INTERNATL MICROBIOL (2000) 3:147–152 © Springer-Verlag Ibérica 2000

REVIEW ARTICLE

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Received 5 May 2000 Accepted 3 July 2000

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Molecular tools for breeding basidiomycetes

Summary The industrial production of edible basidiomycetes is increasing every year as a response to the increasing public demand of them because of their nutritional properties. About a dozen of fungal species can be currently produced for food with sound industrial and economic bases. Notwithstanding, this production is threatened by biotic and abiotic factors that make it necessary to improve the fungal strains currently used in industry. Breeding of edible basidiomycetes, however, has been mainly empirical and slow since the genetic tools useful in the selection of the new genetic material to be introduced in the commercial strains have not been developed for these fungi as it was for other organisms. In this review we will discuss the main genetic factors that should be considered to develop breeding approaches and tools for higher basidiomycetes. These factors are (i) the genetic system controlling fungal mating; (ii) the genomic structure and organisation of these fungi; and (iii) the identification of genes involved in the control of quantitative traits. We will discuss the weight of these factors using the oyster mushroom *Pleurotus ostreatus* as a model organism for most of the edible fungi cultivated industrially.

Key words $Pleurotus ostreatus \cdot Basidiomycetes \cdot Breeding fungi \cdot Mating factors \cdot Fungal genome structure$

Introduction

Basidiomycetes, one of the four branches of the monophyletic group of Eumycota (true fungi), account for about 35% of the fungal species currently described, and are of both ecological and industrial importance. Their ecological impact varies according to their different life styles: saprotrophs (feeding on the remains of dead organisms or wastes), which play a central role in the recycling of organic material because of their ability to degrade some molecules especially reluctant to biodegradation (i.e. lignin breakdown by white rot fungi); symbionts, forming ectomycorrhizae with vascular plants, which facilitate nutrient absorption; or fungal, plant or animal pathogens, responsible for crop losses (Ustilago maydis) or serious human diseases (Criptococcus neoformans). Besides, some basidiomycetes have been traditionally used for human consumption because of their organoleptic characteristics (Boletus edulis, Lactarius spp.), their hallucinogenic properties (Amanita muscaria) or, even, as poisons (A. phalloides).

There is a growing industry of edible mushroom production based on a process of solid fermentation of pasteurized or sterilized substrates inoculated with the appropriate spawn that proceeds under controlled conditions of temperature and humidity. This

control, however, is far from strict and, in practical terms, the overall process shares more characteristics with open-air composting processes, where different populations of microorganisms compete and establish successions, than with industrially-controlled axenic fermentations. The many factors (both biotic and abiotic) involved in this process very often cause instability in yield or in the quality of the product, as it has been the case for the production of the oyster mushroom, Pleurotus ostreatus, over the last few years. Hence, there is a market pressure to improve the yield and quality of the mushrooms currently produced, and to increase the number of cultivable fungi that has fuelled research aimed to develop breeding programs for edible fungi and to formulate appropriate substrates and culture conditions for new species. Furthermore, this pressure is based on three main reasons: (i) the economic value of some highlydemanded fungal species; (ii) their use to produce enzymes or chemicals useful in industry or pharmacy; and (iii) their application in processes aimed to recycle industrial or agricultural wastes.

The development of breeding programs for edible basidiomycetes, however, has been hampered by the difficulties in performing directed crosses between fungal strains, due to incompatibility barriers, by the contradictory data about size and organization of the genetic material, and by the lack of linkage maps to localize genes of interest. In our laboratory, we have used the oyster mushroom *Pleurotus ostreatus* as a model system to study these three aspects.

