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Genome sequence of *Aspergillus luchuensis* NBRC 4314

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Abstract

Awamori is a traditional distilled beverage made from steamed Thai-Indica rice in Okinawa, Japan. For brewing the liquor, two microbes, local kuro (black) koji mold *Aspergillus luchuensis* and awamori yeast *Saccharomyces cerevisiae* are involved. In contrast, that yeasts are used for ethanol fermentation throughout the world, a characteristic of Japanese fermentation industries is the use of *Aspergillus* molds as a source of enzymes for the maceration and saccharification of raw materials. Here we report the draft genome of a kuro (black) koji mold, *A. luchuensis* NBRC 4314 (RIB 2604). The total length of nonredundant sequences was nearly 34.7 Mb, comprising approximately 2,300 contigs with 16 telomere-like sequences. In total, 11,691 genes were predicted to encode proteins. Most of the housekeeping genes, such as transcription factors and *N*-and *O*-glycosylation system, were conserved with respect to *Aspergillus niger* and *Aspergillus oryzae*. An alternative oxidase and acid-stable α-amylase regarding citric acid production and fermentation at a low pH as well as a unique glutamic peptidase were also found in the genome. Furthermore, key

biosynthetic gene clusters of ochratoxin A and fumonisin B were absent when compared with *A. niger* genome, showing the safety of *A. luchuensis* for food and beverage production. This genome information will facilitate not only comparative genomics with industrial kuro-koji molds, but also molecular breeding of the molds in improvements of awamori fermentation.

Key words: Aspergillus luchuensis, kuro (black) koji mold, genome sequence

1.Introduction

Aspergillus luchuensis is a kuro (black) koji mold that is used widely for brewing Japanese traditional spirits, awamori, in the Okinawa islands, which are located in the most southern part of Japan.[1–3] Recently, *A. luchuensis* has also been employed to produce shochu, a distilled beverage manufactured in the Kyushu islands. *A. luchuensis* produces vast amounts of enzymes, which facilitate the maceration and saccharification of raw materials, such as rice, wheat, and sweet potato. Similar to *A. niger*, *A. luchuensis* produces an abundance of citric acid, which maintains the fermentation mash at a low pH to prevent contamination by wild microorganisms. Interestingly, the glycosidases produced by *A. luchuensis* have higher catalytic activity in a more acidic pH range than those secreted by *A. oryzae*, which is used for brewing Japanese sake, rice wine.[4,5] These characteristics enable highly effective and reliable fermentation in warm latitudes, such as Okinawa and the Kyushu islands. There are approximately 50 awamori brewers in Okinawa who originally used their own strains, but recently, they have employed koji-seed (conidiospores) supplied by companies. The market size of awamori is 13,000 Myen (130 Mdollar), and the total market size of Japanese traditional spirits, including Shochu, is 470,000 Myen (4,700 Mdollar). Furthermore, the high potential of its secretory enzymes and the safety of *A. luchuensis* make this microorganism extremely important for modern biotechnology.

Historically, *A. luchuensis* was isolated primarily from awamori koji in the Okinawa islands by Inui in 1901,[6] who stated that *A. luchuensis* is the major fermentation agent in awamori production and that it possesses uniseriate conidial heads. In the same year, Usami also isolated two major kuro koji molds from awamori koji and reported that black *Aspergillus* No. 1 must be *A. luchuensis* Inui.[7] In 1913, Nakazawa obtained α and β strains from awamori koji, and the α strain is an important mold for awamori fermentation.[8] He designated the α strain as *A. awamori* and rejected *A. luchuensis* as a kuro koji mold because the α strain possessed biseriate conidial heads, although its morphological characteristics were similar to those of *A. luchuensis*. These kuro koji molds exhibit clear differences from standard *A. niger* strains, where the conidial surface is smooth and unable to assimilate nitrate in its early culture stage.[9,10] In 1980, Al-Musallam revised the taxonomy of black *Aspergillus*, and *A. awamori* was synonymized as *A. niger* var. *awamori* based on NRRL4948 from the Instituto Ozwaldo Cruz, Brazil, rather than Nakazawa's α strain.[11] A recent phylogenetic analysis of several known genes, including the RTS of ribosomal DNA, demonstrated that the *A. awamori* strains in NRIB and NITE (NBRC) formed two major clusters, one of which co-clustered with *A. niger*, whereas the other clade co-clustered with *A. kawachii* and industrial kuro koji molds.[1] Thus, the classification of kuro koji molds is in dispute. In 2012, Hong et al. re-described *A. luchuensis* as an industrially important black *Aspergillus* in East Asia with biseriate conidial heads, and *A. kawachii* was synonymized as *A. luchuensis*.[12,13] Extrolite analysis of *A. luchuensis* strains showed that they do not produce mycotoxins, and thus, they can be considered safe for use in food and beverage fermentation. Hong et al. also proposed that *A. awamori* is a cryptic phylogenetic species in the section *Nigri*, and they rejected the species name, *A. awamori*.

In this study, we sequenced *A. luchuensis* NBRC 4314 (RIB 2604), which was received from Usami as *A. awamori* No. 1, and it was stored at the IFO (NBRC).[14] The genome size was approximately 34.7 Mb, which is almost the same as that of *A. niger* CBS 513.88, and the average shared identity at the nucleic acid level was 88.9%. We identified some genes that are important for awamori fermentation, and we analyzed the protein *N*-and *O*-glycosylation systems in detail. We also determined the mating locus and confirmed the nonproduction of mycotoxins.

