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Short Communication

Analysis of the fungal population involved in Katsuobushi production

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Naturally occurring fungi have been used in the traditional production of dried bonito, Katsuobushi, in Japan. In this study, we analyzed the fungal population present during Katsuobushi production. Amplicon sequence analysis of ITS1 indicated that *Aspergillus* spp. are predominant throughout the production process. In addition, culture-dependent analyzes identified three species *Aspergillus chevalieri*, *Aspergillus montevidensis*, and *Aspergillus sydowii*, based on sequencing of *benA*, *caM*, and *rpb2* genes. *A. chevalieri* isolates were classified into teleomorphic and anamorphic strains based on morphological analysis. *A. chevarieri* was the dominant species throughout the production process, whereas *A. montevidensis* increased and *A. sydowii* decreased in abundance during Katsuobushi production. Our study will enhance the understanding of fungal species involved in traditional Katsuobushi production.

Key Words: *Aspergillus chevalieri*; *Aspergillus montevidensis*; *Aspergillus sydowii*; fungi; Katsuobushi

Katsuobushi is a dried bonito, which is essential for traditional Japanese food. It is sliced as dried bonito flakes and used for "*dashi*," which is a stock. For Katsuobushi production, molding and sun-drying processes are repeated several times. Molding enhances the drying of Katsuobushi. In addition, it plays a significant role in the decomposition of proteins and lipids, as well as the flavor formation of Katsuobushi (Aoki et al., 2013; Dimici and Wada, 1994; Doi and Shuto, 1995; Doi et al., 1989, 1992; Kaminishi et al., 1999; Kunimoto et al., 1996).

The fungal starter *Aspergillus glaucus* (formerly *Eurotium herbariorum*) is available from the Japan Katsuobushi Association (Tokyo, Japan) for molding (Miyake et al., 2009); however, traditional Katsuobushi production is performed using naturally occurring fungi in a room for molding. Prior studies reported that species from the *A. glaucus* group such as *Aspergillus ruber* and *Aspergillus pseudoglaucus* (formerly *Aspergillus repens*) were dominant in Katsuobushi based on the morphological characteristics of the fungi (Nakazawa et al., 1934; Yoshikawa and Kosugi, 1937). However, the recent classification method based on DNA sequence (Chen et al., 2017; Samson et al., 2014) has not been applied for the classification of the fungal population in Katsuobushi. In this study, we characterized the fugal population in Katsuobushi production using DNA sequencing.

The Katsuobushi samples with different molding steps were prepared at a Katsuobushi manufacturing factory (Makurazaki, Kagoshima, Japan) using the same method used to make the commercial product. Briefly, the head and viscera of bonito, *Katsuwonus pelamis*, were removed and fileted, then further separated into the back and stomach sides. Thus, Katsuobushi can be distinguished into "*obushi*" and "*mebushi*," which are made from back side and stomach side of bonito, respectively. Then, the filets were dipped in hot water at 90–95°C for 60–90 min. After cooling down, bones and a part of the skin were removed. Then, the filets were repeatedly dried in a room with burned firewood from 10 to 15 times. The surface layer was removed and preserved in the room for molding for

