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USING SEQUENCE ANALYSIS TO IDENTIFY CULTURES DERIVED FROM AIRBORNE SPORES

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The purpose of this project was to evaluate rDNA sequencing in identifying airborne fungal spores. Universal fungal small-subunit (SSU) ribosomal RNA primers were used with the polymerase chain reaction (PCR) to amplify the SSU rRNA gene from each isolate for sequencing. Sequences were analyzed through the ribosomal database project to identify the most closely related known rDNA sequence. Restriction fragment length polymorphisms (RFLPs) were obtained from three cultures to be used to detect each culture in mixed samples. Of the five stock cultures studied, one common fungus could be identified to genus with some confidence (*Mucor*). For three cultures, sequence data yielded closest matches that were probably closely related genera (*Penicillium, Aureobasidium, Spongipellis*), but the phylogenetic tree lacked the resolution for genus identification, whereas sequence results from the one culture were more ambiguous. The RFLP analysis could detect the presence of some cultures in mixed DNA isolates, with varying degrees of sensitivity. Although there are not yet enough published sequences to identify less common airborne fungi using sequencing of the SSU rDNA gene, our results indicate that the molecular methods evaluated in this study could have the potential to identify fungal spores from common genera and detect them in mixed environmental samples.

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PREFACE

The work described in this report was authorized under Project No. 10262384A553, Non-Medical CB Defense. This work was started in July 1996 and completed in July 1997.

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CONTENTS

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USING SEQUENCE ANALYSIS TO IDENTIFY CULTURES DERIVED FROM AIRBORNE SPORES

INTRODUCTION

Many medically or ecologically significant microbes are spread predominantly through the air. These airborne microbes are of interest for a variety of reasons, including human, animal and plant health and forest ecology. The purpose of this study is to investigate methods of characterizing airborne biological particles, with the ultimate goal of developing real-time, culture free means of detecting and identifying unique particles. Flow cytometry, gas chromatography and molecular techniques were deemed to be the most promising techniques (Edmonds, 1994; Wick, *et al.*, 1994, 1995, 1997).

It was proposed that the detection of airborne biological particles could be based on their physical characteristics as detected by flow cytometer analysis and identification of biochemical profiles by gas chromatography. Initial research involving flow cytometry and gas chromatography were discussed in a previous report (Wick, *et al.*, 1997). A combination of gas chromatography and mass spectometry has been used successfully to determine sugar composition in the walls of cysts of *Pneumocystis carinii* (DeStefano *et al.* 1990) and prespore specific antigens of a cellular slime mold *Dictyostelium discoideum* (Zachara *et al.* 1996).

Recently, our focus has been upon investigating molecular methods of identifying fungal airborne spores, to be used in combination with physical and biochemical techniques of characterizing field samples of airborne particles. The chief advantage of using a molecular approach is that it is possible to achieve a highly reliable and detailed identification of individual isolates. There are some significant challenges and limitations associated with molecular techniques. Published sequence databases are still quite limited, making it difficult to find a match, especially when studying fungi that are not medically significant; also, molecular techniques generally require a minimum of several days, although turn-around times are becoming increasingly rapid as the technology becomes more refined.

Molecular techniques can be quite powerful when used in combination with physical or chemical separation criteria. For example, real-time flow cytometry and gas chromatography could be used to produce biochemical profiles which would indicate the presence of an unusual biological particle. Molecular methods could then be used to determine the phylogenetic identity of the biological particle e.g., the most-closely related known species. The power of this approach is that it can be used to study a completely unknown organism, without prior knowledge of its identity, and without the necessity of culturing it.

Molecular techniques have recently become common in the study of fungal systematics, resulting in the development of reliable protocols for DNA isolation from fungal cultures and analysis of molecular markers or sequence data. In particular, the ribosomal RNA genes have received considerable attention, resulting in development of an increasingly detailed phylogenetic tree based on sequence-level variation in the rRNA genes. As rRNA genes from more and more fungal species are sequenced, the expanding database becomes increasingly attractive as a tool for characterizing unknown cultures. Briefly, the typical approach is to isolate genomic DNA from the unknown culture (or single spore), amplify (copy) the rRNA gene using short DNA strands (18-20 nucleotides long) known as primers with the polymerase chain reaction (PCR), and then sequence the amplified rRNA gene. The sequence is aligned with rRNA sequences from known organisms, and the organism whose gene is most similar to the unknown's is identified.

In selecting a strategy, we needed to choose a gene for which reliable universal primers and an extensive database of fungal sequences had already been determined. Also, the sequence of the gene had to be variable enough to distinguish to the genus level. The small-subunit (SSU, or 18S) rDNA gene evolves relatively slowly, and is therefore useful for studying distantly related organisms (White *et al.*, 1990), and has been used by many researchers to study fungal phylogenetics (e.g. White *et al.*, 1990; Spatafora *et al.*, 1995). Therefore, we chose to evaluate sequencing of the SSU rDNA gene as a means of identifying fungal isolates from airborne spores.

To assess the feasability of this approach, we began by working with stock cultures of fungi obtained from common airborne spores. Our objectives were: 1) to determine whether sequencing of the SSU rDNA gene could identify common airborne fungi at the genus level, and 2) to evaluate the use of molecular markers in detecting unique fungi after they had been identified by sequencing.

METHODS

Stock Cultures

The stock cultures discussed in this report were obtained in October 1993 from airborne spores collected on open agar plates from various locations on the University of Washington campus, and maintained in pure culture on agar slants. Cultures were inoculated onto thin films of agar under glass coverslips on microscope slides, grown at 22° C for 5-10 days, observed at 100x and 400x under a light microscope, and photographed. In addition, stock cultures were grown on agar plates, agar blocks were dissected out and placed on mounts for use in an environmental scanning electron microscope (SEM), and prints were made of characteristic features from each culture.

DNA Extraction

Genomic DNA was isolated according to a protocol provided by George Mueller (University of Washington, personal communication, 1996). Randomly selected cultures were inoculated in 0.5 ml of liquid medium in 1.5 ml epindorf tubes, and incubated at 22° C for 2 days. Hyphae were pelleted in a microcentrifuge at 12,000 rpm for 60 seconds, culture medium was decanted, and hyphae were rinsed once with sterile water. For samples in which the volume of the pellet exceeded approx. 25 µL, the excess volume was removed, and the pellet was washed again in sterile water. The sample was then immersed in liquid nitrogen to freeze, and ground to a fine powder with a sterile micropestle. Freezing and grinding was repeated once, 30 µL of 2x CTAB (Appendix C) was added, and the sample was mixed using the micropestle. Samples were frozen and thawed repeatedly (3x) in liquid nitrogen and a 65° C waterbath. Samples were incubated in a 65° C water bath for one hour. Proteins and other cell components were removed via chloroform extraction, and DNA was precipitated in isopropanol and washed in Wash Buffer (Appendix C) and 95% ethanol.

