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Proliferation of *Tricholoma matsutake* Mycelial Mats in Pine Forest Using Mass Liquid Inoculum

Won-Ho Lee[1], Sang-Kuk Han[1], Beom-Seok Kim[1], Bhushan Shrestha[1], Soo-Yong Lee[2], Cheol-Soon Ko[3], Gi-Ho Sung[4] and Jae-Mo Sung[1]*

[1]*Department of Applied Biology, Kangwon National University Chuncheon 200-701, Korea*
[2]*Mushtech, Keunhwa-Dong 798-3, Chunchoen 200-931, Korea*
[3]*Yangyang Agriculture Technology, Yangyang, Korea*
[4]*Department of Botany and Plant Pathology, Oregon State University, Corvallis, USA*

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Two isolates of *Tricholoma matsutake* T-008 and T-034, preserved in Entomopathogenic Fungal Culture Collection (EFCC) of Korea, were used in the present study. The isolates had 100% Bootstrap homology with *Tricholoma matsutake* U62964 and *T. matsutake* AB188557 and AF309538 preserved in Gene Bank of NCBI. Mycelial growth of *T. matsutake* was highest in TMM and MYA at 25°C. The highest dry wt. of mycelium was obtained after 65 days of culture, when 6 mycelial discs were inoculated in 100 *ml* of broth in 250 *ml* shaking flask. Mycelial mats were observed in clumped condition at the inoculation sites of pine forest after two weeks of inoculation. After 5 months of inoculation, mycelia mats were observed growing inside soil and walls of a few inoculation sites, while mycelial mats growth up to 5~8 cm were observed in the roots of pine tree after 6 months. The survival rate of the inoculum was about 40% of the total inoculation sites. The survival rate was found below 20% when the mycelium was inoculated in the summer. The reasons for low survival rates of the mycelium were mainly due to dry season and the soil-borne small animals such as earthworm and mole. After one year of inoculation, no external difference was observed between the artificially inoculated mycelia and the naturally existing mycelia of *T. matsutake*. The present study showed that fruiting bodies of *T. matsutake* could be produced by artificial inoculation under the appropriate environmental conditions.

KEYWORDS: Liquid inoculum, Mycelial mats, *Tricholoma matsutake*, Phylogenetic analysis

Tricholoma matsutake forms ectomycorrhizae in the roots of pine tree and is distributed in East Asian countries including Korea, China and Japan. In general, *T. matsutake* mushroom is big and strong in texture. Due to its fragrance and peculiar taste, it has been regarded as the most prized mushroom. It is difficult to derive its isolates from the fruiting body and culture them *in vitro*. The technique for its artificial cultivation included the fruiting body is still not developed. However, a lot of researches have been going on in Korea, Japan and China to develop the techniques for its artificial cultivation (Yamada *et al*., 2001).

The economic importance of *T. matsutake* is believed to have started in the late 1960s when its export started. Despite the hardship, the local farmers could develop the mushroom as a good economic source in the autumn. But, the natural production of *T. matsutake* could not be maintained and its production decreased after 1980s. Past researches have shown that changes in the climate during the fruiting period and in the surrounding areas greatly affect the productivity of *T. matsutake*. Besides that, damage in the pine forest due to fire and attack by *Thecodiplosis japonensis* also cause the low production of the mushroom. Furthermore, thick growth of vegetation in the forest area also dominates the ectomycorrhizal growth of the fungus in the soil.

In Korea, research has been going on since 1960s for the production and artificial cultivation of *T. matsutake* (Ryoo *et al*., 1980; Na and Ryu, 1992). Specially, a large amount of results have been obtained about the environmental factors governing the fruiting body production of the mushroom and the ecological conditions of pine forest (Kang *et al*., 1989; Lee, 1991; Cho and Lee, 1995; Park *et al*., 1995, 1997; Koo and Bilek, 1998). Besides them, researches have been conducted on the ectomycorrhizal growth of *T. matsutake* since 1980s (Lee *et al*., 1997, 1998). Different methods for management of pine forest for the production of the mushroom have been utilized (Lee *et al*., 1986; Song and Min, 1991; Park *et al*., 1998; Hur *et al*., 2001). Recently, molecular techniques have been reported for the production of mushroom (Lee and Hong, 1998; Lee *et al*., 1999; Lian *et al*., 2003; Chapela and Garbelotto, 2004).

