Agaricus bisporus cultivars: hidden diversity beyond apparent uniformity ?

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AGARICUS BISPORUS CULTIVARS: HIDDEN DIVERSITY BEYOND APPARENT UNIFORMITY?

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ABSTRACT

Agaricus bisporus, commonly known as the button mushroom, is the most widely cultivated species of edible fungi. The cultivars used by growers over the world are suspected to come from the same restricted pool of strains, and the genetic base of all the present day hybrids is very narrow. The aim of this study was to assess the genetic variability among traditional and modern commercially used *A. bisporus* strains. Fourteen codominant microsatellite markers (*AbSSR*) were used to characterize 75 cultivated genotypes from European spawn makers, maintained in the Collection of *Agaricus* in Bordeaux (CGAB) since 1990. To our knowledge, it is the most extensive sample ever studied. Seven main groups were identified which corresponded to the six ancestral lineages and the hybrids belonging to either U1 or U3 sub-group of strains. Thirty-three U1-like cultivars could not be differentiated. Very few strains have a distinct and typical SSRs pattern. Based on our results, we proposed also a cultivar identification key with a limited number of markers in order to optimize forthcoming SSRs fingerprinting. For three hybrids that seemed to be genetically identical to Horst-U1 at heterokaryotic level, the analysis of each constituting nuclei has demonstrated allelic rearrangement, suggesting essentially derived varieties. The efficiency of microsatellite markers and implications of these results for germplasm management, breeding strategy and variety identification are discussed.

Keywords: genetic diversity; cultivar identification, strain protection, essentially derived varieties

INTRODUCTION

Agaricus bisporus is the most widely cultivated species of edible mushrooms. However, despite its economical importance, few efforts have been done in terms of breeding and strain improvement. As a result, all currently grown cultivars are assumed to be related to a limited number of traditional genotypes [1] and *A. bisporus* appears to be nearly a monolineage crop. Several studies using various molecular markers have demonstrated that most of the current hybrids are either identical of very similar to the first hybrids U1 and U3 released in the 80's [2, 3, 4]. Microsatellites that stand for being one of the most valuable molecular markers for genetic studies have been recently developed for *A. bisporus* [5]. We have already demonstrated that these SSR markers are useful tools for analysing intraspecific genetic variation, but the characterisation of a large cultivar set with such markers has not been done yet. The collection of Germplasm of Agaricus in Bordeaux (CGAB) encompasses at present time near 1000 strains among which we can find cultivars and mainly wild strains from various origins [6]. To improve

germplasm management, the molecular characterisation of this collection is indispensable but raises the question of duplicates. Indeed, due to the typical life cycle and the meiotic behaviour of *A. bisporus* (see the review of Sonnenberg et al. in the present issue [7]) it is impossible, at the molecular level, to distinguish vegetative copies or post-meiotic heterokaryotic mycelia.

The objective of this study was to use 14 *AbSSR* loci to evaluate the genetic diversity in 75 strains representing traditional and modern commercially cultivars. To our knowledge, this is the most extensive sample of *A. bisporus* cultivars for such analyses. Beyond the characterisation of the cultivars diversity, we have also analysed each constituting nuclei of three hybrids that seemed to be genetically identical to U1 at heterokaryotic level. By this way, we could distinguish copies from essentially derived varieties. Consequences for germplasm management, breeding strategy and strain protection will be discussed.

MATERIALS and METHODS

Fungal strains. Seventy-five fungal cultivar strains originated from various European spawn makers were analysed. Eight of them (Bs002, Bs003, Bs034, Bs061, Bs063, Bs089, U1, U3) have been already used for genetic analysis in a previous study [5] and were included in the present sampling as controls. The detailed listing of the 75 strains and their origin is available upon request.

Six homokaryons were obtained from 3 U1-like heterokaryotic strains (Bs508, Bs516, Bs681) by the protoplast method of deheterokaryotisation described in Kerrigan et al. [8].

All fungal strains are maintained in the "Collection du Germplasm des Agarics à Bordeaux" (CGAB) [6].

SSR genotyping. Total DNA was extracted from freeze dried mycelium with a classical CTAB-chloroform-isoamyl alcohol protocol. In routine use, DNA concentration was adjusted to 25ng/µl.

