Genetic homogeneity of cultivated strains of shiitake (*Lentinula edodes*) used in China as revealed by the polymerase chain reaction

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Nineteen strains of shiitake (*Lentinula edodes*) which are used for spawn production for farms throughout mainland China were characterized by three arbitrarily-primed polymerase chain reaction (AP-PCR) profiles, seven random amplified polymorphic DNA marker (RAPD) profiles and five restriction patterns (restriction fragment length polymorphism patterns of the PCR-amplified ribosomal DNAs; rDNA-RFLPs). For AP-PCR, 4 – 14 DNA bands were amplified for a particular strain while 1 – 9 DNA bands were amplified using RAPD. Among them only three of the strains tested showed different amplification profiles with most of the primers used. The others showed small differences in three or fewer DNA amplification profiles. All strains showed identical rDNA-RFLPs. The study indicates that cultivated strains of shiitake in China are genetically very homogeneous, very like cultivated strains of *Agaricus bisporus* and *Volvariella volvacea*. However, our collection of *L. edodes* covers an enormous geographical area (approx. 1700 km N to S, 700 km E to W) and our results demonstrate that the shiitake industry in China depends on an extremely small gene pool. In comparison, three wild strains collected from the same ecosystem in Fujian Province (SE China) showed more variable amplification profiles. This emphasises the importance of conservation of wild shiitake strains as a reserve of biodiversity for this important industry.

Lentinula edodes (Berk.) Pegler (syn. *Lentinus edodes* (Berk.) Singer), the black oak mushroom (shiitake) was first cultivated in China more than 800 years ago (Chang & Miles, 1987) and has become the most famous edible mushroom in China, Japan, Taiwan and Korea. It is the third or fourth most popular cultivated edible mushroom in the world (Royse, 1995). China is the major producing as well as exporting country for shiitake (Royse, 1995). The mushroom is valued for its unique flavour which derives from the production of lenthionine and guanine-5-monophosphate (Mizuno, 1995), but dietary value is high as the fruit bodies are rich in minerals as well as essential amino acids, especially lysine and leucine. They are high in fibre content as well as vitamins (thiamine, riboflavin, niacin, ascorbic acid) and contain less than 10% crude fat (75% as linoleic acid and low in saturated fatty acids) (Ito, 1978; Mizuno, 1995). Besides the mushroom crop, lentinan (a protein-bound polysaccharide) has been extracted from *L. edodes* fruit bodies and found to have immunomodulatory effects; it is one of the top-selling anticancer drugs in Japan (Chihara, 1993).

There is an international market for this mushroom and, as a result, tremendous efforts, especially in Japan, have been devoted to research on: cytology (Nakai, 1986); genetics (Itavaara, 1990; Bowden, Royse & May, 1991; Hasebe, 1991); degradation of wood/substrate (Tsuneda, Maekawa & Ohira, 1991); population genetics (Tokimoto, Komatsu & Takemaru, 1973; Shimomura *et al.*, 1992; Fukuda *et al.*, 1994; Hibbett *et al.*, 1995); mycoparasitism and pathogen resistance (Komatsu, 1976; Tsuneda & Thorn, 1994); breeding (Mori, Fukai & Zennyoji, 1974; Tokimoto & Momatsu, 1995); *in vitro* fruiting (Terashita, Kono & Murao, 1980; Leatham, 1983; Tan & Moore, 1992); and molecular studies (Hori *et al.*, 1991; Kulkarni, 1991; Kajiwara *et al.*, 1992; Kwan *et al.*, 1992*b*; Chiu, Kwan & Cheng, 1993; Endo *et al.*, 1994; Kondoh *et al.*, 1995). Considerable information has also been obtained about mushroom cultivation (Ito, 1978; Chalmers, 1989; Royse, Bahler & Bahler, 1990) and biochemistry especially during fruiting (Leatham, 1985; Tokimoto, Fukuda & Tsuboi, 1984; Matsumoto, 1988; Takagi, Katayose & Shishido, 1988; Leatham & Hasselkus, 1989; Tan & Moore, 1995).

