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Oyster Mushrooms Species Differentiation Through Molecular Markers RAPD

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Abstract: A RAPD methodology for differentiation and characterization of the fungal edible species *Pleurotus ostreatus* and *Pleurotus sajor-caju* was developed. Two of the most usual methods of DNA extraction were studied. The first was based in the simultaneous precipitation of proteins and polysaccharides by the presence of SDS (sodium dodecyl sulphate) and sodium acetate and the second by the use of cetyltrimethylammonium bromide (CTAB). Both methods presented satisfactory results and the best extraction products were obtained by the one that used SDS and sodium acetate. The study with RAPDs produced consistent DNA fragments, reproducible in different gels, serving as good markers for characterization and genetic differentiation of the studied species of mushrooms. The PCR conditions for obtaining RAPD markers were optimized with good interpretation.

Key words: DNA extraction, PCR, RAPD, *Pleurotus ostreatus*, *Pleurotus sajor-caju*

INTRODUCTION

The genus *Pleurotus* (Jacq.: Fr.) Kumm. (Pleurotaceae, higher Basidiomycetes) is one of the most diverse groups of cultivated mushrooms with high nutritional value, therapeutic properties and various environmental and biotechnological applications. However evolutionary connection among species in the genus *Pleurotus* is still not clear and many taxonomic issues remain controversial (Cohen *et al*., 2000).

There are significant problems in classifying Pleurotus isolates using only morphological characters (which are often unreliable or inconclusive mainly due to the large influence exerted by environmental factors) or compatibility experiments (which are based on the application of the controversial biological species concept) (Zervakis *et al*., 2001). Moreover, considering that starting point in the cultivation of an edible mushroom is usually a pure culture or spores, the use of molecular tools is almost essential to ensure that the inoculum used is from the correct species. Molecular tools provide more accurate methods for identification than the few characters afforded by traditional morphological features. These tools will soon become widespread in following the survival of inoculant fungi, detecting contaminant species, the degree of competition from contaminant and presence of hyperparasites (Amicucci *et al*., 2001; Hall *et al*., 2003).

The random amplified polymorphic DNA technique (RAPD) (Williams *et al*., 1990; Welsh and Mclelland, 1990; Manaf *et al*., 2006) has some advantages, as the efficiency to generate a large number of markers for genomic mapping without any previous knowledge about the organism genetics, the requirement of small amount of DNA, the quickness, simplicity and reproducibility in the data acquisition, the low cost and accessibility of this technology and the potential automation, with the

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possibility of being used in programs of quality control as HACCP (Hazard Analysis Critical Control Point) for controlling and monitoring of critical points of contamination in a process. The aim of this study was to optimize conditions, in order to obtain high quality DNA extraction and develop the RAPD methodology for differentiation and characterization of the edible mushrooms species *Pleurotus ostreatus* and *Pleurotus sajor-caju*.

MATERIALS AND METHODS

The present research was conducted in the Laboratory of Marine Biochemistry, from Federal University of Rio Grande, Brazil, during the year of 2007.

Microorganisms

Pleurotus ostreatus and *Pleurotus sajor-caju* strains were obtained from EMBRAPA (Brasília, DF, Brazil) and UNESP (Botucatu, SP, Brazil) in lyophilized form, preserved in malt extract agar at 4°C and cultivated through culture methods in plate.

Methods of DNA Extraction

Two methods of DNA extraction were studied. The first, based on the method proposed by Delidow *et al.* (1993) and Makimura (1994) (method 1), is based on the simultaneous precipitation of proteins and polysaccharides in the presence of SDS and high concentrations of sodium acetate. The other method was proposed by Weising *et al.* (1995) and González and Labarère (2000) (method 2) and is based on the use of the detergent CTAB.

Molecular Markers RAPDs

Pleurotus ostreatus and *Pleurotus sajor-caju* were characterized genetically by differentiation through the method RAPD consisting basically of 3 stages: DNA extraction, PCR and PCR analysis of the products in electrophoresis gel.

PCR

The PCR protocols also proceeded also proceeded, with certain modifications, according to Delidow *et al.* (1993) and Cushwa and Medrano (1996). The amplifications were carried out in a thermal cycler PTC-100/110V (MJ Research, Inc.) adjusted for the program PCR-1 to 40 cycles for the complete DNA synthesis. The method consisted of 3 stages: denaturation of the DNA chain in simpler sequences of 92°C for 1 min, during regular cycles (the initial denaturation was longer: 5 min); hybridization of the primers to complemental sequence sites in the denatured DNA chain at 35°C for 1 min and primers extension for the synthesis of the DNA complemental sequences at 72°C for 2 min. The PCR reaction took 5 h and the reagents concentrations, the temperature profiles in the thermal cycler and the electrophoresis gel conditions according to Cushwa and Medrano (1996). The tubes can be left at 4°C in the thermal cycler for indefinite time or stored in freezer at -20°C, before the electrophoresis gel analysis, being added shipment lid with EDTA (loading dye) to inhibit any action of DNAses on the PCR products (Ferreira and Grattapaglia, 1996).