2. Materials and methods

2.1. Strain and DNA preparation

A. luchuensis NBRC 4314 (RIB 2604) was used as the DNA donor. Genomic DNA preparation and removal of mitochondrial DNA were performed as described previously.[15,16]

2.2. Genome sequencing

The genome of *A. luchuensis* was sequenced using the whole-genome shotgun (WGS) approach by accumulating raw sequence reads with a depth of coverage of approximately 5.7×. The linkage between contigs was analyzed by fingerprinting and PCR, as well as by optical mapping (OpGen).

2.3 Gene prediction and annotation

GeneDecoder[17] and GlimmerHMM[18] were used for gene prediction. Automated annotation was performed using NCBI BLASTP with the *A. niger* CBS 513.88 genomic database.[19] Interproscan[20] was used for functional analysis of the predicted proteins. The number of genes in each cluster of orthologous group (COG)[21] category was analyzed by BLASTP using the amino acid sequences in the COG set with bit scores ≥60. Comparative analysis of *A. luchuensis* and *A. niger* was conducted with MUMmer tools.[22] AntiSmash[23] was used to search for secondary metabolite biosynthesis gene clusters.

Results and discussion

3.1. Genome sequence and gene prediction

The *A. luchuensis* genome was sequenced using the WGS approach. The 34.7 Mb genome was predicted to contain 11,691 genes that encoded proteins with a length greater than 100 amino acid residues (Table 1). The genome was confirmed as comprising eight chromosomes (chromosomes 1–8 in decreasing order of size).

The average shared identity at the nucleic acid level between *A. luchuensis* NBRC 4314 and *A. niger* CBS 513.88 was estimated as 88.9% using the dnadiff wrapper script in the MUMmer 3.0 package. MUMmer also showed that the correspondence between the

Table 1. Properties of the genomes of *Aspergillus luchuensis* NBRC 4314, *A. niger* CBS 513.88, and *A. kawachii* NBRC 4308

	A. luchuensis	A. niger	A. kawachii
Genome size (Mb)	34.7	33.9	36.6
G + C content (%)	49.7	50.4	49.9
Gene models	11,691	13,160	11,255
Protein length (amino acids)	483.6	466.4	500.1
Exons per gene	2.8	3.7	3.1
Exon length (bp)	660.2	533.0	660.1
Intron length (bp)	113.8	94.6	80.7

Genes that encoded polypeptides longer than 100 amino acids were used in the analysis.

chromosomes of *A. luchuensis* and *A niger* was extremely low compared with that between those of *A. niger* CBS 513.88 and *A. niger* ATCC 1015,[24] which were almost the same (Fig. 1). For example, chromosome 1 of *A. luchuensis* appeared to be composed of portions of chromosome 6, 2 and 4 of *A. niger*. These results are consistent with the suggestion that *A. luchuensis* is a different species from *A. niger*.

The COG classification detected no major differences between *A. niger* and *A. oryzae* (Table 2). For instance, most of the transcription factors were found in the genomes of *A. luchuensis*, as well as *A. niger*, and *A. oryzae* (Supplementary Table S1).[25,26]

3.2. Glycosidases and citric acid production

During the fermentation of awamori and shochu, the most important role of the kuro koji mold *A. luchuensis* is to produce sufficient glycolytic enzymes to convert starch in materials, such as glucose, as well as generating citric acid, which maintains the fermentation mash at a low pH. Previously, it was reported that *A. luchuensis* secretes a characteristic acid-stable α-amylase (AA1_SCon_015_0343), which can function at an acidic pH due to citric acid production. This acid-stable α-amylase has a starch-binding domain in the C-terminus. By contrast, *A. oryzae* possesses three copies of the taka-amylase gene, where the *amyA* gene comprises an *amy* gene cluster with the *amyR* (transcription factor of amylase genes) and *agdA* (α-glucosidase) genes. *A. luchuensis* was also predicted to possess an *amy* gene cluster comprising AA1_SCon_020_0630 (*amyR*), AA1_SCon_020_0632 (α-glucosidase), and AA1_SCon_020_0632.5 (α-amylase). Interestingly, the homology between AA1_SCon_020_0632.5 and the *amyA* gene product (taka-amylase) was relatively low at 73.5%, but a gene almost identical to taka-amylase was found on contig AA1_SCon_049.Con002, which was not assigned to the chromosome. Similarly, *A. kawachii* has a taka-amylase orthologue, AKAW_11452, in addition to an *amy* gene cluster and acid-stable α-amylase. Three other putative α-amylase genes (AA1_SCon_008_0403, AA1_SCon_018_0661, and AA1_SCon_011_0021) were also found, which shared 39%–46% identity with taka-amylase according to a BLASTP search. They possessed three conserved amino acid residues in the catalytic active site of α-amylase. *A. oryzae* possesses two glucoamylase genes, where *glaA* is expressed in submerged culture and *glaB* is expressed in solid-state culture.[27] We found that *A. luchuensis* contained only one glucoamylase gene (AA1_SCon_031_0271), which had a starch-binding domain in the C-terminus, whereas *A. oryzae* lacks this domain. More detailed studies are required to clarify the functions of these glycolytic enzymes in awamori and shochu fermentation.