Microsatellite studies were based on markers previously developed in our lab [5]. The 14 SSR loci used here for genotyping 75 cultivars were chosen on the basis of their unambiguous allele scoring, their level of polymorphism revealed in the six morphotype lineages and their multiplex compatibility. Thus, 3 sets of combined SSR loci were developed according to their expected allele size range and/or fluorescent primer labels (Table 1). Repeat units, primer sequences, fluorescently dye labelling, amplification conditions, have been previously described [5, 9]. Capillary electrophoresis and fragment size determination were performed on a CEQTM 8000 Genetic Analysis System Sequencer (Beckman Coulter). The analysis of possible allelic rearrangement in the 6 homokaryotic strains was performed with 8 microsatellite markers known for being heterozygous in the U1 strain. The haplotypes of the two nuclei U1-2 (equivalent to H97) and U1-7 (equivalent to H93) were taken as reference.

Data analysis. SSR data were scored as several alleles per locus distinguished by their size. For each SSR locus, we computed with PowerMarker 3.25 [10] several genetic diversity parameters as the number of alleles per locus (*N*), the number of genotypes (*G*), the observed heterozygosity (*Ho*), the polymorphic information content (*PIC*) and the power of discrimination ($PD=1-\Sigma g_i^2$ where g_i is the frequency of the *i*th genotype). The probability PI that two distinct strains share by chance the same genetic profile was computed using Identity 4.0 (Centre of Applied Genetics, University of Agricultural Sciences, Vienna).

Genetic relationship between cultivars was assessed with the unweighted pair-group method analysis (UPGMA) using genetic distance based on the proportion of shared alleles as $DAS=1-(\Sigma S)/2u$ where *S* is the number of shared alleles and *u* the number of loci analysed.

RESULTS AND DISCUSSION

SSR informativeness. An illustration of genotyping output was shown on Figure 1. The 14 SSR markers used in the present study amplified 44 alleles, ranging in size from 107 to 332 bp. The number of alleles per locus ranged from 2 to 5 with an average of 3.14 alleles (Table 1). Allele frequencies ranged from 0.02 (*AbSSR06*-allele 196 bp) to 0.92 (*AbSSR64*-allele 107 bp) with a mean value of 0.3. The most and the least informative locus were *AbSSR36* (*PD* =0.83) and *AbSSR49* (*PD*=0.17) respectively. The average value of PD parameter over all loci reached 0.53. Several variability parameters estimated in the present study were lower than those previously reported [5]. This result was not surprising due to our sample restricted to cultivated strains. Indeed, the level of polymorphism among cultivars was described lower compared to wild strains and near 43% of *AbSSR* alleles were never found in cultivars [5].

Table 1. Locus name, multiplex and genetic parameters of the 14 SSR markers estimated in the 75 cultivar strains sample.

AbSSR locus*	Multiplex set	*N*	allele size range	*G*	*Ho*	*PIC*	*PD*	*PI*
AbSSR05	3	2	329-332	2	0.93	0.37	0.40	0.40
AbSSR06	3	3	181-202	4	0.97	0.40	0.45	0.27
AbSSR23	2	2	174-180	2	0.73	0.36	0.50	0.48
AbSSR31	2	3	160-170	3	0.00	0.20	0.27	0.55
AbSSR33	2	2	190-193	2	0.00	0.25	0.26	0.58
AbSSR36	1	5	148-161	8	0.81	0.69	0.83	0.13
AbSSR39	1	3	173-192	4	0.17	0.34	0.57	0.38
AbSSR42	1	4	160-189	6	0.66	0.50	0.79	0.19
AbSSR45	1	5	196-206	7	0.97	0.63	0.82	0.15
AbSSR49	3	2	176-184	2	0.00	0.17	0.17	0.72
AbSSR57	2	4	250-262	8	0.16	0.49	0.79	0.17
AbSSR60	3	3	206-210	4	0.83	0.57	0.34	0.29
AbSSR64	2	2	107-113	3	0.12	0.13	0.52	0.48
AbSSR65	3	4	190-215	4	0.88	0.59	0.71	0.20

* color of locus name referred to fluorescent dye (black=D2, green=D3, blue=D4)

