

DISTINGUISHING LEVEL OF PLOIDY IN *TRICHOLOMA MATSUTAKE*

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ABSTRACT

In order to analyse the ploidy level of the *Tricholoma matsutake* strain NBRC30773 which was obtained by fruit body tissue method, a molecular marker based on the sequence of the region (about 1760bp) upstream of homeodomain protein gene (*Tmhox1*) was developed. Genomic DNA was prepared from *T. matsutake* NBRC30773 strain and was used as template to amplify fragment with primers (*Tm-dis1* and *Tm-dis2*). We obtained two different nucleotide sequence (*Tm-1* and *Tm-2*) with 92% of similarity between each other. These results indicated that the *T. matsutake* strain NBRC30773 was heterokaryotic. On the other hand, in *Tm-1* and *Tm-2*, different cutting sites of restriction enzymes (*Pst*I, *Bam*HI, *Bgl*II and *Eco*T22I) were also investigated. Base on these results, the technique of restriction fragment length polymorphism (RFLP) can be used to distinguish the level of ploidy in *T. matsutake*. In the future, the *T. matsutake* strains which were obtained by spore germination and protoplast regeneration will be distinguished.

Keywords: *Tricholoma matsutake*; level of ploidy; heterokaryon; homokaryon; strain

INTRODUCTION

An ectomycorrhizal fungus, *Tricholoma matsutake* (S. Ito & Imai) Sing. is the most prized and the most expensive mushroom in Japan. The annual harvest of *T. matsutake* was 12,000 tons in 1941, but the harvest in the past decade has reduced to between 30 to 100 tons per year and it became 24 tons in 2009 [1]. Most progress towards understanding the sexuality of some ectomycorrhizal fungi has been made with the germination of the basidiospores and the establishment of homokaryotic cultures [2]. Moreover, genetic analysis and selective breeding of cultivated mushrooms require the isolation of homokaryons from heterokaryotic stocks [3]. It means that level of ploidy of strain is important information for study of fungus. Obtain of homokaryotic strain is important for the study of genetics and breeding application of *T. matsutake*.

In the study of *Tuber melanosporum*, Bonfante Fasolo and Brunel showed that only heterkaryotic mycelium resulting from plasmogamy is able to form mycorrhizas [4], but for ascomycetes, microscopic determination of homokaryotic or heterokaryotic mycelium is difficult. One major reason was that unlike the basidiomycetes, no clamp connections can be found in vegetative hyphae in

ascomycetes [5]. Similarly, less clamp connections can be found in hyphae of *T. matsutake* [6, 11]. So determination of homokaryotic or heterokaryotic mycelium by microscope is nearly infeasible. In most heterothallic mushroom species, inbreeding is avoided by a sexual incompatibility system determined by two loci each with multiple alleles (the A and B mating-type loci) [12]. In tetrapolar mushroom, the A locus comprises multigenes encoding homeodomain proteins. On the basis of the homeodomain sequence, the mating-type proteins of the A locus are divided into two subgroups: HD1 and HD2 [13], [14]. The N-terminal region of the homeodomain (HD1) protein displays higher level of sequence variation between alleles than C-terminal [15]. The high levels of sequence variation in N-terminal region of the HD1 makes this region a candidate for restriction fragment length polymorphism (RFLP) analysis between different level of ploidy.

On the other hand, however, in *T. matsutake*, little is known about the level of ploidy in *T. matsutake* strain which were isolated by either fruit body tissue method, spore germination or protoplast regeneration. So study of the level of ploidy in *T. matsutake* will provide the basic knowledge for *T. matsutake* genetics and breeding application. In this study, we have developed a molecular marker based on the sequence near the homeodomain gene that could be used to resolve this question of level of ploidy in *T. matsutake* strain.

MATERIALS AND METHODS

Fungal strains and culture conditions. *T. matsutake* NBRC 30773 was used in this study. *T. matsutake* NBRC 30773 was obtained from the National Institute of Technology and Evaluation of Japan and isolated by fruit body tissue method. The mycelium of *T. matsutake* NBRC 30773 were routinely cultured on modified Hamada's agar medium (0.5% KH₂PO₄, 0.2% yeast extract, 2% glucose, and 1.5% agar, pH 5.1) prepared with tap water [7]. For the preparation of genomic DNA, 3 square agar blocks (5 x 5 x 5 mm) of fungal mycelium culture were cut and transferred into 20 ml modified Hamada's liquid medium in a 100-ml Erlenmeyer flask and were then incubated at 25 °C for 30 days. Mycelia were then collected, lyophilized, and used for genomic DNA extraction.

Preparation of DNA. Genomic DNA was prepared from lyophilized mycelia of *T. matsutake* strain NBRC 30773 using a GENEALL Plant SV Mini kit (Toyobo Co., Osaka, Japan) according to the manufacturer's instructions.

