

Tiffany's drawings, fungal spots and phylogenetic trees

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ABSTRACT: Samples from fungal spots on Tiffany's drawings were removed and DNA was isolated from these fungal spots. The polymerase chain reaction (PCR) was then used to amplify the internally transcribed spacer (ITS) of the nuclear ribosomal RNA genes. The PCR products were cloned and sequenced and compared to existing ITS sequences of a large database of fungal species. DNA directly extracted from six fungal spots was analyzed for fungal species identification using a phylogenetic tree approach. Our results indicate that several different fungal species have colonized the Tiffany drawings. In particular, species from the genera *Cladosporium*, *Madurella*, *Mycosphaerella*, and *Thielavia* were identified as existing in the fungal spots we removed from the Tiffany Drawings. This study, while specific for art works made with material such as paper, outlines procedures for identification of the uncultured fungal communities infecting art works down to species of origin.

1 INTRODUCTION

The identification of the fungal species on infected art pieces is an important part of restoration and conservation work. In a previous publication we showed that the polymerase chain reaction (PCR) could be used to amplify fragments from a marker DNA molecule the internally transcribed segment (ITS) region of the ribosomal RNA gene (Di Bonaventura et al. 2002). The previous study demonstrated that several species of fungi could be found in different fungal spots either dormant or actually growing on art work. The present study elaborates on these findings and outlines procedures for identification of the fungi infecting these pieces of art down to species of origin. We utilize two approaches relevant to the identification of species using PCR and DNA sequencing techniques. These two approaches are based on phylogenetic principles used in systematic studies (Kretzer et al. 1996, Goodwin et al. 2001).

2 MATERIALS AND METHODS

2.1 DNA sequences

DNA sequences were generated for selected fungal spot isolates as described in Di Bonaventura et al. (2002) and as summarized in Table 1. The art works included

Table 1. Clone sequences generated from selected fungal spots. In particular the brown, gray to dark fungal spots were focused on. The works included Angel and Three Mary's, Mosaic Panel and Vassar College.

Drawings	Fungal spots	Clone sequences
Angel & Three Mary's	Gray to dark	MP90
	Brown	MP74/MP90
	Mixed 1	MP74
Mosaic Panel	Mixed 2	MP35/MP65
	Brown to dark	MP10/MP28/ MP65/MP74
Vassar College	Gray to dark	MP7/MP90

Angel and Three Mary's (MMA Acc.No. 67.654.206), Mosaic Panel (MMA Acc. No. 67.654.53) and Vassar College (MMA Acc. No. 67.654.307). DNA was isolated from the scrapings using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc.) (Pennanen et al. 2001). Three primer pairs were used to amplify regions of the ITS in the rRNA genes (White et al. 1990, Travis et al. 2000). Primer pair A amplified a region of the ribosomal RNA genes approximately 550 base pairs in length, that spans the ITS and includes the 5.8S ribosomal RNA (ITS 1 × 4). Primer pair B amplifies a region of the ITS approximately 250 base pairs in length, that is 5prime of the 5.8S rRNA gene and

include part of the 5.8S gene (ITS 1 × 2). Primer pair C amplifies a region of the ITS approximately 300 base pairs in length, that is 3 prime of the 5.8S rRNA gene and includes part of the 5.8S gene (ITS 3 × 4).

2.2 Cloning and sequencing

Due to the fact that each fungal spot used could contain more than one species of fungi, we cloned the PCR products from the three primer pairs using the TOPO TA cloning kit (Invitrogen). On average about 10 clones from each cloning reaction were sequenced using the Big Dye Chain Terminator (Applied Biosystem Inc.) method. Sequences were run on an ABI 3700 DNA Analyzer (PE Applied Biosystem). Sequences were compiled and corrected using the Sequencer Software (Gene Codes Corporation). Table 1 shows the number of unique rRNA types obtained from each fungal spots.

