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Analysis of genomic characteristics and their influence on metabolism in *Aspergillus luchuensis* albino mutants using genome sequencing

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ABSTRACT

Black *Aspergillus luchuensis* and its white albino mutant are essential fungi for making alcoholic beverages in Japan. A large number of industrial strains have been created using novel isolation or gene/genome mutation techniques. Such mutations influence metabolic and phenotypic characteristics in industrial strains, but few comparative studies of inter-strain mutation have been conducted.

We carried out comparative genome analyses of 8 industrial strains of *A. luchuensis* and *A. kawachii* IFO 4308, the latter being the first albino strain to be isolated. Phylogenetic analysis based on 8938 concatenated genes exposed the diversity of black koji strains and uniformity among albino industrial strains, suggesting that passaged industrial albino strains have more genetic mutations compared with strain IFO 4308 and black koji strains.

Comparative analysis showed that the albino strains had mutations in genes not only for conidial pigmentation but also in those that encode N-terminal acetyltransferase A and annexin XIV-like protein. The results also suggest that some mutations may have emerged through subculturing of albino strains. For example, mutations in the genes for isocitrate lyase and sugar transporters were observed only in industrial albino strains. This implies that selective pressure for increasing enzyme activity or secondary metabolites may have influenced the mutation of genes associated with environmental stress responses in *A. luchuensis* albino strains.

Our study clarifies hitherto unknown genetic and metabolic characteristics of *A. luchuensis* industrial strains and provides potential applications for comparative genome analysis for breeding koji strains.

1. Introduction

Food fermentation using *Aspergillus* fungi is widespread in East Asia, particularly in Japan (Nout and Aidoo, 2010; Tamang et al., 2016). "Koji," which refers to specific *Aspergillus* species, are essential fungi used throughout the history of fermented Japanese foods (for approximately 600–800 years), including sake, miso, soy sauce, vinegar, and other foodstuffs (Machida et al., 2008; Murooka and Yamshita, 2008; Tamang et al., 2016). Due to their importance in Japanese food culture, these species have been designated "national fungi" by the Scientific Conference of The Brewing Society of Japan (2006) and are generally recognized as safe by the US Food and Drug Administration.

Three *Aspergillus* species are recognized as koji: *A. oryzae*, *A. sojae*, and *A. luchuensis* (Ichishima, 2016). *A. oryzae* is the major koji species used to produce sake, soy sauce, and miso (Machida et al., 2008). *A. sojae* is widely used in soy sauce production (Murooka and Yamshita, 2008). They produce green-yellow conidia and belong to *Aspergillus* section Fravi (Fig. 1A). *A. luchuensis*, a black koji, is used to make alcoholic beverages such as shochu spirits and awamori (Hong et al., 2013). The albino mutant of *A. luchuensis*, referred to as white koji, is also used in the production of shochu spirits (Hong et al., 2013). As it belongs to section Nigri, to which *A. niger* also belongs (Fig. 1A), *A. luchuensis* has physiological and biochemical characteristics that differ from *A. oryzae* and *A. sojae* (Murooka and Yamshita, 2008; Park et al., 2017).

Historically, wild koji strains were collected from rice plants and

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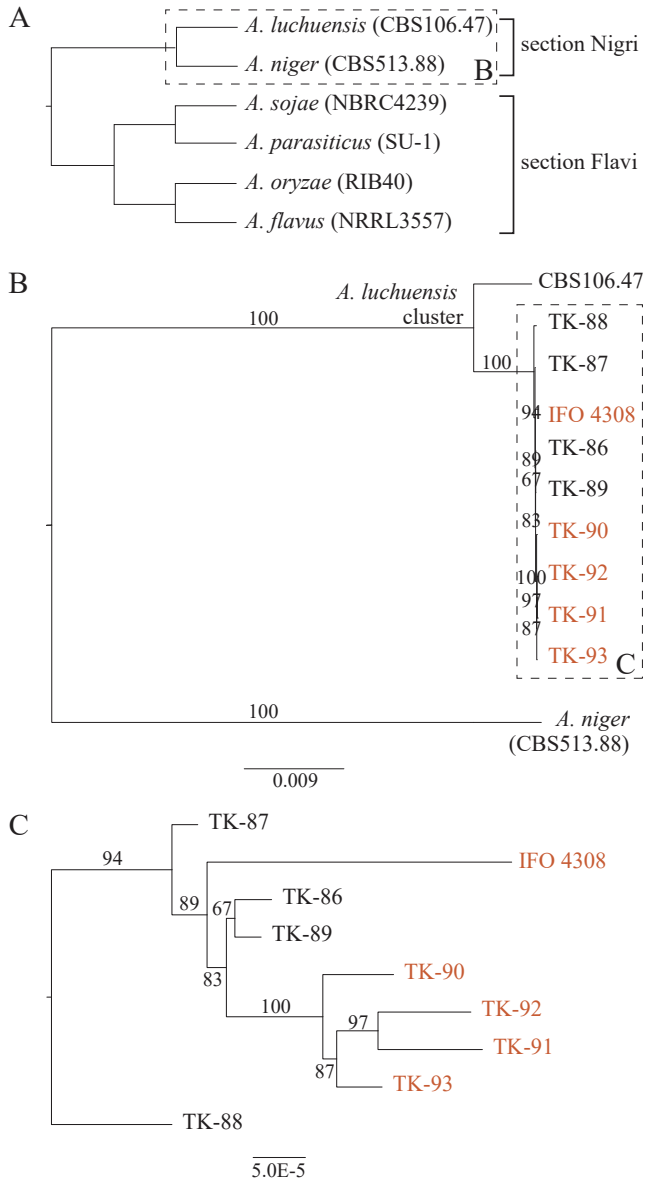

