CHAPTER 6

NEW PERSPECTIVES ON THE GENETICS OF *PLEUROTUS*

J. F. Peberdy, Aidah M Hanifah and Jian-hua Jia

Department of Life Science, University of Nottingham, Nottingham UK, NG7 2RD.

1. INTRODUCTION

The production of cultivated mushrooms has a long history and up until the recent past has not been greatly influenced by the major developments of biotechnology that have occurred in the past two decades. However, research on the genetics and molecular biology, and on biologically important metabolites has started to have an impact on this major multi-million dollar earning industry.

The production of *Pleurotus* spp. (the Oyster mushroom) along with all other forms has increased significantly in the last seven years. In 1990 a 538% increase in production took *Pleurotus* to the second position in world production (Table 1). To meet demand it has been necessary to exploit the metabolic and physiological diversity of the fungus with respect to substrate utilisation. Utilisation of these materials is clearly dependent on the ability of *Pleurotus* to secrete a range of enzymes including peroxidases, laccases, cellulases, hemicelluloses, xylanases and ligninases (Toyama & Ogawa, 1974; Ulezlo *et al*, 1975; Dauglis & Bone, 1977).

TABLE 1. World production of the major edible mushrooms in 1989/1990.

Species	Fresh Weight of Mushrooms*		% Increase
	1986	1989/90	
Agaricus bisporus	1,227	1,424	16.1
Lentinula edodes	314	393	25.5
Volvariella volvacea	178	207	16.3
Pleurotus spp.	169	909	537.9
Auricullaria spp.	119	400	336.1
Flammulina velutipes	100	143	43.0

* Metric tons x 1000
(After Chang, personal communication).

Improved substrate utilisation and conversion leading to higher mushroom yields are clear goals in all areas of mushroom production. Whilst empirical strategies undoubtedly play a role the existence of a natural system for breeding based on the reproductive process (of which the mushroom itself is a key part) provides an obvious rational alternative. In the medium to longer term other strategies using recombinant DNA technology may also play a role particularly in enzyme engineering to improve substrate utilisation.

The reproductive strategies of several basidiomycetes, including some commercial species, have been investigated. Three fertility mechanisms have been described (Table 2). With the exception of *Agaricus bisporus* the other edible species that have been studied exhibited a heterothallic system. Thus *Pleurotus ostreatus* and *P. cystidiosus* are typically tetrapolar with two multi-allelic loci. In such fungi crosses between compatible mycelia derived from single spores can provide a practical method for hybridisation and strain improvement. Fruiting of the dikaryon is an obvious indication of fertility and the progeny of successful crosses may be propagated by tissue culture from the fruit bodies. Interstrain crosses using compatible monokaryons derived from fruit bodies from divergent sources provide an obvious source of potentially useful hybrids.

The advent of protoplast technology in fungi in the early 1970's (Peberdy, 1979) led two useful areas of applications in the basidiomycetes. Isolation and subsequent regeneration of protoplasts derived from mycelium of dikaryons gave rise to a mixed population of dikaryotic and monokaryotic progeny. In the latter case, although the progeny were of only two mating types diversity in genotype was observed. Such de-dikaryotisation has been shown to be a useful procedure in generating genetically variable monokaryons for crossing purposes. Dikaryons derived as protoplast regenerants may have some advantages over the original strain. Magae *et al.* (1985) showed that some regenerants from *P. ostreatus* yielded a 5.7 - 8.1% increase in fruit body weight.

Protoplast fusion has been used as a means of establishing crosses in several edible mushrooms. The value of this technique lies in the potential to generate hybrids. Several reports of hybrids produced in this manner have been published, however, the realisation that some of the so-called species used can be mated by conventional techniques (see later) casts doubt on their validity as interspecies hybrids. Furthermore better substantiation of hybridity is necessary and is possible

TABLE 2. Genetic structures of mating systems identified in agaric basidiomycetes.