Since *L. edodes* has economic importance, typing of the strains is becoming essential and various methods have been assessed. Use of isozymes is relatively straightforward (Ohmasa & Furukawa, 1986; Royse & May, 1987) but only functional genes are evaluated and the expression and activities of enzymes will be affected by the physiological age of the cultures as well as by environmental factors. In comparison, DNA markers (Kulkarni, 1991; Kwan *et al.*, 1992*a*; Chiu *et al.*, 1993; Zhang & Molina, 1995) have the advantage of sampling the genome non-selectively and permit evaluation regardless of the physiology of the cultures and influence of the environment. Although analysis of restriction fragment length polymorphisms (RFLPs) has proved useful with cultivated mushrooms (Loftus, Moore & Elliott, 1988;

Kulkarni, 1991; Fukuda *et al.*, 1994), simpler protocols based on the polymerase chain reaction (PCR) have been developed (Rafalski & Tingey, 1993). We have concentrated so far on applying arbitrarily-primed polymerase chain reactions (AP-PCR; Welsh & McClelland, 1990) to the study of mushrooms (Kwan *et al.*, 1992*a*; Chiu *et al.*, 1993; Chiu, Chen & Chang, 1995) while others have attempted to use random amplified polymorphic DNA markers (RAPD; Williams *et al.*, 1990; Khush, Becker & Wach, 1992; Zhang & Molina, 1995). In this paper we present the results of a study which employs AP-PCR, RAPD and RFLPs of the PCR-amplified rDNAs as the DNA fingerprinting method for cultivated strains of *L. edodes* used in China. It was found that both AP-PCR and RAPD are reliable and convenient methods for typing strains. However, the cultivated strains in China are genetically homogeneous, although genetic variation was readily demonstrable in wild isolates even when obtained from the same ecosystem. Consequently, protecting the natural habitat is a crucial issue for conservation of biodiversity of germplasm for future exploitation of this important crop.

MATERIALS AND METHODS

Organism

Nineteen cultivated strains and three wild strains of *Lentinula edodes* collected in China were employed in this study (Table 1). The cultivated strains were supplied as being currently cultivated ones (not just cultures from a collection) used in the regions around the institute which provides the spawns (seed cultures) to the farmers who then do the mass cultivation using plastic bags of sawdust or logs. The preferred substrates for the strains are shown in Table 1.

Media and cultivation conditions

Cultures were maintained on potato dextrose agar at 25°C. To obtain sufficient biomass for DNA extraction, mycelia were scraped from a plate culture and inoculated into 100 ml potato dextrose broth. The liquid cultures were then incubated at 25° on a rotary shaker operating at 100 rpm for 14 days.

DNA extraction

Genomic DNA was extracted from tissue frozen in liquid nitrogen, and was purified following a protocol of White *et al.* (1990) with the addition of RNAse treatment and caesium chloride further to purify the DNA (Yoon, Glawe & Shaw, 1991; Yoon & Glawe, 1993). The concentration and purity of a DNA sample were measured by the spectrophotometric absorbance and the ratio of OD_{260}:OD_{280} (Sambrook, Fritsch & Maniatis, 1989). A sample having a ratio greater than 1·8 was considered suitable for use, and its concentration and purity were further checked by agarose gel electrophoresis using ethidium bromide staining.

