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REDEFINING *PLASMOPARA VITICOLA* EPIDEMIOLOGICAL CYCLE

BY MOLECULAR GENETICS

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DEDICA *ET AL.*

E qui si chiude non solamente un dottorato, ma anche un quinquennio di vita. Che dire? Ho pagato il mio tributo alla scienza, computabile in un annetto di frustrazione (roba da tagliarsi le vene) ma in cambio ho ottenuto quattro anni tra i più belli della mia vita. Non ho mai viaggiato tanto (e così a scrocco) come in questo periodo. Ho iniziato col pupillo in Germania, per poi recarmi a Geisenheim dal Bernd, a Oppenheim dal Georg, a Epernay dalla Marie-Laure (che poi ha mollato l'osso), a Bordeaux dal Marc e soci e poi in Trentino, Veneto (cavolo, ho mancato il Piemonte, sono uno stupido!) dall'Ilaria (ricordo con piacere le gite in rampichino sulle Dolomiti... e le FUNIVIE soprattutto). Quando pensavo fosse tutto finito, ecco che la Marina m'invita a Bologna e il Mauro nella leggendaria Cugnasco. Come poi dimenticare i congressi? Quello in Portogallo è stato il più bello, abbiamo persino cantato in piedi sulle sedie "se mi sum cioc menim a ca la biciclèta", ma non da meno è stato quello a Napa (che strage e che sbragoooo!) con annesse le visite ai parchi nazionali. E le escursioni fitopatologiche? Abbiamo cavalcato il Trentino, la Grecia e la Romania. Abbiamo aperto gli occhi su realtà che mai ci saremmo immaginati. Questi ultimi tre viaggi ci sono stati più utili dal profilo umano che da quello scientifico. Devo aver dimenticato qualche posto in questa lista, ma fa niente, tanto avete capito l'antifona. Dal profilo umano ho conosciuto persone che ammiro (e invidio) per la loro determinazione e forza di carattere, come Cesare, Ilaria e Mauro, che (in momenti di alcolemia) potrei addirittura acquisire come modelli di vita. A parte gli scherzi, è bello avere frequentato persone tanto affidabili, schiette e oneste come loro. Ma la persona che più mi ha seguito col cuore e con la mente è certamente una sola, l'unica che un cervello esclusivista come il mio abbia saputo amare, apprezzare e stimare. E dal canto suo, colei che cinque anni fa ha osato entrare al Polyball dai sotterranei del Poly sfidando correnti torride e polveri radioattive, lei mi ha dato tutto il sostegno che un uomo possa desiderare. Rispetto, motivazione, incitamento, o forse, con una parola sola, amore. Grazie cara Marianna, per tutto quello che mi hai dimostrato, per esserci sempre stata, per avere respirato con me, per avermi abbracciato e mai più mollato nell'anno di frustrazione, per avere scacciato con tanta forza le tentazioni disequilibranti del destino. Per avere riconosciuto tanta forza in te, questo lavoro te lo dedico interamente. Se solo tu avessi lavato i piatti un po' più spesso, mi avresti reso davvero felice....

TABLE OF CONTENTS

ABSTRACT

The Oomycete *Plasmopara viticola* is the causal organism of downy mildew on grapevine (*Vitis* spp.). A set of four polymorphic microsatellite markers for *P. viticola*, amplifying the loci ISA, GOB, CES and BER, were developed. A semiautomatic high throughput DNA extraction method and sequencer-based microsatellite genotyping protocol were established.

In a survey of European grapevine downy mildew populations from 2000 to 2002, 10158 *P. viticola* samples were collected 1-22 times from 39 vineyards (41 populations). Using a first data subset (4685 lesions collected in central Europe from 18 plots), microsatellite markers revealed a new picture of the downy mildew epidemiological cycle. First, we discovered that numerous primary infections occur during a prolonged period from May to August. Genotypes identified once throughout the surveying period always constituted the dominant class (71% of all genotypes) independently from the collection date. Only seven genotypes were identified more than 50 times throughout the epidemic. Only one or two genotypes per epidemic underwent secondary cycles and generated a high number of progeny. A detailed epidemiological survey in a newly established Italian mountain vineyard confirmed that genotypes participating in the epidemic showed a highly variable and strain-specific aptitude in generating secondary lesions.

In order to examine the levels of genetic differentiation within and among *P. viticola* populations, we used a second subset of 9229 lesions from 34 downy mildew populations (France, Germany, Switzerland, Italy and Greece). 4328 site-specific genotypes were identified. The most polymorphic SSR marker GOB showed 101 alleles in Europe. We highlighted mainly significant differences between European population pairs, as well as a clearly differentiated genetic and phylogenetical population substructuring.

Knowledge furnished after genetic analysis challenges the actual unverified historical assumptions by providing solid bases targeted to redefining the role of primary and secondary cycles in disease dynamic and by downgrading the role of secondary sporangia for disease propagation.

RIASSUNTO

L'oomicete Plasmopara viticola è l'agente causale della peronospora della vite (*Vitis* spp.). Un set di quattro marcatori molecolari (microsatelliti), amplificanti i rispettivi loci ISA, GOB, CES e BER sono stati sviluppati per uno studio di popolazione. Sono state sviluppate un'estrazione di DNA semiautomatica altamente performante e un sistema di analisi del genoma basata sulla *fragment analysis* al sequenziatore.

Nel corso degli anni 2000-2002 sono stati raccolti 10158 campioni di *P. viticola* da 39 vigneti selezionati in cinque paesi europei produttori di vino (Francia, Germania, Grecia; Italia e Svizzera). Usando un subset di dati (4685 lesioni raccolte in Europa centrale da 18 vigneti), l'analisi ai microsatelliti ha rivelato un quadro rivoluzionario del ciclo epidemiologico della malattia. Dapprima è stata evidenziata una continua emissione di lesioni primarie dal mese di maggio a quello di agosto. I genotipi identificati una volta soltanto nel corso dell'epidemia si sono rivelati i più frequenti (71% di tutti i genotipi trovati), indipendentemente dalla data di raccolta dei campioni. Solamente sette genotipi sono stati identificati più di 50 volte nel corso dell'epidemia. Solo uno o due genotipi per epidemia hanno mostrato di riprodursi sessualmente in modo significativo rispetto alla moltitudine di altri genotipi. Un'analisi epidemiologica in un vigneto appena impiantato nella Val di Fiemme (Italia, 1005 m s/l/m) ha mostrato quanto ogni genotipo abbia un diverso successo riproduttivo.

Al fine di analizzare la differenza genetica tra popolazioni del patogeno su vasta scala, è stato selezionato un secondo data set comprendente 9229 lesioni raccolte da 34 popolazioni europee. Sono stati identificati la bellezza di 4328 genotipi specifici per ogni parcella. Il marcatore microsatellite più polimorfo si è rivelato GOB (101 alleli). A livello europeo, le popolazioni si sono rivelate per la maggior parte significativamente diverse dal profilo genetico, dal quale è risultata una filogenia piuttosto differenziata.

La nuova conoscenza fornita da questo studio ci permette di ridiscutere le speculazioni fatte in passato sul ciclo epidemiologico e sull'importanza delle infezioni primarie e secondarie nella conduzione di un'epidemia. Mentre le infezioni primarie sono più numerose del previsto, le progenie secondarie sono poco frequenti e poco persistenti nel tempo. La migrazione di sporangi secondari si è mostrata ridotta persino scala locale (vigneto).
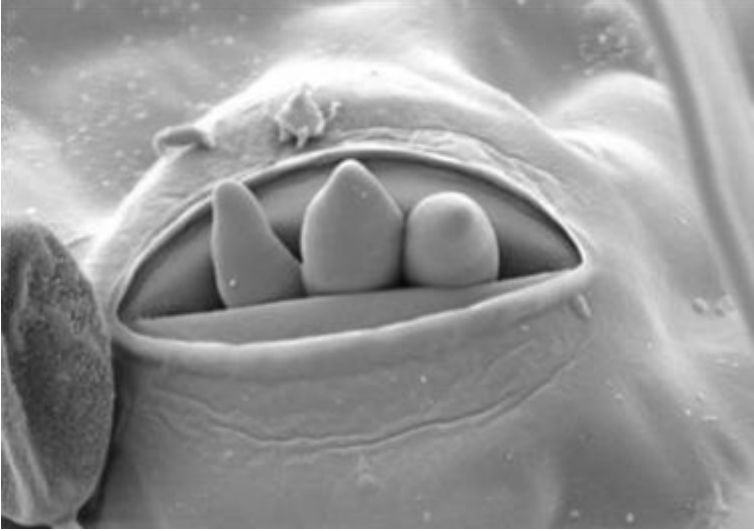
P. viticola exiting from stomata. Picture by: HH Kassemeyer

General Introduction

The diploid obligate biotrophic Oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni (Order: *Peronosporales*, Family: *Peronosporaceae*), the causal agent of grapevine downy mildew disease, was introduced to Europe (France) from North America in 1878, carried by American rootstocks. The dispersal of the pathogen was surprisingly fast: in 1879, it was reported to be 'all over France and Italy', the year after in Germany, Mosel region, (Müller & Schleumer 1934) and, in 1881, in Greece (Messinia region, Gennadios, 1889). Twenty years passed, however, until the occurrence of the first really disastrous epidemic in 1900 when, in a short time, the two-thirds of the expected yield was destroyed (Müller & Schleumer, 1934, Sarejanni, 1951). Since then, downy mildew constitutes the most damaging disease of grapevine of the European humid regions.

The introduction of *P. viticola* to Europe can be described as a founder event, with the American population being the parental population of the European one. Consequently, the population that developed was characterized by genetic drift, which followed the immigration of the pathogen. Despite the probably relatively low number of individuals that initially immigrated to Europe, in comparison to the large American effective population size, the Oomycete succeeded in colonizing many different susceptible host varieties and adapting to very different climatic conditions. Generalized susceptibility of the *Vitis vinifera* and favorable meteorological conditions, in combination with the diverse reproductive abilities of the organism, both sexual and asexual reproduction, (McDonald & Linde 2002) facilitated the rapid increase of the number of individuals to an epidemic level.

In European areas with high humidity, the disease destroys a crop without protection. Historically, protection was first achieved in 1880 with fungicides based on copper (Bordeaux mixture, Pscheidt & Ocamb, 1999) and then with specific compounds more recently discovered (such as Metalaxyl, Cymoxanil and Azoxystrobine). In Switzerland, for example, 7-9 fungicide treatments are regularly applied according to a calendar schedule against the disease. A large part of the sprays, however, are only applied as insurance against the highly erratic appearance of the disease and of the damages it causes (Jermini M, personal communication). In organic production, the control is currently based on the use of copper and sulfur combined eventually with new interspecific cultivars that have a quantitative resistance against downy mildew inherited from resistant American varieties.

To limit applications of the necessary sprays, most of the viticultural nations have developed downy mildew forecasting models. Those models are based upon a good knowledge of the climatic conditions necessary for infections. They provide decision aids in disease management that are the groundwork for any control strategy not based solely on regular, calendar-scheduled application of fungicides. For *P. viticola*, various models or prediction programs have been developed and are used in wide areas of France (Strizyk, 1984; Tran Manh sung *et al.,* 1990; Magnien *et al.,* 1991), Germany (Hill, 1993), Switzerland (Viret *et al.,* .2001) or are still in the developmental stage in Italy (Rosa *et al.,* 1995), Australia (Magarey *et al.,* 1991), Switzerland (Blaise *et al.,* 1999) and the USA (Park *et al.,* 1997). Those prediction aids, in combination with a correct application curative strategy, guarantee, under a severe disease pressure, a healthy crop, even for highly susceptible cultivars in humid years (Chardonnay, Riesling, a.s.o). Models, however, often fail to predict the real quantitative development of epidemics because none of them is able to quantify precisely the intensity and the spread of the inoculum at the plot level. Generally, they tend to overestimate the risk of infection and, therefore, unnecessary fungicide sprays are applied.

The biology of the pathogen has also been studied with much detail starting around 1900. It was recognized early on that *P. viticola* overwinters overwhelmingly, if not exclusively, as oospores in leaf or berries debris (Gregory, 1915). It is assumed that the maturation of oospores is distributed within a relatively short window (mid-to-end of May) and that after mid-June germination does not occur anymore (Cortesi & Zerbetto, 1994). Under suitable microclimatic conditions in the spring those oospores germinate and produce macrosporangia containing up to 60 biflagellated zoospores (Hill G, personal communication) which, once splash-dispersed, may cause primary infections. Incubation time is very variable, but it lasts from five to ten days on average. Symptoms appear as yellowish round spots (also called oil spots), which may produce sporulation. Suitable conditions for sporulation are: saturating humidity and temperatures around 18 $^{\circ}$C. Sporulation can be observed on the abaxial side of the leaf and on the surface of the young grapes. Secondary disease cycles can happen under suitable infection conditions, which are similar to those valid for oosporic-derived (primary) infections (Blaeser & Weltzien, 1979; Lafon & Clerjeau, 1988; Schruft & Kassemeyer, 1999). Secondary sporangia can be released

from sporangiophores and are wind-, dew- or splash-dispersed. Zoospores, released from sporangia, can infect other green tissues (Figure 1).
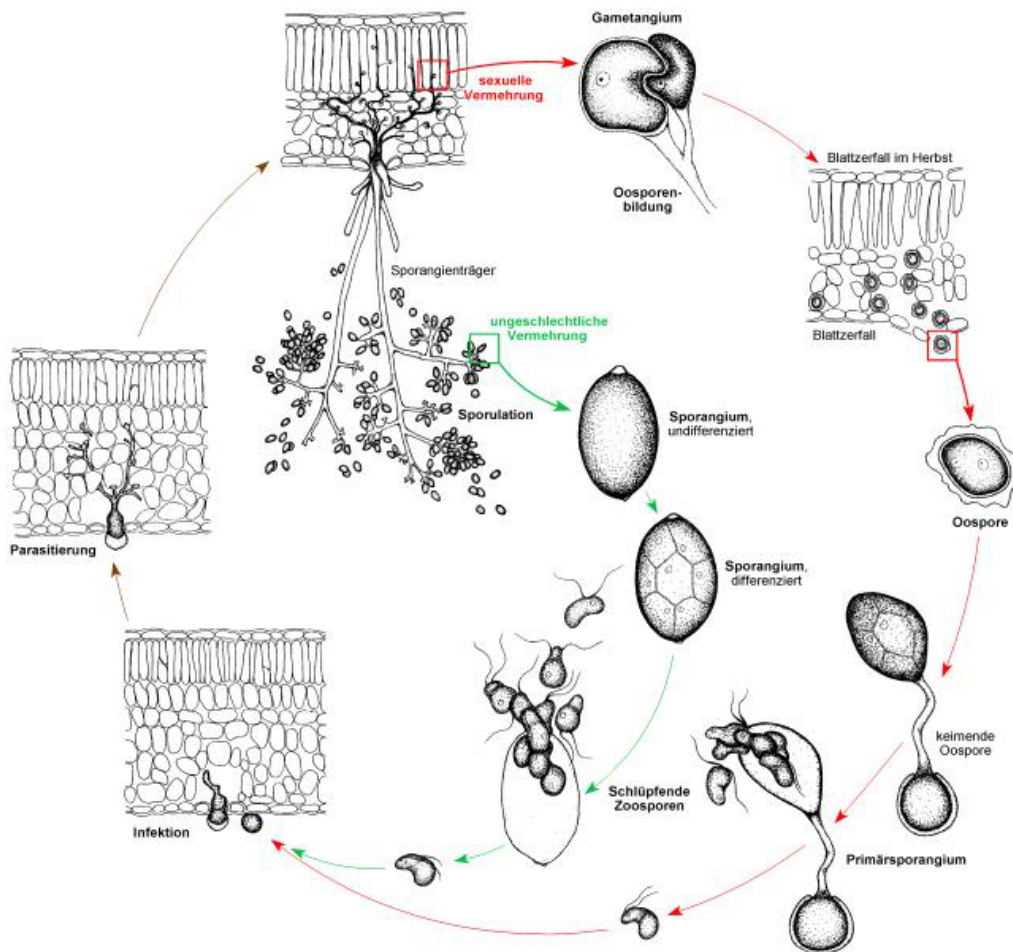
Figure 1. *Plasmopara viticola* life cycle.

One hundred years old opinions in viticulture state that epidemics start following a very small number of primary infections occurring within a relatively small time-window. After the rapid exhaustion of the infective capability of oospores, generally in May-June, secondary infections are supposed to continue and lead to the explosive increase of the epidemic until leaf fall in autumn. Therefore primary lesions are supposed to contribute extremely little to the disease severity while secondary lesions are consequently given the major responsibility

of it. Nevertheless, no evidence of the real involvement of primary and secondary sporangia to a naturally occurring epidemic has ever been experimentally proven or demonstrated.

This dissertation was conducted in order to test the above-mentioned historical assumptions and to overcome the lack of basic quantitative knowledge of the primary and secondary epidemiological cycles. In detail, we investigated 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. From the point of view of population genetics, 5) we also examined and compared patterns of genetic subdivision within and among oosporic populations at the European level. Multiallelic, neutral, variable and co-dominant DNA markers, such as microsatellites, were developed to address these issues (Ashley & Dow, 1994; Bruford & Wayne, 1993; Rafalski *et al.,* 1996). In the second chapter we will describe the establishment of a reliable and fast molecular method that allows the genotyping of the *P. viticola* strains, representing the basis of the whole study. In the third chapter, we will address the first four issues raised by surveying a very mild downy mildew population. In the fourth chapter we explore more deeply the first four issues by determining the relevance of primary and secondary cycles and the spread (migration) of secondary sporangia based on the survey of 18 central European epidemics. Finally, in chapter five, we will examine the genetic variability of European oosporic *P. viticola* populations and the relevance of gene flow at European scale.

The value of this dissertation consists in two major and synergistically interacting aspects. On one hand, the acquisition of quantitative data will contribute to better redefine the epidemiological disease cycle and consequently will help improving downy mildew forecasting models by indicating the magnitude of primary and secondary infection and their periods of occurrence. On the other hand, the same knowledge may lead disease management strategies to be targeted to specific epidemiological phases that can be critical for the pathogen. Furthermore, the exploration of the pathogen genetic diversity will allow a prediction of its ability to adapt to selection pressures, such as fungicide applications, and to environmental conditions, such as climate and host variety.

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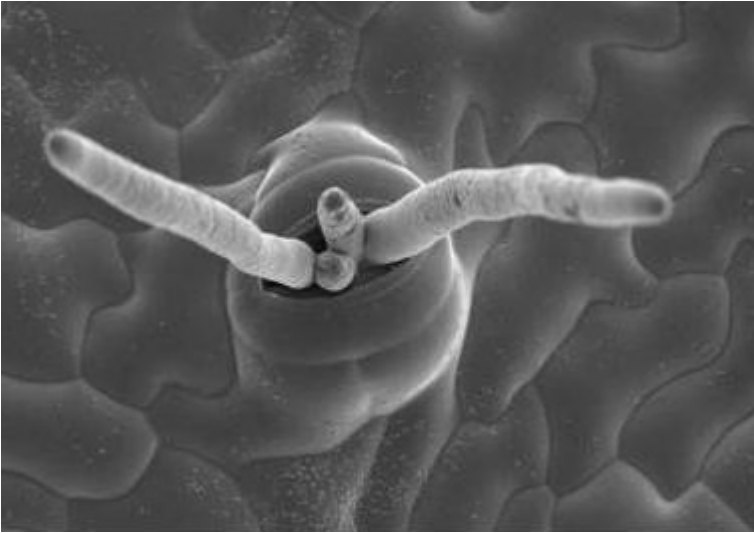
P. viticola exiting from a stoma. Picture by: HH Kassemeyer

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

ABSTRACT

The Oomycete _Plasmopara viticola_ is the causal organism of downy mildew on grapevine (_Vitis_ spp.). In order to set up the techniques for forthcoming investigation of unknown aspects of downy mildew disease dynamics and genetic structure, codominant, 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. 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 allows a molecular analysis of this obligate pathogen directly in the host without any isolation procedure, was developed. Quality and quantity of oil spots did not influence the SSR analysis in a remarkable manner. Amplified SSR loci were separated by electrophoresis onto a Beckman-Coulter 2000XL sequencer and automatically analysed. The objective of this study was to develop molecular biologic tools and methods that allow high throughput analysis of the downy mildew populations.

INTRODUCTION

Downy mildew, caused by the heterotallic (Wong *et al.*, 2001) diploid Oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni, is one of the most important grape (*Vitis vinifera*) diseases world-wide. 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 they often fail in predicting the quantitative development of epidemics for unknown reasons, which impedes their use in practice (Hill, 1990; Lalancette *et al.*, 1988a; Lalancette *et al.*, 1988b).

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 optimise 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 to 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 are firstly, the development of reliable SSR DNA markers for disease diagnosis and genotypic identification of *P. viticola* strains in an infected vineyard. Second, the establishment of a high throughput method that allows DNA extraction from *P. viticola* directly from infected grapevine leaves. Our efforts were directed to provide a methodology allowing examination of disease dynamics and genetic structure of *P. viticola* populations from within and between grape orchards.

MATERIALS AND METHODS

Identification of SSR markers specific for *Plasmopara 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: 2X reaction buffer (Pharmacia Biotechnlogy, 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 to 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 1X 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.

High throughput method (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, 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 cm², 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 manufacturer's protocol with the following modifications: samples were lysed in gCTAB 2X extraction buffer (2% CTAB, 2% PVP (MW 40.000), 1.4M NaCl, 20mM EDTA pH 8.0 and 100 mM TrisCl 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 5600g 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 2X extraction buffer. Five separate PCR amplifications per DNA were performed in a 10 µl volume containing 5 µl of a DNA solution (not quantified), 1x 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}: 0.014µM, 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 5 SSR markers simultaneously. As a size marker, 0.25 µl of a D1-labelled 60-420 base pair 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 base pairs) 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 sec; injection: 2 kV, 30 sec; 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:

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

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} * 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 base pairs). 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 hours 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 fifteen 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, forty-six 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).

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 *Plasmopara viticola* collected in Navicello (Italy) on May 30, 2000.

