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Analysis of Genetic Relatedness in *Alternaria* species Producing Host Specific Toxins by PCR Polymorphism

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Twenty universal rice primers (URPs) were used to detect PCR polymorphisms in 25 isolates of six different *Alternaria* species producing host specific toxins (HST). Eight URPs could be used to reveal PCR polymorphisms of *Alternaria* isolates at the intra- and inter-species levels. Specific URP-PCR polymorphic bands that are different from those of the other *Alternaria* spp. were observed on *A. gaisen* and *A. longipes* isolates. Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis using 94 URP polymorphic bands revealed three clustered groups (*A. gaisen* group, *A. mali* complex group, and *A. logipes* group).

Keywords : *Alternaria* species, host specific toxins, URP, genomic polymorphism

The genus *Alternaria* is composed of about 60 species, the vast majority of which are plant pathogens that cause the diseases on many kinds of plants worldwide (Rotem, 1994). Among them, seven *Alternaria* species have been known to produce host-specific toxins (HSTs) as determinant factors of pathogenicity (Nishimura and Komoto, 1983; Otani and Komoto, 1992). Since each pathogen has distinct host ranges, they cause destructive diseases on defined plant species or varieties. The following *Alternaria* spp. are known to produce HSTs: *A. gaisen* (=*A. kikuchiana*), an AK toxin producer causing black spot of Japanese pear; *A. mali*, an AM toxin producer causing brown spot of apple; *A. longipes*, an AT toxin producer causing brown spot of tobacco; and two biotypes of *A. citri*, a producer of ACRL toxin causing brown spot of rough lemon and a producer of ACTC or ACT toxin causing brown spot of tangerines. As pathogenic variants within *A. alternata*, *A. alternata* f. sp. *lycopersici* and strawberry pathotype of *A. alternata* produce HSTs, named AAL and AF toxins, which are the causal agents of stem canker of tomatoes and black spot of strawberry.

Despite the fact that each *Alternaria* spp. produces unique toxic substance, they are considerably difficult to distinguish among HST-producing *Alternaria* species or between them and non-pathogenic *A. alternata* based on morphological characteristics such as conidial shape, color, septation, and beak.

Molecular tools such as DNA hybridization and PCR techniques have been employed to provide taxonomic profiles of fungi. In the past, ribosomal RNA analyses using restriction fragment length polymorphism (RFLP) were applied in assessing genetic diversity among HST-producing *Alternaria* spp. (Adachi et al., 1993; Kusaba and Tsuge, 1994; Kusaba and Tsuge, 1995). However, the method did not differentiate the populations of HST-producing fungi from one another nor from non-pathogenic *A. alternata*. In addition, the nucleotide sequence analyses of Internal Transcribed Spacer (ITS) regions in ribosomal DNA did not provide critical differences among the fungi (Go et al., 1997; Pryor and Gilbertson, 2000). On the basis of morphological similarity and analytical data of rDNA, it was proposed that HST-producing *Alternaria* spp. are variant strains of *A. alternata* that acquired the ability to produce HST against certain susceptible host plants and, thus, the fungi should be named as pathotypes of *A. alternata* on the basis of the host plants attacked (Kusaba and Tsuge, 1995). Nevertheless, Simmons (1992) and Yu (1992) suggested that some morphological characters such as three-dimensional structure of sporulation and conidial shapes are critical to characterize some HST-producing *Alternaria* spp.

PCR based marker techniques have been extensively applied in genotypic identification of phytopathogens at the species and subspecies level. Random amplified polymorphic DNA (RAPD) method has been used for analyzing phylogenic and taxonomical relationships of *Alternaria* spp. (Kim et al., 1998; Robert et al., 2000; Morris et al., 2000).

Primers named as universal rice primer (URP) were

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developed from repetitive sequences derived from the rice genome and universally have been used in PCR based genomic DNA fingerprinting of various organisms including plants, animals, and microorganisms (Kang et al., 2002). URP produced reproducible PCR polymorphisms under a highly stringent PCR condition. In addition, it was demonstrated that the URP-PCR technique is a useful tool for phylogenic analysis of fungi at intra- and inter-species level (Kang et al., 1998; Kang et al., 2001; Kim et al., 2002; Seo et al., 2002). This study analyzed the phylogenetic relationship among HST-producing *Alternaria* spp. using PCR polymorphic bands amplified by URPs.

