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# Sequence analysis of ITS ribosomal DNA in five *Phaeoacremonium* species and development of a PCR-based assay for the detection of *P. chlamydosporum* and *P. aleophilum* in grapevine tissue

Stefania Tegli, Emanuela Bertelli and Giuseppe Surico

Dipartimento di Biotecnologie Agrarie – Patologia vegetale, Università,
P.le delle Cascine 28, 50144 Firenze, Italy

**Summary.** *Phaeoacremonium aleophilum* and *P. chlamydosporum* are two recently described mitosporic fungi that are involved in the development of symptoms of esca disease and of a decline of young grapevines previously named "black goo". The Internal Transcribed Spacers (ITS) 1 and 2, plus the interveining 5.8S gene, of ribosomal DNA (rDNA) of representative isolates of the two species and, for comparison, of isolates of the congeneric species *P. angustius*, *P. inflatipes* and *P. rubrigenum* were amplified by Polymerase Chain Reaction (PCR) using the ITS4 and ITS5 universal primers. The size of the entire ITS region (ITS1-5.8S-ITS2), plus the 3' end of 18S rDNA and the 5' end of 28S rDNA, was estimated to be about 620 bp, on gel electrophoresis, for all the *Phaeacremonium* species tested. Eleven restriction enzymes were used singularly in the digestion of the ITS region of 30 isolates of *P. chlamydosporum*, 16 of *P. aleophilum*, 2 of *P. angustius*, 2 of *P. inflatipes* and 1 of *P. rubrigenum*. No length-polymorphism could be detected within species (except for *P. aleophilum*), but there were quite strong differences between species. The PCR products of ITS region of ten representative isolates for the five *Phaeoacremonium* species were sequenced, and the sequences aligned and compared. Two main groups were clearly distinguishable, one formed by *P. chlamydosporum*, and the other by *P. aleophilum*, *P. angustius*, *P. inflatipes* and *P. rubrigenum*, with an homology between the two groups ranging from 64.5% to 66.5%. The sequences of ITS region were used to design two pairs of primers, Pal1N/Pal2 and Pch1/Pch2, each of which was subsequently shown to be specific for the amplification of predicted-size fragments from genomic DNA of *P. aleophilum* and *P. chlamydosporum*, respectively. The identity of the amplified fragments was confirmed by sequencing. The primer pairs were further tested using as template DNA extracted from healthy grapevines and from other fungi commonly isolated from esca-diseased grapevine plants but no amplification was observed. The PCR protocol was shown to be quite sensitive (10 pg of DNA) and able to specifically detect *P. chlamydosporum* and *P. aleophilum* in artificially inoculated grapevine plants.

**Key words:** *Phaeoacremonium*, PCR, rDNA, ITS, molecular diagnosis.

## Introduction

Apart from the known pathogenic role of *Phaeoacremonium chlamydosporum* (*Pch*) W. Gams, Crous, M.J. Wingf. & L. Mugnai (Crous *et al.*, 1996), *P. aleophilum* (*Pal*) W. Gams, Crous, M.J. Wingf. & L. Mugnai (Crous *et al.*, 1996) in both esca and Petri grapevine decline ("black goo"), very little is known about the epidemiology of these fungi including the sources of inoculum and their ways of spreading. However, on the basis of the biology of *Phaeoacremonium* spp. (Crous *et al.*, 1996) and some recent findings (Larignon, 1999), it has been supposed that the inoculum of both fungi is produced: i) during saprophytic growth on the residues of grapevine plants; ii) in the soil and/or, iii) on the bark of stand-

Corresponding author: S. Tegli
Fax: + 39 055 354786
E-mail: tegli@ipaf.fi.cnr.it

ing vines. Alternatively, these fungi could well be already present in the propagation material, in mother-plants, in scions or rootstock, or in rooted cuttings, which become infected during preparation in the nursery (Morton, 1995, 1997; Scheck *et al.*, 1998). *Pch* and *Pal* have been isolated from rooted vine cuttings and from shoots of both esca-diseased and healthy-looking vines (Bertelli *et al.*, 1998; Surico *et al.*, 1998; Larignon, 1999). Latent infections may thus facilitate the spread of *Pch* and *Pal* before they can be detected by cultural methods, which have another important limitation: it may take *Pch* as much as two months to grow out of infected wood chips in Petri dishes and spread to the agar medium (Mugnai *et al.*, 1996, 1999).

In this context and since there are at present no chemical treatments, safe for men and the environment, against esca and grapevine decline, there is evidently a need for a rapid, sensitive and specific test to detect *Pch* and *Pal* in grapevine propagation material. In this study we examine the use of the Polymerase Chain Reaction (PCR) technique not only to detect *Pch* and *Pal* in infected grapevine plants and rooted vine cuttings, but also to distinguish between individual *Phaeoacremonium* spp. We used the Internal Transcribed Spacers (ITS) of the ribosomal RNA (rRNA) genes to design PCR primers for the specific identification of esca and grapevine-decline pathogens in infected vine tissue. Diagnostic molecular methods are rapid, specific and sensitive (Hu *et al.*, 1993; Coelho *et al.*, 1997; Lacourt and Duncan, 1997; Mesquita *et al.*, 1998; Murillo *et al.*, 1998; Nicholson *et al.*, 1998; Voigt *et al.*, 1998), and ITS are an attractive target for the development of PCR-based assays (Nazar *et al.*, 1991; Brown *et al.*, 1993; Bonants *et al.*, 1997; Faggian *et al.*, 1998; Lindqvist *et al.*, 1998; Kim *et al.*, 1999). The PCR detection of *Pch* and *Pal* would be especially useful in rooted cutting tests, where speed and sensitivity of the procedure are essential.

## Materials and methods

### Fungal isolates and DNA extraction

A total of 46 isolates, 16 of *Pal* and 30 of *Pch*, were analysed (Table 1). Forty-three isolates came from various Italian regions and were identified in our laboratory. The remaining 3 (1 *Pal* and 2 *Pch*) came from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. Isolates of *P. angustius* (*Pang*), *P. inflatipes* (*Pinf*) and *P. rubrigenum* (*Prub*) (from CBS) were used for comparison. Growth conditions and extraction of fungal DNA were described in Tegli *et al.* (this issue).

### PCR amplification of ITS region

The universal primers ITS4 and ITS5 (White *et al.*, 1990), whose sequences are shown in Table 2, were used to amplify the ITS region of nuclear ribosomal DNA (rDNA), containing ITS1, ITS2 and the intervening 5.8 rRNA gene, plus small portions of 18S and 28S rDNA.

The PCR reactions were carried out in a total volume of 25 µl, in thin-walled, 0.5-ml Eppendorf tubes (Alpha Laboratories Ltd., Eastleigh, Hampshire, UK). The reaction mixture contained 10 ng of DNA template, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 2 µM of each primer and 2.5 U Taq DNA Polymerase (Polymed s.r.l., Florence, Italy). Negative controls were included in all PCR amplifications to test for contaminants in the reagents. Amplification was carried out in an automated thermal cycler (Delphy 1000, Oracle Biosystems, MJ Research Inc., Watertown, MA, USA) according to the following programme: an initial denaturation at 95°C for 3 min, after which 30 cycles of denaturation (2 min at 95°C), primer annealing (25 sec at 50°C) and primer extension (2 min at 72°C) were performed. Amplification reactions were conducted at least twice, in two separate experiments, for each fungal isolate.

Aliquots (2.5 µl) of PCR products were analysed by electrophoresis in 1.4% (w:v) agarose gels, with 1xTBE buffer (Sambrook *et al.*, 1989), stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet (UV) light. The length of the DNA fragments was estimated by comparison with a "1 Kb Plus DNA Ladder" (GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA).

### Restriction analysis and sequencing of the ITS region

Approximately 100 ng of the PCR-amplified ITS region were used directly for the digestion with each of the following restriction enzymes, according to the manufacturer's instructions: *Cfo*I, *Dra*I, *Eco*RI, *Hind*III, *Mbo*I, *Msp*I, *Pst*I, *Rsa*I, *Sma*I, *Taq*I, *Xba*I (GIBCO-BRL, Life Technologies). Enzyme-digested PCR products were then analysed on 2.5% agarose gels.