Locus	primer sequence 5'-> 3'	fpl	N a	repeat type[a]	all. size range
BER	BER$_f$[b]: AATGCAATGGTCTTCATCTCG BER$_r$: CTCTGCGGTAAAAGCCTGTC	D3	3	perfect $(TC)_n$	179-185 b
CES	CES$_f$: CTTGTCGGTAGGTAAGCGTG CES$_r$: GCTGTACTTACAACTTCATCAG	D2	14	compound $(TC)_n(AC)_n$	143-186 bp
GOB	GOB$_f$: CTTGGAAGTTATACCATGCTACC GOB$_r$: TTGAGAAATCGCACAGCTTA	D4	43	compound $(CT)_n(CTAT)_n$[c]	210-434 bp
ISA	ISA$_f$: ATTAGCGGCATGGACGTT ISA$_r$: GAGAAGTTCCGCCAAGTACA	D3	4	perfect $(TC)_n$	118-144 bp
REX	REX$_f$: CGTGTGCGATAGCAAAACTT REX$_r$: TTGCATTCGCACTCCCTTAC	D4	1	compound $(TC)_n(AC)_n$	164 bp

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

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, over 15 isolates of 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 ngµl^{-1}). Even with such low amounts of DNA, double PCR amplifications (total: 57 cycles) were overwhelmingly (97%) successful for each of the 5 SSR markers selected.

Eight times repeated PCR reactions from the same four DNA samples yielded always the same banding pattern for each of the 5 SSR markers.

On polyacrylamide gels the loci CES, ISA and REX (Fig. 1) produced amplicons that were easily scored. Locus BER (Fig. 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 (Fig. 1) were difficult to score. In 83% of all amplifications (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 (Fig. 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 (Fig. 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.

Marker CES

Marker GOB

Marker ISA

Marker REX

Marker BER
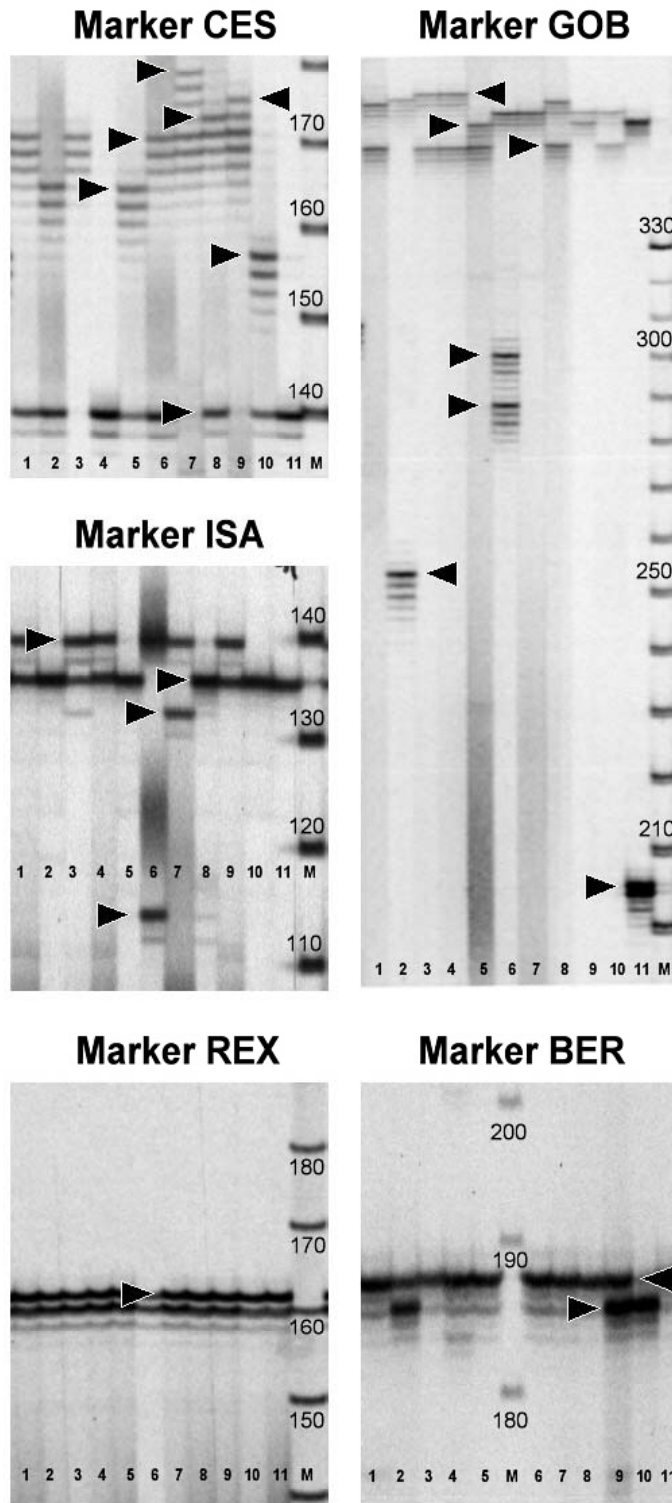
Fig. 1. Microsatellite analysis of 11 *Plasmopara viticola* strains of the 1999 collection (Mezzocorona, Volano and Salorno populations) performed with the five SSR markers CES, ISA, REX, GOB and BER on polyacrylamide gels. Examples of alleles are indicated by arrows. Allele sizes for the CES, ISA, REX, GOB and BER are 140-179, 114-140, 162, 205-approximately 380 and 186-188 bases, respectively. On each panel numbers 1 to 11 indicate gel lanes loaded with amplicons of the test strain. The lane marked with M contain 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).

25

High throughput method (HTM) for DNA extraction from lesions and *P. viticola* microsatellite PCR amplification

190 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 (~0 to 2 ngμl^{-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 to 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).

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

BER (3[a]/372[b])		CES (14/371)		GOB (43/363)		ISA (4/371)		REX (1/372)	
Size	Frq	Size	Frq	Size	Frq	Size	Frq	Size	Frq
179.2	0.134	143.0	0.456	210.0	0.270	118.3	0.094	163.9	1.000
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)

Nei's expected and observed gene diversities (Nei, 1973) range from 0 (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).

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

Locus	H_{exp}^a	H_{obs}^a	$f_{null\text{-}allele}^b$	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	1.00

a: based on allele frequencies of 190 samples of *Plasmopara viticola* collected in Navicello (Italy) on May 30, 2000.
b: based on allele frequencies of 158 genotypes of *Plasmopara viticola* found in Navicello (Italy) on May 30, 2000.

A comparison among the 178 isolates totally coded (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 5 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" base pairs (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 210.0 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 base pairs). 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 (st. dev.) range from 0.42 bp (fragment 215) to a minimum of 0.1 (fragment 154) (fig. 2). Using the inverse function of the calibration line it is possible to calculate the effective allele length, at least for D3-labelled fragments.
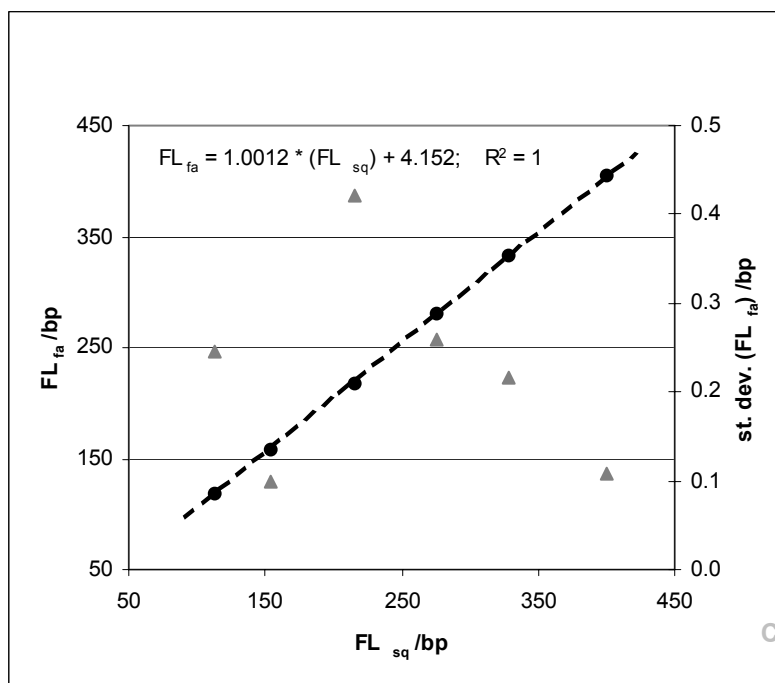
Fig. 2. Correlation between real allele length and allele length determined by fragment analysis. One µl aliquots of a mixture of a set of six D3-labelled PCR products of known sequence (113, 154, 215, 276, 328 and 400 base pairs) were separated by electrophoresis (15 repetitions per fragment set). The linear regression between the two variables (right 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 (left 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}.

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 (Fig. 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).

In EP-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 (Fig. 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.
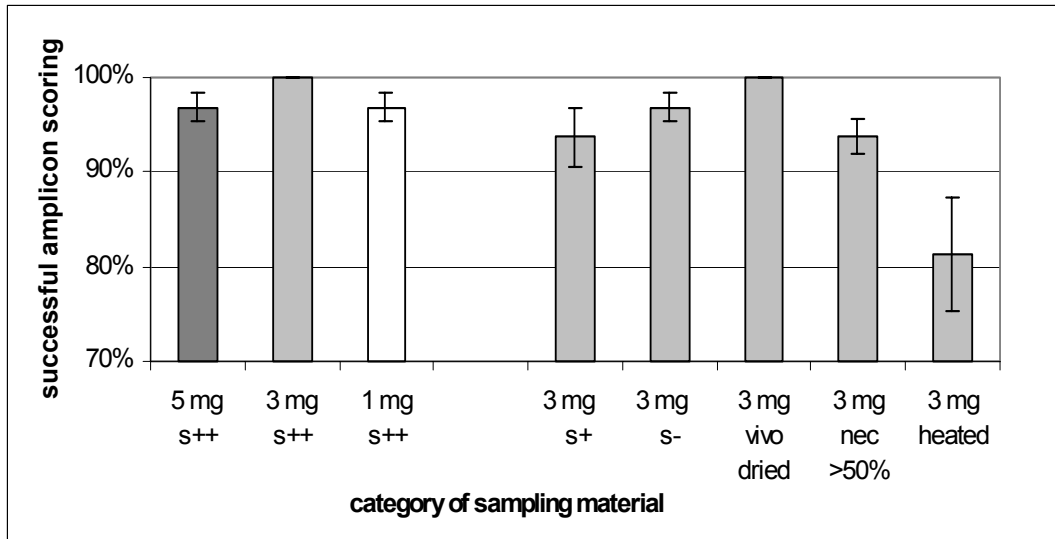
Fig. 3. Influence of quantity and quality of sampling material on PCR amplifications and subsequent electrophoresis. One mg (white bar) ; 3 mg (grey bars); 5 mg (dark grey bar) oil spot quantity classes (dry weight) and five quality classes (s+; s-; dried in vivo; partially necrotic; in EP-heated) were 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); vivo dried (os dried on a wilted grape seedling); nec >50% (os partially (>50%) necrotic); heated (os heated 4 hours in an Eppendorf tube).

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) (Fig. 4).
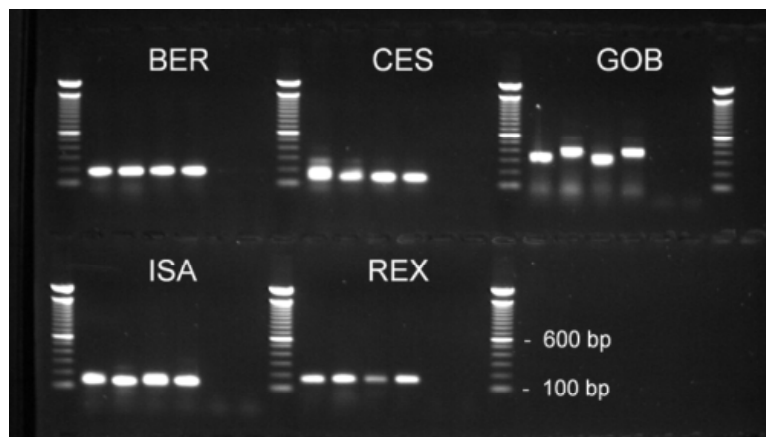
Fig. 4. PCR amplification with the five SSR markers BER, CES, GOB, ISA and REX on a 1.1% agarose gel. DNAs used: lanes 1 and 2 (for each marker): *P. viticola* sporangia DNA; lanes 3 and 4: DNA extracted from two oil spots (*P. viticola* symptom; grapevine: Chardonnay); lanes 5: DNA from one healthy Chardonnay leaf; lanes 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 doesn't affect the reaction.

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 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 appears to be monomorphic in the Trentino populations, therefore totally useless for genotype discrimination. However, this marker could still be very useful in 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 then 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. We thought that 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) nearby 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. Firstly, 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. Secondly, 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.

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P. viticola secondary sporangia. Picture by: HH Kassemeyer

Genetic structure of a *P. viticola* population in an isolated Italian mountain vineyard
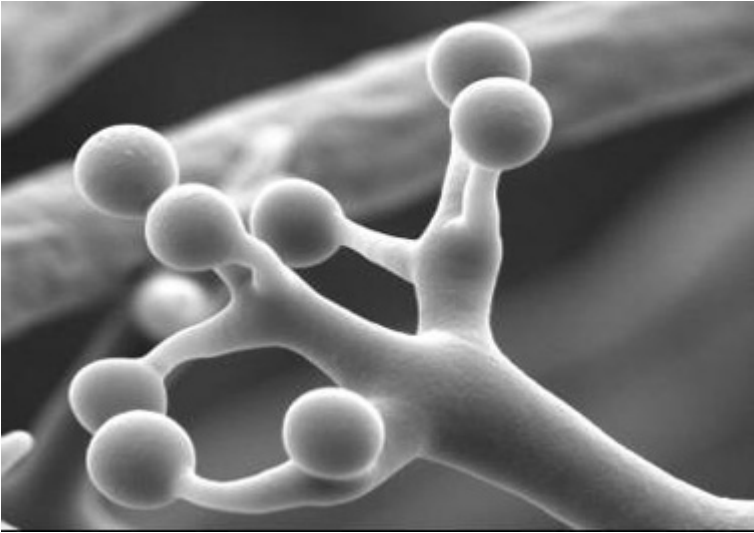
ABSTRACT

In this paper we investigated the genetic population structure of *Plasmopara viticola*, the causal organism of downy mildew on grapevines (*Vitis* spp.). We selected an isolated and newly established mountain vineyard, where the disease was observed for the first time. As soon as disease symptoms appeared, they were collected (3 samplings) and they were genetically analyzed by means of four microsatellite markers. Our study revealed that a human-mediated allochthon oosporic import, rather than naturally immigrating secondary propagules, presumably provided the starting inoculum that initiated the very mild epidemic. Fifteen genotypes initiated the disease during three of the at least seven infection periods. Genotypes participating in the epidemic showed a very variable and strain-specific aptitude in generating secondary lesions. Only two genotypes multiplied asexually and generated some tens of secondary lesions, probably due to the unfavorable climatic conditions of the mountain region. The mountain *P. viticola* population appeared with a poor gene pool, genetically separated and isolated-by-distance from the valley natural stocks as well as recently established by a founder effect event.

INTRODUCTION

Plasmopara viticola (Berk. and Curt.) Berl. and de Toni is the causal agent of downy mildew on grape. This heterothallic Oomycete (Wong *et al.*, 2001) is considered one of the most important grape pathogens. All green plant parts can be attacked. First symptoms generally appear as green-yellow lesions (also called oil spots) on the leaf surface. Suited conditions for sporulation are saturating humidity, darkness and temperatures around 18 °C. Sporulation can be observed on the abaxial side of the leaf and on the surface of the young grapes. The Oomycete overwinters as sexually produced oospore in fallen leaves and berries. In spring, with temperature above 10 °C and saturating humidity, the oospores germinate, produce macrosporangia which release zoospores. Generally, 5-10 days after the infection, depending from the temperature, the Oomycete produces microsporangia containing asexually produced zoospores (optimum: 18 °C and saturating humidity). Secondary disease cycles can happen under suitable infection conditions which are similar to those valid for oosporic-derived (primary) infections (Blaeser and Weltzien, 1979; Lafon and Clerjeau, 1988; Schruft and Kassemeyer, 1999).

Even if no clear proof is yet available, traditionally secondary sporangia have been viewed as the chief cause for disease spreading over time and space (Agrios, 1988; Blaise *et al.*, 1999; Lafon and Clerjeau, 1988). If a small number of secondary sporangia, as well as oospores or macrosporangia, are introduced to disease-free host areas a colonization process may start on disease-conductive plots. Such a phenomenon is known as a founder effect, which is usually characterized by reduced genetic variation in comparison to the parental population. The population established may be featured by a lowered fitness, a higher probability of extinction and a reduced potential for future adaptation (Hedrick *et al.*, 2001). Currently, quantitative investigations of propagules dispersal and its consequences have been greatly facilitated by the use of genetic markers (Knapova and Gisi, 2002), which allow determining the quantitative contribution of distinct genotypes to the epidemic (Flodgren *et al.*, 2000).

Non-genetic approaches, i. e. weather-driven epidemiological models, were developed in order to predict the magnitude of secondary and primary infections. The majority of the models assumed that an epidemic starts from a restricted and simultaneously occurring number of oosporic infections followed by a massive clonal multiplication (Blaise *et al.*, 1999; Lalancette *et al.*, 1988a,b). Other models are mainly based on estimations of relative amounts of oospores germinating during a time course (Hill, 1990; Hill, 2000; Stryzik, 1994). Models, however, often fail to predict the quantitative development of epidemics because none of them is able to precisely quantify the intensity and the spread of the inoculum at the plot level.

This study focused on comprehending the life cycle and genetic structure of *P. viticola* populations in order to help improve disease forecasting models and, consequently, disease management strategies. We investigated: 1) which meteorological factors initiated and fuelled a downy mildew epidemic; 2) the quantitative contribution of oospore-initiated infection to an epidemic by *P. viticola* specific SSR markers (Gobbin *et al.*, 2003), and 3) the magnitude of secondary infections arising from distinct primary infections. We examined these issues in a newly established Italian vineyard after the recent introduction of host and pathogen. Due to the isolated geographical location (30 Km from the nearest vineyard), we speculated that secondary sporangia immigration was extremely limited, and that the oosporic pool for epidemic initiation was restricted. Thus we also investigated 4) if this mountain population was the consequence of a founder effect and, therefore, characterized by low genetic variation. We compared genetic variation of the mountain population with four valley populations collected in locations where the pathogen was long established.

MATERIALS AND METHODS

Location of vineyards and meteorological data collection

The main trial vineyard (not treated with fungicides) was selected in Tesero (Fiemme Valley, Trentino, Italy). It lays 1005 m above sea level and is 30 Km from the nearest cultivated grapevine area (Adige valley, Trentino, Italy, Fig. 1). The Chardonnay grapevines were imported from France (Alsace) at dormant stage and planted in the summer of 1999. No disease carrying leaves or leave residues were visible and no *P. viticola* disease symptoms appeared in 1999, because the vines were treated with fungicides.
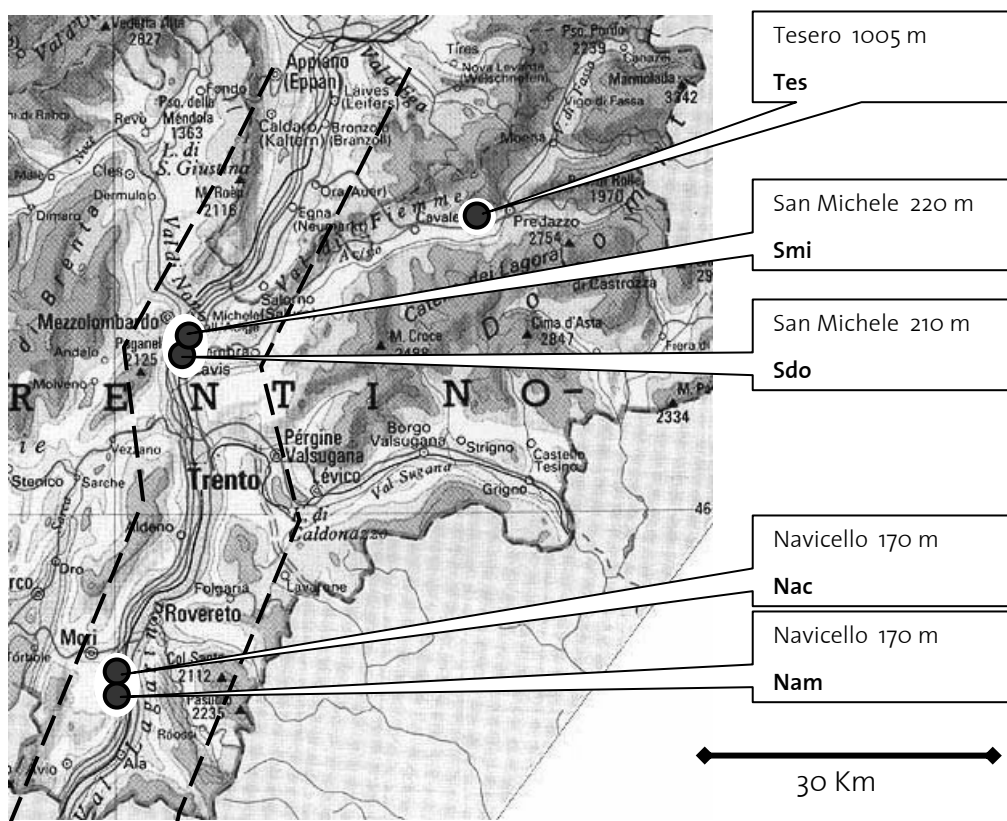
Fig. 1 Geographical map of the Trentino / Alto Adige region (Italy). The five vineyard locations are indicated by black dots. Plot altitude in metres is reported after location names. The grape cultivation area is located approximately within the dotted lines, following the Adige River.

We began our study during the spring 2000. Meteorological data (average daily temperature, amount of rain per hour and relative humidity) were recorded by means of a weather station placed in one corner of the plot. Grape growing stages were recorded weekly according to the Eichhorn-Lorenz stage scale (1977). Incubation periods were estimated using the table for daily cumulative percentage of incubation developed by Goidanich (1964).

