European Journal of Plant Pathology **109:** 153–164, 2003.

Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis

Davide Gobbin[1], Ilaria Pertot[2] and Cesare Gessler[1,*]
[1]*Phytomedicine/Pathology, Institute of plant sciences, Swiss Federal Institute of Technology, Universitätstrasse 2, 8092 Zürich, Switzerland;* [2]*Istituto Agrario di San Michele all'Adige, via E. Mach 1, 38010 S. Michele all'Adige (TN), Italy;* **Author for correspondence (Fax: +41 1 632 15 72; E-mail: cesare.gessler@ipw.agrl.ethz.ch)*

Accepted 10 September 2002

Key words: downy mildew, grapevine, molecular marker

Abstract

The Oomycete *Plasmopara viticola* is the causal organism of downy mildew on grapevine (*Vitis* spp.). In order to set up the techniques for investigating downy mildew disease dynamics and genetic structure, co-dominant, neutral, highly reproducible and polymorphic microsatellite markers for *P. viticola* were developed. Five markers, two with a $(TC)_n$ repeat (loci BER and ISA), two with a $(TC)_n(AC)_n$ repeat (loci CES and REX) and one with a $(CT)_n(CTAT)_n$ repeat (locus GOB), were selected. Simple sequence repeat (SSR) markers revealed different degrees of polymorphism within 190 oil spots (disease symptoms) collected from an infected Italian vineyard. The most polymorphic SSR marker GOB showed 43 alleles (Nei's expected gene diversity $H_e = 0.89$) while CES, ISA, BER and REX showed 14 ($H_e = 0.71$), 4 ($H_e = 0.57$), 3 ($H_e = 0.24$) and 1 allele ($H_e = 0$), respectively. A high throughput DNA extraction method, that allowed molecular analysis of this obligate pathogen directly in the host without any isolation procedure, was developed. The quality and quantity of oil spots did not influence the SSR analysis. Amplified SSR loci were separated by electrophoresis on a Beckman–Coulter 2000XL sequencer and automatically analysed. The objective of this study was to develop molecular biological tools and methods that allow high throughput analysis of the downy mildew populations.

Introduction

Downy mildew, caused by the heterotallic diploid Oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is one of the most important grape (*Vitis vinifera*) diseases world-wide (Wong et al., 2001). Early symptoms appear as yellowish, oily lesions on the upper leaf surface. Sporulation of the organism characteristically occurs on the lower leaf surface when humidity tends to saturation and temperatures approach 18 °C at night. Infection of young inflorescences and berries cause the most relevant disease losses. Oospores, which overwinter in leaf debris, can give rise to primary infections. Asexual sporulation of the first few primary infections is thought to furnish the driving inoculum of the epidemic (Blaeser and Weltzien, 1979; Lafon and Clerjeau, 1988; Schruft and Kassemeyer, 1999).

Forecasting models have been developed to predict the development of epidemics. They usually consider primary infections as the starting date for epidemics and secondary infections as driving inoculum for the entire epidemic. Models can usually accurately predict the conditions for new secondary infections and therefore the risk of epidemics. However, for unknown reasons, they often fail to predict the quantitative development of epidemics which impedes their use in practice (Hill, 1990; Lalancette et al., 1988a,b).

Despite extensive research dealing with oospore maturation (Burruano et al., 1990; Laviola et al., 1986), or effects of environmental conditions on each phase, crucial biological questions are still to be addressed, in

order to rectify epidemiological forecast models and to optimize disease control strategies. For instance, it is not known: (1) what the quantitative contribution of oospore-initiated infection is to an epidemic; (2) during which period of the epidemic oospore-derived infections are possible and probable; (3) how many secondary infections arise from a single primary infection; (4) how far sporangia can migrate; (5) whether an oil spot is caused by a single or multiple zoospore infection; or (6) what the frequency is of heterokaryosis. Multiallelic co-dominant DNA markers, such as microsatellites, are suitable tools to solve these problems (Ashley and Dow, 1994; Bruford and Wayne, 1993; Rafalski et al., 1996).

Microsatellites, or simple sequence repeats (SSRs), are stretches of tandemly repeated nucleotide motifs. Core units generally consist in 1–6 nucleotide repeats. Microsatellite arrays are grouped into three classes: (1) perfect repeats, where each repeat follows the next without interruptions, (2) imperfect repeats, where repeats are interrupted by non-repeat bases, and (3) compound repeats, where two or more repeat units are adjacent to each other (Gupta et al., 1996; Weber and May, 1989). Microsatellites are widely distributed in eukaryotic genomes and they are highly polymorphic due to the variability of the number of repeat units. They are inherited in a simple Mendelian manner and are likely to be selectively neutral (Ashley and Dow, 1994). SSR loci are individually amplified by PCR using primers designed on the conserved and unique flanking regions. The amplified products usually exhibit different levels of length polymorphism characteristic of the considered locus, which result from the variation in the number of tandemly repeated units. In diploid organisms microsatellite markers are co-dominant, simultaneously revealing allele sizes of the SSR locus on both chromosomes (Rafalski et al., 1996). The mutation rate at SSR loci has been estimated between 1×10^{-4} and 5×10^{-6} per meiosis event, considerably more variable than most coding sequences, but more stable than hypervariable minisatellites with mutation rates exceeding 10^{-2} per generation. The main means by which new length alleles are generated is thought to be the intra-allelic polymerase slippage during replication. However, a limitation to the direction and total number of repeat alleles may exist (Bruford and Wayne, 1993). For the cited large availability, the length polymorphism and co-dominance of their alleles, microsatellites are widely used as molecular markers for genotypic identification, genome mapping and population genetics (Groppe et al., 1995).

The objectives of this study were (1), the development of reliable SSR DNA markers for disease diagnosis and genotypic identification of *P. viticola* strains in an infected vineyard; and (2), the establishment of a high throughput method (HTM) that allows DNA extraction from *P. viticola* directly from infected grapevine leaves.

Materials and methods

Identification of SSR markers specific for P. viticola

Sporangia of *P. viticola* were collected by suction from hundreds of sporulating lesions randomly selected in infected Swiss vineyards (mainly from Stäfa and Wädenswil). DNA (10 µg) for constructing the genomic library was extracted from freeze-dried sporangia following the protocol described by Aldrich and Cullis (1993) (CTAB method). The genomic library was enriched for microsatellite repeats, and microsatellite sequences were determined following the protocol described by Tenzer et al. (1999) with the following modifications: size selected fragments were enriched for $(TC)_n$ only, oligonucleotide primer sequences were determined using the program Primer (version 3.0; Whitehead Institute for Biomedical Research, Cambridge, MA) and chosen with a melting temperature of 60 °C.

