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...
Molecular analysis of Fomitiporia mediterranea

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 prodroomic 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 MiniProtean 3 Electrophoresis Cell (Bio-Rad Laboratories). Single-stranded DNA fragments were dyed with silver staining (Amersham, Uppsala, Sweden).
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. 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 \textit{F. robusta} (between nucleotides 235 and 240, Fig. 3C).

The two deletions common to \textit{F. robusta} and \textit{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 \textit{F. mediterranea} (94\%) than for \textit{F. robusta} (89\%) and \textit{F. punctata} (87\%).

**Conclusions**

Sequencing of the ITS regions indicated that the four \textit{Fomitiporia} isolates obtained from esca-affected grapevines in southern Italy belong to \textit{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, \textit{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 \textit{Fomitiporia} isolates from grapevines.

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**Literature cited**