2.3 Analysis of fungal sequences

We analyzed the fungal sequence data by constructing a large fungal ITS sequence data base followed by phylogenetic analysis of the data base including the sequences from the fungal spots (listed in Table 1). The fungal ITS data base was constructed in the following way. Each unique sequence listed in Table 1 was BLASTed (Altschul et al. 1990) through the NCBI data base and the 500 best hits were retained. After all unique sequences were BLASTed the lists of 500 best hits were compared and a final list of best hits for all of the unique sequences was constructed that 1) eliminated redundant hits; 2) eliminated multiple representatives of the same species; and 3) maximized the number of taxa in the ITS data base. In this way a list of over 500 Ascomycetes and over 100 Basidiomycetes (at <http://research.amnh.org/molecular/>) were placed into two matrices one for the Ascomycetes and one for the Basidiomycetes. The sequences were then aligned in CLUSTAL W (Thompson et al. 1997) using default alignment parameters and saved as GCG-MSF files. These files were then imported into the Phylogenetic Analysis Using Parsimony* program (PAUP*; Swofford 2002). Sequences from the fungal spots listed in Table 1 were then aligned in the two matrices. Heuristic searches were performed with a single random addition of taxa using the tree bisection reconnection (TBR) option in PAUP (Swofford 2002). Due to the large number of taxa in the Ascomycetes data set, only 8600 trees were saved during the heuristic search. A Jackknife analysis using 1000 replicates was performed to obtain a tree with any node present in over 50% of the replicates. Jackknife searches were performed to assess the robustness of the phylogenetic inferences from the heuristic searches. The

Basidiomycetes data phylogenetic analysis was rooted with *Cladosporium cladosporioides* and the Ascomycetes phylogenetic analysis was rooted with *Rhodotorula dairenensis*. The positions of the fungal spot sequences listed in Table 1 in the parsimony and jackknife trees were noted and are discussed in the results section. Phylogenetic searches were performed with gaps scored as missing.

3 RESULTS AND DISCUSSIONS

The ITS databases and phylogenetic trees constructed to perform the analysis in this study can be found at the following web site: <http://research.amnh.org/molecular/>. Preliminary analysis of the sequences from the fungal spots we analyzed indicated that one of the ITS sequences from our analysis was more closely allied with the Basidiomycetes and five of the fungal spots were more closely allied with the Ascomycetes. Consequently we placed the Basidiomycete-like sequence into the Basidiomycetes matrix and the five Ascomycete-like sequences into the Ascomycetes data matrix.

3.1 Basidiomycete analysis

The placement of the clone sequence MP10/28, when analyzed using the Basidiomycetes reference matrix, indicated that this sequence was most closely related to a clade of *Kockovaella* and *Fellomyces* species (Figure 1). Precise affiliation was not possible using the phylogenetic tree building approach, and in addition, jackknife support for the placement of the ITS sequence to these three species was low (47%).

3.2 Ascomycetes analysis

Five clone sequences showed preliminary affinity to the Ascomycetes as deduced from the BLAST search (Figure 2). Using phylogenetic analysis, spots MP35 and MP90 were placed into a large relatively unresolved group of fungi including species of *Podospora*, *Thielevia*, *Chaetomium*, *Humicola* and other genera. MP35 was most closely related to *Madurella mycetomatis* while MP90 could not be placed as closely related to any of the species in this clade (Figure 2A). Spots MP65 and MP74 were placed into different strongly supported clades of *Cladosporium*. Spot MP65 was placed at the base of a clade containing three different species of *Cladosporium* and *Mycosphaerella africana* (Figure 2B). Spot MP74 was most closely related to *C. sphaerospermum* (Figure 2D). Spot MP7 was placed into a large unresolved subclade of fungi consisting of species from *Dermea*, *Neofrabraea* and several other genera of fungi. The placement of spot MP7 is ambiguous with respect to its closest relative

