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Molecular characterisation of *Phaeomoniella chlamydospora* isolated from grapevines in Castilla y León (Spain)

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Summary. One of the major causal organisms of grapevine decline is the plant pathogenic fungus *Phaeomoniella chlamydospora*. Sequencing of the internal transcribed spacer (ITS) regions has been useful to classify this species. So far the fungus has shown only slight genetic variation. The aim of the present work was to determine the degree of genetic variation of *Pa. chlamydospora* isolates mainly from grapevines grown in Castilla y León, Spain. Thirty-five isolates of *Pa. chlamydospora* were subjected to PCR in order to amplify the nuclear 5.8S ribosomal RNA gene and its flanking ITS regions (529 bp fragment), the 5' end of the β-tubulin gene (550 bp fragment) and the partial 5'end of the translation elongation factor 1-α gene (337 bp fragment). Nine different RAPD patterns were obtained, two of which were much more common than the others. Despite the great number of RAPD primers screened, only a few reproducible polymorphic fragments were obtained. The 35 isolates fell into two groups depending on two bases at nucleotides 389 and 438 of the ITS4-ITS5 fragment. Sequences of the 5' end of the β-tubulin gene were 100% homologous across all 35 isolates. The 337 bp fragment of the elongation factor 1-α was also 100% homologous across the isolates. These results confirmed the low genetic variation shown by *Pa. chlamydospora*. However, RAPD pattern H was typical of aging grapevines. When two or three isolates were obtained from a single vine, two different *Pa. chlamydospora* genotypes were sometimes found on the same vine.

Key words: grapevine decline, genetic variation, RAPD, β-tubulin, translation elongation factor 1-α, ITS sequences.

Introduction

Phaeomoniella chlamydospora is consistently isolated from both young and mature esca-infected vines with brown wood streaking, as well as from the rootstocks of declining young vines suffering from Petri disease (Surico *et al.,* 2006).

Although the aetiology of these diseases is not completely understood, it appears to involve a succession of different fungi causing infections (Larignon and Dubos, 1997). However the particular role and specific syndromes of each one is still debated. Recently esca was suggested to be a 'complex' disease in the sense that several interacting factors and micro-organisms, acting in association or in succession, concur to produce the overall syndrome (Graniti *et al*, 2000)

The aetiology of both these diseases is not fully understood, but appears to be complex and some authors (Larignon and Dubos, 1997) suggested it involves a succession of fungi causing infections that culminate in wood decay. Grapevines all over the world suffer from these devastating diseases. In recent years much research has been devoted to *Pa. chlamydospora* and to how it is involved in grapevine decline, and this fungus is now the most strongly associated with these diseases in South Africa (Ferreira *et al*., 1994), the United States (Morton, 1997), Italy (Mugnai *et al*., 1999), Australia (Pascoe and Cottral, 2000), New Zealand (Ridgway *et al*., 2002, 2005), and other countries.

Sequencing of the internal transcribed spacer

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(ITS) regions has yielded data that have been helpful in arriving at the current taxonomic classification of *Pa. chlamydospora* (Crous and Gams, 2000). Randomly amplified polymorphic DNA (RAPD) has revealed important taxonomic information on many genera and species of *Penicillium*, *Aspergillus* and *Trichoderma*. It has also been used to examine fungi associated with grapevine decline such as *Eutypa lata* (Péros and Larignon, 1998), and in other studies on *Pa. chlamydospora* (Majer *et al*., 1996). Other molecular analyses, such as RAPD and randomly amplified microsatellite (RAM), have shown that the genetic variation of the Italian *Pa. chlamydospora* isolates is very limited, supporting the hypothesis that reproduction in this pathogen is predominantly asexual (Tegli *et al*., 2000b). A comparative analysis of 39 isolates of *Pa. chlamydospora* from New Zealand and six isolates from Italy using RAPD, RAM, amplified fragment length polymorphism (AFLP), and the universally primed polymerase chain reaction (UP-PCR) also revealed only slight genetic variation. The occurrence of genetic variation in *Pa. chlamydospora* within vineyards and, (although small), within individual vines in New Zealand indicates that multiple introductions of the fungus must have occurred (Pottinger *et al*., 2002). Borie *et al*. (2002) reported that *Pa. chlamydospora* isolates from southern and south-western France did not show any important genetic diversity. Similar results were also reported by Mostert *et al*., 2006. These molecular findings have led to the development of specific molecular markers based on conventional PCR methods that can be used to determine the occurrence of *Pa. chlamydospora* in soil and wood samples (Tegli *et al*., 2000a; Groenewald *et al*., 2001; Ridgway *et al*., 2005).