Table 2. The primers used in this study

Primer	Sequence (5′ → 3′)
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG
OPA-02	TGCCGAGCTG
OPA-04	AATCGGGCTG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPB-07	GGTGACGCAG
OPC-02	GTGAGGCGTC
OPC-05	GATGACCGCC
M13FS	CGCCAGGGTTTTCCCAGTCACGAC
GalK-54	TACGGTCCGGGAGCGCAGCA
M13RS	AGCGGATAACAATTTCACACAGGA

RFLPs of the PCR-amplified rDNA

The 5·8S rDNA with its flanking internally transcribed spacer regions (ITS1 and ITS2) was amplified using primers ITS4 and ITS5 (synthesized by HSC/Pharmacia Biotechnology Service Centre, Toronto) whose sequences are listed in Table 2 (White *et al.*, 1990). In 50 µl of PCR reaction mixture, there were: 1 × PCR buffer II, 10 ng of genomic DNAs, 2 mM $MgCl_2$, 200 µM each of dNTPs (Perkin Elmer Cetus), 1 µM each of the primers and 2·5 U Taq DNA polymerase (Boehringer Mannheim). The mixture was overlaid with mineral oil and incubated for 35 cycles in a thermal cycler (Minicycler, MJ) programmed as follows: 94° for 1 min, 60° for 1 min and 72° for 2 min, and with a 10 min extension at 72° for the last cycle. The amplification product was examined by electrophoresis in 3% NuSieve agarose gel (FMC) and ethidium bromide staining. After purification using a Geneclean II kit (BIO101), the rDNA fragments were digested with the following restriction enzymes according to the manufacturer's instructions (Pharmacia or GIBCO): *Eco* RI (G↓AATTC), *Hpa*

Table 1. Strains of *Lentinula edodes* used in this study

Strain no.	Source and geographic location
Cultivated strains	
BL8107, BK9013, BK089, BL468, BLBC, BKL, BL1	Institute of Vegetables, Academy of Agricultural Sciences, Beijing
Cr03*, Cr10*, Cr12*, Cr18*, Cr33	The Sanming Mycological Institute, Liedong, Sanming, Fujian Province
Nong-1, Nong-2, 856, SL7401*, XN-2*, SX-1	Edible Fungi Research Institute, Shanghai Academy of Agricultural Science, Shanghai
HL7925*	Institute of Applied Mycology, Huazhong Agricultural University, Wuhan, Hubei Province
Natural isolates	
STCL65, STCL67, STCL68	Mt Wuji reserve area, Fujian Province

The cultivated strains were supplied as being representative of those in use by farmers around the institute concerned. The asterisks indicate strains favoured for spawns to be used for log cultivation; cultivated strain numbers without an asterisk are used for spawn intended for 'artificial log' (plastic bags filled with sawdust) cultivation.

I (GTT↓AAC), *Mbo* I (↓GATC), *Msp* I (C↓CGG) and *Rsa* I (GT↓AC).

RAPD

Seven 10-mer arbitrary primers (Operon Technologies) as listed in Table 2 were used to amplify the genomic DNAs. In 25 µl of the reaction mixture, there were: 1 × PCR buffer II (Perkin Elmer Cetus), 1·5 mM $MgCl_2$, 150 µM each of dNTPs (Perkin Elmer Cetus), 1 µM of the primer and 2·5 U Taq DNA polymerase (Boehringer Mannheim) and 50 ng of genomic DNA (protocol modified from Williams *et al.*, 1990; Yoon & Glawe, 1993). The thermal programme was: 40 cycles of 94 for 1 min, 35° for 1 min and 72° for 1 min.

AP-PCR

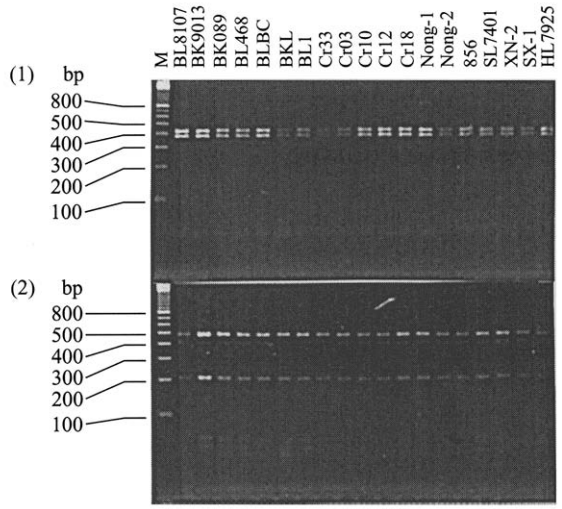
Three arbitrary primers (M13FS, M13RS and GalK-54) were used (Chiu *et al.*, 1993 & 1995). The reaction mixture contained : 1 × PCR buffer II (Perkin Elmer Cetus), 3·5 mM $MgCl_2$, 200 µM each of dNTPs (Perkin Elmer Cetus), 1 µM of the primer and 2·5 U Taq DNA polymerase (Boehringer Mannheim) and 25 ng of genomic DNA. The thermal programme was: 2 low stringency cycles of 94° for 2 min, 35° for 1 min and 72° for 2 min and were followed by 39 cycles of high stringency: 94° for 1 min, 55° for 1 min and 72° for 2 min and with the last extension time lengthened to 10 min.