DNA extraction from sporangia of P. viticola *and microsatellite PCR amplification*

To test SSR markers, pure DNA of *P. viticola* from a sufficient number of isolates (50–100) was required. To reduce the risk of collecting more than one individual, only spatially well delimited putative primary infections were collected from three chemically untreated Italian vineyards (15 individual isolates from Mezzocorona, 30 from Volano and 28 from Salorno) in the time span from 18 to 31 May 1999. To allow DNA extraction from an adequate amount of pure sporangia (10–50 mg fresh weight), isolates were multiplied by artificial inoculation on Chardonnay grape cuttings (Brown et al., 1999). Sporangia were harvested by suction, weighed and frozen. DNA extraction was performed using the DNeasy Plant mini kit (Qiagen, Basel, Switzerland) from freeze-dried sporangia according to

the manufacturer's protocol. DNA was eluted from silica membranes with 200 µl of the supplied elution buffer AE. Due to high costs of fluorescent primer design, it was preferred to perform a preliminary test on high resolving polyacrylamide gels. To obtain a consistent amplification, a 22-cycle PCR pre-amplification for the SSR loci named BER, CES, GOB, ISA and REX was performed in 10 µl volume containing 5 µl of DNA solution (not quantified) and 5 µl PCR mix. PCR mix consisted of: 2× reaction buffer (Pharmacia Biotechnology, Inc.), 0.2 mM of each dNTP, 0.4 µM each of forward (BER_f, CES_f, GOB_f, ISA_f and REX_f) and reverse primers (BER_r, CES_r, GOB_r, ISA_r and REX_r), and 2 U of Taq Polymerase (Pharmacia Biotechnology, Inc., Table 1). A 35-cycle PCR amplification was successively performed in 10 µl volume with 5 µl PCR pre-amplification product and 5 µl PCR mix. One-quarter of forward primer was end-labelled with (γ-^{33}P) ATP (1000–3000 Curie per mmol; Amersham Pharmacia Biotechnology). PCRs were performed in a Gene Amp PCR system 9600 (Perkin Elmer, Foster City, CA) under the following conditions: 5 min at 96 °C, 35 cycles of 30 s at 96 °C, 30 s at 60 °C and 50 s at 72 °C with a final extension of 10 min at 72 °C.

The allele size was determined by loading the PCR products next to a ^{33}P-3′-labelled 30–330 bp AFLP DNA ladder (Gibco BRL) on a 6% denaturing polyacrylamide gel (National Diagnostic, Atlanta) in 1× TBE buffer using an IBI DNA sequencing unit (STS45; Kodak/International Biotechnology Inc., New Haven, CT). After electrophoresis, gels were transferred onto Whatman 3 MM paper, dried at 80 °C for 2 h in a gel dryer (Bio-Rad Laboratories, Richmond, CA), and exposed for 40 h to an X-ray film (X Omat AR; Kodak). The repeat type (perfect, imperfect or compound) of each SSR marker was classified after Weber and May (1989).

Reliability of PCR was tested by PCR amplification of four *P. viticola* DNA samples with the five SSR primer pairs. Eight simultaneously-separated PCRs per marker and per DNA sample were performed and amplicons separated on polyacrylamide gels as described above.

HTM for DNA extraction from lesions and P. viticola *microsatellite PCR amplification*

In a vineyard (not treated with fungicides) in Navicello (Trentino, Italy), 190 oil spots were collected from 45 unequal infected grapevines ordered in 3 rows (avg: 4.2, standard deviations (st. dev.): 4.3; min: 0; max: 14 oil spots per plant) on 30 May 2000 and stored at −20 °C in two deep-well blocks (Macherey-Nagel, Düren, Germany). Except for two cells (A1 in both deep-well blocks), the wells were filled with *P. viticola* oil spots. A single sample consisted of half a sporulating lesion (about 1 cm^2, including some healthy leaf tissue). Collected oil spots were assigned co-ordinates (row and plant number) to identify their exact location in the vineyard. Oil spots were freeze-dried overnight directly in deep-well blocks (Macherey-Nagel, Düren, Germany) and disrupted with a MM300 homogenizer (Retsch, Haan, Germany) equipped with Qiagen mixer mill adapter set 2x96, using carbide beads (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Total DNA extraction was performed on a Tecan Genesis RSP 150 robotic sample processor using NucleoSpin multi-96 Plant kits (Macherey-Nagel, Düren, Germany) according to the

Table 1. SSR primer sequence, forward primer label (fpl), number of alleles (N a), repeat type and allele size range scored by fragment analysis of 190 samples of *P. viticola* collected in Navicello (Italy) on 30 May 2000

Locus	Primer sequence 3′ → 5′	fpl	N a	Repeat type[a]	Approx. allele size range (bp)
BER	BER_f[b]: AATGCAATGGTCTTCATCTCG BER_r: CTCTGCGGTAAAAGCCTGTC	D3	3	Perfect, $(TC)_n$	179–185
CES	CES_f: CTTGTCGGTAGGTAAGCGTG CES_r: GCTGTACTTACAACTTCATCAG	D2	14	Compound, $(TC)_n(AC)_n$	143–186
GOB	GOB_f: CTTGGAAGTTATACCATGCTACC GOB_r: TTGAGAAATCGCACAGCTTA	D4	43	Compound, $(CT)_n(CTAT)_n^c$	210–434
ISA	ISA_f: ATTAGCGGCATGGACGTT ISA_r: GAGAAGTTCCGCCAAGTACA	D3	4	Perfect, $(TC)_n$	118–144
REX	REX_f: CGTGTGCGATAGCAAAACTT REX_r: TTGCATTCGCACTCCCTTAC	D4	1	Compound, $(TC)_n(AC)_n$	164

[a]After Weber and May (1989). [b]f: forward primer, and r: reverse primer. [c]Due to irregularities in the sequence, only an approximated formula is given.