During the production of awamori and shochu koji (solid-state culture of *Aspergillus* mold on grain), the temperature is typically increased to 40 °C in the early cultivation stages to increase the activity of amylase and then lowered to 30 °C in the later stages to promote the production of citric acid. However, the method employed for citric acid production is poorly understood in *A. luchuensis*, and the detailed mechanism is also unclear in *A. niger*. Kirimura reported that a cyanide- and antimycin A-insensitive and salicylhydroxamic acid-sensitive respiratory pathway catalyzed by an alternative oxidase (AOX1) functions in mitochondria, where the cytochrome pathway and inhibition of AOX1 dramatically decreased citric acid production in *A. niger*, although mycelial growth was not affected.[28] The orthologue of AOX1, AA1_SCon_015_0483, may also play an important role in citric acid production by *A. luchuensis*. The members of the glycolytic system and tricarboxylic acid cycle in *A. luchuensis* are listed in Supplementary Table S2. More detailed functional studies of these enzymes are required.

3.3 Protease genes

Genome sequencing of *A. luchuensis* NBRC 4314 showed that this strain contained almost the same protease genes as *A. niger* CBS 513.88. We estimated the putative amino acid sequences from the *A. luchuensis* genome sequence based on the fact that the amino acid sequences around the catalytic residues in the active sites of proteases are conserved.[29] Black *Aspergillus*, *A. luchuensis*, and *A. niger* possess many acidic proteases. The typical acidic proteases, aspartic endopeptidases,[30,31] and serine-type carboxypeptidases[32,33] have an optimum pH of below pH 4.0, and we found 10 and 13 of each, respectively, in the *A. luchuensis* genome. In addition, *A. niger* and *A. oryzae* possess 10 and 11 aspartic protease genes, respectively, and 12 serine carboxypeptidase genes. Each of the 12 serine-type carboxypeptidase genes in *A. luchuensis* had orthologs in the other two *Aspergillus* strains, and one additional gene in *A. luchuensis* was a homologue of gene corresponding to *ocpB*.[34] These two genes might have been generated by gene duplication. The sedolysin family enzymes are also acidic proteolytic enzymes, and the optimum pH range for these enzymes in *A. fumigatus* was reported as pH 5.5–6.0.[35] *A. luchuensis* and *A. niger* have seven sedolysin family enzyme genes, whereas *A. fumigatus* Af 239 and *A. oryzae* RIB 40 have only four and five genes, respectively. The neutral and alkaline protease genes were not as abundant as the acidic proteases. Three serine endopeptidase (kexin,[36] an oryzin[37] ortholog, and a putative vacuole enzyme[38]) genes were found in the *A. luchuensis* genome, similar to other *Aspergillus* strains. Among the metalloendopeptidases, there was one thermolysin-type enzyme gene,[39] but deuterolysins[40] were not found, similar to the *A. niger* genome. The genes of theses enzyme have been found in *A. sojae*, *A. oryzae*,[41] and *A. fumigatus*,[42] and even *Penicillium*.[43] As described above, *A. luchuensis* and *A. niger* share similar protease genes and black *Aspergillus* strains might differ genetically from other *Aspergillus* strains. However, in terms of glutamic peptidase genes,[44] *A. luchuensis* appeared to possess both *A. niger*-type and *A. oryzae*-type genes. *A. niger* and *A. oryzae* possess three glutamic peptidase genes, and *A. luchuensis* has five genes. The phylogenetic tree of the genes for these enzymes in the three *Aspergillus* strains is shown in Figure 2. AA1_SCon_006_0020- and AA1_SCon_019_0031-type genes are found in all three *Aspergillus* strains. *A. niger* lacks a counterpart of AA1_SCon_039_0035, whereas it is present in *A. oryzae*. By contrast, genes corresponding to AA1_SCon_011_0044 are found in *A. niger* but not in *A. oryzae*. AA1_SCon_006_0811 is an orthologue

