in 150 of the 154 DNA sam-

ples that had a single peak, clearly suggesting that fresh tissues are useful for detecting the pathogenic fungi that induce DCS development.

There are no published reports describing the isolation of *Ne. galligena*, which is the pathogen responsible for apple canker disease, in the study region, but it was detected on apples for the first time by an ARISA. Because this analysis is a nontargeted, multiplex fingerprinting technique, pathogenic fungi were detected on apples even though they had not been reported previously as causal agents of DCSs on apple fruits, reflecting the utility of the ARISA. There are several pathogenic fungi that cause DCSs on apples that have not been reported in Japan (Hou et al. 2019; Wenneker et al. 2017). These fungi can be detected by performing an ARISA. In the present study, *Ne. galligena* was detected in the whole tissue, but not in the fresh tissue, of DCSs. Consequently, whether this fungus causes DCSs remains to be confirmed.

A total of 197 DCS samples were analyzed, and the causal pathogen was determined for 79.2% of the samples by a single ARISA trial combined with a PCR amplification using specific primers. Sequencing followed by a BLAST search verified these findings. The results indicated that *P. vagabunda*, *D. mali*, *B. dothidea*, *B. kuwatsukai*, and *Colletotrichum* spp. induce DCS development and that *Ne. galligena* was present in Yokote Basin, Akita Prefecture.

TABLE 2

Automated ribosomal intergenic spacer analyses (ARISAs) using DNA samples extracted from fresh or whole tissues, independently, of dark-colored spots (DCSs) on apple fruits

ARISA trial no.	Apple collection	DCS sampling	No. of apples	No. of DCSs obtained	No. of DCSs with peaks detected	Ratio of DCSs with a single peak (%)	Fragment sizes of standard DNA sample ^a	Fragment sizes of peaks detected in 10% or more of DCSs having peaks (%)	Fragment sizes of peaks detected less frequently than 10% (number of DCSs in which the peak was detected)
Analysis of fresh tissues									
No. 1	Sakae s.f. ^b	15 Dec. 2018	6	29	23	73.9	669	669–670 (87.0), 702–703 (20.4)	632, 660, 689, 693, 709, 713, 711, 752
No. 2	Masuda s.f.	9 Jan. 2019	26	26	20	95.0	667	667–668 (85.0)	658, 1,094, 1,095, 1,096
No. 3	Masuda s.f.	29 Mar. 2021	10	47	37	97.3	668	667–669 (62.2), 686 (18.9)	681 (2), 697 (4), 699, 761
No. 3	Daigo s.f.	29 Mar. 2021	10	47	40	100.0	668	668–669 (90.0)	649, 687, 701, 1,095
No. 4	Nonchemical sprayed orchard	29 Mar. 2021	10	48	44	95.5	668	668–669 (90.9)	683, 687, 691, 697, 700, 749
Analysis of whole tissues									
No. 5	Nonchemical sprayed orchard	26 Nov. 2017	26	44	42	83.3	667	667–668 (55), 658–659 (19), 700–701 (15), 691 (15)	680, 681, 696 (2), 709, 728, 748, 1,095
No. 6	Nonchemical sprayed orchard	9–10 Jan. 2019	88	88	88	78.4	667	667–668 (36), 699–701 (28), 685–686 (18), 689–691 (15)	616, 629 (4), 648, 650–652 (8), 660, 665, 672, 673, 676, 681, 694, 696, 705, 708, 709, 712, 722, 723, 738, 1,045, 1,093–1,095 (8)
No. 6	Masuda s.f.	9–10 Jan. 2019	44	44	41	73.2	667	667–668 (90), 700–701 (17)	629, 658, 690, 691, 692, 722, 723, 1,094, 1,095 (2)
No. 7	Sakae s.f.	10 Jan. 2020	57	57	57	59.6	669	668–670 (83), 702 (19), 690–693 (19), 630 (11)	658, 659, 660, 664, 665, 674–675 (5), 698 (3), 705, 709, 853–854 (4), 1,096
No. 8	Daigo s.f.	10 Jan. 2020	60	60	60	70.0	669	669–671 (98), 702–703 (22)	630 (3), 652, 653, 675, 692–693 (3), 708, 710, 723, 725, 739, 749

^a The DNA was prepared from mycelia of an isolate of *Phlyctema vagabunda*.

