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Comparative mitogenomics of *Pleurotus ostreatus* Gonji7ho and its cap color mutant

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ABSTRACT: *Pleurotus ostreatus* is a globally cultivated mushroom crop. Cap color is a quality factor in *P. ostreatus*. However, cap color can spontaneously mutate, degrading the quality of the mushroom on the market. Early detection and removal of mutant strains is the best way to maintain the commercial value of the crop. To detect the cap color mutant Gonji7ho, molecular markers were developed based on insertion/deletions (InDels) derived from the comparison of mitogenomes of Gonji7ho and Gonji7hoM mushrooms. Sequencing, assembly, and comparative analysis of the two mitogenomes revealed genome sizes of 73,212 bp and 72,576 bp with 61 and 57 genes or open reading frames (ORFs) in *P. ostreatus* Gonji7ho and Gonji7hoM, respectively. Fourteen core protein-encoding genes, two rRNA, and 24 tRNA with some OFRs were predicted. Of the 61 genes or OFRs in the wild type, *dpo*, *rpo*, and two *orf139* were missing (or remnant) in the mutant strain. Molecular markers were developed based on the sequence variations (InDels) between the two mitogenomes. Six polymorphic molecular markers could detect the mutated mitochondria by PCR. These results provide basic knowledge of the mitogenomes of wild-type and mutant *P. ostreatus*, and can be applied to discriminate mutated mitochondria.

KEYWORDS: Cap color, Mitogenome, Molecular marker, *Pleurotus ostreatus*

INTRODUCTION

Pleurotus ostreatus (oyster mushroom) is a main edible mushroom, and its production is second most after oak mushroom (Royse *et al.,* 2017). China is the largest producer of *P. ostreatus*, with 5,633,000 M/T (2011) produced worldwide (Wu *et al.,* 2013), and a total of 48,327 M/T (2019) produced in Korea (MIFAFF, http://www.mafra.go.kr/). This mushroom is easily grown on a wide range of media, including agricultural byproducts (Tuyen *et al.*, 2013), and can provide various nutrients (Bonatti *et al.,* 2004). As the consumption of *P. ostreatus* increases, numerous cultivars with improved traits have been bred. Out of the 52 cultivars registered in the Korea Seed and Variety Service (https://www.seed.go.kr), Gonji7ho, Hwaseong2ho and Heuktari are the major cultivars in Korea (Choi *et al.,* 2021).

Fruiting bodies with dark cap colors are favored by consumers and markets (Choi *et al.,* 2015); therefore, farmers have adopted low temperatures in mushroom cultivation to produce darker cap colors. In some cases, a dark gray or brown cap color can change to a pale color or even white by spontaneous mutagenesis (Kim *et al.,* 2008). Cap color mutants of *P. ostreatus* that develop pale or white fruiting bodies could result in serious economic loss because they are considered out of grade on the market. Moreover, mushroom spawns (mycelial form) may not be distinguishable between the mutant and wild type due to few (or no) morphological differences. Thus, it is important to develop a fast detection of the mutant stains showing abnormal colors.

Mitochondria play roles in the generation of energy molecules, thereby serving as critical organelles in eukaryotic cells. They have their own genome, which is biparentally and/or uniparentally inherited depending on

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where mycelia are isolated (Ye *et al.,* 2020). Mitochondria have been related to programmed cell death and aging in mushrooms, and their genes influence the mycelial growth of *A. bisporus* (De La Bastide *et al.,* 1997). Furthermore, the cap color of *Flammulina velutipes* was influenced by the activity of superoxide dismutase (SOD), which is normally located in the mitochondrial membrane (Cheng *et al.,* 2012, Kitamoto, 1997).

Melanin is main pigment in mushrooms including *P. ostreatus* and *A. bisporus*, is synthesized by many enzymes, 42 genes for *A. bisporus* (Gao *et al.,* 2015; Cherno *et al.,* 2013; Mukherjee *et al.,* 2017). Loss-of-function mutation in the melanin biosynthetic genes may lead to the appearance of albino individuals (Mukherjee *et al.,* 2017; Kawaguchi *et al.,* 2019). It is consisted with that multi QTL loci were involved in mushroom cap color (Gao *et al.,* 2015; Im *et al.,* 2016).

