CHAPTER II

LITERATURE REVIEW

Mushroom, a tasty, meaty-flavor fungus, has long been a staple of exquisite cuisines. Besides being an excellent source of proteins, minerals and vitamins, it also has medicinal properties. Mushroom is an important food item, with the import and export amount and economic value within 1995 to 1999 shown in Appendix I-1. The consumption of shiitake mushroom is on the increase (Kirchhoff and Lelly, 1991) because its tasty, fragrant, thick and meaty cap are preferred. Dried shiitake mushroom is one of the most widely used items among asian markets including Thailand where a large quantity of shiitake mushroom was imported.

Lentinus edodes (Berk.) Sing also known as "shiitake," usually grows naturally on hardwood of dead trees, especially the oak family (Fagaceae) that includes such as oak (*Quercus*), beech (*Facus*), chestnut (*Castenea*), hornbean (*Carpinus*) and Chinapin (*Cantanupsis*) (Diehle and Royse, 1991; Hiromoto, 1991). Shiitake mushroom is the world's second most widely cultivated specialty mushroom with Asian countries are the center for its cultivation and consumption (Delpech and Olivier, 1991). The mushroom is now one of the most popular sources of proteins in Japan, and a major staple in China and other parts of the Pacific Rim (Shiitake Center, 1998). As a food source, beneficial effects of *L. edodes* are the combined attributes of being appetizing, nourishing, dietetics and healthful (Shiitake Center, 1998). Moreover, it contains proteins and all eight essential amino acids in greater proportions than soy beans, meat, milk or eggs; rich in B1, B2, B12 and vitamin D, and are also loaded with Calcium, Phosphorus, Iron, Zinc, Sodium and Potassium (Appendix I-2, I-3).

The shiitake mushroom has amazing healing properties (Appendix I-4). The fruiting body and its extracts are used in traditional Chinese medicine for curing

conditions (Kenneth, n.d.) such as cholesterol reduction, colds and flus, general health maintenance, antiviral and anti-tumor (cancer). Moreover, the fiber of this mushroom contains eritadinine that has a cholesterol reducing effect. Researchers have been reporting that consumption of this mushroom could lower blood cholesterol level by as much as 45% (American Health Association, 1987; Suzuki and Oshima, 1976). There has been reports that this mushroom could lower blood pressure in laboratory animals (Shiitake Center, 1988).

Shiitake is also an excellent source of complex carbohydrates called polysaccharides, some of which are believed to be immune enhancer. Because of several potent immunoactive constituents, these substances stimulate the body's own immune cells to mount an attack against disease (Kenneth, n.d.). That property has proven effective in cancer treatment.

Among shiitake extracts, Lentinan is a highly purified fraction (Shiitake Center, 1998; Solaya Suksa-Ard, 1995). It has an ability to cure cancer. Unlike chemotherapy or radiation which attacks cancer cells directly (Shiitake Center, 1998), lentinan stimulates the body's own defense to mount an attack against the cancer. Suzuki and Oshima (1976) also found that a raw shiitake eaten daily for one week lower serum cholesterol by 12%. Concentrated forms of lentinan have been used in cancer treatment, AIDS, diabetes, fibrocystic breast disease and other conditions with impressive results (American Health Association, 1987).

At present, shiitake has become a popular mushroom that is mainly cultivated and exported among countries in Asia especially in Japan (San Antonio, 1981), China, Taiwan and Korea, with the amount reaching 2,000 –3,000 tons/year. Thailand has been cultivating this mushroom at a commercial and industrial scale in the north and northeast. Fresh and dried shiitake mushrooms can bring in approximately 80-200 baht/kg, and 500-1,000 baht/kg, depending on the quality, respectively (Ban Bruranachonabot, 1992). In 1998, Thailand has imported mushrooms of 137.31 tons with an approximate value of 14 million baht.

