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SCREENING OF HOMOKARYOTIC PROTOCLONES OF *AGARICUS BISPORUS* (J. LGE) IMBACH BY COLONY CHARACTERS AND ISSR MARKERS

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Abstract

Among ten slow-growing protoclones of *Agaricus bisporus* (J. Lge) Imbach, all appressed colonies showed slower growth rate and spawn run, and inability to produce fruiting bodies in substrate. Seven of 40 inter-simple sequence repeat (ISSR) primers amplified 78 reproducible fragments, 48.93% were polymorphic, each producing 7 to 16 bands ranging from 0.10 to 2.10 kbp, sufficient to differentiate the protoclones from each other. Appressed protoclones were homoallelic at a number of loci that were heteroallelic in the parent, suggesting that they represented rare homokaryons. Thus, using morphological characters along with ISSR, polymorphisms could be useful for quick, easy, and accurate in distinguishing homo- and heterokaryotic isolates.

Agaricus bisporus (J. Lge) Imbach is a nutritious edible fungus cultivated industrially world-wide, which has a unique taste and medical properties (Robert *et al.* 2003). The recovery of self-sterile single-spore isolates is laborious and verification through fruiting trials is time consuming (Horgen and Anderson 1992). However, in this study, the techniques for obtaining protoplasts (protoclone), described by Sonnenberg *et al.* (1988) have been employed. In the last two decades, various molecular markers based on nucleic acid polymorphisms have been used in genetic studies of edible fungi (Ma and Luo 2002). Although ISSRs have previously been used to differentiate strains of a variety of homobasidiomycete mushrooms (Guan *et al.* 2008), there have been no reports of their utilization for assessing variability of different homokaryotic strains. We first adopted the ISSR technology to generate more specific and easily recognizable bands for identifying homokaryons of *A. bisporus.* The strain of *A. bisporus* (ACCC50658) was used for protoplast isolation and regeneration was maintained on PDYA (Begin and Spear 1991) medium added with 0.6 M sucrose. After germination started, protoclones were randomly isolated, transferred individually to PDYA slants and incubated at 24° C for further observation. More than 500 protoclones were observed for comparing their growth with parental heterokaryon and 40 slow grown protoclones were selected for further study of their colony morphology and mycelia growth rate and compared with parental heterokaryotic strain (Khush *et al.* 1992). Protoclones, exhibited colony characteristics similar to the parental control (stranded, fluffy and good aerial growth, with or without sectoring) were discarded. The remaining 10 protoclones with growth rate less than parental heterokaryons, which showed different colony morphologies in each sub-culture were selected as putative homokaryons and numbered from 1 to 10 (Table 1). The spawn run, case-run, primordial initiation and maturation all occurred in controlled conditions (Callac *et al.* 1998). The genomic DNA isolation was carried out using method of Sambrook and Russell (2001). Seven ISSR primers (Table 2) were selected for molecular analysis. ISSR-PCR amplifications and analyses were those described by Guan *et al.* (2008). The PCR products were analyzed by electrophoresis on 1.5% agarose gel in 0.5 × TAE and visualized under UV light after staining with ethidium bromide.

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The early regenerates were removed and late regenerated small colonies were isolated and used in this investigation. Twenty five days after inoculation, the protoclones showing colony sizes no more than half that of the parental heterokaryons were selected. Appressed colonized protoclones with growth rates less 0.96 mm/d exhibited slow spawn run and failed to complete the spawn-running process even after 45 days. Results are in agreement with the findings of Singh *et al.* (2007). In our study, all appressed protoclones produced structures like primordia in one or two out of three replicates. The strandy protoclones produced fruits, but number was less than the parental control; this may be due to their moderate spawn run process (Table 1). Appressed colonized single spores are commonly less vigorous and grow more slowly than strandy heterokaryons, inability to produce fruit bodies. Seven primers, selected from 40 primers,

Table 1. Mycelial growth rates, colony characteristics, spawn run and average number of fruit body on rice-straw composted substrate of protoclones of *A. bisporus.*

Isolates	Growth rate (mm/d)*	Colony characteristics	In 2 kg substrate	
			Spawn run**	Fruit bodies***
1	0.79 ± 0.012	Appressed	+	±
2	0.80 ± 0.033	Appressed	+	±
3	0.98 ± 0.026	Strandy	++	4.56 ± 1.13
4	0.84 ± 0.025	Appressed	+	±
5	0.80 ± 0.033	Appressed	+	±
6	0.78 ± 0.009	Appressed	+	±
7	0.79 ± 0.016	Appressed	+	±
8	0.81 ± 0.033	Appressed	+	±
9	0.80 ± 0.042	Appressed	+	±
10	0.96 ± 0.025	Strandy	++	4.66 ± 1.29
C	1.64 ± 0.085	Strandy aerial fluffier	+++	6.34 ± 1.44

*Colony growth rate calculated from the results of three separate experiments with four replications on PDYA medium incubation at 24º C, the values are the means (± standard errors); **Spawn run process in substrate: + = Slow; ++ = Moderate; +++ = Fast. ± = Structures like promordia; C = Parental control; *** Average number of fruit bodies calculated from the results of one set of experiment with three replications.

