MOLECULAR DIAGNOSIS BY PCR-DHPLC TECHNIQUE OF WOOD-DECAY FUNGI IN HISTORICAL BUILDINGS IN ITALY

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ABSTRACT: Wood inhabiting fungi cause real problems in the preservation of wooden surfaces and are responsible for the deterioration of cultural heritage. The identification of fungi based on morphological characteristics are still a topical issue. Nevertheless, they are limited for characterization and identification on an intraspecific level and even sometimes on an interspecific level. It is not always evident and thus many fungi remain unnamed or confused. The objective of this study was to circumvent these limitations by using a new molecular approach allowing fungal detection and identification in historic buildings in Italy. Fungal colonization was assessed by using PCR amplification and amplicons separation by Denaturing High Performance Liquid Chromatography. Due to its high sensitivity, the PCR-DHPLC technique was optimised to profile fungal communities in wood decay as well as ubiquitous contaminants.

Key words: historical building, wood inhabiting fungi, PCR amplification, Denaturing High Performance Liquid Chromatography, PCR-DHPLC

1. INTRODUCTION

Fungi have been playing a considerable role for ecosystem functioning *e.g.* wood-decay and recycling of organic matter in the environment. In contrast to their numerous beneficial effects, fungi were also associated to biodeterioration of indoor wooden materials (Zaremski et *al*. 2005).

Biodeterioration of houses by mould was frequently reported but sometimes this process leads to destruction of historic and cultural value. Although wood persists for long periods of time, accidentally or permanent exposure to humid conditions will favor microbial growth resulting in loss of cultural heritage (Fazio et *al*. 2010).

2. METHOD AND MATERIALS

2.1. Samples collections

Environmental samples analysed in this study were supplied by the CNR IVALSA of Firenze (Italy) and LegnoDoc srl. Most of the samples were collected from Italian historical buildings. There are given in Table 1 and presented in Figure 1 and 2. Each one corresponds to a unique sampling spot. The samples collected were either in the form of decaying wood from buildings. Most of the samples had fungal attack not active at moment of the collecting.

Fig.1. Wood inhabiting fungi Italian historical building in the Church Montalcino, Siena

Fig.2.Sample of wood for analysis

Best hit from BLAST results for sequences collected by PCR-DHPLC analysis Table 1

Nr.	Wood	Origin	Fungal identification
12	Poplar	Chiesa Certaldo, Firenze	*1. Botryobasidium botryosum* *2. Phanerochaete sordida*
13	Chestnut	Church Montalcino, Siena	*1. Scytalidium lignicola* *2. Donkioporia expansa*
16	n.d.	Church S. Stefano	*1. Trametes palisotii*

		Rotondo, Roma	*2. Oidiodendron griseum* *3. Candida digboiensis*
18	Beam	Castello Carrarese, Padova	*1. Sistotrema brinkmannii*

2.2 DNA extraction and PCR amplification

Genomic DNA was extracted from environmental samples using the FastDNA SPIN Kit for soil (MP Biomedicals, Illkirch) with slight modifications to the manufacturer's instructions. Both methods were applied according to the protocols previously described (Sterflinger et *al*.2010).

2.2.1 DNA extraction from wood samples

The timber samples were reduced to chips with a drill. They were handled under the most sterile conditions possible (benzene burner, alcohol, etc.) and the greatest attention was paid to sample heating. The residues were then refined by crushing in liquid nitrogen with a mortar.

DNA was extracted from around 200 mg of chips using a Fast DNA SPIN Kit for Soil (Quiagen MP Biomedicals). Prior to that, the samples underwent lysis (combined action of the beads in the kit and vigorous stirring of the FastPrep in the presence of buffer). Proteins were then precipitated by PPS (Protein Precipitation Solution) and eliminated by centrifugation. DNA was fixed on a soluble matrix (Binding Matrix) retained by a filter after passage of the solution containing DNA by centrifugation. Lastly, DNA was eluted by 60 µL of DES. Thus, the fraction recovered could then be used for different operations such as PCR or quantification by spectrophotometry.

It could also be stored at -20°C.

2.2.2 Total DNA quantification on a Nanodrop 1000

The NanoDrop 1000 UV-Visible spectrophotometer (Thermo Scientific, USA) provides particularly precise measurements of micro-volumes of samples with optimum reproducibility. Just 1µL of the analysis sample is deposited on the inner measuring surface. A magnet system is activated and the arm compresses the drop of liquid, forming a column of liquid. The sample is held between the two reading surfaces by surface tension, enabling the spectrum to be measured. The measurement result is given in ng/µL. It also provides information such as absorbance, and the 260:280 and 260:230 nanometre ratios, which reflect sample contamination by proteins and other compounds respectively.

2.2.3 PCR amplification and CE-SSCP analysis

The ITS region of ribosomal DNA was amplified with ITS 1 and ITS 2 primers. The ITS 2 primer was labelled at 5’ with 6-carboxyfluoresceine (FAM) to analyse the PCR products by SSCP.