***Agaricus bisporus* cultivars identification.** The combination of the allelic pattern obtained with the 14 SSR loci made possible to identify 13 distinct genotypes among the 75 *A. bisporus* cultivated strains analysed (Fig. 2). Thirty-three cultivars could not be differentiated and showed the same genetic profile as U1/U3 hybrid strains. Using a mitochondrial marker (data not shown), these 33 genotypes could be separated into two subgroups that correspond to either U1 or U3 as expected [11]. The small genetic difference, found at a single allele between the group of two strains (Bs005 together with Bs040) and the U1-like group, needed to be further investigated and confirmed with other markers. We could not exclude that minor differences can be due to somatic mutations that may occur in vegetative propagation system such as mycelium maintenance. Comparison of morphological and agronomical data could clarify this slight variability. Our results confirmed that most of the present day hybrids were apparently identical or closely similar to the first hybrids [3].

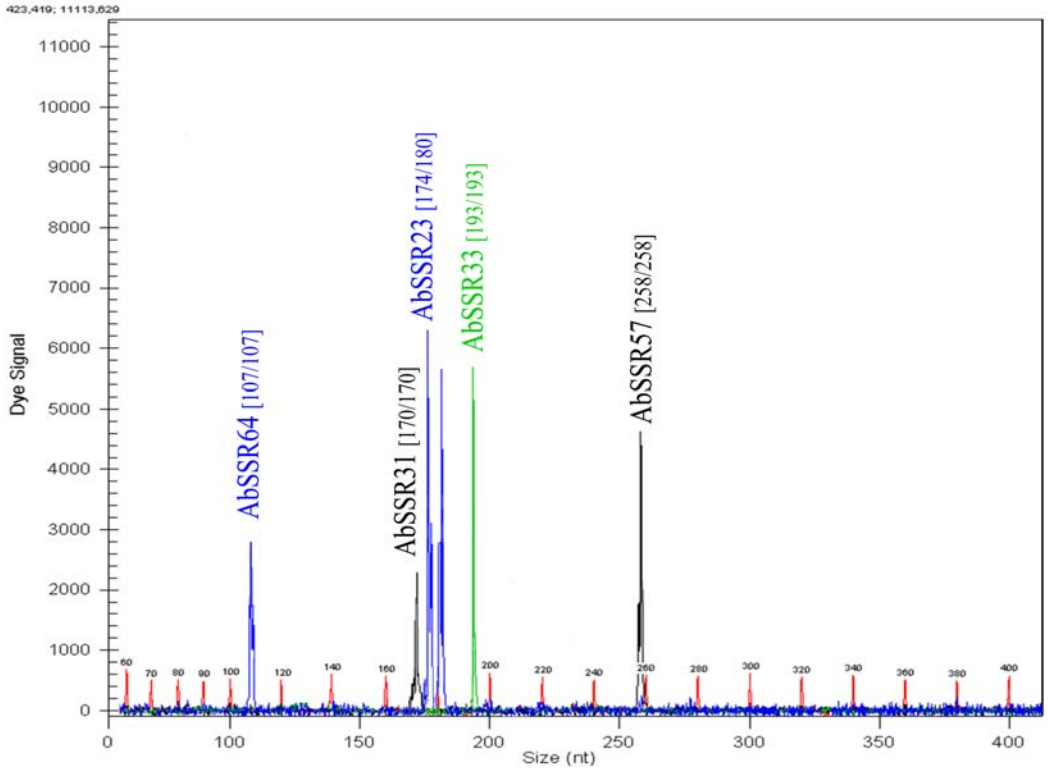

Figure 1. Amplification fragment pattern of five microsatellites (multiplex set n°2) for U1 strain (output from CEQ8000 Genetic Analysis System)

Among the 13 diplotypes, we found the six genotypes that correspond to the six morphotype lineages already described, it is to say "off white", "small white", "white", "brown", "small brown", and "golden white" [5]. However, the off white-like strains were separated into two closely related subgroups. Two strains (Bs022 and Bs668) were singled out. The present data suggested that these two strains were likely to be derived from a cross breeding scheme but it was not possible to clearly set up their pedigrees. Distance values between the 13 diplotypes ranged from 0.04 to 0.78 with an average value of 0.42. The heterozygosity levels were variable among cultivars, ranging from 7.7% (Bs006) to 57.1 % (U1-like hybrids), with an average of 42.2%.