PCR Amplification of the SSU rRNA Gene and Sequencing

A 1150 bp fragment of the SSU rRNA gene was amplified using the universal primers NS1 and NS4 (Figure 1), which have been tested with a wide range of fungal species (White *et al.*, 1990). The reaction conditions were those used by Spatafora *et al.* (1995) with some modifications: 40 cycles of 94° C for 1 min., 53° C for 1 min., and 72° C for 1 min. Genomic DNA extracts were diluted in water at three serial dilutions: 1:100, 1:1000, and 1:10000. Successful amplification was detected on agarose gels. The DNA was purified using QiaQuick PCR purification columns (Qiagen), with a guanidine HCl

modification of the normal protocol to exclude primer dimers. The purified PCR product was submitted to the Biochemistry DNA sequencing facility at the University of Washington for sequence determination.

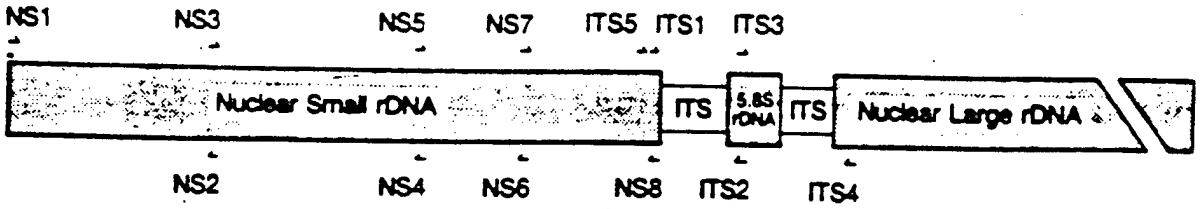


Figure 1: The Ribosomal DNA Gene Cluster and NS1 and NS4 Primers (White *et al.*, 1990)

Analysis of Sequence Data

Sequences were determined in both directions using the NS1 and the NS4 primers, resulting in a region of overlap in the center of the approximately 1050 bp fragment of the SSU gene. Sequence results were confirmed by visual inspection of the sequencing gel readout, and ambiguous bases were designated as N. The sequence of the SSU rRNA gene from each of the selected cultures was compared with known SSU rRNA sequences in the Ribosomal Database Project database (Maidak *et al.*, 1996), resulting in a list of the most closely related sequences in the database and their similarity rank.

Molecular Markers (Restriction Fragment Length Polymorphisms, RFLP s)

For selected cultures, SSU rRNA genes were amplified by the PCR and the resulting amplified sequence was digested with one of two restriction enzymes: Cfo I and Hpa II in React 8 reaction buffer (Gibco BRL). In addition, genomic DNA extracts from each culture were mixed at 1:1 or 1:100 ratios with each of the other cultures to simulate the effect of isolating DNA from a mixed fungal sample. PCR was performed and the amplified product was digested with Cfo I and Hpa II, as previously described. Digested DNA was separated on an agarose gel, stained with ethidium bromide, and digitized by a video analyzer. Migration of each band was measured using NIH-Image software and gel measurement macros, and migration was quantified in reference to the 100 bp band of the 100 bp DNA ladder (Gibco BRL).

RESULTS

Sequences and Direct Observations of Stock Cultures

Sequences were obtained for five stock cultures (Appendix A). Photographs of light microscopy and environmental SEM results are presented in Appendix B. Culture 1 resembled *Penicillium*, but had long chains of conidia not typical of *Penicillium*. The sequence's closest match was *Penicillium notatum*, with a fairly low similarity rank of 0.749 (see Table 1 and Figure 2). The culture appeared to be in the *Penicillium/Aspergillis* cluster, but it could not be identified to genus. Culture 2 had sporangia characteristic of *Mucor*, and the closest matching sequence was *Mucor racemosus*, with a

Table 1. Sequence Analysis of Cultures Derived from Airborne Spores

Culture #	Closest Matches	Similarity Rank (RDP)	GCG-GAP Quality	Morphol. ID
1	*Penicillium notatum*	.749		Penicillium?
	Aspergillus fumagatus	.713		
2	*Mucor racemosus*	.881		Mucor
	Endogone pisiformis	.408		
3	*Aureobasidium pullulans*	.884		Aureobasidium?
	Pleospora rudis	.750		
4	*Spongipellis unicolor*	.807	1010.7	Heterobasidion?
	Heterobasidion annosum *		1008.7	
	Thanatephorus praticola	.765	966.9	
5	*Aureobasidium pullulans*	.831		Cladosporium?
	Blastomyces dermatitidis	.755		

*The *H. annosum* sequence was added to Genbank 10/96. It was not available on RDP at the time of this study, therefore sequences were compared using the GAP program of the Genetics Computing Group (GCG) package.