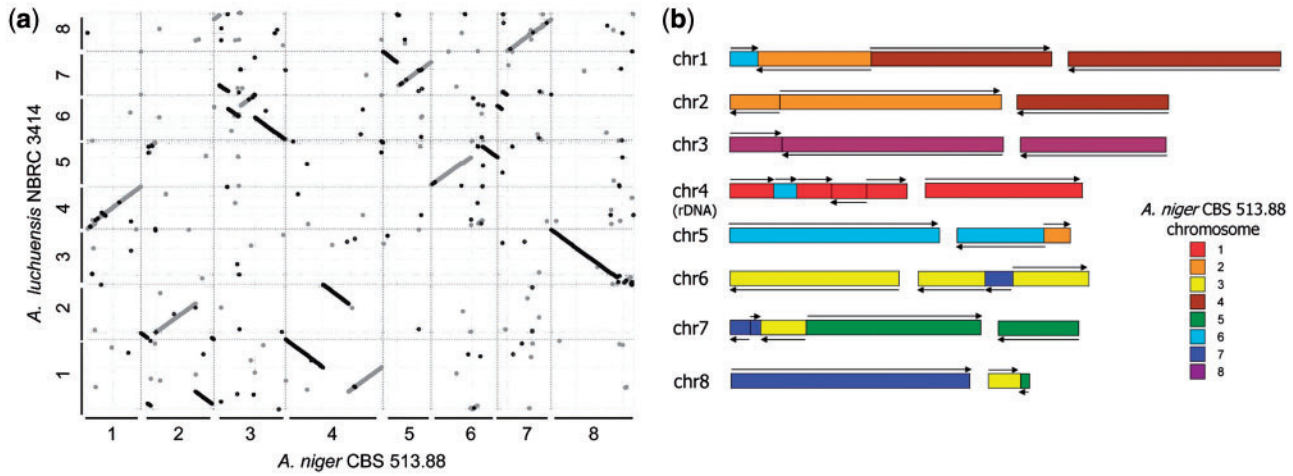

Figure 1. (A) Dot-plot alignments of chromosomes in *Aspergillus luchuensis* NBRC 4314 and *A. niger* CBS 513.88 using MUMmer 3.0. (B) Schematic representation of *A. luchuensis* NBRC 4314 chromosomes. The colored areas refer to the chromosomes assigned to *A. niger* CBS 513.88.

Table 2. Gene numbers for each COG in *Aspergillus luchuensis* NBRC 4314, *A. niger* CBS 513.88, and *A. kawachii* NBRC 4308

		A. luchuensis	*A. niger*	*A. kawachii*
Information storage and processing				
J	Translation, ribosomal structure, and biogenesis	308	302	300
A	RNA processing and modification	210	203	207
K	Transcription	240	238	244
L	Replication, recombination, and repair	193	193	190
B	Chromatin structure and dynamics	93	88	91
Cellular processes and signaling				
D	Cell cycle control, cell division, chromosome partitioning	159	156	152
Y	Nuclear structure	26	27	26
V	Defense mechanisms	50	57	49
T	Signal transduction mechanisms	370	370	372
M	Cell wall/membrane/envelope biogenesis	93	97	92
N	Cell motility	1	1	1
Z	Cytoskeleton	107	111	110
W	Extracellular structures	4	4	5
U	Intracellular trafficking, secretion, and vesicular transport	282	273	284
O	Posttranslational modification, protein turnover, chaperones	453	456	444
Metabolism				
C	Energy production and conversion	393	396	390
G	Carbohydrate transport and metabolism	285	284	291
E	Amino acid transport and metabolism	386	380	373
F	Nucleotide transport and metabolism	87	87	89
H	Coenzyme transport and metabolism	114	116	113
I	Lipid transport and metabolism	384	356	373
P	Inorganic ion transport and metabolism	144	143	142
Q	Secondary metabolite biosynthesis, transport, and catabolism	461	446	433
Poorly characterized				
R/S[a]	General function prediction only/function unknown	1,417	1,448	1,401
X[b]	Gene with unknown function	6,161	7,663	5,806
COG hit		5,530	5,497	5,446
Total		11,691	13,160	11,252

Genes that encoded polypeptides longer than 100 amino acids were used in the analysis. Genes that shared homology with ≥2 COGs were counted redundantly in each COG.

[a]Genes that shared homology with COGs other than R/S were excluded.

[b]Genes that shared no homology with any COGs including the genes sharing homology with R/S.

COG, cluster of orthologous group.

of AA1_Scon_019_0031. These results might indicate that glutamic peptidase genes have been amplified by gene duplication in *Aspergillus* strains, but *A. niger* and *A. oryzae* possess a reduced number of unnecessary genes, whereas they have been conserved in *A. luchuensis*. Thus, *Aspergillus* strains might be divided into *A. oryzae* and black *Aspergillus* groups and black *Aspergillus* might have lost the deuterolysin genes. *A. luchuensis* might have maintained glutamic peptidase genes that resemble the genes in *A. oryzae*. Glutamic peptidases are also acidic proteases, so maintaining genes for both types of enzyme might have improved the ability in acidic condition.

3.4. Protein N-and O-glycosylation

A. luchuensis and other *Aspergillus* species produce large amounts of extracellular proteins, such as glucoamylase and α-amylase. During the secretion process, the proteins are glycosylated in the endoplasmic reticulum (ER) and the Golgi apparatus. Protein glycosylation is believed to play critical roles in various cell activities, such as quality control in secretory proteins, cell wall integrity, environmental adaptation, and pathogenicity in some pathogenic fungi. These roles are related to the function, protein stability, and appropriate localization of secretory proteins, and antigenicity is modulated by protein glycosylation. Analysis of the *A. luchuensis* genome found that the genes involved in the protein *N*- and *O*-glycosylation are conserved relative to those in yeast, *Saccharomyces cerevisiae*, although the *N*- and *O*-glycan structures attached to the proteins differ between these two members of Ascomycota.[45,46]

