HDC CONTRACT REPORT M 17

Strain protection using recombinant DNA technology

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PRACTICAL SECTION FOR GROWERS

Application

This project sought to develop technologies that could be used in the identification and protection of novel commercial strains of the mushroom *Agaricus bisporus,* ie. technologies that would provide a way of 'finger-printing' a spawn.

Two approaches were used. Attempts were made to introduce genetic information, DNA, by using ballistics in which the DNA is literally fired into the mushroom tissue. Secondly, molecular techniques were used to characterise the natural variation present in the DNA of a sample of 26 different strains.

Ballistics has not yet been successful in introducing DNA markers into *Agaricus* mushroom strains but was effective in ink-cap mushrooms. The natural variation present in the 26 strains studied was sufficient to differentiate between them.

The ability to protect strains in the market place is important in encouraging the necessary high level of investment that is required to produce improved spawns for the benefit of all growers. The HDC Mushroom Panel spends some of its levy income on strain development (Project M 4, M 15 and related programmes) and strain marking would provide the ability to have full control over the marketing of any successful strain and would also protect and ensure a return on monies invested.

<u>Summary</u>

Mushroom spawns are in many instances similar in appearance and overall cropping performance. The spawn houses produce a range of similar spawn types and spawns. No reliable and unambiguous method for marking strains is yet available to the mushroom spawn producer; a trading environment which is not conducive to investment in the development of novel strains. In addition, new approaches and strains arising directly from R & D programmes cannot be protected and effectively exploited. The ability to unambiguously mark and therefore recognise a strain would resolve these problems.

There are two possible approaches to strain marking and both have been followed in project M 17. The first approach is to try and alter the DNA of the strain you wish to protect by introducing unique DNA sequences which can then be detected and recognised. The significant advantage of this approach is that it adds something to a strain that wasn't there before and that what has been added can be readily detected. An analogy would be the use of an UV marker pen to put your name/post code on your personal belongings. The second approach depends on the use of natural variation which may be present in spawns to distinguish one from another. This approach does not depend on anything other than an ability to determine the variation present. It is analogous to conventional taxonomy/strain identification which differentiates strains using microscopic and macroscopic characters but it is based on new molecular techniques which can identify small but consistent differences in the DNA sequences.

Introducing unique DNA sequences with DNA ballistics

Ballistics is a relatively recently developed technology used for getting DNA directly into cells and tissues; in essence the DNA is fired into the cell. The mechanics of the process require a macrocarrier ('bullet') which carries microprojectiles bearing the DNA. Given sufficient velocity the microprojectiles penetrate the cell. Firing is carried out in a vacuum. Two DNA guns were used in this work (i) a non-commercial device built in-house at HRI and powered by compressed air and (ii) a commercial device manufactured by Biorad plc, which uses compressed helium. A range of parameters (particle size, target distance, firing pressure, firing vacuum, different types of DNA, and various tissue types, eg. mycelium, fruit-body tissue) were examined to optimise the ballistics process. These 'guns' enabled the introduction of 'foreign' DNA and its subsequent expression and detection in the brewers yeast, *Saccharomyces cerevisiae* and the two ink-cap mushrooms, *Coprinus bilanatus* and *Coprinus cinereus*. If DNA entered the cells of *Agaricus bisporus* it was not integrated and/or expressed.

Natural molecular markers

A molecular technique known as RAPD-PCR was used to assess variation in the DNA of a variety of mushroom spawns and experimental strains. A total of 26 strains were studied of which 23 were commercial in origin. The method detects polymorphisms in the DNA using a range of primers which react with specific sequences in the DNA. Twenty different primers were used and a total of 211 polymorphisms were identified. Overall the data showed that the commercial spawns were very homogeneous showing large numbers of polymorphisms. Individual primers could be used to distinguish brown strains from white but could not separate hybrids or spawns of common origin.

Using information from all the primers it was possible to identify subtle variation that could be used to distinguish each strain from the others in the sample. RAPD-PCR markers are therefore able to differentiate between strains within a given sample and can provide a level of strain marking.

Conclusion

The ability to add a specific DNA sequence to a spawn to provide an unambiguous means of recognising a strain remains the method of choice for strain 'finger-printing'. RAPD-PCR markers though provide a degree of strain 'finger-printing' which could meet the needs of those wishing to protect spawns in the market-place while the more effective DNA transformation technology is being further developed.

4. SCIENCE SECTION

4.1 Introduction

To gain the maximum benefit from R & D investment in strain improvement it is important to be able to protect strains when in the market place from unauthorised use and exploitation; and for this protection to have the force of law. Such protection is usually achieved through patents of plant breeders' rights. Both methods depend on the ability within a crop species to be able to recognise a strain or variety as distinct from all other strains of the species ( ). For the cultivated mushroom this issue is particularly problematic because past and present mushroom varieties (spawns) have a narrow genetic base and whilst major groupings can be readily differentiated, eg. brown-capped verus white capped, individual spawns cannot.

The study described here sought to establish methods for strain protection in the cultivated mushroom *Agaricus bisporus* using the introduction of DNA sequences through transformation and by an investigation of the natural variation present in a range of spawns.

To develop transformation, a DNA delivery system based on ballistics or particle bombardment was evaluated. Procedures were developed using the yeast *Saccharomyces cerevisiae*, and ink cap mushrooms *Coprinus bilanatus* and *Coprinus cinereus* as models. The identification of inherent genetic variation involved screening commercial strains of *A. bisporus* for DNA polymorphisms by polymerase chain reaction amplification of random arbitrarily primed sequences (RAPD-PCR).

4.2 Materials and methods

4.2.1 Mushroom/fungal strains used for transformation studies

The following strains were used as targets for ballistic transformation experiments:-
Saccharomyces cerevisiae 948 *ura3-52, leu2-3,* (Armaleo *et al.,* 1990: Bio Rad 1992) a non-reverting double auxotrophic mutant for uracil and leucine. *Coprinus cinereus* strains FM2 (Casselton & Fuente de la Herce, 1989) and LT2 (Binninger *et al.,* 1987) *trp-2* and *trp-1* auxotrophic tryptophan monokaryons respectively. A *C. cinereus* para-aminobenzoic acid, *paba* mutant, [HRI accession No.R357] (Casselton, unpublished).

Coprinus bilanatus strain Cb1-t2 mating type *A1B1*, a *trp-2* auxotroph derived from from the monokaryon Cb1(*A1B1*) by mutagenesis (Burrows *et al.*, 1990). *A. bisporus* C82 ura, a uracil auxotroph and X25, a commercial strain of *A. bisporus* from the spawn company Le Lion *Agaricus arvensis*, strain R20.

The following strains of *Agaricus bisporus* were used for RAPD-PCR analyses:-

Accession No.	Sporophore type	Spawn company/Source*
C43	Smooth white	A
C54	Rough white	B
C62	Hybrid white	C
C63	Hybrid white	C
C81	Brown	B
C82	Uracil auxotroph	Raper *et al.*, (1972)
C83	Brown	D
C84	Hybrid white	B
C85	Hybrid white	B
C86	Virus breaker	D
C87	Hybrid	D
C88	Hybrid	D
C89	Hybrid	D
C90	Hybrid white	B
C91	Hybrid	E
C92	Brown	E
C93	Hybrid white	E
C94	Hybrid white	E
C96	Hybrid	F
C97	Brown	F
C98	Hybrid	F
C99	Hybrid	F
C100	Hybrid	F
C101	Hybrid white	E
AA0306	Light brown	Wild type
AA0311	Light brown	Wild type

Strains AA0306 and 0311 were wild types from the coastline of California kindly provided by Amycel. Strain C82 *ura* was a monokaryon unable to fruit therefore there was no formation of sporophore tissue.
Spawn companies are coded by letter, ie. same letter indicates spawns from the same company.

4.2.2 Mycological media

S. cerevisiae 948 was grown in a yeast extract, peptone and dextrose (YEPD) based medium (Sherman *et al.*, 1986). A uracil deficient medium (UDM) recommended by BioRad (1992) was used for the selection of putative ballistic transformants.