manufacturer's protocol with the following modifications: samples were lysed in gCTAB 2× extraction buffer (2% CTAB, 2% PVP (MW 40.000), 1.4 M NaCl, 20 mM EDTA pH 8.0 and 100 mM Tris–HCl pH 8.0). Lysed oil spots were cleared by centrifugation for 15 min at 5600*g* on the Sigma 4-15C centrifuge (Sigma GmbH, Oesterode, D), equipped with the Qiagen 2x96 plate rotor, in order to remove polysaccharides and residual cellular debris. 0.3 ml of the cleared supernatant were mixed with 0.3 ml of the binding buffer C4 and 0.2 ml ethanol to create optimal conditions for binding to a silica membrane. Washing was performed firstly with 0.5 ml of the provided buffer C5 and secondly with 0.5 ml of a home-made 70% (v/v) ethanol. Both DNA binding plates were spun for 4 min at 5600*g* and incubated for 20 min at 37 °C in order to remove ethanol traces. The grape – *P. viticola* DNA mixture was eluted in 160 µl TE (10 mM). DNA extraction from 190 oil spots required approximately 3 h. Qiagen DNeasy 96 Plant kits are equally suited to DNA extractions in a 96 wells format only if the provided AP1 buffer is substituted by gCTAB 2× extraction buffer. Five separate PCR amplifications per DNA were performed in a 10 µl volume containing 5 µl of a DNA solution (not quantified), 1× reaction buffer (Amersham Pharmacia, Dübendorf, Switzerland), 0.1 mM of each dNTP, 0.14 µM of total (non-labelled+ dye-labelled) forward primer and 0.14 µM of reverse primer, and 0.7 U of Taq Polymerase (Amersham Pharmacia, Dübendorf, Switzerland) per reaction. Forward primers were labelled with the following dyes: BER_f and ISA_f with the dye D3, GOB_f and REX_f with the dye D4 and CES_f with the dye D2 (Invitrogen, Inchinnan, Scotland). For each PCR reaction different concentrations of forward dye-labelled primer and forward non-labelled primer were used: BER_f^{D3}: 0.028 µM, BER_f: 0.112 µM; CES_f^{D2}: 0.07 µM, CES_f: 0.07 µM; GOB_f^{D4}: 0.056 µM, GOB_f: 0.084 µM; ISA_f^{D3}: 0.014 µM, ISA_f: 0.126 µM and REX_f^{D4}: 0.028 µM, REX_f: 0.112 µM. PCR conditions were the same as described previously for SSR amplification from sporangia DNA but with 38 cycles. For each sample 0.5 µl of PCR product for the markers BER, GOB, ISA and REX, and 1 µl for the marker CES were mixed with 40 µl deionized formamide (Beckman–Coulter, Fullerton, CA) using a Hydra-96 Microdispenser (Robbins, Sunnyvale, CA) in a 96 well sequencing plate (Beckman–Coulter, Fullerton, CA). The different ratio of not-labelled to dye-labelled forward primer and the pipetting of different volumes of each PCR reaction were adjusted in order to obtain fluorescence outputs within the detection limits of the CEQ 2000XL sequencer (10^3–1.25×10^5 counts) for all the five SSR markers simultaneously. As a size marker, 0.25 µl of a D1-labelled 60–420 base pair (bp) ladder (Beckman–Coulter, Fullerton, CA) was loaded in each amplicon mixture. One µl of a in-house calibration standard containing D3-labelled fragments of known length (113, 154, 215, 276, 328 and 400 bp) was loaded in both cells A1 of the 96 well plates to check for reliability of electrophoresis. The aforementioned six fragments were obtained by PCR (same parameters as for HTM) using as template 1 ng of the pBluescript SK(−) phagemid (Stratagene, Inc.) with the shortest allele of the locus ISA cloned into the multiple cloning site. ISA_f^{D3} and six different reverse primers designed on the plasmid, one per reaction, were used. Amplicons were separated by electrophoresis onto Beckman CEQ 2000XL sequencer running the default method 'Frag-1' (denaturation: 90 °C, 120 s; injection: 2 kV, 30 s; separation: 7.5 kV, 35 min) according to the supplied protocol. Data were analysed by the fragment analysis software module CEQ 2000XL provided by Beckman–Coulter, Fullerton, CA (version 4.2.0). The obtained allele sizes were entered into a Microsoft Excel (version 97) spreadsheet. Number of alleles, allele frequencies, Nei's expected and observed gene diversity (Nei, 1973) were calculated with Fstat (Goudet, 2001) (version 2.9.3) using the entire data set. The null allele frequency and the probability to find two identical genotypes (PI) were calculated with the program IDENTITY 1.0 (Wagner and Sefc, 1999) using a clone-corrected data set of individuals completely coded by allele sizes (158 genotypes).

Theoretical maximal number of different genotypes (tG) and theoretical occurrence of the most common genotype ($tG_{o\,max}$) were calculated with the following formulae:

$$tG = \prod_{c=1}^{k} \frac{n_c(n_c+1)}{2}, \quad tG_{o\,max} = \prod_{c=1}^{k} f_{Mc}^2$$

in which c is the cth locus, k is the number of loci, n_c the number of alleles for the locus c and f_{Mc} is the frequency of most abundant allele (f_M) of the locus c. For each locus (for instance: $k = 2$; loci c1 and c2, $n_{c1} = 2$ and $n_{c2} = 4$), the first formula calculates the number of different allele combinations (or genotypes) that could be identified for *P. viticola* or for any other diploid organism (locus c1: $tG_{c1} = 3$, locus c2: $tG_{c2} = 10$). The multiplication of the locus specific number of different allele combinations among them leads to the

theoretical number of different genotypes that can be discriminated by means of a considered number of SSR loci ($tG = tG_{c1} \times tG_{c2} = 30$). The second formula calculates the theoretical occurrence of the genotype that shows homozygosity for the most frequent allele at each SSR locus based on allele frequencies of the genotypes present in the plot.

Reliability and limits of the HTM

Reliability of PCR was tested by PCR amplification of two *P. viticola* DNA samples with the five SSR primer pairs. Sixteen simultaneously-separated PCRs per marker and DNA were performed and amplicons separated on the Beckman CEQ 2000 sequencer as described for the HTM.

Amplicon electrophoretical separation on CEQ sequencer (Beckman–Coulter) and allele length calculation by the fragment analysis software (Beckman–Coulter) was tested for reliability by running 15 times 1 µl aliquots of a mixture of six D3-labelled PCR products of known sequence (113, 154, 215, 276, 328 and 400 bp). The relationship between the real allele length and the allele length computed by fragment analysis software module (Beckman–Coulter) was also determined.

