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Specific SCAR primers for fungi associated with wood decay of grapevine

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Summary. RAPD (Random Amplified Polymorphic DNA) analysis, a technique based on the polymerase chain reaction, was applied to explore variation in 178 isolates of *Fomitiporia punctata*, 94 of *Phaeomoniella chlamydospora* and 34 of *Phomopsis viticola*, selected as being representative of fungal populations from different vineyards and locations. The analysis showed a broad genetic variability in *F. punctata* and a very high genetic uniformity in *P. chlamydospora*. With *P. viticola*, isolates belonging to different vegetative compatibility groups were investigated; the analysis evidenced high genetic similarity among isolates within groups and broad inter-group variation. For each pathogen, specific RAPD markers were selected, cloned and sequenced. The obtained sequences were used to design sequence-characterised amplified region (SCAR) primers specific for each pathogen. These are being used to develop molecular diagnostic tools.

Key words: molecular markers, molecular diagnostics, *Fomitiporia punctata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*.

Introduction

Among the numerous fungal diseases affecting grapevine, wood decay has acquired increasing importance in the last few years in grape-growing areas all over the world. In Southern Italy, the most common and severe diseases of vine wood are esca and Phomopsis cane and leaf spot.

Researches on the aetiology of esca suggest that different pathogens are probably involved. Recently, it has further been suggested that different fungi, causing at least two different diseases (esca and Petri disease), can cause similar foliar symptoms on grapevine (Surico and Mugnai, 2001). Many fundamental aspects of these diseases still remain to be clarified, such as the role and relative importance of each pathogen and the way they develop. The prevalence of one pathogen over the others is influenced by several factors, such as the age of vines, the stage of disease evolution in the vine and the geographical location of the vineyards. In Italy, for example, the pathogens most often found associated with deteriorated wood are *Fomitiporia punctata* (P. Karst.) Murril [= *Phellinus punctatus* (Fr.) Pilàt], and/or other species of the same genus (Fisher, 2001), *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & Gams, and species of the genus *Phaeoacremonium* (Mugnai *et al.*, 1999; Graniti *et al.*, 2000; Pollastro *et al.*, 2000).

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P. viticola is transmitted with grape propagation material (Piglionica, 1981). Recently, it has been hypothesized that some causal agents of esca which also cause Petri disease are transmitted in the same way (Bertelli *et al*., 1998; Morton, 1999; Surico and Mugnai, 2001). A deeper knowledge of the real role played by propagation material in dispersal of wood diseases is urgently needed in order to develop suitable preventive measures and limit pathogen spreading in vineyards. So far, researches on the spread of grapevine diseases by propagation material has been rather difficult since traditional mycological analysis is time-consuming and requires deep expertise on some not yet well-established fungal *taxa*. On this ground, molecular methods can be a useful tool for diagnostic purposes. RAPD (Random Amplified Polymorphic DNA) analysis, a fingerprinting technique based on DNA amplification primed by single nucleotides with short and random sequences (Welsh and McClelland, 1990; Williams *et al*., 1990) has been successfully applied to explore genetic variability in numerous filamentous fungi. Known sequences of species-specific RAPD markers make it possible to design 18-22-mer primer pairs inducing selective amplification of specific loci in genomic DNA, known as a sequence-characterised amplified region (SCAR); SCAR primers yield sensitive and reproducible amplification reactions and are hence suitable for diagnostic purpose (Michelmore *et al*., 1991).

Following a previous study on *F. punctata* (Pollastro *et al*., 2000), the present paper reports results of RAPD analysis in *P. chlamydospora* and *P. viticola*, and the development and testing of specific SCAR primers for each of the three pathogens.

Materials and methods

Handling of nucleic acids and plasmid cloning were in accordance with standard procedures (Maniatis *et al*., 1982). All commercial kits were used according to customer's instructions.

For RAPD analysis, 178 isolates of *F. punctata*, 94 isolates of *P. chlamydospora* and 34 of *P. viticola* sampled in different vineyards and locations were used. Isolates were maintained on potato-dextrose-agar slants at 5°C. Strains E5CS 363, E5AS, E04 11/56 identified as *P. chlamydospora, P. angustius* and *P. inflatipes* at the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) and kindly provide by Salvatorica Serra (University of Sassari, Italy). were used for comparison.