Fig. 1. Phylogenetic trees of *Aspergillus* species related to this study. A: a schematic phylogenetic tree of representative species of *Aspergillus* based on the result of Steenwyk et al. (2019). The dotted rectangle indicates the branch of phylogenetic tree enlarged in panel B. B: a phylogenetic tree of concatenated sequences of *A. niger* and *A. luchuensis* strains used in this study. The scale bar shows phylogenetic distance. Bootstrap values are shown for the nodes. The dotted rectangle indicates the branch of the phylogenetic tree in panel C. C: a phylogenetic tree of concatenated sequences of *A. luchuensis* strains used in this study. The scale bar shows phylogenetic distance. Bootstrap values are shown for the nodes. Strain ID with black indicates black koji and strain ID with orange indicates albino strain in panel B and C.

other natural sources (Machida et al., 2008). However, they were gradually domesticated as industrial strains by starter manufacturers and are now widely used in industrial food production (Kitamoto, 2002). During the extended period of koji domestication in Japan, strain improvements have been made using gene/genome mutation (Machida et al., 2008; Ushijima and Nakadai, 1987), as *Aspergillus* species have been recognized to be asexual organisms (Kück and Pöggeler, 2009). Mutation may occur naturally during subculturing or artificially induced via ultraviolet (UV), gamma irradiation, protoplast-fusion, genome editing or transposon mutagenesis (Fujita et al., 2000; Katayama et al., 2016; Ogasawara et al., 2009; Toyoshima et al., 2012; Ushijima and Nakadai, 1987). In the case of *A. luchuensis*, a natural albino mutant, *A. kawachii*, was discovered in the 1920s and described as a novel species in the 1940s (Kitahara and Kurushima, 1949). Today, *A. kawachii* has been reclassified to *A. luchuensis* mut. kawachii (Hong et al., 2014). Since then, more albino strains have been produced by spontaneous mutation (Sermkiattipong et al., 1999; Takahashi et al., 2011). The mutation greatly influences the variation in metabolic and/or phenotypic characteristics in industrial strains.

The mutations can be evaluated by whole-genome analysis which identifies the genetical characteristics of different variants and strains and their associated metabolic systems. Previous studies using whole-genome analysis suggest that domestication has influenced the genome sequences of *A. oryzae* strains (de Vries et al., 2017; Galagan et al., 2005; Gibbons et al., 2012). The *A. oryzae* RIB40 strain which is the most extensively studied because it is the only strain with a genome that has been completely sequenced, is revealed that *A. oryzae* could possess genes suitable for food fermentation (Machida et al., 2005). *A. sojae* exhibits high gene similarity with *A. oryzae* but some protease genes are unique to *A. sojae*, suggesting that *A. sojae* has been bred specifically for soy sauce production (Sato et al., 2011). A study of the genomes of *A. luchuensis* and *A. luchuensis* mut. kawachii revealed that they possess unique genes linked to citric acid production and acid-stable amylase (Futagami et al., 2011; Yamada et al., 2016). Another study investigated the mutation of a single gene associated with pigment biosynthesis by comparing *A. luchuensis* and *A. luchuensis* mut. kawachii genes (Goto et al., 2014). In contrast to these extensive works using laboratory strains, the knowledge of industrial strains of *Aspergillus* koji is still limited. Also, few comparative analyses of intraspecies mutations in *Aspergillus* koji strains have been reported (Tanabe et al., 1994; Thammarongtham et al., 2018; Umemura et al., 2012; Zhao et al., 2013). Although all albino *A. luchuensis* strains are referred to as white koji, the genetic and/or physiological differences and similarities between natural mutants and artificially induced mutants remain unclear.

In this study, we sequenced and examined the genomes of 8 industrial *A. luchuensis* and its albino strains. Comparative analyses among *A. luchuensis* strains and the *A. luchuensis* mut. kawachii strain facilitated the genetic identification of *A. luchuensis* albino mutants. The albino strains had genetic differences compared with *A. luchuensis* strains besides their conidial color-associated gene. This result is partially supported by the unique flavors and volatile compounds obtained from rice koji made with *A. luchuensis* mut. kawachii (Yoshizaki et al., 2010). In addition, our results indicated that several common gene mutations occurred during the transformation of black koji into white koji, regardless of the method for the mutations. Selective pressures of breeding may have resulted in common mutations in genes that are not directly related to fermentation. The results of an intraspecies mutation analysis revealed novel metabolic changes in koji obtained by breeding.

2. Materials and methods

2.1. Strain collection and culture conditions

TK strains of *A. luchuensis* (TK-86, TK-87, TK-88 and TK-89) and its albino strains (TK-90, TK-91, TK-92 and TK-93) were collected for genomic and metabolic analyses from 3 koji starter-culture manufacturers in Japan. Two albino strains were obtained through artificial mutation. All strains were isolated and sub-cultured independently. The *A. luchuensis* mut. kawachii strain IFO 4308 used in the metabolic analysis was purchased from NITE Biological Resource Center (Chiba, Japan).

Parts of the collected fungal hyphae or freeze-dried agents were suspended in sterilized MilliQ water. Potato dextrose agar (PDA) was inoculated with several drops of the suspension and incubated at 30 °C for 2–3 days. Cultured colonies were suspended in 20% glycerol solution and stored at −85 °C until use. Detailed information on the strains is provided in Table 1.

Table 1
General information and genomic features for fungal strains used in this study.

Strain name	Species	Conidial color	Isolation date	Genome size (Mbp)	GC%	No. of contigs	No. of protein coding genes	No. of OG	No. of genes without OG	Raw sequencing data	WGS/assembly	Source
TK-86	*A. luchuensis*	Black	No data	36.95	48.81%	217	11,490	11,171	10	DRR163639, DRR163640	BJBM01000001-BJBM01000217	This study
TK-87	*A. luchuensis*	Black	1985–1989	36.83	48.81%	148	11,480	11,173	13	DRR163641	BJBN01000001-BJBN01000148	This study
TK-88	*A. luchuensis*	Black	1985–1989	35.23	48.87%	34	11,083	10,896	29	DRR163642	BJBO01000001-BJBO01000034	This study
TK-89	*A. luchuensis*	Black	Around 1970 s	36.62	48.82%	103	11,434	11,140	9	DRR163643	BJBP01000001-BJBP01000103	This study
TK-90	*A. luchuensis*	White	No data	36.83	48.81%	203	11,462	11,169	11	DRR163644, DRR163645	BJBQ01000001-BJBQ01000203	This study
TK-91	*A. luchuensis*	White	No data	36.79	48.81%	232	11,427	11,156	9	DRR163646, DRR163647	BJBR01000001-BJBR01000232	This study
TK-92	*A. luchuensis*	White	Around 1960 s	36.71	48.82%	130	11,463	11,170	8	DRR163648	BJBS01000001-BJBS01000130	This study
TK-93	*A. luchuensis*	White	Around 1960 s	36.79	48.82%	134	11,475	11,166	12	DRR163649	BJBT01000001-BJBT01000134	This study
IFO 4308	*A. luchuensis* mut. kawachii	White	1942	37.11	48.65%	1163	11,480	11,125	21		GCA_000239835.2	NCBI
CBS 106.47	*A. luchuensis*	Black	no data	37.45	48.87%	100	11,664	10,839	324		GCA_001890685.1	NCBI
CBS 513.88	*A. niger*	Black	no data	36.26	50.31%	19	11,021	10,590	102		A_niger_CBS_513_88_s01-m07-r04	AspGD