The *N*-glycan structure in *S. cerevisiae* is characterized as a high mannose type. Protein *N*-glycosylation is initiated in the ER by an oligosaccharyltransferase (OST) complex using $Glc_3Man_9GlcNAc_2$-PP-Dolichol as a sugar donor. The OST complex comprises at least eight subunits in yeast. The orthologue of the catalytic subunit, Stt2, in the OST complex has been characterized in *A. fumigatus*.[47] All of the genes that encode the seven subunits other than yeast-specific *OST5* are conserved in *A. luchuensis* (Fig. 3). After the transfer of $Glc_3Man_9GlcNAc_2$ to Asn residues in a polypeptide, three glucose residues and one mannose residue are removed by the actions of the Gls2–Gtb1 complex and Mns1, respectively. This process is crucial for the quality control of secretory proteins in the ER and for the ER-associated degradation of misfolded proteins via recognition by lectins. The orthologues of yeast *GLS2*, *GTB1*, and *MNS1* have been functionally characterized in *A. oryzae*.[48,49] Thus, the genes (AA1_SCon_023_0105, AA1_SCon_007_0165, and AA1_SCon_010_0597) responsible for the trimming of *N*-glycans in the ER are well conserved in *A. luchuensis*. Glycoproteins that enter the Golgi apparatus are subjected to further elongation with *N*-glycans. Yeast glycoproteins localized in the cell wall and the periplasmic space contain a long branched polymer with approximately 200 mannoses and phosphate in the *N*-glycans. However, the *N*-glycans in *Aspergillus* species are $Man_{5\text{-}24}GlcNac_2$-N, which are shorter in size than those found in yeast.[50,51] The differences in the structure of *N*-glycans between these fungi are due to the presence of the long α-1,6-mannan and a unique phosphorylation pattern in yeast. The long α-1,6-mannan and phosphate residues in *N*-glycans are synthesized via the actions of Och1 and Mnn6, respectively (Fig. 3). The *A. fumigatus OCH1* orthologue compensates for the defective *OCH1* in yeast.[52] *A. luchuensis* also possesses an *OCH1* orthologue (AA1_SCon_006_0409), so this fungus may have the ability to elongate *N*-glycans in the same manner as yeast. Given that *A. luchuensis* possesses all of the genes involved in the elongation of *N*-glycans in the same manner as yeast and that the genes are expressed according to microarray analysis (Supplementary Table S3), then it is likely that the degradation of *N*-glycans by α-mannosidases will yield short *N*-glycans in *Aspergillus* species. *S. cerevisiae* possesses at least two genes, *DCW1* and *DFG5*, encoding putative GPI-anchored α-1,6-mannosidases.[53] We found that the *A. luchuensis* genome contained 11 ORFs with good hits against Dcw1 according to BLASTP searches using *S. cerevisiae* sequence datasets (Supplementary Table S4). Microarray data showed that all of these genes were expressed in the solid-state culture, koji. Among the 11 putative α-mannosidases, 10 α-mannosidases possessed the predicted signal sequence, thereby implying that these are secretory proteins. Four α-mannosidases, that is, AA1_SCon_019_0007, AA1_SCon_027_0100, AA1_SCon_016_0076, and AA1_SCon_020_0838, possessed a potential GPI-modification site as found in yeast, similar to Dcw1 and Dfg5, whereas no GPI-modification site was predicted in the other seven α-mannosidases. Thus, the localizations and functions of these α-mannosidases may differ from those in yeast, Dcw1 and Dfg5. Experimentally based functional characterizations of these mannosidases will allow us to understand the reason why *Aspergillus* species contain short *N*-glycans compared with those in yeast.

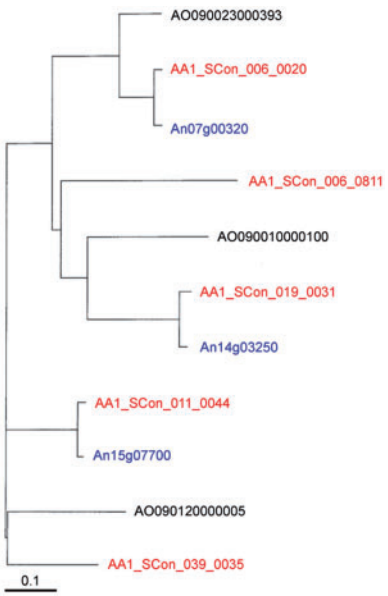

Figure 2. Phylogenetic tree of glutamic peptidase of *Aspergillus luchuensis* NBRC 4314 (red), *A. niger* CBS 513.88 (blue), and *A. oryzae* RIB 40 (black).

O-glycans attached to proteins from *Aspergillus* spp. are characterized by the presence of branching forms of oligosaccharides. The *O*-glycans comprise Man1-*O*, Manα1-2Man1-*O*, Manα1-6Man1-*O*, Man1-6(Glc1-3)Man1-*O*, Man1-6(Gal*p*1-3)Man1-*O*, Man1-6Man1-6Glc1-3Man1-*O*, and Manα1-2(Manα1-6)Man1-*O*.[54] The *O*-glycan found in the glycoproteins of *S. cerevisiae* possesses a linear chain with up to five mannose residues, the structure of which is Manα1-3Manα1-3Manα1-2Manα1-2Man1-*O*. Protein *O*-glycosylation is initiated in the ER by the action of the protein *O*-mannosyltransferase (Pmt) using dolichol phosphate mannose as a sugar donor.[55] Seven *pmt* genes are present in *S. cerevisiae*, which are classified into three protein subfamilies, i.e., Pmt1, Pmt2, and Pmt4 subfamilies. Each member of the Pmt4 subfamily forms a dimer complex with proteins from the same subfamily, whereas each member of the Pmt1 subfamily forms a dimer complex with Pmt2 subfamily members. The simultaneous disruption of more than three *pmt* genes yields a lethal phenotype in yeast. However,

