GENETIC INSTABILITY OF MUSHROOMS AS AFFECTED BY CONTINUOUS SUBCULTURING FOR PRODUCTION

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ความแปรปรวนทางพันธุกรรมของเห็ดอันเนื่องมาจากการถ่ายเชื้ออย่างต่อเนื่องเพื่อการเพาะเห็ด

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การศึกษาความแปรปรวนทางพันธุกรรมของเห็ดด้วยวิธีการทางชีววิทยาระดับโมเลกุลมีความสำคัญคือ มีประสิทธิภาพสูง และ รวดเร็ว จึงมีความเหมาะสมต่อการนำไปใช้ตรวจสอบเชื้อเห็ดก่อนที่จะนำไปใช้เพื่อการผลิตต่อไป การดำเนินงานได้กระทำโดย ศึกษาการเจริญของเชื้อเห็ดบนอาหารเลี้ยงเชื้อ PDA ปริมาณผลผลิตควบคู่ไปกับการวิเคราะห์ดีเอ็นเอ โดยเทคนิค PCR-RFLP ในทุกครั้งของการถ่ายเชื้อ ซึ่งเห็ดที่ใช้ในการศึกษา คือเห็ดนางรม เห็ดนางฟ้า เห็ดเป๋าฮื้อ เห็ดกระด้าง เห็ดขอนขาว เห็ดหอม เห็ดยานางิ เห็ดหูหนู และเห็ดตีนแรด จากการศึกษาพบว่าเห็ดบางชนิดมีแนวโน้มของการเจริญ และปริมาณผลผลิตที่ลดลงอย่างเห็นได้ชัดภายหลังการถ่ายเชื้อหลายครั้ง เช่น เห็ดขอนขาว เห็ดกระด้าง และ เห็ดนางฟ้า นอกจากนี้เห็ดดังกล่าวใช้ระยะเวลาการเจริญบนอาหารเลี้ยงเชื้อนานมากขึ้น เมื่อเปรียบเทียบกับการถ่ายเชื้อครั้งแรก ส่วนการวิเคราะห์ผล PCR-RFLP ของเห็ดทั้ง 9 ชนิด พบว่าเห็ดทุกชนิดหลังจากการใช้ ITS 4 และ 5 primers เพิ่มจำนวนดีเอ็นเอ ได้ขนาดตั้งแต่ 600-800 คู่เบส และภายหลังการตัดดีเอ็นเอ ด้วยเอนไซม์ที่ตัดจำเพาะทั้ง 4 ชนิด พบว่าเห็ดแต่ละชนิดให้ผลของชิ้นดีเอ็นเอ ที่แตกต่างกัน นอกจากนี้ในขณะที่จำนวนการถ่ายเชื้อมากขึ้นเห็ดทุกชนิดยังคงให้ผลของชิ้นดีเอ็นเอ ที่เหมือนเดิม ยกเว้น เห็ดหูหนู ในการถ่ายเชื้อครั้งที่ 3 พบว่ามีผลของชิ้นดีเอ็นเอ ที่เปลี่ยนไปจากเชื้อเริ่มต้น เมื่อตัดด้วย เอนไซม์ตัดจำเพาะ *Hinf* I กล่าวคือในการถ่ายเชื้อครั้งที่ 3 พบตำแหน่งที่ตัดจำเพาะของ *Hinf* I ถึง 2 ตำแหน่ง ผลคือภายหลังการตัดดีเอ็นเอ ให้ขนาด 357 และ 263 คู่เบส แต่ในขณะที่เชื้อเริ่มต้นพบตำแหน่งการตัดจำเพาะเพียง 1 ตำแหน่งที่ 300 คู่เบส ทำให้ภายหลังการตัดดีเอ็นเอ ให้ขนาด 300 คู่เบส 2 ชุด ดังนั้นจึงทำการอ่านลำดับเบสของเห็ดหูหนูนี้ เพื่อตรวจสอบหาตำแหน่งที่ *Hinf* I สามารถตัดได้ พบว่าตำแหน่งในการตัดมีความสอดคล้องกับผลของชิ้นดีเอ็นเอ จากนั้นได้ทำการเปรียบเทียบความเหมือนของลำดับเบสทั้ง 600 คู่เบส พบว่าความเหมือนของลำดับเบสระหว่าง การถ่ายเชื้อครั้งที่ 1 และ 3 มีประมาณ 90 เปอร์เซ็นต์ และเมื่อศึกษาถึงลักษณะของดอกเห็ด พบว่า เชื้อเห็ดจากการถ่ายเชื้อครั้งที่ 3 ให้ลักษณะดอกเห็ดที่ผิดปกติไปจากเชื้อเห็ดเริ่มต้น นอกจากนี้จึงได้ศึกษาถึงยีนอื่นที่เกี่ยวข้องกับการพัฒนาดอกเห็ด คือ β-tubulin gene พบว่าเชื้อเห็ดที่มาจากการถ่ายเชื้อครั้งที่สามนั้น มีผลของชิ้นดีเอ็นเอ ที่แตกต่างไปจากเชื้อเห็ดเริ่มต้นปกติ

ส่วนการทดลองหาสภาพแวดล้อมที่เหมาะสมต่อการเพาะเห็ดตีนแรด จากวัสดุเหลือใช้ทางการเกษตรพบว่าวัสดุที่เหมาะสมต่อการเพาะเห็ดตีนแรด คือการใช้ดินเป็นวัสดุเพาะโดยใช้กระถางดินเป็นภาชนะสำหรับเพาะ ซึ่งสามารถให้ผลผลิตสูงสุดอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่น 99 เปอร์เซ็นต์ ($P< 0.01$) แตกต่างจากการใช้ดินผสมเปลือกถั่วและ เปลือกถั่วอย่างเดียวเป็นวัสดุเพาะ ตามลำดับ และการคลุมวัสดุเพาะด้วยฟางข้าวและแกลบกับการไม่คลุมวัสดุเพาะ นั้นพบว่ามีผลไม่แตกต่างกันทางสถิติ

สาขาเทคโนโลยีชีวภาพ ลายมือชื่อนัก

ศึกษา……

ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่

ปรึกษา……..……..

ลายมือชื่ออาจารย์ที่ปรึกษา

ร่วม…….

ลายมือชื่ออาจารย์ที่ปรึกษา

ร่วม……..…

WANNAPA SATTAYAPHISUT : GENETIC INSTABILITY OF MUSHROOMS AS AFFECTED BY CONTINUOUS SUBCULTURING FOR PRODUCTION

THESIS ADVISOR: PROF. NANTAKORN BOONKERD, Ph.D.101PP. ISBN 974-7359-93-6

This study aimed to investigate the genetic instability of mushroom as affected by continuous subculturing. The DNA techniques were used for investigation since due to high efficiency to detect prior apply to large scale production. The growth rate on PDA medium, average of total yield and detection by using PCR-RFLP techniques were employed. Mushrooms used in this study were *Pleurotus ostreatus*, *P. sajor-caju*, *P. cystidiosus*, *Lentinula polychrous*, *L. squarrossula*, *Lentinus edodes*, *Agrocybe cylindracea*, *Auricularia auricula* and *Tricholoma crassum*. The results found that the growth rate and yield of individual species gave different patterns correspond to individual species. Some species had decreased in growth rate and yield such as *L. squarrossula, L. polychrous* and *P. sajor-caju* when compared with first subculturing. These species had prolonged the growth on PDA medium. For PCR-RFLP analysis, the DNA templates were amplified with ITS 4 and 5 primers. There were different in size about 600-800 bp depend on each of species. The results suggested that individual of species gave different DNA-fingerprint pattern after digested with the 4 restriction enzymes. The DNA patterns were differ individual of species depending on each species and the most of species still gave the same DNA pattern when serial transfers of mycelium were conducted except in *A. auricula* (Ear mushroom). The third subculture of *A. auricula* gave the different fragment size with *Hinf* I when compared with the first subculture. The third subculture of *A. auricula* was digested with *Hinf* I found that 2 recognition sites to digested then fragment gave in size of 357 and 263 bp. While the first subculture found that only one recognition site then fragment gave in size of 300 and 300 bp. Therefore, the DNA sequences were observed further investigated. From the sequences data were aligned between the first and third subcultures showed that recognition site had corresponded with DNA-fingerprint and homology found among 90%. In addition, another gene which related to fruit body development. β- tubulin gene was chosen. The results showed that DNA-fingerprint the third subculture gave different from the first subculture.

For *Tricholoma crassum* production, the appropriate technology was developed on the basis of agricultural wastes utility. The comparison of total yield, found that when using soil as material substrate and clay plot as container able to promote highest yield (575 fresh weight (g)/ 1 container) at significant higher ($P< 0.01$). While using soil mixed with soybean husk (1:1) and soybean husk gave lower yield. In addition, the covering casing with rice-straw, rice husk and non-covering found that were not significantly different ($P< 0.05$) in yield enhancement.

สาขาเทคโนโลยีชีวภาพ ลายมือชื่อนัก

ศึกษา……

ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่

ปรึกษา……..……..

ลายมือชื่ออาจารย์ที่ปรึกษา

ร่วม…….

ลายมือชื่ออาจารย์ที่ปรึกษา

ร่วม……..…

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Wannapa Sattayaphisut

Chapter I

Introduction

Mushrooms are higher filamentous fungi, which perform more advance evaluation than other fungi. The fungi are classified into heterotrophic Protista Kingdom. The most characteristic aspect of filamentous fungi is germinated with spore and grow as a thread of cell (hypha). Main groups of fungi are distinguished on the basis of the structures for sexual reproduction. Almost all mushrooms are classified into Class Basidiomycetes. To basidiomycotina, with some 16,000 species, belonging most of the large and conspicuous fungi found in fields and woods such as toadstools, bracket polypores and puff-balls. The mycelium consists of branched, septate hyphae. Each septum has a central pore of a special kind known as a "dolipore". There is a strong tendency for the cells to be binucleate and "clamp connections" often occur associate with the cross-walls. The distinctive feature of the division is the basidium, a special type of sporangium, which externally produces its spores, usually four in number. However, the resulting haploid nuclei then pass into the developing basidiospores which, unlike ascospores, are produced internally.

Basidiomycetes can be classified into 3 main classes as following.

A. Hymenomycetes

This class contains the largest member. The basidia are in extensive hymenia which are exposed when mature. Spores are shot from the basidium when mature. This group includes agarics, boletes, bracket polypores and coral fungi.

B. Gasteromycetes

The basidia are in hymenia but these are not exposed. The basidium is not a spore-gun, the basidiospores being set free from the basidium by its break down. The basidiocarps remain closed until they disintegrate or only expose their spores after have matured and the basidia disintegrated. The group includes puff-balls, earth-stars, stink-horns and bird's-nest fungi.

C. Teliomycetes

This is one of the large and important orders of obligate biotrophic plant parasites. The basidium is transversely septate into four cells, each of which forms a single basidiospore, which is shot off.

The first two groups are often referred to as "higher basidiomycetes". In their basidiocarps are fairly large and the basidia are unicellular. Hymenomycetes are divided into two orders: Agaricales and Aphyllophorales. In the former the basidiocarp is fleshy and the hymenium covers gills or lines vertical tubes. Toadstool and general mushroom are used by naturalists for the umbrella-shaped type of fungus with a discoid cap supported on a central stalk. However, a common popular conception is that toadstools are poisonous, while several mushrooms are good to eat. For more than 50 years the fungi have major tools for classical genetical research because they have a combination of features unmatched in other eukaryotes.

- They are easy to grow in laboratory conditions and they complete the life cycle in a short time.
- They are haploid so they are easy to mutate and to select for mutants.
- They have a sexual stage for analysis of the segregation and recombination of genes, and all the products of meiosis can be retrieved in the haploid sexual spores.

They produce asexual spores so that genetically uniform populations can be bulked up and maintained.

1.1 Life cycle

The life cycle, pattern of sexuality, and sexual mechanism are three parameters of sexual behavior. Life cycle defines the order and relative durations of successive developmental phases as they involve nuclear and morphological events. Sexual pattern is the nature of events leading specifically to fertility (fruiting, nuclear fusion, meiosis and production of progeny); the progeny may be self-fertile or self-sterile and crossing-fertile in particular combinations. Basidium development in Hymenomycetes: fusion of the two hapoid nuclei in the young basidium produces a diploid nucleus which at once undergoes meiosis to gave four haploid nuclei. At this stage the basidium is full of granular protoplasm. Four little outgrowths (sterigmata) then arise at the top of the young basidium and the tip of each begins to inflate to form a basidiospore. The typical life cycle of Basidiomycetes consists of nine steps (Raper, 1976) which are demonstrated in Figure 1

as follows: (1) germination of a basidiospore initiates the development of (2), a haploid homokaryotic mycelium containing genetically identical nuclei and capable of indefinite independent propagation. The mycelium may or may not go through an asexual cycle the production of oidia or chlamydospores. (3) Mating between two compatible homokaryotic mycelia through hyphal fusion (plasmogamy) establishes (4), the fertile mycelium, which is usually a specialized heterokaryon known as the dikaryon. The two sexually compatible and genetically dissimilar haploid nuclei of the mated parents are associated in pairs, one pair per cell, throughout the mycelium; each septum usually bears called a clamp connection. The dikaryon is capable of independent and indefinite propagation and may or may not go through an asexual cycle. If asexual spores are produced and are uninucleate, homokaryotic mycelia of the parental types are regenerated; if the asexual spores are binucleate, the dikaryon is regenerated. Under appropriate environmental influences the dikaryon produces (5), the fruit body as an outgrowth of specialized tissue. (6) The spore-bearing tissue of fruit body develops as a columnar layer of club-shaped, binucleate cells termed the basidia. (7) Fusion of the paired nuclei of the two parent mating types, karyogamy, establishes the diploid nucleus in a one-cell stage. (8) Meiosis follows immediately, during which genetic material of the mated parents recombines and segregates. Each of the four resulting haploid nuclei moves to the tip of a stalklike structure, the sterigma, on the basidium to form a basidiospore. Typically, four uninucleate spores are formed on each basidium. (9) The spores are discharged. A mitotic division of the nucleus often precedes basidispore germination. The point at which the life cycle is reinitiated.

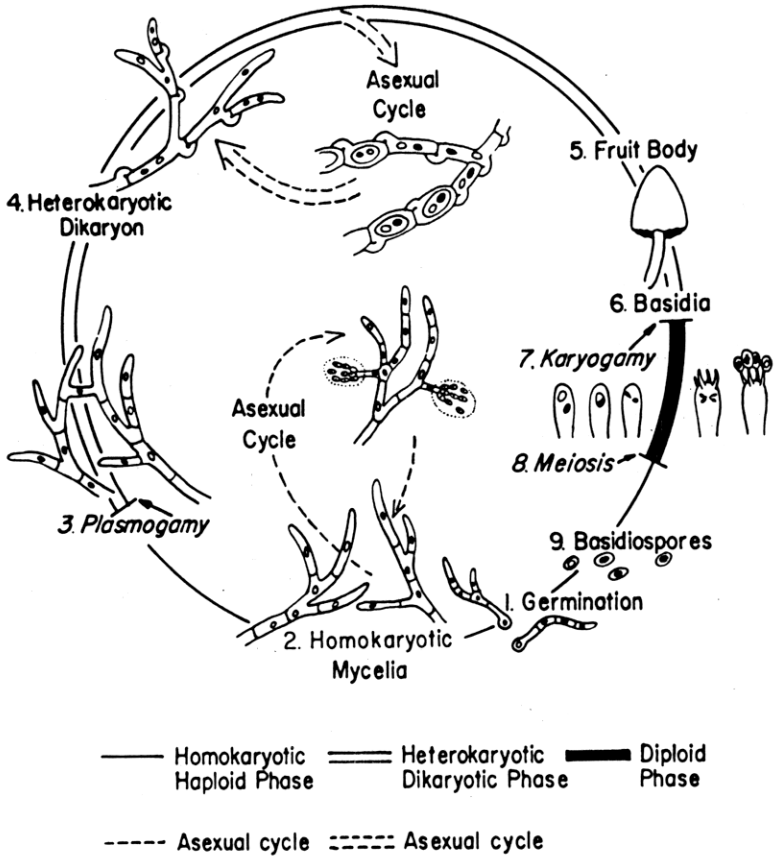


Figure 1. Typical life cycle of Basidiomycetes (Raper, 1976)

1.2 Mushroom Production

Several of the white-rot fungi that can utilize lignocellulose are edible mushrooms. These saprophytic basidiomycetes have been successfully cultivated at a commercial level worldwide using lignocellulosic wastes as the main substrate for their cultivation (Wood and Smith, 1985, quoted in Jones, 1993) *Agaricus bisporus*, known as the "button mushroom", *Lentinus edodes*, known as the "shiitake mushroom" and *Pluerotus ostreatus*, known as the "oyster mushroom" are only three examples of this agricultural crop (Zadrazil and Grabbe, 1983, quoted in Jones, 1993). Nowadays, increasing of world population has affected with increasing demand of food especially proteins but the protein meals have expensive than plants. For the mushroom is an alternative for the consumer which has provided higher protein than other plants. Therefore, the mushroom production has raise attention.

In Thailand, the production has important role to economic of country. Since Thailand is agriculture country, there are abundant waste materials from agriculture. Therefore, the waste is used as spawn preparation, Thailand geographic weather is appropriate for mushroom cultivation. Moreover, they have been reported that mushrooms contain special nutritional properties such as high protein (Table 1). Some strains are also good for the medical such as *Ganoderma lucidum*.

Table 1. The nutritional value of mushrooms (Crisan and Sands, 1978)

Nutrition	*Auricularia auricula* (Ear mushroom)	*Lentinus edodes* (Shiitake mushroom)	*Volvariela volvacea* (Straw mushroom)	*Pleurotus ostreatus* (Oyster mushroom)
Moisture (initial moist %)	87.1	80.3	90.1	90.8
Protein or crude protein (%w/w)	7.7	12.7	21.2	30.4
Fat (%w/w)	0.8	2.0	10.1	2.2
Carbohydrate (%w/w)	87.6	79.6	58.6	57.6
Energy (Kcal/100 g of DW)	347.0	330.0	368.0	345.0
Thiamine (mg/100g of DW)	0.2	7.8	1.2	4.8
Riboflavin (mg/100g of DW)	0.9	4.9	3.3	4.7
Niacin (mg/100g of DW)	1.6	54.9	91.9	108.7
Calcium (mg/100g of DW)	287.0	98.0	71.0	33.0

1.3 Mushroom production at Suranaree University of Technology - Farm

At Suranaree University of Technology-Farm, many varieties of mushroom have been cultivated such as *Pleurotus ostreatus*, *P. sajor-caju*, *P. cystidiosus*, *Lentinus edodes*, *Lentinula squaurrossula*, *L. polychrous*, *Auricularia auricula*, *Tricholoma crassum* and *Agrocybe cylindracea*. Almost fungal strains have identical cultivation. The mycelial form was cultivated on potato dextrose agar and incubated at 30^{o}C for 7-20 days then transferred to cereal grain media spawning and followed with sawdust spawning step. This sawdust spawning cultivation step takes 1-2 months before harvesting the fruiting bodies.

However, problem involving in mushroom production particularly instability of the strain is actually cause unhealthy mycelium and always occurs in large-scale production rendered the decreasing of yields. This problem could be solved by routinely propagate the new mycelial form from tissue culture that often gave lower yields than the original culture. Therefore, maintenance of strains by tissue culture cannot be recommended (Lambert, 1959, quoted in Kirsop and Snell, 1984). Moreover, In commercial production is one of pure culture maintained in refrigerator 4^{0}C during spawn running and next cropping which has reported genetic stability can be achieved by freeze-drying (Onions, 1971 and Heckly, 1978 quoted in Kirsop and Snell, 1984) and liquid nitrogen storage (Prescott and Kernkemp, 1971) both of which prove to be excellent long-term storage technique. However, the causes of instability in the strain have still been uncleared. In addition, it has reported that deviation from the original culture was also found with mycelium transfer, especially if the mother culture was originally a multisporus one. Therefore, this simple method is only practical if the performance of the mycelium is continuously checked. However, not all degenerative symptoms can be detected in the mycelium stage. Therefore, it is necessary to investigate the yield and the shape of the fruit bodies. Symptoms which can be seen in the mycelium stage are for example, sectors of slow-growing mycelium, fluffy mycelium with a normal growth rate (Gandy, 1960). (Zadrazil and Schneidereit, 1972 quoted in Chang and Hayes, 1987) studied that the fruit body development and yield parameter in *Pleurotus* production found that the environment factor is important to fruit body formation as demonstrated in Figure 2.

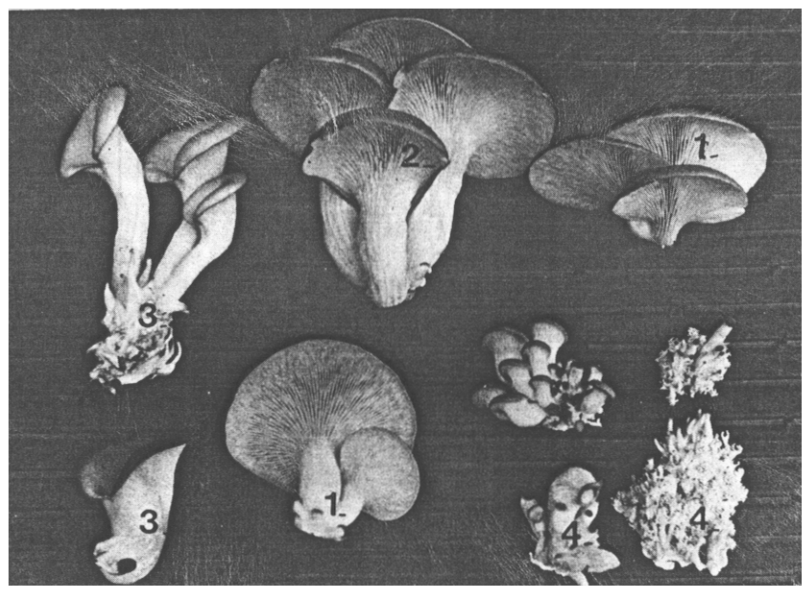


Figure 2. Influence of climatic conditions on the development of fruit bodies of *Pleurotus ostreatus* Zadrazil and Schneidereit, (1972)

In Figure 2, the fungus (1) shows normal fruit body development and appearance. The stipe is greatly reduced and the main mass developed as pileus. The fruit body (2) was grown with less light, the stipe is longer and thicker, and the pileus partly reduced. The effect of light is more plainly seen in (3) and (3a): very marked increase in stipe length and reduced size of pileus. Under extreme conditions are shown in (4). Insufficient ventilation (1-2 vol. % CO_2) and low light exposure have induced bunched growth regeneration. The composition of the air and the development of fruit bodies are directly related; with a greater exchange of air, the amount of fruit bodies per surface unit will reduced. Therefore, the environmental condition within cropping yard should be controlled adequately.

To study the biology of fungi, it would be incomplete without some understanding of the causes of their genetic variability. The vegetative hyphae of fungi are mainly haploid. Mutations arised, are easy to induce and are immediately expressed. There is no system to mask genetic deficiencies, no buffering by heterozygosity or genetic complementation, thus the effects of natural selection are more vigorous and more immediate than in most diploid organisms. The study of

spontaneous mutation is made difficult by the rarity of the event (Bos, 1996). For a given genetic locus, only one individual in 10^5 may carry a new mutation. This occurs at random at low frequency and in the metabolism of an organism, but some mutations have no effect (silent mutations), and others may have different consequences for the phenotype. The adaptive mutation was the reversion of this mutant gene. The experiment was designed so that the placed cells could grow for a period before being arrested. It seems likely that when growth was arrested, the last round of DNA replication may have produced errors at any site in any gene (Cairns et al., 1988 quoted in Bos, 1996). If so, these particular cells may gain the energy required to carry out the processing step (in this case another round of DNA replication) to produce a completed back-mutation. Cells with errors at other sites would not gain this energy, and their DNA would remain unreplicated long enough for repairing to reverse the sequence change. The dikaryon may have a critical selective advantage over the diploid in terms of the establishment of dikaryotic mosaics. This involves the direct exchange of one nucleus of the pair between adjacent dikaryons after hyphal fusion to establish a new dikaryon of different genotype, which Basidiomycotina posses, of eventual nuclear fusion and meiosis to provide still further new recombinations of genes producing further variable genotypes. There are many kinds of mutation, which some kinds may effect to phenotype, or not. For mushroom there are times when reproduce fruiting may appear abnormal fruiting body. So, this problem have been attempted for studying because abnormal quality is important for production such as hollow stems and distorted caps, as occurred in *Agaricus bisporus* (Figure 3)

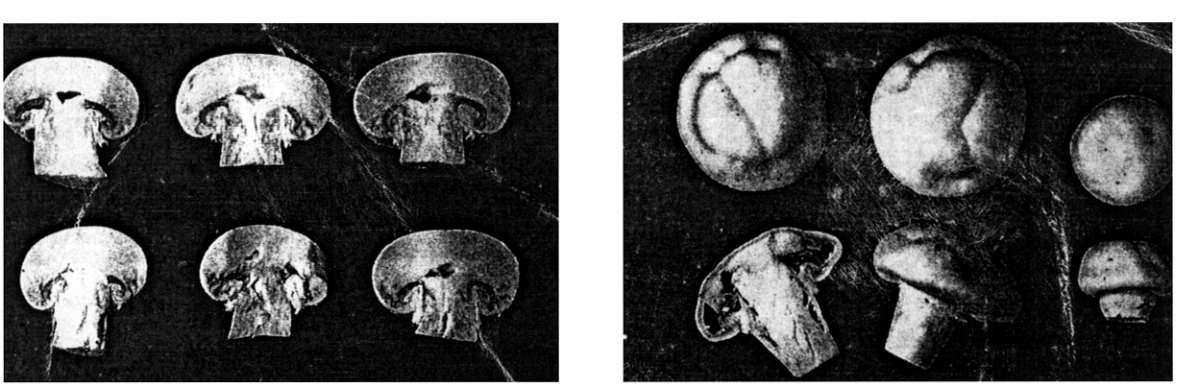


Figure 3. The mushroom showing hollow stem and caps (left) and distorted caps (right) of *Agaricus bisporus* (Gaze, 1999)

body can appear but still unclear probably due to environmental factors such as water, temperature and moisture may effect to fruiting. The appearance of a fruit body is due to genetic or cultural conditions (Eger, 1976, quoted in Chang and Hayes, 1978).

Mutation is the basis of all variations, but mutations are expressed and recombined in different ways depending on the biology of the organism. Fungi are the only major group of haploid eukaryotes; the others are diploid. Haploid organisms express all their genes and thus perpetually expose them to selection pressure. Any mutations in a gene will either cause a loss of fitness, in which case the mutant are eliminated, or it will lead to an increase in fitness in which case the mutant will flourish. This can be beneficial in the short term but the corresponding disadvantage is that haploid organisms cannot accumulate mutations that are not immediate value: they cannot store variation. Mutations often are recessive to the wild type and so they are not immediately expressed; instead, they accumulate and can be recombined in various ways during sexual crossing, so that some of the progeny might have advantageous combinations of mutant genes (Deacon, 1997).

A main point in the life cycle of any organism is the alternation of haploid and diploid "generation". In the diploid phase an organism has two sets of chromosomes (two genomes), and the homologous chromosomes differ when the parents contained different mutation (alleles) on homologous loci. In lower plants (mosses, ferns) the haploid phase is much longer, and in fact the moss plants haploid and only the sporangium and the sporangium stalk are diploid. The haploid phase that ultimately produces the gamete is called the gametophyte, and the diploid phase the sporophyte (Beadle and Tatum, 1941, quoted in Deacon, 1997). The phase alternation consists of plasmogamy-karyogamy-meiosis. In both phases the somatic cells divide in a characteristic way (mitosis). Two different recombination processes occur during meiosis: reassortment of homologous chromosomes, and exchange of genetic material between nonsister chromatids of homologous chromosomes (crossing over).

In fungi the alternation of the haploid and diploid phase can be complex, because there can be a long period of time between plasmogamy and karyogamy (Fincham, Day and Radford, 1979, quoted in Bos, 1996). In general, plasmogamy between different strains results in a heterokaryon. In case of the transition of the gametophytic to the sporophytic phase, plasmogamy results in a dikaryon that is maintained in a specific way of concerted nuclear divisions. In basidiomycetes, the dikaryotic phase is

considerably extended; germ tubes of basidiospores or conidia fuse as soon as they come into contact, exchanging their nuclei. Since often a spore germinates close to others, these hyphal fusions and dikaryotizations are multiplied, leading to different pairs of nuclei (Pontecorvo, 1958, quoted in Deacon, 1997). The germination behaviour of basidiomycetes favours dikaryotization. In *Leccinum* and other genera a mutual stimulation of germination has been found, which can be interpreted as a means of increasing hyphal contacts and thus dikaryotization. The deposition of the major proportion of the spores that are produced in the immediate neighbourhood of the parent mycelium, as is often the case with mushrooms, can not be understood without considering the probable genetic consequences. Although the germination phase may be completed, there is a continuing incorporation of nuclei. Even dikaryotic hyphae continue to incorporate further nuclei. This is achieved by hyphal contact and subsequent fusion. In addition, basidiospores or conidia (mono-or dikaryotic), which settle close to a mycelium of the same species, come into contact with the growing hyphae, which incorporate the nuclei of the propagates. Thus dikaryotization, or the absorption of more nuclei occurs during the entire lifespan of the mycelium.

1.4 Development of detection system for strain instability.

Molecular techniques are now available to study genetic variation within fungi. The techniques include allozymes analysis, restriction fragment length polymorphism (RFLPs) of DNA, and electrophoretic karyotypes (Schwartz and Cantor, 1984, quoted in Oliver and Schweizer, 1999) which involves the separation of whole chromosomes by pulsed-field gel electrophoresis. In the late 1980s, hybridization-based DNA fingerprinting was introduced for the analysis of fungal genomes. Since then, DNA fingerprinting has become one of the favorite methods for diagnostic and epidemiological studies of human, plant, and insect pathogenic fungi; for the characterization of non pathogenic and industrially important fungi; and for the evaluation of taxonomic and phylogenetic problems. The basis of genetic analysis is to have tools to discriminate biological entities at many different levels. Classically, this has been done using morphological and physiological criteria to distinguish between species. With filamentous fungi discrimination at species level using these traits already can be very difficult or even give erroneous results (Samson, Someren and Visser, 1991). At even lower levels (e.g., isolates of one species from different populations), these methods are no longer applicable. Molecular markers, however, can be applied at these levels with great reliability. Molecular markers are not principally different from classical genetic mutations. Both are the results of changes

in the DNA of the organism, but where with classical mutants this resulted in a phenotype that can be distinguished at the cellular level, the phenotype of a molecular marker can only be observed by looking directly at the DNA.

The first molecular markers that have been used, which are simple to interpret and therefore still are very popular (Meyer et al.,1992 quoted in Bos, 1996) are Restriction Fragment Length Polymorphism (RFLPs). An RFLP can be the result of the gain or loss of a restriction endonuclease cleavage site at a given chromosomal location, resulting from point mutations in the DNA. However, deletions and insertions will also lead to alterations in the length of the restriction fragments. RFLP analysis thus requires the isolation of substantial amounts of rather extensively purified DNA. Although several good protocols for DNA extraction from fungi have been published, in many cases obtaining restrictable DNA is quite cumbersome. RFLP analysis is used in taxonomy and phylogenetic, and single-locus RFLP markers can be applied at species level as demonstrated with *Aspergillus*, (Moody and Tyler, 1990) *Phytophthora*, (Foster et al., 1989 quoted in Bos, 1996) and *Fusarium*, as well as at subspecies (formaspecialis) level, e.g., with in *F. oxysporum* and *Leptosphaeria maculans*. However, in some fungal species only very little sequence variation occurs. Since RFLP detects mutations in restriction sites, which are only very short sequences, this might imply that large numbers of restriction enzymes and probes must be used to find sufficient polymorphism in such fungi.

A special application of RFLP analysis, which is particularly well suited for fungi, is the analysis of mitochondrial DNA (mt DNA). Mitochondrial genomes are small (20-180 Kb), and mtDNA can be isolated in quantities sufficient to directly perform restriction analysis with multiple enzymes. This can result in quick and sensitive detection of strain differences, which has made RFLP analysis of mitochondrial DNA very popular in taxonomic studies. Moody and Tyler, (1990) used such methods to discriminate between species within the *A. flavus* group, and Foster et al., (1989) were able to detect differences in *Phytophthora* at both species and subspecies levels. With *F. oxysporum*, mitochondrial RFLPs could discern not only three subspecies within a group of 25 strains, but even different vegetative incompatibility groups within one subspecies (Jacobson and Gordon, 1990). Mitochondria in fungi are usually uniparentally inherited (Tyler, 1986) but they can be transferred independent of the nuclear genome (Collins and Saville, 1990 quoted in Bos, 1996) and mtDNAs do show recombination. Consequently mitochondrial RFLPs might either underestimate or overestimate actual genetic divergence. Therefore, combined RFLP analyses of both mitochondrial and nuclear

DNA sequence can be useful. The relative insensitivity of RFLP analysis as above is partly caused by the fact that analysis is covered by the probe employed. Sensitivity could be enhanced by using probes that detect multiple loci: dispersed repetitive sequences. In many eukaryotic organisms, including several fungi, repetitive sequences are very common. Isolation of such a sequence and using it as a probe on Southern blotes of restriction digests of chromosomal DNA leads to patterns with many bands. This technique, which is called fingerprinting, has been applied to the human pathogen *A. fumigatus* (Loudon et al., 1993 quoted in Deacon, 1997). At least 20 bands were detected, allowing discrimination of individual isolates. Also synthetic simple repeat oligonucleotides can be used as probes, as was demonstrated in typing different strains from the genera *Penicillium, Aspergillus*, and *Trichoderma* and in classification of species within the *Trichoderma* aggregate.

In eukaryotes, rDNA genes are repeated up to several hundreds of times in a clustered manner. The genes are separated by nontranscribed spacer regions (NTS), which contain the signals for rDNA expression. The primary transcript of an rDNA gene is processed to one copy each of 18S, 28S, and 5.8S rRNA. The regions separating these RNAs are called the internal transcribed spacers (ITS) (Bos, 1996). The nuclear rDNA sequences coding for the small subunit (18S) and large subunit (28S) RNAs show very little evolutionary change and can thus be used to compare distantly related organisms (Figure 4).