Fertility Mechanism	Polarity	Structure	Examples
Primary Homothallism			*Coprinus steritirus* (Raper, 1966)
Secondary Homothallism	Bipolar	1 locus/2 alleles	*C. sassii* (Kemp, 1975)
	Bipolar	1 locus/multiple alleles	*C. bisoporus* (Kemp, 1980) *Agaricus bisporus* (Elliott, 1978)
	Tetrapolar	2 loci	*C. plagioporus* (Fincham & Day, 1971)
Heterothallism	Bipolar	1 locus/multiple alleles	*Pholiota nameka* (Arita & Takemaru, 1962)
		2 loci/multiple alleles	*Pleurotus ostreatus* (Eugenio & Anderson, 1968)
			Lentinula edodes (Takemaru, 1961)

TABLE 3. Mating-type alleles in *Pleurotus species*.

Species	Number of Alleles		Reference
	A	B	
P. ostreatus	17	20	Eugenio & Anderson, 1968
P. sapidus	31	36	Anderson *et al,* 1973
P. cystidiosus	6	6	Moore, 1985

through the use of a range of molecular markers such as RFLPs and RAPDs, and the use of molecular karyotypes generated by pulsed-field electrophoresis techniques.

2. MATING TYPE ANALYSIS IN *PLEUROTUS SAJOR-CAJU*

Previous studies of *P. sajor-caju* and other *Pleurotus* species (Eugenio & Anderson, 1968; Roxon & Jong, 1977) indicated that the genus had a typical tetrapolar mechanism of incompatibility with mating factors designated A and B. The number of alleles found for the two mating factors varies in different species (Table 3). However, the numbers obtained will reflect the number of dikaryons analysed. Roxon and Jong (1977) described the mating system of *P. sajor-caju* with only two A and two B alleles. We therefore initiated a study to understand further the mating system of this fungus as a basis for future genetic improvement.

Sixteen dikaryotic strains derived from Europe, North America, the Far East and South East Asia were used in this investigation (Table 4). Mating type testers $A_1\ B_1$, $A_2\ B_2$, $A_1\ B_2$ and $A_2\ B_1$ were obtained from the ATCC. The dikaryons were fruited on wheat straw based substrates and at least 30 single basidiospores were isolated from one sporocarp. Germination of spores was never less than 60%.

The individual monokaryons were identified for mating type and the basis of mating reactions with the master strains on agar plates. Clamp connections were seen in the contact zone of compatible mating after 7-10 days. The formation of clamp connections was consistent with all compatible matings as confirmed by random fruiting trials.

Of the sixteen strains examined, 14 proved to have the $A_1\ A_2\ B_1\ B_2$ genotype. Strains designated 32-8 and 32-15 in our collection, from Thailand and Bhutta (India) respectively were both of different genotypes and therefore indicating the existence of at least 6A and 6B alleles.

In non-complementary crosses several reactions were observed. False clamp connections or pseudoclamps were frequently found in barrage zones where common B alleles were introduced as described by Roxon and Jong (1977) but also in some common A matings as has also been described in *P. ostreatus* (Eger, 1978). The flat mycelium described for common A matings in *Schizophyllum commune* (Raper, 1966) was found in both common A and common B matings in *P. sajor-caju.*

The segregation frequencies of the four mating types was very variable in spore progeny from fruit bodies. In one extreme case, strain 32-4 obtained from France, segregants with the $A_2\ B_2$ alleles were either non-viable, or as in the case of $A_2\ B_1$ of very low viability. This suggests that the A_2 allele may be linked to a deleterious or lethal gene.

The identical nature of the mating type alleles of the majority of the strains investigated raises the question of their origin from a single common progenitor. This perception was confirmed by

TABLE 4. Mating-type analysis of *P. sajor-caju*.

