

Genetic Breeding of Edible Mushrooms: from the Genome to the Production of New Varieties of *Pleurotus ostreatus*

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The breeding of new varieties of industrially cultivated edible mushrooms must proceed in the framework defined by the breeding objectives, the biological characteristics of the material and the legal and cultural constraints imposed to the breeding technology to be used. This last aspect is of the greatest importance in the case of a food that is considered in European countries as high quality and closer to nature than other industrially produced foods. This fact prevents the use of genetic-engineering based technologies for breeding, as the consumers would hardly accept genetically modified mushrooms. Consequently, mushroom breeding should be based on time-consuming processes of classic breeding. Molecular biology, however, can offer to the breeders useful tools for speeding up the selection process, for evaluating the new bred lines and, last but not least, to identify and eventually protect legally the outcome of their breeding programs.

1. Introduction

The breeding of new varieties of industrially cultivated edible mushrooms using genetic-engineering based technologies would be hardly acceptable by the final consumers in the European Union. Consequently, mushroom breeding should be based on low efficient and time-consuming classic-breeding

approaches. Moreover, the lack of mechanisms to protect legally the new bred varieties has limited the research efforts in mushroom breeding programs. Molecular biology, however, can facilitate the selection of elite parentals, the evaluation of the new-bred lines and the determination of molecular signatures useful for identifying the breeding outcomes and for their legal protection (Ramírez et al., 2001). This review summarizes the process that we have followed to produce new varieties of industrially cultivated, edible basidiomycetes using molecular marker-assisted breeding techniques, and discuss some of the results obtained using *Pleurotus ostreatus* (oyster mushroom) (Jacq.: Fr.) Kumm. (*Pleurotaceae*) (Moncalvo et al., 2002) as model organism.

Besides its cultivation for food production, the interest in *P. ostreatus* biochemistry and molecular biology is growing because of its nutritional (Mattila et al., 2001) and health-stimulating (Hossain et al., 2003) properties, its use in paper pulp bleaching (Sigoillot et al., 2005), in recycling agricultural wastes (Aggelis et al., 2003), and in bioremediation protocols (D'Annibale et al., 2005) among other biotechnological applications (Cohen et al., 2002). Moreover, the relatively simplicity of *P. ostreatus*' life cycle, the long experience in its industrial cultivation, its easy manipulation under laboratory conditions, and the availability of transformation protocols (Kim et al., 1999; Honda et al., 2000; Irie et al., 2001a; Irie et al., 2001b; Sunagawa and Magaie, 2002), make this fungus a suitable model organism for studying molecular-based genetic breeding strategies in basidiomycetes (Ramírez et al., 2000a; Ramírez et al., 2000b).

2. Mating factors

The strategy for genetic breeding depends upon the mating systems of the target species. *P. ostreatus* is a bifactorial tetrapolar fungus (Raper, 1966). A dikaryotic individual produces four different classes of meiotic spores as result of the segregation of two unlinked mating factors. Spores belonging to one of these classes are only compatible with those belonging to one of the other three. The lack of identifiable mating phenotypes in the spores and in the monokaryotic mycelia makes difficult the selection of parentals for directed crosses. In order to facilitate determining the mating type of monokaryons,

Larraya *et al.* (Larraya *et al.*, 1999a; Larraya *et al.*, 2001) used the Bulk-Segregant Analysis method previously described by Michelmore *et al.* (Michelmore *et al.*, 1991) to identify RAPD and RFLP markers genetically linked to the two mating factors (MatA and MatB). In his study, they scored six unrelated *P. ostreatus* strains and found 11 different MatA and 14 different MatB types. Moreover, new non-parental MatB types were detected that resulted from genetic recombination of two genetically linked loci (*matB α* and *matB β*) controlling this factor, the linkage distance between them varying in different strains between 0.6 (*MatB7-MatB8*) and 15.8 (*MatB1-MatB2*) cM. In an older study, Eugenio and Anderson (Eugenio and Anderson, 1968) found 17 different MatA and 20 different MatB types in a survey of 22 monokaryons, and reported the appearance of new types in the two mating factors. In our hands, however, this only happens within the MatB factor suggesting that MatA is either controlled by a single gene (*matA*) or by two genes linked below the resolution power of our analysis, that are inherited as a single one. More recently, James *et al.* have described the sequence of the *matA* and *matB* loci in the tropical oyster mushroom *P. djamor* (James *et al.*, 2004), and have found that the *A* mating-type locus in this species consists of only pair of completely linked genes.

3. Genetic linkage maps of *P. ostreatus*

For developing a molecular marker-based breeding program, it is convenient to know the number and structure of the chromosomes present in the species to be bred. In the case of basidiomycetes, the small size of their chromosomes and the occurrence of intranuclear mitosis have hampered classical cytogenetic studies. This small size, however, makes fungal chromosomes amenable to be studied using size-fractionating techniques such as Pulse Field Gel Electrophoresis (PFGE). Our group, in collaboration with the Mushroom Experimental Station in Horst (The Netherlands), separated the two nuclei present in *P. ostreatus* dikaryotic strain N001 by protoplasting and resolved the chromosomes present in each nucleus using PFGE (Larraya *et al.*, 1999b). This study revealed that *P. ostreatus* basic number is $n = 11$ and that the size of the individual chromosomes rang from 1.45 to 4.7 Mbp. Moreover, the comparison of the homologous chromosomes present in the

two nuclei present in the dikaryon revealed prominent (i.e. larger than 10%) length polymorphisms (CLPs) in chromosomes II, VI and VII. This fact, however, did not affect the total amount of DNA per haploid genome that was estimated in 35.3 and 34.7 Mbp for the two N001 nuclei. CLPs have been reported in a diversity of fungal species (Zolan, 1995; Muraguchi et al., 2003).