Complete media for the growth of *C. cinereus, C. bilanatus* and *A. bisporus* were complete yeast extract medium (CYM) described by Raper *et al.* (1972) or the malt, yeast extract, peptone and glucose based medium (MYPG) described by Challen, (1993).

Minimal medium for mushrooms (MMM) described by Raper *et al.*, (1972) was supplemented as necessary to select for putative auxotrophic or resistant transformants of *A. bisporus, A. arvensis, C. bilanatus* and *C. cinereus.*

Plates of *A. bisporus, C. bilanatus* and *C. cinereus* used for bombardment were supplemented with osmotic stabilisers (0.5 M sucrose and 0.5% w/v soluble starch) where appropriate. To distinguish these from normal media the regeneration suffix R was used *eg.* MYPGR.

4.2.2 Bacerial hosts, vectors and culture media

The vector cloning hosts used in this study were *E. coli* HB101 and *E.coli* DH5α.

Tryptophan vectors used for transformation of *C. cinereus* and *C. bilanatus* are listed below.

Plasmid code	Species	Gene	Reference
pAK3	*C. cinereus*	*TRP2*	Burrows *et al.*, 1990
pCC1001	*C. cinereus*	*TRP1*	Binninger *et al.*, 1987
pCBT2-S5	*C. bilanatus*	*TRP2*	Challen *et al.*, 1994

Uracil vectors used for transformation of *A. bisporus* C82 *ura* are tabulated below:-

Plasmid code	Species	Gene	Reference
YEp352	*S. cerevisiae*	*URA3*	Hill *et al.*, 1986
pPL6 & pJR15	*A. nidulans*	*PYR.G*	Oakley *et al.*, 1987
pRS4	*Claviceps purpurea*	*PYR4*	Smit & Tudzynski 1992
pJD7,JD21 & JD32	*Phycomyces blakesleeanus*	*PYR.G*	Diaz Minguez *et al.*, 1990
pURA3.1	*P. chrysosporium*	*URA3*	Alic *et al.*, 1993
pFG1	*Trichoderma reesei*	*PYR.G*	Gruber *et al.*, 1990
pTRura3-2	*T. reesei*	*URA3*	Berges & Barreau, 1991
pTRura5-3	*T. reesei*	*URA5*	Berges & Barreau, 1991
pEF2	*Schizophyllum commune*	*URA1*	Froeliger *et al.*, 1987
pABU3H16 & pABU3H18	*A. bisporus*	*URA3*	Challen, unpublished

4.2.3 Ballistics technologies

Two ballistic devices were used:-

1. An HRI gun driven by compressed air and based on a design from Morikawa *et al.*, (1990). The gene gun consists of a tapered barrel 20 cm long, attached to a plateholder, whose distance from the mouth of the barrel can be adjusted. The plateholder is designed to hold 5 cm diameter Petri dishes. At the top of the barrel is

located a stopper plate, which is attached to the barrel by a screw thread and consists of a hollow tube of smaller diameter than the bore of the barrel. The function of the stopper plate is to stop the macrocarrier, but allows the microcarriers to be shot onto the Petri dish directly above. All these gun parts were constructed from stainless steel and encased in a vacuum chamber. The gun's propellant is a controlled release of compressed air, which is achieved by pulling the level below the base of the vacuum chamber.

2. The commercial available Biorad PDS-1000 system which uses compressed helium. The Bio Rad gun consists of a high pressure helium source connected to a vacuum chamber via a pressure transducer and a vacuum pump. The principle on which the gun operated, is by the controlled release of pressurised He down a gas acceleration tube, for the bombardment of biological tissue under vacuum. The controlled release of He produces a shockwave that breaks a plastic pressure rupture disc and accelerates the plastic macrocarrier disc coated with microcarriers against a metal mesh (stopping screen) which retains the macrocarrier but allows the microcarriers to hit the target tissue.

Fungal material (spores, mycelium and fruit-body tissues) were subjected to bombardment using both these devices. Putative transformants were evaluated using Southern blotting.

4.2.4 RAPD-PCR

RAPD-PCR was done using the method of Williams *et al.,* (1990). The primers used were from Operon technologies, Alameda, USA (10 oligomers from kit A). Their (direction 5' to 3') sequences were:-

OPA-1; CAGGCCCTTC, OPA-2; TGCCGAGCTG, OPA-3; AGTCAGCCAC, OPA-4; AATCGGGCTG, OPA-5; AGGGGTCTTG, OPA-6; GGTCCCTGAC,
OPA-7; GAAACGGGTC, OPA-8; GTGACGTAGG,OPA-9; GGGTAACGCC,
OPA-10; GTGATCGCAG, OPA-11; CAATCGCCGT,
OPA-12; TCGGCGATAG, OPA-13; CAGCACCCAC,
OPA-14; TGTGTGCTGG, OPA-15; TTCCGAACCC,

OPA-16; AGCCAGCGAA, OPA-17; GACCGCTTGT,
OPA-18; AGGTGACCGT, OPA-19; CAAACGTCGG,
OPA-20; GTTGCGATCC.

A typical RAPD-PCR reaction contained the following components:-

5 μl (5 ng μl^{-1}) genomic DNA.
29.85 μl of water (Sigma W-4502).
4 μl X10 buffer (10 mM Tris HCl pH8.8,1.5 mM $MgCl_2$, 50 mM KCl and 0.1% v/v Triton X100).
0.4 μl deoxynucleotides (100 μM dCTP, 100 μM dATP, 100 μM dTTP and 100 μM dGTP).
0.25 μl of primer (100 ng ml^{-1}).
0.5 μl Dynazyme (1 unit) (DyNAzyme Finnzyme, Espoo, Finland).

The components were then held on ice prior to use. The DNA was added to the reaction tube first. Then 35 μl of a bulk reaction mix (containing water, X10 buffer, deoxynucleotides, primer and DyNAzyme) was added to the reaction tube. The reaction mix was made up in bulk to minimise the possibility of pipetting errors and contamination. The reaction was then overlaid with mineral oil (Sigma M5904) before being placed in the thermocycler. All PCR reactions were prepared in a horizontal laminar flow bench and on ice. The reaction was controlled by substituting 5 μl of water (Sigma W-4502) in place of DNA prior to the addition of the reaction mix.

The RAPD-PCR reactions were done using an OmniGene (TR3 CM220, Hybaid Ltd. U.K.) thermocycler. The conditions were, 95^{0}C for 1 minute then 35 cycles of 92^{0}C 1 minute, 35^{0}C 1 minute and 72^{0}C 2 minutes.

4.2.5 Data Analysis

Fragment Interpretation

The interpretation of amplified product sizes was undertaken using a Personal Densitometer SI (Molecular Dynamics Inc, Sunnyvale, California, USA) and scanning the photographic negatives of the RAPD-PCR gels. Precise product sizing of amplified polymorphisms used a Molecular Dynamics software package (FragmeNT analysis, version 1.1). The presence / absence of polymorphisms was then scored and entered into a binomial matrix. The data was then analysed by cluster analysis using the Genstat 5 statistical software package. The dendrograms obtained reflected the level of diversity within the 26 strains studied using all 20 primers and by each primer.

Data analysis of the strains studied was at the individual primer level and by summation of all the data from the primers studied. The amplification products incorporated into the similarity matrix were selected on the basis of strong fluorescence under UV illumination and gave similar banding patterns after experimental repetition using genomic DNA extracted from separate preparations of freeze – dried mycelia.

4.3 Results and discussion

4.3.1 Transformation in *S. cerevisiae*

Ballistic transformants of *S. cerevisiae* 948 were recovered using the PDS1000/He gene gun under a wide range of conditions. The data indicated that the optimum firing pressure and target distance for transformation of *S. cerevisiae* 948 were 1300 psi and 6 cm respectively. The transformants obtained from 900 psi treatment maybe anomalous and more a reflection on the concentration of transforming DNA present. The reason for this is that few transformants were isolated when the firing pressure fell below 1300 psi, at a target distance of 6 cm. Furthermore, no transformants were isolated using firing pressures of 1100 and 1500 psi in conjunction with a DNA level of 415 ng per sample, which was the concentration of DNA delivered at 900 psi. The experimental evidence also showed that as the concentration of transforming DNA decreased the transformation rate dropped rapidly, especially when it fell below 415 ng DNA. Finally when the target distance exceeded 6 cm, no transformants were obtained, although 1 transformant was obtained at a target distance of 3 cm. No Southerns were performed on these isolates as the yeast strain used was constructed so that reversion is not possible (Armaleo *et al.*, 1990).