Strain No	Origin	Mating-type
32-1	Hong Kong	$A_1A_2B_1B_2$
32-2	Korea	$A_1A_2B_1B_2$
32-3	France	$A_1A_2B_1B_2$
32-4	France	$A_1A_2B_1B_2$
32-5	Brunei	$A_1A_2B_1B_2$
32-6	Malaysia	$A_1A_2B_1B_2$
32-7	Taiwan	$A_1A_2B_1B_2$
32-8	Thailand	$A_3A_4B_3B_4$
32-9	China	$A_1A_2B_1B_2$
32-10	Korea	$A_1A_2B_1B_2$
32-11	China (Shanghai)	$A_1A_2B_1B_2$
32-12	India	$A_1A_2B_1B_2$
32-13	Italy	$A_1A_2B_1B_2$
32-14	Italy	$A_1A_2B_1B_2$
32-15	India	$A_5A_6B_5B_6$
32-16	Canada	$A_1A_2B_1B_2$

studies on isozyme profiles (Hanifah, 1993) and molecular karyotypes (Jia, J.H. unpublished data). For enzymes previously identified as showing allozyme variation in *Pleurotus* (May & Royse, 1988) were investigated by polyacrylamide gel electrophoresis. In the cases of alcohol dehydrogenase, malate dehydrogenase and lactate dehydrogenase the band patterns obtained were identical for all sixteen strains. With esterase, however, some polymorphism was evident; the two strains with different mating type alleles (32-8 and 32-15) presented different isozyme patterns. Of the strains with $A_1A_2B_1B_2$ mating type all were identical for esterase except 32-4 which had a unique profile.

3. BREEDING STRATEGY IN *PLEUROTUS SAJOR-CAJU*

Several of the dikaryons produced in the course of mating-type allele analysis were investigated as part of an assessment of the potential of recombination as a breeding tool in this mushroom. Comparisons were made of colony radial growth rate, spawn run, mushroom yield and computed biological efficiency for several of the hybrid dikaryons. The data (Table 5) shows that even in such a limited trial some improvement was possible through such matings. It was interesting to observe that crosses between monokaryons derived from dikaryons from the presumed common progenitor (i.e. 32-1 and 32-4) also gave some improved progeny.

4. MATING INTERACTIONS BETWEEN *PLEUROTUS* SPECIES

There are several reports concerning interspecies crosses involving *Pleurotus* species based on protoplast fusion (see Yoo & Cha, 1993 for review; Ogawa, 1993). In view of the uncertainties in the taxonomy of the genus the status of some of these crosses as interspecies crossed has to be

TABLE 5. Characteristics of *P. sajor-caju*.

Parents Hybrids	Colony Growth[1] (cm)	Spawn Run[1] (days)	Yield[1] (g)	Biological Efficiency[3] (%)
32-1 x 32-2				
32-1	3.68	27a[2]	238.9b	59.7
32-2	3.82	27a	210.0bc	52.5
H1	4.27	46de	114.4fg	28.6
H2	3.90	28ab	119.5fg	29.9
H3	2.90	30ab	106.7g	26.9
H4	4.90	28ab	81.9hi	20.5
32-1 x 32-4				
32-4	4.13	28ab	230.76	57.7
H9	4.50	35bc	235.36	58.8
H10	4.37	29ab	275.2a	68.7
H11	4.07	28ab	231.96	58.0
32-1 x 32-5				
32-5	4.60	32ab	159.2de	39.8
H12	4.18	33abc	47.3ij	11.8
H13	3.18	40cd	31.9j	8.0
H14	2.45	45de	141.9efg	35.5
H15	3.30	55f	109.9g	27.5
32-1 x 32-8				
32-8	2.33	26a	242.4b	60.6
H24	7.93	25a	215.0c	53.7
H25	5.80	27a	256.9b	64.2
H26	6.10	25a	283.0a	70.7
H27	7.63	25a	218.4c	56.0

[1] All measured values are means of 3 replicates
[2] Mean values sharing similar letters (ie a,b, c etc) in the same column do not differ significantly at $P<0.05$.
[3] Biological efficiency was calculated as the percentage of fresh mushroom yield over the dry weight (400g/bag) of the wheat straw/bran substrate.

questioned. There are also several reports of hybrids between supposed species being produced by hyphal anastomosis thus raising the question whether the strains involved are in fact distinct species.

Recent experiments (Jia, J.H. unpublished data) have been directed at the generation of dikaryons between strains identified as different species, and an assessment of the fertility of the products. Differences in these results (Table 6) compared to the published data emphasise the importance of the criteria used in defining speciation in *Pleurotus*. The formation of basidiocarps by a dikaryon must imply that the monokaryotic progenitors are in fact the same species. Based on the results of these experiments we conclude that the strains of *P. ostreatus*, *P. florida*, *P. columbinus* and *P. sapidus* are isolates of a single species.