Materials and Methods

Cultural conditions and fungal strains. All *Alternaria* isolates used are listed in Table 1. Japanese isolates were obtained from Dr. Kohmoto of the Tottori University in Japan, while Korean isolates were purified from lesions of diseased host plants. Pathogenicity of each isolate was confirmed by inoculating them on host plants. On the other hand, non-pathogenic *A. alternata*, *A. brassicicola* isolates from Chinese cabbage, and *A. solani* were included as outgroups for analysis. The *Alternaria* spp. were grown on potato-dextrose agar PDA (Difco Laboratories, Detroit) and preserved as spore suspension in 15% glycerol at -80°C for future use.

DNA extraction. *Alternaria* spp. were grown on PDA media for 7 days at 25°C. Small amount of hypha were taken from fungal colonies on the media and were grown on PD broth by shaking at 25°C for 10 days. The mycelia were harvested by vacuum filtration through Watman paper (No. 2) and lyophilized by freeze dryer. The dried mycelia were ground to a fine powder by toothpick and transferred to a 1.5 ml microfuge tube containing 400 µl of extraction buffer (200 mM Tris-HCl of pH 8.0, 200 mM NaCl, 25mM EDTA, 0.5% SDS) containing 5 µl proteinase K (Promega, 10 mg/ml). After incubation at 37°C for 1 hour, the mixture was extracted with chloroform: isoamylalcohol (24:1, vol/vol) and centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant was transferred to a new tube and 0.6 volume of isopropanol was added to pellet genomic DNA. Precipitated DNA was washed in 70% ethanol and dissolved in TE buffer.

PCR amplification. URPs used in this study are listed inTable 2. Each single URP was used for each PCR reaction, but a high stringent temperature was employed in the annealing step to give high PCR reproducibility. PCR reaction was performed in a 50 µl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each of dNTP, 200 ng primer, 2.5 unit of *Taq* polymerase (Promega), and 50 ng of genomic DNA as template. PCR amplification was carried out in a PTC-100TM (MJ Research, Inc) using the following conditions: one cycle of 4 minutes at 94°C; 35 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C; and one cycle of a final extension for 7 minutes at 72°C. The amplified products were resolved by an electrophoresis on a 1.5% agarose gel in TAE buffer and visualized by staining with ethidium bromide.

Table 1. Alternaria spp. used in this study

Species	Isolates	Host	Soruces (local/country)
A. kikuchiana	K-1	Pear	Koyama/Japan
	K-3	Pear	Koyama/Japan
	K-4	Pear	Koyama/Japan
	AK-11	Pear	Yesan/Korea
	AK-21	Pear	Yesan/Korea
	AK-42	Pear	Yesan/Korea
	No. 15A	Pear	Unknown/Japan
	O-274	Pear	Akasaki/Japan
	O-275	Pear	Akasaki/Japan
A. mali	IFO-8984	Apple	Unknown/Japan
	AM-17	Apple	Taejon/Korea
	AM-28	Apple	Taejon/Korea
	M-62	Apple	Toyama/Japan
	M-69	Apple	Toyama/Japan
	M-87	Apple	Nagano/Japan
	O-154	Apple	Nagano/Japan
A. longipes	AT-16-1	Tobacco	Boeun/Korea
	O-204	Tobacco	Koyama/Japan
	O-205	Tobacco	Koyama/Japan
	O-206	Tobacco	Koyama/Japan
A. alternata. f.sp., lycopersici	As-27	Tomato	Tottori/Japan
	O-227	Tomato	Nagoya/Japan
A. citri	AC-320	Rough lemon	Nagoya/Japan
	AC-325	Rough lemon	nagoya/Japan
A. alternata	EGS35-193	Unknown	SCC
	IMI-147909	Unknown	IMI
A. brassicicola	O-264	Chinese cabage	Unknown/Japan
	BC-1	Chinese cabage	Taejon/Korea
	BC-2	Chinese cabage	Taejon/Korea
A. solani		Potato	Unknown/Korea

IFO: Institute for Fermentation, Osaka, Japan; IMI: International Mycological Institute, Surrey, UK SCC: SCC: Simmons Culture Collection, USA.