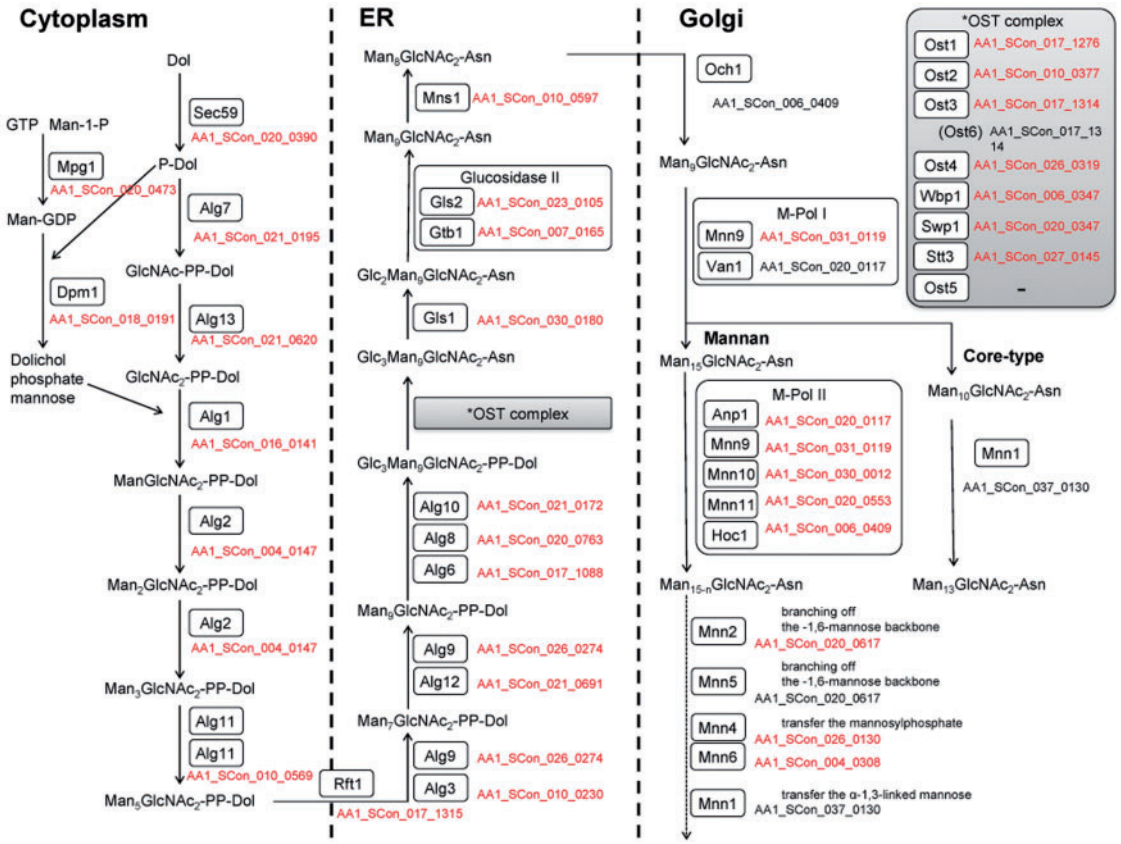

Figure 3. Putative *N*-glycosylation pathway in *Aspergillus luchuensis*. The proteins involved in *N*-glycosylation in *A. luchuensis* were identified by BLASTP search using the amino acid sequences of the components of the N-glycosylation pathway in *Saccharomyces cerevisiae*.[45,46] The proteins detected as reciprocal best BLAST hits are shown in red. The orthologues of Stt3, Gls2, Gtb1, Mns1, and Och1 were also confirmed by BLASTP analysis using the functionally characterized genes from *A. oryzae* and *A. fumigatus*. See Supplementary Table S3 for details of the BLASTP results. Asn, asparagine; Dol, dolichol; ER, endoplasmic reticulum; GDP, guanosine diphosphate; GlcNAc, *N*-acetylglucosamine; GTP, guanosine triphosphate; Man, mannose; OST, oligosaccharyltransferase; P, phosphate.

filamentous fungi only possess three *pmt* genes from each of the Pmt subfamilies. The three *pmt* genes characterized in *A. nidulans* and *A. fumigatus* play crucial roles in hyphal development, morphology, and asexual conidiation in these fungi.[56–60] Our genome analysis demonstrated that *A. luchuensis* also possesses three *pmt* genes, among which *pmtA* has been characterized (Supplementary Table S5).[61] The disruption of *pmtA* in *A. awamori* (*luchuensis*) does not affect the extracellular secretion level of glucoamylase, but it has negative effects on the growth rate, cell morphology, and conidia formation. After initial mannosylation by Pmt in the ER, further glycosylation occurs due to the action of sugar transferases in the Golgi apparatus during the secretory process. Multiple α-1,2- and α-1,3-mannosyltransferases have been characterized in yeast, but a sugar transferase responsible for this elongation process has not been characterized in *Aspergillus* spp. Our genome analysis of *A. luchuensis* detected three orthologues of yeast α-1,2 mannosyltransferase[62] (Fig. 4).

