Distribution of Sterigmatocystin-producing *Aspergilli* in Japan

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Sterigmatocystin is a genotoxic and hepatocarcinogenic mycotoxin that contaminates foods and environments worldwide. Sterigmatocystin is produced as a precursor to aflatoxin B1 or as an end product by certain *Aspergilli*. *Aspergillus* section *Versicolores* is one of the major sections including sterigmatocystin-producing species and is thus a potential health and environmental hazard. Recently, the taxonomy of this section was revised and classified into 14 species on the basis of molecular phylogenetic analysis. However, investigation of the distribution and sterigmatocystin production of each species has been limited; in particular, its distribution in foods has been scarcely reported. In this study, we collected isolates of *Aspergillus* section *Versicolores* from various foods and environments in Japan and investigated their distribution and sterigmatocystin production. The isolates were classified into nine species or species groups, which revealed that *A. creber*, *A. puulaauensis/tennesseensis* and *A. sydowii* are the main species/species groups in Japan. In addition, *A. versicolor sensu stricto* was detected with some frequency, specifically in foods. Furthermore, the two species *A. creber* and *A. versicolor sensu stricto* frequently produced sterigmatocystin. It is therefore important for food safety to intensively monitor these two species and distinguish them from other species, especially *A. sydowii*, which is not considered to produce sterigmatocystin.

Key words: sterigmatocystin, *Aspergillus* section *Versicolores*, fungi, distribution

1. Introduction

Sterigmatocystin (STC) is a toxic polyketide that is a precursor to aflatoxin B1 (AFB1), which is one of the most carcinogenic toxins in nature^{1,2)}. The structure of STC is closely related to AFB1, but the lethal potency of STC is about 1/10 that of AFB1³⁾, and the hepatocarcinogenicity is

one or two orders of magnitude less than AFB1 as assessed in rats⁴⁾. STC has been categorized as a 2B carcinogen by the International Agency for Research on Cancer⁵⁾. Furthermore, genotoxicity and carcinogenicity data were recently compiled by the European Food Safety Agency (EFSA)⁶⁾ demonstrating that STC induces chromosomal damage both *in vitro* and *in vivo* and tumorigenicity was observed after

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Abbreviations: AFB1: aflatoxin B1, EFSA: European Food Safety Agency, STC: sterigmatocystin, TLC: thin-layer chromatography

administration of STC in several animals.

STC contamination has been found in wheat, maize, barley, coffee beans, pistachio nuts and animal feed^{2,7,8}. STC has also been detected in various environmental samples such as carpet dust, dwelling materials within damp buildings and indoor air^{9,10}. Although STC is produced as a precursor to AFB1 in some *Aspergillus* species such as *A. flavus* and *A. parasiticus*, it is the end product in several species, such as *A. nidulans*, *A. versicolor*, *A. aurolatus*, *Chaetomium* spp., *Emericella* spp., and *Penicillium inflatum*^{6,11}.

Aspergillus section *Versicolores* includes the major STC producers, which have been reported to be potential environmental and health hazards^{11–13}. The taxonomy of this section was recently revised, which increased the number of species from four to 14¹⁴. The production of STC by each species in section *Versicolores* except for *A. sydowii* has been tested on two liquid media¹⁵ and revealed that 11 produce STC, namely *A. amoenus*, *A. creber*, *A. cvjetkovicii*, *A. fructus*, *A. jensenii*, *A. protuberus*, *A. puulaauensis*, *A. subversicolor*, *A. tennesseensis*, *A. versicolor* and *A. venenatus*. Two species (*A. austroafricanus* and *A. tabacinus*) were found not to produce STC. Sterigmatocystin production of *A. sydowii* was not tested because this species is generally regarded as a non-STC producer¹⁶. However, the number of strains tested was limited; in particular, only one strain of *A. austroafricanus* and two strains of *A. tabacinus* were tested, and no STC production was reported. Thus, it is not certain whether these two species are indeed non-STC producers. Furthermore, most of the investigated strains were isolated from environmental or clinical samples, although many strains of *Aspergillus* section *Versicolores* were isolated from foods and feeds before the taxonomy was revised^{14,15}.

In this study, we performed molecular characterization of the isolates that had been morphologically associated with *Aspergillus* section *Versicolores* from various foods and environments in Japan, and also tested them for STC production.

