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Botryosphaeria spp. isolated from apple and several deciduous fruit trees are divided into three groups based on the production of warts on twigs, size of conidia, and nucleotide sequences of nuclear ribosomal DNA ITS regions

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We examined the phytopathological and biological characters of *Botryosphaeria* spp. isolated from apples and other deciduous fruit trees, and determined the nucleotide sequences of their rDNA ITS regions. The *Botryosphaeria* isolates from deciduous fruit trees can be divided into three groups based on their production of warts on twigs, size of the conidia, and nucleotide sequences of rDNA ITS 1, ITS 2 and 5.8S rDNA. Isolates of *Botryosphaeria* in ITS group A produced conidia of intermediate size and showed warts on infected twigs prior to the development of ring rot on fruit. This group was common on deciduous fruit trees in Japan as a causal agent of ring rot and wart bark diseases of apples and pears; and it appears similar to the *B. dothidea* from the US that was isolated from apple exhibiting white rot. The ITS group B *Botryosphaeria* produced small conidia and induced shoot blight without wart development prior to the development of ring rot on fruit. This group was localized on pear, persimmon, and kiwi fruit in restricted areas of Japan. The ITS group C *Botryosphaeria* consisted of *B. obtusa*, the causal agent of apple black rot in the US, which produced large dark brown conidia.

Key Words——apple; *Botryosphaeria* spp.; deciduous fruit trees; rDNA ITS region.

Apple ring rot disease, known as *rinmon-byo* in Japanese, occurs throughout the apple-growing areas of Japan. The disease is characterized by ring rot on immature and mature fruit; losses gradually increase as the fruit near harvest. The disease is also referred to as wart or blister bark disease, *ibokawa-byo* in Japanese, because raised blisters or warts occur on infected branches and twigs, which give rise to inocula for fruit rot. The causal fungi of apple ring rot disease, *Botryosphaeria* spp., also infect Japanese and European pears producing similar ring rot and wart symptoms on fruit and twigs. On the other hand, another pathogen that causes a similar ring rot disease on apples and pears occurs in orchards in Japan. The pathogen causes branch or shoot blight on infected trees without producing blisters or warts on branches and twigs, and is referred to as apple dieback or pear dieback (or shoot blight). Yamato (1977) identified the pathogen causing pear shoot blight as *B. dothidea* (Moug. ex Fr.) Ces. & De Not.. Koganezawa and Sakuma (1984), on the other hand, proposed *B. berengeriana* De Not. as the name for the pathogen causing apple dieback and *B. berengeriana* De Not. f. sp. *piricola* (Nose) Koganezawa et Sakuma for the one causing apple wart bark disease, and gave "ring rot" as a common name of the disease. Another disease on Japanese pear showing somewhat similar symptoms, *Physalospora* canker (or wart canker), is considered to be caused by *Physalospora piricola* Nose, the teleomorph of this fungus (The Phytopathological Society of Japan, 1984).

A similar bark disorder called peach blister canker disease is also reported on peach trees in Japan. The disease is associated with reduced yield. Since the pathogen infects peach trees only (Abiko and Kitajima, 1970), Koganezawa and Sakuma (1984) considered it as a forma specialis of *B. berengeriana* and named it *B. berengeriana* De Not. f. sp. *persicae* (Abiko et Kitajima) Koganezawa et Sakuma. Furthermore, similar bark or fruit diseases such as persimmon shoot blight, persimmon canker (Yamato, 1980), grapevine black blight (Kobayashi, 1977; Tanaka and Takahashi, 1976) and kiwi fruit ripe rot (Tachibana et al., 1983), have also been reported to be incited by *B. dothidea.*

As described above, the etiology of the *Botryosphaeria* disorders on deciduous fruit trees in Japan is complicated and not yet clearly understood. Consequently, classification and nomenclature of *Botryosphaeria* spp. in Japan is confusing. For example, the causal agent of apple ring rot, identified as *B. beren-*

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Table 1. Identity and origin of *Botryosphaeria* spp. and characters examined.