TABLE 6. Compatibility of interspecific crosses in *Pleurotus*.

	P.ostreatus	*P.florida*	*P.sajor-caju*	*P.pulmonarius*	*P.colombinus*	*P.sabidus*
P.ostreatus		+	-	-	+	+
P.florida	+		-	-	+	+
P.sajor-caju	+	+		-	-	-
P.pulmonarius	+	+	+		-	-
P.colombinus	+	+	-	-		+
P.sapidus	+	+	-	-	+	

\+ Compatible and fertile mating.
\- Incompatible mating.

TABLE 7. Estimated chromosome size of *Pleurotus* spp. in megabase pairs

Chromosome	*P.ostreatus*	*P.florida*	*P.sajor-caju*	*P.pulmonarius*	*P.colombinus*	*P.sapidus*
IX	5.7	5.1	5.7	5.7	5.7	5.5
VIII	5.3	4.7	5.1	5.3	4.7	4.6
VII	4.6	4.1	3.1	5.1	4.3	4.3
VI	4.3	3.8	2.5	4.5	3.6	3.8
V	3.6	2.7	2.0	3.1	3.1	3.3
IV	3.3	2.2	1.6	2.7	2.5	2.3
III	1.8	1.6		2.0	1.8	1.4
II	1.6	1.1		1.6	1.4	0.9
I	1.1	0.7				
Genome size (Mb)	31.3	26.0	20.0	30.0	27.1	26.1

5. MOLECULAR KARYOTYPES IN *PLEUROTUS*

In view of the observations made in the mating experiments between presumptive different species of *Pleurotus* it was of interest to re-examine and compare the karyotype patterns of strains that proved to be compatible when crossed. Furthermore improvements in the pulsed-field gel electrophoresis procedure, as compared to that developed by Fox (1991) and discussed in Peberdy & Fox (1993), has enabled better resolution of the putative chromosome bands. Compared to the earlier report it has now been possible to resolve more DNA bands in several species of *Pleurotus* thereby obtaining a more reliable estimate of the size of the genome. Of the species (strains) investigated (Table 7) the smallest genome (20Mb) was found in *P. sajor-caju*. Several isolates of this species, of presumed common genetic ancestry, gave identical karyotypes. The three compatible species, *P. florida, P. columbinus* and *P. sapidus* had very similar karyotypes further supporting the conclusion that these isolates could be the same species. However, the genome of the fourth compatible species, *P. ostreatus* is larger. If these are the same species then this observation records possible polymorphism at the gross chromosome level. Current work is directed to the development of a physical map for these species which can then be used to analyze the homology between the different chromosomes and the potential for their recombination.

6. FUTURE OUTLOOK

Unlike other agricultural and horticultural crops most of the edible mushrooms have not been developed through extensive breeding programmes. Conventionally new strains were obtained using tissue culture isolation techniques to recover dikaryons from fruit bodies which had been selected for specific desirable characteristics. More recently the situation has changed and in the last decade the potential of breeding has been recognised for many edible mushrooms including *Agaricus bisporus*, *Lentinula edodes* (Ellingboe, 1993), and *Flammulina velutipes* (Kitamoto *et al*, 1993; Kinugawa, 1993). Clearly with the recent developments in fungal biotechnology and gene technology, combined with a breeding programme based on sexual recombination, a broader strategy for strain development in many edible fungi is now conceivable. Breeding provides the only possibility, certainly in the short to medium term, for improvement of multigenic properties, e.g. growth rate, gross nutritional value, but for more specific characteristics such as improved substrate utilisation, recombinant DNA approaches through the manipulation of specific enzymes involved in the process, has greater potential. The importance of high value medically useful products from the basidiomycete fungi will also provide an impetus for new developments based on genetic engineering strategies.

REFERENCES

ARITA, I. & TAKEMARU, T. (1962). Some problems on the mating system of *Pholiota nameko*. *Report of the Tottori Mycological Institute (Japan)* **2**, 1-10.