However, it is very necessary to adjust the environmental factors of the forest where *T. matsutake* mushroom grows. It is very necessary to introduce new mycelia of *T. matsutake* in the pine forest. Among them, the hurdle is the production of mycelial isolates of *T. matsutake*. A lot of researches are going on but still the success is very low

*Corresponding author <E-mail: jmsung@kangwon.ac.kr>

(Kim, 1984; Min *et al*., 1998). Hence, it is very necessary to prepare mycelial cultures of *T. matsutake* and to find the optimum conditions for the mass mycelial cultures. The mycelia of *T. matsutake*, identified by the DNA analysis, can be directly inoculated in the pine forest. In the mean time, the roots of pine tree can be observed for the growth of ectomycorrhizae and once again make certain that the mycelia are the inoculated ones by the DNA analysis.

Materials and Methods

Preparation of *T. matsutake* isolates. It was necessary to make isolates from fresh *T. matsutake* mushrooms collected from the pine forest and proliferate them before doing any further culture work. *T. matsutake* mushrooms were collected from the forest and recorded for their morphological characters. Spores, mycelia and the ectomycorrhizal growths were observed using the light microscope. It is comparatively difficult to culture *T. matsutake* as it is an obligatory symbiotic fungus with pine roots Isolates of *T. matsutake* were prepared from tissues and spores in the Entomopathogenic Fungal Culture Collection (EFCC) of Kangwon National University, Chuncheon, Korea. The tissue from *T. matsutake* fruiting body was inoculated in Water agar and TMM. After the growth from tissue periphery, it was again transferred to TMM. For spore isolation, the spores were discharged on Water agar from the mature fruiting body. The agar blocks with spores were transferred to TMM.

Optimal conditions for mass culture of *T. matsutake*. Two isolates T-008 and T-034, preserved in EFCC, Kangwon National University, were used in the present study. T-008 was collected from Bonghwa and T-034 was collected from Yangyang regions of Korea in October 2001 and October 2004, respectively. The isolates were inoculated in TMM and incubated at 25°C for 35 days and used as primary inoculum for further medium experiment.

Selection of optimum medium. Ten different agar media including PDA were used to observe the mycelial growth of *T. matsutake* (Table 1). The media were sterilized at 121°C for 20 min and poured in 85 mm Petri dish, 20 *ml* per each plate. Mycelial discs were cut from colonies of *T. matsutake* with the help of 6 mm cork borer and inoculated in the center of agar plates. After inoculation, the plates were incubated at 25°C for 25 days and 90 days and measured for the colony diameter.

Optimum temperature. TMM was prepared in 250 *ml* Erlenmeyer flasks by pouring 150 *ml* of liquid medium with adjusted pH of 5.2 and sterilized at 121°C for 20 min. Mycelial discs, cut with the help of 6 mm cork borer, were inoculated in TMM broths, three in each flask and incubated at 10°C, 15°C, 20°C, 25°C and 30°C for 60 days. The mycelial growth were filtered with the help of Whatman No. 2 and dried at 60°C for 24 hr and weighed for the dry wt.

Liquid culture of isolates. The liquid media were prepared with 100 *ml* per each 250 *ml* Erlenmeyer flasks (Table 2), with adjusted pH of 5.2. After sterilization at 121°C for 60 min, mycelial discs from the Petri dish culture were inoculated and cultured for 35 days. After that, the flask cultures were transferred to 2 *l* and 8 *l* fermenters and cultured for 60 days. The liquid cultures were

Table 1. Composition of media used in this experiment

Composition	Media (g/*l*)									
	HA	PDA	MMM	PDMP	CDY	YMM	TMM	GP	MYA	BM
Potato		200		200						
Dextrose		20		20		10				30
Glucose	20		10		10		20	30		
Malt extract			3	3	3	3			20	
Yeast extract	3				3	3	1.5		2	3
Peptone				1		5		3		
$MgSO_4$	0.5		0.15	0.5	0.5			1	1	1
KH_2PO_4	1		0.5		1			1	1	1
K_2HPO_4				1						
KCl					0.5					
$NaNO_3$				2	2					
$FeSO_4$				0.01	0.01					
$CaCl_2$			0.05	0.5						
NaCl			0.025							
$(NH_4)_2HPO_4$			0.25							
Thiamine HCl			0.1 (mg)							
Soytone							1.5	1.5		
Agar	20	20	20	20	20	20	20	20	20	20