Isolate No.	Identity	Host	Part	Symptom	Location	Collector	Characters examined			
							Nucoleotide seqeunce of ITS region	Conidial characters (size*, color)	Mycelial growth (at 25°C)	Mycelial color group
1	*B.* sp.	apple	fruit	ring rot & warts	Fukushima Pref.	S. Hayashi	Group A	M, hyaline	slow	IV
7	*B.* sp.	apple	fruit	ring rot	Fukushima Pref.	T. Ogata		M, hyaline	slow	IV
12	*B.* sp.	peach	branch	warts	Fukushima Pref.	S. Kanematsu	Group A			
14	*B.* sp.	pear	fruit	ring rot	Akita Pref.	M. Fukaya		M, hyaline		
27	*B.* sp.	apple	branch	warts	Akita Pref.	T. Ogata		M, hyaline	slow	II
29	*B.* sp.	pear	branch	warts	Nagasaki Pref.	T. Ogata	Group A	M, hyaline	slow	III
34	*B.* sp.	apple	fruit	ring rot	Yamagata Pref.	T. Ogata		M, hyaline	slow	IV
37	*B.* sp.	apple	fruit	ring rot	Yamataga Pref.	T. Ogata		M, hyaline	slow	IV
39	*B.* sp.	pear	branch	warts	Fukuoka Pref.	T. Ogata		M, hyaline	slow	II
40	*B.* sp.	pear	branch	warts	Fukuoka Pref.	T. Ogata		M, hyaline		
41	*B.* sp.	pear	branch	warts	Nagano Pref.	T. Ogata	Group A	M, hyaline	slow	III
42	*B.* sp.	apple	branch	warts	Nagano Pref.	T. Ogata		M, hyaline		
43	*B.* sp.	apple	branch	warts	Nagano Pref.	T. Ogata		M, hyaline	slow	IV
44	*B.* sp.	apple	branch	warts	Nagano Pref.	T. Ogata		M, hyaline	slow	
45	*B.* sp.	pear	branch	warts	Hiroshima Pref.	T. Ogata		M, hyaline	slow	
46	*B.* sp.	pear	branch	warts	Hiroshima Pref.	T. Ogata		M, hyaline	slow	
66	*B.* sp.	grapevine	branch	shoot blight	Okayama Pref.	H. Nasu	Group A	M, hyaline	slow	III
67	*B.* sp.	grapevine	peduncle	black blight	Okayama Pref.	H. Nasu	Group B	S, hyaline	slow	I
70	*B.* sp.	quince	fruit	fruit rot	Fukushima Pref.	T. Ogata		M, hyaline		
72	*B.* sp.	peach	branch	warts	Fukushima Pref.	T. Ogata	Group A	M, hyaline	fast	II
73	*B.* sp.	pear	branch	warts	Tokushima Pref.	H. Yamato	Group A		slow	II
74	*B. dothidea*	pear	branch	shoot blight	Tokushima Pref.	H. Yamato	Group B	S, hyaline	fast	III
76	*B. dothidea*	pear	branch	shoot blight	Tokushima Pref.	T. Ogata	Group B	S, hyaline	second fast	III
77	*B. dothidea*	kiwifruit	fruit	ripe rot	Tokushima Pref.	H. Yamato	Group B		fast	III
78	*B. dothidea*	persimmon	fruit	fruit rot	Tokushima Pref.	H. Yamato	Group B		fast	II
79	*B. obtusa*	apple	fruit	black rot	USA	T.B. Sutton	Group C	L, brown		IV
80	*B. obtusa*	apple	fruit	black rot	USA	T.B. Sutton	Group C		slow	IV
81	*B. dothidea*	apple	fruit	white rot	USA	T.B. Sutton	Group A	M, hyaline	fast	IV
82	*B. dothidea*	apple	fruit	white rot	USA	T.B. Sutton	Group A		fastest	II

*L; large, M; intermediate, S; amall, **blank colum; not examined.

geriana (Koganezawa and Sakuma, 1984), has not yet been accepted widely among researchers. Most mycologists consider that *B. berengeriana* is synonymous with *B. dothidea* and that *B. dothidea* has priority (Brown and Britton, 1986; Jones, 1990). Jacobs and Rehner (1998) examined some morphological characters and the nucleotide sequences of nuclear ribosomal DNA ITS (rDNA ITS) regions of anamorphs of the genus *Botryosphaeria* and found that those identified as *Fusicoccum aesculi* Corda, an anamorph of *B. dothidea*, consisted of two ITS groups.

In this study, we examined the phytopathological and biological characters of *Botryosphaeria* spp. isolated from apples and other deciduous fruit trees cultivated in Japan and determined the nucleotide sequences of rDNA ITS regions in selected isolates. We present evidence that *Botryosphaeria* isolates from apples and deciduous fruit trees in Japan and the US can be divided into three groups based on their production of wart symptoms on twigs, size of the conidia, and nucleotide sequences of rDNA ITS 1, ITS 2 and 5.8S rDNA.

