Phytopathol. Mediterr. (2000) 39, 107-111

Usage of molecular markers (PCR-RAPD) for studying genetic variability in *Phellinus* (*Fomitiporia*) sp.

Stefania Pollastro, Angela Abbatecola, Crescenza Dongiovanni and Francesco Faretra

Dipartimento di Protezione delle Piante e Microbiologia applicata, University,
Via Amendola 165/A, 70126 Bari, Italy

Summary. PCR-RAPD was used to explore the genetic variability in *Phellinus* (*Fomitiporia*) sp. isolates from esca-affected vines. The use of 20 random primers yielded 180 polymorphic markers. Cluster-analysis grouped isolates by their origin, geographical location and host plant, but not by their tentative identification as *Phellinus* sp. or *F. punctata*. This shows that all isolates tested belonged to a single species, probably *F. punctata*. Observations carried out on samples of isolates representative of the population of *Phellinus* sp. present in each of two vineyards indicated that clonal plant-to-plant propagation of the fungus did not occur and that infections were probably caused by basidiospores. RAPD markers common to all tested isolates of *Phellinus* sp. (*F. punctata*), but never observed with other grape-associated fungi, were identified and are now being exploited to set up diagnostic techniques based on PCR or molecular probes.

Key words: grapevine, esca, molecular markers, epidemiology.

Introduction

Phellinus igniarius (L.:Fr.) Quél. has for a long time been considered one of the most important pathogens causing esca disease on grapevine. Recently, however, a very similar fungus, *Phellinus punctatus* (P. Karst.) Pilát., was found associated with esca-affected vines in France (Larignon and Dubos, 1997) and in Italy (Mugnai *et al.*, 1999). The latter species was renamed *Fomitiporia punctata* (Fr.) Murrill following a taxonomic revision of the genus *Phellinus* (Fischer, 1996). The similarity between the two species led Mugnai *et al.* (1999) to hypothesise that previous records of *P. igniarius* may have been misidentifications of *F. punctata*. Adequate discrimination between the two fungi is indeed quite difficult and requires examination of the fruiting bodies, because colonies and mycelia do not differ in morphological traits. Moreover, the basidiocarps are not commonly found on grapevine in the field nor are they easily available under laboratory conditions.

Molecular markers may be helpful in investigating numerous aspects of esca that still remain obscure, such as disease etiology and epidemiology, taxonomy of the putative causal agents and their genetic variability, and in improving diagnostic tools. In particular, PCR-RAPD (Polymerase Chain Reaction - Random Amplified Polymorphic DNA) markers have been successfully applied to numerous filamentous fungi in different fields of experimental mycology. The technique is based on DNA amplification primed by single nucleotides with short and random sequences (Welsh and McClelland, 1990; Williams *et al.*, 1990). The present paper reports preliminary results obtained by us-

Corresponding author: F. Faretra
Fax. +30 080 5442911
E-mail: faretra@agr.uniba.it

ing PCR-RAPD markers to study genetic variability in *Phellinus* (*Fomitiporia*) sp. isolates from esca-affected vines.

Materials and methods

Methods of extraction and partial purification of DNA from mycelium, and PCR conditions were optimised for getting reproducible electrophoretic banding patterns. Fungal colonies were grown on cellophane sheets placed on malt extract agar (MEA) for 4 days at 25±1°C, in darkness. Mycelium was collected, transferred to Eppendorf tubes and ground under liquid nitrogen. DNA extraction and purification was carried out according to Murray and Thompson (1980) as modified by Rogers *et al*. (1989) and Kim *et al*. (1990). Briefly, mycelium (200-300 mg fresh weight) was suspended in 600 µl of cold CTAB buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% cetyldimethylethylammonium bromide (w/v), 0.2% β-mercaptoethanol (v/v)); the mixture was transferred threefold to liquid nitrogen and then to warm water (75°C), and afterwards maintained for 1 h at 75°C. After extraction with 600 µl chloroform, nucleic acids were precipitated with isopropanol (2 h at -20°C) and recovered by centrifugation. The pellet, washed with 70% ethanol, was dissolved in 200 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). The solution was amended with 0.1 mg ml^{-1} DNAase-free pancreatic RNAase and kept for 2 h at 37°C. DNA was precipitated with 2 vol. absolute ethanol in presence of 0.6 vol. 5 M ammonium acetate, recovered by centrifugation, and dissolved in water at a final concentration of 25 ng μl^{-1}.