^b s.f. = sorting facility.

TABLE 3

Molecular identification of amplicons corresponding to peaks detected by automated ribosomal intergenic spacer analyses (ARISAs) using DNA samples extracted from fresh and whole tissues, independently, of dark-colored spots (DCSs) from Fuji apples

ARISA trial no., sample ID of DCS	Fragment sizes in ARISA	Fluorescence intensity	Determined nucleotide length (nts)	Nucleotide sequence identity (%)	E value	Closest organism (accession no.)
Analysis of fresh tissues						
No. 1						
E01B	669	4,442	116	95.69	1.00E-43	<i>Phlyctema vagabunda</i> (LC171685.1)
E26B	669	7,417	536	99.81	0.0	<i>P. vagabunda</i> (KT923785.1), <i>Neofabraea alba</i> (KJ396077.1)
E71B	669	2,826	475	95.76	1.00E-169	<i>P. vagabunda</i> (MH855316.1), <i>N. alba</i> (KJ396077.1)
E85B	669	2,597	523	99.24	0.0	<i>P. vagabunda</i> (KY367507.2), <i>N. alba</i> (KJ396077.1)
E28B	702	3,214	450	100	0.0	<i>Aureobasidium pullulans</i> (MT573468.1), <i>Kabatiella microsticta</i> (MT107050.1), <i>Metschnikowia</i> sp. (MG906980.1)
E38B	702	1,659	568	100	0.0	<i>Au. pullulans</i> (MT573468.1), <i>Au. protea</i> (JN712492.1), <i>Dothideomycetes</i> sp. (FN868453.1), <i>K. microsticta</i> (MT107050.1)
E50B	702	6,596	456	100	0.0	<i>Penicillium corylophilum</i> (MK450687.1), <i>Penicillium</i> spp. (MH865722.1, MH858652.1, MH855058.1, MF475914.1)
No. 2						
M42B	667	4,461	535	99.63	0.0	Identical to E26B
M61B	667	2,015	535	99.63	0.0	Identical to E26B
M64B	667	3,454	535	99.63	0.0	Identical to E26B
M85B	1,096	697	401	99.75	0.0	<i>Botryosphaeria</i> sp. (KJ80760.1), <i>B. berengeriana</i> (FJ441665.1)
No. 3						
MF9	668	2,590	536	99.81	0.0	Identical to KJA16
MJ1	668	2,209	480	99.79	0.0	<i>N. alba</i> (KJ396077.1)
MM5	668	2,062	406	99.75	0.0	Identical to MJ1
MI1	686	2,987	556	99.81	0.0	<i>Diplocarpon mali</i> (AB609191.1)
MI2	686	3,581	528	99.60	0.0	Identical to MI1
ML4	686	1,582	490	100	0.0	Identical to MI1
ML5	686	471	555	98.56	0.0	<i>Marssonina coronaria</i> (HM368521.1)
MM1	697	1,167	211	99.63	2.00E-84	<i>B. dothidea</i> (MT611222.1), <i>Botryosphaeria</i> sp. (MT587332.1)
MM3	697	988	258	100	8.00E-124	Identical to MM1
MM4	697	707	325	96.00	1.00E-157	<i>B. dothidea</i> (KC527826.1)
MG9	699	288	570	100	0.0	<i>Colletotrichum nymphaeae</i> (MN744277.1), <i>C. acutatum</i> (KU933355.1), <i>C. guajavae</i> (KJ813590.1), <i>C. simmondsii</i> (JN121202.1), <i>Colletotrichum</i> sp. (LC435466.1), <i>Glomerella acutata</i> (AY266405.1)
No. 3						
DH6	649	2,567	411	99.03	0.0	Ascomycota sp. (HQ166343.1)
DII	668	2,957	481	99.00	0.0	<i>P. vagabunda</i> (LC171685.1a)
DL1	668	7,023	535	100	0.0	Identical to KJA16
DO1	668	2,500	535	100	0.0	Identical to KJA16
DM6	687	1,813	245	100	6.00E-120	<i>D. mali</i> (AB609191.1), <i>Ma. coronaria</i> (MN535182.1)
No. 4						
KJA16	668	3,447	535	99.63	0.0	<i>P. vagabunda</i> (KT923785.1), <i>N. alba</i> (KJ396077.1)
KJB10	668	2,742	326	100	3.00E-168	<i>P. vagabunda</i> (LC171685.1)
KJG1	668	2,671	369	100	0.0	Identical to KJB10
KJA7	687	2,739	452	100	0.0	<i>Ma. coronaria</i> (MG984759.1)
Analysis of whole tissues						
No. 5						
17SD-A	658	2,433	480	100	0.0	<i>Neonectria galligena</i> (JQ434582.1), <i>Ne. major</i> (NR121496.1), <i>Neonectria</i> sp. (HQ166303.1)
17SD-C	658	4,103	486	100	0.0	Identical to 17SD-A
17JO-C	668	6,354	480	100	0.0	<i>P. vagabunda</i> (KY367507.2), <i>N. alba</i> (KJ396077.1)
17JO-E	667	7,360	529	99.81	0.0	<i>P. vagabunda</i> (KT923785.1), <i>N. alba</i> (KJ396077.1)
25-1-9-P	691	1,100	371	78.25	3.00E-77	<i>Alternaria</i> sp. (KM454902.1)
25-11-1	691	2,413	500	100	0.0	<i>Alternaria</i> spp. (MF602685.1, MK311341.1, MK311298.1, MK303973.1, MH237662.1, MG589272.1, LC206592.1)
25-1-9-N	701	1,991	509	100	0.0	<i>Aureobasidium</i> spp. (MK460317.1, MK460806.1, KT6925262.1), <i>K. microsticta</i> (MH858817.1), <i>Metschnikowia</i> sp. (MG906980.1), <i>Dothideomycetes</i> sp. (FN868453.1)
S42	692	1,370	554	100	0.0	<i>A. alternata</i> (MN822657.1), <i>A. tenuissima</i> (MN559435.1), <i>A. yaliinficiens</i> (MK659955.1), <i>Alternaria</i> sp. (MN856304.1)