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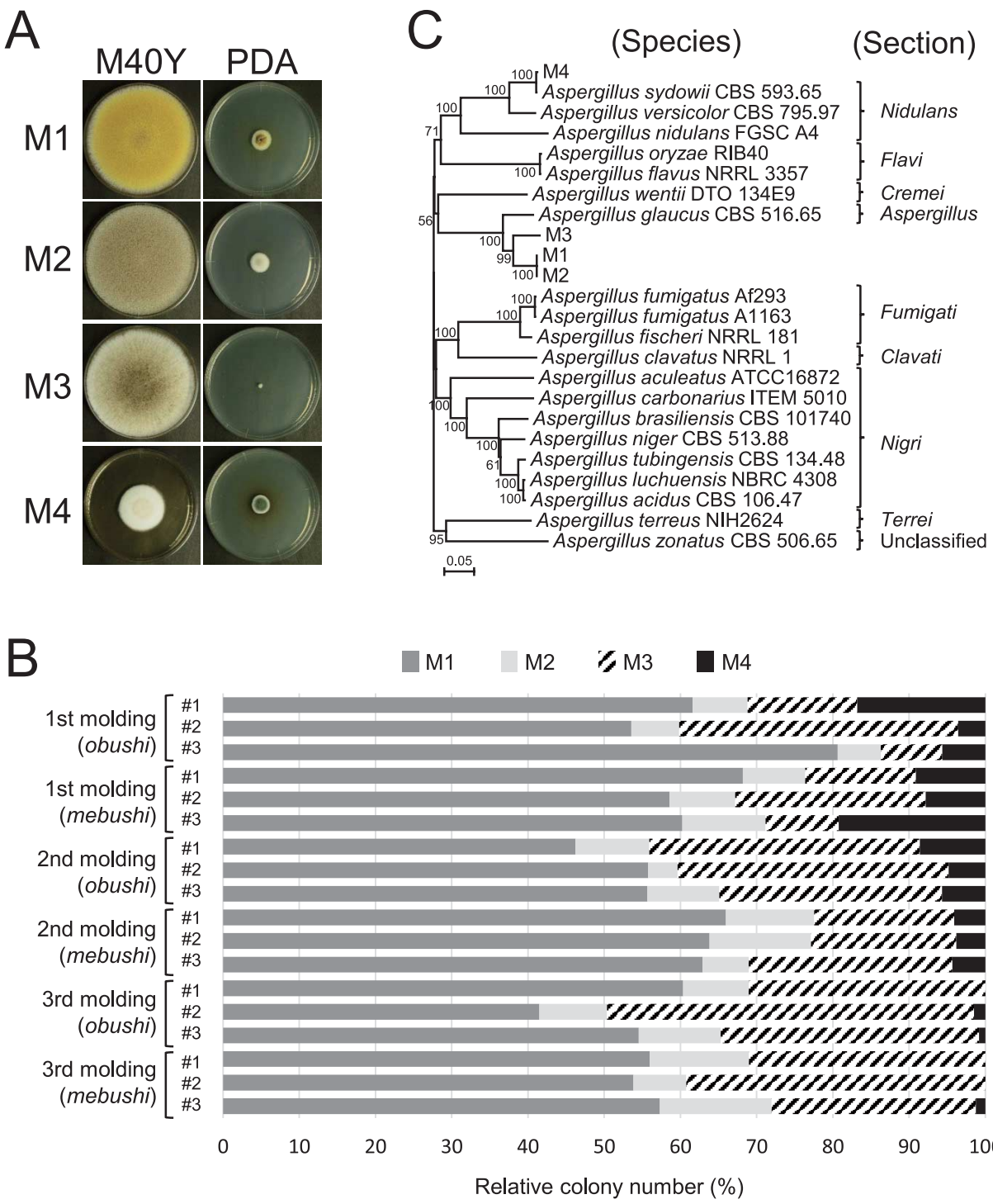

Fig. 1. Fungi isolated from Katsuobushi.

A. Colony formation of strains M1, M2, M3, and M4 isolated from Katsuobushi. Spores of each strain were inoculated onto M40Y agar or PDA medium and incubated at 30°C for 7 days. B. Transition of fungal community structure of Katsuobushi at the first, second, and third steps of molding process. C. Phylogenetic analysis of *benA*, *caM*, and *rpb2* showing the phylogenetic positions of strains M1, M2, M3, and M4. The locus tags of genes are shown in Table S2. The tree was constructed by a neighbor-joining method based on an alignment of concatenated *benA*, *caM*, and *rpb2* sequences with complete gap deletion on MEGA version 6.0 (Tamura et al., 2013). Bootstrap values (1000 replicates) are indicated at the branches. The low bootstrap values (<50) were removed. All the other analytical options were set to default.

2–3 weeks and then sun-dried. The molding and sun-drying steps were repeated up to three times. Each sample was obtained after each sun-drying step, yielding the first, second, and third steps of *obushi* or *mebushi* samples.