P. ostreatus is an edible basidiomycete that grows wildly on decaying wood thanks to its lignin-degrading capacity, and is industrially cultivated on a variety of substrates based on agricultural wastes (such as straw, cotton wastes, sawdust, etc.). *P. ostreatus* is currently the second major mushroom in the world market led by the button mushroom Agaricus bisporus [32]. Besides its importance for food production, it is of interest for industrial applications such as paper pulp bleaching (by the action of its ligninolytic enzymes) and for cosmetics and pharmaceutical industries [2, 3, 12, 13, 20, 21]. The life cycle of P. ostreatus, as well as those of many other higher basidiomycetes, alternates monokaryotic (haploid) and dikaryotic (di-haploid) phases [9]. Two monokaryotic compatible hyphae are able to fuse and give rise to a dikaryotic mycelium in which the two parental nuclei remain independent (dikaryon, heterokaryon) throughout the vegetative growth, and which will fruit under the appropriate environmental conditions. True diploidy occurs at the basidia where karyogamy takes place immediately before the onset of the meiosis giving rise to four uninucleate basidiospores. At this diploid stage, genetic recombination can occur, although some reports have also suggested the occurrence of parasexual somatic recombination in higher basidiomycetes [37]. The basidiospores can germinate when they find the appropriate environmental conditions producing monokaryotic mycelia that reinitiate the fungus life cycle. The monokaryotic or dikaryotic condition of a mycelium can be distinguished by the presence of clamp connections (specialized structures which allow nuclei distribution into daughter cells) in dikaryons and their absence in monokaryons.

Genetic structure of mating genes in higher basidiomycetes

Monokaryon compatibility and mating is controlled by two multiallelic genetically independent loci that ensure the transmission of the two nuclei of the dikaryotic cell during cell division [4]: the genes in locus A are responsible for controlling the pairing of nuclei in the dikaryon, for the formation and septation of clamp cell, and to coordinate cell division, whereas genes at the B locus control the migration of the nuclei towards the hyphal tip, the dissolution septa, and the fusion of clamp cells to ensure a correct dikaryotic stage after cell division. This system of mating control is referred to as bifactorial (two loci) or tetrapolar (as it generates four different incompatibility types in the monokaryotic offspring of a dikaryon) and is common to most of the edible basidiomycetes industrially cultivated with the exception of the unifactorial button mushroom Agaricus bisporus [1, 8, 31]. Molecular analyses of the A and B genes in Coprinus cinereus and Schyzophillum commune have revealed that A genes code for homeodomain proteins that, to be functional, should form heterodimers (with one subunit coded for by each one of the two nuclei forming the dikaryon), whereas B genes code for pheromones and their receptors [4]. The genetic structure of both factors is complex. The factor A gene complex consists of a central motif of two genes (coding for the two protein types present in the heterodimer) transcribed in divergent directions that appears duplicated one to three times in the different A mating types and species [4, 6, 14, 19, 24, 25]. The gene complex for factor B has a central unit formed by a single gene coding for a membrane pheromone receptor and a variable number of genes (from two to seven) coding for pheromones [4, 35]. Again, a variable number of copies of this central motif can be found in different B factors and species.

In P. ostreatus locus A behaves as a single one [16], whereas locus B is a complex of two genes ($matB\alpha$ and $matB\beta$) linked at genetic distances ranging from 17.5 cM to less than 5.0 cM in the different strains, and new B specificities can appear by recombination between the two loci as it occurs in other higher basidiomycetes [9, 26]. The bifactorial mating control system makes it difficult and cumbersome breeding-oriented crossing of monokaryons. In fact, it is first necessary to determine the incompatibility factors present in monokaryons derived from a given strain using testers for the four basic incompatibility types (Ax Bx, Ax By, Ay Bx and Ay By) appearing in the offspring of a dikaryon AxAy BxBy. By using this method, we have studied the mating factors present in P. ostreatus accessions from a variety of origins and have found nine different A and 15 different B mating types, some of which are the result of intra-factorial recombination (Table 1). Moreover, each different strain analysed carried a different pair of A factors, with only one exception, and a different pair of B factors.

The determination of the mating type of a given monokaryon is highly facilitated by the use of molecular markers linked to the mating factors. These markers can be identified, in a first step, using a bulked segregant analysis approach [23] to generate Randomly Amplified Polymorphic DNA (RAPD) markers genetically linked to the genes controlling A and B mating factors in P. ostreatus [16] (Table 2). Due to the number of monokaryons analysed in our study, the minimum linkage distance measurable between markers and the corresponding genes is 1.25 cM (centimorgan). RAPD markers behave as dominant and, in the strain under study, they segregate against a null allele. Consequently, only alleles *matA1*, *matB* α 2, and *matB* β 1 are directly detectable using these RAPD markers. This limitation, as well as those derived from the RAPD methodology, can be avoided by converting RAPD markers in Restriction Fragment Length Polymorphism (RFLP) markers, which allow a quick and certain identification of monokaryons because they distinguish the two alleles present in a dikaryotic individual.