Materials and Methods

Isolates of *Botryosphaeria* spp. Sources of the 29 *Botryosphaeria* isolates used in this experiment are listed in Table 1. Four *Botryosphaeria* isolates were obtained from diseased twigs and fruit collected in orchards in Fukushima Prefecture. Twenty-one isolates were from diseased twigs and fruit kindly collected and provided by researchers in Akita, Yamagata, Nagano, Okayama, Tokushima, Fukuoka, and Nagasaki Prefectures. Some isolates were provided by the Fruit Tree Research Experiment Station of the Ministry of Agriculture, Forestry and Fisheries (MAFF) or by Prefectural Agriculture Research Stations. Two isolates each of *B. dothidea* and *B. obtusa* were kindly provided by Dr. T. B. Sutton (North Carolina State Univ.). These were isolated in the US from ap-

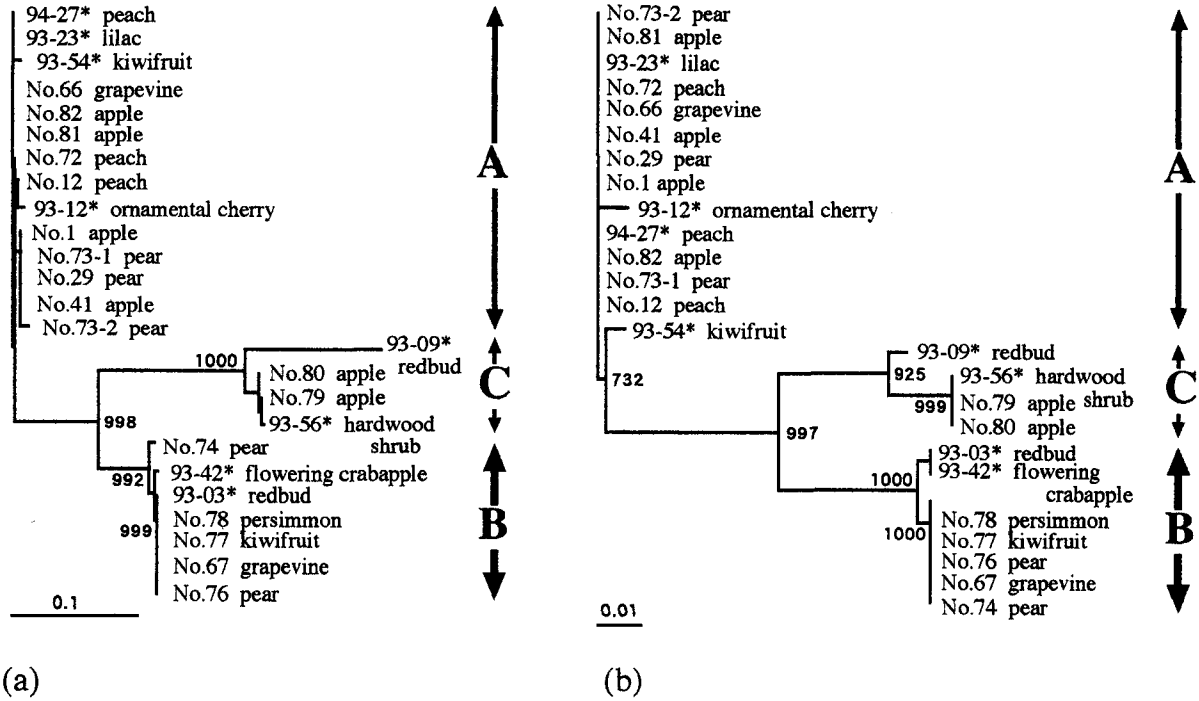

Fig. 1. Unrooted phylogenetic tree of *Botryosphaeria* spp. isolated from several deciduous fruit trees based on nucleotide sequence of nuclear rDNA ITS 1 (a) and ITS 2 (b). The trees were constructed by the neighbor-joining method. The values on nodes are the confidence levels from a 1,000 replicate bootstrap sampling. The numbers of isolates and the name of host plant are indicated in the figures. Details are summarized in Table 1. Those with asterisks were obtained from the DDBJ DNA database deposited by Rehner & Jacob (1998). Arrows with A, B, and C indicate groups based on ITS sequences. DDBJ accession numbers of each isolates are Nos. 1 (AB034808), 12(AB034809), 29(AB034810), 41(AB034813), 66(AB034814), 67(AB034815), 72(AB034816), 73(AB034817), 74(AB034818), 76(AB034819), 77(AB034820), 78(AB034821), 79(AB034822), 80(AB034812), 81(AB034823), 82(AB034811), 93-03(AF027749), 93-09(AF027752), 93-12(AF027746), 93-23(AF027747), 93-42(AF027741), 93-54(AF027750), 93-56(AF027759), and 94-27(AF027748).

ples symptomatic of white rot and black rot, respectively, and imported with the permission of plant quarantine; permission number 9 Yokoshoku No. 1154.