The construction of genetic linkage maps based on molecular markers is facilitated in higher fungi by the availability of monokaryotic mycelia produced by germination of haploid spores. In this material, the products of meiotic recombination are directly testable and the linkage distances between markers can be easily measured. Moreover, the separation of chromosomes by PFGE and the information provided by the molecular karyotype facilitates the construction of the maps by anchoring marker clusters to specific chromosomes. The use of monokaryons for genetic mapping, however, is limited by the reduced number of different phenotypic markers that can be studied and because characters expressed in the dikaryons cannot be directly testable. In order to solve this second drawback, it is necessary to construct dikaryotic populations by mating each one of the individuals of the monokaryotic population to a compatible tester. By this way, all the variation found in this type of dikaryotic population is the result of the genetic variation in the nuclei present in the monokaryotic population as the second nucleus is common in all the individuals. In order to construct the genetic linkage map of *P. ostreatus* N001, we have produced a segregating population formed by 80 monokaryons derived haploid spores. The theoretical linkage resolution power of this population is 1.25 cM (that is: one recombinant in the population of 80 haploid individuals).

3.1. *Scaffold genetic linkage map*

We have constructed two different upgraded versions of the linkage map: a scaffold map based on molecular markers (Figure 1), and two maps identifying the positions of quantitative trait loci (QTLs) on the scaffold map. In a first study, Larraya et al. (Larraya et al., 2000) scored the segregation of 178 RAPD, 23 RFLP markers, eight functional genes and the two mating factors. This study identified 130 map positions non-uniformly scattered across

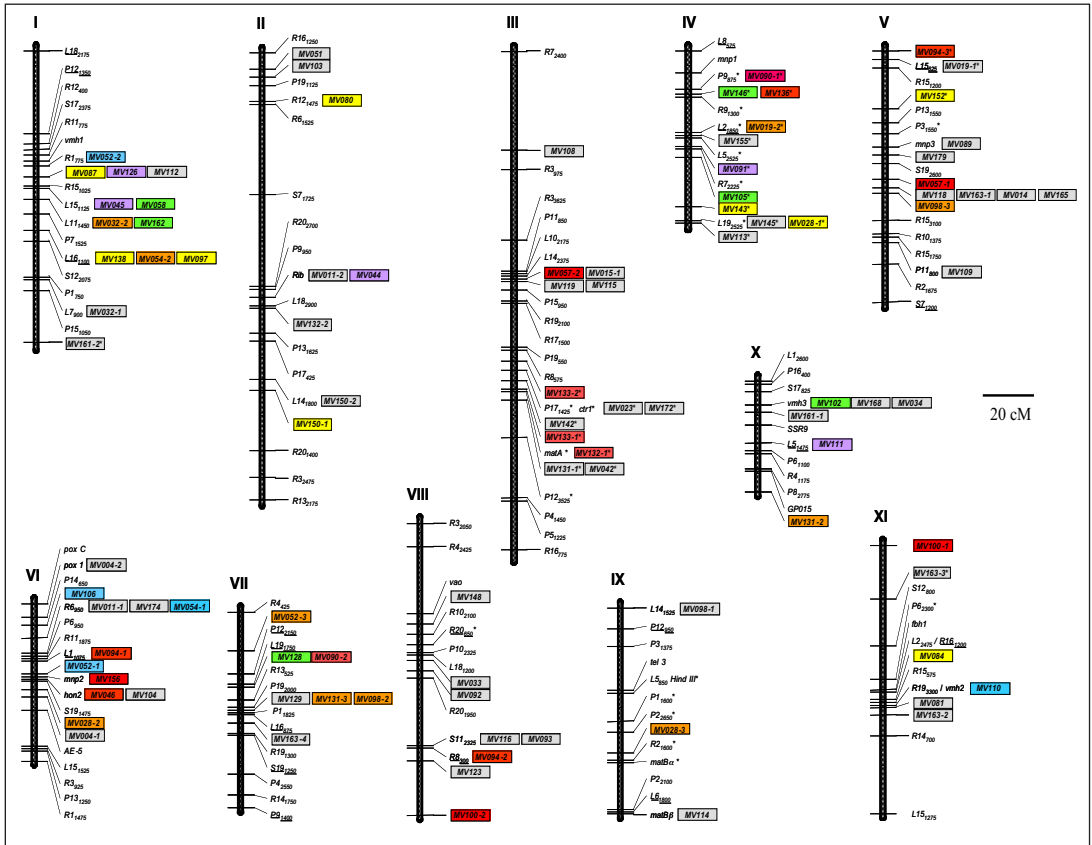
the 11 chromosomes and the total linkage map size was 1000 cM. In a more recent study Park et al. (Park et al., 2006) added to that map 82 functional genes and 12 PCR-based markers that have increased the total number of map positions to 187 and the total map size to 1061 cM (Table 1). Finally, the linkage groups ends are being currently bounded by the mapping of the telomeric sequences (Pérez et al., 2006) that, in this fungus, have the same sequence than the human telomeres.

