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Abstract

It is well known that multiple auxotrophic markers impede fruiting in Coprinus cinereus. Restriction fragment length polymorphisms have been used to advantage (Cassidy et al. 1984 Curr. Genet. 8:607-613; Zolan et al. 1992 Nucl. Acids Res. 20:3993-3999) but the required Southern analysis is both time consuming and laborious.

Quick and reliable method to analyze meiotic segregation patterns in *Coprinus cinereus* using the polymerase chain reaction

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It is well known that multiple auxotrophic markers impede fruiting in *Coprinus cinereus*. Restriction fragment length polymorphisms have been used to advantage (Cassidy et al. 1984 Curr. Genet. 8:607-613; Zolan et al. 1992 Nucl. Acids Res. 20:3993-3999) but the required Southern analysis is both time consuming and laborious. Here we demonstrate a quick and reliable method using the polymerase chain reaction (PCR) (Saiki et al. 1988 Science 239:487-491) to score the presence or absence of a unique insertion among meiotic segregants. To develop the optimum procedure, we dissected tetrads from a cross between a strain that harbored a unique insertion at the trp1 locus and a strain without the insertion. We looked for PCR conditions that gave neither false positives nor false negatives.

Although it is possible to perform PCR reactions on fresh mycelium from *C. cinereus* without first extracting the DNA, this method is sometimes unreliable. Furthermore, one has to rely on wild-type fluffy strains for an adequate supply of mycelium. If the mycelium is first lyophilized, or lyophilized and then boiled, false negatives are still obtained. Accordingly, we simplified the standard mini-prep procedure (Zolan and Pukkila 1986 Mol. Cell. Biol. 6:195-200) and the method that gave consistent results is reported here. With this method, 14 tetrads were analyzed, with 100% accuracy as judged by 2:2 segregation of the PCR-amplified product.

Sterile Eppendorf tubes containing 1 ml YMDT (0.4% yeast extract, 1% malt extract, 0.4% dextrose and 0.01% L-tryptophan) medium were inoculated with a small plug of mycelium and incubated at 37oC. After 5 days, the Eppendorf tubes were vortexed and spun for 20 min in a microcentrifuge at top speed. The supernatant was removed, and the tubes were frozen in a dry ice/ethanol bath for 10 min before being placed in a speed vac for at least 5 h for lyophilization. Typically, 5 mg of tissue was recovered, and was easily pulverized using a steel probe. Three hundred microliters of extraction buffer was added to the dry tissue and thoroughly mixed using a sterile toothpick. The extraction buffer (final concentration) was: 700 mM NaCl, 1% CTAB (hexadecyltrimethylammonium bromide (Sigma)), 50 mM Tris-HCl (pH 8), 10 mM EDTA and 1% 2-mercaptoethanol (Murray and Thompson 1980 Nucl. Acids Res. 8:4321-4325). After 10 min at room temperature, 300 microliters of SEVAG (1 part isoamyl alcohol: 24 parts chloroform) was added and mixed by gentle vortexing. After 10 min at room temperature, the tubes were spun at top speed for 20 min. The supernatant was collected and stored at -20oC. Typically, about 4 micrograms of DNA can be recovered by this procedure at a concentration of about 20 ng/ l, but only 1 microliter of the supernatant is necessary for PCR-amplification.

The DNA sample (1 microliter of supernatant) in 10 microliters of water was incubated in the PCR machine at 94oC in the first step of the PCR cycle to avoid proteolytic degradation of Taq polymerase. The following components were added to the indicated concentrations after the first denaturation step for PCR-amplification (final volume 100 microliters): 67 mM Tris-HCl (pH 8.8), 16 mM (NH4)2SO4, 10 mM 2-mercaptoethanol, 3 mM MgCl2, 2% glycerol, 1 micromole of each primer (see below), dATP, dCTP, dGTP (each 200 uM), dUTP (500 uM), and 2.5 units of Amplitaq DNA Polymerase (Perkin Elmer Cetus). The PCR buffer is a modification of that used by Jeffreys et al. (1988 Nucl. Acids Res 16:10953-10971). The reaction was incubated in a

Perkin Elmer Cetus DNA Thermal Cycler 480 machine at 94oC, 1 min, 58oC, 2 min and 72oC, 3 min for 30 cycles. The primers were as follows: HP (5' AGCTCCATGGAATGTGCAGAC 3') recognized the pBluescript (Stratagene) plasmid integrated in the trp1 locus (Skrzynia et al. 1989 Gene 81:73-82). The other primer, A934 (5' GGACGTACTTGGCA 3'), recognized an internal region of the trp1 gene. This reaction amplified a 1.6 kb fragment only from DNA of segregants containing the plasmid insertion. Twenty microliters of the PCR reaction was run on 0.7% agarose gels and bands were visualized by ethidium bromide staining.

This protocol is far more efficient than Southern analyses. It should allow the segregation patterns of RAPD markers to be monitored, if suitable modifications of $MgCl_2$ concentration and the annealing temperature are used. We routinely use dUTP to permit the destruction of contaminating amplified DNAs. If contamination is detected in a negative control, uracil N-glycosylase can be used since this enzyme cleaves sequences which contain dUTP.

The advantages of our method include: 1. The tissue is grown and lyophilized in the same tube, which facilitates the simultaneous analysis of many segregants. 2. Only one extraction step is necessary. 3. The markers are scored by gel electrophoresis, thereby bypassing Southern analysis.

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