TOWARDS A MOLECULAR APPROACH FOR THE IDENTIFICATION OF FUNGAL TAXA THAT CONTAIN PSILOCYBIN

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BY

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ABSTRACT

The classical taxonomic methodologies for determining taxa of mushrooms are based primarily on morphological traits. If the mushroom specimens are dried or pulverized, the morphological characteristics are frequently disguised or no longer apparent. Included among these fungi are known to produce psilocybin, which are hard to identify by their macroscopic features. Molecular techniques could help to overcome these problems. In this study, twenty samples of two genera, *Psilocybe* and *Panaeolus*, of fungi from genera known to produce psilocybin were analyzed. The profiles of random amplified polymorphic DNA (RAPD) produced by two primer sets were analyzed. Fingerprints of amplified fragment length polymorphism (AFLP) produced by five selective primer pairs were also analyzed. By the analysis of genetic similarity, RAPD and AFLP method were capable of providing genus and species information. AFLP band patterns can provide reliable species test and can be separated in a simple way using PAGE electrophoresis and stained by silver nitrate. The sequencing data of twenty psilocybin-producing mushrooms were established by fluorescent sequencing method on the nuclear small subunit ribosomal DNA (nuc-ssu-rDNA) and the internal transcribed spacer 1 (ITS-1) DNA regions. The sequences of 877 bp DNA fragment of nuc-ssu-rDNA exhibited genus specific DNA sequences. The size (307-344 bp) and sequences variation of ITS-1 showed not only genus-specific but also species-specific DNA motifs. In the results of RNA gene analysis, the internal transcribed spacer DNA has much faster evolving sequences than the small subunit rDNA. A simple method was developed to perform the genus identification using a primer that is specific to either members of the *Psilocybe* or *Panaeolus* genus. A second primer was used to amplify a common product. This multiplex PCR was successfully developed for the quick screening of a large number of samples for identification of the genus. The SSCP

pattern of the common product is sufficient to reveal the species that was present. If dye-labeled primer was used, the accurate size of common product is also a valuable evidence of species. If an automatic sequencer is available in the lab, the best method of species and genus identification is sequencing the RNA gene. The multiallele DNA fragments of ITS-1 DNA in *Panaeolus subalteatus* and *Psilocybe semilanceata* gave strong evidence in species identification. By the phylogenetic analysis of sequence in nuclear small subunit rDNA and the variable internal transcribed spacer 1, even the other species (not included in this study) of the same genus can be easily determined their relationship to the fungal genera known to produce psilocybin. This study developed DNA profiling methods for the identification of members of the genera *Psilocybe* and *Panaeolus*, which can provide information not only in taxon determination but also in forensic identification.

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LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
bp	base pair
CTAB	cetyltrimethylammonium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetate
ETS	external transcribed spacer
GCG	Genetics Computer Group
GS	genetic similarity
IGS	intergenic spacer
ITS	internal transcribed spacer sequences
LSD	lysergic acid diethylamine
MAO	monoamine oxidase
NTS	non-transcribed spacer
nuc-ssu-rDNA	nuclear small subunit ribosomal DNA
OD	optical density
PCR	polymerase chain reaction
RFLP	restriction fragments length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscope
SDS	sodium dodecyl sulphate
SSCP	single-strand conformation polymorphism
TE buffer	Tris ethylenediaminetetraacetate buffer

TBE buffer	Tris borate ethylenediaminetetraacetate buffer
tRNA	transfer ribonucleic acid
UPGMA	unweighed pair group method algorithm

1 INTRODUCTION

1.1 Introduction

Drug trafficking is a major international problem, which requires considerable investment by Customs and Excise, and other law enforcement agencies. When a package containing a sample suspected of being a controlled substance is seized, either as small quantities or as bulk supplies, it is important to identify the seizure as being a Controlled Substance. Secondly, when a number of similar samples are discovered it is useful to link them to larger batches to assist in identifying the supply routes. Information on distribution and supply routes for such drugs can lead to their interception at comparatively few sites in the country of origin rather than at the numerous street outlets. This is considered to be more effective law enforcement.

Under UK Legislation, specially the Misuse of Drugs Act 1971 and the Misuse of Drugs Regulations 1985, the compounds psilocybin and psilocin are classified as controlled substances. Within the legislation these two compounds are classified in the same group as marijuana and lyseric acid diethylamine (LSD). This is due to the hallucinogenic effects of psilocybin without an established medical use. This compound is easily obtained from fungi that produce psilocybin, which grow throughout the world. There are numerous ways of preparing psilocybin from the fungi for consumption. The problems in identifying substances that contain psilocybin and the widespread use of this substance is a cause for concern to Drug Enforcement Agencies. Establishing a method for identifying fungi species that produce the controlled substances will be of benefit.

In forensic identification, the specimens of the psilocybin-containing mushrooms are seldom fresh. They are frequently dried, powdered or mixed with other

edible substances. This study will establish the systematic approaches of psilocybin mushroom identification by DNA techniques.

1.2 Historical Background

In 1957, R. Gordon Wasson published a paper [1] on the use and effect of the magic mushroom, *Psilocybe spp.*. The words magic mushroom became a household term and these fungi were adopted as a symbol of the counterculture of the 1960s. It was widely used in the United States during the 1960s and 1970s and became popular in the United Kingdom and elsewhere about a decade later. No other group of mushrooms reflects society, the culture, and its values as clearly as those producing hallucinations.

Although the popularity of ingesting hallucinogenic mushrooms has waned, it has by no means disappeared. A recent Danish study confirmed that about 7% of Danish students have experience with this group of mushroom [2]. In 1996, honey with *Psilocybe* mushrooms in jars were confiscated at the Dutch-German border [3]. The current incidence of recreational use remains an underground secret.

The term applied to individuals who have ingested these mushrooms depends on one's vantage point: To physicians, they are patients; to law enforcement officers, they are illegal drug users; to parents, they are misguided youth; to toxicologists, they are victims; to scientists they are subjects; to individuals, they are just ordinary people seeking a good time through a perfectly reasonable and harmless pastime [4].

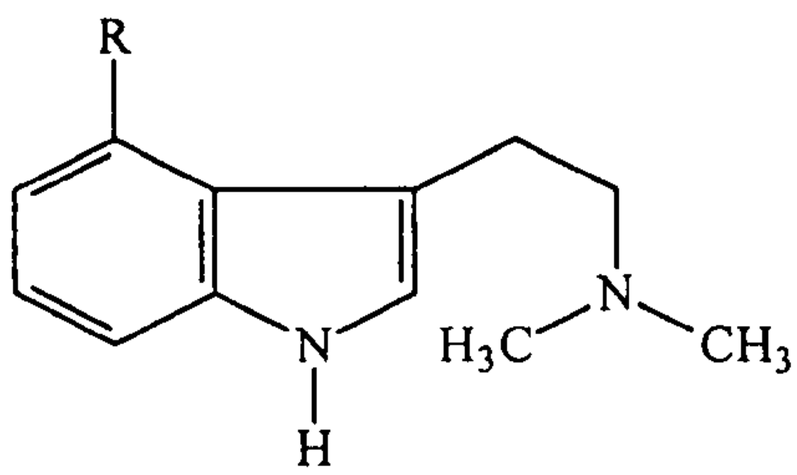
1.3 Toxicology and Pharmacology of Hallucinogenic Mushrooms

Psilocybin and its associated compound psilocin were first isolated from

Psilocybe mexicana. The structure of psilocybin (4-phosphoryloxy-*N,N*-dimethyltryptamine), is shown in Figure 1-1, with the dephosphorylated compound psilocin. Colorless psilocybin can be rapidly oxidized to blue products [5], one of its characteristic features for field identification. Psilocybin is rapidly dephosphorylated to its hydroxyl, psilocin, by alkaline phosphatase, an enzyme present both in the brush border of the intestinal mucosa and in the liver [6].

The rapid degradation of psilocybin suggests that the compound circulating in the bloodstream is primarily psilocin. The usual tryptamine derivatives absorbed from the gastrointestinal tract, such as serotonin, do not normally cross the blood-brain barrier. Psilocin, however, does cross into the brain, probably because of its high lipid solubility as it is unaffected by the enzyme monoamine oxidase (MAO), which is normally responsible for the metabolism and degradation of the biogenic amines.

The pharmacological effects of psilocybin and psilocin are very similar. With a lower dose as little as 4mg, psilocybin will cause a general sense of relaxation and detachment from the environment. As the dose increases to 6-12mg, perceptual changes develop, with alterations in both space and time sense, and the visual effects become prominent. Only when the dose is much higher do patients start experiencing true hallucinations, which frequently involve distortions of space, time and colour perception [6]. Early in the investigation of these effects, it became evident that they resembled those produced by other hallucinogenic drugs also being investigated at the time, most notably, lysergic acid diethylamine (LSD). However, the precise neural mechanism for the effects of the hallucinogenic mushrooms has not been completely defined.



1 R = Phosphate Psilocybin
2 R = OH Psilocin

Figure 1-1 The structures of psilocybin and psilocin [4]

1.4 Legal Status of the Psilocybin-Containing Mushrooms

At the present time, in many countries, any mushroom and preparation containing psilocybin or psilocin is classified under the same status as marijuana and LSD. This classification makes it illegal for both sale and possession without a specific permit from the authority of government. These compounds are classified as Controlled Substances by many countries because: there are no established medical uses for them; the fear of abuse; and there is no consensus from the medical community for their safe or effective use.

1.5 Fungal Species Containing Hallucinogenic Compounds

The hallucinogenic fungi are part of the group of fungi Basidiomycotina, which includes among other species the cultivated mushroom (*Agaricus* spp.). Also present in the Basidiomycotina is the death cap (*Amanita phalloides*), an organism responsible for fatalities in the UK (Watling JFSS 1983 23:53-66). The Basidiomycotina is therefore a

high ranging group of fungi. With one exception (*Schizophyllum commune*) the hallucinogenic fungi all belong to a single group within the Basidiomycotina, the agarics (Agaricales). Member of the agarics all share the familiar structure of a stem (or stipe) upon which is the cap (or pileus) on the underside of which are the gills (or lamellae). The spores, responsible for reproduction, are produced by cells within the lamellae.

Within the agarics the majority of hallucinogenic fungi occur in two genera, *Psilocybe* and *Panaeolus*. Table 1-1 shows part of the hallucinogenic fungi used in this study [4]. The name 'Psilocybe' is Greek and means bald head, which refers to the smooth surface texture of the cap. The genus *Psilocybe* has such close affinities to *Stropharia* and *Hypholoma* that separation of these genera continues to present unique taxonomic difficulties. These genera are clustered within the family *Strophariaceae*, which also includes the more distantly related genus *Pholiot*a. The genus *Panaeolus* belongs to the family *Coprinaceae*. Most psilocybin-producing species of this family are in the genus *Panaeolus*. With the exception of *Psilocybe* and *Panaeolus*, the tryptamine derived compounds like psilocybin and psilocin are widespread throughout many genera of mushrooms. Numerous reports have shown psilocybin and psilocin in *Conocybe*, *Gymnopilus*, *Inocybe*, *Lycoperdon* and *Pluteus* [7]. As the biochemical research continues, many more species are likely to be discovered as active.

Table 1-1 Fungi containing tryptamine derivatives

Psilocybe species			
P. argentipes	*P. mexicana*	*P. baeocystis*	*P. semilanceata*
P. bonetii	*P. quebecensis*	*P. caerulescens*	*P. serbica*
P. caerulipes	*P. sempervira*	*P. coprinifacies*	*P. stuntzii*
P. cambodginiensis	*P. strictipes*	*P. cyanescens*	*P. zapatecorum*
P. cubensis	*P. subaeruginosa*	*P. fimetaria*	
P. cyanofibrillosus	*P. aztecorum*	*P. pelliculosa*	

Panaeolus species			
P. africanus	*P. microsporus*	*P. ater*	*P. papillionaceus*
P. cambodginiensis	*P. retrugis*	*P. castaneifolius*	*P. sphinctrinus*
P. fimcola	*P. subalteatus*	*P. foenisecii*	*P. tropicalis*
Tryptamine derivatives have not been found in all the collections of *Panaelus* examed.			
Conocybe species			
C. cyanopus	*C. smithii*		
Inocybe species			
I. coelestium	*I. corydalina*	*I. haemacta*	*I. aeruginascens*
Gymnopilus species			
G. spectabilis			
Lycoperdon species			
L. mixtecorum	*L. marginatum*		
Pluteus genus			
P. salicinus	*P. nigroviridus*		

The range of species was chosen due to their widespread distribution in the UK and also due to their availability. Some of the species chosen are commonly found, such as *Psilocybe semilanceata*, and others due to their forensic importance. The species *Psilocybe cubensis* is part of a kit available for the cultivation of the fungi.

1.6 Distribution of Fungi Containing Hallucinogenic Compounds

Psilocybin-producing fungi grow throughout the world, and can be found in both fields and forests. Members of the hallucinogenic genera are primarily saprophytic fungi. Some, like *Psilocybe semilanceata* [4], have a penchant for the rich organic

material of dung or manure. More tend to grow in lawns, pastures, gardens, and parks. They can be found along roadsides, along the edge of forested areas, and in open wood.

1.7 Identification of Hallucinogenic Mushrooms Using Macroscopic Features

Identification of hallucinogenic mushrooms is difficult unless a trained mycologist. Microscopic examination and comparison with known species can be a tedious process. However, there are methods for determining whether or not a candidate is part of the psilocybin-containing group. The Stametsian rule [7] for targeting psilocybin mushrooms provides a quick cluster method.

If a gilled mushroom has purplish brown to black spores and the flesh bruises bluish, the mushroom in question is very likely a psilocybin-producing species.

If the unknown sample matches these two conditions, it is highly probable candidate. Once targeted a psilocybin mushroom, it is necessary to compare the mushroom in hand with the species descriptions listed in the reference book.

1.7.1 The Spore Print

Spore printing is an easy and fairly definitive means for separating groups of mushrooms from one another. *Psilocybe* and *Panaeolus*, genera that include the preponderance of psilocybin species, have spore deposits generally purplish brown to black in colour. *Conocybe* and *Galerina* have rusty brown spores. *Inocybe* is yellowish

brown to clay brown to dull brown [7]. The technique for spore printing is very simple and is best done within the first few hours from the time the mushroom has been picked. As the mushroom dries, its production of spores declines, making printing more difficult.

1.7.2 The Bluing Reaction

Many *Psilocybe* and *Panaeolus* species will turn bluish or bluish green when they are bruised. This happens either as a normal response to growing conditions or while they are handled as they are picked. The blue pigmentation is a result of a phenomenon paralleling the degradation of unstable psilocin (dephosphorylated psilocybin) to presently unknown compounds by enzymes within the mushroom cells. However, the bluing feature has limited importance from the taxonomist's point of view when it comes to identifying a mushroom. For instance, many *Psilocybe* and *Panaeolus* species will not blue no matter how much you abuse them, and there are several poisonous and suspect species outside these two genera that exhibit near bluing, although no psilocybin or psilocin is present [7].

1.8 Methods of Consuming the Hallucinogenic Mushrooms

The methods for consuming the mushroom or its extracts are numerous [4]. Some people use the mushrooms fresh; others dry or freeze them for later use. Mushrooms have been powdered and put into capsules for sale and ease of administration. Milkshakes, tea, soup, stews and omelets are but a sample of the ways in which the drugs are ingested. Some methods attempt to alleviate the rather acrid taste of the hallucinogenic mushrooms. Packed in honey, they preserve their potency quite

well. Probably the least noxious way to eat them is to serve them with chocolate.

1.9 Identification Problems in Hallucinogenic Mushrooms

Identification of hallucinogenic mushrooms is difficult unless performed by a trained mycologist. In drug trafficking instances, identification work is more difficult because of the variety of specimen. The macroscopic features usually can not be recovered. Without these features, these fungi are easily disguised and remain unidentified. For law enforcement agencies, the question is only whether the mushroom is belongs to hallucinogenic mushrooms. This is an easy question to answer, because there are many methods available to solve this question. There are methods to identify the active components of hallucinogenic mushrooms, psilocybin and psilocin. These methods include Thin-layer Chromatography [8], High-Performance Liquid Chromatography [8,9,10], and Gas Liquid Chromatography-Mass Spectroscopy [11], which have been reported effective. However, if the questions are concerned about the species and the origin place of hallucinogenic mushrooms, then a rapid test that can readily identify the presence of either *Psilocybe* or *Panaeolus*, and could further provide a method to link such samples as being from the same geographical region, would have many advantages over current conventional testing methods.

In this study, I will use all the possible methods of molecular biology to find out the possible differences in the genetic materials of hallucinogenic mushrooms. These methods can possibly demonstrate that the species identification of hallucinogenic mushrooms in DNA level is reliable. At the end of this study, I will incorporate these methods into a systematic approach for a fast, economic and reliable test method in the routine work.

2 DNA METHODS IN SPECIES IDENTIFICATION

2.1 Introduction

Every animal and sexually reproducing plant has a characteristic physical appearance, or phenotype, due to their composition of genes, or genotype, each inherits. The exception to this rule is genetically identical (or monozygotic) twins, who possess the same unique genotype but, owing to the consequences of complex environmental events, have subtly different phenotypes. The DNA in each nucleated cells of any individual is identical. These principles of individual uniqueness and identical DNA structure within all tissues of the same body provide the basis for DNA profiling. The psilocybin-producing mushrooms, which are classified in the basidomycota of the fungi kingdom, mostly reproduce by sexual reproduction. Every individual possesses also a unique genetic composition. These genetic materials encode all the growth information, which produce individual and species characteristics. Although there is still no DNA analysis reported on the identification of psilocybin-producing mushroom, there are many papers covered on the species identification of microorganisms, fungi and plants. This chapter will review the major DNA methods on species identification.

2.2 DNA Sequencing

Nucleotide sequence of the genome encodes the original information of genetic variation. The information contained within the DNA offers the best method of determining the individual and class characteristics, and also the evolution history of any organism. Since the size of the genome is too great to sequence in a limited time, the approach usually taken is to choose appropriate regions of the genome which are

known to change over evolutionary time periods or are highly conserved. DNA sequencing provides highly reproducible and informative data for a defined region of the genome and can be easily performed. Such DNA sequence data can then be compared between different related organisms to determine the rate of change of the DNA sequence. In recent years, the sequencing approach has been quickly facilitated by the advent of the polymerase chain reaction (PCR) [12,13], making it possible to sequence the DNA region of interest in a short time. Primers are designed on the basis of sequence information for conserved parts of the DNA, and the desired target sequences are amplified. The PCR products can then be sequenced either directly or after cloning.

Since ribosomal DNA (rDNA) sequences have been found the numerous rates of evolution among different regions of rDNA (both among and within genes), the presence of many copies of most rDNA sequences per genome, and the pattern of concerted evolution that occurs among repeated copies [14], provides much information. White et al. [15] used direct sequencing method after amplification of fungal ribosomal RNA genes for phylogenetic studies. Chatterton et al. [16-18] studied the internal transcribed spacer (ITS) region of rDNA in diploid wheat, *Triticum speltoides* L. (Tausch) Gren. Ex Richer (Gramineae), the primitive oat species, *Avena longiglumis* Durieu (Gramineae), and barley, *Hordeum vulgare* L. (Gramineae). They found homology in the nucleotide sequences of 5.8S rRNA genes of closely related species, the lack of homology in non-related species, and variability within the ITS1 and ITS2 regions of related species. The results makes the ITS region especially useful for quantifying relatedness among species. Ritland and Straus [19] found a high evolutionary divergence of the 5.8S ribosomal DNA in *Mimulus glaucescens* (Scrophulariaceae) and the spacer sequences were completely unrelated to other plant taxa. Barbee and Taylor [20] used the 18S ribosomal DNA sequence data to estimate

ascomycete relationships, the time of divergence of major ascomycete lineages, and the history of morphological evolutionary change. Liu and Schardl [21] found a highly conserved sequence in rRNA gene ITS1 among flowering plant species. The rest of ITS1 is highly variable in sequence. The conserved motif within ITS1 may have a key function in the processing of rRNA gene transcripts. Pawlowski et al. [22] sequenced about 400 bp DNA fragment situated at the 5' terminal region of the large subunit ribosomal RNA gene and successfully identified the species of *Glabratella erecta* and *Glabratella elegantissima*. Crease [23] surveyed nucleotide variation of 21 subrepeat arrays from intergenic spacer of 3 *Daphnia pulex* populations to study ribosomal DNA evolution at population level. Fan [24] et al. sequenced the 5S rRNA and the rRNA intergenic spacer of the two varieties of *Cryptococcus neoformans*. They found both varieties of the 5S rDNA genes were identical, but the inter-genic spacer (IGS) showed size variation. Briard et al. [25] studied 28S rDNA sequence divergence within the Pythiaceae, which contains economically important plant pathogens belonging to the genera *Pythium* and *Phytophthora*, and established their phylogenetic relationships. Hibbet [26] discovered inserted group I intron in the nuclear small subunit ribosomal DNA in several species of homobasidiomycetes (mushroom-forming fungi). A phylogenetic study was performed by these intron sequences. Parker and Kornfield [27] developed an improved amplification and sequencing strategy for phylogenetic studies using the mitochondrial large subunit rRNA gene. A primer pair was designed to amplify this variable region in a wide range of taxa. They found most of the informative variation occurs within a 200 bp subset of this segment. Siniscalco Gigliano et al. [28,29] sequenced the internal transcribed spacer I and II, which showed species specific DNA sequences in *Cannabis sativa* L. identification and can be used in forensic analysis. Silva-Hanlin and Hanlin [30] performed small subunit ribosomal RNA gene phylogeny of several loculoascomycetes and its taxonomic implications.

Partial sequences (1002 bp) from this gene were performed to determine the phylogenetic relationships of the ascostromatic fungi. Saenz and Taylor [31] also established the phylogenetic relationships of *Meliola* and *Meliolina* inferred from nuclear small subunit rRNA sequences.

2.3 Restriction Fragments Length Polymorphism

Restriction fragments length polymorphism (RFLP) is an alternative means to detect the DNA sequence variation. This method uses restriction endonucleases to cleave double stranded DNA only at a specific sequence of bases known as a restriction site. Digestion of a particular DNA molecule with such an enzyme results in a reproducible set of fragments of well-defined lengths. Point mutations within the recognition sequence as well as insertions or deletions will result in an altered pattern of restriction fragments that can be screened and visualized between different genotypes. The main steps of RFLP include genomic DNA extraction, DNA digestion with restriction enzymes, separation of digested fragments by electrophoresis, Southern blotting the DNA fragments onto membrane, hybridization with a labeled probe, exposure of the hybridized fragment's signal to X-ray film, and development of the restriction fragment patterns. For a species test by RFLP, single stranded probes, which are species-specific DNA sequences within the ribosomal gene region [32,33], are usually used. Since the development of PCR, most of RFLP methods were performed after PCR amplification for the specific DNA fragment of the interest gene.

Henrion et al. [34] did not sequence the ribosomal RNA gene, they amplified full-length nuclear 17S and 25S ribosomal RNA genes, and ribosomal internal transcribed spacer and intergenic spacer of ectomycorihizal fungi. By restriction endonuclease analysis of nearly 6.0 kbp of amplified rDNA they found interspecific

and intraspecific polymorphism. Most of the polymorphisms were located within the regions corresponding to the internal transcribed spacer and intergenic spacer. Tan et al. [35] used RFLP to discover the rDNA can be used to differentiated the *Gaeumannomyces graminis* and its varieties. The difference among *Gaeumannomyces graminis* isolates were found in the 26S rRNA coding region, where the variation occurs in the IGS. Karvonen et al. [36] discovered length variation in the internal transcribed spacers of rDNA in *Picea abies* and related species by restriction mapping and Southern hybridization. White et al. [37] studied the variations in the ribosomal gene cluster on *Cronartium ribicola*. II. by RFLP. There was no particular restriction site variant or IGS size class from particular geographic areas, and no evidence for geographic races of the fungus was obtained. Cupolillo et al. [38] used PCR-RFLP to study the characterization and evolution of *Leishmania* on rDNA. They amplified 1-1.2 kb internal transcribed spacers between the small subunit and large subunit rRNAs, and followed by restriction enzyme digestion. High levels of intra- and inter-specific variation were observed, and quantitative similarity comparisons were used to associate different lineages. Buscot et al. [39] used PCR-RFLP to study the identification of morels on the ribosomal DNA spacers. The internal transcribed spacer and the intergenic spacer of the ribosomal nuclear DNA appeared to be adequate to assess morel systematics. Siniscalco Gigliano [40] successfully used restriction profiles of the internal transcribed spacer II (ITS2) to identify *Cannabis sativa* L. (Cannabaceae). Fatehi and Bridge [41] Detected the internal transcribed spacer of the rDNA gene cluster in isolates of *Ascochyta* by PCR-RFLP. Mavridou and Typas [42] revealed the intraspecific polymorphism in *Metarhizium anisopliae* var. *anisopliae* by PCR-RFLP analysis of rRNA gene complex and mtDNA. Dresler-Nurmi et al. [43] distinguished the species of lignin degrading corticoid fungi based on PCR-RFLP analysis of 18S rDNA and ITS regions.

2.4 DNA Typing by Restriction Fragment Length Polymorphism (RFLP)

The methodology of DNA profiling of RFLP is only a special case of classical RFLP. The only difference is the probe used in the hybridization. DNA profiling of RFLP uses multilocus probes that can hybridize multiple DNA loci simultaneously and create complex banding pattern. Usually, the multilocus probes are designed to hybridize DNA sequences within regions of DNA that are highly repetitive. There are three kinds of DNA probes used for DNA profiling studies in fungi: (1) anonymous repetitive DNA probes derived from the fungal genome under investigation; (2) minisatellite probe, mostly derived from the human or wild-type M13 phage genome; and (3) synthetic oligonucleotide probes complementary to simple repetitive sequences.

Hamer et al. [44] developed DNA typing of fungal plant pathogen. Hanotte *et al.* [45] identified the Indian peafowl P*avo cristatus*. Scherer and Stevens [46] studied the dispersed, repeated gene family of *Candida albicans* and its epidemiologic applications. Meyer et al. [47,48] also applied the DNA profiling technique to the species identification of fungi. Bierwerth et al. [49] compared the radioactive, colourigenic and chemiluminescent detection methods in the oligonucleotide fingerprinting of plant and fungal genomes.

2.5 DNA Profiling by Random Amplification of Polymorphic DNA (AFLP)

In 1985, PCR was introduced which revolutionized the methodological repertoire of molecular biology [12,13]. This method can amplify any DNA sequence of interest to high copy numbers in a short time. In order to amplify a particular DNA,

two single stranded oligonucleotide primers are designed, which are complementary to the target DNA. The RAPD method [50,51] uses only a single ten-nucleotide primer. This primer is arbitrarily designed with at least 50% GC content, therefore no prior knowledge of DNA sequences is needed and the primer can be universally used for eukaryotes and prokaryotes. The primers will anneal to many sites of both strands through the whole genome. An array of DNA products will be generated on the pairs of sites, which can be extended within the polymerized period of the PCR. RAPD profiles have been observed species specific in many reports [52 – 57].

Khush et al. [52] used RAPD fingerprints to discover the polymorphisms of the cultivated mushroom *Agaricus bisporus*. Meyer et al. [51] applied the RAPD technique to the species identification of fungi. Woods et al. [53] also developed RAPD fingerprints of fungus *Histoplasma capsulatum*. For *Cannabis sativa*, there are Gillan et al. [54] and Jagadish et al. [55] reported their findings on the examination of the cannabis genome by RAPD. Lanza et al. [56] used RAPD markers to study the genetic distance of 18 maize inbred lines and predicted their single-cross performance. Pazoutova and Tudzynski [57] identified *Claciceps purpurea* species by RAPD method.

2.6 DNA Profiling of Amplified Fragment Length Polymorphism (AFLP)

AFLP, developed by Vos et al. [58] in 1995, is based on the selective PCR amplification of restriction fragments from a digested genomic DNA. The technique includes restriction of the DNA and ligation of oligonucleotide adapters, selective amplification of sets of restriction fragments, and gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective

amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. The amplified restriction fragments are separated by electrophoresis and visualized. Usually, 50-100 restriction fragments are amplified and formed a DNA profile. The DNA band pattern generated by AFLP is based on the amplification of restriction fragments. This method combines the advantages of RFLP and PCR. No prior sequence knowledge is needed. The number of fragments will be different when using different selective primer sets. This AFLP profile has been successfully applied to genetic analysis, genetic mapping and linkage analysis.

In genetic analysis, Powell et al. [59] compared four marker systems included: RFLP, RAPD, AFLP and SSR (microsatellitte) markers for germplasm analysis. They found the AFLP method was characterized by a very high multiplex ratio. Mueller et al. [60] used AFLP to screen symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus* for genetic differences. Travis et al. [61] applied AFLP markers to determine the genetic diversity among populations of the endangered plant *Astragalus cremnophylax*. Majer et al. [62] applied in the detection of genetic variation in fungal species. Huys et al. [63] used it to analyze the genus *Aeromonas*. Janssen et al. [64] established the bacterial taxonomy. Rosendahl and Taylor [65] continued the development of AFLP markers for studies of genetic variation in arbuscular mycorrhizal fungi using as little as 0.1-1 ng of DNA. Paul et al. [66] revealed the diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze). Kühn et al. [67] studied the diversity and persistence of coliforms and *Aeromonas* in the water of a Swedish drinking water well. Qi and Lindhout [68] developed AFLP markers in barley. And Donaldson et al. [69] also developed genetic markers for red algae. They found both conservative and variable markers were identified within and between populations, and some markers

were unique to individuals. Hartl and Seefelder [70] developed the genetic diversity of eight hop cultivars by fluorescent AFLPs.

The AFLP technique has also been successfully applied to the genetic mapping and linkage analysis, such as in barley [71], the tomato *Cf-9* gene [72], potato [73], *Arabidopsis* [74], rice [75-77], rat [78], *Brassica oleracea* [79], and the Oomycete *Phytophthora infestans* [80].

2.7 Conclusion

The above-mentioned studies on species identification provided a great deal of information for the identification of psilocybin-producing fungi. The RFLP method is an effective way to reveal genetic variability. However, it requires large quantities of relatively pure DNA, the use of radioisotopes in the detection method and much laborious work. In order to avoid these problems in forensic application, PCR-based techniques are used in this study. The RAPD technique offers advantages over RFLP method because it is not necessary for previous knowledge of the genome to have been acquired and only a small amount of DNA is required. This technique is one of the simplest methods to generate a DNA profile, however, it has proved sensitive to slight variations in reaction conditions and therefore reproducibility is a concern. The AFLP technique from these reports provides a reliable and robust genetic molecular assay. A great advantage of this technique is that it allows simultaneous identification of a large number of amplification products. The applications of AFLP as a technique have increased rapidly. The ribosomal RNA gene has long been used as a means for analyzing phylogenetic relationships over a wide range of species. Sequence data obtained by direct sequencing are frequently used to generate highly resolved classification systems. Due to the functional and structural constraints, the exons of

ribosomal RNA gene show strong evolutionary conservation and they are useful for estimating phylogenetic relationships among organisms. By contrast with them, the ITS regions show much more divergence, suggesting utility for comparisons among closely related organisms.

This study will examine members of the genera of fungi known to produce psilocybin by RAPD, AFLP techniques for the whole genome screening and direct sequencing method to reveal the nucleotide variation in ribosomal RNA gene.

2.8 Aims

The aim of this thesis is to develop a DNA based technique that can identify the presence of fungi belonging to either the genus *Psilocybe* and *Panaeolus*. The methods used will be those currently employed in forensic identification and adapted for this particular problem.

1. In the first instance samples of identified fungi will be collected, from which DNA will be extracted. The method of DNA extraction will isolate whole genomic DNA.

2. RAPD and AFLP will be used to detect polymorphisms within the entire genome of the fungi. A means of scoring the resulting RAPD and AFLP products will be devised. This scoring system will be used to determine whether the presence of key PCR products can be used to detect the presence of particular psilocybin-producing fungi.

3. Genus or species specific DNA sequences will be sought in the polymorphic DNA loci such as the ITS region and the small nuclear ribosomal subunit. In the event of

species or genus specific sequences being identified, a DNA test will be developed to detect the presence of members of the *Psilocybe* or *Panaeolus* genera.

4. The final goal of this thesis is to develop a test that will unambiguously identify the presence of members of the genera *Psilocybe* and *Panaeolus*.

3 SAMPLE PREPARATION

3.1 Sample Collection

Twenty samples of two genera with eleven species (Table 3-1) of psilocybin-producing mushrooms were collected from the collection of the University of Strathclyde. The export (APPENDIX ONE) and import licenses (APPENDIX TWO) were issued by the governments of the United Kingdom and Republic of China, respectively.

Table 3-1 Samples of psilocybin-containing mushrooms in this study

Sample	Species	Sample	species
PS1-1	*Psilocybe semilanceata*	PA1-1	*Panaeolus semiovatus*
PS1-2	*Psilocybe semilanceata*	PA1-2	*Panaeolus semiovatus*
PS1-3	*Psilocybe semilanceata*	PA1-3	*Panaeolus semiovatus*
PS1-4	*Psilocybe semilanceata*	PA1-4	*Panaeolus semiovatus*
PS2	*Psilocybe coprophila*	PA2	*Panaeolus papilionaceus*
PS3	*Psilocybe cyanescens*	PA3	*Panaeolus reckenii*
PS4	*Psilocybe eucalypta*	PA4	*Panaeolus retirugis*
PS5	*Psilocybe montana*	PA5	*Panaeolus semiovatus*
PS6-1	*Psilocybe semilanceata*	PA6	*Panaeolus speciosus*
PS6-2	*Psilocybe semilanceata*	PA7	*Panaeolus subbalteatus*

3.2 Description of Sample

Only few samples still retained their shape (Figure 3-1 and 3-2) after shipping to the laboratory. Most samples were crushed into fragments (Figure 3-3, 3-4 and 3-5) which prevented their identification by their morphological features.

Figure 3-1 Picture of dried *Panaeolus semiovatus* (PA1)

Figure 3-2 Picture of dried *Psilocybe semilanceata* (PS1)

Figure 3-3 Picture of dried *Panaeolus papilionaceus* (PA2)

Figure 3-4 Picture of dried *Panaeolus retirugis* (PA4)

Figure 3-5 Picture of dried *Psilocybe coprophila* (PS2)

3.3 Spore Image by SEM

Spore images of samples were taken by scanning electron microscope (SEM) (Jeol JSM-5410LV) for the observation of their morphological features. Figure 3-6 to 3-15 are 1500X or 2000X image using 20KV.

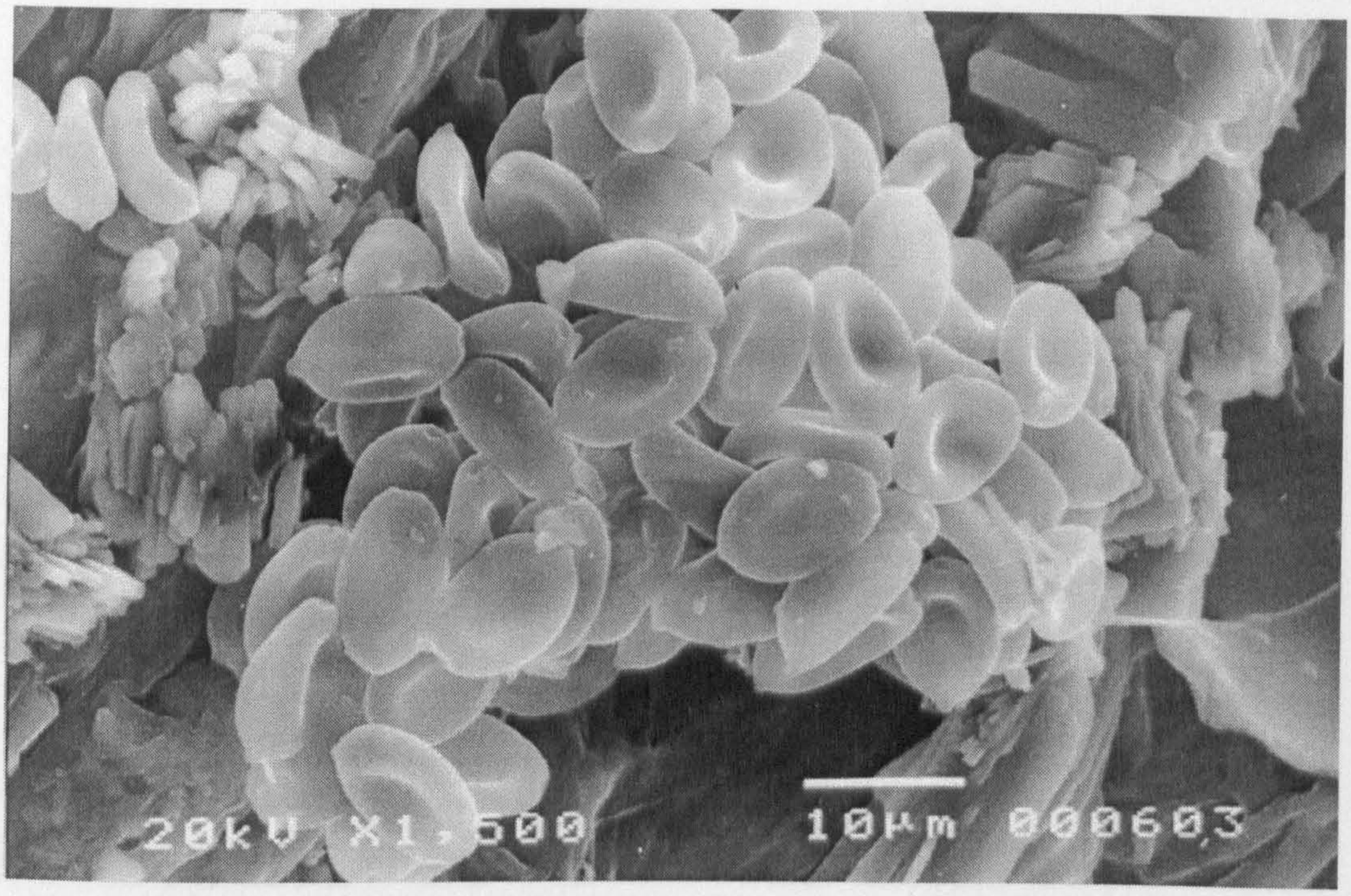


Figure 3-6 Spore image of *Psilocybe semilanceata* (PS1)

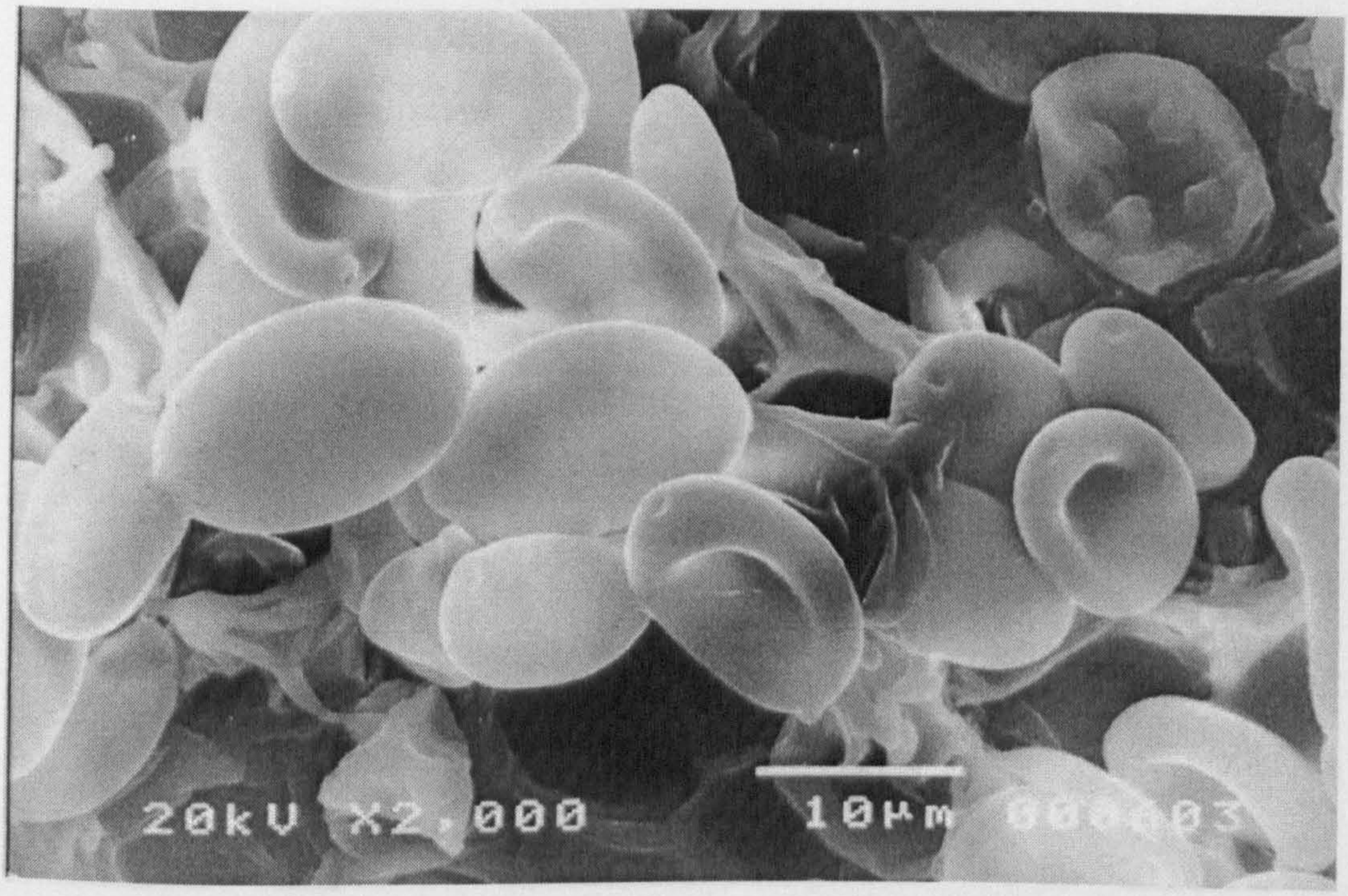


Figure 3-7 Spore image of *Psilocybe coprophila* (PS2)

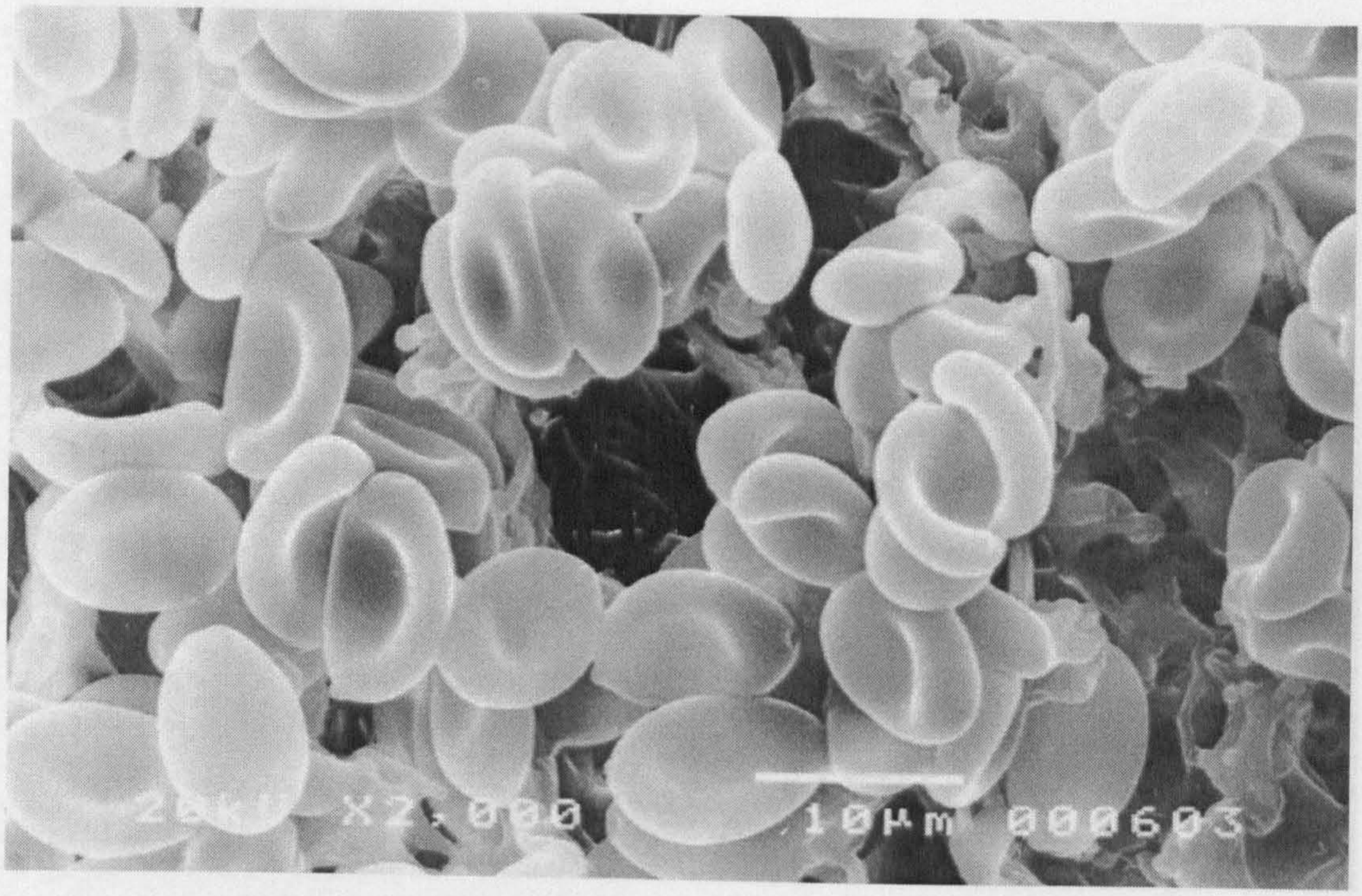


Figure 3-8 Spore image of *Psilocybe cyanescens* (PS3)

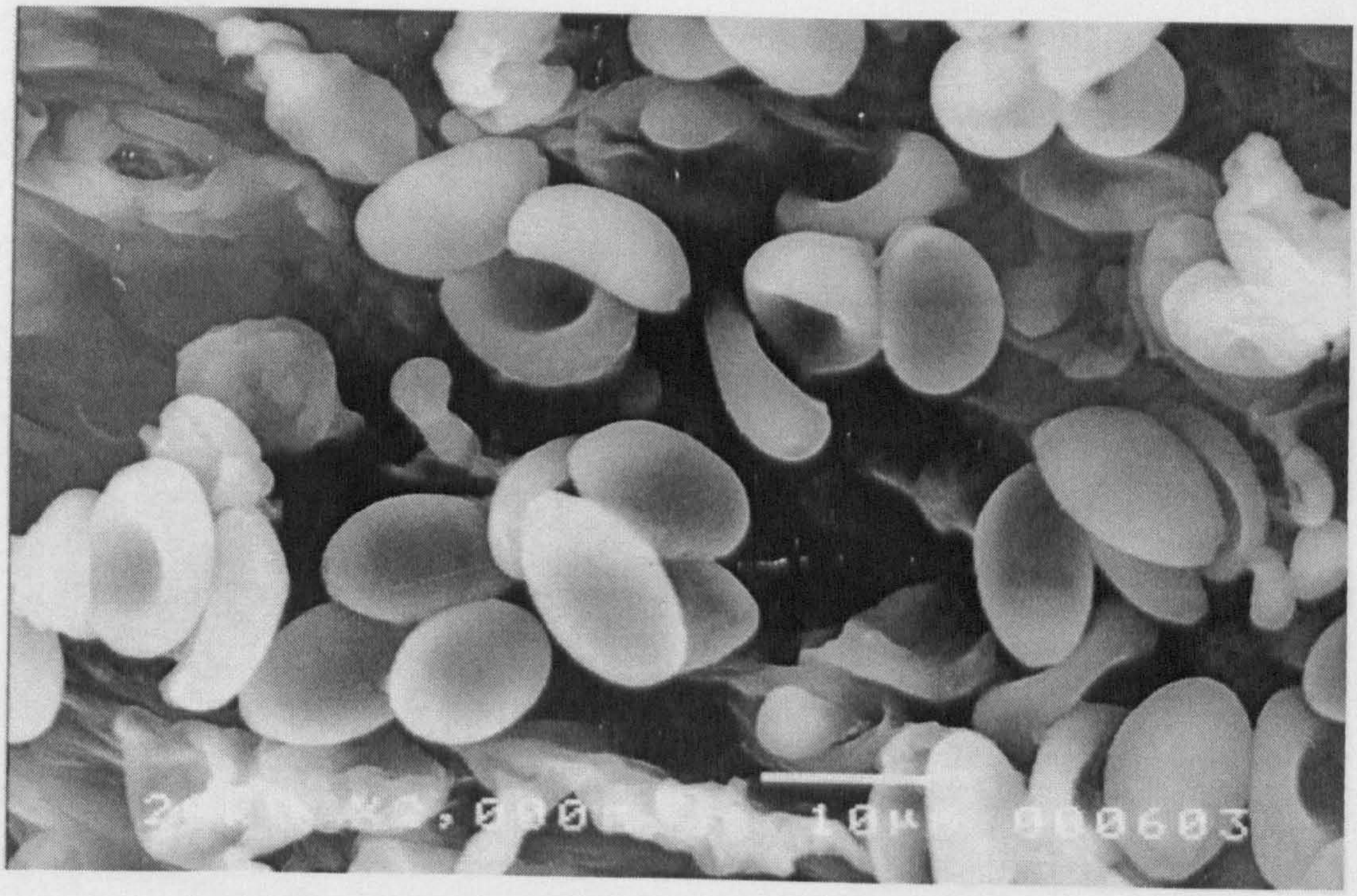


Figure 3-9 Spore image of *Psilocybe eucalypta* (PS4)

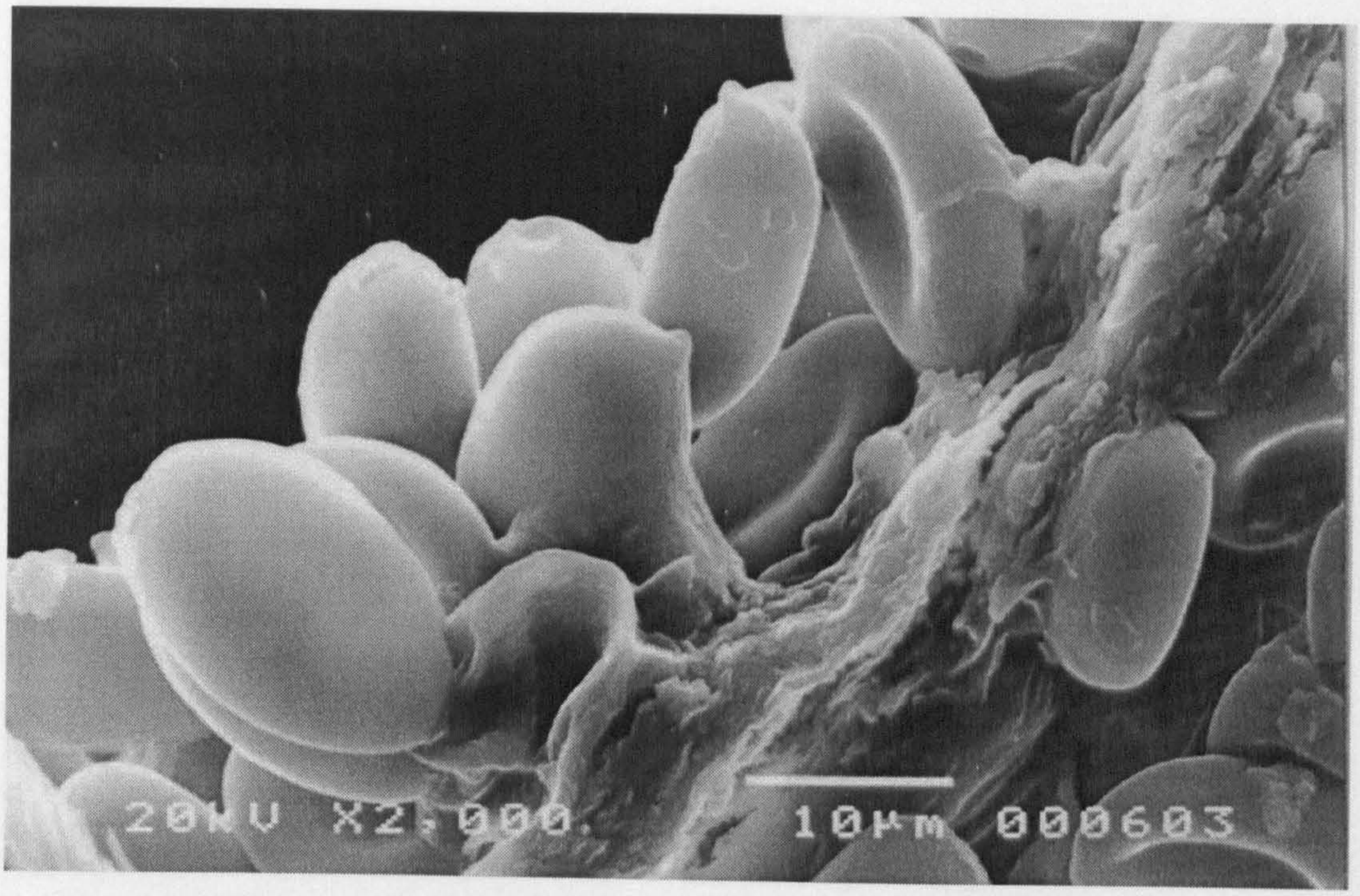


Figure 3-10 Spore image of *Panaeolus semiovatus* (PA1)

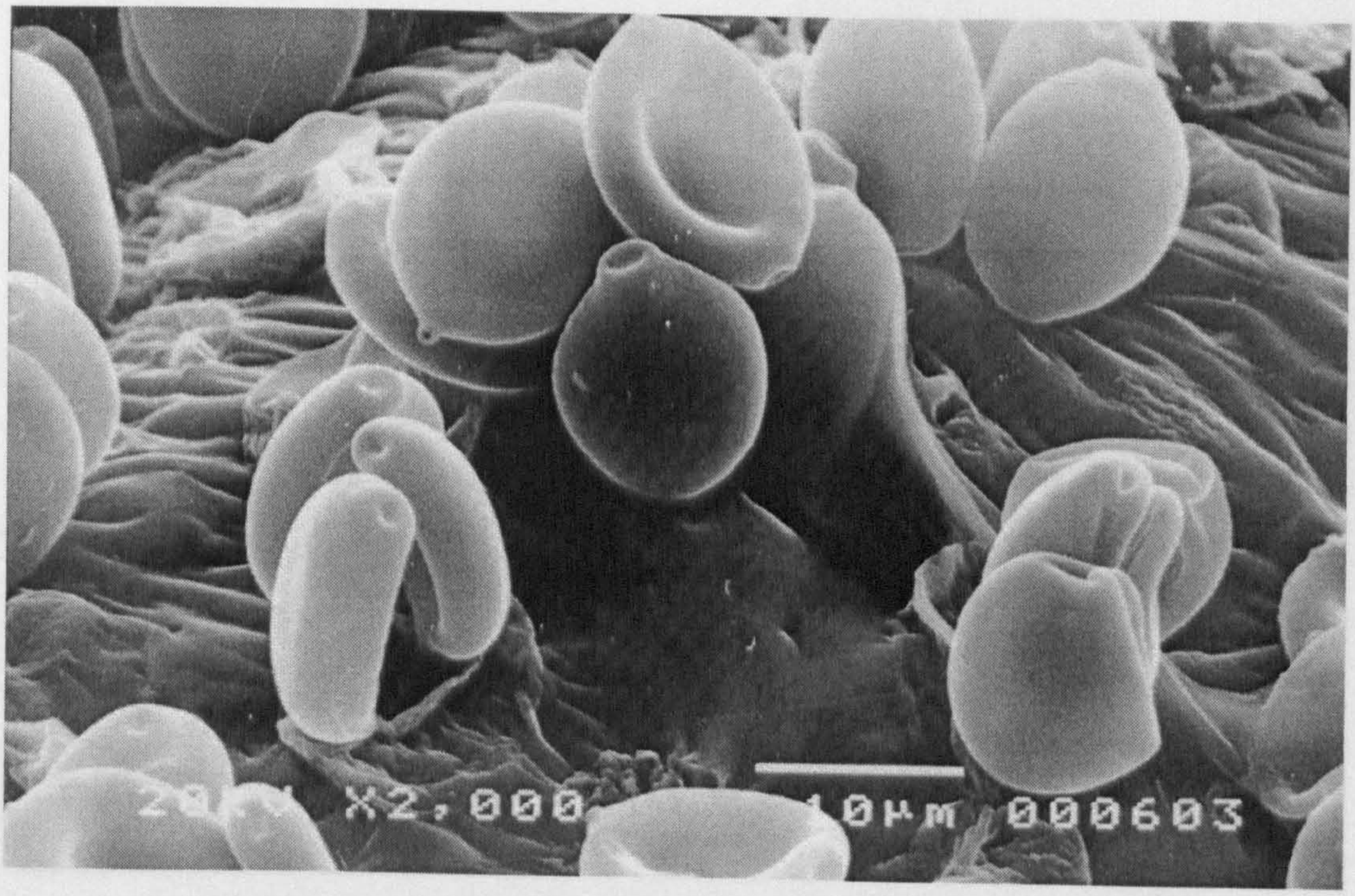


Figure 3-11 Spore image of *Panaeolus papilionaceus* (PA2)

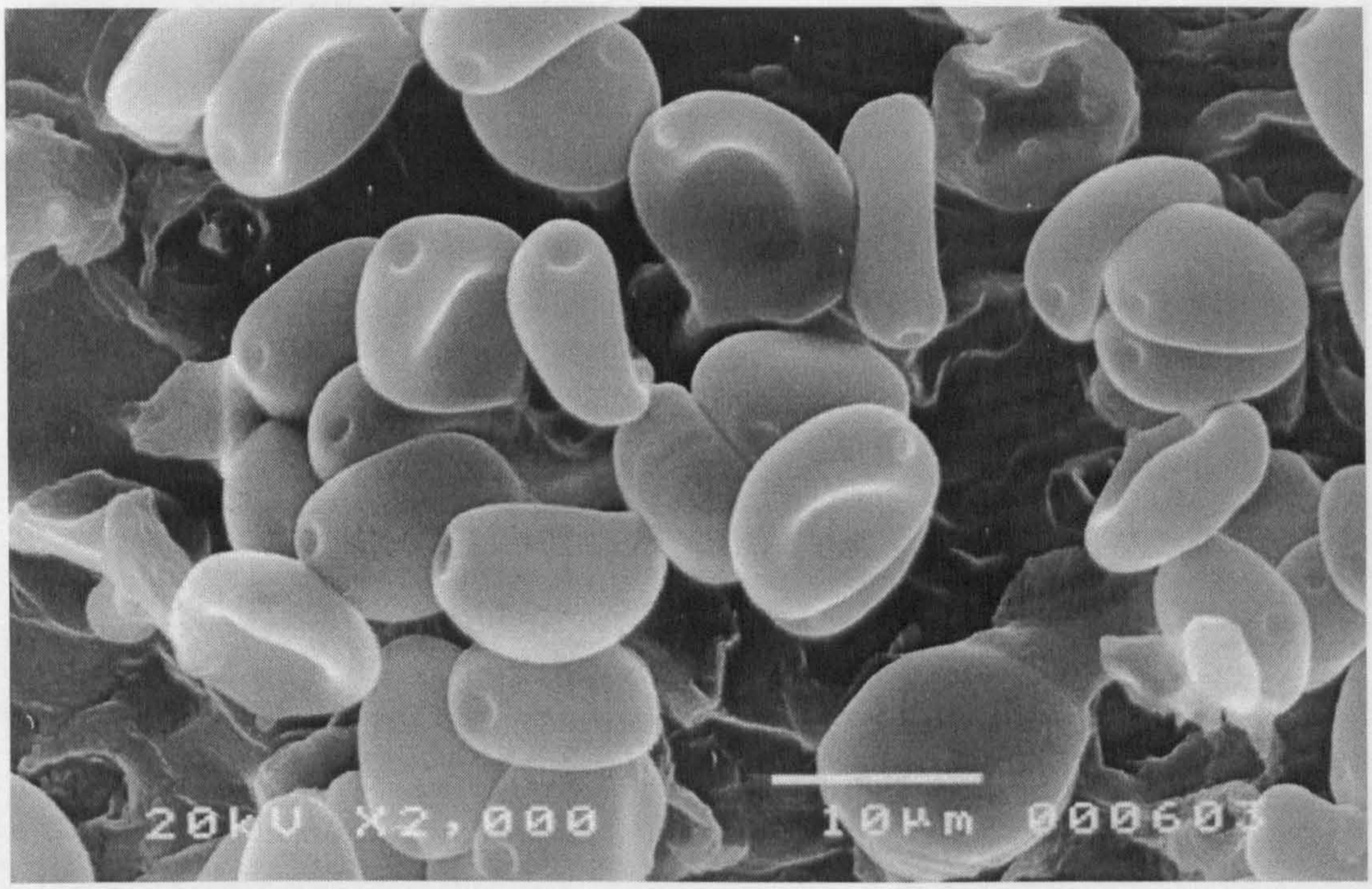


Figure 3-12 Spore image of *Panaeolus reckenii* (PA3)

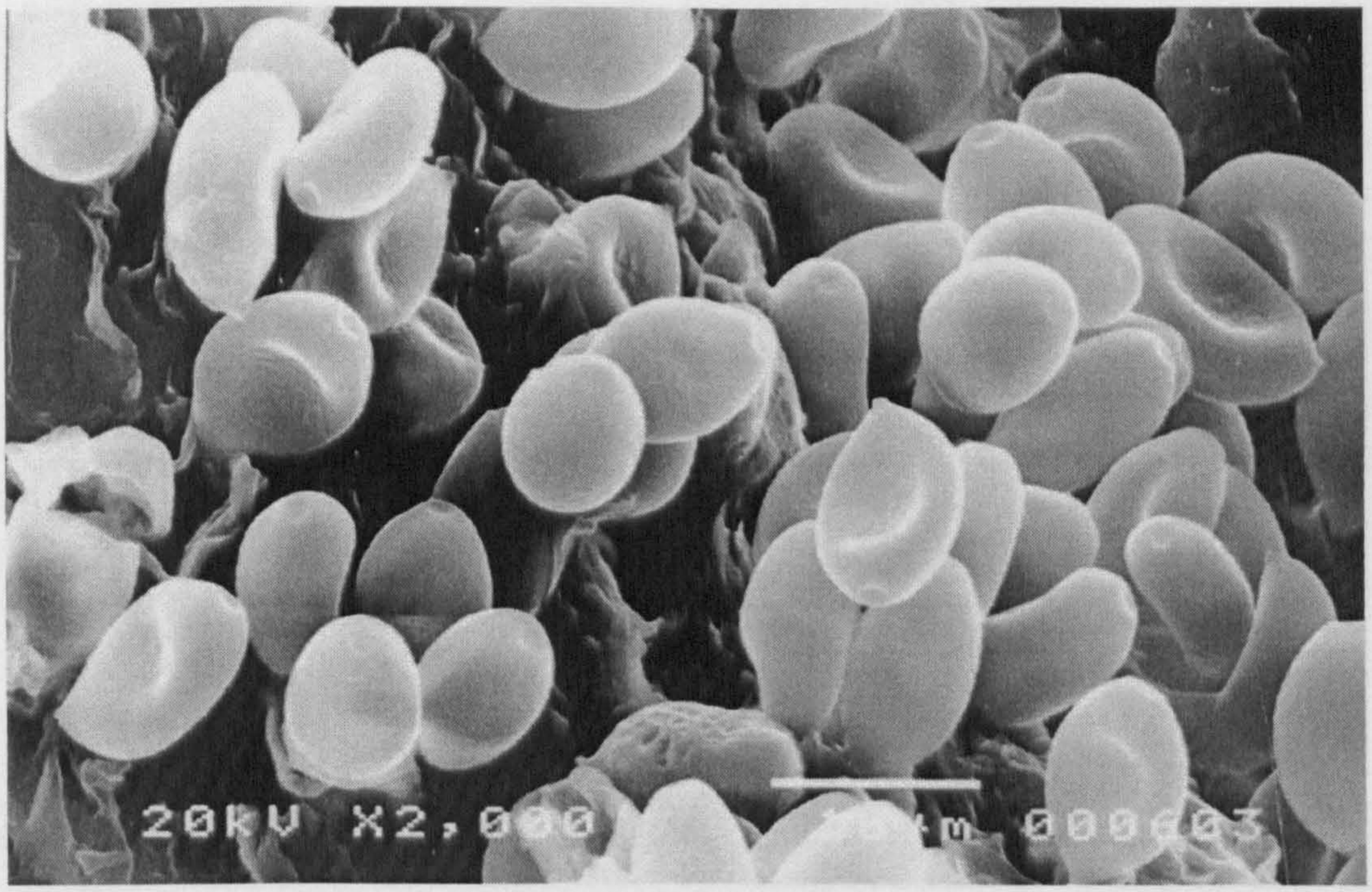


Figure 3-13 Spore image of *Panaeolus retirugis* (PA4)

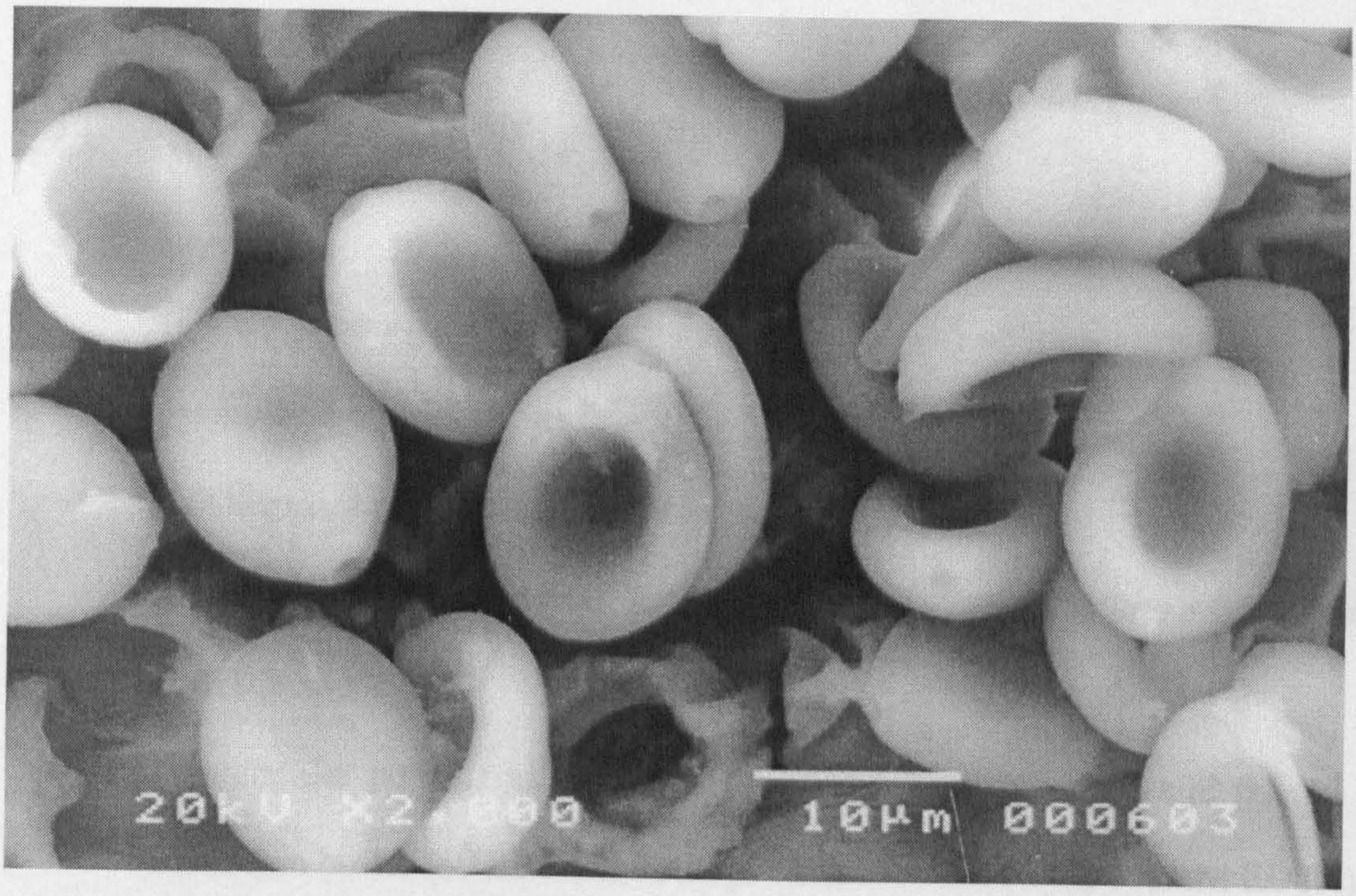


Figure 3-14 Spore image of *Panaeolus speciosus* (PA6)

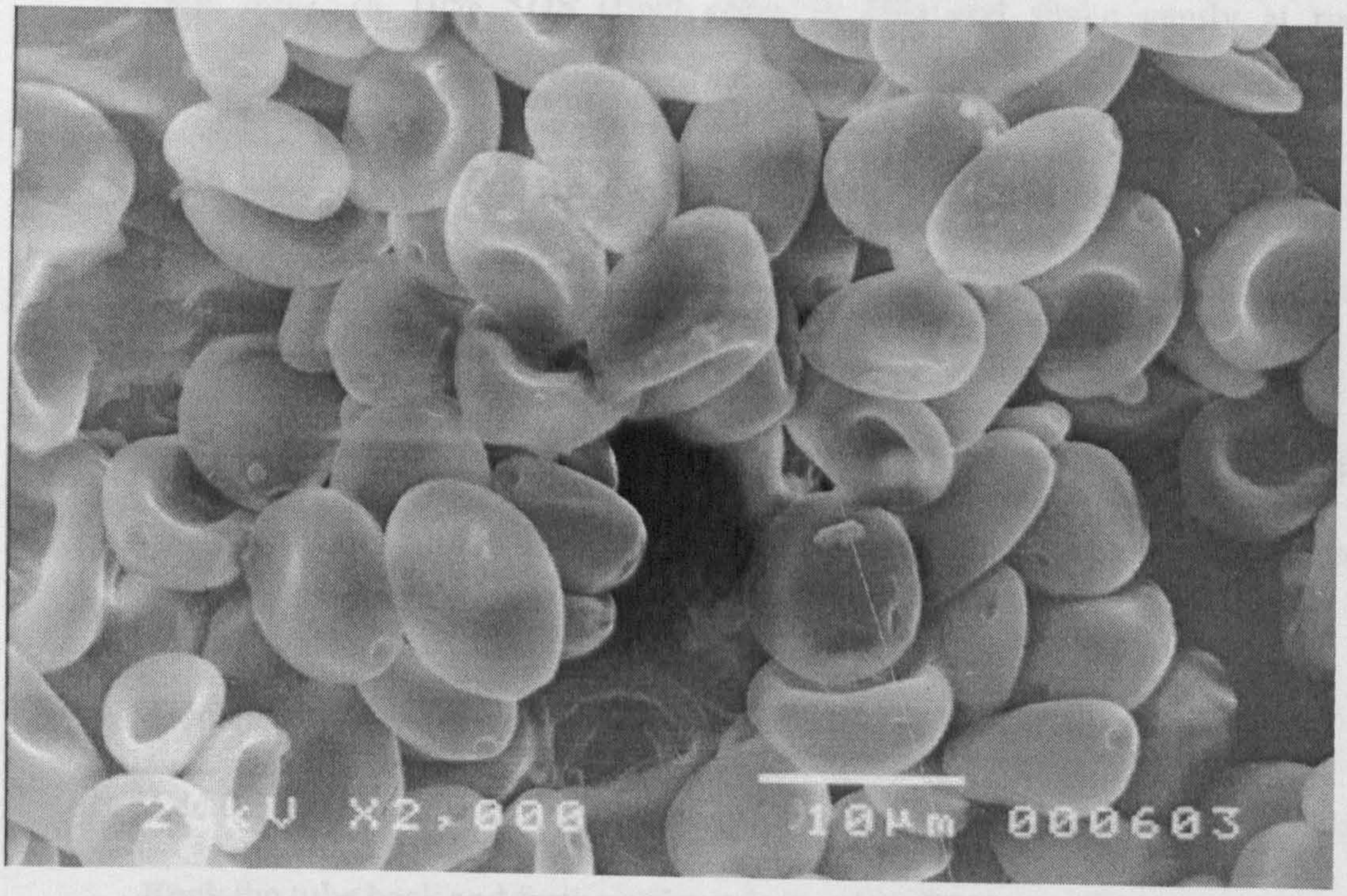


Figure 3-15 Spore image of *Panaeolus subbalteatus* (PA7)

3.4 DNA Extraction

3.4.1 Extraction Protocol

DNA extraction used the CTAB method and modified from Doyle et al. [81].