It has been reported that Gal*f* residues are included specifically in the *N*- and *O*-glycans in *Aspergillus* spp. as well as closely related filamentous fungi.[51,63] A single 1,2-linked Gal*f* residue is present at the nonreducing terminus in *N*-glycans and terminally *β*-1,5-linked Gal*f* residues are present in *O*-mannose-type glycans. The Gal*f* residues present in pathogenic *A. fumigatus* are immunogenic in mammals, and they are assumed to be involved in pathogenicity in humans, so the inhibition and detection of this sugar's biosynthesis might be

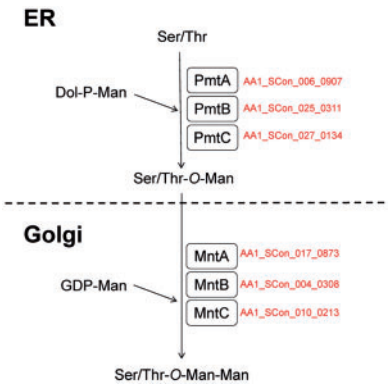

Figure 4. Putative *O*-glycosylation pathways in *Aspergillus luchuensis*. To identify the orthologues involved in *O*-glycosylation in *A. luchuensis*, BLASTP search was performed using the amino acid sequences of the components of the *O*-glycosylation pathway in *A. nidulans* as queries (Oka et al. 2004, Goto 2007, Goto et al. 2009). The protein names of the reciprocal best BLAST hits for the ORFs in *A. nidulans* are shown in red. See Supplementary Table S5 for details of the BLAST results. Dol, dolichol; ER, endoplasmic reticulum; GDP, guanosine diphosphate; Man, mannose; P, phosphate.

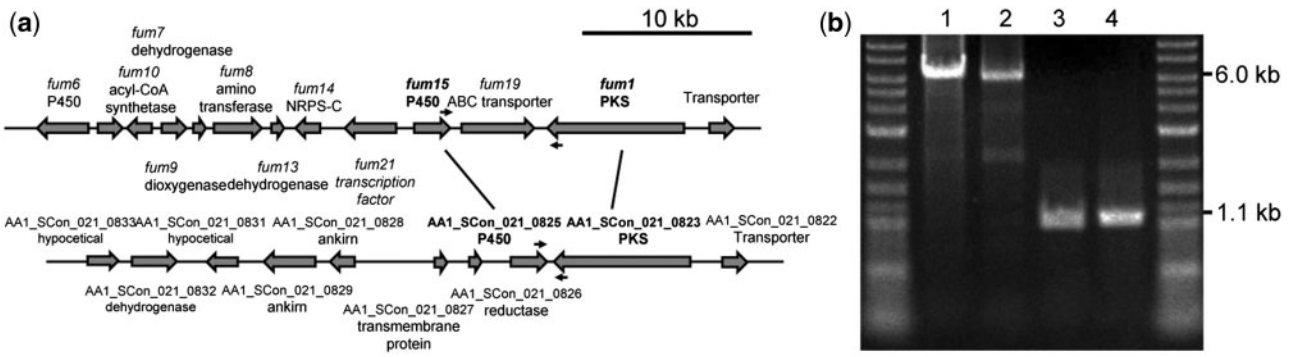

Figure 5. (A) The fumonisin gene cluster in *A. niger* CBS 513.88 and the genes around *fum1* and *fum15* homologues in *Aspergillus luchuensis* NBRC 3414. The predicted proteins are also indicated. Fum1_cons_primer and Fum15_cons_primer are indicated by black arrows. (B) Results of PCR amplification using the primer set Fum1_cons_primer and Fum15_cons_primer: lane 1, *A. niger* CBS 513.88; lane 2, *A. niger* ATCC 1015; lane 3, *A. luchuensis* NBRC 4314; and lane 4, *A. kawachii* genomic DNA as a template.

exploited in the development of chemotherapy and diagnostics related to *Aspergillus* infections.[64] The *gfsA* gene that encodes galactofuranosyltransferases involved in the addition of Gal*f* to *O*-mannose-type glycans has been identified in *A. nidulans* and *A. fumigatus*.[65] We found that *A. luchuensis* genome possesses three homologues of the *A. nidulans gfs* (Supplementary Table S5).

3.5. Mating-type loci

A. luchuensis is considered to be asexual, and it might be heterothallic in the same manner as *A. oryzae*. In heterothallic ascomycetes, the mating type is determined by the alternative presence of either of two genes called idiomorphs, which occupy the same locus in the chromosome, but that shares no sequence similarity with each other. The *MAT1-1* gene encodes a protein with an alpha box domain, and the *MAT1-2* gene encodes a protein with a high mobility group (HMG) domain. We found the *MAT1-2* HMG gene (AA1_SCon_025_0346) in the *A. luchuensis* NBRC 4314 genome. To investigate the possibility of breeding *A. luchuensis* strains by mating, we searched for the opposite mating type gene in 28 strains classified as *A. luchuensis* by PCR using the primer set: MAT1-1F-An (5′-GCGGCCACTGAACA GTTTCATTGCT-3′) and MAT1-1R-An (5′-TGATGGAGTATGCC TTGGCTACGATG-3′). Interestingly, we found no *MAT1-1* strains, and all 28 of the *A. luchuensis* strains that we analyzed had the *MAT1-2* mating type gene. Thus, our previous efforts to mate *A. luchuensis* were unsuccessful because we tried to cross strains with the same mating type. It was also suggested that if we could isolate strains with *MAT1-1*, this would allow the improvement of *A. luchuensis* strains by sexual crossing.