2.2. Genome extraction and fragmentation

Glycerol-stored fungal strains were reintroduced to the PDA and incubated at 30 °C for 2–3 days. Cultured fungal hyphae and spores were suspended in approximately 50 mL of yeast extract–peptone–dextrose (YPD) medium and incubated at 30 °C for 1–3 days with shaking. Cultured fungal cells were then collected, washed with sterilized MilliQ water, and stored at −85 °C.

Genomic DNA was extracted from fungal cells using cetrimonium bromide (CTAB), which was modified for the present study based on several reports (Kikuchi et al., 2009; Rogers and Bendich, 1994; van Burik et al., 1998). First, collected cells were crushed using a sterilized laboratory spoon and the liquid portion was removed by centrifugation at 7340*g* for 2 min. For the TK-86, TK-90, and TK-91 strains, the remaining solid parts of the cells were incubated in 3 × CTAB solution (3% CTAB, 150 mM Tris-HCl, 2.1 M NaCl, 30 mM ethylenediaminetetraacetic acid [EDTA]) containing 1/100 of 20 mg/mL proteinase K at 65 °C for 60 min. Genomic DNA was then separated using a chloroform solution. The solid parts of the TK-87, TK-88, TK-89, TK-92, and TK-93 strains were pre-incubated at 37 °C for 60 min with Yatalase buffer (0.2% Yatalase [Takara BIO Inc., Shiga, Japan], 10 mM Tris-HCl, 50 mM EDTA, and 0.2 M NaCl) before incubation in CTAB. The resulting mixture was centrifuged to obtain a supernatant containing the genome. Genomic DNA was then purified using Tris-EDTA (TE)-saturated phenol, phenol/chloroform/isoamyl alcohol, and a chloroform solution. Obtained crude DNA was precipitated with isopropanol and dissolved with a TE buffer (NIPPON GENE, Co., Ltd., Tokyo, Japan) at pH 8.0. Following this extraction, RNA was degraded using ribonuclease (DNase-free) solution (NIPPON GENE) or RNase Cocktail Enzyme Mix (Thermo Fisher Scientific, MA, USA) at 37 °C for 3 h. Finally, a purified genome solution was obtained using Genomic DNA Clean & Concentrator (Zymo Research Corp., CA, USA).

Fragmented genome libraries were prepared from approximately 2.4 μg of genomic DNA using a TruSeq DNA PCR-Free Library Prep kit (Illumina, Inc., CA, USA), according to the manufacturer's instructions. The average fragment length of the TK-86, TK-90, and TK-91 strain libraries was 350 base pairs (bp), while for the remaining strains it was 550 bp. Genome libraries were sequenced using an Illumina HiSeq 2500 (Illumina, Inc.) with 150-base (for the former library) or 250-base (for the latter library) paired-end runs, at the National Institute of Genetics (Shizuoka, Japan). The Whole-Genome Shotgun projects reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers in Table 1.

2.3. Read assembly, gene prediction, and orthologous group generation

Low-quality regions and adaptor sequences were filtered from the paired-end reads using Platanus_trim v1.0.7 (Kajitani et al., 2014). High-quality reads were then assembled using Platanus v1.2.4, with the default parameters. To eliminate contamination, assembled scaffolds with lengths of at least 1000 bp were aligned to the genomic sequences of *A. oryzae* RIB 40 downloaded from DOGAN (http://www.bio.nite.go.jp/dogan/project/view/AO) using LAST v869, and also to the RefSeq release 89 from NCBI database (https://www.ncbi.nlm.nih.gov) using BLASTn v2.2.8. Scaffolds aligned to bacterial genomes were removed.

For comparative analysis, 3 datasets of scaffold genome sequences and 3 datasets of protein sequences were downloaded from public databases; the scaffold genome sequences of *A. luchuensis* mut. kawachii IFO 4308 and *A. luchuensis* CBS 106.47 from NCBI; the scaffold genome sequences of *A. niger* CBS 513.88 (version: 01-m07-r04) and the protein sequences of *A. oryzae* RIB40 (version: s01-m08-r29), *A. niger* CBS 513.88 (version: 01-m07-r04) and *A. flavus* NRRL 3357 from *Aspergillus* Genome Database (AspGD, http://www.aspgd.org). These datasets were used to carry out gene predictions as follows.

Two strategies of gene prediction were adopted. First, a model-based prediction was performed using GeneMark-ES v4.32 (Ter-Hovhannisyan

et al., 2008) and AUGUSTUS v3.3 (Stanke and Waack, 2003). Second, potential fungal scaffolds of TK strains, IFO 4308, *A. luchuensis* CBS 106.47 and *A. niger* CBS 506.88 were mapped parallel to the protein sequences from the 3 *Aspergillus* strains, including *A. oryzae* RIB40, *A. niger* CBS 513.88, and *A. flavus* NRRL 3357 using GMAP v2017-11-15. The resulting tool-derived and mapping-derived data relating to predicted genes were combined using EVidenceModeler v1.1.1, based on the following weight parameters to give higher priority to the closer species: *A. niger* CBS 513.88, 10; *A. oryzae* RIB40 and *A. flavus* NRRL 3357, 5; AUGUSTUS, 2; and GeneMark-ES, 1. The resulting protein and nucleotide sequences were processed using gffread v0.9.10 to eliminate genes that were frame-shifted or lacked start/stop codons. Raw sequences, assemblies, and annotations are available in the DDBJ/EMBL/GenBank databases under the accession numbers in Table 1. Genomic features for each strain are provided as supplementary general feature format data (Supplementary Data S1).