Summarised below are the conditions under which putative *ura3+* transformants of *S. cerevisiae* 948 were obtained using the PDS1000/He device. The number of transformants isolated is expressed as a mean, each treatment being duplicated.

Firing pressure (psi)	**Target distance (cm)**	**DNA on microcarriers (ng)**	**Mean No. of putative transformants**
1300	6	830	14
1300	6	415	10
1300	6	250	0
1300	6	83	0
900	6	415	3
1100	6	415	0
1500	6	415	0
1300	3	250	1
1300	9	250	0
1300	6	250	0
Control	-	0	0

All putative transformants were isolated using *S. cerevisiae* vegetative cells bombarded with M10 tungsten microcarriers, under a vacuum of 27 inches of Hg and a gap distance of 1 cm (distance between macrocarrier and stopping screen).

4.3.2 Transformation in *Coprinus*

With *C. cinereus* strains LT2 *trp-1* and FM2 *trp-2*, bombardment with vectors pCC1001, containing the homologous *TRP1,* gene for LT2 and pAK3, containing the homologous *TRP2* gene for FM2, produced putative transformants under the following conditions:-

1) A firing pressure ranging between 1000 to 1250 psi.
2) Tungsten grades M5 and G5.
3) Firing vacuum of 500 mm Hg.
4) A target distance of 25 mm.

The coating volume used for the microcarriers with *C. cinereus* was 50 μl, unlike that for *S. cerevisiae*. This may have affected transformation as coating of microcarriers in smaller volumes maybe more efficient. Figures 3.4, 3.5, 3.6 and 3.7 show the Southern blots of putative transformants of *C. cinereus* LT2 and FM2. An additional 40 replicates of *C. cinereus* strain LT2 mycelia were also bombarded using a wet shot preparation. The bombardment conditions were; target distance 2.5 cm, vacuum 500 mmHg and M5 microcarriers.

Additional particle bombardment experiments involved 60 replicates of LT2 with mycelial pellets encased in type E agar 0.7% (w/v). All of these experiments used a 500 mmHg vacuum in conjunction with a target distance of 5 cm and M5 microcarriers fired at 2000 psi. When mycelial pellet tissue was used the target distance was increased to 5 cm as the shockwave produced on impact at 2.5 cm, forced the entire contents of the bombarded plate to be deposited on the floor of the vacuum chamber, irrespective of firing pressure.

Putative *trp+1* ballistic transformants of *C. cinereus* strain LT2 were obtained using the PDS1000/He device under the following conditions:-
The target material selected for bombardment was an overnight culture of oidia plated out at $>1 \times 10^8$ oidia ml^{-1} on cellophane discs resting on MYPGR and subsequently transferred to MMM post-bombardment. The ballistic parameters used for transformation were a firing pressure of 1300 psi, gap distance 1 cm, target distance 6 cm, using M5 microcarriers and a vacuum of 27 inches of Hg.

In total, 8 putative *trp+1* transformants were isolated from the bombardment of 12 plates under these conditions. This indicates that the PDS1000/He device is more efficient than the

HRI device as from a single experiment, 8 putative transformants were obtained, whereas the HRI device only gave 12 transformants from 6 experiments. The evidence would also suggested that coated microcarriers, dried prior to firing may be a more efficient means of transforming mycelia.

The initial procedures used for the transformation of *C. bilanatus* were based on the *S. cerevisiae* and *C. cinereus* data. The experimental evidence indicated that transformation did not occur using the HRI device with *C. bilanatus* strains Cb1-t2 and CbR8. Transformation did not occur even when osmotic stabilisers were incorporated (to minimise cellular trauma due to the disruption of the mycelial cell walls during and post bombardment) in the bombardment medium. A problem observed with osmotic stabilisers and *C. bilanatus* was a poor growth rate. The work of Lorito *et al.* (1993) found little evidence to support an osmoticum presence in the medium for *T. harzianum* and *G. virens* and nor did Bailey *et al.* (1993) with *Phytophthora* spp, both groups using the PDS1000/He device. This suggests that the use of osmotic stabilisers with *C. bilanatus* may serve no useful purpose and that the lack of transformation may be due to other factors.

4.3.2 Transformation in *Agaricus*

Optimal ballistic transformation parameters were not set due to an inability to identify in strains C82 *ura* of *A. bisporus* and R20 of *Agaricus arvensis* any ballistic transformants. Transformation was not observed using the HRI gene gun in conjunction with a range of transforming vectors (listed below) under a wide range of conditions. Only pAN7-1 was used with the PDS1000/He device using the following firing parameters: gap distance 1 cm, target distance 6 cm, microcarriers M5, firing pressure 1300 psi and a vacuum of 27 inches Hg. This did not result in ballistic transformants.

Vectors used to bombard *A. bisporus.*

Vector	Strain	Tissue type	No. of replicates	Transformants
pURA3.1	C82	Mycelia	40	None
pTRURA3-2	C82	Mycelia	40	None
pTRURA5-2	C82	Mycelia	40	None
pABU3H16	C82	Mycelia	40	None
pABU3H18	C82	Mycelia	40	None
pEF2	C82	Mycelia	40	None
pPL6	C82	Mycelia	40	None
pJR15	C82	Mycelia	40	None
pJD7	C82	Mycelia	40	None
pJD21	C82	Mycelia	40	None
pJD32	C82	Mycelia	40	None
pRS4	C82	Mycelia	40	None
pFG1	C82	Mycelia	40	None
pAN7-1	C82	Mycelia	40	None
pGft	C82	Mycelia	40	None
Agphleo	C82	Mycelia	40	None
XAgphleo	C82	Mycelia	40	None
UGHA	C82	Mycelia	40	None
pGft	C88	Lamellae	40	None
Agphleo	C88	Lamellae	40	None
XAgphleo	C88	Lamellae	40	None
UGHA	C88	Lamellae	40	None
pGft	R20	Lamellae	20	None
Agphleo	R20	Lamellae	20	None
XAgphleo	R20	Lamellae	20	None

Ballistic transformation procedures for *A. bisporus* were initially based on the *S. cerevisiae* and *C. cinereus* data, as no transformants were isolated from *C. bilanatus* by ballistics. The bombardment of *A. bisporus* failed to provide any evidence of transformation using either heterologous fungal genes or resistance markers in conjunction with the HRI gene gun. A 40 plate bombardment of C82 *ura* using pAN7-1 in association with the PDS1000/He device also failed to produce any transformants.

4.4.3 Ballistics

The data obtained from the *C. cinereus* experiments suggested that ballistic transformation of *C. bilanatus* tryptophan auxotrophs and the *A. bisporus* uracil auxotroph C82 *ura* was a viable proposition. However, there is no conclusive evidence of transformation using either the HRI device or the PDS1000/He device with these fungi. From the data it would appear that an explanation for the inability to transform *A. bisporus* is complex, involving more than one factor and is probably applicable to either a ballistic or protoplast mediated transformation system

The genetic markers used in this transformation study were based on either auxotrophs or antibiotic resistance. In the transformation experiments on *S. cerevisiae* and *C. cinereus* it was clear that bombardment of auxotrophic strains with wild type genes could restore prototrophy. Transformation could therefore potentially produce marked strains in *A. bisporus* which could be identified by Southern blotting. From this conclusion an extensive number of heterologous and putative homologous fungal genes for *URA3* were obtained and used to bombard the *A. bisporus* strain C82 *ura,* a *ura-3* auxotroph. Two resistance markers have been used in this study, hygromycin and phleomycin, as alternative selection systems. The attraction of resistance markers is their use is not dependant on a pre-existing mutation. Hygromycin was first used as a selectable marker in fungal transformation by Punt *et al.* (1987) in the ascomycete *A. nidulans* and has been used subsequently in other fungal transformations. One of the first reports of using phleomycin resistance as a selectable marker in fungal transformation was in the thermophilic fungus *Talaromyces* sp. CL240. Transformation of this fungus used *ble* constructs linked to an *A. nidulans gpd* promoter or a cloned chromosomal promoter of *T. reesei*, giving transformation rates of 20-50 transformants μg^{-1} DNA (Jain *et al.*, 1992).

