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Characterization of microbial communities in pest colonized books by molecular biology tools

Abstract - This work presents the identification of bacteria and fungi colonies in insect infesting books, by cultural-independent methodologies based on molecular biology techniques. Microbial genomic DNA extraction, *in vitro* amplification of specific target sequences by polymerase chain reactions (PCR), sequencing and sequence analysis were performed. These procedures minimized the samples amount, optimized the diagnostic studies on bacteria and fungi colonization and allowed the identification of many species also in complex microbial consortia. The molecular techniques for sure accomplish and integrate the microbiological standard methods (*in vitro* culture) and morphological analyses (OM, SEM, CLSM), in order to understand the role of microorganisms in bio-deterioration of cultural assets. This monitoring is also indispensable to shed light on the risk for visitors and/or professionals to contract potential illnesses within indoor environments.

Key words: genomic DNA, paper biodeterioration, PCR, termites infestation.

INTRODUCTION

Insects and microorganisms play a significant role in the deterioration of organic substrates, their distribution and development is closely related to environmental parameters (temperature, relative humidity, water activity) and chemical-physical properties of materials (Camuffo, 1998; Valentin, 2003). Particular environments, such as museum, archives, libraries, basements are very often colonized by insects and microorganisms due to the favorable micro-climate, characterized by water condensation phenomena and poor air exchange (Singh *et al*., 1995; Gallo *et al*., 1999; Else *et al*., 2003; Valentin, 2003). There is a huge literature on insects and microbial communities related to biodegradation of historic collections made by organic materials. Specifically, insects as Thysanura (Lepismatidae), Isoptera (Rhinotermitidae, Kalotermidae), Coleoptera (Anobiidae, Cerambycidae, Lictidae, Dermestidae, Ptnidae), bacteria and/or fungal species belonging respectively to *Bacillus*, *Micrococcus*, *Streptomyces*, *Actinomycetes*, and/or *Penicillum*, *Cladosporium*, *Aspergillus*, *Alternaria*, *Trichoderma*, *Rhizopus* have been reported (Florian, 1994; Rust *et al.*, 1996; Valentin, 2003), but many others have still to be identified.

In this study a wide microbial colonization was revealed on books pages, must

likely correlated to the termite infestation (*Reticulitermes lucifugus* Rossi) recognized not only on the collection but even in the wooden cabinets where the books were stored (Not & Guarneri, 2003). Since termites metabolic products (TMP) represent an excellent substrate for microbial growth, we hypothesize a close relationship between the TMP and the complexity of microbial consortia and consequently the clear biodeterioration of books. Moreover by trophallaxis termites can transfer symbiotic bacteria and protozoa in the surrounding environment, where are also able to release chemical compounds that causes physiological changes and behavior in other individuals, usually of the same species (Chiappini *et al*., 2001). As previously described we applied different molecular technologies for the characterization of bacteria and cyanobacteria colonizing cultural assets (Gargano *et al.*, 2009; Palla *et al*., 2010). Results described the identification of bacterial species onto organic substrates.

MATERIALS AND METHODS

Sampling

Sampling on the surfaces of books pages was performed by fragments (20 x 40 mm) of sterile Nylon membrane (Hybond membrane, *Amersham-Biosciences*), as described by Palla *et al.* (2006). This procedure allowed us to isolate single bacterial and fungal colonies.

Colonizing biocenosis were analyzed by Scanning Electron Microscopy and bacteria through molecular protocols.

Microbial DNA extraction

Bacteria colonies isolated on nutrient agar: each colony was dissolved in 20 µl of 1X T.E. (10 mM TRIS-HCl pH 7,5 / 1 mM EDTA) and lysed at 94°C per 2 min.

Aliquots (0.2 g) were utilized for DNA extraction by using QIAamp DNA stool kit (Qiagen); the yield was approximately 500 ng DNA per gram of sample.