To evaluate the benefits of the HTM, the influence of amount and quality of sampling material on PCR amplifications was tested. PCRs for the loci BER, CES, GOB and ISA (REX not tested) have been performed as described for the HTM, but amplicons were run on agarose gels for positive/negative scoring. Three different amounts of highly sporulating oil spots (1; 3; 5 mg dry weight) served to determine the optimum amount of the lesion to be sampled. To determine the sensitivity of PCR on sub-optimal starting material, six classes of oil spot quality (3 mg dry weight each) were tested: (1) well sporulated- (positive control), (2) moderately sporulated-, (3) not sporulated-, (4) in Eppendorf (EP) tubes heated- (lesions stored 4 h at 40 °C in EP tubes), (5) *in vivo* dried- (oil spots collected from a completely wilted infected leaf) and (6) partially (>50%) necrotic lesions. Each experiment was performed with 8 repetitions per class. Statistical analysis on the results was performed using a Microsoft Excel spreadsheet (version 97).

In order to exclude hypothetical non-specific amplifications of grapevine DNA or of any other microbiological 'contaminant' co-extracted with *P. viticola* DNA, pure sporangia DNA, DNA extracted from lesions (mixture of *P. viticola* and grapevine DNA, variety Chardonnay), pure grapevine DNA (varieties Chardonnay, Merlot, Teroldego and Seyval-Blanc) and water (as negative control) were amplified with the five selected SSR primer pairs.

Results

Identification of SSR markers specific for P. viticola *and SSR allele pattern analysis*

Sixty-one *P. viticola* SSR loci were sequenced. In 15 cases primer design was impossible, because the SSR flanking region was too close to the cloning site (<20 bases).

Using the 73 DNAs of the isolates collected in 1999, 46 primer pairs designed to the cloned *P. viticola* SSR sequences were tested. Thirty-six primers pairs designed on the cloned SSR sequences produced very weak or no signals with *P. viticola* DNA. Five primer pairs produced non-specific PCR products beside typical SSR stuttered amplicons, which impeded an objective interpretation (results not shown). Five primer pairs amplified in a specific and reproducible manner and were therefore selected for population genetic studies. Their loci were called BER, CES, GOB, ISA and REX (Table 1).

DNA extraction from sporangia of P. viticola *and microsatellite PCR amplification*

The multiplication of the 73 isolates from the 1999 collection on grape cuttings led to very irregular yields. For instance, for 15 isolates from Mezzocorona, an average of 14.2 mg fresh weight of sporangia (St. dev: 13.2 mg; min: 1 mg; max: 53 mg) was obtained. DNA was extracted in extremely low concentrations that often could not be quantified (<1 ng μl^{-1}). Even with such low amounts of DNA, double PCR amplifications (total: 57 cycles) were overwhelmingly (97%) successful for each of the five SSR markers selected.

Eight times repeated PCR reactions from the same four DNA samples always yielded the same banding pattern for each of the five SSR markers.

On polyacrylamide gels, the loci CES, ISA and REX (Figure 1) produced amplicons that were easily scored. Locus BER (Figure 1) sometimes produced indistinct amplicons very close to each other (2 bp difference). Due to the high level of polymorphism and to size, fragments amplified from the locus GOB (Figure 1) were difficult to score. In 83% of all amplifications

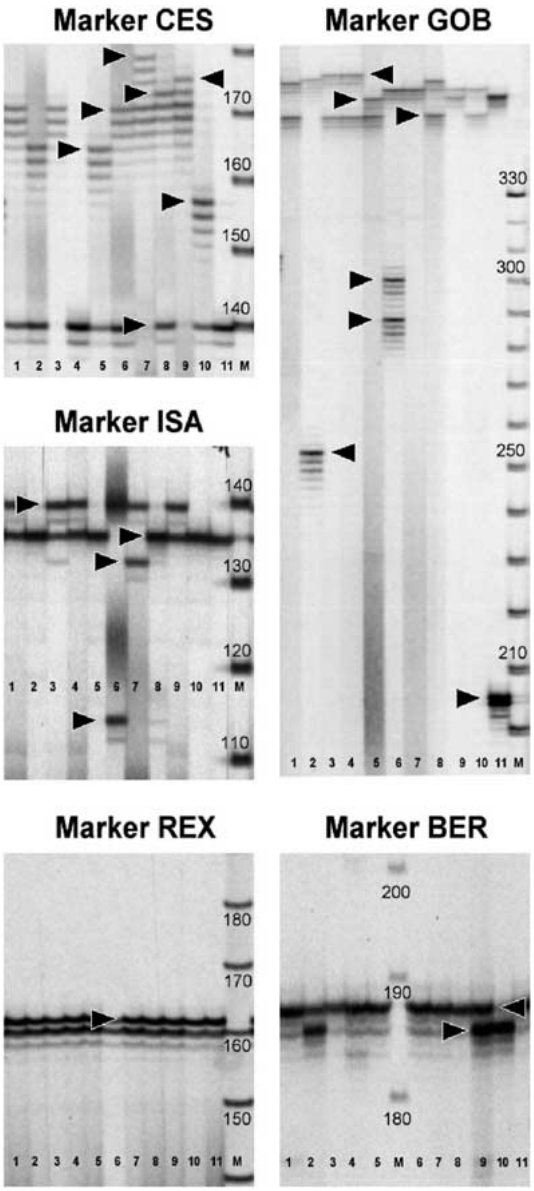

Figure 1. Microsatellite analysis of 11 *P. viticola* strains of the 1999 collection (Mezzocorona, Volano and Salorno populations) performed with the five SSR markers CES, ISA, REX, GOB and BER on polyacrylamide gels. Examples of alleles are indicated by arrows. Allele sizes for the CES, ISA, REX, GOB and BER are 140–179, 114–140, 162, 205–approximately 380 and 186–188 bases, respectively. On each panel numbers 1–11 indicate gel lanes loaded with amplicons of the test strain. The lane marked with M contains markers (30–330 bp DNA ladder). Generally two alleles in case of heterozygosity (marker BER, lane 2) or a single allele in case of homozygosity (marker CES, lane 11) were found. In rare cases more than two alleles were found (marker GOB, lane 6).