Extraction and purification of DNA from mycelium and conditions for the amplification reactions were as previously described (Pollastro *et al*., 2000). Twenty random 10-mer primers (kit A; Operon Technology, Alameda, CA, USA) were used. Each isolate was scored for the presence or absence of each amplicon. As a measure of genetic relatedness among isolates, genetic similarity between all pairs of isolates was calculated using the formula of Dice (1945), as suggested by Nei and Li (1979). Data obtained were submitted to hierarchical cluster analysis using the software package SPSS for Windows (ver. 8.0.1I, SPSS Inc., Chicago, IL, USA).

RAPD markers specific for each pathogen were separated in agarose gel and recovered by mechanical excision of a gel plug. DNA was eluted and purified using the commercial Qiaex II Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). DNA fragments were inserted into an appropriate plasmid vector using the pGEMT/Easy Vector System kit (Promega, Madison, WI, USA), suitable for cloning PCR products. The construct was cloned in the strain DH5a of *Escherichia coli* (Migula) Castellani & Chalmers. Plasmid DNA was extracted and purified using the Wizard Plus SV Minipreps DNA purification System kit (Promega). The correct size of each insert was evaluated by digestion of plasmid DNA with *EcoR*I (Promega), followed by electrophoretic analysis.

Cloned RAPD markers were sequenced by MWG Biotech (Florence, Italy). SCAR primers were designed on the basis of the obtained sequences using Omiga 2.0 software (Oxford Molecular Website, Oxford, UK).

The specificity of each primer pair was tested in amplification experiments with genomic DNA from fungal mycelium. PCR reactions were performed in 25-ml volumes containing 10 mM Tris-Cl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 2 mM $MgCl_2$; 75 mM each of dATP, dCTP, dGTP and dTTP (Promega); 0.5 mM of each 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, Foster City, CA, USA). The conditions of PCR reactions are given below.

Results and discussion

Under the adopted experimental conditions, 16 primers with *F. punctata* and 14 with both *P. chlamydospora* and *P. viticola*, of the 20 tested primers, yielded amplification products which were separated in agarose gel into discrete bands corresponding to molecular size ranging from 200 to 1,500 bps (Fig. 1 and 2). The RAPD markers identified for each pathogen, 123 for *P. chlamydospora*, 127 for *P. viticola* and 180 for *F. punctata*, were used to establish genetic relatedness among isolates.

A broad genetic variation was ascertained in *F. punctata*, even among isolates sampled in single vineyards; this indicates that basidiospores likely play an important role in the pathogen's spreading (Pollastro *et al*., 2000).

Although the 94 isolates of *P. chlamydospora* assayed were collected from 11 vineyards in different locations in the Apulia region, most were definitely identical and very well distinguished from the outgroup isolates of *P. angustius* and *P. inflatipes*. Only the isolates i-6/2-1 and a38 of *P. chlamydospora* were singly separated in the dendrogram generated by cluster analysis (Fig. 3). These results corroborate previous findings (Tegli *et al*., 2000) and suggest that gene flow and gene recombination do not occur into fungal populations, at least in the investigated areas, and hence that the sexual process does not play any role in the pathogen's biology.

The existence of vegetative compatibility groups (VCGs) in *P. viticola* was previously ascertained (Pollastro *et al*., 2001). RAPD analysis was carried out on isolates representative of 14

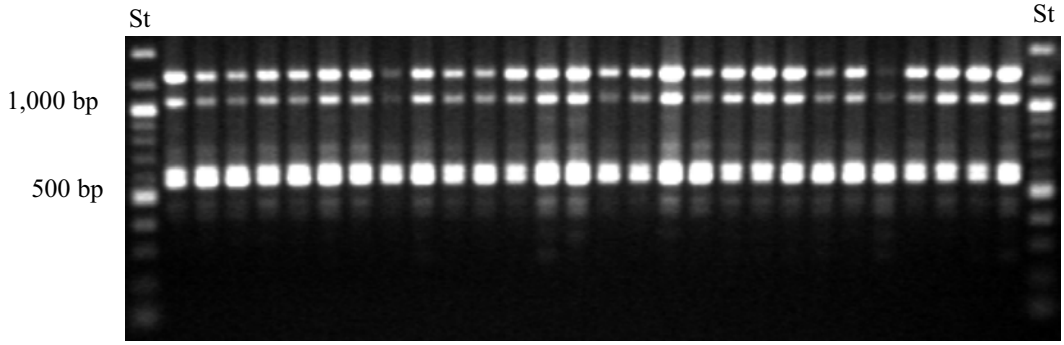

Fig. 1. Example of banding patterns obtained using a single 10-mer primer to amplify the DNA of 28 isolates of *Phaeomoniella chlamydospora* (St, standard).