Table 1. Isolate designation, geographic origin and host or substrate of *Phaeoacremonium* spp. used in this study.[a]

| Species | Isolate[b] | Origin[c] | Host or substrate |
|---|---|---|---|
| *P. aleophilum* | CBS 246.91[d] | Yugoslavia | *Vitis vinifera* |
| | 999.95 | Abruzzi (I) | *Vitis vinifera* |
| | 1002.95 | Abruzzi (I) | *Vitis vinifera* |
| | 1004.95 | Abruzzi (I) | *Vitis vinifera* |
| | 403.Z.95 | Friuli (I) | *Vitis vinifera* |
| | 98.L.95 | Lombardy (I) | *Vitis vinifera* |
| | 93.I.95 | Lombardy (I) | *Vitis vinifera* |
| | Natt.3.95 | Sicily (I) | *Vitis vinifera* |
| | 157 | Tuscany (I) | *Vitis vinifera* |
| | 405.Y.95 | Tuscany (I) | *Vitis vinifera* |
| | 444.J.95 | Tuscany (I) | *Vitis vinifera* |
| | 445.J.95 | Tuscany (I) | *Vitis vinifera* |
| | 321.T2.95 | Apulia (I) | *Vitis vinifera* |
| | 329.U2.95 | Apulia (I) | *Vitis vinifera* |
| | 334.T2.95 | Apulia (I) | *Vitis vinifera* |
| | 334.V1.95 | Apulia (I) | *Vitis vinifera* |
| *P. angustius* | CBS 249.95[d] | California | Vitis vinifera |
| | CBS 777.83 | Buenos Aires (Argentina) | Soil |
| *P. inflatipes* | CBS 391.71[d] | Texas | *Quercus virginiana* |
| | CBS 222.95 | California | *Vitis vinifera* |
| *P. rubrigenum* | CBS 498.94[d] | Bethesda, MD | Human |
| *P. chlamydosporum* | CBS 161.90 | Cape Province (RSA) | *Vitis vinifera* |
| | CBS 239.74 | California | *Vitis vinifera* |
| | 989.95 | Abruzzi (I) | *Vitis vinifera* |
| | 991.95 | Abruzzi (I) | *Vitis vinifera* |
| | 1000.95 | Abruzzi (I) | *Vitis vinifera* |
| | 113.I.95 | Lombardy (I) | *Vitis vinifera* |
| | 229.I.95 | Lombardy (I) | *Vitis vinifera* |
| | 283.U4.95 | Apulia (I) | *Vitis vinifera* |
| | 324.R5a.95 | Apulia (I) | *Vitis vinifera* |
| | 325.R7b.95 | Apulia (I) | *Vitis vinifera* |
| | 330.U2.95 | Apulia (I) | *Vitis vinifera* |
| | 1121.95 | Sicily (I) | *Vitis vinifera* |
| | 1122.95 | Sicily (I) | *Vitis vinifera* |
| | 56 (CBS 229.95)[d] | Tuscany (I) | *Vitis vinifera* |
| | 131.F.95 | Tuscany (I) | *Vitis vinifera* |
| | LT3 | Tuscany (I) | *Vitis vinifera* |
| | RM1 | Tuscany (I) | *Vitis vinifera* |
| | Bb13 | Tuscany (I) | *Vitis vinifera* |
| | Bb16 | Tuscany (I) | *Vitis vinifera* |
| | Bb27 | Tuscany (I) | *Vitis vinifera* |
| | Bb32 | Tuscany (I) | *Vitis vinifera* |
| | Bb36 | Tuscany (I) | *Vitis vinifera* |
| | 191.N7.95 | Umbria (I) | *Vitis vinifera* |
| | 191.P1.95 | Umbria (I) | *Vitis vinifera* |
| | 209.P1.95 | Umbria (I) | *Vitis vinifera* |
| | 217.N3.95 | Umbria (I) | *Vitis vinifera* |
| | 1091.95 | Veneto (I) | *Vitis vinifera* |
| | 1095.95 | Veneto (I) | *Vitis vinifera* |
| | 1096.95 | Veneto (I) | *Vitis vinifera* |
| | 1101.95 | Veneto (I) | *Vitis vinifera* |

[a] Further characterization of isolates is as reported in Tegli *et al.* (this issue).
[b] CBS: Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands); Italian isolates are from culture collection of Dipartimento di Biotecnologie Agrarie - Patologia vegetale, University of Florence, Italy.
[c] I: Italy; RSA: Republic of South Africa.
[d] Holotype.

The amplified ITS regions of some representative isolates (*Pch* 56, *Pch* CBS 161.90, *Pch* CBS 239.74, *Pal* 157, *Pal* CBS 246.91, *Pang* CBS 249.95, *Pang* CBS 777.83, *Pinf* CBS 222.95, *Pinf* CBS 391.71 and *Prub* CBS 498.94) of the five *Phaeoacremonium* species were purified using Qiaquick-spin purification columns (Qiagen, Chatsworth, CA, USA), according to manufacturer's instructions, and sequenced in the Plant Genome Laboratory, ENEA, Rome, Italy. Both strands were sequenced directly using an ABI PRISM Ready Reaction Dye Terminator Cycle Sequencing kit (Perkin Elmer, Applied Biosystems, Norwalk, CT, USA), with the primers ITS4 and ITS5, on an Applied Biosystems model 373A Automated DNA Sequencer Stretch (Perkin Elmer). Multiple sequence alignments and comparisons were performed using the computer package CLUSTAL W (version 1.7; J.D Thompson, D.G. Higgins, and T.J. Gibson, EMBL, Heidelberg, Germany). Alignments were checked visually and modified manually where necessary. A similarity matrix was obtained by a one parameter-model (Jukes and Cantor, 1969) using TREECON software (version 1.3b) (Van de Peer and De Wachter, 1994). The sequence data were also compared with other sequences available through the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) databases.

**PCR primer design and sensitivity**

Two primer pairs, Pal1N-Pal2 and Pch1-Pch2, targeting the ITS regions of *Pal* and *Pch* respectively, were designed and synthesised (GIBCO-BRL, Life Technologies) (EMBL accession numbers: AJ277781 and AJ277782 for Pal1N and Pal2; AJ277779 and AJ277780 for Pch1 and Pch2). The two primer pairs were tested for the amplification of a fragment of known size, according to the sequences of the rDNA-ITS region of *Pal* and *Pch*. Optimal conditions for specific PCR amplification of *Pal* and *Pch* isolates were determined in a total reaction volume of 25 µl, with 10 ng of DNA as template, and containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 50 mM of each of the four dNTPs, 0.5 mM of each of the two specific primers and 2.5 U Taq DNA Polymerase. A negative control without template was used in each PCR experiment. Amplification was carried out as described above except that the annealing temperature was increased to 64°C (for the primer pair Pal1N-Pal2) or to 62°C (for the primer pair Pch1-Pch2).

Aliquots (2.5 µl) of the PCR products were resolved by electrophoresis in 1.4% agarose gels and visualised as already stated above.

Sensitivity was tested by preparing tenfold serial dilutions of purified *Phaeoacremonium* DNA in water, from 10 ng to 1 pg, and checking for amplification with the established PCR protocol.

**Primer specificity**

The specificity of the primer pairs Pal1N-Pal2 and Pch1-Pch2 was tested by attempting amplification using as template the genomic DNA of some pathogenic fungi that are known or thought to be associated with *Phaeoacremonium* spp. in grapevines affected by esca, Petri grapevine decline or similar disorders: *F. punctata*, *Botryosphaeria obtusa*, *Stereum hirsutum* and *Eutypa lata*. The DNA of these fungi was extracted using the same protocol as that described above for *Phaeoacremonium* species.

The specificity of these primers was also tested using grapevine DNA. The DNA was extracted from interveinal mesophyll and petioles of expanded leaves of healthy-looking grapevine plants of cv. Sangiovese, according to the protocol of Lodhi *et al*. (1994).