For ortholog clustering, the predicted protein sequences of all strains and the downloaded protein sequences with gene annotations of *A. niger* CBS 513.88 were clustered in OrthoFinder v2.2.6. Orthogroups (OGs) that contained only one gene from one dataset were removed. After that, the datasets from predicted protein sequences were used for subsequent analyses. OG representative protein sequences were annotated using BLASTp v2.2.8 against RefSeq release 89 (-evalue 0.000001) of the NCBI database unless they were annotated with the gene ID of *A. niger* by OrthoFinder. Of the OGs generated, those containing just a single gene (universal single copy gene, USCG) from all strains were extracted. The protein sequences of OGs defined as USCGs were treated as multiple alignments using MAFFT v7.2.2.2 with the G-INS-I parameters and then concatenated. Phylogenetic analyses were carried out by maximum-likelihood-based phylogenetic inference with RAxML v8.2.11 using options (-m PROTCATDAYHOFFX -N 100), after removal of the columns that contained at least one gap region.

2.4. Metabolic analysis

2.4.1. Amino acid analysis of rice koji

To make koji-grown rice (malted rice or rice koji), stored strains were cultured on PDA as described above. A small piece (approximately 0.4 cm^2) of a cultured colony was suspended in 0.8% NaCl containing 0.1% Tween 20. The suspension was then filtered using a cell strainer with pores 25 μm in diameter. The number of spores in the filtrate was counted using a cell-counting plate (WATSON Co., Ltd., Tokyo, Japan), and the spore solution was adjusted to approximately 1000 spores/μL using 0.8% NaCl/0.1% Tween 20. Commercial, pre-washed rice (Koshihikari, a popular cultivar of Japonica rice) was used as the culture base. Approximately 2 mL of MilliQ water was added to 0.1 g of rice (five grains) and the rice was left to absorb the water at room temperature for 1 h. After removing excess water, the rice was autoclaved to produce sterilized steamed rice and transferred to a 12-well plate for inoculation with 10 μL of spore suspension. The plates were incubated in wet conditions for 2 days at 30 °C. Rice koji of each strain was prepared in triplicate.

Water-soluble components in each rice koji were extracted according to the manufacturer's instructions (Shimazdu Corporation, Kyoto, Japan) with some modifications. MilliQ water and sterilized 10 mm stainless-steel beads (Bio Medical Science Co., Ltd., Tokyo, Japan) were added to the koji, which was then crushed using a bead cell-disrupter (MicroSmash MS-100R; TOMY SEIKO Co., Ltd., Tokyo, Japan) at 2500 rpm for 1 min. Methionine sulfone (10 mg/mL) was used as an internal standard. Amino acid analysis was conducted with a liquid chromatography–tandem mass spectrometry (LC-MS/MS) platform (LC-MS 8050; Shimazdu Corporation), using the LC-MS/MS method package for primary metabolites (Shimazdu Corporation). Glucose concentrations were determined using high-performance liquid chromatography (Chromaster; Hitachi High-Technologies Corporation, Tokyo, Japan). Sequentially diluted amino acid standards (A9906; Sigma-Aldrich, Co, LLC., Tokyo, Japan) mixed with L-asparagine and L-glutamine or glucose standards were analyzed with each run. The concentrations of glucose and each amino acid were calculated from the resulting data, and average concentrations were calculated from triplicate sample data. Concentration values were corrected by the number of inoculated spores to be transformed into the composition ratio. Data analysis was performed using R version 3.2.4 (R core team, 2016).

2.4.2. Culture assays

Three different types of agar were prepared to observe fungal growth under different carbon source conditions. Czapek–Dox (CD) agar contains 2% glucose, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, and 0.05% KCl (at pH 7). Two types of modified CD agar were prepared, one containing 2.1% citrate and the other 2.7% sodium acetate instead of glucose as the sole carbon source. The modified CD agar was adjusted to a pH of 7 using NaOH. Spore suspensions were prepared as described previously, and each plate was inoculated with 2 drops of suspension. The plates were incubated at 30 °C for 7 days.

A sugar utilization assay was conducted using the API 20C AUX system (SYSMEX bioMérieux Co., Ltd, Tokyo, Japan), according to the manufacturer's instructions with some modifications. Spore suspensions were prepared as previously described. Spore solutions were mixed with C media in the kit to obtain 15-fold dilutions. The spore and media mixtures were incubated at 30 °C for 4 days, with 19 types of substrate.

3. Results

3.1. Genome features of A. luchuensis strains

All genomic information is supplied in Table 1. Small differences were recorded in the genome sizes of the *A. luchuensis* TK strains, ranging from 36.62 Mb to 36.95 Mb, except for strain TK-88 (35.23 Mb). Most of the strains contained fewer than 11,500 protein-coding genes.

3.2. Phylogenetic analysis of A. luchuensis strains

Phylogenetic trees from 8938 USCGs revealed that the *A. luchuensis* group could be clearly distinguished from the *A. niger* strain (Fig. 1B). A detailed phylogenetic tree of the species showed that only strain CBS106.47, isolated from Switzerland, was an outgroup, while 9 strains including the IFO strain created a cluster (Fig. 1B). Four *A. luchuensis* strains were placed separately and did not create one group. However, *A. luchuensis* albino strains, apart from strain IFO 4308, formed one cluster (Fig. 1C).