Fungal DNA extraction Each isolate was cultured on PDA (potato, 200 g; dextrose, 20 g; agar, 20 g; and distilled water, 1 L). A 1-cm diam disk with mycelia was removed and incubated in PD broth (potato, 200 g; dextrose, 20 g; and distilled water, 1L) at 20°C for 14 d in the dark. After rinsing with distilled water, 3 g of mycelia was homogenized with a mortar and pestle in 5 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM EDTA, 3% N-lauroyl sarcosinate) and allowed to stand for 1 h at 65°C. The homogenate was extracted with phenol-chloroform, and a cleared lysate was recovered by centrifugation. Then crude DNA was precipitated with ethanol, dissolved in an appropriate amount of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored in a - 30°C freezer. For further purification, 200 μl of the crude DNA suspension was removed, incubated with 2 μg of RNase A (Boefringer Mannheim) for 30 min at 37°C for RNA digestion, then mixed with 120 μl of 20% polyethyleneglycol (PEG #6000) in 2.5 M NaCl. DNA was precipitated by centrifugation after standing for 1 h on ice. After rinsing with 70% ethanol containing 0.2 M sodium acetate, the precipitate was dried and dissolved in 400 μl of STE buffer (0.1 M NaCl, 0.05 M Tris, 0.01 M EDTA, pH 6.8) containing 35% ethanol. The DNA suspension was charged on a cartridge column (Takara, SuprecTM-01) packed with 0.2 g (dry weight) of CF-11 cellulose (Whatman) equilibrated with STE buffer containing 35% ethanol. The column was washed twice by adding 400 μl of STE containing 35% ethanol and centrifuged (ca. 1,500 g for 3 min in a microcentrifuge, Tomy MX-150). Purified DNA was eluted with 100 μl of STE buffer by centrifugation (ca. 1,500 g for 3 min), precipitated with ethanol, and finally dissolved in 20-50 μl of distilled water for sequence analysis.

PCR, DNA cloning and sequencing Five μl of the purified DNA was used as template for polymerase chain reaction (PCR). The fragment between the 3′ terminal region of the nuclear 18S ribosomal RNA gene (18S rDNA) and the 5′ terminal region of the nuclear 25S ribosomal RNA gene (25S rDNA), which included internal transcribed spacer 1 and 2 (ITS 1 and 2), and the intervening 5.8S ribosomal RNA gene (5.8S rDNA), was amplified using the primers ITS-1 and ITS-4 (White et al., 1990). The parameters for PCR amplification consisted of one cycle of heat denaturation at 94°C for 4 min; 35 amplification cycles at 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min; and a final primer extension at 72°C

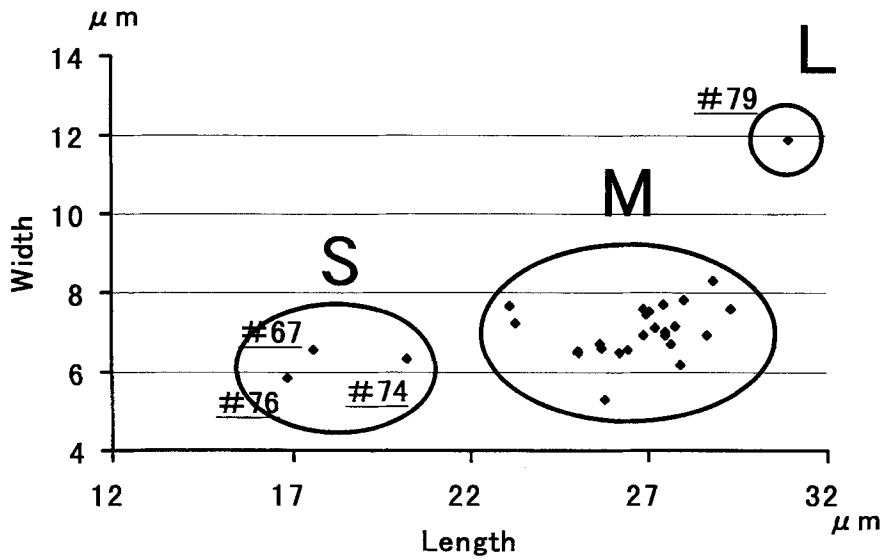

Fig. 2. Distributions of conidial length and width of *Botryosphaeria* spp. in PDA. L, large conidia group; S, small conidia group; M, intermediate conidia group, which includes isolates Nos. 1, 7, 14, 27, 34, 37, 39–46, 66, 70, 72 and 81.