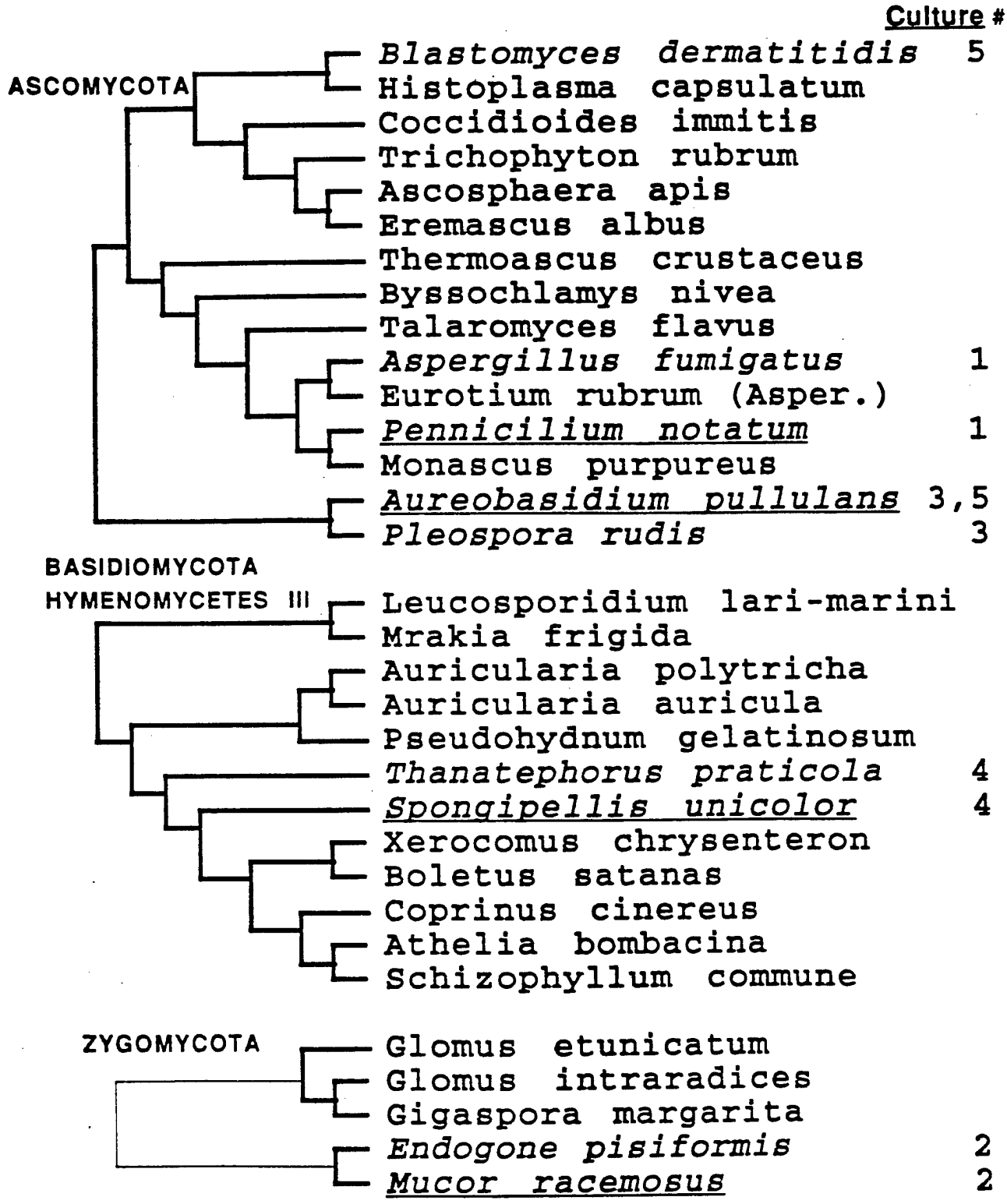


Figure 2. Phylogenetic Tree of Fungal SSU rDNA Sequences (provided by the Ribosomal Database Project, Maidak *et al.*, 1996). Branches without closest matching sequences have been omitted from the figure, but were included in the tree analysis. Closest matching sequences are underlined, next closest matches are italicized, and the stock cultures' number is listed next to each.

similarity rank of 0.881. Culture 3 had a characteristic yeast form, with some pseudomycelium also present. Conidia appeared to resemble *Torula* more than *Aureobasidium* in form. A sequence for the *Torula* SSU rDNA gene was not available on the RDP, but *Aureobasidium* was a close match with a Similarity Rank of 0.884. Culture 4 had previously been tentatively identified as *Heterobasidion annosum*, but no diagnostic morphological features were observed for the stock culture at the time of this study. The SSU rDNA sequence was highly related to *Spongipellis unicolor* (Similarity rank 0.807; GCG-GAP quality index 1010.7) and *Heterobasidion annosum*, a common airborne forest pathogen, was a close second, based on a GCG-GAP quality index of 1008.7. For culture 5, sequence comparisons were indeterminate, as the two closest matches were separated on the phylogenetic tree, and had a relatively low similarity rank (0.831 for *Aureobasidium pullulans* and 0.755 for *Blastomyces dermatitidis*). The culture resembled a *Cladosporium* sp., but directly observed characters were not sufficient to identify the culture with any confidence.

The fungal SSU rDNA sequences available through the ribosomal database project are displayed in Figure 2, arranged according to phylogenetic relationship. Some branches have been omitted due to lack of space, without influencing the arrangement of species. Closest matching sequences are underlined, while next closest sequences are italicized.

Molecular Markers of Cultures 1, 2, and 3

Restriction fragment length polymorphisms (RFLP's) of the SSU rRNA gene for three of the cultures were analyzed. In Figure 3, a gel of RFLP's with the restriction enzyme Cfo I shows a pattern of three bands (750 bp, 260 bp, 170 bp) for Culture 1 and 3, and a pattern of two bands (750 bp and 400 bp) for Culture 2. When the restriction enzyme Hpa II was used (figure **4**), the RFLP pattern had three bands for Culture 1 and 3 (410 bp, 210 bp, and 90 bp) and Culture 2 had two bands (750 bp and 400 bp). While Cultures 1 and 3 are indistinguishable with these two enzymes, Culture 2 can be distinguished with either enzyme.

When genomic DNA extracts from different cultures were mixed before PCR amplification of the SSU gene, the effect of mixing varied. Lanes 2-4 and 6 of the gels in Figures 3 and 4 show the results of mixing gDNA extracts 1:1. The mixture of Culture 2 and 3 (lane 6) has bands from both cultures, while the mixture of Culture 1 and 3 (lane 3) reflects the presence of Culture 1 only. When gDNA extracts were mixed 1:100, the minor component could still be detected with some cultures (lane 11--Culture 1), while with other cultures the minor component was swamped out by the major component (lanes 10 and 12--Culture 2; lane 13--Culture 3).

Lane 1: Culture 1
Lane 2: Culture 1 & 3 (1:1)
Lane 3: Culture 1 & 2 (1:1)
Lane 4: 1 & 2 & 3 (1:1:1)
Lane 5: Culture 3
Lane 6: 2 & 3 (1:1)
Lane 7: Culture 2
Lane 8: Culture 1 & (3) (1:100)
Lane 9: Culture 3 & (1) (1:100)
Lane 10: Culture 1 & (2) (1:100)
Lane 11: Culture 2 & (1) (1:100)
Lane 12: Culture 3 & (2) (1:100)
Lane 13: Culture 2 & (3) (1:100)
Lane 14: 100 bp DNA ladder