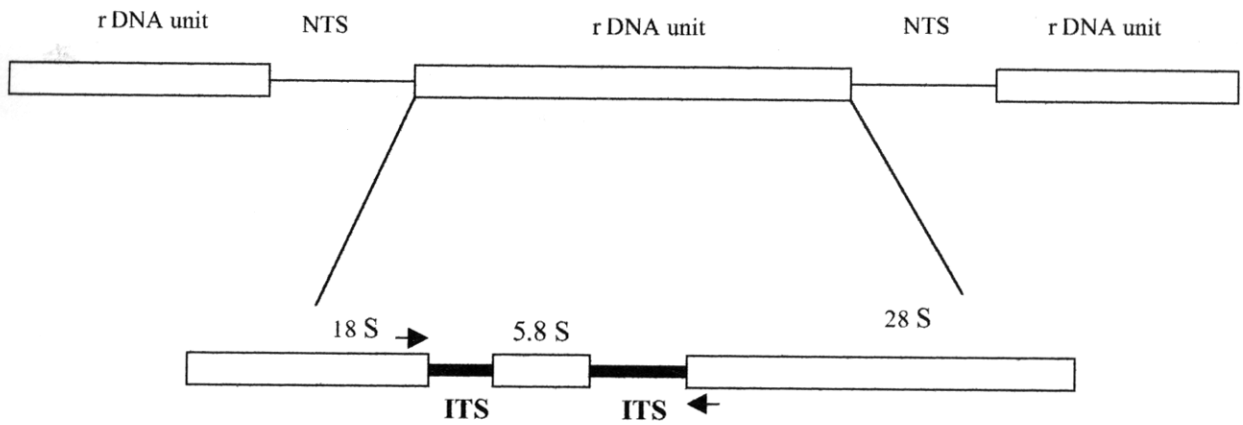


Figure 4. Organization of the eukaryotic ribosomal DNA genes. Several hundred copies of the ribosomal genes are present in a clustered manner. The genes are separated by nontranscribed spacer (NTS) regions, which contain the sequences for rDNA expression. The primary trancript of an rDNA gene is processed to one copy each of 18S, 5.8S, and 28S rRNA. The regions separating these RNAs are called internal transcribed spacers (ITS). Using primer complementary to strongly conserved regions in the 18S and 28S genes (arrows), the highly variable ITS regions of different (sub) species can be amplified by PCR. Analysis of the products reveals the genetic differences (White, Bruns, and Taylor, 1990)

The internal transcribed spacer region as well as the intergenic spacer of the nuclear rDNA repeats evolve much faster, and sequence differnces in these regions occur between species within one genus or even between different populutions of one species. Consequently, analyzing the rDNA repeat is very useful for comparisons over a wide range of taxonomic levels but nevertheless has high resolving power, depending on which part of the ribosomal DNA genes is analyzed. The nucleotide sequences of the rDNA repeat unit has been determined from a large number of eukaryotes. Compilation of these sequence data can identify stretches of nucleotides within the 18S, 5.8S, and 28S regions that are highly conserved (White, Bruns, Lee and Taylor, 1990). This allows the design of primers, which can be used in PCR experiments to amplify regions of the rDNA repeat, including the NTS or ITS regions. Primers may be chosen in such a

primers, which can be used in PCR experiments to amplify regions of the rDNA repeat, including the NTS or ITS regions. Primers may be chosen in such a way that they are specific for a group of genera, e.g all fungi. Because several hundreds of copies of rDNA are present per genome, only very small amounts of chromosomal DNA are necessary for these amplifications. The fragments resulting from such PCR reactions can be directly analyzed on agarose gels for differences in length of the NTS or ITS regions.

A further discrimination can be obtained by digesting the PCR products (Saperstein and Nickerson, 1991) with a number of the restriction endonucleases and analyzing the products. The highest detail is obtained by direct sequencing of the PCR products, which will detect every single base-pair difference of the amplified fragment between samples. Sequencing of PCR-amplified ribosomal DNA genes is widely used in evolutionary genetics to establish phylogenetic relationships among fungi (Bruns et al., 1992 quoted in Bos 1996). As more of these sequence data are generated and put into the data bases, the sensitivity and resolution of the method will be further increased by the possibility of devising class, family, genus, and possibly even species-specific primers (Simon, Lalonde and Bruns, 1992).

In mushroom production for large scale has essential into quality control of phenotype and quality of fruit body such as size, color and satisfy of consumer altogether. The defect of fruit body due to appearance for consumer and efficiency production. Therefore, this study was aimed to genetic instability of mushroom after continuous subculturing for production as quality control prior to production.

1.5 Objectives

1. To investigate genetic variation of mushrooms after continuous subculturing.
2. To develop the nucleic acid for detecting genetic variation in DNA level prior to introduce into commercial scale.
3. To develop the appropriate cultivation technology of *Tricholoma crassum* by using agricultural wastes as cultivation materials.

Chapter II

Materials and Methods

2.1 Materials

A. Mushroom species

1. *Pleurotus ostreatus* (Oyster mushroom)
2. *P. sajor-caju*
3. *P. cystidiosus*
4. *Lentinus edodes* (Shiitake mushroom)
5. *Lentinula squarrossula*
6. *L. polychrous*
7. *Agrocybe cylindracea*
8. *Auricularia auricula* (Ear mushroom)
9. *Tricholoma crassum*

B. Equipment

1. Thermal cycle (PCR Hybaid and Perkin-Elmer-ABI 9700 thermo cycler system (Perkin-Elmer, singapore)
2. Shaker (Innova ™ ; model: 2300 Platform Shaker, New Brunswick Sciencitific, New Jersey, U.S.A)
3. Microcentrifuge (Eppendorf , model: Centrifuge 5415 C, Germany)
4. Water bath (Heto- Hoten; model: maxi-shaker, Denmark)
5. Vortex (Vortex-2 Genie ™; model: G-560 E, Scientific Industry, U.S.A)
6. Autoclave (HICLAVE ™; model: HA- 300 D, Hirayama Manufacturing Cooperation, Japan)
7. Electrophoresis Chamber (Sub-Cell® GT, purchased from Bio-Rad)
   : Horizontal Electrophoresis Chamber

: Vertical Electrophoresis Chamber

8. Gel Documentation (UVP ULTRA-Violet product; model: GDS 7500-Camera, U.S.A)

C. Reagents

1. For cultivation

- PDA (Potato Dextrose Agar): Potatoes 200 g/L
  Bacto Dextrose 20 g/L
  Agar 15 g/L
- PDB (Potato Dextrose Broth): Potatoes 200 g/L
  Dextrose 20 g/L

2. For DNA extraction

- Lysis buffer: 50 mM Tris-HCl (pH 7.2)
  50 mM EDTA (pH 8.0) (w/v)
  3% SDS (w/v)
  1% 2-mercaptoethanol (v/v)
- Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) (v/v/v)
- 3 M NaOAc (pH 8.0) (w/v)
- Isopropanol
- 70% ethanol (v/v)
- TE buffer: 10 mM Tris-HCl (pH 7.2)
  0.1 mM EDTA (pH 8.0)
- TBE buffer: 0.089 M Tris – base
  2.5 mM Na_2EDTA (pH 8.0)
  0.088 M Boric acid
- RNase A solution 100 μg/ml
- DNA marker: 100 bp DNA (purchased from GIBCOBRL®)

3. For PCR (Polymerase Chain Reaction) amplification

- *Taq* DNA polymerase (GIBCO) 5 unit/μl
- 10 X buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl (pH 8.3))

- dNTP (dATP, dTTP, dGTP, dCTP)
- ITS 4 and 5 primer (Internal Transcribed Spacer)

  ITS4: 5' GGAAGTAAA AGTCGTAACAAGG 3'

  ITS5: 5' TCCTCCGCTTATTGATATGC 3'
- β-tubulin gene

  B36F: 5'CACCCACTCCCTCGGTGGTG3'

  B12R: 5'CATGAAGAAGTGAAGACGCGGGAA 3'

4. For RFLP (Restriction Fragment Length Polymorphism) analysis
   - restriction enzyme : *Alu* I, *Taq* I, *Mbo* I and *Hinf* I (purchased from Bio- Rad)
   - 2% Agarose
   - 10 % Polyacrylamide gel: 30% Acrylamide stock solution: acrylamide 29 g, N, N'-methylene bisacrylamide 1 g, bisacrylamide 1 g and distilled water 100 ml
5. For DNA Sequencing
   - 3'- dye labeled dideoxynucleotide triphosphate (dye terminators) fluorescent dyes
6. For Composting
   - sawdust
   - rice bran
   - sucrose
   - gypsum ($CaSO_4$)
   - $MgSO_4$
   - $CaCO_3$
   - water

2. 2 Methods

To attain objective 1 and 2: To investigate genetic variation of mushrooms after continuous subculturing. This study was done as following;

1. The growth rate on PDA, compost and yield were determined every subculturing.
2. The PCR-RFLP analysis were determined every subculturing.

3. The defect fruit body was investigated with ITS4 and 5 RFLP and β-tubulin gene.

A. Culture collection and isolation

The fungal species used throughout this study were obtained from SUT-Farm as followed: *Pleurotus ostreatus*, *P. sajor-caju*, *P. cystidiosus*, *Lentinus edodes*, *Lentinula squaurrossula*, *L. polychrous*, *Auricularia auricula*, *Tricholoma crassum* and *Agrocybe cylindracea*. The pure cultures were prepared from original culture and tissue culture. The tissue culture were done by isolation by fruit body under sterile conditions. Pieces of the interior parts were cut with an inoculating needle and transferred to PDA plates. The mycelial form was cultivated on PDA (potato dextrose agar) and incubated at 30^0C for 7-15 days. Then mycelium form was cultivated continuously until the margin of the colony was 0.6-1.0 cm from the edge of the plate. The growth rate on medium was recorded every day.

B. Spawn preparation

The 1 cm^2 of mycelium form was inoculated into sorghum seed bottle which was sterilized for 1 hour. The bottle was incubated at room temperature about 7-15 days.

C. Mushroom cultivation

The spawn running in sorghum seeds were transferred into compost about 20-30 grains/ 1 compost bag. Then spawn in compost was incubated at room temperature in the growing room. The mycelium reached the end of the compost and grew into it, which generally took 1-2 months. The completely grown bag were taken to moisture controlled room equipped with automatic water sprayer. Before harvesting the fruiting bodies, the growth rate of mycelium in cultivation and spawn running step were determined. The yields of fruiting body were also analyzed in unit of fresh weight (g)/ 1 compost/ 3 months.

D. Maintenance of cultures

The parent cultures were maintained on PDA at 4^0C and supplement in 10% (v/v)glycerol at -70^0C, then check the survival of culture every 2 weeks. The serial transfer mycelium in experiment were maintained at 4^0C for during mushroom cultivation.

E. Production of composting media

The composting media was contained 100 kg of sawdust, 5 kg of rice bran, 1 kg of $CaCO_3$, 0.5-1 kg of gypsum , 0.2 kg of sugar and adjusted moisture content was adjusted about 80% with water. Then the compost were mixed well and filled into each of plastic bag about 0.8 kg per one bag. The compost bags were pasteurized by steaming at 100^0C for 3 hours. The pasteurized compost bags were inoculated with mushroom spawn grown in sorghum seed. When the mycelium was fully grown in compost for 1-2 months then yield was recorded after harvesting.

F. Isolation of DNA

The mycelium was cultivated in PDB. The cultures were incubated at room temperature (30^0C) under shaken at 150 rpm for 3-5 days. Chromosomal DNA was isolated from each fungal strain by the procedure described by Lee and Taylor (1990). The mycelium was ground with liquid nitrogen (-180^0C) and lyzed with 400-500 μl of lysis buffer then mixed to get homogeneous miwture and incubated at 65 ^{0}C for 1 hour. Four hundred μl of phenol: chloroform: isoamyl (25: 24: 1) was added and briefly vortexed (2 times) and then centrifuged at 13,000 X g for 15 mins at room temperature. The aqueous phase was transferred into a new tube and added 40 μl of 3 M NaOAc (pH 8.0) followed with 2 vol of isopropanol and mixed gently. The DNA pellet was rinsed again with 70% ethanol. The pellet was resuspended after 70% ethanol was discorded with 10 to 50 μl of TE buffer and treated DNA template with 1/10 RNase A 100 μg/ml. The DNA template was stained with ethidium bromide after electrophoresis.

G. PCR amplification

The chromosomal DNA were extracted from mycelium and used as DNA template for the amplification. The DNA primers ITS (Internal Transcribed Spacer) (ITS4: 5' GGAAGTAAA AGTCGTAACAAGG 3'), (ITS5: 5' TCCTCCGCTTATTGATATGC 3') primers were used. The reaction mixture contained in total volume of each (for 1 amplification) 27.5 μl of sterile distilled water, 10 μl of 10X amplification buffer (15 mM $MgCl_2$, 500 mM KCl 100 mM Tris-HCl (pH 8.3)), 10 μl of dNTP stock mixture (2 mM each dNTP), 1 μl of primer (50 μM stock), and 0.25 μl of *Taq* polymerase (5 units/μl). The optimal parameter of initial denature was 3 min, 95^0C; denature 30 sec, 95^0C; annealing 30 sec, 53^0C; extension 2 min, 72^0C and final extension 10 min, 72^0C were done in 35 cycles with PCR System PE 9700 (Perkin Elmer Corp., Singapore)

In addition another DNA primers β-tubulin gene (B36F: 5' CACCCACTCCCTCGGTG GTG 3'), (B12R: 5' CATGAAGAAGTGAAGACGCGGGAA 3') primers were also used. The

reactions were performed in a total volume of 25 μl, 2.5 μl of $MgCl_2$ (2.0 mM of $MgCl_2$ stock), 5 μl of dNTP stock mixture (0.2 mM of each dNTP), 1 μl of primer (0.5 mM of each primer) and 1 unit of *Taq* polymerase (GIBCOBRL®). The optimal parameter initial denature 1 min, 94^0C; denature 30 sec, 94^0C; annealing 30 sec, 56^0C; extension 30 sec, 72^0C and final extension 10 min, 72^0C were done in 35 cycles with PCR System PE 9700 (Perkin Elmer Corp.,U.S.A.)

H. RFLP (Restriction Fragment Length Polymorphism) analysis

The restriction enzymes used in this study were *Alu* I, *Taq* I, *Mbo* I and *Hinf* I. The PCR product was digested with the restriction enzymes and analysed on 10 % polyacrylamide gel at 80 volt and 1.30 hours or 2 % agarose gel at 80 volt and 40 mins and stained with < 0.5 mg/ ml of ethidium bromide.

I. Sequencing

Direct sequencing from PCR products were in sizes about 600 bp. The ITS sequences were generated with Perkin Elmer's ABI PRISM™ 377 DNA sequencer. Sequencing reactions were done using ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase, FS (Perkin Elmer). (The DNA sequence by Bio Service Unit)

J. Sequence analysis

In this study, to align between wild type and mutant sequences with ITS 4-5 primers by CLUSTAL X program was used and to search recognition sites for investigated different sequences.

K. *Tricholoma crassum* production

To attain objective 3: To find appropriate cultivation for *Tricholoma crassum* production by using agricultural wastes. This study was done as following;

1. Cultivation

The pure culture were cultivated on PDA (potato dextrose agar) and incubated at 30^0C. Growth rate determination was done by measuring colonial diameter at 20-25 days. Spawn preparation was done by transferring mycelial form into sterilized sorghum seeds containing in flat bottle and incubated for 15-20 days. The spawn was then inoculated into pasteurized sawdust substrate containing in plastic bag at 800 g/bag and incubated for 30-45 days.

2. Casing preparation

The experiment was designed by using completely randomized design having 3 replications. Each replication containing 3 main plots, 2 sub main plots and 3 sub plots. The main plots were (1) rice straw covering (2) rice husk covering (3) non covering. The sub plots were plastic and clay pots. The sub-sub plots were 3 casing materials of (1) clay soil, (2) soybean husk and (3) clay soil and soybean husk mixtures at (1:1,vol/vol). The dimension of clay and plastic pots were 40-47 cm in diameter and 30-37 cm in height. Each container (pot) was contained 4 mushroom blocks (sawdust spawning) having spacing of 10 cm between block. Casing materials were filled into each basket up to 10 cm below brim of container. The total of yield were analyzed with Split-Split Plot Design.

Chapter III

Results and Discussion

3.1 Mushroom Production

To determine the yield of mushroom as affected by subculturing, the growth rates performed both on PDA and spawning steps were investigated and compared with their yield in each generation.

3.1.1 The growth rate of individual species of mushroom on PDA (Potato Dextrose Agar)

The mycelium from each species of mushroom was cultivated on PDA and incubated at 30^0C (3 replications plates) for 10-15 days. Results of growth rate was obtained by measuring the distance of mycelium growth on plate everyday as demonstrated in Figure 5. Each species of mushroom showed the different growth rates on PDA. Most species gave the growth rate in linear relationships when compared within 20 times of subculturing. However, some species prolonged growth times when more times of subculturing (data not shown). The results obtained from this study found that most species gave constant growth rate. Some species of *P. sajor-caju*, *L. polychrous* and *L. squarrossula* gave the highest of growth rate in the first generation about 1.420, 1.778 and 1.670 cm / day, respectively and average of growth rate among 0.91 ± 0.22, 1.34 ± 0.21 and 1.51 ± 0.20 cm / day, respectively. In addition, *P. sajor-caju*, *L. squarrossula*, *L. polychrous* and *Agrocybe cylindracea* showed decreasing intensively growth rate when more times of subculturing while the other species did not. Temperature might provide the growth limiting factor because (Kinugawa and Furakawa, 1965, quoted in Chang and Heyes, 1978) observed that in *Flammulina velutipes* the temperature is one of the important factor in the control of mycelial growth. The optimum temperature is between 22^0C and 26^0C. If temperature raised above 34^0C, the mycelium were slowly grown. For *L. edodes*, if temperature was below 5^0C and above 35^0C the mycelial growth was stopped (Tokimoto and Komatsu, 1975, quoted in Chang and Heyes,

1978). From this experiment, however, the incubated temperature was fixed for all species at 30^0C. Thus, reduction in the growth rate was possibly due to the optimum temperatures.

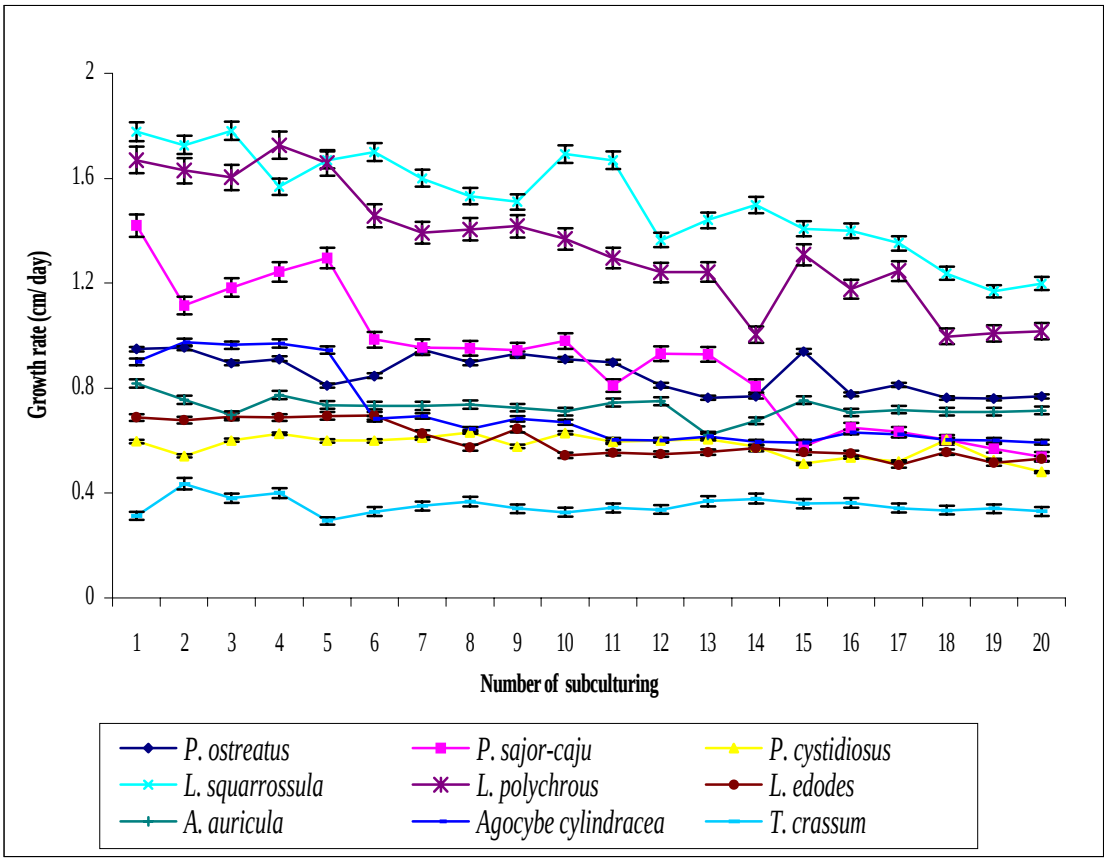


Figure 5. The growth rate of individual species of mushrooms on PDA

3.1.2 The growth rate of individual species of mushrooms in compost

The mycelium form was inoculated on sorghum grains as "spawn run" for 7-15 days at room temperature. The spawn was transferred into compost about 20-30 grains per 1 compost. The mycelium reached the end of plastic bag within 1-2 months in the spawn-growing room. The growth rate was recorded every 5 days in *P.ostreatus*, *P.sajor-caju*, *L. squarrossula*, *L. polychrous*, *Agrocybe cylindracea* and 2 weeks in *P. cystidiosus* and *L. edodes*. It was found that each species gave different in growth rates and spawn run times depended on individual species such as 3.68 ± 0.12 cm/ 5 days of *P. sajor-caju*, 3.36 ± 0.18 cm/ 2 weeks of *L. edodes* and 2.65 ± 0.14 cm/ 5 days of *A. auricula* at significant 95%. However, different species might require

different conditions in the growing room. The average compost temperature during mycelium growth was about 30^0C ventilation in the growing room was ventilated as little as possible resulted in preventing the compost temperature from raising above 30^0C (Vedder, 1975, quoted in Chang and Heyes, 1978).

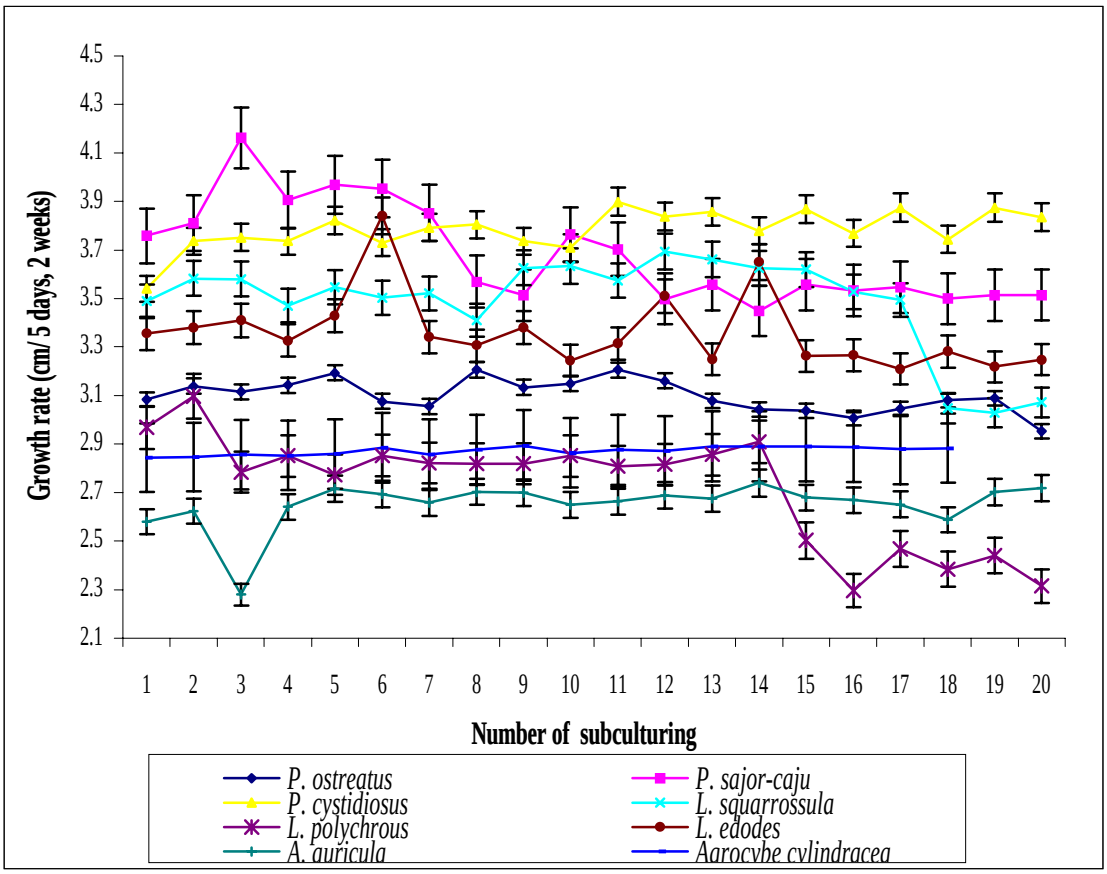


Figure 6. The growth rate of individual species of mushroom in compost

From Figure 6 showed that the most of all species had constant growth rates while some species of *L. squarrossula* and *L. polychrous* were reduced and prolonged of mycelium growth in composts (as shown in Appendix). The growth rate was reduced from the first subculturing about 16.8% and 25.3%, respectively at significantly 95%. From this part, it could be concluded that the growth rates were decreased when more times of subculturing were conducted. But the growth rate was reduced different rates in each of species. For instance, *L. squarrossula* had almost

constant growth rate 3.56 ± 0.10 cm/ 5 days in range 1st-16th subculturing but was decreased 3.05 ± 0.08 cm/ 5 days after 17th subculturing.

3.1.3 Yield of individual species of mushrooms.

When the mycelium reached the bottom of the plastic bag they were carried to production house. Each of species gave different yield depended on characteristics of mushrooms and environment treatments. Most of them gave highest yield in the first cropping. Then yield were reduced when more times of subculturing. The result was agreed with (Tonomura, 1978, quoted in Chang and Heyes, 1978) which phenomena was somewhat similar to *A. bisporus* cultivation that the first crop gave highest yield but the quantity of fruit bodies was small and the quality was also declined with time. The average of total yields, *P. sajor-caju*, *P. ostreatus* and *L. squarrossula* were able to give highest yield among 282.5 g, 279.4 g and 278.0 g (fresh weight/ 1 compost/ 3 months), respectively in the first cropping while 20th subculturing were reduced 32.0%, 27.8%. and 27.8% , respectively. However the other species had constant of total yields as shown in Figure 7.

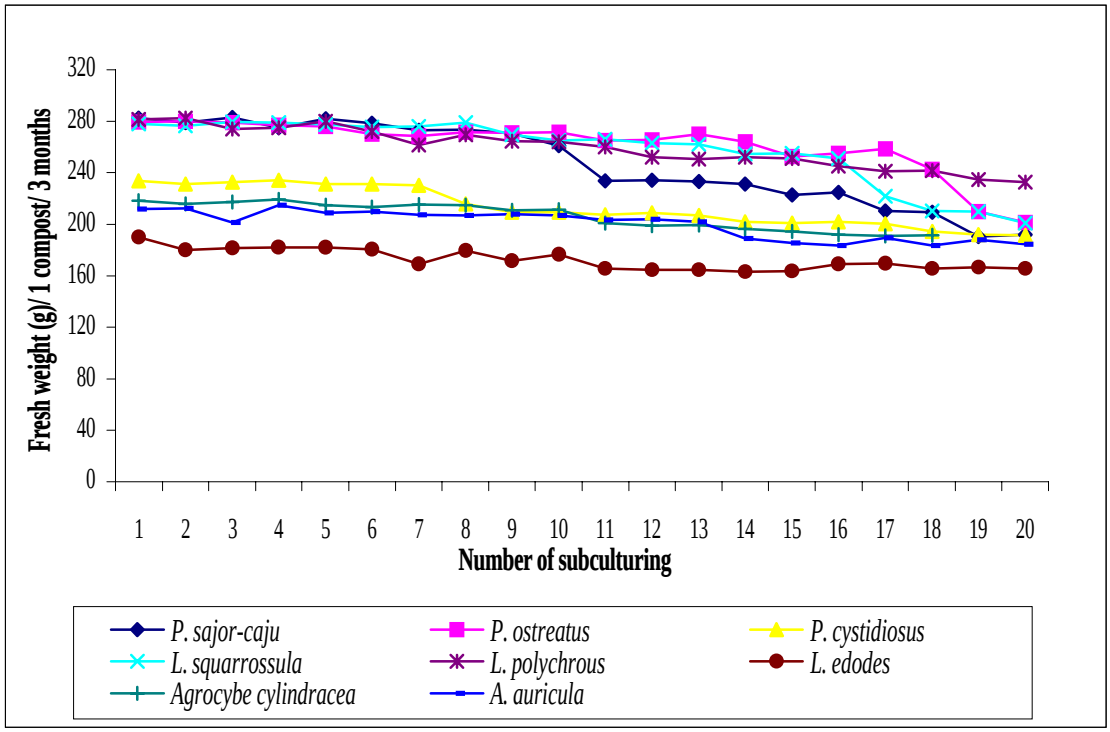


Figure 7. The average yield of different kinds of mushrooms

Although, these species gave high yield but growth rates were reduced with more times subculturing. The yields of *L. squarrossula* was reduced about 27.8% from the first cropping and the growth rates in compost were also reduced. While the yield of *L. polychrous* had constant until 20th subculturing but growth rate in compost was reduced since 14th subculturing. In addition, the average of total yield of *P. ostreatus* was reduced since 18th subculturing 13.1% from the first cropping while the growth rate in compost almost constant since the first subculturing were significant at 95%. From this part, it could be concluded that total yield of mushroom was reduced when more times of subculturing was performed. This result was agreed with Jankowska (1970) quoted in Chang and Heyes, 1978 observed that in *A. bisporus*, it was not possible to find relationships between mycelial growth rate and yield. The other species, *P. cystidiosus*, *Agrocybe cylindracea*, *L. edodes* and *A. auricula* were almost no different in yields and growth rates in compost when more times of subculturing were conducted. In addition, it had been reported that loss of yield were studied by Fritsche (1972, 1974) quoted in Chang and Heyes, 1978 that the maintenance of *A. bisporus* after 2 years found that symptoms of degeneration, poor mycelial growth and loss of yield. Moreover, it had been reported that tissue cultures often gave lower yield than the original culture. Thus, the loss of yield was affected by many factors such as maintenance of culture, mycelium transfer by tissue culture and environment factor. Although, during cropping most of them had constant of total yield but some species were reduced in yields such as *L. squarrossula* and *P. ostreatus*.

The phenotype of fruit body was still normal when more times of subculturing were performed. This experiment was observed the abnormal fruit body of *P. ostreatus*. This abnormal fruit body was obtained from continuous subculturing cropping. It was shown that the defective of fruit body might be due to the effect of continuous subculturing. The defective of *P. ostreatus* was shown the cap diameter above 7.5 cm and stipe length above 8.0 cm while normal fruit body had cap of diameter 6.0 cm and stipe length 6.5 cm as shown in Figure 8. This defective fruit body was further investigated by DNA techniques and found that the DNA pattern gave the same with normal fruit body. Thus, it could be concluded that this defective fruit body was not caused by genetic defective. It has been reported that the cause of abnormal fruit body was caused by diseases and environment (Vedder, 1975). The defective of fruit body found from this study was due to the quality of mushroom. Therefore, in the cultivation it should be managed as adequately

well so that this problem could be reduced. Muller (1989) studied the development of fruit body of *P. ostreatus* he found that the environment factor had effect on gene expression which related with biochemical of mushroom. The temperature had affected on enzyme activity. The fruit body could not develop if raising temperature because enzyme activity will be decreased at high temperature.

a)

b)

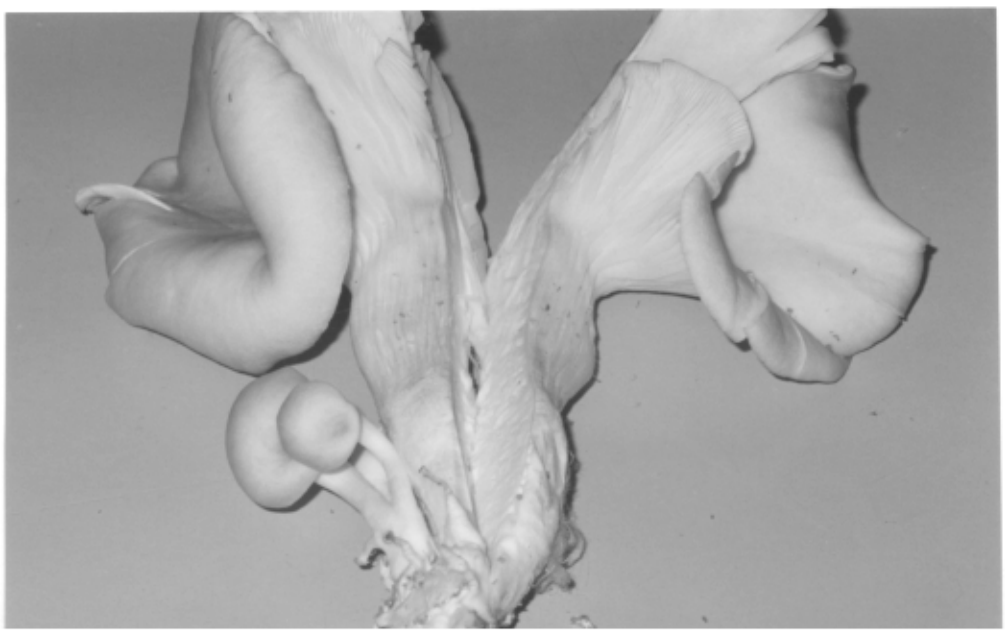


Figure 8. The photo of normal and abnormal fruit body of *Pleurotus sajor-caju* obtained from cropping: a) normal fruit body b) abnormal fruit body

3.2 Analysis of ITS (Internal Transcribed Spacer) 4 and 5 RFLP (Restriction Fragment Length Polymorphism)

To investigate genetic variation of mushroom after continuous subculturing on basis of DNA technique. The PCR products were amplified with ITS 4 and 5 primers by using DNA template from *Lentinula polychrous*, *L. squarrossula*, *L. edodes*, *Pleurotus ostreatus*, *P. sajor-caju*, *P. cystidiosus*, *Auricularia. auricula*, *Agrocybe cylindrace* and *Tricholoma crassum*. Then, the PCR products were digested with the various restriction enzymes such as *Hinf* I, *Alu* I, *Mbo* I and *Taq* I (stained with Ethidium bromide on 2% agarose at 80 V, 40 mins). The fragment sizes of individual species after digested with restriction enzymes as shown in Table 2-5.