Table 2. Details of primers used in this study.

| Primer | Sense | Sequence | Author |
|---|---|---|---|
| ITS5 | Forward | 5'-GGAAGTAAAAGTCGTAACAAGG-3' | White *et al*. (1990) |
| ITS4 | Reverse | 5'-TCCTCCGCTTATTGATATGC-3' | White *et al*. (1990) |
| Pal1N[a] | Forward | 5'-AGGTCGGGGGCCAAC-3' | This study |
| Pal2[a] | Reverse | 5'-AGGTGTAAACTACTGCGC-3' | This study |
| Pch1[a] | Forward | 5'-CTCCAACCCTTTGTTTATC-3' | This study |
| Pch2[a] | Reverse | 5'-TGAAAGTTGATATGGACCC-3' | This study |

[a] EMBL accession numbers: AJ277781 and AJ277782 for Pal1N and Pal2; AJ277779 and AJ277780 for Pch1 and Pch2.

**Detection of *P. aleophilum* and *P. chlamydosporum* in grapevine plants**

Grapevine plants artificially inoculated in 1997 (two years before the present experiment) with *Pch* 56 and *Pal* 157 separately or as a mixture were tested. Wood samples, taken about 30-45 cm above the point of inoculation on the trunk, on the margin of the discoloured wood column caused by the inoculated fungi, were cut into 0.5x0.2 cm segments and transferred to 100-ml Erlenmeyer flasks (0.4 g fresh weight/flask) containing 30 ml potato-dextrose broth (PDB) (Difco Laboratories, Detroit, MI,USA). After incubation (1 to 7 days) at 25°C in the dark on a rotatory shaker (100 rpm), 1.5 ml of the culture medium with fungal growth was harvested and divided into two aliquots of 1.0 and 0.5 ml. The 0.5 ml sample was serially diluted tenfold and plated on MEA (100 ml/plate) to assess fungal growth. The 1-ml sample was centrifuged and the pellet utilised for fungal DNA extraction using the procedure described above.

The DNA extracted from the fungal pellet was used as template in the PCR reactions with primer pairs Pal1N-Pal2 and Pch1-Pch2, performed according to the optimal conditions described above. As positive controls DNA extracted from 1-ml aliquots of sterile culture medium, artificially contaminated with $10^4$ cfu each of *Pch* 56, *Pal* 157 and *Pch* 56 plus *Pal* 157, were used. Aliquots (2.5 µl) of PCR products were analysed on 1.4% agarose gels.

## Results

**PCR amplification of the ITS region**

The ITS4 and ITS5 primers specifically amplified the entire length of the ITS region, including the 5.8S rDNA, plus small portions of the 3' end of 18S and the 5' end of 28S rDNA. Gel electrophoresis of PCR products from *Phaeoacremonium* spp. always yielded a single band approximately 620

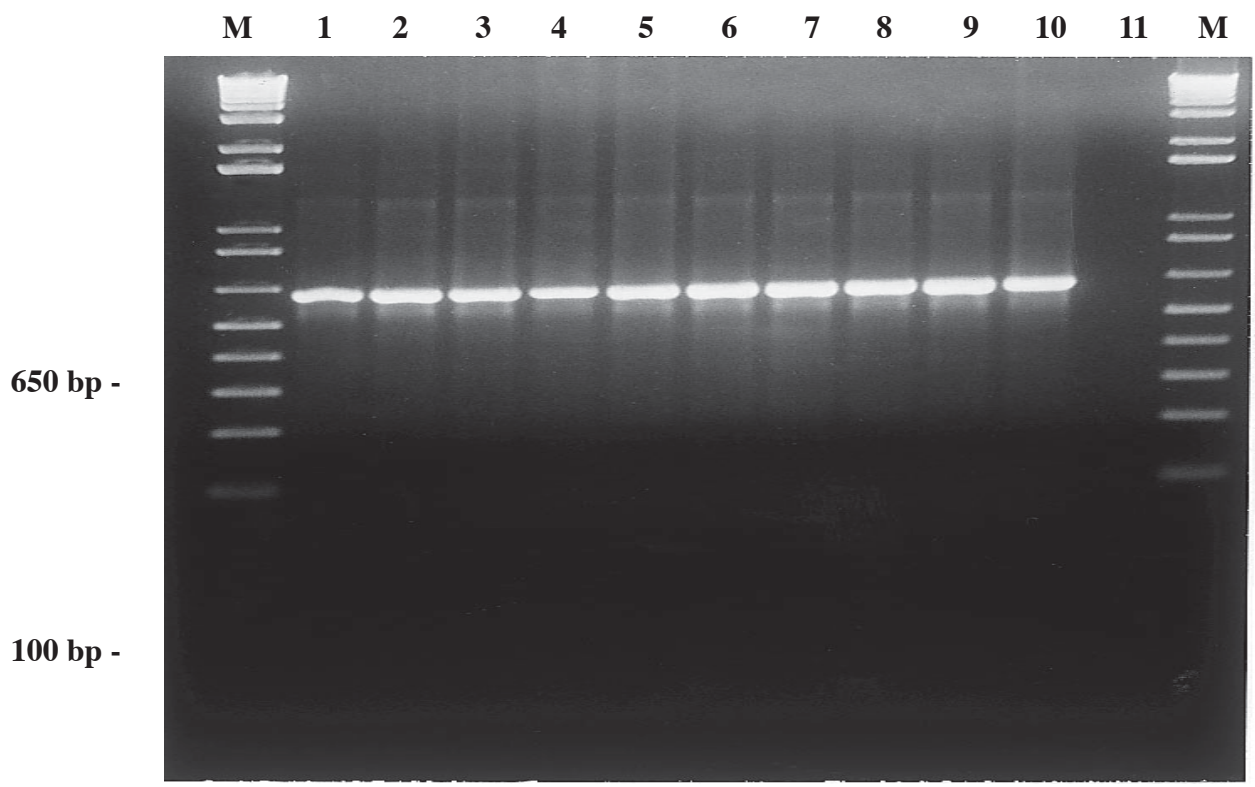


Fig. 1. Agarose gel with PCR amplification products of ITS1-5.8S-ITS2 region of rDNA of *Phaeoacremonium* spp., using ITS4 and ITS5 as primers. Lanes 1-2, *P. aleophilum* CBS 246.91 and 157; lanes 3-5, *P. chlamydosporum* CBS 161.90, CBS 239.74 and 56; lanes 6-7, *P. angustius* CBS 249.95 and CBS 777.83; lanes 8-9, *P. inflatipes* CBS 391.71 and CBS 222.95; lane 10, *P. rubrigenum* CBS 498.94; lane 11, negative control of sterile distilled water; lane M, 1Kb Plus DNA Ladder (Gibco-BRL, Life Technologies). A fragment of about 620 bp is observed with all the samples.

Table 3. Estimated restriction fragments sizes (base pairs) following digestion of ITS region from five *Phaeoacremonium* species with seven restriction enzymes. Fragments which were visible on agarose gel are shown underlined; the remaining fragments are inferred from sequence data for the ITS region (see Fig. 2). Abbreviations: *Pch*, *P. chlamydosporum*; *Pal, P. aleophilum; Pang, P. angustius; Pinf, P. inflatipes; Prub, P. rubrigenum*.

