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Molecular analysis of *Fomitiporia mediterranea* isolates from esca-affected grapevines in southern Italy

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Summary. Four *Fomitiporia* isolates representative of a collection of about 300 isolates from esca-affected grapevines in southern Italy (Apulia, Campania and Abruzzo) were examined by molecular methods. The DNA of each isolate was analysed by means of polymerase chain reaction (PCR amplification) using ITS5 and ITS4 primers. An amplification product of 740 bp was obtained from all isolates. The denaturated products had the same migration pattern when analysed by single-strand conformation polymorphism. The PCR fragments that included the ribosomal ITS1-5.8S-ITS2 region were sequenced. In order to ascertain the taxonomic identity of the isolates, the ITS sequences were compared with those of *Fomitiporia mediterranea, F. punctata* and *F. robusta* deposited in the GenBank. The ITS sequences of the isolates were uniform and homologous with those of the type culture of *Fomitiporia mediterranea* M. Fischer, to which the southern Italian isolates were compared.

Key words: *Fomitiporia punctata, Fomitiporia robusta*, ITS sequences, wood decay.

Introduction

Although it is considered to be a complex disease also involving tracheiphilous fungi (species of *Phaeoacremonium* and *Phaeomoniella*), esca of grapevine (*Vitis vinifera* L.) has been primarily associated with the colonization and decay of the woody tissue by wood-decaying fungi (Graniti *et al*., 2000). And it is true that the trunk or the main branches of grapevines showing esca symptoms often harbour portions of decayed wood in the form of spongy, yellowish masses (white rot). Several species of basidiomycetes have been isolated from this decayed wood and presumed to be associated with the disease. For a long time, two lignicolous fungi, *Phellinus igniarius* (L.) Quél. and, to a lesser extent, *Stereum hirsutum* (Willd.) Gray (1938), were thus thought to cause esca (Galet, 1977; Reisenzein *et al*., 2000). In recent years, however, several studies made clear that *S. hirsutum* has only a negligible or no role at all in esca, and that the mycelial isolates from esca-affected vines, identified as *P. igniarius*, were, in fact, misidentifications of *Fomitiporia punctata* (P. Karst.) Murrill (Mugnai *et al.*, 1999; Cortesi *et al.*, 2000). However, molecular, cultural and genetic studies of a collection of 13 fungal isolates colonising grapevine in central and northern Italy and in Germany, and identified as *F. punctata,* showed that these strains

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differed from those living on other hosts and in other areas. Consequently, a new species, *Fomitiporia mediterranea* M. Fischer was established (Fischer, 2002) and presumed to be widespread, in the Mediterranean area (Fischer and Kassemeyer, 2003).

Actually, the fruiting bodies of *F. mediterranea* are relatively rare on the trunks or branches of grapevines (Fig. 1) and a timely diagnostic characterization of the fungus on the basis of morphological features is not easily performed. At present, reliable identification of the species causing wood decay of grapevines, apart from some characteristics of the mycelium in culture, has to rely mostly on molecular diagnosis (Fischer, 2002).

The aim of the present study was to investigate whether *F. mediterranea* is the main wood-decaying fungus in some vine-growing areas of southern Italy and to set up a protocol for the molecular diagnosis of this fungus.

Materials and methods

Fomitiporia isolates

During the last ten years, in 15 vine-growing areas of southern Italy (Abruzzo, Campania and Apulia) about 300 fungal isolates belonging to the genus *Fomitiporia* were isolated from the decayed wood of grapevines showing symptoms of esca and, occasionally, from *Fomitiporia* carpophores found on the trunks of old vines. On the basis of prodromic naked-eye observations, these isolates were grouped into four phenetic classes according to the characteristics of the colonies in culture (type and speed of mycelial growth, pigmentation, etc.).

Perceived characters were striking enough not to require additional analytic procedures such as image analysis or colorimetric measurements. Four sample isolates, each representing one of the above classes, were selected and subjected to molecular analysis taking into account the procedure described by Fischer (2002).