(averaged over all loci), either two alleles or a single one were found. The existence of two alleles indicates heterozygosity. The presence of a single one indicates either homozygosity (two overlapping signals), or a single allele and a null-allele. Exclusively for the locus GOB, in ten cases (14%) more than two alleles were detected (Figure 1, marker GOB, lane 6). In addition to clear and typical SSR stuttered bands, 7% (avg. over all loci) of shadowed and uncertain bands similar to SSR amplicons were identified (Figure 1; marker ISA, lane 3, lowest signal). Missing amplifications (6% avg. over all loci), unclear signals and the finding of more than 2 alleles, in total 17% of all the amplifications, brought to about 50% of genotypes not coded with a complete set of allele sizes.

HTM for DNA extraction from lesions and P. viticola *microsatellite PCR amplification*

One-hundred and ninty isolates collected in Navicello on 30 May 2000 were analysed following the HTM. From sample to sample, the percentage of infected to total leaf tissue was highly variable. Because of irregular and low yield ($\sim$0–2 ng μl^{-1}), previously determined on test samples, no quantification of DNA mixture (*V. vinifera–P. viticola* DNAs) was done.

A large number of PCR reactions (937/950) were successful. Two DNAs were not amplified by any of the SSR primer pairs, and this corresponds to 10 failed PCR reactions. Three amplicons appeared as uncertain and were not classified as alleles. On electropherograms the large majority of the amplicons generated by the 937 successful PCRs could be scored precisely and unambiguously. Four genotypes appeared as apparently polyploid for one locus only: in three cases (out of 190 genotypes) the SSR marker GOB showed four distinct alleles and in one case the SSR marker CES showed three alleles. Though the five loci of two strains (out of 190) were clearly amplified and the fragments precisely separated by electrophoresis (visible as raw data), the Fragment analysis software module (Beckman–Coulter) failed in computing allele lengths. In one case electrophoretic allele separation was performed incorrectly and the longest allele of the marker GOB could not be scored. Occurrence of non-specific and low fluorescing products has been observed, but that was not affecting the correct analysis of the results. Those uncertainties caused 12 miscoded genotypes (6.3%). Those genotypes could not be considered for population studies because of insufficient

(missing alleles) or excessive (>2 alleles per locus) data set.

Different degrees of polymorphism were observed: the most variable locus is GOB showing 43 alleles. CES and ISA showed a moderate variability, while BER and REX showed low and no variability, respectively. For every SSR marker, one allele always appeared at high frequency (f_{Mc}) (BER: allele 180.9, $f_M = 0.86$; CES: allele 143.0, $f_M = 0.46$; GOB: allele 210.0, $f_M = 0.27$; ISA: allele 144.1, $f_M = 0.59$ and REX: allele 163.9, $f_M = 1$) (Table 2). Nei's expected and observed gene diversities (Nei, 1973) range from 0

Table 2. Allele number, average size and frequency of the five SSR loci based on 190 samples of *P. viticola* collected in Navicello (Italy) on 30 May 2000 scored by fragment analysis

BER (3[a]/372[b])		CES (14/371)		GOB (43/363)		ISA (4/371)		REX (1/372)	
Length (bp)	Frequency	Length (bp)	Frequency	Length (bp)	Frequency	Length (bp)	Frequency	Length (bp)	Frequency
179.2	0.134	143.0	0.456	210.0	0.270	118.3	0.094	163.9	1
180.9	0.860	154.9	0.005	262.8	0.003	137.1	0.051		
184.6	0.005	159.1	0.005	267.6	0.006	140.2	0.261		
		161.1	0.019	269.4	0.008	144.1	0.593		
		167.2	0.005	271.4	0.006				
		169.4	0.005	275.5	0.003				
		171.5	0.019	277.3	0.008				
		173.6	0.073	281.5	0.003				
		175.4	0.243	283.2	0.003				
		177.7	0.124	285.0	0.008				
		179.5	0.019	289.2	0.019				
		181.2	0.005	291.9	0.003				
		183.6	0.005	294.1	0.052				
		185.8	0.016	296.0	0.003				
				298.2	0.039				
				302.3	0.017				
				304.2	0.003				
				306.6	0.014				
				308.4	0.006				
				310.5	0.033				
				314.7	0.036				
				318.9	0.006				
				323.0	0.003				
				329.0	0.003				
				352.2	0.003				
				359.1	0.003				
				361.1	0.019				
				363.1	0.003				
				365.2	0.006				
				367.2	0.003				
				369.3	0.088				
				371.3	0.003				
				373.3	0.080				
				375.6	0.014				
				377.7	0.085				
				379.5	0.006				
				381.6	0.072				
				385.7	0.022				
				389.8	0.014				
				391.8	0.003				
				394.0	0.014				
				396.1	0.008				
				434.0	0.006				

[a]Number of alleles found. [b]Number of alleles used for computations (theoretical maximum: 380 alleles).

Table 3. Expected (H_e) and observed heterozygosity (H_o), estimated frequency of null alleles (f null-allele) and probability of identity (PI) of the five *P. viticola* SSR loci

Locus	H_e^a	H_o^a	f null-allele[b]	PI[b]
BER	0.24	0.11	0.03	0.66
CES	0.71	0.79	−0.04	0.19
GOB	0.89	0.90	0	0.03
ISA	0.57	0.59	−0.01	0.36
REX	0	0	0	1

[a]Based on allele frequencies of 190 samples of *P. viticola* collected in Navicello (Italy) on 30 May 2000. [b]Based on allele frequencies of 158 genotypes of *P. viticola* found in Navicello (Italy) on 30 May 2000.

(locus REX) to 0.9 (locus GOB). The estimated frequency of null alleles showed probabilities ranging around zero for every SSR locus and probabilities of identity for each locus were maximal for the locus REX and minimal for the locus GOB (Table 3).

A comparison of the 178 totally coded isolates (93.7%) revealed the presence of 158 different genotypes in the vineyard studied. Only six genotypes were clonal: three genotypes were found twice, one genotype was found three times, one five times and one twelve times. Based on the allele frequencies of the 158 genotypes, the theoretical occurrence of the genotype homozygote for the five above-mentioned most common alleles ($tG_{o\,max}$) is 0.4%. In the Navicello plot the considered genotype was found once out of 158 genotypes (0.6%).

A spatial analysis revealed that the clonal genotypes were located mainly on the same leaf or on grapevines in close proximity (1–3 plants). Only in sporadic cases clones were located far apart.