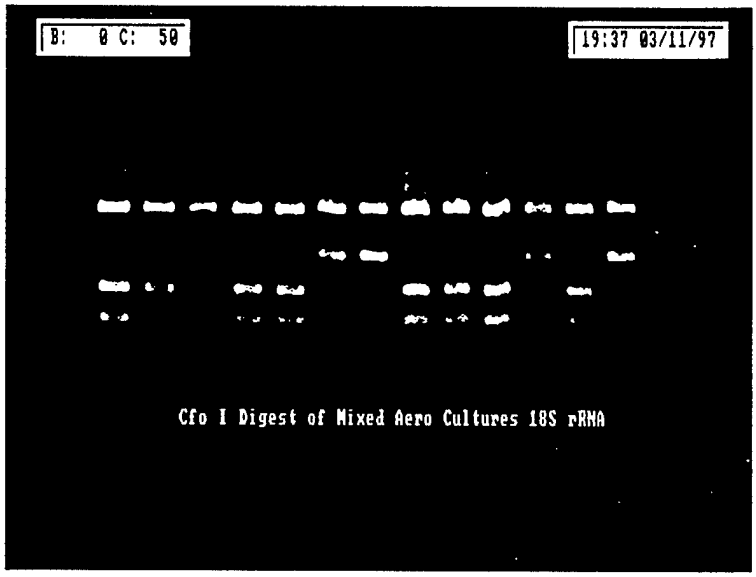
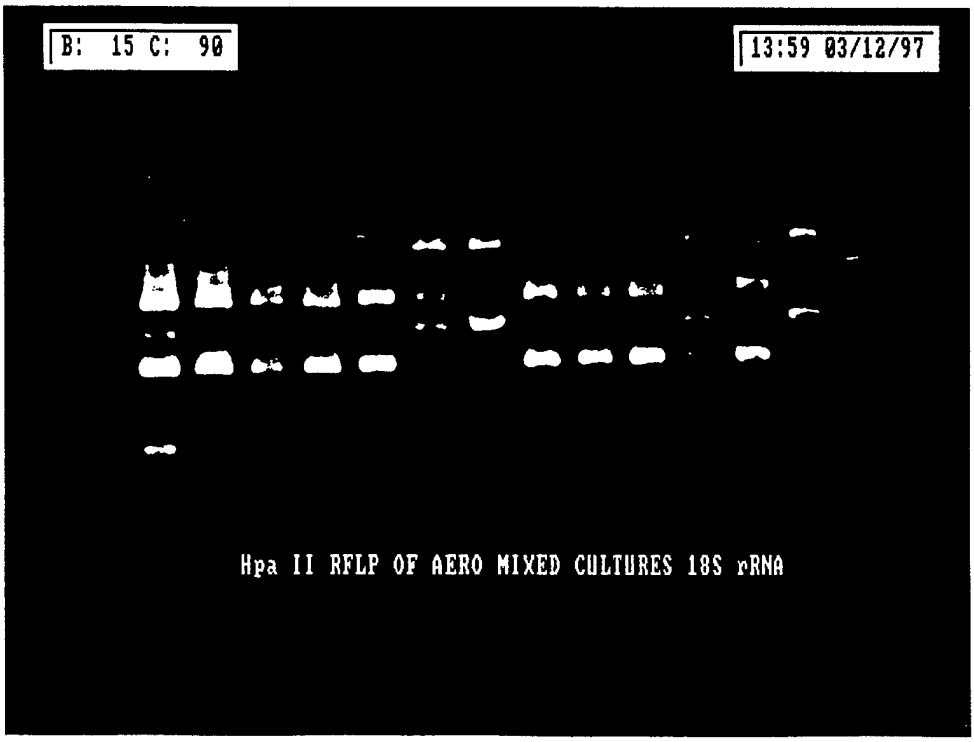


Figure 3. Gel of RFLP's of the SSU rDNA Gene Digested with Cfo I. Genomic DNA samples from stock cultures were diluted 1:1000 in water, then used for PCR directly, or mixed 1:1 or 1:100 with other stock cultures' gDNA.

Figure 4. Gel of RFLP's of the SSU rDNA Gene Digested with Hpa II.
Genomic DNA samples from stock cultures were diluted 1:1000 in water, then used for PCR directly, or mixed 1:1 or 1:100 with other stock cultures' gDNA.

Lane 1: Culture 1
Lane 2: Culture 1 & 3 (1:1)
Lane 3: Culture 1 & 2 (1:1)
Lane 4: 1 & 2 & 3 (1:1:1)
Lane 5: Culture 3
Lane 6: 2 & 3 (1:1)
Lane 7: Culture 2
Lane 8: Culture 1 & (3) (1:100)
Lane 9: Culture 3 & (1) (1:100)
Lane 10: Culture 1 & (2) (1:100)
Lane 11: Culture 2 & (1) (1:100)
Lane 12: Culture 3 & (2) (1:100)
Lane 13: Culture 2 & (3) (1:100)
Lane 14: 100 bp DNA ladder



DISCUSSION

Sequence Analysis

Of the five stock cultures that were selected for study, only one (*Mucor*) could be identified to genus with some degree of confidence. The similarity rank of 0.881 when comparing *Mucor racemosus* and the unknown sequence was high enough to indicate highly related sequences, consistent with both organisms being in the same genus. Direct observation of sporangia in light and scanning electron microscopy confirmed that the isolate was *Mucor*. It is important to note that relatively few sequences are available for zygomycetes (Figure 2), and therefore it was lucky that the *Mucor racemosus* sequence was available. The low resolution on the phylogenetic tree for this branch was reflected also in the low similarity rank of 0.408 for the second most similar organism, *Endogone pisiformis*.

Culture 3 had a high similarity rank of 0.884 to *Aureobasidium pullulans*. Unfortunately, this isolate could not be identified by direct observation in our stock cultures (possibly due to the long period in culture), and therefore it is impossible to evaluate whether or not the culture actually was in the genus *Aureobasidium*. It was an interesting coincidence that culture 5 was also most highly related to *Aureobasidium pullulans*, but with a lower similarity rank of only 0.831. This result emphasizes the importance of paying attention to the degree of similarity instead of merely identifying the most highly related organism. It was also interesting to note that culture 5 had as its next closest match *Blastomyces dermatitidis*, an organism which was on a distant branch from *Aureobasidium pullulans*. This was probably due to the relatively low similarity rank, and can be explained by the rapid decrease in reliability of the alignment as the similarity between the two compared sequences decreases.

Culture 4 was found to be most similar to *Spongipellis unicolor* (0.807), with *Heterobasidion annosum* a close second. The SSU rDNA sequence for *H. annosum* was not yet available on the RDP database, but was submitted to the Genbank database in October of 1996, therefore comparisons were performed using the GAP program of the Genetics Computing Group (GCG) software package (Table 1). Again, visual inspection of the stock culture at the time of DNA isolation did not yield a reliable identification, however this culture had originally been identified as *H. annosum*. It is curious that the similarity rank was quite low (probably just under 0.807), if the stock culture was indeed the same species as the one reported in Genbank. It is possible that the species *H. annosum*, as currently defined, could encompass a wide range of genetic types, or that our stock culture was either misidentified or replaced by a contaminant. To address this

question, further research could look at SSU rDNA sequences from well-identified *H. annosum* cultures.