In vitro amplification of bacteria target sequences (PCR reactions)

The ribosomal intergenic spacer was performed the polymerose chain reaction (ITS-PCR) using the oligonucleotides ITS1 = 5'-gTCgTAACAAggTAgCCgTA-3' and ITS2 = 5'-gCCAAggCATCCACC-3', as primers (Cardinale *et al*., 2004). Genomic DNA extracted from single bacterial colonies (2 µl) or directly from TMP (100 ng) was utilized as template in each reaction. Reaction mixture (up to 50 µl) consisted of: 0.5 Units of Taq DNA Polymerase (Invitrogen) in $MgCl_2$ and salt conditions suggested by the company. The target sequence was amplified for 35 cycles as follows: denaturation for 1 min at 94°C; annealing for 1 min at 50°C; extension for 1 min at 72°C. A final extension step (7 min at 72°C) was added in order to allow the full-elongation of PCR products.

PCR reaction products were resolved by electrophoresis on 2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen).

Sequencing of ITS amplified fragments

ITS-PCR products were purified by Qia-quick PCR purification kit (Qiagen) and sequenced by MWG-Biotech Custom Sequencing Services (http://www.mwg-biotech.com). The research for homology (16S-23S-ITS rDNA) was performed by nucleotide-nucleotide BLAST analyzer (Altschul *et al*., 1997; Pearson *et al*., 1988).

Scanning Electron Microscopy

Samples from books or termites metabolic products (TMP) were coated with gold particles (thickness ≈ 13 nm) by Agar-Auto-Spotter-Coater (B7341) and observed under high vacuum Leica Cambridge-Leo 420.

RESULTS

Fig. 1 - Fragment of wood cabinet infested by *R. lucifugus*.

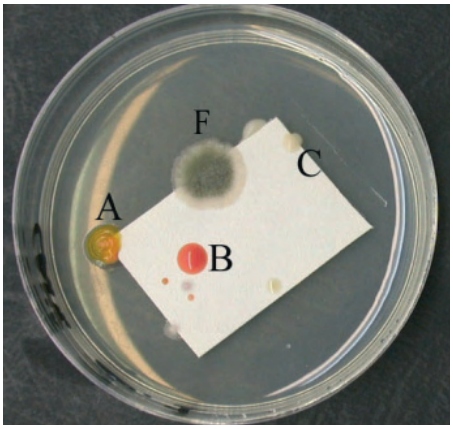

Fig. 2 - Bacteria colonies (A, B, C) growth on Nylon membrane fragments on Nutrien agar plate, after incubation at 30°C per 24 hours. A single fungal colony (F) is also recognizable.

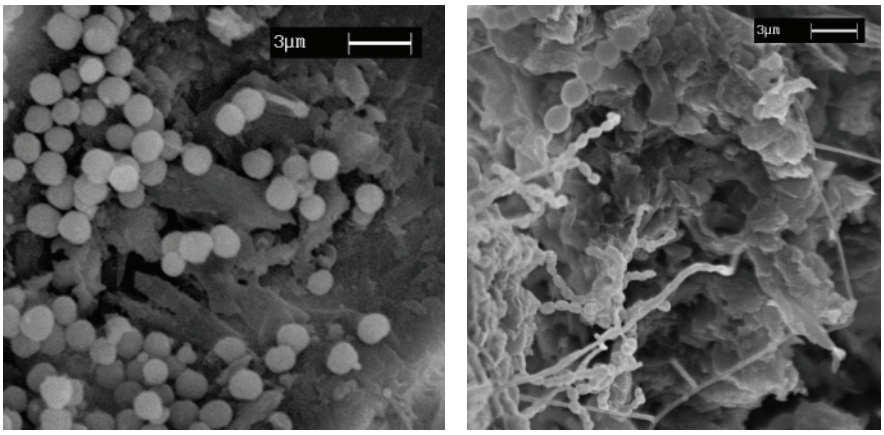

Fig. 3 - SEM micrographs of book fragments colonized by bacteria (left) and actinomyces (right).