Table 2. Media composition of liquid culture used in this experiment

Composition	Media concentration (g/*l*)		
	100 *ml* (Flask culture)	2 *l* liquid culture	8 *l* liquid culture
Glucose	2 g		
Yeast extract	0.15 g		
Yellow sugar		40 g	160 g
Soybean powder		6 g	24 g
Corn oil		3 *ml*	5 *ml*

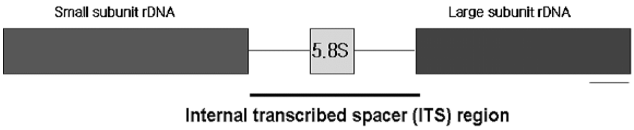

Fig. 1. ITS region used for identification of species.

then used as an inoculum in pine forests.

DNA analysis of *T. matsutake* and ITS region of rDNA sequenced using PCR. DNA analysis was performed to make certain that the *in vitro* mycelial culture is truly pure when compared with the specimens of *T. matsutake* mushrooms. DNA was isolated from the mycelial culture of *T. matsutake*. The sequence was matched with the sequences available in GenBank using maximum parsimony. Bootstrap value above 70% could be considered as same (Fig. 1).

Table 3. Inoculated time and quantity of inoculum cultured 90 days at 8 bottles

	Date of inoculation	Amount of inoculation (*l*)
First inoculation	April 26, 2005	50
Second inoculation	May 6, 2005	200
Third inoculation	July 7, 2005	100
Fourth inoculation	July 25, 2005	100
Fifth inoculation	September 7, 2005	50
Sixth inoculation	September 14, 2005	20
Seventh Inoculation	September 28, 2005	20
Eighth inoculation	November 2, 2005	20

Inoculation of liquid inoculum of *T. matsutake* in pine forest. The liquid culture of *T. matsutake*, cultured for 90 days were inoculated in pine forest from 2005 April 26 to 2005 November 2 for 8 times (Table 3). During inoculation, a lot of care was taken not to damage the roots of pine tree. For inoculation, 20 *l* of liquid inoculum was prepared. During inoculation, the liquid inocula were poured in inoculum tank and sprayed in pine root, 100~150 *ml* per site. In order to observe the ectomycorrhizal formation, the inoculation sites were marked with the colored tapes.