RAPD-PCR Analysis

RAPD-PCR products were analyzed through electrophoresis driven in gel of agarose 1.5%. The bands were visualized with ethidium bromide in ultra-violet light, interpreted and transformed into molecular diagnoses data among individuals (Cushwa and Medrano, 1996). The banding profiles generated by RAPD-PCR were analyzed with the RAPDistance program (Armstrong *et al.*, 1996). The analysis of the diagnoses data was made starting from the presence or absence of bands at a specific molecular weight.

RESULTS AND DISCUSSION

DNA Extraction

The DNA extracted from dried materials, such as root, stem or fruit is often contaminated with proteins, polysaccharides and secondary metabolites, which decrease the reproducibility of method RAPD. Figure 1 shows *P. ostreatus* and *P. sajor-caju* DNA extraction results for the two tested methods. The method 1 allowed a more efficient DNA isolation of the fungal species. Although the good quality extraction presented in the method 2, it was not possible for this method to eliminate the RNA, as verified in the inferior part of gel (Fig. 1).

In this study, a combination of methods reported by Delidow *et al*. (1993) and Makimura (1994), based in the simultaneous precipitation of proteins and polysaccharide in the presence of SDS and high concentrations of sodium acetate, allowed a more efficient DNA extraction (method 1). It was possible to isolate a high purified DNA that certainly permitted a better analysis of the RAPD-PCR products.

Method RAPD

DNA fingerprint patterns might be useful in identifying the species and as an aid to quality control (Cheng *et al*., 2000). Capelari and Fungaro (2003) analyzed the genetic variability by RAPD of isolates of *Pleurotus cystidiosus* and *Pleurotus smithii* which indicated that the criteria used to separate the two species are unsatisfactory and that *P. smithii* should be considered a synonym of *P. cystidiosus*. Shnyreva *et al*. (2003) studied *P. ostreatus* and *Agaricus bisporus*, using molecular markers for differentiation, which allowed differentiating groups of genetically similar and distant strains. *P. ostreatus* strains showed a higher genetic variation while *A. bisporus* strains showed a higher level of homology.

In this study, utilizing the RAPD methodology, 19 different primers were tested for the two species of mushrooms in study, being obtained a large number of DNA fragments (bands) consistent and reproduced in several gels. Among the tested primers, 6 (AC-04, AD-08, AD-09, B-14, D-20, G-02) produced amplifications for both species allowing the differentiation of the same ones, 5 (S-03, D-06, G-07, G-08, B-15) produced amplifications only for *Pleurotus ostreatus*, the primer S-17 produced amplifications only for *Pleurotus sajor-caju* and 7 primers (AC-09, B-02, B-05, B-07, D-05, G-06, G-09) did not amplify any fragment.

The number of amplification products for each primer varied from 1 to 12, totaling 87, being that 77 were polymorphic and 10 were monomorphic (present in both species) (Table 1). The analysis of the data obtained by RAPD methodology was based on the absence or presence of bands from the electrophoresis gel in ultra-violet light. The size of the amplified product ranged from 154 to 4072 bp.

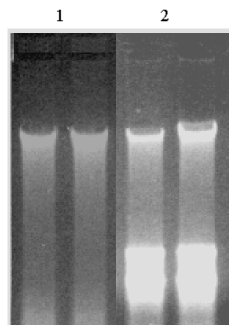

Fig. 1: Results of the extractions of DNA

Table 1: Results of the RAPD methodology in terms of presence or absence of amplification, polymorphism and number of detected bands for each tested primer and oyster mushroom species