Table 2. The fragment sizes of mushroom species that can be seen on the gel after digested with *Alu* I

Mushroom species	PCR products (bp)	PCR-RFLP products digested by *Alu* I (bp)
L. polychrous	700	390, 135
L. squarrossula	700	400, 100
P. ostreatus	700	600, 90
P. sajor-caju	700	600, 100
P. cystidiosus	750	400, 180
Tricholoma crassum	700	500
A. auricula	600	390,123
Agrocybe cylindracea	750	520, 200
Lentinus edodes	800	500, 190, 100

Table 3. The fragment sizes of mushroom species that can be seen on the gel after digested with *Mbo* I

Mushroom species	PCR products (bp)	PCR-RFLP products digested by *Mbo* I (bp)
L. polychrous	700	290, 190
L. squarrossula	700	290, 190
P. ostreatus	700	400, 190, 100
P. sajor-caju	700	400, 200, 100
P. cystidiosus	750	400, 300, 70
Tricholoma crassum	700	390, 130
A. auricula	600	400, 180
Agrocybe cylindracea	750	450, 280
Lentinus edodes	800	400, 190, 100, 90

Table 4. The fragment sizes of mushroom species that can be seen on the gel after digested with *Taq* I

Mushroom species	PCR products (bp)	PCR-RFLP product digested by *Taq* I (bp)
L. polychrous	700	150,100, 90
L. squarrossula	700	300, 180, 100, 70
P. ostreatus	700	350
P. sajor-caju	700	350
P. cystidiosus	750	330
Tricholoma crassum	700	350, 190, 180
A. auricula	600	320, 200
Agrocybe cylindracea	750	350, 210, 120
Lentinus edodes	800	400, 320

Table 5. The fragment sizes of mushroom species that can be seen on the gel after digested with *Hinf* I

Mushroom species	PCR products (bp)	PCR-RFLP products digested by *Hinf* I (bp)
L. polychrous	700	300, 200, 150
L. squarrossula	700	390, 380
P. ostreatus	700	390, 230, 110
P. sajor-caju	700	400, 230
P. cystidiosus	750	300, 250, 150
Tricholoma crassum	700	300, 200, 180
A. auricula	600	300
Agrocybe cylindracea	750	350, 210, 180
Lentinus edodes	800	500

From the Table 2-5 and Figure 9-44 showed that the DNA-fingerprint of each species gave different DNA patterns when digested by the various restriction enzymes. The ITS sequences were studies the distribution of genetic variation in *Lentinula* (Fukada and Tokimoto, 1994) and others higher fungi. There are multiple copies of 18S, 5.8S and 28S rRNA genes which coding sequences of rRNA are highly conserved between species of fungi. Therefore, these species were classified within one genus. But the ITS region could be amplified the DNA template in the highly variable region thus this primers was studied genetic variation after continuous subculture in this experiment. The results of DNA pattern found that the same DNA pattern after digested with various restriction enzymes was obtained when compared with the first subculture except in the third subculture of *A. auricula*. From this part, it could be assumed that ITS 4 and 5 primers were able to detect genetic instability of some mushroom. The genetic variation of *A. auricula* was possible to related with growth rate and total yield (as detail in item 3.3). The internal transcribed spacer region of nuclear rDNA repeat evolve much faster and sequence differences in these regions occurred between species within one genus or even between different populations of one

species (White et al. 1990, Bevan et al. 1992 and Meltzer, 1993). However, this primer could not be detected with every species of mushroom. Therefore, to investigate genetic variation of mushroom ITS 4 and 5 primers were not enough. It should be detected at some deficiency gene which related to growth rate, fruit body formation and lignin-degradation.

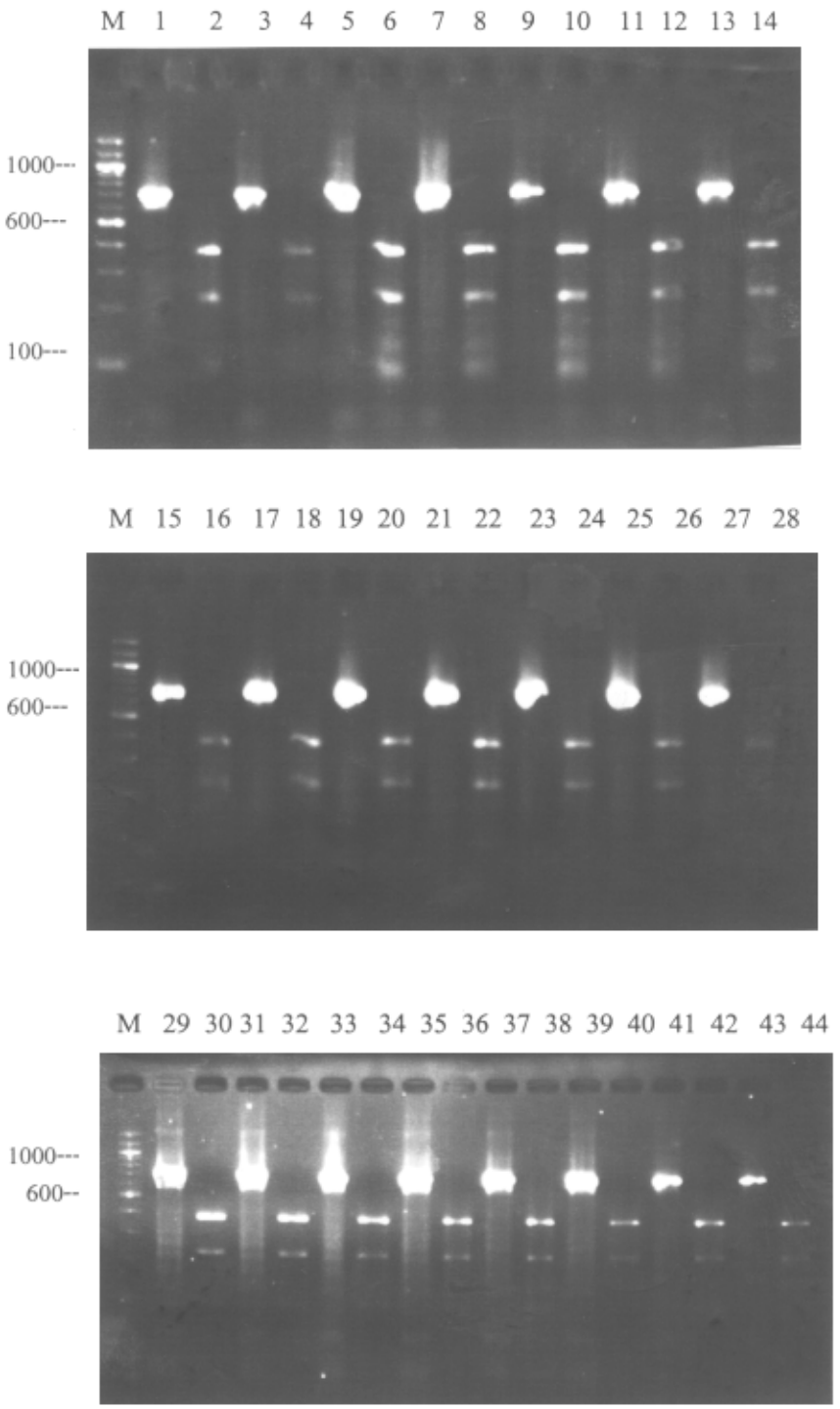


Figure 9. The PCR products of *Pleurotus ostreatus* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

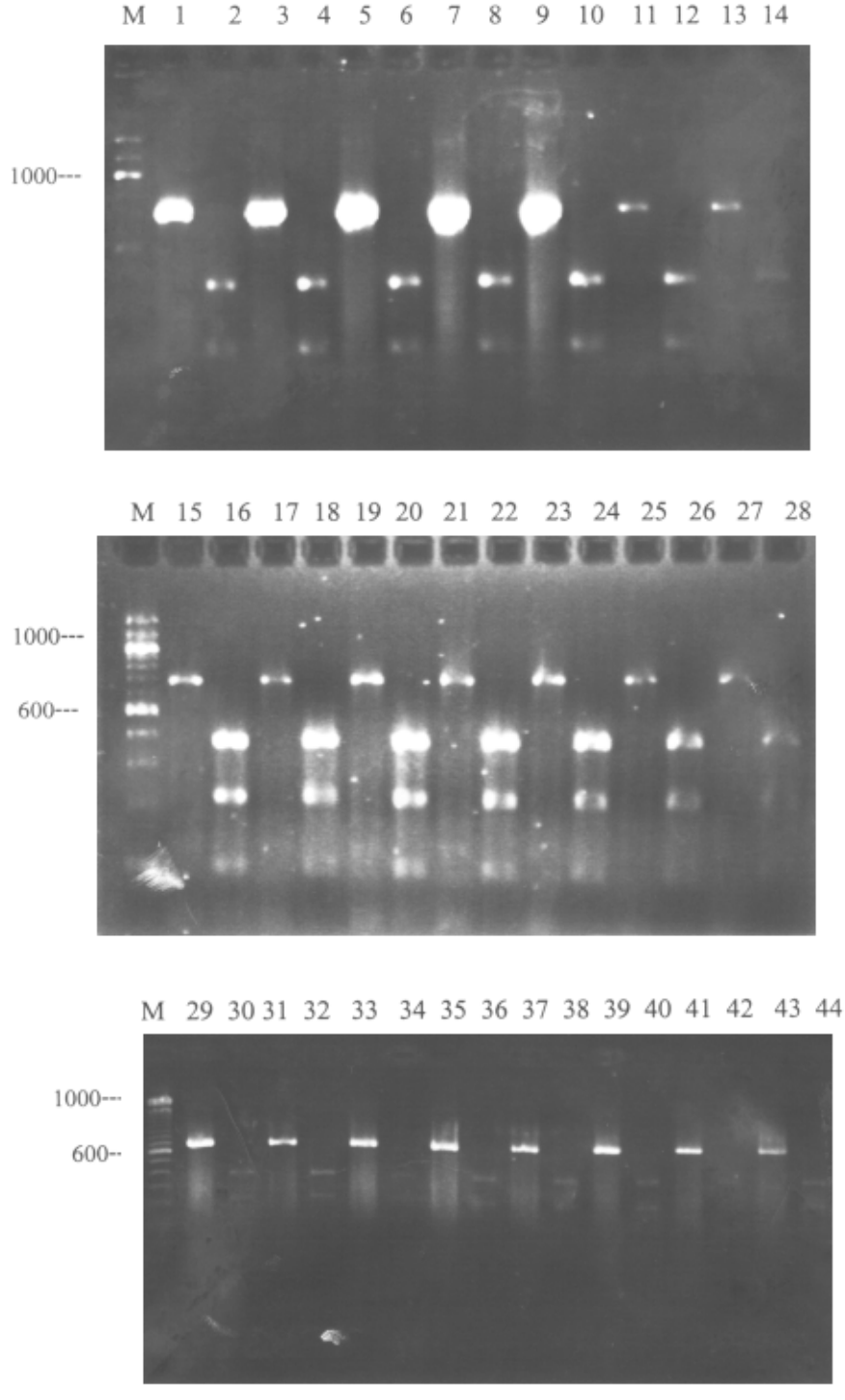


Figure 10. The PCR products of *Pleurotus sajor-caju* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL);lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

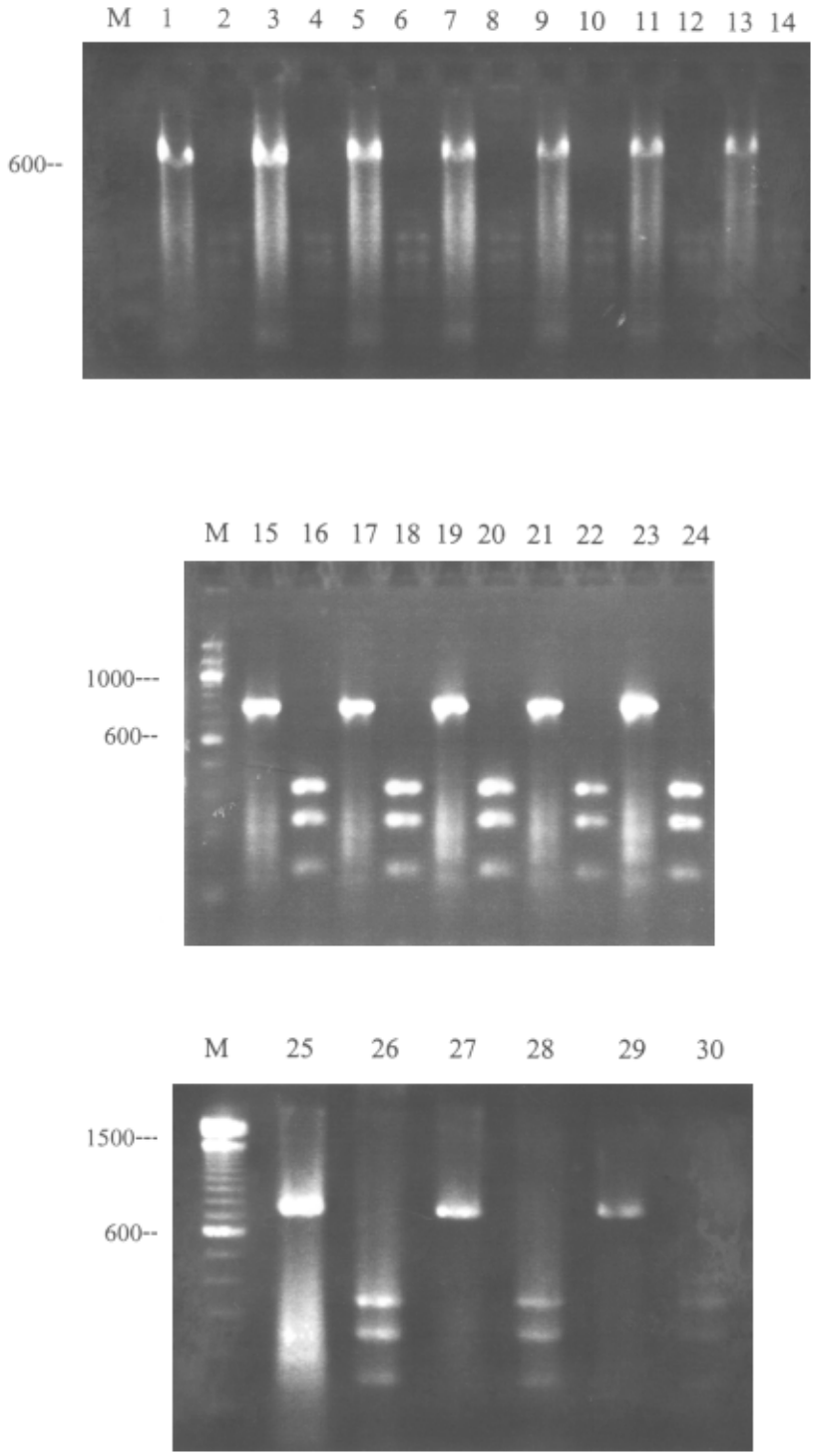


Figure 11. The PCR products of *Pleurotus cystidiosus* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 15th subculturing and lane an even number: digested of PCR product 1st subculturing –15th subculturing

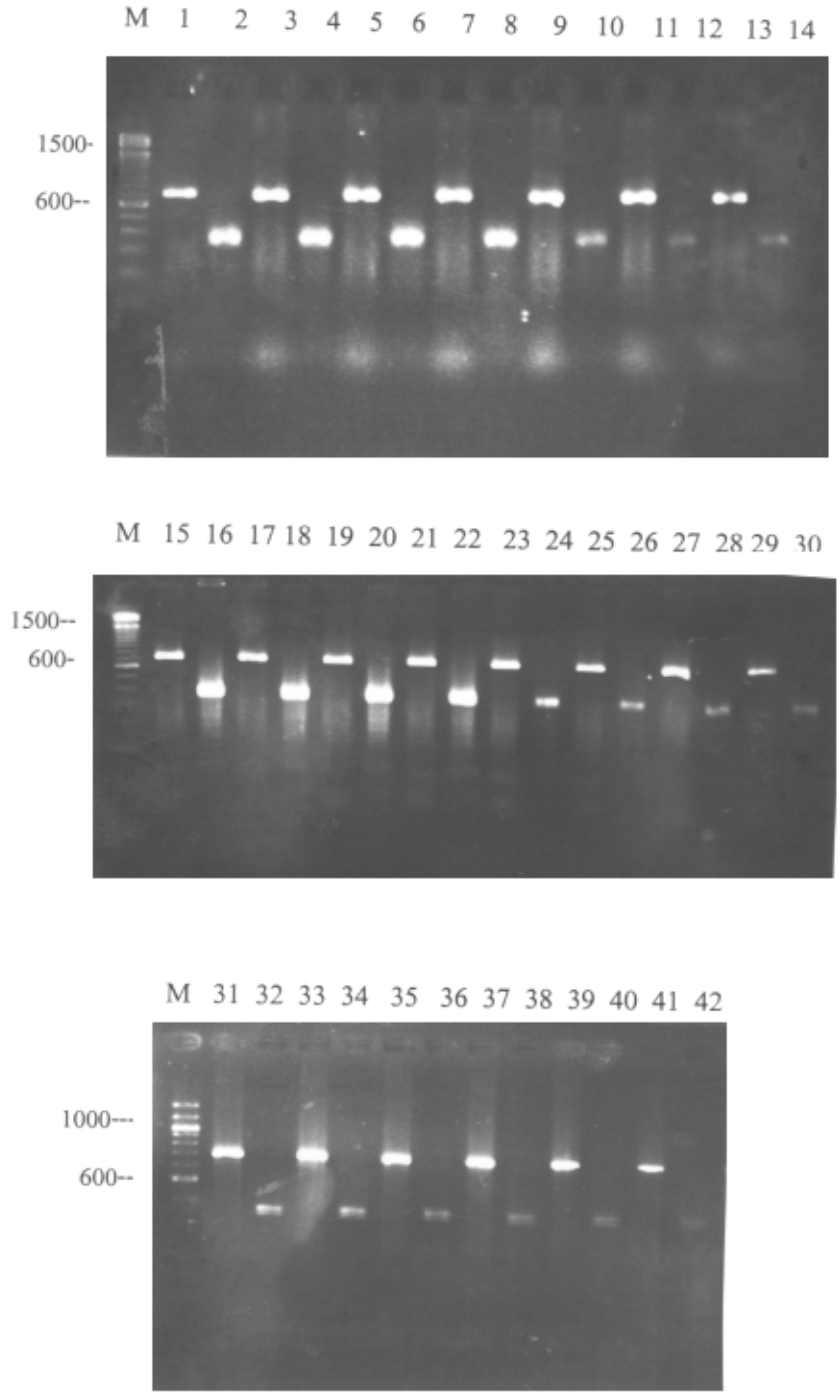


Figure 12. The PCR products of *Lentinula squarrossula* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

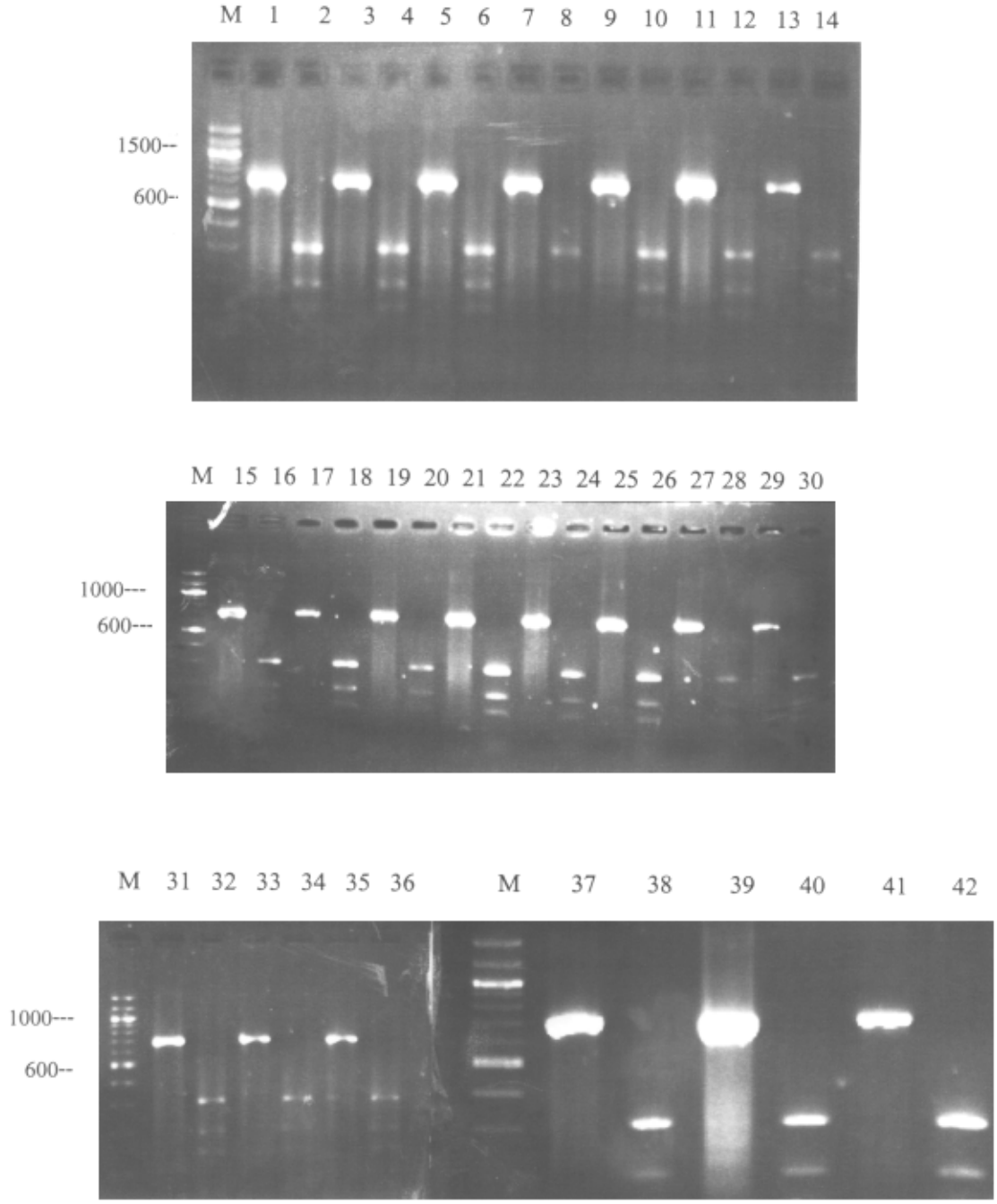


Figure 13. The PCR products of *Lentinula polychrous* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

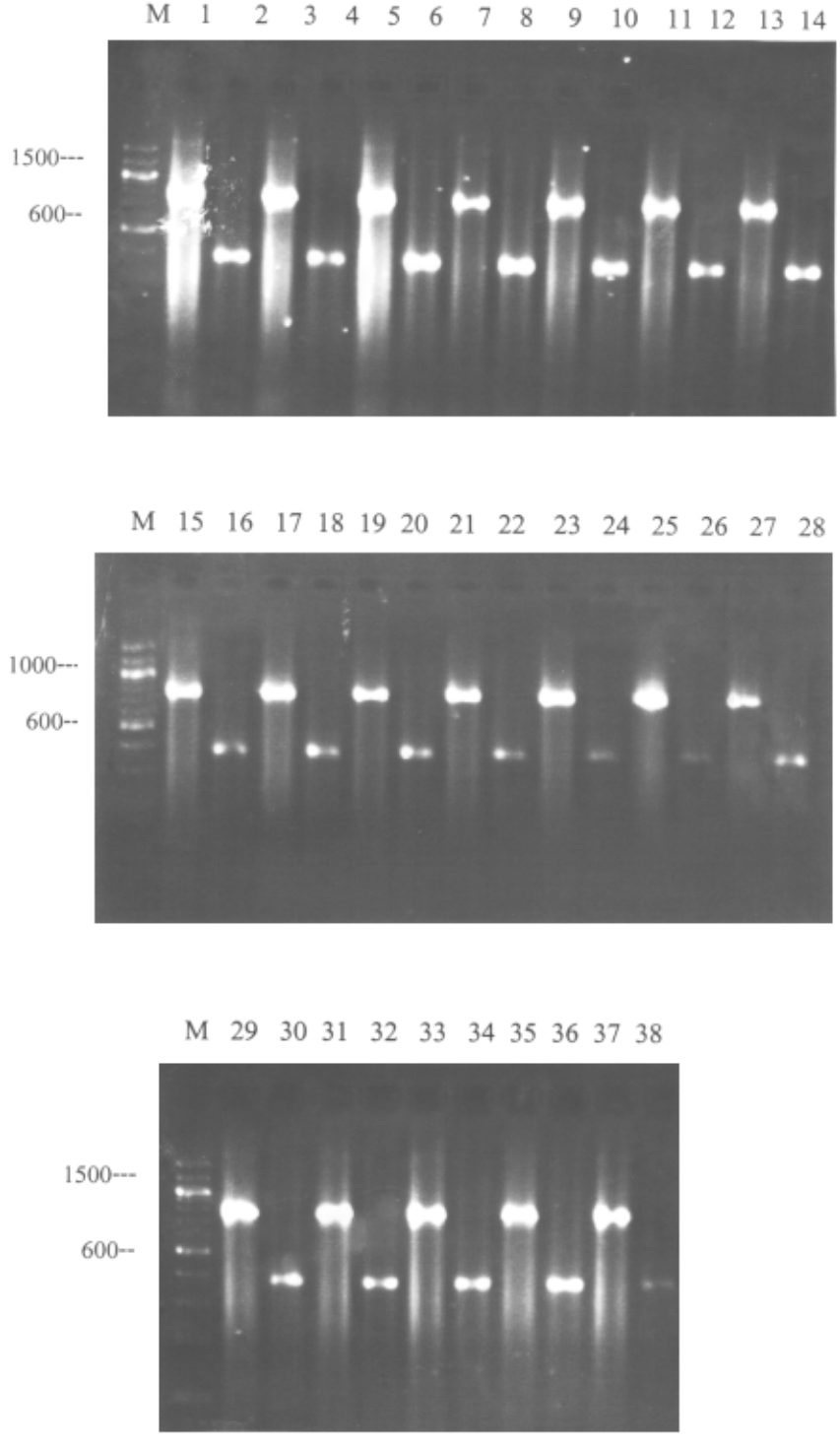


Figure 14. The PCR products of *Lentinus edodes* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane anodd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

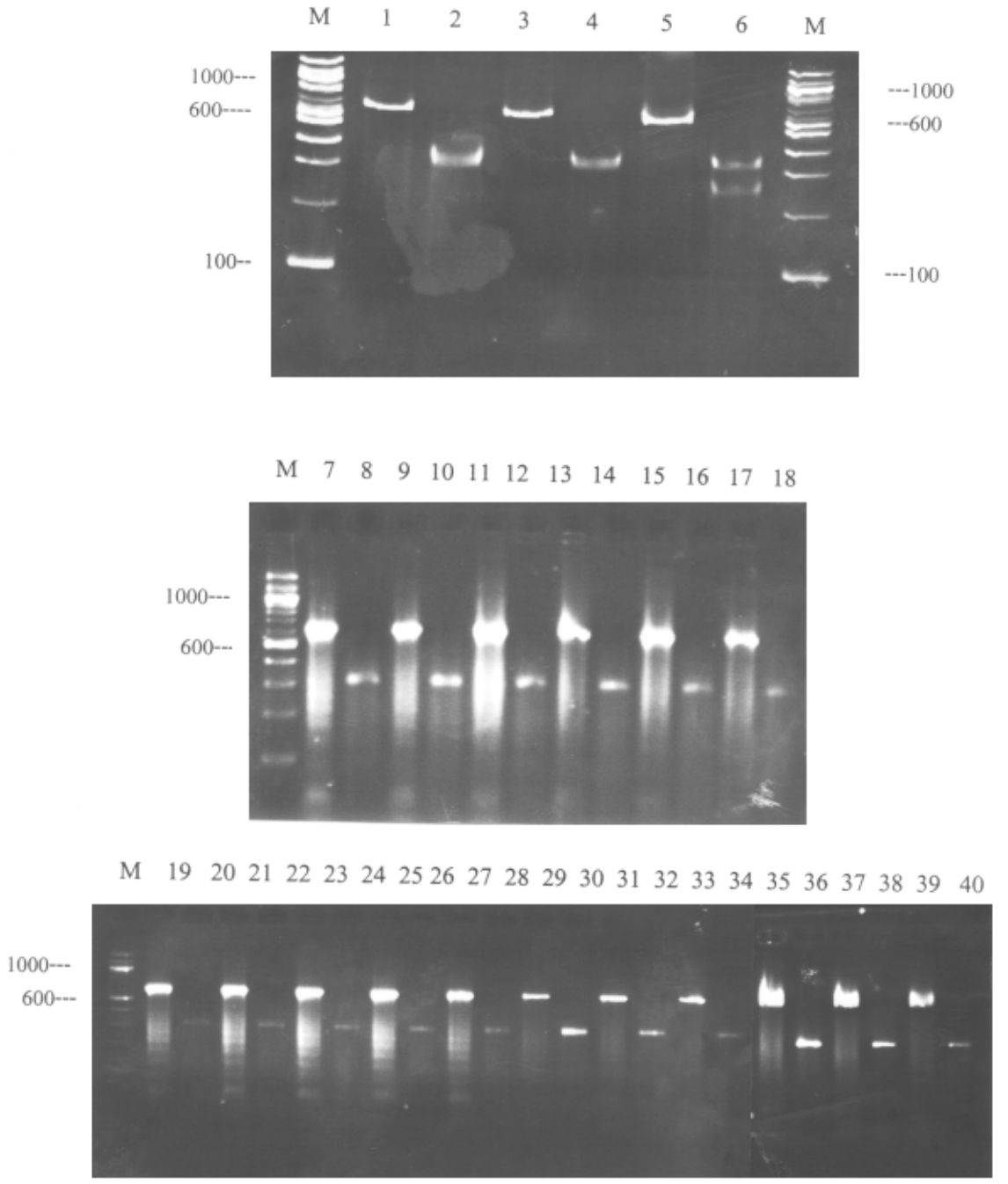


Figure 15. The PCR products of *Auricularia auricula* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

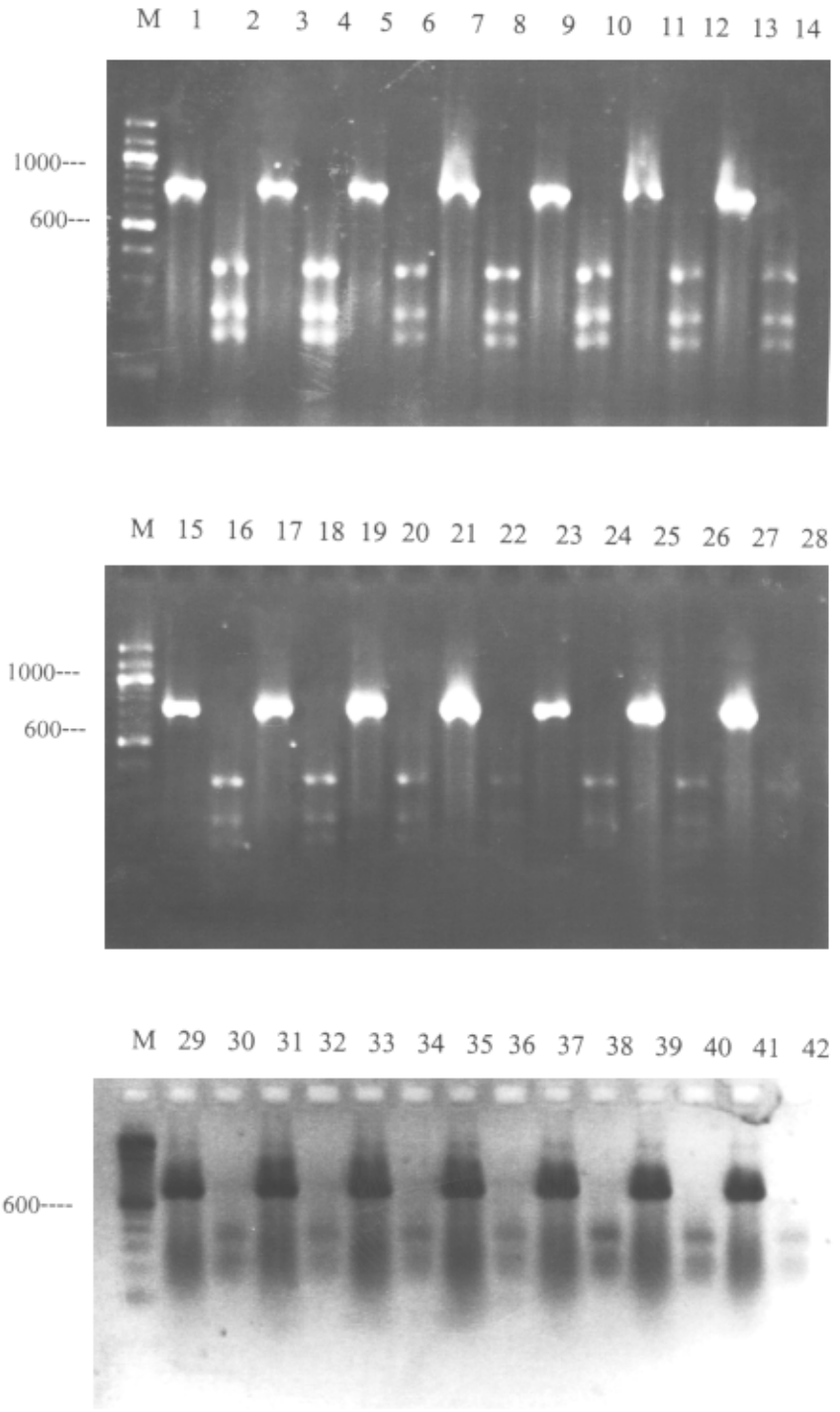


Figure 16. The PCR products of *Agrocybe cylindracea* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

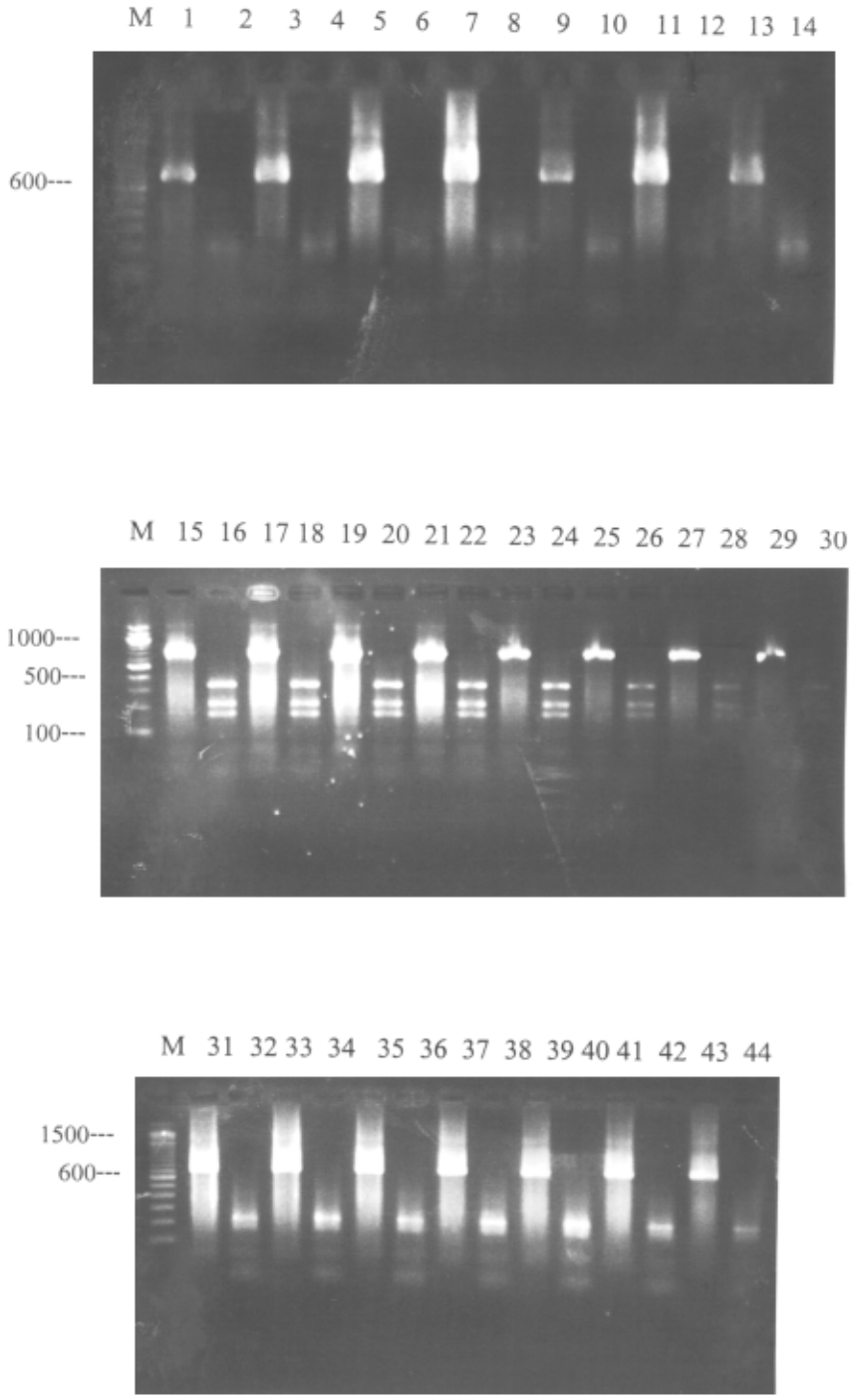


Figure 17. The PCR products of *Tricholoma crassum* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

