Fine-scale genetic analyses reveal unexpected spatial-temporal heterogeneity in two natural populations of the commercial mushroom *Agaricus bisporus*

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This study examined the fine-scale genetic variation of the commercial mushroom, *Agaricus bisporus*, over 2 years at two sites in France. One site was a meadow fertilized with horse manure and disturbed regularly by humans; the other was a Monterey cypress forest free of human disturbance. Altogether, 50 mushrooms were collected and analysed for mitochondrial and nuclear genetic variation marked by RFLPs and multilocus enzyme electrophoretic polymorphisms. Population samples from these two sites were genetically different and both sites contained high levels of genetic diversity. No identical genotypes were found at either site between the 2 years and there was little evidence for extensive vegetative clonality for this species. Contrary to expectations, very limited evidence of pseudohomothallic reproduction was found. Results from tests of Hardy–Weinberg equilibrium and genotypic equilibrium showed that outcrossing and recombination have played significant roles in both populations. The results demonstrated spatial-temporal genetic heterogeneity of *A. bisporus* in natural populations.

Keywords: molecular markers, modes of reproduction, population structure, fungi

INTRODUCTION

The application of molecular markers has had significant impact in addressing questions concerning the natural history and population biology of many organisms, clarifying modes of reproduction, speciation and the spatial-temporal dynamics of populations. For the commercial button mushroom, *Agaricus bisporus* Lange (Imbach) (syn. *A. brunnescens* Peck), large-scale geographic structure has been investigated, primarily due to the potential utility of wild germplasm for commercial breeding purposes (Callac, 1995; Kerrigan, 1995; Xu *et al.*, 1997, 1998). However, relatively little is known about the mode of reproduction and the fine-scale spatial-temporal dynamics of *A. bisporus* in natural environments. The purpose of this study was to use highly polymorphic molecular markers to investigate the potential modes of reproduction and patterns of spatial-temporal genetic variation of *A. bisporus* in two defined locations in France.

The basidiomycete *A. bisporus* is a major agricultural crop in many countries, with a worldwide annual production worth over US\$2 billion (Chang, 1993). This fungus has been cultivated for about three centuries, beginning in France during the reign of Louis XIV. The practice subsequently spread to other European countries. By the early twentieth century, commercial cultivation of *A. bisporus* was widespread on all populated continents. Recent genetic evidence indicated that escapes of cultivar germplasm to the wild accompanied the spread of commercial cultivation in many geographic areas (Xu *et al.*, 1997, 1998).

The mating system in *A. bisporus* is controlled by one locus with multiple alleles (Xu *et al.*, 1993, 1996a; Imbernon *et al.*, 1995). Homokaryons (i.e. mycelium with only one nuclear type) with different alleles at the mating-type locus can mate to form a heterokaryon (i.e. mycelium with two different nuclear types) capable of forming mushroom fruiting bodies. However, the production of homokaryotic basidiospores is limited in most strains of *A. bisporus* (Allen *et al.*, 1992; Kerrigan *et al.*, 1993; Royse & May, 1982; Summerbell *et al.*, 1989). Most often, the majority of basidia produce only

Abbreviations: GE, genotypic equilibrium; HWE, Hardy–Weinberg equilibrium; LG, linkage group.

two basidiospores with each receiving two of the four post-meiotic nuclei. These two nuclei are often non-sisters with respect to meiosis II and carry compatible mating types. Basidiospores from these two-spored basidia germinate to produce fertile heterokaryotic mycelia in which the two genetically different nuclei coexist in each hyphal compartment. This life cycle is termed pseudohomothallism or secondary homothallism (Raper *et al.*, 1972; Royse & May, 1982). Pseudohomothallic reproduction in *A. bisporus* tends to maintain the parental genotype in the offspring (Allen *et al.*, 1992; Kerrigan *et al.*, 1993; Summerbell *et al.*, 1989).

In addition to pseudohomothallic life cycle, heterothallism (outcrossing) can also occur in *A. bisporus*. Some basidia produce three or four basidiospores, many of which receive only one post-meiotic nucleus. Such basidiospores can germinate to form self-sterile homokaryons. Mating among homokaryons with compatible mating types from the same or different mushroom fruiting bodies can result in the formation of a fertile heterokaryon (Xu *et al.*, 1993, 1996a). Therefore, through this life cycle, inbreeding (or selfing) and outcrossing are possible in *A. bisporus*. Because of the two modes of reproduction, pseudohomothallism and heterothallism, *A. bisporus* was also termed an amphithallic species (Lange, 1952). The alternative life cycles of *A. bisporus* depend on the ploidy level of the spores, a trait correlated to the number of spores per basidium.

Despite extensive laboratory studies, few data are available on the modes of reproduction in natural populations of *A. bisporus*. An earlier report based on samples from different areas in California suggested evidence of pseudohomothallic reproduction in the Californian populations of *A. bisporus* (Kerrigan *et al.*, 1998). Those inferences were tentative, not supported by direct observation of mushroom from the same site over time (Kerrigan *et al.*, 1998). Two factors contribute to this lack of understanding. The first is the lack of appropriate samples for such an analysis. Unlike many basidiomycete species, no method is currently available for the direct isolation of *A. bisporus* mycelia from substrates in nature. Collections are limited only to strains obtained from mushroom fruiting bodies either directly from nature, or, rarely, indirectly by placing natural inoculum (e.g. leaf litter or horse manure) onto fruiting substrates under optimal fruiting conditions in the lab and isolating mushrooms from these fruiting experiments (Callac *et al.*, 2000). Unfortunately, fruiting of *A. bisporus* is influenced by many factors (e.g. temperature, moisture and substrate) and is not a commonly observed event in nature.