Table 1
P. ostreatus genetic linkage map statistics

<i>Chrom. No.</i>	<i>Size (Mb)</i>	<i>Length (cM)</i>	<i>Kbp / cM</i>	<i>Crossover events</i>	<i>No. of genes mapped</i>	<i>No. of map positions</i>
I	4.7	107	43.9	0.98	17	18
II	4.4	164	26.8	1.71	6	19
III	4.6	183	25.1	1.75	14	24
IV	3.6	63	57.1	0.59	12	14
V	3.5	92	38.0	0.81	12	18
VI	3.1	58	53.4	0.76	17	19
VII	3.2	72	44.4	0.74	4	16
VIII	3.0	107	28.0	0.84	9	15
IX	2.1	76	27.6	0.74	3	19
X	1.8	41	43.9	0.34	6	12
XI	1.5	98	15.3	0.59	8	13
Total	35.5	1061	36.3 (13.0)	0.90 (0.44)	108	187

Genetic linkage maps are usually interpreted assuming that wide genetic linkage distances reflect wide physical distances in the chromosome; however, the correlation between physical and linkage distances can be distorted by the occurrence of sequences that promote or difficult meiotic recombination (Lichten and Goldman, 1995; Petes, 2001). Regions of repetitive DNA, containing transposons and heterochromatic regions have been associated to recombination frequency bias (Gerton et al., 2000).

Figure 1



Some examples of influence of the sequence structure on the recombination frequency have been recently described in the literature: in an analysis of the genetic structure of the *matB* locus in *S. commune*, Fowler et al. (Fowler et al., 2004) found that the 8 kb region separating the *B α 3* and *B β 2* loci contains 19 different short sequences with imperfect repeats as well as a 1 kb segment where the GC content was highly biased, and that the 5 kb region separating the loci *B β 3* and *B β 3* contains 17 short imperfect direct repeats similar in length and number to those of the *B α 3*-*B β 2* complex. The ratio of physical to genetic distance (up to 1 kb/cM) suggests that this region forms a recombination hotspot. On the contrary, Espeso et al. (Espeso et al., 2005)

have found that recombination frequencies are greatly reduced near the centromeres in two *Aspergillus nidulans* chromosomes altering the physical to genetic distance ratio at these locations.

The uneven distribution of map positions across the *P. ostreatus* linkage map argues in support of the occurrence of recombination-prone genome regions that appear as long empty linkage regions separating marker clusters. Assuming that the RAPD markers, as a whole, are stochastically distributed across the genome sequence, long linkage distances between correlative markers could very well be the result of increased recombination rather than indicate long physical distance. If we consider the DNA elements promoting recombination, little is known about the presence of transposable elements in *P. ostreatus* genome, although their presence has been described in other basidiomycetes (Gaskell et al., 1995; Sonnenberg et al., 1999; Wostemeyer and Kreibich, 2002); and the analysis of a 255 kb genomic sequence allows to estimate that 5.7% of the sequence corresponds to repetitive elements (Palma et al., 2006). This figure, that does not include the rDNA, fits with that of repetitive elements in other higher fungi (Wostemeyer and Kreibich, 2002).

The estimation of the number of crossover events per chromosome per cell (0.90 ± 0.42) reveals that basidia in which no crossovers occur in one or some of the chromosomes are frequent in *P. ostreatus* (Larraya et al., 2000), and poses the question about the mechanisms ensuring an appropriate distribution of the homologous chromosomes to the meiotic products. Besides that, recombination between chromosomes with prominent length polymorphisms questions about the conservation of the genome structure and karyotype stability (Wostemeyer and Kreibich, 2002). A preliminary study of the 880 chromosomes present in the segregating mapping population revealed that 11.4% of the chromosomes were not involved in recombination and that the recombination behavior differs in different chromosomes (Castellón et al., 2006).

3.2. QTL map

We have mapped two different groups of QTLs to the genetic linkage scaffold map described above: (1) QTLs controlling mycelial growth rate and (2) QTLs controlling industrial yield and productivity.

The genetic control of mycelial growth rate is complex and depends on several loci. In a first study, Larraya *et al.* (Larraya et al., 2001) found that

monokaryons carrying *matA2* allele grew significantly ($P = 0.01$) faster than their *matA1* sibs. Moreover, among the *matA2* individuals, those carrying the *matB α 1* allele grew faster than those carrying the *matB α 2* ($P = 0.04$). These differences were not observed when comparing different *matB β* alleles or when the comparisons were made in a *matA1* genetic background. Consequently, the mating type influences the monokaryotic vegetative growth rate in synthetic culture medium. The presence of the *matA1* allele seems to act as a growth rate-limiting factor; *matA2*, on the contrary, releases this limitation and the *matB α* locus acts as a new limiting control. James et al. (James et al., 2004) have found hypothetical genes responsible for cellular energy metabolism in close sequence vicinity to the *matA* gene in *P. djamor*. These genes could explain the differences in growth rate associated to the mating factors that we have found in *P. ostreatus*. However, a direct effect of the mating factor on the growth rate or the occurrence of a selection for balanced gene combinations could not be discarded as responsible for this behavior.

For systematic search of loci controlling growth rate, we studied the correlation between the presence of discrete genome regions (defined by two consecutive map markers) and the vegetative growth rate in monokaryons and dikaryons, cultured on synthetic culture medium (Eger medium, SEM) or on straw (Larraya et al., 2002). For studying the variation in the monokaryotic growth rate, the collection of 80 segregating monokaryons was used. For the corresponding study in dikaryons, four populations were constructed by mating each one of the 80 sib monokaryons to four a different monokaryon unrelated to N001. The study identified two major QTLs controlling monokaryotic growth rate on SEM, three controlling monokaryotic growth rate on straw, and eight major QTLs controlling dikaryotic growth rate on SEM (four of which were detected in two dikaryotic different populations). Three QTL clusters controlling monokaryotic and dikaryotic growth rate were found on chromosomes IV, VII and IX. Chromosome I, on the other hand, bore the two main dikaryotic growth rate QTLs both in terms of individual contribution to the character (R^2 value 25.90% and 11.81%) and robustness of the determination ($P = 2 \times 10^{-5}$ and 3.8×10^{-3} , respectively).