In this study, we assembled and annotated the mitogenomes of *P. ostreatus* Gonji7ho and its cap color mutant with Illumina sequencing. Comparative analysis was conducted to identify physical variations between the two mitogenomes and develop molecular markers for the discrimination of the cap color mutant from the normal cultivar.

MATERIALS AND METHODS

Fungal strains and growth conditions

P. ostreatus Gonji7ho (wild type) and its spontaneous cap color mutant Gonji7hoM were obtained from GARES (Choi *et al.,* 2013). Gonji7hoM was isolated from a mushroom farm. The two strains were cultured on mushroom complete media (MCM; 0.2% peptone, 0.2% yeast extract, 2.0% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, and 0.046% KH_2PO_4), followed by incubation at 25.0°C and periodic transfers on the same media with the same conditions.

Fructification and phenotypic evaluation

Gonji7ho and Gonji7hoM were fruited in the mushroom culturing facility at the KNCAF (Korea National College of Agriculture and Fisheries). The culturing room was under controlled temperature, relative humidity, CO_2 concentration, and light. The strains were grown and inoculated on sterilized media prepared as described (Ryu *et al.,* 2015), with minor modifications. Media consisting of sawdust (poplar):barley bran:cottonseed hulls = 70:20:10 (V/V) were poured into 1,100 ml polypropylene bottles, followed by steam sterilization at 121°C for 90 min. Sixteen repetitions (bottles) were carried out for each strain. The fruiting body was harvested before its cap opened completely (approximately 60-70% open), and then three single mushrooms that were representative of each bottle were chosen to measure weight (Park *et al.,* 2019); the diameters of the cap and stipe were measured using digital calipers (Tokyo, Japan). Yield was measured by weighing all mushrooms from each bottle. The color of the cap was determined at three points in between the center and the margin per cap using a colorimeter (HangZhou CS-10, China) based on the L (lightness), a (redness), and b (yellowness) system. The earliness was estimated by counting the numbers of days required for harvest (from removing old medium to harvest). The statistical significance of traits of interest in Gonji7ho and Gonji7hoM was determined using the two-sample t-test for comparisons among group means. The standard deviation for all 7 traits was calculated. Data analyses were performed with R open-source software (Team *et al.,* 2013) and Microsoft Excel 2007.

Genomic DNA sequencing and *de novo* assembly

Genomic DNA was extracted from lyophilized mycelia using a GenEx Plant plus! Kit (GeneAll, Seoul, Korea) as described (Park *et al.,* 2019). Library construction and paired-end sequencing were performed on an Illumina 2,500 sequencing platform as described (Holm *et al.,* 2019). Raw reads of each sample were trimmed to obtain high-quality PE reads (Phred scores less than 20). Then, *de novo* assembly was conducted using the QIAGEN CLC assembly cell package (v. 4.21, QIAGEN, Hilden, Germany), as described (Lee *et al.,* 2018). Mitochondrial genome contigs were screened by comparison with reference contigs from *Pleurotus platypus* (MG017445), *P. ostreatus* (EF204913), *Pleurotus eryngii* (KX827267.1), *Pleurotus cornucopiae* (NC_038091), and *Tricholomella constricta* (NC_039443) and merged into draft genome sequences. The draft genome sequence was manually polished and gap-filled using PE read mapping. SNP and InDel calling and filtering were performed using the GATK toolkit (McKenna *et al.,* 2010). Two mitogenome sequences were deposited on NCBI (2506878 for Gonji7ho and 2506878 for GOnji7hoM).

Gene annotation, primer design and PCR conditions

The mitochondrial genes were initially annotated using the GeSeq program (Tillich *et al.,* 2017), followed by

Table 1. List of primers for the discrimination of mutated mitochondria

Primer name		Primer sequence (5' → 3')	Expected amplicon size (bp) Gonji7ho/Gonji7hoM	Location (gene)
CAP 1	F	TCCGCAGTTAAAGGTGGAGT	252/ -	*dpo*/genic
	R	GGTTTCAATATCCATGGTTGC		
CAP 2	F	TTAAACCCCCACTCTGCTATG	259/185	orf126~*nad2*/inter genic
	R	GGGATGTTGCTGCCTTTTTA		
CAP 3	F	TTGCTATGCTACTTTCCTCTCCA	150/200	trnT-UGU~trnY-GUA/inter genic
	R	CGTATGTTTGGCTCGGTGTA		
CAP 4	F	CCAAACGTAAGCTATCATTCCA	216/184	trnC-GCA~trnG-UCU/inter genic
	R	TGTTTGTAAGGGCGGATTCT		
CAP 5	F	AGGCAGGTGAAGGACACCTA	295/274	trnW-CCA~nad1/intergic
	R	TTAATGCAATTTCCAAAGAAATAA		
CAP 6	F	AGCAGAGGATGCAGCGTAAT	252/171	cob~cox2/inter genic
	R	AAACGTACGAGTGCGTAGCC		

