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THE VALUE OF DRIED FUNGAL CULTURES FOR TAXONOMIC COMPARISONS USING PCR AND RFLP ANALYSIS

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A DNA fragment of 370 base pairs in size was amplified, using PCR, from eleven-year-old dried culture material of *Ceratocystis adiposa*. This DNA fragment was compared with equivalent DNA amplified from DNA isolated from fresh material of the same isolate. The restriction patterns of both fragments were identical with each of eight restriction enzymes used in the study. It thus appears that the original DNA was preserved intact in the dried material. This technique should prove invaluable for comparisons between type specimens where only small amounts of dried material are available, as in the case of dried cultures.

Every validly described fungal species rests upon a type specimen lodged in an accredited herbarium. The rules of the International Code of Botanical Nomenclature, to which mycologists adhere, dictate that the type specimens including the holotype must be dead. They are thus in a form that will not change (Hawksworth, 1974). Living cultures of fungi are unacceptable as type specimens.

In the case of macrofungi such as mushrooms, puffballs and polypores, herbarium specimens are relatively easily preserved. Type specimens of these larger fungi tend to be relatively reliable. Considerable problems are experienced with type specimens of micro-fungi where sporocarps are minute. Here, herbarium specimens are usually preserved on the original substrate. In many cases, the amounts of material available for examination are extremely limited and may even be insufficient for meaningful morphological comparisons. In order to augment such collections, permanent preparations of characteristic structures on glass slides are often lodged. These can be extremely useful although mounting media do tend to dry out and slides often break. Another means of augmenting herbarium specimens of micro-fungi is to prepare dried cultures of these fungi which can be deposited in the herbarium package.

Where conidial states (anamorphs) sporulate profusely, dried cultures can be of tremendous value in comparisons.

Examination of, and comparisons with, type specimens lodged in herbaria have, until now, been based purely on morphological characteristics. Although these features are of great taxonomic value, they may also present problems such as misleading similarities arising from convergent evolution. Recent trends have therefore been to verify taxonomic conclusions using less subjective comparisons (Bruns *et al.*, 1991). Many techniques have been used including comparisons of metabolites, proteins, enzymes and various nucleic acids (Bartnicki-Garcia, 1970; Otrosina and Cobb, 1987; Kistler *et al.*, 1987; Gueho *et al.*, 1989; Bruns *et al.*, 1990).

The use of molecular and chemotaxonomic techniques in mycological studies has raised considerable problems in terms of the type of material available for comparison. Many of these techniques rely entirely upon living cultures. Such cultures are not available for the majority of fungi, and even when available cannot, as stated above be accepted as typifying a species. It has been estimated that fewer than 20 % of the known fungi are available in live culture collections (Hawksworth, 1990). The advent of the Polymerase Chain Reaction (PCR) has added a new and exciting dimension to the possibilities for comparisons based on minimal amounts of dried tissue. This technique involves the amplification of specific segments of DNA from extremely small amounts of starting material. For example, amplification of DNA from single spores has been reported (Lee and Taylor, 1990). Short fragments of DNA have also been amplified from *Magnolia* species estimated to be 17-20 million years old extremely old (Goldenberg *et al.*, 1990). Fragments of amplified DNA from different individuals can then be compared using Restriction Fragment Length Polymorphisms (RFLPs) (Vilgalys and Hester, 1990) or direct sequencing (Bowman *et al.*, 1992). Theoretically, it should be possible to amplify single copy genes from minute pieces of mycelium or even single spores lodged in herbaria.

Recognizing the power of PCR in fungal taxonomy, Bruns *et al.*, (1990) isolated DNA from 35 dried basidiocarps, representing 31 different species. Using the polymerase chain reaction a specific fragment of the mitochondrial large subunit ribosomal RNA gene was amplified. This region was sequenced and the sequences from the different individuals compared. In the case of micro-fungi, such small amounts of material are available that DNA isolations are far more difficult and sometimes impossible.