| Enzyme | Restriction fragment size of *Phaeoacremonium* spp. ITS region | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Pch* | *Pal* | | *Pang* | | *Pinf* | | *Prub* |
| | 30 isolates[a] | CBS 246.91 | 15 isolates[a] | CBS 249.95 | CBS 777.83 | CBS 222.95 | CBS 391.71 | CBS 498.94 |
| *Cfo*I | | | | | | | 5 | 5 |
| | | | | | | | 14 | |
| | | | | | | | | 15 |
| | | | | | 19 | | | |
| | | 20 | 20 | 20 | 20 | 20 | | |
| | | | | | | | | 26 |
| | | 43 | 43 | 43 | 43 | 43 | 43 | 43 |
| | | 77 | 77 | 77 | 77 | 77 | 77 | 77 |
| | | | | | | | | 101 |
| | | | | | | | 119 | |
| | | | | | | | 127 | |
| | | 128 | 128 | 128 | 128 | 128 | | |
| | | | | | 141 | | | |
| | | 159 | 159 | 159 | | 159 | | |
| | | | | | | | | 169 |
| | | 191 | 191 | 191 | 191 | 191 | | |
| | | | | | | | | 194 |
| | | | | | | | 241 | |
| | 268 | | | | | | | |
| | 351 | | | | | | | |
| *Eco*RI | | | | | | | 304 | |
| | | | | | 305 | | | 305 |
| | 306 | 306 | 306 | 306 | | 306 | | |
| | | 312 | 312 | 312 | | 312 | | |
| | 313 | | | | | | | |
| | | | | | 314 | | | |
| | | | | | | | 322 | |
| | | | | | | | | 325 |
| *Mbo*I | | | | | 44 | | | |
| | | | | | 45 | | | |
| | | 46 | 46 | 46 | | 46 | 46 | |
| | | | | | | | | 47 |
| | 49 | 49 | 49 | 49 | 49 | 49 | 49 | |
| | | | | | | | | 50 |
| | 75 | | | | | | | |
| | 175 | | | | | | | |
| | | 203 | 203 | 203 | | 203 | | |
| | | | | | 206 | | | |
| | | | | | | | 213 | |
| | | | | | | | | 215 |
| | | | | | 275 | | | |
| | | | | | | | 318 | 318 |
| | 320 | 320 | 320 | 320 | | 320 | | |

(*continued on the next page*)

Table 3. (*continued from the preceding page*)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Msp*I | | | | | | | 9 | |
| | | | | | 10 | | | |
| | | | | | 11 | | | |
| | | 12 | 12 | 12 | 12 | 12 | | |
| | | | | | | | 13 | 13 |
| | | | | | 14 | | | |
| | | | | | | | 16 | |
| | | | | | 17 | | | |
| | | | | | | | 18 | 18 |
| | | 25 | 25 | 25 | | 25 | | |
| | | | | | | | | 26 |
| | | | | | 88 | | | 88 |
| | | | | | 111 | | | |
| | | | | | | | 127 | |
| | | | | | | | | 129 |
| | | 135 | 135 | 135 | | 135 | | |
| | 212 | | | | | | | |
| | | | | | 356 | | | 356 |
| | 407 | | | | | | | |
| | | | | | | | 443 | |
| | | 446 | 446 | 446 | | 446 | | |
| *Rsa*I | | | | | 64 | | | |
| | | | 65 | 65 | | 65 | 65 | |
| | | | | | | | | 66 |
| | | | 138 | 138 | | 138 | | |
| | | | | | 141 | | | |
| | | | | | | | 147 | |
| | | 203 | | | | | | 148 |
| | | | | | | | | |
| | | 415 | | | 414 | | 414 | |
| | | | 415 | 415 | | 415 | | |
| | | | | | | | | 416 |
| *Sma*I | | | | | 112 | | - | |
| | | | | | | | | |
| | | | | | 507 | | | |
| *Taq*I | 53 (2) | | | | | | | |
| | | 55 | 55 | 55 | | 55 | 55 | |
| | 59 | 59 | 59 | 59 | 59 | 59 | | 59 |
| | | | | | | | 61 | |
| | | 213 | 213 | 213 | | 213 | | |
| | 216 | | | | | | | |
| | | | | | | | 223 | |
| | 238 | | | | | | | |
| | | | | | 270 | | | |
| | | | | | | | | 281 |
| | | | | | | | 287 | |
| | | | | | 290 | | | 290 |
| | | 291 | 291 | 291 | | 291 | | |

[a] Isolates are as reported in Table 1.

base pairs (bp) in length (Fig. 1). The size of this fragment was about the same in all *Phaeoacremonium* isolates examined, when estimated on agarose gels. No PCR product was obtained in the negative controls.

**Restriction analysis of the ITS region**

The restriction enzymes *Dra*I, *Hind*III, *Pst*I and *Xba*I failed to digest the amplified ITS region of the 51 isolates belonging to the five *Phaeoacremonium* species used in this study. The endonuclease *Sma*I had only one cutting site on the ITS region of the *Pang* isolate CBS 777.83. The other enzymes (*Cfo*I, *Eco*RI, *Mbo*I, *Rsa*I, *Msp*I and *Taq*I) gave distinct restriction profile types resulting in five patterns each for *Cfo*I, *Mbo*I, *Msp*1, *Rsa*I and *Taq*I and four patterns for *Eco*RI, which substantially confirmed the classification of all isolates but CBS 249.95 and CBS 222.95 (Table 3). These two isolates could not be identified as *Pang* and *Pinf* respectively since they gave the same restriction profiles as the *Pal* isolates. *Pal* isolates showed the same restriction pattern with 5 of the 6 enzymes mentioned above. However, with *Rsa*I, 15 isolates had a pattern of three bands of 415, 138 and 65 bp while only one (CBS 246.91) had a pattern of two bands of 415 and 203 bp (Table 3).

All *Pch* isolates showed the same pattern after digestion with each of the following enzymes: *Cfo*I, *Eco*RI, *Mbo*I, *Msp*I, *Rsa*I and *Taq*I. No *Pch* isolate was digested by *Rsa*I. *Eco*RI was the only restriction enzyme out of 6 that did not easily differentiate *Pch* from *Pal* (Table 3). Isolates CBS 777.83, CBS 391.71 and CBS 498.94, belonging to *Pang, Pinf* and *Prub* respectively, showed banding patterns that differed both between each other and from the *Pch* and *Pal* isolates (Table 3).

**Sequencing of the ITS regions**

The ITS regions of 10 isolates of *Phaeoacremonium* spp. were sequenced: three isolates of *Pch*, two each of *Pal, Pang* and *Pinf* and one of *Prub*. These sequences were in accordance with those already obtained by Dupont *et al.* (1998) and Yan *et al.* (1995) (GenBank accession numbers AF017651, AF017652 and U31843). The aligned sequences are illustrated in Fig. 2 (GenBank accession numbers AF266647, AF266648, AF266649, AF266650, AF266651, AF266652, AF266653, AF266654, AF266655, AF266656). Nucleotide sequences of ITS1-5.8S-ITS2 rDNA permitted identification of the restriction sites of the endonucleases used in this study and confirmed the data obtained by restriction analysis for all the *Phaeoacremonium* species examined. Moreover, more cutting sites were detected than were revealed by the number of fragments of the restriction profiles clearly visible on the gels (shown underlined in Table 3). Any fragments too small to be detected on agarose gels but inferred from sequence data for each *Phaeoacremonium* spp. are shown in Table 3 without underlining.

Particularly important is the one-base mutation located at nucleotide 14 of ITS1 of *Pal* isolate CBS 246.91, and corresponding to nucleotide 36 of the alignments in Fig. 2. Since this mutation was within the cutting sequence of *Rsa*I, identical restriction profiles were obtained with 15 of the 16 isolates belonging to this species: only one isolate, CBS 246.91, differed (Table 3).

On the basis of the sequence data of the ITS region, a similarity matrix was constructed (Table 4). The similarity between isolate 157 and *Pal* isolate CBS 246.91 was about 99.6%, not only because of the one-base mutation at nucleotide 36 mentioned above, but also because of another mutation located at nucleotide 138 (Fig. 2). Surprisingly, isolates *Pang* CBS 249.95 and *Pinf* CBS 222.95 not only had identical sequences, as expected from the restriction analysis, but when these isolates were compared with *Pal* isolate 157 the similarity of the ITS region was still 100%. It decreased to 99.6% only with *Pal* isolate CBS 246.91. When *Pang* isolates CBS 249.95 and CBS 777.83 were compared, the similarity of their ITS regions was 94.6%. The same level of similarity was registered when the two *Pinf* isolates, CBS 222.95 and CBS 391.71, were compared.

As regards *Pch*, all the isolates of this species had identical sequences. The similarity of *Pch* with the other species studied was about 66.5% with *Pinf* CBS 391.71, 66% with *Pal* 157, *Pang* CBS 249.95, *Pinf* CBS 222.95, 65.7% with *Pal* CBS 246.91 and 65.2% with *Pang* CBS 777.83 (Table 4).