Detailed protocols are as followings:

1. Dry the mushroom samples at 56°C and weigh about 20mg.
2. Place the sample in a mortar. Add a trace of sand and grind into a fine powder with a pestle.
3. Transfer the powder to a 1.5-mL microcentrifuge tube and add 0.5 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100mM EDTA).
4. Vortex until evenly suspended (no clumps).
5. Add 50μL of 10% SDS (final conc. is 1%) and shake gently at room temperature (RT) for 1 hour.
6. Add 75μL of 6 M NaCl (final conc. is 0.8 M) and mix gently but thoroughly.
7. Add 65μL of 10% CTAB/NaCl solution (10% CTAB in 0.7 M NaCl; final CTAB conc. is 1%). Mix thoroughly and incubate at 65°C for 10 to 20 min.
8. Add equal volume (ca. 690μL) of chloroform/isoamyl alcohol (24:1), mix thoroughly and spin at 12,000 rpm for 10 min, at RT, to separate the aqueous phase from the chloroform.
9. Transfer the upper aqueous phase to a new microcentrifuge tube.
10. Add 0.6 volume (ca. 0.4 ml) of cold isopropanol to precipitate the nucleic acid. Rock the tube back and forth until a stringy white DNA precipitate appears. Let the tube stand at RT for about 10 min until no more precipitation occurs.
11. Centrifuge at 12,000 rpm for 10 min.
12. Discard the solution and rinse the precipitate with cold 70% ethanol (ca. 0.7

ml).

13. Repeat the 70% ethanol wash and then dry the DNA in a dissector for 20-30 min. or in a hood for about 1-2 hrs.
14. After the DNA is dry, dissolve in 0.5 ml of 1X TE buffer.
15. Add 1µl of RNase A (10 mg/ml) to give a final conc. of 20µg/mL. Keep at 4°C overnight to dissolve the DNA completely.
16. Re-precipitate DNA by adding 50µL (1/10 vol.) of 3 M Na acetate, 1 ml (2X vol.) of 95% ethanol.
17. Centrifuge at 12,000 rpm for 10 min. Repeat steps 12 & 13. Dissolve DNA in 100µL of 1X TE buffer. Store at –20 °C

3.4.2 DNA Electrophoresis

Extracted DNA was screened by minigel electrophoresis.

1. Make a 0.7 % agarose gel (in 1X TBE buffer).
2. Mix 5µl of DNA with 2µl of 6X loading tracking dye on a piece of parafilm.
3. Load the sample in the well.
4. Load 3µl of Hind III digested lambda phage DNA (0.01µg/µl) in 1 well.
5. Run the gel at 100 V in 1X TBE buffer.
6. Stain the gel in 100 ml of water plus 5 µl of ethidium bromide (0.5 µg/ml).
7. View the gel with an UV transilluminator (312 nm) and take a Polaroid picture.
8. Estimate the concentration of DNA sample by comparing the stain intensity with that of the standard lambda DNA can obtain semi-quantitative data.
9. More precise DNA quantity was estimated by measuring the absorbance of 260 nm, then transfer OD(260) value to weight. The volume of 25µl of each sample was performed the optical density test.

3.4.3 Results of DNA extraction

DNA from twenty samples was successfully extracted by this CTAB method. Electrophoresis results on agarose gel of extracted DNA were shown in Figure 3-16. DNA quantity were calculated by the absorbance of 260 nm and shown in Table 3-2.

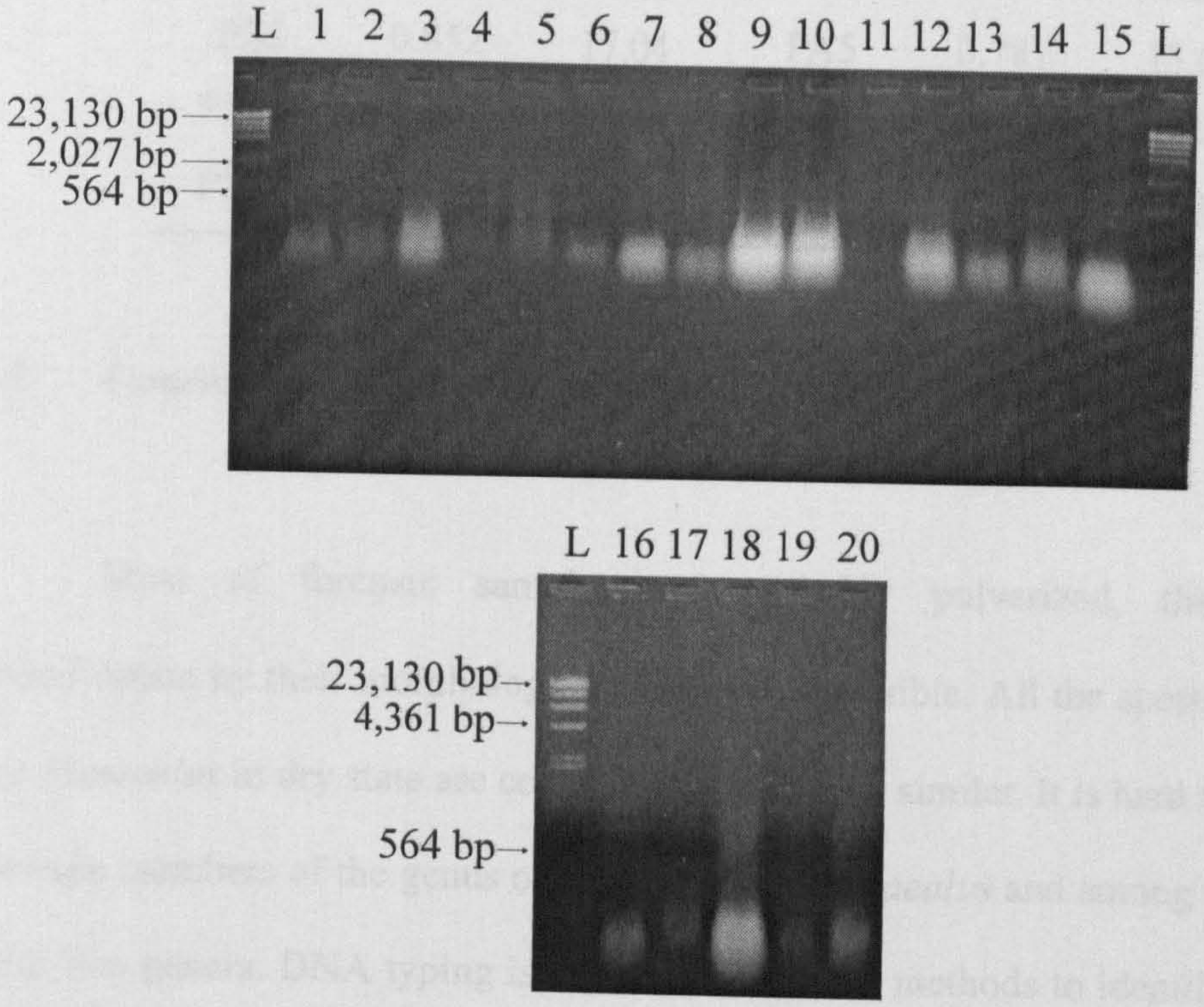


Figure 3-16 Electrophoresis results on 0.7% agarose gel of extracted DNA. Lane L is Hind III digested lambda phage DNA. Lane 1-20 are sample PS1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6-1, 6-2 and PA1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6 and 7.

Table 3-2 Results of DNA extraction in this study

Sample	OD value	DNA(μ g)	Sample	OD value	DNA(μ g)
PS1-1	0.392	7.84	PA1-1	0.138	2.76
PS1-2	0.487	9.74	PA1-2	0.785	15.7
PS1-3	1.119	22.38	PA1-3	0.258	5.16
PS1-4	0.724	14.48	PA1-4	0.279	5.58
PS2	0.410	8.2	PA2	0.573	11.46
PS3	0.586	11.72	PA3	0.212	4.24
PS4	0.526	10.52	PA4	0.391	7.82
PS5	0.852	17.04	PA5	0.781	15.62
PS6-1	1.872	37.44	PA6	0.324	6.48
PS6-2	1.552	31.04	PA7	0.558	11.16

3.5 Conclusion

Most of forensic samples are probably pulverized, therefore visual identification by their morphology becomes less possible. All the spores of *Psilocybe* and *Panaeolus* in dry state are concave oval and look similar. It is hard to differentiate between members of the genus of *Psilocybe* and *Panaeolus* and among species within these two genera. DNA typing is one of the efficient methods to identify members of genera that produce psilocybin. The first step for DNA typing is DNA extraction. In this experiment, DNA was successfully extracted from the all samples. In Figure 3-16, the size of most extracted DNA fragments are distributed smaller than about 4 kb. This showed that DNA was seriously degraded after long time storage despite their intact appearance. From the results of optical density test, there is still enough DNA to perform the PCR-based DNA analysis. For large size DNA analysis, however, it will not be expected to have good results due to this DNA quality.

The condition of sample preparation will affect the yield of DNA and its quality.

Fresh samples can be kept cool and not necessarily in a frozen state for a short period of time, such as up to several hours or a day. For long term storage, samples should be kept in a freezer in –80 °C or lyophilization. This is to avoid the damage of endogenous enzyme in the room temperature after collection. Fresh, frozen and lyophilized sample usually gives higher yields and more intact DNA.

4 DNA ANALYSIS BY RANDOM AMPLIFICATION OF POLYMORPHIC DNA

4.1 Introduction

Since the first use of random amplified polymorphic DNA (RAPD) in 1990 [50,51], it has been used extensively for species identification of bacteria, fungi, plants and animals [52-57]. RAPD has all the advantages of a PCR based technique with the added advantage of requiring no prior DNA sequence knowledge. RAPD produces a "genetic fingerprint" which can be used to determine the genetic relationship, or discriminate, between DNA samples. A characteristic band pattern is produced after RAPD and if the DNA samples are genetically closely related, they will share many bands. The presence or absence of bands in a RAPD reaction is scored to determine this relationship. RAPD has been used in many applications, including in forensic science for the comparison and linkage of *Cannabis sativa* samples [54,55]. RAPD, while being a useful taxonomic tool, has problems due to its poor reproducibility [82]. Despite this problem of poor reproducibility associated with RAPD, this study will use RAPD to determine whether it could be used to determine the genus or species of an unknown fungal sample.

4.2. Materials and Methods

4.2.1 Psilocybin-Containing Mushrooms

DNA samples of the mushrooms extracted in Section 3.4 were used in this analysis. The DNA extraction method is the same as described in section 3.4.

4.2.2 PCR Amplification

In this study two primers, each 10 bp in length, were used to generate two sets of RAPD fingerprints of these samples.

Primer A: 5'-TTCGAGCCAG-3' Primer B: 5'-GTGAGGCGTC-3,'

PCR

Template DNA	15ng
Primer (A or B)	60ng
Taq DNA Polymerase	1 unit (PE Applied Biosystems unless stated)
dNTP	0.4mM dATP, dCTP, dGTP and dTTP
BSA	2.5µg
Reaction Buffer	3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris-HCl pH 8.3
Total Volume	25µl

A PE Applied Biosystems DNA Thermal Cycle 480 was used for amplification.

PCR cycling programme

Denaturation	1 min at 95°C	
Annealing	1 min at 36 °C	45 Cycles
Extension	2 min at 72 °C	

4.2.3 Electrophoresis

In order to maximise the resolution of the RAPD fingerprint, PCR products were separated in 12.5% polyacrylamide gel electrophoresis. Two microlitres of PCR products were electrophoresed in GeneGel Excel 12.5/24 gel (Pharmacia Biotech, Sweden) and run at 600 V, 25mA, 15W, 15 °C for 80 minutes using GenePhor Electrophoresis Unit (Pharmacia Biotech).

4.2.4 Silver Staining

1. After electrophoresis, stain the gel in the Hoefer Automated Gel Stainer together with PlusOne DNA Silver Staining Kit for automatic staining.
2. Run the following staining protocol on the Hoefer Automated Gel Stainer.

DNA silver stain protocol on the Hoefer Automated GelStainer

Step	Solution	Time
1	Fixing sol.	30 min
2	Silver sol.	30 min
3	Water (washing)	1 min
4	Developing sol.	6 min
5	Stopping and preserving sol.	30 min

4.2.5 RAPD Pattern Analysis

4.2.5.1 Band Detection

RAPD band patterns were detected and sized using Image master 1D (Pharmacia Biotech) with parameters of slope 200, noise 10 and width 10. The slope

parameter specifies how pronounced the band must be from its surrounding area in the lane. A high value means that the transition from background lane intensity to a band's peak intensity must be sharp. A lower value allows the gradient to be less severe. Slope 400 is moderate in this software system. The noise parameter specifies the degree to which small local peaks will be ignored on the profile. Noise 10 is designed to eliminate only very low amount of noise in the image. The width parameter specifies the bandwidth to be measured which is useful to measure the relative intensities of the bands. In this study, width 10 is good enough to define the position of bands.

4.2.5.2 Score Assignment

Each gel was divided into 60 equal segments between 50 bp and 1000 bp. The presence or absence of bands was then scored 1 if a band is present in the segment or 0 if absent. This produces a binary data matrix.

4.2.5.3 Cluster Analysis

The genetic similarities (GS) between each sample was calculated using the formula $GS_{ij} = 2Nij/(N_i + N_j)$, where N_{ij} is the number of common loci between i and j [83]. The GS matrix was produced by the comparison of each sample pair. A dendrogram can then be generated using the unweighted pair group method algorithm (UPGMA) clustering procedure using the NTSYS software [84].

4.3. Results and Discussion

4.3.1 Extraction and Amplification

DNA was extracted from the 20 fungal samples as described in Table 3.1. Amplification was performed on the 20 samples using primer A and primer B in separate reactions. The PCR products from primer A were separated on a polyacrylamide gel using a 100bp ladder as a DNA size marker. The gel was stained with silver and the resulting band pattern is shown in Figure 4-1.

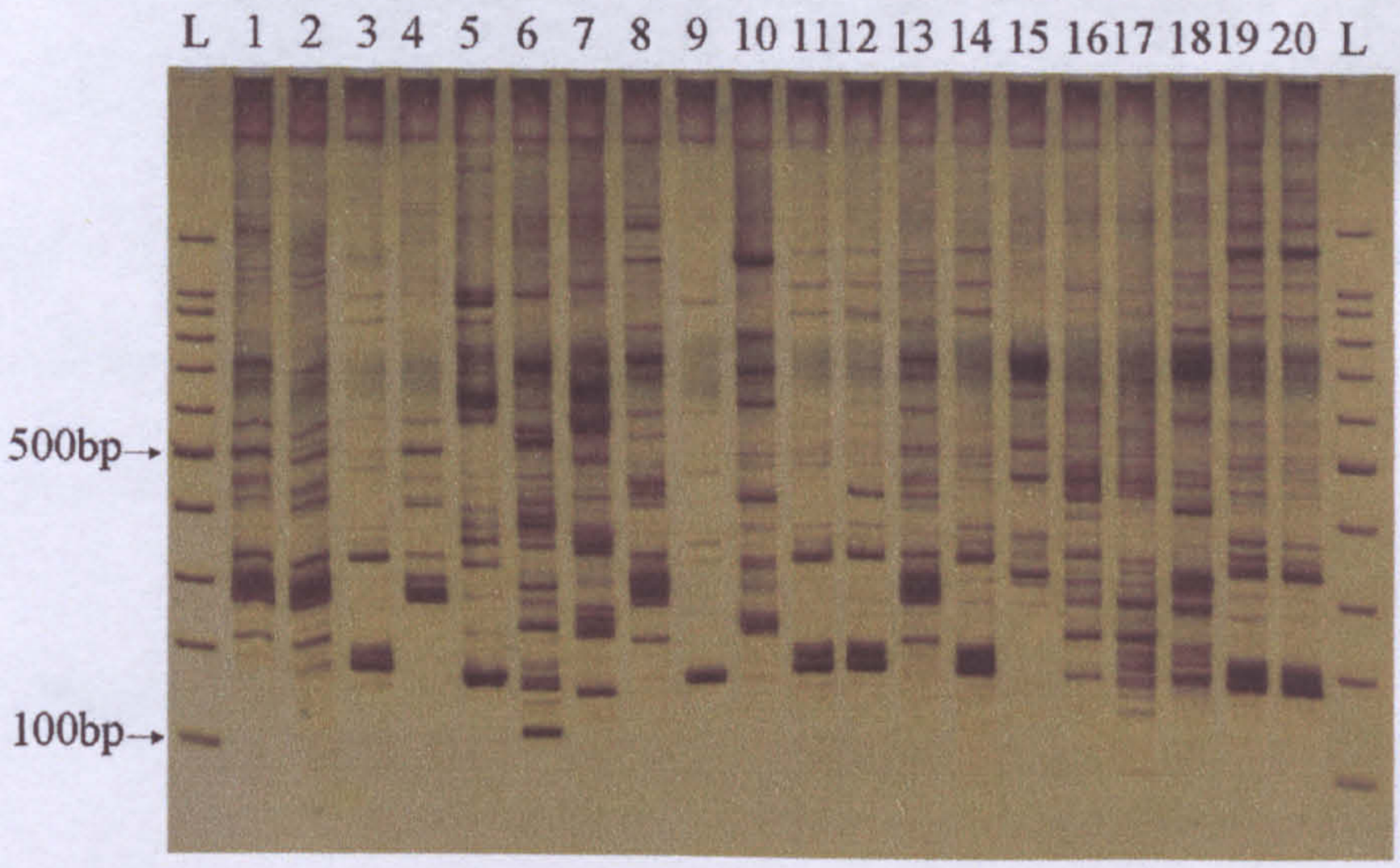


Figure 4-1 RAPD patterns produced by primer A: 5'-TTCGAGCCAG-3'. Lane L is 100 bp ladder. Lane 1 to 20 are samples of PA1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 103, 1-4, 2, 3, 4, 5, 6-1 and 6-2. (file name: 870709OPC1.tif)

Figure 4-2 illustrates the band pattern from primer B, which was separated on a separate polyacrylamide gel and silver stained.

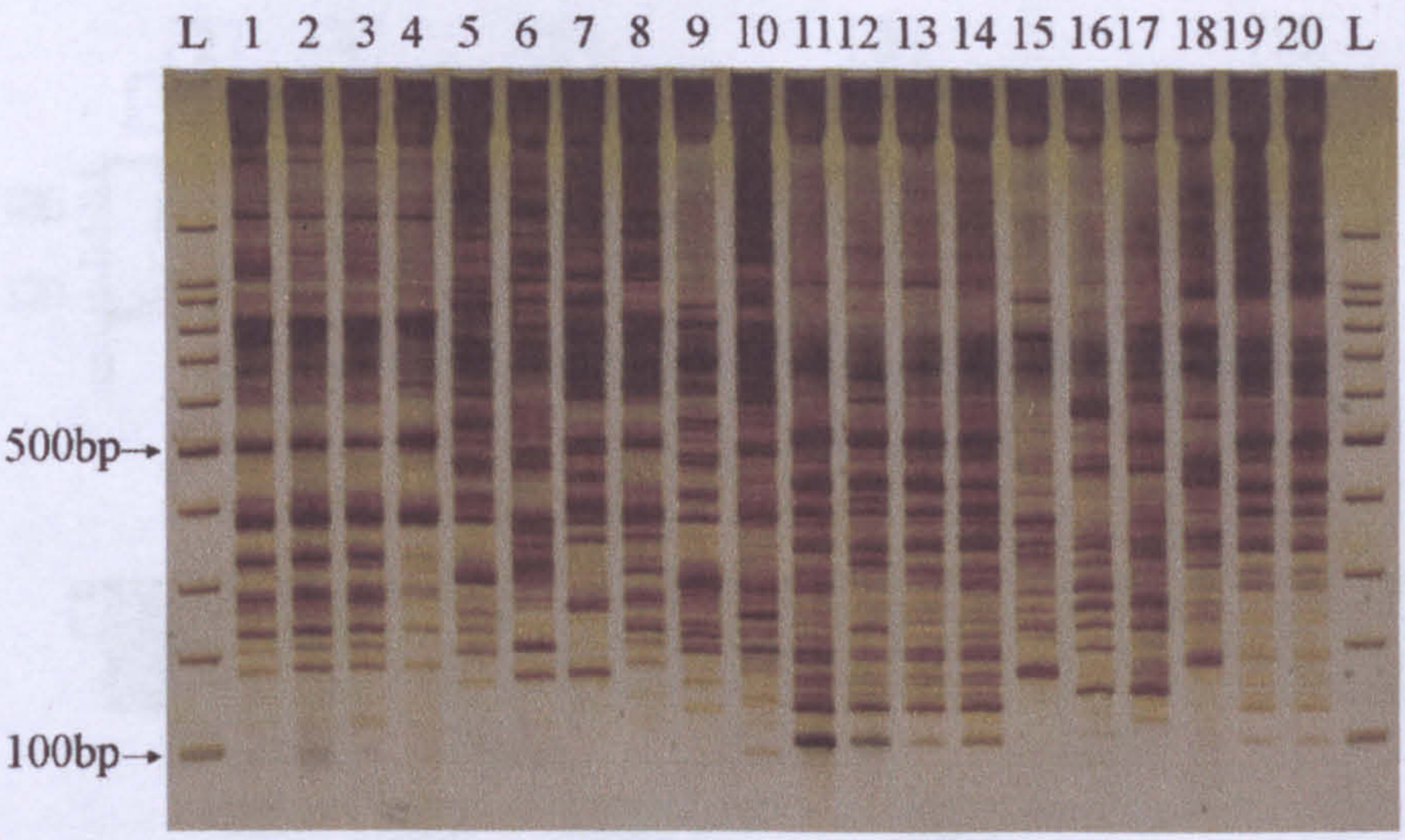


Figure 4-2 RAPD patterns produced by primer B: 5'-GTGAGGCGTC-3'. Lane L is 100 bp ladder. Lane 1 to 20 are samples of PA1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 103, 1-4, 2, 3, 4, 5, 6-1and 6-2. (file name: 870709OPC2.tif)

4.3.2 Image Analysis

In order to analyse the RAPD gel patterns, the position of each band had to be determined. For this a scoring system was used where the gels were scanned into a computer and software would determine the molecular weight using a 100 bp ladder.

The silver stained gels shown in Figures 4-1 and 4-2 were scanned into a computer and saved in tiff image files. The gel files were retrieved using Image Master 1D software for band detection and size determination. Every possible band in the lane was detected automatically using this software under the following parameters: slope 200, noise 10 and width 10. The sizes of ladder were assigned and generated a formula of log curve (Figure 4-3 and 4-4).

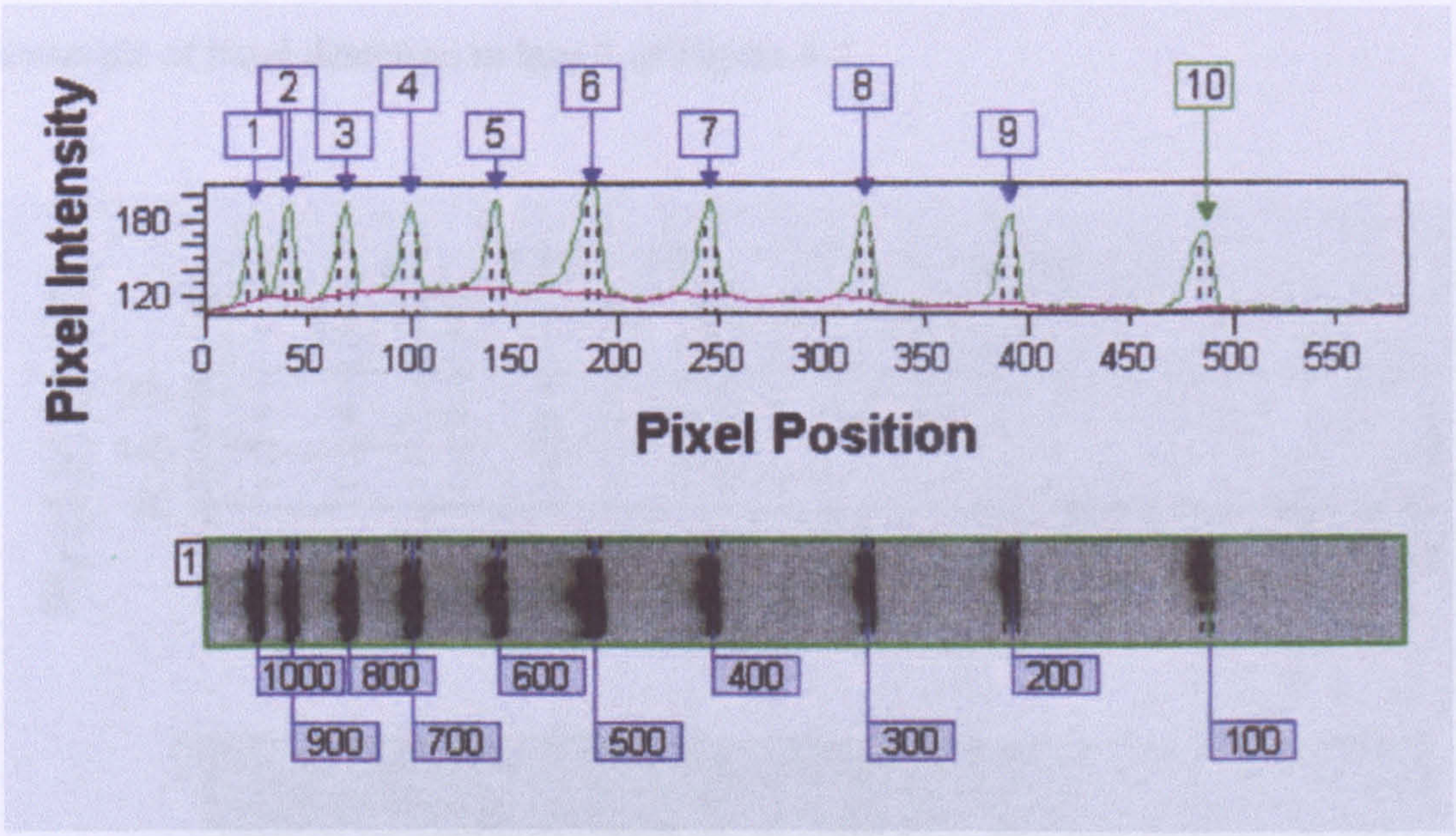


Figure 4-3 The sizes of 100 bp ladder in Figure 4-1 assigned from 1000 bp to 100 bp.

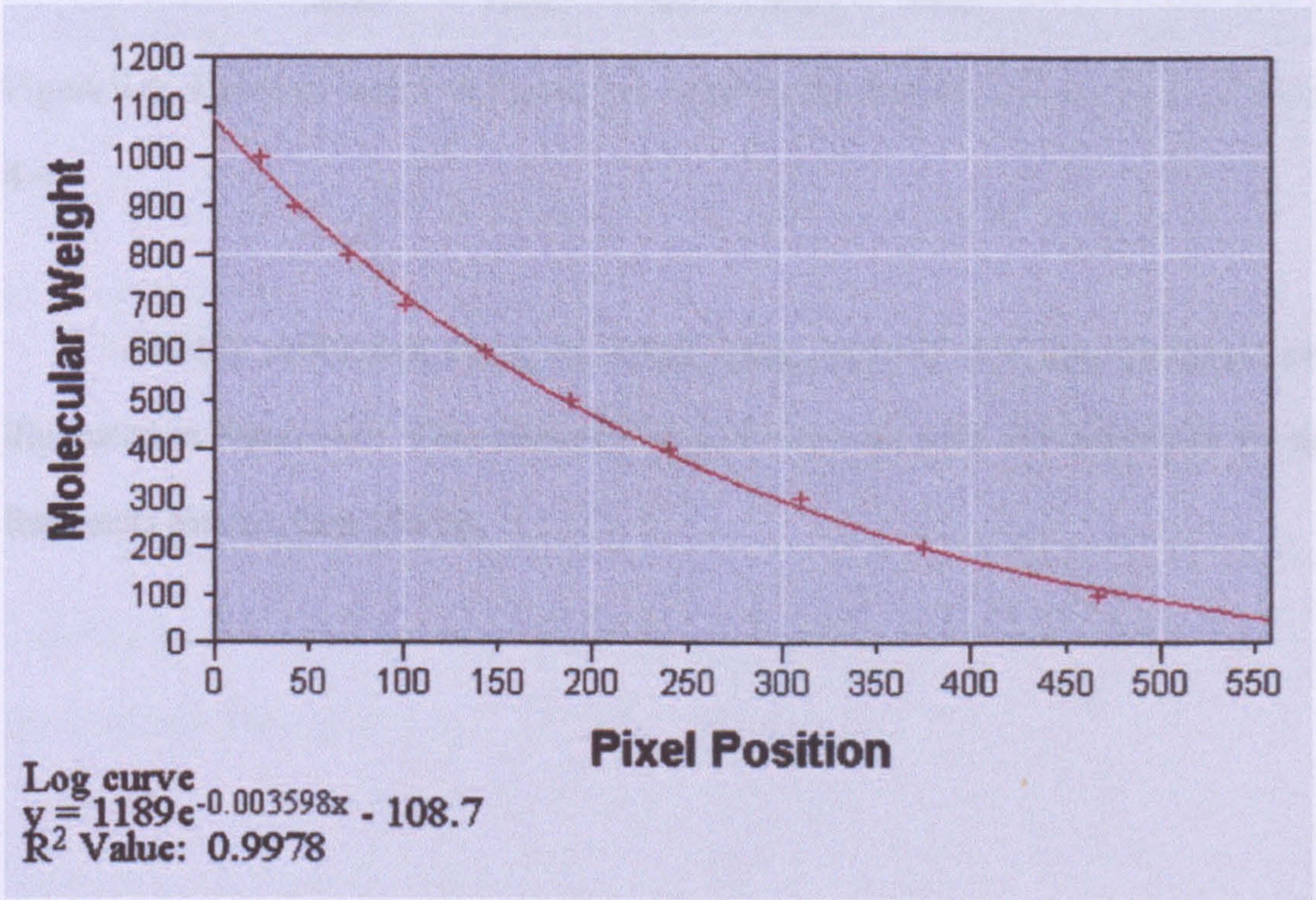


Figure 4-4 A log curve generated by the sizes of 100 bp ladder.

The same formula using the determined molecular weights could be applied to

gel lanes containing each of the 20 PCR samples using primer A. Figure 4-5 is an example of band detection in lane 1 of Figure 4-1.

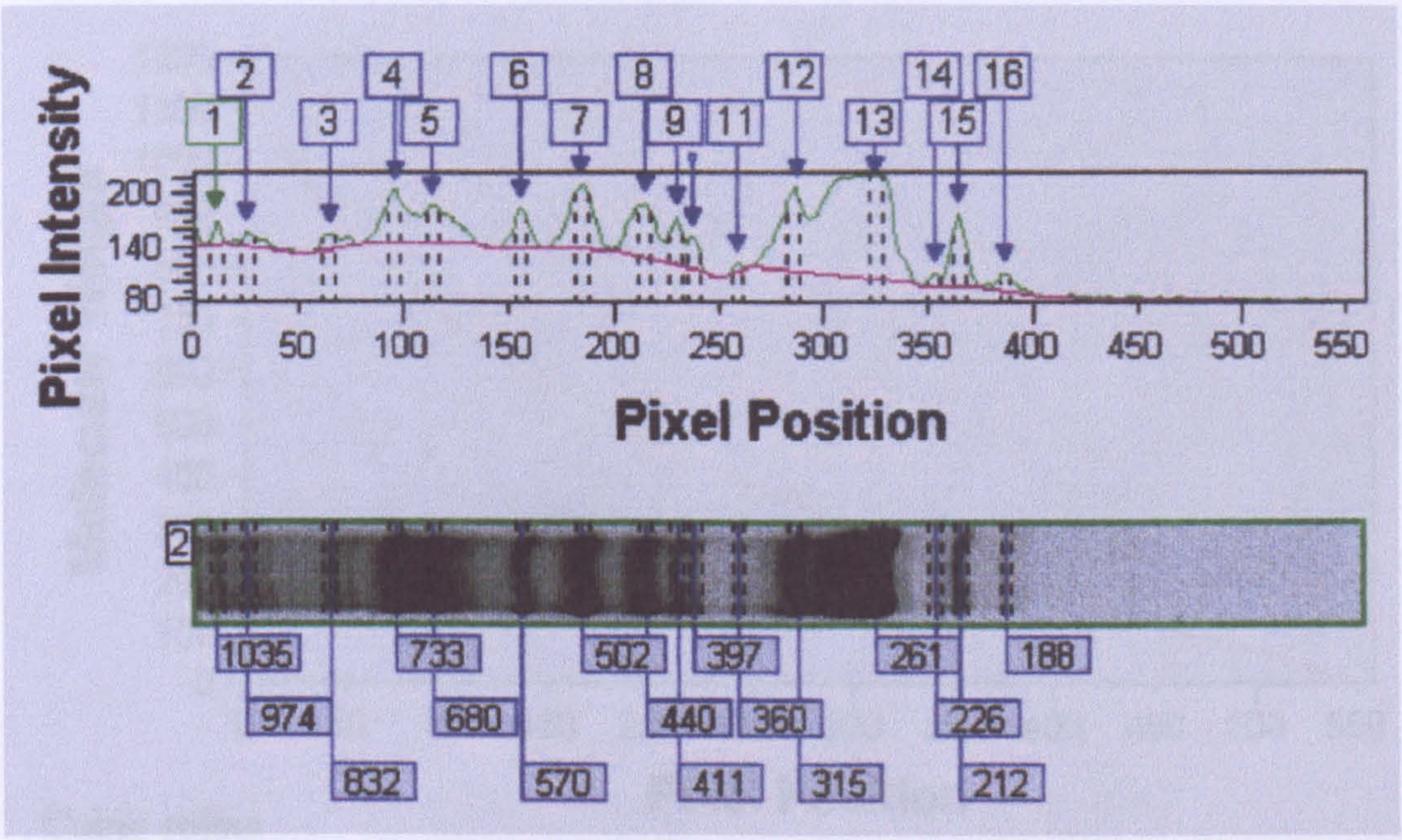


Figure 4-5 Bands of lane 1 in Figure 4-1 sized by the formula of log curve in Figure 4-4.

Another calibration curve by cubic spline method was also produced and illustrated in Figure 4-6. This method can give more accurate size especially for the fragments smaller than 100 bp.

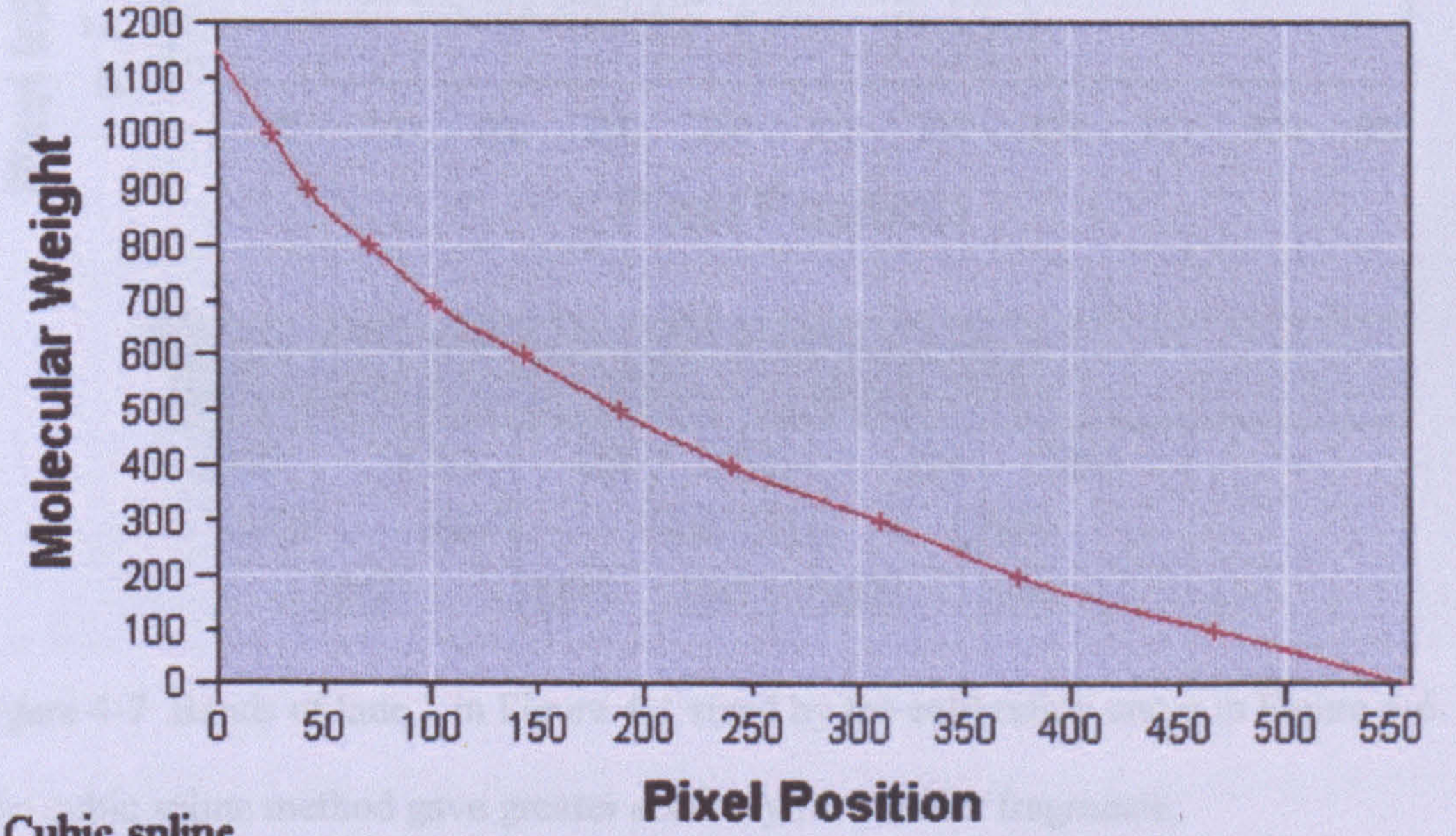


Figure 4-6 A calibration curve generated using the cubic spline method from data of the 100 bp ladder.

In this study, all bands were sized by cubic spline method due to the greater accuracy for sizing PCR products smaller then 100 bp. Figure 4-7 showed DNA sizes of bands in lane 1 of Figure 4-1.

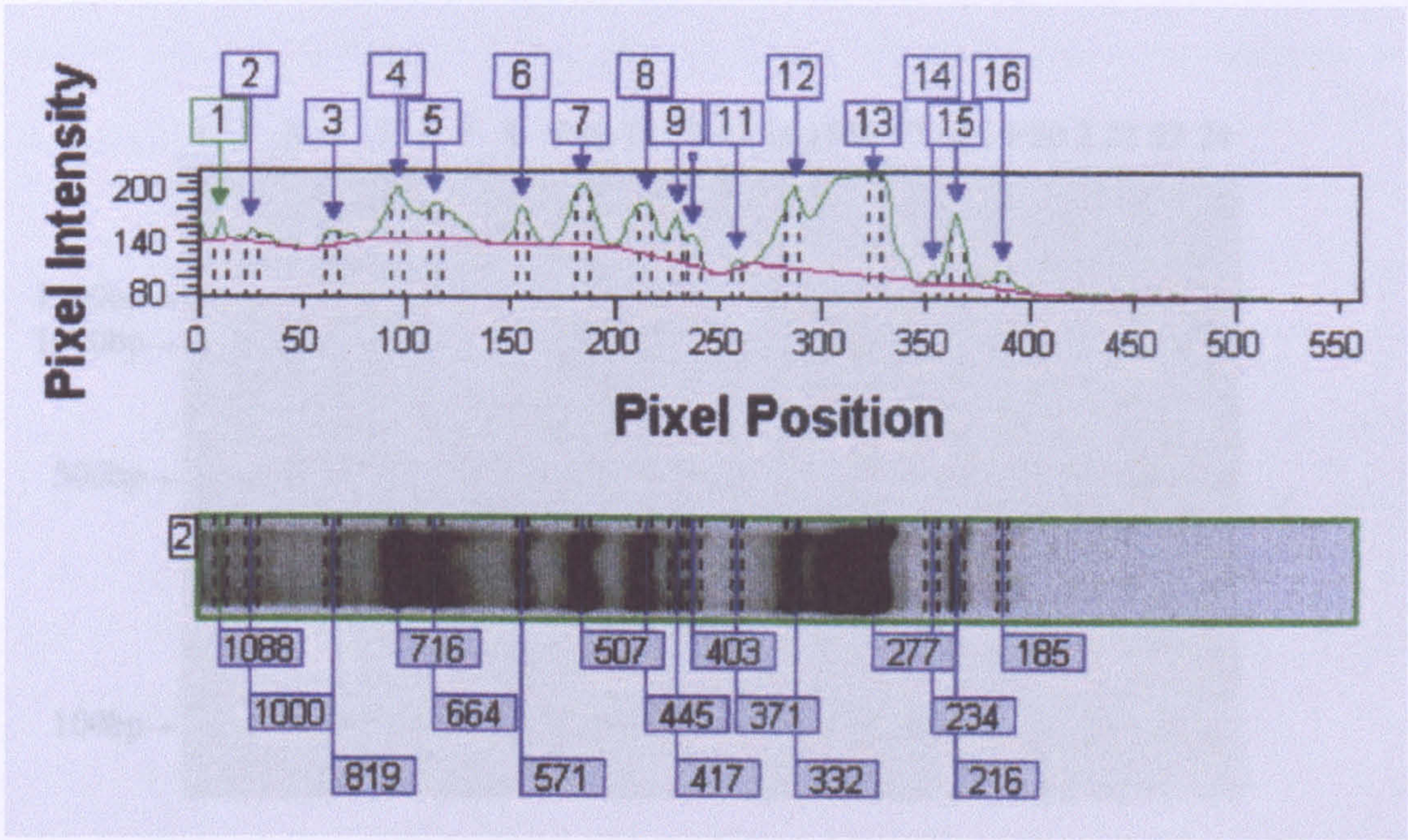


Figure 4-7 Bands of lane 1 in Figure 4-1 sized by the calibration curve in Figure 4-6.

The cubic spline method gave greater accuracy for smaller fragments.

All the gel lanes from primer A and primer B on gels 4-1 and 4-2 were analysed in the same way using the cubic spline method. After analysis of the PCR product bands, the gel was divided into 60 equal segments or bins. The bins ranged from 50 bp to 100 bp. To test the confidence of sizes obtained in this study, the 100 bp ladder was run 24 times on separate lanes of a gel and the position of each of the DNA ladders was measured. Figure 4-8 shows the image of the gel where the 100 bp ladder marker was separated on the polyacrylamide gel. This pattern can be used to establish the error of fragment size due to alterations in eletrophoretic mobility of fragments through a gel. Figure 4-8 illustrates the potential errors in comparing between separate lanes on the same gel due to voltage difference across the gel.

The size of each band was measured with respect to lane 1. Table 4-1 shows the size of each band in the gel as measured by this system. The standard deviation, size

range and average of each band in Table 4-1 are shown in Table 4-2.

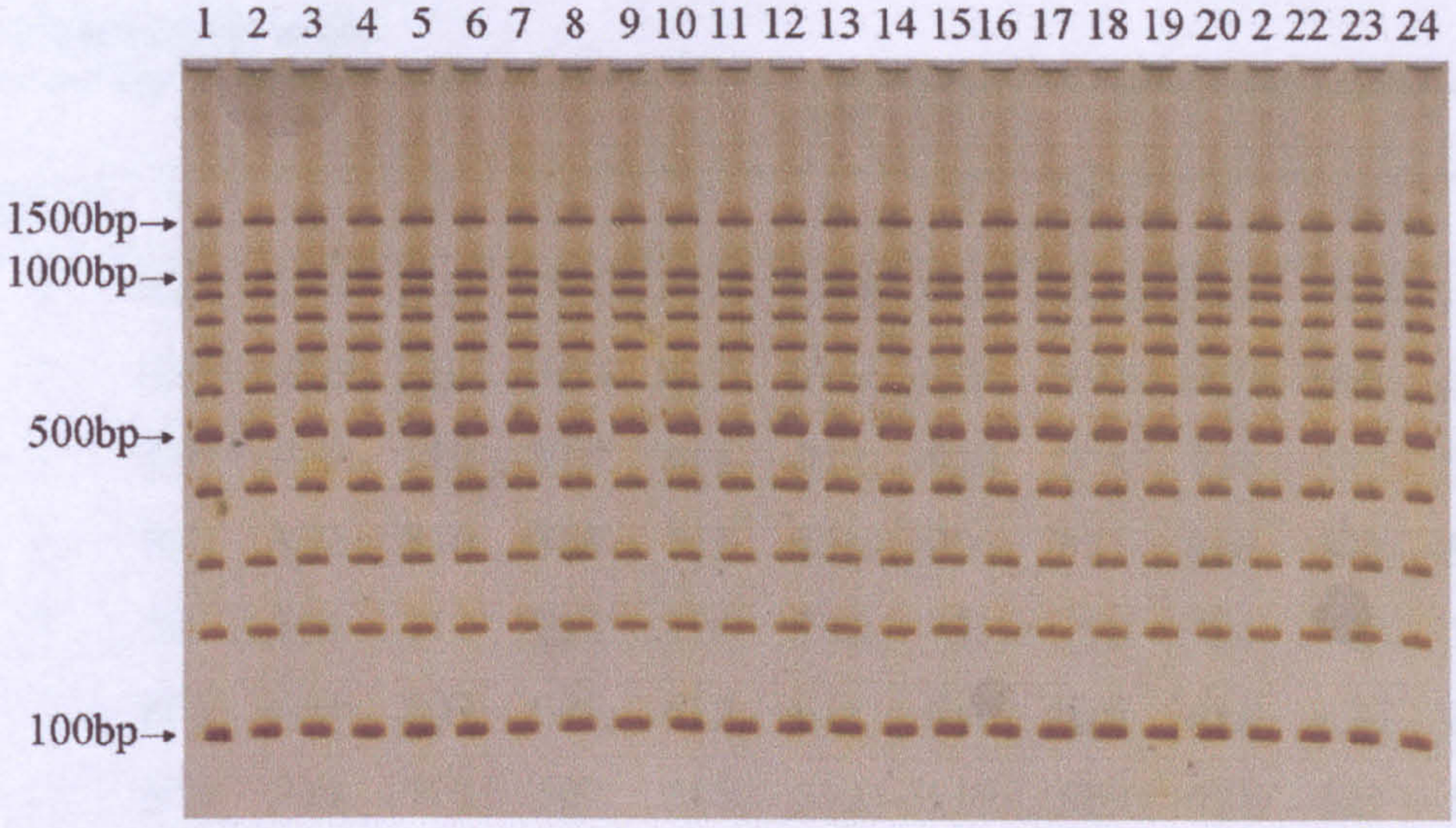


Figure 4-8 Gel image of the 100 bp ladder separated on a polyacrylamide GeneGel Excel 12.5/24 gel and silver stained. The image shows the effect the electrophoretic mobility on bands loading on different lanes on the same gel.

Table 4-1 Sizes of 100 bp ladder in Figure 4-8 estimated by Image master 1D. The size of each band was measured with respect to lane 1. The size range is shown in the last column of the table.

	Lane											
Band	1	2	3	4	5	6	7	8	9	10	11	12
1	1500	1504	1513	1509	1518	1518	1522	1522	1526	1526	1527	1531
2	1000	1003	1017	1014	1021	1014	1028	1018	1025	1039	1028	1039
3	900	905	910	910	918	912	920	915	918	929	920	932
4	800	802	810	808	811	811	816	813	816	818	816	820
5	700	704	710	710	714	712	721	718	720	722	718	723
6	600	603	609	609	612	611	618	616	618	620	614	619
7	500	504	510	507	515	512	519	519	521	521	519	521
8	400	403	408	406	408	409	414	412	416	415	415	412
9	300	302	305	305	307	305	309	311	313	313	311	312
10	200	202	203	203	205	206	210	212	214	212	211	214
11	100	102	103	102	103	104	106	109	111	109	109	107

	Lane											
13	14	15	16	17	18	19	20	21	22	23	24	Range
1531	1526	1527	1540	1522	1527	1518	1522	1522	1509	1513	1500	1500-1540
1028	1032	1021	1032	1035	1032	1039	1032	1028	1017	1014	1000	1000-1039
926	923	915	926	929	923	926	923	921	910	900	900	900-932
818	810	811	813	815	817	817	813	813	807	807	800	800-820
720	716	714	719	720	719	720	717	714	710	707	700	700-723
614	614	614	615	616	616	615	614	611	608	608	600	600-620
516	515	513	515	507	507	507	514	505	511	511	500	500-521
413	408	411	408	406	406	407	408	407	404	403	400	400-416
311	309	311	309	307	307	307	307	308	306	303	300	300-313
211	209	210	210	208	208	209	208	208	207	205	200	200-214
107	107	107	107	106	106	105	106	105	104	104	100	100-111

Table 4-2 Standard deviation of the 100 bp ladder from the data of Table 4-1.

Band	Size (bp)	Standard Deviation	Average	Band	Size	Standard Deviation	Average
1	1500	9.83	1519.70	7	500	6.27	512.04
2	1000	11.44	1023.16	8	400	4.42	408.29
3	900	9.33	917.12	9	300	3.66	307.41
4	800	5.44	811.75	10	200	3.93	207.70
5	700	6.51	714.50	11	100	2.76	105.37
6	600	5.36	6.1225				

4.3.3 Gel Binning and Computation

In order to decide which band should be scored in which segment, the size range of each segment was generated as shown in Table 4-3. This table shows how the gels are divided into 60 bins ranging from 1000 bp to 50 bp. In order to include the error of band shift 95% confident interval was used in this study. If the band was in the line between two segments, both segments were scored. If the band was sufficiently broad due to the intensity of the band or two bands running together at the same point in the gel and therefore covering more than one segment, all occupied segments were scored.

Table 4-3 The size range of each segment of the score system in this study.

No.	Range(bp)	No.	Range(bp)	No.	Range(bp)	No.	Range(bp)
1	1000-940	16	531-512	31	293-281	46	133-124
2	940-885	17	512-493	32	281-269	47	124-116
3	885-845	18	493-474	33	269-257	48	116-108
4	845-811	19	474-456	34	257-246	49	108-100
5	811-780	20	456-438	35	246-235	50	100-93
6	780-750	21	438-420	36	235-224	51	93-86
7	750-722	22	420-404	37	224-213	52	86-80
8	722-695	23	404-388	38	213-202	53	80-75
9	695-667	24	388-373	39	202-191	54	75-70
10	667-640	25	373-359	40	191-181	55	70-66
11	640-615	26	359-345	41	181-171	56	66-62
12	615-593	27	345-332	42	171-161	57	62-59
13	593-571	28	332-319	43	161-151	58	59-56
14	571-551	29	319-306	44	151-142	59	56-53
15	551-531	30	306-293	45	142-133	60	53-50

An analysis system where the gels are divided into 60 bins based on molecular weights has now been developed. This system can now be applied to data from the PCR products separated in Figures 4-1 and 4-2. The presence of absence of bands was scored and a Table produced for each Figure (Table 4-4 and 4-5).

Table 4-4 Genotype scores obtained from Figure 4-1. Columns 1-20 are samples PA1-1, PA1-2, PA1-3, PA1-4, PA2, PA3, PA4, PA5, PA6, PA7, PS1-1, PS1-2, PS1-3, PS1-4, PS2, PS3, PS4, PS5, PS6-1 and PS6-2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
01	1	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
02	0	0	1	0	1	0	0	0	1	0	1	1	0	1	0	1	1	1	0	0
03	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1
04	1	1	1	0	1	0	0	1	0	0	0	0	1	0	0	1	1	1	1	1
05	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
06	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1
07	1	1	0	0	1	0	1	1	1	0	0	0	1	0	1	0	0	1	1	1
08	1	1	1	1	1	1	0	1	0	1	0	1	1	0	1	1	1	1	1	1
09	1	1	1	1	1	1	0	0	0	1	0	1	1	0	1	1	1	1	1	1
10	1	1	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	0	0	0
11	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0
12	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0
13	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0
14	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	1	0	1	1	1
15	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0
16	1	1	0	0	0	1	1	1	0	0	0	1	0	0	1	0	1	0	0	0
17	1	1	1	0	0	1	0	0	0	0	1	1	1	1	1	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1
19	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	1	0	1	1	1
20	0	1	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1
21	1	1	0	1	1	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1
22	1	1	0	0	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	1
23	1	1	0	1	0	1	1	0	0	0	0	0	1	0	1	0	0	1	1	1
24	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0
25	1	1	1	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	0	0
26	0	0	1	0	1	1	1	0	0	0	1	0	0	1	1	0	0	0	1	1
27	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1
28	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1
29	0	0	0	1	0	0	1	1	1	1	0	0	1	0	0	0	1	1	1	1
30	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1
31	1	1	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	1
32	1	1	0	1	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0	0
33	0	0	0	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	1	1
34	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
35	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0
36	1	1	0	0	0	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0
37	1	1	0	0	1	1	1	1	0	0	1	1	0	1	0	1	1	1	0	0
38	1	1	1	0	0	0	0	1	0	0	1	1	0	1	0	0	1	1	1	1
39	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1
40	1	1	1	0	0	1	0	0	0	0	1	1	0	1	0	1	1	1	1	1
41	0	0	1	0	1	1	0	1	1	1	1	1	0	0	0	0	0	1	1	1
42	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	1	1	1	1
43	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1
44	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1

45	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4-5 was generated from Figure 4-2 by primer B. The genetic similarity was calculated by the method of Dice [83].

Table 4-5 Genotype scores obtained from Figure 4-2. Columns 1-20 are samples PA1-1, PA1-2, PA1-3, PA1-4, PA2, PA3, PA4, PA5, PA6, PA7, PS1-1, PS1-2, PS1-3, PS1-4, PS2, PS3, PS4, PS5, PS6-1 and PS6-2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
01	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1
02	1	1	1	0	1	1	1	0	0	1	0	0	0	0	1	0	1	1	1	1
03	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1	0	0	0
04	1	1	1	1	0	0	1	1	0	0	0	0	1	1	0	1	1	0	1	1
05	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	0	0
06	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0
07	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
08	0	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
09	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
10	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	0	0	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1
12	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1
13	1	1	1	0	0	0	1	1	0	1	0	1	1	0	0	1	1	1	1	1
14	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	0	1	1	1
15	0	0	0	0	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1
16	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	0	1	0	0	0
17	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1
18	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1
19	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1
20	0	0	0	0	1	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1
21	0	0	0	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	0	0
22	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1
23	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	1	1	1
24	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	1	1	0	0
25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1
27	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	0	1	1	1
28	0	0	0	0	0	1	1	0	0	1	1	1	1	1	0	1	1	1	1	0
29	1	1	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	0	1	0	1	0	1	0	0	1	1	1	0	0	1
31	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0
32	0	0	0	0	1	1	0	0	1	1	1	0	0	1	0	0	1	0	1	1
33	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	0	1	1
34	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	0	1	1
35	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	1	1	1	0	0
36	1	1	1	0	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0
37	1	1	1	1	0	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1
38	1	1	1	0	1	0	0	0	1	1	0	1	1	1	1	0	0	1	1	1
39	1	1	1	0	0	1	1	1	0	0	1	0	0	0	0	0	1	1	1	1
40	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	0	0	0
41	1	1	1	1	0	0	0	1	0	1	1	1	1	1	0	0	0	1	1	1
42	1	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0	1	1	0	0
43	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1
45	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1

46	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	0	0
47	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	1	1
48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4.3.4 Genetic Matrices

The data of 0's and 1's can be converted into a genetic similarity matrix, which compares the degree of similarity, i.e. number of bands shared between each of the 20 samples. When a sample is compared to itself a score of 1 will be obtained, as all the bands must match. Scores lower than 1 but close to 1 indicate a high degree of similarity. The scores closer to 0 indicate that few bands match, and therefore the two samples are less related. The results of data set from Table 4-4 are shown in Table 4-6.