Culture 1 had been identified as *Penicillium* sp., and had some characteristics of *Penicillium* such as the prodigious production of blue-green spores, however the conidiophore structure was not typical of *Penicillium*. This observation was reinforced by the relatively low similarity rank (0.749) between the stock culture and *Penicillium notatum*, the most closely related sequence in the RDP database.

The SSU rDNA gene appeared to be effective in some cases in identifying unknown stock cultures to the genus level, but the power of the approach was severely limited by lack of resolution in the phylogenetic tree of available known sequences for comparison. Until more sequences become available, it will be difficult to use this approach for any but the most common genera of fungi. However, the recent advent of easily accessible and complete databases like the ribosomal database project should encourage other researchers to contribute new SSU sequences. The addition of a *H. annosum* sequence to the database during the course of this study provides an example of the rapid rate of expansion of the database.

Molecular Markers for Detecting Previously Identified Organisms

Culture 1 and Culture 3 had identical RFLP patterns with both restriction enzymes that were used (Figures 3 and 4). This is not unexpected, since the SSU rDNA gene is quite highly conserved, resulting in similar RFLP patterns among genera that are not closely related. In future research, we plan to use a less highly conserved region of the ribosomal gene cluster, which should result in unique RFLP patterns for most genera of fungi. Specifically, the internal transcribed spacers (ITS-I and ITS-2, see Figure 1) have been used by researchers for molecular markers at the genus, species, or strain level (Gardes and Bruns, 1993).

It was possible to distinguish between Culture 2 and Cultures 1 and 3 using SSU rDNA RFLP's (Figures 3 and 4). This made it possible to examine the effect of mixing gDNA from different stock cultures, to mimic the effect of isolating gDNA from mixed samples of airborne fungal spores. In 1:1 mixtures, Culture 2 could be detected from mixtures with gDNA from Culture 3 (lane 6), but not from Culture 1 (lane 3). In 1:100 mixtures, Culture 2 could be not be detected as a minor component (lanes 10 and 12). In contrast, Culture 1 could be detected both as a major component (100:1, lane 10) and as a minor component (1:100, lane 11) with Culture 2. These differences probably reflect variation in the efficiency of gDNA extraction from each stock culture. Culture 1 appeared to have either a more efficient gDNA extraction from the starting biomass, or more copies

of the rDNA gene in its genome; consequently it was more likely to "swamp out" the other organisms' gDNA in mixtures. Despite the limitation of not knowing the starting concentration of gDNA, these results indicate that PCR amplification could be able to detect SSU rDNA genes even from organisms which represent a minor component in the original fungal spore mixture.

In future research, we would like to attempt to amplify SSU rDNA genes from gDNA isolated from single airborne spores. Lee and Taylor (1990) reported successful amplification from single ascospores of *Neurospora tetrasperma*. It remains to be determined whether single spore amplification will work from conidia and yeast cells dispersed as airborne particles. We propose the following strategy for identifying airborne spores of interest. First, physical or biochemical methods would be used to detect the presence of unusual or interesting fungal spores. Second, gDNA could be obtained from a single spore, and the SSU rDNA gene could be sequenced, providing phylogenetic information about the most closely related known organism. Finally, RFLP's or other molecular markers could be used to detect the presence of that unique spore in gDNA isolated from samples of mixed fungal airborne spores from the environment.

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APPENDIX A

SEQUENCE OF CULTURES DATA

A.1: Sequence of Culture 1 (*Penicillium*?)

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   1  ATNACCANAC ANATCTAAGT ATAAGCAACT TGTACTGTGA AACTGCGAAT
  51  GGCTCATTAA ATCAGTTATC GTTTATTTGA TAGTACCTTA CTACATGGAT
 101  ACCTGTGGTA ATTCTAGAGC TAATACATGC TAAAAACCCC GACTTCAGGA
 151  AGGGGTGTAT TTATTAGATA AAAAACCAAC GCCCTTCGGG GCTCCTTGGT
 201  GAATCATAAT AACTTAACGA ATCGCNTGGC CTTGCNCCGG CGATGGTTCA
 251  TTCAAATTTC TGCCCTATCA ACTTTCGATG GTAGGATAGT GGCCTACCAT
 301  GGTGGCANCG GGNNNCGGGG AATTANGGTT CGATCCCGGA GAGGGAGCCT
 351  GAGAAACGGC TACNACATCC AATGAAGGCA NCTGGCCNCA AATTTCCANT
 401  CCCTATTCNG GGATGTAGTN ACAATAAATA CTGANNCTGG GCTCTTTTGG
 451  GTCTCNTNNN TTGGANTTNA AANCTATTNTATCCCTTA ACGAGGAACA
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 801  CTAGGACCGC CGTAATGATT AATAGGGATA GTCGGGGGCG TCAGTATTCA
 851  GCTGTCAGAG GTGAAATTCT TGGATTTGCT GAAGACTAAC TACTGCGAAA
 901  GCATTCGCCA AGGATGTTTT CATTAATCAG GGAACGAAAG TTAGGGGATC
 951  GAAGACGATC AGATACCGTC GTAGTCTTAA CCATAAACTA TGCCGACTAG
1001  GGATCGGACG GGTTCTATGA TGACCCGTTC GGCACCTTAC GAGAAATCAA
1051  ATTTTTGGTT CTGGGGGATG ATGTCGCANG GTNA
```

A.2: Sequence of Culture 2 (*Mucor*?)