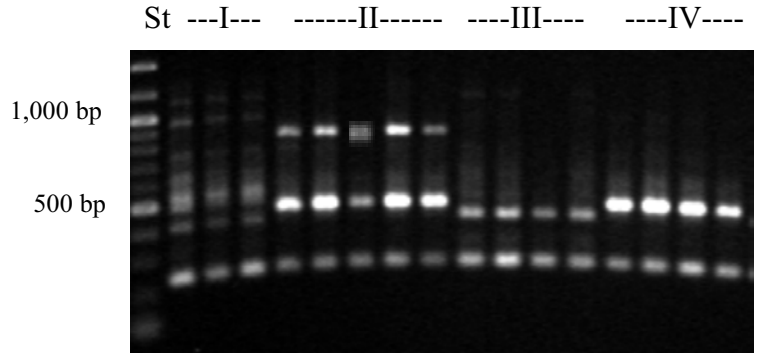

Fig. 2. Example of banding patterns obtained using a single 10-mer primer to amplify the DNA of isolates of *Phomopsis viticola* belonging to four different VCGs (St, standard).

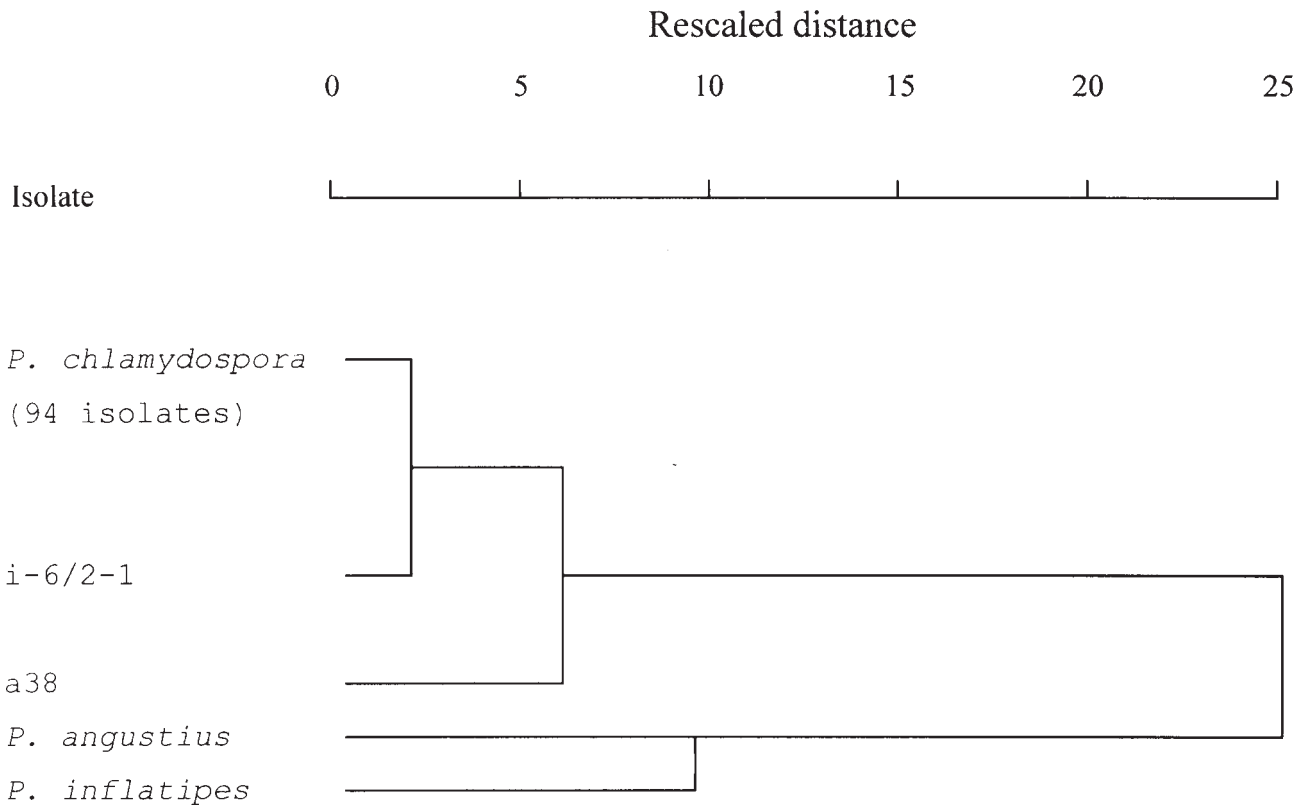

Fig. 3. Dendrogram from cluster analysis of genetic distance among isolates of *Phaeomoniella chlamydospora*.