The second factor contributing to our lack of understanding of reproductive systems in nature is the difficulty in distinguishing outcrossing from inbreeding and pseudohomothallic reproduction. All three processes can generate novel genotypes. However, with highly polymorphic genetic markers, specific predictions from these reproductive modes could be directly compared. Specifically, outcrossing generates random as-

sociation of alleles at the same or different locus and the observed and expected heterozygosities should be comparable. By contrast, long-term strict inbreeding and pseudohomothallic reproduction could not generate random associations of alleles within and between loci. With strict inbreeding, the overall observed heterozygosity should be lower than those expected under random mating. By contrast, on a short time scale, pseudohomothallic reproduction in *A. bisporus* could maintain original parental genotype and heterozygosity, but the maintenance of such heterozygosity is not expected on a long-term scale.

The objective of this study was to examine the mode of reproduction and the fine-scale population structure of *A. bisporus*. Samples from two sites in France were monitored and examined over a 2-year period. One site contained horse manure as substrate and had frequent human disturbances (site 1). The other site was associated with Monterey cypress trees and was without human disturbance (site 2). Highly polymorphic genetic markers were used to examine the genetic structure within and between these two sites.

METHODS

Sites and strains. Favourable mushroom fruiting conditions existed in two sites in France over a 2-year period. These two sites were located about 450 km apart. Site 1 was in Gradignan, near Bordeaux, and covered an area of about 50 × 70 m. Site 2 was in Dinard, near Saint Malo, and covered an area of about 20 × 30 m. The nearest known sample of *A. bisporus* was at least 3 km from site 1 and about 300 m from site 2. The habitat in site 1 was a meadow where horse manure has been spread each May since 1985. Site 2 is near the English Channel in a Monterey cypress (*Cupressus macrocarpa*) forest. The trees were planted in 1947 and the site lacked human disturbance. The numbers of isolates found in each site and year are presented in Table 1. Isolates were identified as *A. bisporus* based on their morphological, and in some cases biochemical, features and on the ability of homokaryons from these heterokaryons to form heterokaryons when paired with known *A. bisporus* homokaryons. Sites 1 and 2 were previously reported as sites 13 and 24, respectively, with respect to the collecting of *A. bisporus* in France (Callac, 1994; Guinberteau *et al.*, 1998). At both sites during the first year, only one representative from each cluster of fruiting bodies was isolated and analysed. Different fruiting bodies from the same cluster (i.e. lumped at the base) were usually identified as genetically identical and likely they resulted from the same vegetative mycelia (see Results). However, to obtain a more complete sample, all the fruiting bodies for the second year at both sites were collected and analysed. In total, 50 fruiting bodies were isolated. No fruiting body from the second-year sample was found at exactly the same location as ones from the previous year at either site. Because of the lack of correlation between physical distances among strains and their genetic distances within each sample (see Results), physical locations of mushroom fruiting bodies are not presented. For genotype analysis, except for strain BS78L where multi-spore culture was used, all other strains were derived directly from fruiting body tissues. The mean spore number per basidium was determined for each of the 50 strains (Callac, 1994; Guinberteau *et al.*, 1998). As far as we know, this collection of 50 fruiting bodies represents the best

material available for fine-scale spatial-temporal analysis of genetic variation of *A. bisporus* from natural environments.

Marker assay. Three types of co-dominant molecular markers were assayed for all 50 strains. The first type was based on Southern hybridization of cloned single-copy DNA fragments to *EcoRI*-digested total genomic DNA. This method screened RFLPs for 10 nuclear loci and 9 mitochondrial probes. The 10 nuclear loci were 1n17, 1n36, 1n38, 1n55, 1n148, 4n6, 4n27, 33n5, 33n16 and 33n18 (Xu *et al.*, 1997; Kerrigan *et al.*, 1993, 1996; Table 1). The 9 mitochondrial probes were B4b, B5, B6, B7, B9, B13, B15, BS1 and BS2(5) (Hintz *et al.*, 1988; Jin & Horgen, 1993; Xu *et al.*, 1998). Allelic determinations and nomenclature followed those of a global population genetic survey by Xu *et al.* (1997).

The second genotyping method was based on PCR of single-copy DNA fragments, followed by restriction digests of the PCR products to detect RFLPs (PCR-RFLP). Five polymorphic PCR-RFLP markers were assayed (PR2, PR3b, PR4, PR5 and PR6). The third type of molecular marker is based on allozyme polymorphism at three loci, alcohol dehydrogenase (ADH), phosphoglucumutase (PGM), and β -glucosidase (BGLU). Locus name and allelic designations for each strain are presented in Table 1. Detailed information for individual loci can be found elsewhere (Callac *et al.*, 1997; Hintz *et al.*, 1988; Jin & Horgen, 1993; Kerrigan *et al.*, 1993, 1996; Mouyna & Brygoo, 1993; Xu *et al.*, 1997, 1998) and are available upon request from J. Xu (jpxu@mcmaster.ca) and P. Callac (callac@bordeaux.inra.fr). Mitochondrial DNA (mtDNA) haplotypes in Table 1 were determined based on the composite Southern hybridization patterns of nine mtDNA probes to *EcoRI*-digested total genomic DNA as described by Xu *et al.* (1998). These nine probes covered about 60% of the mtDNA in a cultivated strain (Hintz *et al.*, 1988).