The intervening spacer regions (ITS1 and ITS2) of the ribosomal RNA (rRNA) gene battery are convenient sections of DNA to amplify

because they have been shown to exhibit a high degree of variation between species (Chambers *et al.*, 1986). However, the regions adjacent to ITS1 and ITS2 are highly conserved (White *et al.*, 1990). This allows specific priming from these areas into the more variable regions using the PCR reaction. The areas are rendered even more suitable by the fact that the organization in the human rRNA genes is such that amplification using these primers is not possible. Contamination is a problem and one of the most likely source of contaminating DNA (human DNA) in these herbarium specimens will not be amplified using these primers.

The aim of this study, was to amplify DNA directly from a dried fungal culture without any complicated DNA isolations, and to compare this with DNA isolated from a living culture representing the same collection. The fungus used in this study was *Ceratocystis adiposa* (Butler) Moreau. A living culture of this isolate was compared with a dried specimen on agar that had been prepared eleven years previously. PCR fragments from both the live culture and the dried material were compared to ascertain that the amplified product of the dried material was not an artifact or amplification of contaminating DNA.

A culture of *C. adiposa* from the culture collection of the junior author, maintained in the Dept. Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, R.S.A., was used in this study. A dried culture prepared from the same isolate of *C. adiposa* in 1979 by the junior author was used as the herbarium specimen for comparative purposes.

C. adiposa was grown in 500 ml of YPD broth (yeast extract (6%), peptone (12%), glucose (12%)) for three weeks. The fungal mycelium (11gm) was collected by centrifugation and washed once in 50 mM EDTA. This material was frozen at -70 °C and lyophilized. The dry material was crushed with a pestle and mortar and the DNA isolated using the method of Chirgwin *et al.*, (1979).

The primers used in the PCR reaction were: 5'-GCATCGATGAAGAAC-3' and 5'-TCCTCCGCTTATTGA-3'. These primers amplify the ITS2 region of the rRNA gene battery which is the larger of the two intervening spacer regions. Total reaction volume was 100 μl and contained the following components: 100 pmoles of each primer, 2 units *Taq* polymerase (Promega) in a solution of 10 mM Tris-HCl (pH 9.0)/50 mM KCl/5.5 mM $MgCl_2$/0.1 mM DTT/0.01% gelatin/0.1% Triton X-100/250 μM of each of the deoxyribonucleotide triphosphates. These reactions were overlaid with liquid paraffin to prevent evaporation during the reaction. The reaction was started with an initial denaturation at 96 °C for 5 minutes. The *Taq* polymerase was then added and this was followed by 35 cycles of the following regime: 20 sec at

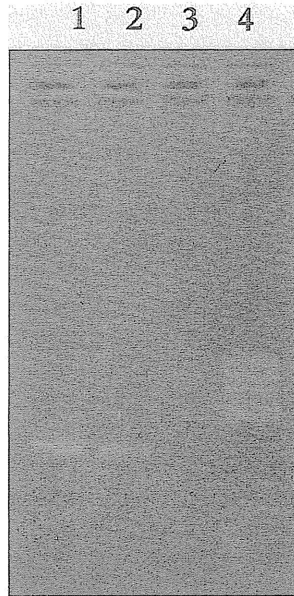

Figure 1. A 2 % agarose gel showing the amplified DNA products. Lane 1. DNA amplified from the DNA isolated from fresh *C. adiposa* material. Lane 2. DNA amplified from the dried *C. adiposa* culture. Lane 3. Control amplification containing no added DNA. Lane 4: pBR322 DNA cut with *Hae* III as size standard, the fragments sizes are 587, 540, 504, 458, 434, 267, 234, 213, 192 and 184 bp respectively.

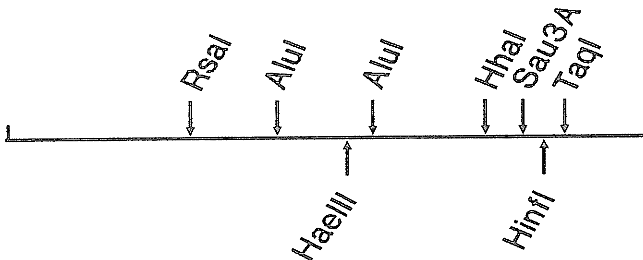

Figure 2. A diagrammatic representation of the restriction map of the ITS 2 region of *C. adiposa*. The fragment is 370 bp in size and the indicated cut sites are drawn to scale.