In reversion experiments on C82 *ura*, it was observed that this strain when plated directly onto a selective or non-selective medium *ie.* MMM, MMM supplemented with uracil and phleomycin/hygromycin or CYM supplemented with phleomycin /hygromycin, showed no evidence of growth after 30 days. These results though conflicted with the results obtained when C82 *ura* was bombarded by phleomycin and hygromycin constructs. Here background growth was observed on selective and non-selective media. This was more pronounced when using the phleomycin constructs and has been linked to media composition (Punt & Hondel, 1992). Phleomycin resistance is higher in complex media *eg.* CYM due to the action of the yeast extract which forms complexes with phleomycin rendering it inert (Drocourt *et al.*, 1990); furthermore, when using osmotic stabilised MMM, reduced antibiotic activity is observed with hygromycin and phleomycin (Punt & Hondel, 1992). This background growth though was not observed when C82 *ura* was just bombarded with either hetologous or homologous *URA3* genes.

Schuren *et al.*, (1994) showed that in protoplast transformation of *S. commune* ascomycete regulatory sequences, linked to the *ble* gene of *Streptoalloteichus hindustanus,* did not yield transformants, the implication being that ascomycete regulatory sequences inhibited transcription in basidiomycetes, by acting as premature terminators in gene transcription (Schuren *et al.*, 1994) and contradicting Jain *et al.* (1992). Earlier, the work of Casselton & Fuente de la Herce (1989) on *C. cinereus,* suggested that transformation would not occur when using constructs containing an ascomycete promoter. This would appear to support the proposition of Schuren *et al.* (1994). Finally the evidence of Christiansen *et al.* (1995) with *Erysiphe graminis* indicated that transformation was most efficient using a homologous rather than a constitutive heterologous derived promoter. van de Rhee *et al.* (1994 & 1996a) demonstrated that protoplast mediated transformation of *A. bisporus* can be achieved by flanking the *hph* gene with ascomycete derived regulatory sequences *ie.* pAN7-1 but efficiency was poor. van de Rhee *et al.* (1996b) recently indicated that transformation is enhanced in *A. bisporus* by the incorporation of a 3.2 kb fragment of *A. bisporus* genomic DNA in the transforming construct. This, though probably indicates that integration is most likely to occur due to homologous recognition rather than being an indicator of efficient expression. The evidence of Peng *et al.* (1992 & 1993) who reported transformation in *P. ostreatus* using pAN7-1, although the gene did not integrate into the genome, also conflicts with the proposal of Schuren *et al.* (1994). The issue of homologous or heterologous sequences in transformation for *A. bisporus* has been complicated by attempts to reproduce transformation in other laboratories, using identical conditions to those described by van de Rhee *et al.,* (1996a): one laboratory being unable to reproduce transformation (van der Lende pers. comm.) and another obtaining unstable transformants but in very low numbers (Challen pers.comm.). A more pertinent point, is that the technique of van de Rhee *et al.* (1996a) maybe to a greater or lesser extent operator and possibly strain and lab dependent due *eg.* to water air humidity *etc.* There may be many other reasons why reliable (reproducible and efficient) transformation has not been achieved in *A. bisporus*. One of those reasons maybe linked to what occurs to the transforming DNA post - entry into the cell *eg.* methylation.

Methylation of integrated transforming DNA might have affected the results obtained. There is evidence from mammalian studies, which may apply to fungi, that methylation acts by either inhibiting the binding of a repressor or facilitating the binding of a methylation dependent activator to DNA (Tilghman, 1993). This suggests that if transformation occurs and the transforming DNA is over methylated or incorrectly methylated, repression of the transforming DNA may occur. Work by Mooibroek *et al.* (1990) on *S. commune,* reported low levels of transformation when the *hph* gene was used. These low levels of transformation were believed to be linked to failure of the gene to integrate and poor gene expression due to methylation, which would support the hypothesis of Tilghman (1993). Gessner & Raeder (1994) identified, in the transformation of *P. chrysosporium* with the *ble* gene, that phleomycin resistance was lost soon after mycelial regeneration. They proposed that *de novo* methylation of the vector DNA, inhibited further expression of the *ble* gene. Thus providing a potential explanation for the identification of so many putative phleomycin transformants in *A. bisporus*, subsequently found to be negative by Southern analysis. Correct methylation, *ie.* methylation of the transforming DNA, must be comparable with the host fungal genomic DNA for efficient integration and expression. Genomic DNA of *A. bisporus* contains low levels of 5-methyl-cytosine (Wilke & Wach, 1993) suggesting that in an effective transformation system for *A. bisporus* a construct containing similar levels of 5-

methylcytosine to those present in the genomic DNA would be useful. Another aspect to be considered in resolving the transformation of *A. bisporus* is the type of gene to be used.

When considering the extensive range of vectors used it was initially considered unusual to recover no transformants by ballistics. Therefore, there was the possibility that the HRI device, used for most of the work, may have been a partial cause of the problem. Unfortunately the BioRad PDS1000/He system was not available for most of the programme of work. This prevented exhaustive comparisons being made between the two systems; in particular, in making comparisons between delivery and vector composition. Brief experiments undertaken using the BioRad system have shown promise, in the recovery of *C. cinereus* LT2 transformants, (although none were recovered from *A. bisporus* C82 *ura*).

This suggests a weak link in the transformation of *A. bisporus* by ballistics or protoplasts or may be the structure of the transforming construct. Most of the constructs used to transform C82 *ura* contained heterologous fungal genes encoding for orotidine-5'- monophosphate decarboxylase (OMPdecase) an evolutionary conserved gene (Radford & Dix 1988; Radford, 1993). Two plasmid subclones though, contained putative *URA3* genes (pABU3H16 and H18), obtained from a genomic library of *A. bisporus* described by Challen *et al.*, (1996). These clones were identified using heterologous *S. commune* and *P. chrysosporium ura3* genes as probes. Therefore, the problem could be orientated around the identification of the *URA3* gene of *A. bisporus*. Subsequent seqence analysis of the pABU3H16 and H18 has revealed that they do not possess any sequence homology with related *URA3* genes (Challen pers. comm.).

4.4.4 RAPD-PCR

Listed below are the strains found to have unique genomic fingerprints with particular primers.

Primer No.	Strains with unique fingerprints
1	C43, 81, 82, 83, 98, 99, 100, AA0306, 0311
2	C43, 63, 92,93, 98, AA0311
3	C81, 82, 83, AA0306
4	C94, 97, AA0311
5	C43, 62
6	AA0311
7	C54, 81, 83, 86, 88, 96, 99, 100, 101, AA0311
8	C43, 62, 81, 82, 97, 98, 101, AA0306, 0311
9	C81, 82, 83, 92, AA0311
10	C43, 54, 81, 82, 83, 92, 93, 96, 97, 98, 99, AA0306, 0311
11	C43, 54, 81, 82, 83, 85, 86, 87, 90, 92, 96, 98, 101, AA0306, 0311
12	C43, 54, 81, 82, 83, 84, 85, 92, 97, 98, AA0306, 0311
13	C43, 81, 82, 86, 91, 98, 100, 101, AA0306, 0311
14	C54, 62, 63, 81, 82, 83, 84, 86, 89, 91, 92, 93, 98, 99, AA0306, 0311
15	C82, 86, 89, 92, 96, 97, 98, 100, 101, AA0306, 0311
16	C43, 54, 81, 82, 92, 99, 100, AA0306, 0311
17	C43, 54, 81, 82, 85, 91, 94, 96, 97, 101, AA0306
18	C43, 54, 62, 83, 85, 86, 92, 97, 98, 99, 100, 101, AA0306, 0311
19	C43, 54, 63, 81, 83, 85, 86, 87, 90, 98, AA0306, 0311
20	C43, 54, 82, 83, 84, 91, 92, 97, 99, AA0306, 0311

Dendrograms for all 20 primers are available and five are included here for illustrative purposes in the Appendices. In Dendrogram 1, the strains are grouped based on the data from the use of Primer 1. Many of the hybrids cannot be distinguished.