Experiments were repeated to verify consistency, and controls without DNA were amplified in parallel to check for contamination or carry-over. The DNA fingerprints were resolved on NuSieve or metaphor gel (FMC) together with molecular size marker consisting of *Bst* II digests of bacteriophage lambda DNA or 100 bp ladders (Pharmacia or GIBCO BRL; the Pharmacia product has a bright band of double intensity at 800 bp, and the GIBCO BRL product, a bright band at 600 bp).

RESULTS AND DISCUSSION

The polymerase chain reaction has proved to be a major breakthrough in molecular studies because it relieves the experimenter of the necessity of obtaining an abundance of highly purified DNA. By exponential amplification *in vitro*, sufficient DNA is generated from trace amounts of starting material. Using fungal-specific primers, specific genes can be amplified such as the rDNAs in this study. Endonuclease digestion can be applied to detect for restriction polymorphism, a simple alternative to comparison of the DNA sequences of various isolates.

In *L. edodes*, previous studies have already demonstrated polymorphisms in the 5·8S rDNA and the flanking ITS regions of three monokaryotic strains which are meiotic progeny of two fruit bodies collected from the wild in China, and six cultivated isolates in Japan and Thailand (Kwan *et al.*, 1992*a*, *b*; Hibbett *et al.*, 1995). These studies revealed four nucleotide differences (substitution and deletion) in the ITS1 region, five in 5·8S rDNA and nine in ITS2 region. Yet the nineteen strains examined here show no restriction polymorphisms as detected with five endonucleases (Figs 1 & 2);



Figs 1–2. The RFLPs of the PCR-amplified ribosomal DNAs (5·8S rDNA and its flanking ITS1 and ITS2 regions) of the cultivated strains of *Lentinula edodes* in China. Restriction enzymes: **Fig. 1.** *Eco* RI; **Fig. 2.** *Mbo* I. M, 100 bp ladder as molecular size marker.

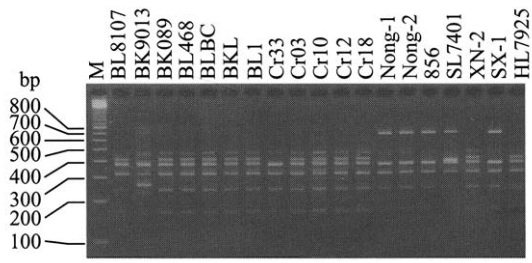
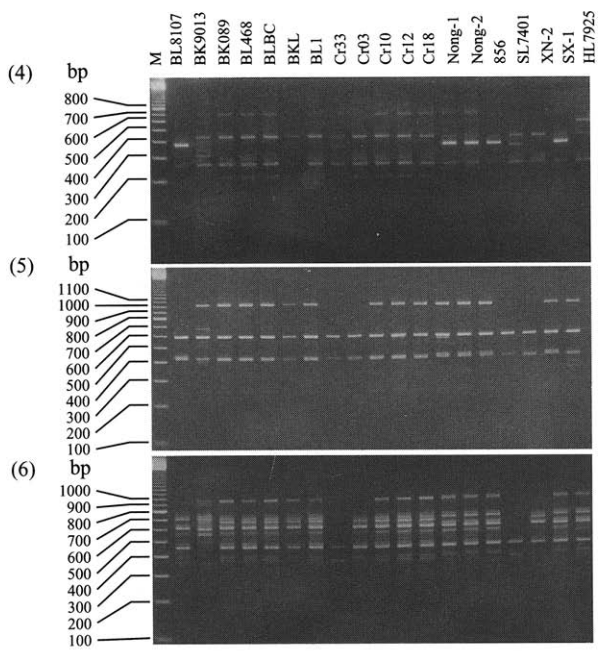