The evaluation of the quantitative effect of the QTLs can distinguish between the effect of different QTLs and the effect of different haplotypes in a given QTL. The effect of different QTLs on the variation of the growth

rate seems to be additive since the sum of the individual effects of different QTLs roughly equals the proportion of the total variation of the character that can be genetically explained at the sensitivity level used in this analysis (see R^2 values in Table 2, as an example). In a study on the interaction between specific QTLs in homologous chromosomes, we have constructed dikaryons which were completely homozygous or completely heterozygous for chromosome VIII (which contains a cluster of QTLs controlling monokaryotic and dikaryotic growth rate) and we have measured the variation in the dikaryotic growth rate as a function of the chromosomal constitution. The preliminary results suggest that the action of the QTLs tested is dominant (i.e., the presence of a “fast” chromosome is enough to produce a fast growing dikaryon) rather than additive (i.e.: dikaryons heterozygous for chromosome VIII present growth rate values intermediate between those of the two types of chromosome VIII homozygotes) (Castellón et al., 2006).

Table 2
QTLs for mycelial growth rate on SEM

<i>Name</i>	<i>Linkage group</i>	<i>Position</i>	<i>Probability</i>	<i>Absolute effect</i>	R^2	<i>Total R^2</i>
<i>Qmgre1</i>	IV	$L5_{2525} + 1.4$	0.017	3.830	9.90	
<i>Qmgre2</i>	VIII	$P10_{2325}$	0.00011	5.030	20.27	38.46
<i>Qdgre1</i>	IV	$R7_{2225} + 3.8$	0.00001	2.240	23.17	
<i>Qdgre2</i>	VIII	$R10_{2100} + 3.8$	0.011	1.030	11.31	
<i>Qdgre3</i>	XI	<i>fbh1</i>	0.035	1.041	8.18	41.19

Besides monokaryotic and dikaryotic growth rate, we mapped quantitative traits related to industrial production (and its components) and quality (Larraya et al., 2003) using the compatible monokaryon PC21 as provider of the common nucleus. The experiment was carried out at two different incubation temperatures (15 and 21 °C), and the parameters scored were yield, flush precocity, first flush yield, fruit body average weight, clean fruit-body weight, stipe weight loss, fruit-body fleshness (texture) and fruit-body color. Most of the parameters displayed a normal distribution that allowed the mapping. Table 3 shows a summary of the mapping results. The analysis revealed that the (1) QTLs controlling production could explain a large por-

tion of the variation of the character. Particularly, nearly the half of the variation in precocity and yield at 21 °C could be explained by the corresponding QTLs. (2) For all the production characters studied, a strong QTL effect could be mapped to the central region of chromosome VII. This QTL could explain roughly half of the variation of the main production traits. Moreover, this QTL maps to a position where a QTL signal for dikaryotic growth had been detected. (3) The QTLs controlling quality traits explained a more reduced portion of the character variation than the QTLs for production do and are scattered across different chromosomes. Moreover, no quality QTLs have been mapped to chromosome VII.

The control of quantitative traits complicates even more if the interactions between the different loci are considered. As an example, 17 and 39 different significant ($P < 0.005$) digenic interactions were observed between loci controlling growth rate and various quality and production traits (respectively) and other genome regions. Consequently, the molecular bases explaining these results are far from being completely understood.

Table 3
Summary of QTL effect for production and quality

<i>Trait</i>	<i>QTL chromosome VII R²</i>	<i>Probability</i>	<i>Other Linkage groups</i>	<i>Total R²</i>
P-21	31.83	$<10^{-5}$	I, VI, VIII	47.96
P-15	36.30	$<10^{-5}$	–	
Y-21	45.47	$<10^{-5}$	IV	47.69
Y-15	32.07	$<10^{-5}$	–	
FFY-21	48.36	$<10^{-5}$	IV	49.26
FFY-15	28.77	$<10^{-5}$	VI	34.28
CFW	13.83	$< 4 \times 10^{-4}$	III	18.13
SWL	–	–	I, II	14.27
FLE	–	–	II, V, VI	26.87
COL	–	–	IV, X	17.31

Precocity (P), Yield (Y), flush, first flush yield (FFY), clean fruit-body weight (CFW), cold-storage weight loss (SWL), fruit-body fleshness (texture) (FLE), and fruit-body color. 21 and 15 indicate the incubation temperature (°C) of the experiment.

Table 4
Summarizes the most relevant genes (quantitative or qualitative)
mapped to the *P. ostreatus* chromosomes.

<i>Chrom. No.</i>	<i>Quantitative trait</i>	<i>Gene or function</i>
I	dgre*, P ₂₁ , SWL	–
II	dgre, SWL*	rDNA
III	CFW, FLE*	MatA
IV	mgre, mgrs*, dgre, Y ₂₁ , FFY ₂₁ , NUM, COL*	Lignocellulolytic enzymes
V	CWL, FLE	–
VI	dgre, P ₂₁ , FFY ₁₅ , CWL, FLE*	Lignocellulolytic enzymes
VII	dgre, P ₂₁ *, P ₁₅ *, Y ₂₁ *, Y ₁₅ *, FFY ₂₁ *, FFY ₁₅ *, NUM*, CFW*	–
VIII	mgre*, mgrs, dgre, P21, CWL*	–
IX	–	MatB
X	COL	–
XI	mgrs, dgre	Hydrophobins

Legend for the QTLs: Growth rate: mgre, monokaryotic on SEM; mgrs, monokaryotic on straw; dgre, dikaryotic on SEM. Industrial Production: P₂₁, precocity at 21 °C; P₁₅, precocity at 15 °C; Y₂₁, yield at 21 °C; Y₁₅, yield at 15 °C; FFY₂₁, first flush yield at 21 °C; FFY₁₅, first flush yield at 15 °C; NUM, number of mushrooms; CFW, clean fruit body weight. Industrial Quality: SWL, stipe weight loss; CWL, storage weight loss; FLE, texture; COL, color. The asterisk indicates the principal QTL for each class.