We calculated periods of potential risk by soilborne (oosporic) inoculum using the SIMPO simulator. The spreadsheet-based computer model calculates a flexible index based on daily relative humidity, temperature and rainfall collected by the weather station. The output value indicates the number of days required by the oospores to germinate and, therefore, the potential risk of primary infections (Hill, 1990; Hill, 2000).

Four additional vineyards (not treated with fungicides) were selected within the wine producing Adige Valley (two in Navicello and two in San Michele). They are good representatives of valley vineyards because they are constituted by Chardonnay and Merlot grapevines and are trained by a pergola and Guyot system, respectively (Table 1 and Fig. 1).

Table 1. Vineyard locations, altitudes, years of plantation (yp), training systems, grape variety, rows (R), distance between rows (dr), vines per row (V), distance between grapes in a row (dv), total vines (Nv), plot area, sampling dates, sampling size (SS) and sampling densities (SD).

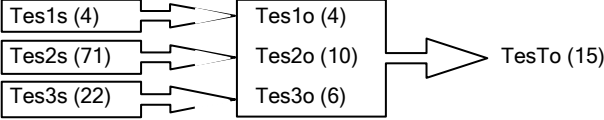| vineyard loc | altitude | training syst | R | V | Nv | sampl date | SS | SD |
	yp	grape variety	dr	dv	A			
Tesero (Tes)	1005 m	Guyot	16	18-23	328	23.06.00	4	0.010[a]
	1999	Chardonnay	1.5 m	0.8 m	394 m^2	02.07.00	71	0.180[a]
						14.07.00	22	0.056[a]
Navicello (Nac)	170 m	Single pergola	4	20-33	106	21.06.00	45	0.190
	1990	Chardonnay	2.8 m	0.8 m	237 m^2	06.07.00	80	0.338
Navicello (Nam)	170 m	Guyot	5	21	105	21.06.00	57	0.271
	1993	Merlot	2 m	1 m	210 m^2	06.07.00	86	0.410
San Michele (Sdo)	210 m	Double pergola	2	15	30	27.06.00	45	0.455
	1995	Chardonnay	5.50 m	0.6 m	99 m^2	18.07.00	76	0.768
San Michele (Smi)	220 m	Single pergola	4	11	44	26.07.00	75	0.758
	1980	Chardonnay	2.8 m	0.8 m	99 m^2			

[a] Sampling density has to be considered as maximal (as far all the oil spots were recognized by the observer), and it is calculated as: SS / plot area.

Sampling

In Tesero, sampling was performed as described in Gobbin *et al.,* (2003). Every identifiable disease symptom was collected (apical part only) as soon it was visible and after every new symptom appearance (total: 3 samplings within the same vineyard). After the last sampling (14.07.2000), chemical treatment was performed in the Tesero vineyard to prevent the epidemic phase of the pathogen (Table 1). Data from this mountain plot were used for epidemiological studies and for population differentiation assessment. Concerning the four valley *P. viticola* populations, Smi, Sdo, Nac and Nam (two located in San Michele and two in Navicello), samplings were performed employing the same methods and during the same period as done for the Tesero collection, but only a regular sampling (1-3 half lesions per vine collected) was possible because of high disease severity (10-100 lesions / vine). Data from those four valley plots were used for assessing and comparing population differentiation, only. Sampling densities (samples/m^2) for Navicello and San Michele were normally higher than for Tesero plot (Table 1).

Populations collected at a specific location on one sampling date, were coded by an abbreviation of the sampled location, followed by a sampling number ("s"-populations, Table 2). After SSR analysis, populations were clone-corrected ("o"-populations, Table 2); only the first identified individual per genotype was considered, while all the clones of the same genotype were removed. Afterwards, "o"-populations collected within the same plot were subjected to a pairwise test of differentiation using the overall loci G-statistic (FSTAT software, vers. 2.9.3.1, Goudet, 2001). Because we obtained exclusively non significant differences at the 5% nominal level, we pooled together "o"-populations, as they all were portions of the same population, we clone-corrected them again and we obtained the total oosporic populations (suffix "To"; Table 2).

Table 2. Vineyard locations, sampling dates, population names and sampling sizes (SS), names of the clone-corrected populations and corresponding number of genotypes (Ngen) and total population names and corresponding number of genotypes (Ngen).

vineyard loc	sampl date	Pop name[a] (SS)	Pop name[b] (Ngen)	Tot pop name[c] (Ngen)
Tesero (Tes)	23.06.00	Tes1s (4)	Tes1o (4)	
	02.07.00	Tes2s (71)	Tes2o (10)	TesTo (15)
	14.07.00	Tes3s (22)	Tes3o (6)	
Navicello (Nac)	21.06.00	Nac4s (45)	Nac4o (45)	
	06.07.00	Nac5s (80)	Nac5o (78)	NacTo (120)
Navicello (Nam)	21.06.00	Nam3s (57)	Nam3o (47)	
	06.07.00	Nam4s (86)	Nam4o (81)	NamTo (127)
San Michele (Sdo)	27.06.00	Sdo3s (45)	Sdo3o (45)	
	18.07.00	Sdo4s (76)	Sdo4o (68)	SdoTo (104)
San Michele (Smi)	26.07.00	Smi3s (75)	Smi3o (64)	SmiTo (64)

a: Sampled populations ("s"-populations)

b: Oosporic populations ("o"-populations) were obtained after clone-correcting the sampled populations. Only the first identified individual per genotype was considered, while all the clones of the same genotype were removed.

c: Total oosporic populations were obtained after pooling and subsequently clone-correcting "o"-populations.

Sample processing and genotype identification

For genotyping the collected samples, we used automated high throughput DNA extraction, PCR amplification of the four *P. viticola* specific SSR loci BER, CES, GOB and ISA, sequencer based fragment analysis and automatic allele scoring. Every step was performed exactly as described in Gobbin *et al.* (2003). *P. viticola* genotypes were defined as a unique SSR allele pattern after PCR amplification with the four microsatellite markers BER, CES, GOB and ISA.

For reasons of convenience, genotypes were named by merging an 8-letter code, each letter or letter set according to the SSR allele pattern. The detailed coding methodology is not described because not relevant to this study. The same 8-letter code means "same genotype", while different codes mean "different genotypes". We assumed that each oospore contains a single nucleus (Gobbin *et al.*, 2003; Gregory, 1915) that it is asexually inherited to the progeny (secondary lesions), and that asexual cycles did not alter substantially the microsatellite allele pattern. Therefore, two lesions showing identical allele patterns (same genotype) were interpreted as clonal progeny, deriving from the same primary lesion. Conversely, two oil spots showing different genetic profiles were interpreted as deriving from independent oosporic infections. Those oil spots may have experienced four different life histories: they can be the result of infections derived from macrosporangia (oospores), either previously sexually produced within the considered vineyard (autochthon primary lesions) or immigrated to the considered vineyard (allochthon primary lesions). Alternatively, they can derive from secondary sporangia either released from unobserved lesions issued within (autochthon secondary lesions) or outside the considered plot (allochthon secondary lesions).

Population differentiation

Unless noted otherwise, statistical analyses were conducted using GENEPOP on the web (Raymond and Rousset, 1995). For each of the four loci, we estimated allele frequencies and observed and expected heterozygosities for clone-corrected and non clone-corrected populations (Table 2). We estimated probabilities of departure from Hardy-Weinberg equilibrium under the null hypothesis H_o of random union of gametes, within each population for each locus. Markov Chain Monte Carlo tests were performed with 1000 dememorization steps and 1000 batches of 1000 iterations of each.

The occurrence of null alleles, as a consequence of deletions or mutations at primer hybridisation sites, 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 then the expected heterozygosity. If undetected, it can lead

to genotype miscoding and loss of information. To account for this problem, the presence of "null" (also called nonamplyfing) alleles within populations was calculated with the software Identity 1.0 (Wagner and Sefc, 1999).

We used pairwise F_{st} estimates (Wright, 1969) to quantify the magnitude of differentiation in allele frequencies among populations. Differences among populations in their genetic composition were examined by testing heterogeneity in allele frequencies under the null hypothesis of no difference among populations for pairwise and overall differences. Significance in pairwise comparisons was evaluated with a sequential Bonferroni adjustment of critical probabilities. For computing F_{st} significance (4500 permutations), we used the software FSTAT vers. 2.9.3.2 (Goudet, 2001).

The theoretical number of different genotypes (tG) that can be discriminated by means of the four SSR loci and the theoretical occurrence of the genotype that shows homozygosity for the most frequent allele at each SSR locus ($tG_{o\,max}$) were calculated according to Gobbin *et al.* (2003). Very roughly, a high value of tG can be interpreted as high allelic diversity and indicates a high "resolution" ability of the employed SSR markers. The probability that two individuals, carrying the most frequent alleles for each locus, may have been generated by two independent recombination events is indicated by $tG_{o\,max}$.

Isolation by distance

We constructed a graph showing the relationship between pairwise F_{st} and the natural logarithm of geographical distance. We used ten independently sampled and clone-corrected data sets to compute the test; the seven valley populations and the three mountain populations ("o"-populations, Table 2). Distances between clone-corrected populations collected in different times but within the same plot were set to 10 m. Pairwise distances among the three collection sites were calculated by means of geographical maps of the Trentino region (San Michele – Navicello: 42 Km, San Michele – Tesero: 31 Km and Tesero – Navicello: 62 Km, San Michele (Smi) – San Michele (Sdo): 500 m and Navicello (Nac) – Navicello (Nam): 100 m). Along the two locations in the Adige valley, grapevine cultivation covers approximately 1/3 of the cultivation area. On the contrary, the linear path from each

valley location to Tesero follows a non-host, highly mountainous region (max altitude = 2500 m). Significance in the isolation by distance relationship was tested statistically using a Mantel test (555 permutations) available within the GENEPOP package (Rousset, 1997). This test assesses whether the pairwise genetic distance matrix is correlated with the pairwise geographic distance matrix.

RESULTS

Meteorological conditions, disease initiation and development

During the period 31.05.00 – 20.07.00, 136 mm of rain fell on the Tesero plot. The rains were unequally distributed over time. Rain intensities varied within the range 0.2-12 mm/h, and the average daily temperature was always between 10 °C and 20 °C (Fig. 2).
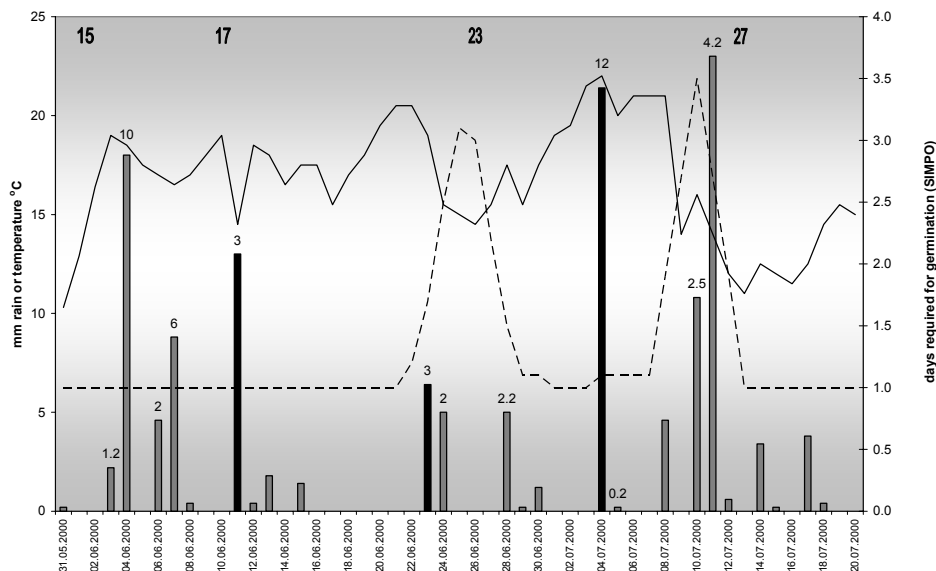
Fig. 2. SIMPO simulation of oosporic maturation (dotted line), rain amount (bars, mm), rain intensity (numbers above bars if >1 mm/h), average temperature (line, °C) recorded in the Tesero plot during the period 31.05.00 - 20.07.00. White circles indicate oil spot appearance; black columns indicate infective rains. Vertical fat arrows indicate sampling dates; horizontal arrows indicate the days required to fulfill the process of incubation after Goidanich (1964), followed either by oil spot identification (black arrows) or no symptom identification (dotted grey arrows); bold top numbers describe grapevine phenological stage (Eichhorn-Lorenz, 1977); patterned segment indicate the protective effect of the applied fungicide.

Both on 04.06.00 and 07.06.00 conditions for primary infections were fulfilled (Goidanich, 1964; Hill, 2000). At the end of the previewed incubation period, however, on 11.06.00 and 15.06.00, respectively, no symptoms were recognized on our experimental plot.

The rain of 11.06.00 (maximal intensity: 3 mm/h; total: 6,6 mm in 7 hours) was supposed to be the activating event to initiate the downy mildew epidemic. According to the SIMPO simulation (Hill, 2000), oospores were mature to germinate and generate the very first primary infections. As the Goidanich table estimates, 10 days after the rain of 11.06.00, incubation time was over and first symptoms were recognized by the observer within the poor grapevine vegetation (Eichhorn-Lorenz stage 21-22). Rains of 23-24.06.00 allowed both new primary infections and sporulation of the recently appeared oil spots, which was followed by secondary sporangia dispersal. Unlike the first infection date, SIMPO simulated 1.5 and 2 days for oosporic maturation on 23.06.00 and 24.06.00, respectively. The Goidanich table (1964) estimates 9 days to complete incubation after each date. The rainy period 28-30.06 did not induce infections, even though humidity could have been sufficient at least for secondary cycles. On 04.07.00, heavy rains allowed both new primary infections and secondary infections. Symptom appearance was expected on 14.07.00 following Goidanich (1964), because oospores were mature to germinate (Hill, 2000) during the rain (Fig. 2). On 23.06.00, the Tesero vineyard showed 4 oil spots (Tes1s). From 9 to 21 days later, the cumulative number of lesions increased to 75 and 97 lesions, respectively. Disease incidence on vines (defined as number of infected vines per vineyard) already reached its maximum on June, 2 (Table 3).

The epidemics in Navicello and San Michele started about one month earlier than in the Tesero plot, and were characterized by more explosive development (data not shown).

Table 3. Genotype-specific contribution to the epidemic: identification date, *P. viticola* population, number of observations and frequencies of the 15 genotypes identified in Tesero. Sampling size (SS), cumulative sampling size (cum SS), number of infected vines (N inf vines), incidence (inc on v) and cumulative incidence (cum inc on v) on vines.

genotype name	23.06.00 Tes1s[a]	02.07.00 Tes2s	14.07.00 Tes3s	total
evuzerca	1 (25)[b]			1
ezezomca	1 (25)			1
abewimna		1 (1.4)[b]		1
abewisma		1 (1.4)		1
aguverre		1 (1.4)		1
evuzemco		1 (1.4)		1
ezazinta		1 (1.4)		1
ezezumca		1 (1.4)		1
abegasra			1 (4.5)[b]	1
ebezemca			1 (4.5)	1
ezezemca			1 (4.5)	1
evivurro	1 (25)	20 (28)		21 (22)[c]
evuzemca	1 (25)	43 (60)	15 (68)	59 (61)
alovurma		1 (1.4)	1 (4.5)	2
evivusro		1 (1.4)	3 (14)	4
total genotypes	4	10	6	15
SS	4	71	22	97
Cum SS	4	75	97	97
N inf vines	4	18	7	22
Inc on v[d]	1.2%	5.5%	2.1%	6.7%
Cum inc on v	1.2%	6.7%	6.7%	6.7%

a: population name

b: frequency in % (100% refers to population size)

c: frequency in % (100% refers to 97 lesions)

d: infected grapes / tot grapes (N=328)

Genotype identification and population structure and dynamic

In the period 23.06.00 – 14.07.00, fifteen *P. viticola* genotypes were discriminated among 97 lesions collected (Table 3). Tes1s population consisted in 4 different genotypes causing 1 oil

spot each. Only two of the four genotypes identified were able to generate progenies, while the remaining two were not re-identified again. *Evuzemca* is the genotype that contributed the most to the epidemic: it was responsible for 60% of the total lesions on 02.07.00 and 68% on 14.07.00. *Evivurro* caused 20 lesions at the beginning of July (28% of total lesions) but, afterwards, it was not able to multiply asexually again. 83% of the total lesions were due to the combined pathogenicity of the two latter genotypes. On 02.07.00, eight new genotypes (putative primary infections) contributed with a single lesion each to the epidemic. Two of them were re-identified at the last sampling date whereas six genotypes disappeared from the plot. Three new genotypes were identified on 14.07.00, causing minor injury (13.5% of total lesions; Table 3). None of the genotypes identified in the Tesero populations were identical to the valley populations (cross comparison not shown). While in Tesero only 15 genotypes were identified among 97 lesions, in the valley populations almost all lesions collected revealed a different genotype. The maximal number of clones was observed in San Michele (Sdo) on 18.07.00, when 68 distinct genotypes were identified among 76 sampled lesions (1.12 lesions/genotype).

Genetic variation

SSR analysis revealed different degrees of polymorphism among microsatellite markers of the Tesero populations. Low diversities were observed at the loci BER and ISA (number of alleles: k=2), while more alleles were recorded with the markers CES and GOB (k_{CES}=6 and k_{GOB}=13; Table 4).

In Tesero, Nei's (1973) expected heterozygosity ranged from a minimum of 0.25 (locus BER, Tes3s) to a maximum of 0.75 (locus GOB and CES, Tes1s). Oosporic data sets (Tes2o, Tes3o and TesTo) revealed similar (loci ISA and BER) or higher H_{exp} values than complete data sets. In all the mountain data sets, a substantial heterozygosity excess for the loci ISA, GOB and CES was observed, whereas a heterozygosity deficiency was exhibited by the locus BER. Although the four valley populations do not differ considerably in H_{obs} and H_{exp} values from mountain populations, we found a higher allele diversity (k_{ISA}=4; k_{GOB}=30-38; k_{CES}=10-15; k_{BER}=2-3). This pattern occurred even though we collected much less representatively (fewer oil spots in

regard to population size) in the valley populations than in the mountain populations (Table 4).

Considering the mountain "s"-populations (Tes1s, Tes2s and Tes3s), genotype frequencies at all loci were in accordance with Hardy-Weinberg (HW) expectations only for the Tes1s population (constituted by four individuals). Tes2s and Tes3s were obviously not in equilibrium because of the effect of clonal multiplication of essentially two genotypes that highly increased allele frequency of a sub set of alleles (Table 4). Clone-corrected total populations (suffix "To"), comprehending valley populations, were generally in HW equilibrium. Mostly the locus BER in Tes2o, TesTo, NamTo, SdoTo and SmiTo, and the locus CES in TesTo, showed significant deviations from HW expectations.

Table 4. Summary of genetic variation within each of the three sampled *P. viticola* populations, the clone-corrected Tesero data sets and the four total oosporic valley populations. Total N refers to the number of individuals per population surveyed. SSR markers are listed in the headings. Stars represent significant ("*": p<0.05) and highly significant ("***": p<0.001) deviations from Hardy-Weinberg equilibrium.

Population		number of alleles (k)				heterozygosity (observed/expected)				
	N	k_{ISA}	k_{GOB}	k_{CES}	k_{BER}	ISA	GOB	CES	BER	overall
Tes1s	4	2	4	3	2	1.00/0.50	1.00/0.75	0.75/0.75	0.00/0.50	0.69/0.63
Tes2s	71	2	11	6	2	0.94/0.50***	1.00/0.66***	0.70/0.69***	0.01/0.44***	0.67/0.57
Tes3s	22	2	6	4	2	0.91/0.50***	0.96/0.65***	1.00/0.67***	0.00/0.25***	0.71/0.51
Tes2o	10	2	11	6	2	0.60/0.43	1.00/0.91	0.80/0.82	0.10/0.50*	0.63/0.67
Tes3o	6	2	6	4	2	0.67/0.47	0.83/0.85	1.00/0.79	0.00/0.33	0.63/0.61
TesTo	15	2	13	6	2	0.67/0.45	0.93/0.88	0.87/0.79*	0.07/0.39*	0.63/0.63
NacTo	120	4	34	15	2	0.50/0.50	0.81/0.90	0.78/0.75	0.13/0.17	0.56/0.59
NamTo	127	4	38	13	3	0.52/0.50	0.91/0.93	0.69/0.75	0.08/0.13*	0.55/0.58
SdoTo	104	4	30	10	2	0.57/0.49	0.90/0.94	0.66/0.60	0.12/0.16*	0.56/0.55
SmiTo	64	4	30	10	2	0.34/0.37	0.87/0.94	0.61/0.67	0.13/0.22*	0.49/0.55

Null allele frequency estimation

For the loci ISA, GOB and CES, null allele frequency for any population is seldom estimated above 5%; ensuring a correct genotyping of the samples for those markers. The locus BER shows instead about 25% probability of having null alleles in the clone-corrected mountain populations and a maximum of 8% for the valley populations. All negative values for allele frequencies were considered as equal to "zero" (Table 5).

Based on allele frequencies of oosporic populations, the theoretical number of different genotypes (tG) that can be discriminated by means of the four SSR loci is approximately 10^4 (order of magnitude) for the Tesero total oosporic population and reaches about 100 times higher values for the valley populations (Table 5). The theoretical occurrence of the genotype that shows homozygosity for the most frequent allele at each SSR locus ($tG_{o\ max}$) is for all populations about $3*10^{-3}$ (Table 5). This genotype was identified exclusively in San Michele on 27.06.00 (Smi3s).

Table 5. Null allele frequency estimation of the 8 clone-corrected *P. viticola* Trentino populations for all loci, theoretical maximal number of different genotypes (tG) and theoretical occurrence of the most common genotype ($tG_{o\ max}$).