```
   1  TTNANGANTG ACGATGNAAG TATAAATAAA TTTATATTGT GAAACTGCGA
  51  ATGGCTCATT AAATCAGTTA TGATCTACGT GACATATTTC TTTACTACTT
 101  GGATAACCGT GGTAATTCTA GAGCTAATAC NTGCAAAAAA ACCCTGACTT
 151  ACGAAAGGGT GCACTTATTA GATAAAGCCA ACGCTGGGTA AAACCAGTTT
 201  CCCTTGGTGA TTCATAATAA TTTAGCGGAT CGCNTGGCCT TGTGCTAGCG
 251  ACAGTCCACT CGATTTTCTG CCCTATCATG GTTGAGATTG TAAGATAGAG
 301  GCTTACAANG CCTACAACGG GTANCGGGGA ATTAGGGTTC GATTCCGGAG
 351  AGGGAGCCTG AGAAACGGCT ACCACNTCCA ANGAAGGCAG CTTGCGCGCA
 401  AATTACCCAA TCCCGACTCG GGGAGGTAGT GACAATAAAT AACAATGCAG
 451  GGCCTTTAAG GTCTTGCAAT TGGAATGAGT ACAATTTAAA TCCCTTAACG
 501  AGGATCAATT GGAGGGCAAG TCTGGTGCCC AGCAGCCGCG GTAATTCCAG
 551  CTCCCATAGC GTATATTAAA GTTGTTGCAG TTAAAACGTC CGTAGTCAAA
 601  TTTTAGTCTT TAGATGAGGT GGCCTGGTCT TCATTGATCA AGCTCGCTTT
 651  TATCGAGACT TTTTTTCTGG TTATGCTATG AATAGCTTCG GTTGTTTATA
 701  GTCTCTAGCC AGATGATTAC CATGAGCAAA TCAGAGTGTT TAAAGCAGGC
 751  TTTTAAGCTT GAATGTGTTA GCATGGAATA ATGAAATATG ACTTTAGTCC
 801  CTATTTCGTT GGTTCAGGAA CTTAAGTAAT GATGAATAGA AACGGTTGGG
 851  GACATTTGTA TTTGGTCGCT AGAGGTGAAA TTCTTGGATT GACCGAAGAC
 901  AAACTACTGC GAAAGCATTT GATCCAGGAC GTTTTCATTG ATCAAGGTCT
 951  AAAGTTAAGG GATCGAAGAC GATTAGATAC CGTCGTAGTC TTAACCACAA
1001  ACTATGCCGA CTAGAGATTG GGCTTGTTTA TTATGACTAG CTCAGCATCT
1051  TAGCGAAAGT AAATTTTTGG TTCTGGGGGG TGTNTAACAG GGTGNNNNNN
1101  NNN
```

A.3: Sequence of Culture 3 (*Aureobasidium*?)

```
   1  CTTCAGGATT GACGGANGAA GGTGTGGCGC TNTTCGGTGA AACTGCGAAT
  51  GGCTCATTAA ATCAGTTATC GTTTATTTGA TAGTACCTTA CTACTTGGAT
 101  AACCGTGGTA ATTCTAGAGC TAATACATGC TAAAAACCCC AACTTCGGAA
 151  GGGGTGTATT TATTAGATAA AAAACCAACG CCCTTCGGGG CTCCTTGGTG
 201  ATTCATAATA ACTAAACGAA TCGCATGGCC TTGCGCCGGC GATGGTTCAT
 251  TCAAATTTCT GCCCTATCAA CTTTCGATGG TAGGATAGTG GCCTACCATG
 301  GTATCAACNG GTAACGGGGA ATTAGGGTTC TATTCCGGAG AGGGAGCCTG
 351  AGAAACGGCT ACCACATCCA AGGAAGGCAG CAGGCGCGCA AATTACCAAT
 401  CCCGACACGG GGAGGTAGTG ACAATAAATA CTGATACAGG GCTCTTTTGG
 451  GTCTTGTAAT TGGAATGAGT ACAATTTAAT CCTTAAACGA GGAACAATTG
 501  GAGGCAAGTC TGGTGCCAGC AGCCGCGGNA ATTCCAGCTC CCATTAGCGT
 551  ATATTAAAGT TGTTGCAGTT AAAAAGCTNC GTAGTTGAAC CTTGGGCCTG
 601  GCTGGCCGGT CCGCCTCACC GCGTGTANTG GTCCGGCCGG GCCTTTCCTT
 651  CTGGGGAGCC GCATGCCCTT CACTGGGCGT GTCGGGGAAC CAGGACTTTT
 701  ACTTTGAAAA AATTAGAGTG TTCAAAGCAG GCCTTTGCTC GAATACATTA
 751  GCATGGAATA ATAGAATAGG ACGTGCGGTT CTATTTNGTT GGTTTCTAGG
 801  ACCGCCGTAA TGATTAATAG GGATAGTCGG GGGCATCAGT ATTCAATTGT
 851  CAGAGGTGAA ATTCTTGGAT TTATTGAAGA CTAACTACTG CGAAAGCATT
 901  TGCCAAGGAT GTTTTCATTA ATCAGTGAAC GAAAGTTAGG GGATCGAAGA
 951  CGATCAGATA CCGTCGTAGT CTTAACCATA AACTATGCCG ACTAGGGATC
1001  GGGCGATGTT ATCATTNTGA TCGTTCGNCA CCNTACGAGA AATCAAATCT
1051  ACGGATCNGG TGATGNATCA CAGTTTCNNN NGN
```

A.4: Sequence of Culture 4 (*Heterobasidion annosum*?)