4. *P. ostreatus* genes

The information about the *P. ostreatus* genome structure provided by the karyotype and the linkage maps is complemented with that corresponding to the structure of genes in this species. We harvested this knowledge from two main sources: (1) genes isolated or identified by direct cloning or during EST screenings, and (2) genes identified or predicted in long stretches of genomic DNA. Currently (Nov. 2005) there are more than 2100 *P. ostreatus* sequences deposited in the genetic databases. Out of them, less than the 10% correspond to core nucleotides and the rest to ESTs. If we estimate the total number of *P. ostreatus* genes as 12.000 (see below), the sequences deposited represent 17% of the expected genes and the sequences corresponding to core nucleotides represent 1.2% of the expected.

4.1. *EST analysis*

In order to contribute to identify new genes expressed differentially during vegetative and reproductive growth in *P. ostreatus*, to study their expression in other developmental stages, and to increase the density of the existing genetic linkage map of this fungus, we collected a number of ESTs isolated from mature lamellae, mapped the corresponding genes and analyzed their expression in different developmental phases (Park et al., 2006). The total number of lamellae-expressed genes identified and mapped was 82. The manual annotation of the genes based on BlastN and BlastX similarities revealed that 56.1% of the genes identified in this study were highly similar to databases entries whereas 34.1% of them corresponded to entirely new sequences. This value fits with those previously reported for other developmental studies carried in basidiomycetes (Ospina-Giraldo et al., 2000; Lacourt et al., 2002; Lee et al., 2002; Guettler et al., 2003; Posada-Buitrago and Frederick, 2005).

The mean GC content of the EST collection was 53.35% (s.d. 4.72). This value is slightly higher than the estimated for the general genome (50.53%) suggesting a higher GC content in the coding regions in comparison with the non-coding ones. In the white-rot fungus *Phanerochaete chrysosporium* strain *RP78*, the GC content of the coding regions (59%) was also higher than the overall value (57%) (Martinez et al., 2004). GC values were, however, higher in *P. chrysosporium* than in *P. ostreatus*.

Table 5 and Figure 2 summarize the genes expressed in the lamellae. In *P. ostreatus*, 30.8% of the genes identified in this study as expressed in the lamellae were also expressed in vegetative mycelia (pattern A), this number raises to 67.9% in the case of the subpopulation of genes expressed in fruit bodies, whereas Lee et al. (Lee et al., 2002) found that only 5.3% of the genes were expressed simultaneously in both developmental stages, and Ospina-Giraldo et al. found that 12% of the genes were simultaneously detected in primordia and basidiome *Agaricus bisporus* samples (Ospina-Giraldo et al., 2000). These authors conclude that gene expression must be quite different quantitatively and qualitatively during fruit body formation. We consider that the statistical sampling process involved in cDNA cloning can be responsible for their results, and that Northern analysis is more accurate in detecting gene expression. Consequently, our results suggest that the number of genes differentially expressed in the two developmental stages is smaller

than that previously reported. These expression results partially support the conclusion put forward by Zanzinge et al. (Zantinge et al., 1979) indicating that RNA sequences isolated from *Schizophyllum commune* fruiting and non-fruiting mycelia were identical for at least 90%.

Table 5
Genes expressed in the lamellae

<i>Expression pattern</i>	<i>Genes with assigned function</i>	<i>Hypothetical proteins</i>	<i>New genes</i>	<i>Total</i>
A	17 (70.8)	3 (12.5)	4 (16.7)	24 (30.8)
B	5 (26.3)	4 (21.1)	10 (52.6)	19 (24.4)
C	8 (28.6)	8 (28.6)	12 (42.9)	28 (35.9)
AB	3 (75.0)	-	1 (25.0)	4 (5.1)
D	-	-	1	1 (1.3)
E+F	1	-	1	2 (2.6)
Total	34 (43.6)	15 (20.7)	29 (35.4)	78

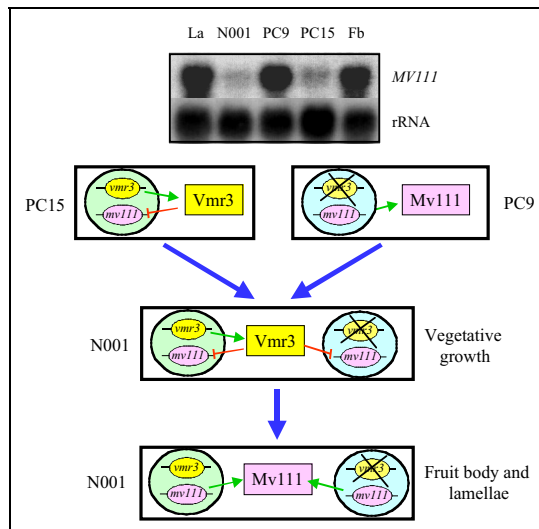
The expression patterns correspond to those described in Figure 2

Besides the major expression patterns, the expression of two genes (*mv111* and *mv123*) was detected in fruit body and in one of the two *P. ostreatus* N001 parental protoclones, whereas it was not detected in the dikaryotic mycelium and in the second parental protoclone. This expression pattern suggests the existence of diffusible gene expression repressors active during the vegetative growth and inactive after the growth phase change (Figure 3).