Population	ISA	GOB	CES	BER	tG	tG_{omax}
Tes1s	$<0^a$	<0	<0	0.27	$5.4*10^2$	$2.78*10^{-3}$
Tes2o	<0	<0	<0	0.24	$1.2*10^5$	$1.16*10^{-3}$
Tes3o	<0	<0	<0	0.22	$1.9*10^4$	$3.80*10^{-3}$
TesTo	<0	<0	<0	0.21	$1.7*10^4$	$1.68*10^{-3}$
NacTo	0	0.05	<0	0.03	$4.3*10^6$	$4.19*10^{-3}$
NamTo	<0	0.06	0.01	0.05	$4.0*10^6$	$1.83*10^{-3}$
SdoTo	<0	0.02	<0	0.04	$1.5*10^6$	$2.68*10^{-3}$
SmiTo	0.01	0.03	0.03	0.08	$1.5*10^6$	$2.61*10^{-3}$

a: slightly negative probabilities were noted by "<0".

Population differentiation

No private allele was found in the Tesero oosporic population (Table 6). In 5 cases of 38, the rarest alleles of the valley populations were detected within the Tesero populations. High differences in allele frequencies were observed among valley and mountain pairwise comparisons (as for GOB-385.7, CES-143.0, BER-179.2).

Table 6. Average allele sizes (bp) and allele frequencies of the total Tesero oosporic population (TesTo) and of the four oosporic valley populations. Bold values refer to alleles occurring at the highest frequency.

	TesTo	NacTo	NamTo	SdoTo	SmiTo		TesTo	NacTo	NamTo	SdoTo	SmiTo
Locus GOB						**Locus ISA**					
205.8[a]	-	-	-	0.005(-)	0.008(-)	118.3[a]	-	0.071	0.075(-)	0.096	0.063(-)
210.0	0.133	**0.263**	**0.177**	**0.144**	**0.148**	137.1	-	0.054(-)	0.098	0.043(-)	0.070
260.7	-	-	0.004(-)	-	-	140.2	0.333(-)	0.204	0.146	0.178	0.078
265.1	-	-	0.004(-)	-	-	144.1	0.667	0.671	0.681	0.683	0.789
267.6	-	-	-	0.019	-						
269.4	-	0.008	0.012	-	-						
271.4	-	0.004(-)	0.004(-)	0.005(-)	-	**Locus CES**					
273.4	-	0.008	0.020	-	0.016						
275.5	-	0.008	0.008	-	-	143.0[a]	0.100	**0.404**	**0.382**	**0.577**	**0.500**
277.3	-	0.017	0.016	0.010	0.023	145.1	-	0.008	0.008	-	-
279.3	-	0.004(-)	-	0.005(-)	-	154.9	-	0.004(-)	-	-	-
281.5	-	0.013	0.012	-	0.008(-)	159.1	-	0.008	0.016	-	0.008(-)
285.0	-	0.017	0.008	0.043	0.047	161.1	-	0.004(-)	-	0.029	0.016
289.7	-	0.017	0.028	0.005(-)	-	163.1	-	-	0.008	-	-
291.9	-	0.004(-)	-	-	-	165.1	-	0.004(-)	-	0.005(-)	-
294.1	0.033	0.042	0.055	0.034	0.016	167.2	-	0.017	0.016	0.014	0.023
298.2	-	0.050	0.067	0.029	0.039	169.4	-	0.017	-	0.005(-)	-
300.2	-	0.017	0.016	-	-	171.5	-	0.025	0.043	0.005(-)	0.008(-)
302.3	0.033	0.038	0.035	0.048	0.055	173.6	0.267	0.063	0.071	0.038	0.117
304.2	-	-	0.016	0.010	-	175.4	**0.300**	0.254	0.303	0.250	0.258
306.6	-	0.013	0.020	0.043	0.039	177.7	0.067	0.108	0.083	0.058	0.047
310.5	0.033	0.038	0.039	0.043	0.023	179.5	0.233	0.063	0.051	0.019	0.008(-)
312.6	-	-	0.004(-)	-	-	181.2	0.033	0.008	0.008	-	0.016
314.7	-	0.029	0.016	0.010	0.008(-)	183.6	-	-	0.008	-	-
318.9	-	-	0.016	-	-	185.8	-	0.013	0.004(-)	-	-

	TesTo	NacTo	NamTo	SdoTo	SmiTo			TesTo	NacTo	NamTo	SdoTo	SmiTo
Locus GOB							**Locus BER**					
323.0	-	-	-	0.014	-		179.2[a]	0.233(-)	0.092(-)	0.067	0.087(-)	0.125(-)
327.0	-	0.004(-)	-	-	-		180.9	**0.767**	**0.908**	**0.929**	**0.913**	**0.875**
330.5	-	-	0.004(-)	0.005(-)	0.008(-)		184.6	-	-	0.004(-)	-	-
339.0	-	0.004(-)	-	-	-							
343.0	-	-	-	-	0.008(-)							
349.0	-	-	-	-	0.031							
355.9	-	-	-	-	0.016							
357.4	-	0.008	-	-	-							
359.1	-	-	-	-	0.008(-)							
361.1	-	0.008	0.004(-)	-	0.008(-)							
365.2	-	0.017	0.028	0.024	-							
367.2	-	-	0.004(-)	0.005(-)	0.008(-)							
369.3	-	0.075	0.055	0.130	0.078							
371.3	-	-	0.004(-)	-	-							
373.3	-	0.058	0.098	0.058	0.023							
375.6	0.033	0.013	0.012	0.058	0.070							
377.7	0.067	0.079	0.102	0.053	0.086							
379.5	-	-	0.004(-)	-	0.016							
381.6	0.200	0.075	0.063	0.072	0.055							
383.9	0.033	-	-	0.010	-							
385.7	**0.267**	0.025	0.016	0.063	0.078							
388.1	0.033	-	0.004(-)	0.014	0.008(-)							
389.8	0.033	0.021	0.016	0.034	0.047							
391.8	0.033	0.008	0.004(-)	0.005(-)	-							
394.0	-	0.008	-	-	0.016							
396.1	-	-	-	0.005(-)	-							
398.3	0.067	0.004(-)	-	-	-							
400.5	-	0.004(-)	0.004(-)	-	-							
412.3	-	-	-	-	0.008(-)							
414.4	-	-	0.004(-)	-	-							

(-) rarest allele sampled.

a: Average allele size was obtained after automatic allele scoring by the fragment analysis software module (CEQ 2000XL, Beckman - Coulter, Fullerton, CA, version 4.2.0).

Fixation indices are measures of differentiation, which incorporate information on both the frequency and identity of the alleles. In this study, F_{st} ranged from a low of 0 (estimates lower than zero were treated as zero) to 0.179 (Table 7). In pairwise comparisons, low differences were recorded among mountain "s"-populations (Tes1s, Tes2s and Tes3s; F_{st} max =0.019). Pairwise tests of population differentiation calculated by FSTAT revealed no significant differentiation among the three mountain "s"-populations sampled. P-values obtained after 4500 permutations were all >0.05. This means that the null hypothesis (no differentiation between populations) can not be rejected (Table 7).

Considering total oosporic populations (suffix "To"), high diversity estimates among mountain and valley populations were recorded (F_{st} max=0.086; TesTo/SdoTo). The lowest diversity estimates were obtained after comparison between valley populations collected at the same locality (SdoTo/SmiTo, F_{st} =0.005 and NacTo/NamTo, F_{st} =0.001). Significant differences were obtained essentially when we compared populations collected from different locations, but never when collected at the same location (SdoTo/SmiTo; NamTo/NacTo; Tes1o/Tes2o/Tes3o/TesTo). Total Tesero oosporic population (TesTo) was always significantly different from valley oosporic populations (Table 7).

Table 7. Estimates of pairwise F_{st} averaged over loci. "*" and "**" correspond to significance at the 5% and 1% nominal level after standard Bonferroni corrections, respectively .

	Tes1s	Tes2s	Tes3s	Tes2o	Tes3o	TesTo	NacTo	NamTo	SdoTo
Tes2s	-0.046								
Tes3s	-0.011	0.019							
Tes2o	-0.034	0.020	0.054						
Tes3o	-0.059	0.006	-0.004	-0.037					
TesTo	-0.041	0.020	0.024	-0.031	-0.051				
NacTo	0.105	0.146**	0.118**	0.058*	0.026	0.052**			
NamTo	0.106	0.151**	0.121**	0.061*	0.027	0.056**	0.001		
SdoTo	0.147*	0.179**	0.149**	0.092**	0.060	0.086**	0.010**	0.012**	
SmiTo	0.132*	0.170**	0.151**	0.067*	0.044	0.068**	0.011**	0.009**	0.005

Isolation by distance

There was a highly significant (p=0.003) positive relationship between pairwise F_{st} and geographical distance or natural logarithm of geographical distance. Linear correlation among the logarithm of geographical distance and the diversity estimator was low (R^2=0.32; y-intercept = 0.0205; slope = 0.0085; R^2=0.32; Fig. 3). When mountain populations were removed from the analysis, the relationship between F_{st} and geographical distance was still statistically significant (p=0.000). Nevertheless the increase of F_{st} per difference of geographical distance was six times lower than when including the Tesero populations (R^2=0.34; y-intercept = 0.0073; slope = 0.0014).
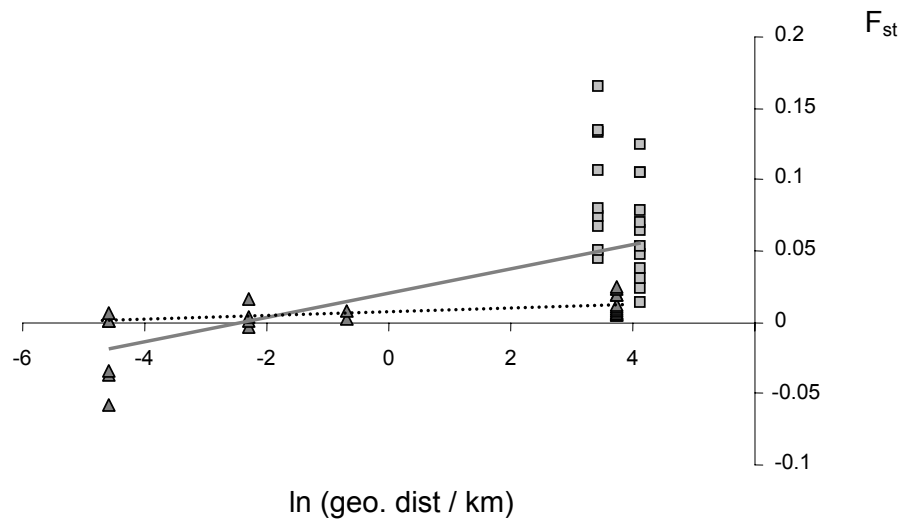

Fig. 3. Genetic distance (F_{st}) plotted against the natural logarithm of the geographical distance (km). The dotted and the solid lines represent the best fit linear regressions when including valley population only, and both valley and mountain populations, respectively. Triangles and squares refer to pairwise comparisons within valley and between valley and mountain populations, respectively.

DISCUSSION

Impact of meteorological conditions to disease initiation and development

Even if meteorological conditions of the period 30.05.00 – 08.06.00 were suited for primary infections (about 40 mm rain and a maximal rain intensity of 10 mm/h, temperature between 10 and 20 °C), we did not observe any yellowish symptom after incubation time on the Tesero plot. To explain this infection lack, we speculate that oospores were not yet mature, even if SIMPO estimates one day to germination. Alternatively we could assume that some few mature oospores did germinate, but macrosporangia were splash- or wind-dispersed to plant material where infections are unlikely (cork, youngest leaves) and not toward the poor vegetation. We observed one other rainy period with no impact on primary and secondary cycles (28-30.06.00). Nevertheless, SIMPO simulation calculated about two days for oospore germination. Probably the time lap required for germination was too long and macrosporangia were produced when microclimatic conditions were not anymore suitable for infection (no rains anymore). Rainy periods that played a major role both for mildew initiation and asexual multiplication were characterized by at least 3 mm/h rain with a maximum of 12 mm/h on 04.07.00. Rain intensity, combined with air turbulence, may represent a key factor for macrosporangia dispersal. We assume that the majority of germinating oospores lays trapped within at least 3/4 year old infected plant material (leaves or berries) in the upper rhizosphere and that a raindrop with an impact higher than an unknown threshold is required to disperse macrosporangia to the grapevine leaves. This speculation seems to be disproved by data recorded during two rainy periods, on 04.06.00 and 07.06.00, when rain intensities were largely above 3 mm/h but, as already discussed, oospores may not have been mature or were dispersed to non-targets.

Patterns of population differentiation

As expected for sexually recombining populations, the results in Table 4 confirm that all the oosporic populations were mostly in Hardy-Weinberg equilibrium, with the exceptions of the

loci CES and BER. Locus CES had a heterozygote deficit and locus BER a heterozygote excess, possibly generated by the presence of undetected null alleles in the sample of colonizers. Indication of null allele presence is strongly suggested after appropriate analysis (Table 5) for the locus BER. As soon as the probability of the presence of BER-null alleles reached 0.03, a significant deviation from HW equilibrium was manifest (exception: Tes30, which has only 6 individuals). Both populations Tes2s and Tes3s showed highly significant deviations from Hardy-Weinberg expectations because of the dramatic increase in frequency of 1-2 genotypes (*evivurro* and *evuzemca*), indicating asexual reproduction and dissemination.

Three lines of evidence suggest that the heterogeneity of allele frequencies we observed is, at least in part, the result of low gene flow on the spatial scale separating most sampled populations. We first consider the observed pattern of isolation by distance, then the results of the pairwise test of differentiation and, finally, some highlights of our more recent study.

The Mantel test detected an association between geographic and genetic distances in *P. viticola* populations. Isolation by distance slopes departed from random expectations, indicating that a significant pattern of isolation by distance applied to those populations. The pattern resulted primarily from comparisons with the mountain populations. Twenty-one of the 45 significant pairwise comparisons and 20 of the 22 F_{st}>0.02 involved the Tesero populations. Although we clearly need to sample between the two valley localities to determine the scale at which consistent differentiation becomes apparent, there seems to be much differentiation at spatial distances of > 30 Km in the Trentino region. This initial statistical approach provides the first indication of a geographically-restricted gene flow, not only between valley and mountain, but also along the cultivated vineyard-rich valley.

Consistent with the isolation by distance test, the pairwise test of differentiation highlighted significant genetic subdivisions between populations collected at different locations (among Tesero, Navicello and San Michele). The finding of significant differences among the Tesero populations and valley populations is likely an effect of the peculiar geographical location. These findings suggest a nearly absent immigration (gene flow) from the nearest neighbouring infected vineyards (30 Km) that would regularize allele number and frequency among sites. Therefore we may consider all the genotypes of the Tesero population as a single demographical unit. On the other hand, to find that the valley populations at a 42 Km

distance were genetically subdivided was unexpected, given that barriers to movements between these locations are absent, that the grapevines cover 1/3 of the cultivated area and that the disease has been established for a long time.

First disease identification was made in 1907 in San Michele (Rigotti, 1932). Again, but more imperatively, this implies that the gene flow even between the two valley locations must be limited. If gene flow exists, considering that 26 alleles out 80 were shared among the valley populations, it may not be sufficient to avoid genetic isolation between populations settled at 42 Km distance. It may, instead, play a major role in establishing patterns of genetic homogeneity at more restricted local ranges (from ~0 to 500 m).

The findings of appropriate statistical analysis, finally, are consistent with our most recent study, which surveyed 18 downy mildew epidemics in four central European countries (4685 lesions were collected, Gobbin *et al.*, 2003, unpublished results). It essentially revealed that secondary lesions caused by the same genotype are often very aggregated, such as at vine or vineyard level (one or few disease clusters were observed). Only in a six-vine plot (Nv=6) did clones of the same genotype succeed in colonizing the totality of the vines (at least one lesion per vine), while, in the remaining 17 epidemics (of 18), we observed a colonization of maximally 2/3 of the total number of vines (4 < Nv < 414; average surveying time: 1.5 months). Based upon this aggregated spatial pattern of secondary lesions, we consider the microsporangia wind-dispersal to be a very rare event, already at modest distances (> 50 m). Rather, we speculated that the oospore-mediated gene flow may be more efficient, since oosporic long viability period (at least five years, Hill GK, personal communication) would permit more extensive disease propagation.

Consequently, both statistical tests and our more recent findings contradict current theories about massive and long distance microsporangia wind-dispersal (Agrios, 1988; Blaise *et al.*, 1999; Lafon and Clerjeau, 1988) and suggest that primary and secondary lesions are likely of autochthon origin or of spatially-restricted allochthon origin.

Given these issues, the simplest and more plausible explanation for the disease establishment in Tesero is that it relies on the oospore import either from France with the young plantlets or from the cultivated Adige valley through any kind of human transport. In both cases, the disease-originating propagules would have experienced a human-mediated-

rather than a wind-mediated allochthon introduction. Having no indication of Alsatian downy mildew alleles or allele frequencies at the studied four loci, we can neither support nor discard the first hypothesis. Instead, considering that all the alleles found in Tesero were identified in the valley, an import from the valley natural stock seems at least partially supported.

Founder effect analysis

Although the small sample size of the TesTo population (15 individuals) may have created a biased allele frequency distribution, several differences among TesTo and the four valley populations are due to alleles that occur at high frequencies (Table 5). For instance, allele GOB-385.7 occurs with high frequency in the TesTo population, but is almost deficient in the four valley populations. Another example is CES-173.6: it is very abundant in TesTo but present in lower frequency in the other populations. GOB-385.7 is the 11th most frequent GOB allele (of 34) in NacTo but the 3rd most frequent (of 30) in SmiTo. Similarly for CES-173.6, it is the 3rd and 4th most frequent in SmiTo (of 10) and NacTo (of 15), respectively. Microsatellite variability of the Tesero populations is also characterized by substantial reductions of allelic diversity in terms of the number of alleles per locus. Our case study revealed the loss of 33 of 38 low-frequency alleles (for instance: GOB-277.3; ISA-137.1; CES-165.1 absent in TesTo), probably absent within the small number of effective population founders. Such abrupt differences in allele frequencies and loss of alleles are typical consequences of founder effects.

Implications for downy mildew epidemiology

In Tesero, 15 genotypes were identified within a vineyard area of 394 m² (0.04 genotypes/m²) while valley populations revealed a higher occurrence of genotypes: from 0.50 gen/m² (NacTo) to 1.05 gen/m² (SdoTo). In the valley we applied a grid sampling contrary to the mountain plot were all lesions were sampled. A possible explanation may be that in the grid sampling we failed in detecting clones and we biased consequently genetic diversity toward an underestimation of lesions per genotype. This presumes that the secondary lesions are

restricted to an area smaller than the resolution of the grid (they are highly aggregated) or that the secondary infections are a rare event. In any case those findings suggest that in San Michele and Navicello autochthon oospore stocks may play a much more relevant role in an epidemic than what is reported in the literature (Agrios, 1988; Blaise *et al.*, 1999; Lafon and Clerjeau, 1988) and generally assumed: primary infections are very numerous and occur probably not exclusively during a short period at the beginning of the epidemic, as observed in Tesero.

We observed a genotype-specific aptitude in producing secondary lesions. Thirteen out of 15 genotypes did not produce a relevant damage (1-3 lesions each), while only two genotypes were responsible for 88% of the total lesions on 02.07.00 and one of them for 68% of the total lesions on 14.07.00. It remains unclear why one of the most aggressive genotypes (*evivurro*) on 02.07.00 was not able to undergo a further secondary disease cycle after 02.07.00. If epidemics in less atypical conditions show similar apparently stochastic and genotype-specific asexual multiplications, it will be difficult to forecast the magnitude of progeny simply by counting the number of sporulating lesions. Thus, it may be due to this apparently stochastic asexual multiplication factors that epidemiological models often fail in predicting the real relevance of secondary cycles. This research and future research, which consider epidemics occurring in different locations, may bring new data to improve those models and, consequently, disease management strategies.

ACKNOWLEDGMENTS

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P. viticola secondary sporangia. Picture by: HH Kassemeyer

The importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine

downy mildew

ABSTRACT

It is generally accepted, that epidemics of grapevine downy mildew, caused by *Plasmopara viticola,* begins from a few germinating oospores. Once sporulating lesions from primary infections are present, the pathogen has the ability to spread through secondary sporangia. It is postulated from the literature, that the number of lesions generated by the secondary phase greatly outweighs the oospore-derived primary lesions, but no quantitative data about the population genetic structure are yet available. We assessed the magnitude of asexual multiplication and quantified the spatial dispersion of secondary sporangia as function of epidemic progression, by genotyping *Plasmopara viticola* samples. In a survey of downy mildew populations from 2000 to 2002, 4685 *P. viticola* samples were collected from 18 plots spread across central Europe. The samples were examined with a semi-automatic microsatellite (SSR) analysis that identified more than 2300 site-specific *P. viticola* genotypes. About 85% of all genotypes were sampled once or twice throughout the various epidemics. In the 18 plots, only seven genotypes (0.3%) were identified more than 50 times. Three particularly successful genotypes in causing disease through secondary cycles, showed mostly a clustered distribution and a marked spatial autocorrelation. A further genotype showed a random plot-scaled dispersion. Secondary sporangia migration distances per secondary cycle were in the range from ~0 to 20 metres. We conclude that the secondary sporangia have a generally low and heterogeneous degree of success in generating secondary infections, which challenges the existing assumptions about disease epidemics.

INTRODUCTION

D Downy mildew, caused by the heterotallic (Wong *et al.,* 2001) diploid Oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is the most important grape (*Vitis vinifera*) diseases in temperate climates with spring rains. *P. viticola* overwinters as sexually produced oospores in fallen leaves and berries. The current conception about the pathogen's epidemiology postulates that the disease starts from a few germinating oospores that cause primary/oosporic infections early in the grapevine vegetative season (Blaeser & Weltzien, 1979; Lafon & Clerjeau, 1988). After 5-18 days, depending on the temperature, the organism produces microsporangia containing asexually produced zoospores (optimum: 18°C and saturating humidity) (Agrios, 1988). The first infections are therefore followed by successive asexual cycles, where the secondary sporangia cause secondary/clonal infections and lead to an explosive disease progress (Schruft & Kassemeyer, 1999). The dispersal of the disease is believed to rely on the migration of secondary sporangia, which are thought to travel long distances in a short time (Blaise *et al.,* 1999; Lafon & Clerjeau, 1988). To our knowledge, no quantitative data about migration distances of specific genotypes are yet available.