Amplification of the *T. matsutake* homeodomain protein gene (Tmhox1). Initially, fragments of genomic DNA encoding the mitochondrial intermediate peptidase protein (Tmmip) were amplified by PCR with the degenerate oligonucleotide primer pair MIP2F and MIP2R. The MIP2F and MIP2R primers were designed based on the amino acid sequences GLGEPKF and FDLWEEI, respectively, which are conserved in the mitochondrial intermediate peptidase protein of *Coprinopsis cinerea* and *Schizophyllum commune* [16], [17]. PCR was performed in a 50- μ l reaction containing 1 x Ex Taq buffer (Takara Bio Co.), 50 ng genomic DNA, 50 pmol each primer, 0.2 mM each dNTP, and 1.25 U Ex Taq polymerase (Takara Bio Co.). The PCR reaction was performed with a Takara PCR Thermal Cycler Personal (Takara Bio Co.) and consisted of an initial denaturation for 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 2 min at 50 °C, and 45 s at 72 °C, and a final elongation step at 72 °C for 10 min. The amplified PCR fragment (approximately 250 bp) was directly cloned into the pT7Blue (R) T-vector (Novagen, Madison, WI, USA), generating pTMGLU1. To amplify three segments of genomic DNA covering complete sequence of the Tmhox1 gene and its upstream region, 3 oligonucleotide primer pairs (Tmmip1nd1/ Tmmip1nd2, Tmmip2nd1/ Tmmip2nd2, Tmmip3nd1/

Tmmip3nd2) were designed based on the partial Tmmip sequence acquired and the nucleotide sequences of the DNA fragments amplified by the cassette PCR method. Template DNA for cassette PCR was prepared with a Takara LA PCRTM In vitro Cloning Kit (Takara Bio) according to the manufacturer's instructions. Genomic DNA from *T. matsutake* was digested with restriction endonuclease, ligated with nucleotide linker and used as templates for PCR.

Amplification of the *T. matsutake* fragments Tm-1 and Tm-2. Nucleotide sequencing of fragments Tm-1 and Tm-2 were conducted using cassette amplification by PCR with one primer sets (Tm-hox-up1: 5'-ATCAACCATCTGGAGACT-3' and Tm-hox-up2: 5'-TTTGACTGCTCAACATAGACCT-3'). The primer sets were designed based on the nucleotide sequences of upstream of Tmhox. Template DNAs for cassette PCR were prepared with a Takara LA PCR In Vitro Cloning kit (Takara Bio Co.) according to the manufacturer's instructions [18]. Genomic DNA from *T. matsutake* was digested with XbaI and the fragments were ligated with nucleotide linkers and used as templates for PCR. For fragments Tm-1 and Tm-2, an approximately 2.0-kbp PCR product from Tm-hox-up2 to a XbaI site were cloned into the pT7Blue (R) T-vector (Novagen), generating pTMGLU1. The sequences for Tm-1 and Tm-2 were obtained. To amplify the entire genomic sequence of Tm-1 and Tm-2 respectively, oligonucleotide primers Tm-dis1/Tm-disF and Tm-dis2/Tm-disF were designed based on the nucleotide sequence of DNA fragment amplified by the cassette PCR method. PCR reactions were performed in a 100- μ l mixture containing 1 \times Ex Taq buffer, 100 ng extracted genomic DNA, 100 pmol each primer, 0.2 mM of each dNTP, and 2.5 U Ex Taq polymerase. PCR reactions were conducted using an initial denaturation at 94 $^{\circ}$ C for 3 min, followed by 30 cycles of 30 s at 94 $^{\circ}$ C and 2 min at 55 $^{\circ}$ C. DNA sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a BigDye Terminator Cycle Sequencing version 3.1 kit (Applied Biosystems) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

To investigate the genomic structure of the region of upstream of Tmhox1 in *T. matsutake*, two nucleotide region containing fragment Tm-1 and Tm-2 were amplified and sequenced. The characterization data for fragments are listed in Table 1.

In process of isolation of *T. matsutake* strain, the *T. matsutake* hyphae was described as "slow-growth, sterile, clamp-less and dikaryotic hyphae" [6]. In this present study, we obtained two different nucleotide sequences that showed 92% of identity between each other. These results indicated that the *T. matsutake* strain NBRC30773 was heterokaryotic. Based on the sequence of the two fragments Tm-1 and Tm-2, the different cutting sites of restriction enzymes (PstI, BamHI, BglII and EcoT22I) were also investigated in them. The difference of cutting sites of restriction suggested that in distinguishing the level of ploidy in *T. matsutake*, the technique of either restriction fragment length polymorphism (RFLP) or PCR-RFLP was feasible.

On the other hand, the two fragments sequence Tm-1 and Tm-2 with different cutting sites of restriction were investigated in *T. matsutake* strain NBRC30773, but the existence of this character in most of *T. matsutake* strain need to be investigated. At the same time, the feasibility of RFLP or PCR-RFLP with restriction enzyme in distinguishing level of ploidy in most of *T. matsutake* strain will also be checked. Then, based on these results, in the future, the *T. matsutake* strains which were obtained by spore germination and protoplast regeneration will be distinguished.