Table 4-6 Genetic similarity matrix from the RAPD Scores in Table 4-4. The data came from the gel of PCR amplification using primer A on 20 fungal samples. Columns 1-20 are PA1-1, PA1-2, PA1-3, PA1-4, PA2, PA3, PA4, PA5, PA6, PA7, PS1-1, PS1-2, PS1-3, PS1-4, PS2, PS3, PS4, PS5, PS6-1 and PS6-2.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.000																			
0.837	1.000																		
0.470	0.487	1.000																	
0.758	0.611	0.370	1.000																
0.450	0.468	0.526	0.424	1.000															
0.391	0.566	0.318	0.358	0.560	1.000														
0.400	0.425	0.263	0.303	0.500	0.480	1.000													
0.526	0.622	0.222	0.451	0.619	0.541	0.523	1.000												
0.068	0.222	0.370	0.090	0.424	0.256	0.363	0.387	1.000											
0.588	0.585	0.375	0.444	0.368	0.454	0.421	0.444	0.222	1.000										
0.242	0.400	0.451	0.076	0.324	0.418	0.270	0.285	0.307	0.258	1.000									
0.512	0.608	0.432	0.375	0.465	0.571	0.279	0.487	0.187	0.486	0.722	1.000								
0.540	0.545	0.400	0.466	0.439	0.468	0.390	0.512	0.333	0.514	0.294	0.500	1.000							
0.421	0.488	0.444	0.258	0.476	0.458	0.333	0.400	0.322	0.222	0.685	0.682	0.512	1.000						
0.400	0.523	0.303	0.357	0.512	0.488	0.512	0.540	0.357	0.242	0.187	0.368	0.611	0.486	1.000					
0.523	0.530	0.550	0.400	0.608	0.538	0.478	0.545	0.285	0.450	0.410	0.666	0.604	0.636	0.487	1.000				
0.409	0.509	0.523	0.270	0.625	0.518	0.458	0.521	0.324	0.380	0.341	0.510	0.444	0.521	0.465	0.680	1.000			
0.536	0.583	0.666	0.352	0.533	0.392	0.444	0.558	0.470	0.410	0.473	0.500	0.571	0.511	0.500	0.638	0.571	1.000		
0.545	0.588	0.571	0.378	0.500	0.518	0.458	0.565	0.378	0.380	0.536	0.595	0.622	0.565	0.511	0.600	0.538	0.775	1.000	
0.577	0.615	0.604	0.368	0.530	0.509	0.448	0.553	0.368	0.372	0.523	0.583	0.652	0.595	0.545	0.627	0.566	0.800	0.981	1.000

The genetic similarity matrix in Table 4-6 can be converted into a dendrogram, which more graphically illustrates the degree of relatedness between the samples. The cluster method called UPGAM was used to produce the dendrogram.

Table 4-7 was generated from the genetic similarity matrix obtained from the RAPD scores in Table 4-5. These genetic similarity matrices were then further clustered by UPGAM method.

Table 4-7 Genetic similarity matrix from the RAPD Scores in Table 4-5. The data came from the gel of PCR amplification using primer B on 20 fungal samples. 1- 20 are PA1-1, PA1-2, PA1-3, PA1-4, PA2, PA3, PA4, PA5, PA6, PA7, PS1-1, PS1-2, PS1-3, PS1-4, PS2, PS3, PS4, PS5, PS6-1 and PS6-2.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.00																			
0.96	1.00																		
0.94	0.98	1.00																	
0.73	0.72	0.75	1.00																
0.64	0.63	0.62	0.63	1.00															
0.54	0.53	0.55	0.56	0.74	1.00														
0.74	0.69	0.71	0.61	0.68	0.70	1.00													
0.82	0.81	0.84	0.78	0.70	0.63	0.81	1.00												
0.57	0.55	0.58	0.67	0.83	0.63	0.64	0.65	1.00											
0.67	0.63	0.65	0.59	0.77	0.63	0.74	0.69	0.69	1.00										
0.52	0.50	0.49	0.59	0.68	0.60	0.58	0.59	0.75	0.69	1.00									
0.58	0.57	0.59	0.63	0.72	0.61	0.62	0.66	0.80	0.77	0.89	1.00								
0.61	0.60	0.56	0.63	0.62	0.50	0.61	0.62	0.69	0.69	0.83	0.86	1.00							
0.57	0.55	0.58	0.62	0.64	0.59	0.60	0.61	0.74	0.68	0.85	0.84	0.86	1.00						
0.59	0.53	0.56	0.52	0.70	0.58	0.59	0.57	0.68	0.53	0.54	0.61	0.53	0.56	1.00					
0.52	0.49	0.48	0.48	0.60	0.61	0.63	0.60	0.53	0.53	0.58	0.61	0.61	0.60	0.58	1.00				
0.75	0.71	0.70	0.61	0.70	0.68	0.75	0.70	0.63	0.70	0.67	0.67	0.61	0.63	0.59	0.57	1.00			
0.68	0.63	0.62	0.51	0.65	0.63	0.71	0.65	0.55	0.69	0.66	0.66	0.69	0.61	0.60	0.57	0.63	1.00		
0.64	0.63	0.65	0.60	0.62	0.63	0.68	0.69	0.69	0.66	0.77	0.74	0.78	0.79	0.64	0.57	0.69	0.69	1.00	
0.65	0.64	0.67	0.64	0.63	0.64	0.62	0:70	0.67	0.64	0.72	0.72	0.73	0.75	0.65	0.59	0.67	0.63	0.96	1.00

Figures 4-9 and 4-10 are dendrograms produced by the genetic similarity matrix in Table 4-6 and 4-7, respectively.

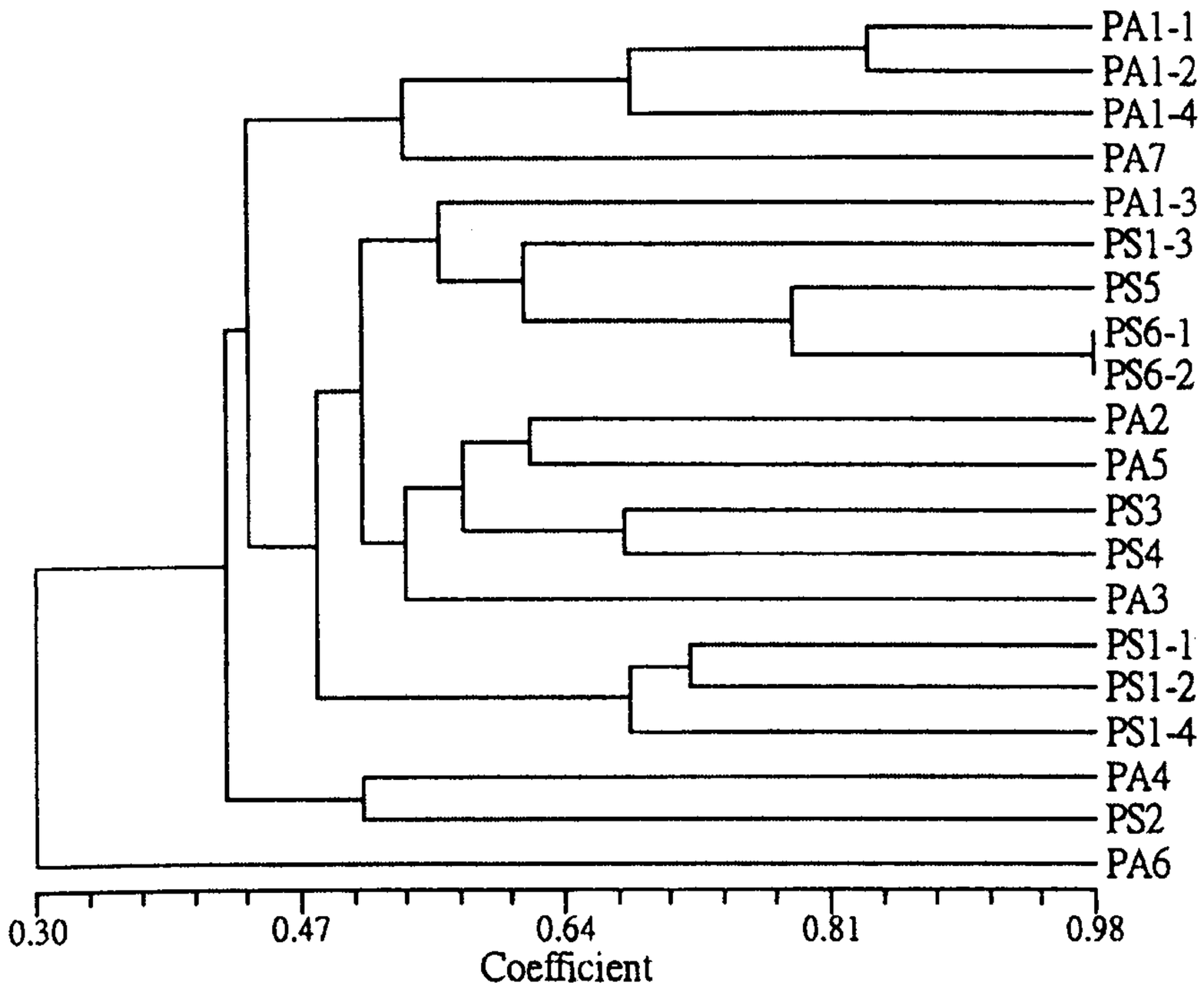


Figure 4-9 Dendrogram generated by the genetic similarity matrix in Table 4-6

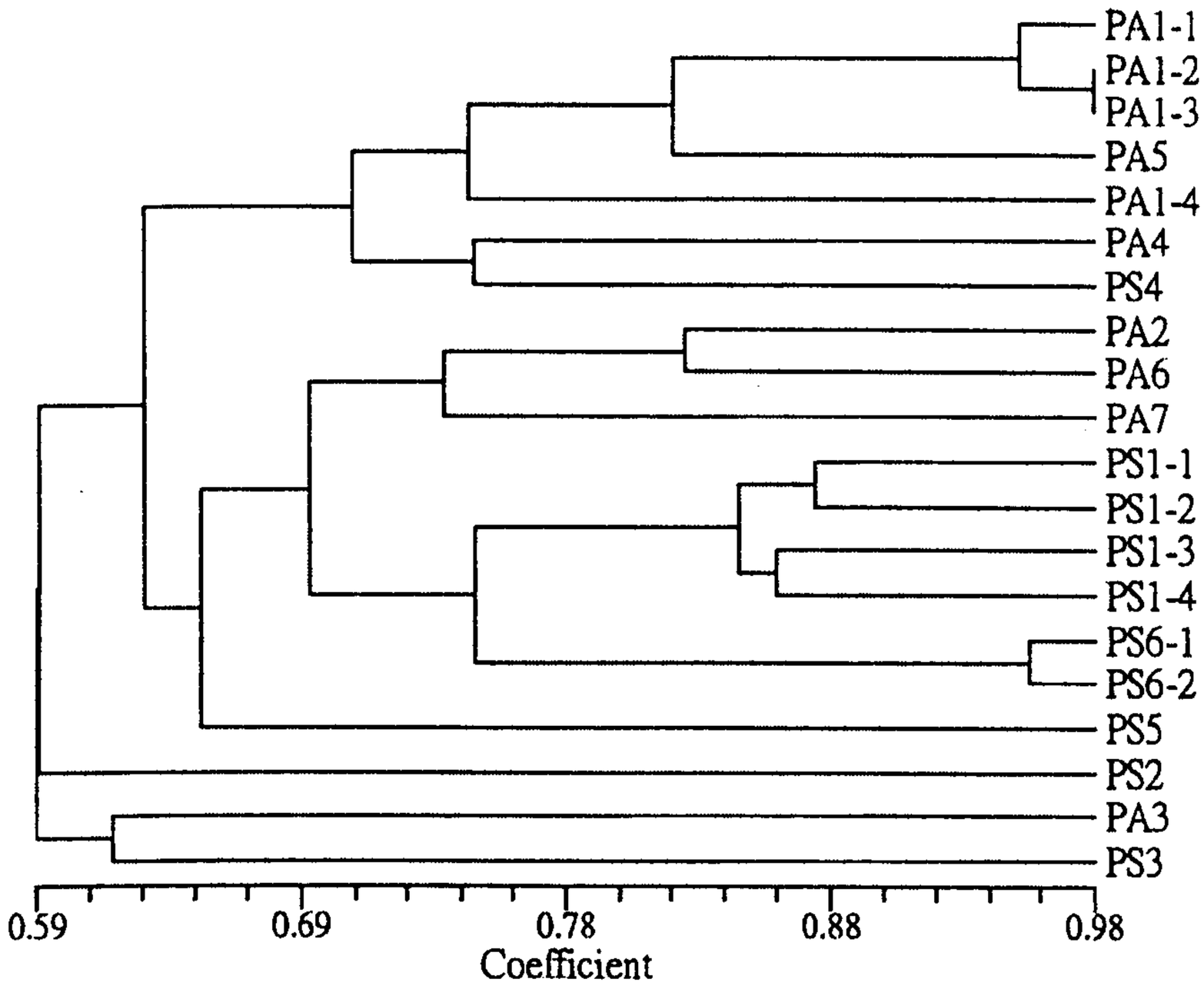


Figure 4-10 Dendrogram generated by the genetic similarity matrix in Table 4-7.

It can be seen from Primer A (Figure 4-9) that the RAPD cannot correctly group all the *Panaeolus* and *Psilocybe* respectively. This would be expected given that one RAPD pattern is usually not enough to provide patterns for the identification of a species. The dendrogram for primer B (Figure 4-10) shows that there is incomplete separation of the two genera with PA 3 showing closer relatedness to *Psilocybe* samples than to other samples from the genera *Panaeolus*. This primer has clustered the *Psilocybe* samples from the same species together.

In order to demonstrate the cluster effect of these two RAPD patterns, The RAPD scores in Table 4-4 and 4-5 were combined. The genetic similarity matrix was generated and shown in Table 4-8.

Table 4-8 Genetic similarity matrix from the RAPD scores in Table 4-4 and 4-5.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.000																			
0.905	1.000																		
0.758	0.760	1.000																	
0.743	0.674	0.613	1.000																
0.562	0.554	0.580	0.547	1.000															
0.475	0.547	0.448	0.471	0.654	1.000														
0.597	0.568	0.531	0.494	0.601	0.592	1.000													
0.702	0.727	0.593	0.658	0.660	0.590	0.693	1.000												
0.400	0.422	0.512	0.493	0.681	0.479	0.543	0.561	1.000											
0.645	0.612	0.555	0.543	0.626	0.557	0.620	0.597	0.545	1.000										
0.421	0.460	0.478	0.433	0.554	0.528	0.470	0.484	0.622	0.551	1.000									
0.557	0.587	0.534	0.543	0.618	0.591	0.486	0.592	0.606	0.672	0.825	1.000								
0.583	0.574	0.516	0.571	0.549	0.485	0.524	0.580	0.571	0.626	0.653	0.727	1.000							
0.510	0.524	0.526	0.488	0.576	0.532	0.495	0.529	0.602	0.514	0.796	0.785	0.730	1.000						
0.511	0.527	0.457	0.459	0.608	0.536	0.559	0.555	0.567	0.426	0.417	0.520	0.565	0.531	1.000					
0.516	0.510	0.511	0.444	0.606	0.576	0.560	0.577	0.431	0.500	0.510	0.635	0.606	0.613	0.539	1.000				
0.609	0.618	0.627	0.473	0.666	0.603	0.625	0.623	0.520	0.574	0.545	0.605	0.540	0.584	0.534	0.629	1.000			
0.618	0.607	0.638	0.447	0.601	0.518	0.596	0.613	0.521	0.580	0.588	0.594	0.640	0.571	0.559	0.600	0.607	1.000		
0.603	0.612	0.621	0.510	0.571	0.581	0.584	0.636	0.574	0.550	0.684	0.683	0.714	0.701	0.588	0.587	0.628	0.725	1.000	
0.622	0.630	0.640	0.531	0.589	0.581	0.548	0.636	0.554	0.532	0.648	0.666	0.696	0.684	0.607	0.605	0.628	0.707	0.967	1.000

Figures 4-11 is dendragrams produced by the genetic similarity matrix in Table 4-8

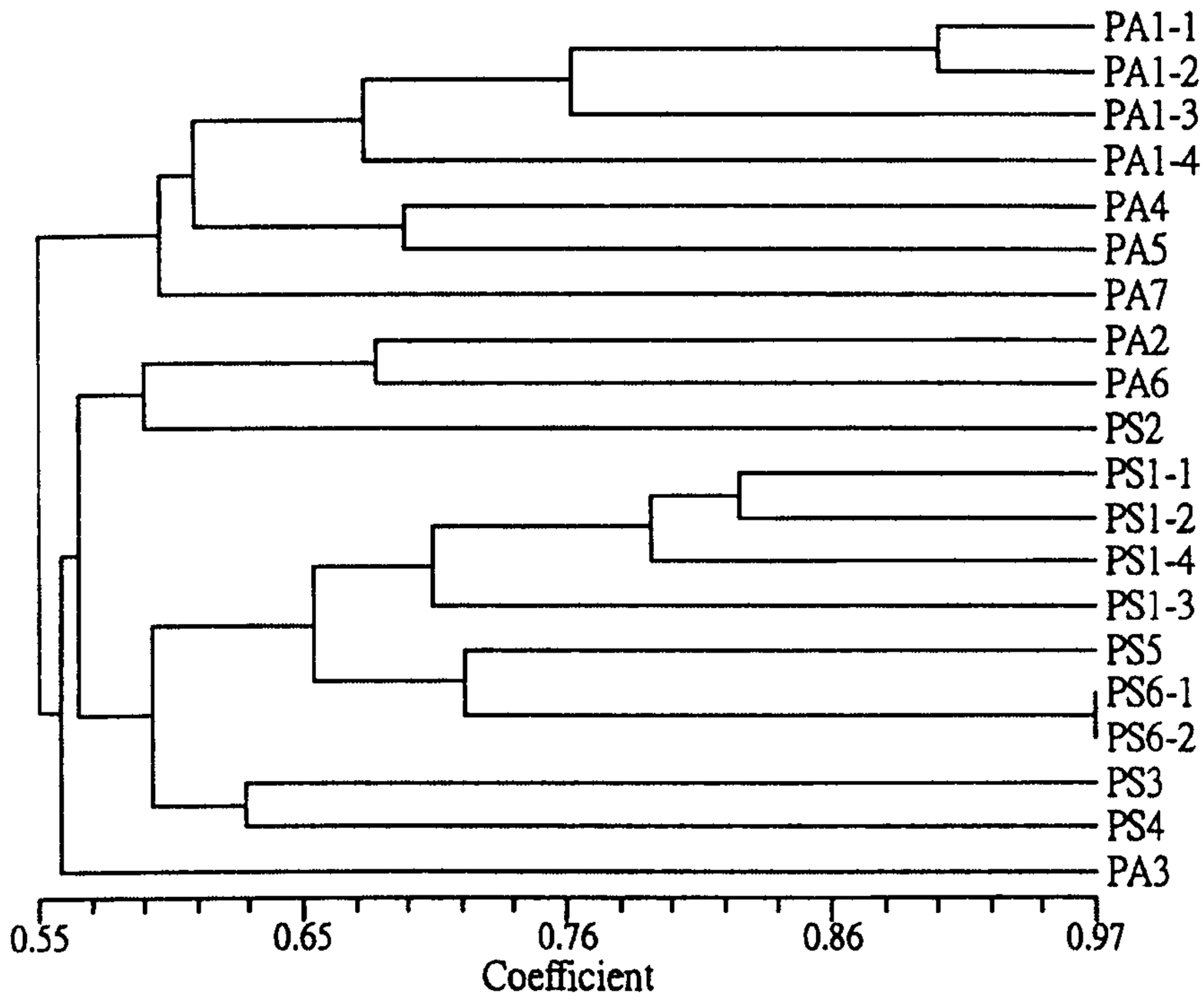


Figure 4-11 Dendrogram generated by the genetic similarity matrix in Table 4-8.

In Figure 4-11, the RAPD has grouped most of the *Panaeolus* samples together separate from all the *Psilocybe* samples except PA2 and PA6. Samples from the same species PA1-1, PA1-2, PA1-3 and PA1-4, PS1-1, PS1-2, PS1-3 and PS1-4, PS6-1 and PS6-2 were clustered together respectively. This would be expected given that intergenic variation would be expected to be greater than intragenic variation. The more RAPD patterns provide more accurate and discriminatory method for systematic analysis.

4.4 Review of RAPD on Fungi

This study has shown that RAPD, using two primers, can separate 20 samples from 5 different species of *Psilocybe* and 6 species of *Panaeolus* into 2 genera. Only two primer sequences were investigated and it is possible that other primers would be more effective at separating firstly genera and then species. The use of RAPD does require controlled standard conditions, which are not always available in forensic cases. Additionally RAPD could not detect mixtures of samples. It is, however, a fast, cheap, and simple method that requires no prior sequence information. A test that is more specific will be investigated in the next chapter.

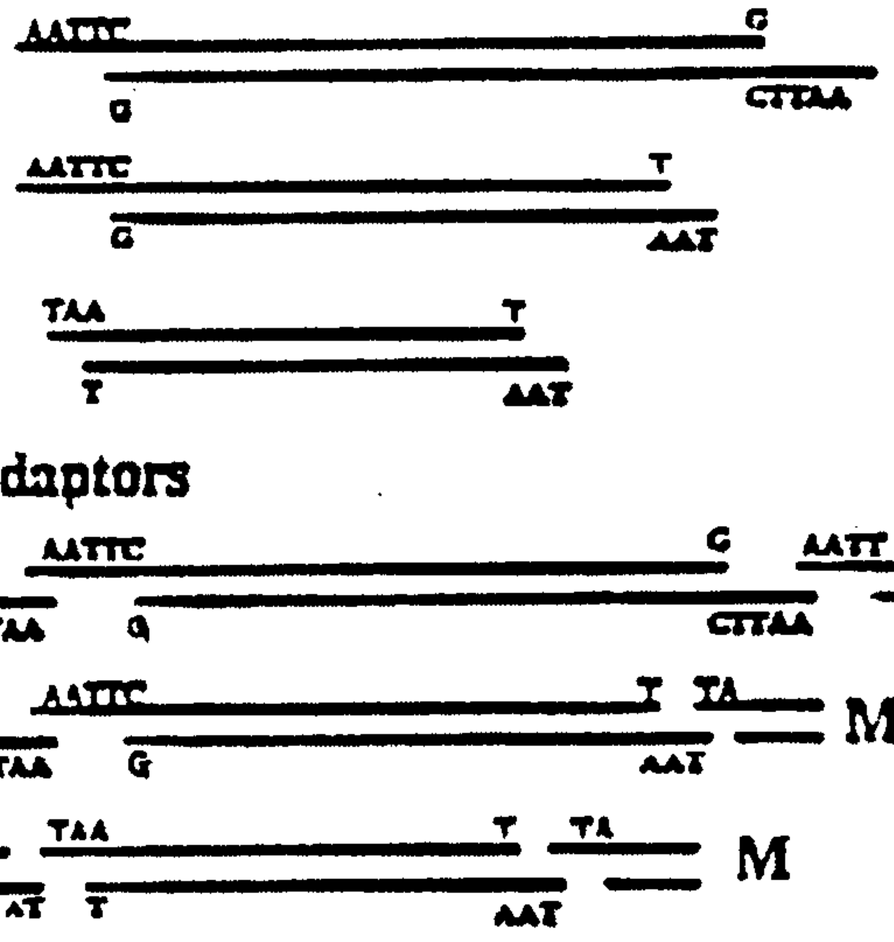
5 DNA ANALYSIS BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

5.1 Introduction

The technique of Amplified Fragment Length Polymorphism (AFLP) was developed by Vos et al.[58] in 1995, and is based on the selective PCR amplification of restriction fragments from a digested genomic DNA. This technique combines the advantages of the RFLP and PCR. No prior sequence knowledge is needed. This pattern has proveN useful in taxonomic studies [59-70].

In AFLP analysis, DNA extracted from the sample is cut with two restriction enzymes to produce overhangs of single stranded DNA. The two restriction enzymes are EcoRI, which leaves an 'AATTC' overhang on the 3' end, and MseI, which leaves an 'AATT' single strand overhang at the 3' end. Adapters made to the overhanging single stranded DNA are ligated to the ends of the restriction fragments. The adaptors are up to 20 bases in length and are made to known DNA sequences. The sequence of the adapters and adjacent restriction site serve as primer binding sites for subsequent amplification of the restrictions (Figure 5-1)[85]. Primers for PCR are made to the adaptor sequence and the restriction recognition sequence. Additionally the primers are increased in length by up to three bases up the addition of selective nucleotides. These selective nucleotides are added to the 3' ends of the PCR primers, reducing the number of restriction fragments to which the primer can bind. The addition of one extra base will reduce the number of restriction sites to be amplified by approximately one quarter, with the addition of two extra bases being found in one sixteenth of the fragments by chance. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified.

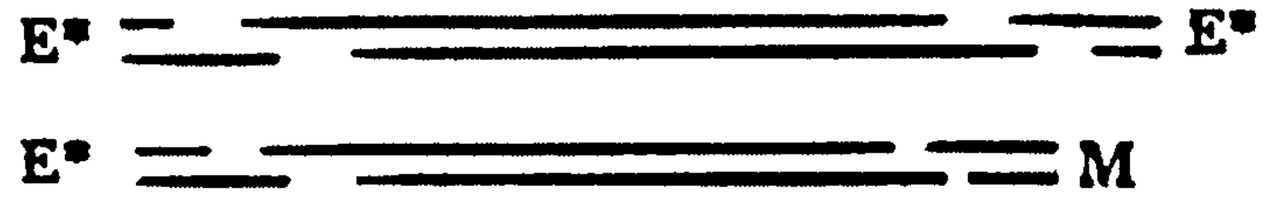
1) Digestion with *EcoRI* and *MseI*



2) Ligation of adaptors

E*: biotinylated adaptor

3) Selection of biotinylated fragments



4) Preamplification with 1 selective nucleotide

Primer 1: (E ADAPTOR) -(AATTC) -(N)

Primer 2: (M ADAPTOR) -(TAA) -(N)

5) Amplification with 3 selective nucleotides

Primer 1: (^{32}P E ADAPTOR) -(AATTC) -(NNN)

Primer 2: (M ADAPTOR) -(TAA) -(NNN)

Figure 5-1 Major steps of AFLP analysis [85]

The aim of this chapter AFLP was designed to determine the genetic relatedness of fungal species and examine whether this technique could form the basis of a DNA test to either determine the genus or species present. Fungi from members of the *Psilocybe* and *Panaeolus* genera and three kinds of edible mushrooms were analyzed. Three selective primers on Mse I site and five selective primers on EcoR I site were used to generate the AFLP fingerprints. Image analysis of AFLP fingerprints was similar to the method used in RAPD study of chapter 4.

5.2 Materials and Methods

5.2.1 Extraction of Genomic DNA

DNA samples of the mushrooms extracted in section 3.4 were used in this analysis. In order to compare the AFLP patterns beyond the psilocybin-containing mushroom, this study chose three edible mushrooms encountered commonly in Taiwan. There are *Volvariella volvacea* (VO), *Agaricus bisporus* (AG), and *Lentinus edodes* (LE). The extraction method is the same as described in section 3.4.

5.2.2 Restriction Digestion of Genomic DNA

Genomic DNA was digested with EcoR I and Mse I enzymes (Life Technologies Kit) described as following:

Add the following to a 0.5-mL microcentrifuge tube:

Component	Control	Sample
5X reaction buffer	5 μl	5μl

Tomato control DNA (100 ng/µl)	2.5 µl	
Sample DNA (250 ng in18µl)		18µl
EcoR I/Mse I	2µl	2µl
ddH2O	15.5µl	To 25µl
Total volume	25µl	25µl

2. Mix gently and collect the reaction by brief centrifugation. Incubate the mixture 2 hrs at 37 °C.
3. Incubate the mixture for 15 min. at 70 °C to inactivate the restriction endonucleases. Place tube on ice and collect contents by brief centrifugation.

5.2.3 Ligation of Adapters (Life Technologies Kit)

Add the following to the digested DNA from step 6.2.3.

Component	Volume
Adapter ligation solution	24µl
T4 DNA ligase	1µl

2. Mix gently at room temperature, centrifuge briefly to collect contents, and incubate at 20 °C ±2 °C for 2 hrs.
3. Perform 1:10 dilution of the ligation mixture as follows:

a. Take 10µl of the reaction mixture and transfer to a 1.5-mL microcentrifuge tube.

b. Add 90 µl TE buffer and mix well.

c. the unused portion of the reaction mixture may be stored at -20 °C.

5.2.4 Preselective Amplification of Target Sequences (Perkin Elmer Kit)

1. Combine the following in a PCR tube:

   4.0µl of the diluted restriction-ligation DNA from step 6.2.5.

   1.0µl of the AFLP Preselective Primer pairs

   EcoR I primer: 5'-GACTGCGTACCAATTC-3'

   Mse I primer: 5'-GATGAGTCCTGAGTAA-3'

   15.0µl AFLP Core Mix

   20µl of light mineral oil

2. Run the following PCR protocol on the DNA Thermal Cycler 480.

3. Store at 4 °C.

PCR temperature control parameters for Preselective Amplification

Hold	Cycle	Hold	Number of Cycles
72 °C 2 min	-	-	1
94 °C 1 sec	56 °C 30 sec	72 °C 2 min	20
60 °C 30 min	-	-	1
4 °C Forever			

5.2.5 Selective Amplification of Target Sequences (Perkin Elmer Kit)

1. Prepare a template for selective AFLP reactions:

   a. Combine the following in a sterile 0.5-mL microcentrifuge tube:

   10 µl Preselective Amplification reaction product

   190 µl TE buffer

b. Mix thoroughly, then spin down in a microcentrifuge for ten seconds.

c. Store at 4 °C.

2. Perform Selective amplification:

a. Combine the following in a PCR reaction tube:

3.0µl diluted Preselective Amplification reaction product

1.0µl MseI [Primer -Cxx] at 5µM

1.0µl EcoRI [Dye-primer-Ax/Tx] at 1µM

15.0µl AFLP Core Mix

20µl of light mineral oil

3. Run the following PCR protocol on the DNA Thermal Cycler 480.

4. Store at 4 °C.

PCR temperature control parameters for Selective Amplification

Hold	Cycle	Hold	Number of cycle
94 °C 2 min	65 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	64 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	63 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	62 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	61 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	60 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	59 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	58 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	57 °C 30 sec	72 °C 2 min	1
94 °C 1 sec	56 °C 30 sec	72 °C 2 min	23

60 °C 30 min	1
4 °C forever	

5. There were six primer pairs used in this study, primer pair A+5, A+7, B+4, B+8, H+6 and H+8. The sequence of the primers is shown in the table below.

Sequences of selective primers

Primer name	Sequence
	Primers on Mse I site
A	5'-GATGAGTCCTGAGTAACAA-3'
B	5'-GATGAGTCCTGAGTAACAC-3'
H	5'-GATGAGTCCTGAGTAACTT-3'
	Primers on EcoR I site
4	5'-GACTGCGTACCAATTCACC-3'
5	5'-GACTGCGTACCAATTCAGC-3'
6	5'-GACTGCGTACCAATTCAAG-3'
7	5'-GACTGCGTACCAATTCAGG-3'
8	5'-GACTGCGTACCAATTCACG-3'

5.2.6 Electrophoresis

1. After PCR amplification, mix 1.5 µl of PCR products and 3.5µl bromophenol blue dye (glycerol 50%, bromophenol blue 0.1%, 10 mM Tris base and 0.1 M EDTA, pH 7.5) for the loading mixture.

2. The loading mixtures were loaded on the GeneGel Excel 12.5/24 gel (Pharmacia Biotech, Sweden), which is a precasted 12.5% polyacrylamide gel, and run on GenePhor Electrophoresis Unit. Electrophoreses at 600V, 25mA and 15W for 80 minutes until bromophenol blue reaches 1 cm margin ahead the anode buffer strip.

5.2.7 Detection

1. After electrophoresis, stain the gel in the Hoefer Automated Gel Stainer together with PlusOne DNA Silver Staining Kit for automatic staining.

2. Run the following staining protocol on the Hoefer Automated Gel Stainer.

DNA silver stain protocol on the Hoefer Automated GelStainer

Step	Solution	Time
1	Fixing sol.	30 min
2	Silver sol.	30 min
3	Water (washing)	1 min
4	Developing sol.	6 min
5	Stopping and preserving sol.	30 min

5.2.8 Image Analysis of AFLP Band Pattern

5.2.8.1 Band Detection

AFLP band patterns were detected and sized using Image master 1D (Pharmacia Biotech) with parameters of slope 200, noise 10 and width 10.

5.2.8.2 Score Assignment

Each gel was divided into 60 equal segments between 50 bp and 1000 bp. The presence or absence of bands was then scored 1 or 0 to produce a binary data matrix.

5.2.8.3 Cluster Analysis

The genetic similarities (GS) between each sample was calculated using the formula $GS_{ij} = 2Nij/(N_i + N_j)$, where N_{ij} is the number of common loci between i and j [83]. The GS matrix was produced by the comparison of each sample pair. A dendrogram can then be generated using the unweighted pair group method algorithm (UPGMA) clustering procedure using the NTSYS software [84].

5.3 Results and Discussion

The technique of AFLP produces three types of product; MseI double digests are expected to be the most common as this enzyme has a four base recognition sequence. EcoRI-MseI fragments would be expected to be less common than the double four cutter and more common than the EcoRI double digests, which would be expected to be the rarest. Using the simple silver stain technique all three products will be detected. The number of bands detected by any primer pair is important, as sufficient bands must be produced to allow analysis to be performed, but too many bands will produce bands that match by chance although they are unrelated. The number of bands detected is affected by the primer pairs and by adding additional bases to the primer at the 3' end. By altering the length of the primers to include two additional bases on the MseI end (MseI +2) and one additional base on the EcoRI end (EcoRI +1) the number of PCR products are reduced. More bases could have been incorporated to the primers to reduce further the number of PCR products, but for this study sufficient discriminatory bands were produced with the primer pairs chosen.

5.3.1 Calibration

Three species of agriculturally produced fungi namely the Padi straw mushroom *Volvariella volvacea*, the cultivated white mushroom *Agaricus bisporus* and Shiitake *Lentinus edodes* were tested at the same time as the *Psilocybe* and *Panaeolus spp*. This was to ensure that the AFLP test could discriminate between *Psilocybe spp, Panaeolus spp* and other commonly occurring fungi. The reproducibility of the banding pattern was tested by performing the same AFLP test twice. On each gel the 100 bp size ladder was run at each end of the gel. The ladder was calibrated by separating the size marker 24 times on a gel to determine the range of size for each ladder as determined by the imaging software as described in chapter 4. From this a band size with 95% confidence interval was assigned and addedto the scoring system. The size range of 60 equal segments was the same as described in chapter 4.

5.3.2 Reproducibility

The effect of varying the concentration of template DNA was tested using primer pairs A+7. Four extracts from a member of the genus *Panaeolus* and four from a member of the genus *Psilocybe* were tested using four different concentrations of starting material (estimated at 12.5ng, 62.5ng, 125ng and 250ng). Additionally four amplifications were performed using 250ng of starting template DNA to examine the reproducibility using the same template DNA concentration. The AFLP results are shown in Figure 5-2. The banding patterns produced by the four different concentrations from each species were visually similar. The banding pattern of the different concentration of DNA compared to the four samples all at the same concentration were again visually similar. From this initial test to determine the variability in the AFLP products produced by different concentrations of starting

material it is evident that there is a high degree of reproducibility in the AFLP method.

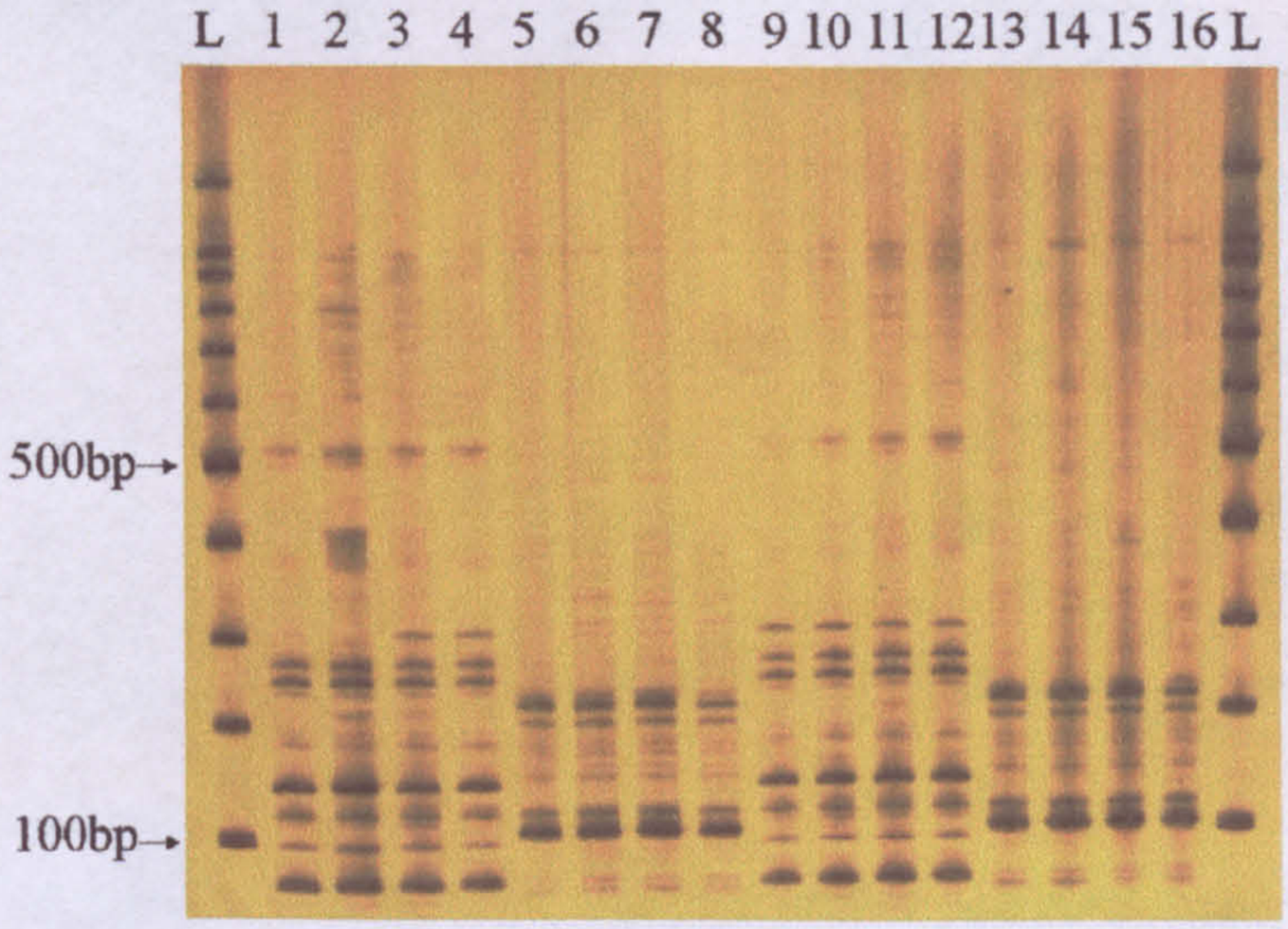


Figure 5-2 AFLP patterns produced by selective primer pair A+7. Eight extracts for each species *Psilocybe semilanceata* and *Panaeolus semiovatus* were tested using different DNA quantity in the AFLP analysis. Lanes 1-4 are approximately 12.5, 62.5, 125 and 250ng of sample PS1-2. Lanes 9-12 are all 250ng of sample PS1-2. Lanes 5-8 are approximately 12.5, 62.5, 125 and 250ng of sample PA1-1. Lanes 13-16 are all 250ng of sample PA1-1.

5.3.3 Image Analysis

In this study, four different primer pairs A+5, B+4 B+8, H+6 and H+8 were used to generate the AFLP patterns from members of the genera *Psilocybe* and *Panaeolus*. Additionally *Volvariella volvacea*, *Agaricus bisporus* and *Lentinus edodes*, were tested and their data combined. Figures 5-3 to 5-7 show AFLP patterns produced by the five selective primer pairs, respectively. The methods of band detection, size determination and score assignment were the same as used in RAPD image analysis in chapter 4.

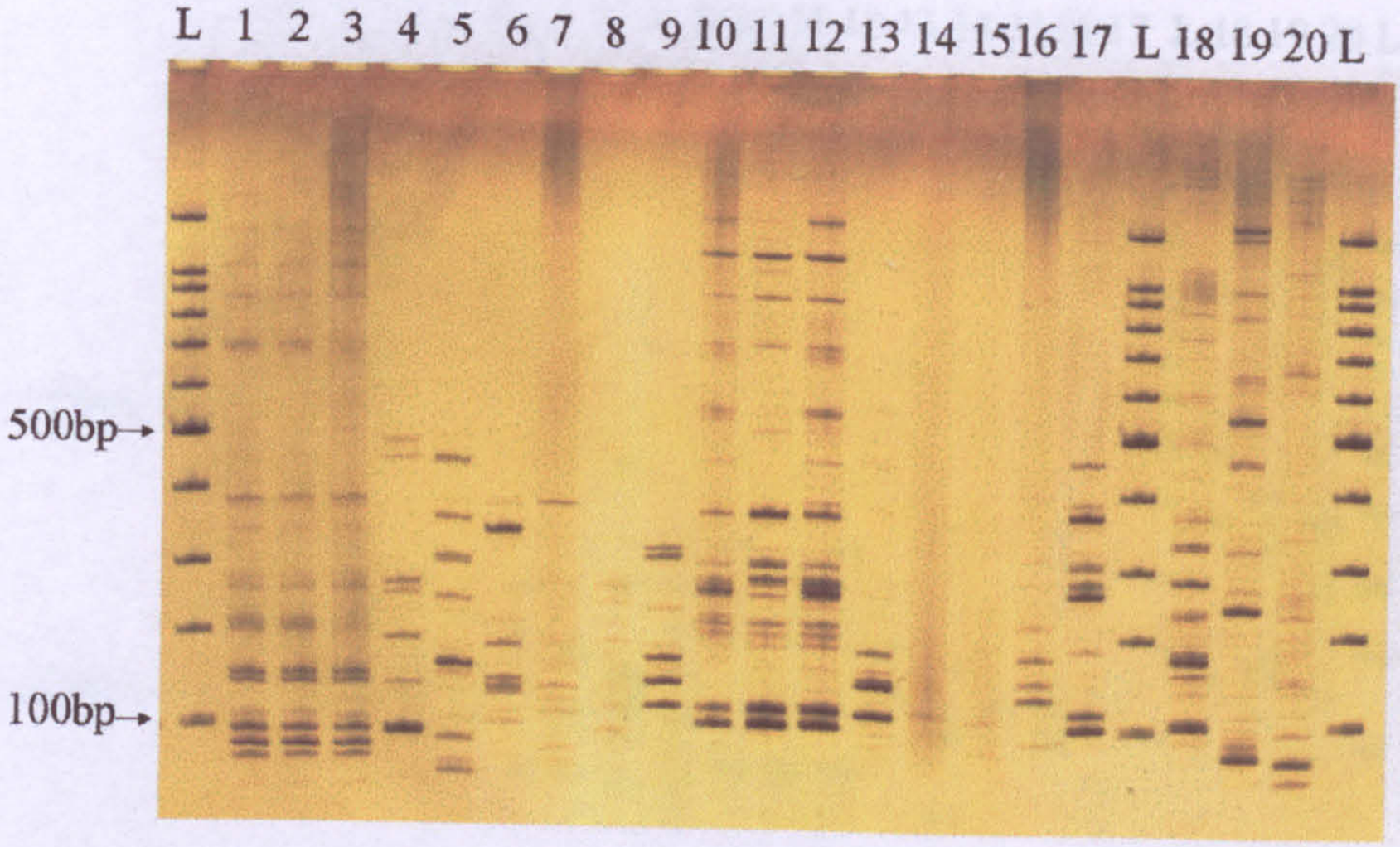


Figure 5-3 AFLP patterns produced by selective primer pair A+5. Lane M is 100 bp ladder. Lanes 1 to 17 are samples of PA1-1, 1-2, 1-3, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 1-3, 2, 3, 4, 5, 6-1, *Volvariella volvacea, Agaricus bisporus and Lentinus edodes.*

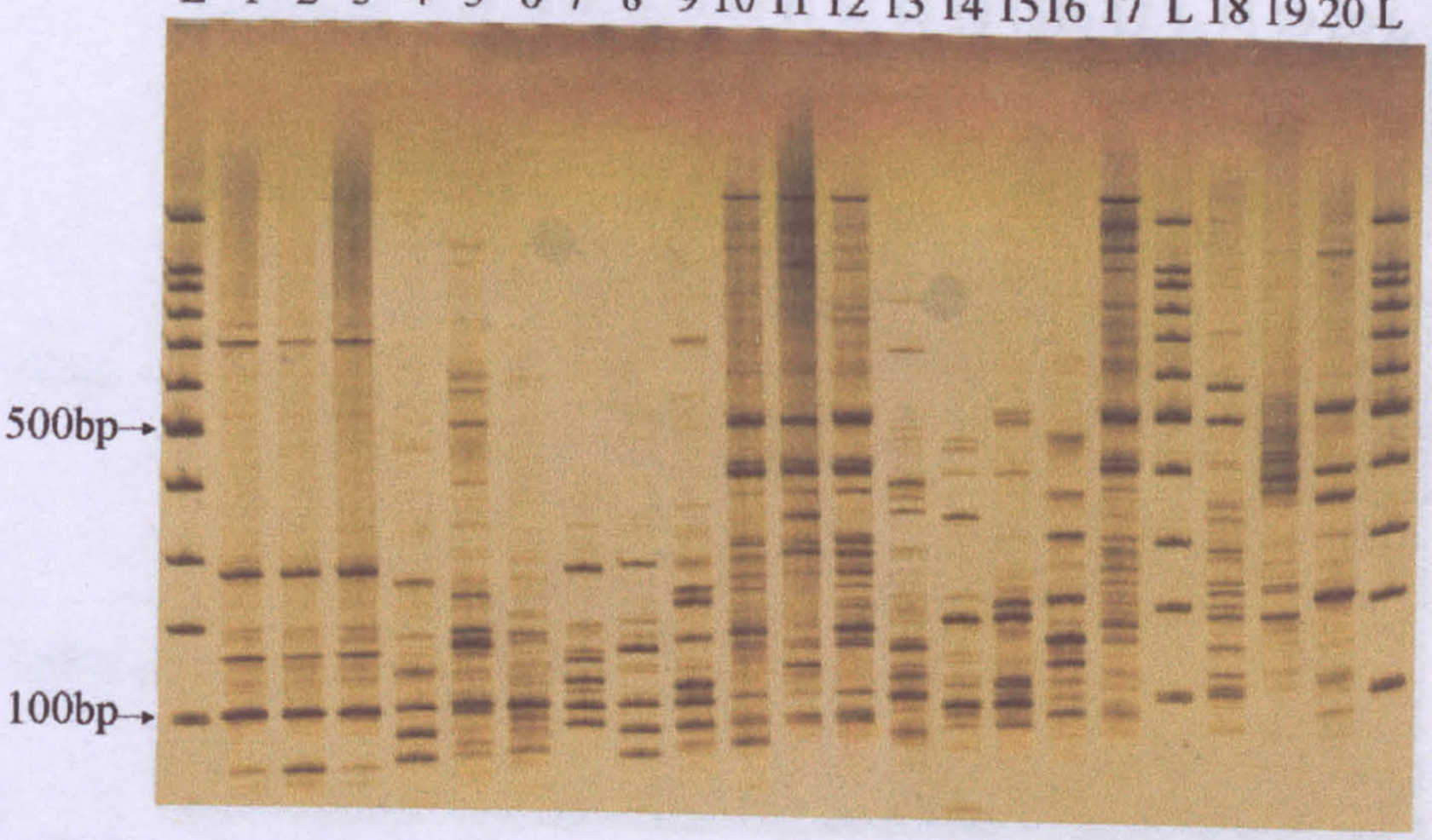


Figure 5-4 AFLP patterns produced by selective primer pair B+4. Lane M is 100 bp ladder. Lanes 1 to 17 are samples of PA1-1, 1-2, 1-3, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 1-3, 2, 3, 4, 5, 6-1, *Volvariella volvacea, Agaricus bisporus and Lentinus edodes.*

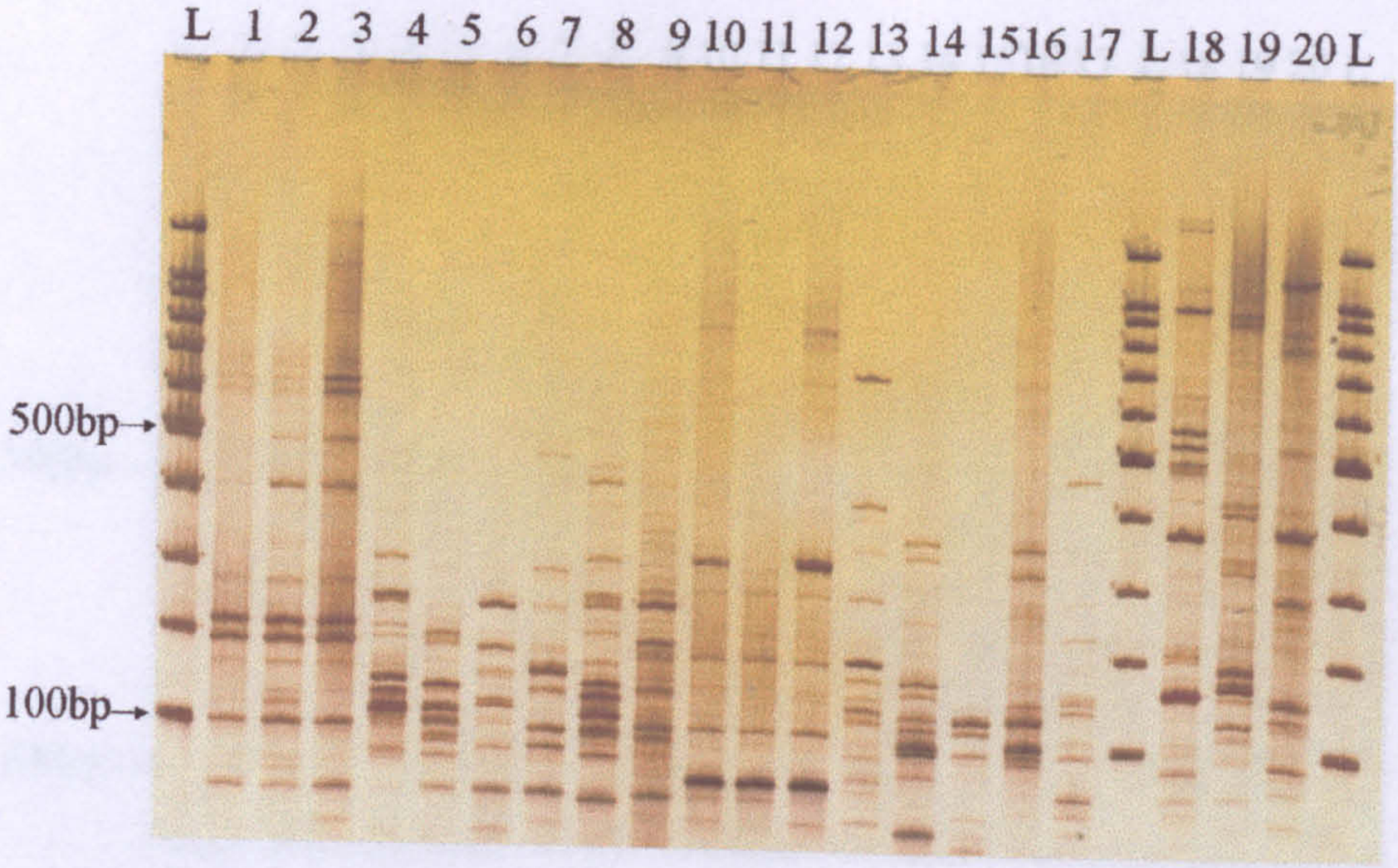


Figure 5-5 AFLP patterns produced by selective primer pair B+8. Lane M is 100 bp ladder. Lanes 1 to 17 are samples of PA1-1, 1-2, 1-3, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 1-3, 2, 3, 4, 5, 6-1, *Volvariella volvacea, Agaricus bisporus and Lentinus edodes.*

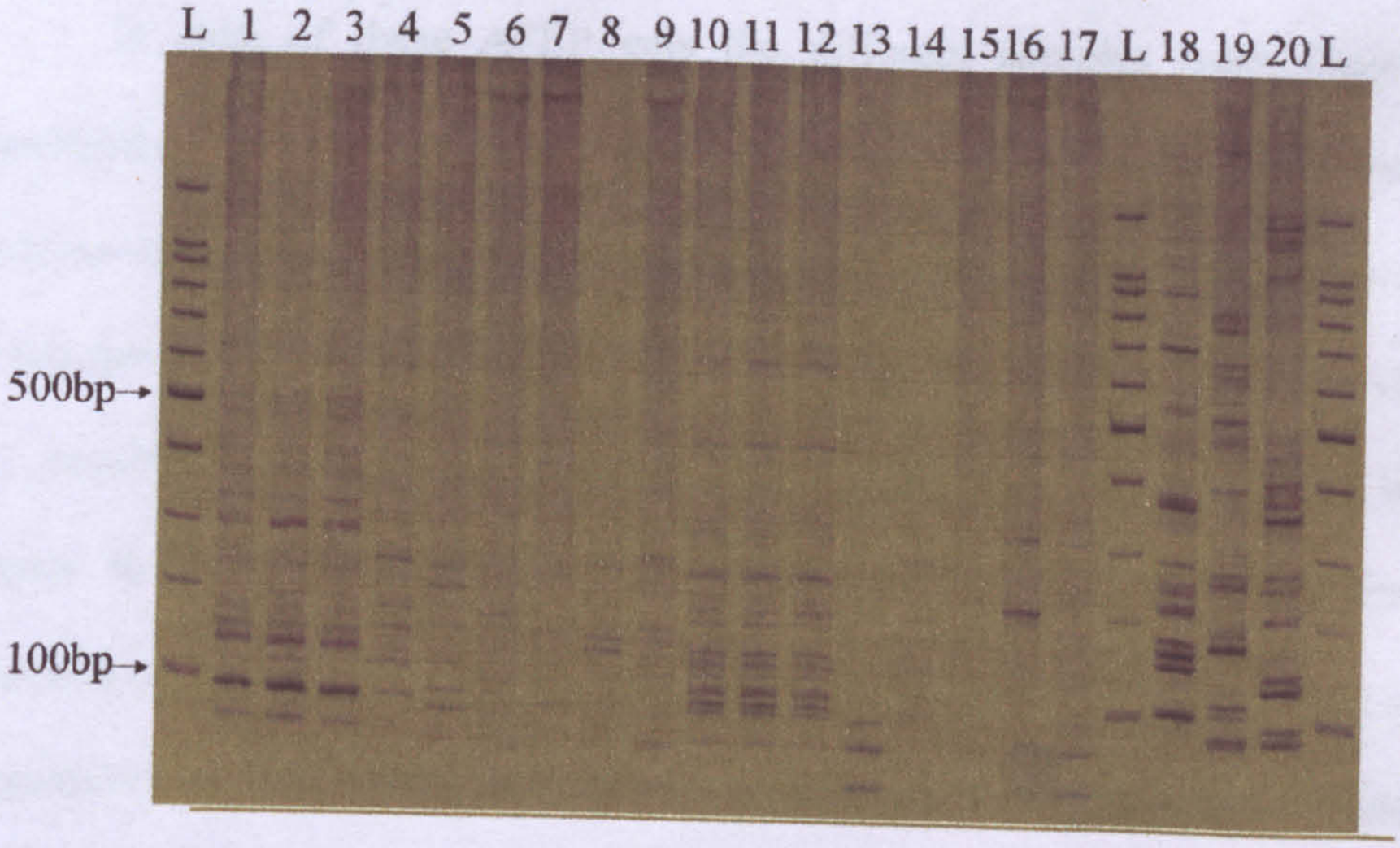


Figure 5-6 AFLP patterns produced by selective primer pair H+6. Lane M is 100 bp ladder. Lanes 1 to 17 are samples of PA1-1, 1-2, 1-3, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 1-3, 2, 3, 4, 5, 6-1, *Volvariella volvacea, Agaricus bisporus and Lentinus edodes.*

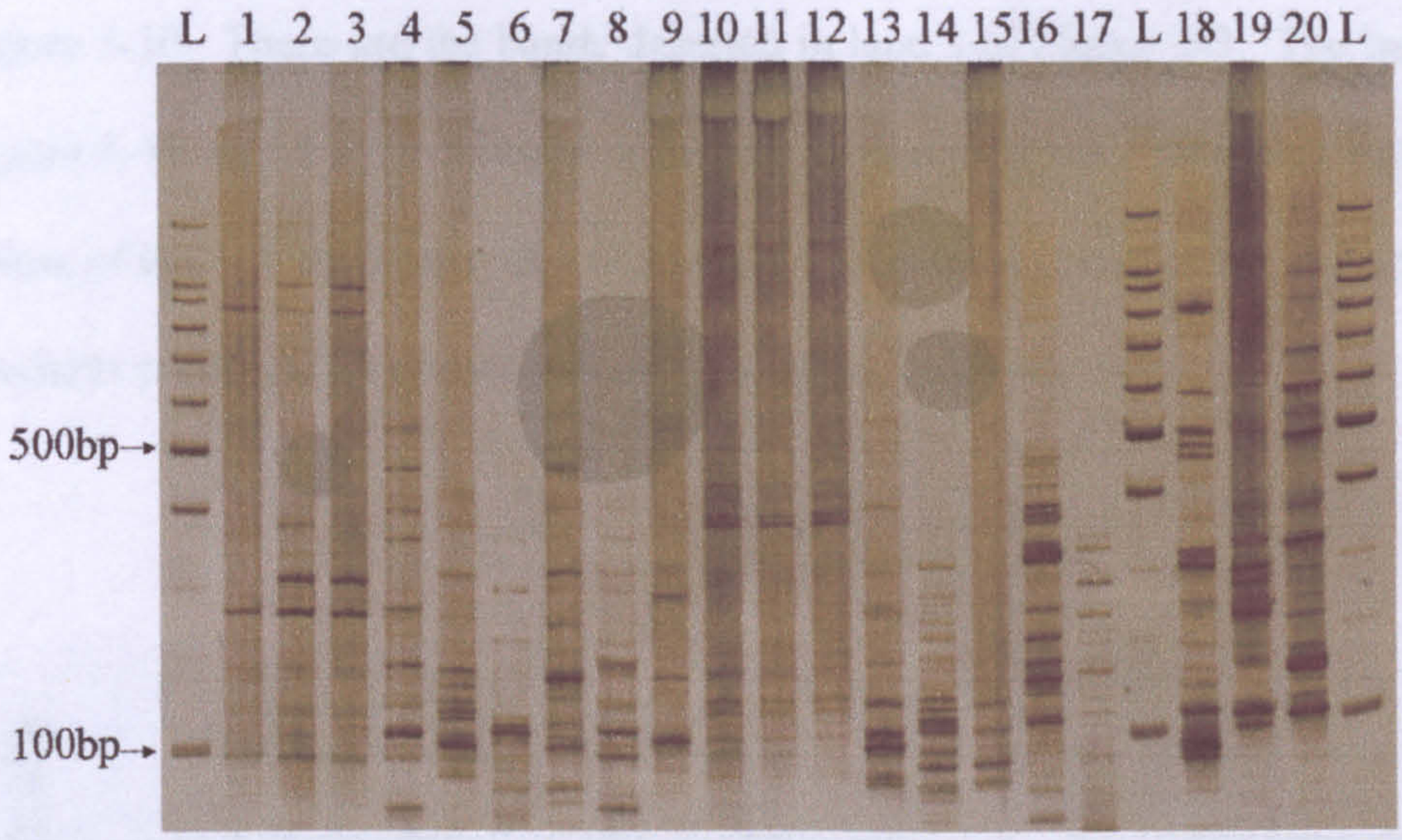


Figure 5-7 AFLP patterns produced by selective primer pair H+8. Lane M is 100 bp ladder. Lanes 1 to 17 are samples of PA1-1, 1-2, 1-3, 2, 3, 4, 5, 6, 7, PS1-1, 1-2, 1-3, 2, 3, 4, 5, 6-1, *Volvariella volvacea, Agaricus bisporus and Lentinus edodes*.

In each of these AFLP gels the different samples from *Panaeolus* were separated in the first lanes of the gel, followed by members of the *Psilocybe* genus and then the three additional fungal species. Visually for all AFLP gels (Figures 5-3 to 5-7) the patterns for each of the members of the *Panaeolus* genus are similar compared to the members of the *Psilocybe* genus. For members of the same genus more bands appear to be of the same size than compared to samples from a different genus. Members of these two genera appear again to be different to the three agricultural important samples. Image analysis was performed on all the samples from these five gels to produce an accurate representation of the genetic similarity of the samples based on the AFLP data.

Figures 5-8 and 5-9 show the sizes of the DNA size ladder and a formula of log curve generated by the ladder of Figure 5-3. These data from the size markers could be

applied to AFLP products separated on the same gel. An example of this is shown in Figure 5-10. These are the bands detected in lane 1 of Figure 5-3. The bands sizes in Figure 5-10 could be accurately produced by applying the calibration curve of cubic spline of the 100 bp ladder; this is shown in Figure 5-11. A further example of AFLP products produced by primer pair A+5 is shown in Figure 5-12.

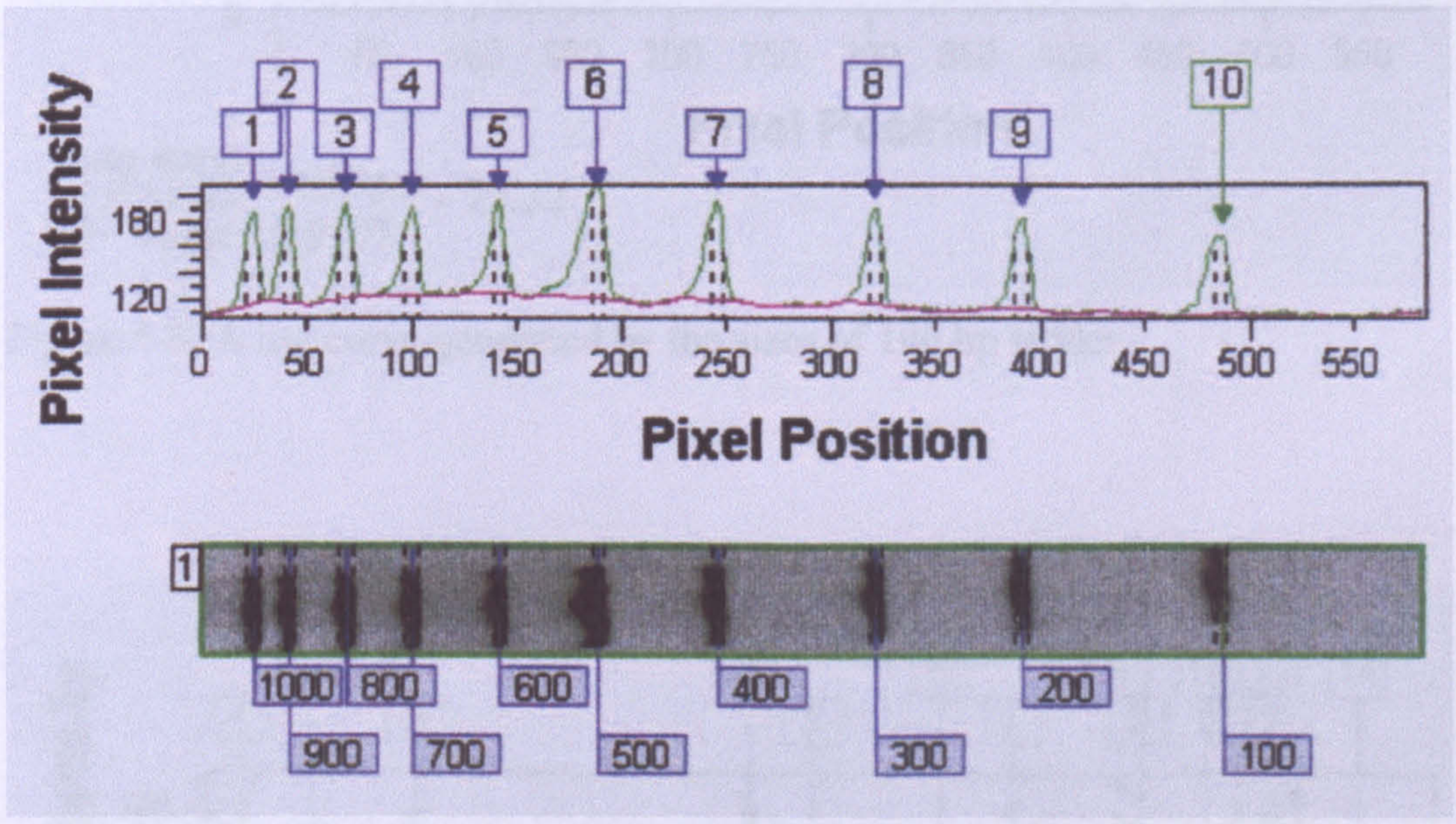


Figure 5-8 The sizes of 100 bp ladder in Figure 5-3 assigned from 1000 bp to 100 bp.