for 7 min. A PCR product of ca. 570-bp was recovered by 7.5% polyacrylamide gel electrophoresis, ligated into pGEM-T vector (Promega), and the resultant plasmid DNA was used for transformation of *Escherichia coli* (strain JM 109). Recombinant DNA clones containing a 570-bp insert were selected, and plasmid DNA was purified from them by using QIAprep-spin Plasmid Kit (QIAGEN). Nucleotide sequencing was performed using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Life Science), according to the manufacturer's instructions. Sequencing reactions were run on a Li-Cor DNA sequencer Model 4000L and analyzed by use of Base ImagIR Software Ver. 2.10.

Phylogenetic analysis DNA sequences were edited, joined, and aligned by DNASIS Ver. 3.6 (Hitachi Software Engineering), using Multiple Alignment, Multi Edit Mode, Automatic (Higgins and Sharp, 1989). Phylogenetic relationships were determined from the aligned sequences by using the neighbour-joining method (Saitou and Nei, 1987) contained in CLUSTAL W, which was released by DNA Data Bank of Japan (DDBJ, Mishima, Japan; http://www.ddbj.nig.ac.jp). The reliability of the phylogenetic relationship was evaluated statistically by using 1000 bootstrap replicates. Phylogenetic trees were drawn by use of TreeView 68K (Ver. 1.5.1).

Morphology and size of conidia Each *Botryosphaeria* isolate was placed on two PDA dishes and was stimulated to produce conidiomata and conidia by incubating them for 10 to 14 d at 25°C under black light (Toshiba, FL20S.BLB). Details of morphology, color, and the presence of septa in conidia were examined under the light microscope. Microscopic images of conidia were also taken by use of a CCD camera system and the enlarged image was visualized on a CRT display. Length of the major axis (length) and the minor axis (width) of 50 conidia from each isolate were measured by using a caliper. The length, width and length-to-width ratio of the conidia were compared statistically by cluster analysis utilizing mean separation procedures and the Analysis of Variance or General Linear Model Procedures (SAS, 1992).

Color of mycelial colony Two agar discs, 5 mm in diam, were removed from the margin of colonies growing on PDA dishes in the dark and placed individually in the center of PDA dishes (9 cm in diam) with the mycelium side down on the agar surface. After incubating the dishes for 14 d at 25°C in the dark, the color of the colony was determined using the Methuen Handbook of Colour (Kornerup and Wanscher, 1984) as a guide. The color of the colony was also measured by using a Chroma Meter (Minolta CR-300) connected with a data processor (DP-300) and expressed as numeric data. Measurements by the Chroma Meter were performed at four points selected arbitrarily in each of the two dishes. Values were compared statistically by cluster analysis (SAS, 1992).

Mycelial growth at different incubation temperatures The effect of temperature on the growth of mycelia was examined by measuring the diam of colonies growing on duplicated PDA (Difco) dishes for 3 d at 15, 20, 25, and 30°C, and for 14 d at 10 and 35°C. Two axial diameters were measured on each of the two independent colonies. Colony diameters were compared statistically by cluster analysis (SAS, 1992).

Results

PCR amplification and nucleotide sequence of nuclear rDNA ITS regions Electrophoresis of PCR products in 7.5% polyacrylamide gels revealed that a DNA fragment of *ca.* 570 bp in length was amplified from all the samples examined. Because the size of the DNA fragment was almost the same among the isolates and was hard to discriminate by electrophoretic analysis, we further analyzed the nucleotide sequence of 16 isolates,

selected after considering host and area from which the pathogen was isolated. The property of producing warts on twigs was also taken into consideration for selection as an important phytopathological character. The 16 isolates included those from apple, peach, pear, grapevine, kiwi fruit, and persimmon trees showing fruit rot with/without accompanying warts on the bark, collected from various fruit producing areas in Japan, and those isolated in the US from apples showing symptoms characteristic of white rot (*B. dothidea*) and black rot (*B. obtusa*).