VCGs and on a sample of isolates representative of the five most frequent VCGs. Of 127 RAPD markers, 111 were polymorphic. The average genetic distances ranged from 0.17 to 0.46 among isolates of different VCGs, but they were much lower (0.03–0.05) among isolates belonging to a same VCG (Fig. 4).

Species-specific markers common to all assayed isolates of each pathogen were searched. The following markers were selected: *F. punctata*, $OPA2_{673}$; *P. chlamydospora*, $OPA1_{291}$ and $OPA13_{844}$; and *P. viticola*, $OPA1_{791}$ and $OPA10_{721}$. They were cloned and sequenced. The sequences obtained were exploited to design SCAR primer pairs. A total of 20 pairs were tested in amplification experiments with fungal DNA to establish appropriate operative conditions. Reactions were then carried out according to the following basic scheme: 4 min at 95°C (initial denaturation), followed by a variable number of cycles of 30 sec at 94°C (denaturation), 30 sec at the annealing temperature, 30 sec at 72°C (extension) and a final extension phase of 7 min at 72°C.

The experiments made it possible to select two primer pairs each for *F. punctata* and *P. chlamydospora,* and three pairs for *P. viticola*, which yielded the most reproducible results and a single band with strong fluorescence. The sequences of each primer pair, optimal PCR conditions and the size of the amplification products are shown in Table 1.

The specificity of each primer pair was tested in amplification experiments with DNA of fungi associated with wood decay of grapevine, such as *Phaeoacremonium angustius* (W. Gams, Crous & M.J. Wingf), *Phaeoacremonium inflatipes* (W. Gams, Crous & M.J. Wingf.), *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul., *Botryosphaeria obtusa* (Schwein.) Shoemaker and *Botryosphaeria dothidea* (Moug:Fr.) Ces & De Not., as well as with other grape-associated micro-organisms [*Botryo-*