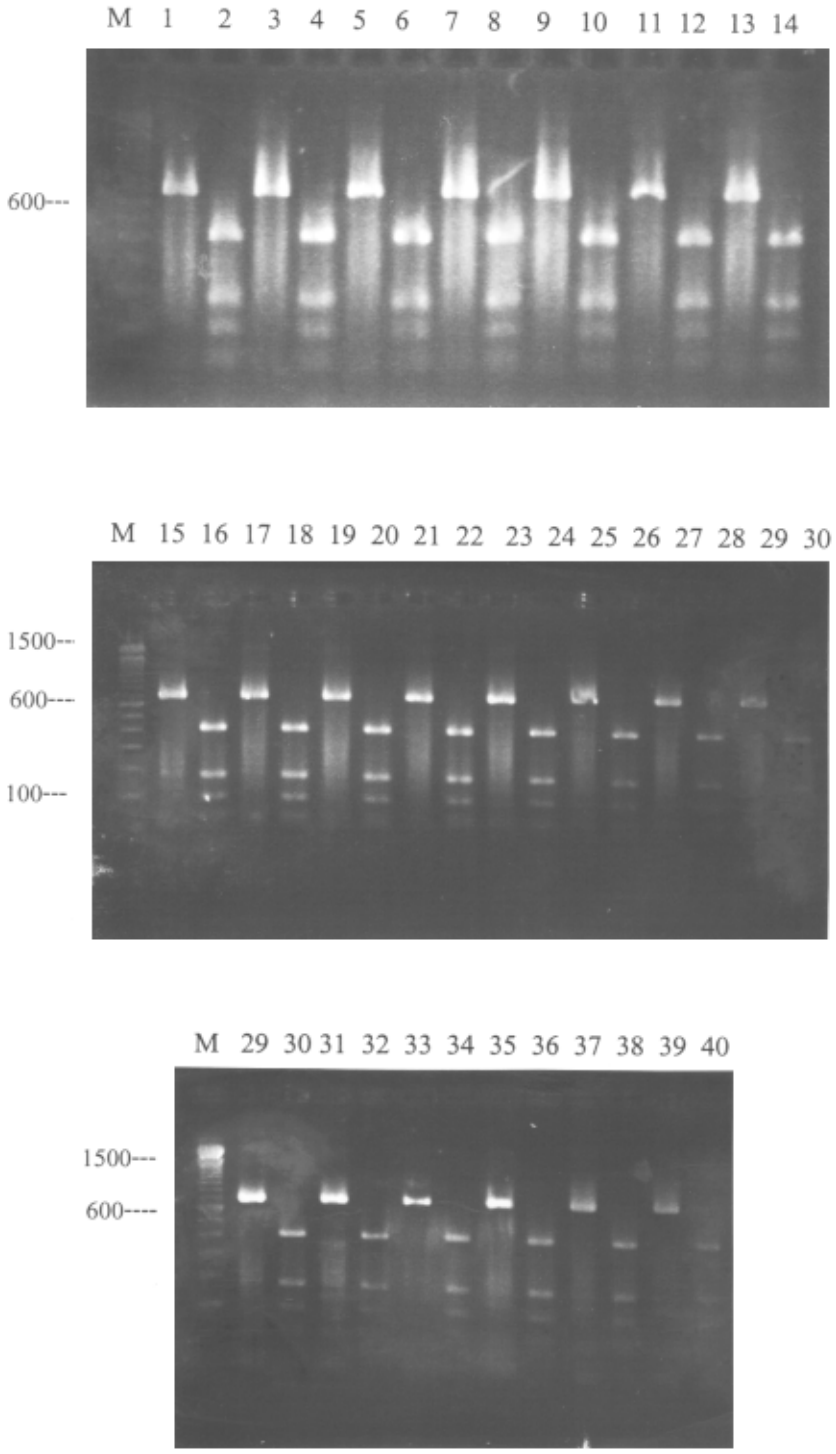


Figure 18. The PCR products of *Pleurotus ostreatus* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

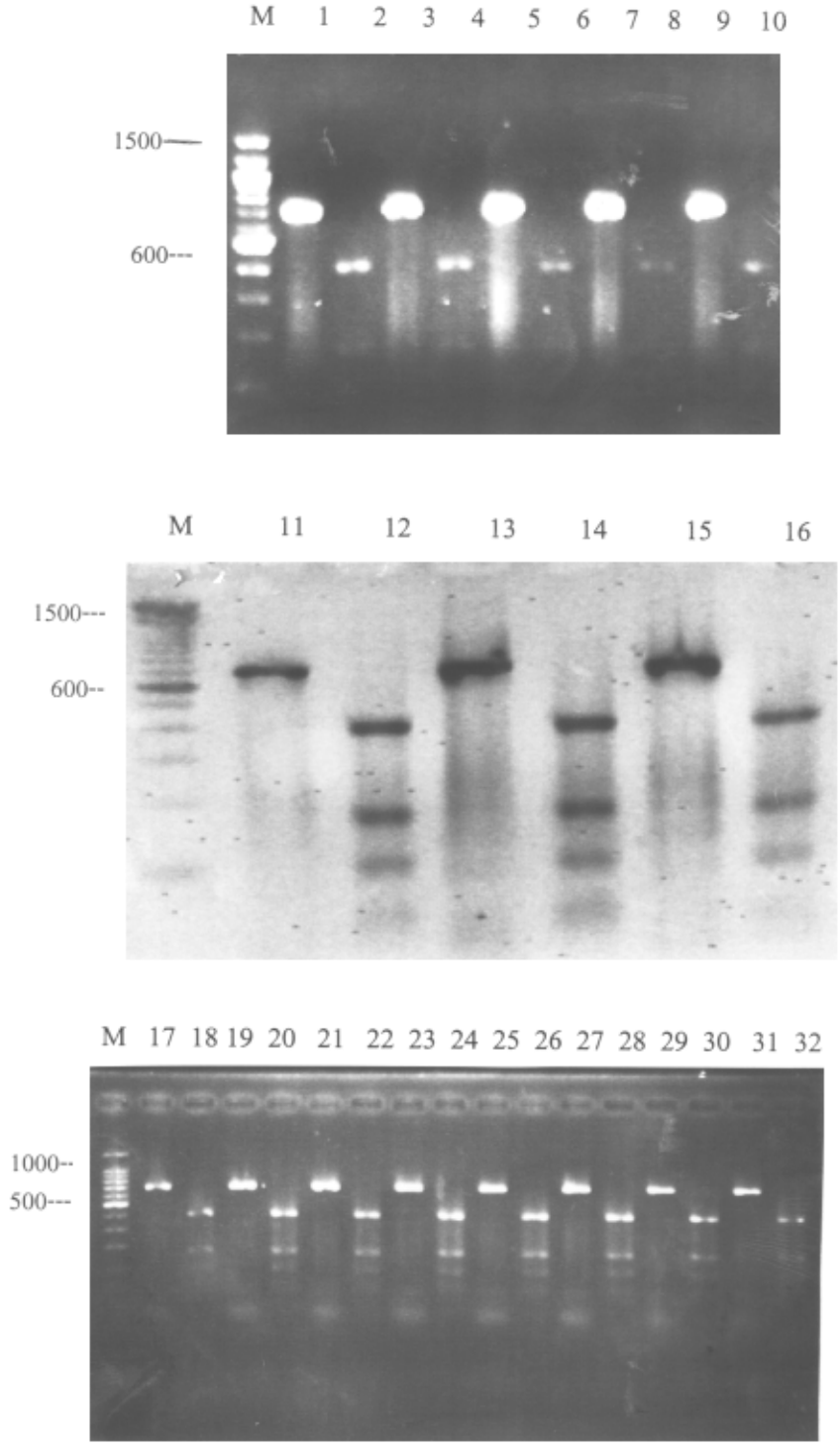


Figure 19. The PCR products of *Pleurotus sajor-caju* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 16th subculturing and lane an even number: digested of PCR product 1st subculturing – 16th subculturing

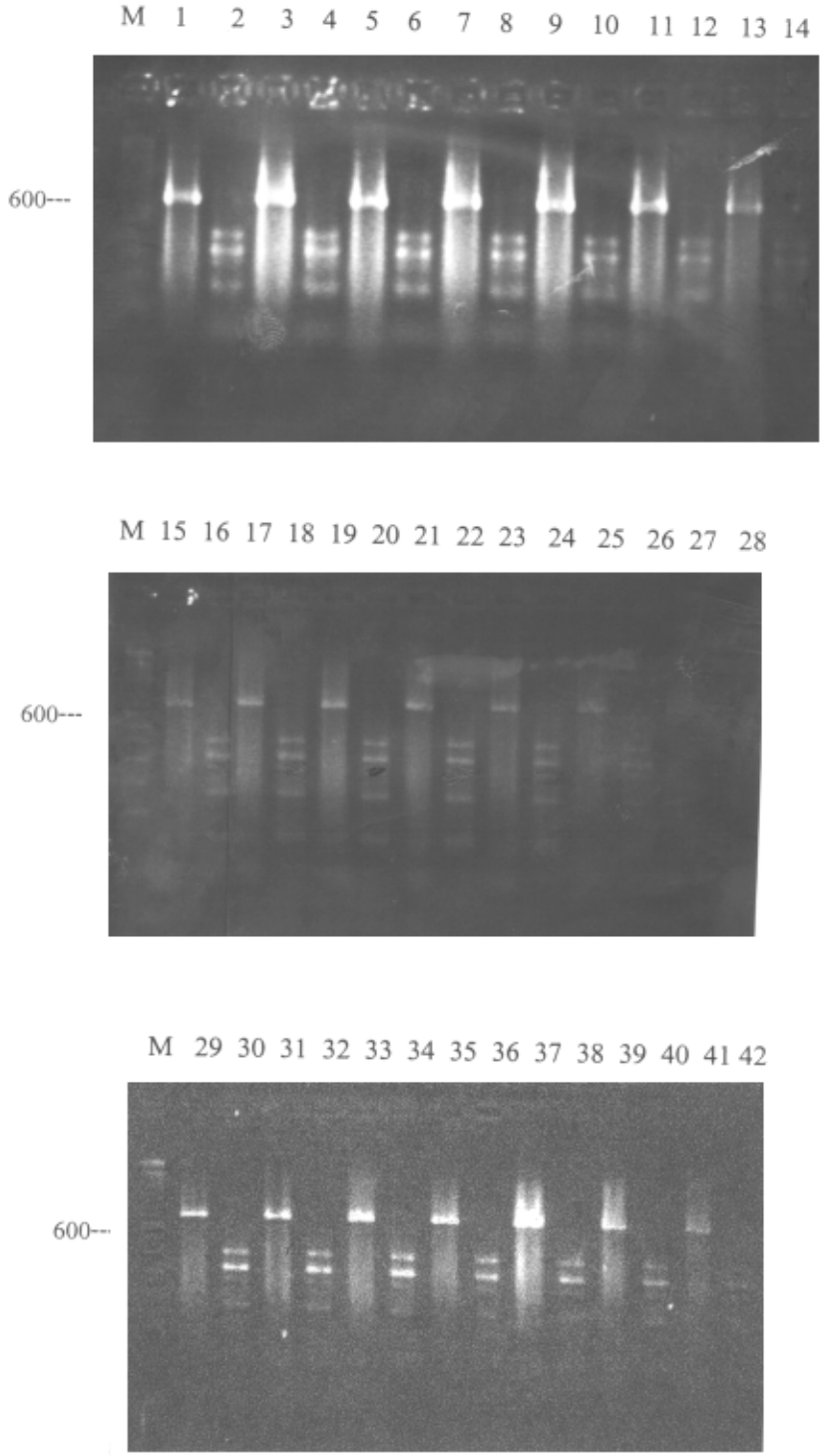


Figure 20. The PCR products of *Pleurotus cystidiosus* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subcultureing– 20th subculturing

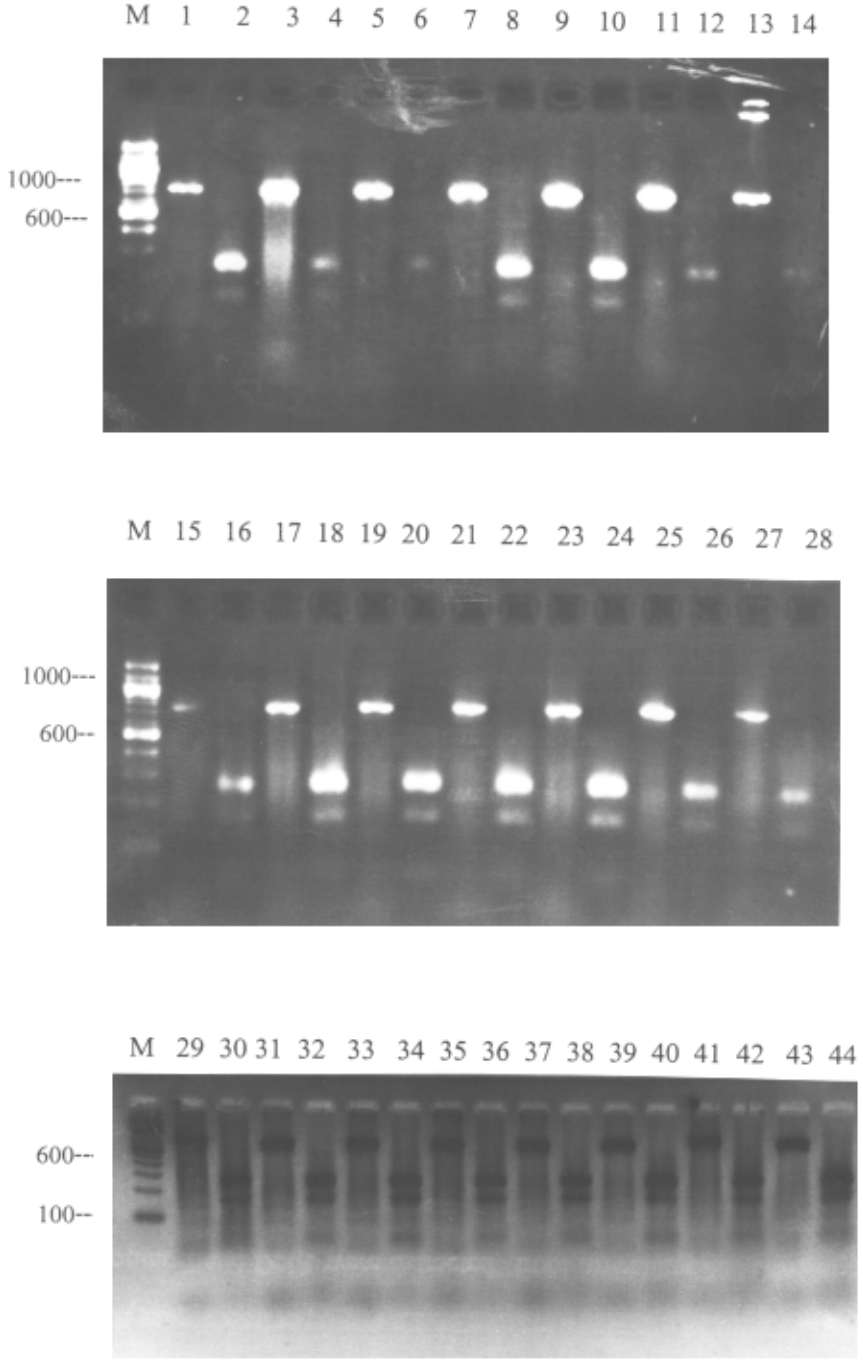


Figure 21. The PCR product of *Lentinula squarrossula* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

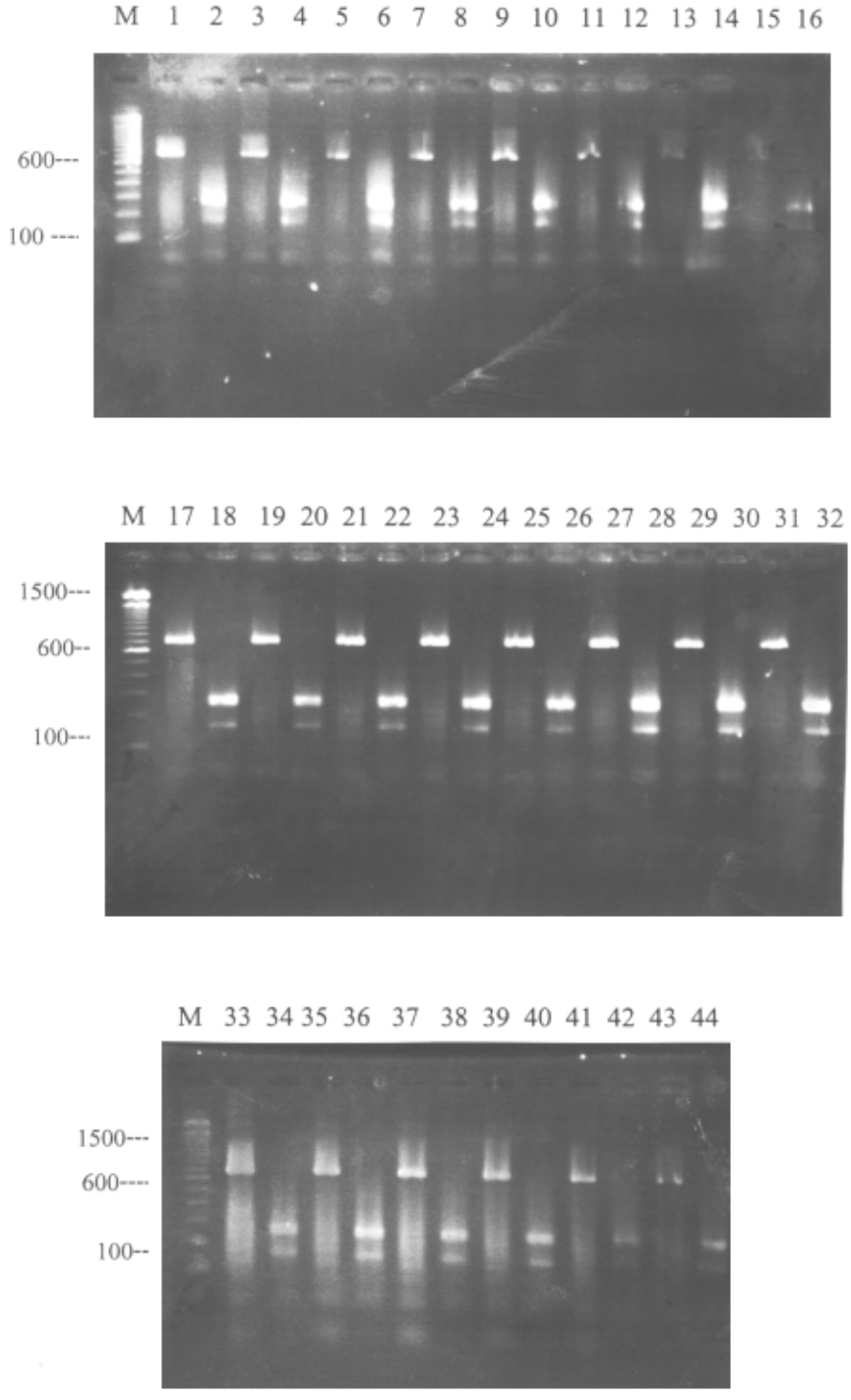


Figure 22. The PCR product of *Lentinula polychrous* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

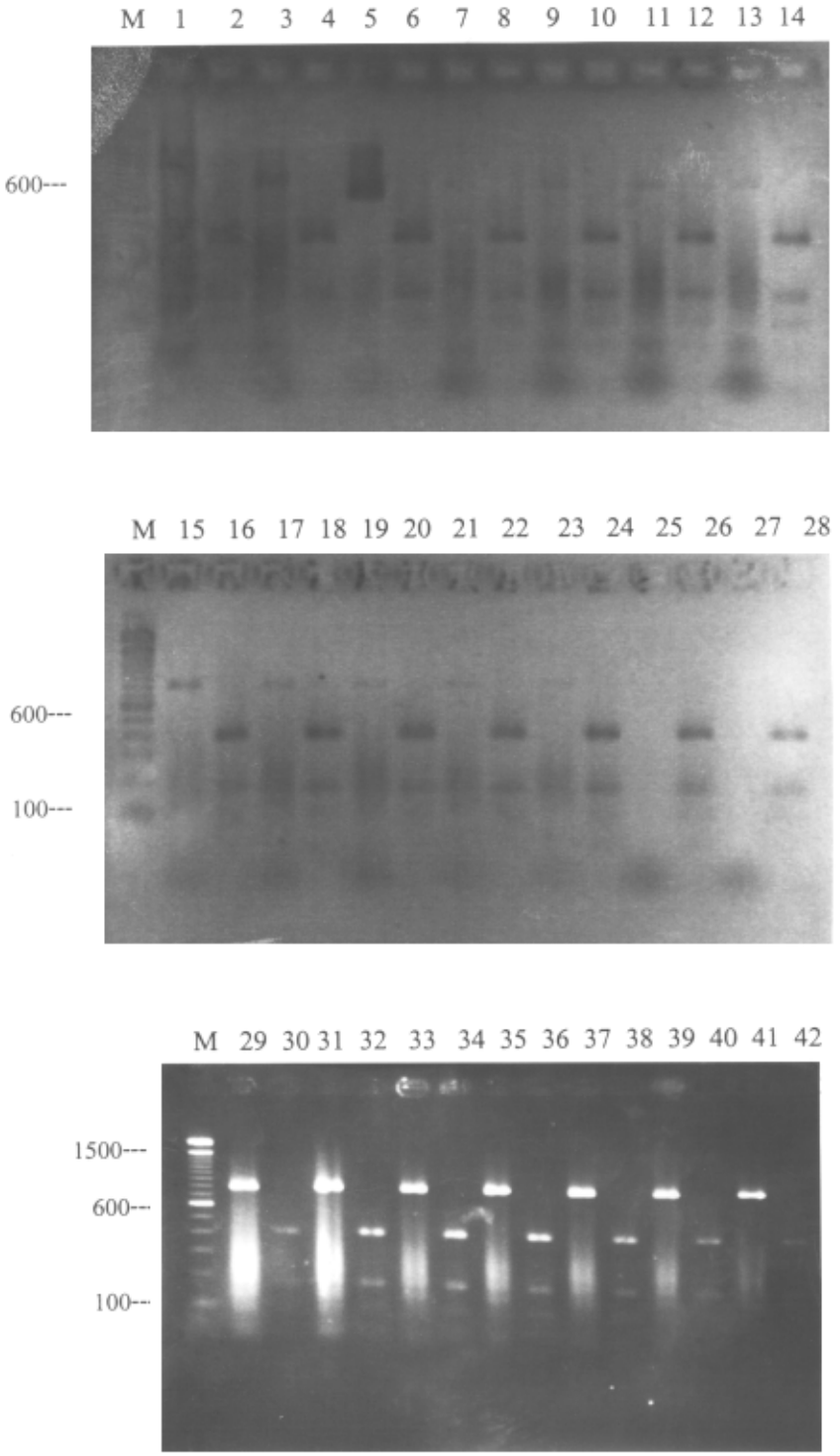
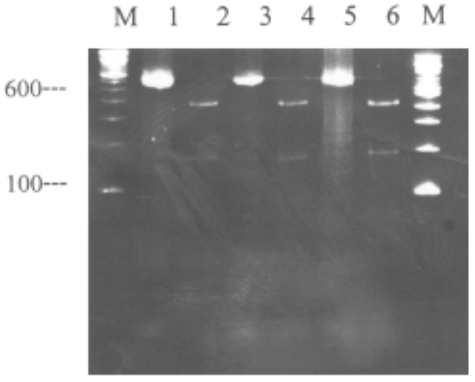
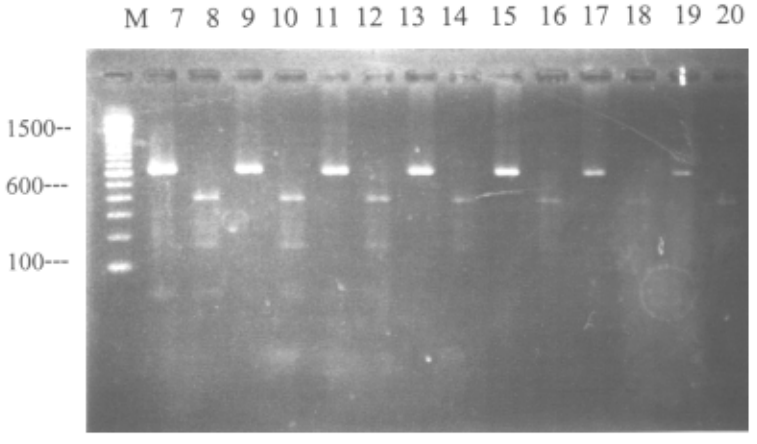


Figure 23. The PCR product of *Lentinus edodes* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing





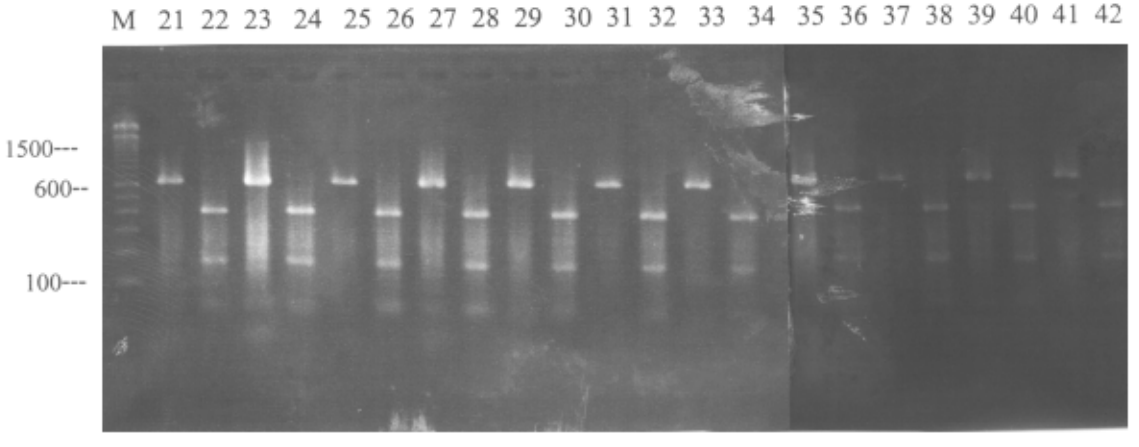


Figure 24. The PCR products of *Auricularia auricula* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

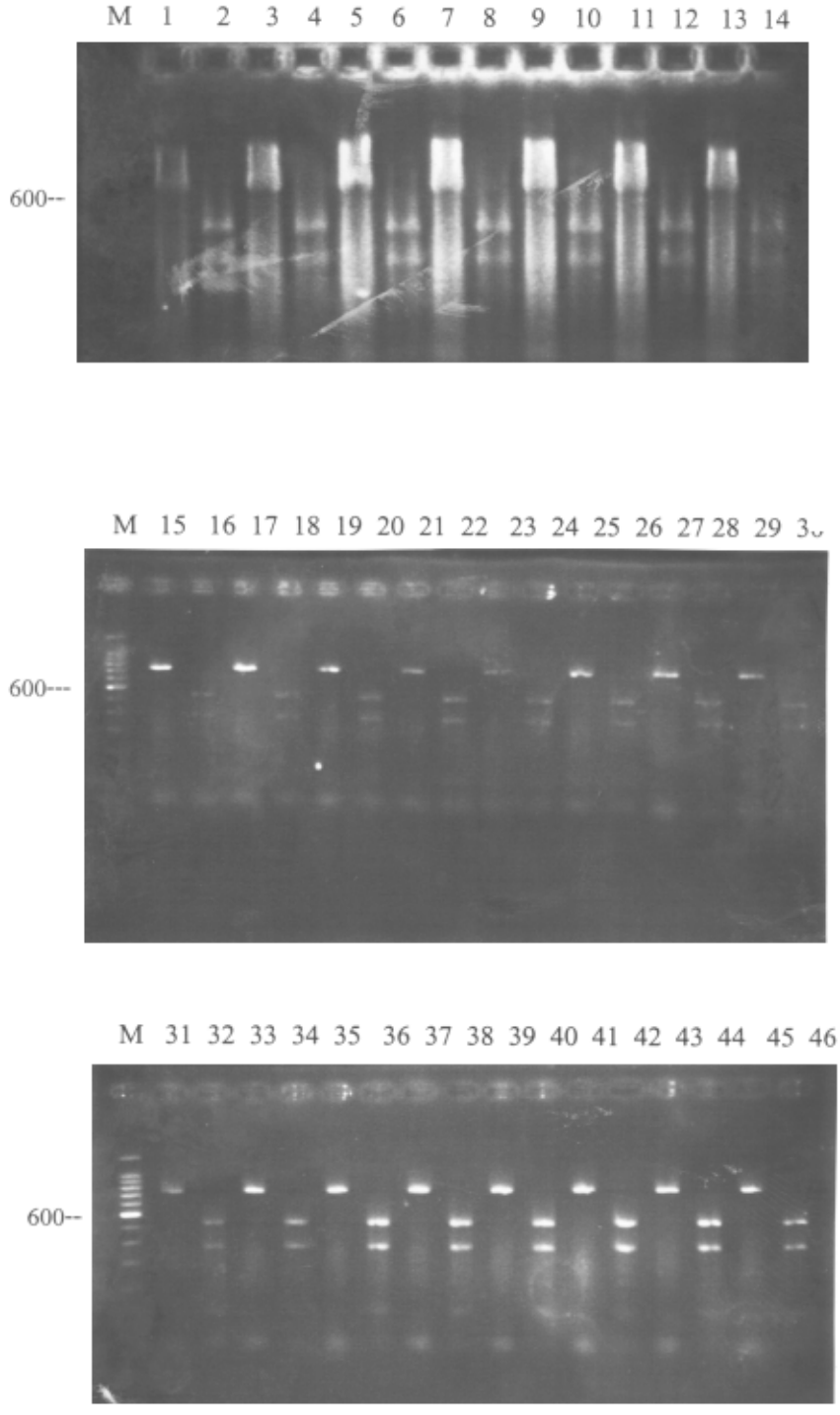


Figure 25. The PCR products of *Agrocybe cylindracea* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing– 23rd subculturing and lane an even number: digested of PCR product 1st subculturing – 23rd subculturing

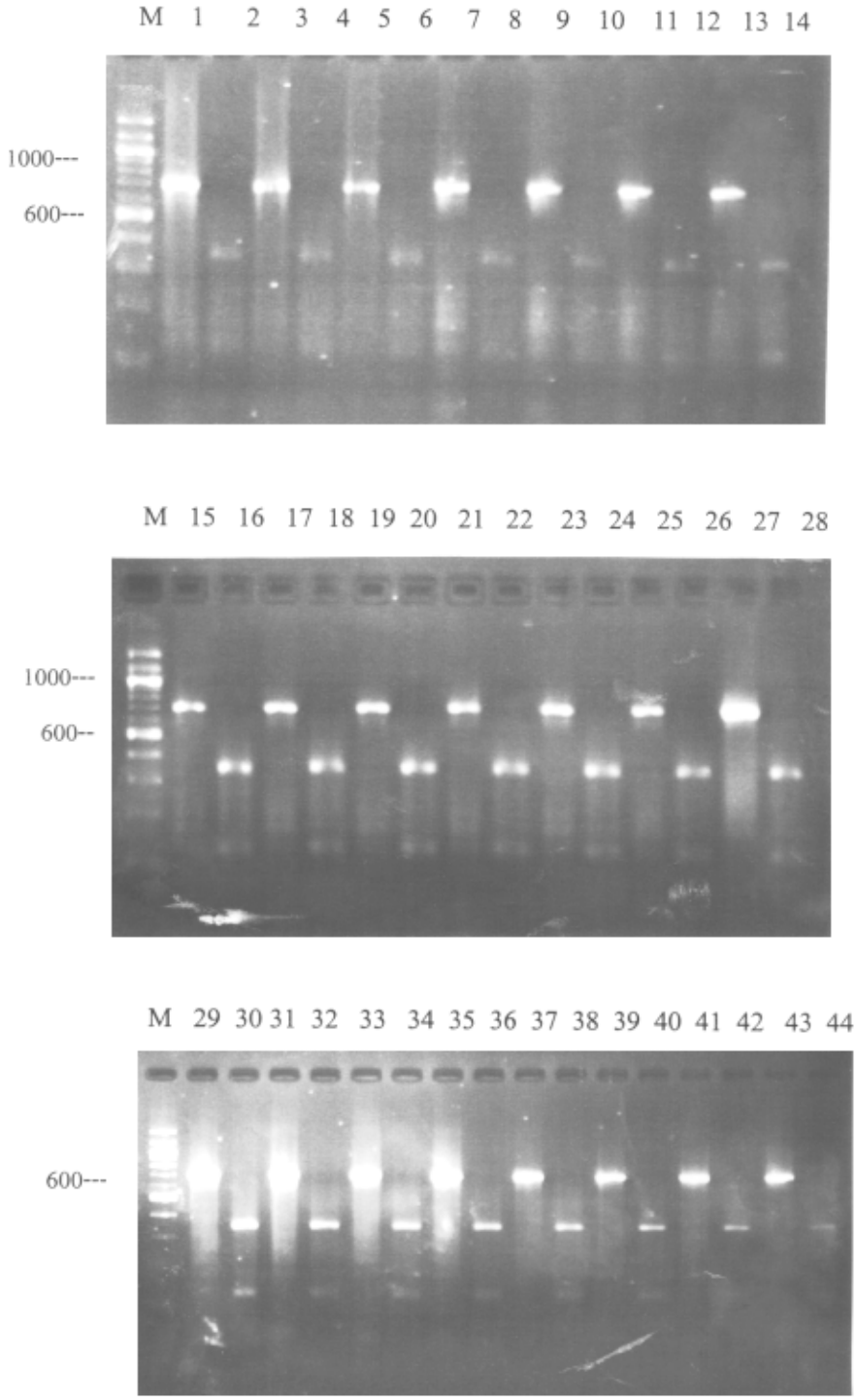


Figure 26. The PCR products of *Tricholoma crassum* digested with the *Mbo* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22rd subculturing and lane an even number: digested of PCR product 1st subculturing – 22rd subculturing