*Prub* CBS 498.94 appeared more closely related to either of the two isolate groups of *Pal* than to *Pch*, with similarities in the ITS region of 93.5-91.3% and 64.5% respectively (Table 4).

**Primer design and sensitivity**

On the basis of the sequence data of the ITS

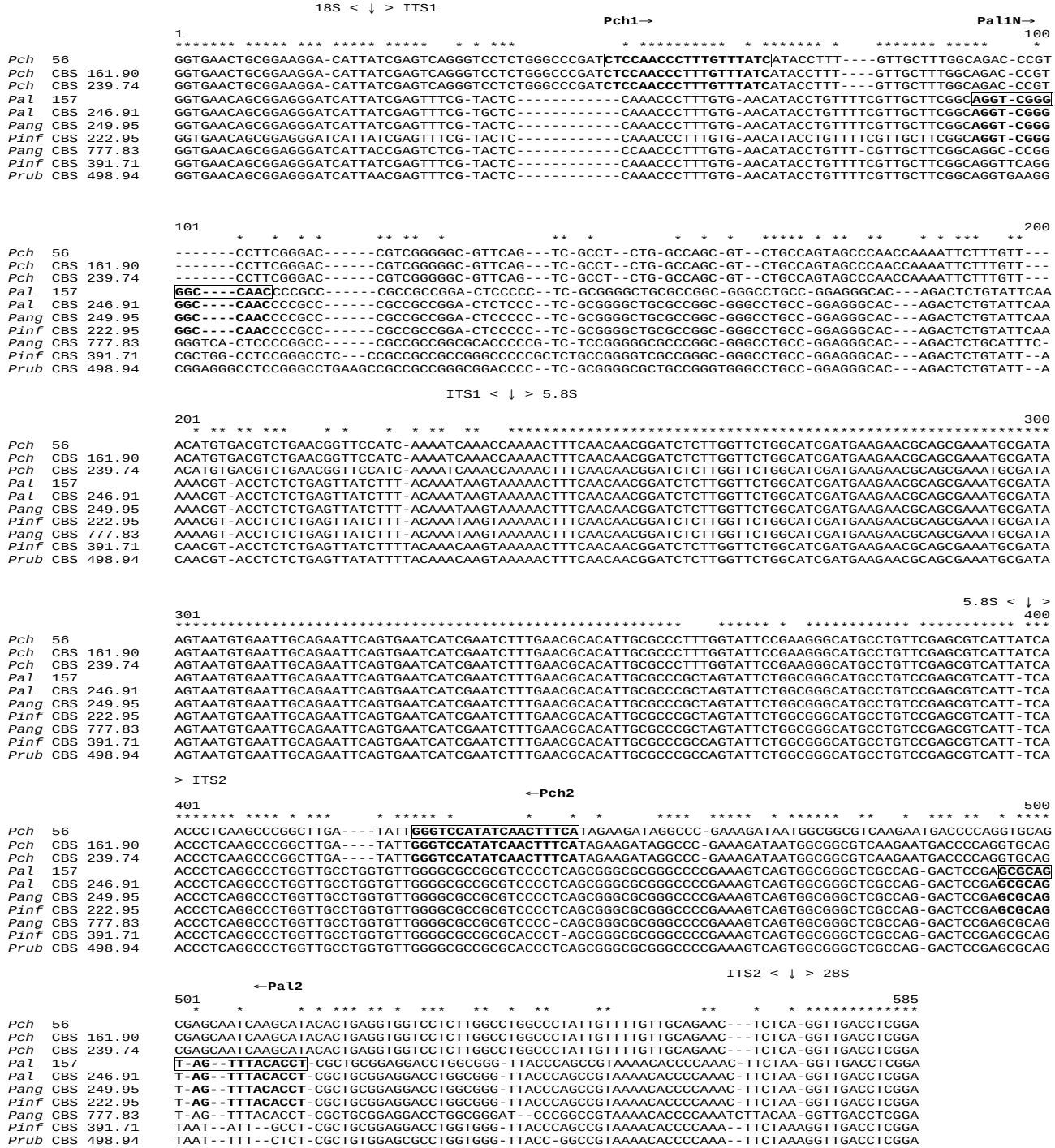


Fig. 2. Multiple alignment ITS1-5.8S-ITS2 sequences, plus small portions of 3' end of 18S and of 5' end of 28S rDNA, of five *Phaeoacremonium* species. The start (>) and the end (<) of the ITS1, ITS2 and 5.8S are indicated, as are the end of the 18S and the start of 28S rDNA. In the aligned sequences, asterisk = match, dash= gap. The localization of the primer pairs Pal1N-Pal2 and Pch1-Pch2 designed for the detection of *P.aleophilum* and *P. chlamydosporum*, respectively, are indicated by large blocks. Bold characters indicate the annealing sites for both the pairs. The sense of each primer is in accordance to the arrow reported above the sequences. Abbreviations: *Pch, P. chlamydosporum*; *Pal, P. aleophilum*; *Pang, P. angustius*; *Pinf, P. inflatipes*; *Prub, P. rubrigenum*.

Table 4. Similarity matrix derived from the sequence data of ITS region in the *Phaeoacremonium* species examined. Abbreviations: *Pch*, *P. chlamydosporum*; *Pal, P. aleophilum; Pang, P. angustius; Pinf, P. inflatipes; Prub, P. rubrigenum*.

| Isolate | *Pch* 56 | *Pch* CBS 161.90 | *Pch* CBS 239.74 | *Pal* 157 | *Pal* CBS 246.91 | *Pang* CBS 249.95 | *Pang* CBS 777.83 | *Pinf* CBS 222.95 | *Pinf* CBS 391.71 | *Prub* CBS 498.94 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Pch* 56 | 100 | | | | | | | | | |
| *Pch* CBS 161.90 | 100 | 100 | | | | | | | | |
| *Pch* CBS 239.74 | 100 | 100 | 100 | | | | | | | |
| *Pal* 157 | 66 | 66 | 66 | 100 | | | | | | |
| *Pal* CBS 246.91 | 65.7 | 65.7 | 65.7 | 99.6 | 100 | | | | | |
| *Pang* CBS 249.95 | 66 | 66 | 66 | 100 | 99.6 | 100 | | | | |
| *Pang* CBS 777.83 | 65.2 | 65.2 | 65.2 | 94.6 | 94.2 | 94.6 | 100 | | | |
| *Pinf* CBS 222.95 | 66 | 66 | 66 | 100 | 99.6 | 100 | 94.6 | 100 | | |
| *Pinf* CBS 391.71 | 66.5 | 66.5 | 66.5 | 94.6 | 94.2 | 94.6 | 93.1 | 94.6 | 100 | |
| *Prub* CBS 498.94 | 64.5 | 64.5 | 64.5 | 93.5 | 93.1 | 93.5 | 91.3 | 93.5 | 94.7 | 100 |

regions, the primer pairs Pal1N-Pal2 and Pch1-Pch2 were designed to amplify specific DNA fragments using genomic DNA from *Pal* and *Pch* isolates respectively. The sequences of these primers are shown in Table 2, and their location in the ITS region in Fig. 2. Primer lengths were 15 bp for Pal1N, 18 bp for Pal2 and 19 bp each for Pch1 and Pch2. The G+C content varied from 73% for Pal1N, to 50% for Pal2 and 42% each for Pch1 and Pch2. To accomodate the molecular characteristics of the primers selected, appropriate PCR conditions were adopted (see Materials and methods).

PCR experiments with the specific primer pairs and using as template genomic DNA produced bands in accordance with those predicted from the sequence analysis for all the *Phaeoacremonium* species (Fig. 3). The primer pair Pal1N-Pal2 specifically amplified a fragment of about 400 bp in all *Pal* isolates tested, as shown for isolates 157 and CBS 246.91 (Fig. 3). The same fragment was amplified using genomic DNA of *Pang* CBS 249.95 and *Pinf* CBS 222.95 as template, because of the 100% homology of their ITS region with that of *Pal*, but no amplification was detected when *Pang* CBS 777.83 and *Pinf* CBS 391.71 were tested. Moreover, the primer pair Pal1N-Pal2 did not amplify any fragment when the genomic DNA of any *Pch* isolate studied or that of *Prub* CBS 498.94 was used as template (Fig. 3).