Fig. 3. RAPD profiles of cultivated strains of *Lentinula edodes* in China using OPA-02 as the arbitrary primer. M, 100 bp ladder as molecular size marker.

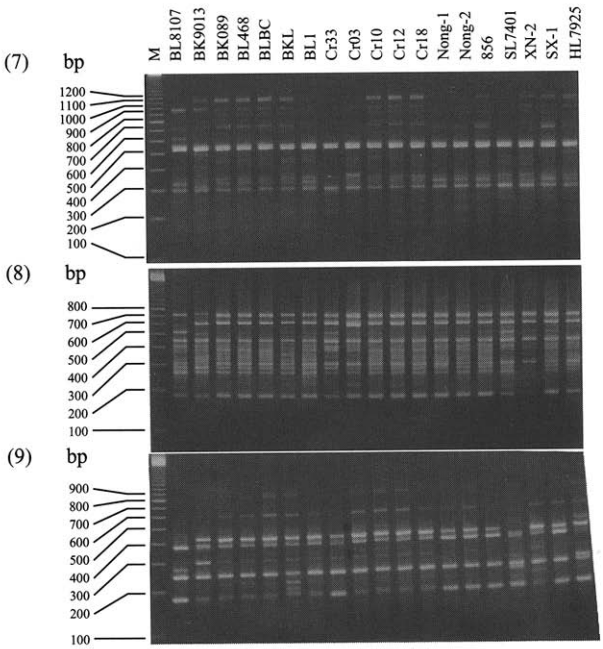
in all cultivated strains tested, two restriction sites for *Mbo* I, single restriction sites for *Eco* RI, *Hpa* I and *Msp* I but no site for *Rsa* I were found in the rDNAs.

Using RAPD and AP-PCR removes any requirement for information about genetic sequence of the target genes and enhances detection of DNA polymorphisms in partial genomes of organisms (Welsh & McClelland, 1990; Williams *et al.*, 1990). Both RAPD and AP-PCR were devised in the same year (Welsh & McClelland, 1990; Williams *et al.*, 1990) and have been further adapted to RNA fingerprint analysis, also called differential gene expression analysis (Liang & Pardee, 1992; Welsh *et al.*, 1992). In terms of the components of the reaction mixture and thermal programmes the two are similar. There are various sources supplying commercial kits (usually a set of ten) of RAPD primers while for AP-PCR, the primers used are arbitrary in sequence, and so commercially available primers for sequencing or cloning can be employed. Overall, both AP-PCR and RAPD are convenient and effective. In our experience, AP-PCR generates more bands; For AP-PCR, 4–14 DNA bands were amplified for a particular strain while 1–9 DNA bands were amplified using RAPD (Figs 3–8).

Pair-wise comparisons of amplification profiles are commonly used to identify polymorphic DNA bands which are further employed to estimate genetic relatedness among individuals. However, some problems have been identified,



Figs 4–6. RAPD profiles of cultivated strains of *Lentinula edodes* in China. Arbitrary primers: **Fig. 4.** OPA-04; **Fig. 5.** OPB-07; **Fig. 6.** OPC-02. M, 100 bp ladder as molecular size marker.