3.3. Analysis of orthologous genes

OGs were created from the 8 TK strains and 3 reference strains to generate a total of 12,048 OGs (Supplementary Table S1). Almost all the predicted genes from TK strains sequenced in the present study were grouped into OGs with genes from other strains. For the genomic data of *A. niger* strain CBS 106.47 processing our assembly pipeline, 102 genes were not assigned OGs. Of the generated OGs, 622 were shared only with *A. niger* gene data from AspGD. Among the above OGs were parts of the ochratoxin and fumonisin biosynthetic clusters, including An15g07890, An15g07910, and An15g07920 for ochratoxin, and An01g06820, An01g06840, An01g06850, An01g06870–An01g06900, and An01g06920 for fumonisin (Susca et al., 2016). Of the remaining toxin-related genes, 4 (An15g07880, An15g07900, An01g06910 and An01g06930) were homologous to the genes of all *A. luchuensis* TK strains. The proportion of OGs common to the 8 strains sequenced in the present study ranged from 96.1% to 98.6%. However, the proportion of OGs common to each strain and *A. niger* was relatively low (87.7%–89.2%).

All *A. luchuensis* TK strains and IFO 4308 shared 10,690 OGs. A total of 10,560 OGs contained a single gene from each strain. OGs with USCGs

were extracted according to the following criteria: (i) OGs with identical amino acid sequences among the 4 *A. luchuensis* strains, the IFO 4308 strain, and the 4 albino strains; (ii) OGs with different amino acid sequences among the 4 *A. luchuensis* strains and the IFO 4308 strain with the 4 albino strains; (iii) OGs with different amino acid sequences among the 4 *A. luchuensis* strains, the IFO 4308 strain, and the 4 albino strains; (iv) OGs with different amino acid sequences among the 4 *A. luchuensis* strains with the IFO 4308 strain and the 4 albino strains; and (v) others. The numbers of OGs screened for each of these groups were (i) 8704, (ii) 7, (iii) 9, (iv) 187, and (v) 1653, respectively (Fig. 2). OGs in group (ii) had just 1 amino acid substitution in each sequence, while group (iii) included OGs with deletion, insertion and substitution mutations. In group (iv), 5 OGs had deletion mutations, 2 OGs had an insertion, 2 OGs had a frame-shift mutation and 3 OGs contained multiple types of mutations (Fig. 2). Detailed information about the OGs from groups (ii) to (iv) and the variants found in albino strains are shown in Supplementary Table S2.

3.4. Metabolism assays

The composition of amino acids was calculated from the concentrations of each acid produced by each strain following 2 days of incubation with rice. Averages and standard deviations are supplied in Supplementary Table S3. A heat map was generated using hierarchical clustering that revealed the amino acid production patterns of *A. luchuensis* albino strains differed from that of the IFO 4308 strain (Fig. 3).

All TK strains and the IFO 4308 strain inoculated onto standard CD agar grew following 7 days of incubation. Only *A. luchuensis* strains formed colonies on modified CD agar when acetate was provided as the sole carbon source (Table 2). Strain IFO 4308 was confirmed to grow on CD agar under both standard and acetate-added conditions (Table 2). When 2 *A. luchuensis* strains and strain IFO 4308 were grown with citrate, lower growth rates were observed (data not shown).

In the sugar utilization assay, *A. luchuensis* strains and the IFO 4308 strain exhibited similar growth characteristics over 4 days of incubation (Table 3). They either could not survive or exhibited lower growth rates with adonitol, galactose, methyl alpha-D-glucopyranoside, and lactose. *A. luchuensis* albino strains were also unable to grow on these 4 substrates. Although more than 75% of the albino strains could grow on glucose, arabinose, xylose, sorbitol, and saccharose, only 25% or 50% of the albino strains could grow on the other 10 substrates.

4. Discussion

Aspergillus koji is a key organism in traditional fermented foods in Japan. Spontaneous mutations in *Aspergillus* koji species created strains with diverse characteristics, and facilitated the development of the fermented food sector in Japan. Recently, it was reported that *Aspergillus oryzae* may be able to reproduce sexually (Wada et al., 2012). This finding prompted further attempts to breed a new strain through mating (Okabe et al., 2018; Tsukasaki et al., 2014). However, most industrial koji strains have been produced by spontaneous mutations. *A. luchuensis*, which is of limited use in the preparation of shochu spirits and awamori, is most commonly isolated in Japan (Hong et al., 2014). *A. luchuensis* albino strains, which arose through either natural or artificially induced mutations, have been sub-cultured following these mutations, again mostly in Japan. The loss of the capacity to synthesize pigments due to mutations in the pigment-synthesis gene resulted in phenotype changes (e.g., the lack of conidial color in albino strains). In addition, mutant strains reportedly exhibit enhanced amylolytic activity and acid production rates in comparison with the original strains (Iizuka and Yamaguchi, 1955). The characteristics of each strain depend on which genes are mutated, and how the mutations influence the phenotype. Although numerous studies have investigated mutations among species variants in *Aspergillus*, and between two strains, few comparisons of the genomes of 3 or more strains within a species have been reported, despite advances in genetic technologies.

The intraspecific differences in *A. luchuensis* species reported in the present study may have valuable industrial applications. We compared genomes among 8 newly identified strains of *A. luchuensis*, a much larger number than previous studies used. The present study also explored differences among *A. luchuensis* albino mutants and black strains, based on ortholog clustering and sequence alignments, with the aim of detecting common key mutations in whitening and passaging. Differences among strains were evaluated at the level of a single amino acid residue for each gene.

Phylogenetic analysis of genomic data, including strain IFO 4308, revealed diversity among black koji strains and uniformity among albino industrial strains. The genomes of albino strains that had been passaged independently were more similar compared with those of non-mutants. Comparisons of OGs among all strains found few strain-specific genes in the albino strains. Our results revealed that mutations in strains occurred via minor sequence variations, rather than extensive mutations in the form of gene transfer. However, strain IFO 4308 was the only strain distantly located from the other albino strains, suggesting that this strain is more closely related to black koji than passaged albino strains. This phylogenetic difference among white koji strains appears to indicate that strain IFO 4308 was stored without subculturing at the culture collection center after discovery, whereas other albino strains were passaged repeatedly at manufacture. This suggestion is partly supported by a previous study that reported different physiological properties between IFO 4308 strain and albino strains isolated from rice koji (Tanabe et al., 1994).