Table 2. ISSR primers used, total number of bands scored for each primer, polymorphic bands, per cent polymorphism and the size range of the amplified fragments.

Primers	Sequence (5´-3´)	Total bands	Polymorphic bands	Polymorphism (%)	Size range (bp)
P_3	$(GA)_8T$	16	5	31.25	1500-400
P_8	$(CT)_8AGA$	14	5	35.71	1800-250
P_{22}	$(AG)_8YC$	12	4	33.33	1500-350
P_{30}	$(GA)_8C$	9	5	55.55	2050-300
P_{31}	$(CT)_8RG$	11	5	45.45	2100-250
P_{38}	$(CTC)_6$	7	6	85.71	2000-350
P_{39}	(CAAGG)3	9	5	55.55	2100-100
	Total	78	35		

generated excellent results (Table 2), which were used for scoring in our experiments. Primers P3, P8 and P39 produced the clearest, rarely overlapping bands, and their combination was sufficient to differentiate all the tested strains (Fig. 1). It may be that the genome of *A. bisporus* has more micro-satellite sequences related to these seven primers than the other primers. The seven selected primers generated a total of 78 reproducible fragments, of which 48.93% were polymorphic with an average of 10 bands per primer and products size ranged from 100 to 2100 bp (Table 2). The primers based on (GA)n and (GT)n repeats, produced good amplification products, while (AT)n primers gave no amplification products, despite the fact that poly (AT) dinucleotide repeats are

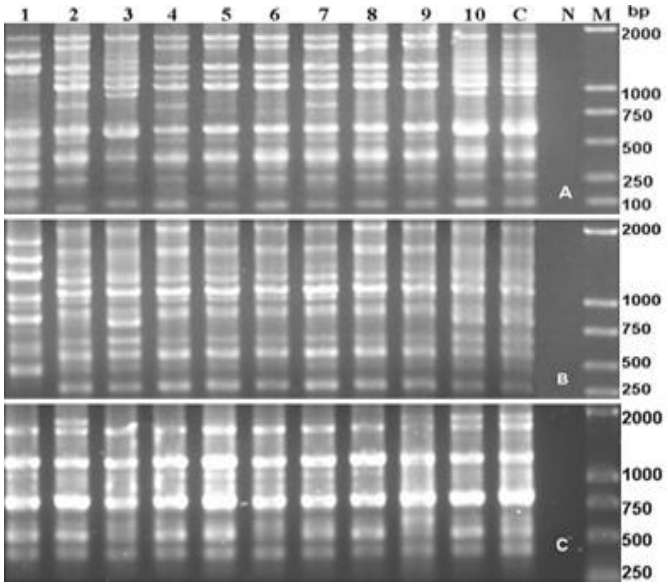

Fig. 1. Banding pattern of 10 putative homokaryotic protoclones (lane 1-10) with their parental control (lane C) of *A. bisporus*: (A) primer P3, (B) Primer P8, (C) Primer P39, N = Negative control, M = Molecular weight markers.

thought to be the most abundant motif in plant species (Depeiges *et al.* 1995). A possible explanation of these results is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity and would form dimers during PCR amplification (Blair *et al.* 1999). As expected, each homokaryon displayed a subset of the polymorphic DNA bands found in the parental heterokaryon. In ISSR fingerprints of the 10 putative homokaryotic protoclones, (- lacked amplification products at multiple loci) grew slowly and all of them had appressed colony morphology. These may be either homokaryons derived from uninucleate basidiospores or heterokaryons composed of post-meiotic sister nuclei. The other isolates displaying amplification products at all loci are likely heterokaryons that received non-sister post-meiotic nuclei. The homokaryotic isolates derived from any heterokaryotic parent display a subset of the parental bands in dominant RAPD profiles (Khush *et al.* 1992). The study revealed losses of ISSR polymorphic patterns in non-fertile homokaryotic protoclones compared to the parental control or fertile heterokaryotic protoclones. ISSR technique is probably an easier way of identifying homokaryons than restriction fragment length polymorphisms of DNA or isozyme banding patterns (Sonnenberg *et al.* 1988). The results in this paper indicated that ISSR could be an alternative molecular marker to identify homokaryons of *A. bisporus*.

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