A New Protocol for the Detection of Sterigmatocystin-producing Aspergillus Section Versicolores Using a High Discrimination Polymerase

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Aspergillus section Versicolores species, except Aspergillus sydowii, produce a carcinogenic mycotoxin sterigmatocystin (STC). Since these fungi are found in varied environmental milieu including indoor dust and food products, our aim was to develop a sensitive and convenient assay to detect STC producing fungal strains. We made use of a high discrimination DNA polymerase (HiDi DNA polymerase), for single nucleotide polymorphism (SNP)-based PCR amplification. Using specific primer pairs based on the SNPs between A. sydowii and other strains of Aspergillus section Versicolores, we succeeded in amplifying the genomic DNA all target strains except A. sydowii. These results confirm that the SNP-based PCR amplification technique, using a high discrimination DNA polymerase, was a reliable and robust screening method for target fungal strains.

Key words: Aspergillus section Versicolores / SNP-based PCR amplification / High discrimination polymerase.

Aspergillus section Versicolores show a wide environmental distribution all over the world (Despot et al., 2016). They have been reported to be environmental contaminants and are detected in various environmental samples including house dust, indoor air, and building materials within damp spaces (Bloom et al., 2009). They produce mycotoxins such as sterigmatocystin (STC), which are transported on very small airborne particles and fungal cells, and therefore, considered to be a potential source of allergens (Belanger et al., 2009; Despot et al., 2016; Piontek et al., 2016). Although several fungal genera, such as Aspergillus flavus, A. parasiticus, A. nidulans, A. aurolatus, Chaetomium spp., Emericella spp., and Penicillium inflatum have been reported to produce STC. Aspergillus section Versicolores strains including A. creber, A. jensenii, A. protuberus, A. versicolor, A. venenatus, A. puulaeensis, and A. tennesseensis are the major STC producers (Jurjevic et al., 2013; Rank et al., 2011). The taxonomy of this section was recently revised, which increased the number of constituent species from 4 to 14 (Jurjevic et al., 2012). In our previous study, we molecularly characterized isolates that have been morphologically associated with Aspergillus section Versicolores obtained from various foods and environmental samples in Japan (Kobayashi et al., 2018). The production of STC by each species in section Versicolores was assessed by thin layer chromatography (TLC) and revealed that 18 isolates in section Versicolores, namely,
A. creber, A. jensenii, A. versicolor sensu stricto, A. venenatus, A. puulaauensis, and A. tennesseensis produced STC, and all 22 A. sydowii isolates did not.

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation among species. The SNP-based PCR amplification technique is widely used as a traditional genotyping method and is based on the principle that PCR amplification by a primer only occurs when its 3’-end is perfectly complementary to the template DNA. Although SNPs can be detected using a PCR primer that corresponds to a specific SNP site on the 3’ terminal nucleotide, only this does not reliably discriminate between SNPs. To solve this problem, SNP specific primers with an additional base pair change within the four bases closest to the SNP site have been proposed (Lefever et al., 2019). However, since the artificial mismatch may negatively affect on the primer–template hybridization, the decision to optimize the position of the mismatch to maximize discrimination power while minimizing the effect on hybridization between primers and samples, is controversial.

Therefore, this study attempted to develop a novel SNP-based PCR amplification method using a newly available DNA polymerase called the HiDi DNA polymerase. This allows us to easily make SNP primers without any additional mismatches, and thus, screen Aspergillus section Versicolorae strains with STC production. Recent biotechnological advances have led to the development of tailor-made DNA polymerases, which were produced by a combination of site-specific mutagenesis and screening techniques (Aschenbrenner, and Marx, 2017; Huber and Marx, 2017). HiDi DNA polymerase is one such engineered polymerase, which efficiently amplifies primers that are completely matched at the 3'-end and discriminates against primers that are mismatched (Drum et al., 2014). In this study, the HiDi DNA polymerase was evaluated for its specificity and tested for screening of STC producing fungal species.