Figure 2
Expression patterns of lamellae-expressed genes in *P. ostreatus*

	La	N001	PC9	PC15	F.b	Expression pattern	Number of genes	% of genes
						<i>MV094</i>		
						rRNA	A	24
						<i>MV019</i>		
						rRNA	B	19
						<i>MV091</i>		
						rRNA	C	28
						<i>MV087</i>		
						rRNA	AB	4
						<i>MV123</i>		
						rRNA	D	1
						<i>MV103</i>		
						rRNA	E	1
						<i>MV111</i>		
						rRNA	F	1
						Total	78	100

Figure 3
Hypothetical model for the repression of the expression of *mv011* by a diffusible factor produced by PC15



The genes identified in this study provide tags for the study of house-keeping, fruit body specific and lamellae specific promoters. Other genes studied in our laboratory were found to be expressed in vegetative mycelium and repressed in fruiting bodies had been previously found [hydrophobin genes *vmh1* and *vmh2* (Peñas et al., 2002)] or expressed in fruit body but not in lamellae [hydrophobin *fbh1* (Peñas et al., 1998) and copper transporter *ctr1* (Peñas et al., 2005)]. Leaving aside more bizarre expression patterns theoretically possible (for instance, genes expressed in vegetative mycelium and lamellae but repressed in fruit body), altogether, the main promoter types expected in this type of fungus are available for sequence comparative studies using these genes as tags.

4.2. Genomic sequence

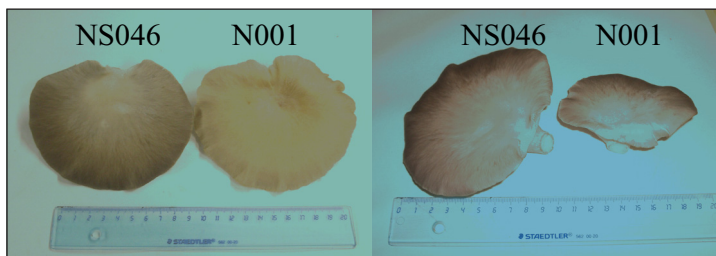
We have cloned and sequenced a 255 kb genomic region of *P. ostreatus* chromosome VII where the major QTL cluster for mushroom production maps to. This sequence is the longest genomic stretch sequenced in *P. ostreatus* up to date. A preliminary manual annotation based on BlastX and BlastN similarities identified 61 putative genes; however, if we take into account that nearly one third of the *P. ostreatus* genes correspond to sequences without counterpart in the databases, the expected gene number in this region raises to nearly 90. By extrapolation of the number of genes expected in this region, and assuming an even distribution of genes in this genome, we can expect that the complete *P. ostreatus* genome would consist in around 12.000 genes. The region sequenced included two short direct, two short inverted, two long direct and two long inverted repeated sequences that account for nearly 11.6 kb (4.5% of the region). The search for microsatellite sequences identified 588 elements involving 3.1 kb (1.22% of the sequence). Taken together, these two types of repetitive sequences add up to 5.7% of the sequence (Palma et al., 2006).

5. Selection of parentals and construction of new bred lines

The information derived from the genetic and molecular studies described above was used to select parentals for constructing new dikaryotic strains

with improved traits. Basically, the parents for the new hybrid lines were monokaryons derived from N001 and selected on the basis of their performance on those characters controlled by the QTLs described. Different types of hybrids were constructed: outbred dikaryons produced by mating of monokaryons belonging to the mapping population to compatible monokaryons derived from other *P. ostreatus* strains unrelated to N001, and inbred dikaryons constructed by mating of compatible elite monokaryons present in the mating population. *P. ostreatus* commercial strain HK35, and the model strain used in our laboratory N001, were the controls for the breeding field trials that were carried out under the production conditions used by commercial producers in Spain. The parameters scored in these experiments were yield (Y), precocity (P), clean fruit body weight (CFW), and stipe weight lost (SWL). Five repetitions of each strain and an at random design were used in the field trials which were tested in autumn winter, spring and summer time. The performance of the hybrids was tested in successive trials: hybrids scoring high in a trial run into the next scaled-up one. At the end of the process two new strains scoring better than the parents for CFW, SWL and P were produced. Figure 4 shows a comparison of the selected new strain 046 in comparison with the parental N001

Figure 4
NS046, a new *P. ostreatus* inbred strain produced by Molecular marker assisted selection of parents