confirmation using the Artemis annotation tool (Carver *et al.,* 2012). A gene map was drawn by the OrganellarGenomeDRAW program (Greiner *et al.,* 2019) with basic parameters. Comparative mitogenome analysis was conducted with LASTZ (Schwartz *et al.,* 2003).

PCR primer sets (CAP 1~6) were designed using PRIMER3 (Rozen and Skaletsky, 2000) based on the consensus sequences flanking the variable InDel regions and *in silico* ePCR using GMATA (Wang *et al.,* 2016). Polymorphic amplicons with 100~300 bp in size were expected in the wild type and mutant mitochondria (Table 1). CAP 1 was expected to amplify the *dpo* gene region, which was expected to produce a single amplicon only in Gonji7ho, while the remaining markers were located in intergenic regions and were expected to generate polymorphic fragments in the wild type and mutant mitochondria (Table 1).

PCR was performed using *e-Taq* DNA polymerase (SolGent, Korea) under the following conditions: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 30 s, and one cycle of final extension at 72°C for 5 min. The PCR products were resolved on a 1.5% agarose gel containing SafeView Classic (Intron Biotechnology, Seongnam, Korea).

RESULTS AND DISCUSSION

Phenotypic differences between Gonji7ho and its cap color mutant Gonji7hoM

P. ostreatus Gonji7ho (wild type) and Gonji7hoM (mutant) were cultivated using a typical method and evaluated for weight, morphological characteristics and cultivation properties (Table 2). Though similar in appearance except cap color (Fig. 1), the two strains were statistically difference in most phenotypes (Table 2). Stipe thicknesses were 16.8±2.1 mm and 13.7±1.9 mm, and cap diameters were 46.6±8.3 and 39.1±4.7 mm in the wild type and mutant, respectively. The wild type had more weight per stipe (122.6%, data not shown) and yield (total weight per bottle) (111.6%) than the mutant. The cap color (L, lightness) was 55.1 and 69.5 in the wild type and mutant, respectively, with a significant difference (p-value < 2.2e-16 in a two sample t-test). The earliness (required days to harvest) of the wild type was 9.6 days, which was 0.6 days later than that of the mutant (p-value < 8.66e-5 in the two-sample t-test). This change is only valuable in the mutant, as enhanced earliness might increase productivity. Clearly, the morphological characteristics, including cap color, were highly dependent on environmental

Fig. 1. Phenotypic characteristics of the *P. ostreatus* cultivar Gonji7ho (right) and its cap color mutant Gonji7hoM (left).

Table 2. Morphological characteristics of Gonji7ho and its cap color mutant Gonji7hoM

Cultivar	Length (mm)	Stipe thickness (mm)	Cap (pileus) diameter (mm)	Yield (g)	Cap color			Earliness
					L*	a	b	
Gonji7ho	64.4±8.6	16.8±2.1	46.6±8.3	120.8±7.3	55.1±2.6	0.6±1.2	7.7±1.6	9.6±0.6
Gonji7hoM	64.0±4.2	13.7±1.9	39.1±4.7	107.5±2.5	69.5±1.8	0.6±0.6	8.2±2.2	9.0±0.0

conditions and culture stage. To remove bias, we harvested mushrooms twice a day based on cap morphology, with approximately 70% of caps being open and cultivated under homogeneous conditions with sufficient air flow. The CV (coefficient of variance) of traits ranged from 2.4 (yield) to 17.9 (cap diameter), with values of 2.5 (mutant) and 4.7 (wild type) for cap lightness. These values were reliable in terms of mushroom fruiting body data (Im *et al.,* 2016).

Yield represents the total weight of the fruiting body in a bottle. Means are presented with standard deviations. *The cap color of the two strains is significantly different (p-value < 2.2e-16 in a two sample t-test). Other traits except length, show statistically different (maximum p-value is < 0.02 for yield). Earliness was estimated by counting the numbers of days required for harvest (from removing old medium to harvest).