In Dendrogram 2 based on Primer 2 many of the strains cannot be distinguished indicating the homogeneity of the *A. bisporus* genome.

In Dendrogram 3 based on primer 12 the brown strains (C81, 83, 92 and 97) are delimited into a specific grouping.

In Dendrogram 4 based on primer 11 many of the strains can be distinguished.

In Dendogram 5 based on primer 13 there are six polymorphisms linked to cap colour.

Finally, Dendrogram 6 combines data from all primers and show sthat each strain can be recognised individually.

Preliminary indications gave the impression, from the high number of common polymorphisms identified, that commercial *A.bisporus* strains were homogeneous. This supported anecdotal evidence of Kligman (1950) and Fritsche (1978) who both implied that many commercial white strains were derived from a chance cluster of pure smooth capped white mushrooms, found in a bed of cream coloured *A. bisporus* in 1927 and that hybrid strains were similar because of common parents and piracy.

All 26 strains of *A. bisporus* when screened by the 20 primers used, gave products, ranging in size from 2200 bp to 305 bp. Within the 26 strains screened there was a wide variation in the number of polymorphisms produced by individual primers *e.g.* primer OPA-11 gave 14 scorable polymorphisms, whereas primer OPA-5 only 7 scorable polymorphisms. Furthermore, the primers identified many common polymorphisms across the 26 genomes. Only three primers OPA-15, 16 and 20 did not share common polymorphisms across the genomes of the 26 strains.

A closer examination of the data showed that individual primers were unable to differentiate between all 26 strains. In fact one primer, Primer 6, was only able to differentiate 1 strain, AA0311 from the 26 screened. In contrast primer OPA-14 provided sufficient polymorphisms for 16 of the strains studied to be differentiated. Further analysis indicated that the wild type strains AA0306 and AA0311, the strains of long established varieties (historical) C43 and C54 and the monokaryon C82 *ura* gave consistently unique fingerprints with the majority of primers used.

Cluster analysis of the RAPDs appeared at first not to be linked to any discernible phenotypic feature. Closer study showed that some of the primers were identifying polymorphisms linked to the brown phenotype (Dendrogram 3).

The most informative primer that identified a link to the brown phenotype was OPA-13. This primer provided clear evidence for a polymorphism that linked pigmentation, a genetically regulated event, to a specific strain fingerprints. The primer identified 6 shared polymorphisms in the browns that contributed to their grouping away from the hybrids. The most important factor that linked the browns was the presence of a 1085 bp polymorphism not present in any other strain, indicating that the brown phenotype was linked to this polymorphism. The differences in the wild strains were either due to their wild germplasm or geographical origin. Three of the commercial strains C83, 92, 97 showed 100% similarity, yet C81 showed the most dissimilarity from both the commercial and wild type strains. This dissimilarity could be an indicator that C81 has been exposed to less breeding manipulation than the other commercial brown strains.

When cluster analysis was undertaken on the hybrid strains the evidence indicated that many of these hybrids, if not identical, shared a common point of origin. This was supported by the frequent grouping of many hybrid strains at a 100% similarity using any one of the 20 primers. Furthermore the consistent polymorphic uniformity of the hybrid genomes supports this proposition.

In particular this was seen with the hybrid strains C62 and C63 which have a common ancestry (Fritsche, 1983). Therefore the level of similarity seen in the sharing of molecular polymorphisms acts as an indicator of there commonality. Evidence from primers OPA-3, 4, 6, 7, 9, 10, 13, 15, 16, and 20, consistently grouped strains C62 and 63 at a 100% similarity due to their polymorphic commonality.

When primers OPA-1, 4, 8, 12 and 19 were examined only one common band of variable size was observed in both hybrids and brown strains. The relevant dendrograms though indicated a high level of homogeneity within the hybrids, as the vast majority of strains were grouped at 100 % similarity. The supporting evidence provided by the spawn companies, implied that hybrid strains *eg.* C62, 93, 94 and 101 were interrelated. The evidence from primer OPA-15 showed a 100 % similarity between strains C93 and 94, yet it grouped strains C62 and 101 separately. When the data from the other primers was studied, strains C93, 94 and 101 derived from the same spawn company were grouped separately from those of C62 and 63.

The differences were seen in the absence of 1480, 1350, 675 and 570 bp polymorphisms, in C93, 94 and 101, using primer OPA-20. This implies that the right primer will detect subtle genomic variations between the genomes of commercial strains. Primer OPA-3 gave a similar result, the difference being, only 1 polymorphism of 540 bp was absent in C93, 94, and 101. When primers OPA-7, 10, 14 and 18 were used they identified two common polymorphisms of variable size.

The results obtained by primers OPA-11 and 17 identified 3 common polymorphisms of variable size. Cluster analysis of this data was unable to discern any clear relationship between spawn companies, hybrid or brown strains. Cluster analysis of the primers OPA-5 and OPA-13 identified little of diagnostic value within the hybrid strains. The information derived from the binomial matrix and dendrograms reflected more upon the genomic homogeneity of the commercial strains of *A. bisporus* than any other characteristic.

RAPD screening using the primers OPA-2, 3, 6 and 9 identified 5 common polymorphisms of variable size. The data from the binomial matrix and dendrograms had the vast majority of these strains grouped at a 100 % similarity and were therefore, considered of little value for the purposes of strain fingerprinting.

Cluster analysis of the 26 strains using all 20 primers from the binomial matrix was able to find sufficient variation to discriminate between all the strains. This implies that using RAPDs for fingerprinting commercial strains of *A. bisporus* was a viable possibility. The evidence also acts as an indicator to the industry that there must be an investment in new strain development to reverse the trend of decreased genetic variation in the genomes of commercial strains.

When the matrix data was summarised, using all 20 primers the RAPDs provided sufficient information for the cluster analysis programme to differentiate between the commercial strains. The dendrogram indicated, that many commercial strains were probably interrelated, with 17 strains showing 90% and greater similarity. Furthermore, the common point of origin for the 17 strains exhibiting 90% and greater similarity is 84%. This could be an indication of the problems of current breeding programmes. There is only a small pool of germplasm to draw upon. In addition it reflects on the low number of polymorphic dissimilarities identified in the commercial strains.

An interesting point identified by this study was that wild types AA0306 and AA0311, had a genome 75% similar to commercial *A. bisporus* strains. The differences are a possible reflection of the secondary homothallic life cycle which has prevented out-crossing and restricted the level of genetic variation both within wild strains and strains derived from commercial breeding programmes. The data were unable to group commercial strains according to their point of origin *ie.* spawn company. It was noted that the commercial brown strains C81, 83, 92 and 97 were grouped with the wild strains away from all the hybrid strains, other than C98 and the historical strain C43. This supported the evidence discussed earlier which linked a polymorphism, using OPA-13 to the brown phenotype. The out-grouping of C43 and 98 maybe an indicator that these strains have been subjected to less intensive genetic manipulation.

4.4 Conclusions

Two approaches were used to try and establish methods for strain protection in *Agaricus bisporus* (i.) transformation (ii.) appraising natural variation.

Ballistic transformation was successful in the yeast, *Saccharomyces cerevisiae* and in the ink-cap mushroom *Coprinus cinereus* (and in a prior study in the ink-cap mushroom *Coprinus bilanatus*). Transformants were not obtained using ballistic mediated delivery in the cultivated mushroom, *A. bisporus*. The attraction of transformation for strain protection is that it permits the unambiguous marking of a strain. A specific and unique DNA sequence can be inserted into the genome which will not be present in any other strains. The only potential disadvantage of this method is that there could be consumer resistance to a transgenic mushroom. However, it is only a question of time before transformation becomes a routine technology in *Agaricus bisporus*.