To investigate the population of fungi in Katsuobushi, we performed sequence analysis of the ITS1 (Internal Transcribed Spacer 1) region amplified from DNA isolated from Katsuobushi. Approximately 5 mm of the surface layer of *obushi* or *mebushi* samples were scraped with a chisel and treated with bead beating at 4.5 m/s for 30 s

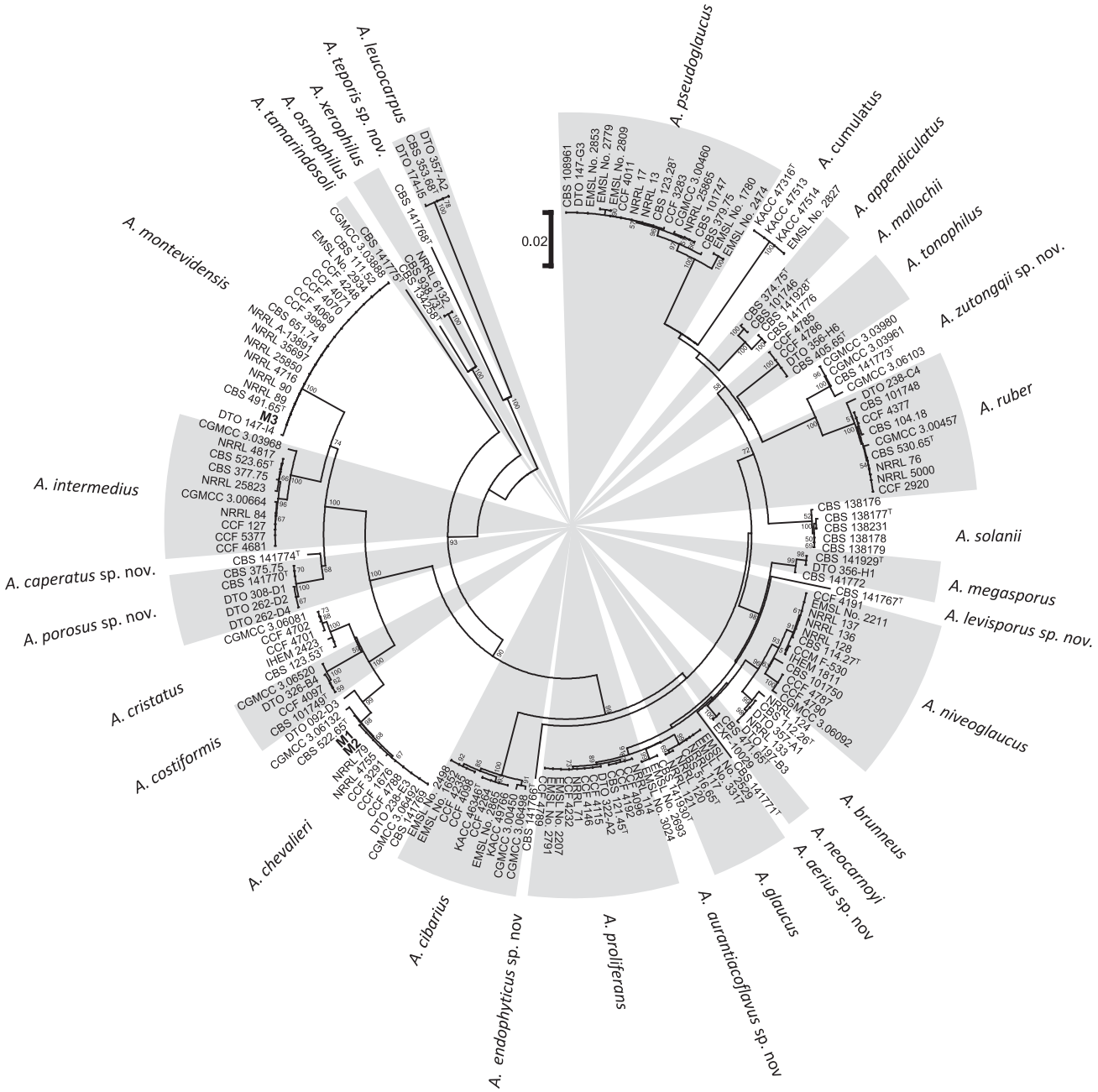

Fig. 2. Phylogenetic analysis of *benA*, *caM*, and *rpb2* showing the phylogenetic position of strain M1, M2, and M3 isolated from Katsuobushi. The gene accession numbers are shown in Table S3. The tree was constructed by a neighbor-joining method based on an alignment of concatenated *benA*, *caM*, and *rpb2* sequences with complete gap deletion on MEGA version 6.0 (Tamura et al., 2013). Bootstrap values (1,000 replicates) are indicated at the branches. The low bootstrap values (<50) were removed. The positions of strains M1, M2, and M3 are indicated by bold type. All the other analytical options were set to default.