The Pch1-Pch2 primer pair specifically amplified a fragment of about 360 bp in all *Pch* isolates but did not give rise to any amplification product when other species of *Phaeoacremonium* were tested (Fig. 3).

Fragments amplified with primer pairs Pal1N-Pal2 and Pch1-Pch2 were sequenced and their se-

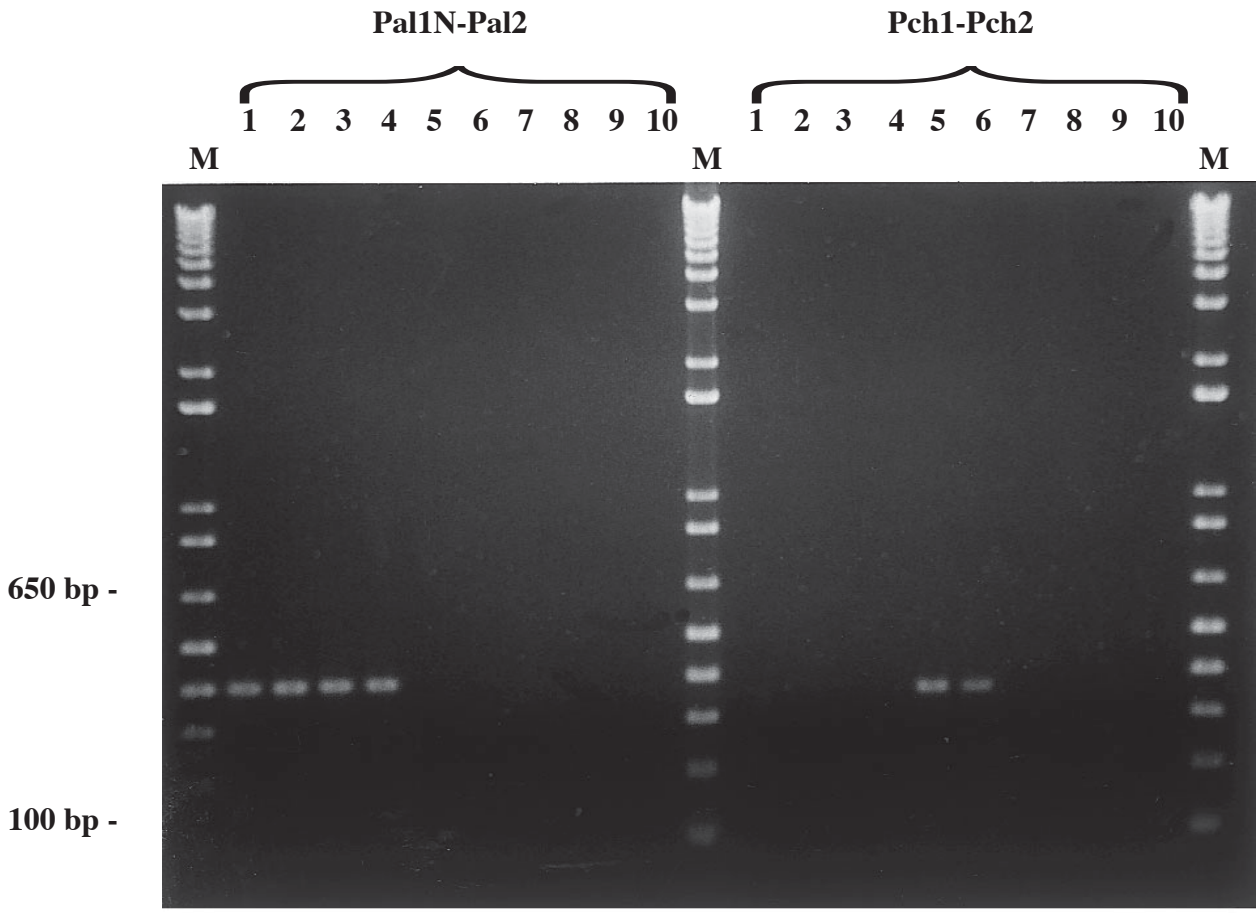


Fig. 3. Agarose gel of the PCR products using primer pairs Pal1N-Pal2 and Pch1-Pch2. Lanes 1-2, *P. aleophilum* 157 and CBS 246.91; lane 3, *P. angustius* CBS 249.95; lane 4, *P. inflatipes* CBS 222.95; lanes 5-6, *P. chlamydosporum* 56 and CBS 161.90; lane 7, *P. angustius* CBS 777.83; lane 8, *P. inflatipes* CBS 391.71; lane 9, *P. rubrigenum* CBS 498.94; lane 10, negative control of sterile distilled water; lane M, 1Kb Plus DNA Ladder (Gibco-BRL, Life Technologies).

quences confirmed the specificity of the amplification. Each primer pair was able to amplify the appropriate fragment from 10 ng down to 10 pg of DNA template (Fig. 4).

**Primer specificity.**

The DNA from vine plants gave no amplification products with either primer pair Pal1N-Pal2 or Pch1-Pch2 (Fig. 5, lanes 2 and 9) nor were PCR products obtained with genomic DNA from *B. obtusa*, *E. lata*, *F. punctata* and *S. hirsutum* (Fig. 5, lanes 3 to 6 and 10 to 13, respectively).

**Detection of *P. aleophilum* and *P. chlamydosporum* in grapevine wood**

Wood chips from vine artificially inoculated with *Pch* and/or *Pal* were colonised by a number of other fungal organisms, but *Pch* and/or *Pal* were the most prevalent.

Two-day-old fungal cultures plated on MEA yielded $10^4$ cfu/ml of *Pch* and/or *Pal* and the fungal mass increased slightly with time. Other fungi grown on MEA after inoculation with 0.5-ml samples of culture medium with fungal growth from wood chips were *B. obtusa*, *Alternaria* spp., *Gliocladium* spp., *Fusarium* spp.

Products of 360 and 400 bp, identical in size to those amplified from *Pch* and *Pal* DNA, were produced when primer pairs Pch1-Pch2 and PalN1-Pal2 were used in PCR experiments with purified DNA extracted from 2-day-old and older liquid cultures of wood chips from grapevine plants artifi-