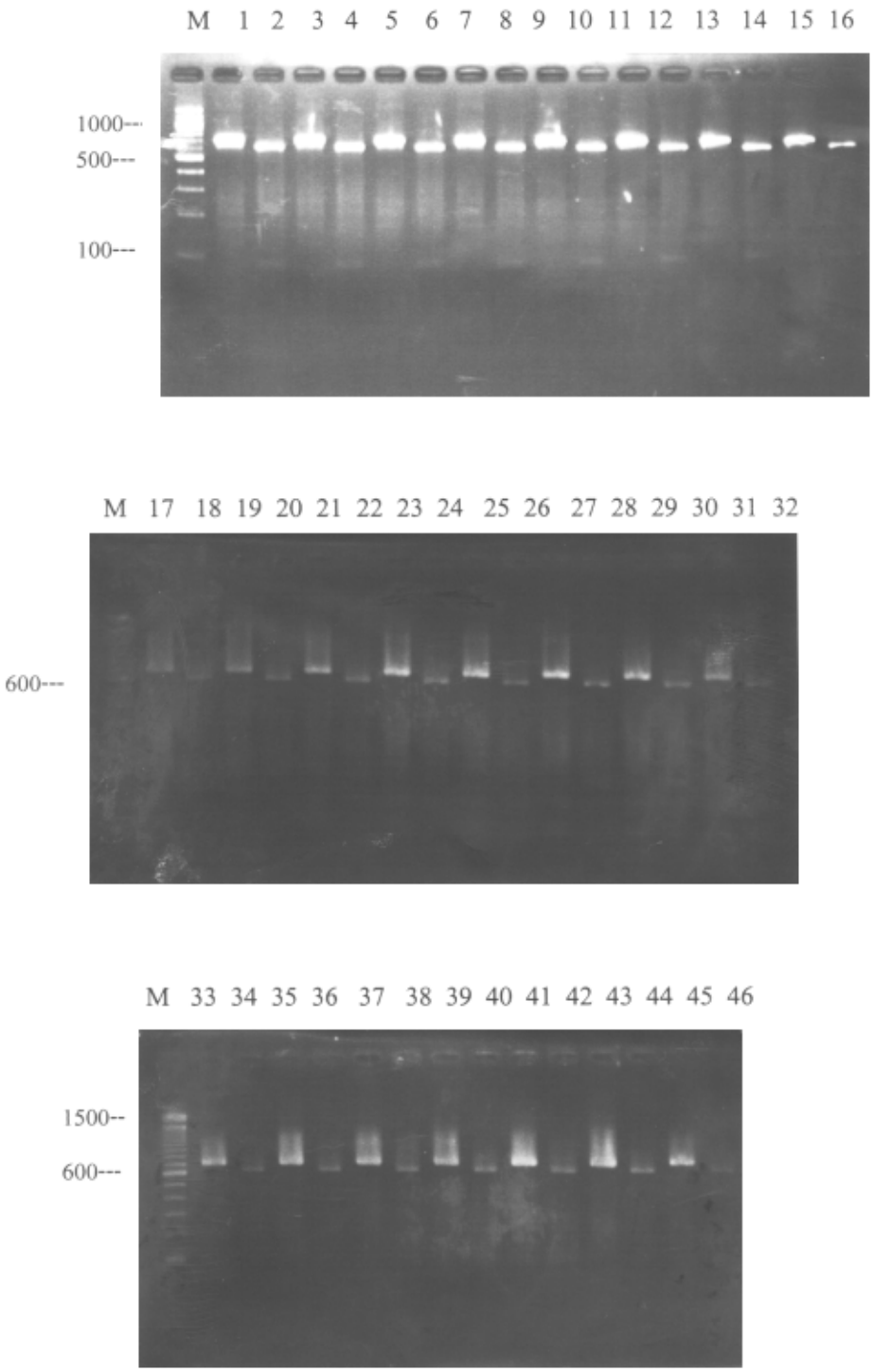


Figure 27. The PCR products of *Pleurotus ostreatus* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 23rd subculturing and lane an even number: digested of PCR product 1st subculturing – 23rd subculturing

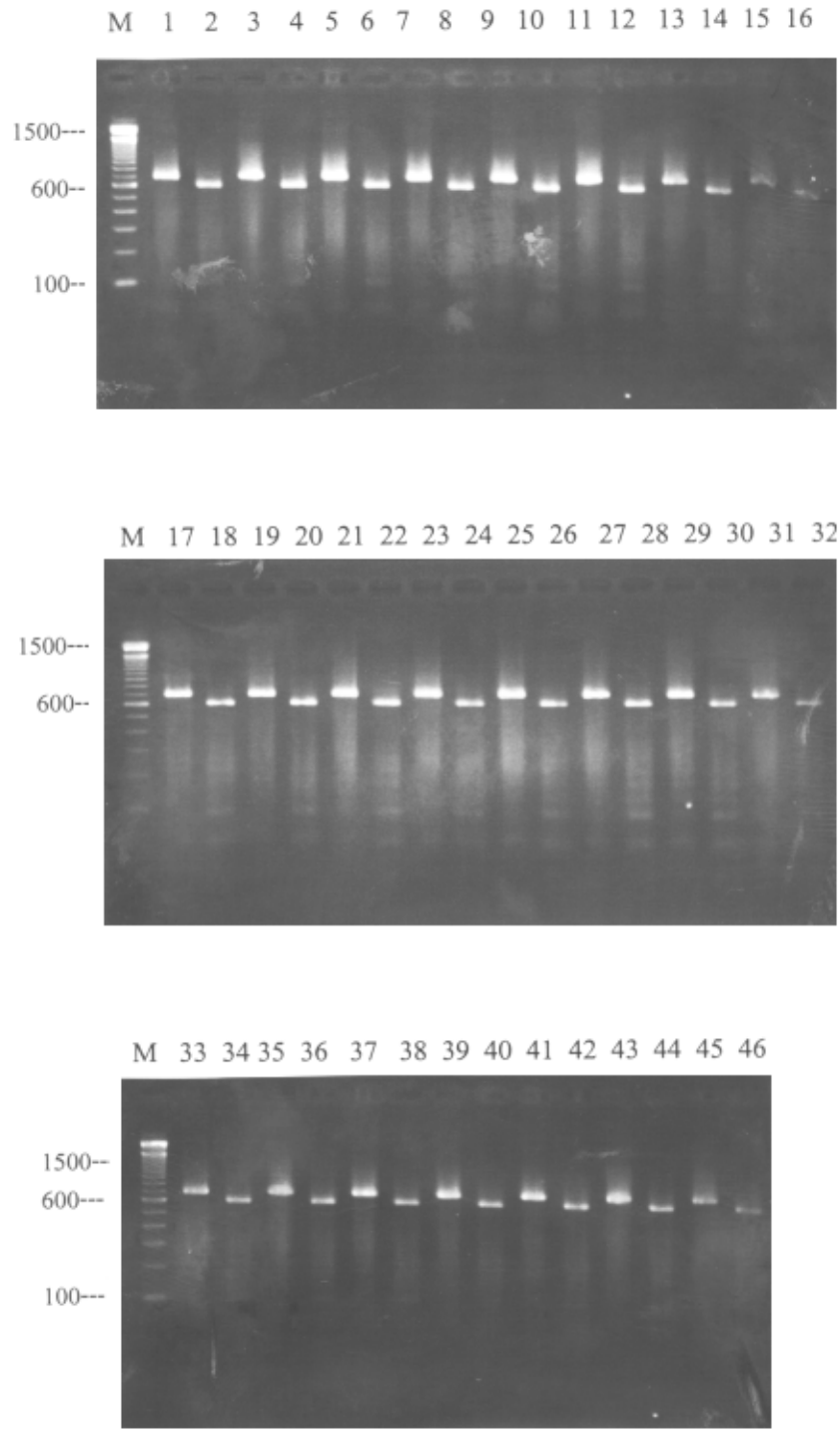


Figure 28. The PCR products of *Pleurotus sajor-caju* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 23rd subculturing and lane an even number: digested of PCR product 1st subculturing – 23rd subculturing

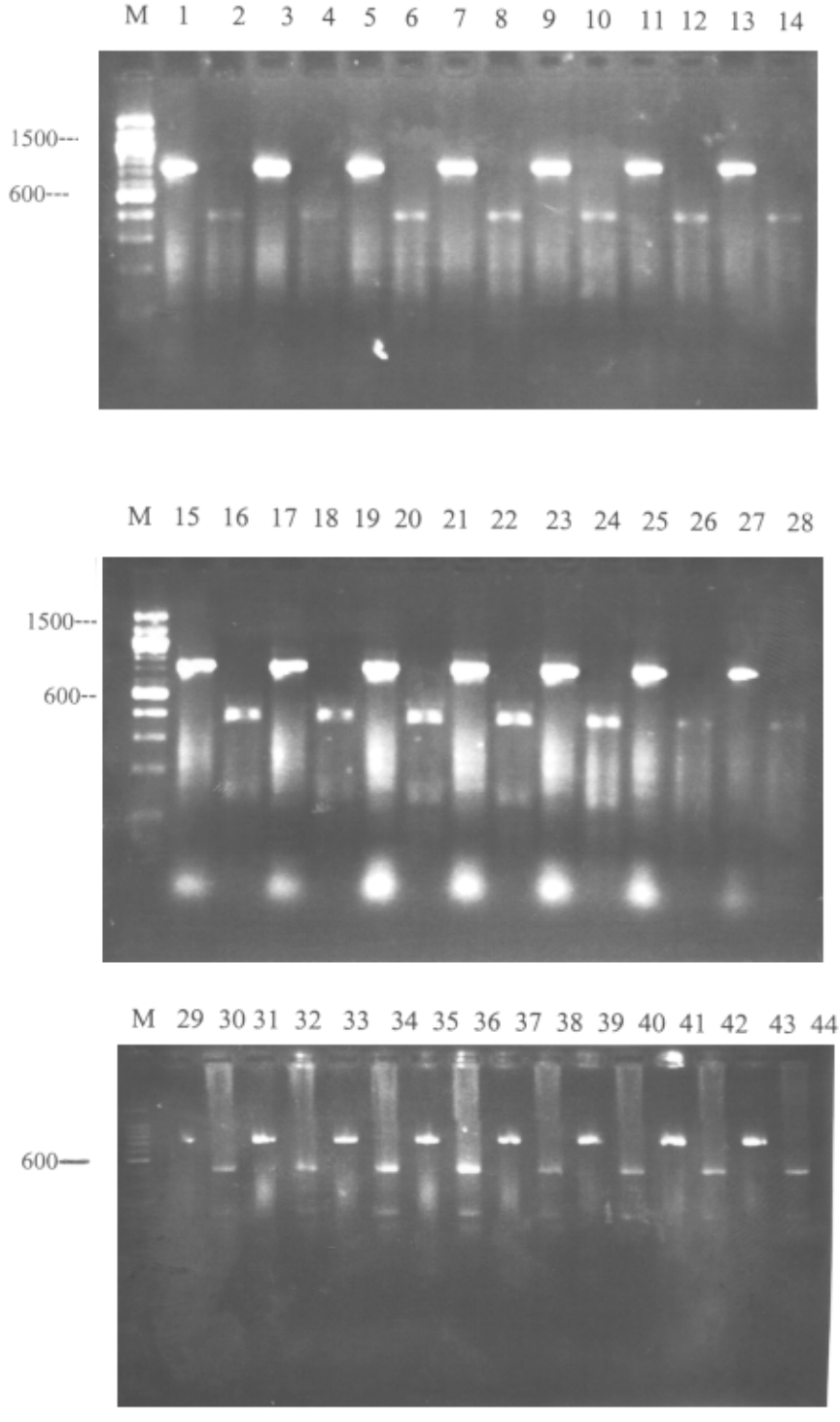


Figure 29. The PCR products of *Pleurotus cystidiosus* digested with the *Alu* I; lane M: 100 bp; lane an odd number: uncut of PCR product 1st subculturing – 22rd subculturing and lane an even number: digested of PCR product 1st subculturing – 22rd subculturing

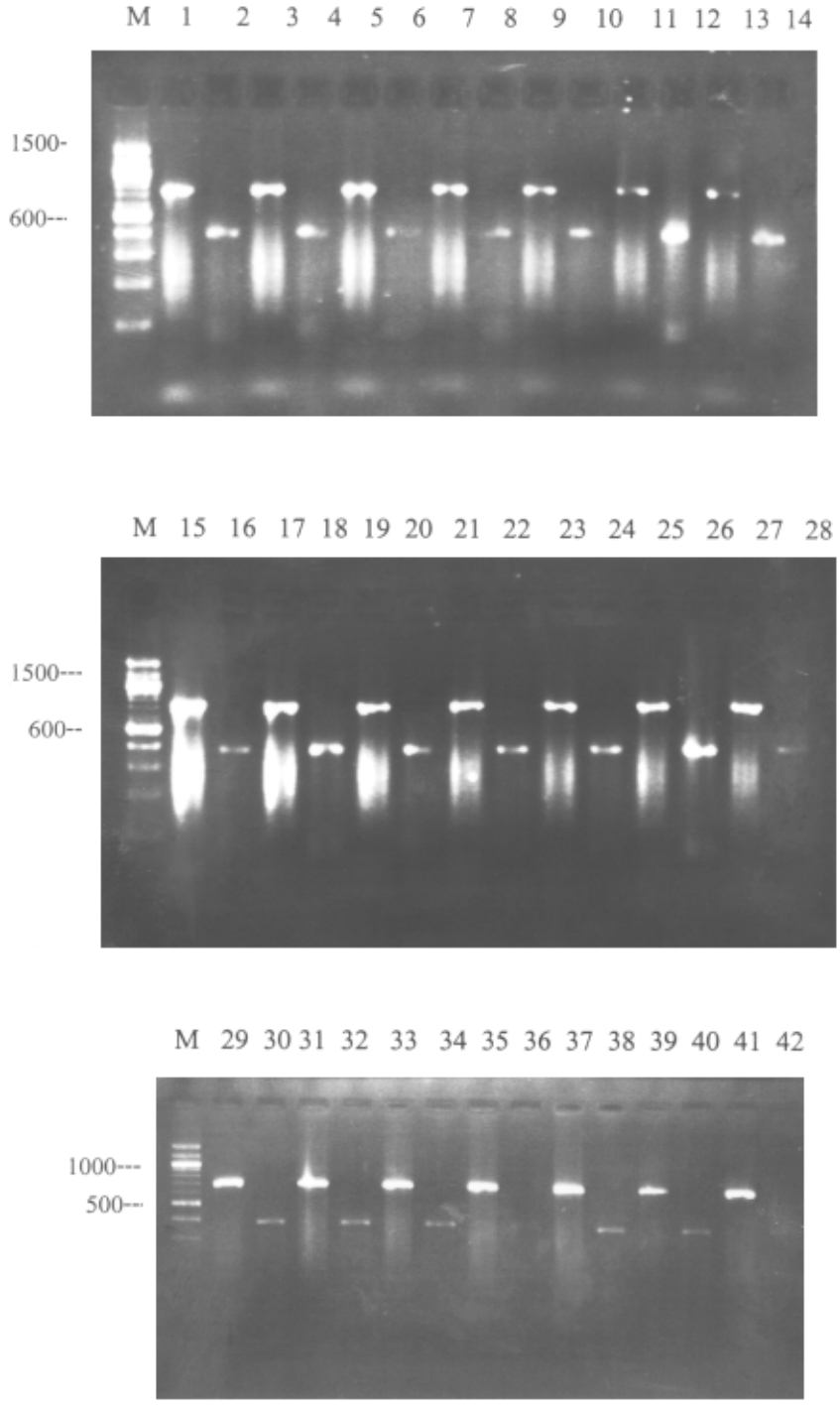


Figure 30. The PCR products of *Lentinula squarrossula* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

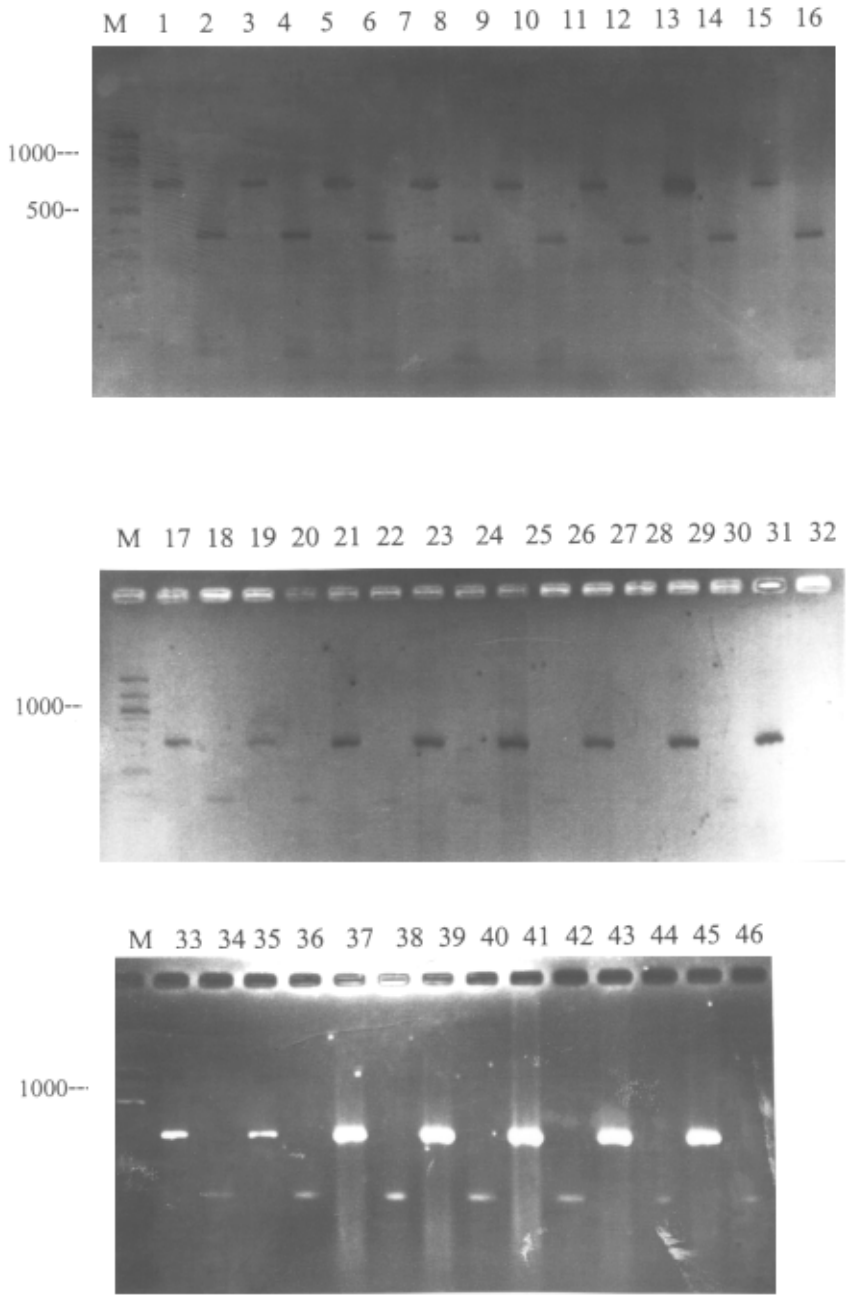


Figure 31. The PCR products of *Lentinula polychrous* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 23rd subculturing and lane an even number: digested of PCR product 1st subculturing – 23rd subculturing

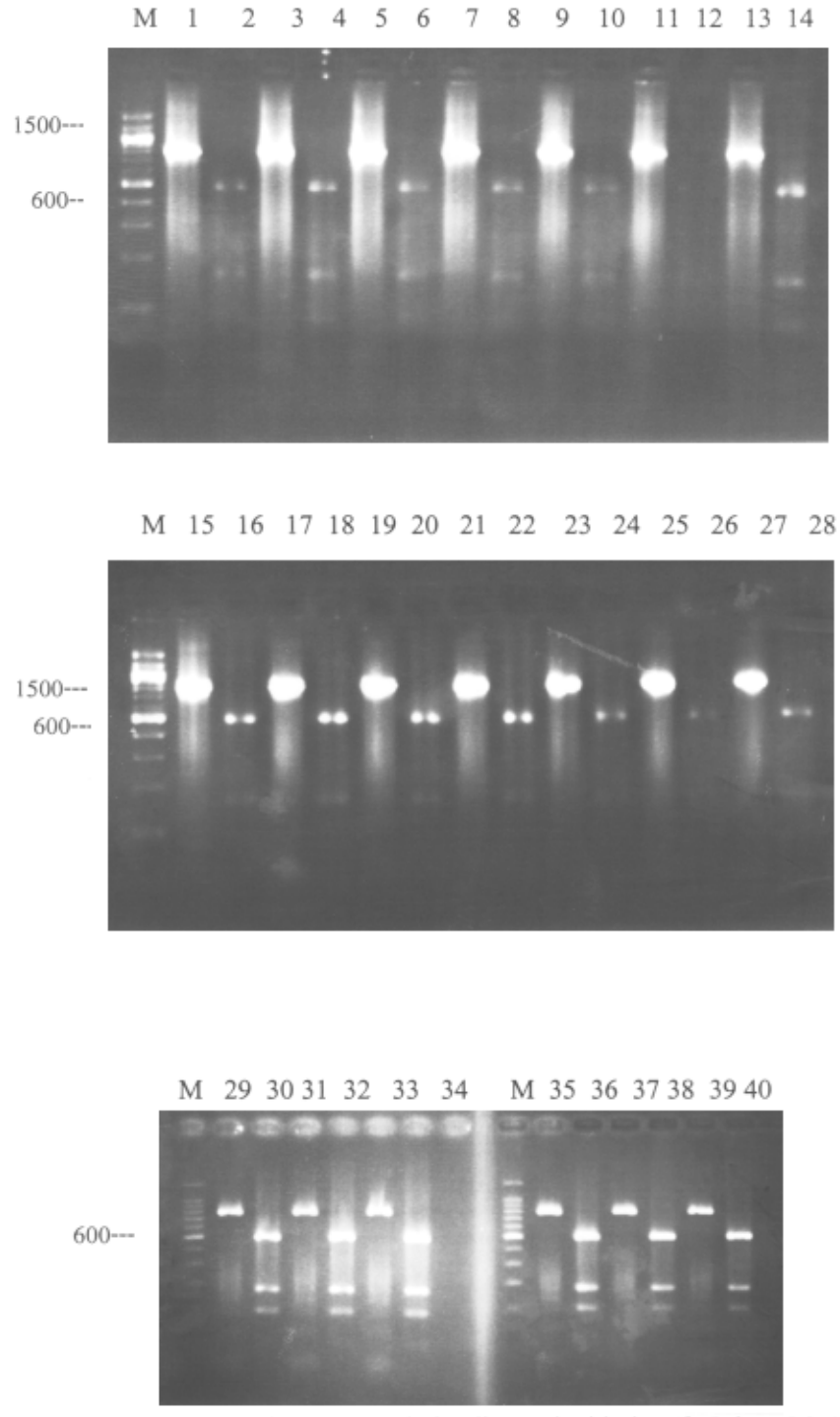


Figure 32. The PCR products of *Lentinus edodes* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

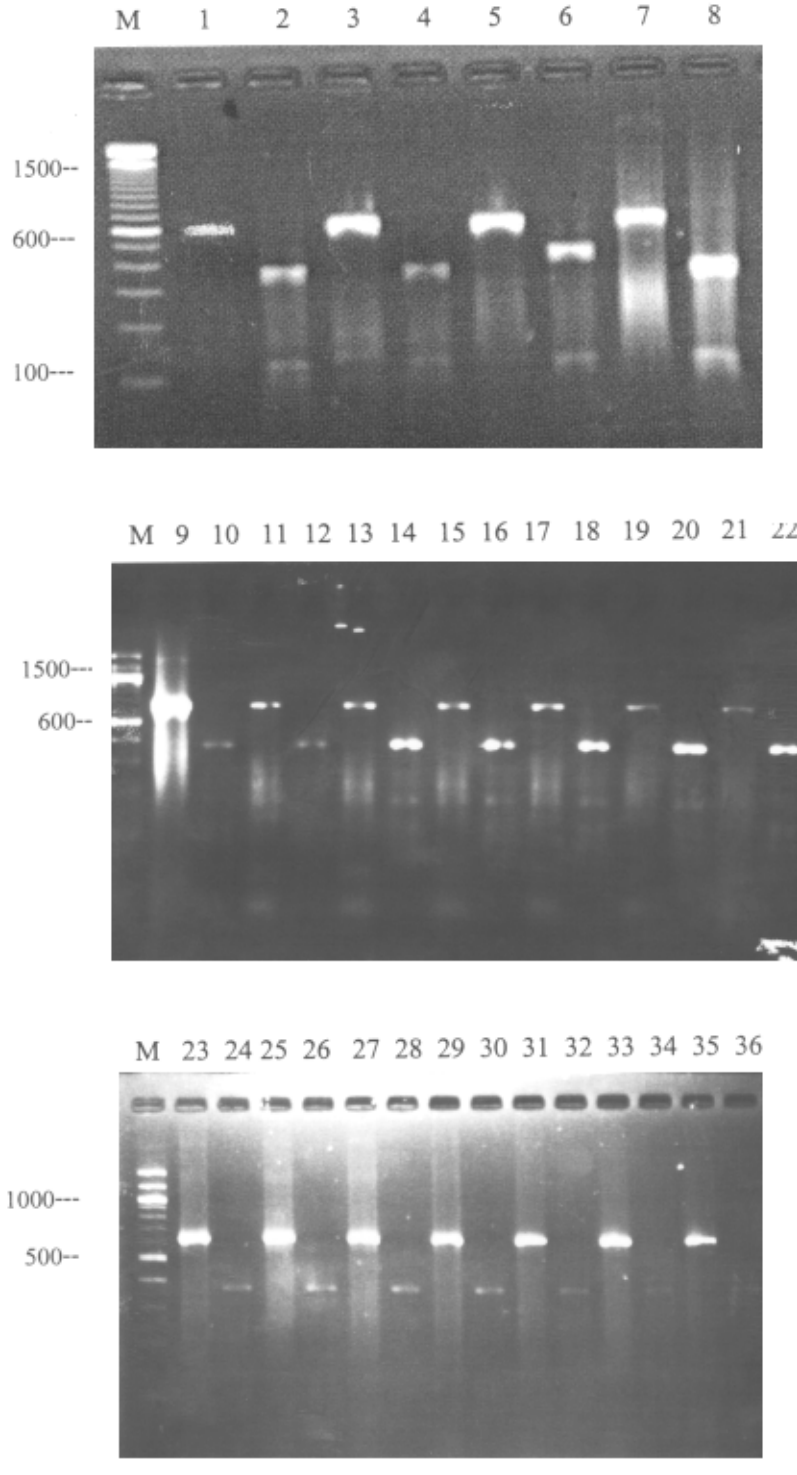


Figure 33. The PCR products of *Auricularia auricula* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subculturing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

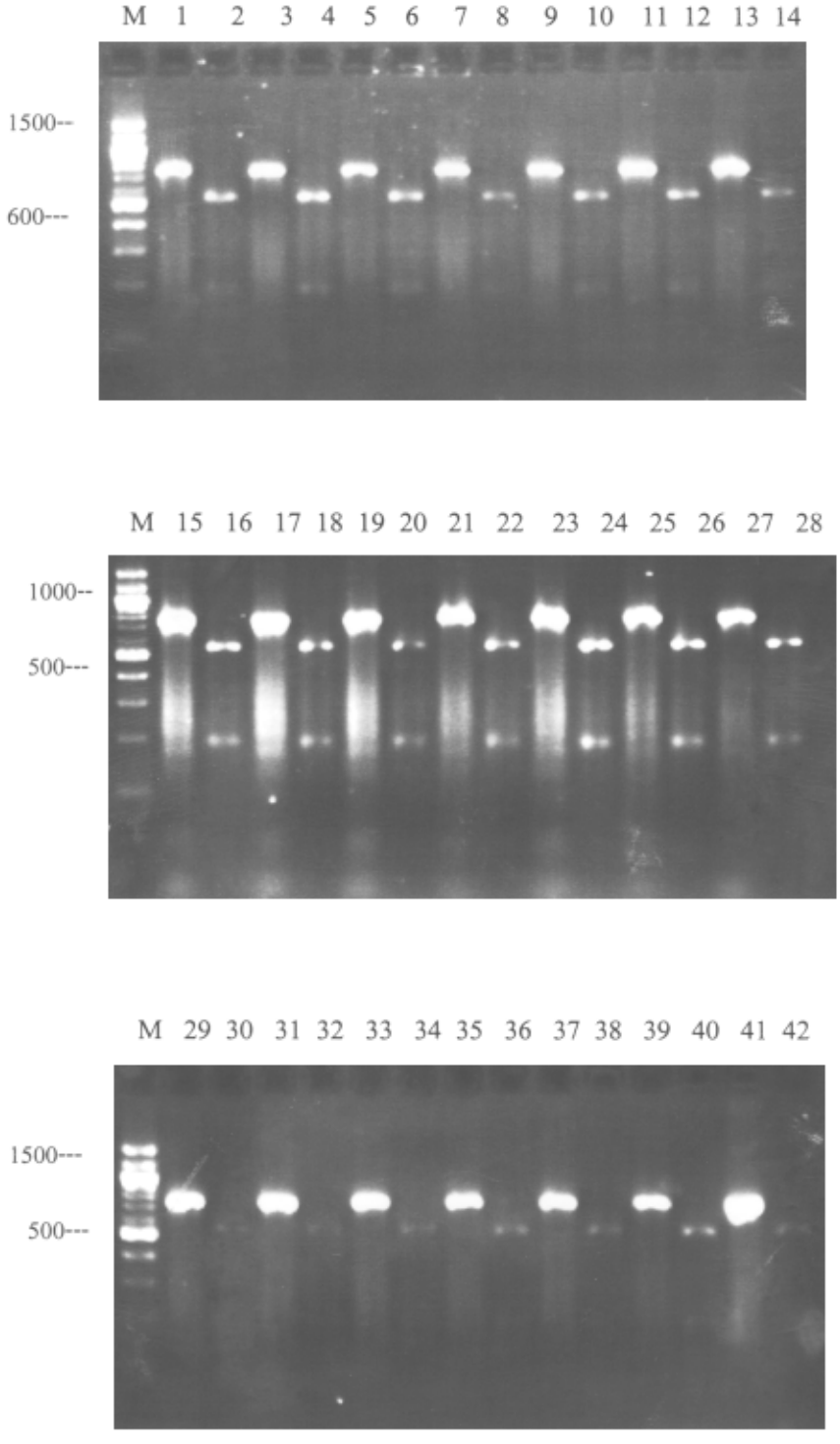


Figure 34. The PCR products of *Agrocybe cylindracea* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

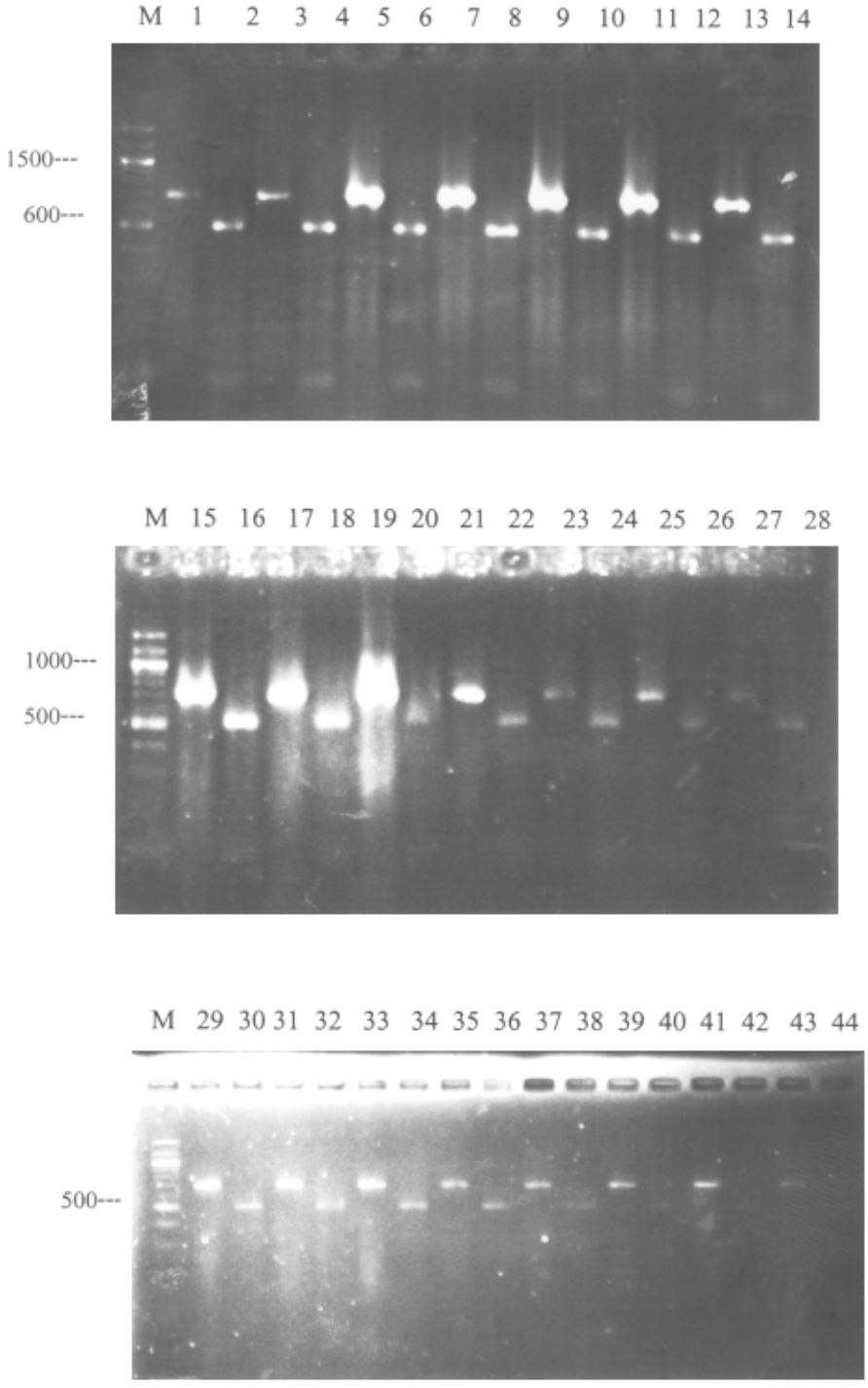


Figure 35. The PCR products of *Tricholoma crassum* digested with the *Alu* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