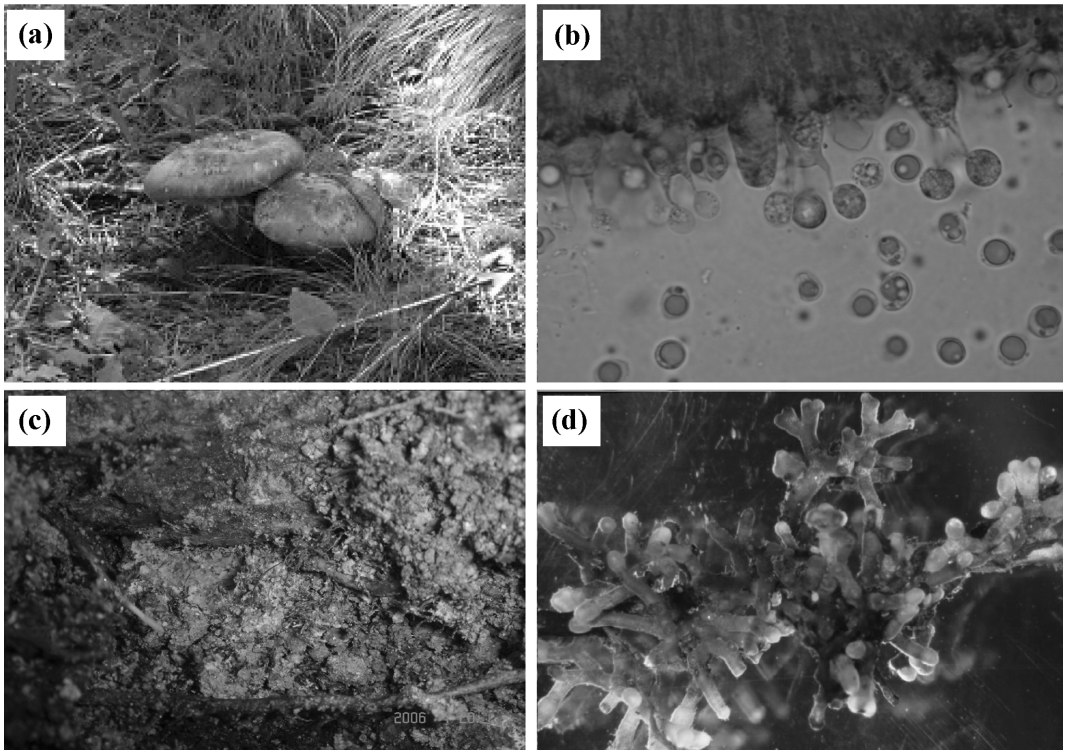

Fig. 2. Life cycle of *Tricholoma matsutake*. a, fruit body in nature; b, basidiospores; c, mycelia and d, mycorrhizae on pine root-hairs.

Observation of ectomycorrhizal formation. In order to observe the mycelial growth of *T. matsutake* in the inoculation sites, the pine roots were brought to the lab and observed under the light microscope.

DNA analysis of ectomycorrhizae of *T. matsutake* collected from the forest. The inoculated mycelia of *T. matsutake* formed the ectomycorrhizae inside the soil in pine forest, which was also confirmed by the DNA analysis. For DNA analysis, ITS region was sequenced. ITS2t primer was prepared and used to detect *T. matsutake* and other mushrooms belonging to genus *Tricholoma*.

Primers used: ITS2t 5'-TGCACGCCTGACGCCAATC-3'
ITS4 5'-TCCTCCGCTTATTGATATGC-3'

Maximum parsimony method was used with the sequences deposited in GeneBank. If the Bootstrap value is higher than 70%, the isolates were regarded as a single one (Fig. 1). ITS region of DNA was sequenced to identify the sample of *T. matsutake*. Specific primer was prepared for the sequencing. The primer thus prepared, ITS2t, could be applied to other species of *Tricholoma* genus.

Results

Collection of *Tricholoma matsutake*: Its identification and isolation. There are 44 isolates of *T. matsutake* being preserved in EFCC, Kangwon National University. The isolates were collected from 2000~2005 from Bonghwa and Yangyang. Photographs were taken of *T. matsutake* before collection and were brought to lab. Spores were discharged and observed with the help of microscope and taken photographs at the same time. Photographs were also taken of the mycelial growth of *T. matsutake* and its ectomycorrhizal formation in pine roots (Fig. 2).

For isolation, the outer layer of the *T. matsutake* mushroom was peeled off and the inner part of the tissue of 5 × 5 mm size was inoculated in Water agar. For spore isolation, the pileus region of the mushroom of 10 × 10 mm size was kept over the water agar and waited until the spore discharge. The agar blocks with discharged spores were inoculated in TMM (Fig. 3).

Basic medium experiment of *T. matsutake* mycelium. Selection of optimum medium: For the selection of optimum medium, isolates T-008 and T-034 were grown in 10 different media including PDA. Both isolates T-008 and T-034 showed the highest growth in TMM, 70 mm and 65 mm colony diameter for 90 days, respectively (Fig. 4). MYA also showed better growth based on colony diameter and mycelial density.

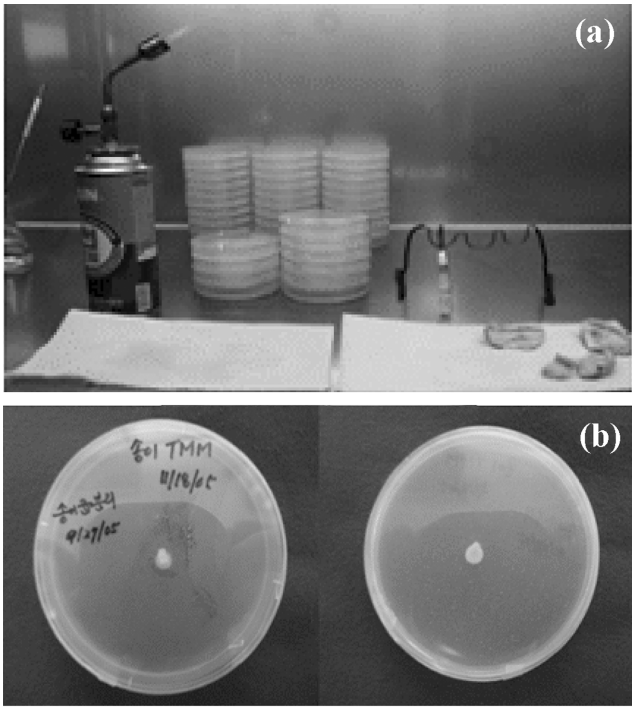

Fig. 3. Procedure of *Tricholoma matsutake* fruit bodies isolation using WA and TMM. a, preparation of a pieces of fruit bodies in WA and b, inoculation of agar blocks with fruit bodies in TMM.