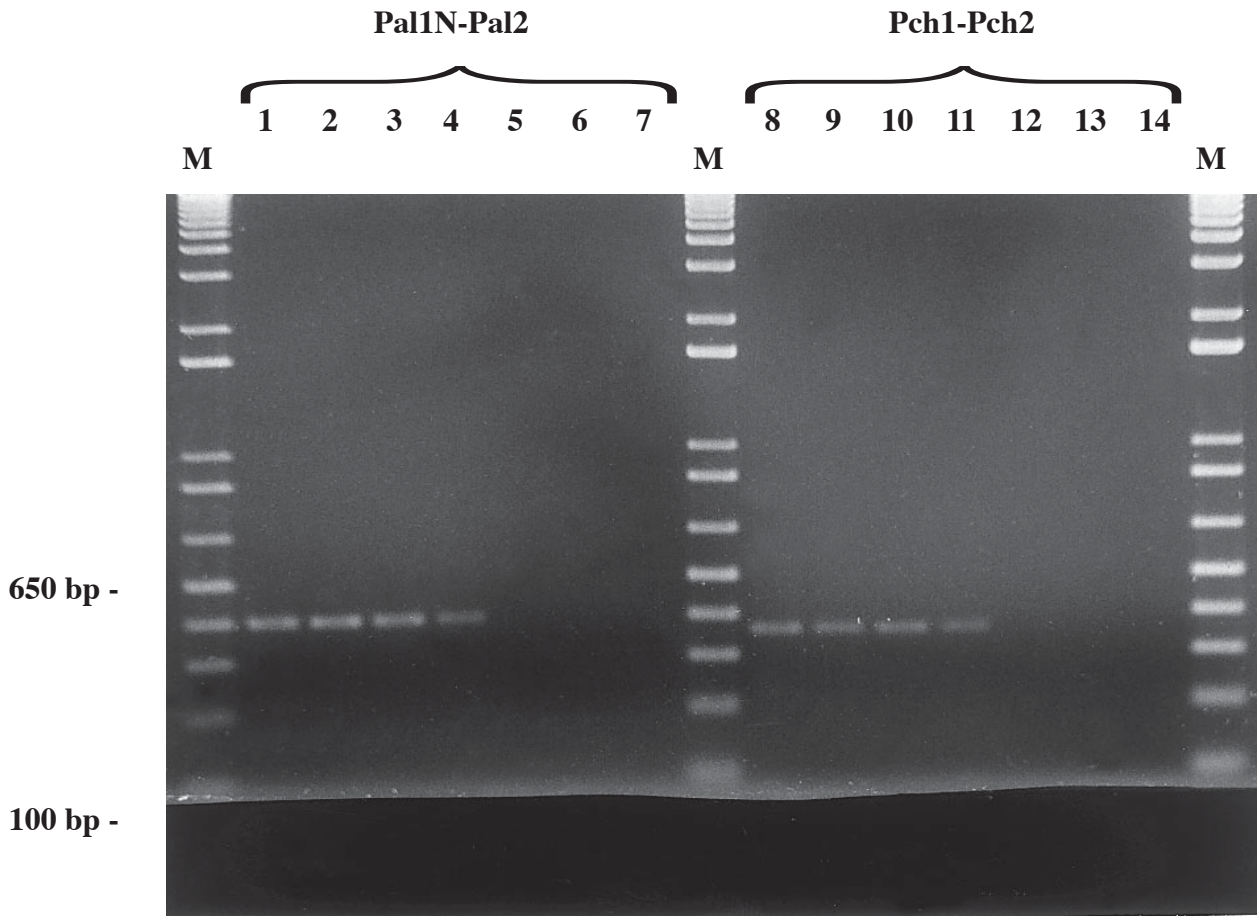


Fig. 4. Gel electrophoresis of the PCR products obtained with primer pairs Pal1N-Pal2 (lanes 1-7) and Pch1-Pch2 (lanes 8-14), using a tenfold dilution series of DNA template. Lanes 1-5, 10 ng to 1 pg of *P. aleophilum* 157 DNA; lane 6, 10 ng of *P. chlamydosporum* 56 DNA; lanes 8 to 12, 10 ng to 1 pg of *P. chlamydosporum* 56 DNA; lane 13, 10 ng of *P. aleophilum* 157 DNA; lanes 7 and 14, negative control of sterile distilled water; lane M, 1Kb Plus DNA Ladder (Gibco-BRL, Life Technologies).

cially infected with *Pch* and/or *Pal* (Fig. 6). Sequencing of the PCR products confirmed that the amplicons contained the target sequences of the fungal DNA. PCR products of the expected size were obtained in the positive controls.

## Discussion

This work shows that there were consistent differences among *Phaeoacremonium* species in the sequence of the ITS1-5.8S-ITS2 rDNA region. It was possible to detect species-specific restriction profiles from the ITS region in the genus *Phaeoacremonium* with the enzymes *Cfo*I, *Mbo*I, *Rsa*I and *Taq*I: these distinguished the five *Phaeoacremonium* species. Potentially, restriction analysis of ITS region provides a rapid and specific method for the identification of species in the genus *Phaeoacremonium*. Some further isolates of *Pang* and *Pinf* should be examined to confirm the findings for these species.

The two pairs of primers, designed and used with PCR conditions established here showed the potential of this method as a diagnostic test for *Pal* and *Pch* in grapevine wood and rooted vine cuttings. The results showed that each of the PCR primer pairs Pal1N-Pal2 and Pch1-Pch2 amplified a specific fragment from the ITS region of *Pal* and

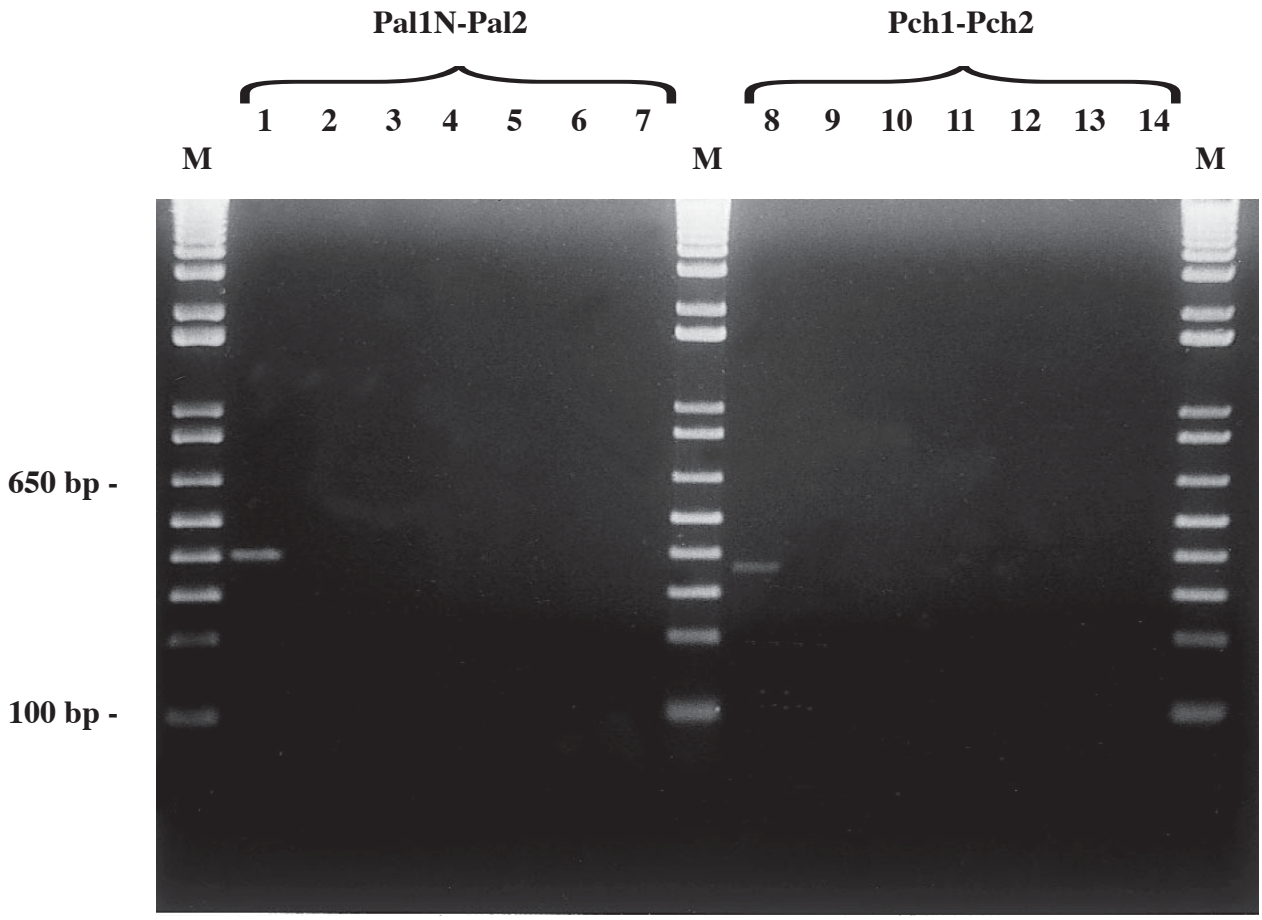


Fig. 5. Gel electrophoresis of the PCR products obtained with primer pairs Pal1N-Pal2 (lanes 1-7) and Pch1-Pch2 (lanes 8-14), using as template 10 ng of fungal or grapevine DNA. Lane 1, *P. aleophilum* 157; lane 8, *P. chlamydosporum* 56 DNA; lanes 2 and 9, grapevine DNA; lanes 3 and 10, *Botryosphaeria obtusa*; lanes 4 and 11, *Eutypa lata*; lanes 5 and 12, *Fomitiporia punctata*; lanes 6 and 13, *Stereum hirsutum*; lanes 7 and 14, negative control with distilled sterile water; lanes M, 1Kb Plus DNA Ladder (Gibco-BRL, Life Technologies).