Local cultivated shiitake mushrooms have a low market share because of its interior quality, insufficient amount, uncertainly of the product availability. Most consumers perfer to consume import products. Shiitake mushrooms can be grown in the north because of a suitable climate, e.g. low temperature and higher humidity. It's essential to create tolerant strain which is suitable to the country environment and has a high yield. Therefore, studies on strain improvement, strain collection and hybrid strain aimed at developing shiitake mushroom varieties for better fruiting body, high yield with all the desirable characteristics are important.

Normally, shiitake mushroom production is dependent on the climate, e.g. high humidity, low temperature with a suitable period of cultivation from August to March in the north of Thailand. The optimal temperature for shiitake mushroom mycelial growth is about 25°C (Delpech and Olivier, 1991), and will stop growing at temperature below 5 °C and above 35 °C, and mycelia will die at 40-45 °C.

Life cycle of shiitake mushroom is divided into 3 stages: spore formation stage, mycelium formation stage, and fruiting body formation stage. Usually, shiitake mushroom was classified on the basis of morphological characteristics of fruiting body. It cannot be classified based on spore formation stage or mycelium stage because some morphological characteristics, e.g. clamp connection, color of mycelia, are similar. The fruiting body formation stage is too short and quick decay, in some case may take a long time in fruiting body development growth (more than 6 months to 1 ½ years). Moreover, if the environment such as temperature, moisture, air, light, nutrients and pH, limited growth area or the condition of cultivating logs are not suitable, it cannot form fruiting bodies. Therefore, it is important to develop techniques for classification of the mushrooms based on mycelia which are, easily obtained and independent on the environment.

In the past decade, molecular markers have become fundamental tools for mycologists (Yu, Deynze and Pauls, 1993); they are useful for studying genetic variation by genetic analysis such as fungal systematic, molecular evolution,

population genetics or plant-fungus interaction (Möller *et al.*, 1992). Genetic analysis has been important in the development of molecular biology, because genetic arguments have often led to testable model of molecular process and molecular interaction (Freifelder, 1935). Several methods for comparison at a molecular level have been developed, including the identification of isozymes, restriction fragment length polymorphism, or polymerase chain reaction (PCR) markers.

Isozymes and restriction fragment length polymorphism (RFLP) provide a source of selectively neutral codominant markers for mapping (Chaparro *et al.*, 1994). Isozyme analysis was the first molecular markers used to reveal genetic variation among isolates of *Lentinus edodes* (Royse *et al.*, 1983 a; Royse *et al.*, 1983 b). Goodwin and Annis (1991) demonstrate that this technique could be used to distinguish fungi pathotypes of *Leptosphaeria maculans,* in combination with certain cultural characteristics. As reliable markers, isozymes have been used to distinguish A and V pathotypes. Changtragoon *et al.* (1996) studied genetic diversity of rattan (*Azadirachta* sp.) by using isozyme analysis. The result showed that rattan in Thailand differred from others. However, isozyme analysis is limited by the techniques (Demeke and Adams, 1994; Yu *et al.*, 1993), specific to growth stage and environment (Somsak Apisitwanich *et al.*, 1995).

Currently, Restriction fragment length polymorphism (RFLP) assay is the preferred method in the genetic diversity studies in many species. RFLP is based on differences in fragment lengths obtained by digesting DNA samples with one or more restriction endonucleases, and separating the resulting fragments according to molecular weight by gel electrophoresis (Dayhoff and Eck, 1968; Yu *et al.*, 1993) Direct DNA polymorphisms are observed through restriction endonuclease digestion, and coupled with DNA blot hybridization (Tingey and del Tufo, 1993). Polymorphisms are due to the presence or absence of restriction sites in the genome being compared (Yu *et al.*, 1993). RFLPs have been used extensively to develop maps (Helentijaris *et al.*, 1986; Landry *et al.*, 1991, cited in Yu *et al.*, 1993), establish linkages to traits (Osborn *et al.*, 1987; Lee *et al.*, 1988, cited in Yu *et al.*, 1993),