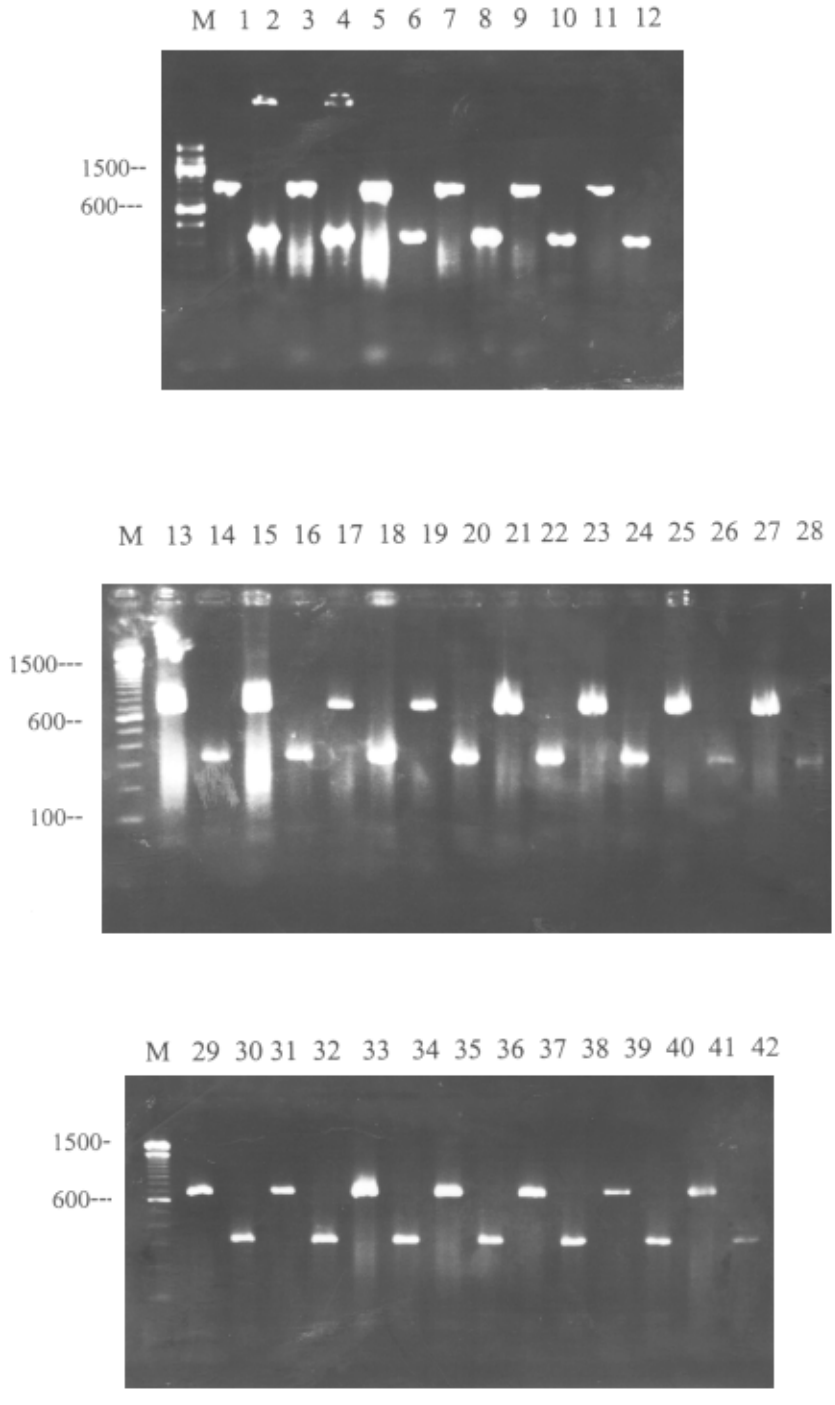


Figure 36. The PCR products of *Pleurotus ostreatus* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing– 21st subculturing and lane an even number: digested of PCR product 1st subculturing– 21st subculturing

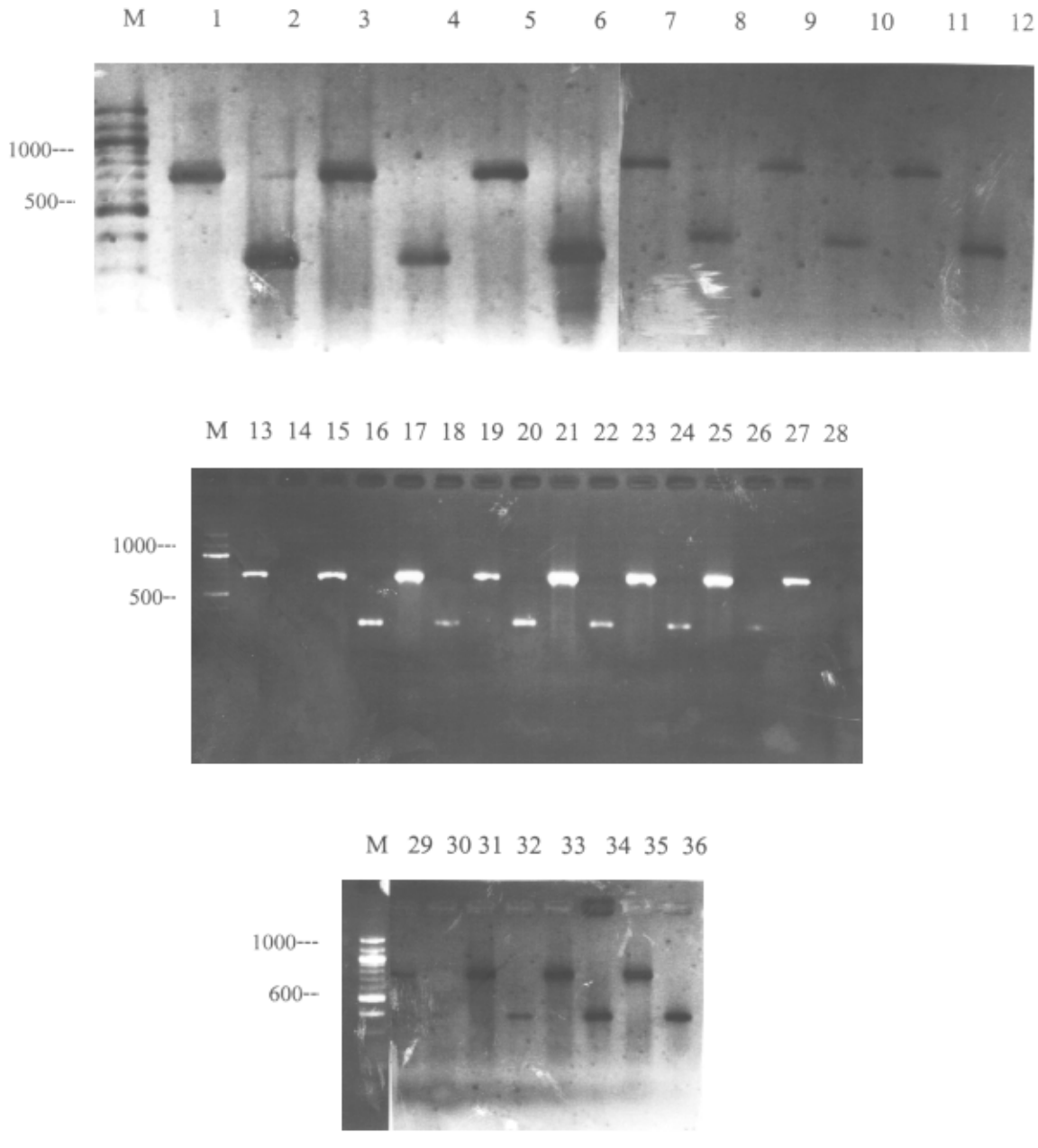


Figure 37. The PCR products of *Pleurotus sajor-caju* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 18th subculturing and lane an even number: digested of PCR product 1st subculturing – 18th subculturing

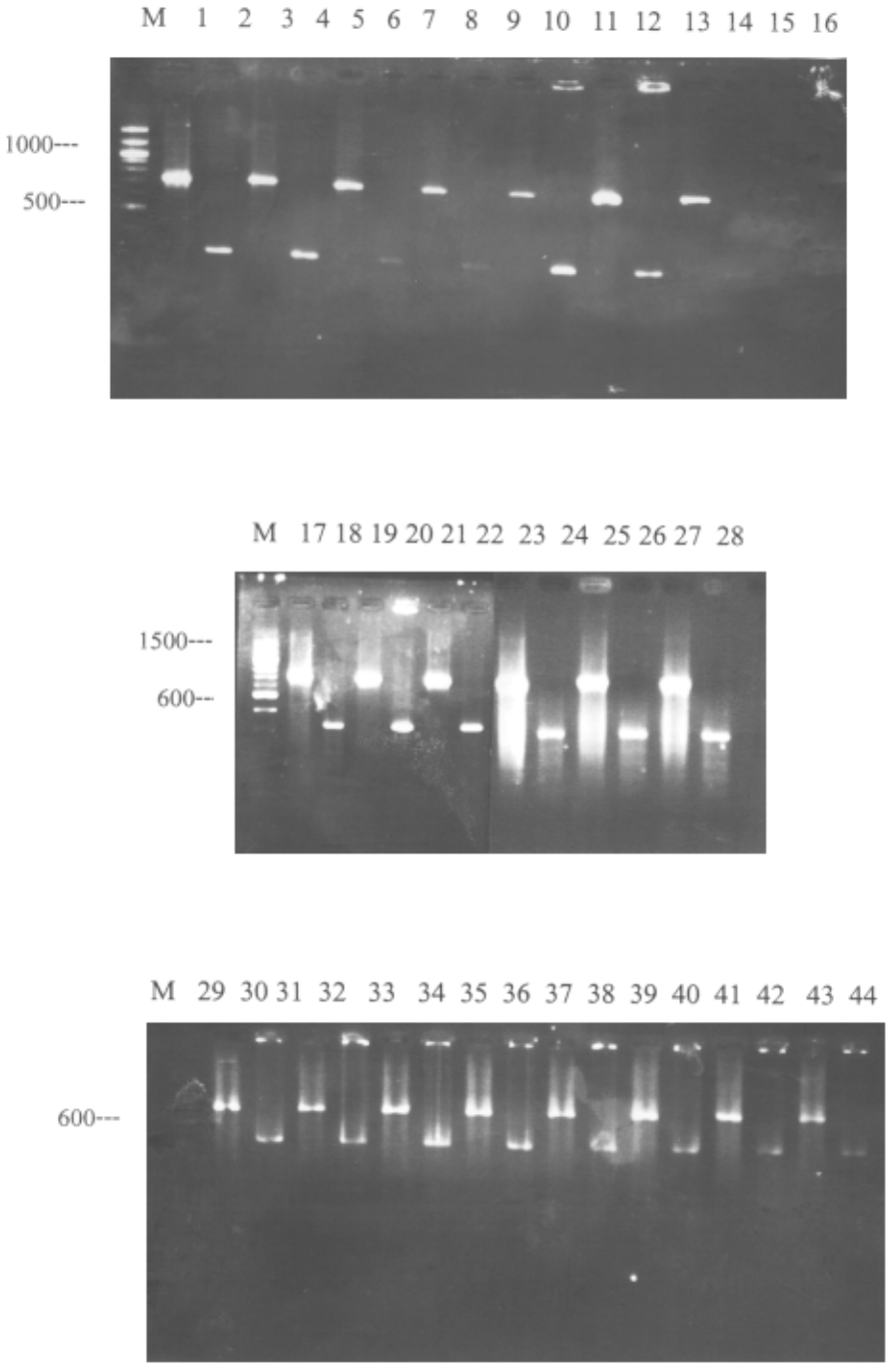


Figure 38. The PCR products of *Pleurotus cystidiosus* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

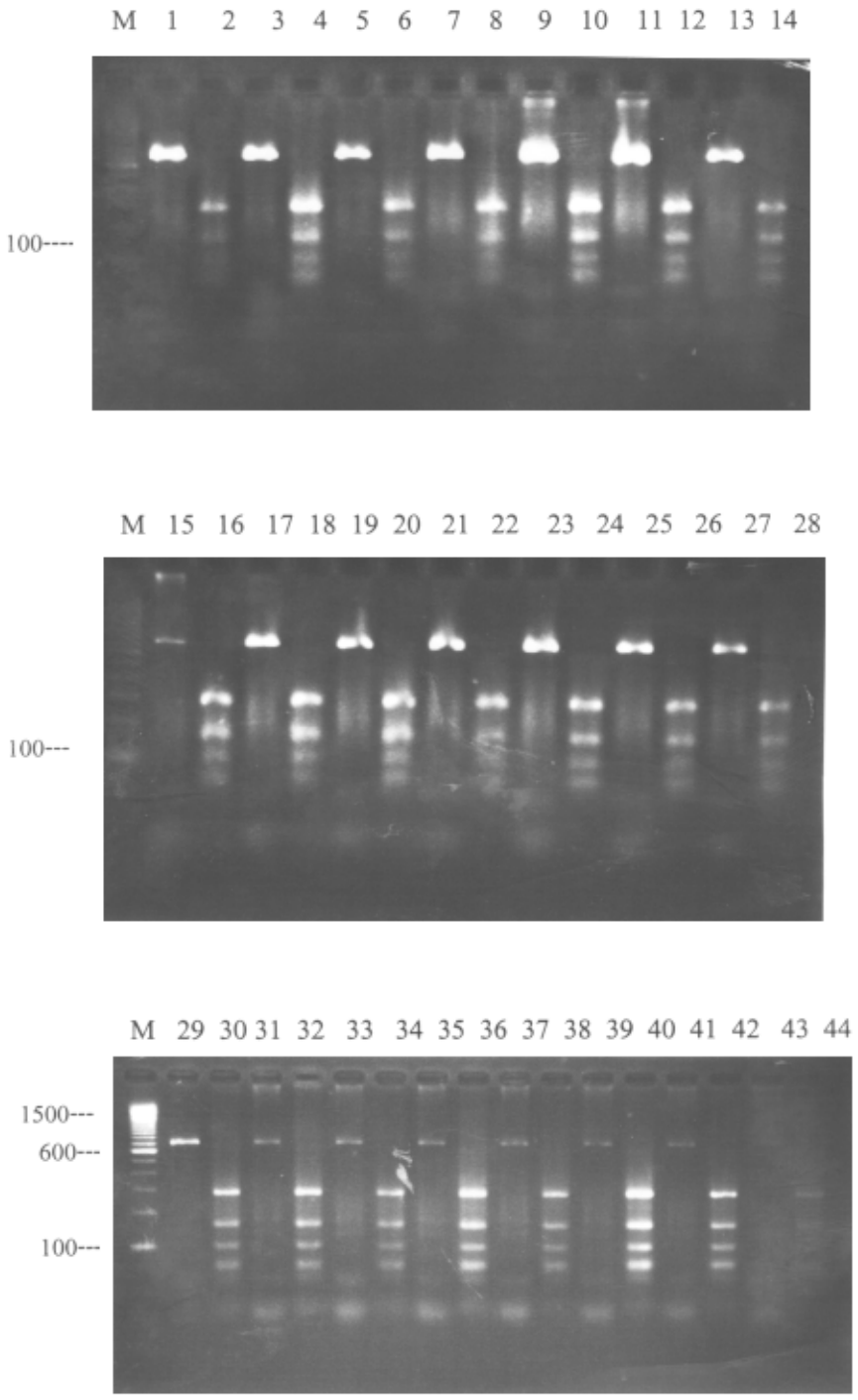


Figure 39. The PCR products of *Lentinula squarrossula* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

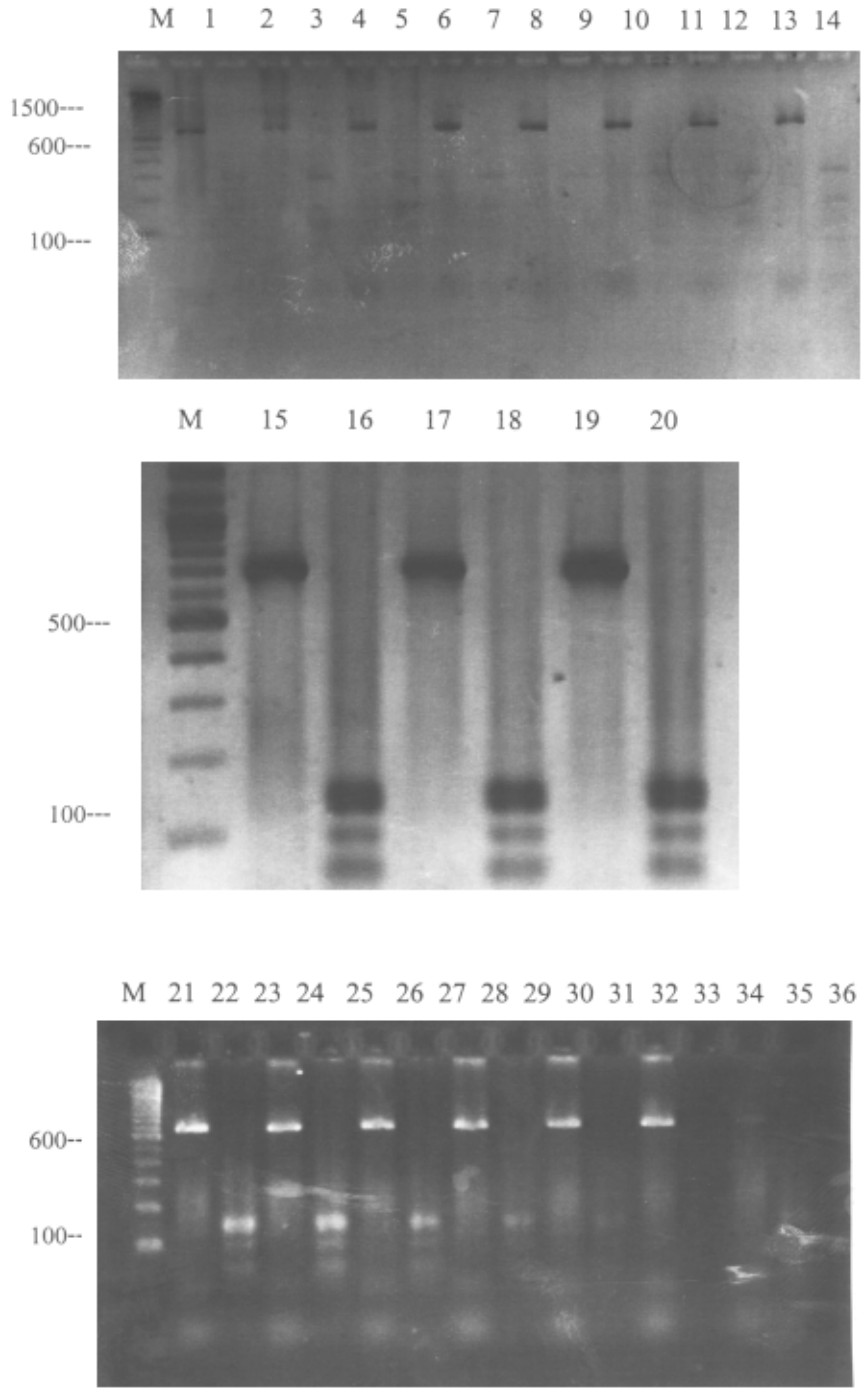


Figure 40. The PCR products of *Lentinula polychrous* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 20th subcultureing and lane an even number: digested of PCR product 1st subculturing – 20th subculturing

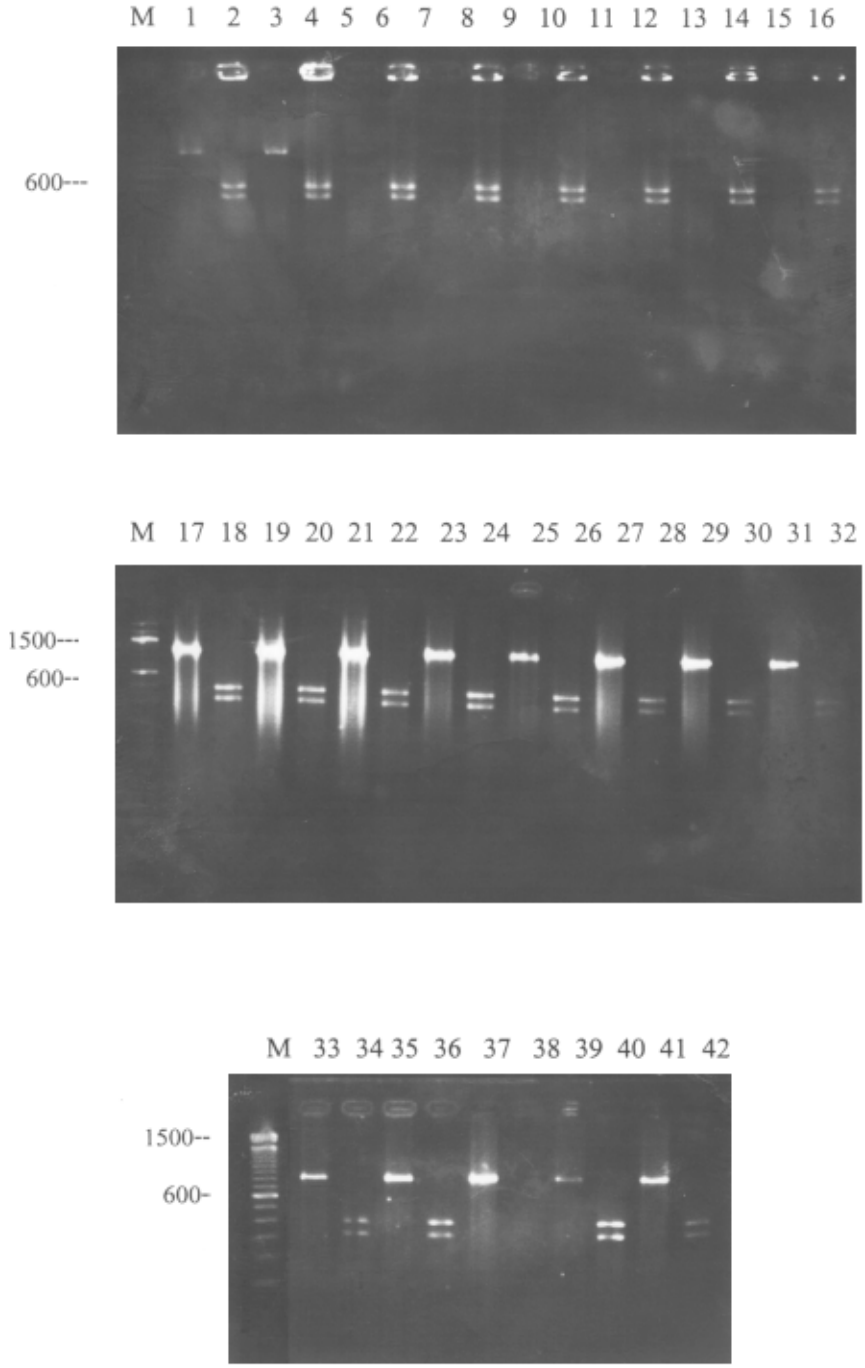


Figure 41. The PCR products of *Lentinus edodes* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 21st subculturing and lane an even number: digested of PCR product 1st subculturing – 21st subculturing

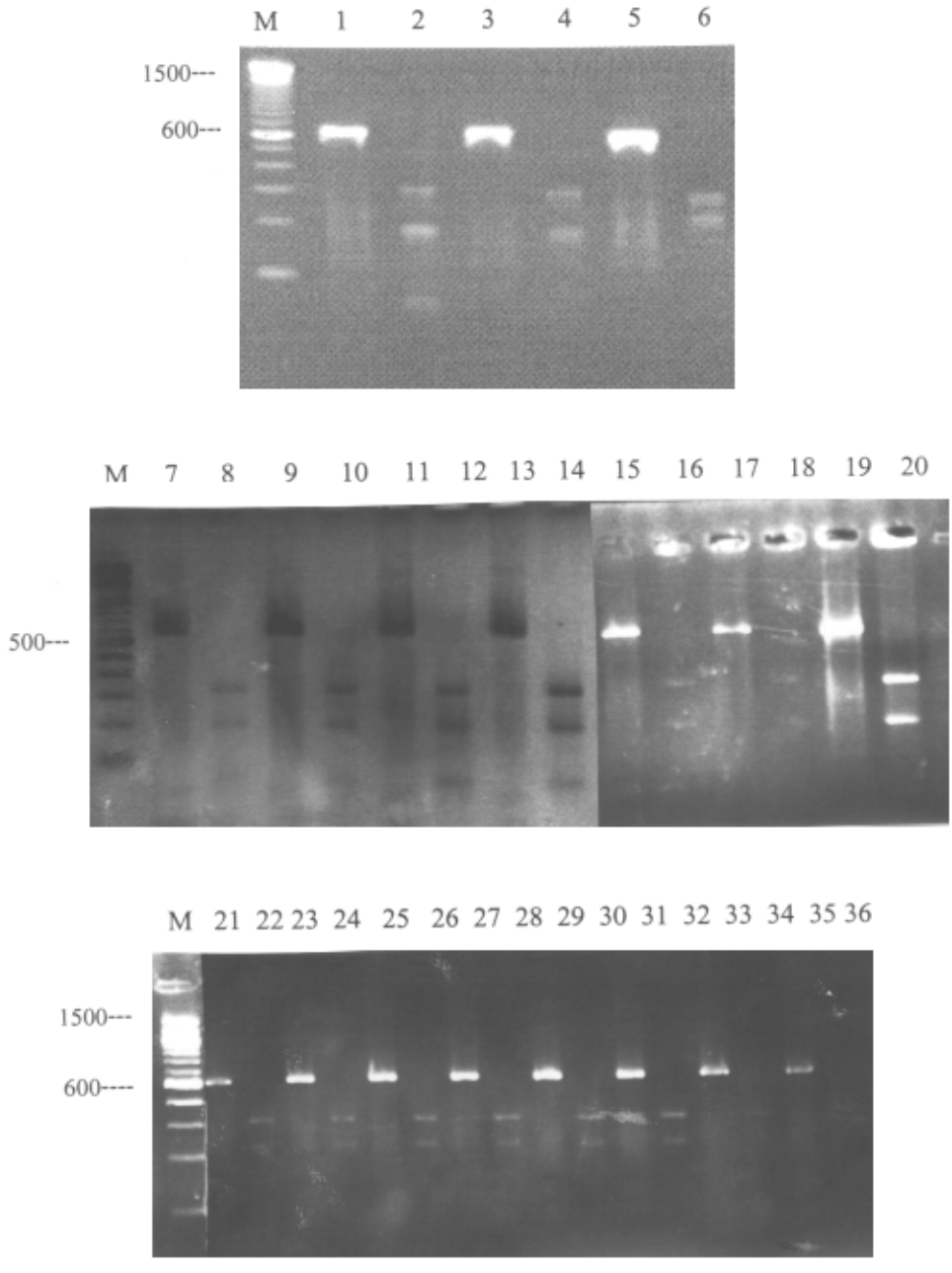


Figure 42. The PCR products of *Auricularia. auricula* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 18th subculturing and lane an even number: digested of PCR product 1st subculturing – 18th subculturing

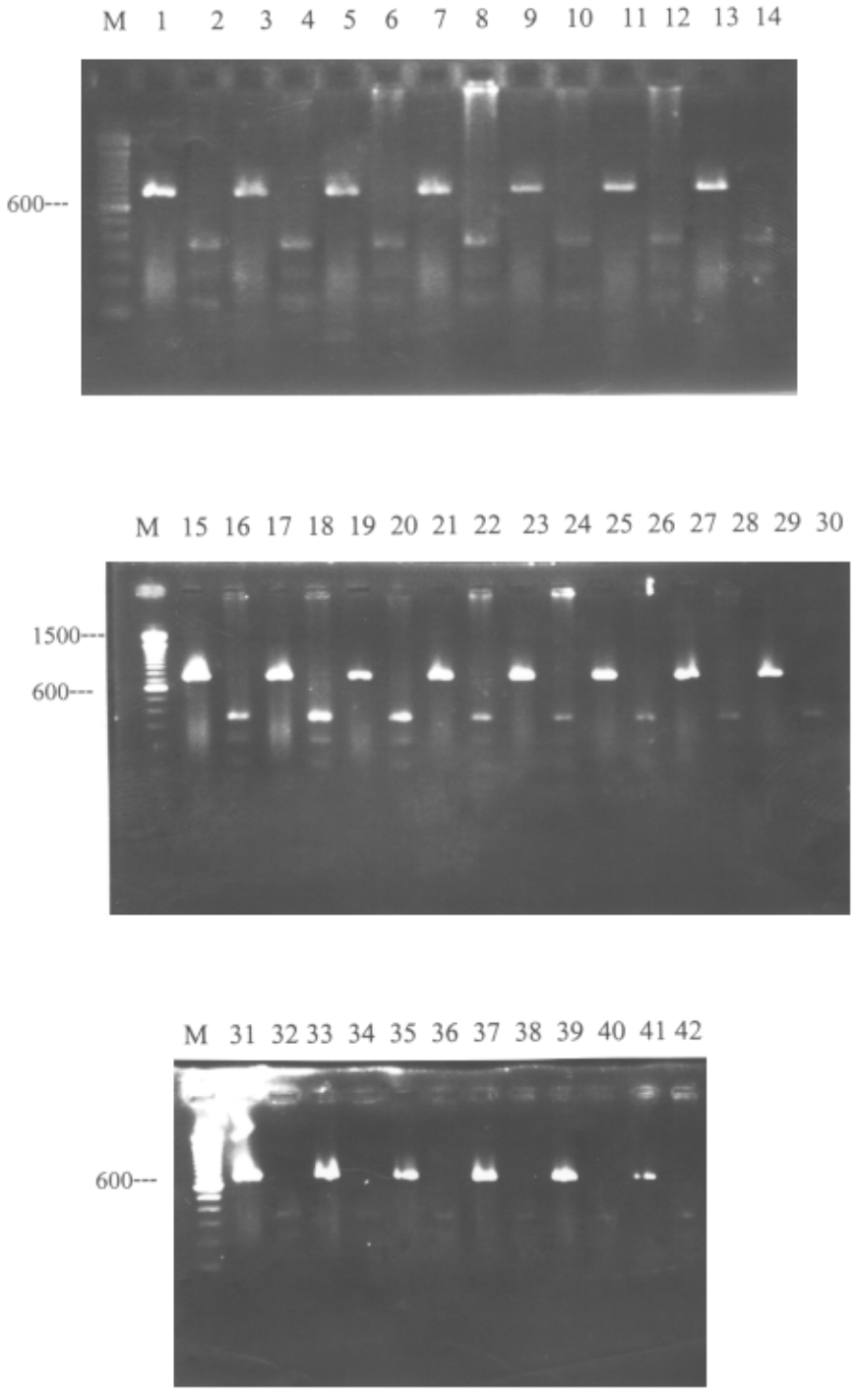


Figure 43. The PCR products of *Agrocybe cylindracea* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 22nd subculturing and lane an even number: digested of PCR product 1st subculturing – 22nd subculturing

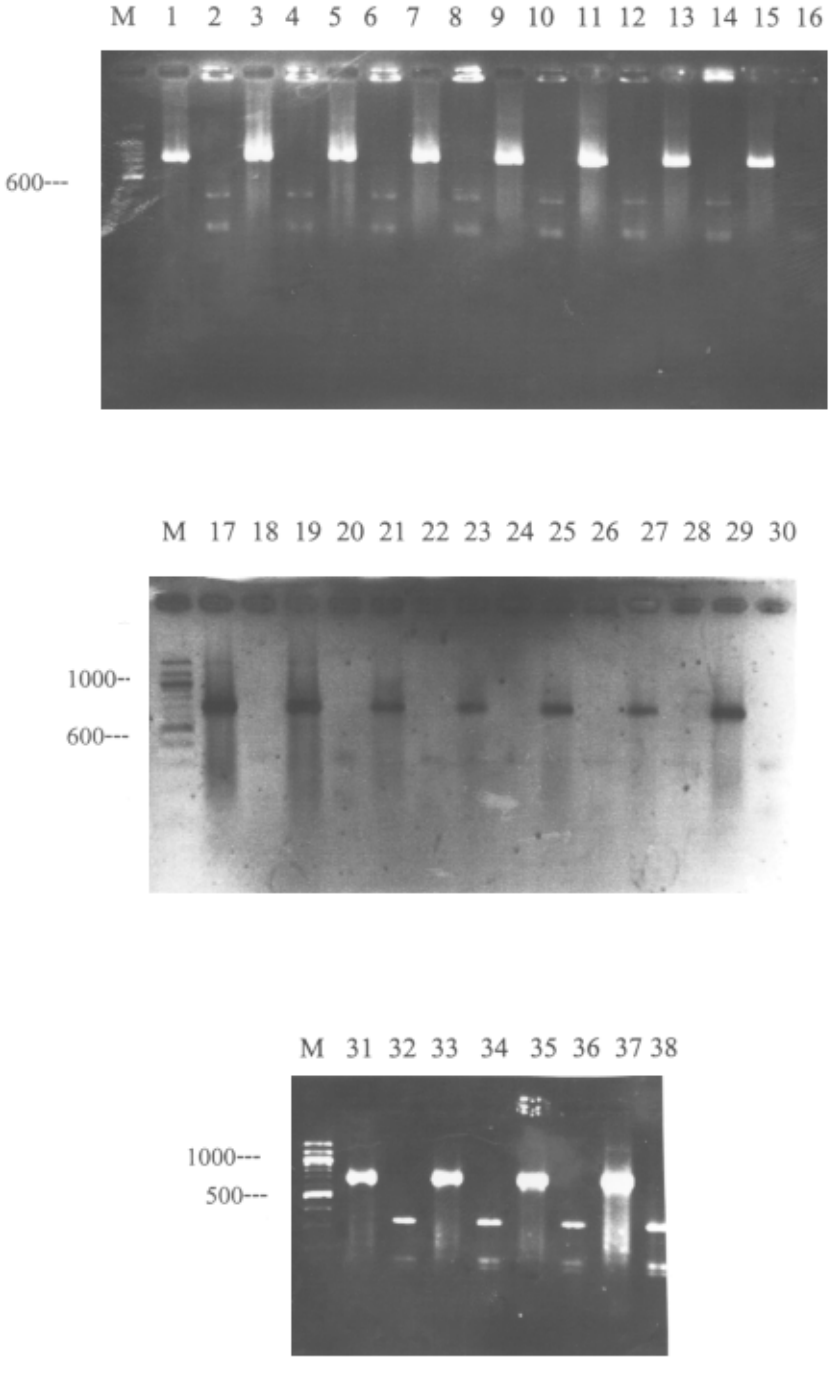
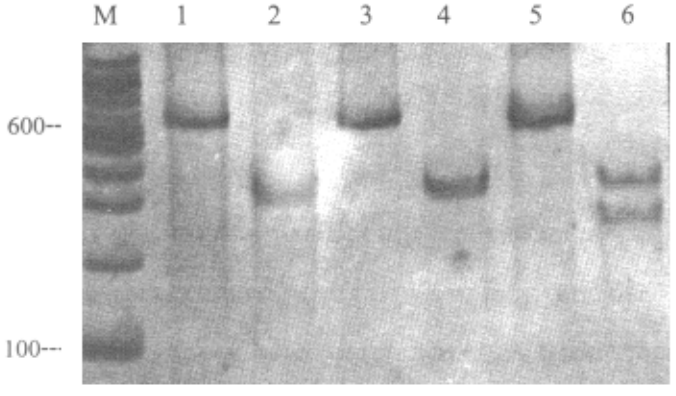


Figure 44. The PCR products of *Tricholoma crassum* digested with the *Taq* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1st subculturing – 19th subculturing and lane an even number: digested of PCR product 1st subculturing – 19th subculturing

3.3 Analysis of *Auricularia auricula* by ITS 4 and 5 primer RFLP

The PCR product generated by ITS 4 and 5 primers by using DNA template from *A. auricula* gave PCR products in size of 600 bp. Both PCR products were amplified from wild type and mutant were further conducted by PCR-RFLP analysis. The alignment sequences were compared homology by CLUSTAL X program for different sequences. The percent homology of ITS-primers sequence between wild type and mutant was 90%. The results suggested that recognition sites were digested among 600 bp at one site 300 bp (wild type) and 2 sites at 357 bp and 263 bp (mutant) with *Hinf* I as demonstrated in Figure 45 (A).