(Continued on next page)

The fragment sizes of the peaks varied a little among the three runs of the genetic analyzer (Table 1). The fragment sizes of the peaks for the DNA sample of a *P. vagabunda* isolate, which was used as the standard sample in each trial, were between 667 and 669 bases during the eight runs of the genetic analyzer, indicative of the high reproducibility of the data (Table 2). However,

considering that the fragment size of a peak for individual fungal species can vary among trials (Table 1), a DNA sample extracted from an authentic fungal isolate should be used as a standard to determine the fragment size variation among trials.

The analysis of the collected apple fruits revealed that *P. vagabunda* is by far the most dominant causal agent of DCSs

TABLE 3 (Continued from previous page)

ARISA trial no., sample ID of DCS	Fragment sizes in ARISA	Fluorescence intensity	Determined nucleotide length (nts)	Nucleotide sequence identity (%)	E value	Closest organism (accession no.)
Analysis of whole tissues						
No. 6						
AK-20	667	1,756	479	100	0.0	<i>P. vagabunda</i> (LC171685.1)
AK-35	667	2,268	535	99.81	0.0	<i>P. vagabunda</i> (KR859275.1), <i>N. alba</i> (KJ396077.1)
AK-81	668	3,475	535	99.63	0.0	<i>P. vagabunda</i> (KT923785.1)
AK-1	685	4,346	248	96.75	6.00E-112	<i>D. mali</i> (AB609191.1)
AK-2	686	7,217	547	100	0.0	Identical to AK-1
AK-14	686	4,256	545	99.62	0.0	Identical to AK-1
AK-30	686	3,375	536	99.81	0.0	Identical to AK-1
AK-69	686	3,223	557	100	0.0	Identical to AK-1
AK-74	686	4,306	556	98.20	0.0	<i>Ma. coronaria</i> (HM36852.1)
AK-36	689	3,185	315	92.74	5.00E-134	<i>A. tenuissima</i> (MG569636.1), <i>A. alternata</i> (JQ625592.1)
AK-45	689	745	422	97.16	0.0	<i>C. siamense</i> (MG751903.1), <i>C. gloeosporioides</i> (KR704206.1)
AK-65	689	932	374	99.73	0.0	<i>C. siamense</i> (MK141761.1), <i>C. gloeosporioides</i> (KR704206.1)
AK-94	689	1,127	557	100	0.0	<i>C. siamense</i> (LC260490.1), <i>C. gloeosporioides</i> (KR704206.1)
AK-33	699	3,448	558	100	0.0	<i>C. fiorinae</i> (MN077457.1), <i>C. acutatum</i> (MH931266.1), <i>C. lupini</i> (LC206489.1), <i>G. acutata</i> (JF288551.1), <i>G. fiorinae</i> (JN943453.1)