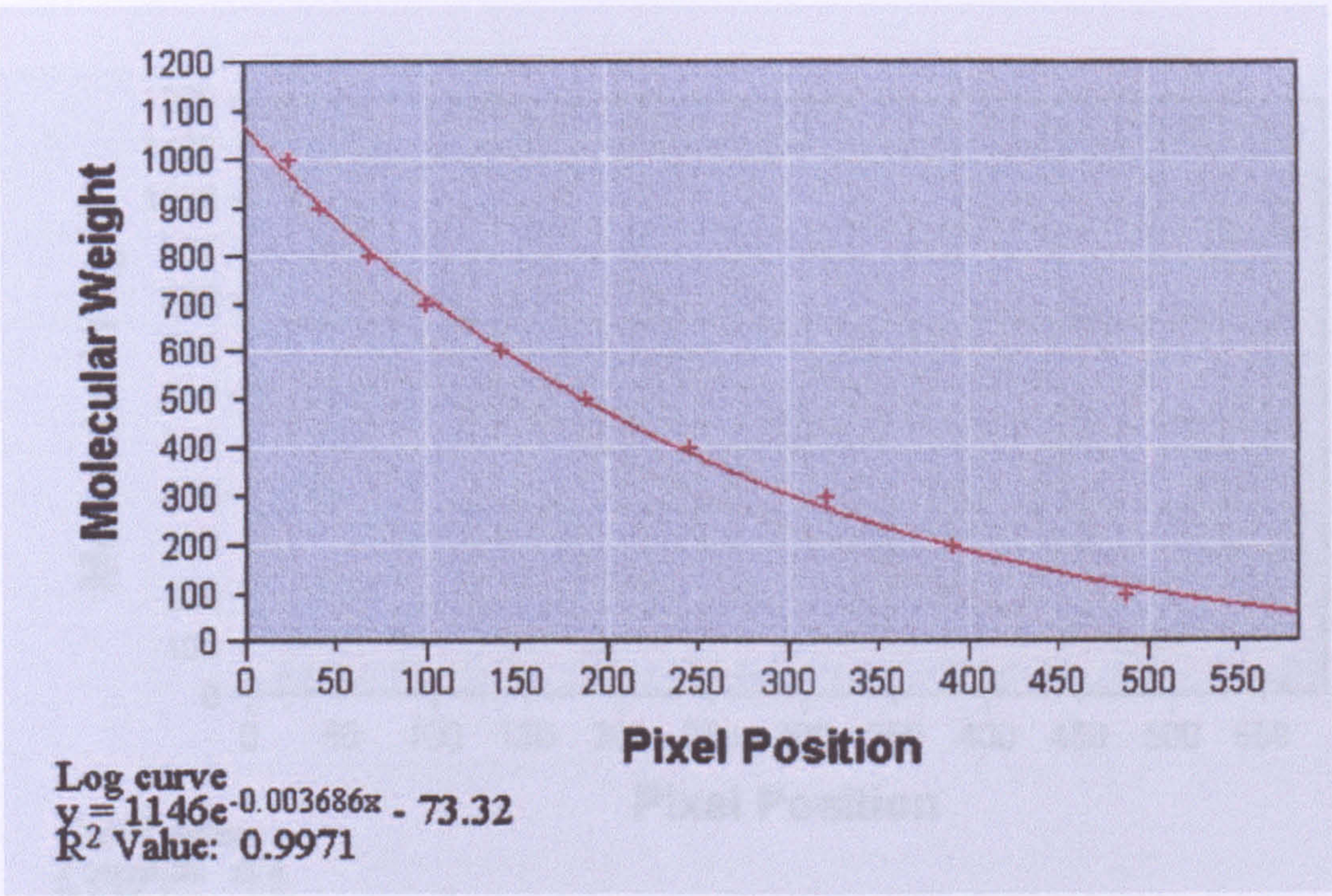


Figure 5-9 A log curve generated by the sizes of 100 bp ladder.

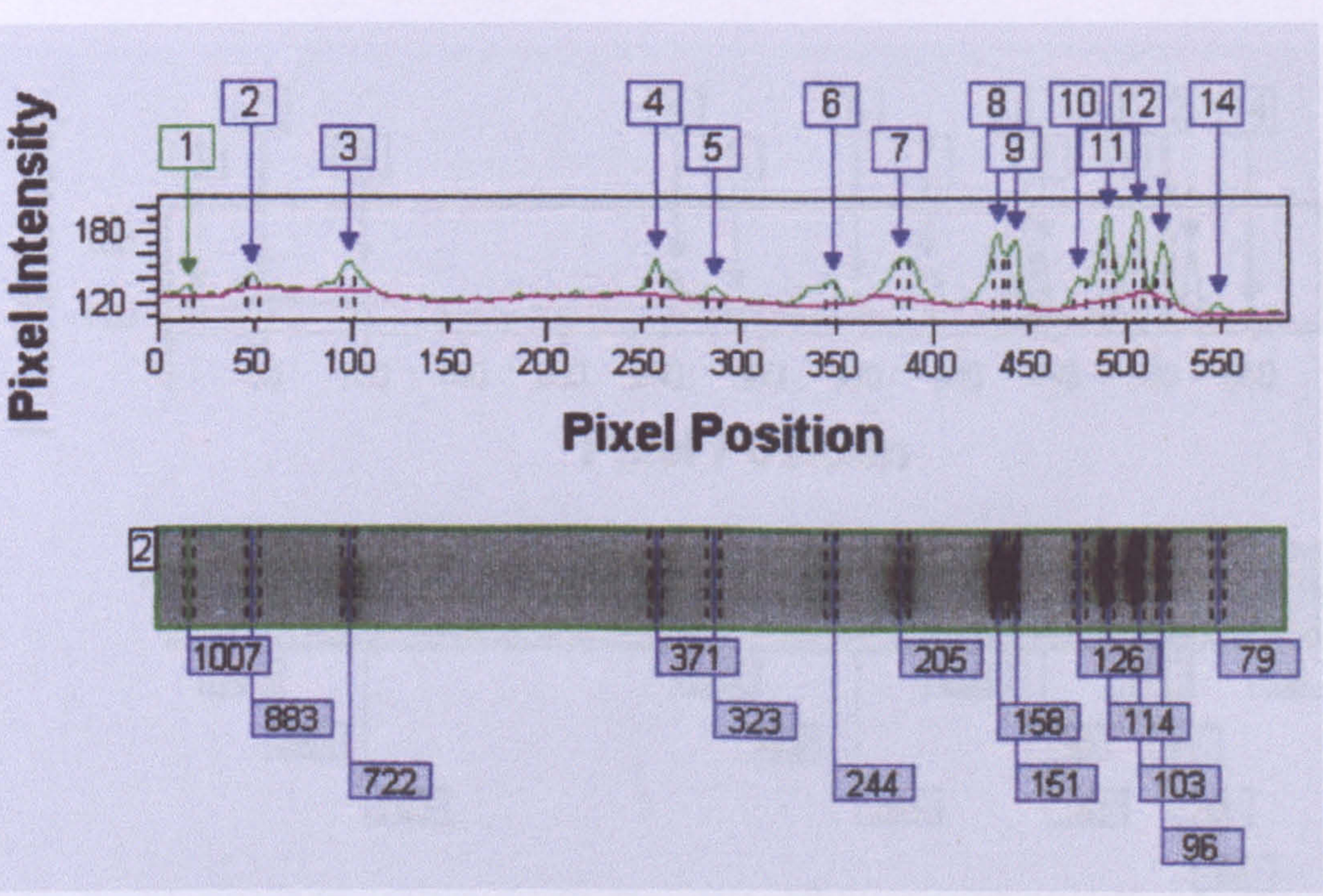


Figure 5-10 Bands of lane 1 in Figure 5-3 sized by the formula of log curve in Figure 5-9.

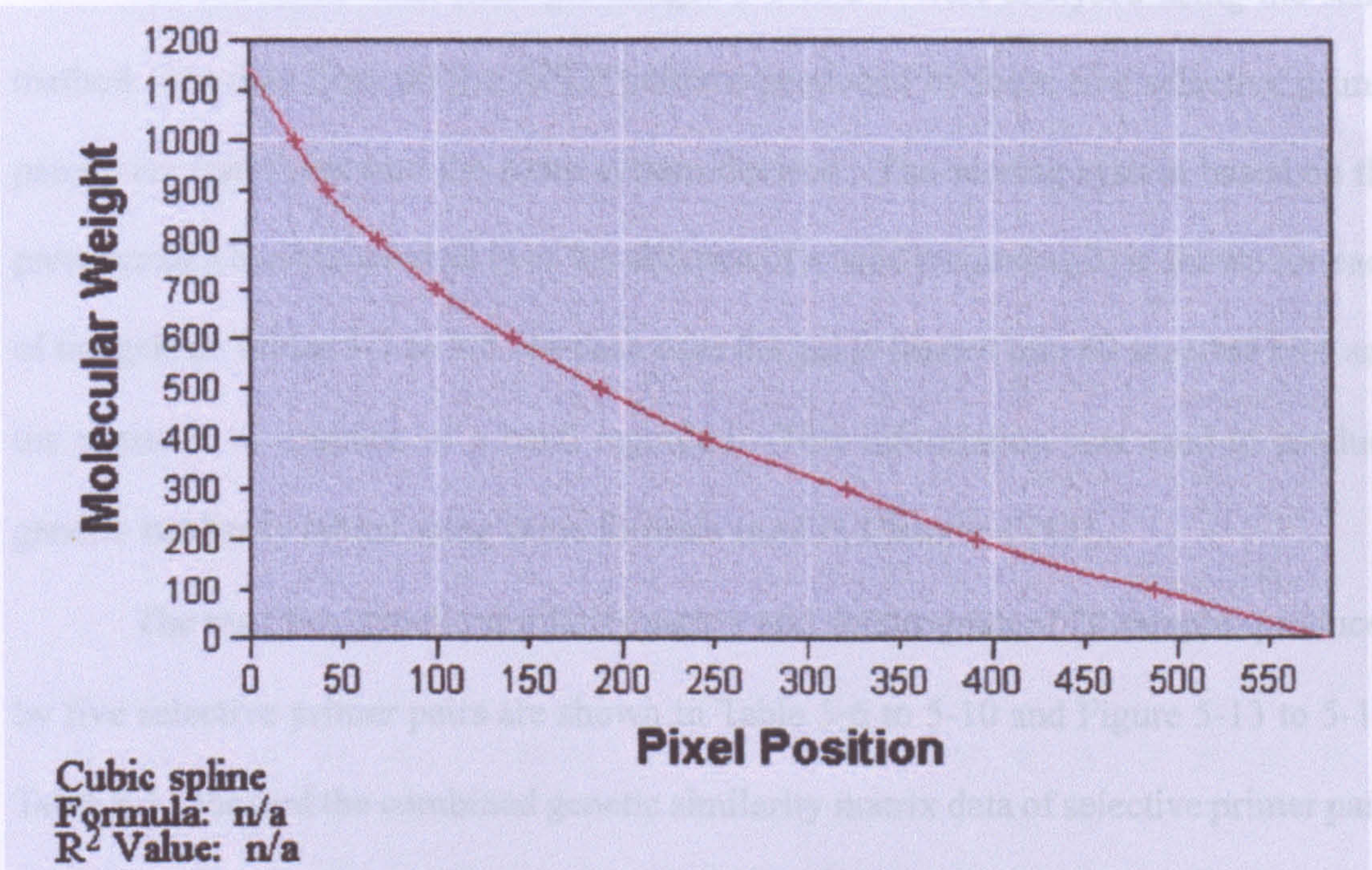


Figure 5-11 A calibration curve generated using the method of cubic spline by the sizes of 100 bp ladder.

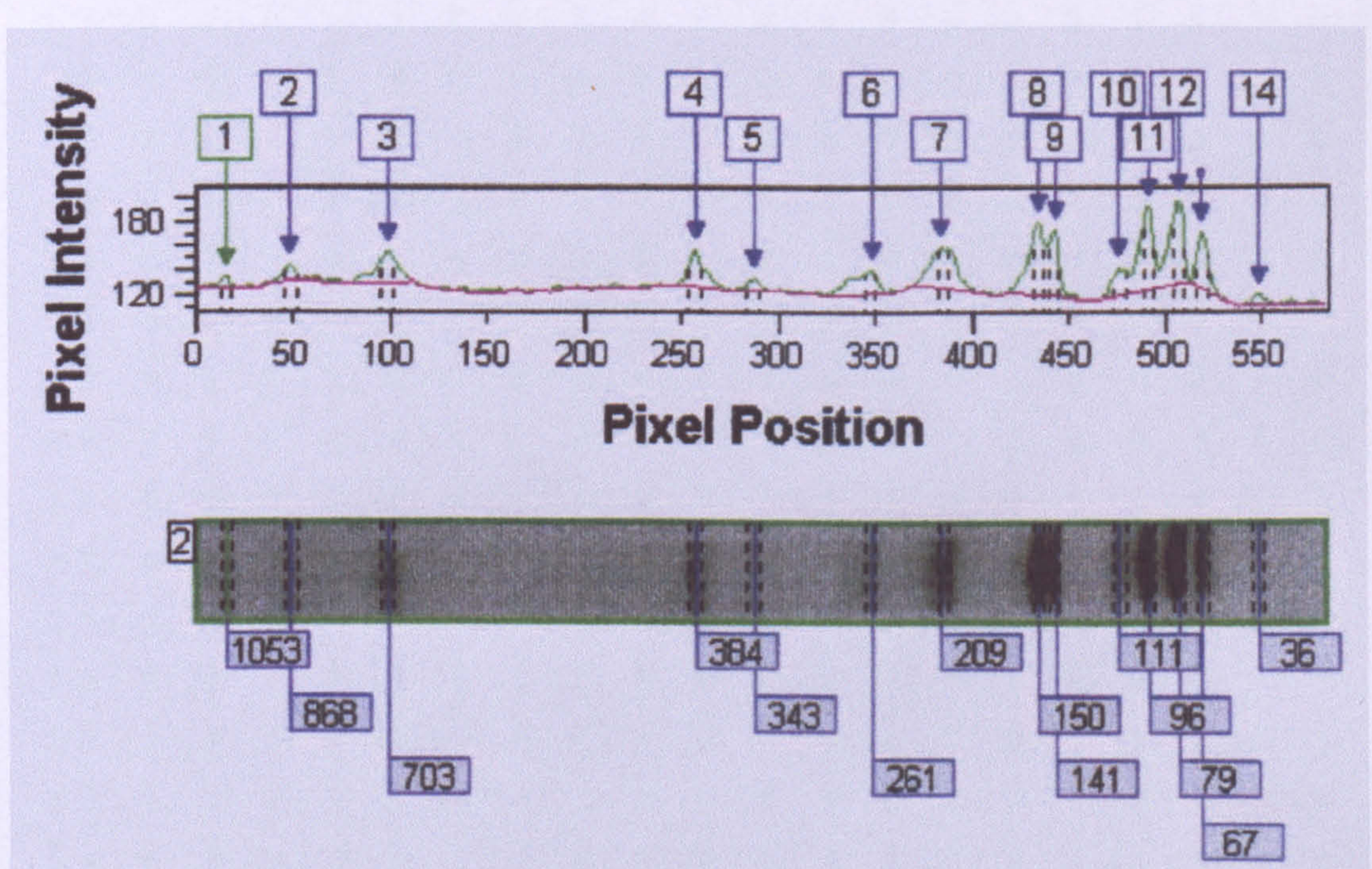


Figure 5-12 Bands of lane 1 in Figure 5-3 sized by the calibration curve in Figure 5-11.

All the AFLP data from gel images 5-3 to 5-7 were analysed using the same method. The data from all the AFLP patterns produced by these five selective primer pairs were transfered into the score system devised. The scoring system based on the presence of a band (scored as 1) or the absence of a band (scored as 0) is shown for each of the gels in Tables 5-1 to 5-5. In each case the gel is binned into 60 separate bins and the presence or absence of a band recorded. This information was used to produce genetic similarity tables using same formula used in Chapter 4 [83].

The resulting genetic similarity matrix and dendrogram of 20 samples produced by five selective primer pairs are shown in Table 5-6 to 5-10 and Figure 5-13 to 5-17. Table 5-11 showed the combined genetic similarity matrix data of selective primer pairs A+5, B+4, B+8 and H+6. Figure 5-18 shows the dendrogram established by the genetic similarity matrix in Table 5-11.

Table 5-1 Genotypes of score system based on AFLP patterns obtained by selective primer pair A+5. Columns 1-20 are sample PA1-1 PA1-2 PA1-3 PA2 PA3 PA4 PA5 PA6 PA7 PS1-1 PS1-2 PS1-3 PS2 PS3 PS4 PS5 PS6-1 VO (*Volvariella volvacea*) AG (*Agaricus bisporus*), and LE (*Lentinus edodes*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
0	2	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1
0	3	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	8	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1
0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	5	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
1	6	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0
1	7	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
1	8	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
1	9	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
2	1	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0
2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
2	3	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	4	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
2	6	1	1	1	0	0	1	1	0	0	1	1	1	0	0	0	0	1	0	0	0
2	7	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0
2	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	9	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1

30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
31	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	
32	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	
33	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
34	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	0	0	
35	0	0	0	1	0	0	1	1	0	1	1	1	0	0	0	0	1	0	1	0	
36	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0	1	
37	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	
38	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	
39	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	1	0	0	0	0	
40	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	
41	0	0	0	1	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	
42	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	0	0	
43	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	
44	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	
45	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0	
46	0	0	0	1	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	
47	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
48	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	
49	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	1	0	1	0	
50	1	1	1	0	1	1	0	0	0	1	1	1	0	0	1	0	1	1	0	0	
51	1	1	1	1	0	0	0	1	0	0	0	0	1	1	0	0	1	0	1	1	
52	1	1	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	1	0	
53	1	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	
54	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	
55	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
56	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Table 5-2 Genotypes of score system based on AFLP patterns obtained by selective primer pair B+4. Columns 1-20 are sample PA1-1 PA1-2 PA1-3 PA2 PA3 PA4 PA5 PA6 PA7 PS1-1 PS1-2 PS1-3 PS2 PS3 PS4 PS5 PS6-1 VO (*Volvariella volvacea*) AG (*Agaricus bisporus*), and LE (*Lentinus edodes*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
01	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0
02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
03	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
04	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0
05	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0
06	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
07	1	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0
08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
09	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	0
10	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
12	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
13	1	1	0	0	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0	1
14	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
16	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
17	1	1	1	0	0	0	0	1	0	1	1	1	0	0	1	0	1	0	1	1
18	0	0	0	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	1	1
19	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0
20	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0
21	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0
22	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0
23	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	0
24	0	0	0	0	0	0	1	0	0	1	1	1	0	1	1	0	1	1	0	1
25	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	1	0	1	1
26	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0
27	0	0	0	0	1	0	0	0	0	1	1	1	1	0	0	1	1	0	1	1
28	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1
29	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0

30	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0
31	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	0	0	1
33	1	1	1	0	1	0	0	0	1	0	1	1	1	0	0	0	1	0	1	0	
34	1	1	1	0	1	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	
35	1	1	1	0	0	1	1	0	0	0	0	1	0	0	0	1	0	1	0	0	
36	0	0	0	1	0	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	
37	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	
38	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1	1	1	
39	0	0	0	0	0	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	
40	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1	1	0	1
41	1	1	1	1	0	0	1	1	1	0	1	1	0	1	1	1	0	0	1	1	0
42	1	1	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	0
43	0	1	1	0	0	1	0	1	1	1	1	1	1	0	0	1	0	0	1	0	
44	1	1	1	1	0	0	1	1	0	1	1	1	1	0	0	1	1	0	0	0	
45	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	1	1	0	
46	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	0	0	0	0	
47	0	0	0	0	0	1	1	0	1	0	1	0	1	0	0	1	1	0	0	0	
48	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1	
49	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	0	1	0	0	1	
50	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	0	1	0	0	
51	0	0	0	0	0	1	1	0	0	0	1	1	1	1	1	0	0	1	0	1	
52	0	0	1	1	0	1	1	1	1	1	1	1	0	1	0	1	0	0	0	1	
53	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	1	0	0	0	
54	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	
55	1	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	
56	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	
67	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
59	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
60	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Table 5-3 Genotypes of score system based on AFLP patterns obtained by selective primer pair B+8. Columns 1-20 are sample PA1-1 PA1-2 PA1-3 PA2 PA3 PA4 PA5 PA6 PA7 PS1-1 PS1-2 PS1-3 PS2 PS3 PS4 PS5 PS6-1 VO (*Volvariella volvacea*) AG (*Agaricus bisporus*), and LE (*Lentinus edodes*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
11	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
12	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
17	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
18	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
19	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
20	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
21	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
22	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1
23	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
27	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0
28	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0

29	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0
30	0	0	0	1	0	0	0	1	1	1	0	1	0	0	0	1	0	0	0	0
31	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
33	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	1	1
34	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
35	0	0	0	1	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1
36	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
37	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0
38	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
39	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0
40	1	1	1	0	1	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0
41	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0
42	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	1	0
43	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1
44	0	0	0	1	1	0	0	1	1	1	0	1	1	1	0	1	1	0	0	1
45	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0	1	0	0	1
46	0	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1
47	1	1	0	1	1	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0
48	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0
49	1	1	1	1	0	0	1	1	1	0	0	0	0	1	0	1	0	1	0	0
50	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1
51	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1	0	1	0	0
52	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
53	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1
54	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	1	0
55	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0
56	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	1	1	0	1
57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
58	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0
59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0

Table 5-4 Genotypes of score system based on AFLP patterns obtained by selective primer pair H+6. Columns 1-20 are sample PA1-1 PA1-2 PA1-3 PA2 PA3 PA4 PA5 PA6 PA7 PS1-1 PS1-2 PS1-3 PS2 PS3 PS4 PS5 PS6-1 VO (*Volvariella volvacea*) AG (*Agaricus bisporus*), and LE (*Lentinus edodes*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
07	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
12	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	0
13	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
17	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
18	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
20	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
21	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	1
22	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	1
23	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
24	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1
25	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
26	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
27	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
29	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0

30	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0
32	1	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	1	0	1	0
33	1	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0
34	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	1
35	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	1
36	0	0	0	1	0	0	0	1	1	1	1	1	0	1	0	0	0	1	1	1
37	1	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0
38	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
39	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	1
40	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	1	1
41	0	1	1	1	0	0	0	0	0	1	1	1	0	1	0	0	1	1	1	0
42	1	1	1	1	1	1	0	1	0	1	1	1	0	0	0	0	1	1	1	0
43	1	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	1
44	1	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0
45	1	1	1	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	0	1
46	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1
47	1	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	1
48	1	1	0	1	1	0	0	1	1	1	1	1	0	0	0	0	1	0	0	0
49	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1	0
50	0	0	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0
51	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	0	0	0	0	1
52	0	0	1	0	0	0	1	1	0	1	1	1	1	1	0	0	1	0	1	1
53	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1
54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
55	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0
56	0	0	0	0	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
59	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0

Table 5-5 Genotypes of score system based on AFLP patterns obtained by selective primer pair H+8. Columns 1-20 are sample PA1-1 PA1-2 PA1-3 PA2 PA3 PA4 PA5 PA6 PA7 PS1-1 PS1-2 PS1-3 PS2 PS3 PS4 PS5 PS6-1 VO (*Volvariella volvacea*) AG (*Agaricus bisporus*), and LE (*Lentinus edodes*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1
0	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	4	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0
0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
1	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1	7	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
1	8	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	1	1
1	9	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1
2	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	1	1
2	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0	1
2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1
2	3	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	1
2	4	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0
2	5	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0
2	6	0	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
2	7	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0	0	1	0
2	8	0	0	0	1	0	1	1	0	0	1	1	1	0	0	0	1	0	1	1	0
2	9	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0

3	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
3	2	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1
3	3	0	1	1	0	0	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0
3	4	1	1	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	1
3	5	1	1	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1
3	6	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
3	7	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1
3	8	0	0	0	1	0	0	1	0	1	0	0	0	1	1	0	1	0	1	1	1
3	9	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	1	1	1
4	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	1	0	1
4	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	1
4	2	0	1	1	1	1	0	0	1	1	1	0	1	0	0	0	1	0	1	0	0
4	3	0	0	0	1	0	0	1	1	0	1	0	0	1	1	0	1	0	0	1	1
4	4	0	0	0	1	1	0	1	0	1	1	1	1	1	1	0	1	1	0	1	1
4	5	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1
4	6	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	0	1	0	1
4	7	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0	1
4	8	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	0	1
4	9	0	0	0	1	1	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1
5	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	0	1	1	1
5	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
5	2	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1
5	3	0	0	0	0	1	1	0	1	0	1	0	0	1	1	1	1	1	1	0	0
5	4	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	1	1	0	0
5	5	0	0	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	1	0	0
5	6	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0
5	7	0	0	0	1	0	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0
5	8	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0
5	9	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
6	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

Table 5-6 Genetic similarity matrix from the AFLP scores of selective primer pair A+5 in Table 5-1.

PA1-1	PA1-2	PA1-3	PA2	PA3	PA4	PA5	PA6	PA7	PS1-1	PS1-2	PS1-3	PS2	PS3	PS4	PS5	PS6-1	VO	AG	LI
1.000																			
0.965	1.000																		
0.814	0.857	1.000																	
0.190	0.181	0.200	1.000																
0.250	0.240	0.260	0.235	1.000															
0.454	0.434	0.380	0.266	0.111	1.000														
0.363	0.434	0.476	0.400	0.111	0.375	1.000													
0.200	0.190	0.210	0.615	0.000	0.285	0.571	1.000												
0.181	0.173	0.190	0.266	0.222	0.375	0.375	0.571	1.000											
0.266	0.258	0.137	0.260	0.307	0.250	0.333	0.181	0.166	1.000										
0.266	0.258	0.206	0.260	0.307	0.333	0.416	0.272	0.250	0.750	1.000									
0.250	0.242	0.129	0.320	0.357	0.307	0.384	0.250	0.230	0.882	0.823	1.000								
0.521	0.583	0.636	0.250	0.210	0.235	0.470	0.400	0.352	0.160	0.160	0.222	1.000							
0.333	0.315	0.352	0.181	0.142	0.000	0.333	0.400	0.166	0.200	0.200	0.181	0.615	1.000						
0.000	0.117	0.133	0.000	0.166	0.200	0.200	0.000	0.000	0.111	0.111	0.100	0.181	0.000	1.000					
0.416	0.400	0.260	0.117	0.200	0.444	0.111	0.125	0.333	0.307	0.307	0.357	0.421	0.142	0.000	1.000				
0.230	0.222	0.240	0.421	0.454	0.200	0.300	0.222	0.100	0.571	0.571	0.666	0.285	0.250	0.142	0.363	1.000			
0.342	0.333	0.352	0.071	0.322	0.206	0.068	0.148	0.275	0.216	0.270	0.256	0.333	0.080	0.086	0.322	0.242	1.000		
0.322	0.375	0.400	0.333	0.148	0.080	0.320	0.347	0.240	0.242	0.121	0.228	0.384	0.285	0.105	0.148	0.275	0.315	1.000	
0.384	0.370	0.400	0.315	0.181	0.200	0.200	0.222	0.100	0.285	0.214	0.200	0.190	0.125	0.000	0.090	0.166	0.303	0.344	1.000

Table 5-7 Genetic similarity matrix from the AFLP scores of selective primer pair B+4 in Table 5-2.

PA1-1	PA1-2	PA1-3	PA2	PA3	PA4	PA5	PA6	PA7	PS1-1	PS1-2	PS1-3	PS2	PS3	PS4	PS5	PS6-1	VO	AG	LE
1.000																			
0.903	1.000																		
0.838	0.866	1.000																	
0.428	0.370	0.444	1.000																
0.285	0.222	0.222	0.000	1.000															
0.500	0.514	0.514	0.437	0.125	1.000														
0.516	0.533	0.600	0.370	0.148	0.628	1.000													
0.666	0.689	0.689	0.538	0.153	0.588	0.482	1.000												
0.400	0.411	0.470	0.322	0.258	0.564	0.411	0.424	1.000											
0.380	0.390	0.439	0.315	0.263	0.391	0.292	0.450	0.311	1.000										
0.428	0.439	0.487	0.368	0.263	0.391	0.390	0.450	0.266	0.769	1.000									
0.380	0.390	0.439	0.315	0.263	0.347	0.292	0.350	0.311	0.846	0.807	1.000								
0.311	0.363	0.363	0.243	0.292	0.448	0.318	0.418	0.500	0.509	0.545	0.472	1.000							
0.200	0.206	0.275	0.307	0.076	0.411	0.551	0.357	0.303	0.150	0.300	0.200	0.465	1.000						
0.303	0.312	0.312	0.206	0.206	0.432	0.375	0.451	0.333	0.418	0.372	0.465	0.434	0.516	1.000					
0.312	0.387	0.451	0.428	0.071	0.555	0.580	0.400	0.457	0.380	0.380	0.380	0.444	0.400	0.303	1.000				
0.391	0.355	0.355	0.238	0.476	0.320	0.355	0.318	0.367	0.607	0.607	0.642	0.576	0.227	0.553	0.304	1.000			
0.388	0.342	0.342	0.187	0.312	0.550	0.628	0.352	0.461	0.260	0.217	0.304	0.408	0.411	0.540	0.333	0.480	1.000		
0.235	0.303	0.303	0.200	0.266	0.315	0.242	0.312	0.378	0.409	0.409	0.454	0.510	0.437	0.457	0.470	0.416	0.368	1.000	
0.193	0.200	0.200	0.148	0.296	0.342	0.266	0.275	0.294	0.439	0.439	0.536	0.409	0.413	0.687	0.322	0.577	0.457	0.424	1.000

Table 5-8 Genetic similarity matrix from the AFLP scores of selective primer pair B+8 in Table 5-3.

PA1-1	PA1-2	PA1-3	PA2	PA3	PA4	PA5	PA6	PA7	PS1-1	PS1-2	PS1-3	PS2	PS3	PS4	PS5	PS6-1	VO	AG	LE
1.000																			
0.923	1.000																		
0.880	0.962	1.000																	
0.307	0.357	0.296	1.000																
0.272	0.250	0.173	0.500	1.000															
0.300	0.363	0.380	0.272	0.444	1.000														
0.363	0.333	0.347	0.333	0.400	0.666	1.000													
0.413	0.387	0.333	0.516	0.444	0.320	0.444	1.000												
0.378	0.358	0.368	0.410	0.400	0.424	0.400	0.571	1.000											
0.347	0.320	0.333	0.240	0.476	0.315	0.190	0.285	0.444	1.000										
0.315	0.285	0.300	0.095	0.470	0.400	0.235	0.083	0.312	0.777	1.000									
0.190	0.173	0.181	0.260	0.421	0.235	0.105	0.153	0.352	0.900	0.750	1.000								
0.480	0.518	0.461	0.370	0.434	0.285	0.260	0.266	0.315	0.416	0.300	0.454	1.000							
0.333	0.384	0.320	0.538	0.363	0.300	0.272	0.413	0.378	0.173	0.105	0.190	0.480	1.000						
0.111	0.200	0.105	0.200	0.250	0.142	0.000	0.086	0.064	0.117	0.153	0.133	0.315	0.444	1.000					
0.105	0.190	0.200	0.380	0.352	0.266	0.235	0.333	0.375	0.333	0.142	0.375	0.300	0.526	0.307	1.000				
0.250	0.230	0.240	0.230	0.545	0.300	0.272	0.344	0.378	0.782	0.736	0.761	0.480	0.166	0.111	0.210	1.000			
0.235	0.277	0.285	0.222	0.250	0.266	0.312	0.410	0.468	0.242	0.275	0.193	0.000	0.058	0.000	0.137	0.294	1.000		
0.333	0.375	0.322	0.250	0.214	0.307	0.214	0.285	0.465	0.206	0.240	0.222	0.451	0.266	0.166	0.080	0.266	0.350	1.000	
0.193	0.242	0.250	0.303	0.275	0.296	0.275	0.277	0.409	0.266	0.230	0.214	0.375	0.322	0.240	0.230	0.322	0.243	0.216	1.000

Table 5-9 Genetic similarity matrix from the AFLP scores of selective primer pair H+6 in Table 5-4.

PA1-1	PA1-2	PA1-3	PA2	PA3	PA4	PA5	PA6	PA7	PS1-1	PS1-2	PS1-3	PS2	PS3	PS4	PS5	PS6-1	VO	AG	LE
1.000																			
0.900	1.000																		
0.820	0.918	1.000																	
0.413	0.444	0.307	1.000																
0.400	0.357	0.296	0.470	1.000															
0.137	0.148	0.153	0.250	0.470	1.000														
0.222	0.160	0.250	0.142	0.133	0.142	1.000													
0.484	0.387	0.400	0.600	0.380	0.200	0.333	1.000												
0.375	0.333	0.275	0.315	0.400	0.210	0.235	0.434	1.000											
0.454	0.523	0.487	0.387	0.437	0.322	0.206	0.400	0.411	1.000										
0.454	0.523	0.487	0.387	0.437	0.322	0.206	0.400	0.411	1.000	1.000									
0.454	0.523	0.487	0.387	0.437	0.322	0.206	0.400	0.411	1.000	1.000	1.000								
0.074	0.080	0.166	0.000	0.266	0.428	0.333	0.111	0.352	0.275	0.275	0.275	1.000							
0.258	0.344	0.428	0.444	0.105	0.111	0.250	0.636	0.380	0.363	0.363	0.363	0.125	1.000						
0.083	0.090	0.095	0.000	0.000	0.181	0.222	0.133	0.142	0.076	0.076	0.076	0.222	0.153	1.000					
0.000	0.000	0.000	0.000	0.142	0.153	0.181	0.000	0.125	0.142	0.142	0.142	0.000	0.000	0.250	1.000				
0.444	0.470	0.424	0.347	0.333	0.173	0.285	0.296	0.230	0.631	0.631	0.631	0.190	0.240	0.222	0.200	1.000			
0.468	0.444	0.454	0.294	0.228	0.117	0.125	0.315	0.216	0.408	0.408	0.408	0.062	0.277	0.000	0.064	0.243	1.000		
0.291	0.304	0.311	0.285	0.166	0.228	0.242	0.358	0.210	0.440	0.440	0.440	0.181	0.324	0.000	0.125	0.333	0.339	1.000	
0.434	0.409	0.418	0.242	0.176	0.181	0.193	0.432	0.388	0.416	0.416	0.416	0.129	0.457	0.071	0.133	0.250	0.352	0.538	1.000

Table 5-10 Genetic similarity matrix from the AFLP scores of selective primer pair H+8 in Table 5-5

PA1-1	PA1-2	PA1-3	PA2	PA3	PA4	PA5	PA6	PA7	PS1-1	PS1-2	PS1-3	PS2	PS3	PS4	PS5	PS6-1	VO	AG	LE
1.000																			
0.769	1.000																		
0.615	0.875	1.000																	
0.285	0.352	0.352	1.000																
0.344	0.400	0.457	0.378	1.000															
0.260	0.206	0.275	0.387	0.375	1.000														
0.400	0.444	0.444	0.578	0.256	0.424	1.000													
0.307	0.375	0.437	0.411	0.514	0.551	0.333	1.000												
0.347	0.482	0.551	0.322	0.562	0.230	0.303	0.551	1.000											
0.214	0.235	0.294	0.444	0.594	0.451	0.315	0.529	0.387	1.000										
0.250	0.200	0.266	0.375	0.545	0.444	0.294	0.333	0.370	0.875	1.000									
0.230	0.250	0.312	0.470	0.571	0.413	0.333	0.375	0.413	0.882	0.933	1.000								
0.240	0.322	0.387	0.363	0.352	0.357	0.514	0.451	0.428	0.484	0.344	0.322	1.000							
0.400	0.388	0.333	0.473	0.512	0.484	0.550	0.611	0.484	0.421	0.294	0.277	0.571	1.000						
0.333	0.250	0.333	0.230	0.444	0.476	0.285	0.416	0.380	0.384	0.363	0.333	0.434	0.500	1.000					
0.318	0.400	0.400	0.538	0.603	0.382	0.518	0.560	0.468	0.576	0.500	0.560	0.408	0.481	0.238	1.000				
0.250	0.266	0.266	0.437	0.545	0.444	0.294	0.333	0.444	0.375	0.357	0.333	0.275	0.529	0.545	0.416	1.000			
0.186	0.285	0.367	0.392	0.500	0.478	0.490	0.489	0.434	0.470	0.382	0.448	0.416	0.528	0.292	0.686	0.425	1.000		
0.222	0.380	0.380	0.363	0.311	0.307	0.478	0.333	0.410	0.363	0.350	0.333	0.341	0.391	0.058	0.566	0.300	0.508	1.000	
0.325	0.326	0.367	0.431	0.423	0.304	0.490	0.408	0.434	0.431	0.382	0.408	0.416	0.490	0.195	0.626	0.425	0.606	0.542	1.000

Table 5-11 Genetic similarity matrix from the AFLP scores of selective primer pairs A+5, B+4, B+8 and H+6 in Table 5-6 to 5-9.

PA1-1	PA1-2	PA1-3	PA2	PA3	PA4	PA5	PA6	PA7	PS1-1	PS1-2	PS1-3	PS2	PS3	PS4	PS5	PS6-1	VO	AG	LE
1.000																			
0.920	1.000																		
0.836	0.901	1.000																	
0.346	0.346	0.320	1.000																
0.307	0.269	0.240	0.292	1.000															
0.355	0.373	0.368	0.329	0.258	1.000														
0.372	0.372	0.428	0.325	0.200	0.506	1.000													
0.464	0.428	0.425	0.555	0.266	0.387	0.454	1.000												
0.349	0.333	0.344	0.346	0.326	0.429	0.372	0.500	1.000											
0.374	0.388	0.370	0.307	0.358	0.333	0.260	0.352	0.345	1.000										
0.385	0.400	0.396	0.300	0.353	0.362	0.324	0.330	0.311	0.837	1.000									
0.345	0.359	0.340	0.324	0.358	0.316	0.260	0.304	0.330	0.907	0.864	1.000								
0.333	0.383	0.396	0.244	0.306	0.376	0.333	0.320	0.400	0.375	0.372	0.375	1.000							
0.271	0.310	0.343	0.395	0.172	0.261	0.379	0.449	0.330	0.224	0.267	0.241	0.432	1.000						
0.153	0.197	0.183	0.144	0.173	0.305	0.238	0.233	0.175	0.230	0.220	0.250	0.352	0.382	1.000					
0.217	0.257	0.247	0.278	0.177	0.414	0.337	0.252	0.356	0.298	0.272	0.315	0.357	0.307	0.242	1.000				
0.348	0.333	0.328	0.290	0.454	0.265	0.314	0.305	0.303	0.634	0.624	0.662	0.444	0.220	0.350	0.280	1.000			
0.355	0.364	0.200	0.276	0.300	0.296	0.318	0.368	0.290	0.298	0.303	0.368	0.419	0.217	0.188	0.220	0.329	1.000		
0.293	0.335	0.330	0.264	0.198	0.241	0.252	0.325	0.335	0.346	0.328	0.358	0.394	0.333	0.203	0.220	0.335	0.343	1.000	
0.313	0.313	0.323	0.250	0.232	0.260	0.236	0.316	0.328	0.367	0.349	0.367	0.296	0.360	0.303	0.201	0.357	0.337	0.397	1.000

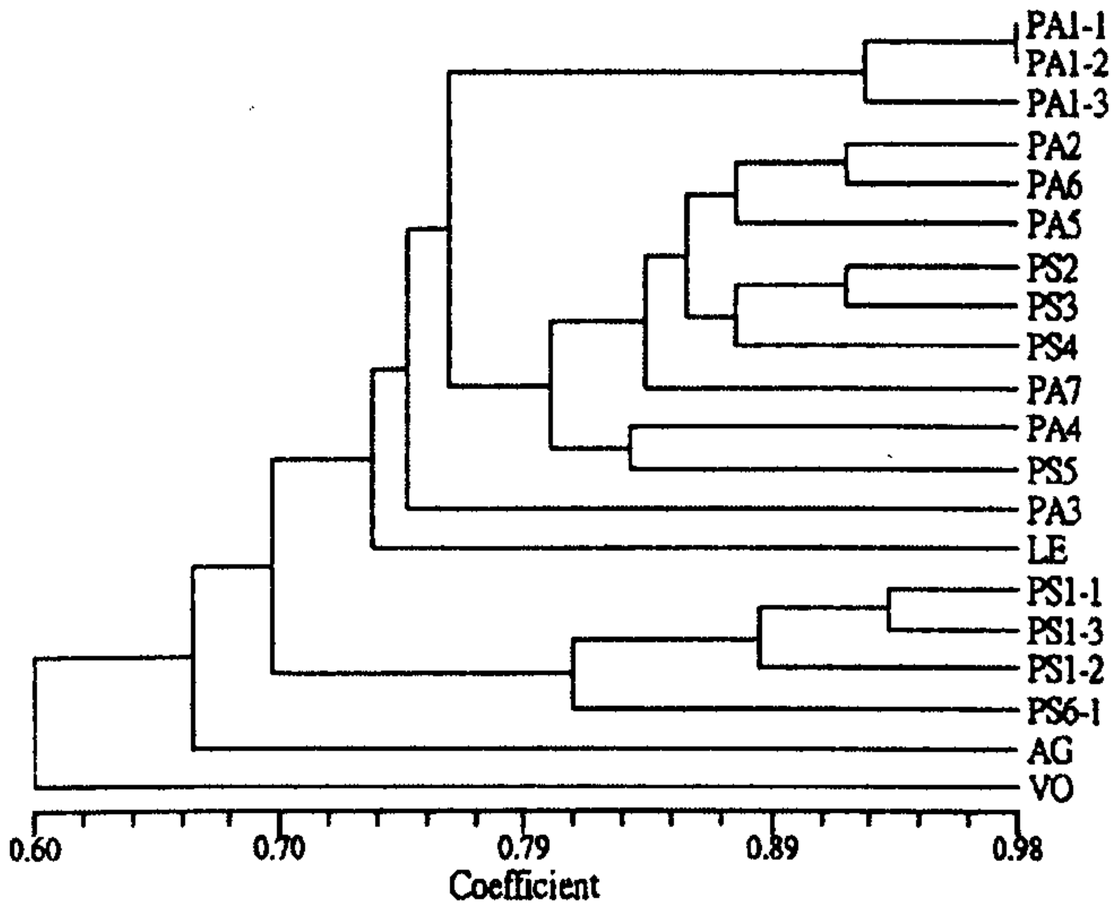


Figure 5-13 Dendrogram established by the genetic similarity matrix in Table 5-6.

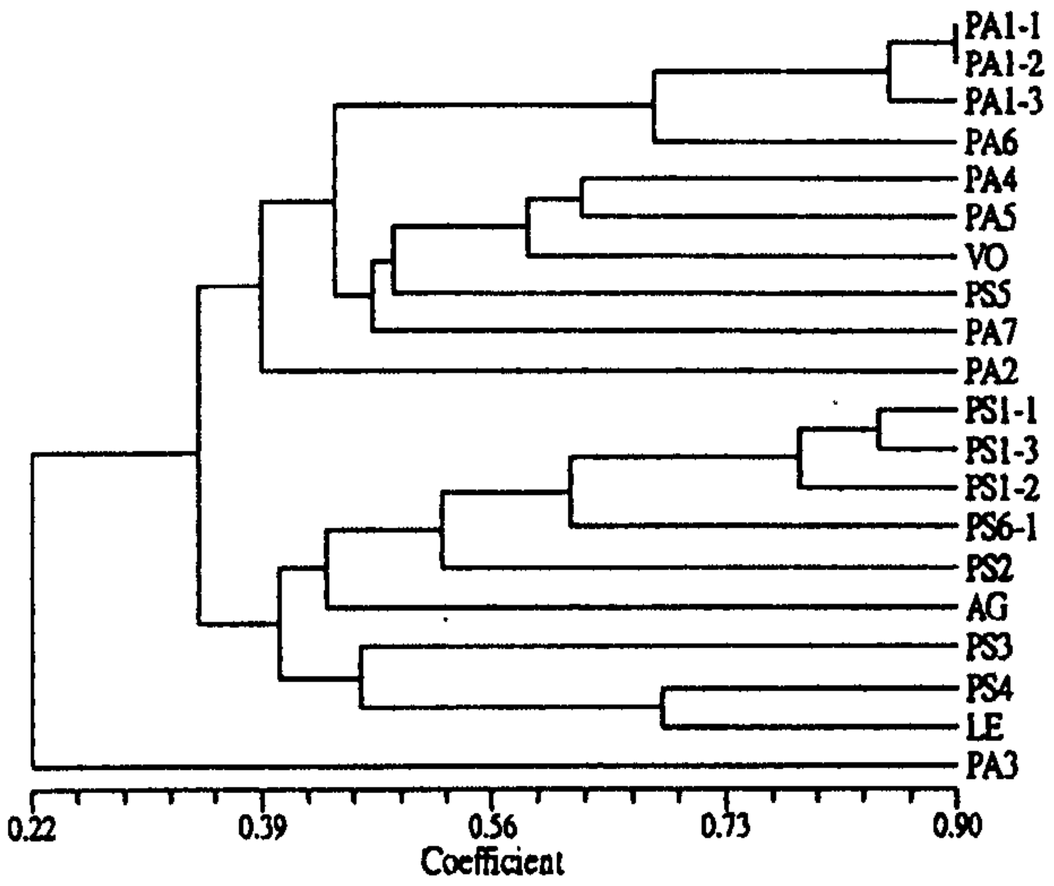


Figure 5-14 Dendrogram established by the genetic similarity matrix in Table 5-7.

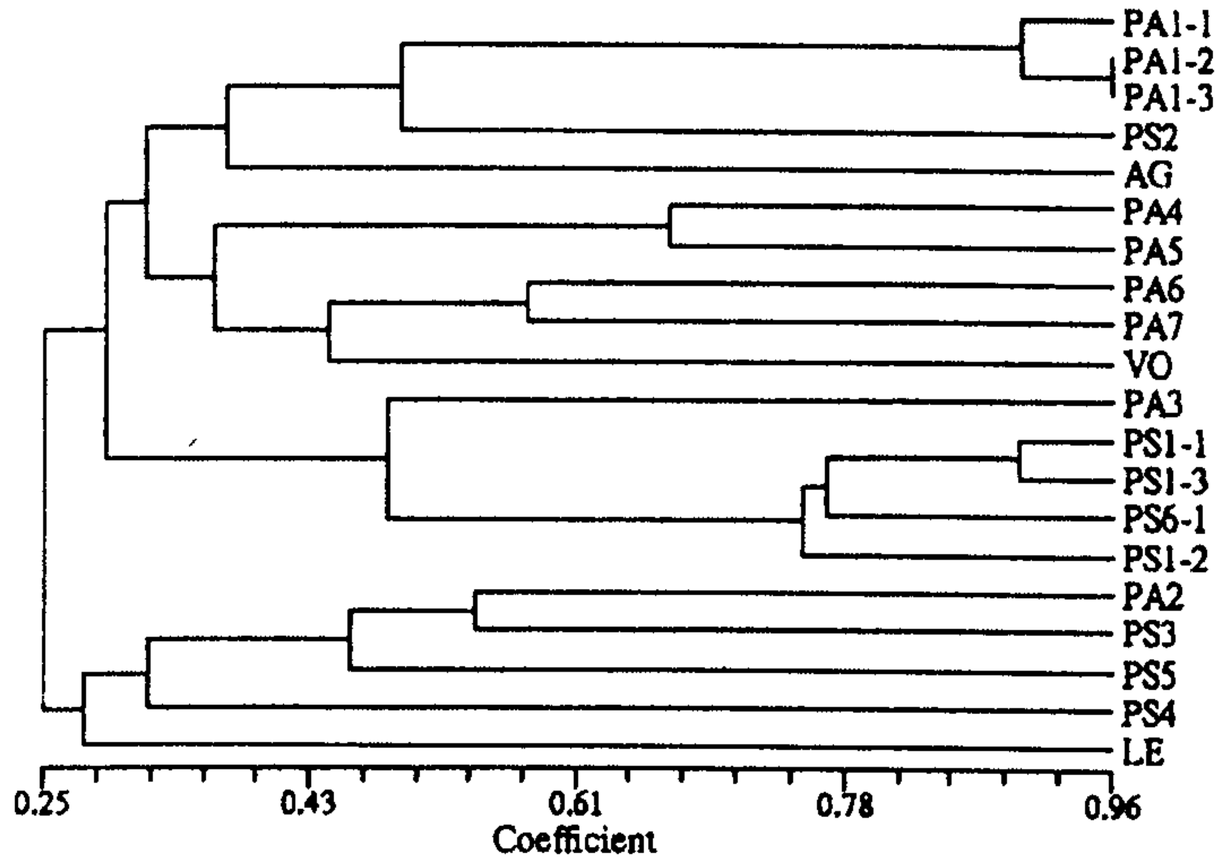


Figure 5-15 Dendrogram established by the genetic similarity matrix in Table 5-8.

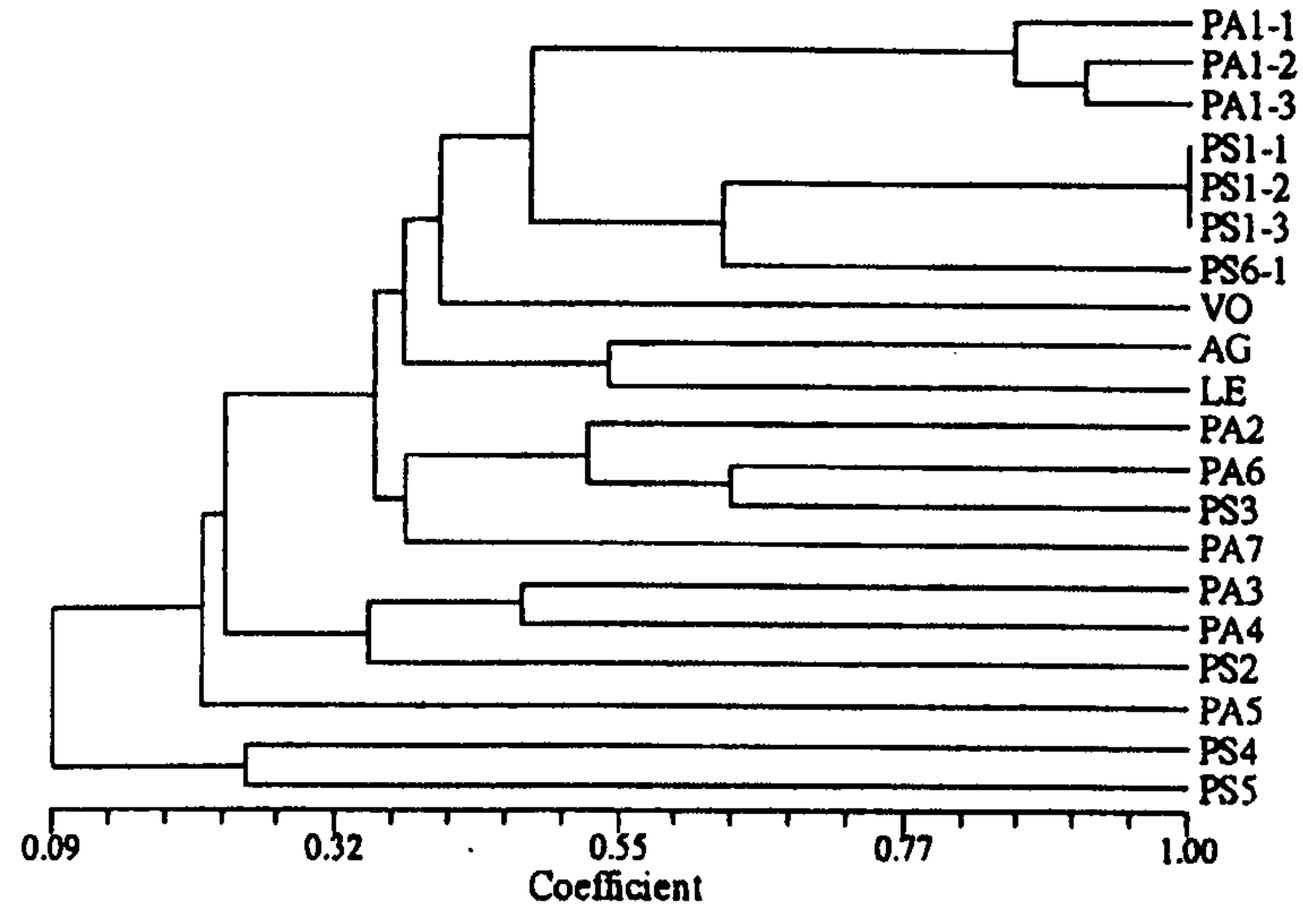


Figure 5-16 Dendrogram established by the genetic similarity matrix in Table 5-9.

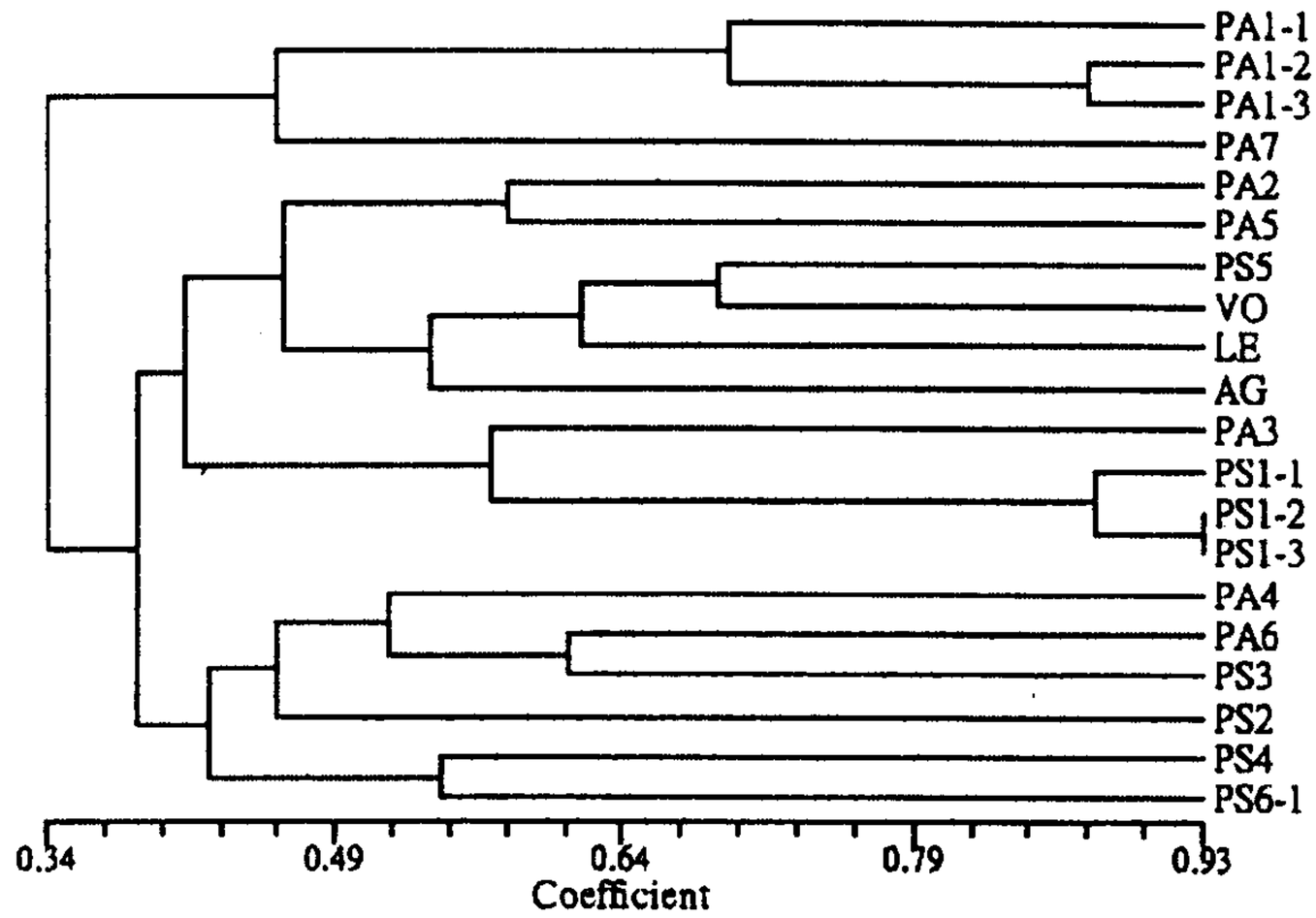


Figure 5-17 Dendrogram established by the genetic similarity matrix in Table 5-10.

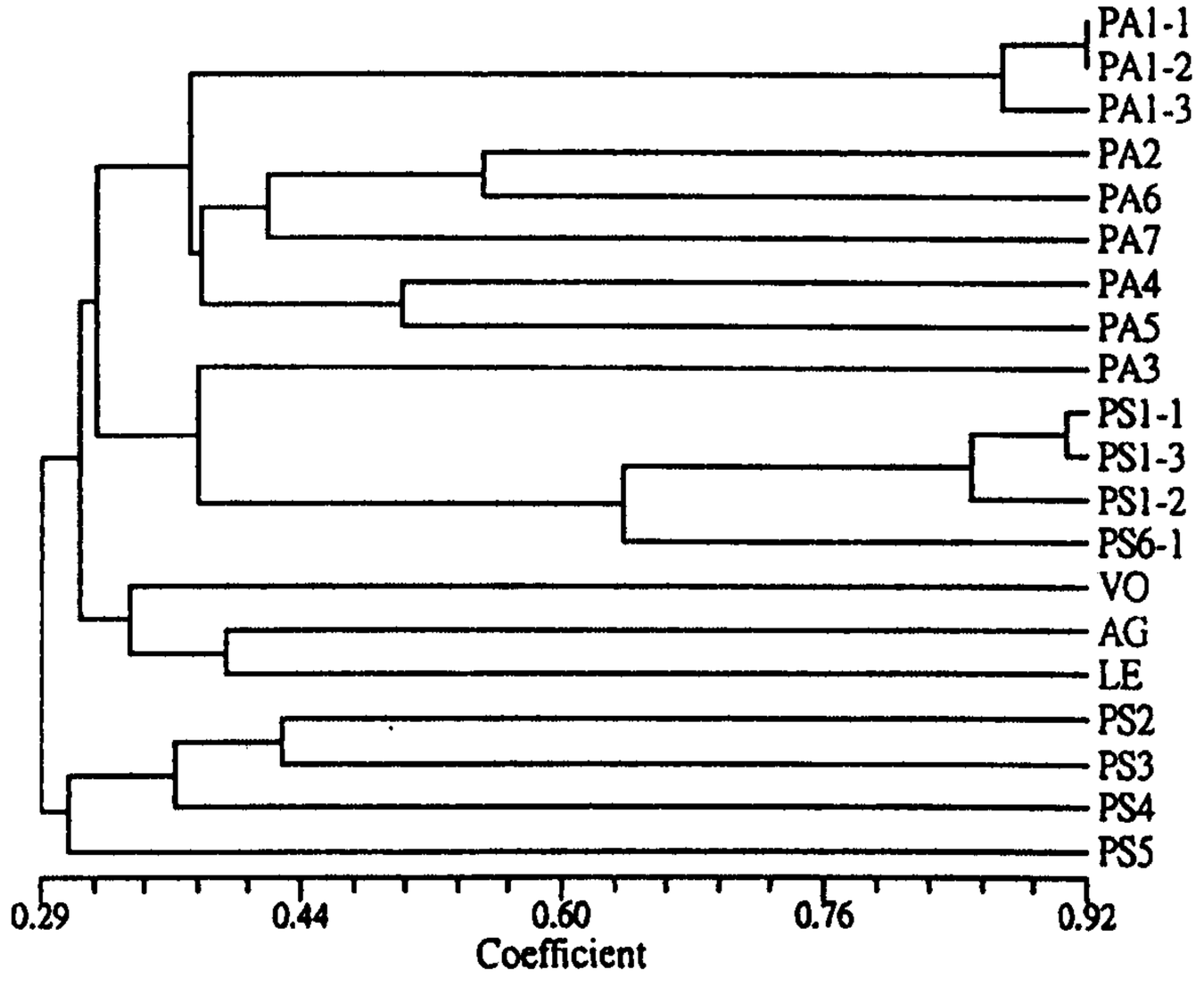


Figure 5-18 Dendrogram established by the genetic similarity matrix in Table 5-11.

5.3.4 Species Discrimination

For AFLP to be of value in this study it must either discriminate species or genus. From the cluster results of Figure 5-13 to 5-17, AFLP grouped members of the same species together, and therefore would be of value in species comparison.

AFLP samples from members of the same species (PA1-1, 1-2 and 1-3, and PS1-1, 1-2, 1-3 and PS6-1 were clustered in the same group due to the high GS score based on matching AFLP bands. An exception was sample PS6-1 in Figure 5-17, which should have been grouped with other members of the same species (PS1-1, PS1-2 and PS1-3).

It can be noted that each primer pair used in AFLP produce different GS scores due to the band matching criteria. For any study it is necessary to test a number of primer pairs to determine which are of greater value.

Figures 5-18 showed the cluster results produced by combining the four AFLP patterns. The resulting dendrogram clustered all the members of the *Panaeolus* genus together by these AFLP patterns. PA1-1, PA1-2 and PA1-3 are samples of the same species (*Panaeolus semiovatus*) and therefore have a high GS score (0.836), as expected. This is not the case with PA1 and PA5 where the GS score is 0.372 despite the samples being identified morphologically as being of the same species. It is possible that an error was made in the original identification of specimen PA5.

On the basis that PA1 and PA5 are not members of the same species, the lowest GS score for members of the same species was between samples PS1-2 and PS6. This produced a score of 0.624. A GS score of lower than 0.6 could be used as a threshold, below which the samples are from different species. A greater number of tests may be needed to confirm this.

For genus specific evaluation in this system, the GS score between any two

species in *Panaeolus* were from 0.200 to 0.555, and in *Psilocybe* were from 0.220 to 0.432. The three additional species tested consistently showed low GS scores compared to any of the *Psilocybe* or *Panaeolus* samples. The highest score for any of these three samples was 0.397, being between *Lentinus edodes* and *Agaricus bisporus*. This clearly shows that the score obtained within genera is higher than that obtained between genera. By this system, however, the AFLP patterns show neither genus specific band nor genus specific GS score range.

AFLP using silver staining is simple, cheap and easy to perform. By use of selecting primer pairs and extending the primers by up to three bases specific reproducible bands can be produced. AFLP can discriminate between species of the genus *Psilocybe*. In summary, this technique offers identification of fungi from the genera *Psiloctybe* and *Panaeolus* to species level where it is not possible using examination of the morphology and / or chemical constituents of the sample.