2. Materials and Methods

2-1. Strains

The 60 strains used in this study are detailed in **Table 1**. Thirty strains were isolated from foods, and 30 were isolated from various environmental sources in regions of Japan such as Tokyo, Miyagi and Kumamoto prefectures. All strains were stored on potato dextrose agar (Eiken, Tokyo, Japan) slant medium at 25°C.

2-2. DNA Extraction and Sequencing

Conidia from slant cultures were inoculated into 1 mL

potato dextrose broth medium (Difco Laboratories, Franklin Lakes, NJ, USA) in a microfuge tube and incubated at 25°C for 3 days. The mycelia were clumped by centrifugation at $18,000 \times g$ for 10 min. Genomic DNA from each strain was extracted using either the sodium dodecyl sulfate method¹⁷ or a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Partial sequences of the β -tubulin gene (*tub2*) were selected as the region for molecular phylogenetic analysis. For amplification and sequencing, primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTA GTGACCCTTGGC-3') were used as described¹⁸. Amplification reactions were carried out in a thermal cycler (LifeECO; Bioer Technology, Hangzhou, China) with *Ex Taq* (TaKaRa Bio, Shiga, Japan). The PCR program for the amplification of *tub2* consisted of an initial denaturing step at 95°C for 3 min, 35 amplification cycles (95°C for 30 sec, 60°C for 45 sec, 72°C for 30 sec) and an additional extending step at 72°C for 2 min. The amplified fragments were sequenced using an ABI 3700 Analyzer and the BigDye Terminator Sequencing reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2-3. Identification of Species

Isolate identities were confirmed or refuted based on the *tub2* genomic region. Twenty-six sequence datasets of *Aspergillus* section *Versicolores* were downloaded from GenBank and used as reference data for our phylogenetic inference. *A. asperescens* and *A. multicolor* were used as out-group taxa, and their *tub2* sequences were also downloaded from GenBank. The sequences were automatically aligned using MEGA6¹⁹, and the alignments were manually adjusted. A phylogenetic tree was constructed with the neighbor-joining method by the maximum composite likelihood model using MEGA6. The stability of clades was assessed with 1,000 bootstrap replications.

2-4. STC Analysis by Thin-layer Chromatography (TLC)

One-dimensional TLC was carried out as previously described²⁰ with some modifications. In brief, isolates were cultured on potato dextrose agar at 25°C for 14 days. Using a 1 mL pipette tip, agar plugs were removed from cultures and placed into 1.5 mL sample tubes. The plugs were treated with methanol-chloroform [1:2 (vol/vol)], and an aliquot of each extract was directly transferred onto a TLC plate (Silica gel 60, Merck, Darmstadt, Germany) that had been previously activated for 15 min at 120°C. The plates were developed in methanol-chloroform [2:98 (vol/vol)] and dried. Metabolites were visualized under UV light at 366 nm and compared to a STC standard (Major Chemicals Ltd, Rehovot, Israel). Me-

Table 1. *Aspergillus* strains used in this study

Species/Species group	Strain	Origin	STC production ^a	Accession No. ^b
<i>A. amoenus</i>	NIHS6481	House dust	–	LC367586
<i>A. creber</i>	FSSN0002	Canned Truffle	–	LC367551
	NIHS0585	Hair of cattle	–	LC367554
	NIHS0586	House dust	+	LC367555
	NIHS1302	Grape	+	LC367559
	NIHS2991	Indoor air	+	LC367561
	NIHS3057	Indoor air	+	LC367562
	NIHS4459	Environment	+	LC367571
	NIHS6467	House dust	+	LC367579
	NIHS6718	Dust of bed	+	LC367592
	NIHS6806	Cocoa powder	–	LC367599
	Tokyo-AV-54	Olive oil	+	LC367605
	Tokyo-AV-63	Barley tea	–	LC367610
<i>A. jensenii</i>	NIHS3160	Indoor air	+	LC367563
	NIHS3253	Indoor wall	–	LC367565
	NIHS6501	House dust	–	LC367591
	Tokyo-AV-19	Pilled millet	+	LC367604
<i>A. protuberus</i>	NIHS6474	House dust	–	LC367582
	NIHS6482	House dust	–	LC367587
<i>A. puulaquensis/A. tennesseensis</i>	NIHS0581	Vinyl-coated cloth	–	LC367553
	NIHS6259	Polished rice	–	LC367572
	NIHS6262	Polished rice	+	LC367574
	NIHS6270	Polished rice	+	LC367577
	NIHS6466	House dust	–	LC367578
	NIHS6480	House dust	–	LC367585
	NIHS6805	Lemon juice	–	LC367597
	NIHS6807	Cocoa powder	–	LC367598
	Tokyo-AV-15	Soy beans	+	LC367602
	Tokyo-AV-16	Jasmine tea leaf	+	LC367603
	Tokyo-AV-55	Turmeric	–	LC367606