The present study focused on single-nucleotide polymorphisms in protein-coding sequences to investigate potential differences among black koji, strain IFO 4308 and white koji. Mutations were observed in 7 genes of group (ii), which might influence the phenotypes. The proteins responsible were identified as N-terminal acetyltransferase A (*nat1*), annexin XIV-like protein, cytochrome P450, R3H domain protein, RTA1 domain protein, oxidoreductase and a short-chain dehydrogenase. The effect of mutations in *nat1* on the ecology of *Aspergillus* is not clear, although *nat1* has been linked with some phenotypes in yeast species (Park and Szostak, 1992). It has been proposed that members of the annexin family play roles in endocytosis, exocytosis, and stress response (Khalaj et al., 2015). Fungal annexins belong to the C family of proteins,

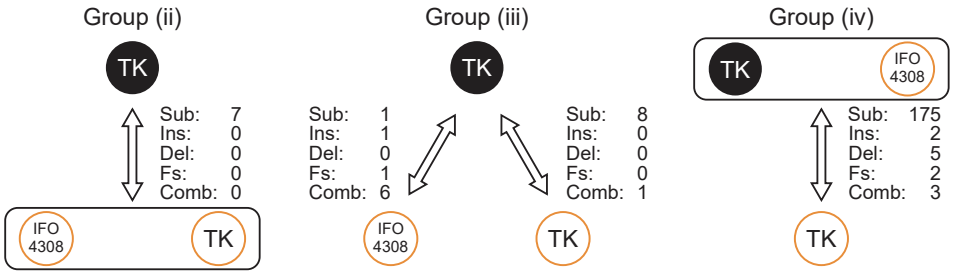

Fig. 2. Summary of mutated OGs classified into group (ii), (iii) and (iv). A black circle indicates black koji strains. A white circle with an orange line indicates albino strains. The name of the strain or the strain group are shown in the circle. The strain or the strain group sharing same protein sequences are enclosed with rounded squire. An arrow describes the target of comparison. The number of mutations detected are shown beside the corresponding arrow. Sub, substitution; Ins, insertion; Del, deletion; Fs, frame-shift; Comb, combination of multiple types of mutations.

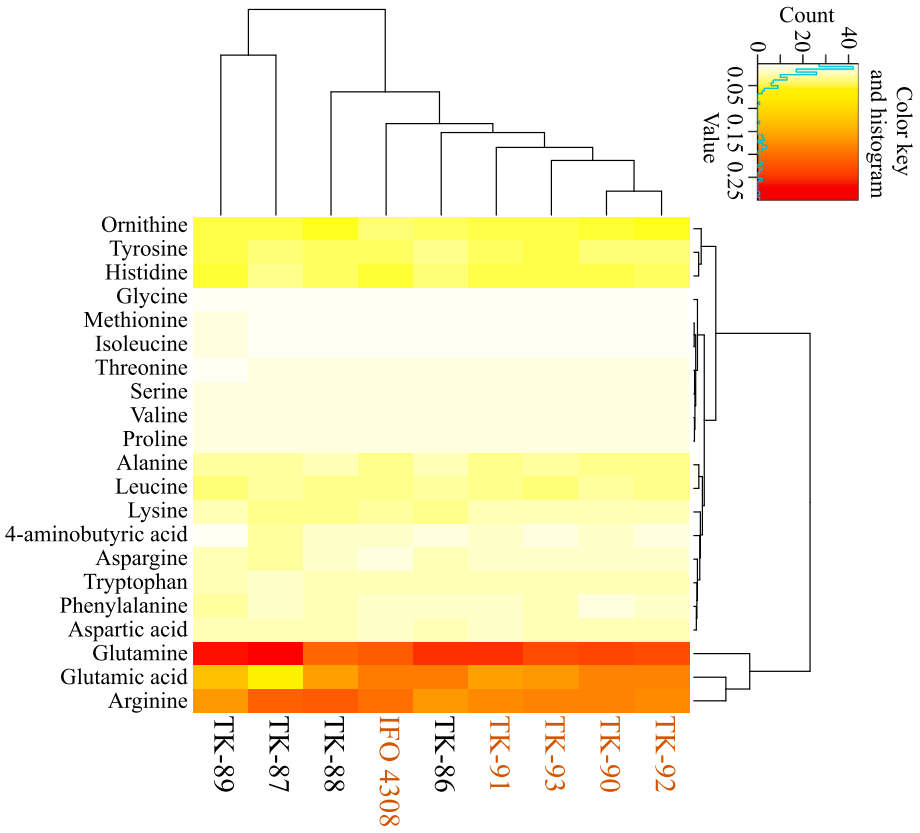

Fig. 3. Heat map and hierarchical clustering for the relative proportion of 21 amino acids originated from rice koji. Clustering was performed using an average-linkage method. Strain name with orange indicates albino strain. The color from yellow to red indicates relative ratio from low to high.

Table 2
Growth of *A. luchuensis* strains in Czapek–Dox (CD) medium with glucose or acetic acid as a sole carbon source.

Carbon source	Black koji strains				Albino strains				
	TK-86	TK-87	TK-88	TK-89	TK-90	TK-91	TK-92	TK-93	IFO 4308
Glucose (Standard CD medium)	++	++	++	++	++	++	++	++	++
Acetic acid (Modified CD medium)	+	+	+	+	–	–	–	–	+

++, rapid growth; +, moderate growth; -, no growth.

and AnxC3.1, part of a C-subfamily, reportedly have no influence on growth or protein secretion in *A. niger* (Khalaj et al., 2004). Annexin C4 may assist with resistance to oxidative stress in *A. fumigatus* (Khalaj et al., 2011). However, the annexin gene isolated in our study was grouped with anxC3.2, the function of which remains unknown in *Aspergillus* koji species. Cytochrome P450 is an enzyme which has numerous functions in fungi such as toxin biosynthesis and denitrification (Crešnar and Petrič, 2011). Further studies into the effects of such genetic mutations in albino strains on growth and metabolism are required, using gene-knockout methods.