Table 1: Character of fragments Tm-1 and Tm-2 in *T. matsutake*

Fragment	Length (bp)	cutting sites of restriction enzymes	identity
Tm-1	1760	<i>Pst</i> I, <i>Bam</i> HI, <i>Eco</i> T22I and <i>Bg</i> III	92%
Tm-2	1763	<i>Pst</i> I, <i>Bam</i> HI and <i>Eco</i> T22I	

CONCLUSIONS

In the present study, two fragments sequence Tm-1 and Tm-2 with different cutting sites of restriction were investigated in *T. matsutake* strain NBRC30773. Thus, the conclusion of that level of ploidy in *T. matsutake* strain NBRC30773 was heterokaryon was indicated. Base on these results, the technique of restriction fragment length polymorphism (RFLP) or PCR-RFLP can be used to distinguish the level of ploidy in *T. matsutake*. These results will provide the basic knowledge of genetics and breeding application for *T. matsutake*.

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REFERENCES

- [1] Forestry Agency (2010). Ministry of Agriculture, Forestry and Fisheries: Japan Annual report on trends in forest and forestry in Japan, Fiscal year 2009 (Summary). <http://www.rinya.maff.go.jp/> (In Japanese)
- [2] Kope HH. (1992). Interactions of heterokaryotic and homokaryotic mycelium of sibling isolates of the ectomycorrhizal fungus *Pisolithus arhizus*. *Mycologia* 84(5): 659-667.
- [3] Kerrigan RW. et al. (1992). Strategies for the efficient recovery of *agaricus bisporus* homokaryons. *Mycologia* 84:575-579
- [4] Fasolo-Bonfante P. & Brunel A. (1972). Caryological features in a mycorrhizal fungus: *Tuber melanosporum*. *Vitt Allionia* 18: 2-11.
- [5] Roth-Bejerano N. et al. (2004). Homokaryotic and heterokaryotic hyphae in *Terfezia*. *Antonie van Leeuwenhoek* 85:165-168.
- [6] Yamada A. et al. (2001). Isolation of *Tricholoma matsutake* and *T. bakamatsutake* cultures from field-collected ectomycorrhizas. *Mycoscience* 42: 43-50.
- [7] Hiromoto K. (1960). Isolation and pure culture of the mycelia of *Armillaria matsutake* S.Ito et Imai, the most important edible mushroom in Japan. *Bio. Mag.* 73:326-333. (In Japanese)
- [8] Hamada M. (1964). Method of pure culture of the mycelium of *Armillaria matsutake* and its allies. In: *Matsutake (Tricholoma matsutake Singer): Its fundamental studies and economic production of the fruit-body*, The Matsutake Research Association Eds. Nakanishi Printing, Kyoto. pp. 97-100. (In Japanese)
- [9] Tominaga Y. (1963). Studies on the life history of Japanese pine mushroom, *Armillaria matsutake*

- Ito et Imai. Bulletin of the Hiroshima Agricultural College 2:105-145. (In Japanese)
- [10] Ohta A. (1990). A new medium for mycellial growth of mycorrhizal fungi. *Trans. Mycol. Soc. Japan* 31: 323-334.
- [11] Yamada A. & Terasaki M. (1998). Characterization of *Thricholoma matsutake* isolation taken from Ibaraki Prefecture. *Trans. Jpn. For. Sci.* 109: 521-522. (In Japanese)
- [12] Timothy YJ. et al. (2004). The genetic structure and diversity of the A and B mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*. *Fungal Genet. Biol.* 41:813-825
- [13] Kües U. et al. (1994). Two classes of homeodomain proteins specify the multiple A mating types of the mushroom *Coprinus cinereus*. *Plant Cell* 6:1467–1475
- [14] Kües U. & Casselton LA. (1992). Homeodomains and regulation of sexual development in basidiomycetes. *Trends Genet* 8:154–155
- [15] Badrane H. & May G. (1999). The divergence-homogenization duality in the evolution of the b1 mating type gene of *Coprinus cinereus*. *Mol Biol Evol.* 16 (7): 975-986.
- [16] Stajich JS. et al. (2010). Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). *Proc Natl Acad Sci USA* 107: 11889-11894.
- [17] Giasson L. et al. (1989). Cloning and comparison of A alpha mating-type alleles of the basidiomycete *Schizophyllum commune*. *Mol. Gen. Genet.* 218 (1):72-77.
- [18] Isegawa Y. et al. (1992). Selective amplification of cDNA sequence from total RNA by cassette-ligation mediated polymerase chain reaction (PCR): Application to sequence 6-5 kb genome segment of hantavirus strain B-1. *Molecular and Cellular Probes* 6:467-475.