RAPD-PCR was able to distinguish between all the starins in the sample of 26 but only when all the data was pooled. As it depends on natural variations in the DNA its application is limited to where such variation can be detected. The use of other primers may detect further polymorphisms. For strains within the sample of 26 the data produced here provides a means of identification. For strains outside the sample the specific data is less valuable but new data using the same primer set could be obtained to specify the strain.

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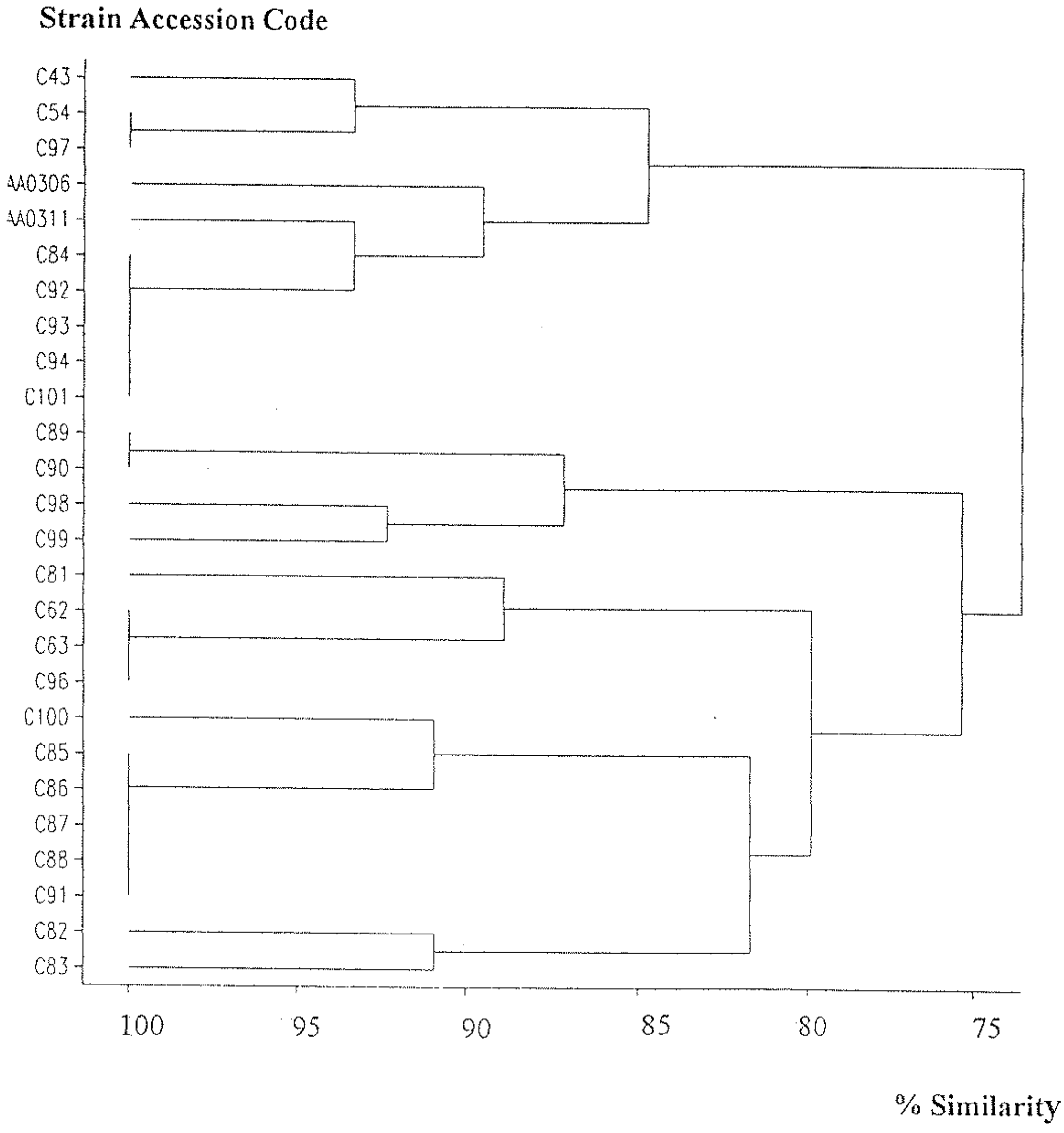
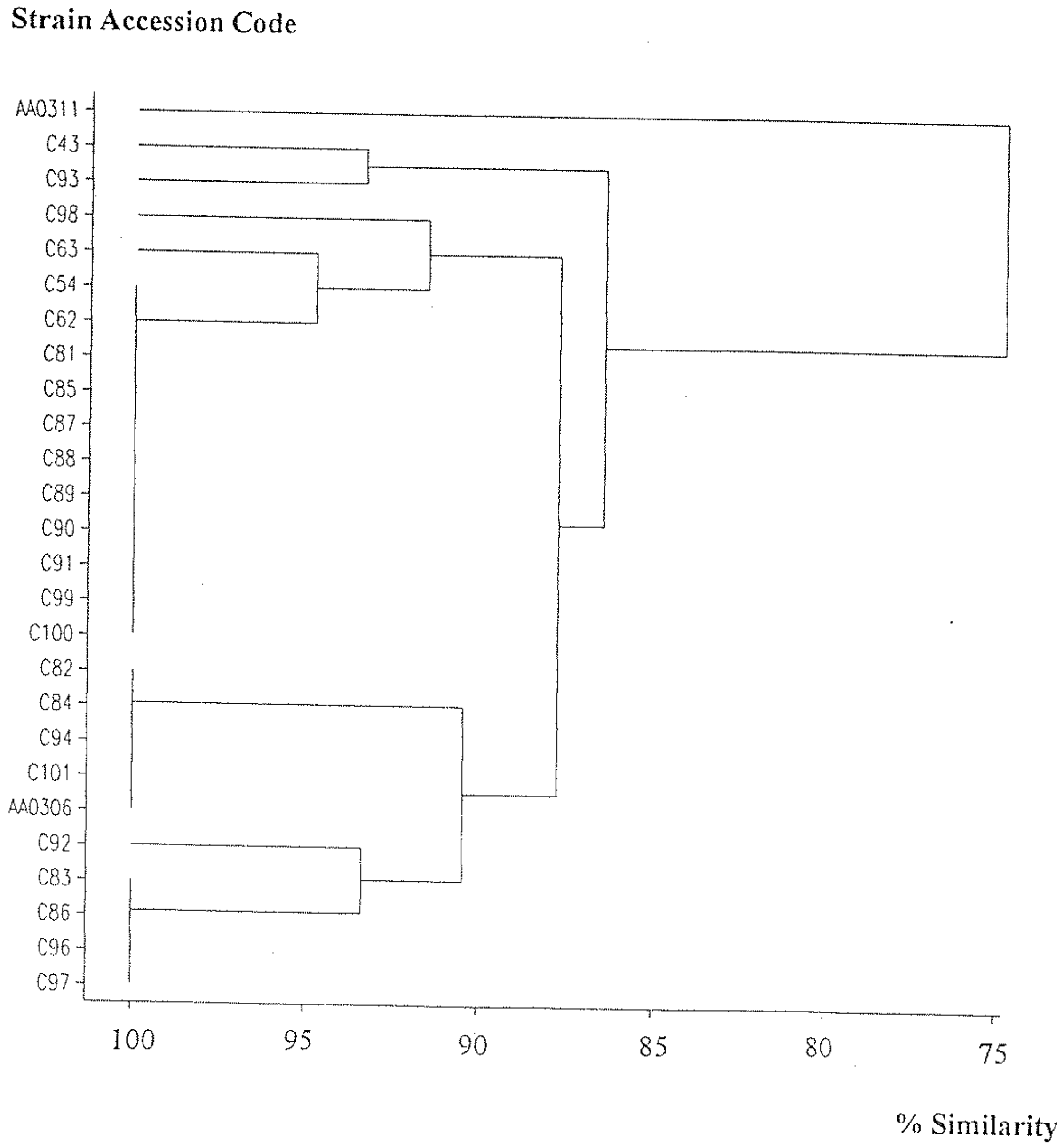
APPENDICES