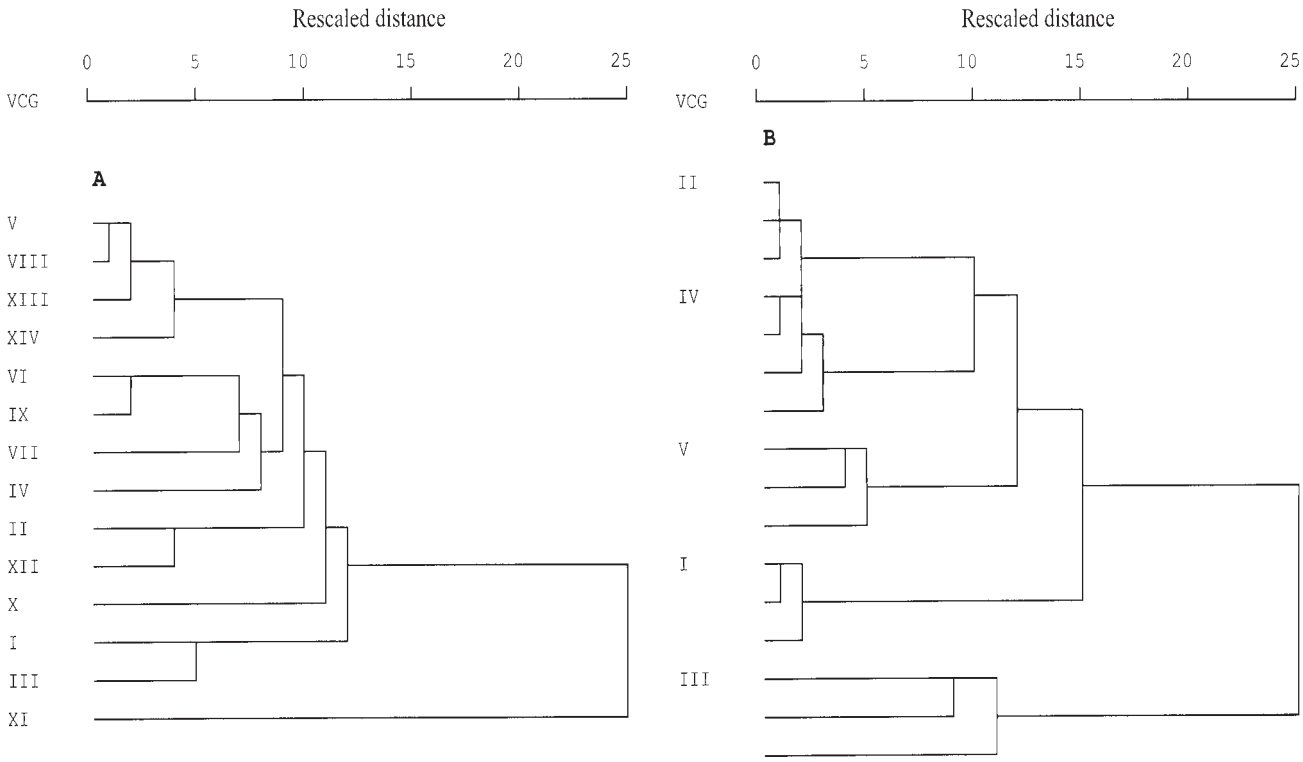

Fig. 4. Dendrograms from cluster analysis of genetic distance among isolates of *Phomopsis viticola* belonging to different VCGs. A, reference strains of different VCGs; B, representative isolates of the most common VCGs.

Table 1. SCAR primers and PCR amplification conditions.

Pathogen	RAPD marker	Sense	Sequence	Product size (bps)	PCR condition	
					Cycles No.	Annealing temperature °C
Fomitiporia punctata	$OPA2_{673}$	Forward	5'-TGACCAACGCTGAATGAC-3'	295	25	60
		Reverse	3'-GGTTCAACTCAGGCAGT-5'			
		Forward	5'-CAGTCCACTTCATAATAACC-3'	581	30	57
		Reverse	3'-GCAAATACCTGTTATCTCG-5'			
Phaeomoniella chlamydospora	$OPA1_{291}$	Forward	5'-AGCTCAAGAGCTTATTCC-3'	217	30	53
		Reverse	3'-CCAGTGTCACTTCAGTAG-3'			
	$OPA13_{844}$	Forward	5'-TGACAGAAGATTGACTGCAC-3'	191	30	55
		Reverse	3'-CCCTTCCCACTAACTAAC-5'			
Phomopsis viticola	$OPA1_{791}$	Forward	5'-GCCAGTGTGCTAGTCATAATGG-3'	194	30	55
		Reverse	3'-GAAAGGGGTTACAAAGGA-5'			
	$OPA10_{721}$	Forward	5'-TCCATCAGCAGAGTCTGTTTC-3'	399	25	60
		Reverse	3'- GCTAAGTGTTCTATCGGTCCTT-5'			
		Forward	5'-AGAGACGATAATGACTCG-3'	609	30	55
		Reverse	3'-CCTCAGAGACATTCCATTCA-5'			