Primer	Species	Sequence 3'-5'	G+C (%)	Amplification	Polymorphism	N° Bands
AC-04	*P. ostreatus*	ACGGGACCTG	70	Yes	Yes	10
AC-04	*P. sajor-caju*	ACGGGACCTG	70	Yes	Yes	3
AC-09	*P. ostreatus*	AGACGGTACC	60	No	-	-
AC-09	*P. sajor-caju*	AGACGGTACC	60	No	-	-
AD-08	*P. ostreatus*	GGCAGGCAAG	70	Yes	Yes	5
AD-08	*P. sajor-caju*	GGCAGGCAAG	70	Yes	Yes	1
AD-09	*P. ostreatus*	TCGCTTCTCC	60	Yes	Yes	3
AD-09	*P. sajor-caju*	TCGCTTCTCC	60	Yes	Yes	2
B-02	*P. ostreatus*	TGATCCCTGG	60	No	-	-
B-02	*P. sajor-caju*	TGATCCCTGG	60	No	-	-
B-05	*P. ostreatus*	GTAGACCCGT	60	No	-	-
B-05	*P. sajor-caju*	GTAGACCCGT	60	No	-	-
B-07	*P. ostreatus*	CCTTGACGCA	60	No	-	-
B-07	*P. sajor-caju*	CCTTGACGCA	60	No	-	-
B-14	*P. ostreatus*	ACCCCCGAAG	70	Yes	Yes	5
B-14	*P. sajor-caju*	ACCCCCGAAG	70	Yes	Yes	5
B-15	*P. ostreatus*	GGACCCAACC	70	Yes	No	6
B-15	*P. sajor-caju*	GGACCCAACC	70	No	-	-
D-05	*P. ostreatus*	TCTGGTGAGG	60	No	-	-
D-05	*P. sajor-caju*	TCTGGTGAGG	60	No	-	-
D-06	*P. ostreatus*	TTGGCACGGG	70	Yes	Yes	6
D-06	*P. sajor-caju*	TTGGCACGGG	70	No	-	-
D-20	*P. ostreatus*	CTGGGGACTT	60	Yes	Yes	7
D-20	*P. sajor-caju*	CTGGGGACTT	60	Yes	Yes	2
G-02	*P. ostreatus*	GGCACTGAGG	70	Yes	Yes	6
G-02	*P. sajor-caju*	GGCACTGAGG	70	Yes	Yes	2
G-06	*P. ostreatus*	GGTCTACACC	60	No	-	-
G-06	*P. sajor-caju*	GGTCTACACC	60	No	-	-
G-07	*P. ostreatus*	GAACCTGCGG	70	Yes	No	2
G-07	*P. sajor-caju*	GAACCTGCGG	70	No	-	
G-08	*P. ostreatus*	TCACGTCCAC	60	Yes	No	2
G-08	*P. sajor-caju*	TCACGTCCAC	60	No	-	-
G-09	*P. ostreatus*	CCGAGGGGTT	70	No	-	-
G-09	*P. sajor-caju*	CCGAGGGGTT	70	No	-	-
S-03	*P. ostreatus*	CAGAGGTCCC	70	Yes	Yes	12
S-03	*P. sajor-caju*	CAGAGGTCCC	70	No	-	-
S-17	*P. ostreatus*	TGGGGACCAC	70	No	-	-
S-17	*P. sajor-caju*	TGGGGACCAC	70	Yes	Yes	8
Total						87

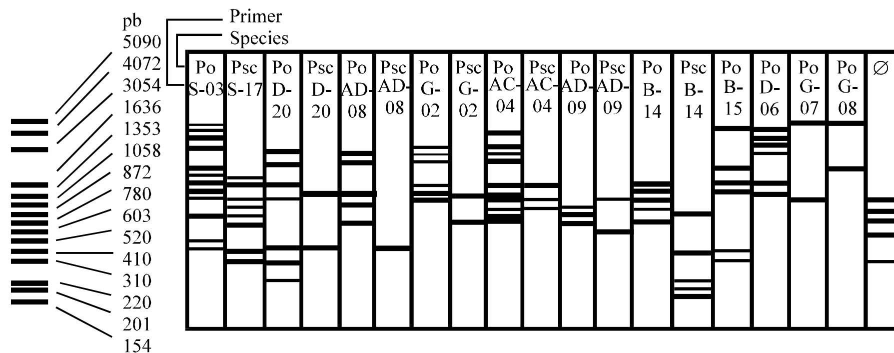

Fig. 2: Representation of products of PCR-RAPDs visualized in ultra-violet light

The percentages of GC in the utilized primers (60% and 70%) did not influence the amplification patterns. The high polymorphism with markers RAPD is probably due to the preferential amplification of repetitive areas of the genome (Fig. 2).

The methodology RAPD produced consistent fragments of DNA, which were reproducible in different gels. However, the fragments amplification from different similar sizes that occupy the same position in a gel after electrophoresis can happen, but the risk of erroneous interpretations of these fragments with similar size is minimized by the use of several primers, such a way that the genetic analysis is based on a high number of markers RAPD. The analysis based on RAPD-PCR fingerprinting obtained in this study was clear enough to allow discrimination, characterization and differentiation of the fungal species *P. ostreatus* and *P. sajor-caju*.

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