Data analysis. Each mushroom fruiting body was considered an independent isolate. Multilocus genotypes were identified for each of the 50 strains. Standard population genetic measures of mean alleles per locus, observed heterozygosity (H_o) and expected heterozygosity (=gene diversity, H_e) were calculated. The distribution of genetic diversity within and among samples and the degree of correlation among uniting gametes were evaluated through F -statistics. F -statistics were calculated by using the ANOVA approach presented by Weir & Cockerham (1984). Inbreeding coefficients were calculated as recommended by Reynolds *et al.* (1983). The program Genetic Data Analysis GDA1d16c (version 1.0D16c) by P. O. Lewis and D. Zaykin was used for the above calculations of population genetic parameters. This program was kindly provided by Paul Lewis of the University of Connecticut, USA.

To determine if there was evidence for outcrossing in natural populations of *A. bisporus*, we performed two tests. The first compared the observed genotype frequencies with those expected under the assumption of random mating (test of Hardy–Weinberg equilibrium, HWE). The lack of significant difference between observed and expected genotypic counts would be consistent with HWE and the hypothesis that outcrossing and recombination have played a significant role in these populations. The second test was to determine the associations of genotypes between loci [test of genotypic equilibrium (GE)] for each sample. For the GE tests, diploid genotypes with each locus were preserved to ensure that disequilibrium within a locus (i.e. the Hardy–Weinberg disequilibrium) does not influence the tests between loci.

Because of multiple tests, Bonferoni correction was used to adjust significance values for the tests of genotypic disequilibrium (Zaykin *et al.*, 1995). In this test, random associations of genotypes between loci would also be consistent with recombination and suggest evidence of outcrossing. Fisher's exact tests were used to determine both HWE and GE for each sample. Statistical significances of both tests were based on the observed genotypic frequency and compared to the null distribution of genotype frequencies. The null genotype frequency distributions were derived from permutation with 1000 repeats using empirical allele frequencies and under the null hypothesis of random mating. Through permutation, the random effects of small sample sizes on both HWE and GE are reduced. Tests were done for each individual locus and between all pairs of loci. The program GDA1d16c was used for these two disequilibrium tests (Lewis & Zaykin, 2001).

While outcrossing and recombination were tested against the null hypotheses of random mating, the alternative hypotheses of clonality, inbreeding and pseudohomothallic reproduction were tested directly through pairwise comparisons of multi-locus genotypes. To determine whether inbreeding and pseudohomothallic reproduction are present and if so, their prevalence, we compared all pairs of strains between the two years from each site and looked for evidence of highly similar or identical genotypes. Specifically, identical genotypes and loss of heterozygosity shown in the second-year sample would suggest such reproductions in nature. Beyond mutation, no gain of heterozygosity should be expected for pseudohomothallic reproduction or inbreeding. In addition, vegetative clonality could also contribute to identical genotypes between the years. However, loss of heterozygosities for nuclear loci was only consistent with inbreeding or pseudohomothallic reproduction.

RESULTS

Molecular genetic variation

The genotypes of all strains are presented in Table 1. There were high levels of genetic variation in all four samples (Table 2). At site 1, all 18 nuclear loci in the 1991 sample were polymorphic, while 16 were polymorphic in the 1992 sample. At site 2, all 18 loci were polymorphic for both the 1992 and 1993 samples. The mean number of alleles among the 18 nuclear loci was 2.9 (site 1, 1991), 2.7 (site 1, 1992), 3.5 (site 2, 1992) and 2.9 (site 2, 1993) (Table 2).

From Southern hybridization of nine mtDNA probes, a total of nine mtDNA haplotypes (A, B, C, D, E, F, G, H and I) were found among the 50 strains analysed in this study. Briefly, there were two mtDNA haplotypes (A and C) in the site 1, 1991, sample; four haplotypes (A, C, D and E) in the site 1, 1992, sample; seven haplotypes (A, B, C, D, F, G and H) in the site 2, 1992, sample; and five haplotypes (A, B, G, H and I) in the site 2, 1993, sample. mtDNA haplotype A was present in all four samples. This haplotype is one of the two mtDNA haplotypes in the cultivar strains used for at least the last two decades worldwide for commercial cultivation (Kerrigan, 1995; Xu *et al.*, 1998). Haplotype B is the other cultivar mtDNA haplotype. This haplotype was found in both samples from site 2, but not site 1 (Table 1).

Table 1. Multilocus genotypes of 50 *A. bisporus* strains analysed in this study

See Methods for references to loci information.