Table 1. *Aspergillus* strains used in this study (continued)

Species/Species group	Strain	Origin	STC production ^a	Accession No. ^b
<i>A. sydowii</i>	NIHS0733	Fennel	–	LC367557
	NIHS1013	Agar weed	–	LC367558
	NIHS3261	Outdoor air	–	LC367566
	NIHS3404	Outdoor air	–	LC367567
	NIHS3941	Indoor air	–	LC367568
	NIHS4021	Indoor air	–	LC367569
	NIHS4095	Indoor wall	–	LC367570
	NIHS6261	Polished rice	–	LC367573
	NIHS6265	Polished rice	–	LC367576
	NIHS6469	House dust	–	LC367580
	NIHS6472	House dust	–	LC367581
	NIHS6478	House dust	–	LC367583
	NIHS6479	House dust	–	LC367584
	NIHS6497	House dust	–	LC367588
	NIHS6498	House dust	–	LC367589
	NIHS6499	House dust	–	LC367590
	NIHS6801	Peanuts	–	LC367593
	NIHS6802	Peanuts	–	LC367594
	NIHS6803	Beverage	–	LC367595
	NIHS6804	Coconut oil	–	LC367596
	Tokyo-AV-58	Dried fish	–	LC367608
	Tokyo-AV-60	Roasted green tea	–	LC367609
<i>A. tabacinus</i>	NIHS0587	Cellophane	–	LC367556
<i>A. venenatus</i>	NIHS3237	Indoor wall	–	LC367564
	NIHS6808	Soy beans	+	LC367600
	NS270272	Rice grain	–	LC367601
<i>A. versicolor sensu stricto</i>	NIHS0093	Rice grain	+	LC367552
	NIHS2500	Rice grain	+	LC367560
	NIHS6264	Polished rice	+	LC367575
	Tokyo-AV-57	Noodle	–	LC367607

^a Sterigmatocystin production: + is producing, – is non-producing^b GenBank accession number for partial sequences of β -tubulin genes

tabolites demonstrating similar Rf and color to the standard were regarded as STC.

3. Results

3-1. Identification of Species

To assign isolates into the 14 species in *Aspergillus* section *Versicolores* described by Jurjevic et al.¹⁴⁾, the isolates were subjected to molecular phylogenetic analysis based on *tub2* with the 26 sequences of *Aspergillus* section *Versicolores* strains used in the previous study¹⁴⁾. We obtained the neighbor-joining tree using 88 sequences (Fig. 1). Each reference species formed a monophyletic clade, and seven of the clades were supported with high bootstrap values (BP, >80%). Of the 60 strains isolated in this study, 22 were identified as *A. sydowii* and 12 as *A. creber*. The support values for both clades were very high (>99% BP). We also identified the following strains with high bootstrap support, i.e., >80% BP: four strains of *A. versicolor sensu stricto*, three strains of *A. venenatus*, two strains of *A. protuberus*, one strain of *A. amoenus*, and one strain of *A. tabacinus*. Among the 15 remaining strains, four strains formed a monophyletic clade with *A. jensenii* (69% BP) and 11 formed a monophyletic clade with *A. puulaauensis* and *A. tennesseensis* (66% BP).

3-2. Distribution of Species

Among the 30 strains isolated from food, there were 10 strains of *A. sydowii*, eight in the species group of *A. puulaauensis* and *A. tennesseensis*, five of *A. creber*, four of *A. versicolor sensu stricto*, two of *A. venenatus*, and one of *A. jensenii* (Table 1). Among the 30 strains isolated from the environment, there were 12 strains of *A. sydowii*, seven of *A. creber*, three of *A. jensenii*, three in the species group of *A. puulaauensis* and *A. tennesseensis*, two of *A. protuberus*, one of *A. amoenus*, one of *A. tabacinus* and one of *A. venenatus*.