We sequenced two independent cDNA clones for each isolate. Although a minor sequence heterogeneity was found in the ITS 1 (Nos. 73-1 and 73-2 in Fig. 1a) and 5.8S rDNA (data not shown) regions of the No. 73 isolates, no heterogeneity was found in the other 15 isolates. Phylogenetic analysis was performed by using the ITS 1, ITS 2, and 5.8S rDNA sequence sets separately. Sequence data of *Fusicoccum aesculi*, an anamorph of *B. dothidea*, and *Sphaeropsis* sp., an anamorph of *B. obtusa* reported by Jacobs and Rehner (1998), were obtained from a DNA database (DDBJ), and aligned with the sequence sets constructed in this experiment. All of the phylogenetic trees based on the ITS 1, ITS 2, and 5.8S rDNA sequence sets were consistent with each other. *Botryosphaeria* spp. isolated from apples and several deciduous fruit trees in Japan and in the US were clearly separated into three groups (A, B, and C), which received bootstrap support above 90% in ITS 1 and 2 (Fig. 1a, b). Although the bootstrap support (above 60%) was not as high in the case of the 5.8S rDNA sequence set, combinations of four base substitutions found in residues 120 (T or C), 125 (T or C), 133 (A or G), and 158 (A or T) of the 5.8S rDNA sequence were consistent with the grouping based on ITS 1 and 2 sequence sets. Concretely, the sequences of the 120th, 125th, 133rd, and 158th are: T, T, A, and A in group A; C, T, G, and T in group B; and C, C, G, and A in group C.

Group A, the largest group, consisted of those isolates from apples showing fruit rot and bark warts in Fukushima (No. 1) and Nagano (No. 41), peaches showing bark warts in Ibaraki (No. 12) and Fukushima (No. 72), pears showing bark warts in Nagasaki (No. 29) and Tokushima (No. 73), and grapevines showing shoot blight (No. 66) in Okayama. A characteristic feature of the members in this group was that all except No. 66 showed wart symptoms on twigs. The host plants and the areas from which the pathogen was isolated were variable in this group. Two *B. dothidea* isolates Nos. 81 and 82 from US apples showing white rot symptoms were also included in this group.

Group B consisted of isolates from pears showing shoot blight without accompanying warts (Nos. 74, 76), kiwi fruit showing fruit rot (No. 77), and persimmon showing fruit rot (No. 78), which were all identified as *B. dothidea* (Yamato, 1977) and were all from Tokushima. One isolate from grapevines showing black rot (No. 67; Nasu, unpublished) from Okayama was also included in this group. None of the isolates showed wart symptoms on twigs. Although four of the five isolates in this group were from Tokushima Prefecture, the host plants were variable.

Group C consisted of *B. obtusa* (Nos. 79, 80) isolates from the US from apples from a tree exhibiting black rot symptoms. None of the Japanese isolates used in this experiment was included in this group, which was genetically more distant from the first and the second groups.

Morphology, length and width of conidia on PDA agar Twenty-three of the 29 isolates used in this experiment formed conidiomata and produced sufficient conidia for analysis. Under the conditions employed, the central region of the colony began to turn grayish brown to dark grayish black and started to form conidiomata after 3 to 4 d of incubation. Because conidia produced in the conidiomata mature in 10 to 14 d under our conditions, they were harvested after 14 d of incubation. *Botryosphaeria obtusa* produced unicellular, ellipsoidal conidia, dark brown in color, which were morphologically distinct from and larger than those of the other isolates. The other isolates produced unicellular, hyaline, long ellipsoidal to spindle-shaped conidia. A few of the conidia observed in these specimens had one or two septa, although the latter were rare. Cluster analysis of the data on length, width, and length to width ratio of the conidia revealed that these isolates were separated into three spore size groups (L, M, and S in Fig. 2), which were significantly different at $P = 0.005$. *Botryosphaeria obtusa* (No. 79), as mentioned above, had the largest conidia. Isolates Nos. 67, 74, and 76 constituted the smallest conidia group. Isolates Nos. 1, 7, 14, 27, 29, 34, 37, 39–45, 46, 66, 70, 72, and 81 formed a cluster located between them, although they were much closer to the smaller group. Clusters based on the size of conidia were consistent with those established by nucleotide sequence of nuclear rDNA ITS regions (including 5.8S rDNA).

Color of mycelial colonies grown on PDA in the dark Cluster analysis of numeric data obtained by the Chroma Meter revealed that the isolates could be divided into four groups; I (No. 67), II (Nos. 27, 39, 72, 73, 78, 82), III (Nos. 29, 41, 66, 74, 76, 77), and IV (Nos. 1, 7, 34, 37, 43, 44, 79–81). The clusters were not consistent with those based on rDNA sequence or their biological characters.