In the present study, two molecular techniques were used to determine degrees of genetic variation in *Pa. chlamydospora* isolates mainly from the region of Castilla y León, Spain. The isolates were morphologically characterised and identification was confirmed by PCR using the specific primers described by Tegli *et al*. (2000a). Variation among the isolates was explored by sequencing part of the β-tubulin (Groenewald *et al*., 2001) and the translation elongation factor 1-α genes (Carbone and Kohn, 1999), as well as an amplicon of the internal ITSs of the ribosomal DNA (rDNA) produced with primers ITS4 and ITS5 (White *et al*., 1990).

Materials and methods

Isolation and preservation of fungi

Thirty-five isolates of *Pa. chlamydospora* were studied (Table 1), 21 of which came from 12 diseased grapevines of the cultivars Tempranillo, Verdejo, Juan García, Malvasia and Prieto Picudo, all growing in the region of Castilla y León, Spain. All the 21 isolates from diseased vines were collected in 2004 and 2005 from vines aged 1–80 years showing symptoms of grapevine decline. Five isolates were collected from nursery material with no external symptoms. Four isolates were obtained from three young diseased grapevines of the cultivar Cencinel growing in Extremadura. One isolate was provided by R. Raposo (INIA, Madrid, Spain). Two *Pa. chlamydospora* isolates identified in 1996 (LR9 from Grenache-d'Assignan and LR47 from Grenache-St. Christol, both in the Dépt. de L'Hérault, France) were kindly provided by J.P. Péros (INRA, Villeneuve-les-Maguelone, France), and two strains, *Pa. chlamydospora* CBS 161.90 from South Africa and CBS 239.74 from California, were obtained from the *Centraalbureau voor Schimmelcultures* (CBS, Utrecht, The Netherlands).

The fungi were isolated as described by Martin and Cobos (2007). Isolates were identified by standard morphological techniques; identifications were confirmed by DNA sequencing of the ITS region of the rDNA and subsequent BLAST searches. Six of the vines were found to be infected with two isolates each: Y80 (Y80-6-1 and Y80-10-2); Y82 (Y82-11-4 and Y82-15-1); Y89 (Y89-4-2 and Y89-7-1); Y106 (Y106-3-3 and Y106-4-3); Y116 (Y116-18-3 and Y116-35L4); and Y122 (Y122-3-6 and Y122-14-3), and two vines had three isolates each: Y87 (Y87-5-3, Y87-7-1 and Y87-9-1); and Y121 (Y121-20L1, Y121-27L1 and Y121-29-6). All other infected vine samples had only one isolate each. All 35 isolates were cultured on potato-dextrose agar (PDA) (Merck, Darmstadt, Germany) at 25ºC.

DNA extraction

Genomic DNA was isolated from mycelium using the DNeasy plant mini kit (QIAGEN, Cologne, Germany). All DNA samples were diluted to a working concentration of 10 ng μl^{-1}. PCR amplifications were performed using a Gene Amp® 7200 thermocycler (Applied Biosystems, Foster City, CA, USA) using the primers detailed below.

RAPD analysis

Each amplification reaction involved 0.5 units of Taq polymerase (Biotools Biotechnological and Medical Laboratories SA, Madrid, Spain), 2 μl of 10× buffer purchased with the enzyme, 1 mM of $MgCl_2$, 150 μM of each dNTP, 0.5 μM primer, and 100 ng of template DNA in a final volume of 20 μl. The reaction mix was denatured at 94ºC for 5 min, followed by 45 cycles of denaturing at 95ºC for 1 min, annealing at 34ºC for 1.5 min and 72ºC for 2 min, followed by a final extension step of 72ºC for 10 min. Each run included a reaction without DNA (negative control). Each primer-template combination was amplified at least twice. After amplification, the total volume of the reaction of each PCR product was separated by electrophoresis on 1% agarose gels in 1× TBE. The gels were stained with ethidium bromide, visualised and photographed using a UV transilluminator. A pre-selection of the 20 OPERON primers (Operon Technologies, La Jolla, CA, USA) of the OPA, OPC, OPD and OPE series was made using the DNA of six isolates. OPA-2, 8, 11 and 16, OPC-8 and OPE-20 were selected to study single-spore cultures of the 35 *Pa. chlamydospora* isolates.