6 ANALYSIS OF THE NUCLEAR SMALL SUBUNIT RIBOSOMAL DNA

6.1 Introduction

In eukaryotic organisms the nuclear ribosomal RNA genes are arranged in three clusters, which are a small subunit gene (16S to 18S), a large subunit gene (26S to 28S), and the 5.8S gene. Between these genes there are two internal transcribed spacer sequences (ITS1 and ITS2) and an external transcribed spacer (ETS) at the 5' end of the transcribed RNA. These six components make up the basic gene cluster (Figure 6-1). This gene cluster is repeated in a tandem array up to hundreds or thousands of times. Between each cluster in the array is a non-transcribed spacer (NTS) that serves to separate individual repeats from one another [86]. From previous studies it has been found that the genes sequences are more conserved than the transcribed spacers [14]. Equally the transcribed spacers are more highly conserved than the non-transcribed spacers [14]. The small subunit rDNA sequences evolve relatively slowly, resulting in a high degree of sequence similarity between closely related organisms. The internal transcribed spacer region and intergenic spacer of the nuclear rDNA repeat units evolve fast and may vary among species of the same genus or between varieties of the same species [15]. These gene sequences has been used in taxonomic studies due to their evolutionary rates [14].

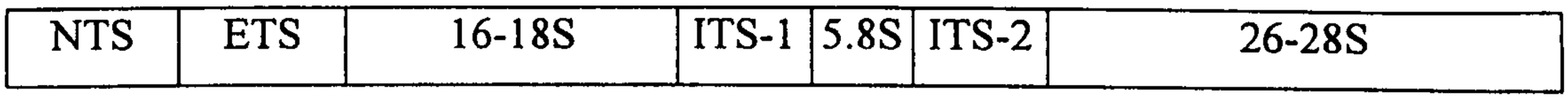


Figure 6-1 Map of the nuclear rDNA

NTS is the Non-transcribed spacer that separates the rRNA gene sequence. ETS is the external transcribed spacer that is outside of the main encoding region. ITS is the Internal Transcribed Spacer, of which there are two. The gene sequences 16, 18, 26 and 28 S gene sequences. The S relates to the Svedberg Unit of density.

In this study the nuclear small subunit ribosomal DNA (nuc-ssu-rDNA) from members of the genera *Psilocybe* and *Panaeolus* will be examined for DNA sequences that are genus or species specific. The complete DNA sequence from this locus will be compared to other fungi. To perform this study, primers were designed to amplify the nuc-ssu-rDNA from *Psilocybe* and *Panaeolus* fungal samples. The resulting polymerase chain reaction products will be sequenced and by comparing the resulting sequences of these fungi sample to each other and to previously known sequences, this study will try to reveal the sequence variation among members of the genera *Psilocybe* and *Panaeolus*.

6.2 Materials and Methods

6.2.1 *Psilocybe* and *Panaeolus* Fungi

DNA samples of fungi extracted in section 3.4 were used in this analysis.

6.2.2 PCR Amplification

Two primers (SR1c and NS6) were used as described by Hibbett [26] which had previously amplified DNA products of 877 bp from nuc-ssu-rDNA in fungal species. The sequences of the forward primer SR1c are 5'-AGCAGCCGCGGTAAT-3', and reverse primer NS6 in 3' end are 5'-GCATCACAGACCTGTTATTGCCTC-3'.

PCR amplification

Reaction Buffer	10mM Tris-HCl, pH8.8 at 25 °C, 1.5mM MgCl2, 1.5mM KCl, 0.1% Triton X-100
dNTPs	200μM
DNA Polymerase	1 unit of DyDNAyme™ II
Template DNA	approximately 15 ng
Total Volume	50 μl

Amplification Conditions

Temperature	Time	
94 °C	1 min	
58 °C	1 min	Number of Cycles 35
72 °C	2 min	

The PCR amplifications were performed in a DNA thermal cycler 480 (Perkin-Elmer, NJ, USA).

6.2.3 Electrophoresis of Samples

PCR products were separated by electrophoresis on a 3% agarose gel (FMC

BioProducts, Rockland, ME, USA) with 0.5μg/ml ethidium bromide in TBE buffer (0.89M Tris-HCl, 0.889M borate, 0.002M EDTA, pH8.0) at 100V for 30 min. PCR products were visualized by 312nm UV light and photographed using Polaroid 667 film.

6.2.4 DNA Sequencing

The PCR products of nuc-ssu-rDNA fragments were separated by 2% low melting agarose gel electrophoresis to remove excess dNTPs and primers. The amplified fragments run in low melting gel were sliced from the gel and placed in a separate microcentrifuge tube. The DNA in the gel slice was purified by alcohol precipitation. Approximately 50ng of purified PCR products were used to perform the sequencing reaction using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems CA, USA). Two sequencing primers were used, one using the primer SR1c and the other using the primer NS6. According to the sequencing results of primer SR1c, primer SR1c2 was designed and used to reveal the sequences in the middle region of the fragments, since there are instances that can not be read through the whole fragment only by forward and reverse primers. The sequences of primer are 5'-ATGATTAATAGGGATAGTTGGG-3'. Sequencing electrophoresis was carried out in PE Applied Biosystems PRISM 310 Genetic Analyzer according to the manufacturer's instructions.

6.2.5 Sequence Analysis

Sequence comparison was performed using the Pileup and Pretty programs within the GCG (Genetics Computer Group) software. Unweighted pair-group method

with arithmetic averaging (UPGMA) and Neighbor-Joining tree [87] were constructed to display the phylogenetic relationships.

6.3 Results and Discussion

DNA fragments of nuclear small subunit ribosomal gene were successfully amplified by primer pairs SR1c and NS6, and produced a DNA product of approximately 900 bp (Figure 6-2) estimated by the 100 bp ladder marker. From this gel image it is clear that there is very little difference in size between the PCR fragments for all the fungal samples examined. The PCR products were removed from the gel, purified, and the complete DNA sequence determined for both strands. The PCR products were too large to permit complete DNA sequencing using primers from either end. The internal DNA sequences were confirmed by using the primer SR1c2. Figure 6-3 to 6-5 show examples of sequencing results by sequencing primer SR1c, NS6 and SR1c2 of sample PA1-1, respectively.

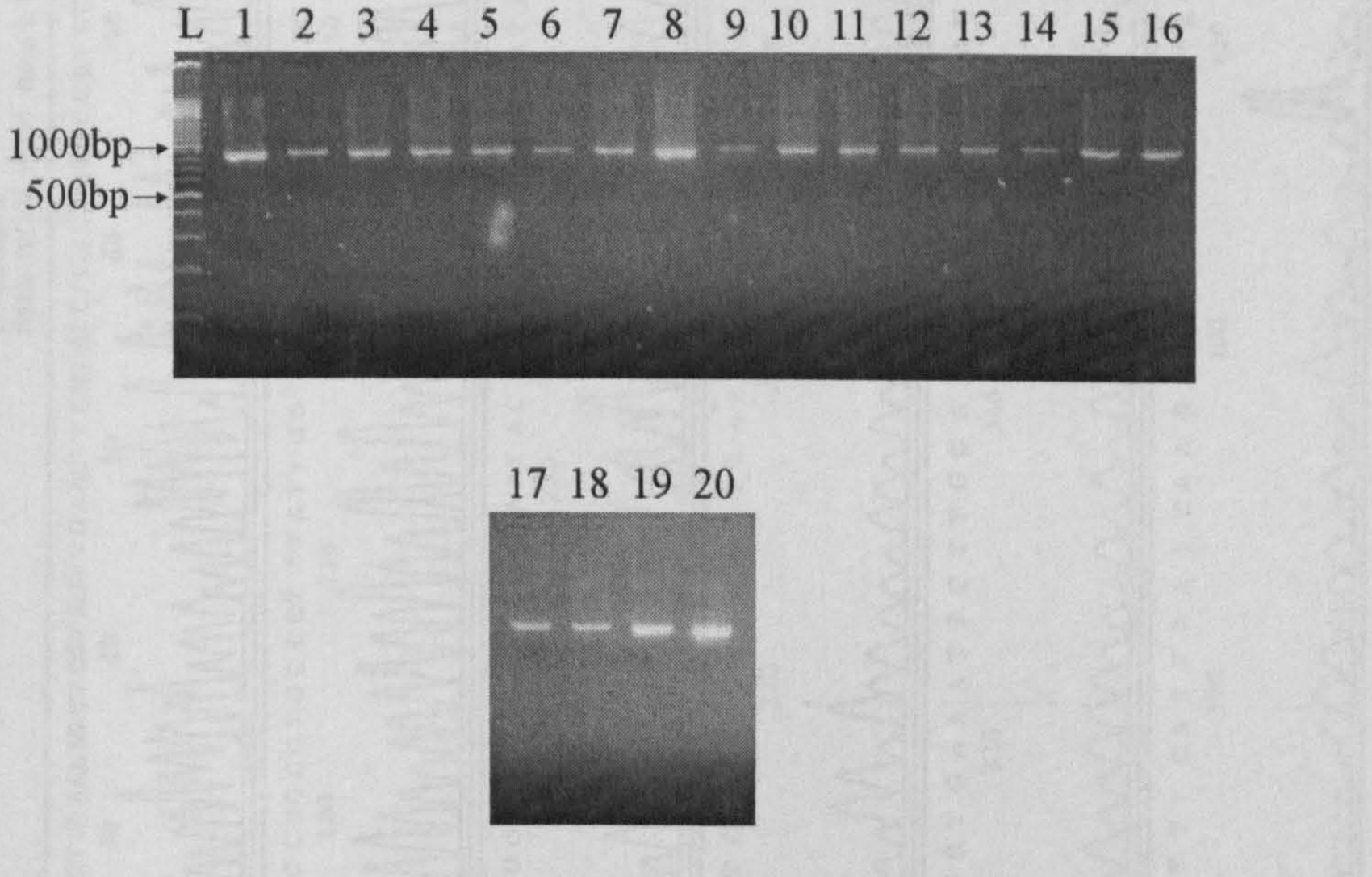


Figure 6-2 PCR products of this nuc-ssu-rDNA fragments amplified by primer SR1c and NS6. L is 100 bp ladder. Lanes 1-10 are samples PS1, PS2, PS3, PS4, PS5, PA1, PA2, PA3, PA4, PA5.

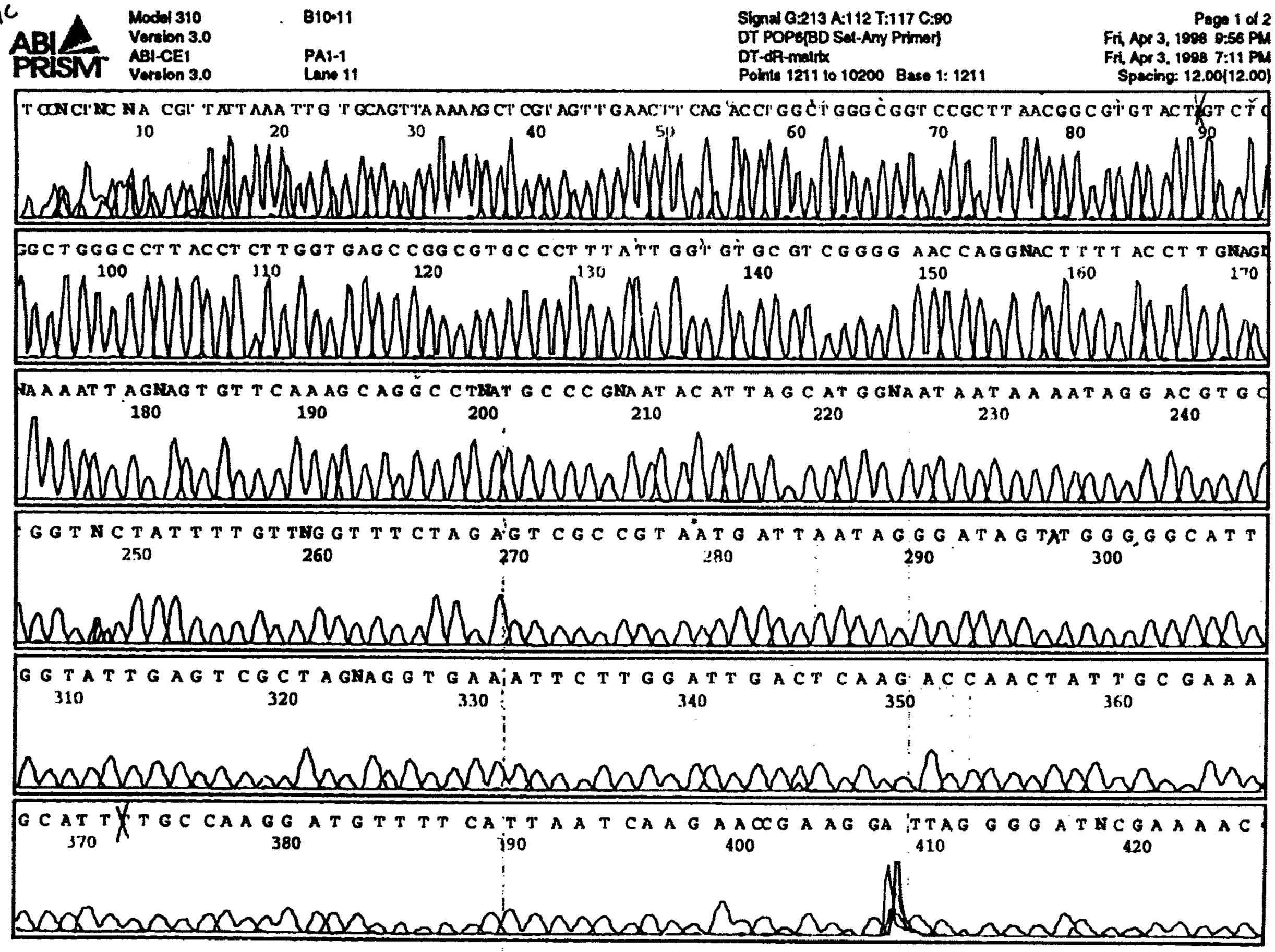


Figure 6-3 Sequencing electropherogram of sample PA1-1 from DNA fragment of the nuclear small subunit rDNA by primer SR1c.

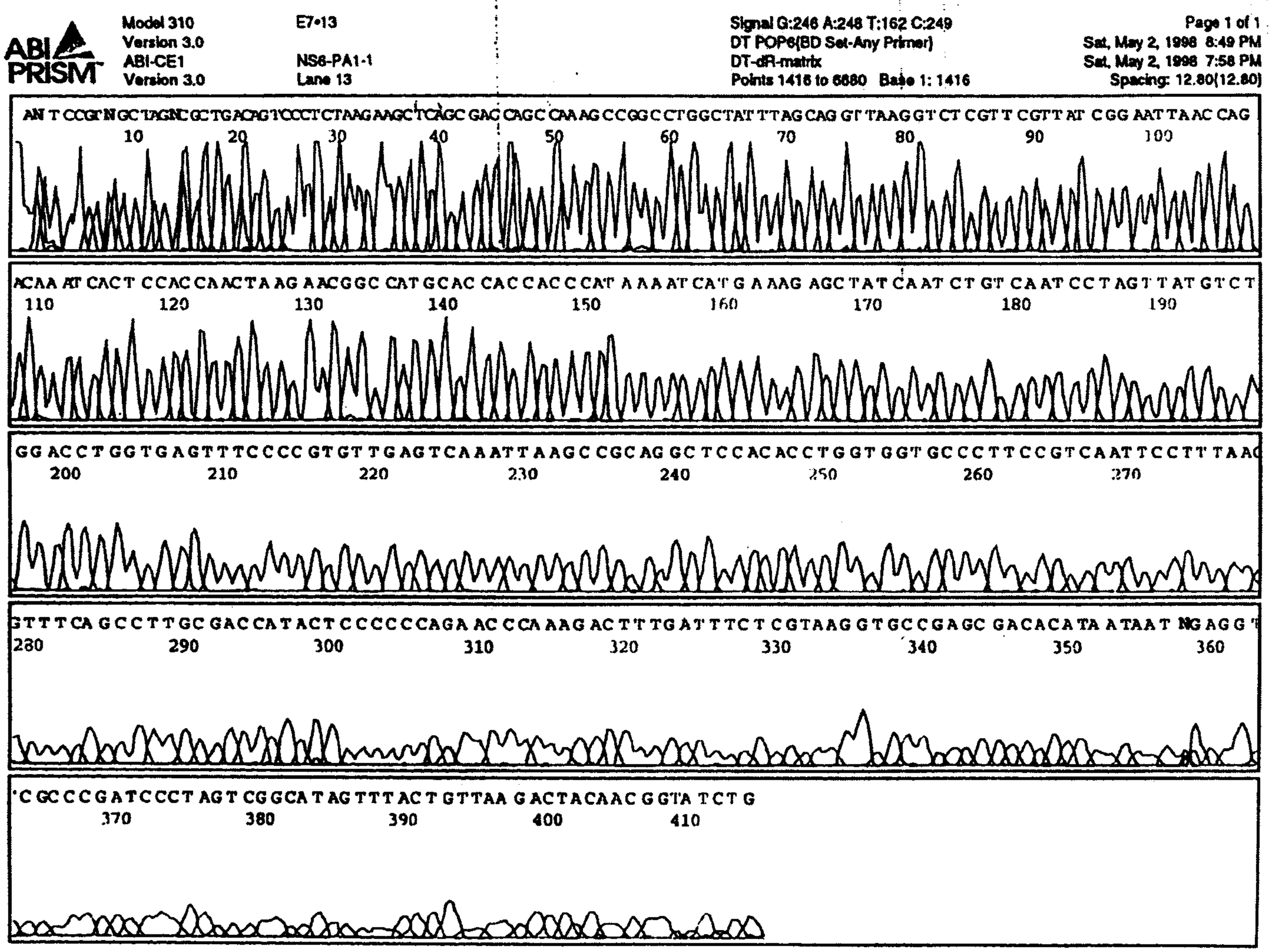


Figure 6-4 Sequencing electropherogram of sample PA1-1 from DNA fragment of the nuclear small subunit rDNA by primer NS6.

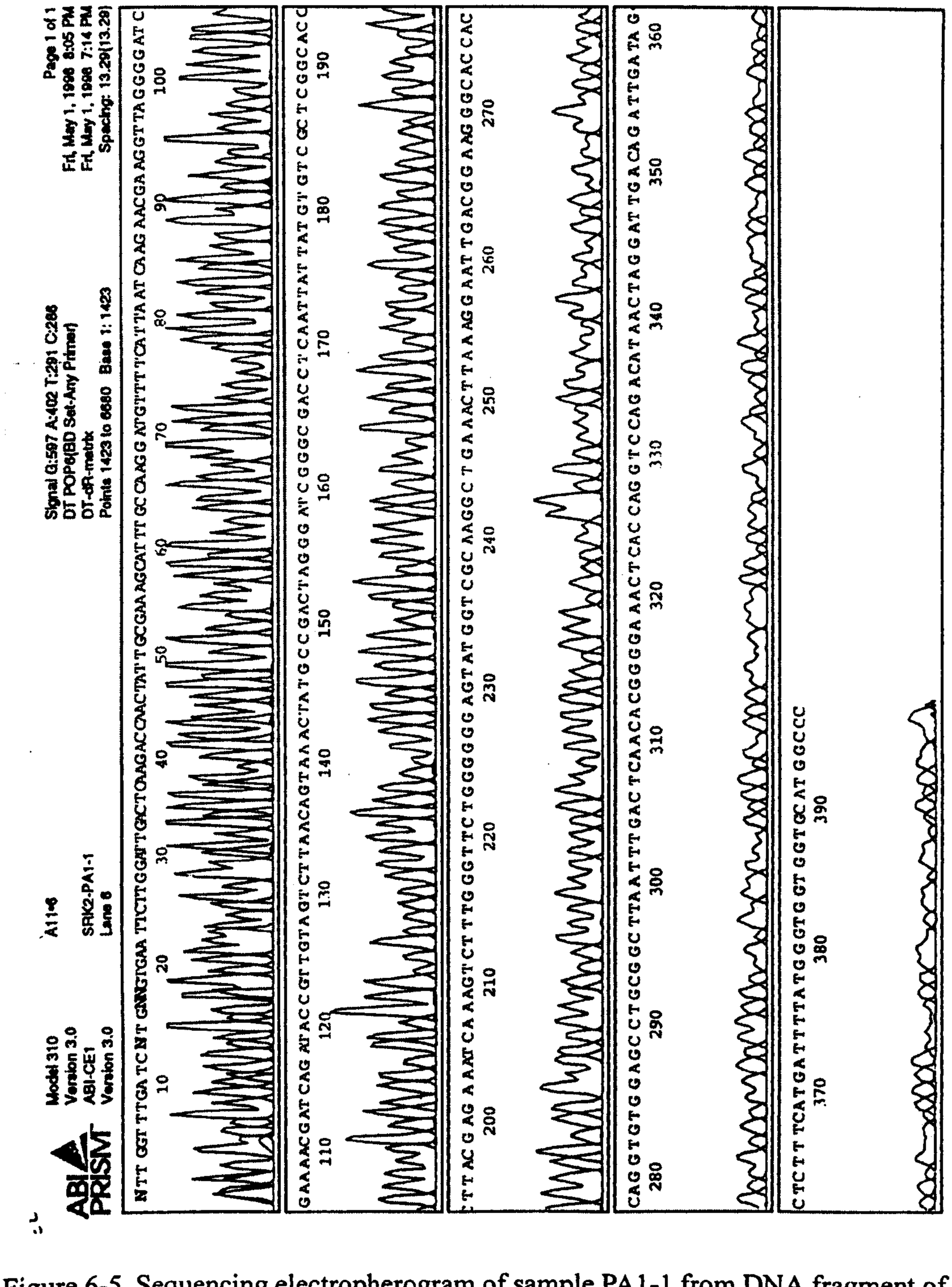


Figure 6-5 Sequencing electropherogram of sample PA1-1 from DNA fragment of the nuclear small subunit rDNA by primer SR1c2.

The complete nucleotide sequence of the nuc-ssu-rDNA locus for the *Panaeolus* sample PA1-1 is shown in Figure 6-6. The DNA sequence of two fungal (samples PA1-1 and PS-1) were compared to the EMBL databank of DNA sequences to determine their homology to previously reported sequences. All 877 bases of PA1-1 and PS1-1 were compared to the EMBL databank and a comparison performed using the FASTA program. The species exhibiting the nearest similarity to sample PA1-1 of *Panaeolus* genus was *Calvatia gigantea*, which was found in the partial sequence of 18S small subunit ribosomal RNA gene (locus AF026622) from the EMBL database. In this instance there was an identity of 98.6% over the 878 nt overlap. The most similar species to sample PS1-1 of *Psilocybe* genus is *Panellus serotinus*, which was found in the partial sequence of 18S small subunit ribosomal RNA gene (locus PSU59088) from the EMBL database with 99.0% identity in 877 nt overlap. Figure 6-7 and 6-8 showed the FASTA results for query sequences in the EMBL.

This high homology among *Panaeolus*, *Psilocybe* and the other genera of fungi are a demonstration of the slow evolution rate in the exon area of rDNA locus. This slowly evolving pattern can be used to differentiate the genera of *Panaeolus* and *Psilocybe*. Twenty samples from these two genera were sequenced and these sequence data were aligned by Pileup and Pretty software with sequences of *Calvatia gigantea* and *Panellus serotinus*. Figure 6-9 shows the results of sequence alignment of 22 sequences with the polymorphic sites in the nuc-ssu-rDNA gene for the species tested being shown in Figure 6-10.

```
   1 AGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAG
  51 TTAAAAAGCTCGTAGTTGAACTTCAGACCTGGCTGGGCGGTCCGCTTAAC
 101 GGCGTGTACTGTCTGGCTGGGCCTTACCTCTTGGTGAGCCGGCGTGCCCT
 151 TTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGAG
 201 TGTTCAAAGCAGGCCTATGCCCGAATACATTAGCATGGAATAATAAAATA
 251 GGACGTGCGGTTCTATTTTGTTGGTTTCTAGAGTCGCCGTAATGATTAAT
 301 AGGGATAGTTGGGGGCATTGGTATTGAGTCGCTAGAGGTGAAATTCTTGG
 351 ATTGACTCAAGACCAACTATTGCGAAAGCATTTGCCAAGGATGTTTTCAT
 401 TAATCAAGAACGAAGGTTAGGGGATCGAAAACGATCAGATACCGTTGTAG
 451 TCTTAACAGTAAACTATGCCGACTAGGGATCGGGCGACCTCAATTATTAT
 501 GTGTCGCTCGGCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGA
 551 GTATGGTCGCACGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCA
 601 GGTGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGG
 651 TCCAGACATAACTAGGATTGACAGATTGATAGCTCTTTCATGATTTTATG
 701 GGTGGTGGTGCATGGCCGTTCTTAGGTTGGTGGAGTGATTTGTCTGGTTA
 751 ATTCCGATAACGAACGAGACCTTAACCTGCTAAATAGCCAGGCCGGCTTT
 801 GGCTGGTCGCTGAGCTTCTTAGAGGGACTGTCAGCGTCTAGCTGACGGAA
 851 GTTGAGGCAATAACAGGTCTGTGATGC
```

Figure 6-6 Sequencing result of sample PA1-1 from nuc-ssu-rDNA gene. The sequences presented are the PCR amplified region which are 877 bp. Bases underlined are polymorphic sites except on the two ends which are primer sequences in this study.

```
                                                  10        20        30
PA1-1.dat                                         AGCAGCCGCGGTAATTCCAGCTCCAATAGC
                                                  ||||||||||||||||||||||||||||||
AF026622  GAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGC
                  540       550       560       570       580       590

                   40        50        60        70        80        90
PA1-1.datGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACTTCAGACCTGGCTGGGCGG
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  GTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACTTCAGACCTGGCTGGGCGG
                  600       610       620       630       640       650

                  100       110       120       130       140       150
PA1-1.dat TCCGCTTAACGGCGTGTACTGTCTGGCTGGGCCTTACCTCTTGGTGAGCCGGCGTGCCCT
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  TCCGCTTAACGGCGTGTACTGTCTGGCTGGGCCTTACCTCTTGGTGAGCCGGCGTGCCCT
                  660       670       680       690       700       710

                  160       170       180       190       200       210
PA1-1.dat TTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGAGTGTTCAAAGC
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  TTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGAGTGTTCAAAGC
                  720       730       740       750       760       770

                  220       230       240       250       260       270
PA1-1.dat AGGCCTATGCCCGAATACATTAGCATGGAATAATAAAATAGGACGTGCGGTTCTATTTTG
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  AGGCCTATGCCCGAATACATTAGCATGGAATAATAAAATAGGACGTGCGGTTCTATTTTG
                  780       790       800       810       820       830

                  280       290       300       310       320       330
PA1-1.dat TTGGTTTCTAGAGTCGCCGTAATGATTAATAGGGATAGTTGGGGGCATTGGTATTGAGTC
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  TTGGTTTCTAGAGTCGCCGTAATGATTAATAGGGATAGTTGGGGGCATTGGTATTGAGTC
                  840       850       860       870       880       890
```

```
                340        350        360        370        380        390
PA1-1.dat GCTAGAGGTGAAATTCTTGGATTGACTCAAGACCAACTATTGCGAAAGCATTTGCCAAGG
          ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
AF026622  GCTAGAGGTGAAATTCTTGGATTGACTCAAGACCGACTATTGCGAAAGCATTTGCCAAGG
                900        910        920        930        940        950

                400        410        420        430        440        450
PA1-1.dat ATGTTTTCATTAATCAAGAACGAAGGTTAGGGGATCGAAAACGATCAGATACCGTTGTAG
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  ATGTTTTCATTAATCAAGAACGAAGGTTAGGGGATCGAAAACGATCAGATACCGTTGTAG
                960        970        980        990       1000       1010

                460        470        480        490        500        510
PA1-1.dat TCTTAACAGTAAACTATGCCGACTAGGGATCGGGCGACCTCAATTATTATGTGTCGCTCG
          ||||||||||||||||||||||||||||||||||||||||||||||||| | | ||||||||||||
AF026622  TCTTAACAGTAAACTATGCCGACTAGGGATCGGGCGACCTCAAATTTGATGTGTCGCTCG
                1020       1030       1040       1050       1060       1070

                520        530        540        550        560        570
PA1-1.dat GCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCACGGCTGAAA
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
AF026622  GCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAA
                1080       1090       1100       1110       1120       1130

                580        590        600        610        620        630
PA1-1.dat CTTAAAGGAATTGACGGAAGGGCACCACCAGGTGTGGAGCCTGCGGCTTAATTTGACTCA
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  CTTAAAGGAATTGACGGAAGGGCACCACCAGGTGTGGAGCCTGCGGCTTAATTTGACTCA
                1140       1150       1160       1170       1180       1190

                640        650        660        670        680        690
PA1-1.dat ACACGGGGAAACTCACCAGGTCCAGACATAACTAGGATTGACAGATTGATAGCTCTTTCA
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF026622  ACACGGGGAAACTCACCAGGTCCAGACATAACTAGGATTGACAGATTGATAGCTCTTTCA
                1200       1210       1220       1230       1240       1250
```

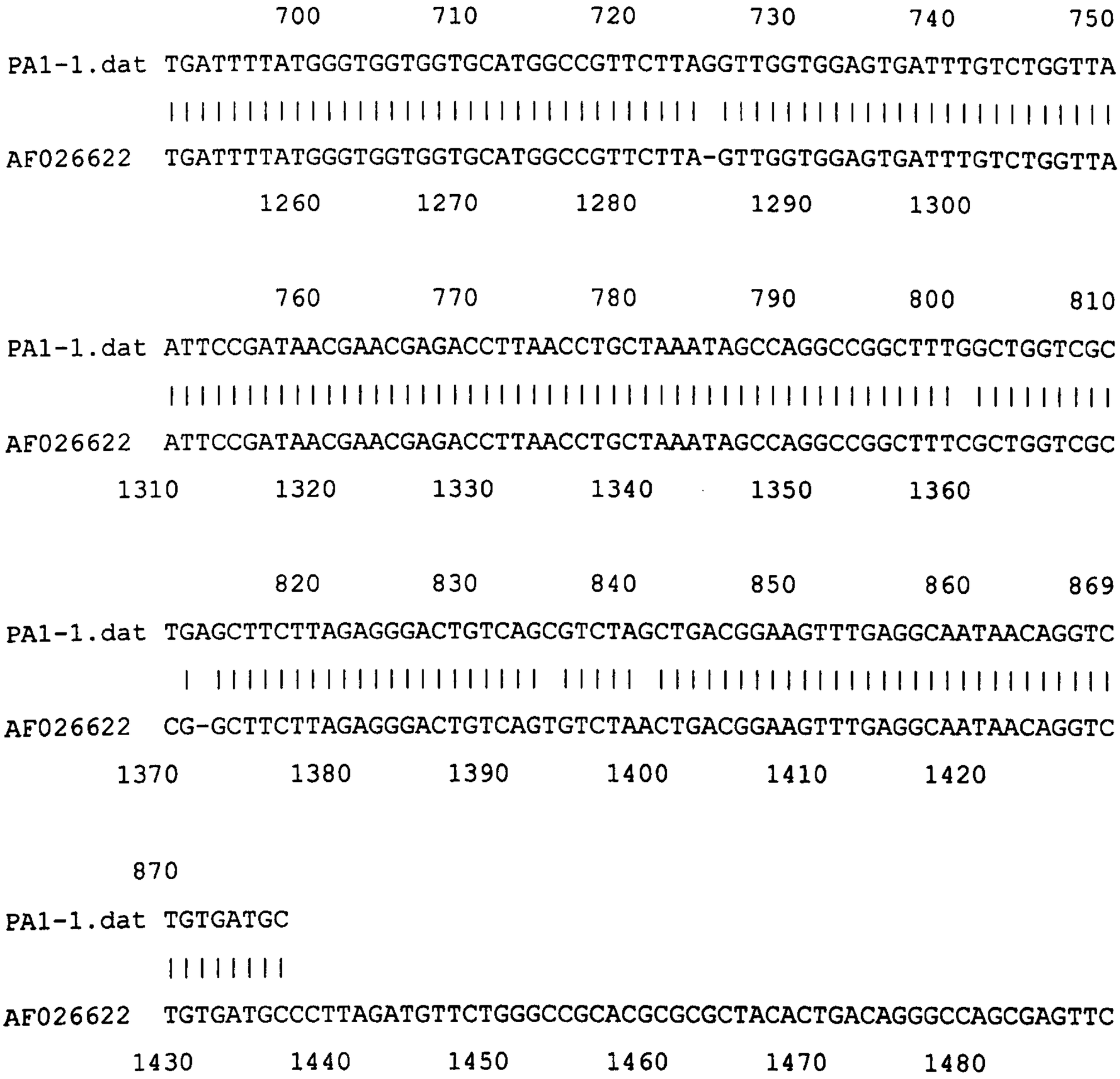


Figure 6-7 The comparison of the PA1-1 sequence data to the closest species (*Calvatia gigantea*) in the partial sequence of 18S small subunit ribosomal RNA gene (locus AF026622) from the EMBL database with 98.6% identity in 878 nt overlap.

```
                                                  10        20        30
PS1-1.dat                                         AGCAGCCGCGGTAATTCCAGCTCCAATAGC
                                                  ||||||||||||||||||||||||||||||
PSU59088  GAGGAACCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGC
                510       520       530       540       550       560

                 40        50        60        70        80        90
PS1-1.dat GTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACTTCAGACCTGGCCGGGCGG
          ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
PSU59088  GTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACTTCAGACCTGGTCGGGCGG
                570       580       590       600       610       620

                100       110       120       130       140       150
PS1-1.dat TCCGCTTAACGGCGTGTACTGTCTGGCTGGGCCTTACCTCTTGGTGAGCCGGCGTGCCCT
          |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
PSU59088  TCCGCTTAACGGCGTGTACTGTCTGACTGGGCCTTACCTCTTGGTGAGCCGGCGTGCCCT
                630       640       650       660       670       680

                160       170       180       190       200       210
PS1-1.dat TTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGAGTGTTCAAAGC
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PSU59088  TTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGAGTGTTCAAAGC
                690       700       710       720       730       740

                220       230       240       250       260       270
PS1-1.dat AGGCCTATGCCCGAATACATTAGCATGGAATAATAAAATAGGACGTGCGGTTCTATTTTG
          |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
PSU59088  AGGCCTATGCCCGAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTG
                750       760       770       780       790       800

                280       290       300       310       320       330
PS1-1.dat TTGGTTTCTAGAGTCGCCGTAATGATTAATAGGGATAGTTGGGGGCATTGGTATTGAGTC
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PSU59088  TTGGTTTCTAGAGTCGCCGTAATGATTAATAGGGATAGTTGGGGGCATTGGTATTGAGTC
                810       820       830       840       850       860
```

```
                340        350        360        370        380        390
PS1-1.dat GCTAGAGGTGAAATTCTTGGATTGACTCAAGACCAACTATTGCGAAAGCATTTGCCAAGG
          ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
PSU59088  GCTAGAGGTGAAATTCTTGGATTGACTCAAGACCAACTACTGCGAAAGCATTTGCCAAGG
                870        880        890        900        910        920

                400        410        420        430        440        450
PS1-1.dat ATGTTTTCATTAATCAAGAACGAAGGTTAGGGGATCGAAAACGATCAGATACCGTTGTAG
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PSU59088  ATGTTTTCATTAATCAAGAACGAAGGTTAGGGGATCGAAAACGATCAGATACCGTTGTAG
                930        940        950        960        970        980

                460        470        480        490        500        510
PS1-1.dat TCTTAACAGTAAACTATGCCGACTAGGGATCGGGCGACCTCAATTATTATGTGTCGCTCG
          |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
PSU59088  TCTTAACAGTAAACTATGCCGACTAGGGATCGGGCGACCTCAATTATGATGTGTCGCTCG
                990       1000       1010       1020       1030       1040

                520        530        540        550        560        570
PS1-1.dat GCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCACGGCTGAAA
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
PSU59088  GCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAA
               1050       1060       1070       1080       1090       1100

                580        590        600        610        620        630
PS1-1.dat CTTAAAGGAATTGACGGAAGGGCACCACCAGGTGTGGAGCCTGCGGCTTAATTTGACTCA
          |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
PSU59088  CTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCA
               1110       1120       1130       1140       1150       1160

                640        650        660        670        680        690
PS1-1.dat ACACGGGGAAACTCACCAGGTCCAGACATAACTAGGATTGACAGATTGATAGCTCTTTCA
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PSU59088  ACACGGGGAAACTCACCAGGTCCAGACATAACTAGGATTGACAGATTGATAGCTCTTTCA
               1170       1180       1190       1200       1210       1220
```

```
                  700        710        720        730        740        750
PS1-1.dat TGATTTTATGGGTGGTGGTGCATGGCCGTTCTTAGGTTGGTGGAGTGATTTGTCTGGTTA
          ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
PSU59088  TGATTTTATGGGTGGTGGTGCATGGCCGTTCTTA-GTTGGTGGAGTGATTTGTCTGGTTA
                 1230       1240       1250       1260       1270

                  760        770        780        790        800        810
PS1-1.dat ATTCCGATAACGAACGAGACCTTAACCTGCTAAATAGCCAGGCCGGCTTTCGCTGGTCGC
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PSU59088  ATTCCGATAACGAACGAGACCTTAACCTGCTAAATAGCCAGGCCGGCTTTCGCTGGTCGC
       1280       1290       1300       1310       1320       1330

                  820        830        840        850        860        869
PS1-1.dat CGGCTTCTTAGAGGGACTGTCAGCGTCTAGCTGACGGAAGTTTGAGGCAATAACAGGTCT
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PSU59088  CGGCTTCTTAGAGGGACTGTCAGCGTCTAGCTGACGGAAGTTTGAGGCAATAACAGGTCT
       1340       1350       1360       1370       1380       1390

           870
PS1-1.dat GTGATGC
          |||||||
PSU59088  GTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAGCCAGCGAGTTTT
    1400       1410       1420       1430       1440       1450
```

Figure 6-8 The comparison of the PS1-1 sequence data to the closest species (*Panellus serotinus*) in the partial sequence of 18S small subunit ribosomal RNA gene (locus PSU59088) from the EMBL database with 99.0% identity in 877 nt overlap.

```
                    1                                                  50
  1.msf{Cal}  ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
   Consensus  AGCAGCCGCG GTAATTCCAG CTCCAATAGC GTATATTAAA GTTGTTGCAG

                   51                                                 100
  1.msf{Cal}  ---------- ---------- ---------- ---t------ ----------
  1.msf{Pan}  ---------- ---------- ---------- --tc------ ----------
1.msf{PS1-1}  ---------- ---------- ---------- ---c------ ----------
1.msf{PS1-2}  ---------- ---------- ---------- ---c------ ----------
1.msf{PS1-3}  ---------- ---------- ---------- ---c------ ----------
1.msf{PS1-4}  ---------- ---------- ---------- ---c------ ----------
  1.msf{PS2}  ---------- ---------- ---------- ---c------ ----------
  1.msf{PS3}  ---------- ---------- ---------- ---c------ ----------
  1.msf{PS4}  ---------- ---------- ---------- ---c------ ----------
1.msf{PS6-1}  ---------- ---------- ---------- ---c------ ----------
1.msf{PS6-2}  ---------- ---------- ---------- ---c------ ----------
1.msf{PA1-1}  ---------- ---------- ---------- ---t------ ----------
```

```
                51                                                  100
1.msf{PA1-2}  ---------- ---------- ---------- ---t------ ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---t------ ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---t------ ----------
  1.msf{PA2}  ---------- ---------- ---------- ---t------ ----------
  1.msf{PA3}  ---------- ---------- ---------- ---t------ ----------
  1.msf{PA5}  ---------- ---------- ---------- ---t------ ----------
  1.msf{PA6}  ---------- ---------- ---------- ---t------ ----------
  1.msf{PA7}  ---------- ---------- ---------- ---t------ ----------
  1.msf{PA4}  ---------- ---------- ---------- ---t---t-- ----------
  1.msf{PS5}  ---------- ---------- ---------- --tc------ ----------
   Consensus  TTAAAAAGCT CGTAGTTGAA CTTCAGACCT GGC-GGGCGG TCCGCTTAAC

                101                                                 150
  1.msf{Cal}  ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}  ---------- -----a---- ---------- ---------- ----------
1.msf{PS1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}  ---------- ---c------ ---------- ---------- ----------
  1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}  ----a----- ---------- ---------- ---------- ----------
   Consensus  GGCGTGTACT GTCTGGCTGG GCCTTACCTC TTGGTGAGCC GGCGTGCCCT
```

```
                151                                                 200
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---c---.-- ---------- ---------- ---------- ----------
   Consensus    TTATTGGTGT GCGTCGGGGA ACCAGGACTT TTACCTTGAG AAAATTAGAG

                201                                                 250
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- -----g----
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
```

```
                201                                                 250
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    TGTTCAAAGC AGGCCTATGC CCGAATACAT TAGCATGGAA TAATAAAATA

                251                                                 300
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    GGACGTGCGG TTCTATTTTG TTGGTTTCTA GAGTCGCCGT AATGATTAAT
```

```
                301                                                 350
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    AGGGATAGTT GGGGGCATTG GTATTGAGTC GCTAGAGGTG AAATTCTTGG

                351                                                 400
  1.msf{Cal}    ---------- ----g----- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------c ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
```

```
                    351                                                 400
1.msf{PA1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
   Consensus  ATTGACTCAA GACCAACTAT TGCGAAAGCA TTTGCCAAGG ATGTTTTCAT

                    401                                                 450
  1.msf{Cal}  ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
   Consensus  TAATCAAGAA CGAAGGTTAG GGGATCGAAA ACGATCAGAT ACCGTTGTAG
```

```
                451                                                 500
  1.msf{Cal}    ---------- ---------- ---------- ---------- ---a-t-g--
  1.msf{Pan}    ---------- ---------- ---------- ---------- -------g--
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    TCTTAACAGT AAACTATGCC GACTAGGGAT CGGGCGACCT CAATTATTAT

                501                                                 550
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
```

```
                501                                                 550
1.msf{PA1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
   Consensus  GTGTCGCTCG GCACCTTACG AGAAATCAAA GTCTTTGGGT TCTGGGGGGA

                551                                                 600
  1.msf{Cal}  ---------- -a-------- ---------- ---------- ----------
  1.msf{Pan}  ---------- -a-------- ---------- ---------- ----------
1.msf{PS1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}  ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}  ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
   Consensus  GTATGGTCGC ACGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
```

```
                601                                                  650
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    --c------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    GGTGTGGAGC CTGCGGCTTA ATTTGACTCA ACACGGGGAA ACTCACCAGG

                651                                                  700
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
```

```
                651                                                   700
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    TCCAGACATA ACTAGGATTG ACAGATTGAT AGCTCTTTCA TGATTTTATG

                701                                                   750
  1.msf{Cal}    ---------- ---------- ----.----- ---------- ----------
  1.msf{Pan}    ---------- ---------- ----.----- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    GGTGGTGGTG CATGGCCGTT CTTAGGTTGG TGGAGTGATT TGTCTGGTTA
```

```
                751                                                 800
  1.msf{Cal}    ---------- ---------- ---------- ---------- ----------
  1.msf{Pan}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS4}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-1}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-2}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-3}    ---------- ---------- ---------- ---------- ----------
1.msf{PA1-4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA2}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA3}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA5}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA6}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA7}    ---------- ---------- ---------- ---------- ----------
  1.msf{PA4}    ---------- ---------- ---------- ---------- ----------
  1.msf{PS5}    ---------- ---------- ---------- ---------- ----------
   Consensus    ATTCCGATAA CGAACGAGAC CTTAACCTGC TAAATAGCCA GGCCGGCTTT

                801                                                 850
  1.msf{Cal}    ---------- --.------- ---------- ----t----- a---------
  1.msf{Pan}    ---------- --.------- ---------- ---------- ----------
1.msf{PS1-1}    ---------- --.------- ---------- ---------- ----------
1.msf{PS1-2}    ---------- --.------- ---------- ---------- ----------
1.msf{PS1-3}    ---------- --.------- ---------- ---------- ----------
1.msf{PS1-4}    ---------- --.------- ---------- ---------- ----------
  1.msf{PS2}    ---------- --.------- ---------- ---------- ----------
  1.msf{PS3}    ---------- --.------- ---------- ---------- ----------
  1.msf{PS4}    ---------- --.------- ---------- ---------- ----------
1.msf{PS6-1}    ---------- --.------- ---------- ---------- ----------
1.msf{PS6-2}    ---------- --.------- ---------- ---------- ----------
1.msf{PA1-1}    g--------- t--------- ---------- ---------- ----------
```

```
                    801                                                 850
1.msf{PA1-2}  g--------- t--------- ---------- ---------- ----------
1.msf{PA1-3}  g--------- t--------- ---------- ---------- ----------
1.msf{PA1-4}  g--------- t--------- ---------- ---------- ----------
  1.msf{PA2}  g--------- t--------- ---------- ---------- ----------
  1.msf{PA3}  g--------- t--------- ---------- ---------- ----------
  1.msf{PA5}  g--------- t--------- ---------- ---------- ----------
  1.msf{PA6}  g--------- t--------- ---------- ---------- ----------
  1.msf{PA7}  g--------- t--------- ---------- ---------- ----------
  1.msf{PA4}  g--------- t--------- ---------- -------t-- ----------
  1.msf{PS5}  t--------- --.------- ---------- ---------- ----------
   Consensus  CGCTGGTCGC CGAGCTTCTT AGAGGGACTG TCAGCGTCTA GCTGACGGAA
```

```
                851                                 878
  1.msf{Cal}  ---------- ---------- --------
  1.msf{Pan}  ---------- ---------- --------
1.msf{PS1-1}  ---------- ---------- --------
1.msf{PS1-2}  ---------- ---------- --------
1.msf{PS1-3}  ---------- ---------- --------
1.msf{PS1-4}  ---------- ---------- --------
  1.msf{PS2}  ---------- ---------- --------
  1.msf{PS3}  ---------- ---------- --------
  1.msf{PS4}  ---------- ---------- --------
1.msf{PS6-1}  ---------- ---------- --------
1.msf{PS6-2}  ---------- ---------- --------
1.msf{PA1-1}  ---------- ---------- --------
1.msf{PA1-2}  ---------- ---------- --------
1.msf{PA1-3}  ---------- ---------- --------
1.msf{PA1-4}  ---------- ---------- --------
  1.msf{PA2}  ---------- ---------- --------
  1.msf{PA3}  ---------- ---------- --------
  1.msf{PA5}  ---------- ---------- --------
  1.msf{PA6}  ---------- ---------- --------
  1.msf{PA7}  ---------- ---------- --------
  1.msf{PA4}  ---------- ---------- --------
  1.msf{PS5}  ---------- ---------- --------
  Consensus   GTTTGAGGCA ATAACAGGTC TGTGATGC
```

Figure 6-9 Sequence alignment of 22 specimens by Pretty program of GCG software. The left column is the sample name in the file name: 1.msf. Cal is *Calvatia gigantea*, Pan is *Panellus serotinus*. Consensus sequences were automatically produced by the same program. Symbol "." is deletion, "-"is the same sequence with the consensus sequence. There are three sequence blocks from left to right. These designate primer positions used to amplify and sequence this nuc-ssu-rDNA fragments and are described in the text.

	83	84	88	105	114	154	158	801	811-3	838
PA1-1	G	G	C	T	G	G	G	C	G	G	C	G	G	G	T	G	G	C	T	G	A	G	T	C	T
PA1-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA1-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA1-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA4	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
PA5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PA7	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PS1-1	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS1-2	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS1-3	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS1-4	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS2	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS3	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS4	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS5	.	.	T	.	.	.	.	.	A	.	.	.	C	.	.	.	-	.	.	T	.	.	C	G	.	.	.
PS6-1	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.
PS6-2	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.	.	.

Figure 6-10 Polymorphic sites in nuc-ssu-rDNA gene of twenty samples in this study. The numbers on first line are nucleotide numbers from 5' end of the amplified region. Dot means the same base as PA1-1; short dash means deletion compared to PA1-1 and an underline denotes a polymorphic site.

From the alignment results, this study showed that among the genus *Panaeolus* samples PA1-1, 1-2, 1-3, 1-4, 2, 3, 5 and 6 shared the same 877 bp sequences, which included the 15 bp of primer SR1c and 24 bp of primer NS6. Among these samples, PA1-1, 1-2, 1-3, 1-4 and PA5 are from the same species (*Panaeolus semiovatus*). Sample PA2, 3, and 6 were from different species of *Panaeolus* (*Panaeolus papilionaceus*, *Panaeolus reckenii*, and *Panaeolus speciosus*, respectively). Even from different species, these samples have identical 877 nucleotides in this amplified DNA fragment. For the other two species of *Panaeolus* samples, sample PA4 exhibits a two base difference and PA7 only a single base difference. Sample PA4 has two different bases compared to sample PA1-1 and the other 7 same sequence samples. The polymorphic sites in PA4 are two substitutions found at nucleotide positions 88 and 838 from the 5' end of amplified region; each substitution was of T/C. Sample PA 7 has only one different base substitution compared to all the other *Panaeolus* samples in the nucleotide 114 by C/T substitution.

Among the samples from the genus of *Psilocybe*, sample PS1-1, 1-2, 1-3, 1-4, 2, 3, 4, 6-1 and 6-2 shared the same 876 nucleotides, while sample PS5 has six different bases when compared to the other *Psilocybe* samples. The differences were in nucleotide position 83(T/C), 84 (T/C), 105 (A/T), 154 (C/T), 158 (T deletion) and 801 (T/C). Among the *Psilocybe* samples, sample PS1-1, 1-2, 1-3, 1-4, 6-1 and 6-2 are the same species (*Psilocybe semilanceata*). Sample PS2, 3, 4,and 5 are from other species of the *Psilocybe* genus, although they share 100% homology at this DNA locus. Of the samples tested, only sample PS5 was found to exhibit DNA sequence differences when compared to the other *Psilocybe* samples.

From these sequence data, there are highly conserve sequences preserved among the same genus but there are observable differences between the two genera. Between genera *Psilocybe* and *Panaeolus*, there are three different bases in the

nucleotide 811, 812 and 813 with C/T, deletion/G and G/A substitution respectively. These three nucleotide sequences were present in every *Psilocybe* sample tested. There are still two highly consistent nucleotides in the nucleotide 84 and 801 among samples in the genus of *Psilocybe* except sample PS5 with T/C and G/C substitution.

An examination of this locus for polymorphic DNA sequences that may differentiate *Panaeolus* and *Psilocybe* samples shows that the nucleotide positions 811, 812 and 813 present possible opportunities. Additionally, nucleotide positions 84 and 801 were also conserved within this genus, with the exception of sample PS5. Besides the possible genus specific sequences in the 877 bp DNA fragment of nuc-ssu-rDNA, this study cannot reveal any species specific sequences due to the sample size of the same species and only few sequence variations existed which cannot be a strong proof to make conclusions.

Using the data generated from the DNA sequences analysis, genetic distances generated by Kimura 2-parameter method [88] produced by the Distance software in GCG were produced. These are shown in Table 6-1, and using this data a dendrogram was produced and shown in Figure 6-11. This dendrogram of UPGMA generated by genetic distance data clearly separates the three fungi genera into their respective groups indicating that the complete nuc-ssu-rDNA DNA sequence can separate members of the *Panaeolus* genus from members of the *Psilocybe* genus, with the exception of *Psilocybe montanae* (sample PS5). Figure 6-12 shows the Neighbor-Joining tree from the genetic distance in Table 6-1. Within the *Panaeolus* genus the greatest genetic distance between two species (*Panaeolus retirugis* and *Panaeolus subbalteatus*) was 0.34, indicating a very high degree of homology. All the species of *Psilocybe* genus except species *Psilocybe montanae* (sample PS5) have same nuc-ssu-rDNA sequences in this study. In Table 6-1, the closest genetic distance among these fungi and their closest species on the basis of the nuc-ssu-rDNA locus, *Calvatia*

gigantea and *Panellus serotinus*, was 1.39. This was the distance between *Panellus serotinus* and *Panaeolus retirugis* (sample PA4). This data can be a strong proof to demonstrate the unique sequences of members of the genera *Psilocybe* and *Panaeolus*.

Hibbet [26] reported a group I intron inserted at the same position in the nuclear small-subunit ribosomal DNA in several species of homobasidiomycetes (mushroom-forming fungi). In the data presented in this chapter, there are no insertions found in this gene in either *Psilocybe* or *Panaeolus*. It had been suggested from previous phylogenetic analysis of nuc-ssu-rDNA sequences that the group I introns could be used as phylogenetic markers [26]. In this study, however, no group I intron was found in these samples indicating that this is not the case. From the results presented in this chapter, the DNA sequences of nuc-ssu-rDNA gene represent a possible genus specific marker.

Table 6-1 Genetic distance matrix of 22 nucleotide sequences generated by Kimura 2-parameter method produced by the Distance software in GCG. Cal is *Calvatia gigantea*, Pan is *Panellus serotinus*. Distances are estimated number of substitutions per 100 bases.

Matrix Part 1

	Cal	Pan	PS1-1	PS1-2	PS1-3	PS1-4	PS2	PS3	PS4	PS6-1	PS6-2	PA1-1
Cal	0.00	1.27	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92	1.04
Pan		0.00	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.81	1.15
PS1-1			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34
PS1-2				0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34
PS1-3					0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34
PS1-4						0.00	0.00	0.00	0.00	0.00	0.00	0.34
PS2							0.00	0.00	0.00	0.00	0.00	0.34
PS3								0.00	0.00	0.00	0.00	0.34
PS4									0.00	0.00	0.00	0.34
PS6-1										0.00	0.00	0.34
PS6-2											0.00	0.34
PA1-1												0.00
PA1-2												
PA1-3												
PA1-4												
PA2												
PA3												
PA5												
PA6												
PA7												
PA4												
PS5												

Table 6-1 Genetic distance matrix of 22 nucleotide sequences generated by Kimura 2-parameter method produced by the Distance software in GCG. Cal is *Calvatia gigantea*, Pan is *Panellus serotinus*. Distances are estimated number of substitutions per 100 bases.

Matrix Part 2

	PA1-2	PA1-3	PA1-4	PA2	PA3	PA5	PA6	PA7	PA4	PS5
Cal	1.04	1.04	1.04	1.04	1.04	1.04	1.04	1.15	1.27	1.39
Pan	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.27	1.39	1.04
PS1-1	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS1-2	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS1-3	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS1-4	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS2	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS3	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS4	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS6-1	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PS6-2	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.46	0.57	0.46
PA1-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.23	0.69
PA1-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.23	0.69
PA1-3		0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.23	0.69
PA1-4			0.00	0.00	0.00	0.00	0.00	0.11	0.23	0.69
PA2				0.00	0.00	0.00	0.00	0.11	0.23	0.69
PA3					0.00	0.00	0.00	0.11	0.23	0.69
PA5						0.00	0.00	0.11	0.23	0.69
PA6							0.00	0.11	0.23	0.69
PA7								0.00	0.34	0.81
PA4									0.00	0.92
PS5										0.00

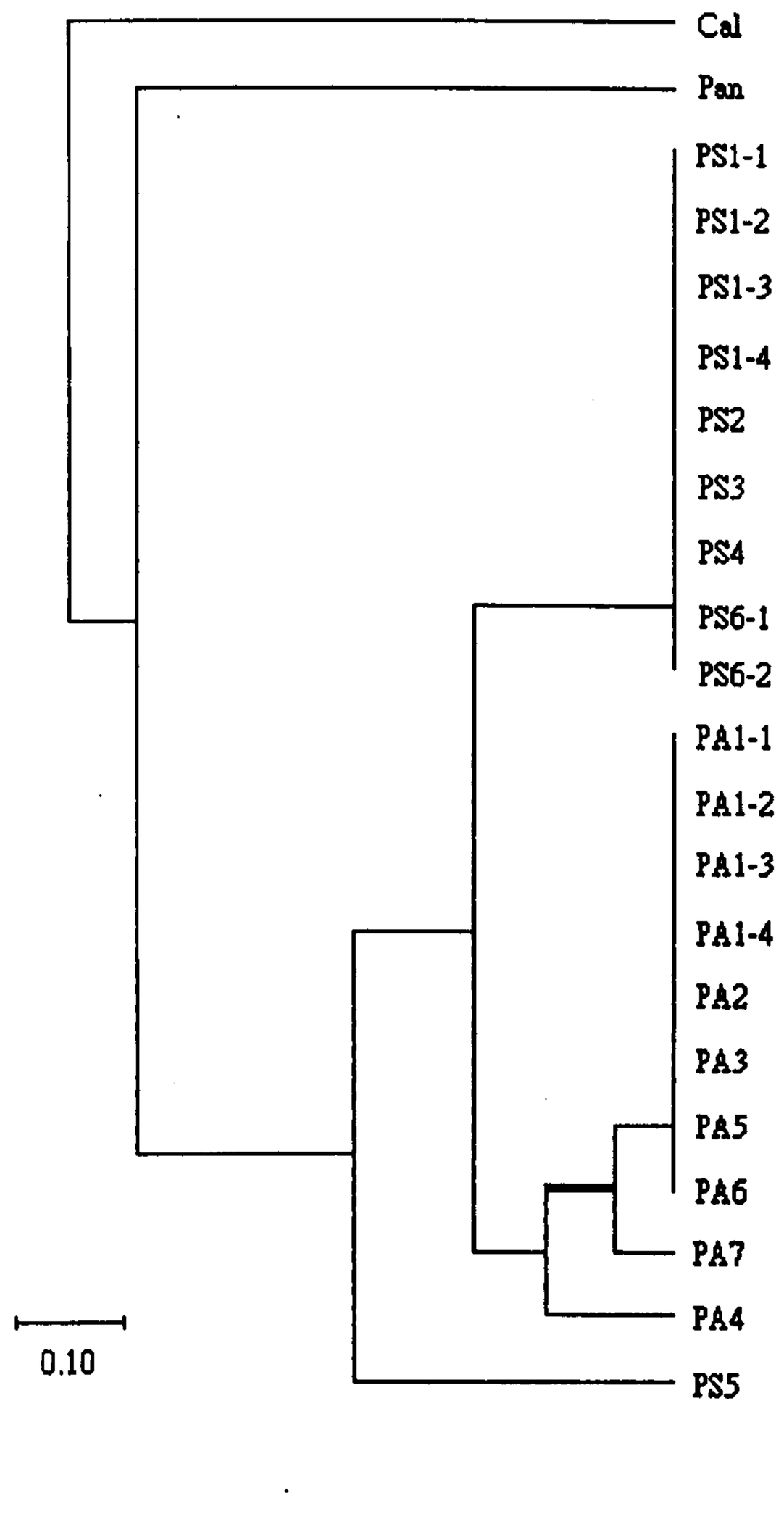


Figure 6-11 Dendrogram established by the genetic distances in Table 6-1 produced by UPGMA method using the Growtree software in GCG. Cal is *Calvatia gigantea*, Pan is *Panellus serotinus*.

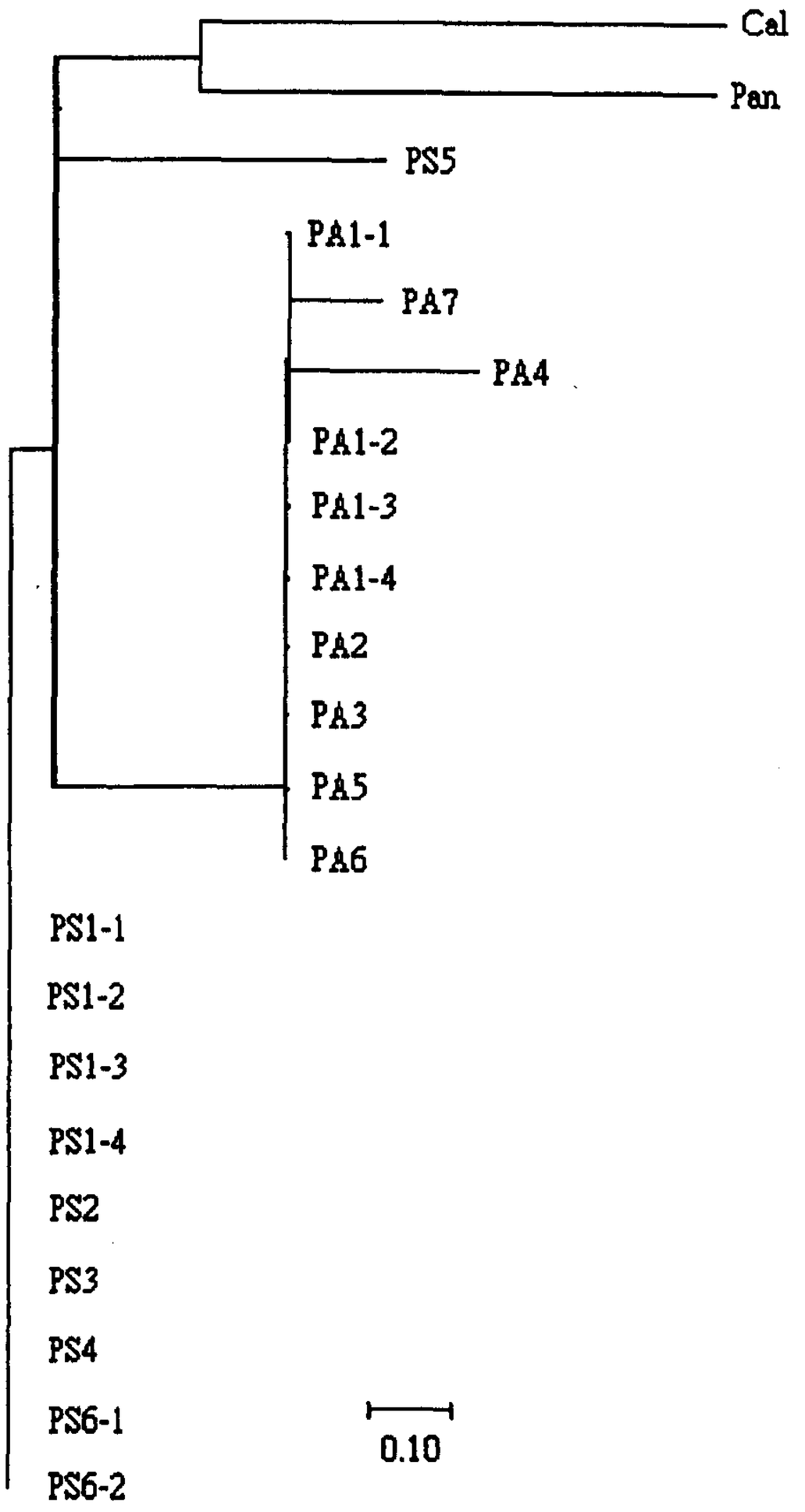


Figure 6-12 Dendrogram established by the genetic distances in Table 6-1 produced by NJ method using the Growtree software in GCG. Cal is *Calvatia gigantea*, Pan is *Panellus serotinus*.

7 ANALYSIS OF THE INTERNAL TRANSCRIBED SPACER 1 DNA (ITS-1) IN NUCLEAR RIBOSOMAL RNA GENE

7.1 Introduction

In eukaryotes the genes for 18S, 28S and 5.8S ribosomal RNA (rDNA) are usually arranged as tandem repeats. In fungi the number of rDNA repeats ranges from approximately 60 in the mushroom genus *Coprinus* [89] to 220 in the mould *Neurospera* [90]. These gene sequences are highly conserved, as they evolve very slowly, and are consequently used in evolutionary studies for distantly related organisms [15]. Such conserved gene sequences are unlikely to separate or distinguish more closely related genera such as *Psilocybe* and *Panaeolus*. Chapter 6 detailed the study of the nuclear small subunit ribosomal RNA gene, in which the farthest genetic distance between these two genera was only 0.92 (estimated number of substitutions per 100 bases) using the method of Kimura 2-parameter. This chapter will study more variable area in ribosomal RNA gene.

The rDNA gene sequences are separated by the variable Internal Transcribed Spacer (ITS) and a non-transcribed intergenic spacer (IGS) [15]. Fungi have multiple copies of this rDNA gene complex, which makes this gene locus amenable to PCR, even from highly degraded samples [91]. The ITS region is removed after transcription and is therefore non-coding, which results in a high degree of polymorphism. This polymorphic locus, both of sequence and length, within the ITS region has been used in the identification of fungal species [34,92,43,93,39] and separation of species into pathogenicity groups [94]. In some fugal genera, such as *Colletotrichum*, the ITS locus varies by only a few nucleotides [95]. Species within other genera have been shown to be more variable [96].

The internal transcribed spacers (ITS-1 and ITS-2) lie on each site of the 5.8S gene respectively and separate the small subunit gene (16S to 18S) and the large subunit gene (26S to 28S) in eukaryotes [86]. They are considered to evolve quickly and may vary among species within a genus or among populations [14]. This study is designed to discover the differences among the genera *Psilocybe* and *Panaeolus* in ITS-1 locus.