Strain	Nuclear*																		mtDNA* haplotype
	1n148	PR5	PR2	PR6	1n17	4n6	33n5	33n16	33n18	1n38	1n55	1n36	4n27	Adh	Pgm	Glu	PR3b	PR4	
Site 1, Gradignan, 1991 sample																			
BS78A	3/6	3/3	2/2	1/3	2/4	2/3	8/8	6/6	3/3	1/7	6/6	4/4	1/2	3/3	2/2	6/6	1/1	2/2	A
BS78B	3/6	1/5	1/2	1/1	2/4	2/4	3/8	6/6	3/3	1/7	6/8	4/4	2/3	3/5	2/5	5/5	1/2	1/2	A
BS78C	3/6	3/4	2/2	1/1	4/4	3/3	8/8	6/6	3/3	1/7	6/8	3/6	1/2	4/5	2/5	6/6	1/2	2/3	A
BS78D	4/6	5/7	1/2	1/1	2/4	2/3	3/3	6/7	3/4	1/7	6/8	5/5	1/2	3/5	2/5	6/6	2/2	1/2	A
BS78E	3/6	1/5	1/2	1/1	4/4	3/3	4/8	4/7	3/3	1/1	8/8	5/5	1/2	3/7	5/5	6/6	1/2	2/2	C
BS78F	3/4	5/7	1/2	1/4	2/4	3/4	5/8	6/6	3/3	1/7	8/8	4/4	1/2	2/5	2/5	5/5	2/2	1/2	C
BS78G	3/6	3/3	2/2	1/3	2/4	2/4	8/8	6/6	3/4	1/7	6/8	4/4	2/3	3/5	2/5	5/5	1/1	2/2	A
BS78H	3/6	1/5	1/2	1/1	4/4	3/3	4/8	6/6	3/3	1/1	8/8	4/4	1/2	3/7	5/5	6/6	1/2	2/2	C
BS78I	3/6	1/5	1/2	1/1	2/4	2/3	8/8	6/6	3/3	1/7	6/6	4/4	1/2	3/3	2/2	6/6	1/2	1/1	A
BS78J	3/6	1/5	2/2	1/3	2/4	3/4	8/8	6/6	3/3	1/7	6/8	4/6	1/2	3/5	2/5	6/6	2/2	1/2	A
BS78K	3/4	3/3	1/2	1/4	2/2	3/4	5/5	6/6	3/4	1/7	6/8	4/7	1/2	3/4	2/5	6/6	1/1	2/2	A
Site 1, Gradignan, 1992 sample																			
BS78L	3/6	3/3	2/2	1/3	4/4	3/3	5/8	6/6	3/3	1/1	8/8	4/4	1/2	3/7	5/5	6/6	1/1	2/2	C
BS78M	3/6	3/3	2/2	1/3	4/4	3/3	5/8	6/6	3/3	1/1	8/8	4/4	1/2	3/3	5/5	6/6	1/1	2/2	C
BS78N	3/6	3/3	2/2	1/3	4/4	3/3	5/8	6/6	3/3	1/1	8/8	4/4	1/2	3/3	5/5	6/6	1/1	2/2	C
BS78P	4/6	5/7	1/2	1/1	2/4	2/3	4/4	6/6	3/4	1/7	5/8	4/4	2/2	5/7	2/2	6/6	1/2	2/2	A
BS78Q	4/6	5/7	1/2	1/1	2/4	2/3	4/4	6/6	3/4	1/7	5/8	4/4	2/2	5/7	2/2	6/6	1/2	2/2	A
BS78R	4/6	5/7	1/2	1/1	2/4	2/3	4/4	6/6	3/4	1/7	5/8	4/4	2/2	5/7	2/2	6/6	1/2	2/2	A
BS78T	3/6	3/4	2/2	1/1	3/3	3/4	4/6	6/6	3/3	1/6	6/6	6/6	1/2	3/5	2/5	6/6	1/2	1/1	D
BS78U	3/6	3/4	2/2	1/1	3/3	3/4	4/6	6/6	3/3	1/6	6/6	6/6	1/2	3/5	2/5	6/6	1/2	1/1	D
BS78V	3/6	3/4	2/2	1/1	3/3	3/4	4/4	6/6	3/3	1/6	6/6	6/6	1/2	3/5	2/5	6/6	1/2	1/1	D
BS78Y	3/6	1/5	1/2	1/1	3/3	2/3	3/8	6/6	3/3	3/5	6/6	4/7	1/2	3/3	2/2	6/6	1/1	1/2	A
BS78Z	3/6	3/3	2/2	1/3	4/4	3/3	5/8	6/6	3/3	1/1	8/8	4/4	1/2	3/3	5/5	6/6	1/1	2/2	E
Site 2, Dinard, 1992																			
BS257	3/6	3/3	2/2	1/4	4/4	3/4	4/8	6/6	3/3	1/5	5/8	4/4	1/3	2/5	4/5	6/6	1/2	1/2	A
BS258	3/4	5/7	1/2	1/4	2/4	3/4	3/3	6/6	3/4	1/7	6/8	4/6	2/5	5/7	2/5	5/5	1/2	2/2	B
BS259	3/3	1/6	2/2	3/4	2/4	3/4	4/4	3/6	3/4	7/9	6/8	4/6	1/2	3/3	2/5	2/6	1/2	2/2	A
BS260	3/6	3/3	2/2	1/4	4/4	3/4	4/4	6/6	3/3	1/5	5/8	4/4	1/3	2/5	4/5	6/6	1/2	1/2	G
BS261	3/3	5/7	1/2	1/4	2/4	2/6	4/8	6/6	3/3	1/3	8/8	5/9	1/5	3/3	3/5	2/2	1/2	2/2	A
BS262	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	7/9	8/8	4/4	2/5	3/3	2/3	6/6	2/2	1/2	H
BS263	3/3	1/6	2/2	3/4	2/4	3/4	4/4	3/6	3/4	7/9	6/8	4/6	1/2	3/3	2/2	3/3	1/2	2/2	A
BS264	3/6	1/5	2/2	1/3	2/4	3/4	6/6	6/6	3/3	1/7	6/8	4/6	1/2	3/5	2/5	6/6	2/2	1/2	A
BS265	3/6	1/5	2/2	1/3	2/4	3/4	6/6	6/6	3/3	1/7	6/8	4/6	1/2	3/5	2/5	6/6	2/2	1/2	A
BS266	3/6	1/5	2/2	1/3	2/4	3/4	6/6	6/6	3/3	1/7	6/8	4/6	1/2	3/5	2/5	6/6	2/2	1/2	A
BS267	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	1/7	8/8	4/4	2/5	3/3	2/2	6/6	2/2	1/2	C
BS268	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	1/7	8/8	4/4	2/5	3/3	2/3	6/6	2/2	1/2	C
BS269	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/7	3/4	1/7	8/8	4/4	2/5	3/3	2/2	6/6	2/2	1/2	C
BS270	3/6	3/3	2/2	1/4	4/4	3/4	4/4	6/6	3/3	1/5	5/8	4/4	1/3	2/5	4/5	6/6	1/2	1/2	A
BS271	3/4	5/7	1/2	1/4	2/4	3/3	5/5	4/6	3/3	1/6	5/8	4/6	1/5	7/7	2/2	3/3	1/2	1/1	D
BS272	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/7	3/4	7/7	8/8	4/4	1/5	3/3	2/2	6/6	2/2	1/2	F
BS274	3/4	5/7	1/2	1/4	2/4	4/4	3/3	6/6	3/4	1/7	6/8	4/6	2/5	5/7	2/5	5/5	1/1	2/2	B
Site 2, Dinard, 1993																			
BS355	3/4	5/7	1/2	1/4	2/4	4/4	3/3	6/6	3/4	1/7	6/8	4/6	2/5	5/7	2/5	5/5	1/2	2/2	B
BS357	3/4	1/5	1/2	1/1	2/4	2/4	8/8	6/6	3/4	1/7	6/8	4/4	2/3	3/5	2/5	5/5	1/2	1/1	A
BS358	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	1/7	8/8	4/4	2/5	3/3	2/2	6/6	2/2	2/2	I
BS359	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	1/7	8/8	4/4	2/5	3/3	2/2	6/6	2/2	1/2	I
BS361	3/4	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	1/7	8/8	4/4	2/5	3/3	2/5	6/6	2/2	1/2	H
BS363	3/3	1/5	1/2	1/4	2/4	3/3	4/6	6/6	3/4	1/1	8/8	4/4	1/1	3/3	5/5	6/6	2/2	2/2	I
BS364	3/6	5/7	2/2	1/4	4/4	3/4	4/8	6/6	3/4	1/7	8/8	6/6	1/2	5/5	2/5	6/6	2/2	1/1	G