RAPD and RFLP markers, in addition, allow the study of the genomic areas flanking the mating factors that have been reported to be highly conserved [4]. The sequences of the RAPD markers linked to the mating factors in *P. ostreatus* show no homology with any other entry in the gene databank, and no

Table 1 Mating factors found in different Pleurotus ostreatus strain
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Strain (mating genotype)	Variety	Origin	B factor	Occurrence of B factor	Sample size ^(a)	Recombination frequency (%)
N001	florida	USA	B1	63	120	15.8
(A1A2 B1B2)			B2	38		
			B3	11		
			<i>B4</i>	8		
N017	florida	UPNA ^(b)	<i>B3</i>	45	102	15.7
(A1A2 B3B4)			B4	41		
			B1	8		
			<i>B2</i>	8		
N002	ostreatus	Germany	<i>B5</i>	39	98	8.2
(A5A6 B5B6)		•	B6	51		
			B15	6		
			B16	2		
N018	ostretaus	UPNA ^(b)	B15	41	105	4.8
(A5A6 B15B16)			B16	59		
· · · · · ·			B5	3		
			<i>B6</i>	2		
N003	ostreatus	Spain	<i>B7</i>	86	170	0.6
(A7A8 B7B8)		1	<i>B8</i>	83		
× ,			<i>B17</i>	1		
N005	colombinus	Italy	B11	_	_	_
(A8A11 B11B12)		5	B12	-		
N006	sajor-caju	India	B13	_	_	_
(A13A14 B13B14)	<i>v</i> 3		B14	_		

^(a)Number of individuals studied.

^(b)UPNA: Public University of Navarra, Spain.

obvious open reading frames were found to suggest that they may correspond to coding sequences rather than to intergenic regions. Notwithstanding, when those probes were used in Southern experiments on genomic DNA purified from other *P. ostreatus* strains, a strong hybridization signal was obtained, whereas, in the same conditions, only a weak signal on DNA from other species of the same genus, and no signal on genomic DNA purified from other agaricales appeared; this suggests a high degree of species-specificity [16].

 Table 2 RAPD and RFLP alleles genetically linked to the mating alleles identified in *Pleurotus ostreatus* var. *florida*. Alleles placed in the same row are in coupling phase

Mating allele	RAPD marker	RFLP marker
matA1	S11,900	$rS11_{900}\alpha$
	S18 ₁₃₀₀	$rS18_{1300}\alpha$
matA2	_	$rS11_{900}\beta$
	-	$rS18_{1300}\beta$
mat $B\alpha$ 1	-	rL3 ₁₃₀₀ 1
matB α 2	L3 ₁₃₀₀	rL3 ₁₃₀₀ 2
mat $B\beta$ 1	$L6_{1800}$	rL6 ₁₈₀₀ 3
matBβ 2	_	rL6 ₁₈₀₀ 2

Genome structure in higher basidiomycetes

Cytogenetic study in higher basidiomycetes has been difficult because of intranuclear mitosis and the small size of fungal chromosomes [5]. In the case of *P. ostreatus*, both chromosome number and genome size have been under discussion for many years [5, 27, 33]. Chromosome separation by Clamped Homogeneous Electric Field (CHEF) electrophoresis is the method of choice to study fungal molecular karyotypes and their evolution [11, 34, 38, 39]. We have used this approach to find out the chromosome number and to study the molecular karyotype in *P. ostreatus* [17]: eleven chromosomes were resolved per haploid (monokaryotic) genome, which accounted for a total genome size of 35.1 Mbp in average (Figure 1, Table 3).