Efficient combination of SSR markers as molecular identification key of *A. bisporus* cultivars. Considering the 14 markers, the probability that two distinct diplotypes share by chance the same genotype was estimated to be 8.3×10^{-8}. To optimize SSR fingerprinting, we would like to determine the combination(s) of *AbSSR* markers that could be sufficient to discriminate each diplotype. For some subgroups, it was possible to establish a molecular identification key by identifying specific *AbSSR* alleles. For instance, *AbSSR*36-148 bp allele was found only in small white-like strains. The signature of small-brown cultivars was the *AbSSR*39-173 pb allele (Table 2). The U1-like hybrids were characterized by specific heterozygous profiles at the two loci *AbSSR*36 (152/160) and *AbSSR*42 (179/181). The use of three selected markers (*AbSSR*36-*AbSSR*42-*AbSSR*45) appeared to be sufficient to distinguish the 13 distinct genotypes. Using this combination, we expected a probability of identity of 3.7×10^{-3} to find, by chance, two strains that showed the same profile.

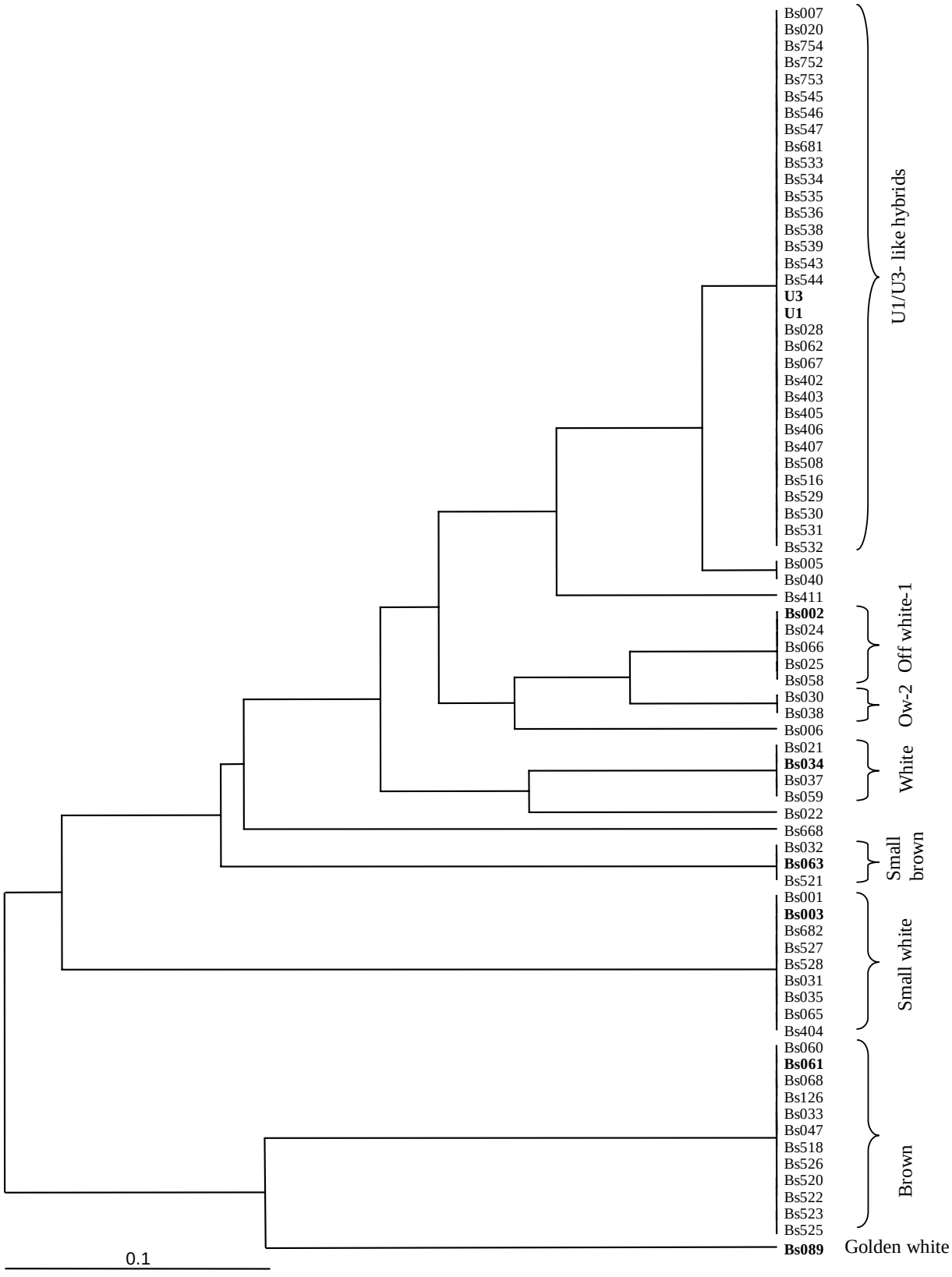

Figure 2. UPGMA dendrogram of the 75 cultivars of *Agaricus bisporus* based on distance of shared alleles estimated with 14 SSR loci. Cultivar lineage morphotypes already described in Foulongne-Oriol et al. [5] are in bold characters.

Table 2. Allelic profile at the 14 *AbSSR* loci for the traditional lineages and the U1-like hybrid.

Lineage	reference strain	AbSSR05	AbSSR06	AbSSR23	AbSSR31	AbSSR33	AbSSR36	AbSSR39	AbSSR42	AbSSR45	AbSSR49	AbSSR57	AbSSR60	AbSSR64	AbSSR65
white	Bs034	329/332	181/202	180/180	170/170	193/193	160/160	188/188	179/179	198/206	176/176	252/258	208/210	107/107	190/215