Table 1 (cont.)

Strain	Nuclear*																		mtDNA* haplotype
	1n148	PR5	PR2	PR6	1n17	4n6	33n5	33n16	33n18	1n38	1n55	1n36	4n27	Adh	Pgm	Glu	PR3b	PR4	
BS365	4/6	5/7	1/2	1/4	2/4	3/4	4/4	6/6	3/4	1/5	8/8	4/4	2/5	3/3	2/2	6/6	2/2	1/2	H
BS368	3/3	5/7	1/2	1/4	2/3	3/4	4/5	6/6	??	1/7	5/8	4/6	2/2	3/3	2/5	6/6	2/2	3/3	H
BS369	3/4	5/7	1/2	1/4	2/4	3/4	4/5	6/6	3/4	1/7	5/8	4/6	1/2	3/3	2/5	6/6	2/2	2/2	H
BS370	3/3	1/6	2/2	3/4	2/4	3/4	4/4	3/6	3/4	7/9	6/8	4/6	1/2	3/3	2/2	6/6	1/2	2/2	A

* For descriptions of types of markers, see Methods. Allelic nomenclatures of the nuclear and mitochondrial RFLP loci followed those established by Xu *et al.* (1997) during a global survey of genetic variation for *A. bisporus*. These data are available upon request from J. Xu (jpxu@mcmaster.ca). Primer information and nomenclature of alleles for PCR-RFLP and allozyme loci are available from P. Callac (callac@bordeaux.inra.fr). The loci are arranged according to their linkage relationships. Loci 1n148, PR5, PR2, PR6 and 1n147 are on linkage group (LG) I; locus 4n6 on LG II; loci 33n5 and 33n16 on LG III, locus 33n18 on LG V; locus 1n38 on LG VI; and locus 1n36 on LG X. Linkage relationships for the remaining 7 loci (4n27, Est1, Adh, Pgm, Glu, PR3b and PR4) are not known at present.

Genotype distributions and vegetative clonality

Vegetative clonality in *A. bisporus* is inferred from multilocus genotypic analysis of fruiting bodies. Based on the combined nuclear and mitochondrial genotypic data, no evidence of vegetative clonality was found in two of the four samples: the site 1, 1991, sample and the site 2, 1993, sample. All fruiting bodies from each of these two samples were genetically unique (Table 1). In the site 1, 1992, sample, three genotypes were shared among seven strains. However, each shared genotype was from mushrooms clustered together with almost no physical distance from each other. The first cluster contained isolates BS78M, BS78N and BS78Z. The second contained isolates BS78P, BS78Q and BS78R. The third contained BS78T, BS78U and BS78V. Therefore, isolates from site 1, 1992, did not provide any robust evidence for extensive vegetative clonality of *A. bisporus* (Table 1). In fact, physically clustered fruiting bodies can have different genotypes either in the mitochondrial genome (BS78M and BS78N versus BS78Z) or in the nuclear genome (BS78T and BS78U versus BS78V) (Table 1). The total diameters of the basal areas for each of the three clusters in the site 1, 1992, sample did not exceed 10 cm.