DNA extraction

Total DNA was extracted, following Aljanabi and Martinez (1997), from 50–100 mg of mycelium of each fungal isolate and resuspended in 300 μl of TE buffer pH 8 (Tris-HCl 10 mM, 1 mM EDTA). The extracted DNA was analysed by electrophoresis in 1.5% agarose gel in TAE running buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8.0) and viewed using the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, CA, USA).

The concentration of DNA was determined by comparison with the molecular weight marker 1 kb ladder (Invitrogen, Carlsbad, CA, USA), using the Quantity One Software (Bio-Rad Laboratories).

PCR (Polymerase Chain Reaction)

Primers ITS5 and ITS4 (White *et al*., 1990) were used for amplifying the ITS1 (Internal Transcribed Spacer) and ITS2 regions, and the RNA 5.8S encoding gene. Reactions were performed in a volume of 100 μl containing sterile bidistilled water, 10 μl of 10× buffer, 200 μM of each of the nucleotides dATP, dGTP, dCTP, dTTP, 0.5 mM of each primer, 4 Units of Red Taq DNA polymerase (Sigma, Missouri, USA) and about 10 ng of template DNA. A negative control with sterile bidistilled water was carried out for each PCR. A thermalcycler (PCR-Express Hybaid Ltd., Middlesex, UK) was used with the following program: 1 cycle of 2 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C; a final step of 7 min at 72°C. Amplification products were analysed by electrophoresis at 5 V cm^{-1} in 1.5% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 μg μl^{-1}. The molecular weight of the fragments was determined using the 1kb DNA ladder.

Single-strand conformation polymorphism

For the single-strand conformation polymorphism (SSCP) analysis, 1 μl of PCR product was mixed with 9 μl of 95% formamide, 20 mM EDTA and 0.05% bromophenol blue, denatured for 10 min at 99°C and quickly chilled on ice. Different conditions were tested to optimize the migration of single-stranded DNA fragments (Glava and Dean, 1993). Denatured products were analysed by electrophoresis at 300 V at 4°C for 4 h, in 12% poly-acrylamide non-denaturing gel, with an acrylamide/bis-acrylamide ratio of 37.5:1, in TBE running buffer (89 mM Tris-borate) using a Mini-Protean 3 Electrophoresis Cell (Bio-Rad Laboratories). Single-stranded DNA fragments were dyed with silver staining (Amersham, Uppsala, Sweden).