PCR reactions were performed in 25-µl volumes containing 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 2mM $MgCl_2$; 75 µM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI, USA); 0.5 µM primer; 50 ng of target DNA and 1.5 units of Taq polymerase (Promega). Reactions were carried out in a thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer, Norwalk, USA) programmed as follows: 5 min at 94°C (initial denaturation); 40 cycles of 30 sec at 94°C (denaturation), 30 sec at 35°C (annealing), 30 sec at 72°C (extension); and 7 min at 72°C (final extension). Aliquots (10 µl) of the reaction mixtures were loaded on 1.4% agarose gel (Amplisize agarose; Bio-Rad Laboratories, Hercules, CA, USA) and run in TBE buffer at 110 V for 55 min (Sub-Cell Electrophoresis Cell, Bio-Rad Laboratories). The 100 bp DNA Ladder (New England Bio-Labs, Beverly, USA), giving 12 bands from 100 to 1500 bp, was used as a standard. The gel was stained with 1 µg ml^{-1} ethidium bromide. Gel images were acquired and analysed by using a Gel Doc 1000 system (Bio-Rad Laboratories).

Twenty random 10-mer primers (kit A; Operon Technology, Alameda, CA, USA) have so far been used to analyse DNA from 178 fungal isolates, identified as a *Phellinus* sp. or as *F. punctata*, most of which derived from esca-affected grapes (Table 1).

To provide a quantitative measure of relatedness between isolates, similarity matrices were constructed from the RAPD-PCR patterns, using the software package SPSS for Windows (ver. 8.0.1I, SPSS Inc., USA). The genetic similarity (GS) between each pair of fungal isolates was calculated according to the formula $2b_{ij}/(b_{ij} + b_i + b_j)$, where b_{ij} is the number of common bands, and b_i and b_j are the numbers of unshared bands (Dice, 1945; Nei and Li, 1979). Data obtained were submitted to hierarchical cluster analysis.

Results and discussion

Under the adopted conditions, 16 out of 20 primers yielded amplicons separable in agarose gel into discrete bands corresponding to molecular weights ranging from 200 to 1500 bp (Fig. 1). About 180 polymorphic RAPD markers were identified and used to establish genetic relatedness among fungal isolates. Cluster analysis grouped isolates by their origin, geographical location and

Table 1. Fungal isolates investigated with PCR-RAPD analysis.

No. of isolates[a]	Identified as	Host	Region
2	*F. punctata*	Grapevine	Venetia
3	*P. igniarius*	Grapevine	Lombardy
96 (A)	*Phellinus* sp.	Grapevine	Puglia
79 (B)	*Phellinus* sp.	Grapevine	Puglia
2	*F. punctata*	Citrus	Puglia

[a] A and B indicate sets of samples, each of which was representative of a distinct population of *Phellinus* sp. in a vineyard of table grape cv. Italia.

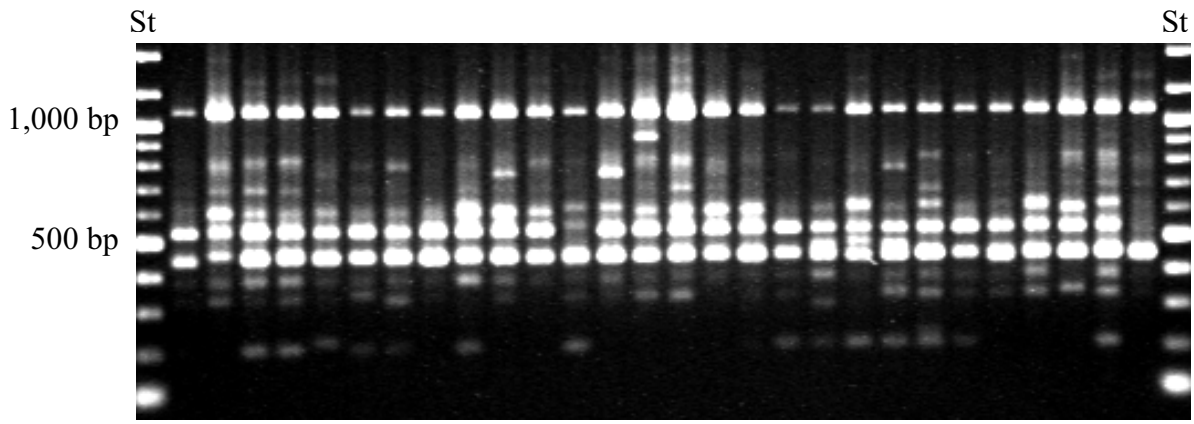

Fig. 1. Banding patterns obtained using a single 10-mer primer to amplify the DNA from 28 isolates of *Phellinus* sp. (St = standard). Common bands as well as polymorphic markers are obvious.