This chapter details the isolation and complete sequencing of the ITS-1 locus from some members of the *Psilocybe* and *Panaeolus* genera of fungi. Sequence data from this region was used to design a DNA based test with the aim to identify unambiguously the presence of the members of these two genera, and further identify the genus.

7.2 Materials and Methods

7.2.1 *Psilocybe* and *Panaeolus* Mushrooms

DNA samples of mushrooms extracted in section 3.4 were used in this study.

7.2.2 PCR Amplification

Due to the highly polymorphic character of the internal transcribed spacers, this study used the primers designed to span each of the ribosomal genes as described by White et al. [15]. Primer ITS5 is located on the 3' end of the nuclear small subunit rDNA that is more conserved. The sequences are 5'-GGAAGTAAAAGTCGTAACAA GG-3'. Primer ITS2 is located on the 5' end of the 5.8S rDNA, and the sequences are 5'-GCTGCGTTCTTCATCGATGC-3'. The size of amplified DNA cannot be expected

due to its highly polymorphic and no previous reference can be followed. PCR amplification was accomplished in 50 µl of reaction mixture, which contained about 15ng extracted genomic DNA, reaction buffer (10mM Tris-HCl, pH8.8 at 25 °C, 1.5mM $MgCl_2$, 1.5mM KCl, 0.1% Triton X-100), 200µM of dNTPs, 1 unit of DyDNAyme™ II DNA polymerase (Finnzymes Oy, Finland) and 0.5µM each of primers. The PCR amplifications were carried out using the program of 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min in a DNA thermal cycler 480 (Perkin-Elmer, NJ, USA). PCR products were electrophoresed on a 3% agarose gel (FMC BioProducts, Rockland, ME, USA) with 0.5µg/ml ethidium bromide in TBE buffer (0.89M Tris-HCl, 0.889M borate, 0.002M EDTA, pH8.0) at 100V for 30 min. PCR products were visualized by 312nm UV light and photographed using Polaroid 667 film.

7.2.3 DNA Sequencing

The PCR products of amplified DNA fragments were separated by 3% low melting agarose gel electrophoresis to remove excess dNTPs and primers. The amplified fragments were sliced from the low melting gel and purified by alcohol precipitation. Approximately 50ng of purified PCR products were used to perform the sequencing reaction using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems CA, USA). Primer ITS5 and ITS2 were used for the forward and reverse sequencing direction on each site of 5' end. Sequencing electrophoresis was carried out in PE Applied Biosystems PRISM 310 Genetic Analyzer according to the manufacturer's instructions.

7.2.4 Sequence Analysis

Sequence comparison was performed using the Pileup and Pretty programs of.GCG (Genetics Computer Group) software. Genetic distances were generated by Kimura 2-parameter method [88]. Unweighted pair-group method with arithmetic averaging (UPGMA) and Neighbor-Joining tree [87] were constructed to display the phylogenetic relationships. And Confidence values for internal lineages were assessed with the bootstrap analysis [97] by Phylip [98].

7.2.5 Single-Strand Conformation Polymorphism (SSCP)

PCR products obtained by primers ITS5 and ITS2 were separated using a polyacrylamide gel to reveal their features of single-strand conformation polymorphism (SSCP). In SSCP analysis, a DNA sequence is amplified by PCR, denatured, and run in a nondenaturing polyacrylamide gel. If fragments differ in their nucleotide sequences, they will migrate at different mobilities due to conformational differences. Sometimes even single nucleotide differences can be detected by the SSCP analysis [99-102]. In this study, the electrophoresis and silver staining protocol were similar to section 4.2.2 and 4.2.3 except the loading products were denatured for 5 minutes and instantly cooled on ice before loading.

7.3 Results and Discussion

This amplification performed on the extracted genomic DNA using PCR with the primers ITS5 and ITS2 produced DNA fragments of approximately 300 bp to 400 bp, estimated by the 100 bp ladder (see Figure 7-1). The size of the product appeared to show size variation between samples. The whole fragments of amplified products were

fully sequenced from forward and reverse primers of ITS5 and ITS2.

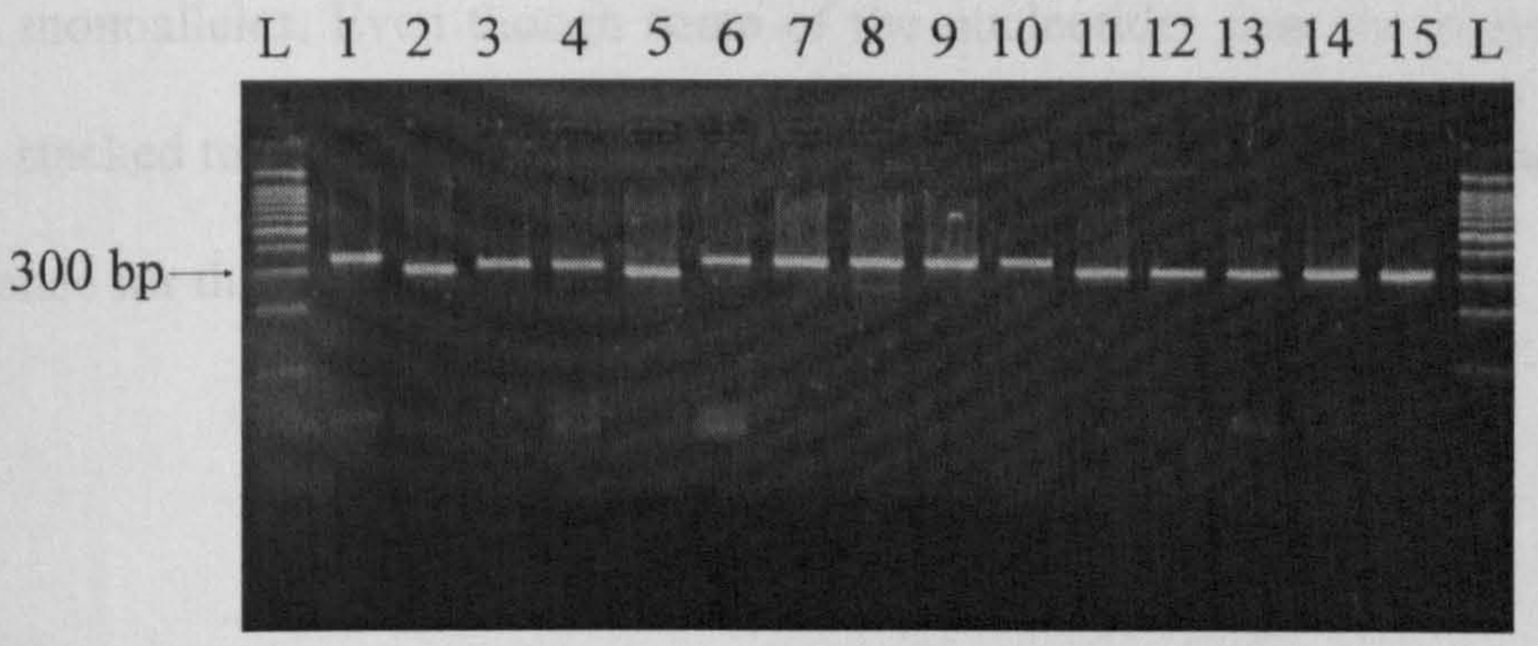


Figure 7-1 PCR products of ITS-1 DNA fragments amplified by primer ITS2 and ITS5. L is 100 bp ladder. Lane 1-20 are sample PS1-1, PS2, PS3, PS4, PS5, PS6-1, PS6-2, PS1-2, PS1-3, PS1-4, PA1-1, PA2, PA3, PA4, PA5, PA6, PA7, PA1-2, PA1-3 and PA1-4.

7.3.1 Monoallele

Thirteen samples out of twenty samples were sequenced with clear signals

which can be identified as homozygous copies of ITS-1 DNA fragments. That is, there was only one sequence detected at this locus. Samples PA1-1, PA1-2, PA1-3, PA1-4, PA2, PA3, PA4, PA5, PA6, PS2, PS3, PS4 and PS5 were found to be monoallelic in this study. Figure 7-2 to 7-5 showed examples of electropherograms of DNA sequences from monoalleles. Even though some of the nucleotides near the sequencing primer were stacked together, they can be clearly identified by the reverse sequence. The full sequence for these samples was determined.

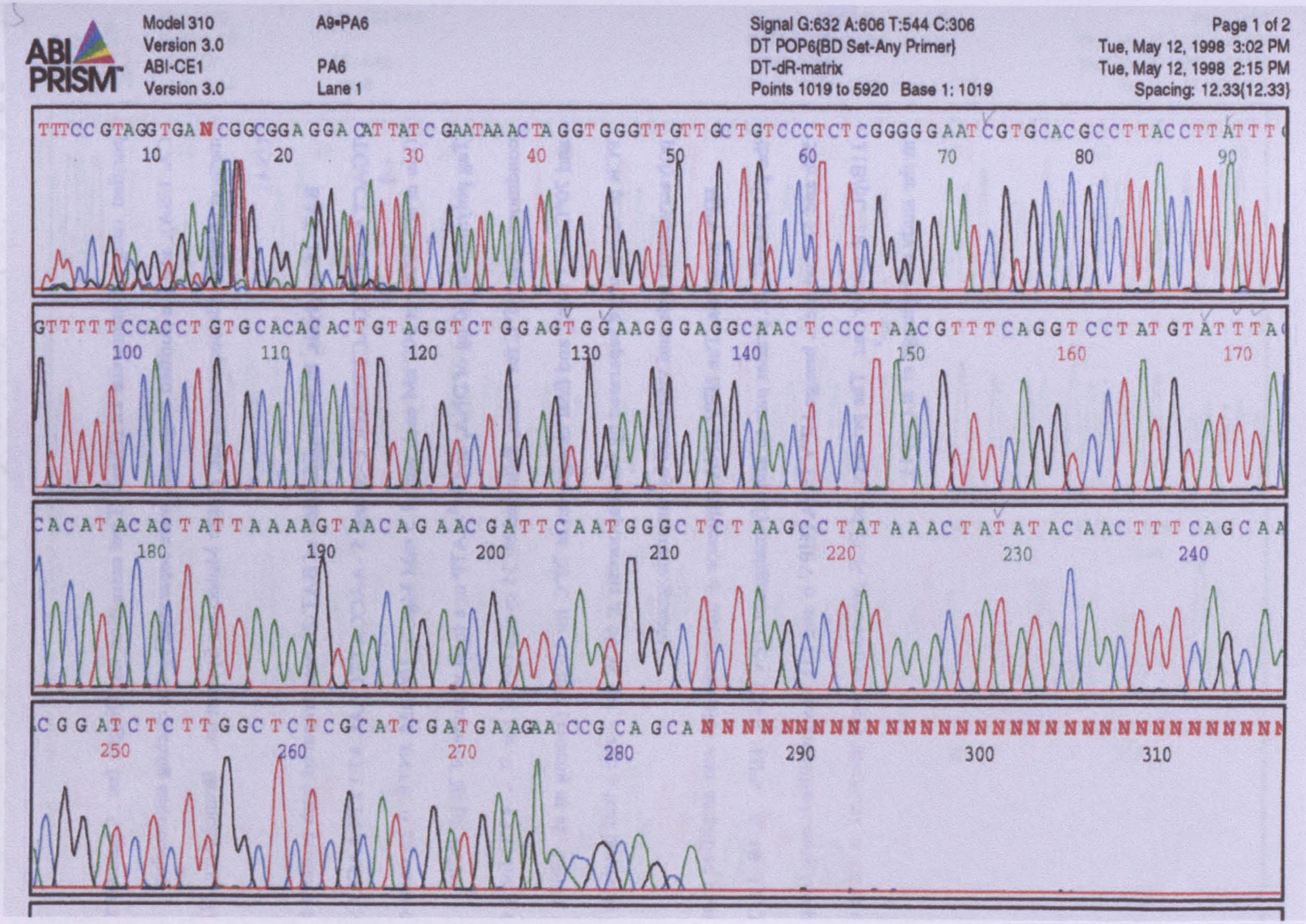


Figure 7-2 Sequencing electropherogram of sample PA6 from DNA fragment of the ITS-1 rDNA by primer ITS5.

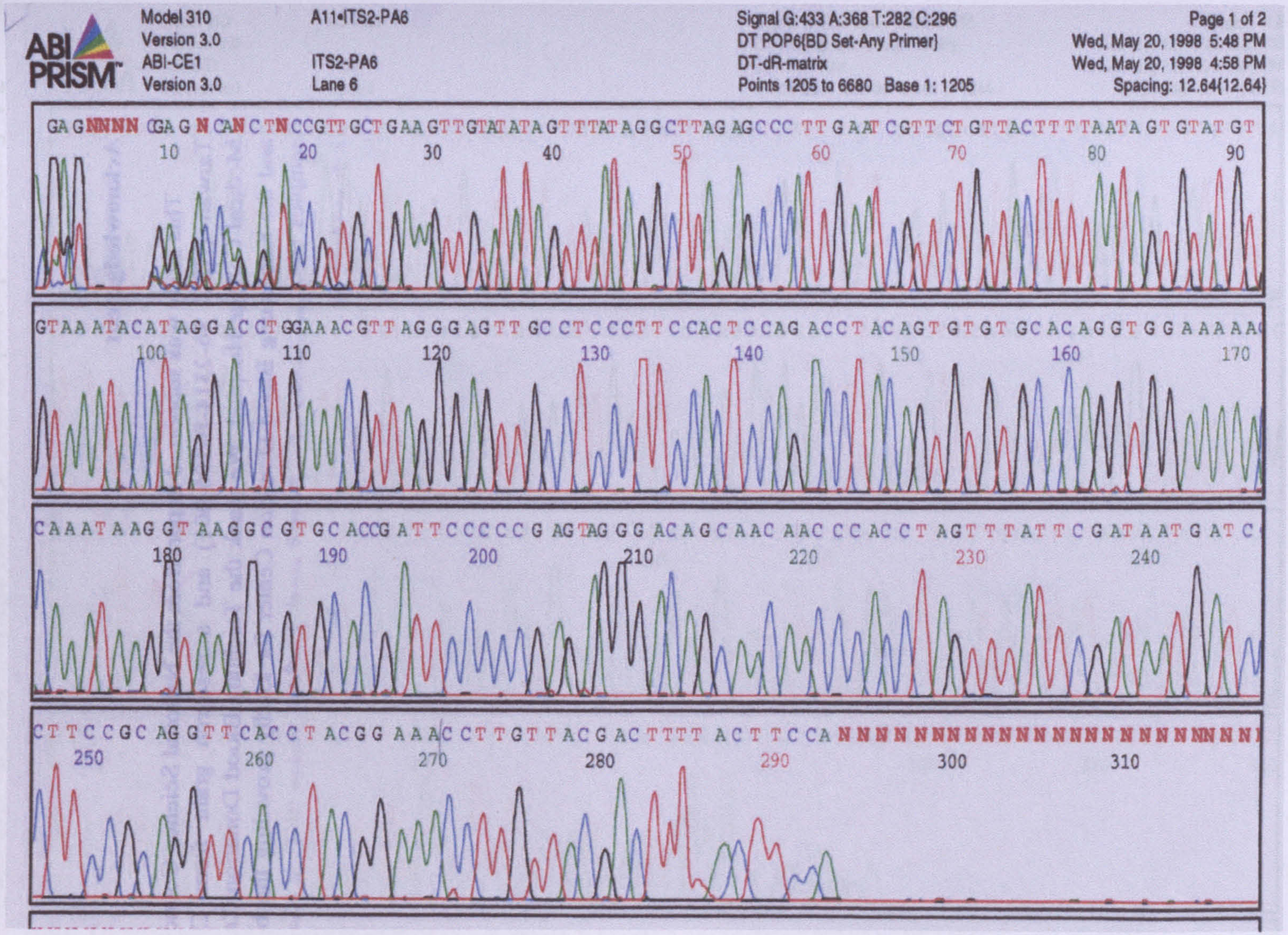


Figure 7-3 Sequencing electropherogram of sample PA6 from DNA fragment of the ITS-1 rDNA by primer ITS2.

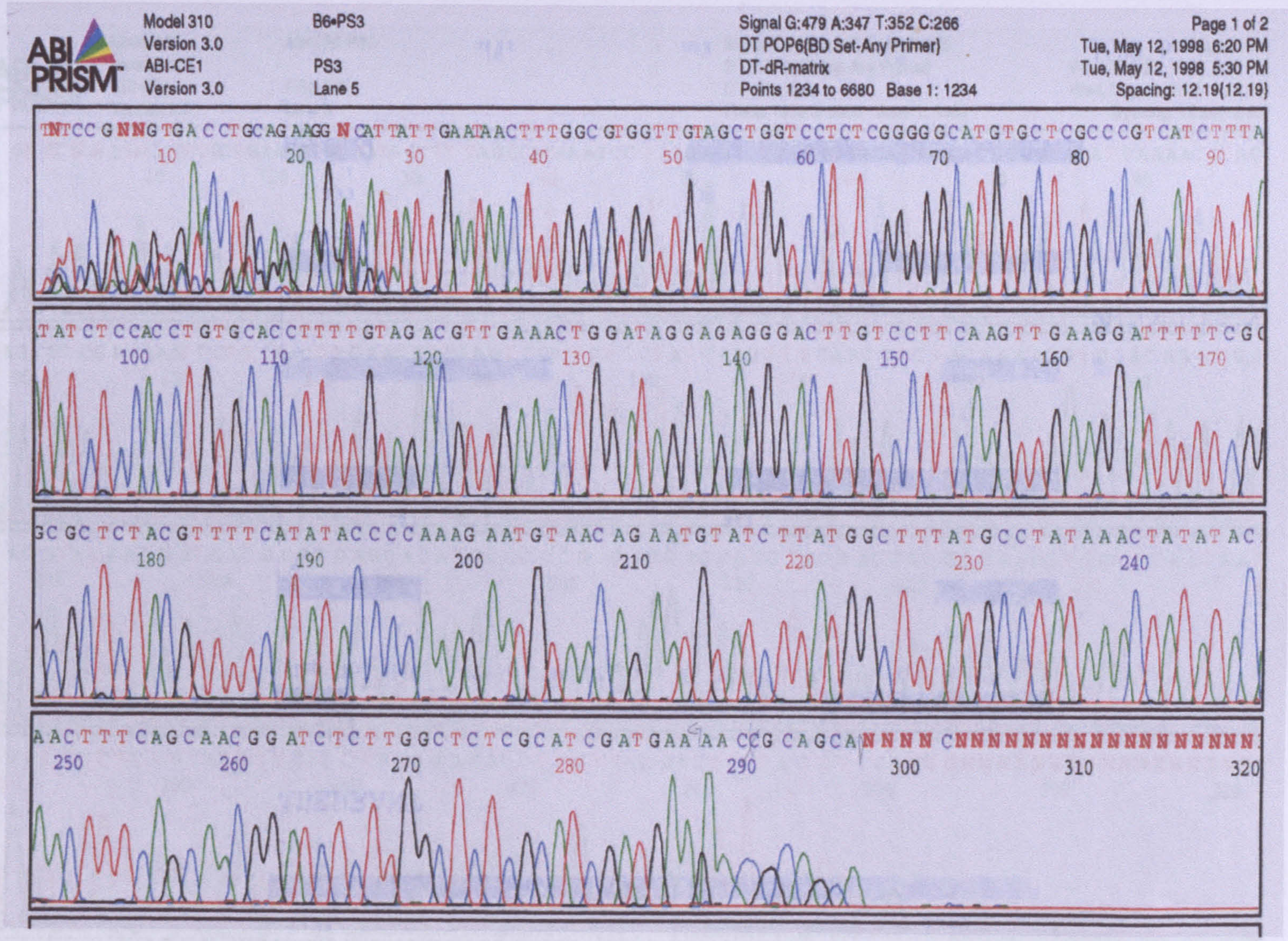


Figure 7-4 Sequencing electropherogram of sample PS3 from DNA fragment of the ITS-1 rDNA by primer ITS5.

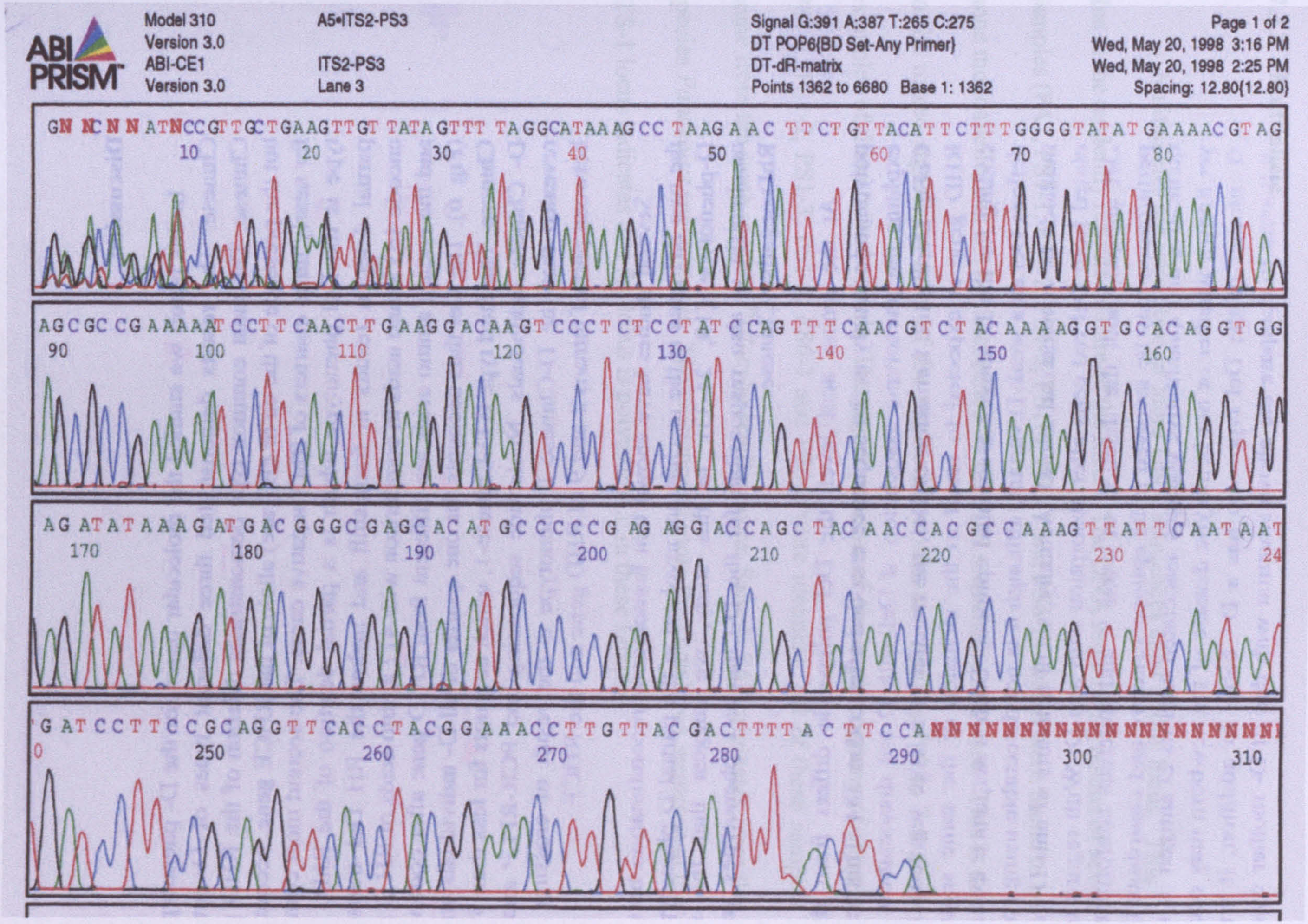


Figure 7-5 Sequencing electropherogram of sample PS3 from DNA fragment of the ITS-1 rDNA by primer ITS2.

7.3.2 Multiallele

Multialleles are when two or more DNA sequences exist at this locus. This is due to the tandemly repeated nature of the locus. The DNA sequencing results of seven samples (PA7, PS1-1, PS1-2, PS1-3, PS1-4, PS6-1 and PS6-2), which were found to be none monoallelic, can not be determined by the ABI sequencing software. This is due to the mixed signals on most of the nucleotide positions. Figures 7-6 to 7-9 show examples of these mixed signals. The signal patterns of electropherograms of sample PS1-1, PS1-2, PS1-3, PS1-4, PS6-1 and PS6-2 were identical. All of these samples come from the same species, *Psilocybe semilanceata.* Sample PA7 originates from the species *Panaeolus subbalteatus.* The existence of more than one DNA sequence at the ITS-1 locus indicates that the locus is polymorphic in these samples.

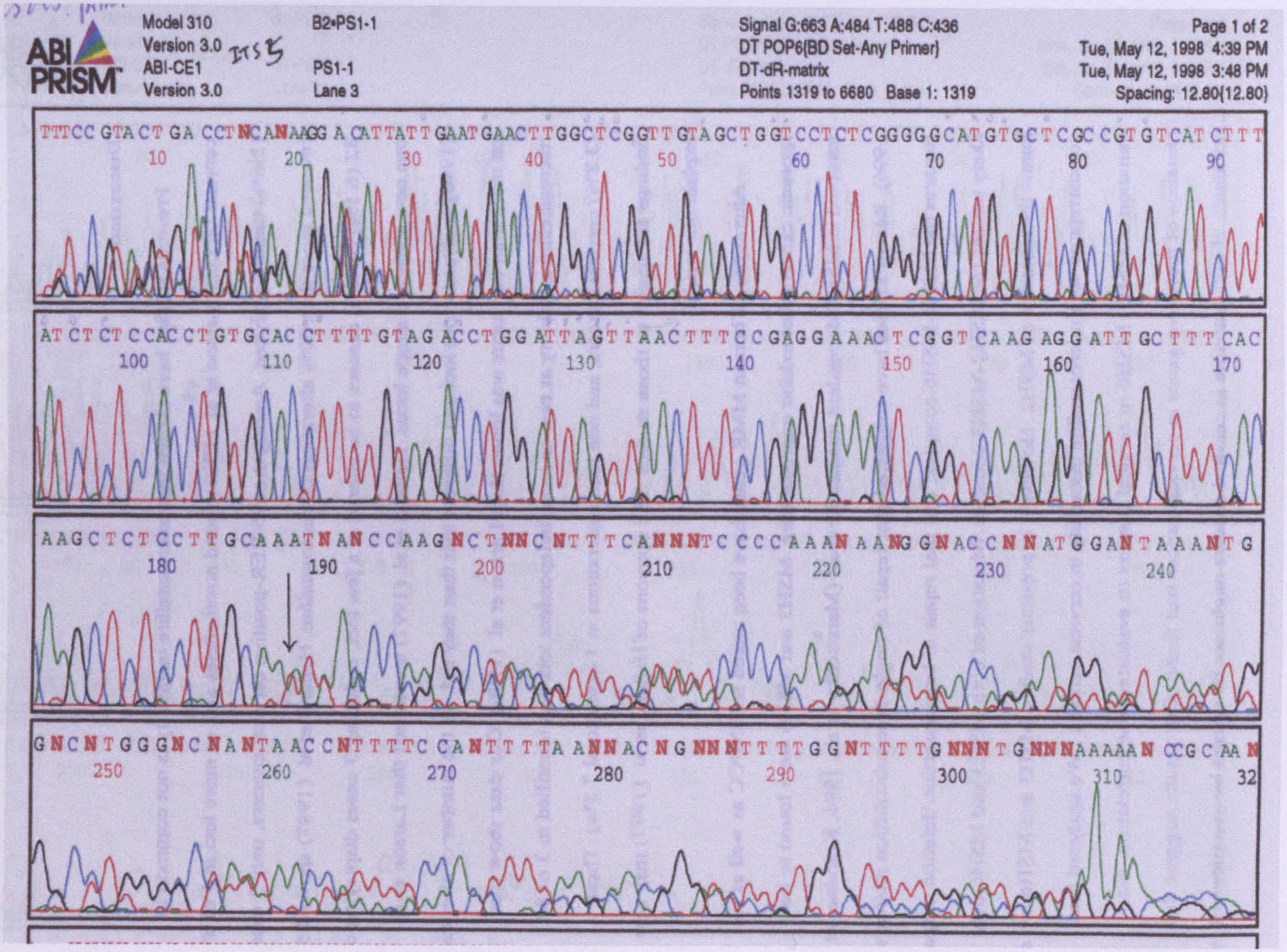


Figure 7-6 Sequencing electropherogram of sample PS1-1 from DNA fragment of the ITS-1 rDNA by primer ITS5. After position 188 (indicated by arrow) in this electropherogram, there are mixed signals which shows multialleles in this sample.

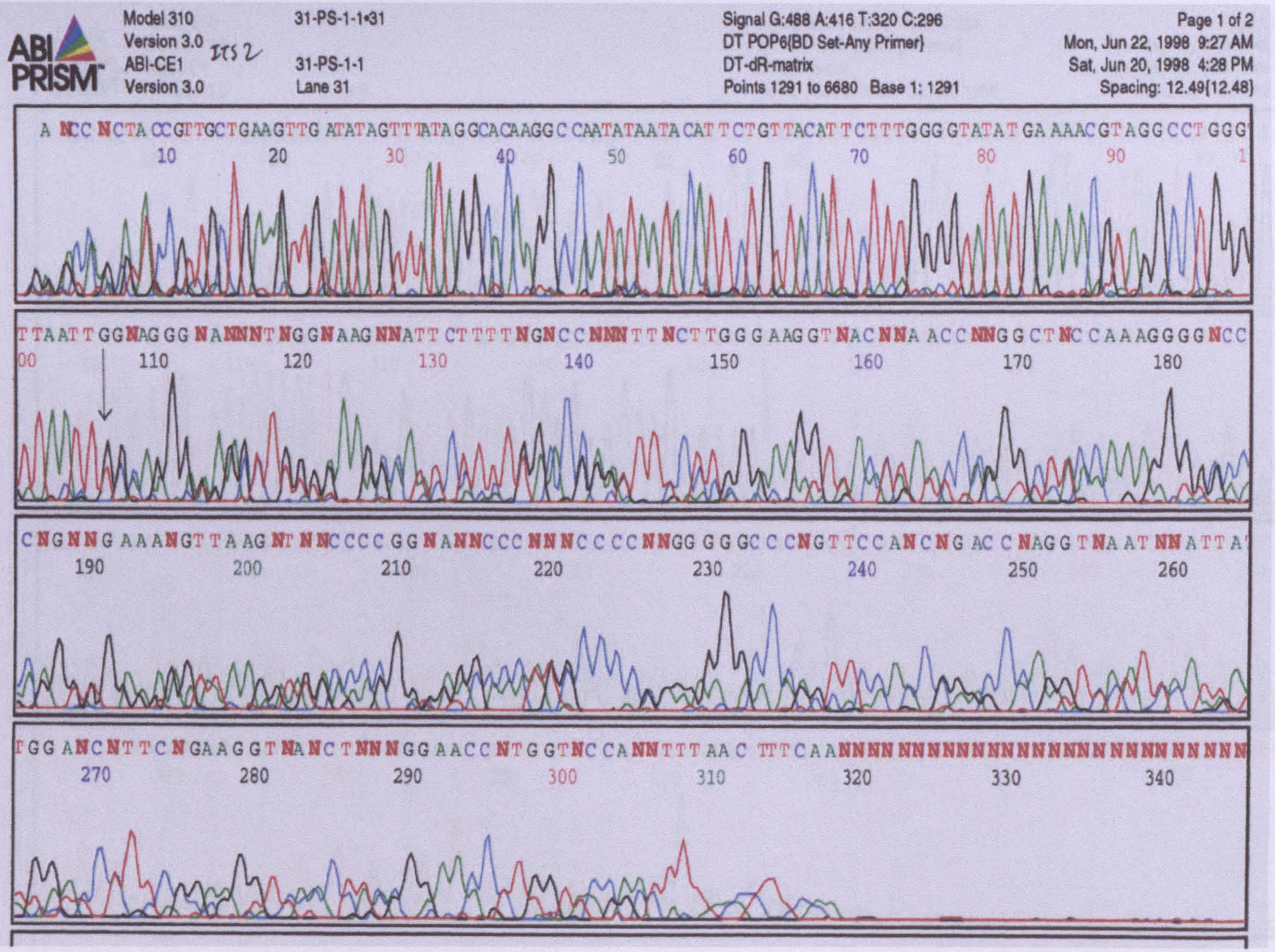


Figure 7-7 Sequencing electropherogram of sample PS1-1 from DNA fragment of the ITS-1 rDNA by primer ITS2. After position 106 (indicated by arrow) in this electropherogram, there are mixed signals which shows multialleles in this sample.

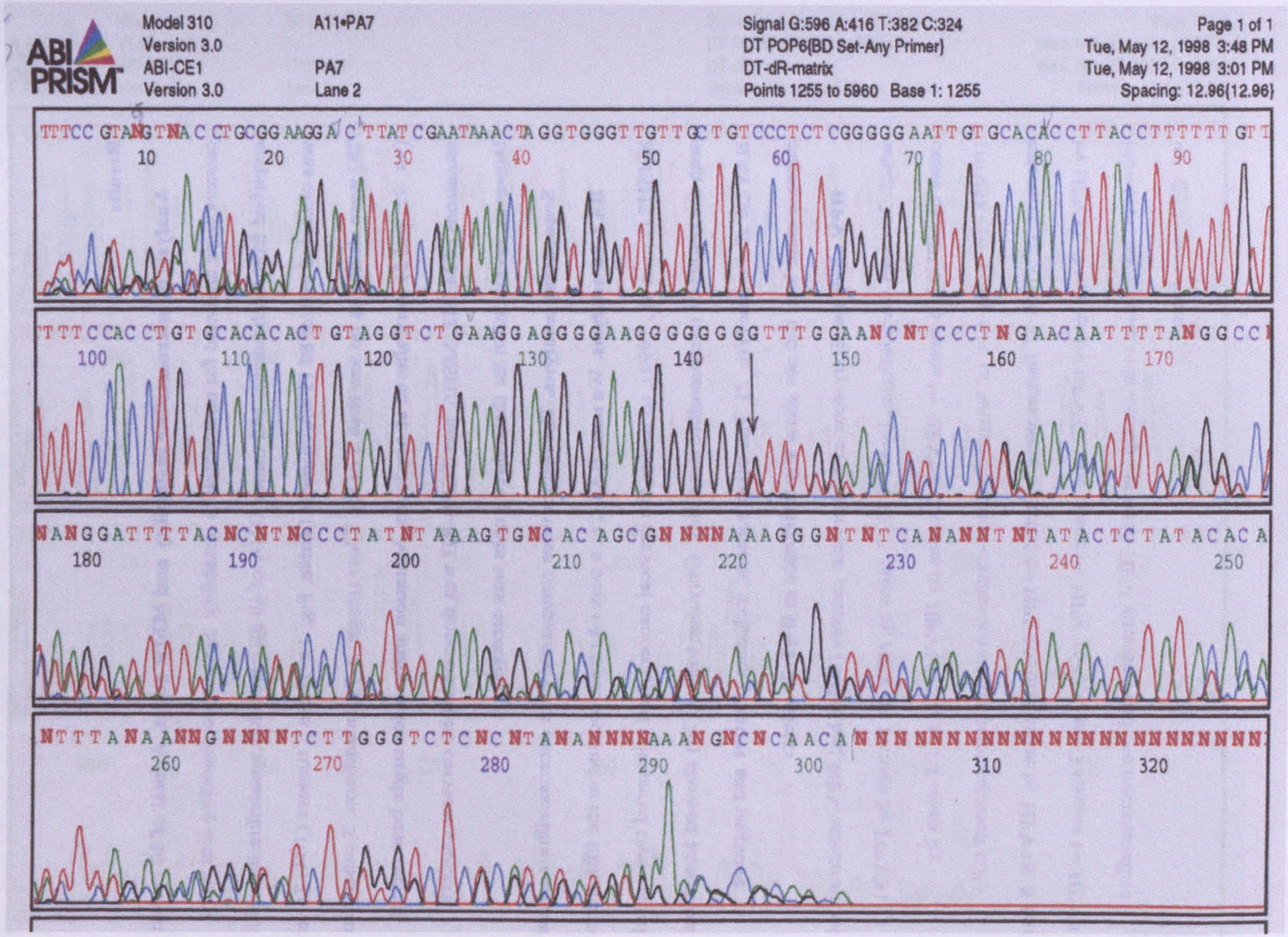


Figure 7-8 Sequencing electropherogram of sample PA7 from DNA fragment of the ITS-1 rDNA by primer ITS5. After position 144 (indicated by arrow) in this electropherogram, there are mixed signals which shows multialleles in this sample.

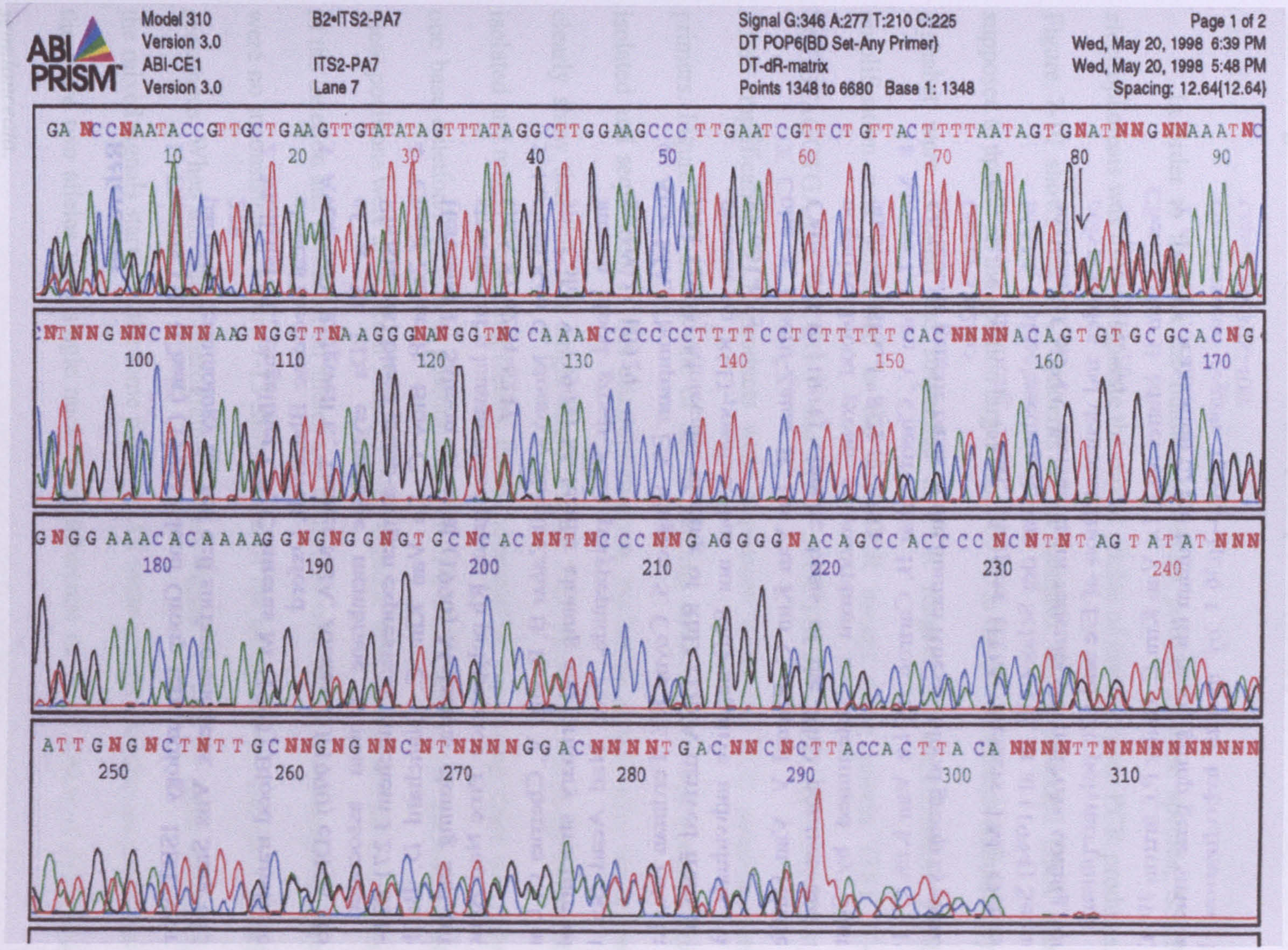


Figure 7-9 Sequencing electropherogram of sample PA7 from DNA fragment of the ITS-1 rDNA by primer ITS2. After position 80 (indicated by arrow) in this electropherogram, there are mixed signals which shows multialleles in this sample.

7.3.3 Single-Strand Conformation Polymorphism (SSCP)

In order to find out the number of alleles present in these species, SSCP electrophoresis was used to separate the mixed alleles of heterozygous PCR products. Figure 7-10 shows the SSCP patterns of all samples. Single stranded DNA was supposed to appear on the position larger than 500 bp in the gel. The bands indicated by alphabet were isolated and suspended in PCR reagent for amplification. PCR amplification was performed as the first PCR except using primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2: 5'-GCTGCGTTCTTCATCGATGC-3'. After amplification, PCR products were sequenced using the PCR amplification primers. Figure 7-11 shows one of the sequencing results which was successfully isolated and sequenced. In this electropherogram, nucleotide signals after the arrow clearly show that this is a single allele. Figure 7-12 shows the close-up image of isolated and mixed sequencing results. From the results of sequencing, there was only one base deletion/insertion at position 213 (or 214, 215, because the nucleotides at these positions were all A) from 5' end of this fragment. When combined the sequences of the deletion and non-deletion alleles and compared with the electropherogram, they were no inconsistency. Figure 7-13 shows some of the mixed signals and their mixed sequences. When the electropherograms between Figure 7-6 and 7-7 were compared, the mixed signals started at the same position. All these analysis results can prove that there are two alleles with single nucleotide difference in ITS-1 DNA of *Psilocybe semilanceata*.

The sequencing results after isolation of sample PA7 (*Panaeolus subbalteatus*) still produced mixed sequences. The SSCP pattern in this study can not separate the mixed products efficiently. By comparison both direction of sequencing (Figure 7-14

and 7-15), however, two nucleotides at the same positions indicate a mixture, position 164 and 223 (or 243, if aligned with other species) from 5' end of this fragment. When combined the sequences of the deletion and non-deletion alleles at these two positions and compared with the electropherogram, they were matched. Figure 7-16 and 7-17 showed some of the mixed signals and their mixed nucleotides. According to this evaluation, four alleles appeared in this mixture by the size difference of one or two bases.

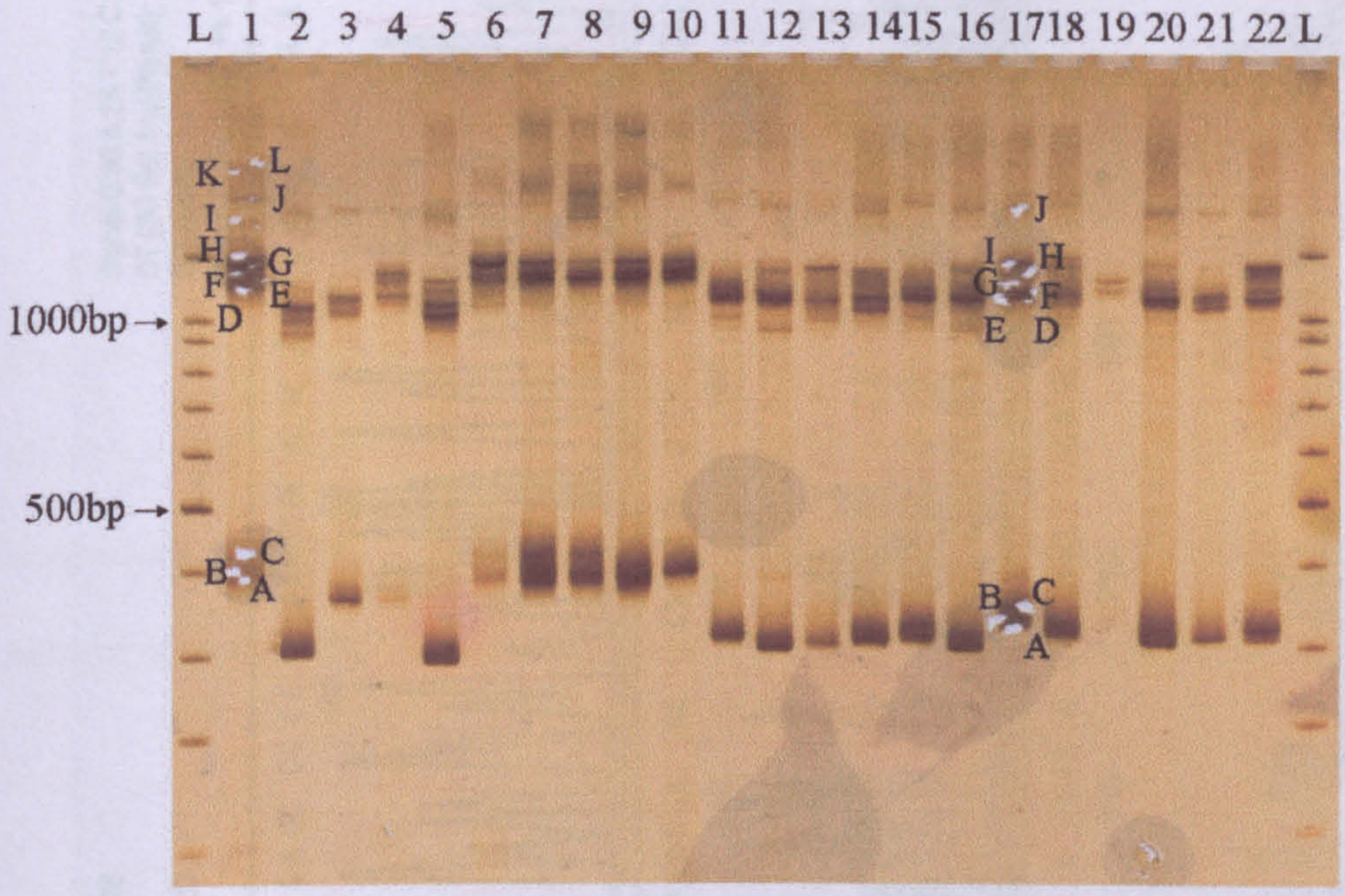


Figure 7-10 SSCP patterns of ITS-1 DNA fragments separated by 12.5% polyacrylamide gel electrophoresis. L is 100 bp ladder. Lane 1-22 are PCR products amplified by primer ITS2 and ITS5 of mushroom samples. Lane 1 is sample PS1-1 and Lane 17 is sample PA7. Alphabets on gel indicated isolated area of SSCP bands.

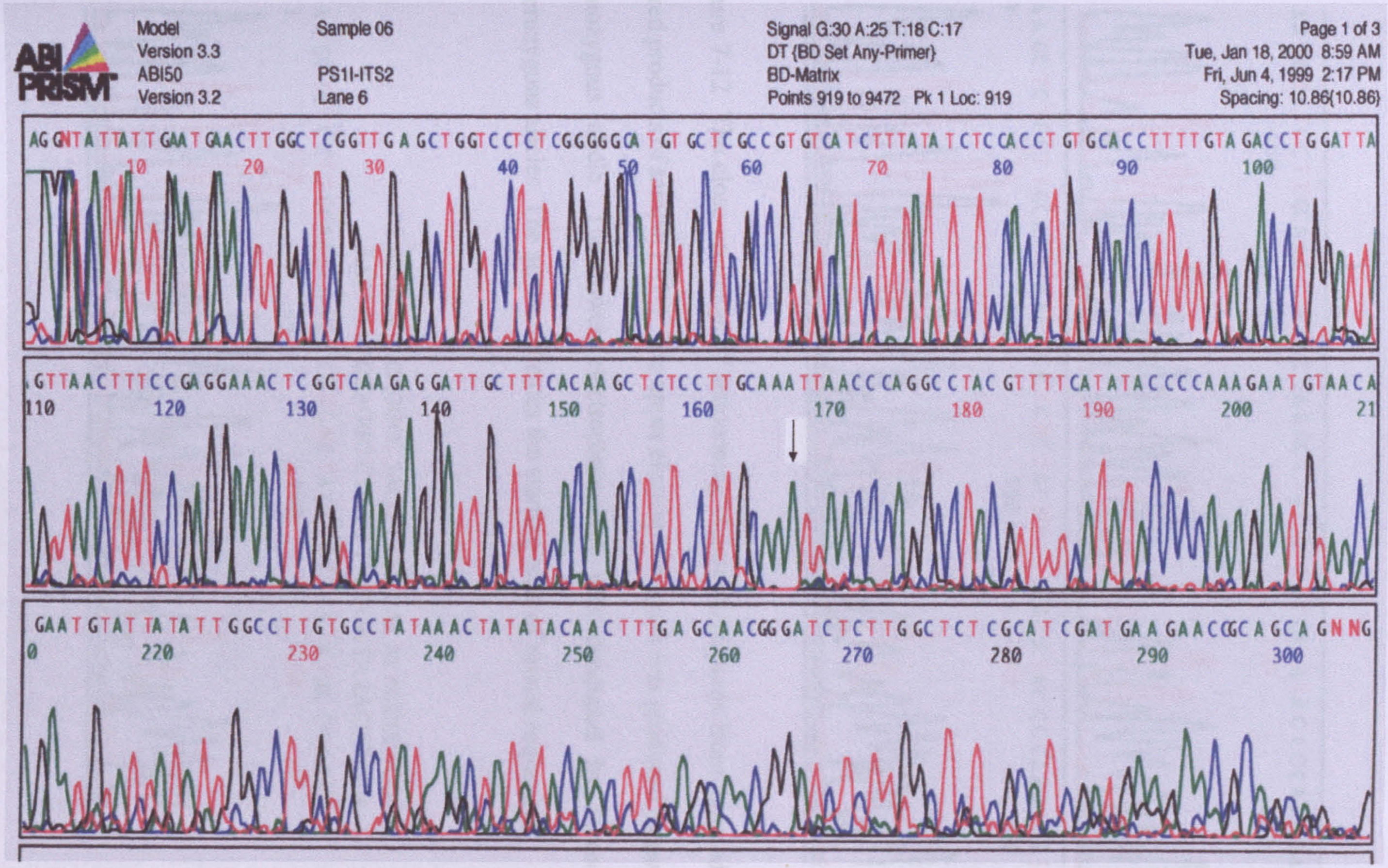


Figure 7-11 Sequencing electropherogram of DNA fragment PS1I isolated from sample PS1-1 on SSCP gel of Figure 7-10. Sequencing primer was primer ITS2.

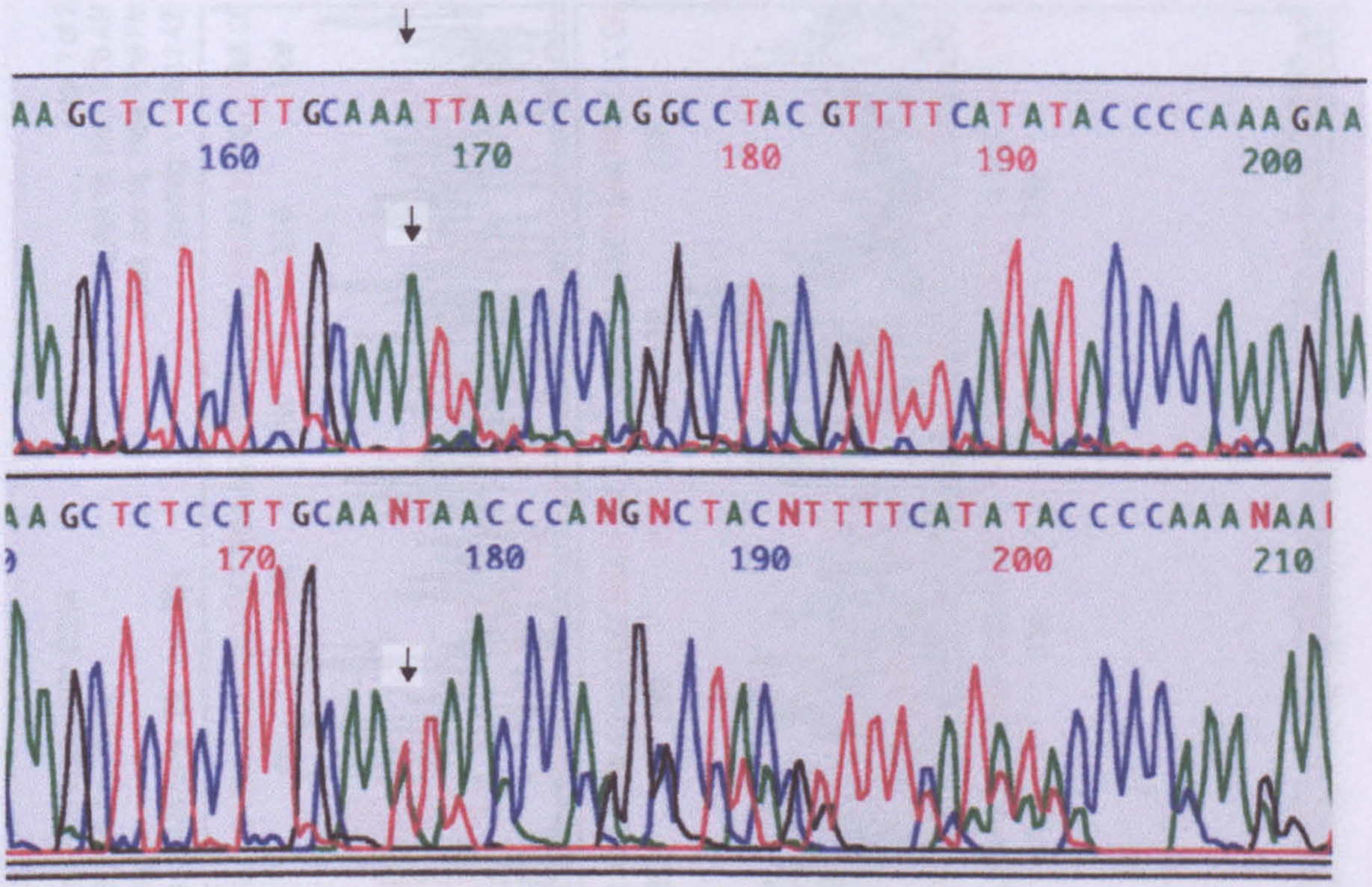


Figure 7-12 The close-up image of sequencing electropherograms from isolated and mixed products of sample PS1-1. The upper electropherogram was produced by pure or homozygous allele. The lower electropherogram was produced by mixed or heterozygous alleles. The arrow indicates the starting base of mixed sequences.

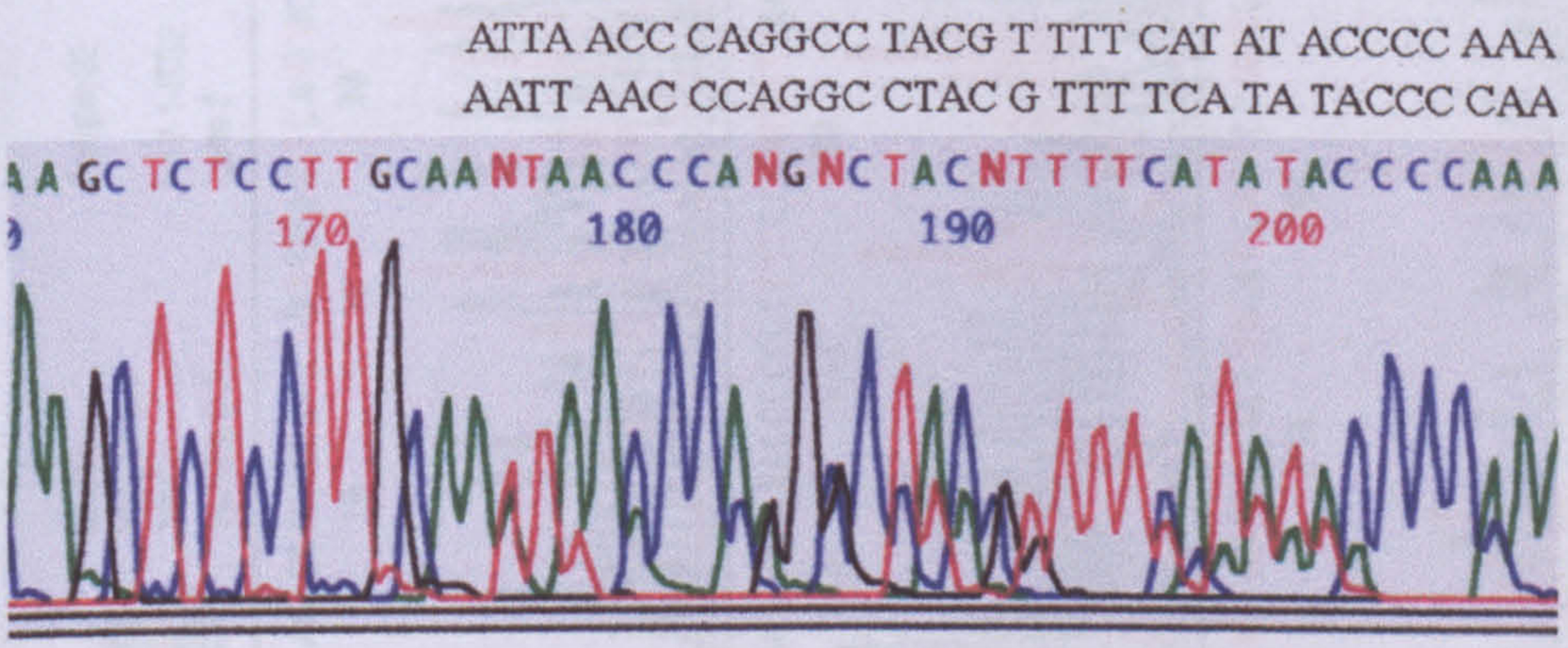


Figure 7-13 Part of the mixed signals and called sequences of sample PS1-1 in ITS-1 DNA.

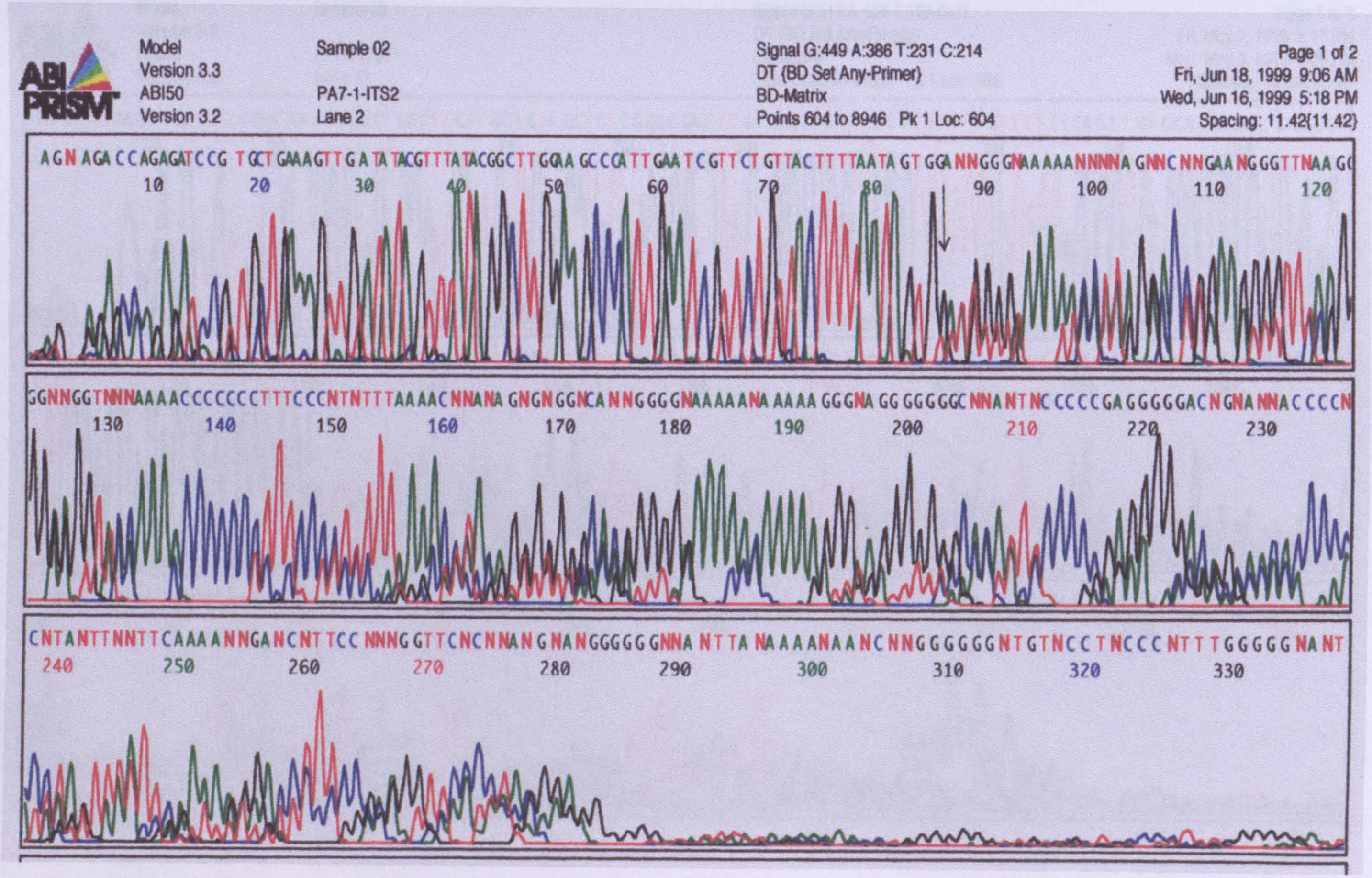


Figure 7-14 Sequencing electropherogram of sample PA7 from DNA fragment of the ITS-1 rDNA by primer ITS1. After position 85 (indicated by arrow) in this electropherogram, there are mixed signals which shows multialleles in this sample.

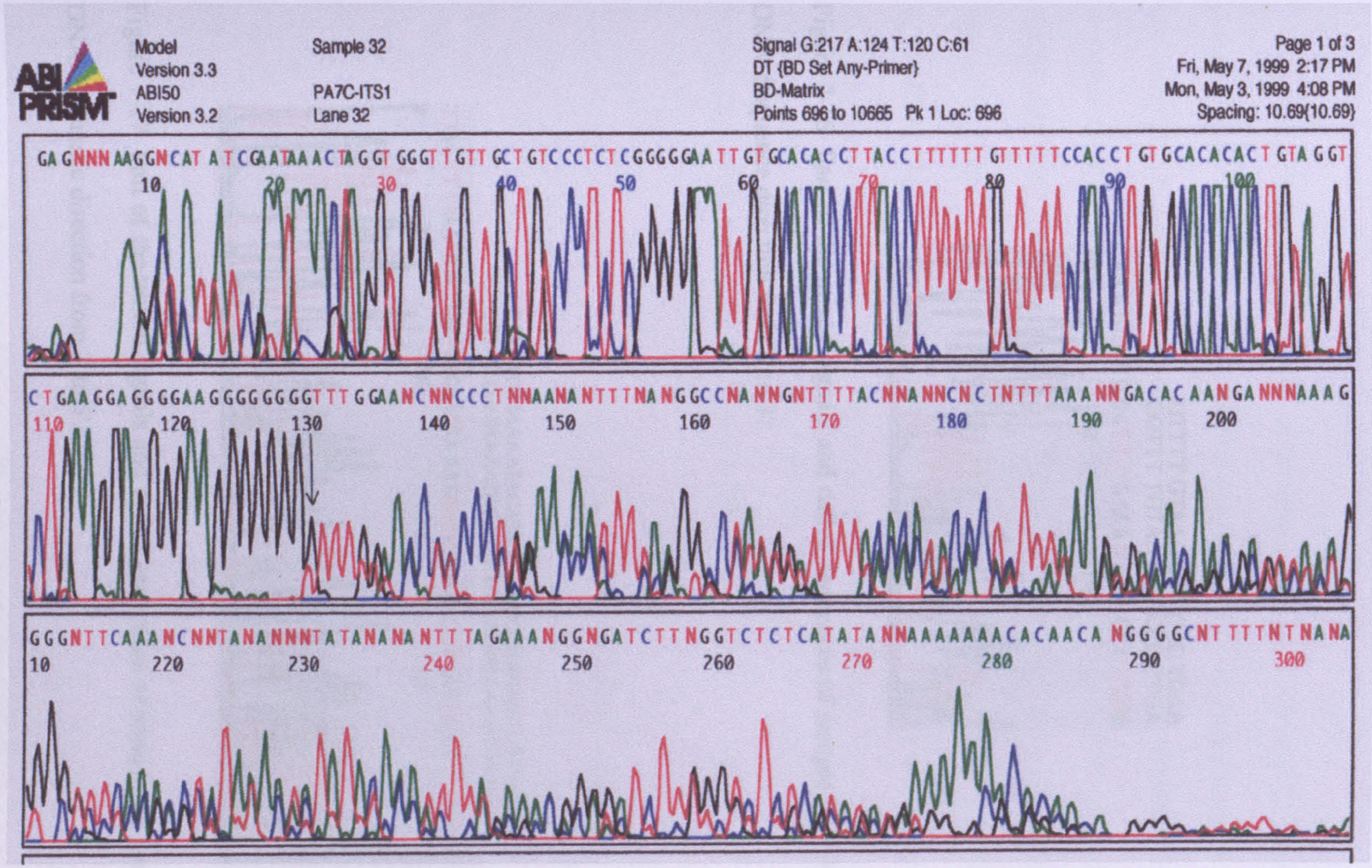


Figure 7-15 Sequencing electropherogram of sample PA7 from DNA fragment of the ITS-1 rDNA by primer ITS2. After position 129 (indicated by arrow) in this electropherogram, there are mixed signals which shows multialleles in this sample.