The only evidence of potential vegetative clonality came from the site 2, 1992, sample. The three isolates BS264, BS265 and BS266 were found to have identical multi-locus genotypes (Table 1). BS264 was 0.4 m from BS265; BS265 about 1.0 m from BS266; and BS264 about 1.3 m from BS266. Aside from these clusters of mushrooms, there was no correlation between genetic distance and physical distance among the strains at either site (data not shown).

Difference between the two sites

Statistical analysis confirmed that populations from sites 1 and 2 were significantly differentiated from each other (Table 3). The overall F_{ST} value (inbreeding coefficient between sub-populations) was 0.0727 with 95 % confidence interval of 0.0402–0.1061, not overlapping with 0. Within each site, the differences between years were statistically not significant; the 95 % confidence intervals all overlapped 0 (Table 3). At site 2, both samples had higher observed heterozygosity than those expected under random mating (Table 3). However, the two samples from site 1 differed in patterns of heterozygosity, with the 1991 sample having higher observed

Table 2. Descriptive population genetic statistics for samples of *A. bisporus*

Site	Year	P^*	$A^\dagger$	$He^\ddagger$	$Ho^\S$	$IC^\parallel$
1. Gradignan	1991	1.0	2.9	0.52	0.56	−0.079
	1992	0.89	2.7	0.46	0.41	0.102
2. Dinard	1992	1.0	3.5	0.55	0.63	−0.151
	1993	1.0	2.9	0.49	0.58	−0.209

* P , proportion of polymorphic loci (maximum value is 1.0).

† A , mean number of alleles per locus.

‡ He , mean expected heterozygosity per locus (= gene diversity).

§ Ho , mean observed heterozygosity per locus.

|| IC , inbreeding coefficient estimated based on the method-of-moments (Reynolds *et al.*, 1993).

Table 3. Similarity and difference among samples of *A. bisporus* in two French sites

Mean and 95% confidence interval (CI) for each estimate are presented. F_{IS} , F_{IT} , F_{ST} , inbreeding coefficients within a sub-population, within the total population and between sub-populations, respectively; NS, not statistically significant.

	F_{IS}	F_{IT}	F_{ST}
Site 1, between years 1 and 2			
Mean	0.0067	0.0316	0.0251 (NS)
95% CI	−0.1566 to 0.1784	−0.1411 to 0.2123	−0.0083 to 0.0621
Site 2, between years 1 and 2			
Mean	−0.1710	−0.1714	−0.0004 (NS)
95% CI	−0.3430 to 0.0366	−0.3392 to 0.0289	−0.0123 to 0.0151
Between sites 1 and 2			
Mean	−0.0857	0.0042	0.0727*
95% CI	−0.2499 to 0.0910	−0.1449 to 0.1631	0.0402 to 0.1061

* $P < 0.05$, significant difference between sites 1 and 2.

heterozygosity than expected and the 1992 sample showing the reverse (Table 2).

Lack of identical genotypes between the two years at either site

We were interested in whether mushrooms with the same multilocus genotypes could be recovered repeatedly at a site over a 2-year period. If so, then there was potential evidence for vegetative mycelial longevity at these sites. However, mushroom fruiting bodies from neither site showed identical genotypes between the two consecutive years.

Limited evidence for pseudohomothallic reproduction and inbreeding at either site

The mean numbers of basidiospores per basidium were 2.18 (range 2.03–2.87 among the 22 strains) for site 1; and 2.34 (range 2.03–3.77 among the 28 strains) for site 2. One strain, BS261 from site 2, had a mean spore number per basidium of 3.77. For all other 49 strains, the majority of the basidiospores were bi-nucleate and self-fertile. Our prediction therefore was that many pairs of strains reflecting pseudohomothallic reproduction or inbreeding should be found at both sites. Such strains are marked by two genetic characteristics: (i) both isolates carry the same mitochondrial haplotype; and (ii) compared to the first-year strains, the second-year strains should show identical genotype or loss of heterozygosity at certain loci. These two criteria assumed that mutations at the examined genomic regions are negligible over the 2-year span, the duration of available sample. This assumption is most likely valid. Monthly laboratory transfers of the same strain over a 2-year period revealed that transferred clones had identical genotype to the parental clone at the examined RFLP loci (J. Xu, unpublished data).

Two pairs of strains from site 2 are consistent with the predictions of pseudohomothallic reproduction in nature. The first pair consisted of strains BS258 and BS355; with strains BS355 showing loss of heterozygosity at locus 4n6. The second pair consisted of strains BS259 and BS370, with strain BS370 showing loss of heterozygosity at locus Pgm (Table 1). No evidence for pseudohomothallic reproduction was found at site 1 (Table 1). Aside from the multilocus genotype comparisons, the higher observed heterozygosity than those expected under random mating in three of the four samples were also inconsistent with significant pseudohomothallic reproduction of *A. bisporus* in nature.

Evidence for outcrossing and recombination in all four samples

Table 4 presents the summary of HWE and GE tests. Overall, more than half of the 18 loci in each sample had genotype frequencies not significantly different from those expected under random mating. More strikingly, the genotypic disequilibrium tests showed that a significant proportion of loci were in GE (Table 4). Results from both tests were consistent with the hypothesis that outcrossing and recombination have played significant roles in the populations of *A. bisporus* at the two sites.