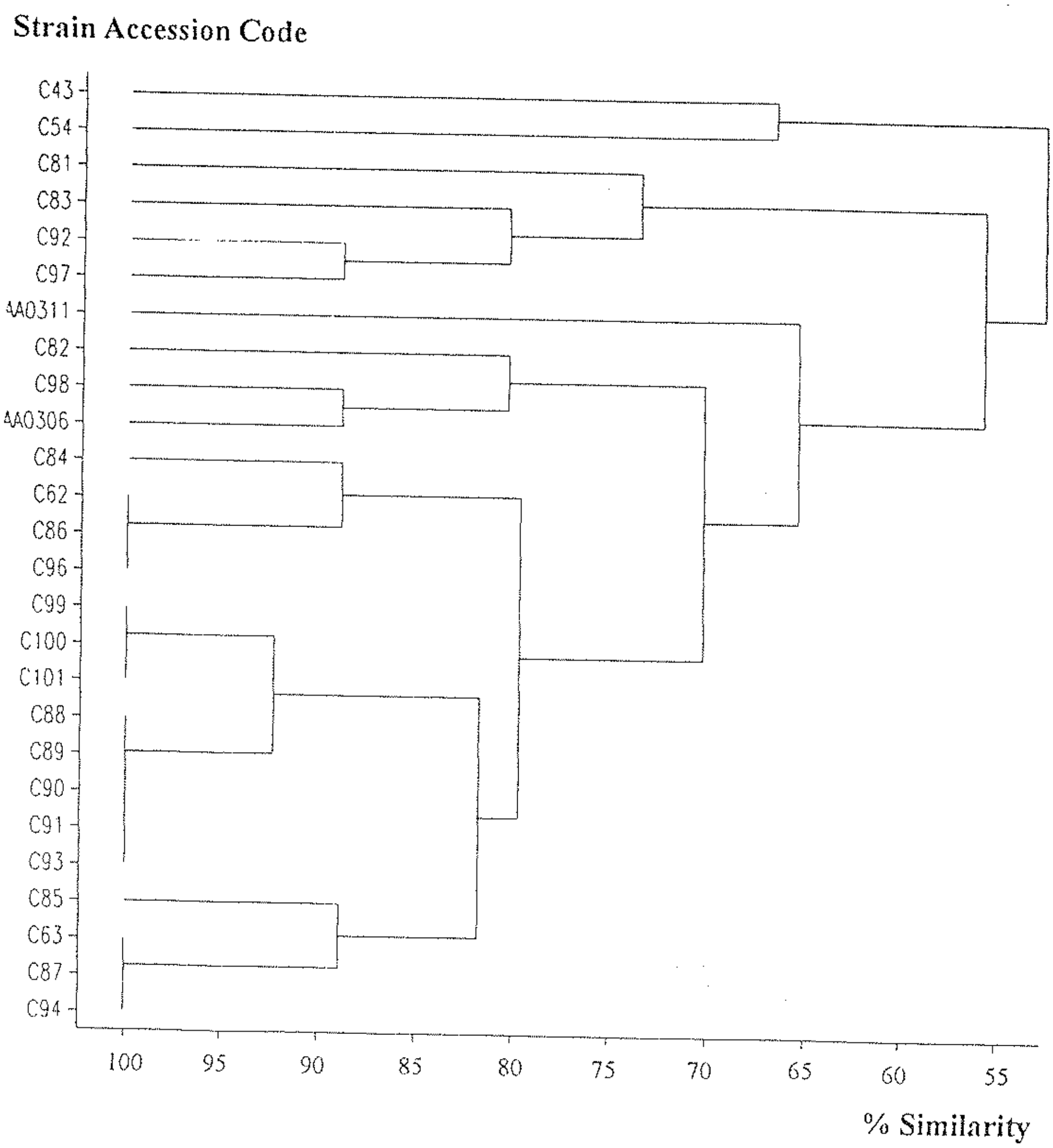
Dendrogam 1 – Similarity matrix for *A. bisporus* strains based on Primer 1.



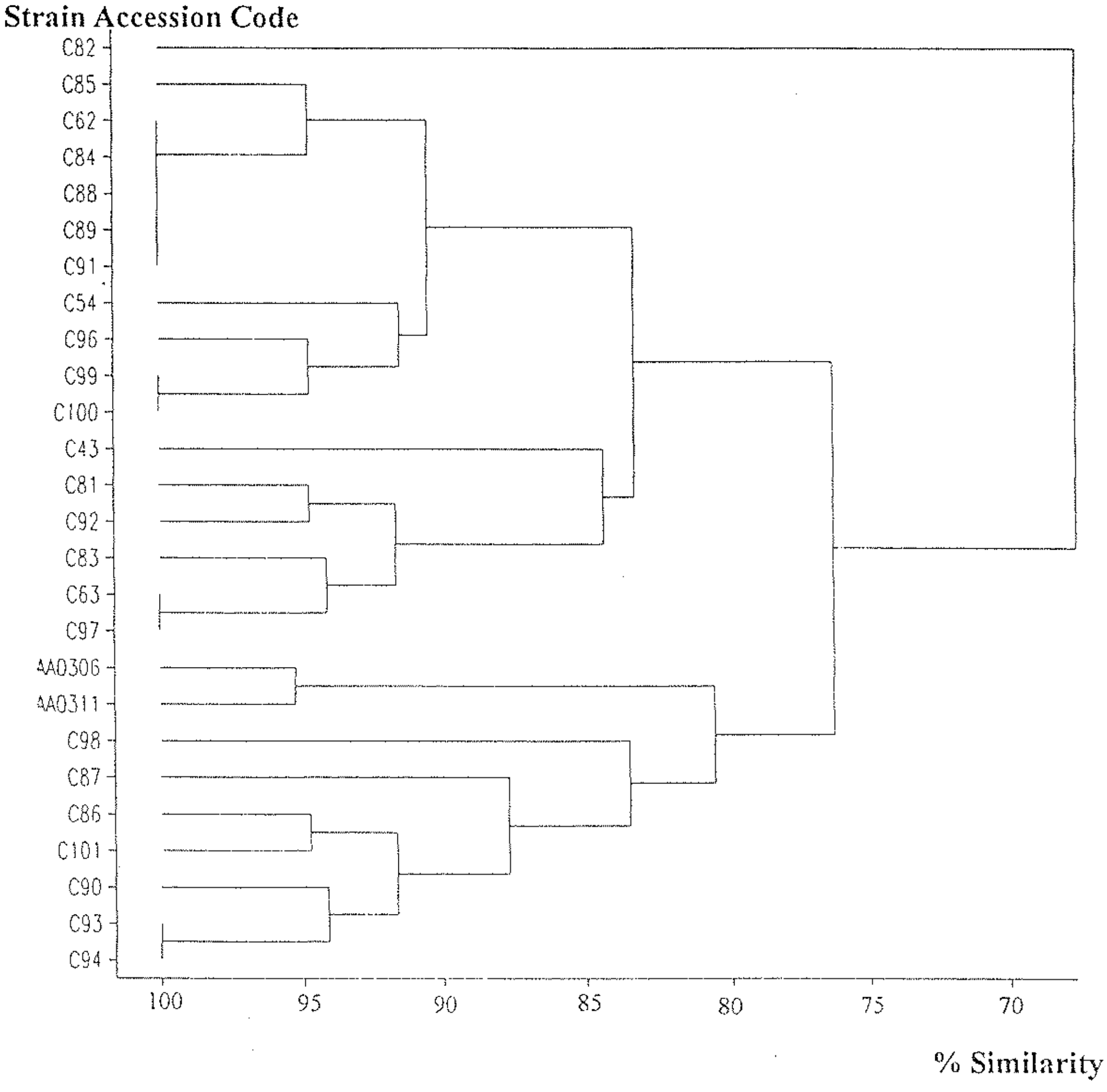
Dendrogam 2 – Similarity matrix for *A. bisporus* strains based on Primer 2.