In order to predict the risk of secondary or primary infections, weather-driven epidemiological models have been proposed. Most of these models are based on the assumption that an epidemic starts from a restricted number of oosporic infections, followed by massive clonal multiplication (Lalancette *et al.,* 1988a,b; Blaise *et al*., 1999). A few models are based primarily on the simulation of the germination dynamic of oospores and consider them as the only responsible for the whole epidemic (Hill 1990; Hill 2000; Strizyk 1994). However, models often fail in predicting the real quantitative development of epidemics, which restricts their use in practice or leads to overestimation of infection risk. At the current knowledge status, models can not be significantly improved without separate quantitative modelling of sexual and asexual phases (M Jermini and M Raynal, personal communication).

Spatial pattern of disease results from the interaction between physical, biological and environmental factors. Several statistical methods for defining spatial relationships, such as indexes of dispersion or calculation of variances of blocked quadrats, have been developed

(Charest *et al.*, 2002). These calculations do not consider the physical location of the quadrat or its relationship with other sample sites. To overcome this deficiency, geostatistical analyses have been developed (Charest *et al.*, 2002). Geostatistic, originally developed for mining (Barker & Lally, 1993), has already been applied in plant pathology to quantitatively characterize spatio-temporal patterns of disease (Xiao *et al.*, 1997; Turecheck & Madden, 1999; Van der Lande & Zadocks, 1999). Geostatistical analysis can be used to detect spatial dependency for continuous and discrete variables. Spatial dependence could be analysed directionally or omnidirectionally with semivariograms, which quantify spatial dependence by measuring variations between samples separated by a vector: the "lag distance" (Savary *et al.*, 2001, Wu *et al.*, 2001).

In this study, we combined the genetic (Gobbin *et al.,* 2003a) and statistical knowledge, in order to 1) determine which proportion of total *P. viticola* genotypes undergoes secondary disease cycles, 2) quantify the magnitude of secondary sporangia-derived asexual progeny, and 3) quantify the spatial dispersion of particularly successful genotypes in generating secondary lesions as a function of epidemic progression. In order to reduce single site variability, in terms of particular macro and microclimatic conditions, grape responses to disease attack, pathogen aggressiveness etc., we selected 18 sites across central Europe where to survey downy mildew epidemics.

MATERIALS AND METHODS

Vineyard locations and epidemics surveyed

Eighteen plots (17 not treated and one, Cum, treated four times with fungicides) were selected within four European-wine-producing countries (Germany, France, Italy and Switzerland; Fig. 1). Naturally occurring downy mildew epidemics were surveyed during years 2000-2002 within those plots. The survey period started as soon as the first lesions (also called oil spots) were noticed and stopped when downy mildew generated a mosaic pattern which impeded the collection of distinct lesions. On average, the survey period lasted 1.5 months and ended generally in July or August. One sample consisted in 0.5 - 1 cm^2 of every identifiable lesion (corresponding to approx. 1/3 to 1/2 of the total lesion surface) including up to 0.5 cm^2 of healthy leaf tissue. The remaining part (1/2 to 2/3) of the lesion was not sampled to allow the survival of the genotype in the plot. The exact location of each lesion within the plot was recorded according to vine and row number. Oil spots were collected on 2 to 22 sampling dates per plot throughout the 18 epidemics; in total, for all plots and all sampling dates, 83 independent samplings were performed (Table 1). Two different sampling strategies were used: total- and partial- sampling strategies (TSS and PSS, respectively). The total sampling strategy was used when, on average, less than 5 oil spots per vine were present. Using this strategy all oil spots were collected as soon they were visible. A partial sampling strategy was performed as an alternative to total sampling, when more than 5 oil spots per vine were present. Using PSS, 1 to 3 oil spots per vine were sampled.

When the total sampling strategy was applied at least on two sampling dates within the same plot throughout one epidemic, we considered the total downy mildew population (pooled individuals collected on all sampling dates) to be totally sampled (TSS; Table 1). When the TSS was used on one sampling date (or none, as in Wad) and the PSS on all the remaining sampling dates, we considered the total *P. viticola* population as partially sampled (PSS, Table 1). Hereafter TSS or PSS will refer to the sampling strategy applied to the 18 total populations and not to the 83 independent samplings.

As in Was a single vine was infected, we sampled all lesions (TSS) on any sampling date and we recorded their positions according to a reference system defined by six sectors (top, medium, low and left, right vine sectors, Fig. 4).

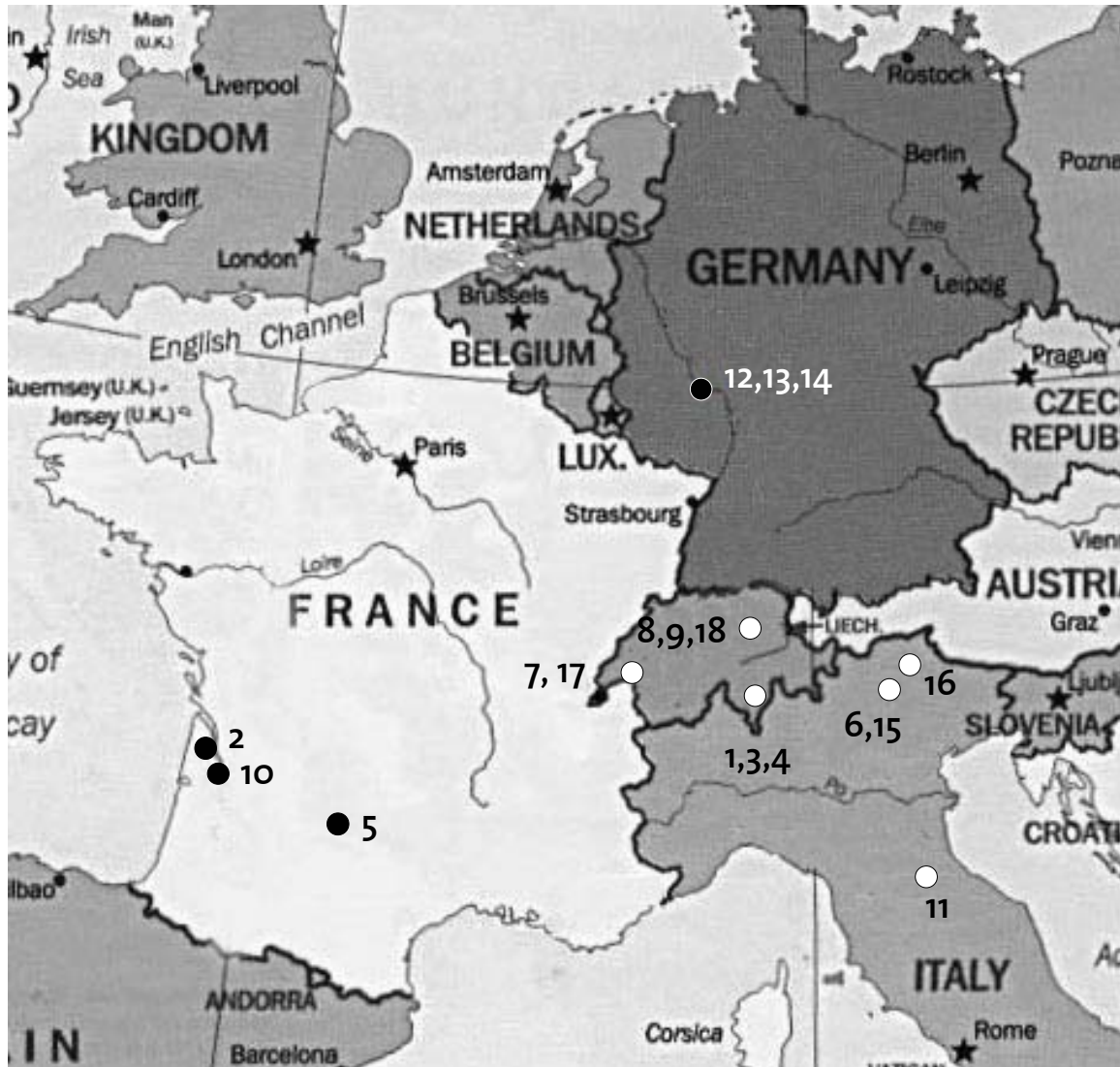Figure 1 Locations of the 18 downy mildew populations surveyed during years 2000-2002. 1: Biasca (Bia), 2: Blanquefort (Bla), 3;4: Cugnasco (Cut and Cum), 5: Gaillac (Gai), 6: Navicello (Nam), 7: Perroy (Per), 8: Stäfa (Sta), 9: Wädenswil (Wad), 10: Bommes (Bom), 11: Carpineta (Car), 12: Erbach (Erb), 13: Geisenheim (Gei), 14: Lorch (Lor), 15: Navicello (Nac), 16: Tesero (Tes), 17: Vic (Vic) and 18: Wädenswil (Was).

Table 1 Vineyard location (site number, site abbreviation, site name in full and nation), training system, grape variety, number of rows (R), distance between rows (dr), number of vines per row (V), distance between vines in a row (dv), total vines (N v), plot area (A), sampling dates (S date), sampling strategy (SS), sampling size (N_{obs}), number of different genotypes (N_{gen}), number of single genotypes (genotypes that occurred once all over the surveying period; N_{sgen}), name of the most frequent genotype (MFG name), occurrence of the most frequent genotype ($N_{obs\ MFG}$), name of the (SMFG name) and occurrence of the SMFG ($N_{obs\ SMFG}$). Bold characters refer to the main sampling strategy applied, to the total number of lesions collected (tN_{obs}), of genotypes identified (tN_{gen}), of single genotypes identified (tN_{sgen}) of MFG identifications ($tN_{obs\ MFG}$) and of SMFG identifications ($tN_{obs\ SMFG}$).

Vineyard loc	training syst	R	V	N v						*MFG name*	*SMFG name*
	grape var	dr	dv	Aa	S date	SS [b]	N_{obs}	N_{gen}	N_{sgen}	$N_{obs\ MFG}$	$N_{obs\ SMFG}$
1- Bia										*evawasma*	*ibevasca*
Biasca	Guyot	2	21	42	26.5.00	TSS	27	26	18	0	1
Switzerland	Merlot	1.75 m	1.25 m	92 m²	18.6.00	PSS	96	70	48	5	5
					30.6.00	PSS	101	64	36	12	8
					13.7.00	PSS	90	67	42	3	2
					tot pop	**PSS**	**314**	**190**	**144**	**20 (6.4%)[c]**	**16 (5.1%)[d]**
2- Bla										*ebebissa*	*ebetassa*
Blanquefort	Doub Guyot	4	70	280	10.5.00	TSS	36	30	22	0	2
France	Merlot	1.5 m	1 m	420 m²	31.5.00	PSS	100	85	53	2	0
					16.6.00	PSS	189	151	94	7	5
					07.7.00	PSS	106	85	45	1	2
					07.8.00	PSS	126	93	62	4	0
					tot pop	**PSS**	**557**	**363**	**276**	**14 (2.5%)**	**9 (1.6%)**
3- Cut										*alovispa*	*ibetosna*
Cugnasco	Guyot	1	6	6	07.6.01	TSS	1	1	1	0	0
Switzerland	Merlot	2 m	1.2 m	14 m²	25.6.01	PSS	42	17	10	10	14
					23.7.01	PSS	135	56	39	34	21
					tot pop	**PSS**	**178**	**69**	**50**	**44 (23.5%)**	**35 (18.7%)**
4- Cum										*alovispa*	*ebebesra*
Cugnasco	Guyot	4	61	244	07.6.01	TSS	17	9	5	0	6
Switzerland	Merlot	2 m	1.2 m	586 m²	25.6.01	PSS	41	21	17	0	20
					23.7.01	PSS	340	137	78	45	0
					29.8.01	PSS	86	50	27	10	0
					tot pop	**PSS**	**484**	**193**	**127**	**55 (11.4%)**	**26 (5.4%)**

5- Gai										*abebessa*	*aveyusla*
Gaillac	C Royat	4	21	79	05.6.00	PSS	21	10	5	10	2
France	Duras	2.5 m	1 m	210 m²	14.6.00	PSS	26	16	12	9	3
					tot pop	PSS	47	22	17	19 (40%)	5 (10.6%)
6- Nam										*avavissa*	*igigomma*
Navicello	Guyot	5	21	105	22.5.00	TSS	2	2	1	0	0
Italy	Merlot	2 m	1 m	210 m²	30.5.00	PSS	18	17	12	0	0
					21.6.00	PSS	57	47	40	5	3
					06.7.00	PSS	86	81	74	0	0
					tot pop	PSS	163	142	127	5 (3.0%)	3 (1.8%)
7- Per										*ebesossa*	*efoganta*
Perroy	S Guyot	4	26	88	30.5.00	PSS	85	41	18	17	2
Switzerland	Chasselas	2 m	0.8 m	166 m²	29.6.00	PSS	131	53	26	26	17
					11.8.00	PSS	109	49	22	19	4
					tot pop	PSS	325	108	66	62 (19.0%)	23 (7.1%)
8- Sta										*evivusme*	*abevisme*
Stäfa	Guyot	4	20	73	27.6.00	PSS	72	44	22	7	11
Switzerland	RxS	2.1 m	0.9 m	134 m²	26.7.00	PSS	117	65	28	4	9
					18.8.00	PSS	139	60	29	30	11
					tot pop	PSS	328	129	79	41 (12.5%)	31 (9.5%)
9- Wad										*ozewisra*	*ebezesma*
Wädenswil	Guyot	5	3-15	47	21.6.00	PSS	54	21	10	19	6
Switzerland	RxS	1.9 m	1 m	89 m²	26.7.00	PSS	73	29	17	35	3
					tot pop	PSS	127	43	27	54 (42.5%)	9 (7.1%)
10- Bom										*übetosca*	*azowirma*
Bommes	D Guyot	7	40-69	414	<27.6.01[e]	-	1	1	0	1	0
France	Sémillon	1.5 m	0.9 m	559 m²	27.6.01	TSS	63	3	1	60	0
					10.7.01	TSS	18	9	2	4	0
					24.7.01	PSS	58	15	5	25	5
					27.8.01	PSS	53	27	20	22	5
					tot pop	TSS	193	41	28	112 (58.0%)	10 (5.2%)
11- Car										*afevissa*	*afowusba*
Carpineta	wild	3	23	57	25.5.01	TSS	18	15	11	1	0
Italy	Sangiovese	2.5 m	2 m	345 m²	08.6.01	TSS	49	31	17	8	1
					27.8.01	TSS	57	45	31	0	3
					tot pop	TSS	124	84	59	9 (7.3%)	4 (3.2%)

										afolonna	*dgulepma*
12- Erb											
Erbach	Halbbogen	3	12	36	18.5-6.7.00 [f]	TSS	230	199	136	0	9
Germany	Riesling	2 m	1.1 m	79 m^2	10.7.00	TSS	11	9	5	2	0
					27.7.00	TSS	1	1	1	0	0
					01.8.00	TSS	45	21	6	7	0
					tot pop	**TSS**	**287**	**192**	**148**	**9 (3.1%)**	**9 (3.1%)**

										egigusra	*jlomaspa*
13- Gei											
Geisenheim	Halbbogen	3	13	39	26.5.00	TSS	7	2	0	3	0
Germany	RxS	1.8 m	1.2 m	84 m^2	31.5.00	TSS	7	2	0	6	0
					05.6.00	TSS	30	8	2	10	4
					19.6.00	TSS	141	25	15	55	31
					06.7.00	TSS	21	8	3	5	5
					tot pop	**TSS**	**206**	**33**	**20**	**79 (38.2%)**	**40 (19.4%)**

										afevimca	*avuzomme*
14- Lor											
Lorch	Rundbogen	3	12	36	05.6.00	TSS	14	9	5	6	0
Germany	Riesling	2 m	1.3 m	93 m^2	06.6.00	TSS	3	3	3	0	0
					16.6.00	TSS	57	33	19	16	0
					19.6.00	TSS	45	38	24	2	0
					20.6.00	TSS	148	107	81	16	4
					21.6.00	TSS	34	22	16	0	13
					27.6.00	TSS	60	49	36	7	1
					tot pop	**TSS**	**361**	**228**	**184**	**47 (13.0%)**	**18 (5.0%)**

										ymatosmo	*abebessa*
15- Nac											
Navicello	Sin.Pergola	4	33	132	15.5.00	TSS	6	6	5	0	0
Italy	Chardonnay	2.8 m	0.8 m	296 m^2	22.5.00	TSS	27	19	12	6	2
					30.5.00	TSS	414	351	315	13	6
					tot pop	**TSS**	**447**	**370**	**332**	**19 (4.3%)**	**8 (1.8%)**

										evuzemca	*evivurro*
16- Tes											
Tesero	Guyot	16	18-23	328	23.6.00	TSS	4	4	2	1	1
Italy	Chardonnay	1.5 m	0.8 m	394 m^2	02.7.00	TSS	71	10	6	43	20
					14.7.00	TSS	22	6	3	15	0
					tot pop	**TSS**	**97**	**15**	**11**	**59 (60.8%)**	**21 (21.6%)**

										abedussa	*abegisma*
17- Vic											
Vic	Pinot noir	6	25	136	11.8.00	TSS	74	45	15	8	3
Switzerland	Guyot S	1.65 m	0.8 m	180 m^2	25.8.00	TSS	262	97	40	25	16
					tot pop	**TSS**	**336**	**114**	**55**	**33 (9.8%)**	**19 (5.7%)**

18- Was										*ezazerma*	*emizorma*
Wädenswil	Guyot	1	4	4	01.6.02	TSS	1	1	0	1	0
Switzerland	RxS	1.9 m	1 m	8 m^2	10.6.02	TSS	25	1	0	25	0
					16.6.02	TSS	85	6	4	79	2
					tot pop	**TSS**	**111**	**6**	**4**	**105 (94.6%)**	**2 (1.8%)**

a: Plot area was calculated as R*dr*V*dv [m^2].

b: TSS: total sampling strategy; PSS: partial sampling strategy.

c: Pecentage refers to the MFG-derived disease severity: $DS_{\%MFG}$ ($tN_{obs\ MFG}$ / tN_{obs}).

d: Pecentage refers to the SMFG-derived disease severity: $DS_{\%SMFG}$ ($tN_{obs\ SMFG}$ / tN_{obs}).

e: No sampling was performed prior to 27.06.01. An "open" date refers to the putative appearance of the very first primary infection caused by the MFG übetosca.

f: A summary of the first 19 samplings performed in Erbach is shown. SMFG *dgulepma* was identified 6 times on 6.06.00, once on 09.06.00 and twice on 06.07.00.

Sample processing and genotype identification

For genotyping the collected samples, we used automated high throughput DNA extraction, PCR amplification of the four polymorphic *P. viticola* specific SSR loci BER, CES, GOB and ISA, sequencer based fragment analysis and automatic allele scoring (Gobbin *et al.,* 2003a). *P. viticola* genotypes were defined as strains that differed by at least one allele in their SSR profiles. Genotypes were named by merging 8-letters (for instance: *evawasma* and *ibevasca* identified in Biasca, Table 1), according to a SSR allele coding methodology not described in this paper. We considered that each oospore contains a single nucleus (Gregory, 1915; Gobbin *et al.,* 2003a) that it is asexually inherited by the progeny (secondary lesions). We assume that asexual cycles did not alter the microsatellite allele pattern. Therefore, two oil spots showing identical allele pattern (same genotype) were interpreted as clonal progeny, deriving from the same oospore. Different genetic profiles of two oil spots were interpreted as derived from independent oosporic infections. The first appearance of a genotype was considered as an oosporic-derived primary lesion. Any subsequent observation of the same genotype was interpreted as a secondary lesion.

Data and distribution analysis

Independently for each of the 18 plots we pooled all the individuals collected on the 2 to 22 sampling dates. The total number of individuals (tN_{obs}) and the total number of genotypes (tN_{gen}) were calculated. The number of genotypes that were identified the same number of times (tN_{obs}=X; $1 \leq X \leq 112$) throughout the survey period was determined and the frequency of every occurrence class ($F_{tNobs=x}$) was calculated. The genotypes occurring once throughout the survey period (tN_{obs}=1) were defined as single genotypes. The genotypes occurring at the highest frequency were defined as most frequent genotypes (MFGs), while genotypes occurring at the second highest frequency were defined as second most frequent genotypes (SMFGs). In this study, disease severity was defined as the number of lesions, or observations (N_{obs}), identified in a spatial unit (plot or vine). The contribution of any genotype(s) to the total disease severity, was calculated by dividing the number of lesions generated by the considered genotype(s) by the total severity and expressed in percentage ($DS_{\%}$). Scatterplots showing data distribution and MFG locations in the plots were constructed by means of

Systat (version 10, SPSS Inc., 2000). The same software was used to construct ELL Gaussian bivariate confidence ellipses (p=0.68). Two-sided t-tests were performed in a Microsoft Excel spreadsheet (version 2002). In order to evaluate the oil spot distribution pattern, we employed the variance-to-mean ratio (v/m), which gives an indication of the type of distribution (regular, random or clustered) of the data (Campbell & Madden, 1990). To calculate this index, we used individual grapevines as spatial units, or quadrats, because they are regularly planted within plots. For every vine, the cumulative number of lesions caused by the MFG at a defined date ($cN_{obs\ MFG}$) or by any other genotype (cN_{obs}) was calculated. Variance and mean variables of $cN_{obs\ MFG}$ and cN_{obs} per vine were calculated for every plot. We analysed the variation of the v/m ratio over time for three plots Bom, Gei and Tes, where clones of MFG were identified at least 50 times ($tN_{obs\ MFG} > 50$) and a total sampling strategy (TSS) was performed (Table 1). When v/m is <1, =1, or >1, the pattern of distribution is considered regular, random or aggregated, respectively. The Poisson probability distribution was used to test whether a MFG spatial pattern of distribution was random or non-random, based on $cN_{obs\ MFG}$ per vine. When the frequency of lesions distribution corresponded to the expected frequency distribution of Poisson, determined by the ^{2} test, the spatial distribution was considered to be random. Alternatively, a non-random distribution was interpreted either as aggregated or regular (Charest *et al.*, 2002).