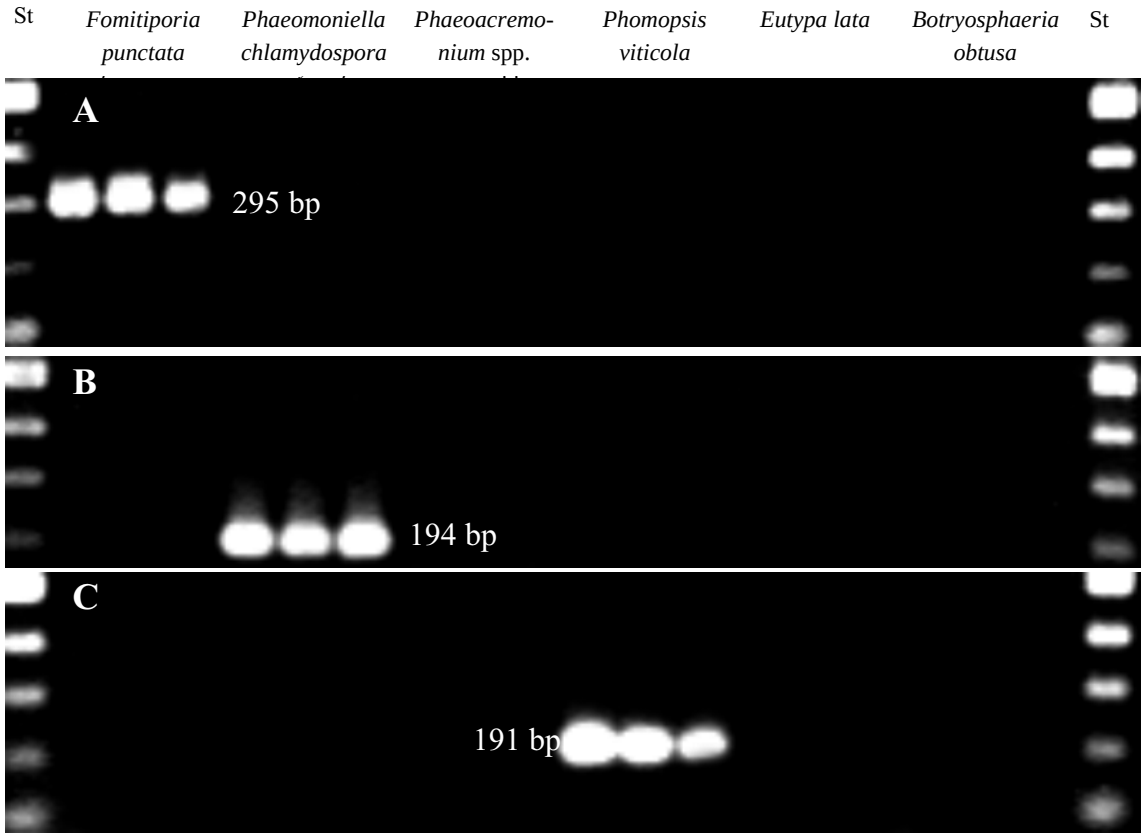

Fig. 5. Example of SCAR primer specificity. Each electrophoretic line represents a different isolate. The PCR amplifications depended on the specific primer pair used for the detection of *Fomitiporia punctata* (A), *Phaeomoniella chlamydospora* (B) and *Phomopsis viticola* (C). St, standard.

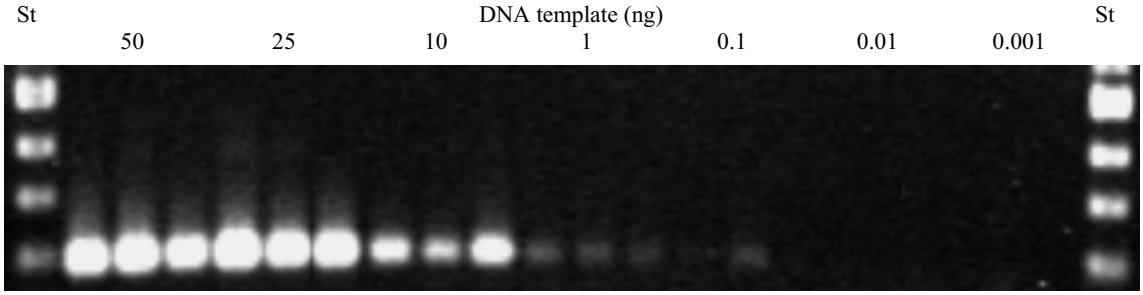

Fig. 6. Example of SCAR primer sensitivity. Three *Phaeomoniella chlamydospora* isolates were tested at each concentration of DNA template. The PCR amplifications were carried out using the primer pair specific for *P. chlamydospora*, yielding a 191 bp amplification product. St, standard.

tinia fuckeliana (de Bary) Whetz., *Uncinula necator* (Schw.) Burr., *Aspergillus niger* Van Tieghem, *Aspergillus carbonarius* (Bainier) Thom]. All primer pairs proved specific for the target pathogen (Fig. 5).

The sensitivity of SCAR primers was ascertained in PCR reactions with decreasing concentration of the target DNA. In all instances, a clear band remained visible down to 0.1–1 ng DNA, depending on the primer pair (Fig. 6).

As a result of the work, specific SCAR primers became available for *F. punctata*, *P. chlamydospora* and *P. viticola*. The primers can be used to identify fungi isolated from decaying wood. Work is in progress to exploit the primers for developing molecular sensitive detection methods for the three pathogens. New diagnostic techniques are indeed expected to increase dramatically the number of analyses that can be performed, allowing deeper understanding on disease epidemiology and easier evaluation of the sanitary status of grapevine rootstocks, cuttings and standing vines.

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