Moreover, this study revealed the occurrence of relevant chromosome length polymorphisms (CLPs) between the homologous chromosomes in each of the two nuclei occurring in the dikaryon. CLPs are common in fungal genomes [38], and have been related to variations in the copy number of sequences such as the ribosomal DNA (rDNA) [28, 34], subtelomeric repeats [10, 18], and to the result of mitotic and meiotic recombination processes [38, 39]. The molecular basis of the CLPs found in *P. ostretaus* is not clear yet, although the



Fig. 1 Molecular karyotype of *Pleurotus ostreatus*. A) Clamped Homogeneous Electric Field (CHEF) separation of the chromosomes present in the dikaryon (N001) and in each of the two nuclei (PC9 and PC15). B) Idiotype of the two nuclei (PC9 and PC15) indicating the chromosome length polymorphisms. (Figure from Larraya et al. [17]. Reproduced with permission.)

most prominent CLP found corresponds to chromosome II, where the rDNA as well as a polymorphism of repetitive DNA have been mapped (L. Larraya, unpublished results).

The molecular karyotype should be complemented with information on the genomic position of genes of interest. This positioning is facilitated by the use of genetic linkage maps, which are a powerful tool for the design of breeding strategies and for positional gene cloning. The number of linkage maps available is low in filamentous fungi [7, 22, 29, 36]. Only one linkage map of edible fungi is currently available: the map for the button mushroom, Agaricus bisporus [15]. We have developed a map for P. ostreatus based on 196 markers (RAPD and RFLP markers, as well as functional characters) studied in a population of 80 monokaryons derived from single spores produced by the commercial strain florida (L. Larraya, unpublished results). P. ostreatus linkage map associated the markers into eleven linkage groups corresponding to the chromosomes resolved by PFGE analysis and which span a total of 1000.7 cM (Table 3). Table 3 shows the correlation between physical (measured in Mbp) and genetic (measured in cM) sizes, which suggests a random distribution of the anonymous molecular markers used for this study. The comparison between the two linkage maps available for edible mushrooms reveal several similarities as well as some obvious differences. Among such differences it is particularly relevant that the level of estimated polymorphism (average number of segregating markers per RAPD primer used) was higher in P. ostreatus (an heterothallic fungus) than in A. bisporus (a secondary homothallic fungus).

The availability of genetic linkage maps allows, in addition, the study of the meiotic behavior of these fungi, which is of the greatest importance to the breeding process. An example of this is the measure of the average number of crossovers per chromosome, because one of the functions of crossovers during meiosis is to ensure a correct chromosome disjunction whenever a correct chromosome alignment occurs. In *P. ostreatus*, an average of 0.89 crossovers per chromosome and individual occurs. This low value combined with the CLPs found in the genome of this fungus could have an effect on the sorting of chromosomes during meiosis and thus reducing the rate of germination of basidiospores by the formation of odd meiotic products or by the production of chromosome deletions, which would render nonviable spores.

Table 3 Characteristics of the molecular karyotype and linkage map of Pleurotus ostreatus

Chromosome	Size ^a (Mbp)	Size ^b (cM)	Markers number	kbp/cM	Average Marker Interval (cM)	Cross-over events
Ι	4.70	103.0	23	45.6	4.5	0.98
II	4.35	173.6	23	25.1	7.5	1.71
III	4.55	178.7	25	25.5	7.1	1.75
IV	3.55	59.2	14	60.0	4.2	0.59
V	3.45	82.0	13	42.1	6.3	0.81
VI	3.10	76.7	20	40.4	3.8	0.76
VII	3.15	74.4	18	42.3	4.1	0.74
VIII	2.95	85.3	14	34.6	6.1	0.84
IX	2.10	74.5	16	28.2	4.7	0.74
Х	1.75	33.8	13	51.8	2.6	0.34
XI	1.45	59.5	10	24.4	5.9	0.59
Average	3.19	91	16.7	35.1	5.3	0.89
Total	35.1	1000.7	189			

^a Average of the two homologous chromosomes [17].

^b Sum of the linkage distances between the markers placed on the corresponding chromosome. Sizes in centimorgans (cM) correspond to the sum of all the distances between adjacent chromosome markers.