DAUGLIS, A. J.& BONE, D. H. (1977). Submerged cultivation of edible white rot fungi on tree bark. *European Journal of Applied Microbiology* **4**, 156-168.

EGER, G. (1978) Biology and breeding of *Pleurotus*. In *The Biology and Cultivation of Edible Mushrooms,* pp. 497-519. Edited by S.T. Chang & W.A. Hayes. New York: Academic Press.

ELLIOTT, T. J. (1978). Comparative sexuality in *Agaricus* species. *Journal of General Microbiology*

107, 115-122.
EUGENIO, C. P. & ANDERSON, N. A. (1968). The genetics and cultivation of *Pleurotus*. *Mycologia* **60**, 627-634.
FINCHAM, J. R. S. & DAY, P. R. (1971). *Fungal Genetics* 3rd ed. Oxford: Blackwell.
HANIFAH, A. H. M. (1993). Studies on the mating biology of *Pleurotus sajor-caju*. M.Phil. Thesis, University of Nottingham.
KEMP, R. F. O. (1975). Breeding biology of *Coprinus* species in the section *Lanatuli*. *Transactions of the British Mycological Society* **75**, 29-35.
KEMP, R.F.O. (1980). Bifactorial incompatibility without clamp connections in the *Coprinus patouillardii* group. *Transactions of the British Mycological Society* **74**, 357-362.
KINUGAWA, K. (1993). Physiology and the breeding of *Flammulina velutipes*. In *Genetics and Breeding of Edible Mushrooms,* pp.86-109. Edited by S.T. Chang, J. A.Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
KITAMOTO, K., NAKAMATA, M. & MASUDA, P. (1993). Production of a novel white *Flammulina velutipes* by breeding. In *Genetics and Breeding of Edible Mushrooms,* pp.65-86. Edited by S.T. Chang, J. A. Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers,
MAGAE, Y., KAKIMOTO, Y., KASHIGAWI, Y. & SASAKI, T. (1985). Fruiting body formation from regenerated mycelium of *Pleurotus ostreatus* protoplasts. *Applied and Environmental Microbiology* **49**, 441-442.
MAY, B. & ROYSE, D. J. (1988). Interspecific allozyme variation within the fungal genus *Pleurotus*. *Transactions of the British Mycological Society* **90**, 29-36.
OGAWA, K. (1993). Interspecific and Intergeneric hybridization of edible mushrooms by protoplast fusion. In *Genetics and Breeding of Edible Mushrooms,* pp.193-206. Edited by S.T. Chang, J. A. Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
PEBERDY, J. F. (1979). Fungal protoplasts: isolation reversion and fusion. *Annual Review of Microbiology* **33**, 21-39.
PEBERDY, J. F. & FOX, H. M. (1993). Protoplast technology and edible mushrooms. In *Genetics and Breeding of Edible Mushrooms,* pp. 125-155. Edited by S.T. Chang, J. A. Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.
RAPER, J. R. (1966). *Genetics of Sexuality in Higher Fungi*. New York: Ronald Press.
ROXON, J. E. & JONG, S. C. (1977). Sexuality of an edible mushroom, *Pleurotus sajor-caju*. *Mycologia* **69**, 202-205.
TAKEMARU, T. (1961). Genetical studies on fungi. IX. The mating system of *Lentinus edodes* (Berk.) Sing. *Report of the Tottori Mycological Institute (Japan)* **1**, 61-68.
TOYAMA, N. & OGAWA, K. (1974). Comparative studies on cellulolytic and oxidising enzymes of edible and iedible wood rotters. *Mushroom Science* **9**, 745-760.
ULEZLO, I. V., UPCROVA, V. & FENISKOVA, R. (1975). Oxidative enzymes of the lignin-degrading fungus, *Pleurotus ostreatus*. *Microbiology* **11**, 535-538.
YOO, Y.B. & CHA, D. Y. (1993). Gene transfer in edible fungi using protoplasts. In: *Genetics and Breeding of Edible Mushrooms,* pp.157-192. Edited by. S.T. Chang, J.A.Buswell & P. G. Miles. USA: Gordon and Breach Science Publishers.