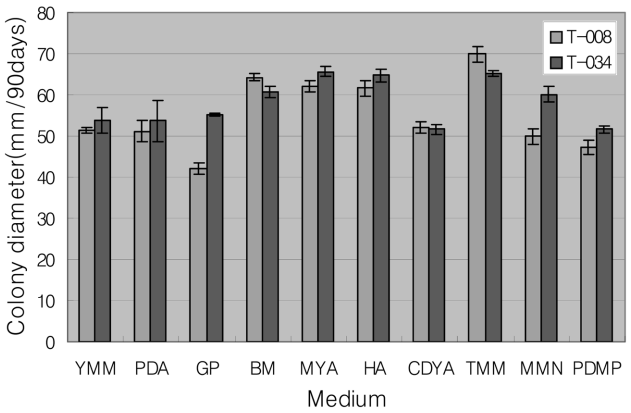

Fig. 4. Effects on various media on mycelial growth of *Tricholoma matsutake* T-008 and T-034 after 90 days.

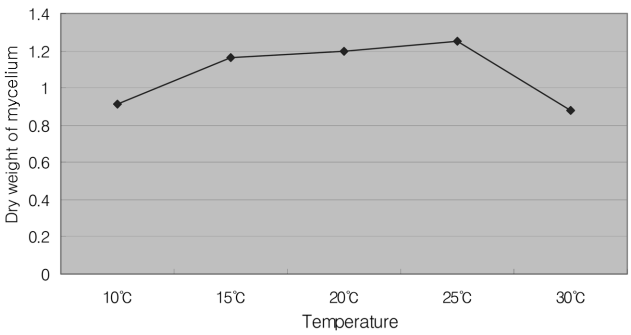

Fig. 5. Effects of various temperature on mycelial growth by *Tricholoma matsutake* T-034 after 60 days.

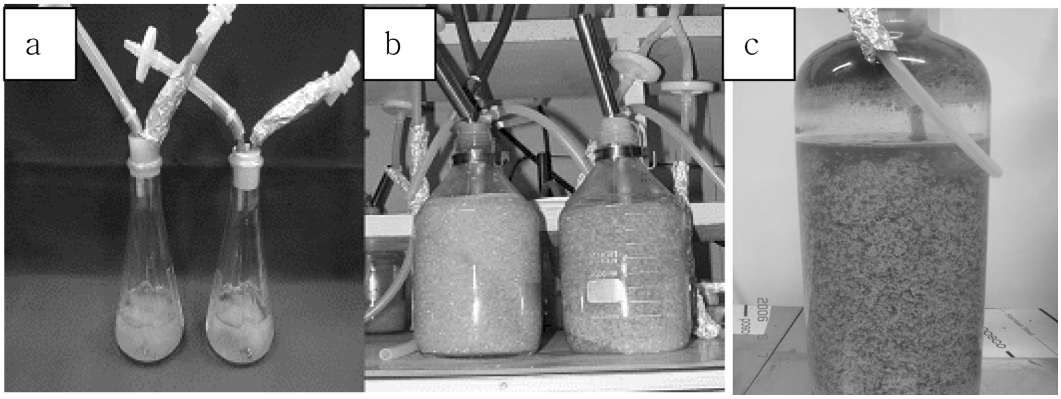

Fig. 6. Liquid culture of *Tricholoma matsutake*. a, flask culture; b, 2 *l* bottle culture and c, 8 *l* air-lift fermenter culture.

Selection of optimal temperature: Mycelia of *T. matsutake* showed growth at 10~25°C, but no growth at 30°C. The highest growth was observed at 25°C (Fig. 5). The lower the temperature, the less the growth was.