Nine genes in group (iii) had different sequences among black strains, the IFO 4308 strain, and the other white strains. In 2 of the 9 genes of the TK albino strains, a mutation in another site was found in addition to the mutation in IFO 4308. Additional mutations could have occurred in these genes during subculturing, after the mutations in the black strains, and are thought to influence gene expression. A previous study reported that polyketide synthase (*pksP*) for conidial yellow-pigment biosynthesis was mutated in white koji (Goto et al., 2014). Considering that *A. niger pksP*-mutant strains had a fawn color (Jørgensen et al., 2011), this gene could be a major reason for the whitening seen in *A. luchuensis*. The last domain of *pksP*, the thioesterase domain, is presumed to be missing from the genome of strain IFO 4308 (Goto et al., 2014). In the present study, however, complete domains were maintained in the genome sequence of strain IFO 4308, while inter-domains were deleted (Fig. 4A, Fig. S1). In addition to these inter-domain deletions, the deletion of terminal sections from phospho-pantetheine attachment sites of the *pksP* gene in albino TK strains was observed. Our result supports a previous report that important domains are deleted from *pskP* gene (Goto et al., 2014). Further gene deletion occurred during repeated passages and the mutated region was expanded, although the *pksP* gene had already lost its ability. This gene is important in spore coloration (melanin synthesis). Melanin is not only a pigment component for *Aspergillus* species, but it is advantageous for survival, providing stress protection to cell walls (Heinekamp et al., 2012), increased UV resistance (Singaravelan et al., 2008), and protection against reactive oxygen species (Langfelder et al., 1998). In the case of the albino strain, a state of weakness against various stresses may control its fermentation ability, and further gene mutations may have been caused. For example, a mutation in the allantoate permease gene that is associated with the allantoin metabolic pathway may be occurred because this pathway is activated by nutrient limitation in yeast.

Numerous genes in group (iv) showed identical sequences among strain IFO 4308 and black strains, but were mutated in the other albino strains. Because the gene mutation sites were similar in the strains, regardless of mutation method or strain origin, we concluded that passaging without the capacity to synthesize pigments led to the

Table 3
Growth of *A. luchuensis* strains under sugar substrates for confirmation of sugar transporter activity.

Strain name	None	GLU	GLY	2 KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF
TK-86	-	++	++	+	++	++	-	+	-	++	++	-	++	++	-	++	++	++	++	++
TK-87	-	++	++	++	++	++	-	+	-	++	++	-	++	++	-	++	++	++	++	++
TK-88	-	++	++	++	++	++	-	++	-	++	++	-	++	++	-	++	++	++	++	++
TK-89	-	++	++	+	++	++	-	++	-	++	++	-	++	++	-	++	++	++	++	++
TK-90	-	++	+	-	-	+	-	-	-	-	++	-	-	-	-	-	++	-	-	+
TK-91	-	++	++	+	++	++	-	+	-	+	++	-	++	++	-	++	++	++	++	++
TK-92	-	++	+	-	++	++	-	-	-	-	++	-	+	-	-	-	++	-	+	++
TK-93	-	++	-	+	++	++	-	-	-	-	-	-	-	-	-	-	++	-	-	-
IFO 4308	-	++	++	++	++	++	+	++	-	++	++	-	++	++	+	++	++	++	++	++

++, rapid growth; +, moderate growth; -, no growth; GLU, D-glucose; GLY, glycerol; 2 KG, calcium 2-keto-gluconate; ARA, L-arabinose; XYL, D-xylose; ADO, adonitol; XLT, xylitol; GAL, D-galactose; INO, inositol; SOR, D-sorbitol; MDG, methyl alpha-D-glucopyranoside; NAG, N-acetyl-glucosamine; CEL, D-cellobiose; LAC, D-lactose; MAL, D-maltose; SAC, D-saccharose; TRE, D-trehalose; MLZ, D-melezitose; RAF, D-raffinose.

mutation of particular genes. Examples of mutations during passage have been reported for the rice blast fungus, *Magnaporthe oryzae* (Jeon et al., 2013). Genetic mutations in *M. oryzae* accumulated rapidly before any change in phenotype became visible during subculturing, suggesting a buffering capacity of the fungus (Jeon et al., 2013). Jeon et al (2013) also speculated that oxidative damage and UV radiation were major sources of mutation in the sub-cultures. Similarly, our results demonstrate that mutations occurred with the passage of albino strains. Notably, subculturing affected common mutations in more than 100 genes.

Some genes in group (iv) were associated with the mitogen-activated protein kinase–signaling pathway with amino acid substitution. This pathway regulates adaptation to various environmental stress factors (Ma and Li, 2013: May et al., 2005). In addition, 3 mutated genes were associated with chitin synthesis. Because these genes can influence fungal growth and responses to stress (Rogg et al., 2012), the mutant genes in passaged albino strains are assumed to have adapted to environmental factors such as osmotic pressure or nutritional conditions. As no mutations occurred in these genes in passaged black koji, gene mutations that resulted in whitening may influence properties associated with fungal growth and stress responses. Further investigations are required to reveal potential genome mutations during serial passage of black strains following *pksP* mutation because 58 other OGs have unknown functions.

Although most mutations were single-residue mutations, 12 genes with deletion, insertion or frame-shift mutations were observed. Because these mutations may exert considerable influence over metabolic functions in albino strains, metabolic assays associated with 2 of these genes from group (iv) were conducted for all strains. Isocitrate lyase (OG0001075 in Table S2, ICL), an enzyme that catalyzes the production of glyoxylic acid from isocitrate (Dunn et al., 2009), is critical in the gluconeogenesis glyoxylate cycle pathway for the synthesis of glucose. *A. luchuensis* has the capacity to grow using carbon sources other than glucose, and in the present study we showed that albino strains, except for strain IFO 4308, could not grow with acetate as a sole carbon source. This result indicates that the gluconeogenesis is inhibited in albino TK strains. The studies about gene expression activity in *Aspergillus* suggest that ICL is relevant to metabolic control under hypoxic conditions and germination (Ebel et al., 2006; Meijer et al., 2009; Terabayashi et al., 2012). In other genera, ICL mutations were found to affect cell growth, germination, virulence and abiotic stress tolerance (Dubey et al., 2013; Dunn et al., 2009). Furthermore, the previous study (Dubey et al., 2013) reported that the strains with ICL mutation showed conidial decolorization under specific conditions. In the case of albino strains in the present study, the loss of the ability to synthesize pigment occurred first, followed by the mutation of ICL. Therefore, the ICL mutation in albino strains possibly influence on not only growth and environmental responses but also pigmentation. The specific reason for mutation in the ICL gene is unknown, but it is presumed to be due to a decrease in UV resistance by the mutation of the *pksP*, or long-term passage under the conditions where ICL is unnecessary.