```
   1  TNTTGGACCG ACANATCTAA GTATAAACAA GTTTGTACTG TGAAACTGCG
  51  AATGGCTCAT TAAATCAGTT ATAGTTTATT TGATGGTGCT TTGCTACATG
 101  GATAACTGTG GTAATTCTAG AGCTAATACA TGCAATCAAG CCCCGACTTC
 151  TGGAAGGGGT GTATTTATTA GATAAAAAAC CAACGCGGTT CGCCGCTCCA
 201  TTGGTGATTC ATAATAACTT CTCNAATCGC ATGGCCTTGT GCCGGCGATG
 251  CTTCATTCAA ATATCTGCCC TATCaACTTT CGATGGTAGG ATAGAGGCCT
 301  ACCATGGTTT CAACGGGTAA CGGGGAATNA GGGTTCGATT CCGGAcAGGG
 351  AGCCTGAAAA ACGGCTACCA CNTCCAAGGA AGGCNGCAGG CGCGCAAATT
 401  NCCCANTCCC GACCGGGGAG GTAGTGACAA TAAATAACAA TATAGGGCTC
 451  TTTCGGGTCT NATAATTGGA ATNAGTACAA TTTAAATCTC GAGGA
 501  ACAATTGGAG GGCAAGTCTG GTGCCAGCAG CCGCGGTAAT TCCAGCTCCA
 551  ATAGCGTATA TTAAAGTTGT TGCAGTTAAA AAGCTCGTAG TTGAACTTCA
 601  GGCCTGGCTG GGCGGTCTGC CTAACGGTAT GTACTGTCTG GCTGGGTCTT
 651  ACCTCTTGGT GAGCCGGCAT GCCCTTCACT GGGTGTGTCG GGGAACCAGG
 701  ACTTTTACCT TGAGAAAATT AGAGTGTTCA AAGCAGGCTT ATGCCCGAAT
 751  ACATTAGCAT GGAATAATAA AATAGGACGT GCGGTTCTAT TTTGTTGGTT
 801  TCTAGAGTCG CCGTAATGAT TAATAGGGAT AGTTGGGGGC ATTAGTATTC
 851  CGTTGCTAGA GGTGAAATTC TTGGATTTAC GGAAGACTAA CTACTGCGAA
 901  AGCATTTGCC AAGGATGTTT TCATTAATCA AGAACGAAGG TTAGGGGATC
 951  GAAAACGATC AGATACCGTT GTAGTCTTAA CAGTAAACTA TGCCGACTAG
1001  GGATCGGGCG AANTCAATTN GATGTGTCGC TCGGCACCNN ACGAGAAATC
1051  AAATCNCNGG NTCCCGTGNT NNNNATTCAT ATGCTNTTCC
```

A.5: Sequence of Culture 5 (*Cladosporium*?)

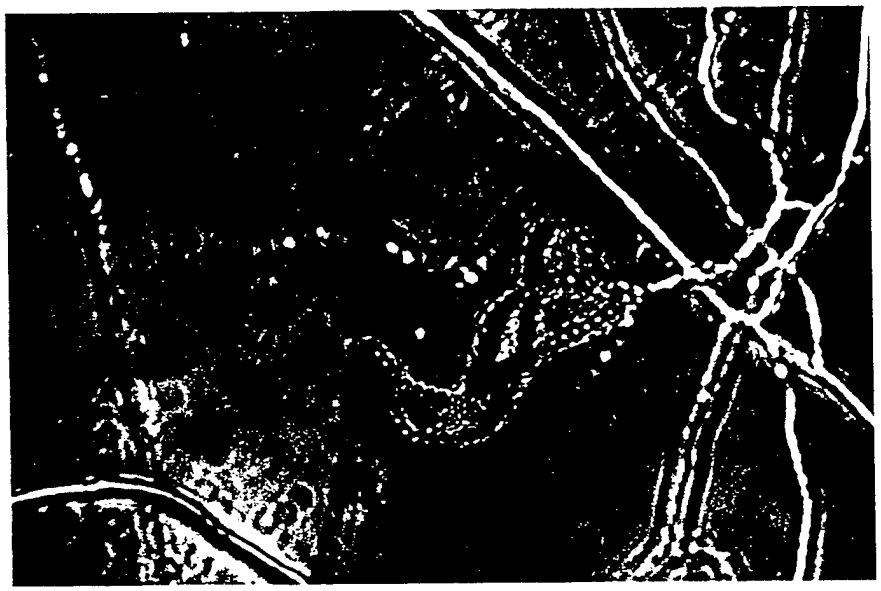
```
   1  AAAGATGANC GNCNNTCTAA GTATAAGCAA CTATACGGTG AAACTGCGAA
  51  TGGCTCATTA AATCAGTTAT CGTTTATTTG ATAGTACCTT ACTACTTGGA
 101  TAACCGTGGT AATTCTAGAG CTAATACATG CTAAAAACCT CGACTTCGGA
 151  AGGGGTGTNT TTATTANATA AAAAACCAAT GCCCTTCGGG GCTCCTTGGT
 201  GATTCATAAT AACTTAACGA ATCNCATGGC CTTGTGCCGG CGATGGTTCA
 251  TTCAAATTTC TGCCCTATCA ACTTTCGATG GTAGGATAGT GGCCTACCAT
 301  GGTTTCCAAC GGGTNACGGG GAATTAGGGT TCTATTCCGG ANANGGACCT