AK-40	700	259	567	99.65	0.0	<i>Aureobasidium</i> spp. (MN077446.1, KX100335.1, JN712492.1), <i>K. microsticta</i> (MH858817.1), <i>Dothideomycetes</i> sp. (FN868453.1)
AK-92	701	1,003	459	98.47	0.0	<i>Aureobasidium</i> spp. (MK937952.1, KY436128.1), <i>Kwoniella</i> sp. (MK613238.1), <i>K. microsticta</i> (MH858817.1), <i>Metschnikowia</i> sp. (MG906980.1)
AK-47	1,045	217	888	99.32	0.0	<i>Venturia inaequalis</i> (DQ887774.1)
No. 6						
M91A	667	5,584	487	99.79	0.0	Identical to 17JO-E
M59A	668	3,363	481	100	0.0	<i>N. kienholzii</i> (KR859083.1), <i>Neofabraea</i> sp. (KU712233.1)
M72A	667	4,375	487	100	0.0	<i>P. vagabunda</i> (KT923785.1), <i>N. alba</i> (KJ396076.1)
M65A	692	471	557	100	0.0	<i>A. alternata</i> (MH844771.1)
No. 7						
S12	669	2,325	536	99.81	0.0	<i>P. vagabunda</i> (KT923785.1), <i>N. alba</i> (KJ396077.1)
S35	669	2,128	535	100	0.0	Identical to S12
S49	669	5,635	535	99.81	0.0	Identical to S12
S55	669	2,955	535	100	0.0	Identical to S12
S2	692	5,018	556	100	0.0	<i>A. alternata</i> (MN944587.1), <i>A. tenuissima</i> (MH-824257.1), <i>Alternaria</i> sp. (MT035960.1)
S14	692	1,214	556	100	0.0	Identical to S2
S19	692	1,122	556	99.82	0.0	Identical to S2
S20	693	236	557	100	0.0	<i>A. alternata</i> (KY026591.1), <i>A. tenuissima</i> (MF405157.1), <i>A. brassicola</i> (KF542.557.1), <i>A. mali</i> (AB470849.1), <i>Alternaria</i> sp. (KT264732.1)
S30	691	1,370	554	97.82	0.0	<i>A. alternata</i> (KY026591.1)
S32	693	1,886	558	99.82	0.0	<i>A. alternata</i> (KY026591.1), <i>A. tenuissima</i> (MF405157.1), <i>A. brassicola</i> (KF542.557.1), <i>Alternaria</i> sp. (KJ527009.1)
S42	692	1,370	554	100	0.0	<i>A. alternata</i> (MN822657.1), <i>A. tenuissima</i> (MN559435.1), <i>A. yaliinficiens</i> (MK659955.1), <i>Alternaria</i> sp. (MN856304.1)
No. 8						
D6	669	4,101	535	99.63	0.0	<i>P. vagabunda</i> (KT923785.1), <i>N. alba</i> (KJ396077.1)
D26	670	2,938	535	99.63	0.0	Identical to D6
D35	670	4,696	535	99.81	0.0	Identical to D6
D30	692	2,069	561	99.82	0.0	<i>C. siamense</i> (LC260490.1), <i>C. gloeosporioides</i> (KR704204.1)
D11	702	661	902	99.86	0.0	<i>Au. pullulans</i> (MH9312627.1), <i>K. microsticta</i> (EU167608.1)
D46	703	1,272	823	99.86	0.0	<i>Au. protea</i> (JN712492.1), <i>K. microsticta</i> (EU167608.1)