Geostatistical analyses

Semivariogram analysis was performed on the natural logarithm of the cumulative number of MFG-derived lesions incremented by one ($\ln[cN_{obs\ MFG} +1]$) and associated spatial locations (row direction and direction perpendicular to row, or, by convention, North and East directions, respectively; all extensions were expressed in metres defining by co-ordinate (0 m ; 0 m) the location of vine 1 in row 1), using software GS+ for Windows (version 5.1, RockWare Inc., 2003). We applied a transformation from $cN_{obs\ MFG}$ to $\ln[cN_{obs\ MFG} +1]$ in order to reduce semivariance among lag increment classes and to obtain better fit of models. Lag distances were set to 8 m (Gei) and 10 m (Bom and Tes), which are less than half the maximum distance between sample pairs, while lag increments were set to the plot specific distances between vines increased by 10 cm (dv, Table 1). In GS+, both anisotropic and isotropic

semivariograms were generated. For anisotropic semivariograms, four conventional directions were considered: North (row direction, angle = 0°), North-East (45°), East (direction perpendicular to rows, 90°) and South-East (135°), while for isotropic semivariograms, all angles between 0° and 360° were considered. The best-fitting models were selected among the five provided by GS+, according to the highest R^2 value and the lowest sum of squares.

RESULTS

Differential genotype contribution to the epidemic

Although plot locations were spread across central Europe, sampling was performed in different years (mostly in 2000) and meteorological conditions differed remarkably (data not shown), downy mildew symptoms appeared approximately at the same time period (mid May to end of May). In a few cases, the disease first appeared in June (Bom, Wad, Tes and Sta) or even in August (Vic). The survey periods lasted on average 1.5 months and ended generally in July or August, when downy mildew started generating mosaic patterns which impeded the collection of lesions. From the nine plots where TSS was applied, 2162 half oil spots were successfully genetically analysed, revealing the presence of 1083 genotypes. From the remaining nine plots where PSS was applied, 2523 symptoms (half oils spots) were analysed and 1259 different genotypes were distinguished. In summary, among the 4685 oil spots considered, 2342 genotypes were identified (Table 1).

After grouping of the genotypes occurring the same number of times throughout one epidemic (tN_{obs}=X) into genotype frequency classes ($F_{tNobs=x}$), we showed that single genotypes (tN_{obs}=1) often constituted the greatest fraction of the individuals collected. On average, 71% of all genotypes of the 18 total populations, assessed both after PSS (Fig. 2a) and TSS (Fig. 2b), were identified only once throughout the survey period. The lowest percentage was recorded in Vic, where single genotypes were 48% of all genotypes; the highest was at Nac, when single genotypes comprised 90% of all genotypes. On average, over the nine populations sampled after PSS, single genotypes were responsible for 37% of the total disease severity (st.dev: 19%; Fig. 2c). In the other populations (TSS, Fig. 2d), single genotypes caused an average of 31% of the total disease severity (st.dev: 25%). A high proportion of single genotypes was observed independently from the sampling date (Fig. 3). Even on the latest sampling dates, in seven populations (Bia, Bla, Gai, Nam, Car, Lor and Nac) more than 40% of new single genotypes were found.

Genotypes that were identified twice (tN_{obs}=2) in the same plot throughout the season (either twice on the same sampling date or once on two sampling dates), were, on average, 15% and 13% of the total number of genotypes identified, after PSS and after TSS, respectively

(Fig. 2a and 2b). The proportion of disease severity caused by those genotypes was on average 13.7% (st.dev: 2.2%) after PSS and 11.0% (st.dev: 8.7%) after TSS (Fig. 2c and 2d).

The Euclidean distances between the two clones of the genotypes that were identified on the same sampling date were shorter than the distance between the clones of the genotypes collected once on two sampling dates (tN_{obs}=2). 107 genotypes were identified twice on the same sampling date (tN_{obs}=2; 214 lesions). The two clones of each of the 107 genotypes were located in 22 case (20.6%) on the same leaf, in 19 cases (17.8%) on two different leaves of the same vine, in 12 cases (11.2%) on two neighbouring vines of the same row, in 2 cases (1.9%) on two vines of the same row with a vine in-between and in 2 cases (1.9%) on two vines laying *vis-à-vis* of two adjacent rows. That indicates approximately the half of all genotypes (49.5%) are located maximally at about 1m apart while the other half at farther distances. The longest distance between two clones of the same genotype, 15 m, was measured in Vic, where the genotype *ewavisna*, was identified on 25.08.00 (2nd sampling) on the vines 21/4 and 2/5 (vine/row). 268 genotypes were identified once on two sampling dates (tN_{obs}=2; 536 lesions). OIn none of the 268 cases, none were colonizing the same leaf (just because we never sampled on previously sampled leaves), in 24 cases (8.9%) the two clones of the same genotype were colonizing two different leaves of the same vine, in 20 cases (7.5%) they were colonizing two neighbouring vines of the same row, in 11 cases (4.1%) they were colonizing two vines of the same row with a vine in-between and in 9 cases (0.3%) they were colonizing two *vis-à-vis* vines of two adjacent rows. The remaining 76.1% of the clones was at more than 3m distance. The longest distance between two clones of the same genotype, 67 m, was measured in Blanquefort, where the genotype *ebenossa*, first identified on 10.05.00 on vine14/6, was found on 07.08.00 on vine 81/3.

Based on TSS, only 15 of 1083 genotypes (1.4%) were identified more than 10 times, while 8 (0.7%) were identified more than 20 times. The same trend was found after a partial pathogen collection (PSS), when, out of 1259 genotypes identified, 23 (1.8%) and 11 (0.9%) genotypes, were identified more than 10 times and more than 20 times, respectively. The proportion of total disease severity generated by those particularly successful genotypes reached 20% in seven cases and 50% in three cases.

MFGs were uniformly distributed within the populations surveyed, independent from the collection strategy applied. Twelve genotypes that were identified between 21 and 50 times belonged to 8 populations (avg: 0.67; min: 0; max: 3 genotypes per population). Seven genotypes that were identified more than 50 times belonged to seven distinct populations. Populations that included the most successful genotypes ($tN_{obs} \geq 21$) were: Gei (the MFG was identified 79 times + the SMFG was identified 40 times), Tes (59 + 21), Per (62 + 23), Cum (55 + 26) and Cut (44 + 35) (Table 1 and Fig. 3).

$F_{tNobs=X}$ and $DS_\%$ data distribution within occurrence classes ($tN_{obs}=X$) are equally described independently from the sample collection method (TSS or PSS; Fig. 2). Non significant differences (p>0.05 after t-tests) were always obtained when comparing the averages of $F_{tNobs=X}$ and $DS_\%$ for $1 \leq tN_{obs} \leq 9$. No comparisons were possible after $tN_{obs} \geq 10$ because of the lack of a sufficient data set.
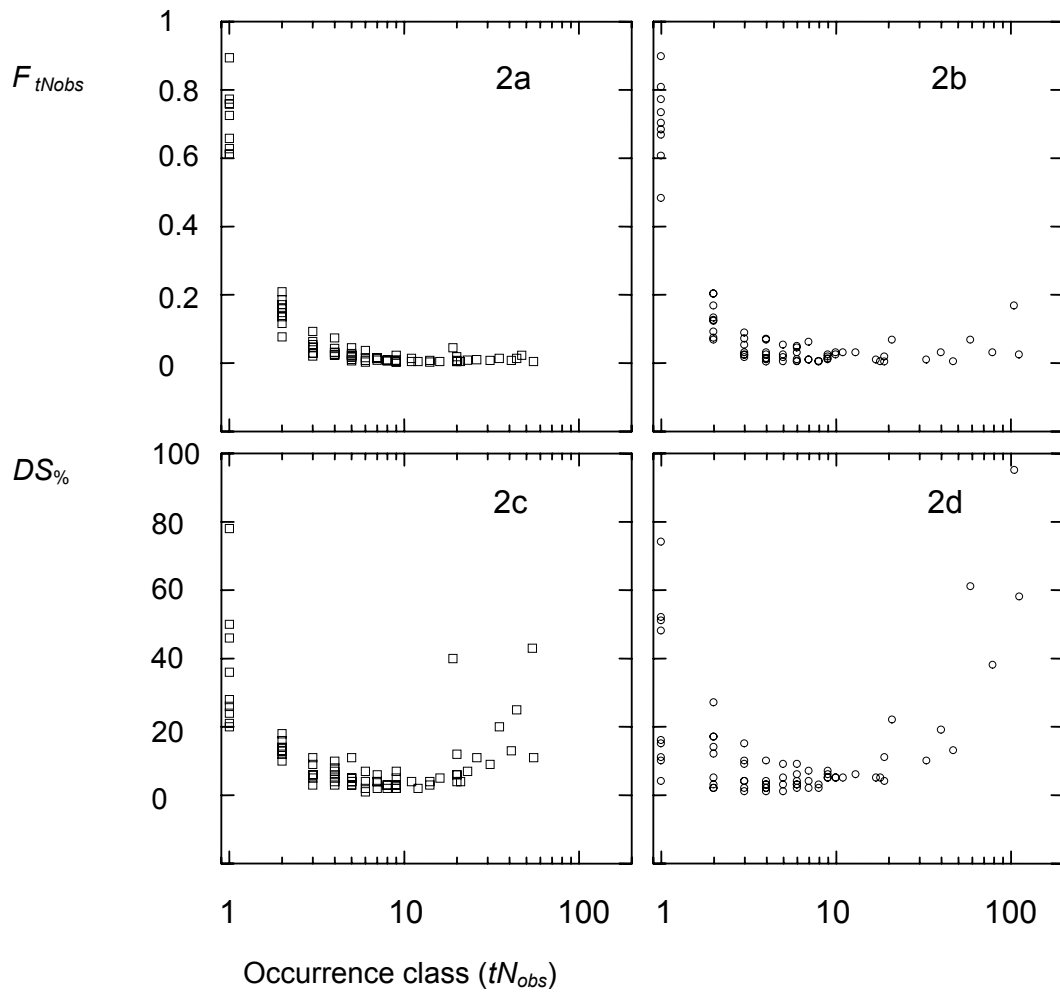
Figure 2abcd. Frequency ($F_{tNobs=x}$, Fig. 2a and 2b) and percentage of total disease severity ($DS_{\%}$; Fig. 2c and 2d) of genotype occurrence classes (x-axis). The genotypes that were identified the same number of times throughout the survey period were classified into occurrence classes (tN_{obs}=1,2,3…112). Square and round symbols refer to data collected by PSS and TSS, respectively. All data points were calculated independently for each of the 18 total populations surveyed.
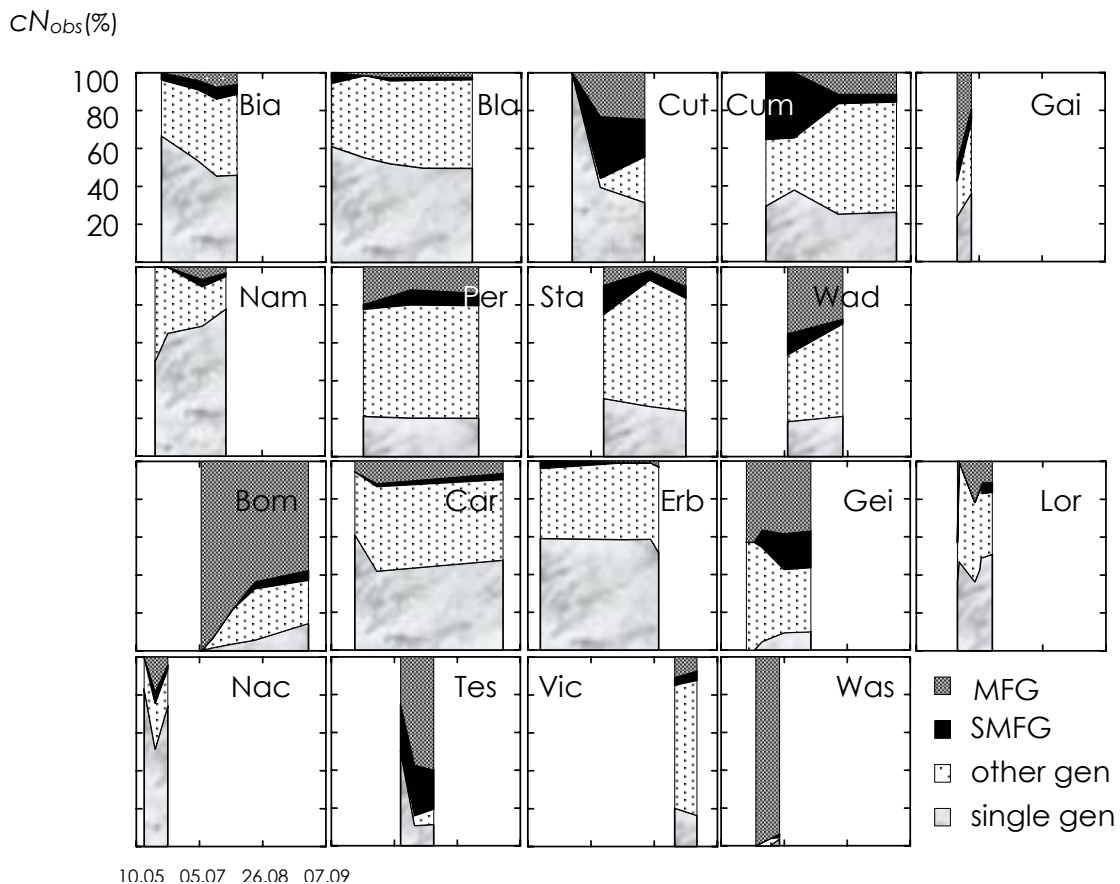
$cN_{obs}(\%)$

Figure 3. Cumulative proportion of disease severity (y-axis; $DS_\%$) caused by the most frequent genotype (MFG), the second most frequent genotype (SMFG), all other genotypes (other gen) and single genotypes as a function of epidemic progress. The survey periods (x-axis) spans from 10.05 to 27.08 on the sampling year, either 2000 or 2001 or 2002 (Table 1).

Downy mildew dispersion strategies

Based on the epidemics studied, *P. viticola* genotypes showed five distinct dispersal patterns: 1) clonal multiplication on single, or few, vines (Car: *afevissa*, Cum: *ebebesra*, Gei: *jlomaspa*, Lor: *avuzomme*, Tes: *evivurro* and Was: *ezazerma*); 2) clonal multiplication on single, or few, vines followed by plot-scaled dispersion (Bia: *ibevasca* and *evawasma*, Bom: *übetosca*, Cum and Cut: *alovispa*, *ibetosna*, Nac: *ymatosmo*, Per: *efoganta*, Vic: *abegisma*); 3) multi-cluster plot-scaled dispersion without previous clonal multiplication on a few vines (Lor: *afevimca*, Tes: *evuzemca*); 4) random plot-scaled dispersion without previous clonal multiplication on a

few vines (Gei: *egigusra*), and 5) minor clonal multiplication and dispersal. The last class included 99% of the genotypes. The MFGs found in Gai, Per, Sta, Vic and Wad were not strictly classified into any of these four categories because, on the first sampling date, they were already found to be partially plot-scale dispersed. Therefore, due to the lack of a restricted focus, they would eventually be classified into the third or fourth class.

In the following four case studies, we examined four dispersed patterns by genotypes that were identified at least 50 times each: *ezazerma* (strategy 1), *übetosca* (strategy 2), *egigusra* (strategy 3) and *evuzemca* (strategy 4).

Strategy 1: clonal multiplication on a single vine

In Wädenswil (Was) a heavily sporulating oil spot (genotype *ezazerma*) was detected on 01.06.02 on a leaf located within the medium-right sector of a vine (Fig. 4 and Table 1). After an asexual disease cycle, on 10.06.02, we observed a spatially restricted infection focus generated by the same genotype in the immediate vicinity of the first infected leaf. A total of 25 new oil spots were detected on six adjacent newly infected leaves: 14 lesions on two leaves within the left-medium sector (12 and 2 lesions, respectively) and 11 lesions on four leaves within the right-medium sector (3, 3, 3 and 2 lesions, respectively). No other genotype was identified among the 25 oil spots. On 16.06.02, presumably after a new secondary cycle, 85 lesions were detected within the same vine. Five additional genotypes were identified, suggesting the occurrence of new primary infections. One of them (*emizorma*) was already present twice (2 clones) on a unique leaf, suggesting a double infection by two (or more?) zoospores released from the same macrosporangium (oospore). The other four single genotypes were found on four distinct leaves. *Ezazerma* was found 79 times, colonizing 23 new leaves and sharing two of them with other two genotypes. On average, it produced 3.43 lesions per leaf (st. dev:1.8; max: 8 m; min: 1 lesions/leaf). Forty-one lesions were distributed within the medium and the highest left sector, while the remaining 38 were located within the right sectors. In summary, 50 lesions (on 14 leaves) were found on the left side of the wine, while on the right vine side 55 lesions (on 16 leaves) were found, mostly (68%) distributed within the medium sectors. Secondary sporangia did not migrate more than 1 meter after two secondary cycles, never reaching nearby vines that were perfectly healthy.
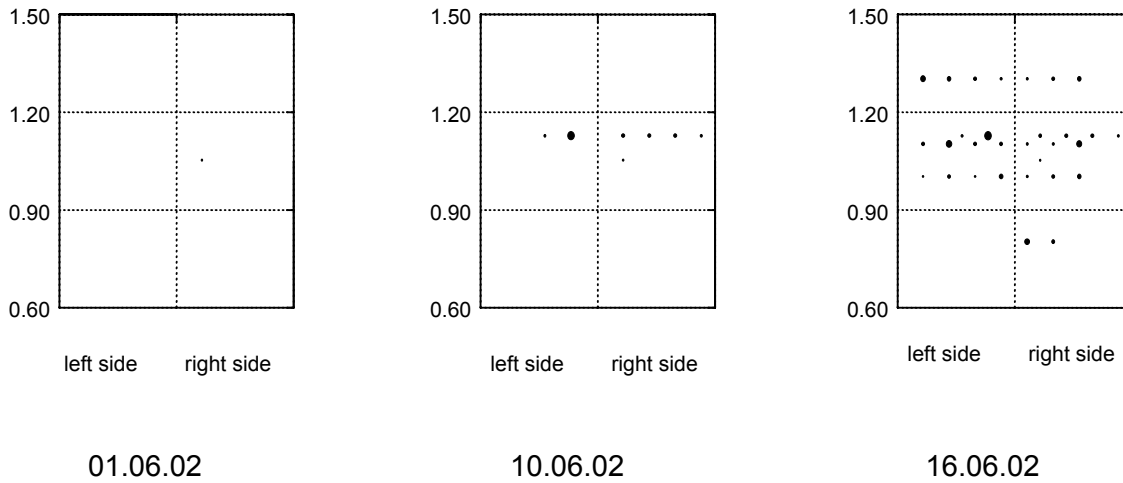
Figure 4. Cumulative spatio-temporal dispersion pattern of the most frequent genotype in Wädenswil (ezazerma). The single vine infected is represented by a bi-dimensional scaled rectangle, as if it was observed from a neighboring vine on the same row. Dimensions on x-axis represent left-side and right-side vine extensions, while y-axis represents vine height (in metres). The grapevine was divided into six sectors (left and right, top, medium and low sectors). Dot size is proportional to the natural logarithm of ($N_{obs\ MFG}$+1) and represents the number of MFG observations per infected leaf. The position of infected leaves was assigned randomly within the corresponding sector. Uninfected leaves and leaves infected by other genotypes are not shown. From left to right, plots describe the cumulative genotype spread as a function of time.

Strategy 2: clonal multiplication on few vines followed by vineyard-scaled dispersion

Among the 64 lesions collected in Bommes on 27.06.01, three distinct genotypes were identified. One, *übetosca* (MFG), was identified 61 times, while the other two, once and twice, respectively. At the time of collection, one oil spot out of the 61 lesions successively genotyped as *übetosca* wad visibly larger and more necrotized than the remaining 63 lesions. Therefore, we speculated that this oil spot may have been older (<27.06.01) than the other 60 ones, and that it may have been the lesion that generated the progeny (60 lesions until 27.06 + 51 lesions from 28.06 until 27.08) by successive secondary cycles (Fig. 5a; Table 1).

On 27.06, 13 vines (6,5 and 2 vines on rows 6,7 and 8, respectively) were unequally attacked by *übetosca* (causing 94% of total disease severity): 60% of the lesions were localised on the

tenth vine located in the seventh row (vine 10/7), 13% on the vine where the above-mentioned partially necrotized lesion was found (11/7), while the remaining 16 lesions were found on 11 immediately surrounding vines. Assuming the vine 11/7 to be the location of the very first primary infection (secondary inoculum source), secondary sporangia would have migrated, on average, 1.37 m (st. dev: 1.33 m). A maximal airborne dispersion of 6.5 m was found. All the lesions generated were dispersed approximately in a southern direction. During the next two weeks, one (or few) very mild secondary cycles occurred, which slightly increased the occurrence of the MFG (four new lesions found). The next two samplings revealed that *übetosca* colonised intensively in the northern part of the vineyard, with 25 and 22 new lesions, respectively. The MFG *übetosca* colonised a vineyard surface of approximately 133 m^2 (area of the confidence ellipse) on 27.08.01. Roughly, that corresponds to an average vineyard colonisation rate of about 2 m^2/ day or about the 24% of the total vineyard surface in about two months.

Throughout the survey period, the v/m ratio of the MFG was always >0; this suggested a aggregated pattern of distribution (Table 2). From a beginning ratio of 20.36 (prior to 27.06.01), we observed an exponential v/m decrease as a function of time or, stated differently, it was a consequence of the increasing number of secondary disease cycles. On 27.08.01, it reached the minimum of 7.05, which indicated that the MFG did not succeed in colonizing every grapevine with regular success. The frequencies of the different classes of $cN_{obs\ MFG}$ did not follow the Poisson pattern of distribution (P<0.001), as tested by χ^2. This indicates that the MFG was never randomly distributed within the vineyard. Considering all genotypes infecting the vines at any date in Bommes, we still detected a clustered pattern of distribution. When the MFG was excluded from the population, the χ^2 test a random pattern of distribution was found (P>0.05). Therefore, disease clustering appeared to be an effect of the limited spatial expansion of a sole genotype (MFG), while the remaining 40 genotypes (Table 1) did not confer any aggregation pattern to the population.