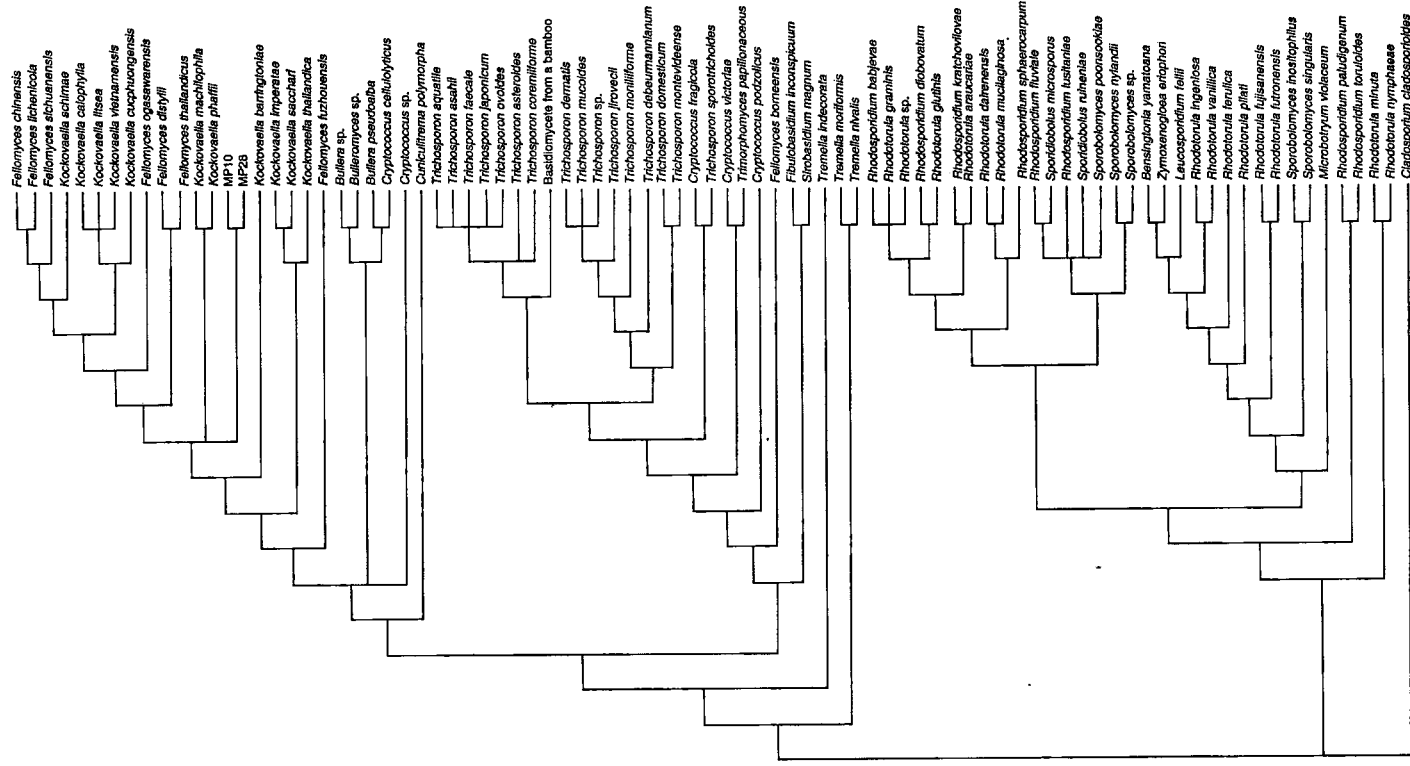


Figure 1. Phylogenetic tree generated using the Basidiomycetes data matrix and the ITS sequence from fungal spot MP10/28. The tree is a parsimony consensus tree of 21675 equally parsimonious solutions with a length of 3530 and a consistency index of 0.30 and a retention index of 0.71.