using a FastPrep 120 Cell Disrupter System (Thermo Savant; Carlsbad, CA) and glass beads BZ-4 (0.35–0.5 mm; AS ONE Corporation, Osaka, Japan), then subjected to DNA extraction. DNA was extracted from 70 mg of each sample using the ZR fecal DNA MiniPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Sequencing analysis of ITS1 amplified using the ITS1F_KYO1 and ITS2_KYO2 primer set (Toju et al., 2012) was performed using MiSeq (Illumina, San Diego, CA) at the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). The data were deposited to the DNA Data Bank of Japan (DDBJ) under the accession numbers DRR186742-DRR186744. The sequence reads were subjected to quality filtering using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and low quality reads with quality values of less than 20 or with lengths of 40 bases were removed after trimming the primer sequences. Then, the pair-end reads were generated and subjected to chimera check using UCHIME (Edgar et al., 2011) with a 97% representative operational taxonomic units (OTUs) set of the UNITE database (Kõljalg et al., 2013). After the quality filtering and chimera check of sequencing reads, the OTUs were predicted using a Quantitative Insight Into Microbial Ecology (QIIME) (Caporaso et al., 2010) without using any external set of reference sequences. The QIIME analysis was performed with default settings. The

result indicated that approximately 99.9% of ITS1 amplicons were classified into genus *Aspergillus* at the first, second, and third molding steps (Table S1). This result was inconsistent with the previous report that *Penicillium* spp. were also present with *Aspergillus* spp. during Katsuobushi production (Yoshikawa and Kosugi, 1937). Conceivable explanations for this difference could be: (i) taxonomic identification difficulties by morphology (e.g., misidentification in the previous study) and (ii) the fungal populations might depend on Katsuobushi samples (e.g., factory-dependent fungal population).

A culture-dependent approach was used to perform species-level identification. Approximately 5 mm of the surface layer of *obushi* and *mebushi* samples were scraped with a chisel and dissolved in sterilized water. Then, the suspension solutions were spread on Potato Dextrose Agar (PDA) (BD Difco, Franklin Lakes, NJ); single colonies were transferred to new media and classified based on colony morphology with a focus on size, shape, and color (spore color and bottom color of petri dish). Approximately 100 colonies (from 82 to 156 colony for each sample, 2110 total colonies) were morphologically classified from each obushi and *mebushi* sample, and four different types of colonies, namely strains M1, M2, M3, and M4, were obtained (Fig. 1A). Strains M1, M2, M3, and M4 showed better growth in M40Y medium (40% [wt/vol] sucrose, 2% [wt/vol] malt extract, 0.5% [wt/vol] yeast extract, 2% [wt/vol] agar) (DSMZ-Medium 187), indicating that they are osmophilic fungi. There is no significant difference in the relative abundance of strains M1, M2, M3, or M4 between *obushi* and *mebushi* samples. However, the relative colony numbers of strain M1 and M2 are stable at the first, second, and third steps of molding, whereas those of strains M3 and M4 tend to increase and decrease over the molding step, respectively (Fig. 1B).

For the identification of strains M1, M2, M3, and M4, the ITS1-5.8S rDNA-ITS2 region was amplified by colony PCR analysis using a primer set of ITS1 and ITS4 (White et al., 1990), and subsequent sequencing analysis indicated that all strains were classified into genus *Aspergillus* (data not shown). This was consistent with the result that nearly all sequence reads of the ITS1 amplicon sequence analysis were classified into genus *Aspergillus* (Table S1). The *benA*, *caM*, and *rpb2* genes, which encode β-tubulin, calmodulin, and the second largest subunit of RNA polymerase II, respectively, were then sequenced to further identify strains M1, M2, M3, and M4. The *benA*, *caM*, and *rpb2* genes have been used for species-level identification of *Aspergillus* (Chen et al., 2017; Samson et al., 2014). Colony PCR analysis was performed using the following primer sets: Bt_2a and Bt_2b for *benA* (Glass and Donaldson, 1995); CMD5 and CMD6 for *caM* (Hong et al., 2005); 5F and 7CR for *rpb2* (Liu et al., 1999). The results of sequence analysis were deposited to the DDBJ under the accession numbers LC494250-LC494265.