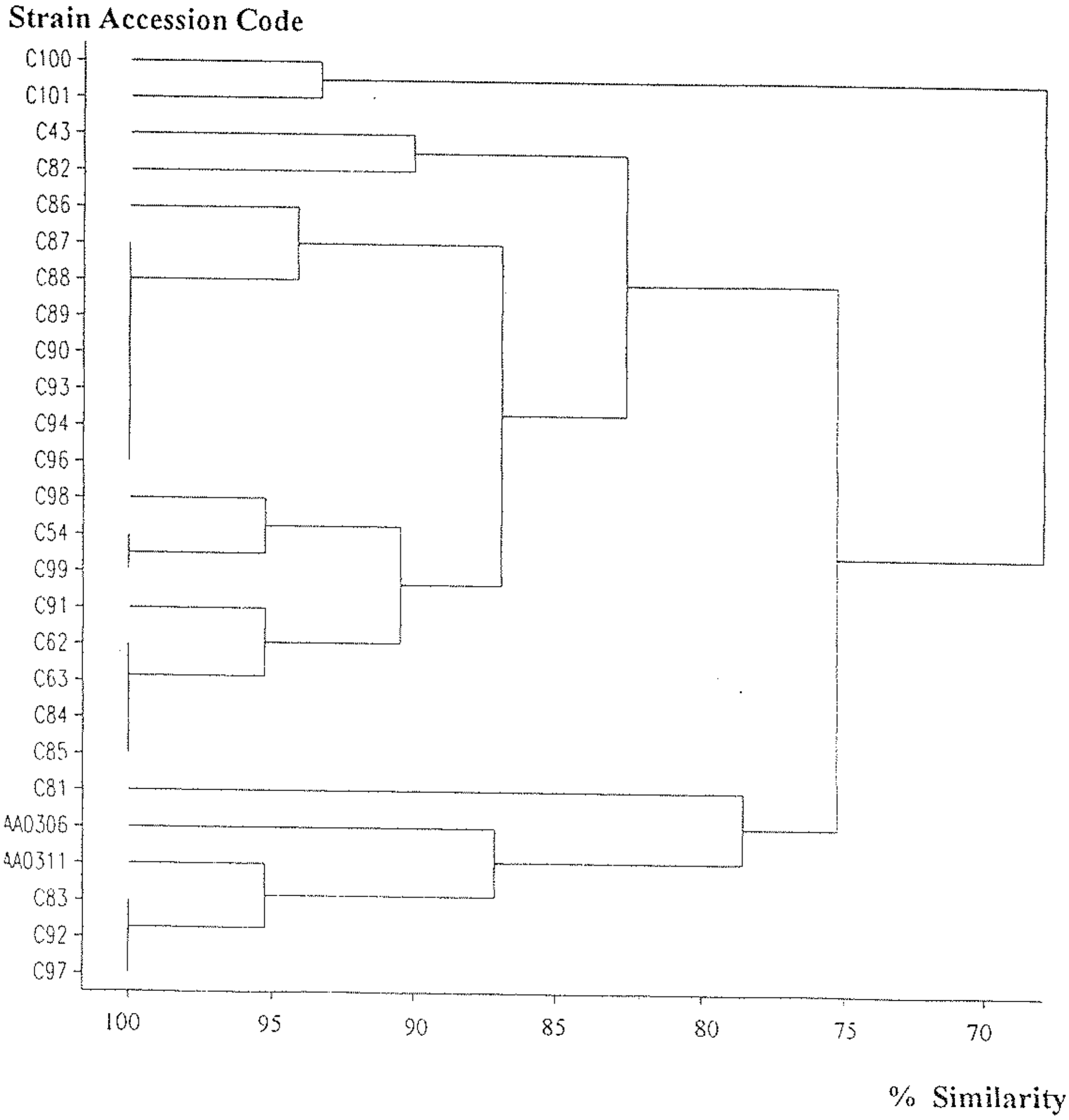
Dendrogam 3 – Similarity matrix for *A. bisporus* strains based on Primer 12.



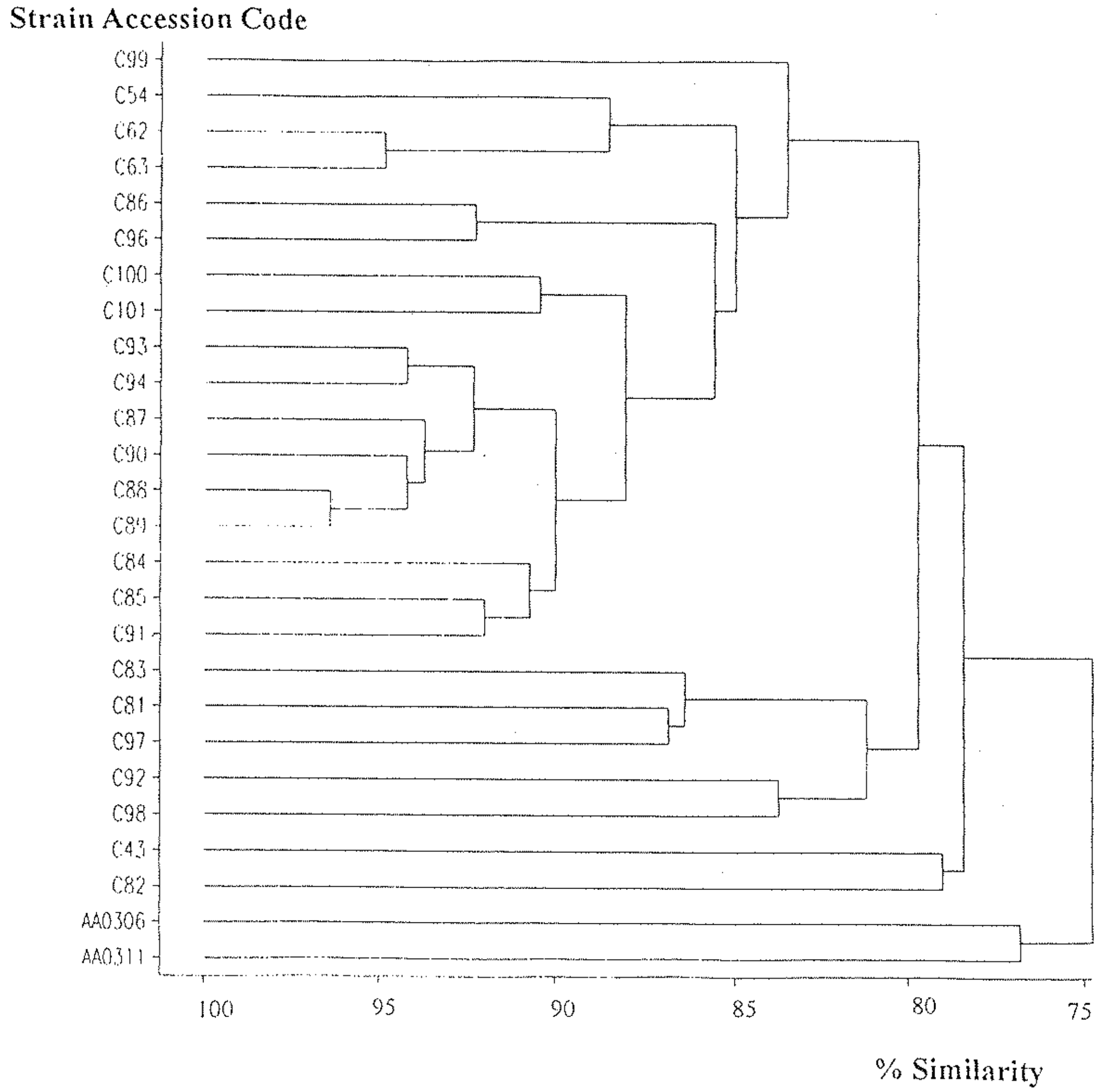
Dendrogam 4 – Similarity matrix for *A. bisporus* strains based on Primer 11.



Dendrogam 5 – Similarity matrix for *A. bisporus* strains based on Primer 13.



Dendrogam 6 – Similarity matrix for *A. bisporus* strains based on all primers.



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