Each RAPD marker was treated as a unit character either present in (coded 1) or absent from (coded 0) single-spore cultures. To verify the homogeneity of the populations of each isolate, the RAPD patterns (with all primers) of the corresponding single-spore cultures were compared. The resulting data were analysed with distance-based methods. The presence or absence of reproducible bands was converted to binary data and incorporated into the analysis. A similarity matrix was constructed using the method of Nei and Li (1979). Cluster analysis was performed with the unweighted pair-group method using arithmetic averages (UPGMA). Binary data were analysed with Treecon 1.3b software for Windows (Van de Peer and De Wachter, 1994) and a dendrogram was constructed with a distance scale. The significance of the branch order was tested with 1,000 replications of bootstrap sampling (data not shown).

Sequence analysis

The nuclear 5.8S ribosomal RNA gene and its flanking ITS regions were amplified using the primers ITS4 and ITS5 (White *et al*., 1990) and the REDExtract-N-Amp kit (XNAP) (Sigma, St. Louis, MO, USA) following manufacturer's instructions. The amplification conditions were: initial denaturing for 5 min at 95ºC, followed by 25 cycles of 1 min at 94ºC, 1 min at 58ºC, 1 min at 72ºC, and a final extension step of 10 min at 72ºC. After amplification, 5 μl of each PCR product was separated by electrophoresis on 1% agarose gels in 1× TBE. The gels were stained and visualised as above. The amplified PCR fragments were purified using a GFX PCR DNA Gel band purification kit (Amersham, Chalfont St. Giles, UK) before sequencing.

PCR fragments (about 650 bp) were sequenced by the Instrumental Techniques Laboratory of the University of León. The DYEnamic ET dye terminator kit (MegaBACE, Amersham) was used for the sequencing reaction; amplification was performed using an MJ Research PTC-200 thermocycler (Amersham). The DNA sequences were analysed with a MegaBACE 500 sequencer. The complete sequences of the ITS region were read and edited using Chromas v.1.45 software (Southport, Australia). Alignments were checked visually and modified manually where necessary. Phylogenetic trees were produced using the Clustal program.

A similar procedure was followed to sequence a 550 bp fragment of the 5' end of the β-tubulin gene amplified with the T1 (O'Donnell and Cigelnik, 1997) and the Bt-2b primers (Glass and Donaldson, 1995). These primers amplified partial sequences of intron 1 and exons 2 to 6 of the β-tubulin gene. The amplification conditions were: initial denaturing for 5 min at 95ºC, followed by 32 cycles of 1 min at 94ºC, 1 min at 56ºC, 1 min at 72ºC, and a final extension step of 10 min at 72ºC. Visualisation, sequencing and sequence analysis were performed as described above for the ITS4-ITS5 amplicon.

The elongation factor 1-α amplicon (about 280 bp) was obtained with the EF1-728F and EF1-986R primers (Carbone and Kohn, 1999). The amplification conditions were: initial denaturing for 5 min at 95ºC, followed by 35 cycles of 30 s at 94ºC, 45 s at 53ºC, 90 s at 72ºC, and a final extension step of 10 min at 72ºC. Two new primers were designed: (EFE-2F 5'TGATCTACAAGTGCGGTGGT3' and EFE-3R 5'GGGCGATATCGATGGTGATA3'). These primers amplified a partial sequence of exon 2, the complete sequence of intron 2, and a partial sequence of intron 3. The amplification conditions were: initial denaturing for 5 min at 95ºC, followed by 25 cycles of 1 min at 94ºC, 30 s at 58ºC, 1 min at 72ºC, and a final extension step of 10 min at 72ºC. Visualisation, sequencing and

sequence analysis were performed as described for the ITS4-ITS5 amplicon.

All primers used for sequencing were provided by IZASA (Barcelona, Spain).