Data analysis. URP-PCR polymorphic bands were scored on their presence (value = 1) or absence (value = 0). The similarity coefficient was calculated by rearranging the scored bands of each isolate. On the basis of the similarity coefficient, a dendrogram was constructed with the statistical program NTSYSpc (Rohlf, 2000) using the unweighted pair-group method with arithmetic mean (UPGMA).

Results and Discussion

URP-PCR polymorphism. This study aimed at providing a novel method focused on URP-PCR assay for genetic relatedness of *Alternaria* fungi producing HST dependent on host plants.Twenty five isolates of six different *Alternaria*

Table 2. Oligonucleotide characteristics of 12 URP primers

Primers	Sequences (5'-3')	GC content (%)	*PCR bands
URP1F	ATCCAAGGTCCGAGACAACC	50	15
URP2F	GTGTGCGATCAGTTGCTGGG	50	12
URP2R	CCCAGCAACTGATCGCACAC	50	14
URP4R	AGGACTCGATAACAGGCTCC	50	13
URP8R	GCTAGGTTGCCGAAACACGG	60	9
URP9F	ATGTGTGCGATCAGTTGCTG	50	12
URP13R	TACATCGCAAGTGACACAGG	50	7
URP17R	AATGTGGGCAAGCTGGTGGT	55	No amplification
URP25F	GATGTGTTCTTGGAGCCTGT	50	No amplification
URP30F	GGACAAGAAGAGGATGTGGA	50	12
URP32F	TGCACGTCTCGATCTACAGG	50	No amplification
URP38F	AAGAGGCATTCTACCACCAC	50	No amplification

*PCR bands indicate average numbers of total PCR bands amplified by each URP primer on 31 Alternaria isolates tested in this.

species producing HSTs of different isolates and two non-pathogenic *A. alternata* isolates from various geographical regions were used as collections of small-spored *Alternaria* species. Medium-spored *A. brassicicola* isolates and large-spored *A. solani* isolates were used as control species for comparing genomic PCR patterns against small-spored *Alternaria* spp. Twenty URPs were used to reveal PCR polymorphism on *Alternaria* species. On the average, seven primers, URP1F, URP2F, URP2R, URP4R, URP8R, URP9F, and URP30F, amplified 12 distinct PCR polymorphic bands ranging in size from 150 to 5,000 bp. The representative PCR profiles produced by primers URP8F, URP1F, and URP9F are shown in Fig. 1. Primer URP8R amplified unique bands of 2,000 bp on *A. gaisen* isolates, except for O-274, 15A, and O-275 isolates (Fig. 1A). PCR polymorphisms shared among *A. alternata* f. sp. *lycopersici*, *A. mali*, *A. citri*, and non-pathogenic *A. alternata* isolates, showed closely genetic background among them. Nevertheless, all isolates of *A. longipes* produced a characteristic PCR band of around 3,000 bp that is distinguishable from the other isolates of *Alternaria* species tested. On the other hand, *A. brassicicola* and *A. solani* isolates showed unique PCR profiles that are not shared by small-spored *Alternaria* spp. producing toxins and non-pathogenic *A. alternata*.