The 18 nuclear loci analysed here were located on different linkage groups (LGs, see footnote to Table 1), with loci 1n148, PR5, PR2, PR6 and 1n147 located on linkage group I (LG 1), the same LG as the mating-type locus. Loci linked to mating-type locus could show high levels of heterozygosity because of the potential hitchhike effects of frequency-dependent selection on the mating-type locus. Therefore, we tested if loci on LG I have significantly elevated heterozygosity as compared to loci on other LGs. However, no statistically significant differences were observed for either observed or expected heterozygosities ($P > 0.1$). Heterogeneity was

Table 4. Tests of HWE and GE in four samples of *A. bisporus* from two sites in France

Allelic association indices	Site 1, Gradignan		Site 2, Dinard	
	1991	1992	1992	1993
Number of loci in HWE (out of 18 total loci)	13	11	10	12
Number of pairs of loci in GE (out of 153 total possible pairs)	152	127	131	153

observed among loci from both within and between different LGs. The four samples also varied greatly in their patterns of heterozygosity.

Changes of genes and gene frequencies from one year to the next

Aside from the lack of identical genotypes between the two years at both sites, there were significant changes in genes and gene frequencies. Most notable was the number of alleles present in one year but absent in the other. For example, at site 1, 10 of the 53 nuclear alleles in the 1991 sample were absent in the 1992 sample, but the 1992 sample contained six additional alleles not found in the 1991 sample. Similar results were observed at site 2. Compared to the 1992 sample, 13 alleles were not observed but two new alleles were found in the 1993 sample. Aside from the allelic differences, a range of allele frequency changes was also observed. For example, at site 1, allele 4 at locus 4n6 decreased by 40% and allele 3 increased by 48% (Table 1).

The genetic differences in the nuclear genome from one year to the next were paralleled by the changes in the mitochondrial haplotypes at both sites. For example, at site 1, two additional mtDNA haplotypes (D and E) were found in the 1992 sample but absent in the 1991 sample. At site 2, three mtDNA haplotypes (C, D, F) present in the 1992 sample were not found in the 1993 sample. However, one new mtDNA haplotype (I) was found in the site 2, 1993 sample (Table 1).

DISCUSSION

In this study, we examined a collection of *A. bisporus* natural isolates and determined their multilocus genotypes at 18 polymorphic nuclear loci and the mitochondrial genome. Though our sample sizes are relatively small, this unique collection allowed us to infer the mode of reproduction and the fine-scale spatial-temporal genetic variation of *A. bisporus* in its natural environment. A high level of genetic diversity was found within each of the samples. The large number of highly polymorphic loci examined here allowed unambiguous identification of genotypes.

The results here demonstrated limited evidence for vegetative clonality of *A. bisporus* in nature. Among the four samples, two (the site 1, 1991 and site 2, 1993) had mushroom fruiting bodies all with different multilocus

genotypes. While fruiting bodies with identical genotypes were found in the other two samples, those from the site 1, 1992 sample were all clustered. The largest potential genet (consisting of strains BS264, BS265 and BS266 from the site 2, 1992 sample) was 1.3 m in one dimension. Interestingly, this genotype was not recovered in the second year at this site. In addition, when we examined the *Cupressus* leaf litter around mushroom fruiting bodies, we generally observed discoloured litter less than 50 cm in diameter, suggesting that *A. bisporus* mycelia were poorly extended in nature (Callac, 1994). Taken together, the data here suggest that vegetative clonality is limited for *A. bisporus* in nature.

Such an apparent lack of vegetative clonality at both sites contrasts with many other basidiomycete species (Hansen & Hamelin, 1997). For example, a single clone of the root-rot mushroom, *Armillaria gallica*, can occupy several hectares in area and over 1500 years in age (Smith *et al.*, 1992). However, basidiomycete species lacking extensive vegetative clonality are not uncommon (Hansen & Hamelin, 1997). For example, the typical genet size of the ectomycorrhizal mushroom *Hebeloma cylindrosporum* was about 50–70 cm² (Guidot *et al.*, 2001). In addition, most studies where large genets were found used only few and often genetically undefined markers to distinguish genets (see review by Hansen & Hamelin, 1997). If more genetically well-defined and highly polymorphic markers were used, genet sizes in some of those species could be smaller. We would also like to point out that the small genet size as discovered here for *A. bisporus* did not exclude the possibility that basidiospores and possibly mycelia in *A. bisporus* could persist for long periods of time in nature.

Interestingly, there was only limited evidence of pseudohomothallic reproduction or inbreeding in *A. bisporus* in nature. This result was surprising given our current understandings of the life cycle of *A. bisporus* and what we observe of basidiospore numbers from these isolates. Furthermore, this limited evidence for pseudohomothallic reproduction or inbreeding contrasts with the abundant evidence for outcrossing and recombination at both sites (Table 4). There are several possible explanations for the observed patterns of genetic variation. The first is that outcrossing is more common than previously thought. Even though only a small proportion (typically less than 10%) of homokaryotic basidiospores were produced by most fruiting bodies (Kerrigan *et al.*, 1993; Summerbell *et al.*, 1989), if

suitable mating partners existed, outcrossing among these self-sterile homokaryons could generate novel genotypes capable of out-competing strains from heterothallic spores (Xu *et al.*, 1996a; Xu, 1995). Given that one mushroom fruiting body can produce about 10^9 spores, if 5% of those were homokaryotic, 5×10^7 self-sterile spores is a big number and could provide abundant opportunity for outcrossing. In addition, some strains (e.g. BS261, see Callac *et al.*, 1998) had predominantly four-spored basidia and a significant proportion of those spores were capable of mating with other homokaryons carrying compatible mating types.