Results

The high resolution achieved with RAPD enables sufficient genomic variation to be detected to characterise individual members of a species. However, this technique fails to provide consistently reproducible results. To partially overcome this disadvantage, and to guarantee the homogeneity of the population, two or three single-spore cultures of each isolate were examined under strict conditions of DNA quantification and reaction substrate concentration. All reactions were repeated at least twice to verify the patterns obtained. In an initial screening, those primers that provided reproducible patterns were selected; of the 20 primers in each of the OPA, OPC, OPD and OPE kits, six decamer primers were selected. Amplification with the six OPERON primers provided 2–4 clear bands. The product sizes obtained ranged from approximately 450 to 2060 bp. OPA-2, OPA-11 and OPA-16 revealed only one clear genetic group. OPC-8 amplified a band of 1220 bp in 80% of the isolates. OPE-20 amplified two bands, 1500 bp and 870 bp in 90% and 50% of the isolates respectively, and it revealed three genetic groups based on the presence or absence of these two fragments (Fig. 1). OPA-8 amplified a band of around 2060 bp in 60% of the isolates. Figure 2 shows the combined results for all the 35 isolates. The combined results for the three RAPD primers identified 9 genetically distinct groups of isolates. Most isolates belonged to group A or group D. Group A with 9 isolates (including CBS 16190b and the two isolates from France) were found on vines of all ages. Group D isolates, also very common, were found on mature vines (Y116-35L4c, Y121-29-6c and Y121-20L1c), on young vines (VR3-2, VR6-1 and VR39) that had been supplied by nurseries for planting in vineyards in Castilla y León, and on vines Y105-3-2 and Y41-2-2 planted in another Spanish region (Extremadura). Five isolates belonging to group H occurred only in old vines (50–80 years). Moreover, the two isolates found on the single vine Y89, Y89-4-2b and Y89-7-1b, both belonged to group E, and the three isolates from vine Y87, Y87-5-3c, Y87-7-1c and Y87-9-1b, all belonged to group H. The two isolates per vine found on the vines Y80, Y82, Y106, Y116, and Y122, on the other hand, belonged to two different groups, one to group E, F, A, or H, the other to group G, A, D or I. Of the three

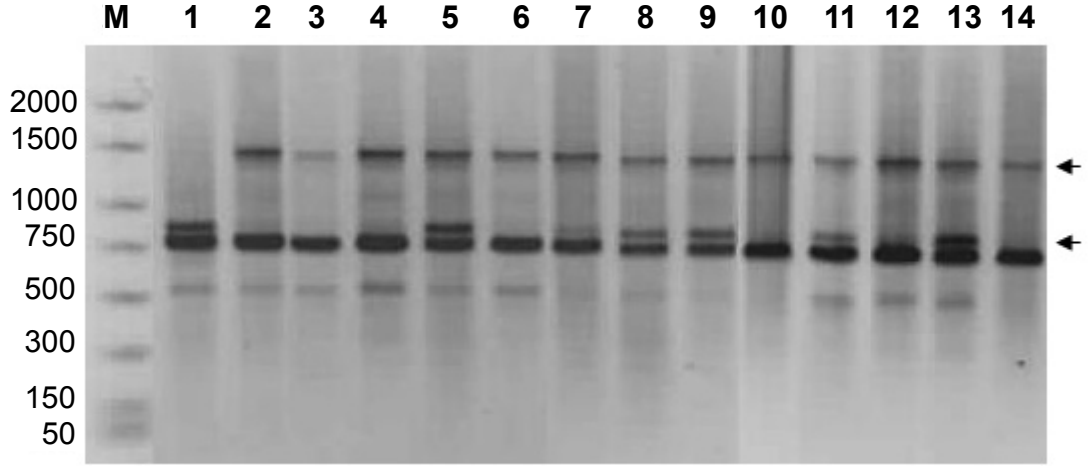

Fig. 1. Random amplified polymorphic DNA of *Phaeomoniella chlamydospora* using primer OPE-20; amplified fragments were separated by 1% agarose gel electrophoresis. Lane 1, reference isolate CBS 161.90b. Lanes 2 to 14: isolates VR3-2b, VR6-1b, VR39c, Y38-8-1c, Y41-2-2c, Y87-5-3c, Y89-4-2c, Y83-8-1c, Y106-3-3c, Y112-42-3c, Y119-8-5b, Y116-18-3c and Y122-3-6c. Lane M, DNA molecular weight marker (2 Kb); the fragment size is indicated on the left. Markers E-20 1500 bp and E-20 870 bp are indicated by arrows.

isolates found in vine Y121, one belonged to group H, and the other two to group D (Fig. 2).