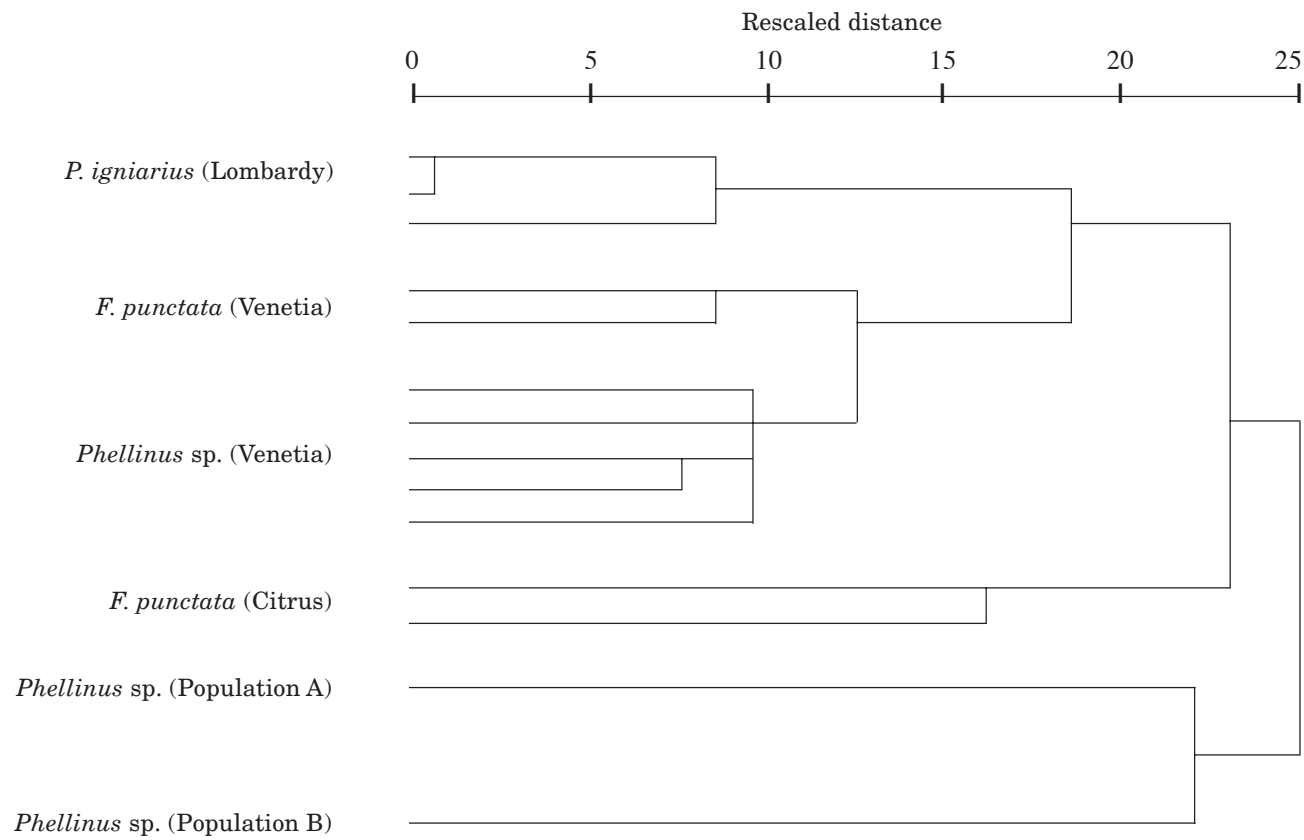

Fig. 2. Dendrogram from cluster analysis of genetic distance among isolates.