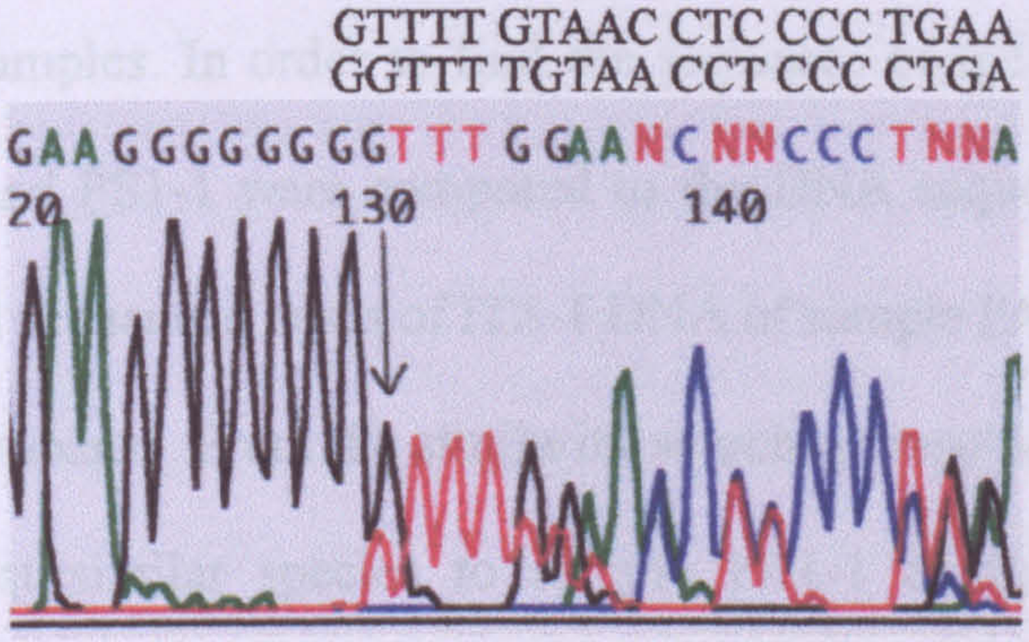


Figure 7-16 Part of the mixed signals and called sequences of sample PA7 in ITS-1 DNA. Sequence direction from 5' to 3'.

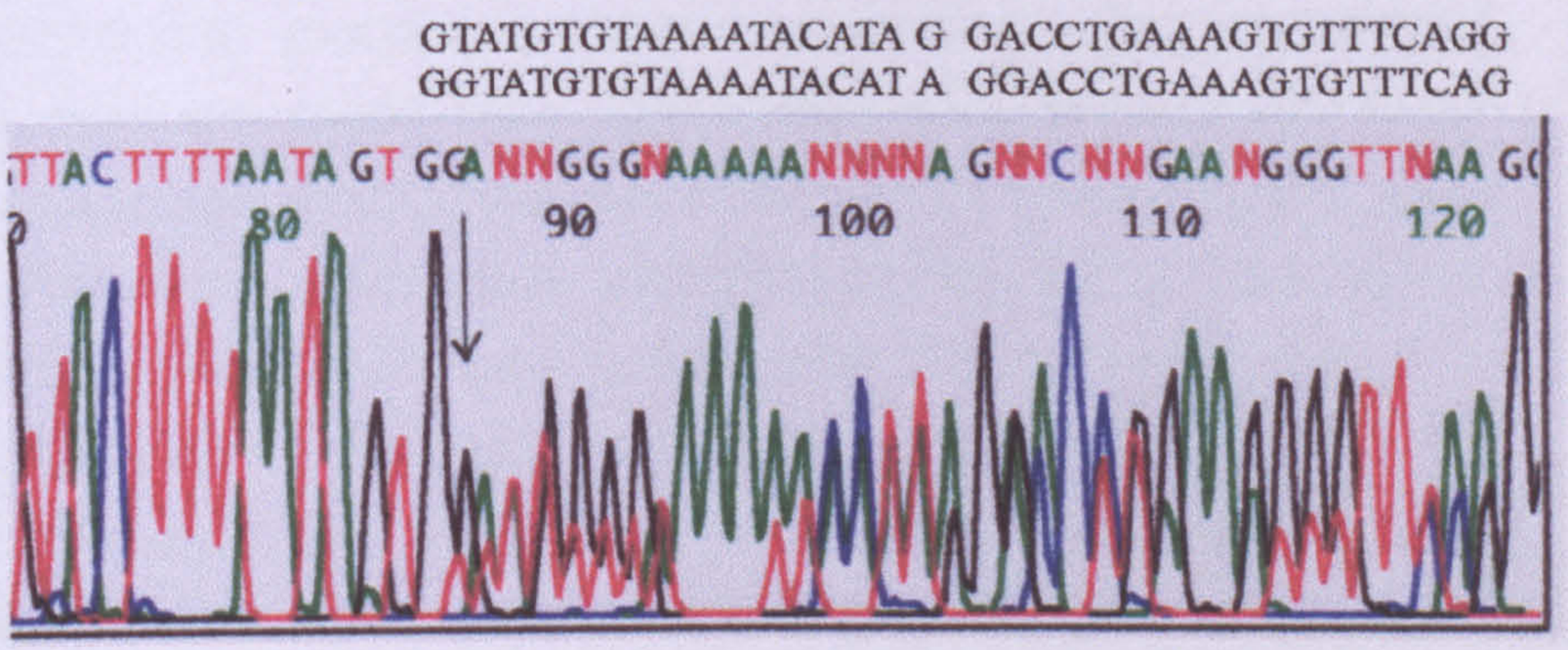


Figure 7-17 Part of the mixed signals and called sequences of sample PA7 in ITS-1 DNA. Sequence direction from 3' to 5'.

7.3.4 Sequence Analysis

From the sequencing results of 20 samples, sequence data of 18 alleles were listed in Figure 7-18. Table 7-1 showed the sizes of ITS-1 DNA fragments sequenced in this study of these samples. In order to find the sequence of the closest species, the sequence of PA1-1 and PS1-1 were compared to the DNA sequences lodged on the EMBL databank. All sequenced bases of ITS-1 DNA of sample PA1-1 and PS1-1 were sent to the EMBL databank. From the similarity searching results in EMBL databank by FASTA, the most similar species to sample PA1-1 of *Panaeolus* species is *Tomentella sublilacina* which has similarity of 73.651% (Figure 7-19). And the most similar species to sample PS1-1 of *Psilocybe* species is *Dermocybe olivaceopicta* which has similarity of 78.345% (Figure 7-20).

```
      1                                                            50
 PA1  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PA2  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PA3  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PA4  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PA5  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PA6  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PA7a  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PA7b  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PA7c  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PA7d  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PS1a  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PS1b  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PS2  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PS3  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PS4  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
 PS5  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PS6a  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
PS6b  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC

      51                                                           100
 PA1  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
 PA2  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATCG
 PA3  ATTATCGAAT AAACTGGGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
 PA4  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTA GGGGGAATTG
 PA5  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
 PA6  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATCG
PA7a  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
PA7b  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
PA7c  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
PA7d  ATTATCGAAT AAACT.AGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG
PS1a  ATTATTGAAT GAACTTGGCT CGGTTGTAGC TGGTCCTCTC GGGGGCAT.G
PS1b  ATTATTGAAT GAACTTGGCT CGGTTGTAGC TGGTCCTCTC GGGGGCAT.G
 PS2  ATTATTGAAT AAACCTGATG TGGTTGTTGC TGACTCTTTC GGGAGTAT.G
 PS3  ATTATTGAAT AACTTTGGCG TGGTTGTAGC TGGTCCTCTC GGGGGCAT.G
 PS4  ATTATTGAAT AACTTTGGCG TGGTTGTAGC TGGTCCTCTC GGGGGCAT.G
 PS5  ATTATTGAAT AAACCTGATG TGGTTGTAGC TGGCTCTCTC GGGAGTAT.G
PS6a  ATTATTGAAT GAACTTGGCT CGGTTGTAGC TGGTCCTCTC GGGGGCAT.G
PS6b  ATTATTGAAT GAACTTGGCT CGGTTGTAGC TGGTCCTCTC GGGGGCAT.G
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```
      101                                                        150
 PA1  TGCACGCCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
 PA2  TGCACGCCTT ACCTTATTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
 PA3  TGCACGCCTT ACCCTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
 PA4  TGCACGCCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
 PA5  TGCACGCCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
 PA6  TGCACGCCTT ACCTTATTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
PA7a  TGCACACCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
PA7b  TGCACACCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
PA7c  TGCACACCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
PA7d  TGCACACCTT ACCTTTTTTG TTTTTC.CAC CTGTGCACAC ACTGTAGGTC
PS1a  TGCTCGCCGT GTCATCTTTA TCTCTC.CAC CTGTGCACCT TTTGTAGACC
PS1b  TGCTCGCCGT GTCATCTTTA TCTCTC.CAC CTGTGCACCT TTTGTAGACC
 PS2  TGCACGCC.C GTCATCTTTA TATCTC.CAC CTGTGCACCT TTTGTAGACC
 PS3  TGCTCGCC.C GTCATCTTTA TATCTC.CAC CTGTGCACCT TTTGTAGACG
 PS4  TGCTCGCC.C GTCATCTTTA TATCTC.CAC CTGTGCACCT TTTGTAGACG
 PS5  TGCACGCC.C GTCATCTTTA TATTTC.CAC CTGTGCACCT TTTGTAGACC
PS6a  TGCTCGCCGT GTCATCTTTA TCTCTC.CAC CTGTGCACCT TTTGTAGACC
PS6b  TGCTCGCCGT GTCATCTTTA TCTCTC.CAC CTGTGCACCT TTTGTAGACC
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```
      151                                                        200
 PA1  T...GGAGGG AAAGGGAGGC AACTCCCTAA C.........  ...GTTTCAG
 PA2  T...GGAGTG GAAGGGAGGC AACTCCCTAA C.........  ...GTTTCAG
 PA3  T...GGAGTG GAAGGGAGGC AACTCCCTAA C.........  ...GTTTCAG
 PA4  T...GAAGGG GAAGGGAGGC AACTCCCTGA T.........  ...GTTTCAG
 PA5  T...GGAGGG AAAGGGAGGC AACTCCCTAA C.........  ...GTTTCAG
 PA6  T...GGAGTG GAAGGGAGGC AACTCCCTAA C.........  ...GTTTCAG
PA7a  TGAAGGAGGG GAAGGGGGGG GTTTTGTAAC CTCCCCTGAA ACACTTTCAG
PA7b  TGAAGGAGGG GAAGGGGGGG GTTTTGTAAC CTCCCCTGAA ACACTTTCAG
PA7c  TGAAGGAGGG GAA.GGGGGG GTTTTGTAAC CTCCCCTGAA ACACTTTCAG
PA7d  TGAAGGAGGG GAA.GGGGGG GTTTTGTAAC CTCCCCTGAA ACACTTTCAG
PS1a  TGGATTAGTT AACTTTCCGA GGAAACTCGG TCAAGAGGAT TGCTTTCACA
PS1b  TGGATTAGTT AACTTTCCGA GGAAACTCGG TCAAGAGGAT TGCTTTCACA
 PS2  TGGAGATTGG AGAGTA.... .......... .......ATC TCTTTTCTTT
 PS3  TTGAAACTGG ATAGGAGAGG GACTTGTCCT TCAAGTTGAA GGATTTTTCG
 PS4  TTGGAACTGG ATAGGAGAGG GACTTGTCCT TCAAGTTGAA GGTTTTTTCG
 PS5  CAG.GCGATT TGAGCA.... .......... .......ATC AAGTCATTTG
PS6a  TGGATTAGTT AACTTTCCGA GGAAACTCGG TCAAGAGGAT TGCTTTCACA
PS6b  TGGATTAGTT AACTTTCCGA GGAAACTCGG TCAAGAGGAT TGCTTTCACA
```

```
      201                                                        250
 PA1  GTCCTATGT. ......... ......... TTA.CACACA TA..CACTAT
 PA2  GTCCTATGT. ......... ......... ATT.TACACA TA..CACTAT
 PA3  GTCCTATGT. ......... ......... CTT.TACACA TA..CACTAT
 PA4  GTCCTATGT. ......... .........C TTT.TACACA TA..CACTAT
 PA5  GTCCTATGT. ......... ......... TTA.CACACA TA..CACTAT
 PA6  GTCCTATGT. ......... ......... ATT.TACACA TA..CACTAT
PA7a  GTCCTATGT. ......... .........A TTT.TACACA TA.CCACTAT
PA7b  GTCCTATGT. ......... .........A TTT.TACACA TA..CACTAT
PA7c  GTCCTATGT. ......... .........A TTT.TACACA TA.CCACTAT
PA7d  GTCCTATGT. ......... .........A TTT.TACACA TA..CACTAT
PS1a  AGCTCTCCTT GCAAATTAAC CCAGGCCTAC GTT.TTCATA TA.CCCCAAA
PS1b  AGCTCTCCTT GC.AATTAAC CCAGGCCTAC GTT.TTCATA TA.CCCCAAA
 PS2  GG........ .......... ...GCCTATG TTT.ATCATA TA.CCCCATA
 PS3  GC........ .......... ...GCTCTAC GTT.TTCATA TA.CCCCAAA
 PS4  GC........ .......... ...GCTCTAC GTT.TTCATA TA.CCCCAAA
 PS5  GG........ .......... ...CCTACGA TTT.ATCATA TA.CCCCA.A
PS6a  AGCTCTCCTT GCAAATTAAC CCAGGCCTAC GTT:TTCATA TA.CCCCAAA
PS6b  AGCTCTCCTT GC.AATTAAC CCAGGCCTAC GTT.TTCATA TA.CCCCAAA
      251                                                        300
 PA1  TAAAAGTAAC AGAACGATTC AATGGGCTCT AAGCCTAT.. AAACTAAATA
 PA2  TAAAAGTAAC AGAACGATTC AATGGGCTCT AAGCCTAT.. AAACTATATA
 PA3  TAAAAGTAAC AGAACGATTC AATGGGCTCT AAGCCTAT.. AAACTATATA
 PA4  TAAAAGTAAC AGAACGATTC AATGGGCTCT AAGCCTATAA AAACTATATA
 PA5  TAAAAGTAAC AGAACGATTC AATGGGCTCT AAGCCTAT.. AAACTAAATA
 PA6  TAAAAGTAAC AGAACGATTC AATGGGCTCT AAGCCTAT.. AAACTATATA
PA7a  TAAAAGTAAC AGAACGATTC AATGGGCTTC CAAGC.CTAT AAACTATATA
PA7b  TAAAAGTAAC AGAACGATTC AATGGGCTTC CAAGC.CTAT AAACTATATA
PA7c  TAAAAGTAAC AGAACGATTC AATGGGCTTC CAAGC.CTAT AAACTATATA
PA7d  TAAAAGTAAC AGAACGATTC AATGGGCTTC CAAGC.CTAT AAACTATATA
PS1a  GAATGTAACA GAATGTATTA TATTGGCCTT GTGCCTAT.. AAACTATATA
PS1b  GAATGTAACA GAATGTATTA TATTGGCCTT GTGCCTAT.. AAACTATATA
 PS2  GTATGTAACA GAATGTA.TC AATGGGCTTC GTGCCTAT.. AAACTAAATA
 PS3  GAATGTAACA GAATGTA.TC TTATGGCTTT ATGCCTAT.. AAACTATATA
 PS4  GAATGTAACA GAATGTA.TC TTATGGCTTT ATGCCTAT.. AAACTATATA
 PS5  GTATGTATTA GAATGTA.TC AATGGGCTTC GTGCCTAT.. AAACATTATA
PS6a  GAATGTAACA GAATGTATTA TATTGGCCTT GTGCCTAT.. AAACTATATA
PS6b  GAATGTAACA GAATGTATTA TATTGGCCTT GTGCCTAT.. AAACTATATA
```

```
      301                                                        350
PA1   CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA2   CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA3   CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA4   CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA5   CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA6   CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA7a  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA7b  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA7c  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PA7d  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PS1a  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PS1b  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
 PS2  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
 PS3  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
 PS4  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
 PS5  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PS6a  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
PS6b  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
```

Figure 7-18 Sequence data of 18 alleles out of 11 species. Allele PA1 was from samples PA1-1, PA1-2, PA1-3 and PA1-4. Allele PA7a, PA7b, PA7c and PA7d were from samples PA7. Allele PS1a and PS1b were from sample PS1-1, PS1-2, PS1-3 and PS1-4. Allele PS6a and PS6b were from samples PS6-1 and PS6-2.

Table 7-1 The sizes of DNA fragments amplified from the ITS-1 DNA in nuclear ribosomal RNA gene

Sample	Size	Sample	size
PS1-1	343/344 bp	PA1-1	307 bp
PS1-2	343/344 bp	PA1-2	307 bp
PS1-3	343/344 bp	PA1-3	307 bp
PS1-4	343/344 bp	PA1-4	307 bp
PS2	300 bp	PA2	307 bp
PS3	321 bp	PA3	308 bp
PS4	321 bp	PA4	310 bp
PS5	298 bp	PA5	307 bp
PS6-1	343/344 bp	PA6	307 bp
PS6-2	343/344 bp	PA7	323/324/325 bp

```
                    10         20         30         40         50
PA1,        GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCG
            :::::::::::::::::::::::::::::::::::::::::::::::::::: ::
EM_FUN AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCG
                10         20         30         40         50         60

          60         70         80         90         100        110
PA1,    AATAAACTAGGTGGGTTGTTGCT-GTCCCTCTCGGGGGAATTGTGCACGC-CTTACCTTT
        :::   : :   :::::::::: :::: :    :::::  ::::::::: ::
EM_FUN AATTGTC-AANCGGGTTGTTGCTGGTCCTTGAAAGGGGACATGTGCACGCTCTGTTTACA
               70         80         90         100        110

          120        130        140        150        160        170
PA1,    TTTGTTTTTCCACCTGTGCACACACTGTAGGTCTGGAGGGAAAGGGAGGC---AACTCCC
           :    : :::::::::: : :::::: :::  : ::   ::::: :     :: ::
EM_FUN CATCCACTCACACCTGTGCACCCTCTGTAGTTCT--ATGGTCTGGGAGACCTTGTCTTCC
      120        130        140        150        160        170

                180        190        200        210        220        230
PA1,    TAACGTTTCAGGTCCTATGTTTACACACATACACTATTA-AAAGTAACAGAACGATTCAA
        :  :::    ::: ::: :: :  ::::: ::::: : : :::::  :  :: :: :  :
EM_FUN TGCCGT----GGTTCTACGTCTTTACACACACACTGTAACAAAGT--CTTAAGGAATGTA
       180        190        200        210        220        230
```

```
                    240         250         260         270         280
PA1,     TG--GGCTCTAAGCCTATAAACTAAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC
         .:  .  .....  . ...     ..............................  .....
EM_FUN TGCAGCGTCTAACGCAATA----CAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC
               240         250             260         270         280


       290         300
PA1,     ATCGATGAAGAACGCAGC
         ::::::::::::::::::
EM_FUN ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
       290         300         310         320         330         340
```

Figure 7-19 Similarity searching result by FASTA on EMBL database (EM_FUN) with the closest species *Tomentella sublilacina* (locus TSU83481). PA1 is *Panaeolus semiovatus*. DNA sequence is located in ITS1 region of RNA gene. There is 73.651% identity in 315 nt overlap.

```
                    10         20         30         40         50         60
PS1,    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAAT
                    :::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN              TTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAAA
                              10         20         30

                   70         80         90         100        110        120
PS1,    GAACTTGGCTCGGTTGTAGCTGGTCCTCTCGGGGGCATGTGCTCGCCGTGTCATCTTTAT
          ::    : : :::::  ::::::: : :: ::: :::::::: :  : ::::::::::::
EM_FUN  TAAACCTGATGGGTTGCTGCTGGTTCCCTAGGGAGCATGTGCAC-ACTTGTCATCTTTAT
        40         50         60         70         80         90

                  130        140        150        160        170
PS1,    CTCTCCACCTGTGCACCTTTTGTAGACCTGGATTAGTTAACT---TTCCGAGGAAACTCG
          :::::::::::::::::::::::::::::  :  ::  :  ::   : :: : : :: ::
EM_FUN  ATCTCCACCTGTGCACCTTTTGTAGACCCTGGATATCTTTCTGAATGCCTACGCAATTCA
        100        110        120        130        140        150
```

```
          180        190        200        210        220        230
PS1,      GTCAAGAGGATTGCTTTCACAAGCTCTCCTTGCAAATTAACCCAGGCCTACGTT--TTCA
          :    :::::::: ::  :   :: ::::: ::  :    ::::::::: :::  ::::
EM_FUN    GGTTTGAGGATTGACTTTGCTGTCTTTCCTTACATTT----CCAGGCCTATGTTTCTTCA
          160        170        180        190            200        210

            240        250        260        270        280        290
PS1,      TATA-CCCCAAAGAATGTAACAGAATGTATTATATTGGCCTTGTGCCTATAAACTATATA
          :::: :::::: : :::: :::::::::: :: :: ::::::    ::::::::  ::::
EM_FUN    TATACCCCCAATGTATGTTACAGAATGTAATAAATGGGCCTTTGTGCTATAAAC-CTATA
            220        230        240        250        260        270

            300        310        320        330        340
PS1,      CAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGC
          :::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN    CAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGAT
              280        290        300        310        320        330

EM_FUN    AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCC
              340        350        360        370        380        390
```

Figure 7-20 Similarity searching result by FASTA on EMBL database (EM_FUN) with the closest species *Dermocybe olivaceopicta* (locus DOU56050). PS1 is ***Psilocybe semilanceata***. DNA sequence is located in ITS1 region of RNA gene. There is 78.354% identity in 328 nt overlap.

In order to reveal the similarity of sequences, we use the Pileup and Pretty programs of GCG software to perform the sequence comparison study. Figure 7-21 showed the results of Pretty program analysis. From the data of Figure 7-21, there are 12 alleles in twenty samples. The DNA sequence data show that 5 samples of *Panaeolus semiovatus*, sample PA1-1, PA1-2, PA1-3, PA1-4, and PA5, share the same sequences in ITS-1 DNA of nuclear ribosomal RNA gene. Sample PA2 and PA6 are different species of *Panaeolus papilionaceus* and *Panaeolus speciosus*, respectively. However, these two species shared the same sequences in this DNA area. Sequences in ITS-1 DNA of sample PA3, PA4 and PA7 show unique among 20 samples. There are four alleles observed in sample PA7, *Panaeolus subbalteatus*, with two nucleotide's deletions/insertions. The rare feature of multialleles in sample PA7 is a good evidence of the presence of the species *Panaeolus subbalteatus*.

```
                1                                                    50
1.msf{PA7a}     ---------- ---------- ---------- ---------- ----------
1.msf{PA7b}     ---------- ---------- ---------- ---------- ----------
1.msf{PA7c}     ---------- ---------- ---------- ---------- ----------
1.msf{PA7d}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA1}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA5}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA2}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA6}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA3}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA4}     ---------- ---------- ---------- ---------- ----------
1.msf{PS1a}     ---------- ---------- ---------- ---------- ----------
1.msf{PS6a}     ---------- ---------- ---------- ---------- ----------
1.msf{PS1b}     ---------- ---------- ---------- ---------- ----------
1.msf{PS6b}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS3}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS4}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS2}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS5}     ---------- ---------- ---------- ---------- ----------
 1.msf{Tom}     ---------- ---------- ---------- ---------- ----------
 1.msf{Der}     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ---------- ----------
  Consensus     GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC

                51                                                   100
1.msf{PA7a}     ---------- -----.---- ---------- ---------- ----------
1.msf{PA7b}     ---------- -----.---- ---------- ---------- ----------
1.msf{PA7c}     ---------- -----.---- ---------- ---------- ----------
1.msf{PA7d}     ---------- -----.---- ---------- ---------- ----------
 1.msf{PA1}     ---------- -----.---- ---------- ---------- ----------
 1.msf{PA5}     ---------- -----.---- ---------- ---------- ----------
 1.msf{PA2}     ---------- -----.---- ---------- ---------- --------c-
 1.msf{PA6}     ---------- -----.---- ---------- ---------- --------c-
 1.msf{PA3}     ---------- -----gg--- ---------- ---------- ----------
 1.msf{PA4}     ---------- -----.---- ---------- ---------a ----------
1.msf{PS1a}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
1.msf{PS6a}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
1.msf{PS1b}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
1.msf{PS6b}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
```

```
                51                                                    100
1.msf{PS3}   -----t---- --ct--g-cg t------a-- --gt------ -----c--.-
1.msf{PS4}   -----t---- --ct--g-cg t------a-- --gt------ -----c--.-
1.msf{PS2}   -----t---- ----c-gatg t--------- --a-t--t-- ---a-t--.-
1.msf{PS5}   -----t---- ----c-gatg t------a-- --g-t----- ---a-t--.-
1.msf{Tom}   ----c----- tgt-.a--cg --t-gt-gct g----t-gaa a-----ca--
1.msf{Der}   -----t---a t--acct-a- ------c--- --gtt-c--a ---a-c--.-
 Consensus   ATTATCGAAT AAACTTAGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG

               101                                                    150
1.msf{PA7a}  -----a---- ac---t---- ------.--- --------a- a---------
1.msf{PA7b}  -----a---- ac---t---- ------.--- --------a- a---------
1.msf{PA7c}  -----a---- ac---t---- ------.--- --------a- a---------
1.msf{PA7d}  -----a---- ac---t---- ------.--- --------a- a---------
 1.msf{PA1}  ---------- ac---t---- ------.--- --------a- a---------
 1.msf{PA5}  ---------- ac---t---- ------.--- --------a- a---------
 1.msf{PA2}  ---------- ac---a---- ------.--- --------a- a---------
 1.msf{PA6}  ---------- ac---a---- ------.--- --------a- a---------
 1.msf{PA3}  ---------- ac-c-t---- ------.--- --------a- a---------
 1.msf{PA4}  ---------- ac---t---- ------.--- --------a- a---------
1.msf{PS1a}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
1.msf{PS6a}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
1.msf{PS1b}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
1.msf{PS6b}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
 1.msf{PS3}  ---t----.c gt-a-----a -a-c--.--- --------ct tt-----acg
 1.msf{PS4}  ---t----.c gt-a-----a -a-c--.--- --------ct tt-----acg
 1.msf{PS2}  --------.c gt-a-----a -a-c--.--- --------ct tt-----ac-
 1.msf{PS5}  --------.c gt-a-----a -a----.--- --------ct tt-----ac-
 1.msf{Tom}  -------tc- gtt-a-acat ccac--a--- --------c- t------t--
 1.msf{Der}  -----a-.-- gt-a-----a -a-c--.--- --------ct tt-----ac-
 Consensus   TGCACGCCTT --CTTCTTTG TTTTTC-CAC CTGTGCAC-C -CTGTAGGTC
```

```
                151                                                   200
1.msf{PA7a}  --a------- g-----g--g -tt-tg-a-c -tcccc---- acac------
1.msf{PA7b}  --a------- g-----g--g -tt-tg-a-c -tcccc---- acac------
1.msf{PA7c}  --a------- g--.--g--g -tt-tg-a-c -tcccc---- acac------
1.msf{PA7d}  --a------- g--.--g--g -tt-tg-a-c -tcccc---- acac------
 1.msf{PA1}  -...------ a--------c a-----ct-- -......... ...g------
 1.msf{PA5}  -...------ a--------c a-----ct-- -......... ...g------
 1.msf{PA2}  -...----t- g--------c a-----ct-- -......... ...g------
 1.msf{PA6}  -...----t- g--------c a-----ct-- -......... ...g------
 1.msf{PA3}  -...----t- g--------c a-----ct-- -......... ...g------
 1.msf{PA4}  -...-a---- g--------c a-----ctg- t......... ...g------
1.msf{PS1a}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
1.msf{PS6a}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
1.msf{PS1b}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
1.msf{PS6b}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
 1.msf{PS3}  -t--aact-- at---aga-g ----tg-cct t----t---- g-a----tc-
 1.msf{PS4}  -t-gaact-- at---aga-g ----tg-cct t----t---- g-t----tc-
 1.msf{PS2}  -----att-- ag--ta.... .......... .......atc tct---cttt
 1.msf{PS5}  ca-.-cgatt tg--ca.... .......... .......atc aag-ca-tt-
 1.msf{Tom}  -.at--tct- gg--accttg tct----gcc gtggttct-c gtc---a--c
 1.msf{Der}  ct-gat-tct ttct-a-t-c ctacg-aatt -aggtt---g gat-gacttt
  Consensus  TGGAGGAGGG -AAGGGAGG- GACTCCT-AA CCAAG-TGAA -G-TTTTCAG

                201                                                   250
1.msf{PA7a}  ---------. .......... .........a ---.----c- --.----t--
1.msf{PA7b}  ---------. .......... .........a ---.----c- --..---t--
1.msf{PA7c}  ---------. .......... .........a ---.----c- --.----t--
1.msf{PA7d}  ---------. .......... .........a ---.----c- --..---t--
 1.msf{PA1}  ---------. .......... .......... --a.c---c- --..---t--
 1.msf{PA5}  ---------. .......... .......... --a.c---c- --..---t--
 1.msf{PA2}  ---------. .......... .......... a--.----c- --..---t--
 1.msf{PA6}  ---------. .......... .......... a--.----c- --..---t--
 1.msf{PA3}  ---------. .......... .......... c--.----c- --..---t--
 1.msf{PA4}  ---------. ...:...... .........- ---.----c- --..---t--
1.msf{PS1a}  ag-tctcc-- ---------- ----g----- g--.-t--t- --.--c-a-a
1.msf{PS6a}  ag-tctcc-- ---------- ----g----- g--.-t--t- --.--c-a-a
1.msf{PS1b}  ag-tctcc-- --.------- ----g----- g--.-t--t- --.--c-a-a
```

```
                201                                                   250
1.msf{PS6b}  ag-tctcc-- --.------- ----g----- g--.-t--t- --.--c-a-a
 1.msf{PS3}  -c..... ..... ...--t---- g--.-t--t- --.--c-a-a
 1.msf{PS4}  -c..... ..... ...--t---- g--.-t--t- --.--c-a-a
 1.msf{PS2}  -g..... ..... ...---tatg ---.at--t- --.--c-ata
 1.msf{PS5}  -g..... ..... ...c-tacga ---.at--t- --.--c-a.a
 1.msf{Tom}  aca-ac---a a----g-ctt aag-aatgta -gc.ag-gtc --.a-g-a--
 1.msf{Der}  -ctg-c-t-c ctt-ca-tt- -ag---tatg ---c-t--t- --c--c-a--
  Consensus  GTCCTATGTT GCAAATTAAC CCAGCCCTAC TTT-TACA-A TA-CCAC-AT

                251                                                   300
1.msf{PA7a}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
1.msf{PA7b}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
1.msf{PA7c}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
1.msf{PA7d}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
 1.msf{PA1}  ---------- -g-a-g---- --------c- --------.. ------a---
 1.msf{PA5}  ---------- -g-a-g---- --------c- --------.. ------a---
 1.msf{PA2}  ---------- -g-a-g---- --------c- --------.. ----------
 1.msf{PA6}  ---------- -g-a-g---- --------c- --------.. ----------
 1.msf{PA3}  ---------- -g-a-g---- --------c- --------.. ----------
 1.msf{PA4}  ---------- -g-a-g---- --------c- --------a ----------
1.msf{PS1a}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
1.msf{PS6a}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
1.msf{PS1b}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
1.msf{PS6b}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
 1.msf{PS3}  g--tgta-ca ga-tgt-.-- ttat------ -t------.. ----------
 1.msf{PS4}  g--tgta-ca ga-tgt-.-- ttat------ -t------.. ----------
 1.msf{PS2}  gt-tgta-ca ga-tgt-.-- ---------c gt------.. ------a---
 1.msf{PS5}  gt-tgtatta ga-tgt-.-- ---------c gt------.. ----at----
 1.msf{Tom}  ac--...t-- -actttcag- --c--atc-c tt-g--c-cg c-t-g--ga-
 1.msf{Der}  gt-tgt--ca ga-tgt-a-a --------c-- tgtg----.. .--acc----
  Consensus  TAAAAGTAAC A-A-C-ATTC AATGGGCTTT AAGCCTATAT AAACTATATA
```

```
                301                                                 350
1.msf{PA7a}  ---------- ---------- ---------- ---------- ----------
1.msf{PA7b}  ---------- ---------- ---------- ---------- ----------
1.msf{PA7c}  ---------- ---------- ---------- ---------- ----------
1.msf{PA7d}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA1}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1a}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6a}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1b}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6b}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS3}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS4}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS2}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
 1.msf{Tom}  g---gcagc~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 1.msf{Der}  ---------- ---------- ---------- ---------- ----------
  Consensus  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
```

Figure 7-21 Sequence alignment of 20 alleles by Pretty program of GCG software. The left column is sample name in the file name: 1.msf. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*.. Consensus sequences were automatically produsedby the same program. Symbol "." is deletion, "-"is the same sequence with the consensus sequence. Symbol "~" is not shown in the EMBL database. Sample PA7, PS1 and PS6 show multiple allleles. There are two blocked sequences from left to right were primer positions to amplify sequence this ITS-1 DNA fragments described in the text.

The sequencing data from the *Psilocybe* samples also showed heterozygous in this locus. Samples PS1-1, PS1-2, PS1-3, PS1-4, PS6-1 and PS6-2 are *Psilocybe semilanceata*. They shared the same sequences and contained two alleles with only one nucleotide difference of deletion/insertion to each other. The sequencing results of the other different species of *Psilocybe*, sample PS2, PS3, PS4 and PS5, showed sequence deviation. The sequencing data from the ITS-1 region showed that except *Panaeolus papilionaceus* and *Panaeolus speciosus*, there are variations at this locus among different species and also genus variations. This region of DNA provides the basis of a genus specific test. The internal transcribed spacer region of the nuclear rDNA repeat units exhibit a higher degree of polymorphism compared to the small subunit rDNA within the animals and plant tested, probably due to greater evolutionary pressures. The sequence variation in ITS-1 DNA showed that these sequences can be possibly used in the differentiation of not only genus but also species.

In this study, GCG software was also used to evaluate the similarity between samples on the bases of these 18 sequence data of twenty samples from the genera *Psilocybe* and *Panaeolus* and two other species. Table 7-2 showed the similarity of 20 sequences. Dendrograms generated by cluster analysis of unweighted-pair group method algorithms (UPGMA) and neighbor-joining method were computed by GCG software and shown in Figure 7-22 and 7-23, respectively. Figure 7-24 showed unrooted phylogenetic tree computed by Phylip, and a neighbor-joining tree with 100 bootstrap replications is shown in Figure 7-25 computed by Phylip.

Table 7-2 Genetic distance matrix of 20 nucleotide sequences generated by Kimura 2-parameter method produced by the Distance software in GCG. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*. Distances are estimated number of substitutions per 100 bases.

Matrix Part 1

	PA7a	PA7b	PA7c	PA7d	PA1	PA5	PA2	PA6	PA3	PA4	PS1a	PS6a
PA7a	0.00	0.00	0.00	0.00	7.57	7.57	7.56	7.56	7.58	8.21	39.05	39.05
PA7b		0.00	0.00	0.00	7.57	7.57	7.56	7.56	7.58	8.21	39.21	39.21
PA7c			0.00	0.00	7.60	7.60	7.59	7.59	7.61	8.24	38.69	38.69
PA7d				0.00	7.60	7.60	7.59	7.59	7.61	8.24	38.85	38.85
PA1					0.00	0.00	2.65	2.65	2.66	2.66	34.78	34.78
PA5						0.00	2.65	2.65	2.66	2.66	34.78	34.78
PA2							0.00	0.00	1.65	2.65	33.27	33.27
PA6								0.00	1.65	2.65	33.27	33.27
PA3									0.00	2.66	33.10	33.10
PA4										0.00	33.08	33.08
PS1a											0.00	0.00
PS6a												0.00
PS1b												
PS6b												
PS3												
PS4												
PS2												
PS5												
Tom												
Der												

Table 7-2 Genetic distance matrix of 20 nucleotide sequences generated by Kimura 2-paramet method produced by the Distance software in GCG. Der is *Dermocybe olivaceopicta*. Tom *Tomentella sublilacina*. Distances are estimated number of substitutions per 100 bases.

Matrix Part 2

	PS1b	PS6b	PS3	PS4	PS2	PS5	Tom	Der
PA7a	39.05	39.05	33.33	34.35	30.55	32.36	59.97	45.99
PA7b	39.21	39.21	33.47	34.49	30.69	32.50	59.52	46.20
PA7c	38.69	38.69	33.47	34.49	30.69	32.50	60.30	45.60
PA7d	38.85	38.85	33.60	34.63	30.82	32.65	59.85	45.81
PA1	34.78	34.78	30.88	30.88	26.64	29.82	58.98	42.87
PA5	34.78	34.78	30.88	30.88	26.64	29.82	58.98	42.87
PA2	33.27	33.27	30.38	30.38	28.15	28.76	58.15	41.62
PA6	33.27	33.27	30.38	30.38	28.15	28.76	58.15	41.62
PA3	33.10	33.10	30.23	30.23	28.06	28.66	59.56	42.08
PA4	33.08	33.08	29.23	29.23	27.52	29.69	58.82	41.45
PS1a	0.00	0.00	16.30	16.68	18.87	22.77	79.59	36.26
PS6a	0.00	0.00	16.30	16.68	18.87	22.77	79.59	36.26
PS1b	0.00	0.00	16.30	16.68	18.87	22.77	80.07	35.90
PS6b		0.00	16.30	16.68	18.87	22.77	80.07	35.90
PS3			0.00	0.63	16.76	19.46	73.72	31.30
PS4				0.00	16.77	19.45	73.76	30.29
PS2					0.00	11.20	60.23	22.67
PS5						0.00	64.50	24.89
Tom							0.00	89.57
Der								0.00

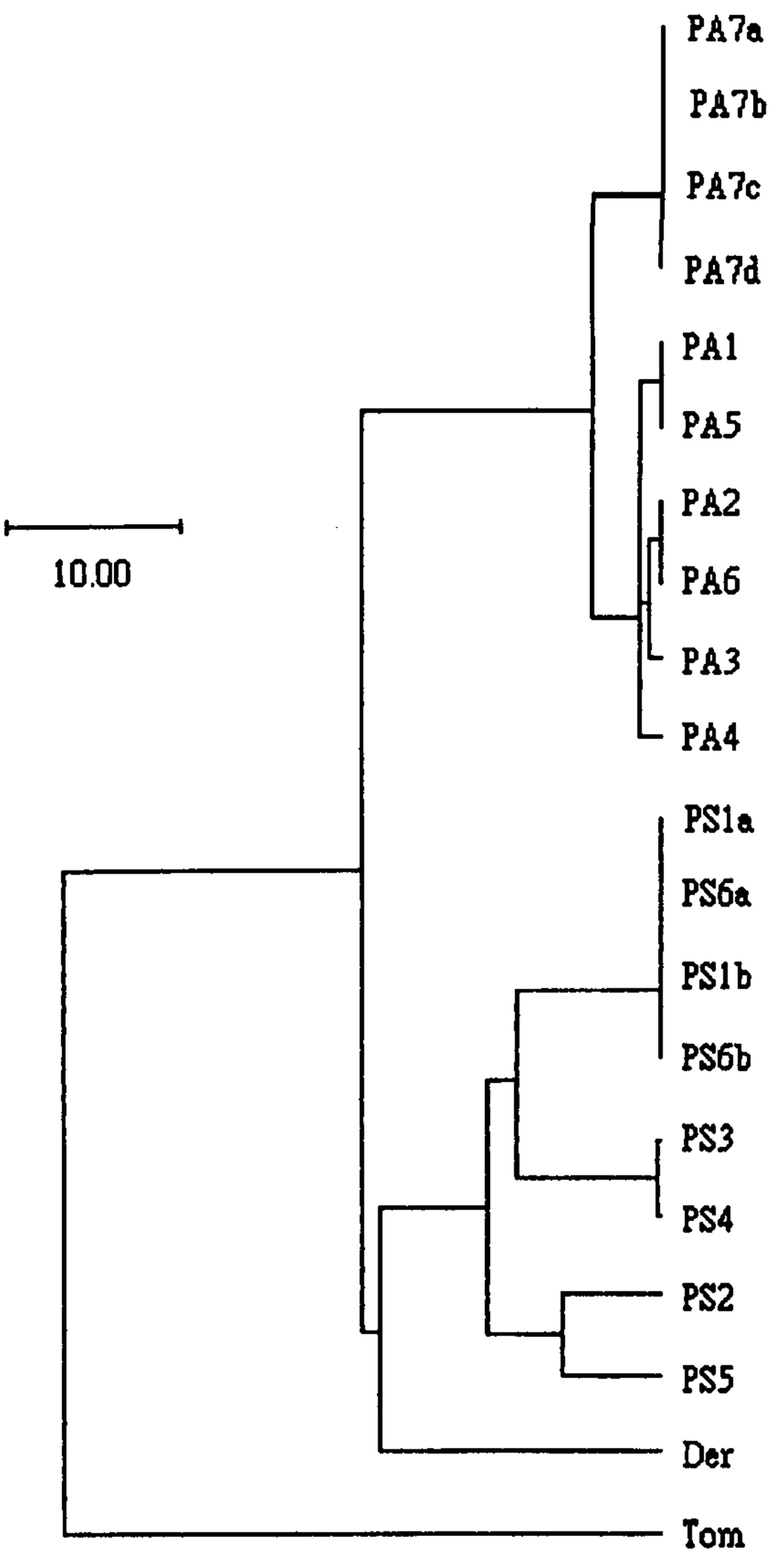


Figure 7-22 Dendrogram established by the genetic distances in Table 7-2 produced by UPGMA method using the Growtree software in GCG. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*.

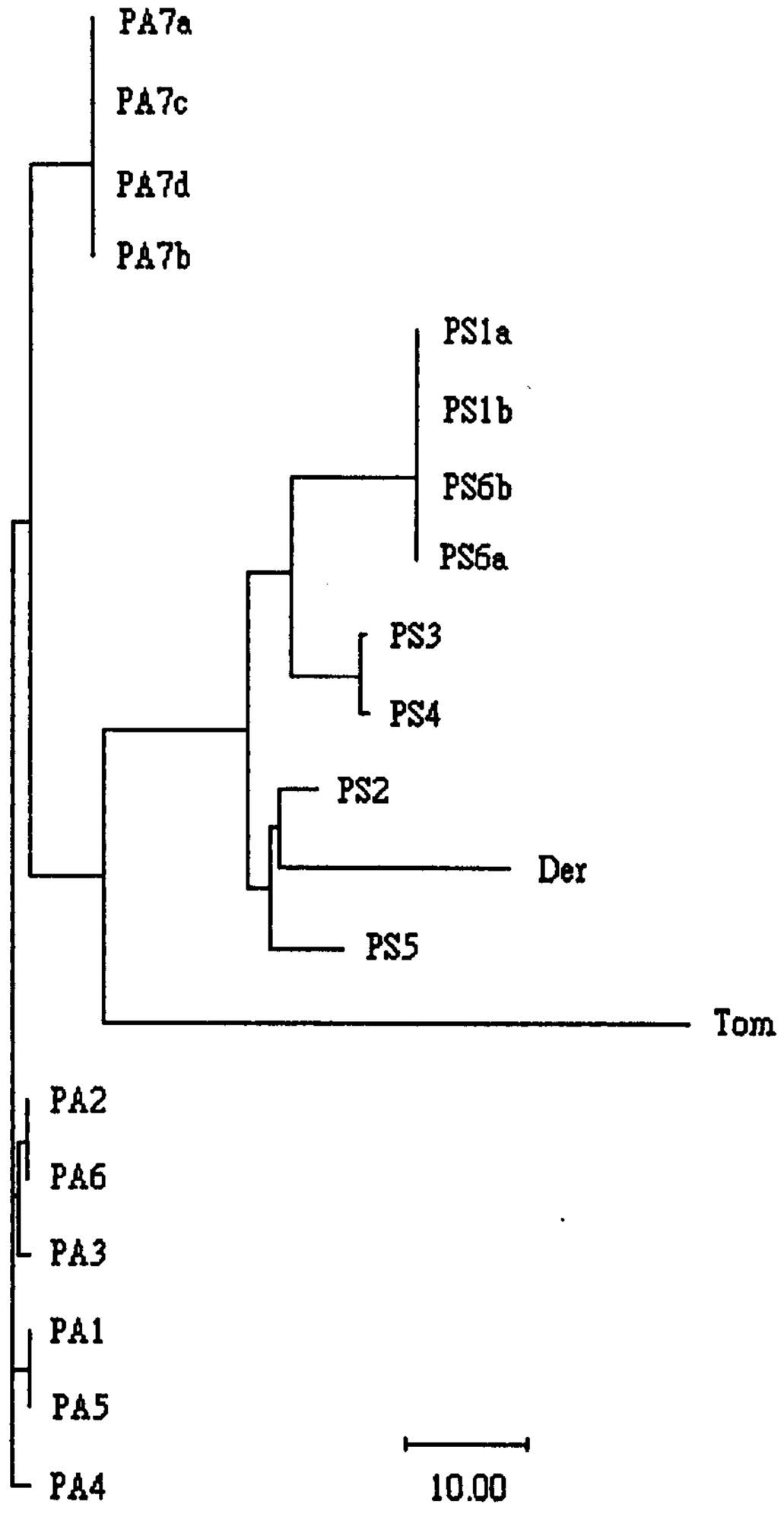


Figure 7-23 Dendrogram established by the genetic distances in Table 7-2 produced by NJ method using the Growtree software in GCG. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*.

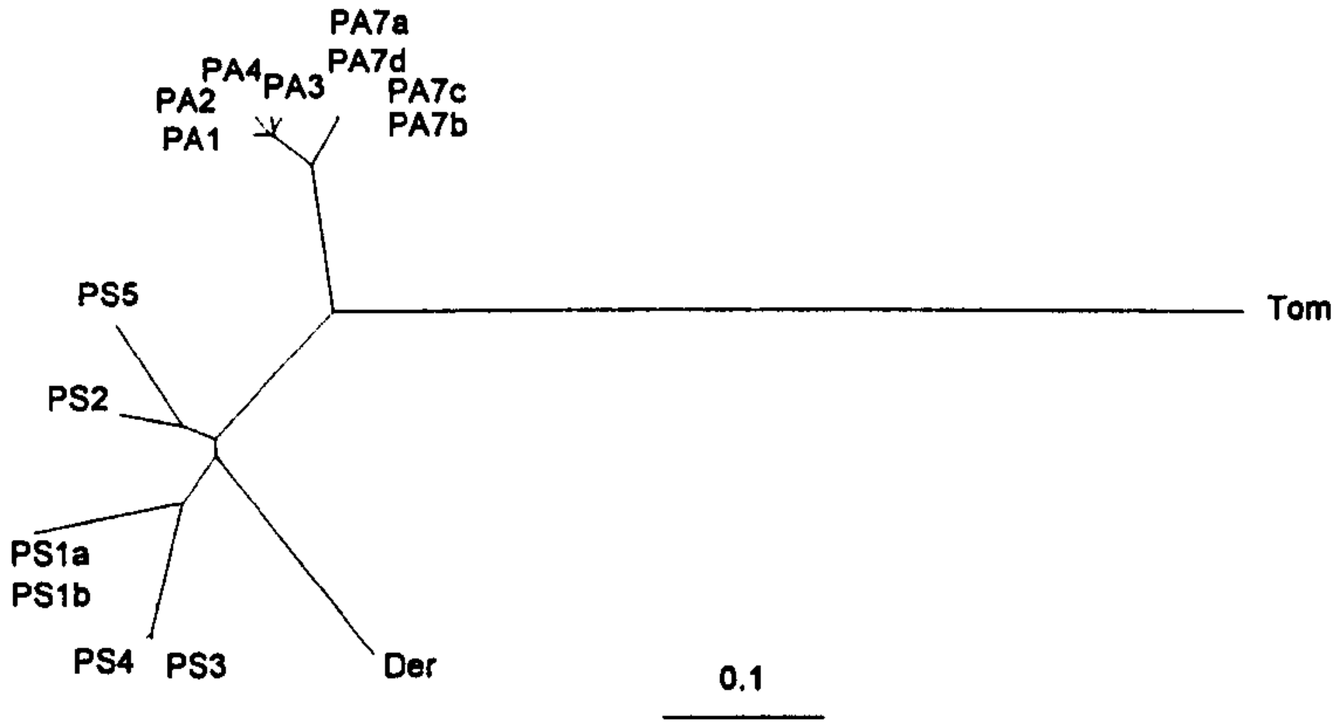


Figure 7-24 Unroot neighbor-joining tree established by the genetic distances in Table 7-2 using Phylip. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*.

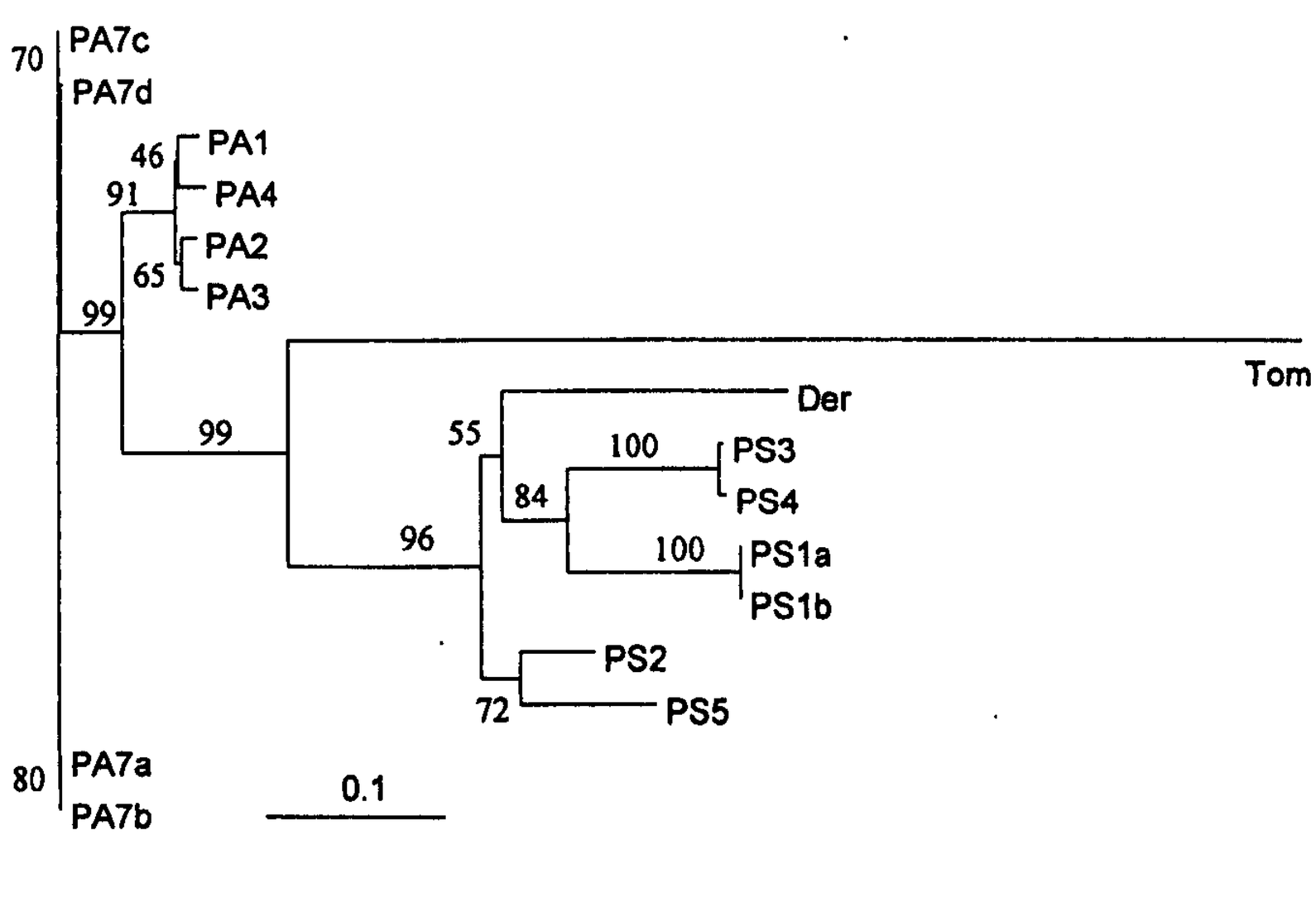


Figure 7-25 Neighbor-joining tree constructed by 100 bootstrap trials using Phylip. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*.

There are two major groups clustered, *Panaeolus* and *Psilocybe*, which showed the genetic distance estimated number of substitutions per 100 bases from 26.64 to 39.21 among each pair between any two samples. Within each group the higher genetic distance that were obtained were no more than 22.77. These data are obtained among genus *Psilocybe* between *Psilocybe semilanceata* (sample PS1 and PS6) and *Psilocybe montana* (sample PS5). In genus *Panaeolus*, the farthest genetic distance is between *Panaeolus subbalteatus* and *Panaeolus retirugis*. It is 8.24 of genetic distance. The genetic distance in Table 7-2 and dendrogram in Figure 7-23 shows that species in genus *Panaeolus* are close in sequence variation. In genus *Psilocybe*, however, there was a wide range of sequence variation among species. The most distant species is *Psilocybe montanae*. In Figure 7-22, the dendrogram established by UPGMA, sample *Dermocybe olivaceopicta* and *Tomentella sublilacina* were clustered beyond the *Psilocybe* and *Panaeolus* groups. In neighbor-joining tree (Figure 7-23 to 7-25), however, sample *Dermocybe olivaceopicta* was clustered in the *Psilocybe* group. The reason for this is the high sequence similarity between *Psilocybe montana* (sample PS5) and *Dermocybe olivaceopicta*. In the bootstrapping trials (Figure 7-25), there was only 55 times supported in the connection node between sample *Dermocybe olivaceopicta* and sample PS1, PS3 and PS4. It showed insufficient evidence to support this topology. Usually, more than 70 supports in 100 bootstrapping replications are reliable. This cluster analysis demonstrated that genus test for *Panaeolus* can obtain highly conclusive result. For *Psilocybe*, this is still a good method, particularly if the genetic distance greater than 22.67.

Using the homology and diversity of sequence in psilocybin-containing mushroom, this study demonstrated that the ITS1 DNA can be used to perform a genus or species test.

8 GENUS IDENTIFICATION BY THE INTERNAL TRANSCRIBED SPACER 1 DNA (ITS-1) IN NUCLEAR RIBOSOMAL RNA GENE USING PCR AMPLIFIED SPECIFIC FRAGMENTS

8.1 Introduction

In chapter 7, the sequences of the ITS-1 ribosomal gene locus in 11 species of the genera *Psilocybe* and *Panaeolus* have been established. The DNA sequence data revealed both sequence and length polymorphisms. These polymorphisms were shown to be specific to either the *Panaeolus* or *Psilocybe* genus with sequences being specific to members of species within each of these two genera. This study will use the genus specific DNA sequences, for both *Panaeolus* and *Psilocybe*, which will produce a DNA product in the presence of DNA from either of these two genera. A common set of primers, that will amplify a product in all fungi, will be used in tandem with the genus specific primers. This second amplification will act as a control to ensure that a negative result is due to the absence of either *Psilocybe* of *Panaeolus*, rather than a failed amplification. The position of the primers is shown in Figure 8-1. The primer sequences were determined by examining the sequence data in Chapter 7. The sequence data was used to determine DNA sequences that are specific to either *Panaeolus* or *Psilocybe*. The position of these primer sites is shown in Figure 8-2.

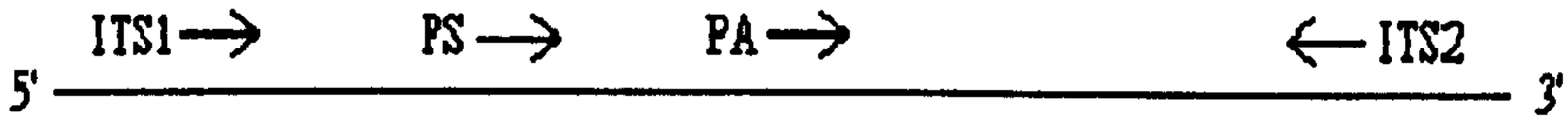


Figure 8-1 The location of primers on ITS-1 region used in this study. PA position includes primers from PA1 to PA5.

```
                1                                                    50
1.msf{PA7a}     ---------- ---------- ---------- ---------- ----------
1.msf{PA7b}     ---------- ---------- ---------- ---------- ----------
1.msf{PA7c}     ---------- ---------- ---------- ---------- ----------
1.msf{PA7d}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA1}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA5}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA2}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA6}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA3}     ---------- ---------- ---------- ---------- ----------
 1.msf{PA4}     ---------- ---------- ---------- ---------- ----------
1.msf{PS1a}     ---------- ---------- ---------- ---------- ----------
1.msf{PS6a}     ---------- ---------- ---------- ---------- ----------
1.msf{PS1b}     ---------- ---------- ---------- ---------- ----------
1.msf{PS6b}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS3}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS4}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS2}     ---------- ---------- ---------- ---------- ----------
 1.msf{PS5}     ---------- ---------- ---------- ---------- ----------
 1.msf{Tom}     ---------- ---------- ---------- ---------- ----------
 1.msf{Der}     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ---------- ----------
  Consensus     GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC

                51                                                   100
1.msf{PA7a}     ---------- -----.---- ---------- ---------- ----------
1.msf{PA7b}     ---------- -----.---- ---------- ---------- ----------
1.msf{PA7c}     ---------- -----.---- ---------- ---------- ----------
1.msf{PA7d}     ---------- -----.---- ---------- ---------- ----------
 1.msf{PA1}     ---------- -----.---- ---------- ---------- ----------
 1.msf{PA5}     ---------- -----.---- ---------- ---------- ----------
 1.msf{PA2}     ---------- -----.---- ---------- ---------- --------c-
 1.msf{PA6}     ---------- -----.---- ---------- ---------- --------c-
 1.msf{PA3}     ---------- -----gg--- ---------- ---------- ----------
 1.msf{PA4}     ---------- -----.---- ---------- ---------a ----------
1.msf{PS1a}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
1.msf{PS6a}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
1.msf{PS1b}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
1.msf{PS6b}     -----t---- g-----g-c- c------a-- --gt------ -----c--.-
```

```
                51                                                    100
1.msf{PS3}   -----t---- --ct--g-cg t------a-- --gt------ -----c--.-
1.msf{PS4}   -----t---- --ct--g-cg t------a-- --gt------ -----c--.-
1.msf{PS2}   -----t---- ----c-gatg t--------- --a-t--t-- ---a-t--.-
1.msf{PS5}   -----t---- ----c-gatg t------a-- --g-t----- ---a-t--.-
1.msf{Tom}   ----c----- tgt-.a--cg --t-gt-gct g----t-gaa a-----ca--
1.msf{Der}   -----t---a t--acct-a- ------c--- --gtt-c--a ---a-c--.-
 Consensus   ATTATCGAAT AAACTTAGGT GGGTTGTTGC TGTCCCTCTC GGGGGAATTG

               101                                                    150
1.msf{PA7a}  -----a---- ac---t---- ------.--- --------a- a---------
1.msf{PA7b}  -----a---- ac---t---- ------.--- --------a- a---------
1.msf{PA7c}  -----a---- ac---t---- ------.--- --------a- a---------
1.msf{PA7d}  -----a---- ac---t---- ------.--- --------a- a---------
 1.msf{PA1}  ---------- ac---t---- ------.--- --------a- a---------
 1.msf{PA5}  ---------- ac---t---- ------.--- --------a- a---------
 1.msf{PA2}  ---------- ac---a---- ------.--- --------a- a---------
 1.msf{PA6}  ---------- ac---a---- ------.--- --------a- a---------
 1.msf{PA3}  ---------- ac-c-t---- ------.--- --------a- a---------
 1.msf{PA4}  ---------- ac---t---- ------.--- --------a- a---------
1.msf{PS1a}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
1.msf{PS6a}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
1.msf{PS1b}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
1.msf{PS6b}  ---t----g- gt-a-----a -c-c--.--- --------ct tt-----ac-
 1.msf{PS3}  ---t----.c gt-a-----a -a-c--.--- --------ct tt-----acg
 1.msf{PS4}  ---t----.c gt-a-----a -a-c--.--- --------ct tt-----acg
 1.msf{PS2}  --------.c gt-a-----a -a-c--.--- --------ct tt-----ac-
 1.msf{PS5}  --------.c gt-a-----a -a----.--- --------ct tt-----ac-
 1.msf{Tom}  -------tc- gtt-a-acat ccac--a--- --------c- t------t--
 1.msf{Der}  -----a-.-- gt-a-----a -a-c--.--- --------ct tt-----ac-
 Consensus   TGCACGCCTT --CTTCTTTG TTTTTC-CAC CTGTGCAC-C -CTGTAGGTC
```

```
                151                                                   200
1.msf{PA7a}  --a------- g-----g--g -tt-tg-a-c -tcccc---- acac------
1.msf{PA7b}  --a------- g-----g--g -tt-tg-a-c -tcccc---- acac------
1.msf{PA7c}  --a------- g--.--g--g -tt-tg-a-c -tcccc---- acac------
1.msf{PA7d}  --a------- g--.--g--g -tt-tg-a-c -tcccc---- acac------
 1.msf{PA1}  -...------ a--------c a-----ct-- -......... ...g------
 1.msf{PA5}  -...------ a--------c a-----ct-- -......... ...g------
 1.msf{PA2}  -...----t- g--------c a-----ct-- -......... ...g------
 1.msf{PA6}  -...----t- g--------c a-----ct-- -......... ...g------
 1.msf{PA3}  -...----t- g--------c a-----ct-- -......... ...g------
 1.msf{PA4}  -...-a---- g--------c a-----ctg- t......... ...g------
1.msf{PS1a}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
1.msf{PS6a}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
1.msf{PS1b}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
1.msf{PS6b}  ----tt--tt a-ctttcc-a -gaaa--cgg t----ag--t t-c---caca
 1.msf{PS3}  -t--aact-- at---aga-g ----tg-cct t----t---- g-a----tc-
 1.msf{PS4}  -t-gaact-- at---aga-g ----tg-cct t----t---- g-t----tc-
 1.msf{PS2}  -----att-- ag--ta.... .......... .......atc tct---cttt
 1.msf{PS5}  ca-.-cgatt tg--ca.... .......... .......atc aag-ca-tt-
 1.msf{Tom}  -.at--tct- gg--accttg tct----gcc gtggttct-c gtc---a--c
 1.msf{Der}  ct-gat-tct ttct-a-t-c ctacg-aatt -aggtt---g gat-gacttt
  Consensus  TGGAGGAGGG -AAGGGAGG- GACTCCT-AA CCAAG-TGAA -G-TTTTCAG

                201                                                   250
1.msf{PA7a}  ---------. .......... .........a ---.----c- -.----t--
1.msf{PA7b}  --------. .......... .........a ---.----c- --.---t--
1.msf{PA7c}  ---------. .......... .........a ---.----c- --.----t--
1.msf{PA7d}  ---------. .......... .........a ---.----c- --..---t--
 1.msf{PA1}  ---------. .......... .......... --a.c---c- --..---t--
 1.msf{PA5}  ---------. .......... .......... --a.c---c- --..---t--
 1.msf{PA2}  ---------. .......... .......... a--.----c- --..---t--
 1.msf{PA6}  ---------. .......... .......... a--.----c- --..---t--
 1.msf{PA3}  ---------. .......... .......... c--.----c- --..---t--
 1.msf{PA4}  ---------. .......... .........- ---.----c- --..---t--
1.msf{PS1a}  ag-tctcc-- ---------- ----g----- g--.-t--t- --.--c-a-a
1.msf{PS6a}  ag-tctcc-- ---------- ----g----- g--.-t--t- --.--c-a-a
1.msf{PS1b}  ag-tctcc-- --.------- ----g----- g--.-t--t- --.--c-a-a
```

```
                201                                                   250
1.msf{PS6b}  ag-tctcc-- --.------- ----g----- g--.-t--t- --.--c-a-a
 1.msf{PS3}  -c..... ..... ...--t---- g--.-t--t- --.--c-a-a
 1.msf{PS4}  -c..... ..... ...--t---- g--.-t--t- --.--c-a-a
 1.msf{PS2}  -g..... ..... ...---tatg ---.at--t- --.--c-ata
 1.msf{PS5}  -g..... ..... ...c-tacga ---.at--t- --.--c-a.a
 1.msf{Tom}  aca-ac---a a----g-ctt aag-aatgta -gc.ag-gtc --.a-g-a--
 1.msf{Der}  -ctg-c-t-c ctt-ca-tt- -ag---tatg ---c-t--t- --c--c-a--
  Consensus  GTCCTATGTT GCAAATTAAC CCAGCCCTAC TTT-TACA-A TA-CCAC-AT

                251                                                   300
1.msf{PA7a}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
1.msf{PA7b}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
1.msf{PA7c}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
1.msf{PA7d}  ---------- -g-a-g---- ---------c c-ag-.c--- ----------
 1.msf{PA1}  ---------- -g-a-g---- --------c- --------.. ------a---
 1.msf{PA5}  ---------- -g-a-g---- --------c- --------.. ------a---
 1.msf{PA2}  ---------- -g-a-g---- --------c- --------.. ----------
 1.msf{PA6}  ---------- -g-a-g---- --------c- --------.. ----------
 1.msf{PA3}  ---------- -g-a-g---- --------c- --------.. ----------
 1.msf{PA4}  ---------- -g-a-g---- --------c- ---------a ----------
1.msf{PS1a}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
1.msf{PS6a}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
1.msf{PS1b}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
1.msf{PS6b}  g--tgta-ca ga-tgt---a t--t---c-- gt------.. ----------
 1.msf{PS3}  g--tgta-ca ga-tgt-.-- ttat------ -t------.. ----------
 1.msf{PS4}  g--tgta-ca ga-tgt-.-- ttat------ -t------.. ----------
 1.msf{PS2}  gt-tgta-ca ga-tgt-.-- ---------c gt------.. ------a---
 1.msf{PS5}  gt-tgtatta ga-tgt-.-- ---------c gt------.. ----at----
 1.msf{Tom}  ac--...t-- -actttcag- --c--atc-c tt-g--c-cg c-t-g--ga-
 1.msf{Der}  gt-tgt--ca ga-tgt-a-a --------c-- tgtg----.. .--acc----
  Consensus  TAAAAGTAAC A-A-C-ATTC AATGGGCTTT AAGCCTATAT AAACTATATA
```

```
                301                                                  350
1.msf{PA7a}  ---------- ---------- ---------- ---------- ----------
1.msf{PA7b}  ---------- ---------- ---------- ---------- ----------
1.msf{PA7c}  ---------- ---------- ---------- ---------- ----------
1.msf{PA7d}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA1}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA5}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA2}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA6}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA3}  ---------- ---------- ---------- ---------- ----------
 1.msf{PA4}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1a}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6a}  ---------- ---------- ---------- ---------- ----------
1.msf{PS1b}  ---------- ---------- ---------- ---------- ----------
1.msf{PS6b}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS3}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS4}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS2}  ---------- ---------- ---------- ---------- ----------
 1.msf{PS5}  ---------- ---------- ---------- ---------- ----------
 1.msf{Tom}  g---gcagc~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 1.msf{Der}  ---------- ---------- ---------- ---------- ----------
  Consensus  CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
```

Figure 8-2 The ITS-1 sequence alignment by Pretty program of GCG software. The left column is sample name in the file name: 1.msf. Der is *Dermocybe olivaceopicta*. Tom is *Tomentella sublilacina*. Consensus sequences were automatically produced by the same program. Symbol "." is deletion, "-"is the same sequence with the consensus sequence. Symbol "~" is not shown in the EMBL database. There are eight blocked sequences. They were named ITS1 (25-43), PS (120-142), PA1 (242-268), PA2 (203-252), PA3 (255-278), PA4 (245-266), PA5 (191-209) and ITS2 (331-350). The numbers in parentheses are nucleotide positions. ITS2 was used to be a 3' end primer and the rest were 5' end primers in PCR amplification. Primer ITS1 and ITS2 were used

to amplify a common product. Primer PS and PA1, PA2, PA3, PA4, PA5 were used to amplify specific fragments of *Psilocybe* and *Panaeolus* samples with primer ITS2 described in the text.