Mitogenome structure and organization

Raw reads of 4.1~5.6 Gb were trimmed to obtain high-quality PE reads (Phred scores less than 20). Then, 87.9~211.1 Mbp of clean data were subjected to *de novo* assembly using the QIAGEN CLC assembly cell package (v. 4.21, QIAGEN, Hilden, Germany), as described (Lee *et al.,* 2018). There were 20,173 aligned reads for Gonji7ho and 38,096 for Gonji7hoM. The contigs were confirmed as mitochondrial origin by comparison with other contigs. The assembled mitochondrial genome lengths of Gonji7ho and Gonji7hoM were 73,212 and 72,576 bp with 79.9x and 155.6x depths, respectively. The GC/AT ratios were 26.5% (Gonji7ho) and 26.6% (Gonji7hoM). These results are similar to the previously reported mitogenome of *P. ostreatus* (Wang *et al.,* 2008). A sequence alignment analysis showed that Gonji7ho and Gonji7hoM have similar sequence orders, with approximately 2.6~2.9 kb insertions and deletions (Supplemental Table S2). Numerous large-scale rearrangements (inversions) of gene order were detected within the mitogenomes of Gonji7ho and other species of the genus *Pleurotus* (Supplemental Figure S1), was as has been previously observed within the genus (Qieng *et al.,* 2018). These inversions are considered natural biological events during the mitochondrial evolution of *Pleurotus* spp. (Qieng *et al.,* 2018). The relationship between the massive genome-wide shuffling and phenotypes has not yet been determined.

Gene content and order

Many CDSs (> 92%) were identified on the forward sequence in both Gonji7ho and Gonji7hoM contigs (Fig 2, Supplemental Table S1). Core protein-coding genes (PCGs) were identified: three cytochrome c oxidase subunits 1, 2, and 3 (cox1, cox2, and cox3); apocytochrome b (cob); three subunits of ATP synthase (atp6, atp8, and atp9); and seven subunits of NADH dehydrogenase (nad1, nad2, nad3, nad4, nad4L, nad5, and nad6). These gene numbers and orders were consistent with those of other *Pleurotus* species (Wang *et al.,* 2008, Li *et al.,* 2018). In addition, 2 ribosomal RNA genes (rnl and rns) located on the opposite side and 24 tRNAs were identified. Gonji7ho also had two nucleotide-dependent DNA (*dpo*) and RNA polymerase (*rpo*) sequences. Additionally, 17 hypothetical proteins (9 intronic ORFs and 8 non-intronic ORFs) were detected, which is different between species or strains (Li *et al.,* 2018). *nad4L* was found to overlap with the nad5 gene by 1 nucleotide (at the stop and start codons); this arrangement has been frequently observed in various fungal species (Li *et al.,* 2018, Wang *et al.,* 2008). The gene orders of *dpo* were different from those of *rpo* in Gonji7ho and not similar to those of previously reported *P. ostreatus* varieties (Li *et al.,* 2018, Wang *et al.,* 2008), in which *dpo* was adjacent to *rpo*.

On the other hand, Gonji7hoM contained 4 fewer genes than the wild type: DNA polymerase (*dpo*), RNA polymerase (*rpo*) and two hypothetical proteins (orf139). A pseudo *dpo* gene was found in Gonji7hoM and showed 80% similarity with that in Gonji7ho due to a deletion and an early stop codon (data not shown).