(A)



(B)

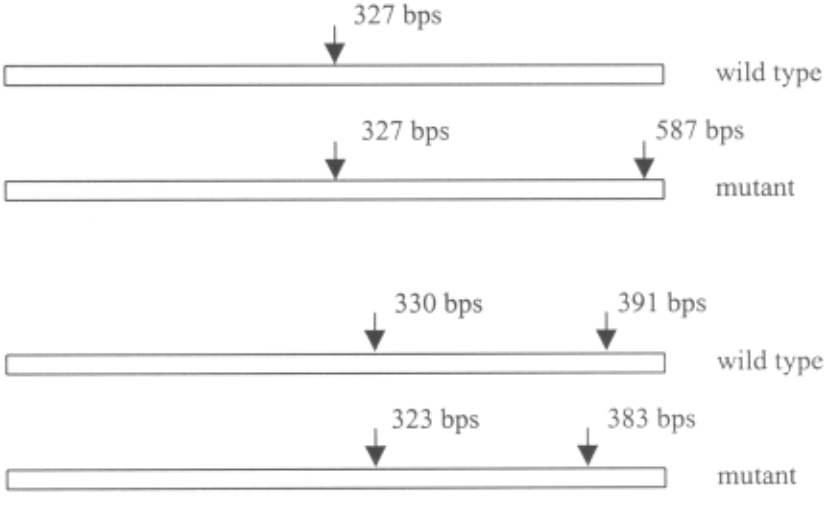


Figure 45. (A): The PCR products of *Auricularia auricula* digested with the *Hinf* I; lane M: 100 bp (GIBCOBRL); lane an odd number: uncut of PCR product 1^{st} –3^{rd} subculturing and lane an even number: digested of PCR product 1^{st} –3^{rd} subculturing; (B):The restriction maps of *Auricularia auricula* digested by a): *Hinf* I and b): *Taq* I

From Figure 45 (B), it was shown that the restriction maps of *A. auricula* were corresponded to the results obtained from 10 % polyacrylamide gel as shown in Figure 45 (A) that gave intense band of wild type while the PCR-RFLP product of 600 bp which was digested by *Hinf* I at middle site of sequence gave 2 equal fragments in size of 327 bp. While the third subculture as mutant had 2 sites digested by *Hinf* I. The first recognition sites were digested at 327 bp into 2 equal fragments and one at 587 bp but small fragment could not detected in 10 % polyacrylamide gel. Thus, the fragment size of mutant gave 327 and 260 bp when digested by *Hinf* I. From this part, it could be concluded that using *Hinf* I able to detect genetic instability of mushroom while other restriction enzymes could not. Moreover, when the sequence were compared between wild type and mutant. It was found that the DNA sequence of mutant suggested that recognition site at 587 bp with *Hinf* I could be digested because it has recognition site for *Hinf* I while the wild type sequence no recognition site to be digested. It was possible that the DNA sequence between wild type and mutant were different at 587 bp. Therefore, both restriction sites of wild type and mutant gave different site.

Table 6. Comparison of fragment size of *Auricularia auricula* between wild type and mutant when digested with the various restriction enzymes.

Restriction enzymes	PCR product in size of 600 bps	
	wild type (1st subculturing)	mutant (3rd subculturing)
Alu I	390, 120 bp	390, 120 bp
Hinf I	300, 300 bp	357, 263 bp
Mbo I	400, 190 bp	400, 190 bp
Taq I	316, 190 bp	316, 245 bp

From Table 6, the third subculture of *A. auricula* gave different fragment sizes when digested with various restriction enzymes (Figures 15, 24, 33 and 42). It was indicated that both the PCR products of *A. auricula* which were digested with the *Hinf* I and *Taq* I gave intense different fragment sizes which corresponded to DNA-fingerprint. Both sequences of wild type and

mutant were investigated with direct sequencing the PCR product. The sequence data were aligned between wild type sequence as parental and mutant sequence by CLUSTAL X program as shown in Figure 46.

```
CLUSTAL X (1.64b) multiple sequence alignment

Hn1its4         NGNNTTTCCCCGCTTTATTGATATGCTTAAAGTTCGGCTGG-TAGTTCTACCTGATCTGG
Hn3its4         --GNCNTNCCCGCTT-ATTGATATGNTTTA-GTTCAGCGGGGTAGTCCTACCTGATTTGA
                   * ******* ********* ** * **** ** ** **** ********* **

Hn1its4         TGGTCGAAGCTTGCCAAGATAATGCGTTCACTTAGGGACGGTTGTCAGCTGGACCTGTGA
Hn3its4         -GGCCAAAGCTTT--AAAATAGTT-GTCCACA-AGGGACGGCTGTAAGCAGCGCCGCTGA
                 ** * ******   ** *** *  ** ***  ******** *** *** *  **  ***

Hn1its4         AGAGGCCTAGG-CATT-GGCGCATATTATTATTACACCGAT-GCCCAGCACTCTAAAAGC
Hn3its4         AGAGGCCCAGGGCAGTCGGCGCAGATAATTATCACACCGTTCGCCCAGCACTCTAAAAGC
                ******* *** ** * ****** ** ***** ****** * ******************

Hn1its4         GCCAGCTAATGCATTTCAAGACGAGCCGGTTAC-GGCACAGTCCAAGTCCACCACGGGCG
Hn3its4         GCCAGCTAATGCATTTCAAGACGAGCCGATTACCGGCACGGTCCAAGTCCACCGCAGGCG
                ************************** **** ***** ************* * ****

Hn1its4         ACTGTTACATCGCAAGGGTGAGGGTTTACGTGACACTCAAACAGGCATGCTCCATGGAAT
Hn3its4         ACTGTTACATCGCAGGGGTGAGGGTTTACGTGACACTCAAACAGGCATGCTCCATGGAAT
                ************** ******************************************

Hn1its4         ACCAAGGAGCGCAAGATGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
Hn3its4         ACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
                *************** ******************************************

Hn1its4         TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAA
Hn3its4         TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAA
                ************************************************************

Hn1its4         AGTTGTTACTTTTTATGGTTTTGTTAACATTCGAGACTGA--GTTGTTGCATTTGAAAGC
Hn3its4         AGTTGTTACTT--TATAGTTCTCGTCACATTCTAGACTTTTCGTGGTTGCATGTAAAAGC
                ***********  *** *** *  * ****** *****    ** ******* * *****

Hn1its4         GGCAGCGACCGAAGCCGCAACCGAAAAGGTGCACAGGTGTGGGGTCTTGCTCCAGCGTGC
Hn3its4         GGAAGCGGCCGAAACCGCAACCGAAAAGGTGCACAGGTGTGAGAGGTTAT--CGGCGTGC
                ** **** ***** ************************** *   **    * ******

Hn1its4         AGCCCTGTGAAGGGCGCACA-GCTGAACGATCGGGTTAAAAGCCCAA-AATCTTTAATGA
Hn3its4         AGCC---TGA---GCGCACAAGCCGAA--ATTAAGACCGAAGCCTTAGAATCTTTAATGA
                ****   ***   ******* ** ***  **   *    *****  * ************

Hn1its4         TCCTTCCGCAGGCTCACCTAC-NGAAACCTTGTTANGACTTTNTNNANNNCC-
Hn3its4         TCCTTCCGCAGGTTCACCTACCGGNAACCTTGTTACGACTTTTTNTNNNNNNC
                ************ ********  * ********** ****** **  ***
```

Figure 46. Alignment of the PCR products of *Auricularia auricula* were compared between 1st subculturing (as parental) and 3rd subculturing; Hn1its4 means sequence of wild type (1st subculturing) and Hn3its4 means sequence of mutant (3rd subculturing).

3.3.1 DNA extraction from fruit body of the third subculturing of *Auricularia auricula*

The genomic DNA from fruit body of *A. auricula* at 1st and 3rd subculturing were extracted. The PCR-RFLP gave similar with DNA mycelium, which digested in fragment size of 357, 263 bp (mutant) and 300, 300 bp (wild type). Results obtained from this study, it could concluded that genetic instability was caused from continuous subculturing. The ITS region could be used detect to the defective gene by PCR-RFLP technique. The genetic variation was persisted in fruiting as depicted in Figures 47 and 48. The deficiency gene was effect on phenotype of fruit body as demonstrated in Fig ure 50.

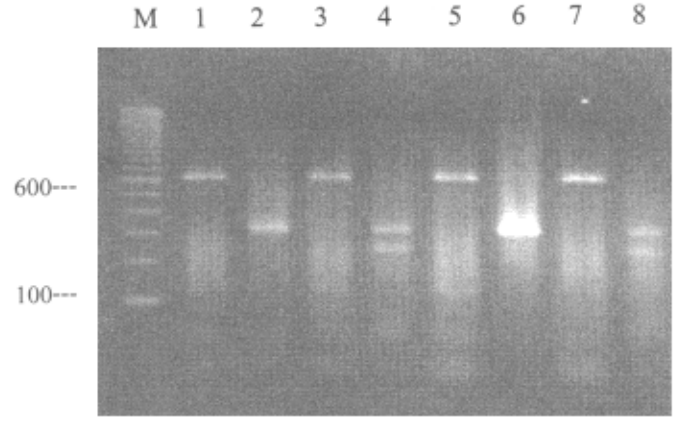


Figure 47. Comparison of PCR products of *Auricularia auricula* digested with the *Hinf* I. lane M: 100 bp; lane odd number: uncut of PCR product; lane even number: digested of PCR product; lane 1,3: PCR products were amplified from mycelium of 1st, 3rd subculturing, respectively; lane 5, 7: PCR products were amplified from fruit body of 1st, 3rd subculturing, respectively

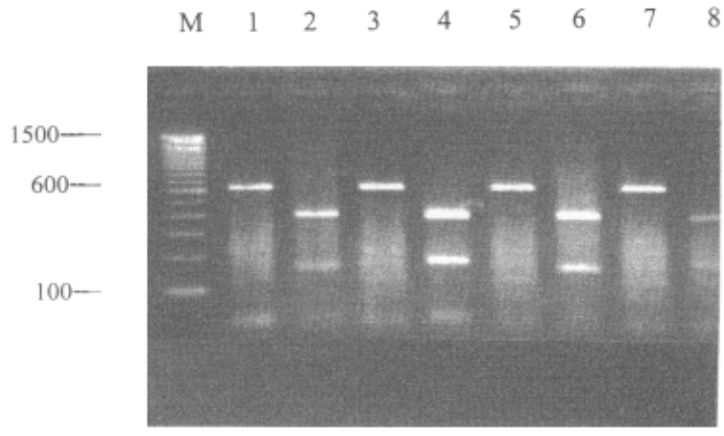


Figure 48. Comparison of PCR products of *Auricularia auricula* digested with the *Mbo* I. lane M: 100 bp (GIBCOBRL); lane odd number: uncut of PCR product; lane even number: digested of PCR product; lane 1, 3: PCR products were amplified from mycelium of 1st, 3rd subculturing, respectively; lane 5, 7: PCR products were amplified from fruit body of 1st, 3rd subculturing, respectively

3.3.2 Analysis of β- tubulin gene between wild type and mutant.

From the restriction map showed that the genetic variation of mushroom was observed by ITS 4-5 PCR-RFLP analysis. Another set of gene which is involved in fruit body formation was investigated. β-tubulin gene was chosen in this experiment. The deficiency gene of 3rd subculturing *A. auricula* was investigated by β-tubulin gene. The DNA templates were amplified by β-tubulin gene. The products gave in size of 100 bps (wild type) and 400, 100 bp (mutant) as shown in Figure 49. The deficiency gene of 3rd subculturing *A. auricula* was cropped by mycelium transfer. When the fruit bodies were compared between 1st and 3rd subculturing. The fruit body of wild type had normal size about 5.0 cm of diameter and brownish which larger than mutant while the mutant had smaller size, distorted and brownish as depicted in Figure 50. It could be concluded that β-tubulin gene were able to distinguish between wild type and mutant and the deficiency gene had effect to phenotype which observed with this gene. Thus, the β-tubulin gene could be studied the fruit body formation. It was shown that this primer were able to be used as monitoring for mushroom production. In addition, the defective fruit body of *P. sajor-caju* was

observed from SUT- Farm as shown in Figure 51. Then, the DNA from defective fruit body of *P. sajor-caju* which were extracted and followed by amplified with β-tubulin gene gave PCR product in size of 150 bp (normal fruit body) and 150, 100 and 90 bp (defective of fruit body). Results showed that although normal growth rate of culture and environment of cropping could be occurred the defective of fruit body during cropping and investigated with β-tubulin gene was also able to detect the defect of fruit body of *A. auricula* and *P. sajor-caju* formation. This finding was agreed with Balduaf and Doolittle (1997) quoted in (Thon and Royse, 1999) who made use of β-tubulin genes to relate among fungi at all levels. The tubulin gene family is composed of three major groups, the α, β and γ tubulins, α- and β- tubulins make up microtubules, that are major components of the cytoskeleton, mitotic spindles and flagella of eukaryotic cells. Thus, this gene seems to play an important role in fruit body structure.

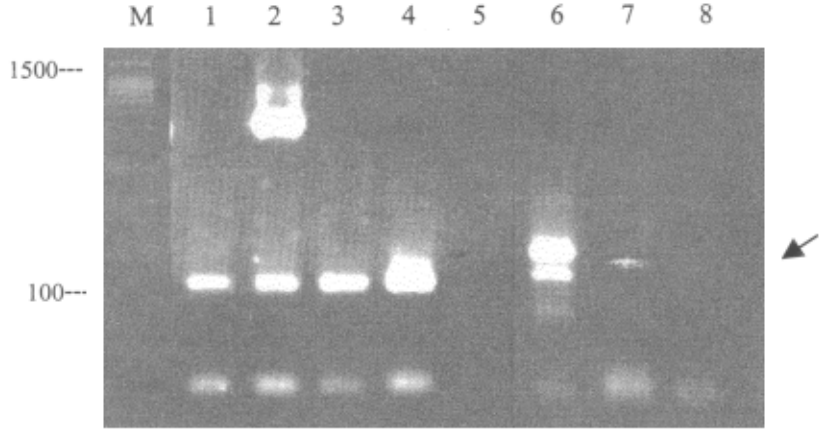


Figure 49. The PCR products of *Auricularia auricula* and *Pleurotus sajor-caju* which were amplified with β-tubulin gene; lane M: 100 bp (GIBCOBRL); lane 1: 1st subuclturing (wild type); lane 2: 3rd subculturing (mutant); lane 3: *Auricularia auricula* from Arunyik farm; lane 4: defective of *Auricularia auricula* from Arunyik farm; lane 5: control; lane 6: defective of *Pleurotus sajor-caju*; lane 7: normal fruit body of *Pleurotus sajor-caju* and lane 8: control

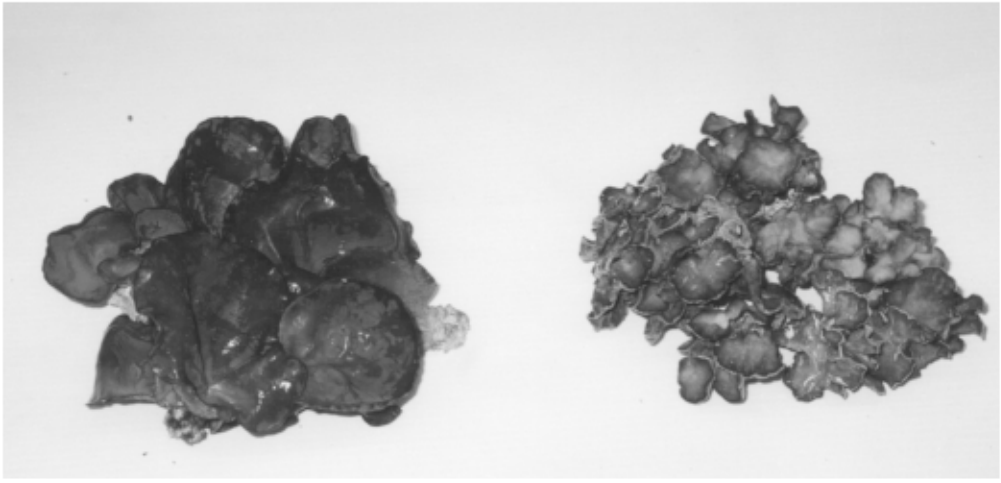


Figure 50. Morphology of *Auricularia auricula* fruit body; left: wild type and right: mutant.

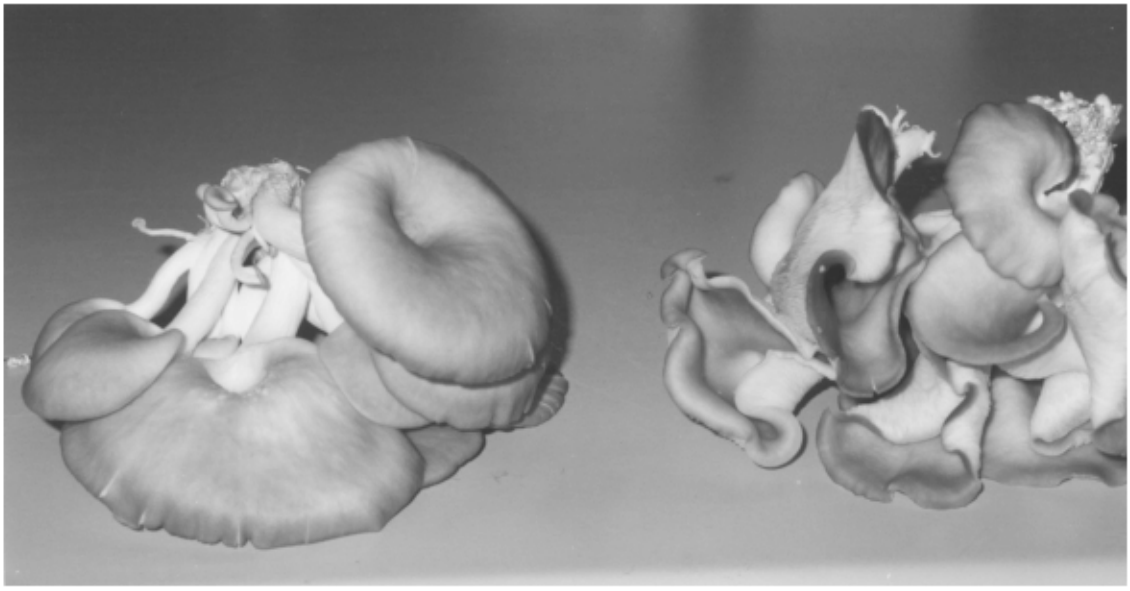


Figure 51. Morphology of defective phenotype of *Pleurotus sajor-caju* cultivated at SUT- Farm.
left: normal fruit body and right: defective fruit body

3.3.3 The growth rate and yield of the third subculturing of *Auricularia auricula*

The results of DNA-fingerprint and restiction map of the 3rd *A. auricula* suculturing were different from the first subculturing. Thus the defective gene of *A. auricula* might relate to the growth rate in compost. Therefore, the growth rate in compost and yield of the 3rd *A. auricula* subculturing were observed. The results showed that the growth rates in compost of the 3rd subculture and yield were decreased (as demonstrated in Figure 52) when compared with the first subculturing. It could be concluded that the deficiency gene of the 3rd subculturing was due to growth rate in compost and yield and indicated that genetic instability was observed during continuous subculturing. Normally, the mushroom productions were maintained in the form of pure culture by serial transfer mycelium on agar. Then the continuous subculturing for production was able to cause genetic instability which affected the growth rate and yield. The growth rate in compost of mutant was reduced about 11.63% from the first subculturing at significant 95% but growth rate on PDA medium still normal.

Figure 52. Comparison of growth rates in compost between the first and the third subculturing; a): slow growth rate of the third subculturing; b): normal growth rate of the first subculturing

Result suggested that determination of growth rates on medium was not enough for quality control because the medium contained with rich nutrient while the compost was sawdust which contain mainly cellulose as nutrient source. Therefore, the mushroom must produced some enzyme such as cellulase, cellohydrolase for decomposition (Zhao and Kwan, 1999). In this case the enzyme activity played an important role in cellulose degradation. In addition, Fritsche (1974) was studied that growth rate of *A. bisporus* found that the maintenance of culture might be due to continue reduced rate and loss of yield. Loss of yield was indicated that the weak points of strain which slow start of cropping and long period between the flushes were observed.

When the 4th subculturing was cultivated by mycelium serial transfer. It was found that both growth rate and DNA pattern gave the same pattern as the first subculture as shown in Figure 15. Results obtained from this study, it was possible that reversible genetic was occurred. The result was agreed with (Hartl, 1988) it has been reported that normal gene is changed into a form that results in mutant phenotype an event called reverse mutation which most occurred in only nature. The reversion might be also result from the second mutation at some other site in the genome. Therefore, it was possible that the 4th subculturing of *A. auricula* was reversion which growth rate and DNA pattern due to changed into wild type characteristic.

3.4 *Tricholoma crassum* production

To find appropriate cultivation for *T. crassum* production by using agricultural wastes. This experiment was designed with Split-Split Plot. The results of yield throughout 4 months for production were analyzed by Split-Split Plot Design as shown in Table 7.

Table 7. The total statistical analysis of *Tricholoma crassum* cultivated under different conditions (MxHxS)

Treatment (Sub-sub plot (S))	Main plot (H) Plastic fresh weight (g)	Clay plot fresh weight (g)	S-MEAN	DIFF
Non-covering				
Soil	220.00[a]	575.00[a]	397.50	-355.00**
Soybean husk	50.00[a]	328.33[b]	189.17	-278.33**
Soil+soybean husk	75.33[a]	233.33[b]	154.33	-158.00*
Rice husk-covering				
Soil	163.33[ab]	241.63[a]	202.50	-78.33[ns]
Soybean husk	0.00[ns]	73.33[b]	36.67	-73.33[ns]
Soil+soybean husk	253.33[a]	0.00[ns]	126.67	253.33**
Rice straw-covering				
Soil	30.00[a]	270.00[a]	150.00	-240.00**
Soybean husk	16.67[a]	16.67[b]	16.67	0.00[ns]
Soil+soybean husk	0.00[ns]	150.00[ab]	75.00	-150.00[ns]
H-MEAN	89.85	209.81	149.83	-119.96

** = significant at 1% level, * = significant at 5% level, ns = not significant

In a column under each M, means followed by a common letter are not significantly different at the 5% level by DMRT.

Comparison	S.E.D.	LSD (5%)	LSD(1%)
2-H means at each M* S	73.50	157.22	218.71
2-S means at each M*H	80.71	166.57	225.75

Table 8. Effect of casing material on *Tricholoma crassum* yield (HxS)

Casing material	Plastic fresh weight (g)	Clay pot fresh weight (g)	S-MEAN	DIFF
Soil	137.78[a]	362.22[a]	250.00	-224.44**
Soybean husk	22.22[a]	139.44[b]	80.83	-117.22**
Soil+soybean husk	109.56[ab]	127.78[b]	118.67	-18.22[ns]
Average	89.85	209.81	149.83	-119.96

** = significant at 1% level, * = significant at 5% level, ns = not significant

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Comparison	S.E.D.	LSD (5%)	LSD(1%)
2-H means at each S	42.43	90.77	126.27
2-S means at each H	46.60	96.17	130.33

The growth rates of *T. crassum* were observed. The growth rates on PDA medium and compost were 0.35 ± 0.07cm/ day, 4.05 ± 0.30 cm/ 2 weeks at significant 95%, respectively. The optimum temperature for cultivated on medium at 30^0C. Then the mycelium had completely grown bag and carried into cropping. The optimum temperature for cultivated in casing materials about 26-30^0C depend on moisture and weather. The mycelium was observed in area below of surface about 5.0 cm after 17 days. The mycelium in soil was fast to developed in soil mixed with soybean husk and soybean husk, respectively because the constituent of the humus and soil had effect to promote growth (Ogawa, 1964 quoted in Chang and Hayes, 1978). Therefore, the mycelium was able to uptake the nutrient from soil (Wason, 1978) better than other casing materials. Some container in casing had not occurred the mycelium in below of surface after 17 days. *T. crassum* production in this experiment since July – October through 4 months which rainy season. In October, yields were harvested. The primordia were appeared at the edge of container after 29 days and size of cap about 0.3-0.5 cm at first day. After that the fruit body was developed until 9-10 days into mature stage for harvesting. The diameter of the mature cap is usually 8-11 cm and length of stipe about 11-13 cm as depicted in Figure 53, 54 and 55. Moreover, almost of fruit bodies were generated with group and the edge of container. The result was agreed with

(Tominaga, 1973 quoted in Chang and Hayes, 1978) *T. matsutake* was observed. Result suggested that the fruit bodies almost grow in typical fairy ring and develop on the outermost of trees.

From Tables 7 and 8, showed the main plots between non-covering and covering were not significantly different ($P<0.05$). This result suggested that between non-covering and covering had no effect on total of *T.crassum* yields. In Table indicated that subplot as plastic and clay plot. The clay plot could be gave higher yield (209.81 g/ 1 container) than plastic (89.85 g/ 1 container) as container with every sub-sub plot in significantly higher ($P<0.01$). It showed that the clay plot had suitable for cultivation. In casing material were observed. The soil as material substrate gave highest of yield with every main plot and sub plot in significantly higher ($P<0.01$) soil mixed with soybean husk and soybean husk, respectively. The non-covering had soil as substrate gave significantly higher yield in clay plot (575 g/ 1 container) than plastic (220 g/ 1 container). For the rice husk covering, the clay plot gave higher yield than plastic containers were not significant. The rice-straw covering, the soil as material substrate and clay plot as container still gave (270 g/ 1 container) higher yield than soil mixed with soybean husk (150 g/ 1 container) and soybean husk (16.67 g/ 1 container), respectively.

From this part, It could be concluded that the soil as the best casing material and clay plot as the best container for *T. crassum*. It was possible that the clay plot could be kept moisture better than the plastic which had around the hole. The optimum temperature for cultivated about 30^0C. In addition, Wason (1978) observed the relationship between the promordia and rainfall. He found that the primordia was developed in rainy season better than other season because high moisture in rainy season. Therefore, humidity and temperature seem to be important in the formation of primordia and fruit bodies.

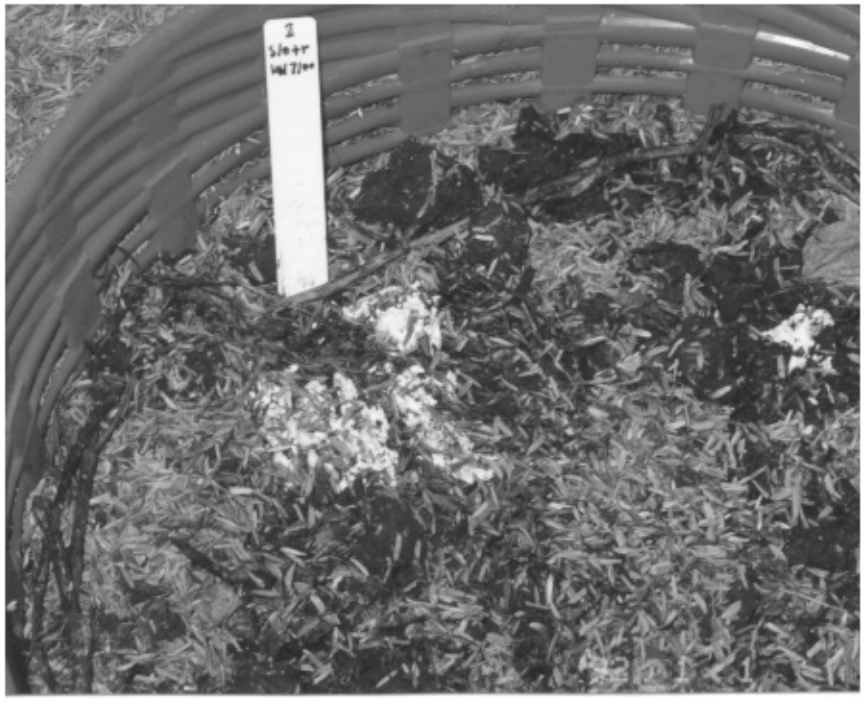


Figure 53. The primordia of *Tricholoma crassum* at first day

Figure 54. The primordia of *Tricholoma crassum* at 4-5 days

Figure 55. Morphology of *Tricholoma crassum* in mature stage at 9-10 days

3.5 Maintenance of individual species in refrigerator 4 ^{0}C and freezer –70 ^{0}C

To determined the effect of preservation of culture for long period for cultivation on growth rates, two preservation methodswere chosen. The pure culture were maintained in refrigerator at a temperature 4 ^{0}C and freezer –70 ^{0}C using 10% glycerol as the cryoprotectant (Smith and Onions, 1994). The cultures were checked the survival every two weeks on PDA medium plates. Then the growth rates of culture were stored at 4^{0}C. It was found that each species of mushroom showed that the different growth rates on PDA (as shown in Figure 56). The most species were not different in growth rate during 80 weeks. Smith and Onions (1994) it has been reported that the preservation of living fungi by use of cold storage could be decreased rate of metabolism and thus increase the intervals between subculture. Thus, the pure culture could be survival for long time which growth rate was not changed.

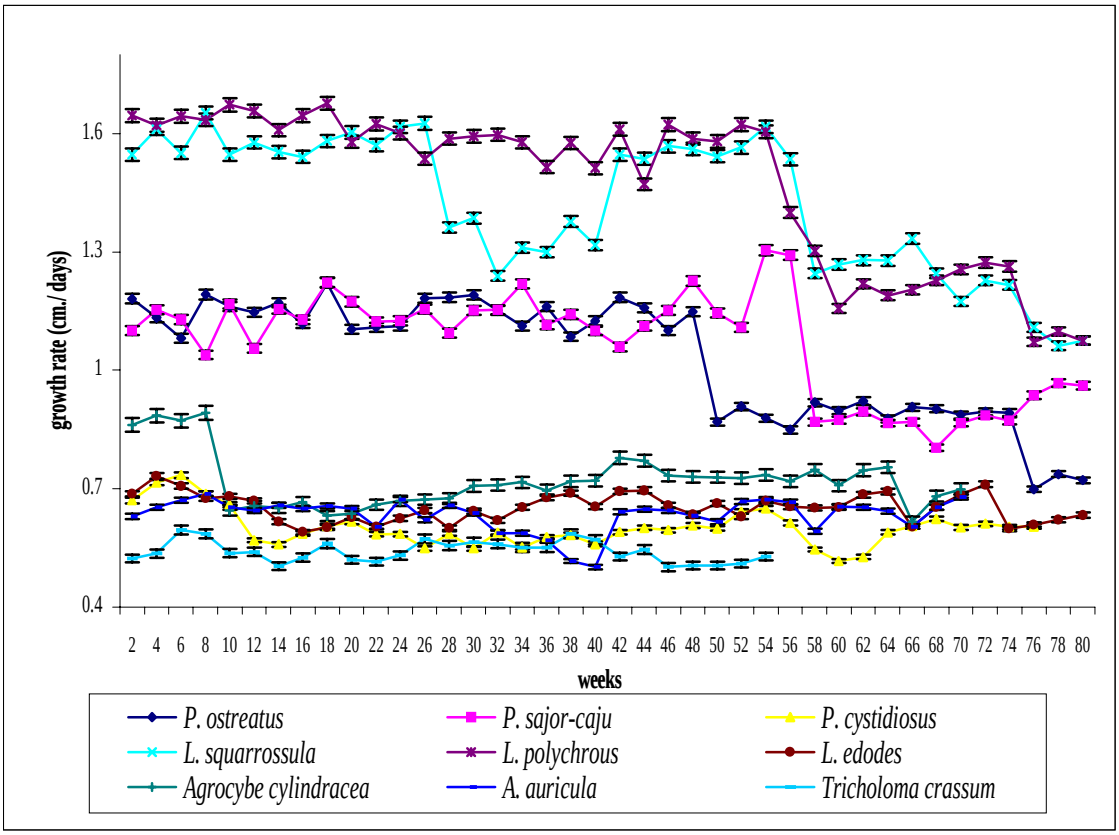


Figure 56. The growth rates of individual of species were stored at 4 ^{0}C

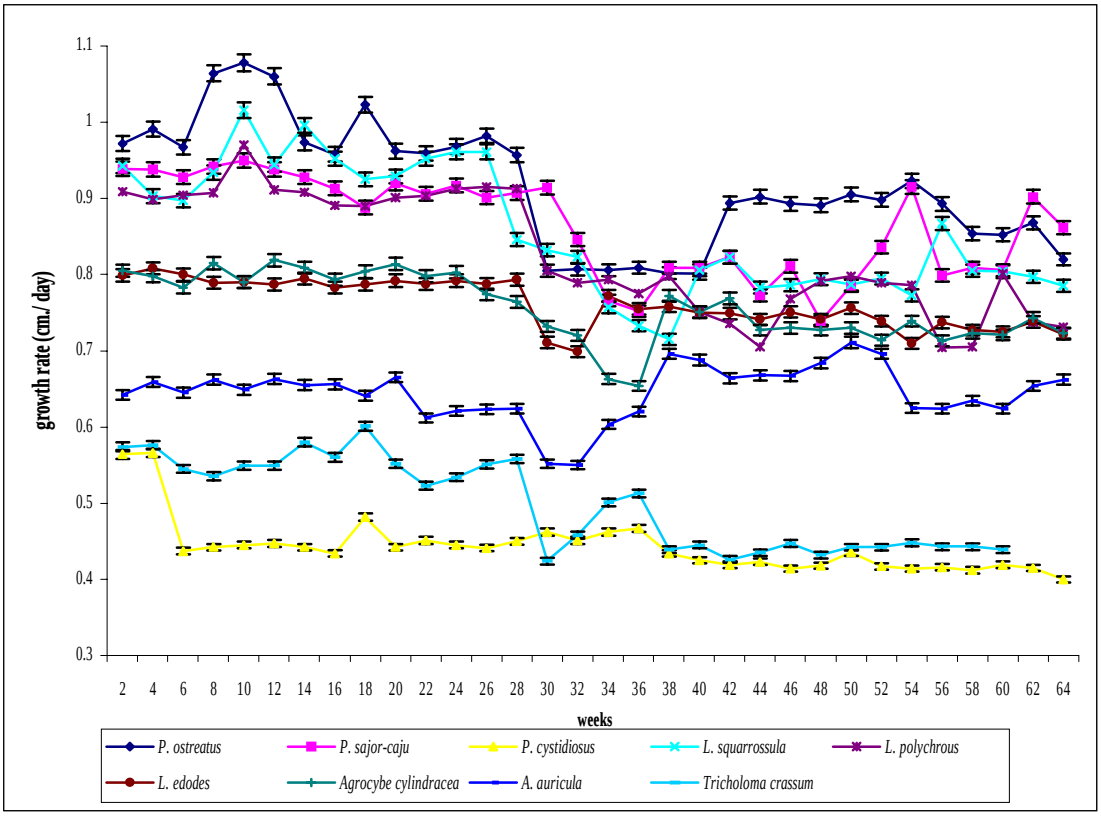


Figure 57. The growth rates of individual of species were stored at -70 ^{0}C

From Figure 57, This result suggested that the most of species performed almost constant growth rate for long period and each species of mushrooms showed that different growth rates. The result showed that several pure cultures were survived with stable growth rate. In addition, there are several preservation method of living fungi such as cold storage, freezing and liquid nitrogen etc. But both the freeze-drying and liquid nitrogen preservation was recommended that genetic stability (Prescott and Kernkemp, 1971) for long-time storage technique. The genetic stability had an important role to quality control of mushroom production. However, the most of preservations were aimed to decreased rate of metabolism on storage for long-time and without contamination.