DNA extracted from the two single-spore cultures of each *Pa. chlamydospora* isolate was amplified by PCR using the ITS4 and ITS5 primers. The sequences of the ITS4-ITS5 amplicons obtained (529 bp) were also compared with the sequences in the GenBank database. All those isolates were very similar. Multiple alignments of the 529 bp sequence showed that only two bases differed at positions 369 and 438. The phylogram generated by Clustal alignment placed the 35 isolates into two groups (Fig. 2) defined by these two base differences: group I (14 isolates) with thymine nucleotides at positions 369 (T369) and 438 (T438), and group II (21 isolates) with an adenine nucleotide at position 369 (A369) and a cytosine nucleotide at position 438 (C438).

A similar procedure was followed to compare the aligned sequences of the 550 bp fragment of the 5' end of the β-tubulin gene amplified with primers T1 and Bt-2b. All partial sequences were identical (100% homology).

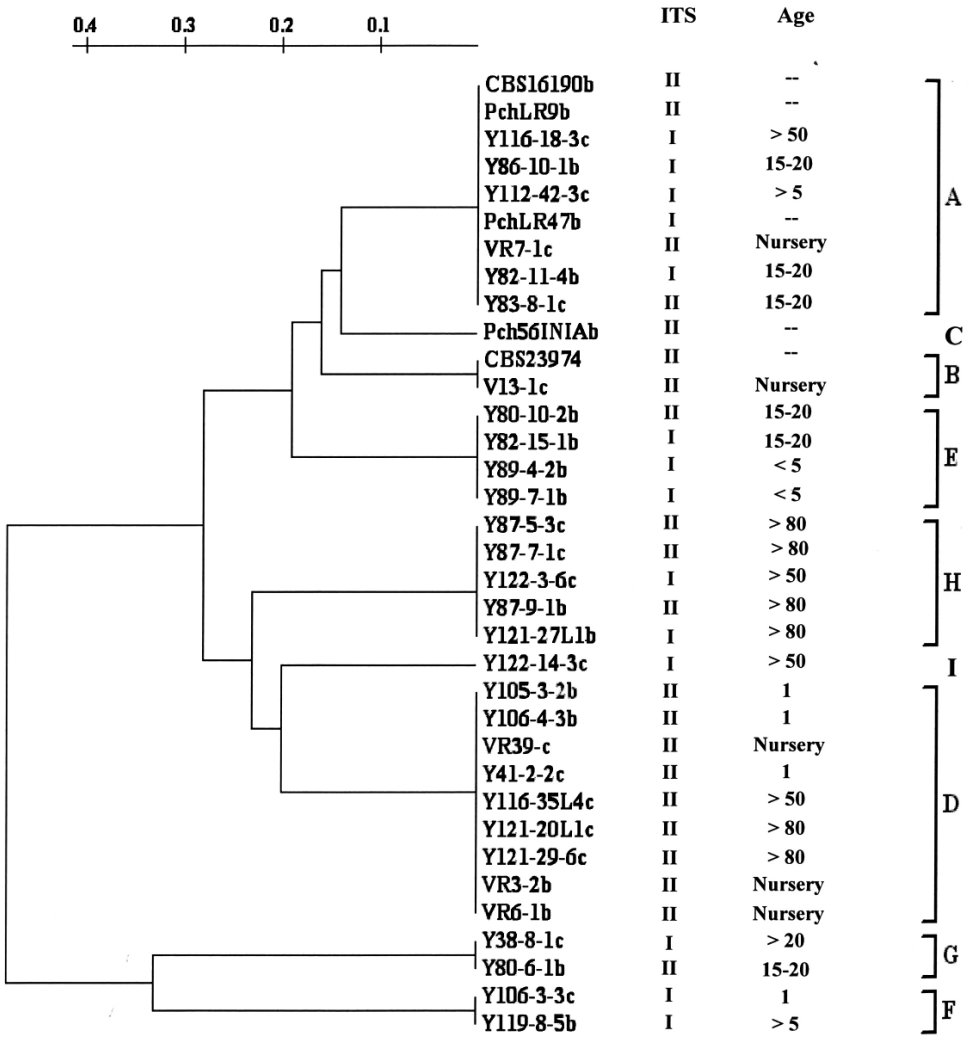

Fig. 2. Dendrogram depicting RAPD patterns of genetic variation among 35 isolates of *Phaeomoniella chlamydospora*. Sequence variations found in the ITS4-ITS5 fragments divided the isolates into two groups: 14 isolates had A369 and C438 (group I), and 21 had T365 and T438 (group II).