Fig. 1B shows PCR profile generated by URP1F primer. Basically, the polymorphisms of the PCR profile were roughly similar to that by primer URP8R. The URP8R-PCR bands with 100 bp, 1.800 bp, and 2.500 bp were uniquely observed on *A. gaisen* isolates, whereas, an intensive band of 1.700 bp was detected on HST-producing *Alternaria* and non-pathogenic *A. alternata* isolates but not on *A. gaisen* isolates. This suggests that the PCR profile of *A. gaisen* produced by primer URP1F can be differentiated from isolates of other *Alternaria* spp. However, *A. mali*, *A. citri*, *A. alternata* f. sp. *lycopersici*, and non-pathogenic *A. alternata* did not show PCR polymorphisms specific to produce different HSTs. PCR using primer URP9F yielded polymorphic bands (around 2,000 bps) from *A. gaisen* isolates, which clearly differentiate them from other *Alternaria* spp. tested (Fig. 1C). In repeated experiments using additional 12 isolates of *A. gaisen* from Japan and Korea, it was confirmed that URP9F-PCR amplification patterns of *A. gaisen* isolates were reliable and reproducible (data not shown). In contrast, URP9F-PCR bands of *A. mali* isolates partially shared with those of isolates of *A. alternata* f. sp. *lycopersici*, *A. citri*, *A. longipes*, and non-pathogenic *A. alternata*, although an intense band with approximately 2,500 bp was observed only on non-pathogenic *A. alternata* isolates. From the results, it was concluded that URP-PCR profiles could be effectively used as a DNA standard index for differentiating genotypes of *A. gaisen* producing AK-toxin and *A. longipes* producing AT-toxin from other *Alternaria* species producing HSTs and non-pathogenic *A. alternata* isolates. Genomic fingerprinting using random amplified polymorphic DNA (RAPD) was performed to analyze genetic diversity of *Alternaria* spp. isolates that cause brown spot of the host plants, citrus species (Jasalavich et al., 1995; Peever et al., 2000; Weir et al., 1998).

PCR method requires little biological materials and provides a rapid method for screening large sample sizes. Accordingly, PCR fingerprinting techniques have been extensively applied for assessing genetic diversity of diverse genomes. RAPD that uses short arbitrary primers consisting of 10 oligonucleotides was developed as a versatile method that produces DNA polymorphism of diverse genomes (Williams et al., 1990), but the method has been recognized to be problematic in PCR reproducibility because of unstable PCR polymorphism caused by PCR conditions such as high numbers of PCR cycles and low annealing