Based on the number of alleles found within the 190 *P. viticola* strains sampled in the Navicello plot, it is potentially possible to distinguish 3×10^6 theoretical *P. viticola* genotypes (e.g. allele combinations) (tG).

Reliability and limits of the HTM

As already observed on polyacrylamide gels, PCR reactions for all loci were 100% reproducible: the banding pattern of all SSR markers for two DNAs were identical. For instance, the two alleles of the heterozygote locus GOB of one DNA were calculated as '209.94' bp (mean of 16 repetitions, st. dev.: 0.2) and as '294.16' bp (mean of 16 repetitions, st. dev.: 0.26), respectively.

However, a small but consistent allele length discrepancy between polyacrylamide gels and sequencer data (fragment analysis) has been observed for all the SSR markers. PCR products loaded on the sequencer appeared about 1.01–1.04 times longer than when loaded on polyacrylamide gels. For instance, the shortest GOB allele is calculated as 210 bp on the sequencer and 205 bp on gel (size ratio: 1.024), the shortest allele of locus ISA, was scored as 118.3 bp on the sequencer, compared to 114 bp on gel (size ratio: 1.038; effective allele size assessed by DNA sequencing: 113 bp) and the single D4-labelled REX allele appears as 163.9 bp on the sequencer and 162 bp on gel (size ratio: 1.012; effective allele size assessed by DNA sequencing: 163 bp).

An additional confirmation of the overestimation of the allele sizes is shown by the electrophoresis of the 15 aliquots of the six D3-labelled PCR products mixture (113, 154, 215, 276, 328 and 400 bp). A superlative ($R^2 = 1$) correlation has been found between real and measured allele length. The overestimation of the real fragment size is about 4 bases but the output values are highly reproducible. Standard deviations range from 0.42 bp (fragment 215) to a minimum of 0.1 (fragment 154) (Figure 2). Using the inverse function of the calibration line it is possible to calculate the effective allele length, at least for D3-labelled fragments.

Averaged over all loci, 95% of PCR amplifications were successful on DNA extracted from 1, 3 and 5 mg (dry weight classes) of well sporulating oil spots (Figure 3). While amplicons of the 3 and 5 mg classes produced intensive bands on the agarose gel, the 1 mg class led to very weak bands (results not shown).

Eppendorf-heated oil spots were difficult to amplify (80% success, average over all loci), whereas well sporulating-, moderately sporulating-, non-sporulating-, partially necrotic- and *in vivo* dried-lesions were successfully amplified in at least 90% of the cases (Figure 3). Locus GOB was generally the most difficult to amplify (14% of failure all over the 8 classes), especially in the case of EP-heated oil spots (50% failure; results not shown). In those cases, double PCRs as described for DNA extracted from sporangia must be performed to amplify the locus GOB.

On 1.1% agarose gel PCR products of the 5 SSR loci were clearly visible exclusively when *P. viticola* DNA was present (DNA extracted from sporangia and DNA mixture extracted from lesions). Grapevine DNA alone was never amplified with any SSR marker (only variety Chardonnay shown) (Figure 4).

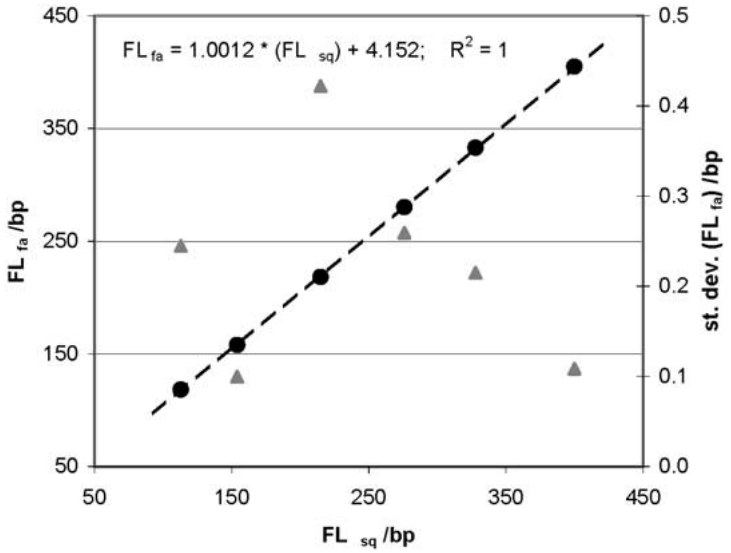

Figure 2. Correlation between real allele length and allele length determined by fragment analysis. One µl aliquots of a mixture of a set of six D3-labelled PCR products of known sequence (113, 154, 215, 276, 328 and 400 bp) were separated by electrophoresis (15 repetitions per fragment set). The linear regression between the two variables (left Y-axis, dots) shows a superlative correlation ($R^2 = 1$). The sequencer tends to overestimate the real fragment size by about 4 bases but output values are highly reproducible. Standard deviations (right Y-axis, triangles) range from 0.42 bp (fragment size: 215 bp) to a minimum of 0.1 (fragment size: 154 bp). FL_{fa}: fragment length determined by fragment analysis, FL_{sq}: fragment length determined by sequencing, st. dev. (FL_{fa}): standard deviation of FL_{fa}.