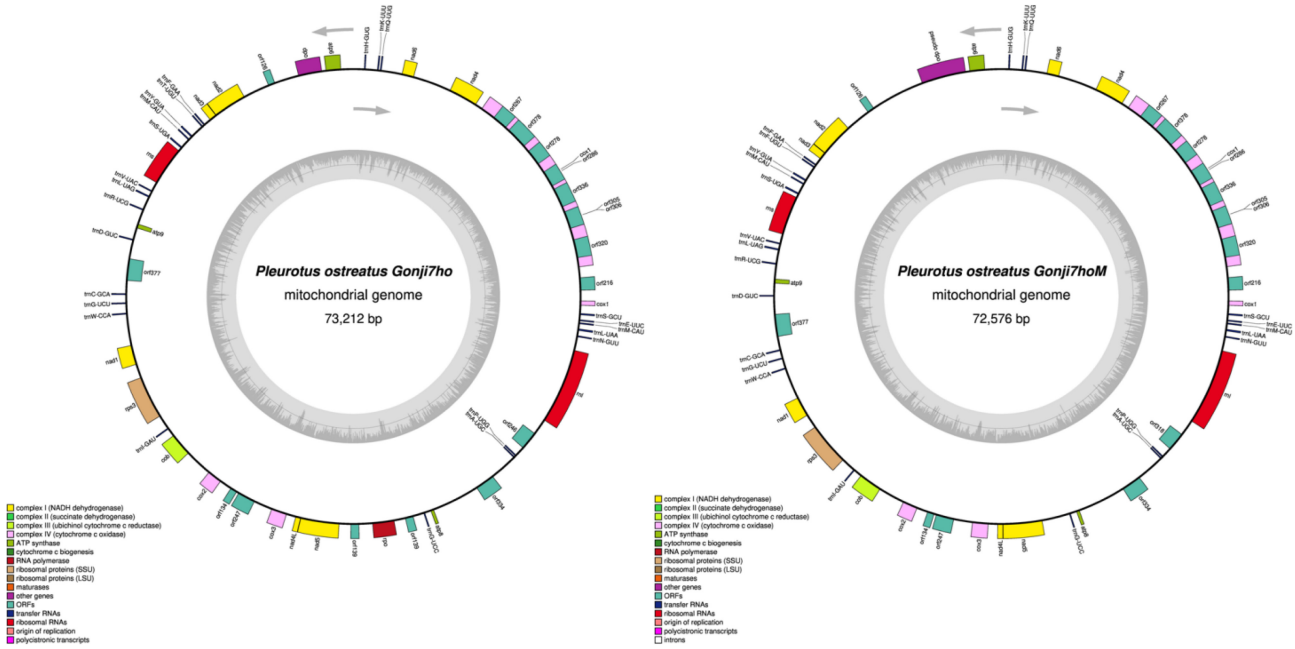

Fig. 2. Physical map of the mtDNA of *P. ostreatus* cultivar Gonji7ho (right) and its cap color mutant Gonji7hoM (left). The mitogenomic map was drawn using OGDraw online (https://chlorobox.mpimp-golm. mpg.de/OGDraw.html) with basic parameters. Genes are shown in the colored box and grouped by function. The protein-coding genes are shown outside and inside the circle with colored boxes. The direction of each gene is indicated by arrows. The inner circle in gray shows the GC ratio (%).

There is no data suggesting that these four genes are involved in cap color traits. The influence of a lack of *dpo* and/or *rpo* in the mitogenome on cell phenotypes has not yet been reported. These two nucleotide polymerase genes were encoded in mitochondrial plasmids in *P. ostreatus* (Wang *et al.,* 2008). Phylogenetic analysis revealed that *dpo* and *rpo* were derived from mitochondrial plasmids by horizontal gene transfer (Robison *et al.,* 2005). Deficiency of *dpo* and *rpo* is not likely to be lethal because the DNA and RNA polymerases in mitochondria are encoded by the nuclear genome, so the replication and transcription of the mitogenome are tightly dependent on the nucleus (Lee and Kocher, 1995). The gene and ORF number, order and organization, including *dpo* and *rpo*, vary in the genus *Pleurotus* and even within *P. ostreatus* and it is mainly depended on ORF number (Li *et al.,* 2018, Wang *et al.,* 2008).

Sequence variation and detection of mutated mitochondria by molecular markers

Sequence variations between the wild type and mutant included 331 SNPs and 225 InDels (Supplemental Table S2). Pyrimidine bases (C and T) were altered more often, 190 locations, than purine bases (A and G), 141 locations. Transition mutations (purine to purine) occurred 144 times, while transversion mutations (purine to pyrimidine or vice versa) occurred 187 times. Theoretically, if the number of transitions is 2 times that of transversions, the TS/TV ratio is 0.5. Here, the ratio was 0.78; thus, bias was observed. This bias is considered to be a natural case and originates from physicochemical and biological causes (Zou *et al.,* 2021). The largest insertion was 2.6 kb, whereas the largest deletion was 2.9 kb (Supplemental Table S2), showing consistency with the genome alignment results (Supplemental Figure S1).