3.6. Safety and nonproductivity of mycotoxins

The secondary metabolites produced by filamentous fungi, especially mycotoxins, are most important from a safety viewpoint. *A. niger* has been reported to produce two types of mycotoxin, i.e., ochratoxin A (OTA) and fumonisin B.[66,67] Previously, we reported that the synthesis of OTA by *A. niger* is mediated partly by polyketide synthase, which is encoded by the An15g07920 gene, and we showed that *A. luchuensis* lacks this gene based on PCR and Southern analyses.[1] Interestingly, in the genome of *A. niger* CBS 513.88, An15g07920 forms a gene cluster with the cytochrome P450 and nonribosomal peptide synthase genes, whereas *A. niger* ATCC 1015 lacks this gene cluster. Genome sequencing demonstrated *A. luchuensis* has also lost a 21-kb region of the OTA cluster in a similar manner to both *A. niger* ATCC 1015 and *A. kawachii* IFO 4308.[3,24] Thus, these results show clearly that *A. luchuensis* lacks the ability to produce OTA.

The fumonisins are a group of polyketide-derived mycotoxins, which were first isolated in 1988 from *Fusarium verticillioides*.[68] In contrast to the OTA gene cluster, the genomes of both *A. niger* CBS 513.88 and ATCC 1015 include putative homologues of the *F. verticillioides* fumonisin gene cluster, and the actual production of fumonisin B2 has been confirmed. The fumonisin gene cluster of *A. niger* contains at least 14 *fum* genes. We searched the *A. luchuensis* genome for orthologues of *A. niger* fumonisin biosynthesis cluster genes using bidirectional best hit analysis. Only the *fum1* and *fum15* gene products shared high identity with AA1_SCon_021_0823 (68% identity, polyketide synthase) and AA1_SCon_021_0825 (72% identity, cytochrome P450) . The other FUM proteins shared low identities of 20%–43%, and these orthologues were distributed throughout the *A. luchuensis* genome. Analyses of the secondary metabolite biosynthesis gene clusters using antiSMASH only predicted that AA1_SCon_021_0823 and AA1_SCon_021_0825 comprised a gene cluster (Fig. 5A). These results indicate that *A. luchuensis* lacks most of the *fum* genes and productivity of fumonisin, even if these two gene products are functional. Interestingly, the orthologues of *fum1* and *fum15* lie next to each other in opposite directions in the *A. luchuensis* genome, whereas the *fum9* gene is located between *fum1* and *fum15* genes in *A. niger*. According to this feature, we designed a primer set, i.e., Fum1_cons_primer (5′-GGCGGCATTGAGATCAGCACATTGGA-3′) and Fum15_cons_primer (5′-GAAGGTAACCCGCACAGTAACTGCCAGGCC-3′), to amplify a 1.1-kb gene fragment from the *A. luchuensis* genome as a template and a 6.0-kb fragment from *A. niger* (Fig. 5B). Using this primer set, all of the *A. luchuensis* strains had the same 1.1-kb signal, thereby indicating that *A. luchuensis* has the same genome structure, and it lacks the ability to produce fumonisin (data not shown). Recently, Shimizu et al. reported that disruption of the *fum8* gene, which encodes α-oxoamine synthase, resulted in the loss of fumonisin B2 from *A. niger*, but *A. luchuensis* appears to lack this gene.[69] Susca et al. suggested that fumonisin production was once more widespread among black aspergilli and that nonrandom partial deletion of this cluster has occurred multiple times based on partial fumonisin gene cluster homologues identified in several black *Aspergillus* species genomes.[70]

The ex-type strain of *A. luchuensis* is NBRC 4281 (RIB 2642), but we used NBRC 4314 in the present study, which is the oldest strain, and it was isolated in 1901. We also confirmed that Nakazawa's α strain was stored as JCM 2261 (IAM 2112)[71] and

that it belongs to *A. luchuensis*. JCM 22320 (IAM 2351) was reported by Sakaguchi in 1949, and it is now known that the kuro koji mold that survived World War II is also *A. luchuensis*.[72] In 1975, Sugama isolated two types of kuro koji mold from awamori koji (awamori type and acid-producing saitoi type), and we confirmed that both of these strains belong to *A. luchuensis*.[73] Tukahara reported that ISH1 (awamori kin, a high glycosidase-producing strain) and ISH2 (saitoi kin, a high acid-producing strain), which are the strains used most widely for awamori fermentation at present, are also *A. luchuensis* according to next-generation sequencing[74] Thus, *A. luchuensis* had been used historically and widely in awamori production, which clearly indicates that *A. luchuensis* is most appropriate for making awamori. The genome sequence obtained in this study will provide a platform to help understand the important characteristics of *A. luchuensis*, such as citric acid production. A transformation system and an efficient gene-targeting system for *A. luchuensis* have already been established.[75] We hope that more advanced molecular biological research will be conducted using the kuro koji mold, *A. luchuensis*.

The sequences and annotations reported in this article have been deposited in DDBJ/EMBL/GenBank under the accession nos BCWF01000001-BCWF01000044. Information about the sequences and gene annotations are also available in the genome database of microorganisms sequenced at NITE (DOGAN; http://www.bio.nite.go.jp/dogan/top).

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Supplementary data

Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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