Figs 7–9. AP-PCR profiles of cultivated strains of *Lentinula edodes* in China. Arbitrary primers: **Fig. 7.** M13FS. **Fig. 8.** M13RS. **Fig. 9.** GalK-54. M, 100 bp ladder as molecular size marker.

including studies showing that homologous DNA fragments amplified in RAPD vary in size among different isolates, implying that some 'amplification polymorphisms' are actually 'length polymorphisms' (Pillay & Kenny, 1995), and the fact that band intensity in the 'fingerprints' not only reflects the competition in binding between different regions of the DNA template with the arbitrary primer under low stringency (Welsh & McClelland, 1990; Williams *et al.*, 1990) but also the number of DNA species having the same mobility (Pillay & Kenny, 1995). Thus, as the thermal programmes of both RAPD and AP-PCR employ low stringency for annealing the primer to the DNA template, and agarose gel electrophoresis only reveals ***mobilities*** of DNA bands in an electric field, the polymorphic DNA bands revealed by this approach are not always equivalent to genetic markers, so genetic relatedness of the isolates cannot be estimated (this is a contrast to studies using RFLPs). In this study, the DNA fingerprint is regarded as a multilocus probe and different profiles are used in concert for comparisons among strains.

The amplification profiles also reveal genetic homogeneity in these cultivated strains. Examples of the data obtained appear in Figs 3–9; other data (not shown) confirmed the homogeneity. Even strain 856 which is sold as a protoplast fusion product, claimed to have better performance than the parental strains (Nong-1 and Nong-2), has a DNA fingerprint nearly identical with its parents. Strains Nong-2 and 856 showed identical restriction profiles and amplification profiles with those of strain Nong-1 except with primer OPB-07; an additional band is present in strain Nong-1 (Fig. 5). Similarly, strains BK089, BL468, BLBC, Cr12 and Cr18 showed highly similar DNA fingerprints. This similarity is reflected in colony morphology and mating type specificities. The appearance of Petri dish cultures of 17 of the strains were at least similar, and most were identical. Only strains BL8107 and BK9013 had distinctive colony morphologies (Fig. 10, Table 3). As far as mating types are concerned, when the component monokaryons were recovered by protoplast technology from twenty-one Chinese cultivated strains (including all those in this study) and mated together, 65% of the pairings were incompatible, suggesting that many of these strains have mating type alleles in common (Lin, unpublished results).

Although the differences are small, some 'strain typing' is feasible and the DNA markers can be used to classify these

Table 3. Grouping of the nineteen cultivated strains of *Lentinula edodes* in China

Group	Strain number	Colony morphology	Origin
1	BL8107	A	Beijing
2	BK9013	B	Beijing
3	BK089, BKL, BL1	C	Beijing
	BL468, BLBC	D	Beijing
	Cr03, Cr10	C	Sanming
	Cr12, Cr18	D	Sanming
	XN-2	D	Shanghai
4	SX-1	C	Shanghai
	Nong-1, Nong-2, 856	D	Shanghai
5	HL7925	C	Wuhan
6	Cr33	C	Sanming
	SL7401	C	Shanghai

Genetic relatedness of the isolates cannot be estimated from RAPD and AP-PCR polymorphisms (in contrast to studies using RFLPs). In this Table, the DNA fingerprint is regarded as a multilocus probe to compare the 19 cultivated strains. The six groups into which they are allocated differ by four or more DNA fingerprints (see Figs 3–9). Isolates within each group are either identical or differ in up to three primers. Colony morphology is illustrated in Fig. 10.

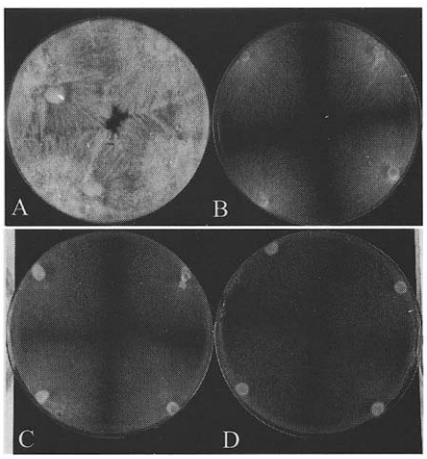


Fig. 10. The four colony morphologies (A, B, C and D) expressed by cultivated strains of *Lentinula edodes* in China. Typing depends on the relative mycelial density of aerial hyphae.