Two types of amplification were carried out: Either in a total volume of 15 µL, comprising 3 µL of 5X buffer, 1.17 µL of $MgCl_2$ at 25 mM, 1.2 µL of dNTP at 2.5 mM, 1µL of each of the primers at 10 µM, 0.75 Units of GoTaq Polymerase, 6.48 µL of sterile water and 1µL of DNA. Or in a volume of 50 µL, comprising 10 µL of 5X buffer, 4 µL of $MgCl_2$ at 25 mM, 4 µL of dNTPs at 2.5 mM, 1 µL of each of the primers at 10 µM, 2µL of DMSO, 25.8 µL of sterile water, 1 Unit of GoTaq Polymerase and 2 µL of DNA.

There then followed an amplification programme starting with three minutes of initial denaturation at 94°C, then 35 denaturation cycles at 95°C for one minute, hybridization at 60°C for one minute and elongation at 72°C for one minute. Amplification ended with ten minutes of final elongation at 72°C.

Amplification quality was then checked by electrophoretic transfer to 8% agarose gel at 100 V for one hour and immersion in BET for twenty minutes to reveal the DNA.

2.2.4 Statistical analysis

The data collected in this way were standardized, i.e. all the values for a sample were divided by the mean of that sample. We then produced the profiles and determined the OTUs. Thirty-one OTUs were characterized, considering all the peaks with fluorescence intensity over 2. A table representing the different samples and OTUs was obtained.

The table was transposed and we exploited the values using StatBox 6.6 software to carry out the principal components analysis. We thus obtained all the data needed to make use of the results (correlation circle, distribution of individuals, etc.)

2.3 DHPLC analysis conditions, fraction collection and sequencing of amplicons

Denaturing High Pressure Liquid Chromatography assay was developed to separate the ITS1 rDNA PCR amplified gene fragments (Transgenomic, Ohama, NE). The amplicons from peak fractions were collected and re-amplified as a template with the same primer pair (ITS1/ITS2). Studies conducted with ITS1 and ITS2 showed that are generally sufficient for characterizing strains on a genus and species (Zaremski et *al*.2005).

Mass sequencing was performed by capillary electrophoresis using a ABI 3130 capillary sequencer (Applied Biosystems). Sequencing was realized in both direction to assure fidelity (Bigdye Terminator V3.1).

Sequence analysis consisted of a manual trimming of low quality ends followed by a revision of each nucleotide with DNA baser software (HeracleSoftware v2.6, Germany). Sequence comparison was based on the results of BLAST (Basic Local Alignment Search Tool) searches for homology comparison with known sequences in GenBank. Only cases where analyses yielded the same hit were taken into account (96 to 100% similarity existing species were retrieved from the database).

3 RESULTS AND DISCUSSION

3.1 Profiling fungal community of wood rot

The optimised conditions in PCR amplification and DHPLC analysis allowed the analysis of fungal populations from decaying-wood samples.

Figure 3 represents the elution profiles of two samples (#12 and #13), which shows differences in elution time and relative intensities related to different peaks.

Normally, each peak corresponds to a species, but many peaks were splitted in two separated peaks due to amplification cycle too important.

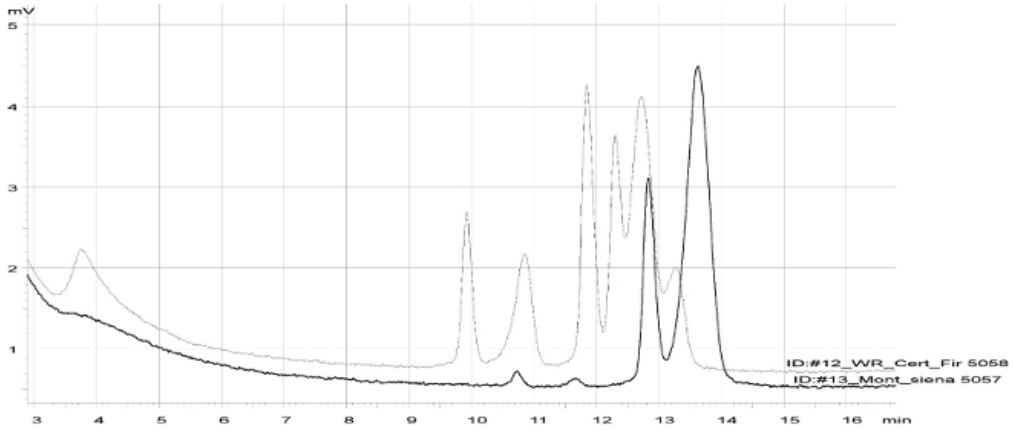

Fig. 3: DHPLC fingerprints of sample 12 (grey) and 13 (black). All the peaks obtained were collected, followed by amplification and sequencing of the ITS1 rDNA.

3.2 Fungal identification by sequencing of collected fractions

Blast results have identified different species belonging to the basidiomycetes, particulary, known fungi to be responsible for wood decay. For a same sample, fungal associations or succession were described for the first time. Generally, wood decay symptôms (brown, soft and white -rot) were associated to a single infestation by a fungus. In sample 16, white rotting fungus *Trametes* was associated with *Oidiodendron griseum,* a soft rotting fungus.

4 SUMMARY

Therefore, it is important to characterize the type of biodeterioration in order to assess the impact on the state of conservation of a wooden object. The setting of molecular markers involved *in situ* molecular detection of some white-rot and brown-rot basidiomycetes infecting historic buildings in Italy will be the next step.

LITERATURE

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