To evaluate the HiDi DNA polymerase for use in a SNP-based PCR assay, the SNPs in RNA polymerase 2 (RPB2) gene among three Aspergillus species were utilized (TABLE 1). First, to design suitable primers, the RPB2 gene information of the selected Aspergillus species (Aspergillus tennesseensis NRRL 229 [Accession No. JN853811], Aspergillus creber NRRL 58587 [Accession No. JN853831], and Aspergillus jensenii NRRL 225 [Accession No. JN853809]) were collected from GenBank, and then the SNPs were identified. A. creber and non-A. creber primers were designed, based on the identified SNPs of the RPB2 gene, for detection of A. creber or non-A. creber strains (A. tennesseensis and A. jensenii) (TABLE 2 and FIG. 1A). The A. creber forward and reverse primers contained ‘A’ and ‘C’ at the 3’-end of the primers, respectively, while ‘G’ and ‘T’ were inserted at the 3’-end of non-A. creber forward and reverse primers, respectively. Next, in order to prepare genomic DNA from A. tennesseensis NIH 6807, A. creber FSSN0002, and A. jensenii NIH 3221 identified in a previous study, these strains were grown on potato dextrose agar (Eiken, Tokyo, Japan) at 25°C (Kobayashi et al., 2018) (TABLE 1). The fungal genomic DNA was isolated from mycelia using NucleoSpin Soil kit with MN Bead Tube Holder, according to the manufacturer’s instructions (Takara, Siga, Japan). Finally, the detection PCR method for Aspergillus species was established. One microliter genomic DNA solution was added to the reaction mixture (25 µL) containing 2.5 µL HiDi buffer, 1.5 µL MgCl2 (25 mM), 0.25 µL HiDi DNA polymerase (myPOLS Biotec, Konstanz, Germany), 2.5 µL dNTPs (2.0 mM), and 1.0 µL (10 µM) of each primer pair. The PCR mixtures were cycled under the following conditions using a C1000 thermal cycler (Bio-Rad, Hercules, CA): initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 10 sec, and 72°C for 45 sec. The PCR products were observed under ultraviolet light after electrophoresis on a 1.5% agarose gel.

As shown in FIG. 1B, the HiDi DNA polymerase with A. creber primers yielded a positive band for genomic DNA from A. creber FNS 0002, but they failed to amplify the genomic DNA of A. tennesseensis NIH 6807 and A. jensenii NIH 3221. Conversely, non-A. creber primers gave an amplification product with genomic DNA of A. tennesseensis and A. jensenii. As expected, A. creber genomic DNA did not give rise to any amplification product with non-A. creber primers. This might be attributed to the ability of HiDi DNA polymerase to discriminate 3’-end mismatches among primers.

Based on the above verification experiment, we decided to use the HiDi DNA polymerase for SNP-based PCR detection of STC-producing species of Aspergillus section Versicolorae. Since in our previous study we reported that Aspergillus section Versicolorae except for A. sydowii were STC-producing species, we wanted to design primers that distinguished these species (Kobayashi et al., 2018). To identify suitable SNPs for PCR primers, DNA sequence of the calmodulin (CaM) gene was used for this experiment (TABLE 1). The CaM gene sequence of the selected Aspergillus species (A. tennesseensis NRRL 229 [Accession No. JN854022], A. creber NRRL 231 [Accession No. JN854024], A. protuberus NRRL 3505 [Accession No. EF652372], A. venenatus NRRL 13147 [Accession No. JN854014], A. sydowii NRRL 5585 [Accession No. JN854039], A. versicolor NRRL 238 [Accession No. EF652354], A. puulaauensis NRRL 58602 [Accession No.
JN854048], A. tabacinus NRRL 4791 [Accession No. EF652390], and A. jensenii NRRL 58984 [Accession No. JN854062]) were downloaded from GenBank. Multiple sequence alignment indicated many SNPs between species, several of which were candidates for primer design. Primer design strategy for specific SNP-based PCR is illustrated in FIG. 2A. Non-A. sydowii specific forward and reverse primers were designed with an ‘A’ at both the 3’-end positions overlapping the SNPs. While, the Versicolores-universal primers were of the same length as the non-A. sydowii specific primers, they were shifted by one base toward the 5’-end (TABLE 2). In addition to A. tennesseensis NIH5807, A. creber FSSN0002, and A. jensenii NIH3221, the mycelia of A. protuberus NIH5550, A. venenatus NIH5808, A. sydowii NIH5817, A. versicolor NIH5400, A. puulauensis NIH5801, A. tabacinus NIH5807, and A. jensenii NIH3253 were prepared (TABLE 1). All strains used have been characterized in our previous study using morphological and molecular techniques (Kobayashi et al., 2018). Genomic DNA purification, PCR, and amplicon check was performed as described above. As shown in FIG. 2B, all Aspergillus section Versicolores samples in yielded 186 bp amplicons with Versicolores-universal primer pairs (lower panel). As expected, all Aspergillus section Versicolores samples except A. sydowii yielded 184 bp bands using non-A. sydowii specific primers (upper panel).