Using the data from the position of the primer sites it is possible to predict the size of the PCR fragments from each of the fungi samples. The predicted sizes are shown in Table 8-1. This table illustrates the larger size range of members of the *Psilocybe* genus compared to the *Panaeolus* genus.

For the members of the *Panaeolus* genus, the genus specific primers are predicted to produce products of similar size for all the samples tested. There is greater variation in the size of the predicted genus specific primers for the members of the *Psilocybe* genus included in this study.

Table 8-1 The sizes of the DNA fragments amplified from the ITS-1 DNA locus using the common primers. These size fragments are as predicted from the sequence information shown in Figure 8-1.

Sample	Common fragment	Sample	Common fragment
PS1-1	319/320 bp	PA1-1	283 bp
PS1-2	319/320 bp	PA1-2	283 bp
PS1-3	319/320 bp	PA1-3	283 bp
PS1-4	319/320 bp	PA1-4	283 bp
PS2	276 bp	PA2	283 bp
PS3	297 bp	PA3	284 bp
PS4	297 bp	PA4	286 bp
PS5	274 bp	PA5	283 bp
PS6-1	319/320 bp	PA6	283 bp
PS6-2	319/320 bp	PA7	299/300/301bp

Table 8-2 The sizes of amplification products from the ITS-1 gene locus using the genus specific primers. The sizes are predicted using the data displayed in Figure 8-1.

Sample	Primer PS+ITS2	Sample	Primer PA1+ITS2	Primer PA2+ITS2	Primer PA3+ITS2	Primer PA4+ITS2	Primer PA5+ITS2
PS1-1	225/226	PA1-1	104	121	94	132	133
PS1-2	225/226	PA1-2	104	121	94	132	133
PS1-3	225/226	PA1-3	104	121	94	132	133
PS1-4	225/226	PA1-4	104	121	94	132	133
PS2	183	PA2	104	121	94	132	133
PS3	204	PA3	104	121	94	132	133
PS4	204	PA4	106	124	96	135	136
PS5	181	PA5	104	121	94	132	133
PS6-1	225/226	PA6	104	121	94	132	133
PS6-2	225/226	PA7	106/107	124/125	95	135	136/137

It is necessary to test primers in amplifications to ensure that the primers will bind to their complementary sequence at the same temperature used for all the primers in the same reaction. The conditions suitable for all the primers used in a multiplex amplification need to be determined, with the temperature of binding being the most important parameter.

The PCR products will be separated by single-strand conformation polymorphism (SSCP). The SSCP method can detect all the possible polymorphic sequences, even single base difference, between two PCR products [99-102].

8.2 Materials and Methods

8.2.1. *Psilocybe* and *Panaeolus* Mushrooms

DNA samples of mushrooms extracted in section 3.4 were used in this study.

8.2.2. PCR Amplification

The PCR was designed to amplify simultaneously one common product of ITS-1 DNA in RNA gene and one DNA fragment specific on *Psilocybe* or *Panaeolus*, respectively. For the common product, primers ITS1 and ITS2 [15] were used. Primer ITS2 is located on the 3' end of the nuclear small subunit rDNA. The primer sequences and names used as shown in the table below (Table 8-3).

Table 8-3 Sequences of specific primers for PCR amplifications of genus *Psilocybe* and *Panaeolus*.

Primer name	Sequences(5'→3')	Positions in ITS-1 sequence of Figure 8-2
ITS1	TCCGTAGGTGAACCTGCGG	25-43
ITS2	GCTGCGTTCTTCATCGATGC	331-350
PS	ATCTCTCCACCTGTGCACCTTT	120-142
PA1	ACCACTATTAAAAGTAACAGAACGAT	242-268
PA2	CCTATGTTATTTTACACATACACTATTA	203-252
PA3	AGTAACAGAACGATTCAATGGGCT	255-278
PA4	CACTATTAAAAGTAACAGAACG	245-266
PA5	ACACTTTCAGGTCCTATGT	191-209

PCR amplification was performed in 50 μl of reaction mixture containing about 15ng extracted genomic DNA, reaction buffer (10mM Tris-HCl, pH8.8 at 25 °C, 1.5mM MgCl2, 1.5mM KCl, 0.1% Triton X-100), 200μM of dNTPs, 1 unit of

DyDNAyme™ II DNA polymerase (Finnzymes Oy, Finland) and 0.5μM each of four primers including primers ITS1 and ITS2 for common product, and one of each genus specific primers. All the PCR amplifications were carried out using the same program of 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min in a DNA thermal cycler 480 (Perkin-Elmer, NJ, USA).

PCR products were electrophoresed on a 3% agarose gel (FMC BioProducts, Rockland, ME, USA) with 0.5μg/ml ethidium bromide in TBE buffer (0.89M Tris-HCl, 0.889M borate, 0.002M EDTA, pH8.0) at 100V for 30 min. PCR products were visualized by 312nm UV light and photographed using Polaroid 667 film.

8.2.3. DNA Size Determination

In order to determine the size of amplified DNA, Gene Scan analysis by fluorescent detection system was used. Primer ITS1 was labeled with a dye of FAM (PE Applied Biosystems), Primer PA5 with TET dye, and PS1 with HEX dye. The same PCR amplification in section 8.2.2 was used to produce fluorescent PCR products. PCR products were analyzed by PE Applied Biosystems PRISM 310 Genetic Analyzer. The data were collected and analyzed by the GeneScan computer software.

8.2.4. SSCP Analysis

PCR products obtained by common primers were separated using a polyacrylamide gel to reveal their features of single strand conformation polymorphism (SSCP). The electrophoresis and silver staining protocol were similar to section 4.2.2 and 4.2.3 except the loading products were denatured for 5 minutes and instantly cooled on ice before loading.

8.3 Results and Discussion

This study used multiplex PCR to amplify a common product and a genus specific product in one PCR tube. The primer set including 4 primers used in the same reaction. The common product must be amplified to show the success of PCR. These primers were designed to work on all fungi used in this study. Primer pair ITS1 and ITS2 produced the product expected in all reactions performed (see Figures 8-3 to 8-4).

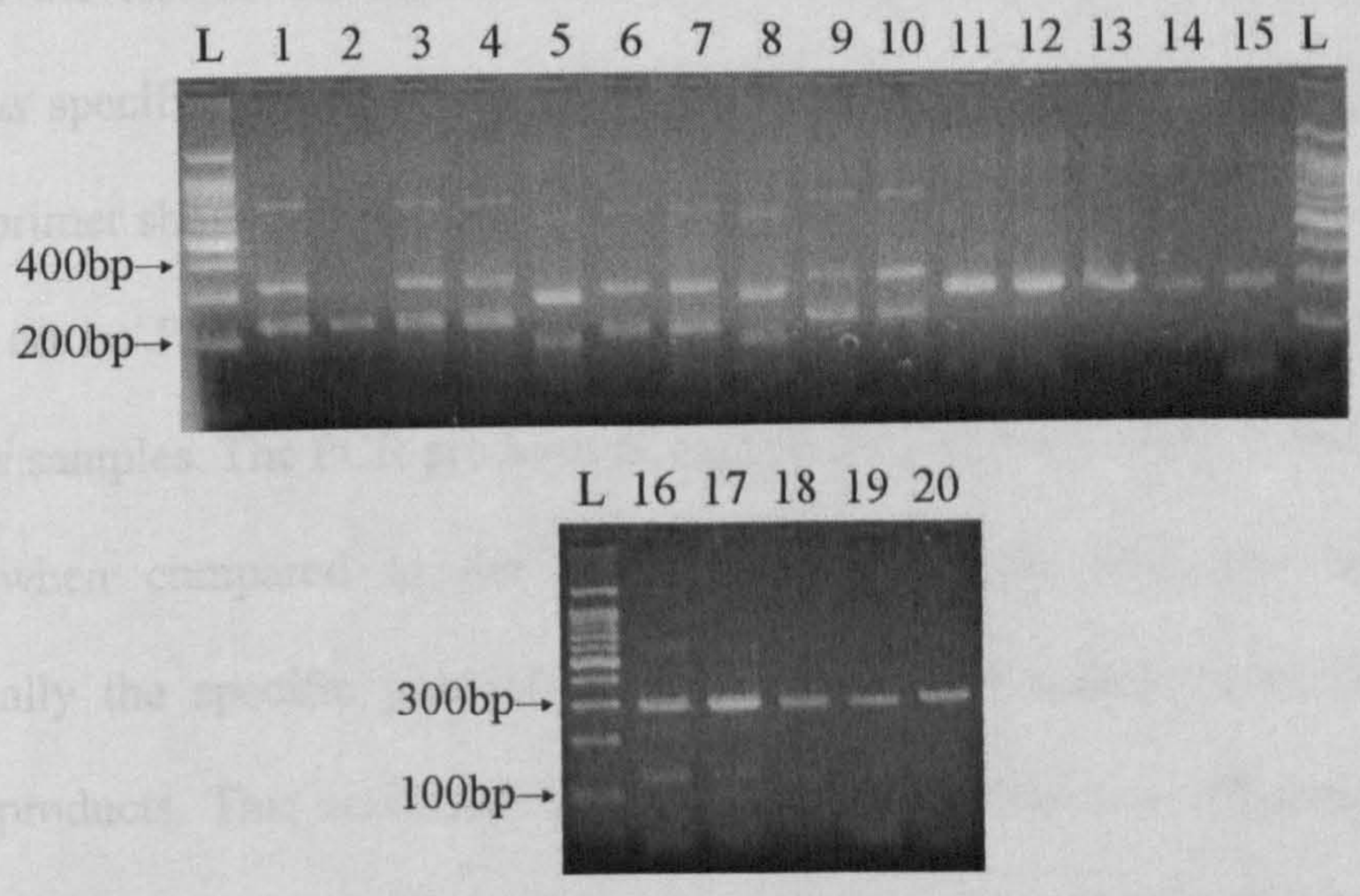


Figure 8-3 Electrophoresis results of PCR products amplified by primer ITS1, PS, PA2 and ITS2. PCR products were run on 3% agarose gel. Lane L is 100 bp ladder. Lanes 1-20 are sample PS1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6-1, 6-2, PA1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6 and 7. PCR products around 300 bp are common products and those around 200 bp and 100 bp are *Psilocybe* and *Panaeolus* specific products, respectively.

The genus specific primers were designed to bind to sequences present only in

members of one of these two genera studied. The genus specific fragment was also amplified from the fungal samples, although with varying degrees of success.

The results of the amplification using primer set ITS1, PS, PA1 and ITS2 are shown in Figure 8-3. Only common products and *Psilocybe* specific fragments from *Psilocybe* samples were amplified. The products of primer PA1 and ITS2 was not observed. The production of a common product from the *Panaeolus* samples indicates that the amplifications were performed correctly and that there were no inhibitors present in the reaction. The conditions required by the common primers were not suitable for this set of genus specific primers.

In the test of the second primer set, common products and *Psilocybe* and *Panaeolus* specific products were amplified simultaneously (Figure 8-3). The genus specific primer showed a high degree of specificity to anneal to their target sequences, i.e. there are no *Psilocybe* product in *Panaeolus* samples and no *Panaeolus* product in *Psilocybe* samples. The PCR products of sample PA1-1, PA1-2, PA1-3 and PA1-4 were weaker when compared to the amplification products from the rest samples. Additionally the specific products of *Panaeolus* were weaker than the *Psilocybe* specific products. This was most likely caused by the different efficiency of primer annealing between primer PS and PA2 to their target sequences and some mismatch nucleotides in some *Panaeolus* species. The third and fourth primer set caused non-specific products in the *Panaeolus* samples. The temperature used was most likely too low, permitting mismatches to form between the primer sequence and non-complementary sequences.

The fifth primer set produced PCR of even amplification and with no detectable non-specific amplification. Figure 8-4 shows the results of *Psilocybe* samples amplified by primer ITS1, PS, PA5 and ITS2. The size of common products is approximately around 300 bp and the *Psilocybe* specific fragments is around 200 bp. Figure 8-5 shows

the results of the *Panaeolus* samples amplified by the same primer set. The specific products, however, are different from Figure 8-4. Except the common products about 300 bp, the *Panaeolus* specific fragments were approximately around 100 bp. The precise sizes are listed in Tables 8-1 and 8-2. These sizes were confirmed by dye-labeled PCR products, which were analyzed by fluorescent detection method. An example of sample PA1-1 is shown in Figure 8-6. Another example for multiple fragments of sample PA7 is shown in Figure 8-7, 8-8 and 8-9. These results confirmed the sequencing results in chapter 7 that *Psilocybe semilanceata* (sample PS1-1, 1-2, 1-3, 1-4, 6-1 and 6-2) had two alleles in ITS-1 and *Panaeolus subbalteatus* (sample PA7) had four alleles. For *Panaeolus subbalteatus* in Figure 8-9, since two alleles have same size (see Figure 7-18), therefore only three fragments were found.

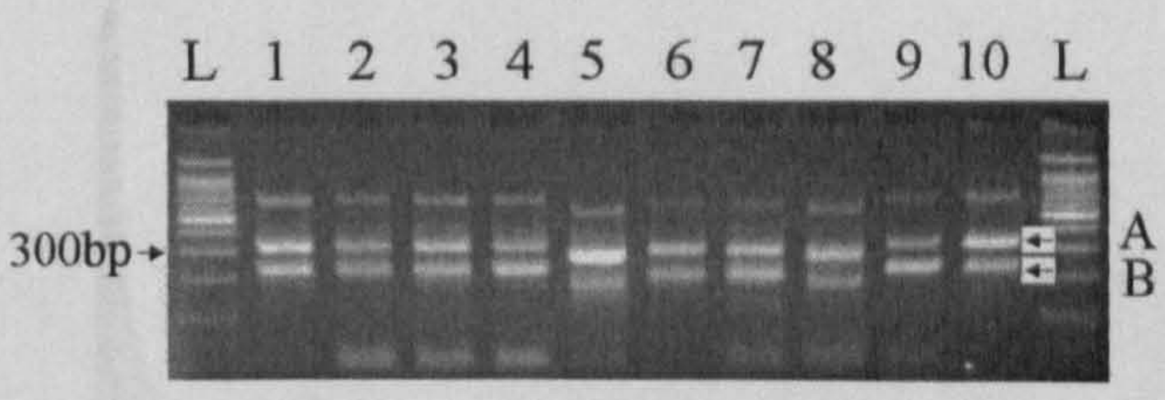


Figure 8-4 Electrophoresis results of PCR products amplified by primer ITS1, PS, PA5 and ITS2. PCR products were run on 3% agarose gel. Lane L is 100 bp ladder. Lanes 1-10 are sample PS1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6-1 and 6-2. A is common products and B is Psilocybe specific products.

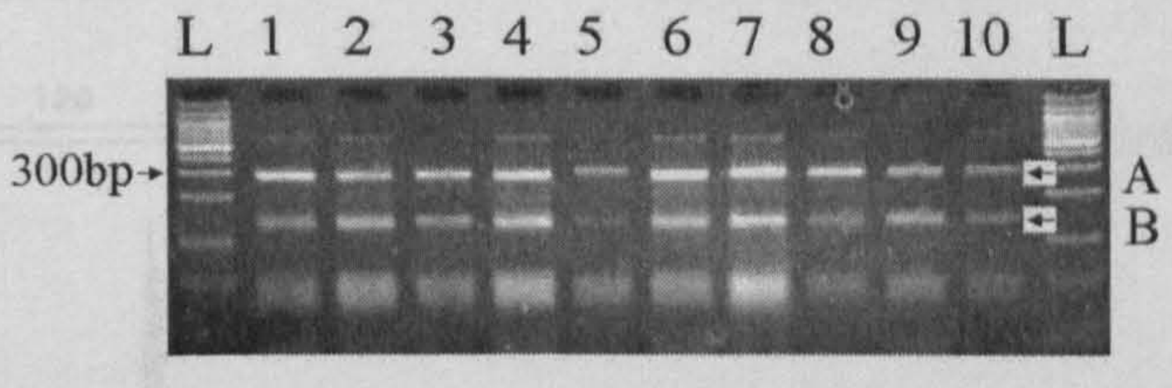
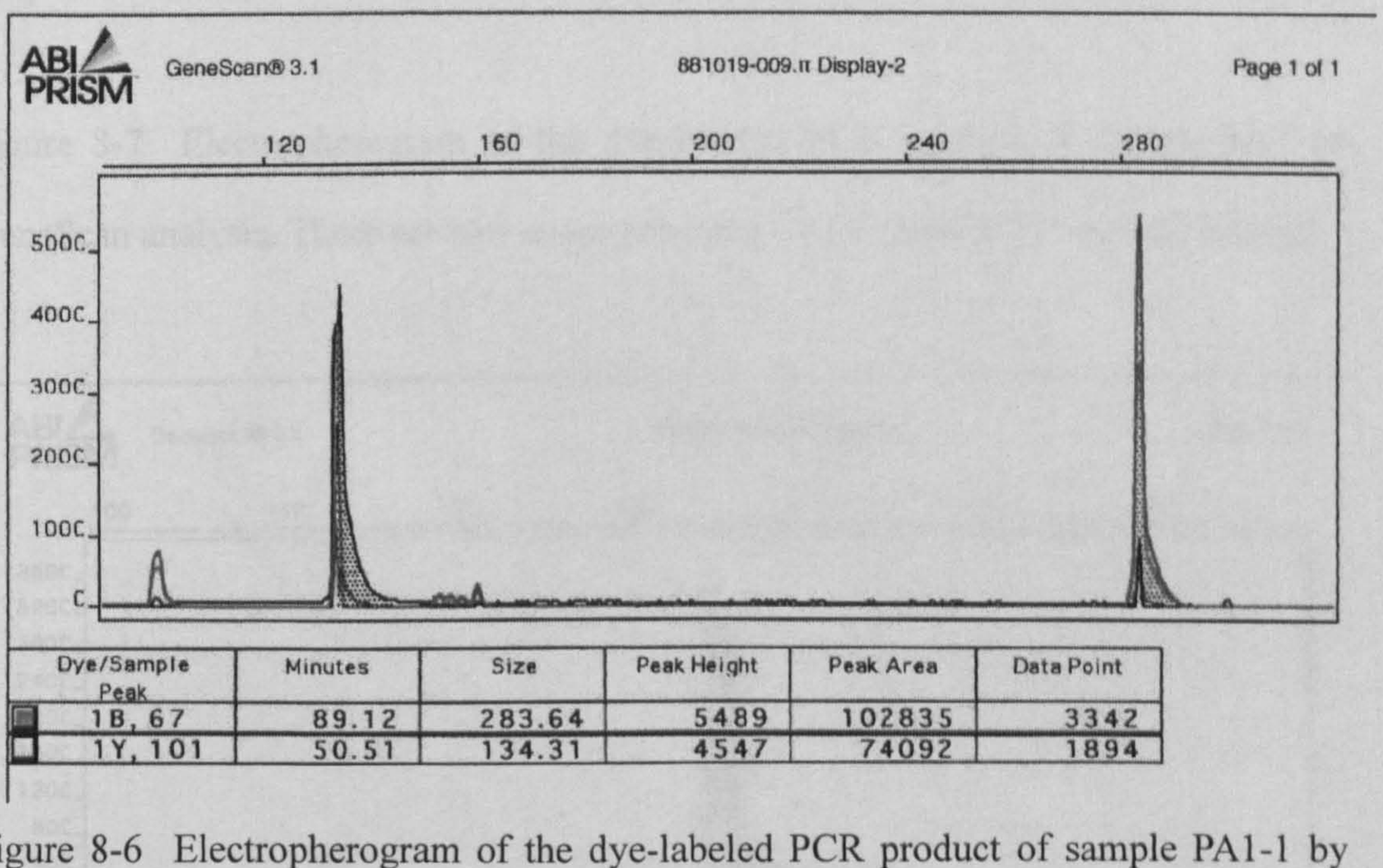
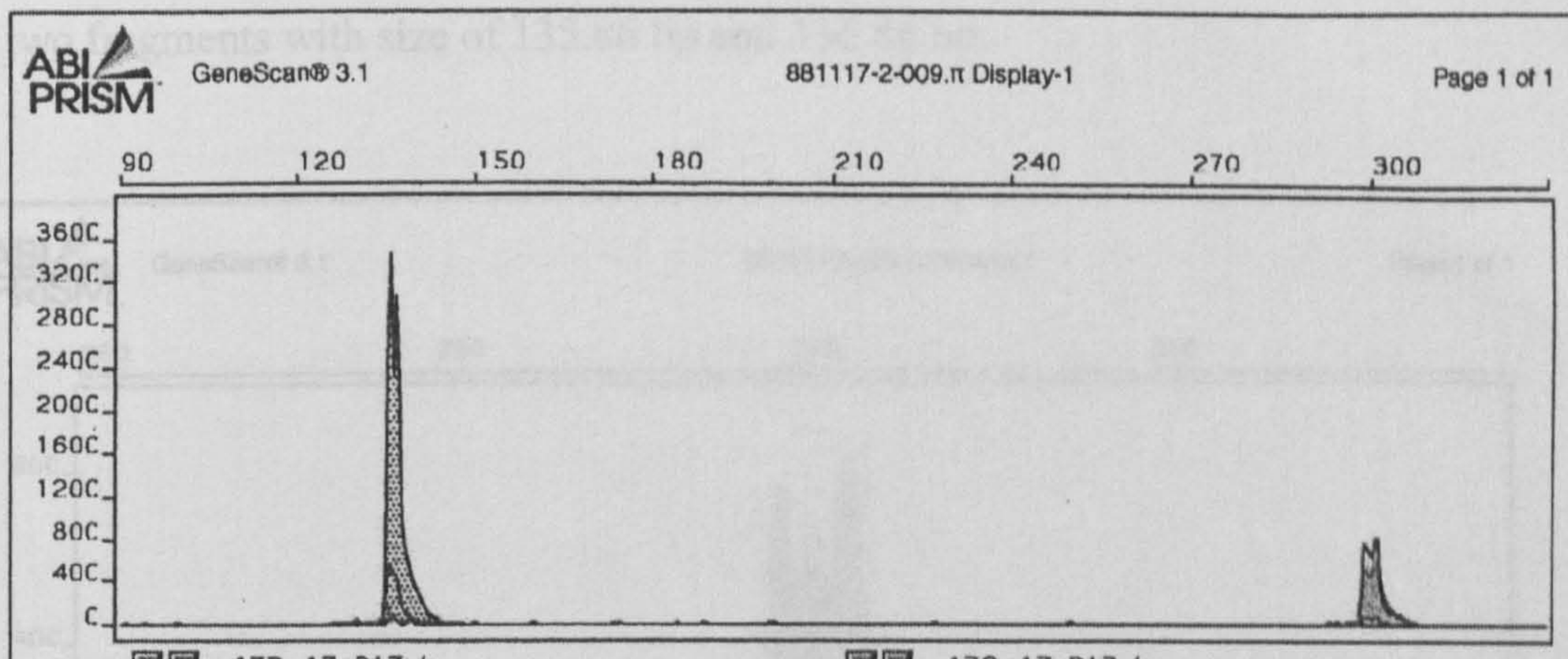


Figure 8-5 Electrophoresis results of PCR products amplified by primer ITS1, PS, PA5 and ITS2. PCR products were run on 3% agarose gel. Lane L is 100 bp ladder. Lanes 1-10 are sample PA1-1, 1-2, 1-3, 1-4, 2, 3, 4, 5, 6 and 7. A is common products and B is Panaeolus specific products.



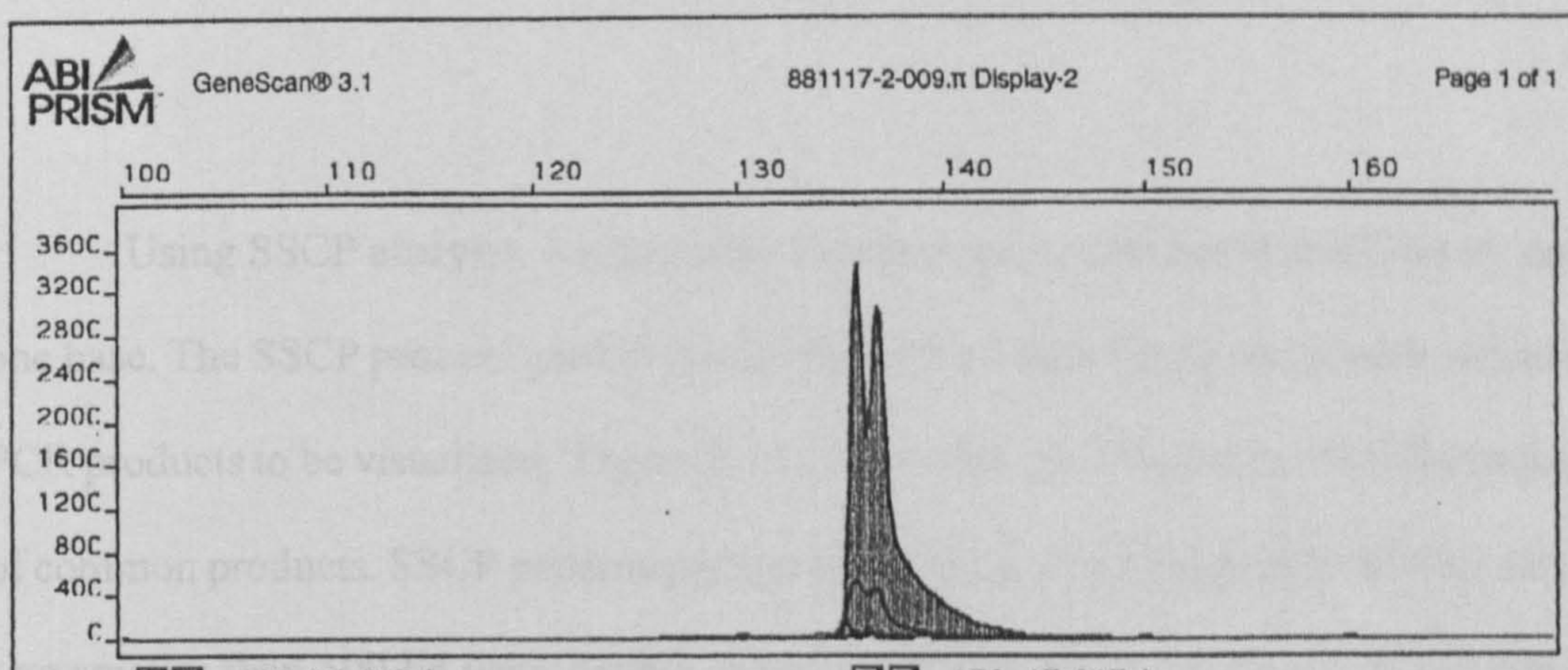
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
1B, 67	89.12	283.64	5489	102835	3342
1Y, 101	50.51	134.31	4547	74092	1894

Figure 8-6 Electropherogram of the dye-labeled PCR product of sample PA1-1 by GeneScan analysis.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
17B, 27	94.03	299.62	777	6913	3526
17B, 28	94.29	300.58	680	5785	3536
17B, 29	94.56	301.54	845	16347	3546
17Y, 27	50.93	135.85	3508	24876	1910
17Y, 28	51.17	136.86	3089	41839	1919

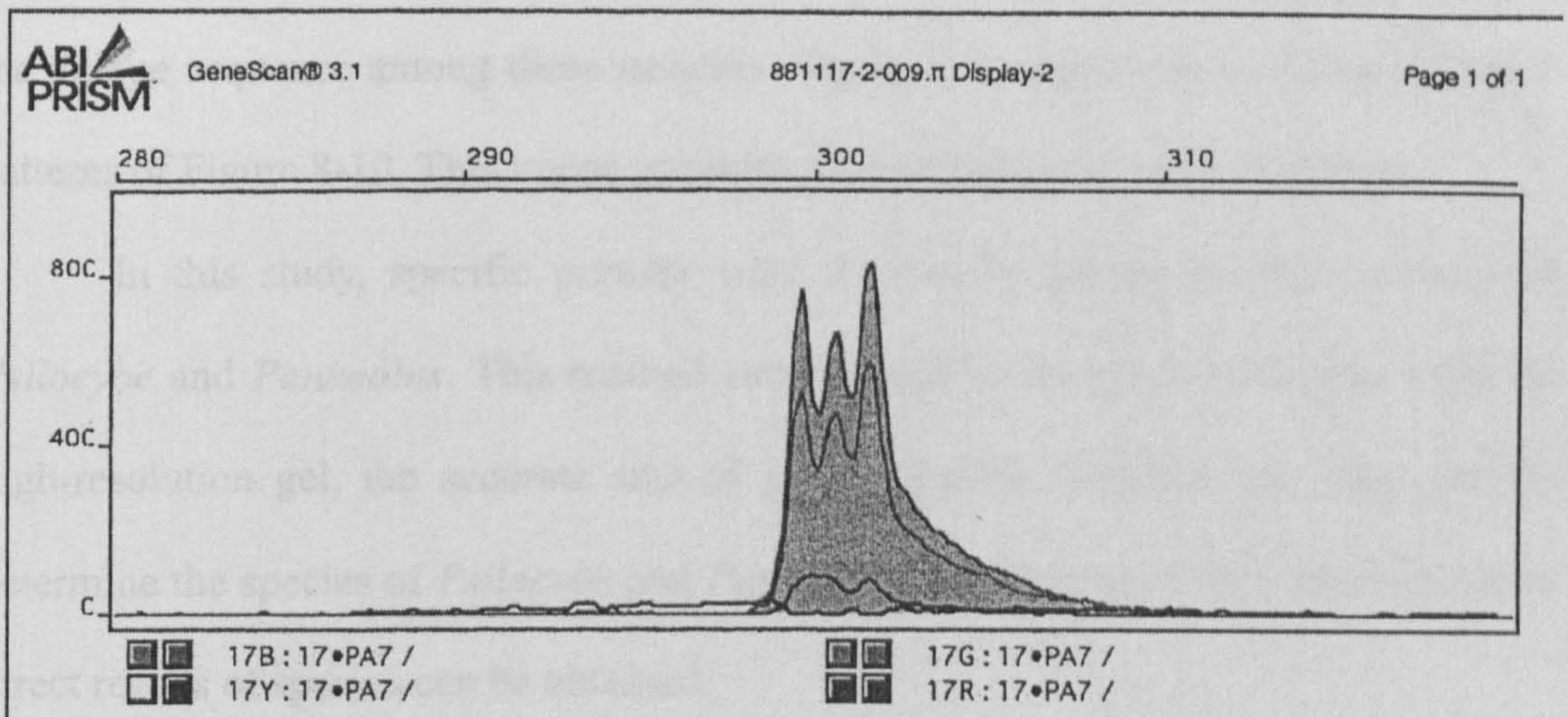
Figure 8-7 Electropherogram of the dye-labeled PCR product of sample PA7 by GeneScan analysis. There are two major products near size scale 135 bp and 300 bp.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
17Y, 27	50.93	135.85	3508	24876	1910
17Y, 28	51.17	136.86	3089	41839	1919

Figure 8-8 Electropherogram of the close-up image in 135 bp area of Figure 8-5. There

are two fragments with size of 135.86 bp and 136.86 bp.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
17B, 27	94.03	299.62	777	6913	3526
17B, 28	94.29	300.58	680	5785	3536
17B, 29	94.56	301.54	845	16347	3546

Figure 8-9 Electropherogram of the close-up image in 300 bp area of Figure 8-5. There are three fragments with size of 299.62 bp, 300.58 bp and 301.54 bp.

Using SSCP analysis, it is possible to reveal polymorphism that differs by only one base. The SSCP process used in this study allowed both single and double stranded PCR products to be visualized. Figure 8-10 showed the SSCP patterns of PCR products of common products. SSCP patterns present in the gel at a size larger than 800 bp. DNA size smaller than 500 bp were double strand DNA fragments in this gel. In the SSCP patterns of common products, lane 1-5 were samples from different species of genus *Psilocybe* that showed unique patterns. These unique SSCP patterns reflect the sequence difference and are consistent with the sequencing results of chapter 7 and shown in Figure 8-2. Lanes 6-12 are samples from genus *Panaeolus*. Lanes 6 and 10 share the same pattern, and the same between lane 7 and lane 11. From Figure 8-2,

sequences in this DNA fragments between samples in lane 6 and 10, 7 and 11 are identical. Except this, the SSCP patterns of lanes 8, 9 and 12 are unique which represent the unique sequence among these samples. Figure 8-11 shows the close-up of SSCP patterns of Figure 8-10. This image provides more details of the SSCP patterns.

In this study, specific primers were to amplify genus specific products of *Psilocybe* and *Panaeolus*. This method can be used in the quick screening work. In high-resolution gel, the accurate size of genus specific fragment can also used to determine the species of *Psilocybe* and *Panaeolus*. By using the SSCP analysis, more direct results of species can be obtained.

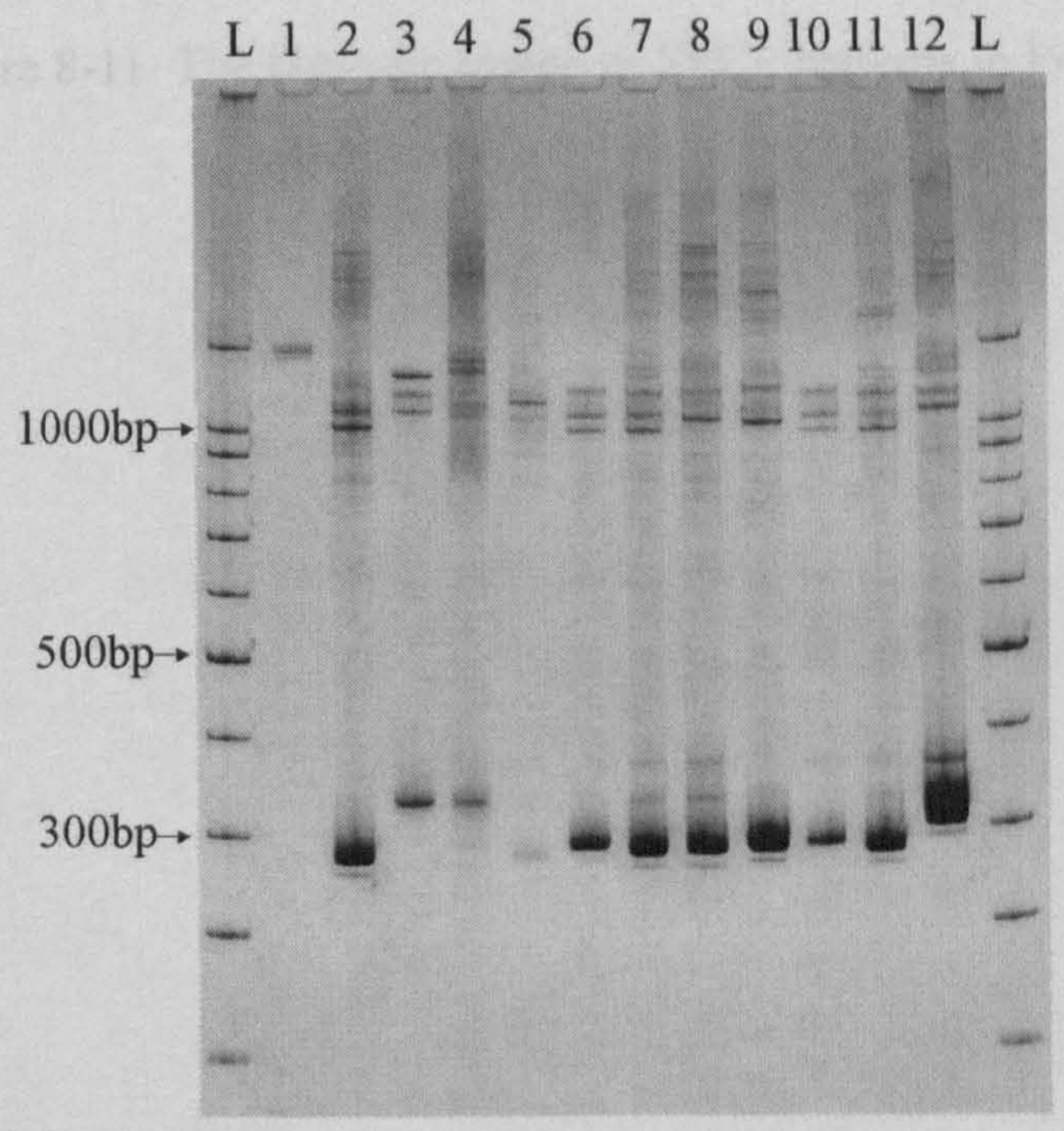


Figure 8-10 SSCP patterns of ITS-1 DNA fragments separated by 12.5% polyacrylamide gel electrophoresis. L is 100 bp ladder. Lanes 1-12 are common product amplified by primer ITS1 and ITS2 of sample PS1-1, PS2, PS3, PS4, PS5, PA1-1, PA2, PA3, PA4, PA5, PA6 and PA7.

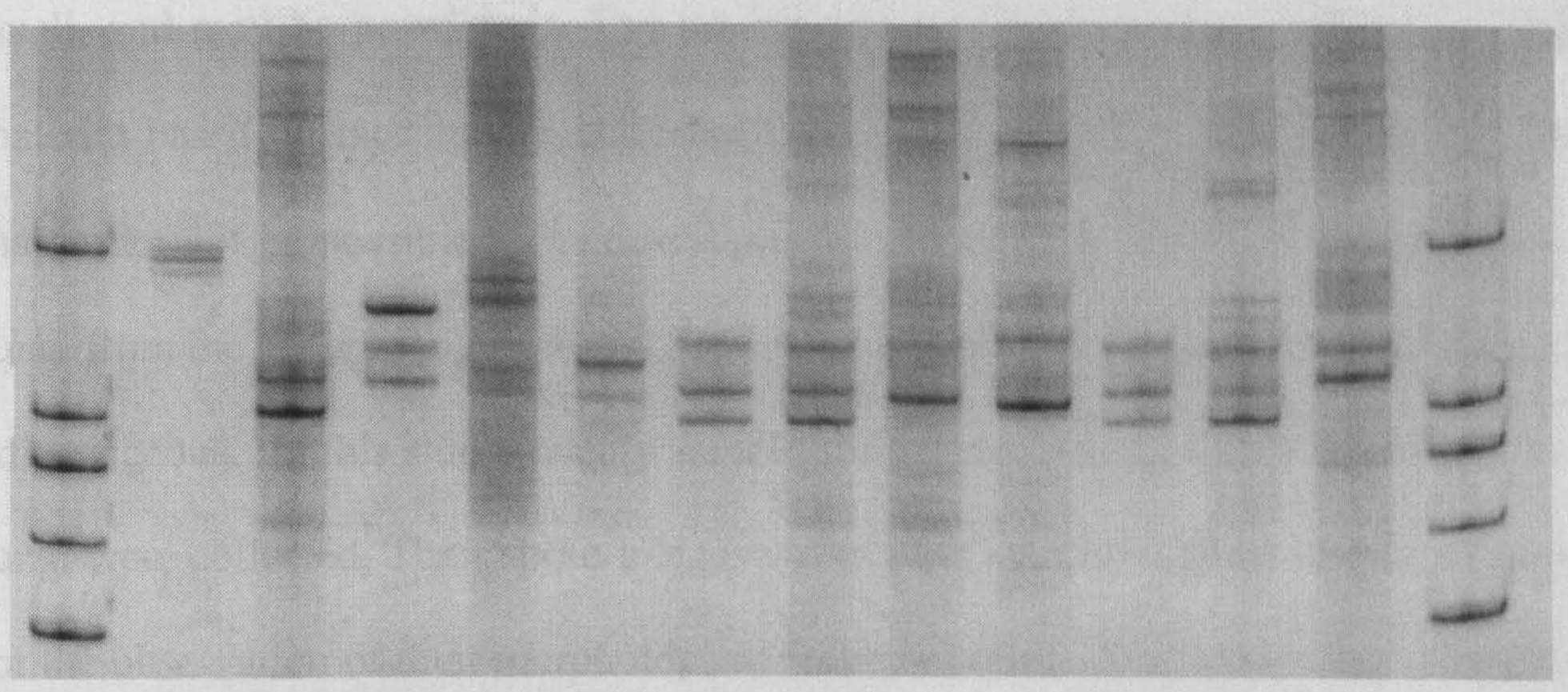


Figure 8-11 The close-up image of SSCP patterns in Figure 8-10

9 CONCLUSION AND GENERAL DISCUSSION

Fungal samples commonly encountered in forensic investigations are often small, and may be powdered. The unambiguous identification of controlled fungal material based on morphological characteristic such as gill structure or spore shape may often not be possible. The development of a test that identifies the presence of fungi from the genera *Psilocybe* and *Panaeolus* based on DNA polymorphisms may be advantageous. In this study, twenty samples of the two genera with eleven species of fungi were collected. Their spore images were taken by scanning electron microscope for the observation of their morphological features before DNA extraction. The DNA extracted from these samples was degraded and of low molecular weight. The samples were collected in the early 1980s as part of a PhD study by Professor Pierre Margot. The methods of random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) were used to establish DNA band patterns of the whole genome. The sequences of the nuclear small subunit ribosomal DNA and the internal transcribed spacer 1 DNA (ITS-1) in the nuclear ribosomal RNA gene were sequenced to develop a simple method in the identification of these fungal species.

9.1 Spore Image

The spore images of members of the genera *Psilocybe* and *Panaeolus* in a dry state taken with a scanning electron microscope were all visually similar. They are all concave oval in appearance making it difficult to determine the species or genus of the samples in this study. According to 'The Genus Psilocybe' [103], the spore form of *Psilocybe cyanescens* is elongate-ellipsoid in both the face and side view or slightly broader in face view. The spore form of *Psilocybe eucalypta* is sub-ellipsoid both in

face and side view. The spore form of *Psilocybe semilanceata* is sub-ellipsoid or ellipsoid both in face and side view, sometimes slightly lemon-shaped. These slight differences among species sometimes can not be recorded correctly in the species identification. It is possible, however, that the spore shape of samples in this study was different from those of other fungus families [104]. Figure 9-1 showed an example of spore image of mushroom *Volvariella volvacea* taken in this study. This image shows much variation in the shape and surface structure of different spores.

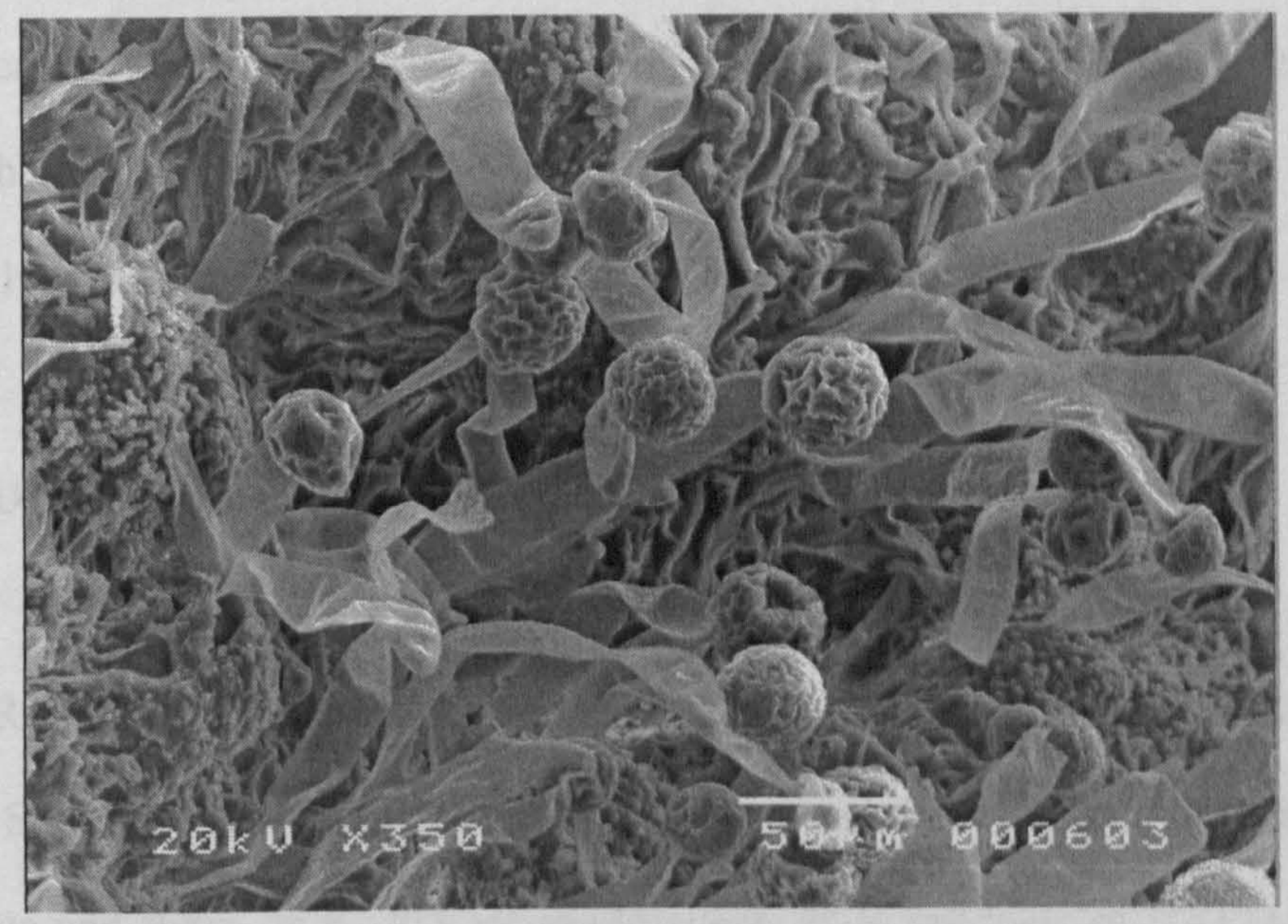


Figure 9-1 Spore image of *Volvariella volvacea*.

If the fungal sample is sufficient to perform a morphological examination, it is possible to provide sufficient information for species identification. If this is not the case, further examination by DNA analysis is important to obtain a distinct conclusion.

9.2 DNA Extraction

The samples analysed in this study dated from 1981, and although stored

desiccated much DNA degradation had occurred. In this study, the extracted DNA fragments were distributed predominantly smaller than about 4 kb in size. This indicated that DNA was seriously degraded after long time storage despite the intact appearance of the fungi. In spite of the degradation of DNA, the analyses of RAPD, AFLP and RNA gene were still performed successfully by the PCR method. Psilocybin-containing mushrooms are usually disguised as legal food products [3] which tends to destroy DNA if not kept in a dry state. The condition of sample preservation will deeply affect the yield of DNA extraction and its quality. Fresh sample can be kept cool, and not necessarily frozen, for a short period of time; such as several hours or a day. For long period storage, samples should be kept in freeze in –80°C or lyophilized.

9.3 DNA Profiling

RAPD and AFLP have been useful methods for species identification [50-57, 59-80]. Since the products of the AFLP method are selective only for the restricted fragments and those of RAPD are randomized, PCR products of RAPD can not be designated to a particular allele and can be affected by DNA quality and PCR conditions. The reproducibility of RAPD is questionable, especially for the forensic samples, which are mostly degraded or at trace levels. RAPD products are usually separated by agarose gel electrophoresis. By scoring for the presence or absence of a band in a particular bin, RAPD can then be transferred to digital code allowing a convenient form for comparison.

In order to increase the resolution of PCR product separation, this study used 12.5% PAGE for PCR product separation. The Image master 1D (Pharmacia Biotech) was used to obtain reproducible results. The band sizing was using cubic method to

establish a calibration curve for more reliable size. A 95% confidence interval was used to transfer band sizes to a bin. Finally, the score or code of RAPD or AFLP fingerprints was established. The genetic similarities between each sample were calculated by the method of Dice [83]. A dendrogram established by UPGMA method can give a quick image of the relationship among samples. The results of the RAPD and AFLP analysis in this study indicated that the two methods provided a potential for species identification. This was especially the case when a combination of patterns of RAPD or AFLP was used. In this study using twenty samples of the genera *Psilocybe* and *Panaeolus* fungi, samples within the same species produced band patterns of high similarity. In RAPD, since only two random primers were used, there is insufficient species specific result obtained. Some of the similarities in different species were higher than those of same species, for instance, *Panaeolus retirugis* (PA4) and *Panaeolus semiovatus* (PA5), *Panaeolus papilionaceus* (PA2) and *Panaeolus speciosus* (PA6), *Psilocybe montana* (PS5) and *Psilocybe semilanceata* (PS6). Samples of the same species, PA1 and PA5 (*Panaeolus semiovatus*), PS1 and PS6 (*Psilocybe semilanceata*), were not clustered in the same group. In the AFLP analysis, since 5 selective primer pairs were used, this method was highly species specific when the data was combined for the four selective primer pairs. In this analysis, the similarity between *Panaeolus retirugis* (PA4) and *Panaeolus semiovatus* (PA5), *Panaeolus papilionaceus* (PA2) and *Panaeolus speciosus* (PA6), was decreased. They did however cluster in the same group. This was more evident between *Psilocybe montana* (PS5) and *Psilocybe semilanceata* (PS6). The similarity was decreased largely and clustered to different group. Samples in the same species, PS1-1, PS1-2, PS1-3, PS6-1 (*Psilocybe semilanceata*), PA1-1, PA1-2, PA1-3 (*Panaeolus semiovatus*) were all clustered to the same group. According to the morphological record, sample PA1 and PA5 were the same species, however, in the results of RAPD and AFLP analysis, it appeared to be

two different species. The high genetic similarity between *Panaeolus papilionaceus* (PA2) and *Panaeolus speciosus* (PA6) was also proved in the ITS-1 analysis. The two species shared the same sequences in the ITS-1 DNA fragment. For genus specific evaluation in this system, the genetic similarity in species level is higher than in genus level. By this system, however, the AFLP patterns show neither genus specific band nor genus specific GS score range.

9.4 Analysis of Nuclear Ribosomal RNA Gene

In eukaryotes the genes for the 18S, 28S and 5.8S ribosomal RNA (rDNA) are arranged as tandem repeats. In fungi the number of rDNA repeats ranges from approximately 60 in the mushroom *Coprinus* [89] to 220 in the mould *Neurospera* [90]. These gene sequences are highly conserved, as they evolve very slowly, and are consequently used in evolutionary studies for distantly related organisms [15]. Such conserved gene sequences are unlikely to separate or distinguish more closely related genera such as *Psilocybe* and *Panaeolus*. The rDNA gene sequences are separated by the variable Internal Transcribed Spacer (ITS) and a non-transcribed intergenic spacer (IGS) [15]. Fungi have multiple copies of this rDNA gene complex, which makes this gene locus amenable to PCR, even from highly degraded samples [14,15]. The ITS region is removed after transcription and is therefore non-coding. In this study, both DNA fragments in coding and non-coding area were analyzed which were nuclear small subunit ribosomal DNA and internal transcribed spacer 1 DNA (ITS-1).

From the sequencing data of nuc-ssu-rDNA, there were 12 polymorphic nucleotides in 877 bp DNA fragment. In genus *Psilocybe*, all samples of species *Psilocybe semilanceata, Psilocybe coprophila, Psilocybe cyanescens* and *Psilocybe eucalypta* shared the same sequence. *Psilocybe montana* (PS5) had unique sequence

having 5 nucleotide substitutions and 1 deletion compared to the other members of the genus *Psilocybe*. In the genus *Panaeolus*, there were only 3 polymorphic nucleotides distributed within this genus, 2 nucleotide substitutions in *Panaeolus retirugis* and 1 in *Panaeolus subbalteatus*. *Panaeolus semiovatus, Panaeolus papilionaceus, Panaeolus reckenii* and *Panaeolus speciosus* shared the same sequence. DNA fragments in this area of each sample were homozygous despite the large numbers of repeats in the genome. Polymorphic nucleotides were not evident, which can be used as genus or species specific test. There were sequences specific to particular species as indicated by the cluster analysis; with the exception of *Psilocybe montana*. The small nuclear sub unit of the rRNA gene is a reliable DNA region to test for the presence of members of the genus *Psilocybe* and *Panaeolus*.

The ITS-1 sequences of the samples tested showed greater diversity compared to the nuc-ssu-rDNA. In the genus *Psilocybe*, each species had a unique sequence. Genetic distances among species in this genus distributed in a wide range. *Psilocybe montana* (PS5) is also a farthest species in genus *Psilocybe*. In the genus *Panaeolus*, there were two of the five species shared the same sequence. These were *Panaeolus papilionaceus* (PA2) and *Panaeolus speciosus* (PA6). Genetic distance among this genus distributed in a small range. Sample PA1 and PA5 are *Panaeolus semiovatus* and share the same sequence of ITS-1 DNA. In RAPD and AFLP typing, however, they were clustered as different species. It is possible that they are close species just like *Panaeolus papilionaceus* (PA2) and *Panaeolus speciosus* (PA6). Although the ITS locus is known to evolve at a high rate compared to other DNA loci, species specific sequences were not detected. RAPD and AFLP analysis scan the polymorphisms in the whole genome and can give information on the genetic relatedness of species. In ITS-1 analysis for *Panaeolus subbalteatus* (PA7) and *Psilocybe semilanceata* (PS1 and PS6) possessed multiple alleles.

When compared the genetic distance between nuc-ssu-rDNA and ITS-1 among samples in this study, 0.92 is the largest number in nuc-ssu-rDNA and 39.21 is the largest number in ITS-1. The genetic distance was an estimated number of substitutions per 100 bases. This great difference demonstrated that the faster evolution rate in ITS-1 than in nuc-ssu-rDNA.

This study used the ITS-1 DNA as a marker for the identification of members of the genera *Psilocybe* and *Panaeolus*. Multiplex PCR with three primers for a common product and genus specific product was designed to be used as a quick way of screening for the presence of *Psilocybe* and *Panaeolus* fungi. A method of SSCP analysis for a common product amplified by a simple PCR was also developed. It is a simple and fast method to screen a large number of samples. This sensitive method is sufficiently sensitive to detect a single base difference between two nucleotide sequences. To perform this SSCP analysis effectively a database of the standard patterns for each species should be established in advance.

9.5 Conclusion

This study demonstrated that *Psilocybe* and *Panaeolus* fungi identification can be successfully performed at the DNA level. Methods of DNA profiling were also established. RAPD can be used as a quick screening for the whole genome of the fungi, particularly when several random primers are used. The band patterns of RAPD showed that it can also be species specific, however due to the parameters in PCR this method suffers from problems of reproducibility. It is not recommended in forensic application, since its poor reproducibility in the unexpected sample quality. The band patterns of AFLP showed better reproducibility. The number of fragment can be restricted in a small number by the selective primers if the mixed sample is tested.

AFLP is a good method for a species test. This method provides more reliable results in the species identification than the RAPD method. By using the score system developed for the comparison of DNA patterns, the results of RAPD and AFLP can be used in the inter gel or even inter lab comparison. In the results of RNA gene analysis, the internal transcribed spacer DNA has much faster evolving sequences than the small subunit rDNA. The size and sequence variation of ITS-1 DNA sequences in *Psilocybe* and *Panaeolus* fungi are both species and genus specific. A simple method was developed to amplify the common product and genus specific product for the genus identification. The SSCP pattern of the common product is sufficient to reveal the species specific sequences. If dye-labeled primer were used, the accurate size of common product would also be a valuable method for species identification. If automatic sequencer is available in the laboratory, the best method of species and genus identification is sequencing the RNA gene. By the phylogenetic analysis of sequence in nuclear small subunit rDNA and the variable internal transcribed spacer 1, even the other species (not included in this study) of the same genus can be easily determined.

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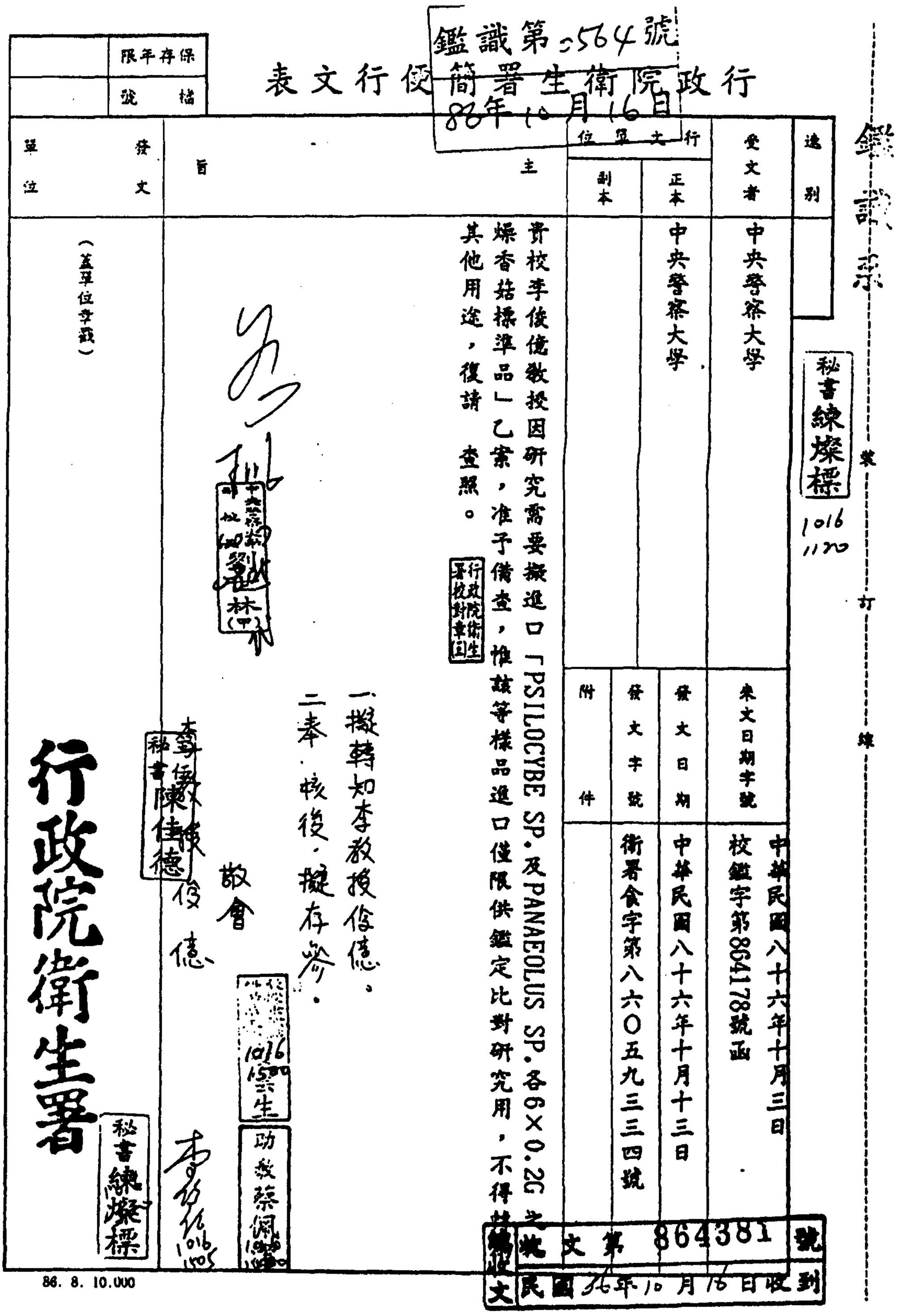

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