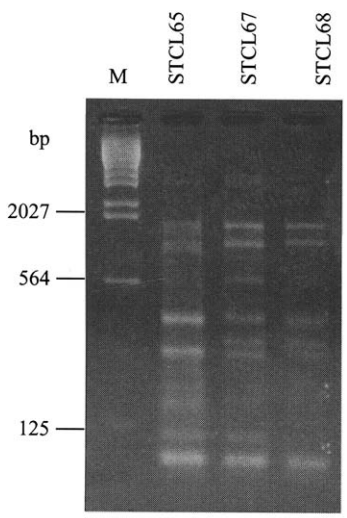


Fig. 11. Amplification profiles of three wild *Lentinula edodes* isolates collected from the Mt Wuji reserve area, Fujian Province, China, using M13FS as the arbitrary primer. M, *Bst* II digest of bacteriophage lambda DNA as molecular size marker.

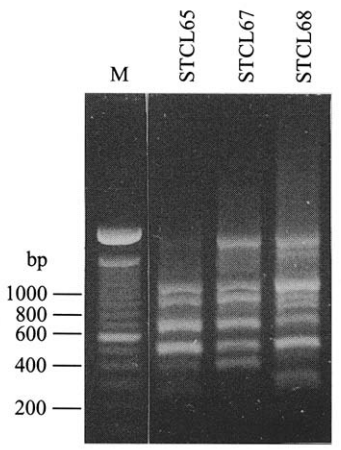


Fig. 12. Amplification profiles of three wild *Lentinula edodes* isolates collected from the Mt Wuji reserve, China using M13RS as the arbitrary primer. M, 100 bp ladder as molecular size marker.

nineteen cultivated strains into six groups (Table 3) which differ by four or more DNA fingerprints (Figs 3 – 9). Within each group, the isolates differ in only a minor fashion in the amplification profiles (zero to three primers). Although cultivated strains can be 'typed' in this way, this grouping should not obscure the fact that all the strains are genetically extremely similar; so similar that they may comprise a closely related family.

The 19 cultivated strains examined here are described and supplied as being different strains by the spawn suppliers (which are the agricultural institutes in this instance) and most are claimed to be products of the institute's own strain improvement programme. They derive from geographically diverse regions of China. Wuhan is in central China, over 1000 km South of Beijing. Sanming is approx. 650 km SE of Wuhan and Shanghai 700 km due East of Wuhan. The Mt Wuji reserve is approx. 100 km NW of Sanming. Approximate geographical equivalents are N-S London to Algiers or Ottawa to Orlando, W-E London to Münich or Indianapolis to Washington. Yet, the observations reported above indicate that most of the strains surveyed are probably close family derivatives of one original genotype and may be either vegetative clones or different generations (McDonald & Martinez, 1990). Japanese strains were introduced into cultivation in mainland China in the 1960s and since then have been widely cultivated. Strain SL7401 was one of the strains commonly cultivated in Japan in the 1960s but cannot be assumed to be native to Japan as Japanese spawn producers collected widely throughout Asia and have done much breeding work.

None of the spawn suppliers can provide definitive evidence of the provenance of their strains. Unlike *Agaricus*, shiitake is mostly sold in dried form, and as the drying process kills the mushroom tissues, recovery of the cultivated strain by subculture or spore isolation from the marketed product is highly unlikely. What seems to be happening is that institutes readily exchange cultures and the receiving institute assigns a different strain number. Intellectual property rights are not a major concern in China. The significance of our study is that it demonstrates the genetic homogeneity of the cultivated strains and emphasises the paucity of the gene pool on which the Chinese shiitake industry depends. In comparison, cultivated strains collected worldwide did show genetic heterogeneity (Kwan *et al.*, 1992*a*; Chiu *et al.*, 1993; Fox *et al.*, 1994; Fukuda *et al.*, 1994).