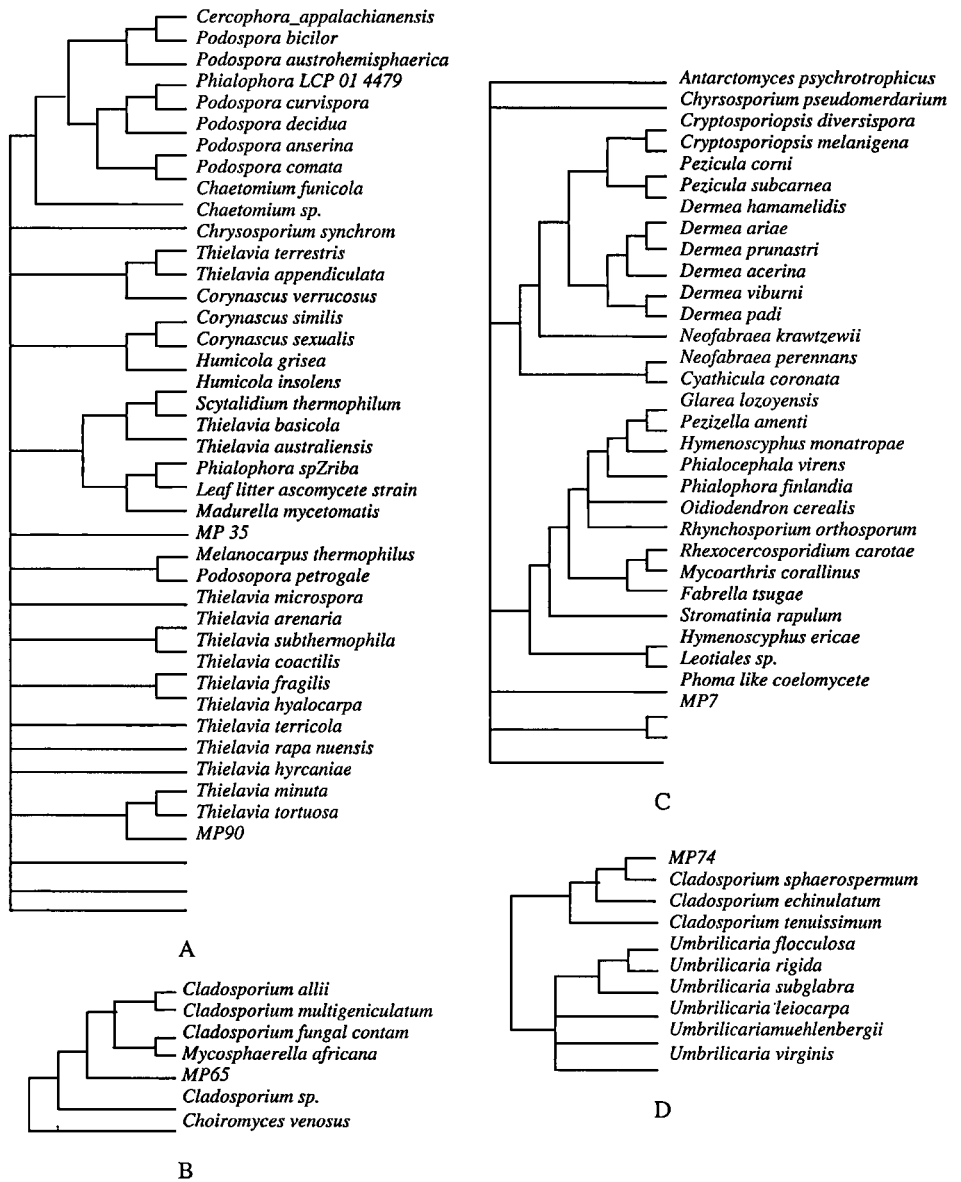


Figure 2. Pruned subtrees from the phylogenetic analysis of the ITS region of 459 fungal species in the Ascomycetes data set. The subtrees in this figure were all supported with at least 50% Jackknife support (see text). Panel A shows the affinity of MP90 and MP35 to several fungal species. Panel B shows the affinity of MP65 to several fungal species. Panel C shows the affinity of MP7 to several fungal species. Panel D shows the affinity of MP74 to several fungal species. The full tree and full ITS sequence matrices can be found at <http://research.amnh.org/molecular/>. The strict consensus tree from which the trees in this figure were pruned resulted from 8600 equally parsimonious trees of length 23535 with consistency index of 0.16 and retention index of 0.68.

in this clade and demonstrates the occasional lack of ability of this approach to diagnose a species origin precisely (Figure 2C).

3.3 Comparison of BLAST designations with phylogenetic analysis

BLAST searches often times gave multiple high scores indicating a high degree of similarity of the spot ITS sequences with sequences in the data base.

For the most part the BLAST scores are consistent with the phylogenetic analysis in that high BLAST scores indicated close phylogenetic affinity in the constructed trees. However, in five of the six spots for which we obtained ITS sequences (MP90, MP35, MP65, MP74, and MP10) the phylogenetic trees indicated more specific and precise species designations than the BLAST searches. The completeness of the ITS sequence database and the robustness of the phylogenetic analysis are important aspects of determining species origin in these kinds of studies. It is interesting that close phylogenetic affinity is shown for some of the fungal spots we examined with specific species already in the data base. In our first report (Di Bonaventura et al. 2002) we outlined that the fungal cultures isolated from the spots could be considered as most recent contaminants, with the exception of *Cladosporium*. This was supported by the fact that the direct extraction of DNA from field samples and the sequencing of the ITS regions – entire ITS region and the ITS 1 and 2 regions individually amplified – showed the involvement of several different fungal species. The investigations indicated that culturing may lead to a partial results in terms of fungal species composition or even a misunderstanding of those microorganisms responsible for the deterioration of art works.

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