Genetic breeding of *P. ostreatus* strains

Many of the characters of interest for either industrial or agronomic production are controlled by series of genes which contribute additively to the final phenotype. The genes responsible for phenotypic traits behaving in this way are located in the Quantitative Trait Loci (QTL). Linkage maps are especially valuable to map QTL and to study these polygenic characters. In our laboratory, we have mapped QTLs responsible for growth rate of monokaryons (Q-grm genes) and dikaryons (Q-grd genes) on the P. ostreatus linkage map described above. A genetic background for explaining linear growth rate variation between monokaryons had been previously observed, as the two nuclei present in the commercial strain *florida* showed remarkable differences in this variable. The linkage analysis allowed the identification of five genomic regions involved in the control of these QTL (Table 4) with one of them (Q-grm1) being responsible for about the 21.4% of the total variation in growth rate. Two other regions (Q-grm5 and Q-grm2) are responsible for the 6.5 and 9.9%, respectively, of the total variation in growth rate. Note that these two regions map to chromosome IV which, in the global linkage analysis, shows the highest number of molecular markers with distorted segregation [30].

 Table 4 Chromosomes carrying Quantitative Trait Loci (QTL) responsible

 for variation in the character growth rate in monokaryons (Q-grm loci) and

 dikaryons (Q-grd loci) of Pleurotus ostreatus

Chromosome	QTL	Significance (%)	\mathbb{R}^2	
Ι	Q-grd1	0.06	17.6	
III	Q-grm4	1.68	7.3	
III	Q-grd3	3.31	8.4	
IV	Q-grm2	0.45	9.9	
IV	Q-grm5	2.33	6.5	
VII	Q-grd6	3.21	6.1	
VIII	Q-grm1	0.01	21.4	
VIII	Q-grd5	0.39	10.5	
Х	Q-grd2	3.84	7.4	
XI	Q-grm3	0.79	8.8	
XI	O-grd4	0.28	8.2	

Significance (%) indicates the level of statistical significance (in all cases it is lower than 5%). R^2 indicates the percentage of the total variation in growth rate that is controlled by a given QTL.

In addition to the identification and mapping of Q-grm genomic regions, we studied the effect of particular nuclei on the dikaryotic growth rate by crossing 80 segregating monokaryons by a compatible monokaryon produced by a different strain which provides a common nucleus. Under these conditions, the variation in growth rate observed in the dikaryons should be due to the nuclei provided by the segregating monokaryons and to the interactions of each one of them with the common nucleus. The results summarised in Table 4 indicate that genomic regions can be associated to a higher dikaryotic growth rate in these particular experimental conditions. Further research should be

carried out to estimate the effect of the interaction factor and the correlation between the growth rates determined in laboratory conditions and those obtained in field conditions. Nevertheless, the identification of the genomic regions contributing to a faster substrate colonization by molecular markers can speed up the selection process by enabling the choice of those monokaryons that will perform better in dikaryotic condition.

Conclusions

The industrial use of fungi requires the initial selection of the strain of interest, and the subsequent breeding of the selected strain to improve its yield in terms of secondary metabolite production or biomass yield. In the case of edible fungi, however, the construction of genetically modified organisms is not a strategy of choice for the improvement of the economic quality of the fungus, and breeding should be based on "classical" genetic approaches. Some molecular techniques, however, can facilitate the design of breeding programs for edible fungi aimed to increase their commercial value facilitating the selection of both quantitative traits such as growth rate, agronomic yield, accumulation of nutrients of interest, as well as qualitative features such as pathogen resistance and spore formation. Besides, the genomic structure of hybrids can be checked by using specific molecular markers, selected among those mapped at critical positions in different chromosomes to verify the occurrence of karyotipic abnormalities that could be responsible for strain unstability during industrial-scale culture. The techniques and results described in this article suggest that P. ostretaus breeding and the positional cloning in higher basidiomycetes could be fuelled by these tools.

Acknowledgments This work has been supported by projects BIO94-0443 and BIO 99-0278 of the CICYT (Spain) and by Institutional Funds of the Universidad Pública de Navarra.

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