Mycelial growth on PDA at different incubation temperatures The optimum temperature for most of the isolates examined was 25°C. Isolates from peach showing wart bark (No. 72), pear showing shoot blight without wart bark (Nos. 74, 76), kiwi fruit showing fruit rot (No. 77), persimmon showing fruit rot (No. 78), and two *B. dothidea* from the US (Nos. 81, 82) tended to grow faster than the others at this temperature. Of these isolates, No. 82 grew the fastest and No. 76 was the second fastest. Other isolates such as those from apples and pears showing fruit rot with wart bark and *B. obtusa* tended to grow more slowly. At lower temperatures, Nos. 66, 67, 74, 76–78, 80, and 81 grew well. Isolates Nos. 72, 81, and 82 exhibited similar growth at the higher temperature to that at 25°C The other isolates

grew significantly more slowly at the lower and higher temperatures tested. The growth of isolate No. 72 was similar at all temperatures examined. We could find no significant correlation between the rate of mycelial growth and the other characters examined in this study.

Discussion

We determined the nucleotide sequences of rDNA ITS 1, ITS 2, and 5.8S rDNA regions for *Botryosphaeria* spp. isolated from apples and several other deciduous fruit trees in Japan and the US and found that they could be divided into three ITS groups. The reliability of this phylogenetic relationship using ITS 1 and 2 sequence sets was supported statistically by bootstrap values above 90%. The relationship was also supported by a 5.8S rDNA sequence set. Although the bootstrap support (above 60%) was a little lower in this case due to the strict conservation on the ribosomal gene, combinations of four base substitutions found in residues 120 (T or C), 125 (T or C), 133 (A or G), and 158 (A or T) of the 5.8S rDNA sequence were consistent with the grouping based on ITS 1 and 2 sequence sets. The finding that the 5.8S rDNA, as well as ITS 1 and 2, sequences of the *Botryosphaeria* spp. examined in this study were heterogeneous and could be divided into at least three groups led us to conclude that the *Botryosphaeria* disease on deciduous fruit trees is a complex of fungi with genetically divergent ITS regions.

The largest group (tentatively named "ITS group A") consisted isolates from apples, peaches, pears, and grapevines collected from various areas of Japan, and *B. dothidea* isolated in the US from apples showing symptoms of white rot disease. All but No. 66 in group A produced wart symptoms on infected twigs and branches irrespective of their host species. The second group (tentatively named "ITS group B") consisted of isolates from pears, grapevine, kiwi fruit, and persimmon, which showed fruit rot accompanied by shoot blight without warts. The third group (tentatively named "ITS group C") consisted of isolates identified as *B. obtusa* from the US, from apples showing symptoms of black rot disease.

The division of the *Botryosphaeria* isolates studied into three ITS groups was also supported by data based on the size of conidia. Cluster analysis of length, width, and length-to-width ratio of the conidia resolved three spore size groups which are consistent with those based on the ITS sequences: the largest spore size group L corresponded to ITS group C, the middle spore size group M to ITS group A, and the small spore size group S to ITS group B. The other biological characters such as morphology of conidia, color of mycelial colony, and mycelial growth were not correlated with the ITS grouping.

In conclusion, our results can be summarized as follows. Isolates of *Botryosphaeria* in group A produced conidia of intermediate size and showed warts on infected twigs and branches prior to the development of ring rot symptoms on fruit. This group is quite common among deciduous fruit trees in Japan as a causal agent of ring rot and wart bark diseases of apples and pears, and it appears similar to the *B. dothidea* isolates from the US which were isolated from fruit exhibiting symptoms of the white rot disease. The group B *Botryosphaeria* isolates produced small conidia and induced shoot blight without wart development prior to the development of ring rot symptoms on fruit. Group B isolates seem to be localized on pear, persimmon, and kiwi fruit in restricted areas of Japan, although more extensive surveys will be necessary to confirm this. The group C *Botryosphaeria* isolates consisted of *B. obtusa*, the causal agent of apple black rot disease in the US, which produced large dark brown conidia that were distinct from those of the other isolates examined. Neither this type of *Botryosphaeria* nor black rot disease has yet been identified in apple production areas in Japan.