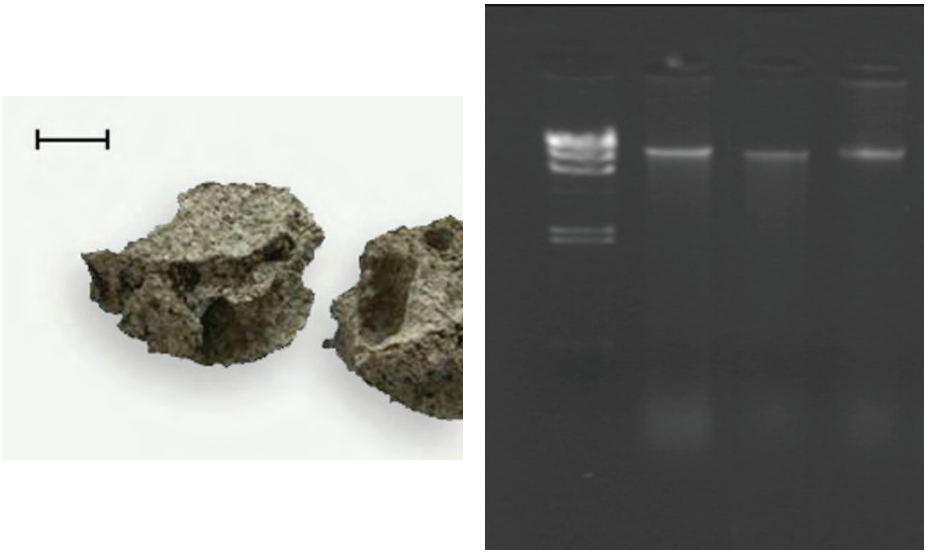

Fig. 4 - Fragments of Termites Metabolic Products (left) utilized for the extraction of microbial DNA (right), analysed on 0.8% agarose gel, stained by SYBR safe (Invitrogen). M = λ Hind III DNA (molecular weight marker). Bar = 0.5 cm.

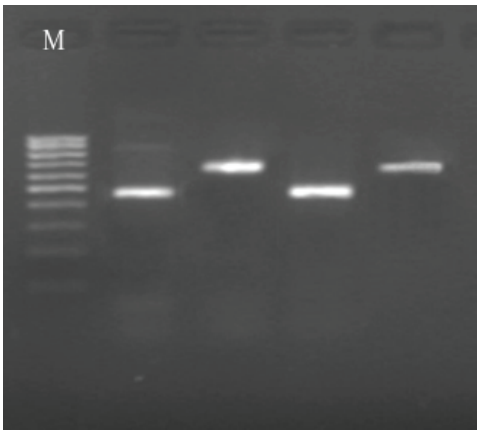

Fig. 5 - Electrophoretic analysis of amplified ITS (16S-23S) bacterial region. M= 100 bp DNA Ladder (Promega).

Biodeterioration by termites *R. lucifugus* was clearly shown in both wood cabinet (Fig. 1) and in most of the books where a noticeable microbial colonization was also identified by a multidisciplinary approach. Bacteria and fungi colonies were revealed by *in vitro* culture (Nutrien agar, Difco) incubating the Petri dish for 24 hours at 30°C (Fig. 2), and scanning electron microscopy (Fig. 3). Bacterial colonization was characterized by ITS-PCR reactions, using as template the genomic DNA obtained from single colonies or extracted from TMP fragments (Fig. 4). Analysis of ITS-PCR products (Fig. 5) allowed to identify *Arthrobacter nicotianae*, *Micrococcus luteus*, *Kocuria rosea* and *Curtobacterium flaccumfaciens* as predominant species.

DISCUSSION AND CONCLUSION

In this work we applied non-destructive sampling procedures, combining SEM analysis with *in vitro* culture and molecular biology techniques in order to characterize microbial, and particularly bacteria colonizing historic book collections. Furthermore, the study point out the potential relationship between termites infestation and microbial colonization. It is to be underlined that *M. luteus* and *K. rosea* are able to produce lytic enzymes and pigmented compounds (Gallo *et al*., 1999; Valentin, 2003; Palla *et al*., 2007) and the high cellulose degradation activity of *C. flaccumfaciens* (Lednicka *et al*., 2000) represents, for this typologies of organic substrates, an high potential risk of biodegradation. In this paper we set up protocols to revealing the presence of bacterial consortia over books surfaces and o TMP, which represent an extraordinary source for microorganisms.

We want also point out that bacteria and fungi colonizing indoor environment are able to damage artifacts and release toxins detrimental to human health (Peltola *et al.*, 2001; Nilsson *et al*., 2004). Molecular technologies could be used for characterizing these dangerous microbes, in order to establish suitable strategies for conservation and fruition of cultural assets.

Finally, we are setting up molecular protocols for fungal identification by using oligonucleotide primers specific for ITS regions or tubulin gene (Glass *et al*., 1995; Palla *et al.*, 2009).

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