 351  GAGAAACGGC TGCCACATCC AAGGAAGGCA GCAGGCGCGC AAATTACCCA
 401  ATCCCGACAC GGGGAgGTag TGACAATAAA TACTGATACA GGGCTCTTTT
 451  GGGTCTTGTA ATTGGAATGA NTACAATTTA AATCCCTTAA CGAgGAACAA
 501  TTGGAgGGCA AGTCTGGTGC CAgCAGCCGC GGTAATTCCA GCTCCAATAg
 551  CGTATATTAA AgTTGTTGCA GTTAAAAAGC TCgTAgTTGA ACCTTGAGCC
 601  TGGCTGGCCG GTCCGCCTCA CCGCGTGCAc TGGTCCGGCC GGGTTTTTCC
 651  TTcTGGGGAG CCGCATGCCC TTCAcTGGGT GTGTCGGGGA ACCAGGACTT
 701  TTACTTTGAA AAAATTAGAG TGTTCAAAGC AGGCCTATGC TCGAATACAT
 751  TAGCATGGAA TAATAGAATA GGACGTGTGG TTCTATTTTG TTGGTTTTCT
 801  AGGACCGCCG TAATGATTAA TAGGGATAGT CGGGGGCATC AGTATTCAAT
 851  TGTCAGAGGT GAAATTTCTT GGATTTATTG AAGACTAACT ACTGCGAAAG
 901  CATTTGCCAA GGATGTTTTC ATTAATCAGT GAACGAAAGT TAGGGGATCG
 951  AAGACGATCA GATACCGTCG TAGTCTTAAC CATAAACTAT GCCGANTAGG
1001  GATCGGGCGA TGNTATTTTT TTGACTCGCT CGGCACCTTA CGAGAAATCA
1051  AATCTTTGGT TCTGGGGGGT ATGTCGCAAG GTGAA
```

Blank

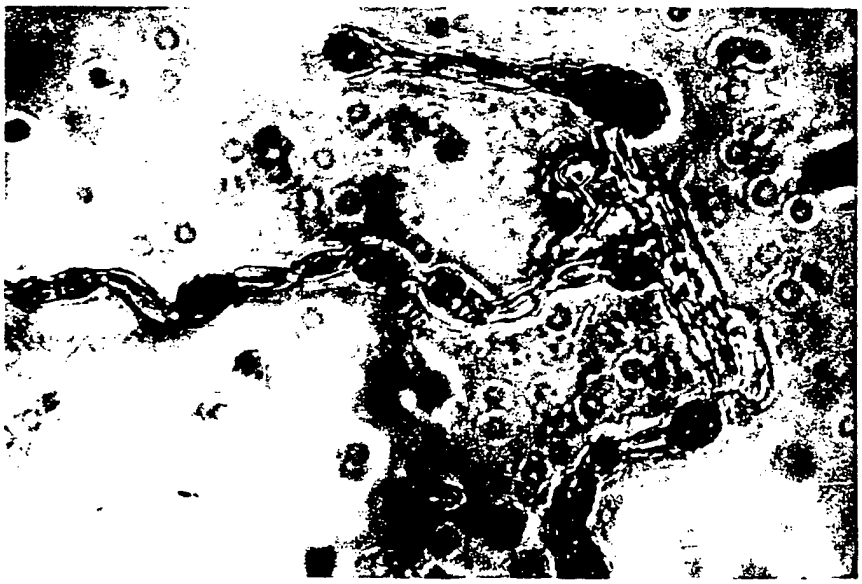
APPENDIX B

PICTURES OF STOCK CULTURES

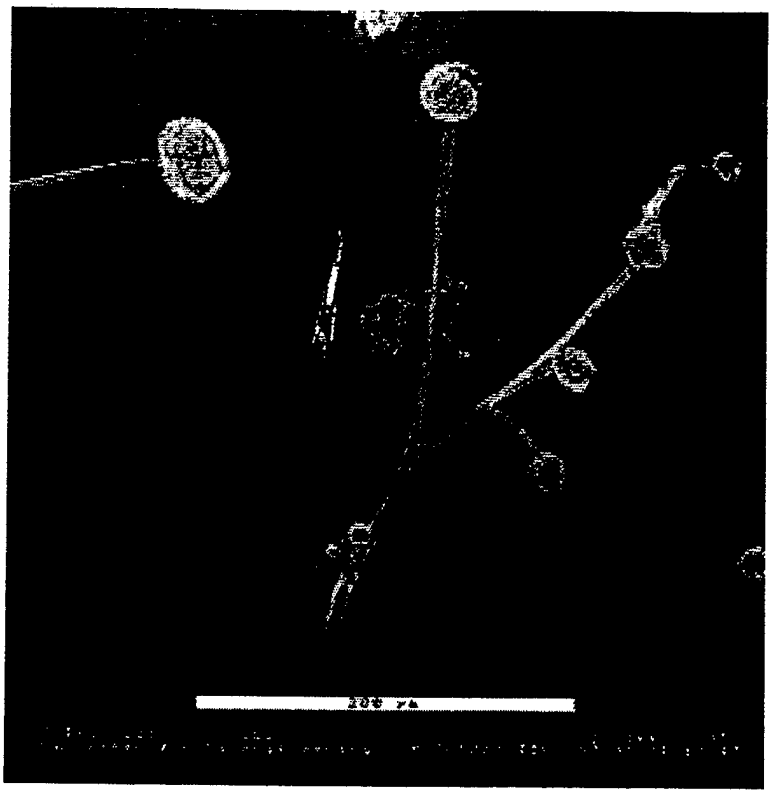
Culture 1: *Penicillium* (?), conidiophore and conidia in long chains, 400x.



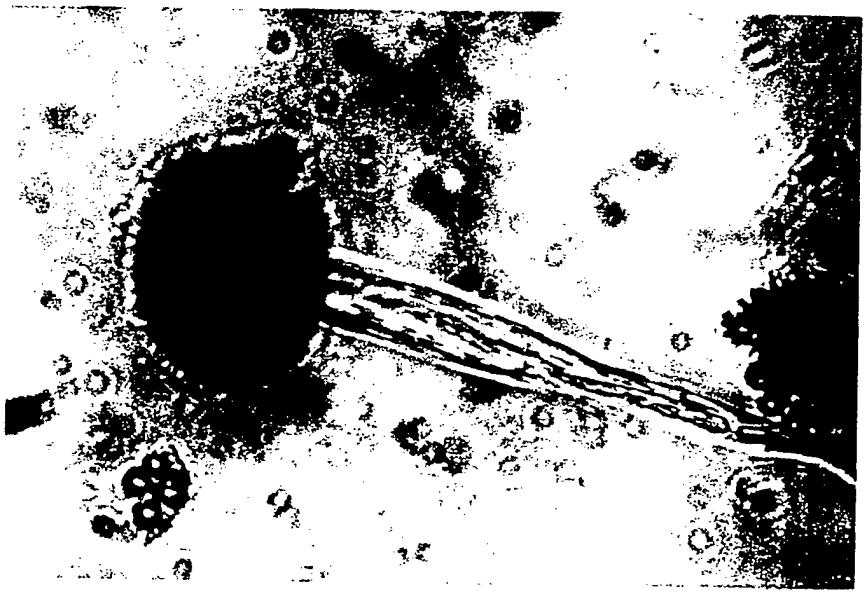
Culture 3: *Aureobasidium* (?), pseudomycelium and yeast growth form, dark conidia?, 400x.



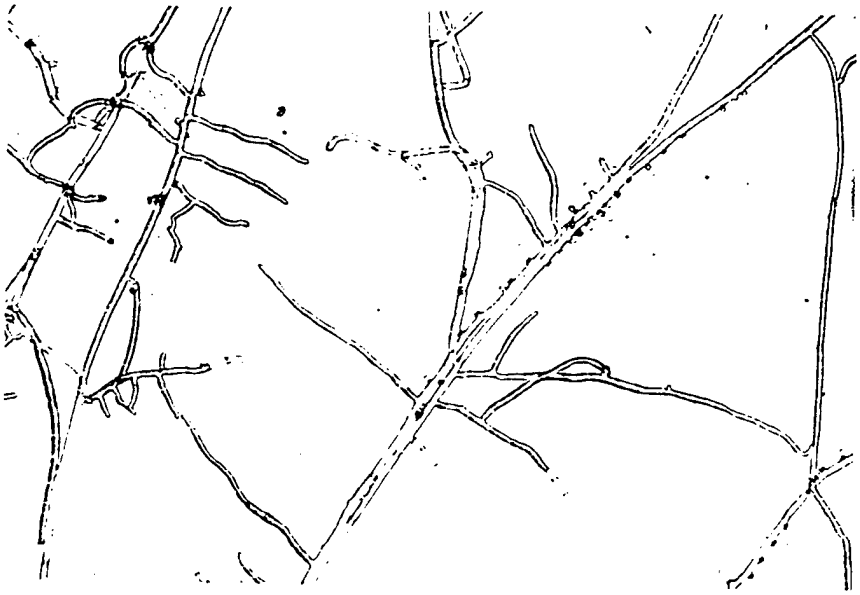
Culture 2: *Mucor*, branched sporangiophore and sporangia, environmental SEM image.



Culture 2: *Mucor*, single sporangiophore and sporangium, 400x light micrograph.



Culture 4: *Heterobasidion annosum* (?), 100x.



Culture 5: *Cladosporium* (?), conidiophore and dark-celled conidia?, 400x light micrograph.

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APPENDIX C

BUFFERS

2X CTAB Lysis Buffer:

100 mM Tris-Cl (pH 8.0)
1.4 M NaCl
20 mM EDTA
2.0% w/v CTAB (Hexadecyltrimethyl-ammonium bromide)

Wash Buffer:

76 % ethanol
10 mM NH4OAc