Several *Botryosphaeria* isolates originally classified into different species or forma specialis were clustered in the same ITS group. *Botryosphaeria dothidea* (the causal agent of apple ring rot; isolate No. 1 in this work), *B. berengeriana* f. sp. *piricola* (the cause of apple wart bark; similar to isolate No. 4 in this work), *Physalospora piricola* (the cause of pear wart canker; similar to isolate No. 29 in this work), and *B. berengeriana* f. sp. *persicae* (the cause of peach blister canker diseases; similar to Nos. 2 and 72 in this work) were all included in group A. Although the isolates were identified by considering the symptoms, host specificities, and the color of the mycelial growth, the results obtained in our study suggested that they are genetically quite similar and raise questions regarding the synonymy of the various species. On the contrary, some *Botryosphaeria* isolates that were originally identified as *B. dothidea* were found to cluster in different ITS groups. Recently, Jacobs and Rehner (1998) also described that *Fusicoccum aesculi*, an anamorph of *B. dothidea* isolated from redbud, ornamental cherry, lilac, flowering crabapple, apple, peach, and kiwi fruit collected in the US, Japan, and New Zealand, was divided into two terminal ITS groups I and III, which correspond to group B and group A, respectively, in the present study.

Smith (1934) reported that fungi included in *Botryosphaeria* showed a wide host range, and Arx and Müller (1954) described *B. dothidea* as a complex fungus. These observations are consistent with our findings and raise questions regarding the classification and nomenclature of *Botryosphaeria* spp. on apple and other deciduous fruit trees. Our results showed that grouping based on the nucleotide sequence of rDNA ITS region (including 5.8S rDNA) would be a strong tool in conducting more detailed pathological and ecological research on *Botryosphaeria*-related fruit rot or bark disorders prevalent in deciduous fruit trees.

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Literature cited

Abiko, K. and Kitajima, H. 1970. Blister canker, a new disease of peach tree. Ann. Phytopathol. Soc. Jpn. **36**: 260–265.

Arx, J. A. von und Müller, E. 1954. Die Gattungen der Amerosporen Pyrenomyceten. Beitr. Kryptogamenfl. Schweiz **11**: 1–434.

Brown, E. A. and Britton, K. O. 1986. *Botryosphaeria* diseases of apple and peach in the southeastern United States. Plant Dis. **70**: 480–484.

Higgins, D.G. and Sharp, P.M. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Comput. Appl. Biosci. **5**: 151–153.

Jacobs, K. A. and Rehner, S. A. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia **90**: 601–610.

Jones, A. L. 1990. Apple Ring Rot. In: Compendium of Apple and Pear Diseases, (ed. by Jones, A. L. and Aldwinckle, H. S.), p. 28, APS Press.

Kobayashi, T. 1977. Ecology and relationship between the causal fungi of stem canker and of shoot blight on woody plants. Kongetu-no-Noyaku **21**: 101–105. (In Japanese.)

Koganezawa, H. and Sakuma,T. 1984. Causal fungi of apple fruit rot. Bull. Fruit Tree Res. Stn. **C11**: 63–73.

Kornerup, A. and Wanscher, J. H. 1984. Methuen Handbook of Colour, Third Editon, Metuen London Ltd.

The Phytopathological Society of Japan. 1984. Pears Rinmon-byo, In: Common Names of Economic Plant Diseases in Japan. Vol. 3, 2nd Ed. p. 30. Japan Pl. Protec. Assoc. Tokyo.

SAS. 1992. SAS Procedures. SAS Institute, Raleigh, NC.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method of reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.

Smith, C. O. 1934. Inoculation showing the wide host range of *Botryosphaeria ribis*. J. Agr. Res. **49**: 467–476.

Tachibana, Y., Sagawa, M. and Ohmori, N. 1983. Occurrence of soft rot of kiwi fruit (I) Isolation of fungi and their pathogenicity. Ann. Phytopathol. Soc. Jpn. **49**: 403. (Abstract, in Japanese.)

Tanaka, S. and Takanashi, S. 1976. Studies on a *Macrophoma* sp. causing black rot-like symptoms of grapes with special reference to its perfect stage. Res. Rep. Facul. Agric. Tamagawa Univ. **16**: 83–89.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols, (ed. by Innis, M. A. et. al.) pp. 315–322. Academic Press, San Diego, California.

Yamato, H. 1977. *Botryosphaeria* spp. associated with shoot blight of Japanese pear. Ann. Phytopathol. Soc. Jpn. **43**: 324. (Abstract, in Japanese.)

Yamato, H. 1980. *Botryosphaeria dothidea* associated with stem canker of Japanese persimon. Ann. Phytopathol. Soc. Jpn. **46**: 98. (Abstract, in Japanese.)