Geostatistical analysis confirmed a spatial dependence was present. The exponential model provided the best-fit for all isotropic semivariograms, describing the four epidemiological stages with a good approximation (R^2 ≈ 0.9). Overall, the autocorrelation decreased with increasing distance between vines. The nugget variance was <40% of the sill. The range of

spatial dependence was between 2 m and 3 m, with a sill of about 0.02, creating a steep slope that revealed a strong spatial dependence (semivariograms not shown). Only anisotropic semivariograms with North direction (along rows, angle=0°) were considered because models fitted with $R^2 > 0.5$ and approximated well isotropic semivariograms. The range of spatial dependence along the northern direction increased from 14 m to 19 m with epidemic progress, indicating a preferential distribution of the MFG along rows rather than a spreading to neighbouring rows. Anisotropic semivariograms constructed in other directions never gave an interpretable pattern with a satisfactory model fitting ($R^2 < 0.5$).
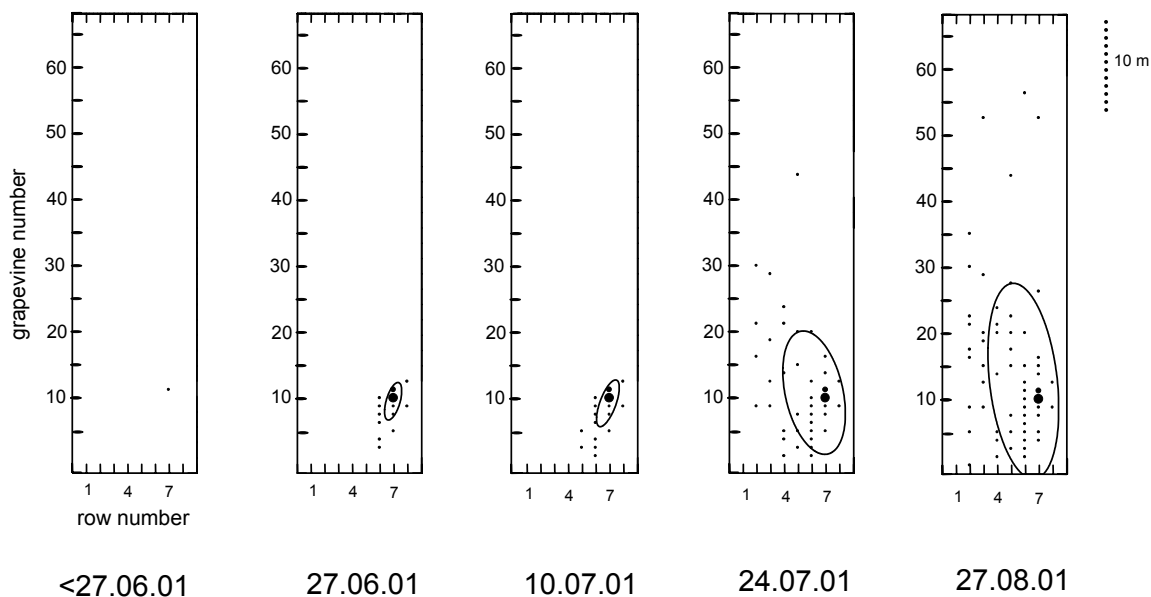
Figure 5a. Spatio-temporal dispersion patterns of the most frequent genotypes, *übetosca* found in Bommes. The vineyard is represented by a scaled rectangle (distances are given by the dotted line on the right hand side). Every vertical x-axis thick describes the beginning and the end of a grapevine row. Each number on x- and y-axis represent grapevine coordinates in the plot (row and grapevine number, respectively). Dot size is proportional to the natural logarithm of ($cN_{obs\ MFG+1}$) and represents the cumulative number of MFG observations per vine. ELL Gaussian bivariate confidence ellipses, centered on the sample means of x and y severities, were specified by a default probability value of 0.68. From left to right, plots describe the genotype spread as a function of time. The first plot describes the putative location of the very first infection that started the epidemic prior to 27.6.01. Row one was chemically treated and no sampling was done. The last 14 vines per row are not shown (12.6 m) because they were MFG-free.

Table 2. Location, sampling date (S date), cumulative number of observations (cN_{obs}), cumulative number of infected vines (cNiv), variance-to-mean ratios (v/m), calculated for population specific MFGs, MFG- (ev. SMFG-) deprived populations and total populations, collected in Bommes (Bom), Geisenheim (Gei) and Tesero (Tes).

Location S date	MFG $cN_{obs\ MFG}$ (%)[a]	cNiv (%)[b]	v/m[c]	MFG (ev. SMFG) deprived pop cN_{obs} (%)	cNiv (%)	v/m	total population cN_{obs} (%)	cNiv (%)	v/m
Bom	*übetosca*[d]			removed: *übetosca*[d,e]			all genotypes considered		
<27 .06.01[f]	1 (100)	1 (0.24)	20.36[nc]	0 (0)	0 (0)	nc	1 (100)	1(0.24)	20.36[nc]
27.06.01	61 (95.31)	13 (3.14)	12.49[*g]	3 (4.69)	3 (0.72)	11.70	64 (100)	15 (3.62)	11.91*
10.07.01	65 (79.27)	16 (3.86)	11.72*	17 (20.73)	17 (4.11)	4.83	82 (100)	31 (7.49)	9.35*
24.07.01	90 (64.29)	39 (9.42)	8.52*	50 (35.71)	49 (11.84)	2.75	140 (100)	81 (19.57)	5.56*
27.08.01	112 (58.03)	58 (14.00)	7.05*	81 (41.97)	77 (18.60)	2.15	193 (100)	126 (30.43)	4.17*
Gei	*egigusra*[d]			removed: *egigusra*[d] and *jlomaspa*[h]			all genotypes considered		
26.05.00	3 (42.86)	2 (5.12)	4.61	4 (57.14)	4 (10.26)	3.00	7 (100)	4 (10.26)	3.35
31.05.00	9 (64.29)	6 (15.38)	2.76	5 (35.71)	5 (12.82)	2.64	14 (100)	8 (20.51)	2.43
05.06.00	19 (43.12)	10 (25.64)	2.15	21 (47.72)	10 (30.77)	2.62	44 (100)	13 (33.33)	2.11*
19.06.00	74 (40.00)	19 (48.71)	1.78*	76 (41.08)	15 (38.46)	1.94*	185 (100)	22 (56.41)	1.91*
06.07.00	79 (38.35)	20 (51.28)	1.73*	87 (42.23)	20 (51.28)	1.72	206 (100)	24 (61.54)	1.80*
Tes	*evuzemca*[d]			removed: *evuzemca*[d] and *evivurro*[h]			all genotypes considered		
23.06.00	1 (25)	1 (0.30)	18.11[nc]	2 (50)	2 (0.61)	12.79	4 (100)	4 (1.22)	9.01
02.07.00	44 (58.67)	15 (4.57)	6.85*	10 (13.33)	9 (2.77)	6.20	75 (100)	22 (6.71)	5.18*
14.07.00	59 (60.82)	16 (4.88)	6.24*	17 (17.53)	13 (3.96)	5.45	97 (100)	22 (6.71)	4.74*

a: cNobs MFG / cN_{obs}.

b: cNiv / total vines. Total vines refer to 414 (Bom), 39 (Gei) and 328 (Tes).

c: Variance-to-mean ratio. v: variance of cN_{obs} per vine stock and m: mean of cN_{obs} per vine stock.

d: Most frequent genotype (MFG).

e: Only the MFG was removed, because the SMFG did not cause a relevant disease severity (10 lesions).

f: No sampling was performed prior to 27.06.01. An "open" date refers to the putative appearance of the very first primary infection caused by *übetosca*.

g: The Poisson distribution was calculated using P(x) = (e (-m) * mx) / x!. Statistical significance was assessed after χ^2 test."*" indicates P= 0.00.

h: Second most frequent genotype (SMFG).

nc: Statistic not calculated because of a lack of sufficient data points.

Table 3. Model parameters (maximal lag distance, lag increment and angle tolerance) and isotropic and anisotropic semivariogram characteristics of occurrences[a] of *P. viticola* MFGs, *übetosca, egigusra* and *evuzemca*, identified in Bom, Gei and Tes, respectively.

	max lag dist	lag incr	angle tol	model[b]	isotropic spc (all directions)				anisotropic spc (northern direction)			
					nugget[d]	sill[e]	range[f]	R^2	nugget[d]	sill[e]	range[f]	R^2
Bom: *übetosca*												
27.06.01	10 m	0.91 m	5°	exponential	0.003	0.016	2.990	0.896	0.005	0.030	14.05	0.584
10.07.01					0.003	0.016	2.790	0.908	0.006	0.031	14.33	0.585
24.07.01					0.006	0.019	2.090	0.911	0.010	0.035	17.89	0.543
27.08.01					0.008	0.022	2.240	0.912	0.012	0.042	18.77	0.536
Gei: *egigusra*												
26.05.00	8 m	1.21 m	5°	exponential	0.000	0.012	1.950	0.723	0.000	0.016	3.07	0.608
31.05.00					0.006	0.025	1.230	0.485	0.000	0.044	5.03	0.504
5.06.00					0.013	0.047	0.020	0	0.039	0.099	32-93	0.525
19.06.00					0.053	0.191	3.680	0.830	0.088	0.349	16-20	0.647
06.07.00					0.063	0.179	7.720	0.859	0.098	0.334	32-39	0.631
Tes: *evuzemca*												
02.07.00	10 m	0.91 m	5°	linear	0.022	0.033	9.307	0.611	0.017	0.065	25.84	0.418
14.07.00					0.029	0.045	9.307	0.632	0.023	0.086	22.75	0.350

a: Occurrence was transformed to the natural logarithm of the cumulative number of MFG observations incremented by one: $\ln(cN_{obs\ MFG}+1)$.

b: Regression model that best fit the semivariogram according to last squares analysis. Models resulting in highest R^2, with the lowest mean square error, indicated best fit.

c: Semivariogram parameters.

d: Nugget variance, also called c_0.

e: Sill, also defined as c_0+c_1.

f: Range of spatial dependence, or A_0 (isotropic semivariograms), is defined as the lag distance at which the semivariance approaches a constant value at which samples are no longer spatially related. Instead of a single value, for anisotropic variograms, a minimal (A_1) and a maximal (A_2) spatial dependence may be obtained (Gei).

Strategy 3: multi-cluster vineyard-scaled dispersion without previous clonal multiplication on a few vines

In Tesero, *evuzemca* was the genotype that contributed the most to the epidemic (MFG): it was identified on vine 1/14 on 23.06.00 and was responsible for 60% of the disease severity on 02.07.00 and 68% on 14.07.00 (Fig. 5b). On 02.07.00, 14 vines were attacked, mostly vines 5/16 (11 lesions on 9 leaves), 7/15 (8 lesions on 7 leaves) and 9/15 (8 lesions on 7 leaves). The other 16 lesions were dispersed on 11 vines. Considering vine 1/14 as the secondary inoculum source, the average dispersion distance was 6.41 m (st. dev: 2.87 m; max: 16.88 m; min: 2.19 m). A weaker dispersion occurred in the period 02 to 14.07.00: the MFG was newly identified 15 times, mostly on the same vines that were already colonised (only vine 4/16 was newly colonized by a MFG clone). The 15 clones were identified on six vines, mostly on vine 11/15, showing seven lesions on four leaves. With an average colonisation rate of about 1.3 m^2/day, the MFG invaded only 7% of the total vineyard area during 3 weeks. The SMFG (*evivurro*) caused 20 lesions at the beginning of July (28% severity) but, afterwards, it was not identified again. It followed strategy 1. 83% of the total disease severity was due to the combined pathogenicity of the two latter genotypes. The detailed epidemic pattern is reported in Gobbin *et al.*, 2003b.

Exactly as shown for *übetosca* (Bom), initially the v/m ratio indicated a high disease clustering that decreased with epidemic progress. Significance (according to χ^2 test) of this simple aggregation index is achieved exclusively when MFG alone or total populations (comprehending MFG) are considered, indicating a non-random pattern of symptom distribution. Considering *evivurro-* and *evuzemca-* deprived populations, significance is no longer detected (p>0.05). Geostatistical analysis confirmed the existence of spatial dependence. The linear model provided the best fit, nevertheless with modest correlations (R^2 =0.6 for isotropic semivariograms and R^2=0.4 for anisotropic semivariograms with angle=0°). For isotropic variograms, the range was 9.3 m long, but the nugget variance was 70% of the sill (both for the second and third samplings). The range of spatial dependence was extended more when we considered anisotropy (North direction), and the nugget variance about ¼ of the sill.
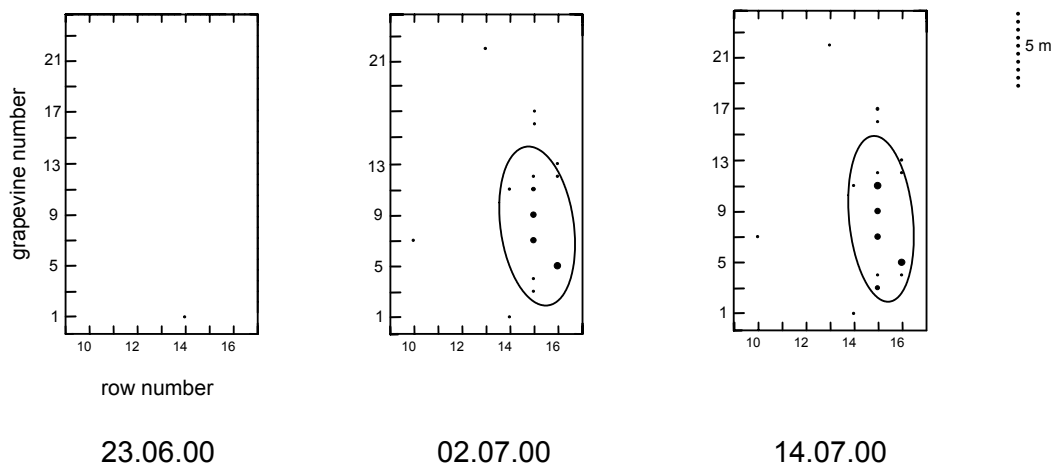
Figure 5b. Spatio-temporal dispersion patterns of the most frequent genotype *evuzemca* found in Tesero. The vineyard is represented by a scaled rectangle (distances are given by the dotted line on the right hand side). Every vertical x-axis thick describes the beginning and the end of a grapevine row. Each number on x- and y-axis represent grapevine coordinates in the plot (row and grapevine number, respectively). Dot size is proportional to the natural logarithm of ($cN_{obs\ MFG}$+1) and represents the cumulative number of MFG observations per vine. ELL Gaussian bivariate confidence ellipses, centered on the sample means of x and y severities, were specified by a default probability value of 0.68. From left to right, plots describe the genotype spread as a function of time. Only the last seven rows (of 16) are shown because of disease hosting.

Strategy 4: random vineyard-scaled dispersion without previous clonal multiplication on a few vines

In Geisenheim on 26.05.00, the MFG *egigusra* was found colonising two nearby vines both located in the third row (Fig. 5c and Table 1). One oil spot was found on vine 7/3 and two lesions on vine 8/3. We speculated that a secondary cycle were already occurred and that the very first primary infection that originated the three MFG clones, was missed by the observers, because it was small or hidden among the canopy. On 31.05.00, *egigusra* was identified on four vines. Assuming that vine 8/3 was the secondary inoculum source, the average migration distance was 4.97 m with a st. dev. of 1.54 m (max: 7.00 m; min: 3.6 m). It took only 10 days after the first appearance date to colonise 10 vines (26%) dispersed throughout the plot. One disease focus was observed on the fourth sampling date on the two vines where the MFG was first observed: on vine 7/3, 13 new oil spots were located on 10 leaves and, on vine 8/3, 11 leaves were infected each by the MFG. Forty days after the first observation, on 06.07.00, lesions were observed on 20 vines (51%) disposed on a diagonal passing from the vine 3/1, to vine 13/3. With a colonisation rate of about 0.8 m²/day, the MFG was found on vines covering 38% of the total vineyard surface. The SMFG (*jlomaspa*) was identified on 05.06.00 four times on vine 12/3, and on 19.06, it was found on three vines (once on 8/2, 7 times on 11/3 and 22 times on 12/3). The secondary sporangia migration distances spanned from a minimum of ≈ 0 m to a maximum of 5.13 m (avg: 0.44 m; st. dev: 1 m), showing a behaviour between strategy 1 and 2. On 06.07.00, it was identified five times in the vicinity of older infections (three times on vine 12/3 and twice on vine 13/3).

The variance-to-mean ratio of the MFG decreased exponentially from 4.61 (26.05.00) to 1.73 (06.07.00). χ^2 statistic suggested a non-random pattern of distribution as soon as the focus localized on vines 7-8/3 appeared (19.06). Variance to mean ratios (always >1) did not differ substantially when considering MFG spatial distribution, MFG and SMFG deprived populations and total populations. This indicates that clustering, when present, was not strictly caused by *egigusra* and *jlomaspa*, but by the other genotypes as well. Geostatistical analysis showed that $\ln(cN_{obs\ MFG}+1)$ was not always spatially autocorrlated. The isotropic distribution of $\ln(cN_{obs\ MFG}+1)$ fit the exponential model best in four of five epidemiological stages, but no model fit the data collected on 05.06.00. The range varied from 1.23 to 7.72 m.

Along the rows (northern direction only), $\ln(cN_{obs\ MFG}+1)$ distribution fit semivariogram data with lower correlations, but showed longer autocorrelation distances (ranges).
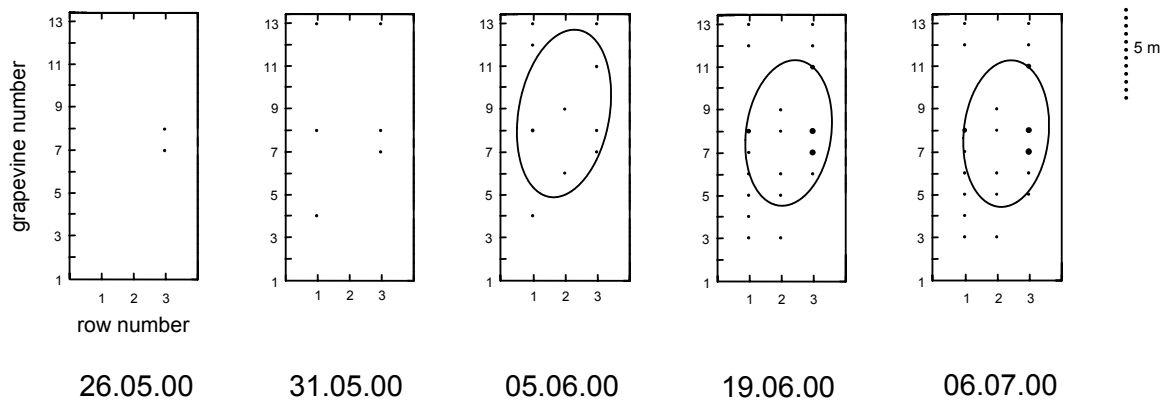

Figure 5c. Spatio-temporal dispersion patterns of the most frequent genotype *egigusra* found in Geisenheim. The vineyard is represented by a scaled rectangle (distances are given by the dotted line on the right hand side). Every vertical x-axis thick describes the beginning and the end of a grapevine row. Each number on x- and y-axis represent grapevine coordinates in the plot (row and grapevine number, respectively). Dot size is proportional to the natural logarithm of $(cN_{obs\ MFG}+1)$ and represents the cumulative number of MFG observations per vine. ELL Gaussian bivariate confidence ellipses, centered on the sample means of x and y severities, were specified by a default probability value of 0.68. From left to right, plots describe the genotype spread as a function of time.

Primary vs. secondary cycle

The percentage of lesions caused by putative oosporic inoculum cumulated showed a similar trend in every of the 18 epidemic surveyed (Fig. 6). If we assume, per definition, the very first lesions exclusively as primary (immigration not accounted), in every epidemic we always started with 100% primary lesions and 0% secondary lesions. Immediately after the identification of the first symptoms, an abrupt decrease of primaries and the consequent inverse increase of secondary lesions were assessed. This period varied from epidemic to

epidemic: it lasted 7 days in Bom, 9 days in Was, 10 days in Tes, 18 days in cut and cum, approximately one month (Bia, Per) or even more (Bla). After this time-lap, a constant level of primary and secondary lesions seems to settle and stabilize for the subsequent surveying period. This plot-specific level was found to be highly variable (20-90% primary lesions).
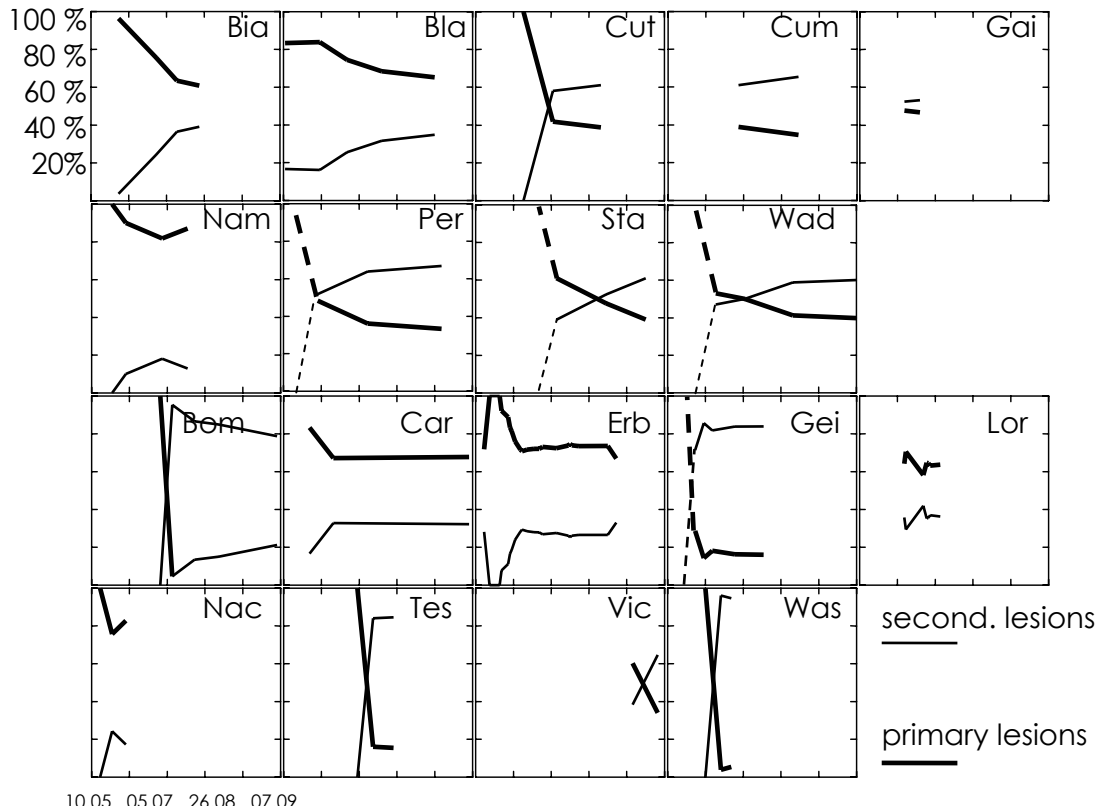

Figure 6. Relative contribution of cumulative primary and secondary inoculum. The first appearance of a genotype was considered as an oosporic-derived primary lesion. Any subsequent observation of the same genotype was interpreted as a secondary lesion. Dotted lines are set to indicate the putative appearance time of the very first primary lesions (100% primary lesions and 0% secondary lesions), missed by the observers.