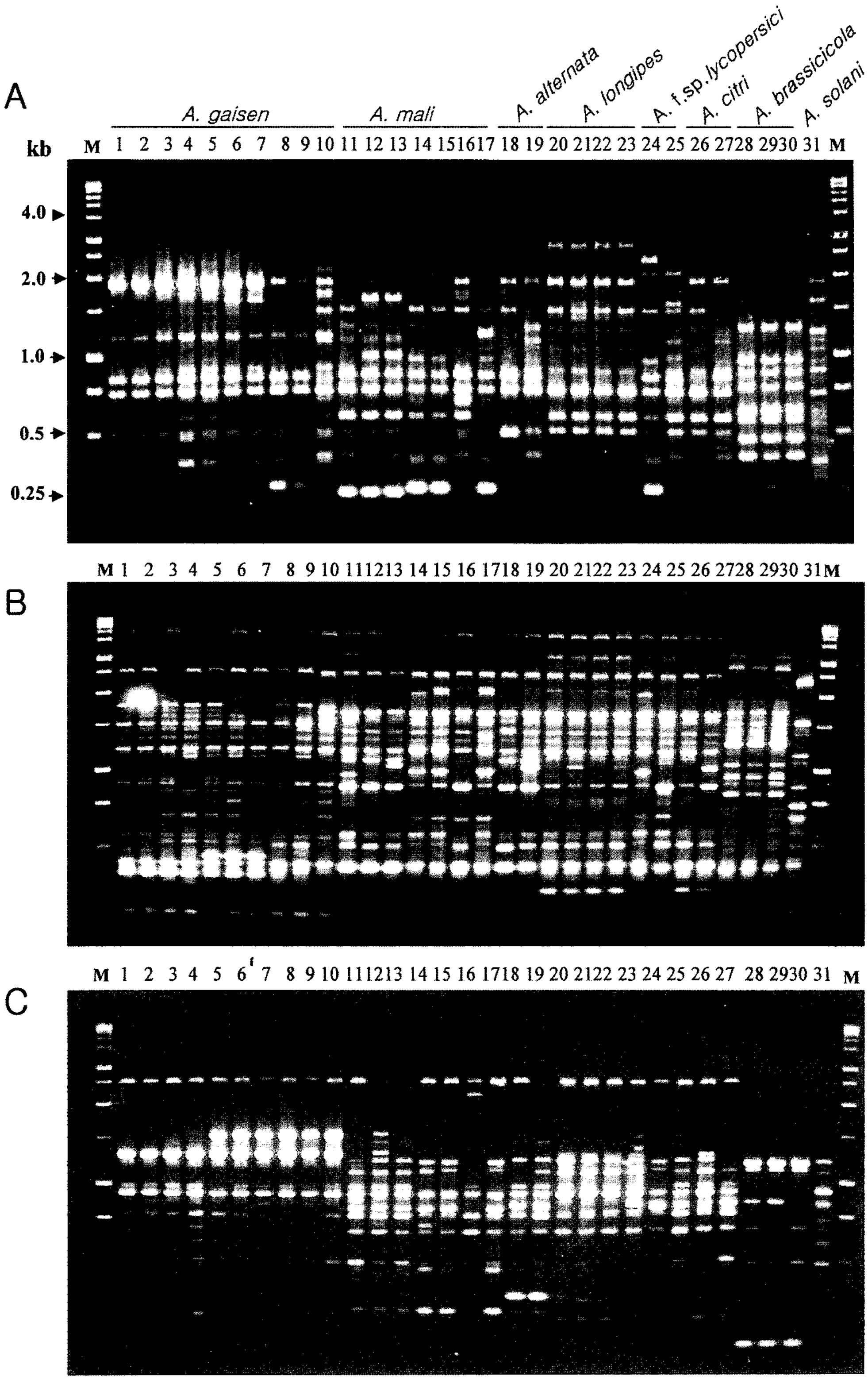

Fig. 1. PCR amplification of HSTs producing *Alternaria* spp. by URP8R (**A**), URP 1F (**B**), and URP9F (**C**) primers. Lane M: 1-kb ladder; Lanes 1-10: *A. gaisen* isolates K-2 K-5 K-3 K-4 AK-11 AK-21 AK-42 O-274 No. 15A; Lanes 11-17: *A. mali* isolates O-275 IFO 8984 AM17 AM28 M62 M69 M87 O-154; Lanes 18-19: non-pathogenic *A. alternata* isolates EGS35-193 CMI-147909; Lanes 20-23: *A. longipes* AT-1b-1,O-204, O-205, O-206; Lanes 24-25: *A. alternata.* f.sp.. *lycopersici* isolates AS-27 O-227; Lanes 26-27: *A. citri* isolates AC320. AC325: Lanes 28-30: *A. brassicicola* isolates O-264 BC-1 BC-2: Lane 31*: A. solani*

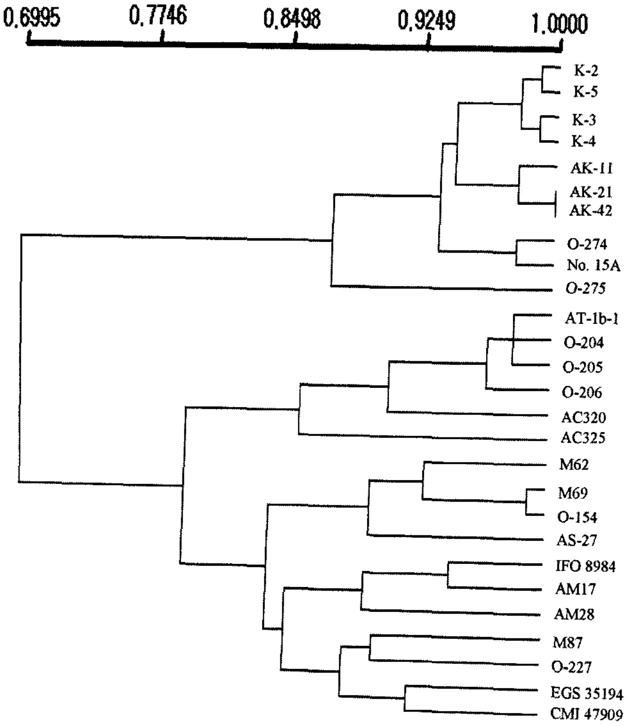

Fig. 2. UPGMA dendrogram of *Alternaria* spp. producing HSTs using PCR polymorphisms obtained by URP-PCR amplification.

temperature. On the contrary, URP-PCR technique uses long primers of 20 mer, designed to detect polymorphisms from organisms including animal, plant, and microbial species at a relatively high annealing temperature (Kang et al., 2002). Generally, long primer and high annealing temperatures improve the specificity between primers and template DNA (Caetano-Anolles et al., 1992; Wu et al., 1991). Thus, URP-PCR condition that started at high annealing temperature may be expected to increase the PCR reproducibility.