The genetic homogeneity we have observed in cultivated strains in China is similar in kind to that observed in other cultivated mushrooms such as *Agaricus bisporus* (Loftus *et al.*, 1988) and *Volvariella volvacea* (Chiu *et al.*, 1995). Loftus *et al.* (1988) could find no RFLP polymorphisms between three commercial cultivars of *Agaricus bisporus*, and noted that the growth conditions and flushing (times of fruiting) patterns for each of the three strains were also very similar, despite the fact that the three strains were marketed by different companies as original and independent products. These authors commented on how remarkable was '... the coincidence of their genetic similarity ...' Since the same remarkable genetic similarity between allegedly different commercial cultivars has been

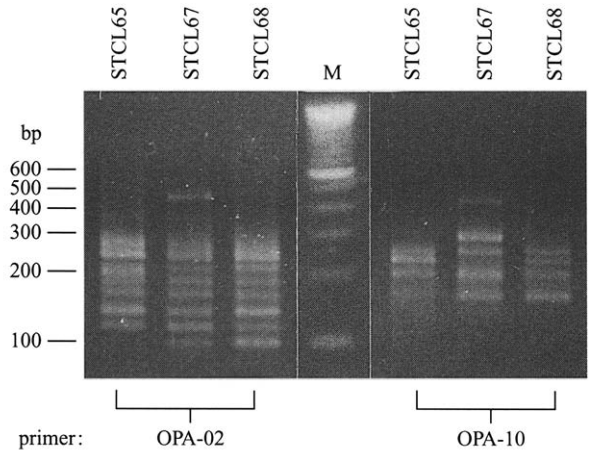


Fig. 13. Amplification profiles of three wild *Lentinula edodes* isolates collected from the Mt Wuji reserve, China using OPA-02 or OPA-10 as the arbitrary primer. M, 100 bp ladder as molecular size marker.

encountered in *Volvariella volvacea* (Chiu *et al.*, 1995) and now in *Lentinula edodes*, we venture to suggest that genetic homogeneity in cultivated mushrooms is unlikely to result from any peculiarity of mushroom genetics but most probably from the commercial morals and behaviour patterns of mushroom growers around the world.

Fortunately, a wide range of DNA variation and/or mating type polymorphisms are present in isolates collected from nature (Tokimoto *et al.*, 1973; Mori *et al.*, 1974; Royse & May, 1987; Shimomura *et al.*, 1992; Chiu *et al.*, 1993; Fox *et al.*, 1994). Heterogeneity of wild isolates is certainly observed in the *L. edodes* isolates collected in China (STCL65, 67 and 68 in Table 1). Even though collected from the same nature reserve area they have different mating type alleles (Fox *et al.*, 1994) and showed distinctive DNA fingerprints (Figs 11–13) and identical rDNA-RFLPs as the cultivated strains (data not shown). It is not our purpose here to compare wild and cultivated strains critically; an extensive analysis of the gene pool of wild shiitake in China is under way and will be reported in due course. We wish only to make the point that genetic homogeneity over a large geographical area is a peculiarity of the cultivated strains; heterogeneity is easily demonstrated even in wild strains from the same locality. As far as is known, only the mating type genes are involved in control of incompatibility reactions in *L. edodes* (Royse & May, 1987; Shimomura *et al.*, 1992; Fox *et al.*, 1994), so cross-breeding using different eco-types would be a feasible way to introduce heterogeneity into the cultivated strains (Mori *et al.*, 1974). The crop is subject to attack by pests (e.g. *Trichoderma*) and, like any agricultural/horticultural monoculture which has been under stabilised selection for a long time, is prone to potentially devastating and widespread losses should a particularly virulent pest or disease arise. This has already happened in China with one of the introduced Japanese strains in the 1980s. It is, therefore, essential that the genetic diversity which still exists in the natural environment is protected, collected and made use of in this important crop.

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