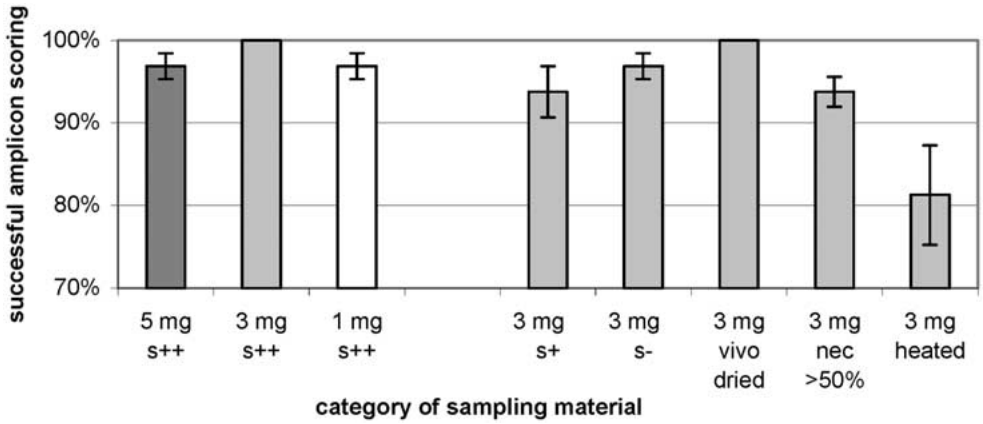

Figure 3. Influence of quantity and quality of sampling material on PCR amplifications and subsequent electrophoresis. One mg (white bar); 3 mg (grey bars); 5 mg (dark grey bar) oil spot quantity classes (dry weight) and five quality classes (s+; s−; dried *in vivo*; partially necrotic; in EP-heated) were tested. Histograms indicate the average percentage of clear and unambiguous electrophoretical analysis of the loci BER; CES; GOB and ISA (32 data points). Percentages less than 100% represent unclear signals or missing amplification. Eight samples per class were used: os: oil spots; s++ (highly sporulated os); s+ (moderately sporulated os); s− (not sporulated os); *in vivo* dried (os dried on a wilted grape seedling); nec >50% (os partially (>50%) necrotic); heated (os heated 4 h in an EP tube).

Discussion

Five of sixty-one SSR loci obtained could be used for population genetic studies. The obtained molecular markers were reproducible, specific, polymorphic, co-dominant and unambiguously scorable. Five other primer pairs produced confusing banding patterns with many artefacts and non-specific amplifications. In

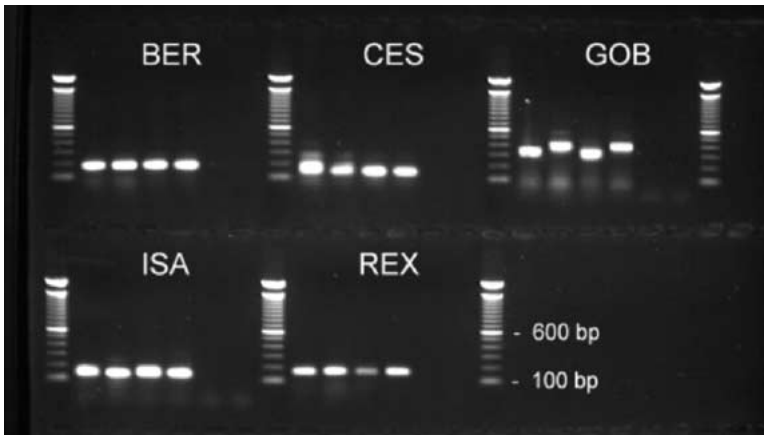

Figure 4. PCR amplification with the five SSR markers BER, CES, GOB, ISA and REX on a 1.1% agarose gel. DNAs used: lanes 1 and 2 (for each marker): *P. viticola* sporangia DNA; lanes 3 and 4: DNA extracted from two oil spots (*P. viticola* symptom; grapevine: Chardonnay); lane 5: DNA from one healthy Chardonnay leaf; lane 6: water as negative control. All the amplicon sizes are comprehended between 100 and 200 bp, with exception of the marker GOB that generates products between 300 and 400 bp. Successful amplifications only occur if the DNA of the pathogen is present, while grapevine does not affect the reaction.

those cases, distinguishing between the typical SSR amplicons and non-SSR amplifications on polyacrylamide gels was impossible. It remains unclear why the majority of the primer pairs designed failed to amplify any DNA sequence. The reasons causing this amplification failure could be following: it is possible that the DNA extracted from sporangia and successively used for constructing the *P. viticola* genomic library was contaminated by DNA belonging to bacteria or other micro-organisms living on grapevine leaves (for instance spores of other pathogens like *Uncinula necator* or *Botrytis cinerea*). The chance of collecting contaminants was high, considering the hundreds of sporulating lesions needed, but the ratio between the amount of contaminants to the amount sporangia of *P. viticola* tended clearly to the latter. Furthermore, each of the 46 primer pairs were tested on the plasmid on which they were designed, on sporangia DNA, on oil spots and on grapevine DNA. An amplification was always observed on plasmids, 10 times both on sporangia DNA and on oil spots (5 selected SSR markers and 5 markers that generate confused amplicon patterns) while it was never observed on healthy leaf tissue. This fact indicates that primer design was correct and that no other 'contaminating' micro-organism was present on sporangia, on oil spots or on leaves. Therefore, it seems more likely that artefacts could have been generated during any of the steps required for generation of the SSR library (digestions, ligations, clonings, PCR, transformations . . .) and not from contaminating DNAs of any origin.

For its huge polymorphism, the low probability of identity, high values of expected and observed heterozygoty, the most informative SSR marker for genotype identification is GOB. Nevertheless amplicon scoring deserves the greatest accuracy and precision. The best method for sizing the amplicons is surely fragment analysis. On polyacrylamide gels it was often impossible to assess the precise allele size of fragments longer than 350 bp because of low gel resolution and/or lack of direct comparison with alleles on nearby lanes. On the contrary the SSR marker REX appeared to be monomorphic in the Trentino populations, and was therefore totally useless for genotype discrimination. However, this marker could still be very useful for identifying interpopulation polymorphism on a larger scale. It could be a useful marker for newly introduced *P. viticola* strains from abroad.

The occurrence of null alleles is a possible problem associated with SSR markers (Callen et al., 1993). The presence of a null allele in an appreciable frequency can be suspected when the observed heterozygosity is markedly less than the expected heterozygosity. If undetected it can lead to genotype miscoding and loss of information. The obtained probability values are about 0 for all SSR markers, therefore miscoded homozygote genotypes should be a seldom event.

A small but consistent difference in allele sizes between polyacrylamide gels and fragment analysis was observed. This effect could be an artefact generated by the different labels used for detection of fragments.

As a consequence a set of alleles of each SSR marker should be verified for the different types of labelling (or detection technology) before the markers can be used in larger population genetics studies.

The genetic analysis showed that in most cases (178/182), a lesion is generated by a single genotype because each locus showed either one (homozygote or an allele and a null-allele) or two (heterozygote) alleles. If more than two alleles per locus were found (4 cases, markers CES and GOB) we speculate that what on the leaf was considered as a single oil spot, was effectively a mixture of two (or more) adjacent oil spots. Alternatively heterokaryosis could have taken place prior to that infection. Other sources of genotype misinterpretation could be cross-contaminations occurring during sample collection in the vineyard or the automated DNA extraction procedure, or amplicon mixing for fragment analysis. Although the Tecan Genesis RSP 150 robot and the Robbins Hydra microdispenser work with high accuracy, it is also possible that some cross-contaminations could have taken place during their operation. Anyway, considering the low number of apparently or real polyploid loci, the entire process from sample collection to fragment analysis is to be considered as robust, safe and reliable.