Table 1. Thirty-five isolates of *Phaeomoniella chlamydospora* obtained from *Vitis vinifera* and analysed in this work, and the ITS, β-tubulin and translation elongation factor 1-α GenBank accession numbers for six representative isolates.

Isolate	Region, Country	GenBank number: ITS, β-tubulin, elongation factor 1-α respectively
CBS161.90	South Africa	
CBS239.74	California, USA	
LR9	Assignan, France	
LR47	St Christol, France	
INIA56	Madrid, Spain	
V13-1	Castilla y León, Spain	
VR3-2	"	
VR6-1	"	
VR7-1	"	
VR39	"	
Y38-8-1	"	
Y41-2-2	Extremadura, Spain	
Y80-6-1	Castilla y León, Spain	
Y80-10-2	"	
Y82-11-4	"	
Y82-15-1	"	
Y83-8-1	"	EU018412, EU078329, EU158820
Y86-10-1	"	EU018413, EU078334, EU158821
Y87-5-3	"	
Y87-7-1	"	
Y87-9-1	"	EU018414, EU078330, EU158822
Y89-4-2	"	
Y89-7-1	"	
Y105-3-2	Extremadura, Spain	
Y106-3-3	"	
Y106-4-3	"	
Y112-42-3	Castilla y León, Spain	EU018415, EU078332, EU158823
Y116-18-3	"	EU018416, EU078331, EU158824
Y116-35L4	"	
Y119-8-5	"	
Y121-20L1	"	EU018417, EU078333, EU158825
Y121-27L1	"	
Y121-29-6	"	
Y122-3-6	"	
Y122-14-3	"	

Primers EF1-728F and EF1-986R did not amplify the target fragment of the translation elongation factor 1-α of *Pa. chlamydospora* at the annealing temperature of 58ºC. A lowering of the annealing temperature to 53ºC gave a short amplicon that had a low sequence homology with any other elongation factor sequence in the GenBank database. With a second pair of primers, designed in the exon II and exon III, EFE-2F and EFE-3R respectively, an amplicon of 337 bp was obtained and sequenced. The sequences obtained with the EFE-2F and EFE-3R primers were compared and showed 100% homology among the *Pa. chlamydospora* isolates. Of the 35 isolates analysed, six were selected and their ITS, β-tubulin and translation elongation factor 1-α nucleotide sequences deposited in the GenBank database (See Table 1 for accession numbers).

Discussion

The aim of this work was to determine the degree of genetic variation in *Pa. chlamydospora* isolates collected in Spain. Three RAPD primers provided different patterns for the 35 isolates, even though the differences were very small. Borie *et al.* (2002) already obtained two polymorphic fragments of around 1500 bp and 870 bp with the OPE-20 primer. *Pa. chlamydospora* showed considerable genetic homogeneity, which was confirmed by the sequencing of three PCR amplicons: a region of the ITS region, the 5' end of the β-tubulin gene, and a partial sequence of the translation elongation factor 1-α gene.

Despite the great number of RAPD primers screened, only four polymorphic fragments were reproducible, defining 9 groups of isolates (A to I). When two or three isolates were obtained from a single vine, most of them had different RAPD patterns. However, six vines each had two or three *Pa. chlamydospora* isolates with different RAPD patterns. This indicates that a double infection may occur even in young vines (as vine Y106), confirming the funding reported in Mostert *et al.* (2006).

The ITS rDNA region is generally thought to be constant within a species, but to vary between species of the same genus (Tegli *et al.*, 2000b). The low genetic variation shown by the RAPD primers plus the low variation suggested by the ITS sequences contrasts with the variation found between isolates from vines Y121, Y106 and Y116. Two of the three isolates from vine Y121 belonged to RAPD pattern group D and to ITS sequence group II, while the third isolate belonged to RAPD pattern group H and ITS sequence group I. The same level of variation was seen with the isolates from vines Y106 and Y116. The low genetic variation suggested by these different techniques should be confirmed using a more sensitive method such as AFLP. Mostert *et al.* (2006) analysed 88 isolates from 8 countries, with only two AFLP primer combinations, and produced 138 scorable markers of which 33% were polymorphic. The authors concluded that the low level of genetic variation indicated that reproduction of *Pa. chlamydospora* in field conditions was asexual.