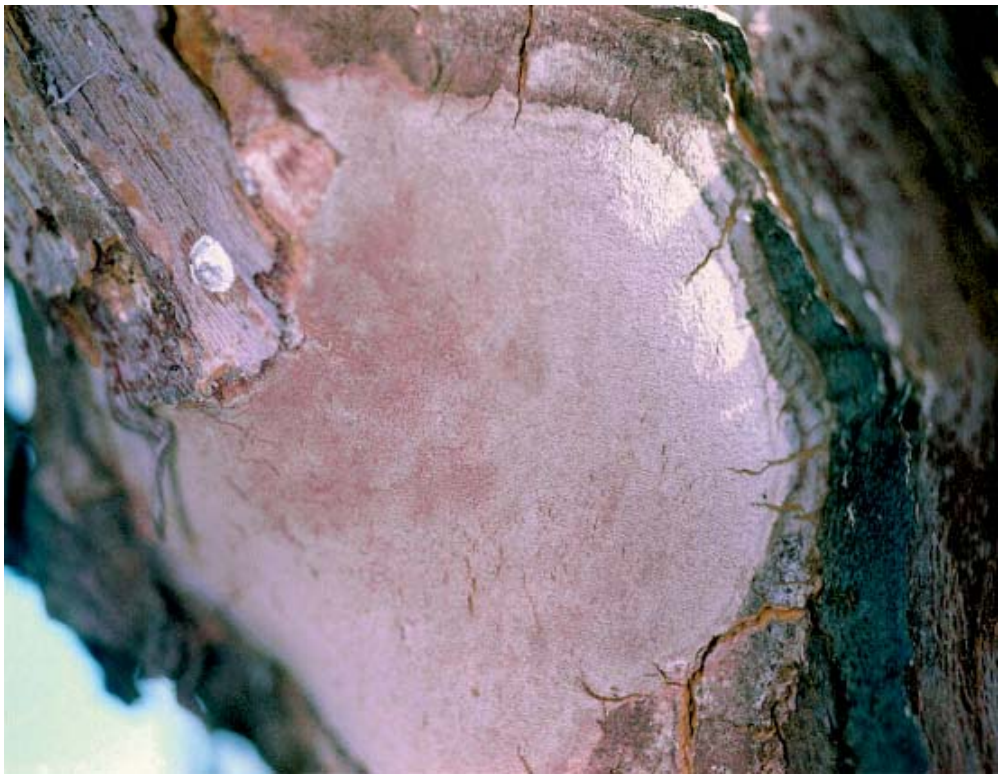

Fig. 1. Fruiting body of *Fomitiporia mediterranea* on *Vitis vinifera* cv. Montepulciano.

Sequencing

The PCR products of the 4 isolates were purified by Mini Elute columns (Qiagen, Hilden, Germany) and sequenced in both directions.

Automatic sequencing was performed by BMR, University of Padua (http://bmr.cribi.unipd.it), using the Big Dye terminator method and a genetic Analyzer ABI Prism 3100 (Applied Biosystems).

Chromas software was used to elaborate the sequences, the BLAST program (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) to compare the sequences and the CLUSTAL W program (www.ebi.ac.uk/clustalw) for multiple alignments.

Results

A fragment of 740 bp was produced by PCR for all samples. No difference was found between samples by SSCP analysis; all fragments were almost uniform, showing a nearly identical migration pattern (Fig. 2).

The ITS1, ITS2 and 5.8S sequences of the four *Fomitiporia* samples under study were compared by alignment with the following sequences deposited in GenBank by Fischer (2002): *Fomitiporia mediterranea* (AF515578), *F. punctata* (AF515563) and *F. robusta* (AF515560).

The ITS1 region of the samples neither contained the typical insertion of *F. robusta* (between nucleotides 49 and 56, Fig. 3A), nor the characteristic deletions of *F. punctata* (between bases 185

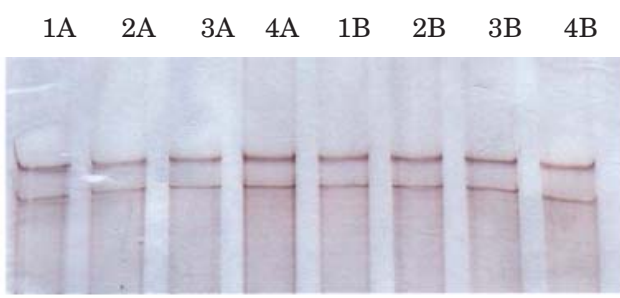

Fig. 2. Single-strand conformation polymorphism (SSCP) profiles of selected *Fomitiporia mediterranea* isolates.