Chapter IV

Conclusion

The mushroom production has raised attention for farmer because mushroom able to use agriculture wastes as material casing that has abundant material in Thailand. The benefits of agriculture wastes are alter to be adding values for wastes. In addition, the production for large scale has necessary to be adequately quality controlled. Since the quality of mushroom production as reduced yield and defect fruit body after continuous subculturing for production. Therefore, this work was studied for monitoring the genetic instability of mushrooms by using DNA techniques prior to scale up the mushroom cultures. ITS 4 and 5 primers were used to detect different DNA fingerprint on the basis of PCR-RFLP analysis. This approach able to distinguish the variation in ITS-region between 1st subculturing of *A. auricula* and 3rd subculturing. While the other species were not found different DNA pattern by continued serial transfer. Additional, the abnormal DNA fingerprint of third subculture had effect to growth rate in compost and phenotype of fruit body. The defect DNA pattern of *A. auricula* was further investigated by DNA sequencing. It was suggested that DNA sequence was indicated different sequence between the first and third subculture. The recognition site of *Hinf* I restriction enzyme could be detect add one site in the third subculture at 263 bp while the first subculture had only one site at 300 bp and homology was 90%. It could be concluded that ITS4 and 5 primers and *Hinf* I as restriction enzyme able to detect genetic instability of mushroom while other restriction enzymes could not. The genetic variation of mushroom could be occurred when serial transfer with mycelium and reversible genetic as indicated in 4th subculturing. This variation of gene had due to growth rate and phenotype of fruit bodies. The recognition site had corresponding with DNA fingerprint and defective of fruit body was also confirmed by β-tubulin gene. The β-tubulin gene could be detected the defective of fruit body as indicated in *A. auricula* of third subculturing and *P. sajor-caju*. For mushroom production, the most of species had trend to be decreased the yield and growth rate prolonged on

PDA medium with continued serial transfer especially in *L. squarrossula*, *L. polychrous* and *P. sajor-caju*. However, the defective of fruit body could be occurred during in cropping which affected from environment as indicated in *P. sajor- caju.* Thus the quality control in cropping had an important role to phenotype of fruit body.

For *T. crassum* production, the soil was used as substrate and clay plot as container is the best cultivation. The soil gave highest of yield (575 fresh weight (g)/ 1 container)in significant higher ($P<0.01$) with every main plot and sub plot. The covering casing with rice husk, straw and non-covering had no effect to yield. In addition, the most of mushroom production in Thailand by conventional method and lack of adequately quality control and cropping yard management. Therefore, the total of yield and quality were not favorable. This means the DNA techniques could be solved this problem.

In the future research work, studies on genetic instability of mushroom should be emphasized more with other genes because development of fruit body since the primordia until mature fruit body has wide ranges in expression for use in breeding.

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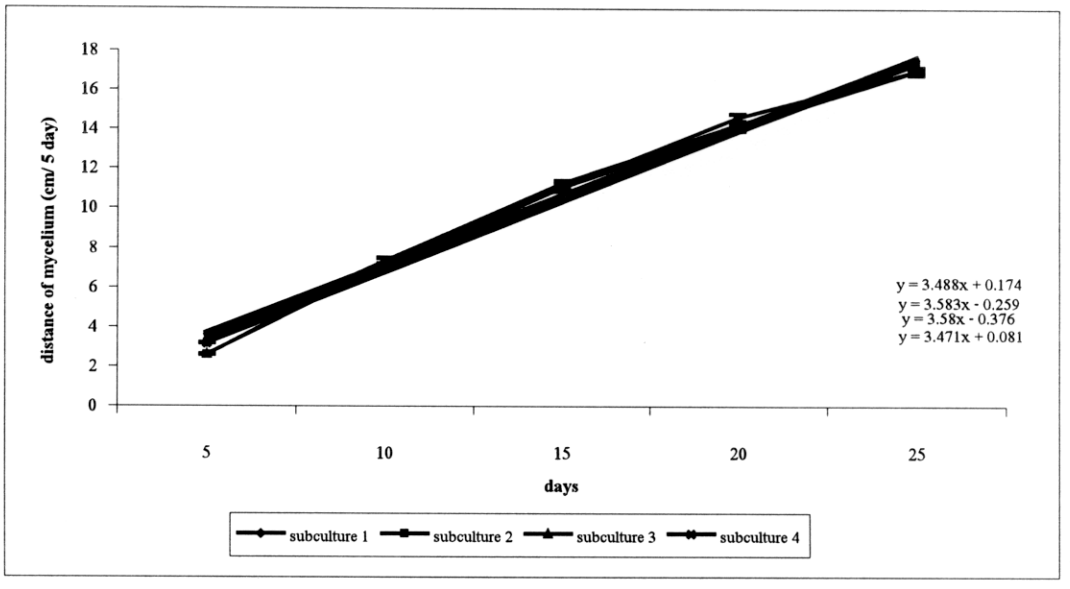
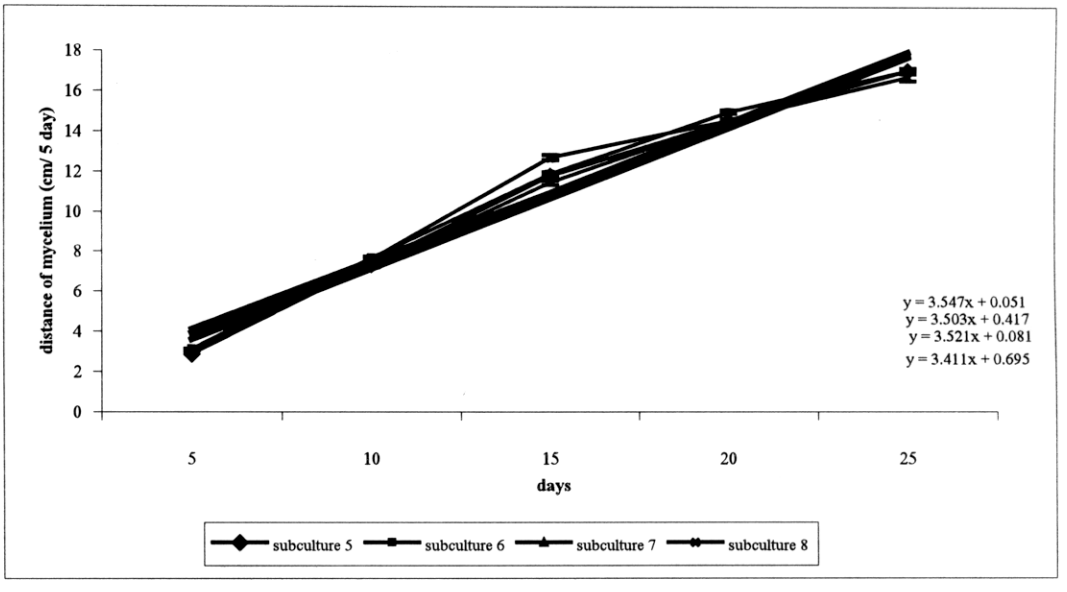
APPENDIX A

Figure 1. The growth rates graph of *L. squarrossula* in compost





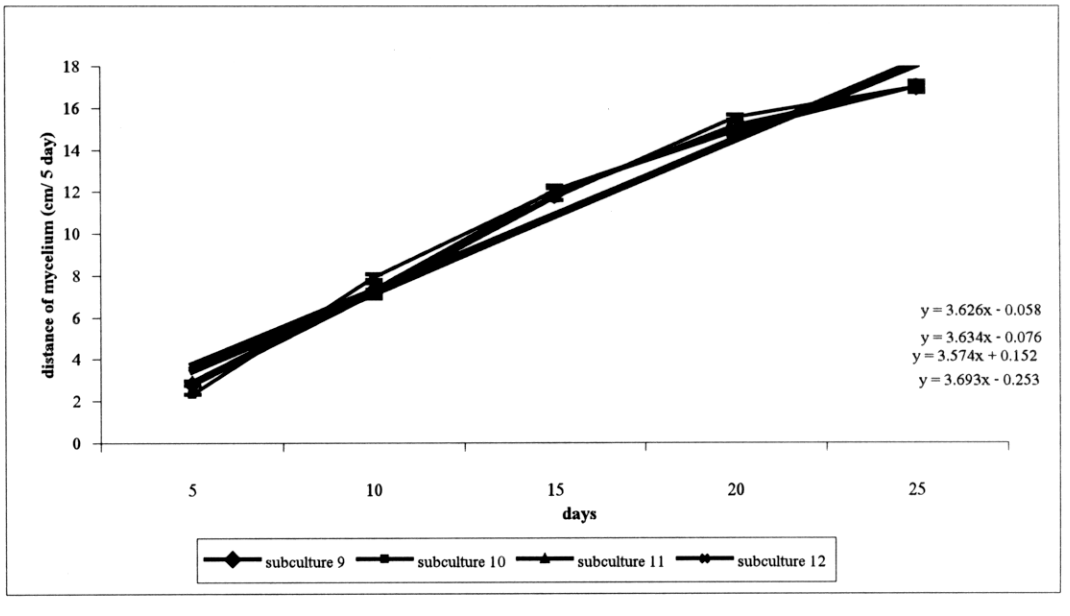
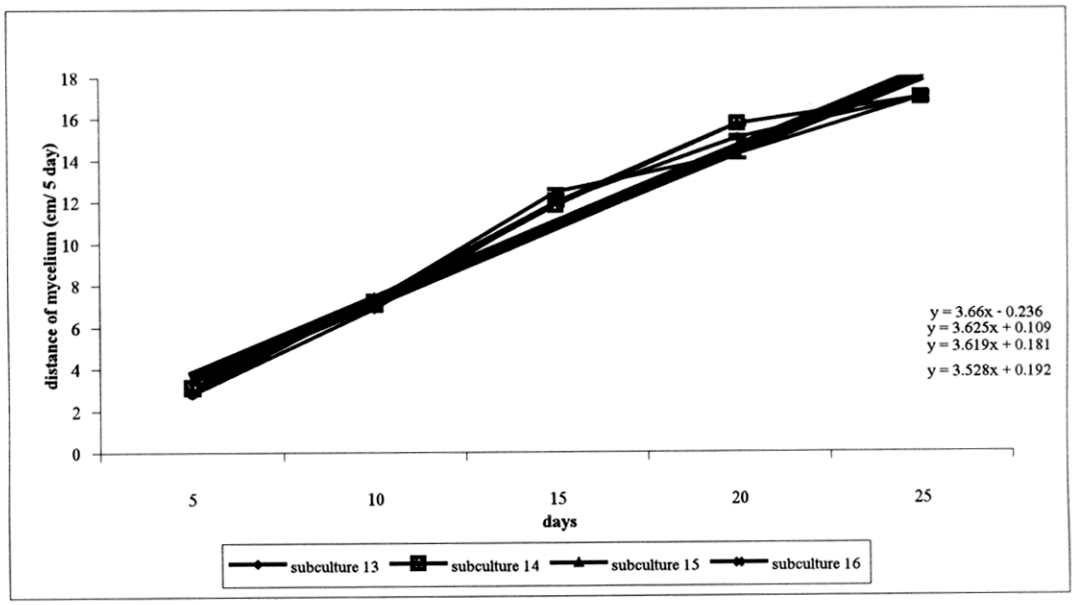


Figure 1. continued



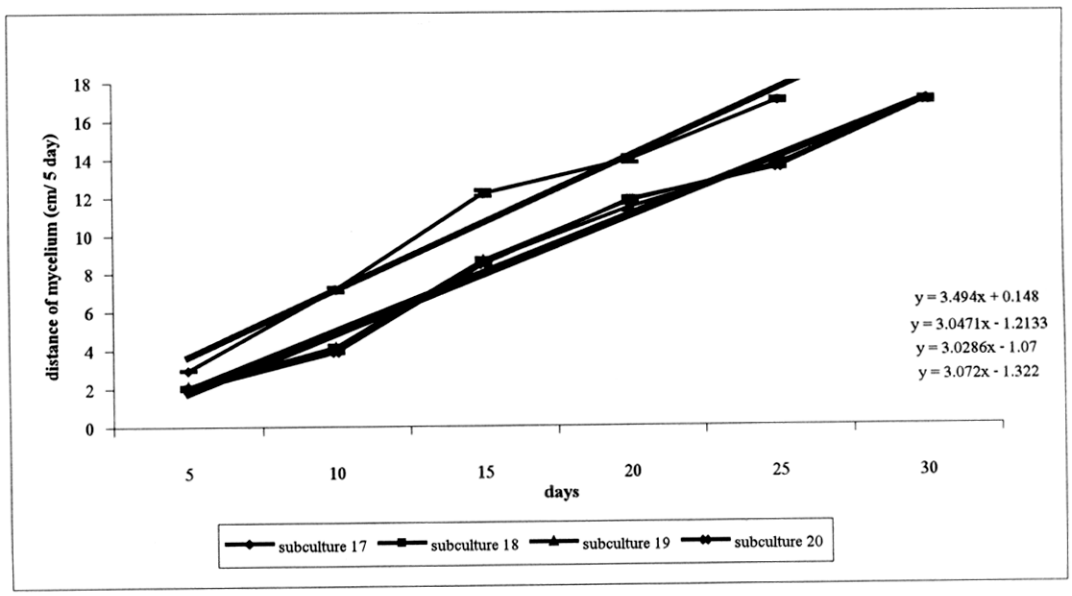
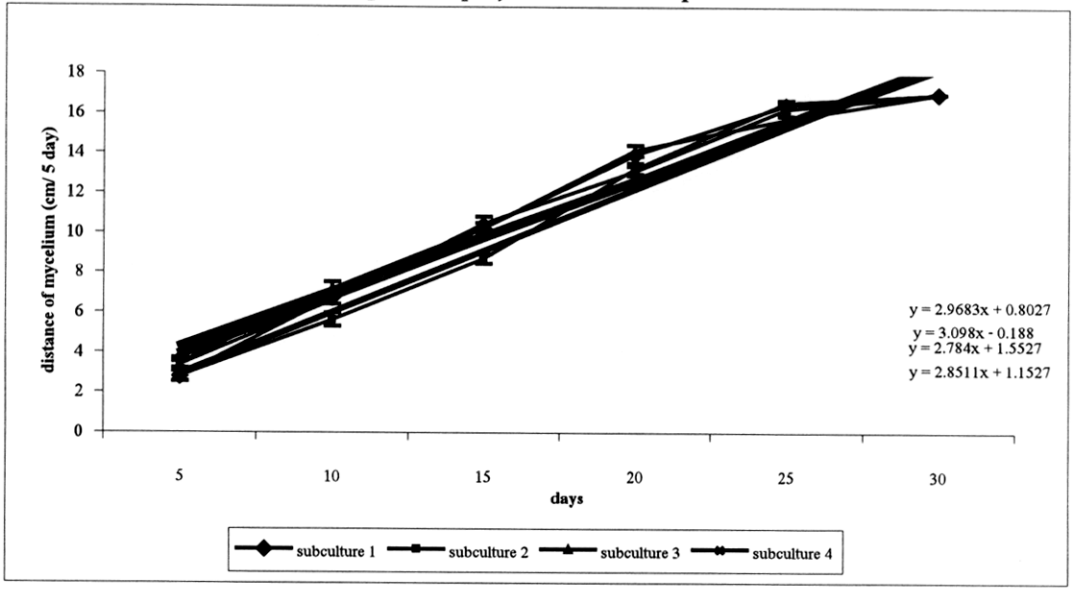
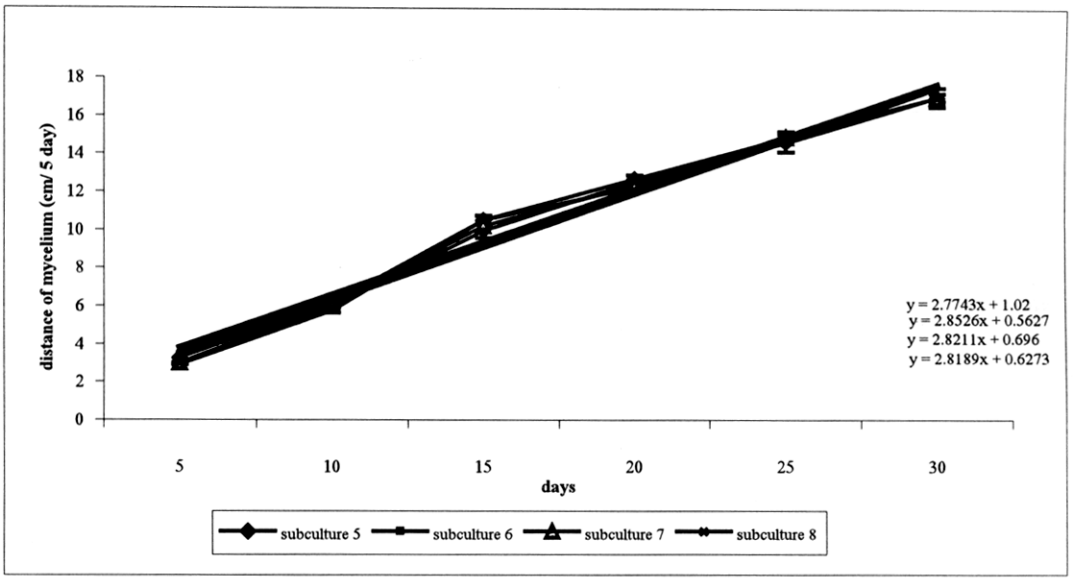


Figure 2. The growth rates graph of *L. polychrous* in compost





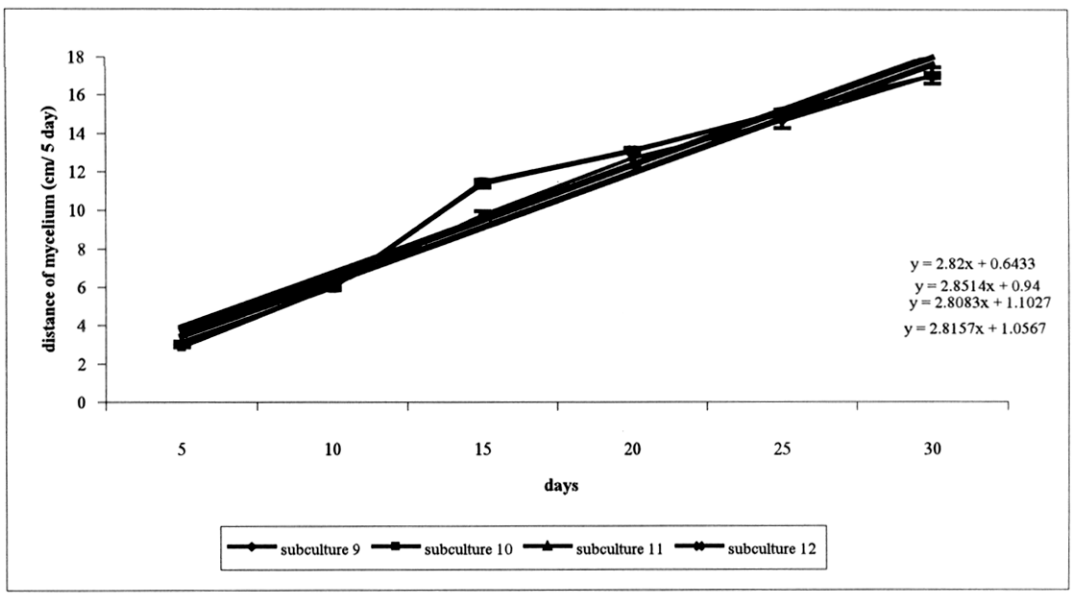
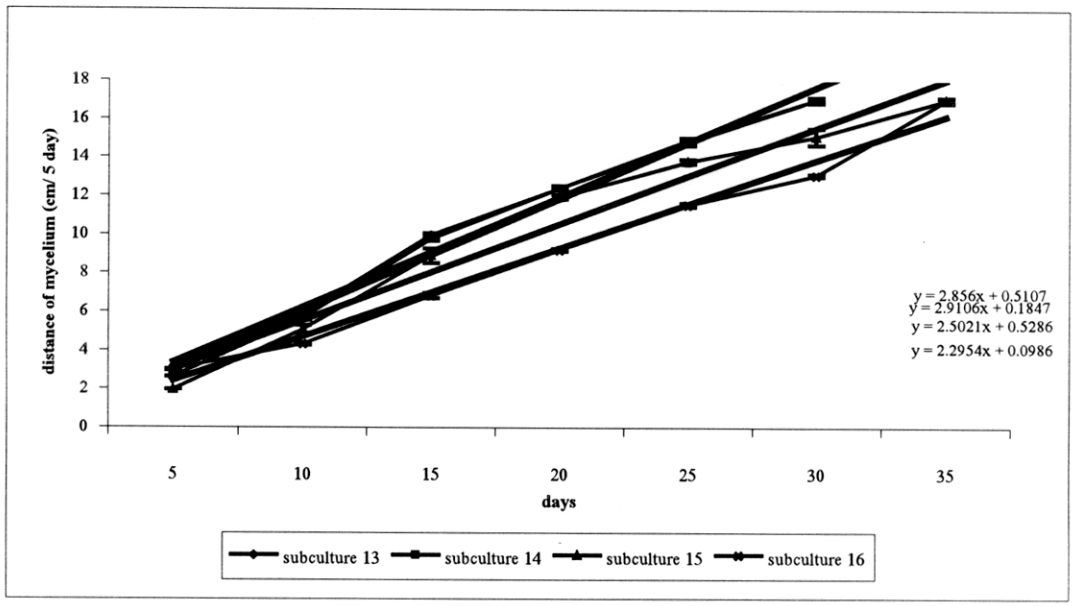


Figure 2. continued



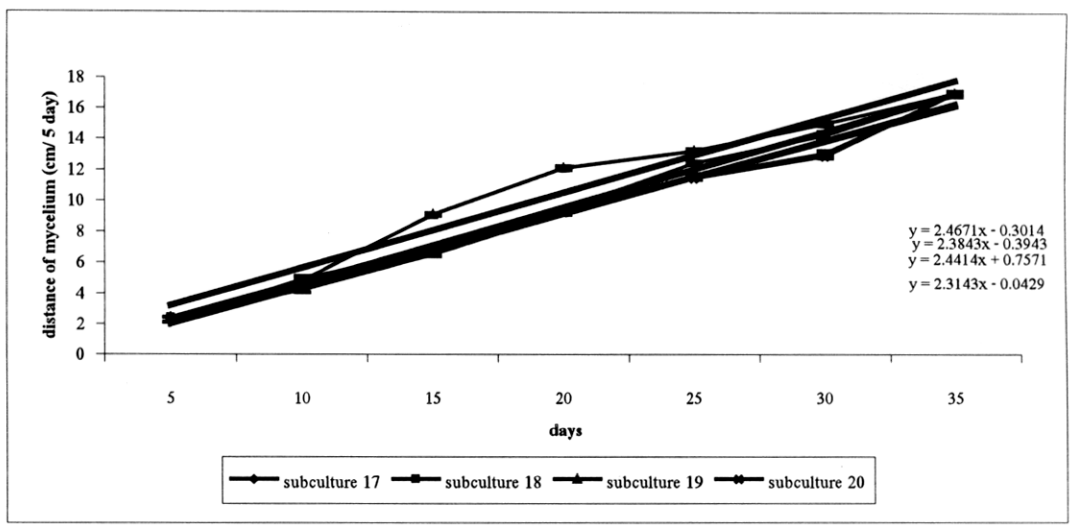
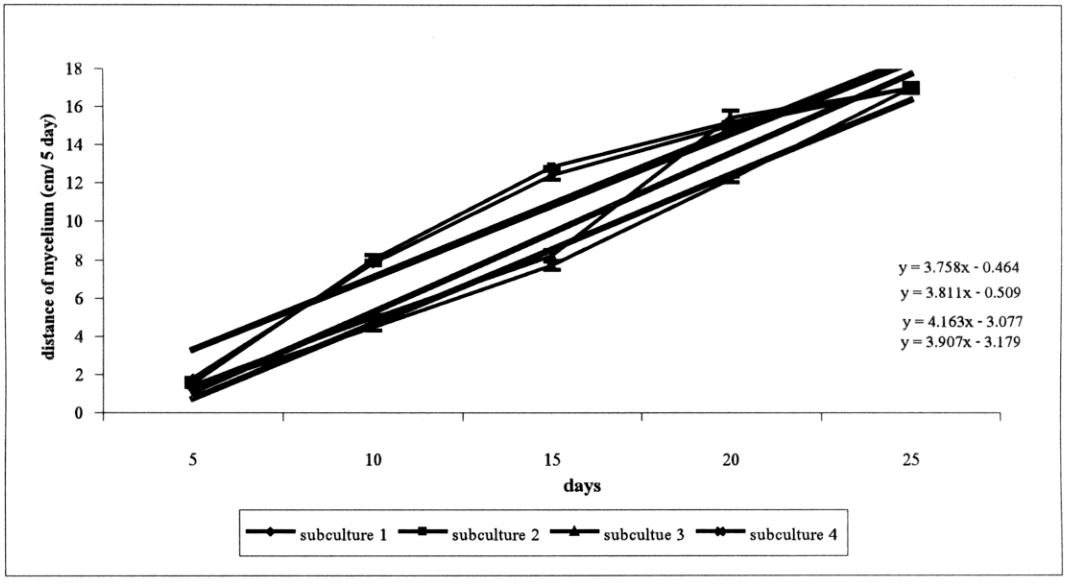
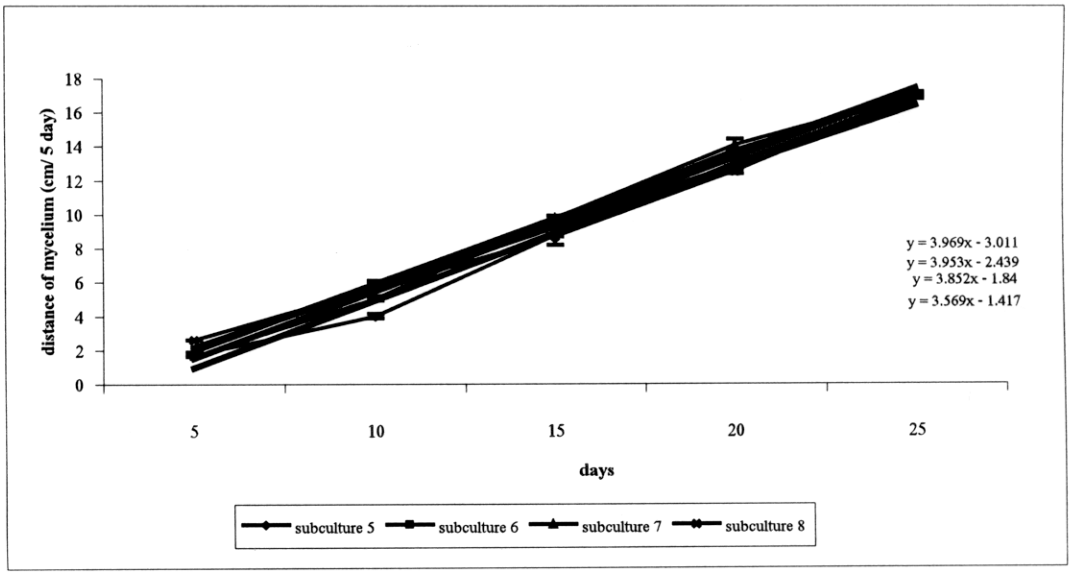


Figure 3. The growth rates graph of *P. sajor-caju* in compost





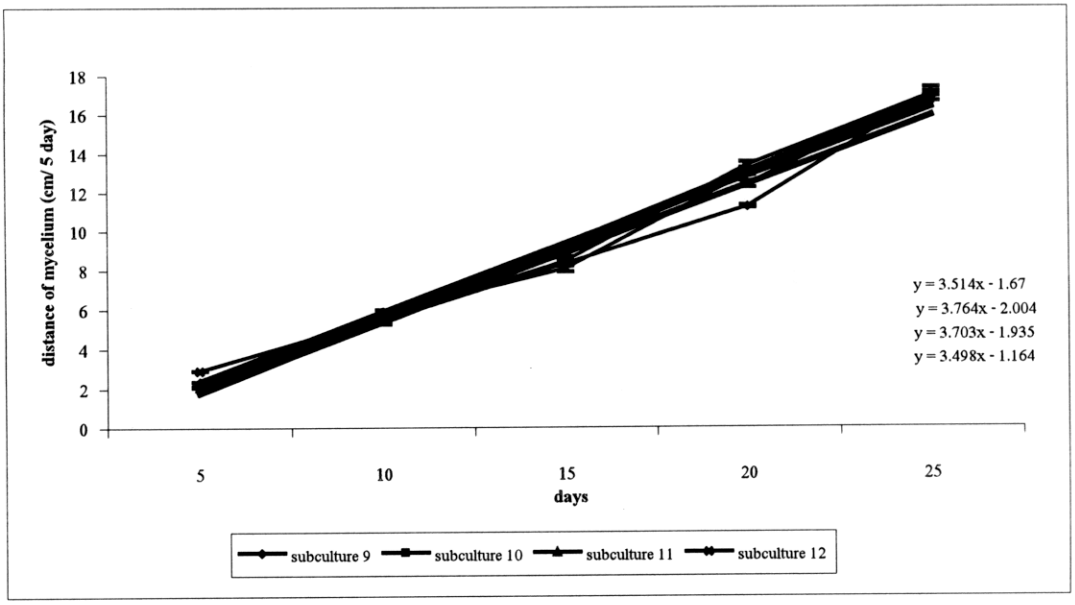
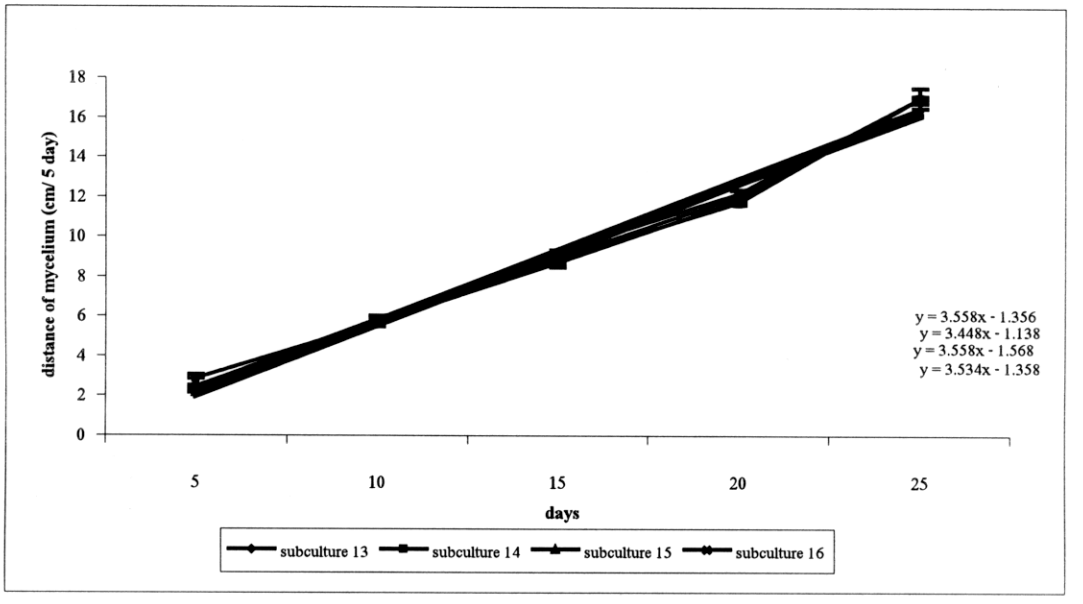
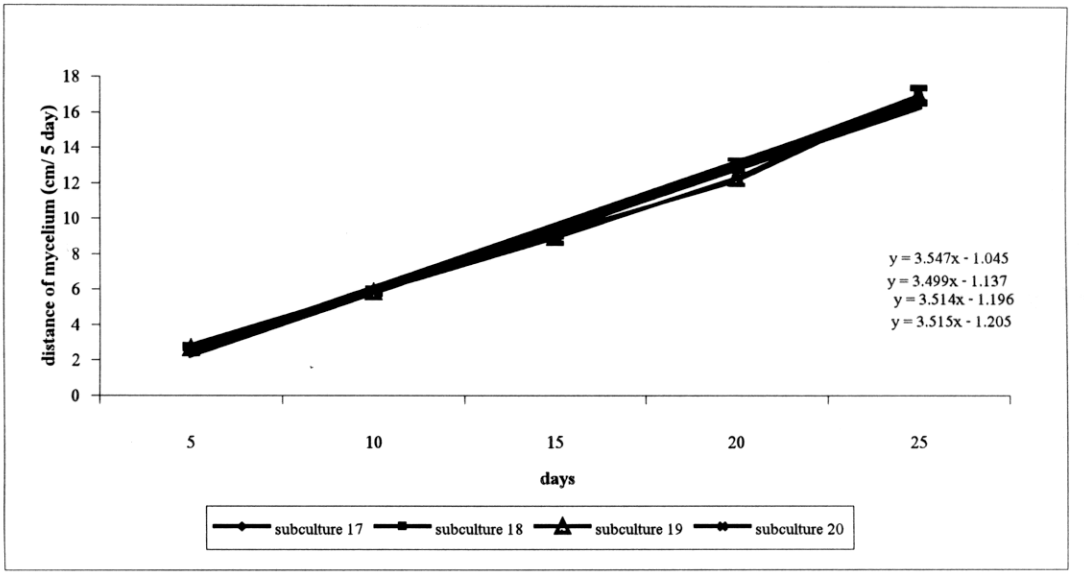


Figure 3. continued





BIBLIOGRAPHY

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