develop phylogenetic trees (Song *et al.*, 1988a; Song *et al.*, 1988b, cited in Yu *et al.*, 1993), and tag chromosomes (McGrath *et al.*, 1990, cited in Yu *et al.*, 1993). These markers have the advantages that they are phenotypically neutral (Yu *et al.*, 1993), codominantly and stably inherited in the Mendelian fashion, disclose unlimited polymorphic markers (Demeke and Adams, 1994), are nonspecific to growth stage, and practically limitless in number (Yu *et al.*, 1993). The development and application of RFLP technology in crop improvement were both reviewed by Tanksley *et al.* (1989).

However, problems with detection of RFLP by Southern blot hybridization was laborious, time-consuming and expensive; the procedure moreover required a large amount of DNA (2 to 10 μg) and suitable specific probes, and may involve the use of radioactive reagent (Goodwin and Annis, 1991; Demeke and Adams, 1994; Lin *et al.*, 1996) which all made it unpopular.

With the emerging of new technology, the polymerase chain reaction (PCR) has been reported to facilitate genetic studies in any organisms (Mullis, 1990; Erlich *et al.*, 1991, cited in Demeke and Adams, 1994). This led to the development of several novel genetic assays based on selective DNA amplification (Tingey and del Tufo, 1993). One variation of PCR analysis is the random amplified polymorphic DNA (RAPD) analysis. William *et al.* (1990) were the first to use the procedure on plant samples and suggested it be name RAPD (pronounced 'rapid'). It was used for molecular biology simultaneously for genetic fingerprinting that was termed "arbitrary-primed polymerase chain reaction (AP-PCR)" by Welsh and McClelland (1990). The methods are based on the observation that fragments of the genomic DNA are amplifiable and polymorphic (Chulee Chalsrisook, 1994) which are generated by amplification of genomic DNA template with a single short synthetic primer of the arbitrary oligonucleotide sequences (10-mers) of many sites or randomly chosen DNA sequences and temperature cycling conditions (Demeke *et al.*, 1992; Yu *et al.*,1993; Chulee Chalsrisook, 1994; Hiltan, Banks and Renn, 1997; Yu and Lin, 1997).

The arbitrary primers used for the procedure are usually up to 10 bp in size: they have a GC content of 50 to 80% and do not contain palindromic sequences. The number of DNA fragments that are amplified depends on the primer and the genomic DNA used (Yu *et al.*, 1993). RAPD are usually dominant markers and are inherited in a simple Mendelian fashion (Demeke and Adams, 1994).

In comparison with RFLP, the major advantages of RAPD analysis are that (1) the procedure is inexpensive, (2) more rapidly since a large number of samples can be processed simultaneously in a short period of time and no southern transfersare needed, (3) only requires a small quantity (ng) of DNA, (4) the procedure is relatively easy to perform, (5) this technique requires neither prior knowledge of DNA sequence information nor specific DNA probes, (6) does not involve the use of radioactive in the assay, and (7) a universal set of primers can be used for all species using genetic markers (Welsh and McClelland, 1990; Wolff *et al.*, 1993; Chaparro *et al.*, 1994; Demeke and Adams, 1994; Fernández and Hamlin, 1996; Hansen and Winding, 1997; Yu and Lin, 1997).

Williams *et al.* (1990) used the simplest system of visualizing fingerprints using agarose in developing the technique that had become the most widely used system. However, if a greater number of bands in a fingerprint is required for a more rigorous identification, the method of Welsh and McClelland using polyacrylamide gel and ethidium bromide staining is a consistently used alternative.

Caetano-Anolles *et al.* (1991) demonstrated a fingerprinting system called DNA amplification fingerprinting (DAF). This technique uses a single random primer between 5 and 21 nucleotides in length in a single stage PCR then separate the amplified products through the polyacrylamide gel and visualize the products using silver stain. The fingerprints generated usually contain more than 10 bands.