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7. References

- Aggelis, G., Iconomou, D., Christou, M., Bokas, D., Kotzailias, S., Christou, G., Tsagou, V. and Papanikolaou, S., 2003. Phenolic removal in a model olive oil mill wastewater using *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process. *Water Res.* 37, 3897-3904.
- Castellón, J., Pisabarro, A.G. and Ramírez, L., 2006. Chromosome structure of a monokaryotic progeny of *Pleurotus ostreatus* N001.
- Cohen, R., Persky, L. and Hadar, Y., 2002. Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. *Appl. Microbiol. Biotechnol.* 58, 582-594.
- D'Annibale, A., Ricci, M., Leonardi, V., Quaratino, D., Mincione, E. and Petruccioli, M., 2005. Degradation of aromatic hydrocarbons by white-rot fungi in a historically contaminated soil. *Biotechnol. Bioeng.* 90, 723-731.
- Espeso, E.A., Coboño, L. and Arst, H.N., 2005. Discrepancies between Recombination Frequencies and Physical Distances in *Aspergillus nidulans*: Implications for Gene Identification. *Genetics* 171, 835-838.
- Eugenio, C.P. and Anderson, N.A., 1968. The genetics and cultivation of *Pleurotus ostreatus*. *Mycology* 60, 627-634.
- Fowler, T.J., Mitton, M.F., Rees, E.I. and Raper, C.A., 2004. Crossing the boundary between the Balpha and Bbeta mating-type loci in *Schizophyllum commune*. *Fungal Genet. Biol.* 41, 89-101.
- Gaskell, J., Van den Wymelenberg, A. and Cullen, D., 1995. Structure, inheritance, and transcriptional effects of Pce1, an insertional element within *Phanerochaete chrysosporium* lignin peroxidase gene lipI. *Proc Natl Acad Sci U S A.* 92, 7465-7469.
- Gerton, J.L., DeRisi, J., Shroff, R., Lichten, M., Brown, P.O. and Petes, T.D., 2000. Inaugural article: global mapping of meiotic recombination hotspots and coldspots in the yeast *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A.* 97, 11383-11390.
- Guettler, S., Jackson, E.N., Lucchese, S.A., Honaas, L., Green, A., Hittinger, C.T., Tian, Y., Lilly, W.W. and Gathman, A.C., 2003. ESTs from the basidiomycete *Schizophyllum commune* grown on nitrogen-replete and nitrogen-limited media. *Fungal Genet. Biol.* 39, 191-198.
- Honda, Y., Matsuyama, T., Irie, T., Watanabe, T. and Kuwahara, M., 2000. Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. *Curr. Genet.* 37, 209-212.
- Hossain, S., Hashimoto, M., Choudhury, E.K., Alam, N., Hussain, S., Hasan, M., Choudhury, S.K. and Mahmud, I., 2003. Dietary mushroom (*Pleurotus ostreatus*) ameliorates atherogenic lipid in hypercholesterolaemic rats. *Clin. Exp. Pharmacol. Physiol.* 30, 470-475.

- Irie, T., Honda, Y., Hirano, T., Sato, T., Enei, H., Watanabe, T. and Kuwahara, M., 2001a. Stable transformation of *Pleurotus ostreatus* to hygromycin B resistance using *Lentinus edodes* GPD expression signals. *Appl. Microbiol. Biotechnol.* 56, 707-709.
- Irie, T., Honda, Y., Watanabe, T. and Kuwahara, M., 2001b. Efficient transformation of filamentous fungus *Pleurotus ostreatus* using single-strand carrier DNA. *Appl. Microbiol. Biotechnol.* 55, 563-565.
- James, T.Y., Liou, S.R. and Vilgalys, R., 2004. The genetic structure and diversity of the A and B mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*. *Fungal Genet. Biol.* 41, 813-825.
- Kim, B.G., Magae, Y., Yoo, Y.B. and Kwon, S.T., 1999. Isolation and transformation of uracil auxotrophs of the edible basidiomycete *Pleurotus ostreatus*. *FEMS Microbiol. Lett.* 181, 225-228.
- Lacourt, I., Duplessis, S., Abba, S., Bonfante, P. and Martin, F., 2002. Isolation and characterization of differentially expressed genes in the mycelium and fruit body of *Tuber borchii*. *Appl. Environ. Microbiol.* 68, 4574-4582.
- Larraya, L., Peñas, M.M., Pérez, G., Santos, C., Ritter, E., Pisabarro, A.G. and Ramírez, L., 1999a. Identification of incompatibility alleles and characterisation of molecular markers genetically linked to the A incompatibility locus in the white rot fungus *Pleurotus ostreatus*. *Curr. Genet.* 34, 486-493.
- Larraya, L.M., Alfonso, M., Pisabarro, A.G. and Ramírez, L., 2003. Mapping of Genomic Regions (Quantitative Trait Loci) Controlling Production and Quality in Industrial Cultures of the Edible Basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 69, 3617-3625.
- Larraya, L.M., Idareta, E., Arana, D., Ritter, E., Pisabarro, A.G. and Ramírez, L., 2002. Quantitative Trait Loci controlling vegetative growth rate in the edible basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 68, 1109-1114.
- Larraya, L.M., Pérez, G., Iribarren, I., Blanco, J.A., Alfonso, M., Pisabarro, A.G. and Ramírez, L., 2001. Relationship between monokaryotic growth rate and mating type in the edible basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 67, 3385-3390.
- Larraya, L.M., Pérez, G., Peñas, M.M., Baars, J.J., Mikosch, T.S., Pisabarro, A.G. and Ramírez, L., 1999b. Molecular karyotype of the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 65, 3413-3417.
- Larraya, L.M., Pérez, G., Ritter, E., Pisabarro, A.G. and Ramírez, L., 2000. Genetic linkage map of the edible basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 66, 5290-5300.
- Lee, S.H., Kim, B.G., Kim, K.J., Lee, J.S., Yun, D.W., Hahn, J.H., Kim, G.H., Lee, K.H., Suh, D.S., Kwon, S.T., Lee, C.S. and Yoo, Y.B., 2002. Comparative analysis of sequences expressed during the liquid-cultured mycelia and fruit body stages of *Pleurotus ostreatus*. *Fungal Genet. Biol.* 35, 115-134.
- Lichten, M. and Goldman, A.S., 1995. Meiotic recombination hotspots. *Annu. Rev. Genet.* 29, 423-444.