The second possibility was that mating between homokaryons and heterokaryons could also occur to generate novel genotypes. In a laboratory investigation, genetic recombination was commonly found from pairings between homokaryons and heterokaryons (Xu *et al.*, 1996b). The third possibility was that mating and recombination could also occur in pairings involving unrelated heterokaryons (Xu *et al.*, 1996b). Indeed, the matings between homokaryons and homokaryons, between homokaryons and heterokaryons and between heterokaryons and heterokaryons could all contribute to the generation of novel genotypes and to the maintenance of high levels of genetic variation in these populations. Aside from the mechanisms of outcrossing, the time and frequency of outcrossing in populations of *A. bisporus* are also contentious issues remaining to be resolved. More extensive sampling of mushroom fruiting bodies as well as spores and mycelia is needed to address these questions. Unfortunately, fruiting is an uncommon event for *A. bisporus* in nature and no method is available to directly isolate and analyse spores and mycelia from its natural habitats. Putative hybrids have also been reported in a coastal Californian population of *A. bisporus* (Kerrigan *et al.*, 1998).

Unlike rhizomorphic fungi where vegetative mycelium could be easily sampled and used for population genetic analysis, our sampling and analyses of *A. bisporus* were limited only to mushroom fruiting bodies. Though we attempted to obtain as many fruiting bodies as possible from these two sites and other sites in France between 1991 and 1994, samples from these two sites represent the best we were able to obtain for fine-scale spatial-temporal analysis. Undoubtedly, what we observed here represented only a small part of the genetic diversity within each site. Significant genetic diversity could exist in the forms of dormant spores and mycelia that escaped our sampling. If more sensitive sampling methods were developed in the future for spores and mycelia of *A. bisporus*, analyses of these samples might reveal a population structure very different from what we observed here for mushroom fruiting bodies. Furthermore, in combination with fruiting body samples, the analysis of mycelia samples over an extended period of time could allow us to address other potential questions regarding the length of reproductive cycle, the maintenance of vegetative mycelium in the natural environments and the roles of natural selections in populations of *A. bisporus*.

Our results also demonstrated that genetic drift within a population and gene flow among neighbouring populations could contribute to gene and genotype changes. However, the significant differentiation between the two sites suggests that, aside from cultivar escapes, long-distance gene flow was relatively limited (Table 3; see also Xu *et al.*, 1997). Interestingly, the overall temporal genetic variations were similar between the two sites. For example, both sites showed abundant genotype and gene frequency changes over the 2-year period. However, there were some differences. First, the overall observed heterozygosity was higher at site 2 than at site 1 (Table 2). Second, while no evidence of pseudohomothallic reproduction or inbreeding was observed at site 1, two pairs of strains at site 2 were consistent with pseudohomothallic reproduction and inbreeding. The frequent disturbance experienced at site 1 could have contributed to these differences.

Observed heterozygosities were higher than expected under random mating in three of the four samples (Table 2). Several factors could have contributed to the excess heterozygotes, including (i) linkage to the mating-type locus, (ii) close proximity to centromeres and (iii) selection for heterozygotes. In *A. bisporus*, the mating-type locus was mapped to LG 1 (Xu *et al.*, 1993). Five of the 18 loci analysed here are on LG 1 (see footnote to Table 1). Because mating-type alleles are under frequency-dependent selection, loci linked to mating type could have a significantly higher observed heterozygosity than expected because of hitchhiking effects. However, our data showed little consistent pattern of heterogeneities among loci from within and between different LGs (Table 1). Another factor that could influence heterozygosity is distances between loci and centromeres. The closer loci are to their respective centromeres, the less likely crossing over will occur and therefore, heterozygosities are more likely to be preserved. However, the positions of centromeres are not known for any of the LGs in *A. bisporus*. The third factor is the potential selection for heterozygotes or against homozygotes. A previous investigation showed that outcrossing and heterozygosity were positively correlated to fitness in *A. bisporus* under laboratory conditions (Xu, 1995). However, there is currently no direct evidence of natural selection for excess heterozygosity in natural populations of *A. bisporus*.

The observation of significant evidence for mating and recombination in natural populations of *A. bisporus* calls into question the origin and maintenance of the predominant pseudohomothallic life cycle in this species. One possible explanation is that heterokaryotic basidiospores were abundantly produced and germinated but the establishment of these heterokaryotic mycelia was soon followed by either homokaryon–heterokaryon or heterokaryon–heterokaryon matings. Heterokaryotic spores generally have higher germination rates than homokaryotic spores and heterokaryotic mycelia have greater vegetative growth rates than homokaryotic mycelia. Therefore, advantages afforded by heterokaryotic spores could contribute to

the origin and maintenance of the pseudohomothallic reproduction in *A. bisporus*.

In conclusion, we found high levels of genetic diversity in all four samples of *A. bisporus* from the two French sites. This high genetic diversity along with the large number of polymorphic loci analysed enabled us to address several questions concerning the modes of reproduction and the fine-scale spatial-temporal patterns of genetic variation of *A. bisporus* in nature. Our analyses indicated that natural vegetative clones were small in *A. bisporus* and that strict pseudohomothallic reproduction was not common in nature. Instead, the data here indicated that outcrossing and recombination have played significant roles in the genetic structure in natural populations of *A. bisporus*.

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