Given the utilization of sugar during culture assays, the effect of mutations in the sugar transporter–associated gene (OG0001467 in Table S2) on the metabolic characteristics of albino strains can be confirmed. The associated gene belongs to a group responsible for the transfer of pentose and glycerol, based on gene clusters identified in *A. niger* (Peng et al., 2018). The deleted portion of the gene in albino strains contained 1 sugar-binding motif (PETxx), which is considered to have led to poor growth following glycerol addition (Fig. 4B, Fig. S2). Cells usually intake or produce to accumulate glycerol intracellularly because of their protective properties against stress, particularly hyperosmotic and thermal stress (Klein et al., 2017). Lack of or minimal growth with glycerol was demonstrated in albino strains except strain IFO 4308 (Table 3). These results suggest that this mutation may result in uncharacterized responses to environmental stress.

Previous studies have focused on genes that play a key role in

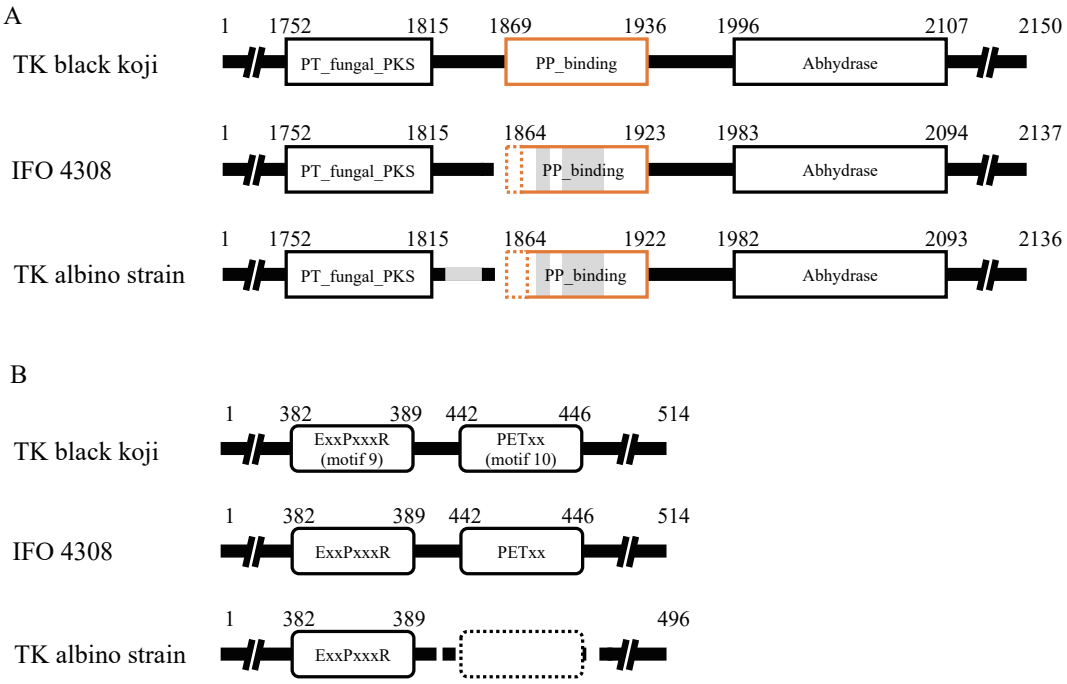

Fig. 4. A schematic illustration indicating mutated regions of strains used in this study. The positions of domains and motifs in amino acid sequence are shown. A double slash describes the omission of the middle part. A: the *pksP* gene; B: sugar transporter gene. In panel A, rectangles indicate the internal domains. The width of rectangles represents the approximate length of the domain. The domain names in the rectangles are defined in SPARCLE (https://www.ncbi.nlm.nih.gov/sparcle) with ID 12176575. PT_fungal_PKS, iterative type I polyketide synthase product template domain; PP_binding, phosphopantetheine attachment site; abhydrolase, alpha/beta hydrolases. A dotted line indicates a deleted region. A gray line and box indicate a mutated region. In panel B, rounded rectangles indicate the motifs reported by Peng et al. (2018). A dotted line indicates a deleted region. The motif names in the rounded rectangles are defined by Peng et al. (2018).

fermentation. Our study, which focused on approximately 200 genes, indicates that even if these mutations are not directly related to fermentation, they may affect important traits for breeding, such as phenotype and growth characteristics in white koji. As shown above, 15 genes other than the pigment-synthesis gene were mutated during the evolution of black koji into white koji. In addition, 187 unique gene mutations in albino strains have emerged through subculturing. We performed culture assays to show the deficient growth of white koji strains except strain IFO 4308 with acetate as a sole carbon source and the difference in sugar utilization among the strains. Based on these results, it is assumed that selective pressure following years of passage influenced the mutation of genes associated with environmental stress responses via increased enzyme activity or secondary metabolites. Further investigation is required to provide quantitative evidence to support our results using RNA-sequencing to clarify the levels of expression under stress conditions. In addition, OGs with multiple genes should be studied because such genes can also influence strain characteristics. The knowledge of the relationship between genetic variants and their metabolic activity under stress conditions can be applied to improve breeding methods.

Koji has been commercially available in Japan for many years, and numerous strains with diverse characteristics have emerged. Our results show the capacity of whole-genome analysis to reveal novel gene mutations between various koji strains. Whole-genome analysis should be effective for *A. oryzae*, the most famous koji species, to reveal the patterns of mutation and unique genes as well as the non-coding regions in each strain. Consequently, these results could facilitate the identification of novel strains with novel characteristics or enhanced production capabilities.

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