55 ° C, 30 sec at 72 ° C and 1 min at 92 ° C. To ensure a completely double-stranded product the reaction was ended by incubating at 72 ° C for 10 min.

The amplified products were assayed from 5 μl aliquots by gel electrophoresis in a 2% agarose gel containing 0.2 μg/ml ethidium bromide. The DNA bands were visualized under UV light and photographed through an orange plexiglass filter with Polaroid 665 positive-negative film.

For amplification from the dried herbarium specimen, a small amount of material was scraped off the dried agar and added, without any further preparation, to the reaction tube. The amount of material used was similar to that which would be used in preparing a slide for light microscopy.

A DNA fragment of 370 base pairs (bp) in size was amplified from both the purified DNA and the small piece of dried material (Fig. 1). The DNA amplified from the dried material was not in as high a concentration as that amplified from the isolated DNA.

The liquid paraffin was removed from the amplified DNA by chloroform extraction. The DNA was precipitated with isopropanol and one-tenth volume 3 M sodium acetate. The DNA was pelleted by centrifugation and the pellet washed twice with 70% ethanol. The DNA was dissolved in sterile deionised water and used for the restriction digests. The restriction enzymes *Alu* I, *Hha* I, *Hae* III, *Hpa* II, *Hinf* I, *Rsa* I, *Sau* 3A, and *Taq* I were used to digest the amplified DNA. These enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) and used according to the manufacturer's instructions. The restriction fragments were electrophoresed on 6% acrylamide gels and the DNA visualized using the silver staining method of Perbal (1988).

Both the amplified fragments gave identical restriction patterns. The compiled restriction map of this DNA representing the ITS2 region of *C. adiposa* is shown in Fig. 2. The restriction enzyme *Hpa* II did not cut the DNA, whereas *Alu* I cut the fragment twice. All the other enzymes cut the DNA once.

In this study we have shown that it is possible to amplify a DNA segment directly from a fungal culture that has been dried for eleven years. This amplification is from the DNA preserved in the dried fungus and not from contaminating DNA because its restriction fragment pattern is identical to that of the fragment amplified from DNA isolated from a fresh culture of the same isolate.

The ITS2 region is known to be highly variable between species (Chambers *et al.*, 1986; Vilgalys and Hester 1990). We have found restriction fragment length polymorphisms using the same enzymes

between *C. adiposa, C. fimbriata* (Ellis and Halst.) and *C. moniliformis* (Hedgcock) Moreau [unpublished data]. This further confirms that the fragment amplified from the dried material is representative of DNA preserved in the herbarium specimen and not from contaminating DNA.

The potential for comparisons between dried fungal cultures and living cultures at the molecular level is great. This technique also has potential in comparisons between fungi that are difficult or impossible to culture. The amount of material needed for these comparisons is the same or potentially even less than that which is needed for classical morphological comparisons. It also has the advantage of not requiring any additional expensive and time-consuming preparation of the material.

One of the greatest problems with PCR is the possibility of contamination. While nothing can be done about the way cultures have been dried in the past, we would recommend that care should be taken to avoid contamination with foreign DNA during the preparation of new type material. The best way to achieve this would be to dry cultures under sterile conditions and avoid handling them as much as possible. If possible, attempts should also be made to store these dry cultures in sterile conditions. It may be necessary to develop new methods for handling and storing type material to ensure that molecular comparisons can be done at a future date.

The maintenance of a live culture collection requires a great deal of care, is labour-intensive, expensive and not always the best method of keeping material for comparative purposes. Fungi vary in the ease with which they can be cultured, and isolates often die out. Many organisms also change considerably with time in culture, making morphological comparisons difficult. The practice of keeping dried material is in many cases the only answer to some of these problems. However, with the availibility of molecular techniques such as PCR, it would also be desirable to isolate and keep the DNA of these cultures for subsequent comparisons. A simple approach might be to add vials of sterile dry mycelium to herbarium packets. Indeed, we recommend that mycologists follow this procedure when describing new species.

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