The results from this study strongly suggest that the SNP-based PCR amplification technique, using HiDi DNA polymerase, is useful for sensitive and convenient profiling of fungal species such as Aspergillus section Versicolores. Notably, the primers used for the no amplification, which both had a mismatch at the 3’-end, should have a significant effect on the reliability and robustness of direct nucleotide variation analysis by HiDi DNA polymerase. To distinguish between A. sydowii and other strains of Aspergillus section Versicolores in this

### TABLE 1. Strains and nucleotide data used in this study

| Species               | Strain Accession No. | Strain Accession No. | Strain Accession No. | Strain Accession No. | Strain Accession No. |
|-----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Aspergillus creber    | NRRL 58587           | JN853831             | NRRL 231             | JN854024             | FSSN0002             |
| Aspergillus jensenii  | NRRL 225             | JN853809             | NRRL 58984           | JN854062             | NIH53221             |
| Aspergillus protuberus| —                    | —                    | NRRL 3505            | EF652372             | NIH55550             |
| Aspergillus puulauensis| —                   | —                    | NRRL 58602           | JN854048             | NIH50581             |
| Aspergillus sydowii   | —                    | —                    | NRRL 5585            | JN854039             | NIH57141             |
| Aspergillus tabacinus | —                    | —                    | NRRL 4791            | EF652390             | NIH50587             |
| Aspergillus tennesseensis | NRRL 229           | JN853811             | NRRL 229             | JN854022             | NIH56807             |
| Aspergillus venenatus | —                    | —                    | NRRL 13147           | JN854014             | NIH56808             |
| Aspergillus versicolor| —                    | —                    | NRRL 238             | EF652354             | NIH5400              |

---: Not analyzed in this study.

### TABLE 2. Primers used in this study

| Primer name                        | Sequences                      |
|------------------------------------|--------------------------------|
| Non-A. creber forward              | 5’-ACTGGGTTCTGGTTGTTGCC-3’      |
| Non-A. creber reverse              | 5’-ACTTCGCTACGATTCACT-3’        |
| A. creber forward                  | 5’-ACTGGGTTCTGGTTGTTCCA-3’      |
| A. creber reverse                  | 5’-ACTTCGCTACGATTCACTG-3’       |
| Non-A. sydowii specific forward    | 5’-GATTCCATTGGATCAATT-3’        |
| Non-A. sydowii specific reverse    | 5’-TTGGTATGCTGATCTGACCTGA-3’    |
| Versicolores-universal forward     | 5’-TGATCCATTGGATCAATT-3’        |
| Versicolores-universal reverse     | 5’-CTTGGTATGCTGATCTGACCTGA-3’   |
In this study, we used SNP information from the CaM gene because of the availability of high-quality sequence data. Recently, massive parallel sequencing platforms such as HiSeq and NovaSeq (Illumina, Inc., San Diego, CA) have significantly reduced the cost of high-throughput sequencing, and thus, significantly increased the availability of genomic DNA sequence information (Jagadeesan et al., 2019). Because the concepts of primer design in this study are versatile, suitable primers might be modified and fine-tuned for the specific SNP-based PCR amplification at hand by using newly available genomic DNA sequence data.

Since the species belonging to *Aspergillus* section *Versiclores* are distributed in a broad range of environments, this method has a wide application for analyzing materials commonly contaminated by them. In order to develop a PCR-based screening method using complex and often mixed DNA derived from various biological species, the reduction of co-amplification of similar target DNA from non-fungal sources such as plant materials should be considered. Although, further experiments are required, the amplification by HiDi DNA polymerase needs to fulfill certain conditions. These include perfect matches at 3’-end of the primers, few primer-template mismatches, and an appropriate distance between primers, and therefore, we believe that a negligible number of unexpected amplicons would be produced.

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FIG. 2. Selective amplification of genetic regions of *Aspergillus* section *Versicolores* except *Aspergillus sydowii*. A: Nucleotide polymorphisms for primer-design matched nucleotide sequences of calmodulin (*CaM*) gene. B: Electrophoretic profiles of PCR products by using primers specific for *Aspergillus* section *Versicolores*. Lane 1, *Aspergillus tennesseensis* (NIHS6807); lane 2, *Aspergillus creber* (FSSN0002); lane 3, *Aspergillus protuberus* (NIHS5550); lane 4, *Aspergillus venenatus* (NIHS6808); lane 5, *Aspergillus sydowii* (NIHS7141); lane 6, *Aspergillus versicolor* (NIHS5400); lane 7, *Aspergillus puulaauensis* (NIHS0581); lane 8, *Aspergillus tabacinus* (NIHS0587); lane 9, *Aspergillus jensenii* (NIHS3221); lane 10, *Aspergillus jensenii* (NIHS3253); and lane M, molecular size marker.
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