Goodwin and Annis (1991) used RAPD markers to screen different strains of *Leptoshaeria maculans*, which is the causal agent of blackleg disease in crucifers.

Differences between avirulent and virulent pathotypes, could be distinguished by calculating similarity coefficients from the collection of RAPD markers. The results of the RAPD analysis agreed with previous classifications based on RFLPs and cultural assays; they also resolved a question of the ancestor of the fungus (*Leptosphaeria*). The major advantage of this technique is its sensitivity and short time requirement.

Kush, Becker and Mark (1992) used RAPD markers to identify distinct genotypes among heterokaryotic strains of *Agaricus bisporus*. These results demonstrated that RAPD markers provided an efficient alternative strain fingerprinting and a versatile tool for genetic studies and manipulation of *A. bisporus*.

Yu and Pauls (1992) studied the optimized PCR program used in RAPD analysis of alfalfa leaflets. The result suggested that in a systematic examination of the effects of the length of each step and the number of cycles on RAPD patterns, the time for the PCR program can be reduced to 2.5 hours without changing the RAPD patterns, and resulted in sharper banding patterns for most of the primers than the original program. There is considerable opportunity for increasing the efficiency of the PCR program for RAPD analysis.

Wilkie *et al.* (1993) used 20 random primers (10-mers) to analyze *Allium* species (and cultivars). The results were in general agreement with classical classification. However, *A. roylei* was shown to be the closest relative of *A. cepal*, contradictory to results from previous work.

Yu and Nguyen (1994) used RAPD method to study DNA polymorphisms and genetic diversity cultivars of rice (*Oryza sativa* L.) that differ in drought-resistance characteristics. The results of PCR products indicated that 80% were polymorphic. In genaral, fewer polymorphisms were found between upland and lowland cultivars within the *indica* subspecies. This study indicated that RAPD analysis is a useful tool in determining the genetic relationship among rice cultivars.

Somsak Apisitwanich *et al.* (1995) studied 16 accessions of rice including 5 species of the genus *Oryza* that were screened with random primer by using RAPD assay to examine the evolution/pedigree relationship among wild species and cultivar.

Mileham (1997) used different primers based on RAPD and standard conditions, and allowed characteristic DNA fingerprints to be generated from any microorganism even in the absence of information on its DNA sequence. Different primers can be used to produce genus-specific, species-specific, or even strain-specific DNA fingerprints. This offers a substantial time saving advantage as the DNA region need not be identified, cloned, and its sequence determined before it can be used as a DNA marker.

Yu and Lin (1997) applied RAPD for analysis of phylogenetic relationships among species, to examine interspecific and intraspecific variation among *Nicotinia* sp. and determined the taxonomic position of *N. sylvestris* is a member of section *Alatae*.

These studies have shown that RAPD is a powerful method for genotype identification, population and pedigree analysis, phylogenetic studies, genetic mapping and determination of genetic purity of hybrid (Hashizume, 1993, cited in Somsak Apisitwanich, 1995; Vaccino *et al.*, 1993). In the past years, most research examined shiitake (*Lentinus edodes*) to determine genetic relationships among strains or isolates and their potential application in mushroom breeding and genetics. Fukuda *et al.* (1994) used mitochondrial DNA RFLPs on the study of wild strains of *L. edodes* to examine genetic relationships among different geographical populations. Nicolson *et al.* (1997) studied RFLP data from PCR amplified regions of the rDNA repeats from *Lentinus* sp. in a phylogenetic analysis. Although a few studies on RAPD analysis of mushrooms had been done, there has been no report on the use of RAPD technique in the analysis of *L. edodes*.

In this study, one of the RAPD analyses, DAF, was used to detect the DNA distinction of shiitake mushroom isolates of *L. edodes*. The main advantages of the DAF analysis are its sensitivity, simplicity and rapid reduction of all laborious steps and accessibility for simultaneous processing of many samples. When this method was combined with the modified rapid DNA preparation, the results could be obtained within 3-4 days.