Phylogenetic analysis was performed using the neighbor-joining method and *benA*, *caM*, and *rpb2* sequences retrieved from the *Aspergillus* genome database (http://www.aspgd.org/) (Fig. 1C). Results indicated that *benA*, *caM*, and *rpb2* of strain M1, M2, and M3 are similar to those from section *Aspergillus*, whereas strain M4 is similar to *Aspergillus sydowii*. The topology was also supported by the maximum likelihood method (Fig. S1). Nucleotide BLAST analysis of *benA*, *caM*, and *rpb2* of strain M4 also showed best hits to those of *A. sydowii* (data not shown), indicating that strain M4 is classified into *A. sydowii*. Because comprehensive DNA sequence information of *benA*, *caM*, and *rpb2* were available for the section *Aspergillus* (Chen et al., 2017), we performed phylogenetic analysis with those from strains M1, M2, and M3 (Fig. 2). The phylogenetic analysis indicated that those of strains M1 and M2 are identical to *Aspergillus chevalieri*, while that of strain M3 are identical to *Aspergillus montevidensis* (formerly *Aspergillus amstelodami*).

A. chevalieri, *A. montevidensis*, and *A. sydowii* were previously identified from Katsuobushi (Hiyama et al., 1995; Nakazawa et al., 1934; Yoshikawa and Kosugi, 1937). Among prior studies on fermented food, *A. chevalieri* was isolated from "Bagoong," a traditional fermented fish prepared with salted anchovy from Luzon Island in the Philippines (Murao et al., 1982); *A. chevalieri* and *A. montevidensis* are isolated from meju, a brick of dried fermented soybeans in Korea (Hong et al., 2011); *A. sydowii* and *A. montevidensis* were isolated from "Jinhua Huotui," a traditional Chinese ham fermented through molding (Wagu et al., 1996). The fungal isolates from fermentation foods have been characterized from the view point of degradation of proteins and lipids, and flavor formation. For example, there is a study comparing the lipases of *A. pseudoglaucus* and *A. glaucus* that are used for Katsuobushi production (Kaminishi et al., 1999). To obtain a better understanding of the differences of *A. chevalieri*, *A. montevidensis*, and *A. sydowii* during Katsuobushi production, we should study the enzymatic properties as well as the physiological characteristics of strains M1, M2, M3, and M4.

Strain M1 formed yellow colonies in M40Y medium, whereas strain M2 formed gray colonies (Fig. 1A); however, both strains M1 and M2 were classified as *A. chevalieri* (Fig. 2), and there was no difference in ITS, *benA*, *caM*, and *rpb2* sequences between M1 and M2. Thus, we performed microscopic characterization of strains M1 and M2. Strains were grown on M40Y agar medium after cultivation for 7 days at 30°C and observed by an optical microscope DMI6000B (Leica Microsystems, Wetzlar, Germany) with Fungi-Tape and MycoPerm Blue (Scientific Device Laboratory, Des Plaines, IL) or an Quanta 400 scanning electron microscope (Field Electron and Ion Company, Hillsboro, OR). Microscopic analysis indicated that strain M1 formed cleistothecia dominantly, whereas strain M2 formed conidial heads dominantly (Fig. S2A–F), indicating that strain M1 and strain M2 show opposite sexual and asexual reproduction; the former is a teleomorphic strain and the latter is an anamorphic strain. This was consistent with an observation that *A. chevarieri* produces yellow ascomata and *en masse* grayish-green conidia (Chen et al., 2017).

In conclusion, based on the sequence analysis of ITS, *benA*, *caM*, and *rpb2*, *Aspergillus* spp. were dominant during the Katsuobushi production process and at least three species *A. chevalieri*, *A. montevidensis*, and *A. sydowii* were identified. Relative abundance of *A.*

chevalieri is stable, whereas *A. montevidensis* increased and *A. sydowii* decreased through the first, second, and third molding steps. Because this is a case study in a Katsuobushi factory at Makurazaki, Japan, further analysis is needed to elucidate the fungal species involved in Katsuobushi (e.g., factory-dependent fungal population) to obtain a better understanding of the fungal population used for Katsuobushi.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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