3-3. STC Production

Of the 60 isolates tested, 18 (30.0%) produced STC as assessed with TLC. Specifically, the following isolates produced STC: 8 of 12 isolates (66.7%) of *A. creber*, 4 of 11 isolates (36.4%) in the species group of *A. puulaauensis* and *A. tennesseensis*, 3 of 4 isolates (75%) of *A. versicolor sensu stricto*, 2 of 4 isolates (50.0%) of *A. jensenii*, and 1 of 3 isolates (33.3%) of *A. venenatus*. The isolates of the other six species did not produce STC. In particular, none of the 22 isolates of *A. sydowii* produced STC.

4. Discussion

Sixty strains, morphologically identified as belonging to

Aspergillus section *Versicolores*, were isolated from various foods and environments in Japan. Using molecular phylogenetic inference, the strains were partitioned into nine species or species groups. Most of the isolates were identified as *A. sydowii* (22 isolates), followed by *A. creber* (12 isolates) and the species group of *A. puulaauensis* and *A. tennesseensis* (11 isolates) (Table 1 and Fig. 1). These three species or species groups made up two-thirds of the isolates, indicating that they likely comprise the majority species of *Aspergillus* section *Versicolores* in Japan. This is the first report of the distribution in Japan of species belonging to *Aspergillus* section *Versicolores*. In previous studies carried out subsequent to the reclassification of *Aspergillus* section *Versicolores*^{13,14)}, *A. creber* was the species most commonly isolated, followed by *A. jensenii* and *A. protuberus*. Although *A. sydowii* cannot be compared with previous results because it was excluded from the analyses, the frequency of *A. creber* in the present study agrees with that reported previously. Our findings, however, differ from previous results in that *A. jensenii* and *A. protuberus* were not frequently isolated in our study. There may have been fewer isolates of *A. jensenii* and *A. protuberus* in our study because different sources were sampled. Although the previous studies mainly used isolates from the environment or clinical specimens, we collected half of the isolates from food. Thus, *A. jensenii* and *A. protuberus* might be abundantly distributed in the environment but rarely in food. On the other hand, *A. creber* was detected in our study as frequently as in previous studies. Although *A. creber* has not previously been isolated from food, we isolated a similar number of *A. creber* strains from food and environmental samples in our study. Thus, it appears that *A. creber* is frequently distributed in both the environment and food. Furthermore, *A. versicolor sensu stricto* was isolated only from foods in both previous studies and our study, indicating that this species is mainly distributed in food. Most of the isolates identified as *A. versicolor sensu lato* from food and feed, prior to reclassification of the species in *Aspergillus* section *Versicolores*, might be *A. versicolor sensu stricto*.

Aspergillus section *Versicolores* is comprised of a major group of STC producers that have been reported to be potential environmental and health hazards^{11,12)}. After reclassification of this section, STC-producing strains were detected in most species of this section. Our finding that none of the 22 *A. sydowii* isolates produced STC supports the belief that this is a non-STC species, but this does not mean that an STC-producing strain of *A. sydowii* does not exist. Therefore, any sampling of *A. sydowii* should be subjected to STC testing. Because *A. versicolor sensu stricto* and *A. creber* frequently produce STC, and because these two species were frequently isolated from food, they are likely to be the main cause of

Fig. 1. Phylogenetic tree based on partial sequences of β -tubulin. The tree was generated by the neighbor-joining method using 1,000 bootstrap replicates. Only the bootstrap values above 60% are indicated at the branches. The tree was rooted with outgroup taxa *A. asperescens* and *A. multicolor*. The strains marked by black circles were isolated from foods; the strains marked by white diamonds were isolated from environmental samples; the sequences of the unmarked strains were downloaded from GenBank.

STC contamination in food and feed in Japan. Therefore, it is important for purposes of food safety to intensively monitor foods for these two particular species. Because we examined STC production only in 2-week cultures at 25°C on potato dextrose agar, STC production of each species from *Aspergillus* section *Versicolores* should be examined under additional conditions. Further understanding of the distribution and STC production by strains of *Aspergillus* section *Versicolores* isolated from additional locations is needed and will lead to improved food safety.

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Conflict of interest

The authors have no conflict of interest.

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