The developed PCR primer sets were tested using Gonji7ho and Gonji7hoM total DNA. For CAP 1~6, a single amplicon each was observed for each strain with the expected size range (Fig 3). Unexpectedly, the CAP 1 primer amplified an weak amplicon with Gonji7hoM. Due to the pseudo *dpo* gene (target region of CAP1) in Gonji7hoM, non-specific band might be amplified. Because the mitochondrial mutation rate was higher than that of the nucleus (de Melo *et al.,* 2021), the combination of 6 primer sets was useful for detecting cap color mutants of Gnji7ho. Cross-reactivity was observed

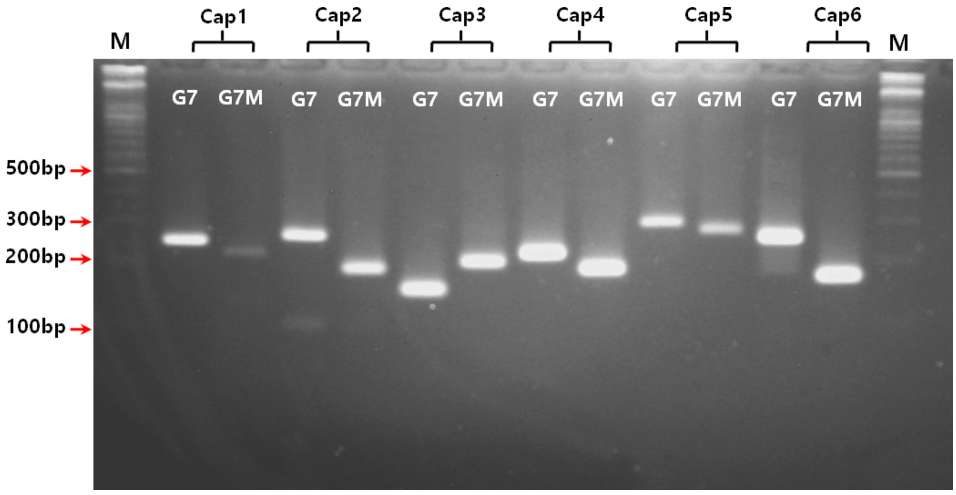

Fig. 3. DNA fragments amplified by PCR using the CAP 1-6 primer set specific for *P. ostreatus* cultivar Gonji7ho and its cap color mutant Gonji7hoM. M, 100-bp plus DNA ladder (Bioneer, Daejeon, Korea); G, *P. ostreatus* Gonji7ho; G7M, *P. ostreatus* Gonji7hoM.

with the other three *P. ostreatus* cultivars (Heuktari, Chunju2o and Suhan1ho) with polymorphic bands, but very weak bands appeared for *Pleurotus eryngii* and *Pleurotus pulmonarius* (data not shown). However, the applicability of the markers for other cultivar is not ensured, due to no test for their cap color mutant.

Mushroom cap color is controlled by multiple factors, melanin biosynthesis pathway (Gao *et al.,* 2015; Mukherjee *et al.,* 2017) and redox signaling agents. Mutation in melanin pathway (42 genes for *A. bisporus*) could lead to the abnormal cap color (Mukherjee *et al.,* 2017; Kawaguchi *et al.,* 2019). In addition, cytochrome P450 and SOD in mitochondria were reported as the candidate genes for the melanogenesis in mushrooms (Kitamoto, 1997; Wang, 2021). Cap color is controlled by many QTLs (Gao *et al.,* 2015; Im *et al.,* 2016), it may reflects various factors. Moreover, melanin production was mediated by mitochondria even that is not a main factor (Rosania, 2005). In this context, it could not exclude that mitochondria involve in color trait in organisms. Linkage between traits and sequence variations of the mitogenomes and genomes of Gonji7ho and Gonji7hoM could be discovered by further studies. Cytochrome 450 and SOD activity in the mitochondria of the wild type and mutant could be a good representation of cap color variation as well.

In conclusion, the mitogenomes of *P. ostreatus* Gonji7ho and its cap color mutant Gonji7hoM contained 61 and 57 genes and ORFs, respectively, and *dpo*, *rpo*, and 2 orf139s were missing in the mutant. Six polymorphic molecular markers could detect the mutated mitochondria by PCR. Our results provide basic knowledge about the mitogenomes of wild-type and cap color mutants in *P. ostreatus.*

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