Mass culture of *T. matsutake*. The mycelial growth of *T. matsutake* depended on the type of conical flask and amount of liquid medium. The highest growth was observed when grown in 100 *ml* of liquid medium in flask with baffles in a shaker. Among different carbon sources such as brown sugar, white sugar, starch syrup, potato, brown sugar gave the satisfactory results. After the medium was prepared in 8L bottle, it was sterilized for 60 min. The mycelial discs were cut using the homogenizer and transferred to 8 *l* bottle and connected to filtered air and cultured for 60 days (Fig. 6).

DNA analysis of liquid mycelium of *T. matsutake*. A total of 28 samples were tested for phylogenetic analysis.

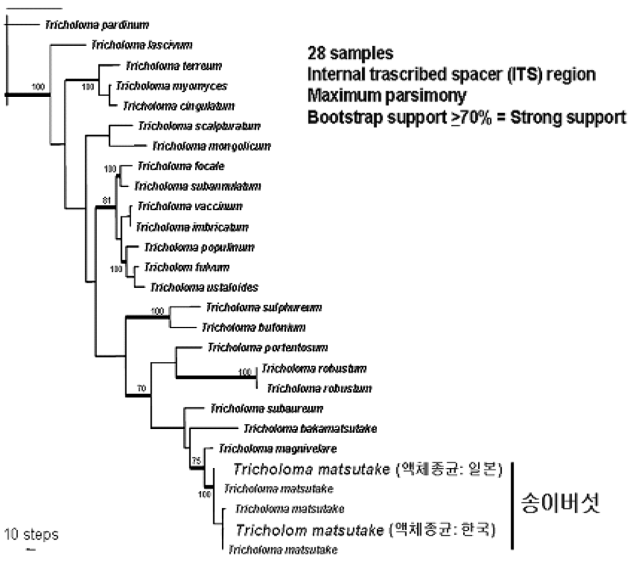

Fig. 7. Bootstrap analysis of *Tricholoma matsutake*.

DNA was isolated from EFCC T-008 (collected from Bonghwa) and T-002 (collected from Japan). Bootstrap analysis was done with two isolates deposited in GeneBank *T. matsutake* U62964 and *T. matsutake* AB188557 and made sure that the liquid cultures are pure *T. matsutake* (Fig. 7).

Periodic Monitoring of ectomycorrhizal formation and growth. After 2 weeks of inoculation, pellet formation or thin ectomycorrhizal formation were observed in the inoculation sites. After 4 weeks the cottony mycelium grew into white mycelium. But, due to earthworm and mole, there was loss due to no growth of mycelium. After five months, the mycelium was observed growing on the walls as well as inside soil pores of inoculated holes in the forest. Mycelium of *T. matsutake* was also observed growing with roots of pine trees. After six months, the mycelium was observed growing up to 5~8 cm from pine roots.

After eight months, the mycelia were found not growing due to high temperature and hot weather, and also due to earthworm and mole. The mycelium could not grow due the low moisture content of soil between the spring season and the rainy season. Due to heavy rain, the mycelium lost its viability as the growing mycelia were dipped in rainy water for a long time. Since *T. matsutake* mycelium grows on ridges of soil particles, so during rainy season, it will grow well under good drainage condition. After fourteen months, the mycelia were observed to be growing. After the hot rainy season, the mycelia start growing with more vitality and grow up to 8~13 cm.

The survival rate of inoculated mycelia in the pine forest depended upon the temperature during the time of inoculation. About 40% of the inoculated sites were survived by the mycelia of *T. matsutake* when inoculated during the low temperature season, while less than 20% of the inoculated sites were survived by mycelia when inoculated in the hot summer. The reason may be due to less existence of harmful microorganisms and insects in