- Martínez, D., Larrondo, L.F., Putnam, N., Gelpke, M.D., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Coutinho, P.M., Henrissat, B., Berka, R., Cullen, D. and Rokhsar, D., 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat. Biotechnol.* 22, 695-700.
- Mattila, P., Konko, K., Euroola, M., Pihlava, J.M., Astola, J., Vahteristo, L., Hietaniemi, V., Kumpulainen, J., Valtonen, M. and Piironen, V., 2001. Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *J. Agric. Food Chem.* 49, 2343-2348.
- Michelmore, R.W., Paran, I. and Kesseli, R.V., 1991. Identification of markers linked to resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genome regions by using segregating populations. *Proceedings of the National Academy of Sciences USA* 88, 9828-9832.
- Moncalvo, J.M., Vilgalys, R., Redhead, S.A., Johnson, J.E., James, T.Y., Catherine Aime, M., Hofstetter, V., Verduin, S.J., Larsson, E., Baroni, T.J., Greg Thorn, R., Jacobsson, S., Clemencon, H. and Miller, O.K., Jr., 2002. One hundred and seventeen clades of euagarics. *Mol. Phylogenet. Evol.* 23, 357-400.
- Muraguchi, H., Ito, Y., Kamada, T. and Yanagi, S.O., 2003. A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNAs and restriction fragment length polymorphisms. *Fungal Genet. Biol.* 40, 93-102.
- Ospina-Giraldo, M.D., Collopy, P.D., Romaine, C.P. and Royse, D.J., 2000. Classification of sequences expressed during the primordial and basidiome stages of the cultivated mushroom *Agaricus bisporus*. *Fungal Genet. Biol.* 29, 81-94.
- Palma, L., Peñas, M.M., Ramírez, L. and Pisabarro, A.G., 2006. Sequence analysis of the production QTL region of *Pleurotus ostreatus*.
- Park, S.-K., Peñas, M.M., Ramírez, L. and Pisabarro, A.G., 2006. Genetic linkage map and expression analysis of genes expressed in the lamellae of the edible basidiomycete *Pleurotus ostreatus*. *Fungal Genetics and Biology* (in the press).
- Peñas, M.M., Asgeirsdóttir, S.A., Lasa, I., Culiañez-Macià, F.A., Pisabarro, A.G., Wessels, J.G.H. and Ramírez, L., 1998. Identification, characterization, and *in situ* detection of a fruit-body-specific hydrophobin of *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 64, 4028-4034.
- Peñas, M.M., Azparren, G., Domínguez, Á., Sommer, H., Ramírez, L. and Pisabarro, A.G., 2005. Identification and functional characterisation of *ctr1*, a *Pleurotus ostreatus* gene coding for a copper transporter. *Mol. Genet. Genom.* 274, 402-409.
- Peñas, M.M., Rust, B., Larraya, L.M., Ramírez, L. and Pisabarro, A.G., 2002. Differentially regulated vegetative mycelium specific hydrophobins of the edible basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 68, 3891-3898.
- Pérez, G., Pisabarro, A.G. and Ramírez, L., 2006. Cloning and mapping of telomeric sequences in *Pleurotus ostreatus*.
- Petes, T.D., 2001. Meiotic recombination hot spots and cold spots. *Nat Rev Genet.* 2, 360-369.

- Posada-Buitrago, M.L. and Frederick, R.D., 2005. Expressed sequence tag analysis of the soybean rust pathogen *Phakopsora pachyrhizi*. Fungal Genet. Biol. 42, 949-962. Epub 2005 Nov 2015.
- Ramírez, L., Larraya, L.M., Peñas, M.M., Pérez, G., Eizmendi, A., Agós, I., Arana, D., Aranguren, J., Iribarren, I., Olaberria, N., Palacios, E., Ugarte, B.E. and Pisabarro, A.G., 2000a. Molecular techniques for the breeding of *Pleurotus ostreatus*. In: Griensven, L.J.L.D.v. (ed.), Science and cultivation of edible fungi. A.A. Balkema, Rotterdam, The Netherlands, Vol. 1, pp. 157-163.
- Ramírez, L., Larraya, L.M. and Pisabarro, A.G., 2000b. Molecular tools for breeding basidiomycetes. Int Microbiol 3, 147-152.
- Ramírez, L., Muez, V., Alfonso, M., Garcia Barrenechea, A., Alfonso, L. and Pisabarro, A.G., 2001. Use of molecular markers to differentiate between commercial strains of the button mushroom *Agaricus bisporus*. FEMS Microbiol Lett 198, 45-48.
- Raper, J.R., 1966. Genetics of sexuality in higher fungi. The Ronald Press Co., New York.
- Sigoillot, C., Camarero, S., Vidal, T., Record, E., Asther, M., Perez-Boada, M., Martinez, M.J., Sigoillot, J.C., Asther, M., Colom, J.F. and Martinez, A.T., 2005. Comparison of different fungal enzymes for bleaching high-quality paper pulps. J. Biotechnol. 115, 333-343.
- Sonnenberg, A.S.M., Baars, J.J.P., Mikosch, T.S.P., Schaap, P.J. and van griensven, L.J.L.D., 1999. *Abr1*, a transposon-like element in the genome of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach. Applied and Environmental Microbiology 65, 3347-3353.
- Sunagawa, M. and Magae, Y., 2002. Transformation of the edible mushroom *Pleurotus ostreatus* by particle bombardment. FEMS Microbiol Lett. 211, 143-146.
- Wostemeyer, J. and Kreibich, A., 2002. Repetitive DNA elements in fungi (Mycota): impact on genomic architecture and evolution. Curr Genet. 41, 189-198. Epub 2002 Jun 2021.
- Zantinge, B., Dons, H. and Wessels, J.G., 1979. Comparison of poly(A)-containing RNAs in different cell types of the lower eukaryote *Schizophyllum commune*. Eur. J. Biochem. 101, 251-260.
- Zolan, M.E., 1995. Chromosome-length polymorphism in fungi. Microbiological Reviews 59, 686-698.