Obviously, the amplified 550 bp fragment of the β-tubulin gene was 100% homologous across all isolates. This confirmed the status of the genus *Phaeomoniella* as that originally proposed by Groenewald *et al.* (2001), based on its ITS sequence information. This is to our knowledge the first report on the partial sequence of the translation elongation factor 1-α gene. The 337 bp fragment sequenced showed 100% homology across the isolates, even with respect to the second intron of the gene.

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Literature cited

Borie B., L. Jacquiot, I. Jamaux-Despréaux, P. Larignon and J.P. Péros, 2002. Genetic diversity in populations of the fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* on grapevine in France. *Plant Pathology* 51, 85–96.

Carbone I. and L.M. Kohn, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91, 553–556.

Crous P.W. and W. Gams, 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., the causal organism of Petri grapevine decline. *Phytopathologia Mediterranea* 39, 112–118.

Ferreira J.H.S., P.S. van Wyk and E. Venter, 1994. Slow dieback of grapevines: association of *Phialophora parasitica* with slow dieback of grapevines. *South African Journal of Enology and Viticult*ure 20, 43–46.

Graniti A., G. Surico and L. Mugnai, 2000. Esca of grapevine: a disease complex or a complex of diseases? Phytopathologia Mediterranea 39, 16–20

Groenewald M., J.C. Kang, P.W. Crous and W. Gams, 2001.

ITS and β-tubulin phylogeny of *Phaeomoniella chlamydospora* species. *Mycological Research* 105, 651–657.

Larignon P. and B. Dubos, 1997. Fungi associated with esca disease in grapevine. European Journal of Plant Pathology, 103,147-157.

Majer D., R. Mithen, B.G. Lewis, P. Vos and R.P. Oliver, 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* 100, 1107–1111.

Martin M.T. and R. Cobos, 2007. Identification of fungi associated with grapevine decline in Castilla y León (Spain). *Phytopathologia Mediterranea* 46, 18–25.

Morton L., 1997. Update on "Black goo." *Wines and Vines* 78, 62–64.

Mostert L., E.C.A. Abeln, F. Halleen and P.W. Crous, 2006. Genetic diversity among isolates of *Phaeomoniella chlamydospora* on grapevines. *Australasian Plant Pathology* 35(4), 453–460.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.

Nei M. and W.H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences* 76, 5269–5273.

O'Donnell K. and E. Cigelnik, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium are nonorthologous. *Molecular Phylogenetics and Evolution* 7(1), 103–116.

Pascoe I. and E. Cottral, 2000. Developments in grapevine trunk diseases research in Australia. *Phytopathologia Mediterranea* 41, 199–211.

Péros J.P. and P. Larignon, 1998. Confirmation of random mating and indication for gene flow in the grapevine dieback fungus, *Eutypa lata*. *Vitis* 37, 97–98.

Pottinger B., A. Stewart, M. Carpenter and H.J. Ridgway, 2002. Low genetic variation detected in New Zealand populations of *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 41, 199–211.

Ridgway H.J., B.E. Sleight and A. Stewart, 2002. Molecular evidence for the presence of *Phaeomoniella chlamydospora* in New Zealand vineyards and its detection in grapevine wood using species specific PCR. *Australasian Plant Pathology* 31, 267–271.

Ridgway H.J., J.M. Steyaert, B.M. Pottinger, M. Carpenter, D. Nicol and A. Stewart, 2005. Development of an isolate-specific marker for tracking *Phaeomoniella chlamydospora* infection in grapevines. *Mycologia* 97(5), 1093–1101.

Surico G., L. Mugnai and G. Marchi, 2006. Older and recent observations on esca: a critical overview. *Phytopathologia Mediterranea* 45 supplement, S68–S86.

Tegli S., E. Bertelli and G. Surico, 2000a. 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. *Phytopathologia Mediterranea* 39, 134–149.

Tegli S., E. Santilli, E. Bertelli and G. Surico, 2000b. Genetic variation within *Phaeoacremonium aleophilum* and *P. chlamydosporum* in Italy. *Phytopathologia Mediterranea* 39, 125–133.

Van de Peer, Y. and R. De Wachter. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Applications in the Biosciences (CABIOS)* 10, 569–570.

White T.J., T.D. Bruns, S. Lee and J.W. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A Guide to Methods and Applications* (M. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, ed.), Academic Press, San Diego, CA, USA, 315–322.

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