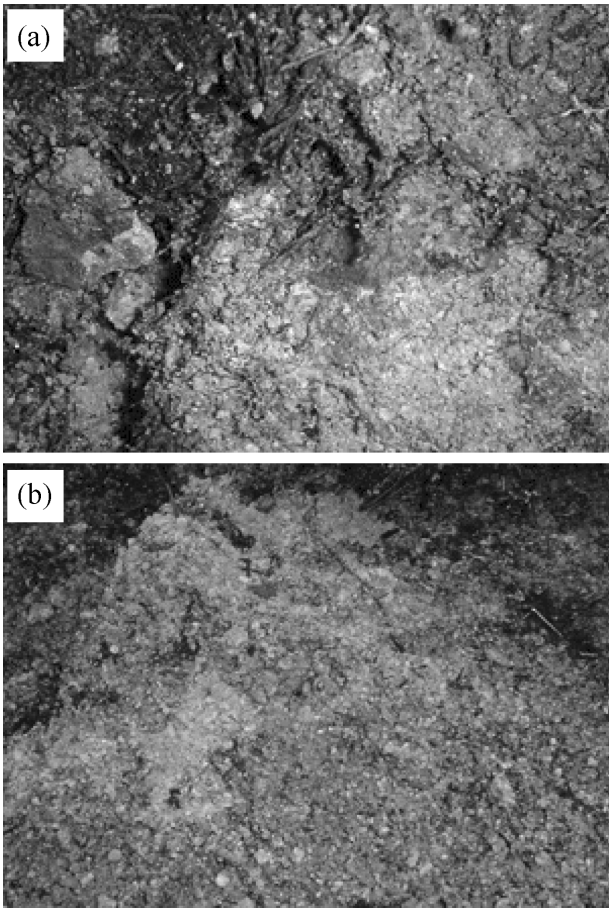

Fig. 8. Mycelial mats formation of *Tricholoma matsutake* in pine forest. a, after 14 months of inoculation with liquid culture and b, natural mycelial mats.

soil during the cool season (Fig. 8).

DNA analysis of mycelial mats of *T. matsutake* obtained from liquid inoculum. The site inoculated with liquid inoculum on 8 May 2005 was collected on different days such as 7 samples on 11 June, 3 samples on 27 June and 2 samples on 10 July. In DNA analysis of all samples using ITS region, the 6 samples of mycelial mats

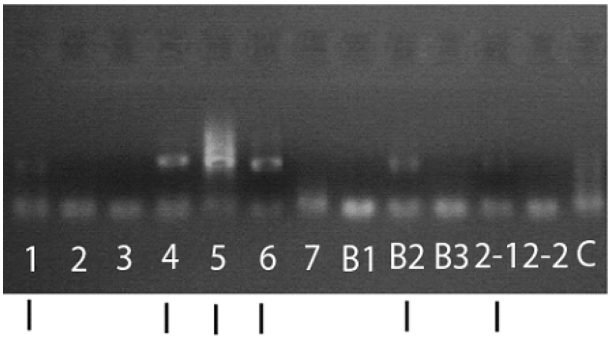

Fig. 9. DNA of *Tricholoma matsutake* using *Tricholoma* specific primers.

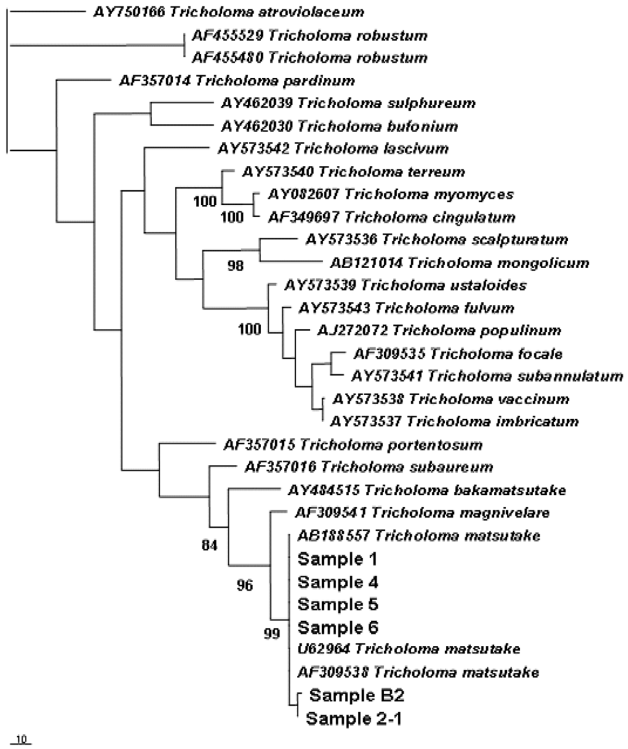

Fig. 10. Tree showing six out of twelve *Tricholoma matsutake* mycelial mats with 99 homology with *Tricholoma matsutake*.

(1, 4, 5, 6, B2 and 2-1) were shown by PCR band (Fig. 9).

Although we used *Tricholoma* specific primer, for further identification, we tried DNA sequencing analysis. The analysis used Maximum parsimony method and statistical significance level was followed by bootstrap method. In bootstrap method, if the value is over 70% it means strong affinity. The Fig. 10 showed that all 6 samples formed bands in PCR analysis were true *T. matsutake* due to their 99% bootstrap support value.