*Pch*, respectively. No amplification was observed with these primers when DNA from vines or from other fungi that commonly colonise grapevine wood concomitantly with *Pal* and *Pch* was used. Thirty cycles of PCR amplification using *Pal*- and *Pch*-specific primers produced a sufficient amount of the predicted-size fragments (400 bp for *Pal* and 360 bp for *Pch*) to visualise them on ethidium bromide-stained gels, when one-tenth of the PCR reaction volume (2.5 μl) was loaded on the gel. In these conditions the detection threshold was found to be 10 pg of fungal genomic DNA, which is an acceptable limit of detection. It thus seems that this method can be employed when a laboratory is asked to verify the health of a batch of rooted vine cuttings. Presently, *Pch* and *Pal* identification is done by traditional methods. Chips of discoloured wood (*i.e.* wood that has brown to black stripes but seems otherwise healthy) are first incubated on a solid medium such as MEA, and then sampled for fungi. The fungal isolates are transferred to fresh solid medium to determine the morphological and biological characteristics. This whole procedure takes up to 2 months (Mugnai *et al.*, 1999). As our data showed, the PCR-based detection of *Pal* and *Pch* in grapevine wood would markedly speed up this process and indicate in a short time whether specific precautions are required to prevent the

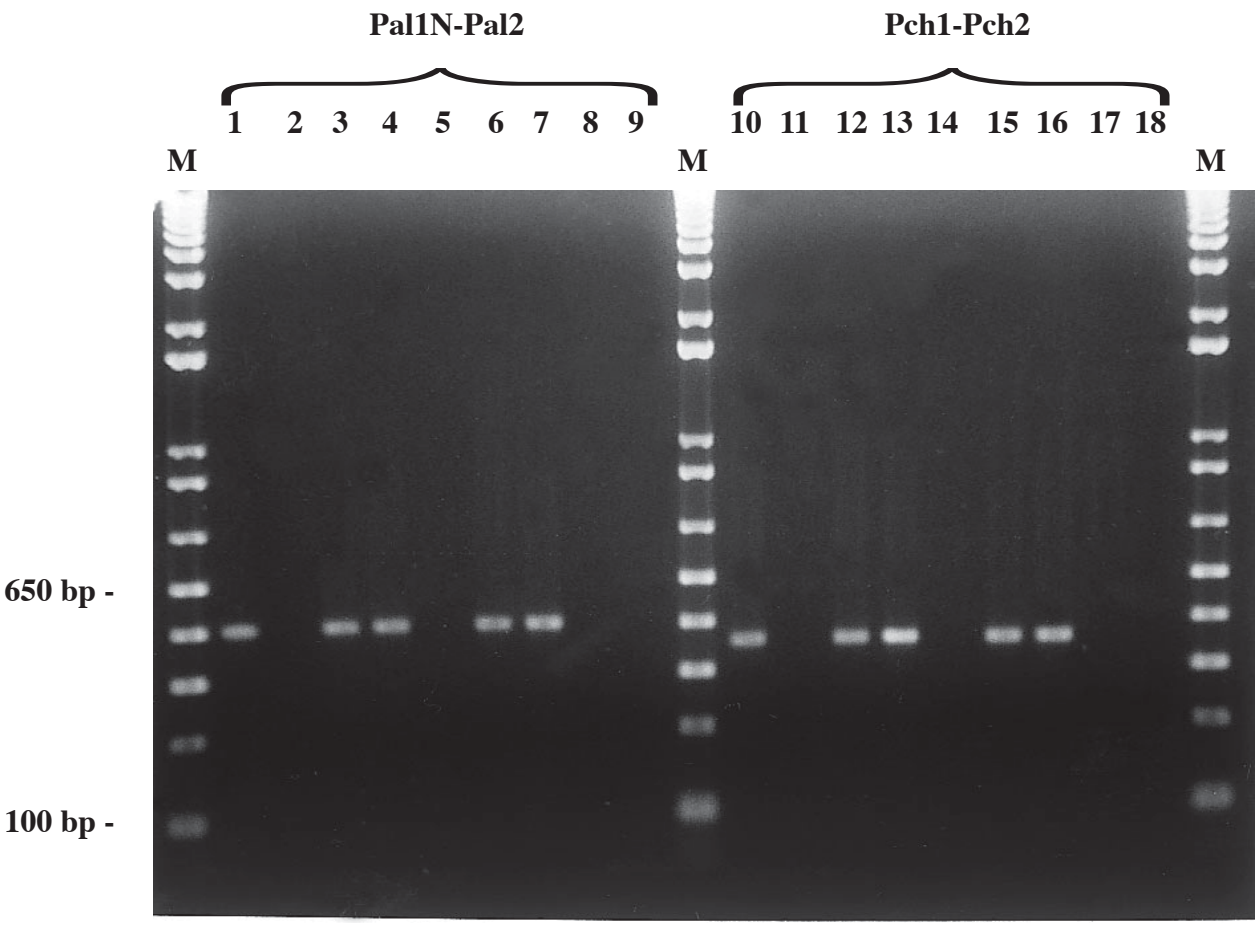


Fig. 6. Gel electrophoresis of the PCR products obtained with primer pairs Pal1N-Pal2 (lanes 1-9) and Pch1-Pch2 (lanes 10-18), using as template fungal DNA or DNA extracted from infected or contaminated grapevine wood. Lanes 1 and 11, *P. aleophilum* 157; lane 2 and 10, *P. chlamydosporum* 56 DNA; lanes 3-5, grapevine wood of plants infected with each of *Pal, Pal* plus *Pch* and *Pch*; lanes 6-8, grapevine wood from healthy plants and artificially contaminated with $10^4$ c.f.u. each of *Pal, Pal* plus *Pch* and *Pch*; lanes 12-14, grapevine wood of plants infected with each of *Pch*, *Pch* plus *Pal* and *Pal*; lanes 15-17, grapevine wood from healthy plants and artificially contaminated with $10^4$ cfu each of *Pch*, *Pch* plus *Pal* and *Pal*; lanes 9 and 18, negative control with sterile distilled water; lane M, 1Kb Plus DNA Ladder (Gibco-BRL, Life Technologies).

spread of esca and related syndromes over short or long distances. Moreover, in the case of sanitised rooted grapevine cuttings this PCR assay would serve to ensure that the sanitation procedures employed had been effective.

The results of our study also prompted some speculations on the structure of the genus *Phaeoacremonium* and the species into which it is currently divided. *Phaeoacremonium* is a new hyphomycete genus, intermediate between the two heterogeneous genera *Acremonium* and *Phialophora* and includes six fungal species. So far four of these species have been isolated from grapevines: *Pal*, *Pch*, *Pang* and *Pinf*. The other two species are *P. parasitica* (ex *Phialophora parasitica*, the type species) and *P. rubrigenum*, a pathogen in man. Species identification is based largely on morphological characteristics and some biological properties such as optimum growth temperature and colony pigmentation.

Our data on ITS sequencing showed a close relationship between *Pal*, *Pang*, *Pinf* and *Prub*, but

revealed a distant relationship between all these species and *Pch*. This suggests that *Pch* should be accomodated in another genus as proposed by Dupont *et al*. (1998).

Moreover, in our study we found complete similarity between the sequences of the ITS regions of *Pang* CBS 249.95, *Pinf* CBS 222.95, and all but one *Pal* isolates. Although extensive divergence in the ITS DNA sequence may be found within the same biological species (Edel *et al*., 1996) this region is generally viewed as conserved within species yet variable among species of the same genus (Cooke and Duncan, 1997; Sequerra *et al*., 1997; Wang and White, 1997; Chillali *et al*., 1998). It therefore seems possible that *Pang* CBS 249.95 and *Pinf* CBS 222.95 were misclassified and should be referred to *Pal*, as already suggested for *Pang* CBS 249.95 by Dupont *et al*. (1998). In any case, it seems that the identification of *Phaeoacremonium* species by their morphological and biological characteristics should be appropriately supported by molecular analysis.

## Acknowledgments

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