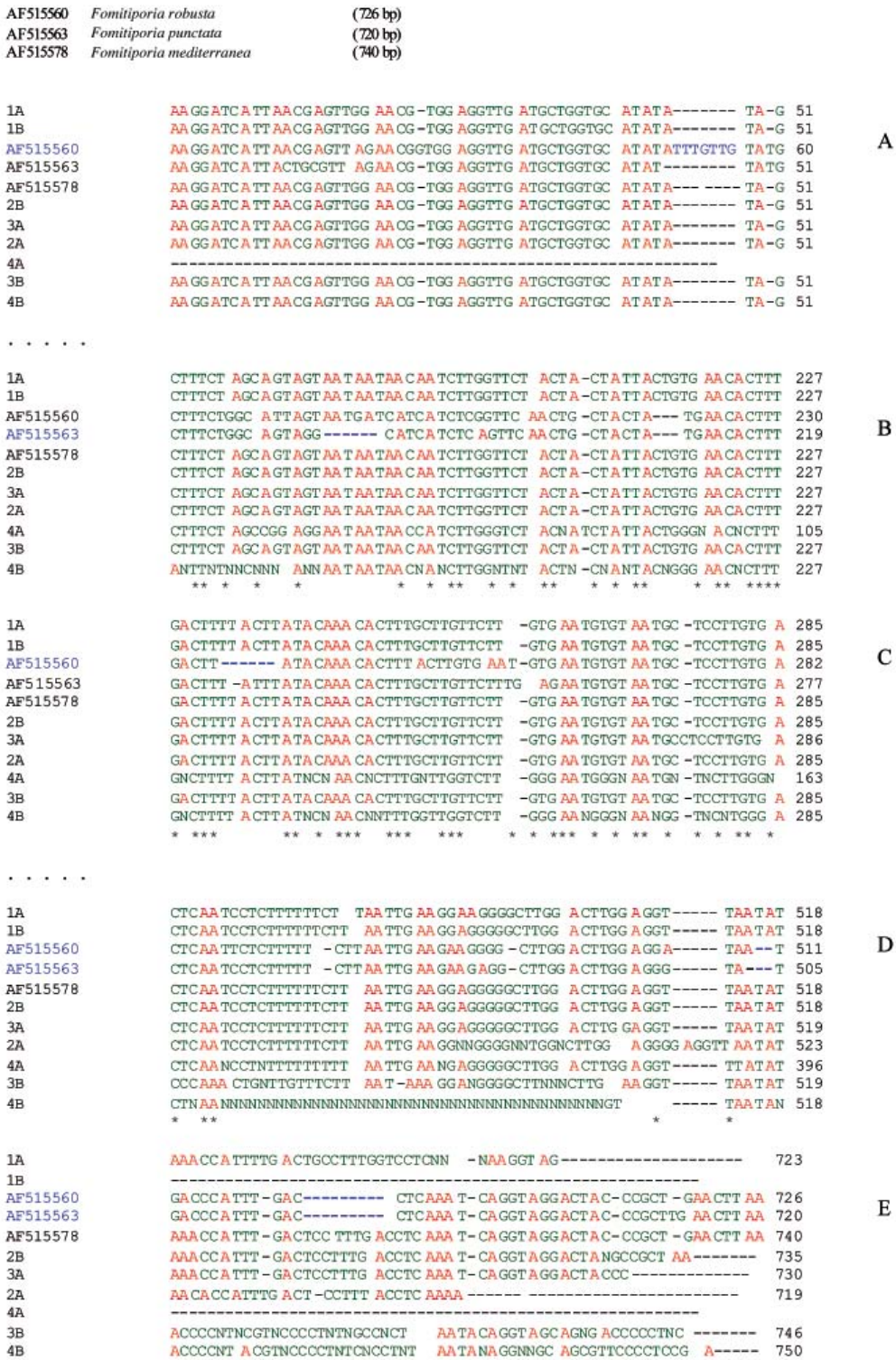

Fig. 3. Allignment of *Fomitiporia mediterranea* ITS 1 and 2 regions with reference sequences deposited in GenBank. Insertions and deletions characteristic of *F. punctata* (AF515563) and *F. robusta* (AF515560) are highlighted in blue.

and 191, Fig. 3B), nor those of *F. robusta* (between nucleotides 235 and 240, Fig. 3C).

The two deletions common to *F. robusta* and *F. punctata* between the bases 508 and 510 and between the nucleotides 694 and 704 in the ITS2 region, were missing in the samples examined (Fig. 3D and 3E). In addition, all samples showed greater homology for *F. mediterranea* (94%) than for *F. robusta* (89%) and *F. punctata* (87%).

Conclusions

Sequencing of the ITS regions indicated that the four *Fomitiporia* isolates obtained from esca-affected grapevines in southern Italy belong to *F. mediterranea*. Since the isolates examined represent a collection of about 300 cultures isolated in different places and seasons over a period of 10 years, it may be assumed that, in the examined area, *F. mediterranea* prevails as the main wood-rot basidiomycete associated with esca of grapevine. Until a reliable diagnosis can be performed with morphological, cultural and biochemical data (work in progress), the protocol for molecular analysis reported here can be used for identification of *Fomitiporia* isolates from grapevines.

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