Discussion

An obligatory symbiotic ectomycorrhizae of *T. matsutake* is produced mainly on root of pine tree (Vaario *et al*., 2002; Gill *et al*., 1999). Although *T. matsutake* uses symbiotic nutrition, its growth is very slow on agar plates. So it is very difficult to isolate and culture it. For above reasons, artificial fruiting body formation has not been reported recently, except on root of pine tree *in vivo*. In Korea, research for culture and sustainable yield of *T. matsutake* was started in late 1960s. Especially, climatic and ecological conditions in pine forests could affect *T. matsutake* growth (Park *et al*., 1995). Beside *T. matsutake*, mycelial mats was important to produce fruiting bodies under ground and sustainable management methods of mycelial mats have been researched since 1980s. These days, yield management of *T. matsutake* by using

genetical methods was reported (Hitoshi Murata *et al*., 2005). Current researches about *T. matsutake* are carried out in the field of cultivation, ecological condition, enzymological view, substrate movement in mycelial mats and molecular biological method.

For cultivation of *T. matsutake*, it is very important to produce inoculum of *T. matsutake* for inoculation in pine forest. In this new research, we could obtain *T. matsutake* isolates from tissue and spore and showed the potentiality of cultivation in pine forest. So this study can help to understand about other mycorrhizae not isolated, such as *Amanita hemibapha* and *Sarcodon aspratus*.

We analyzed the DNA of cultivated pellets of *T. matsutake* to prove *T. matsutake* isolation and to compare with others belonging to *Tricholoma*, before inoculation. Due to demonstration using liquid inoculum, mycelial mats produced on test field in Yangyang was identified as the real *T. matsutake*. If any researcher or farmer harvests *T. matsutake* from inoculated mycelial mats, he could prove the mushroom same as the natural specimens.

In study of optimal medium and temperature, T-008 and T-034, both isolates showed good growth on TMM medium followed by MYA media at 25°C. The optimal medium depends on species, but optimal temperature does not. Due to these optimal conditions reported already by Kim *et al*. (2005), *T. matsutake* grows well under above conditions.

When the pellets of *T. matsutake* were inoculated in pine forest using 8 *l* liquid inoculum, some pellets were observed growing in the soil in clod shape. Although some pellets made symbiotic relation with pine tree and their hyphae followed the root of pine tree, a lot of pellets inoculated were shown weakly growing or dying. Although there is previous report that two *T. matsutake* mushrooms were grown when suspension was inoculated in pine forest and four *T. matsutake* were grown when the soil was inoculated with artificial mycelial mats (Park and Park, 1980), it was not clear due to discontinuous results. Also in the pine seedling inoculum project in cooperation with Japan for production of artificial *T. matsutake* from 1980 to 1984, it was reported that although a lot of pine seedling inoculum had high survival rate, *T. matsutake* died after plantation and the period of artificial inoculation took long time. The results of artificial *T. matsutake* production have not been reported recently but we think that using liquid inoculum of *T. matsutake*, used in our research, are new technique to obtain artificial *T. matsutake*. As a result of morphological characteristics compared to natural *T. matsutake*, the mycelial mats and rootlets were covered by hyphae of *T. matsutake* and they produced black color in natural *T. matsutake*. External characters showed rigid epidermis and shape like branches. It showed *T. matsutake* was more close to parasitic than symbiotic mushroom in only morphological characteristics. There were thin rootlets of pine tree on the external rigid epidermis. In artificial *T. matsutake* mycelial mats, they showed that white or brownish colors and if mycelial mats branches spread, the shape was similar to natural *T. matsutake* mycelial mats. Because external epidermis was little covered by hyphae and soft epidermis, we thought it as the initial stage of *T. matsutake*.

After *T. matsutake* isolates were identified by DNA analysis, they were mass cultured and the liquid inoculum was inoculated in pine tree. They could form *T. matsutake* mycelial mats and their DNA was same as that of natural *T. matsutake*. Hereafter for sustainable yielding of *T. matsutake*, it is necessary to develop mass cultivation techniques of *T. matsutake* and the environment monitoring of pine forest to produce *T. matsutake* mycelial mats. Although we have not been able to produce artificial *T. matsutake* fruit bodies, it needs further researches to approach the problem.

Acknowledgement

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