The use of SSR markers allows the study of the genotypes directly from lesions overcoming cumbersome and often unsuccessful isolation and cultivation of the pathogen *in vitro* (not possible for *P. viticola*) or tedious multiplication on agar plates (as for *P. infestans*). A small part of the infected tissue (3–5 mg dry weight) can be removed from the leaf so that a genetic analysis can be performed. Such a minimal removal of tissue leads to an almost undisturbed continuation of the disease in the field. Repeated observations in vineyards showed that the remaining part of a sampled lesion goes on sporulating under suitable weather conditions. A direct employment of oil spots for genetic analysis has several advantages over *in vivo* sporangia suction and multiplication on grape cuttings. First, since the quantity of sporangia available on an oil spot is extremely low, the entire amount should be aspirated. A removal of the totality of sporangia generates a shift back in the infection capability of the sampled isolate, and that leads to a shift in population dynamics compared to others genotypes. Second, we have to consider that multiplication in controlled conditions bears the risk of losing isolates and cross contaminations, in addition to being laboratory intensive practice. All aforementioned procedures are very time consuming and require great accuracy. This technique is only recommended if non-specific markers like RAPDs are to be applied.

It was shown that a PCR amplification is still possible even with sub-optimal sample quality (dried or partially necrotic lesions) or independently from the level of sporulation of the pathogen. Therefore, the described molecular markers can be used as diagnostic tools, especially BER or REX that often produce a strong signal consisting in two identical overlapping PCR products, in order to identify atypical *P. viticola* disease symptoms.

Preliminary studies performed with the HTM indicated high gene diversity within the 190 samples collected about two weeks after the first infection periods. Therefore, we speculated that the collected spots were mainly primary infections caused by oospores. A higher proportion of identical genotypes is expected among secondary infections, caused by the spread of asexually produced zoospores arising from sporulation of primary infections. In the future a more detailed analysis of population structure can be done. A better knowledge of *P. viticola* population genetics, obtained by the use of SSR markers, will allow quantification of the contribution of oospores produced in the vineyards, and possibly the role of asexual sporangia from distant vineyards in fuelling the epidemics. Moreover, it could lead to research on better and more precise decision aids in the form of forecasting models and control strategies not based on a calendar schedule or solely on risk situations.

References

Aldrich J and Cullis CA (1993) CTAB DNA extraction from plant tissues. Plant Molecular Biology Reporter 11: 128–141

Ashley MW and Dow BD (1994) The use of microsatellite analysis in population biology: Background, methods and applications. In: Schierwater B, Streit B, Wagner GP and DeSalle R (eds) Molecular Ecology and Evolution: Approaches and Applications (pp 185–201) Birkhäuser, Basel, Switzerland

Blaeser M and Weltzien HC (1979) Epidemiologische Studien an *Plasmopara viticola* zur Verbesserung der Spritzterminbestimmung. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 86: 489–498

Brown M, Moore JN, Fenn P and McNew RW (1999) Comparison of leaf disk, greenhouse, and field screening procedures for evaluation of grape seedlings for downy mildew resistance. HortScience 34: 331–333

Bruford MW and Wayne RK (1993) Microsatellite and their applications to population genetic studies. Current Opinion in Genetics & Development 3: 939–943

Burruano S, Conigliaro G and Di Graziano M (1990) Prime indicazioni sull'azione delle basse temperature sulla germinazione

delle oospore di *Plasmopara viticola*. Phytopathologia Mediterranea 29: 73–75

Callen DF, Thompson AD, Shen Y, Philips HA, Richards RI, Mulley JC and Sutherland GR (1993) Incidence and origin of 'null' alleles in the $(AC)_n$ microsatellite markers. The American Journal of Human Genetics 52: 922–927

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Journal of Heredity 86: 485–486

Groppe K, Sanders I, Wiemken A and Boller T (1995) A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichlöe* spp.) in grasses. Applied and Environmental Microbiology 61: 3943–3949

Gupta PK, Balyan HS, Sharma PC and Ramesch B (1996) Microsatellites in plants: a new class of molecular markers. Current Science 70: 45–54

Hill GK (1990) *Plasmopara* Risk Oppenheim – a deterministic computer model for the viticultural extension service. Notiziario sulle Malattie delle Piante 111: 231–238

Lafon R and Clerjeau M (1988) Downy mildew. In: Pearson RC and Goheen AC (eds) Compendium of Grape Diseases (pp 11–13) APS Press, St. Paul, Minnesota, USA

Lalancette N, Ellis MA and Madden LV (1988a) Development of an infection efficiency model of *Plasmopara viticola* on American grape based on temperature and duration of leaf wetness. Phytopathology 78: 794–800

Lalancette N, Ellis MA and Madden LV (1988b) A quantitative model for describing the sporulation of *Plasmopara viticola* on grape leaves. Phytopathology 78: 1316–1321

Laviola C, Burruano S and Strazzeri S (1986) Influenza della temperatura sulla germinazione delle oospore di *Plasmopara viticola* (Berk. et Curt.) Berl. et De Toni. Phytopathologia Mediterranea 25: 80–84

Nei M (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the United States of America 70: 3321–3323

Rafalski JA, Vogel JM, Morgante M, Powell W, Andre C and Tingey V (1996) Generating and using DNA markers in plants. In: Birren B (ed) Nonmammalian Genomic Analysis: A Practical Guide (pp 75–134) Academic press, San Diego, USA

Schruft G and Kassemeyer HH (1999) Rebenperonospora. In: Thomas Mann Verlag (ed) Krankheiten und Schädlinge der Weinrebe (pp 14–17) Gelsenkirchen-Buer, Germany

Tenzer I, Degli Ivanissevich S, Morgante M and Gessler C (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. Phytopathology 89: 748–753

Wagner HW and Sefc KM (1999) IDENTITY 1.0. Centre for applied genetics, University of agricultural sciences, Vienna (available at: *http://www.boku.ac.at/zag/forsch/identity.htm*)

Weber JL and May PE (1989) Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. American Journal of Human Genetics 44: 388–396

Wong FP, Burr HN and Wilcox (2001) Heterothallism in *Plasmopara viticola*. Plant Pathology 50: 427–432