small white	Bs003	329/332	181/202	180/180	167/167	193/193	148/156	188/192	179/179	196/202	184/184	250/250	208/210	107/107	199/213
off-white-1	Bs002	329/332	181/202	174/180	170/170	193/193	152/152	188/188	181/181	196/198	176/176	258/258	210/210	107/113	190/190
off-white-2	Bs030	329/332	181/202	174/180	170/170	193/193	152/161	188/188	181/181	196/198	176/176	258/262	210/210	107/113	190/213
small brown	Bs063	329/329	196/202	180/180	170/170	193/193	156/156	173/192	179/189	196/206	176/176	256/258	210/210	107/107	190/213
brown	Bs061	329/332	181/202	174/180	170/170	190/190	156/161	192/192	160/179	196/200	176/176	252/252	206/210	107/107	190/213
golden white	Bs089	329/332	181/181	180/180	170/170	190/190	160/160	192/192	160/160	198/206	176/176	252/262	208/208	107/107	190/213
U1-like hybrid	U1/U3	329/332	181/202	174/180	170/170	193/193	152/160	188/188	179/181	196/206	176/176	258/258	208/210	107/107	190/215

Allelic rearrangement analysis. For the 3 U1-like strains, the analysis of the homocaryotic nuclei showed a re-assortment of alleles by comparison with the U1-7 and U1-2 nuclei. We observed 2, 3 and 4 allelic differences over the 8 examined loci for Bs516, Bs681 and Bs508 respectively. Furthermore, although these 3 strains are identical at heterokaryotic level, the allelic composition of their nuclei is different (Fig. 3). That indicates an independent assortment of homologous chromosomes in meiosis I and a pairing of non-sister nuclei in meiosis II. No crossing over was observed between loci shared by the same linkage group (*AbSSR*05-*AbSSR*06 on LG VI, *AbSSR*45-*AbSSR*60 on LG VII). Our results demonstrated that these 3 strains apparently genetically identical to U1 were likely to be issued from single spore isolates of U1. These results confirmed those reported by Sonnenberg et al. [7].