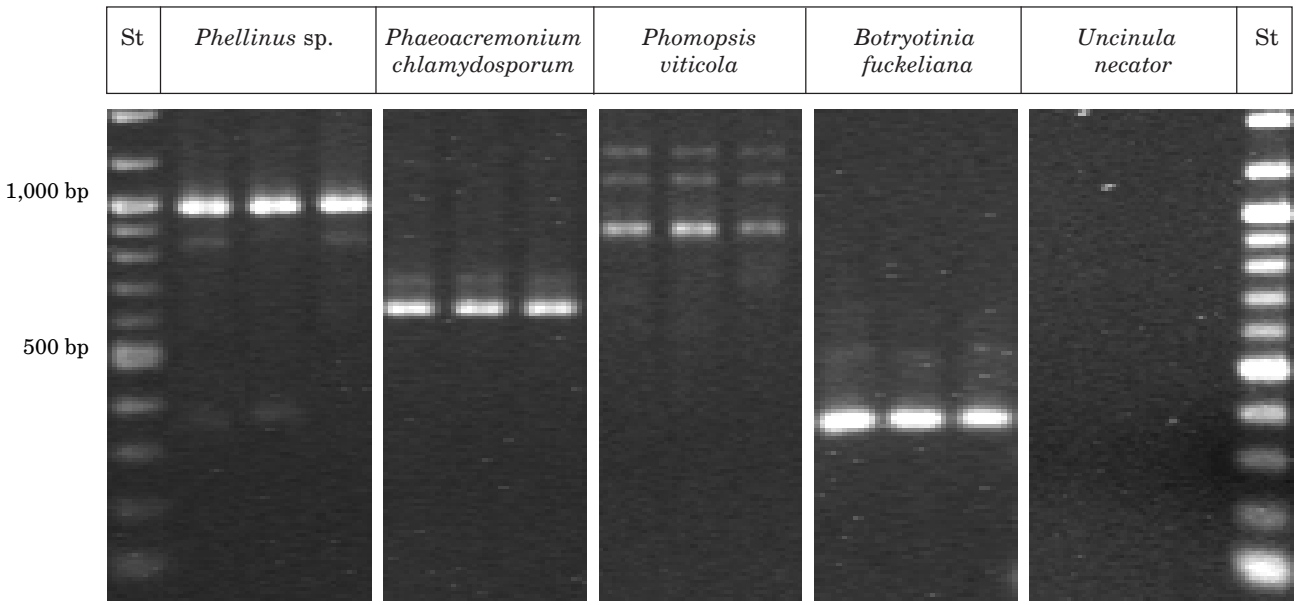

Fig. 3. Example of a PCR-RAPD marker selective for *Phellinus* sp. not found in other phytopathogenic fungi associated with grapevine. Each line represents a different isolate and all reactions were primed by the same 10-mer nucleotide.

host plant, but discrimination was irrespective of the tentative identification of isolates as *Phellinus* sp. or *F. punctata* (Fig. 2). Hence genetic variability was broader among isolates of different origin than among isolates previously identified as one species or another. This was clear evidence that all isolates tested belonged to a single species, probably *F. punctata*. However, numerous basidiocarps that differentiated on grapevine wood are now available and will be exploited for a correct and definitive identification of the basidiomycete associated with esca-affected plants in Apulian vineyards.

Observations were carried out on samples of isolates representative of the population of *Phellinus* sp. present in each of two vineyards. Each sample was made up of isolates from over 20 groups of 4-5 adjacent diseased vines (populations A and B, Table 1). The average genetic distance for each group of neighbouring plants did not differ significantly from the calculated value for the whole population (0.219 for population A and 0.289 for population B). Moreover, no isolates with identical RAPD patterns were detected in individual groups. These findings suggest that clonal plant-to-plant propagation of the fungus did not occur and that infections were probably caused by basidiospores. This conclusion conflicts with an observed tendency for symptomatic plants to cluster, especially in young vineyards, which suggests that in such cases the disease is somehow transmitted from plant to plant (Pollastro *et al.*, this issue). The discrepancy here underlined might be caused by the age of the vines (more than 15 years) in the vineyards surveyed with molecular markers. Further examination will be carried out on fungal populations in vineyards of different ages, and where the disease is at different stages of evolution.

As a result of the study, RAPD markers common to all tested isolates of *Phellinus* sp. (*F. punctata*), but never observed with other grape-associated fungi, such as *Phaeoacremonium* spp., *Phomopsis viticola* (Sacc.) Sacc., *Botryotinia fuckeliana* (de Bary) Whetz. and *Uncinula necator* (Schw.) Burr., were identified (Fig. 3). Such markers will

be exploited in an attempt to set up new diagnostic techniques based on PCR or molecular probes.

Acknowledgements

Work supported by a grant from Regione Puglia, the Italian Ministero delle Politiche agricole e forestali, and the European Union (Programma Operativo Plurifondo Regione Puglia, Misura 4.3.1) to the project "Alterazioni del legno della vite (escoriosi, mal dell'esca e imbrunimenti delle barbatelle)".

Literature cited

Dice L.R., 1945. Measures of the amount of ecological association between species. *Ecology*, 26, 297-302.

Fischer M., 1996. On the species complexes within *Phellinus*: *Fomitiporia* revisited. *Mycological Research*, 100, 1459-1467.

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

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.

Murray H.G. and W.F. Thompson, 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acid Research*, 8, 4321-4325.

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, USA,* 76, 5269-5273.

Rogers S.O., S. Rehner, C. Bledsoe, G.J. Muller and J.F. Ammirati, 1989. Extraction of DNA from Basidiomycetes for ribosomal DNA hybridization. *Canadian Journal of Botany*, 67, 1235-1243.

Kim W.K., W. Mauthe, G. Hausner and G.R. Klassen, 1990. Isolation of high molecular weight DNA and double-stranded RNAs from fungi. *Canadian Journal of Botany*, 68, 1898-1902.

Welsh J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, 18, 7213-7218.

Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalsky and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18, 6231-6235.