in the study region, but it might not be the dominant fungus in every orchard in the region. Pathogens other than *P. vagabunda* were detected in 38% of the DCSs from fruits obtained from the Masuda sorting facility in March 2021 (Table 2). Because of the automated analysis of the PCR products for an ARISA, many samples can be processed simultaneously, thereby enabling the identification of the causal agent(s) in each orchard in a particular region. This allows apple producers to adopt the most appropriate DCS control strategy for each orchard. To limit the damages caused by DCSs, accurate diagnosis at the orchard level is crucial.

ARISA is a nontargeted, multiplex fingerprinting technique that can analyze many samples at the same time.

A single ARISA trial or a single ARISA trial combined with a PCR amplification using specific primers can detect all pathogenic fungi causing DCSs (e.g., eight species and two species complexes). The ARISA-based technique can accurately, reproducibly, efficiently, and comprehensively detect the pathogens responsible for DCSs. It is clearly more useful than other DNA-based fingerprinting techniques for diagnosing DCSs in these respects. It could be applicable for other plant diseases that produce similar symptoms (Supplementary Fig. S3). Furthermore, the method could be effective for the initial screening of quarantined organisms at ports because of its sensitivity and its utility for analyzing numerous samples simultaneously for the presence of multiple pathogenic fungi (Lin et al. 2018).

Pathogenic fungi were undetectable in the fresh tissue of 33 DCSs (16.8% of the examined DCSs). These DCSs might have resulted from physiological disorders, including bitter pit and lenticel blotch pit (Ferguson and Triggs 1990; Raese and Drake 2002). This possibility will need to be experimentally verified.

Peaks with fragment sizes of 689 to 693 and 699 to 703 bases were more frequently detected for the DNA samples obtained from the whole tissue of DCSs than for those from the fresh tissue of DCSs. Additionally, 30% and 20% of these peaks, respectively, were for DNA samples from saprophytic fungi according to the sequence analyses and PCR with specific primers. These fungi, including *Alternaria* spp. and *Aureobasidium* spp., are often present in the phyllosphere of apple orchards (Andrews et al. 2002; Woody et al. 2003). *Au. pullulans* is a potential biocontrol agent that could prevent postharvest diseases in pome fruits (Mari et al. 2012; Vero et al. 2009). *Alternaria* spp. and *Penicillium* sp., which were detected in fresh tissues (Table 3), can cause postharvest decay and produce mycotoxins on apple fruits (Escrivá et al. 2017; Moslem et al. 2013; Sutton et al. 2014; Wenneker and Kohl 2014). Because an ARISA is quite effective for detecting these fungi and for profiling microbial communities, it is useful for ecological studies of phylloplanes or phyllospheres (Shen et al. 2017).

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