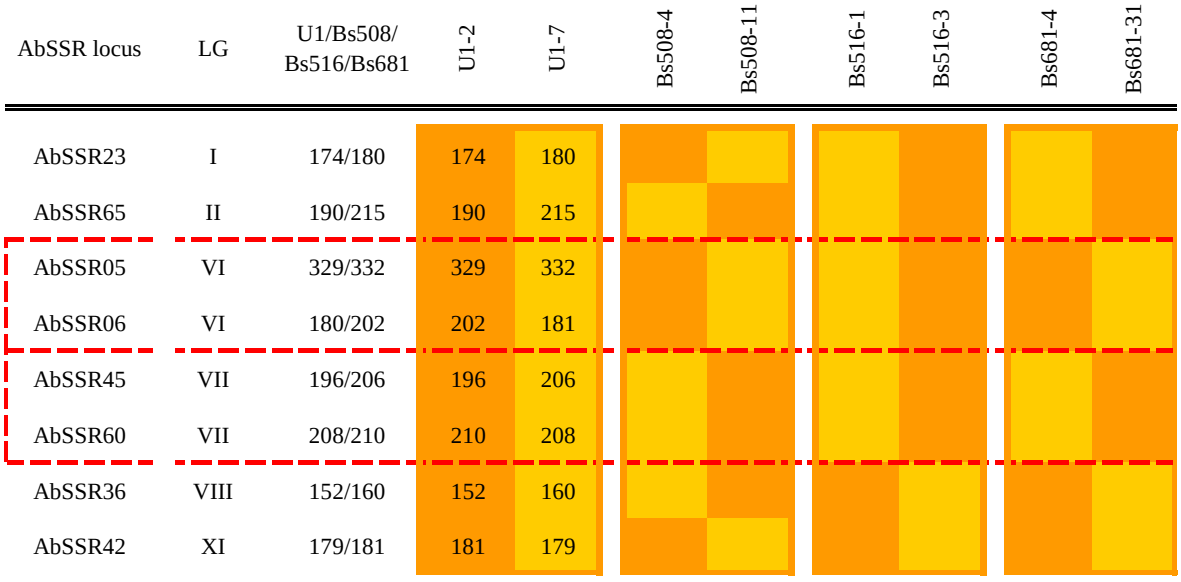

Figure 3. Genotype of homocaryotic nuclei of 3 U1-like strains at 8 *AbSSR* loci and comparison with U1-2 (dark orange) and U1-7 (light orange) nuclei taken as references. Red dotted line signifies genetically linked markers on a same chromosome.

The redistribution of chromosomes has been demonstrated to induce some phenotypic variability [12] and this could be favourable in breeding programs. Thus, the cultivars identical to U1 at the heterozygous level could present advantageous differences for phenotypic traits. Thus, phenotypic characterisation appeared to be inseparable from molecular one to optimize mushroom collection management. Consequently, in parallel to genotypic data, most of the strains in collection were also phenotyped for various agronomic traits (data not shown).

CONCLUSIONS

The SSR markers previously developed are confirmed to be powerful for genetic diversity and cultivar identification in *A. bisporus*. The molecular identification key we propose, particularly the lineage signature, may serve as an efficient tool for the *A. bisporus* community. Multiplex *AbSSR* set coupled with fluorescence-based automated detection systems make also possible the optimisation of this molecular identification key in routine use.

The narrow genetic base of the button mushroom cultivars is confirmed with the identification of six lineages that constitute the parentage of most of the available commercial strains. With this crop uniformity, the mushroom culture would be more vulnerable to pests and environmental stresses. Thus, the exploitation of wild genetic resources to broaden genetic variability is promising to develop new varieties with resistance to diseases or adaptation to climate changes [5, 6].

The homogeneity found within the actual commercial strains is in agreement with other studies but our results also demonstrate that a hidden diversity exist beyond the apparent uniformity. Several consequences should be considered. First, as it appears that the deheterokaryotisation of each strain in collection is unlikely, the molecular characterisation appeared to be needed but not sufficient to maximize the conserved diversity. It can not replace and avoid the phenotypic data, and thus, these two approaches are clearly complementary to optimize germplasm management. Secondly, it raises the question of strain protection and the definition of essentially derived varieties in this economical species, as underlined by Sonnenberg et al. [7].

Our results provide also a sound basis to manage breeding programs. The genetic data collected during this work will guide the choice of parental genotypes to cross according to their lineage belonging or their level of heterozygosity. Subsequent breeding scheme will also be facilitated through potential marker assisted selection.

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