Genetic relationship. Genetic similarity index calculated using 94 PCR polymorphic bands amplified by eight URPs was used to assess the genetic relatedness among 27 *Alternaria* isolates including 6 HST-producing *Alternaria* species. Based on the URP-PCR fingerprint data, genetic distance was used to construct a dendrogram for the *Alternaria* isolates. Fig. 3 shows the genetic relationship of the *Alternaria* isolates on the basis of URP-PCR data. *Alternaria* isolates including *Alternaria* spp. producing HSTs and non-pathogenic *A. alternata* were grouped in three distinct clusters on the dendrogram. All *A. gaisen* isolates formed independently a group with similarity levels ranging 85% to 100%. *A. longipes* isolates were grouped in a separate cluster, showing a high genetic similarity level of more than 95%. However, genetic similarity values ranging from 85% to 90% were observed in both *A. longipes* and *A. citri* isolates. The close relatedness of *A. longipes* and *A. citri* was consistent with a previous report on RAPD analysis using *Alternaria* isolates from brown spot lesions of citrus (Peever et al., 1999). Small-spored *Alternaria* isolates from various hosts were classified by morphological observations and genetic diversity of their morphological groups using RAPD was investigated (Roberts et al., 2000). *A. gaisen* and *A. longipes* isolates were grouped according to distinct branches of dendrogram and supporting two phylogenic groups (*A. gaisen* and *A. logipes* groups) in this study. On the other hand, the remaining *Alternaria* isolates including *A. mali*, *A. alternata*, and *A. alternata* f. sp. *lycopersici* were grouped together as a complex cluster without characteristic pattern dependent on species at similarity levels of 84% and 98%.

In previous studies, ribosomal RNA analyses including sequencing and RFLP were applied in assessing genetic diversity among *Alternaria* spp. known to produce different HSTs (Adach et al., 1993; Kusaba and Tsuge, 1994; Tsuge et al., 1989; Go et al., 1997). However, the methods did not discriminate each *Alternaria* fungi producing HSTs or even from non-pathogenic *A. alternata* strains. The phylogenetic analysis based on rDNA-RFLP pattern showed all *Alternaria* fungi producing HSTs and non-pathogenic *A. alternata* isolates clustered into a single genetic group mixed among them (Kusaba and Tsuge, 1995). Thus, the study strongly supported the hypothesis that *Alternaria* fungi producing HSTs should be characterized as intraspecific variants of *A. alternata* (Nishimura et al., 1983). On the contrary, URP-PCR profiles in this study led to the possibility that at least *A. gaisen* and *A. logipes* are genetically different from other *Alternaria* species producing HSTs and from non-pathogenic *A. alternata*. rDNA is relatively conserved in the genomes of most fungal species and has been useful in molecular evolution study of filamentous fungi at the level of genus or interspecies (White et al., 1990). Thus, it is unsound to conclude that *Alternaria* spp. producing HSTs can not be genetically differentiated based solely upon data from DNA sequence and RFLP-based analysis of rDNA region. Moreover, it is reasonable to assume that genomes of HST-producing *Alternaria* spp. require complex gene assemblies that function to produce different toxic compounds. This suggests that URP-PCR polymorphisms on genomic DNA will be effective for differentiating *Alternaria* species producing HSTs or even for non-pathogenic *A. alternata* isolates.

In conclusion, URP-PCR polymorphic bands will be useful as molecular markers in analyzing genetic diversity of *Alternaria* species at the inter- and intra-species levels, especially in distinguishing isolates of *A. gaisen* and *A. longipes* from small-spored *Alternaria* spp. producing HSTs and non-pathogenic *A. alternata*.

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