DISCUSSION

Sampling strategies

In this study, two kinds of sampling strategies were applied: PSS and TSS. The total sampling strategy (TSS) was practical only when downy mildew epidemics were below a few hundred lesions throughout the survey period. Such epidemics are not typical for central Europe, where a rainy-mild climate favors explosive disease development. In 9 of the 18 epidemics studied, we employed, from the second sampling on, a partial sampling strategy (1-3 lesions per vine), ensuring the collection from throughout the plot. The main difference between sampling strategies is related to their ability to find highly aggregated clones. Partial sampling failed to detect clones restricted to an area smaller than the resolution of the grid and did not allow exhaustive sampling within single vines. As a result, this strategy underestimated the number of progeny per genotype (Fig. 2a and 2b, right side). Nevertheless, differences among data collected by both sampling types were nonsignificant: PSS and TSS equally determined the frequency ($F_{tNobs=x}$) and the disease severity ($DS_\%$) of every occurrence class (tN_{obs}=X).

Downy mildew dispersion strategies

We described four case studies (Wädenswil, Bommes, Tesero and Geisenheim) in which *P. viticola* exhibited essentially four different dispersal patterns. The finding of different dispersion strategies was expected because of regional variation in climatic conditions, cultivation systems and strain-specific aptitude in generating secondary lesions. Different pathogen aptitude in generating secondary lesions was strikingly revealed in Was (*ezazerma*), Tes (*evivurro*), Car (*afevissa*) and Cum (*ebebesra*), where the genotypes colonised, intensively, a single vine but did not spread to other vines. This initial spatially limited aggregation of secondary inoculum (distance among clones < 1 m) seems to be the antecedent step to the genotype dispersing further (strategy 2); this was observed, for instance, in Bom (*übetosca*) and in Cugnasco (Cut and Cum plots; *alovispa* and *ibetosna*). In the Cugnasco case study, the MFGs and SMFGs first originated in the Cut plot, accrued their number on a few vines after a (few) secondary cycle(s), and finally migrated to the Cum plot

located 10 m away (results not shown). Further dispersal patterns follow the examples of Tesero (*evuzemca*), where secondary sporangia generated a few clusters, and Geisenheim (*egigusra*), were sporangia were dispersed at a plot scale generating a random pattern. The last two case study suggest a more effective airborne sporangia dispersal than the Was and Bom cases. In none of the epidemic studied we showed a homogenous plot colonisation by MFG or any other genotype.

Surprisingly, we found that genotypes that generated several secondary lesions at a precise sampling date are not necessarily found throughout the following epidemiological stages. For instance, among the categories of MFGs and SMFGs, we found four genotypes that suddenly disappeared (Erb: *dgulepma*, Tes: *evivurro*, Car: *afevissa*, Nag: *avavissa*) and three genotypes whose progeny drastically fluctuated over time, in terms of number of newly produced secondary lesions (Lor: *avuzomme*, Gei: *egigusra*, Bom: *übetosca*, Table 1).

Aggregation and geostatistical analysis

In this study aggregation analysis was conducted for genotypes identified twice throughout the survey period (tN_{obs}=2) and, with more statistical details, for MFGs. For the first category, we showed that aggregation was higher when both clones were identified the same date than at two distinct dates. The finding of two oil spots with identical genetic profiles may be interpreted in three different manners. They may be either 1) both primary lesions generated by two zoospores produced by the same oospore, or 2) both secondary lesions deriving from a common non-sampled "mother" (primary lesion) or 3) they can be "mother and son" (a primary lesion and a secondary lesion generated by the primary one). We lean toward the first hypothesis to explain the simultaneous identification of the 49.5% of clone pairs (or at least 20.6% of the clone pairs colonizing the same leaf). We may assume that splash-dispersed zoospores combined with further rainfall may disperse the infective propagules until 1 m distance. For explaining spreading distances of more than one metre, the second or third hypotheses may be more reasonable. The finding of two clones at two different sampling dates mainly at distances longer than 3m indicates that clones tend to spread apart after secondary cycles and that new plot areas are colonized.

Spatial analysis of MFG clones showed an aggregation especially in Bommes and Tesero, less markedly in Geisenheim. The degree of aggregation was high at the beginning of the epidemic and decreased as the disease progressed. Significant non-random spatial distribution of clones, which was interpreted as aggregation of the clones, was detected exclusively when we considered the dispersion pattern of the MFG or the total population. The opposite result was obtained when the spatial distribution of MFG-, and SMFG-deprived population was analysed: those populations composed essentially of single genotypes or genotypes found only two or three times, were randomly distributed. This indicates that, if a genotype multiplies asexually, then all the clones may be dispersed in clusters and not randomly within the plot. Thus, downy mildew populations showing a clustered character may therefore hide a single (or a few) dominant genotypes, which are multiplying asexually. Conversely, a random pattern distribution may be interpreted as absence of a dominant genotype or presence of non-dominant genotypes.

Once aggregation of clones of the MFGs in the plots was detected by the index of dispersion, spatial autocorrelation analysis was used to examine directionality of aggregation within the plots. Anisotropic spatial autocorrelation was found exclusively along row direction, where models were fitted with good approximation. No relationship between spatial distance and semivariance could be found in all the other directions examined. Isotropic autocorrelation analysis revealed shorter ranges of spatial dependence than anisotropic analysis, and models, with exceptions for Gei 31.05.00 and Gei 05.06.00, had fairly good correlations ($R^2 > 0.6$). Isotropismus is easily explained by the regular host allocation along rows, which allows the pathogen to spread up and down the rows without discontinuity. A "jump" of about 2 metres instead is required to reach the nearest vine located to the adjacent row. Good spatial dependence along the rows indicates that the pathogen spreads preferentially along the rows; nevertheless, as the Geisenheim case study shows, this is far from to representing a rule.

Implications for downy mildew epidemiology

To our knowledge, this is one of the rare studies to provide a wide-scale quantitative analysis of the proportion of *P. viticola* genotypes that undergoes secondary multiplication and of the genotype-specific distribution within plots during an epidemic (Gobbin *et al.,* 2003b, Rumbou & Gessler, 2004). As a result, one of the most salient consequences of this study consists in the deviation from the generally accepted view that an epidemic starts from a few primary infections, followed by a massive asexual reproduction. Instead, we observed that there was a continuous input of new genotypes to the epidemic, which contributed one or a few lesions each to the total disease severity. Rumbou & Gessler (2004) reached the same conclusions. We assume at least single genotypes to be oosporic-derived primary infections, especially when the sampling was performed exhaustively (TSS). For instance, after analysing 361 oil spots collected in seven periods in the Lorch epidemic, we could always find genotypes that were not collected on the previous sampling(s) date(s). The same trend occurred in every epidemic surveyed, though less markedly in Gei and in Was. About one third of the lesions sampled (or about ¾ of the genotypes sampled), depending on the epidemic and sampling strategy, was composed by genotypes that never gave rise to asexual progeny or gave rise to progeny that were undetected by the observers. A restricted genotype life-time may be an indication of a generalised low fitness (virulence) of secondary sporangia. Some studies have shown that many fungal species, such as the downy mildews, are highly sensitive to environmental conditions. For example, Rotem *et al.,* (1985) have shown that many species are negatively affected by long wavelength UV. Furthermore, unfavourable leaf wetness duration or intolerance to temperatures that deviate from the optimum may be other factors that lead to high mortality of lesions (or very restricted clonal multiplication) and even to disappearance of successful genotypes.

Another finding of this study is that the clones of a genotype are descendents of a primary infection previously originated within the same plot and not the result of secondary sporangia immigration from a neighbour plot. The underpinning of this assumption is based on following facts: 1) surrounding plots are constantly treated with fungicides according to precise schedules. This implies that vines are normally disease-free, providing low (or no) inoculum source, 2) a strong aggregation of the clones of the same genotype (specifically

Was, Bom and Tes) was found in this study. According to dispersion functions (Skogsmyr, 1994; Melé, 2001), we do not expect a large quantity of secondary sporangia immigrating from far distances, 3) the sporangia dispersion rate, even if roughly calculated, was found to be rather moderate, ranging around 1 to 2 m²/day and, 4) generally, the number of successful infections in comparison to the very high number of sporangia produced (Agrios, 1988) is very low, drastically reducing the chances of migration of the considered genotype. Two case studies support the fourth statement: the first, in Navicello, where the surveyed plots are located 50 m away (Nac and Nam), only a genotype among 370 (in Nac) and among 142 (in Nam) was shared among the two total *P. viticola* populations. Second, in the previously cited Cugnasco plots (Cut and Cum), where two successful genotypes (*alovispa* and *ibetosna*) required approximately one month to migrate from Cut to Cum (10 m). Based on those four considerations, we hypothesize that secondary sporangia-mediated gene flow was overestimated in the past. Massive asexual multiplication, as shown for Bom, Tes and Gei, seems, therefore, to be the exception rather than the rule. This may be why epidemiological models based on subsequent and massive secondary disease cycles, are often inaccurate in disease forecasting and risk estimation.

If secondary inoculum has an undergraduate role in pathogen dissemination, it still remains unclear how downy mildew spread rapidly across Europe after its introduction in 1878. We speculate that the oospore-mediated gene flow is more efficient, because the long viability of oospores (at least five years, GK Hill, personal communication) would permit more extensive disease spread. Alternative explanations may rely on multiple introduction events followed by human-mediated transport of infected material (rooted grape cuttings carrying soil that contains oospores) or on unreported pathogen introduction(s) prior to 1878.

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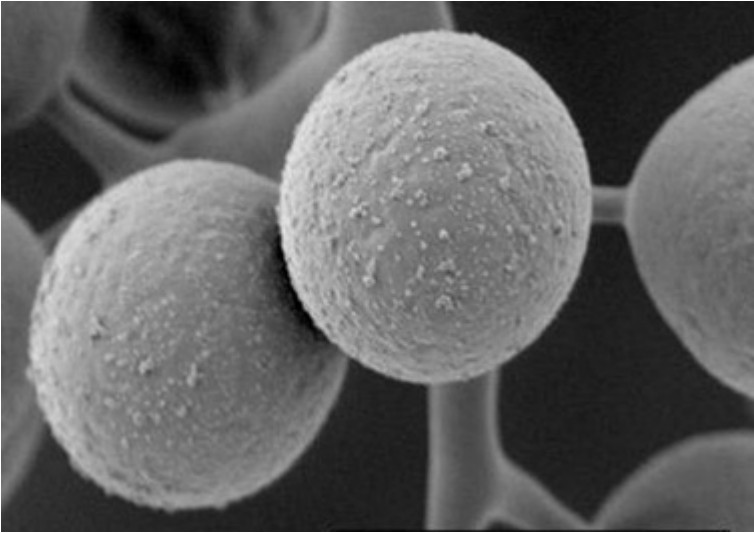
P. viticola secondary sporangia. Picture by: HH Kassemeyer

A population genetic snapshot of *Plasmopara viticola* after 125 years of colonisation in Europe

This chapter was written in collaboration with Artemis Rumbou, Phytopathology, ETH, Zürich.

ABSTRACT

In order to examine the levels of genetic differentiation within and among *P. viticola* populations 125 years after the introduction to Europe of the Oomycete, we collected 9229 lesions from 34 downy mildew populations (France, Germany, Switzerland, Italy and Greece). Four polymorphic microsatellite loci were used for genotyping, and they allowed the identification of 4328 site-specific genotypes. One hundred and thirty nine alleles were found, among which, at population level, 10 were omnipresent and 18 were private. Oosporic populations were often in HWE equilibrium. Within population diversity attained high values in three out of the four markers ($H_o \geq 0.5$, with GOB showing $H_o = 0.92$). F_{ST} ranged from a value of 0 to 0.137, with an average of 0.03. The pairwise test of differentiation highlighted significant differences for 524 out of 561 population comparisons. The highest values were recorded between European and Greek populations, as $F_{ST} = 0.14$ between Blanquefort (F) and Damassi (GR). We found a significant relationship among geographical distance and F_{ST}, when including all 34 populations, indicating a strong evidence of isolation by distance. The Neighbor Joining phylogenetic tree based on Shriver's Dsw revealed a clear substructuring, distinguishing mainly five clusters: the German, the French/Swiss, the South of Alps, the Greek East/mainland and the Greek West. The Neighbor Joining tree topology showed a pattern similar to the geographical distances separating the populations. Those findings led us to the conclusion that *P. viticola* populations are independent demographical units and that gene flow plays a minor role at large geographical scale impeding the homogenization of the communities.

INTRODUCTION

The diploid Oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni (Order: Peronosporales, Family: Peronosporaceae), the causal agent of grapevine downy mildew disease, was introduced to Europe (France) from North America in 1878 through the phylloxera (*Daktulosphaira vitifoliae*) resistant wild American rootstocks. The dispersal of the pathogen was surprisingly fast: in 1879 it was reported to be 'all over France and Italy', the year after in Germany, Mosel region, (Müller and Schleumer, 1934) and in 1881 in Peloponnesus of Greece (Messinia region) (Gennadios, 1889). However twenty years passed until the first really disastrous epidemic in 1900 occurred when, in a short time, two-thirds of the expected yield were destroyed (Sarejanni, 1951). Since then, downy mildew constitutes the most vicious grapevine disease in Europe and, despite numerous studies on the biology and epidemiology of the pathogen, a consistently effective control strategy has yet to be proposed. Evolutionarily, the introduction of *P. viticola* to Europe can be described as a founder event, with the American population being the parental population of the European one. Consequently, the population developed was characterized by a genetic drift, which followed the migration of the pathogen. Despite the relatively low amount of individuals that initially immigrated to Europe, in comparison to the large American effective population size, the Oomycete succeeded in colonizing many different susceptible host varieties covering huge regions of monoculture. Until 1880, when the copper based fungicide called Bordeaux mixture was first applied, the Oomycete was allowed to spread undisturbed from vineyard to vineyard (Psceidt and Ocamb, 1993). These favorable conditions, in combination with the diverse reproductive manners of the organism, both sexual and asexual reproduction (McDonald and Linde, 2002), facilitated the high increase of the number of individuals to an epidemic level.

Until now, it has been assumed that few genotypes are responsible for the epidemic, whose explosive progress is caused by the asexual cycles of the disease. The dispersal of the disease relies on the migration of sporangia, is supposed to attain long distances in a short time,

nevertheless no data that precisely quantify sporangia dispersal rate or distance are available (Agrios, 1988; Blaise *et al.,* 1999; Lafon and Clerjeau, 1988, Zahos, 1959). Given these assumptions, the European population is expected to be homogeneous and characterized by limited substructure and low genotypic diversity. This hypothesis has not been investigated because the appropriate molecular tools were only recently developed (Gobbin *et al.,* 2003a). These tools, microsatellite markers, were now used to investigate the validity of the current assumptions mentioned above. For that aim, we examined the genetic structure of 34 *P. viticola* populations collected in five wine producing European countries. We studied the phylogenetic relationships among them and, finally, we explored the pattern of isolation by distance, to determine whether populations are in equilibrium between gene flow and genetic drift.

MATERIALS AND METHODS

Sample collection

Collections were made during 2000, 2001 and 2002 in Switzerland, Germany, France, Italy and Greece (Fig. 1a and 1b). The 32 selected plots were not treated against downy mildew during the three years of the samplings, with some exceptions (plots at Cugnasco, Lefkada, Rapsani and Naoussa). In Greece, the first sampling in each plot was performed approximately one week after the first rain in May. In central Europe, the selected plots were checked weekly for primary lesions after the 1st of May, and all the detected lesions were collected as soon as they appeared. The dates of the next samplings depended on the rain events during the grapevine-growing period. On average, four samplings took place in each plot (min: 1, max: 22, total: 137 independent samplings). Two different sampling strategies were used: total- and partial- sampling strategies (TSS and PSS, respectively). Applying a total sampling strategy, part (about ½ to 1/3) of every identifiable disease symptom on the leaves was collected as soon it was visible and the rest of the lesion remained within the population. Partial sampling strategies were performed as an alternative to total sampling only when a high disease severity was observed (on average > 10 oil spots per vine) that impeded an exhaustive sampling. In general, one to two lesions per vine were sampled by cutting part of each lesion. In four plots the samplings were repeated for a second continuous year (rap, nao, agh and kef, Table 1). The exact site of the plots, the number and dates of the samplings, the sampling strategies, and the total number of oilspots collected in each plot are shown in Table 1.
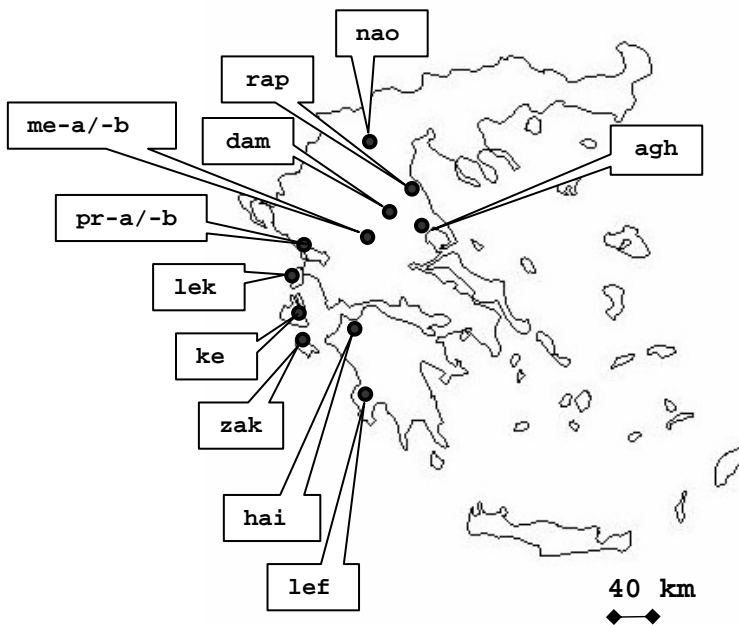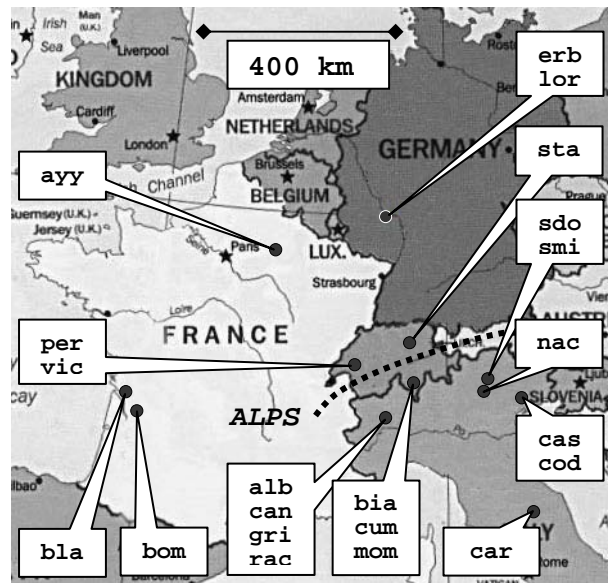
Figures 1ab. Geographical maps of central Europe (1a) and Greece (1b) showing the locations from which the 34 *P. viticola* populations were sampled.

Table 1. Vineyard location (nation and plot), grape variety, *P. viticola* population name, sampling period, number of samples, number of individuals collected, number of genotypes identified and Shannon's equitability (E_H).

Nation	plot	*P. vit* pop	Sampling period	N sam (TSS+PSS)	N ind	N gen	E_H
France	Ay (Champagne)	ayy	26/05/00-20/06/00	3 (2+1)[a]	121	89	0.90
France	Blanquefort (Bordeaux)	bla	09/05/00-07/08/00	6 (1+5)	557	363	0.89
France	Bommes (Bordeaux)	bom	20/06/01-27/08/01	4 (2+2)	193	41	0.39
Switzerland	Perroy (Ct. Vaud)	per	30/05/00-11/08/00	3 (0+3)	325	108	0.66
Switzerland	Vic (Ct. Vaud)	vic	11/08/00-25/08/00	2 (2+0)	336	114	0.72
Switzerland	Stäfa (Ct. Zürich)	sta	27/06/00-18/08/00	3 (1+2)	328	129	0.72
Switzerland	Biasca (Ct. Ticino)	bia	26/05/00-13/07/00	4 (1+3)	314	191	0.85
Switzerland	Cugnasco (Ct. Ticino)	cum	07/06/01-29/08/01	4 (1+3)	484	193	0.73
Switzerland	Montalbano (Ct. Ticino)	mom	26/05/00-07/08/00	2 (0+2)	238	190	0.93
Italy	Navicello (Trentino)	nac	15/05/00-06/07/00	5 (3+2)	572	464	0.94
Italy	San Donà (Trentino)	sdo	22/05/00-18/07/00	4 (2+2)	148	122	0.94
Italy	San Michele (Trentino)	smi	22/05/00-26/07/00	3 (2+1)	104	91	0.95
Italy	Alba (Pedimont)	alb	24/05/00-13/07/00	2 (1+1)	81	65	0.89
Italy	Canale (Pedimont)	can	24/05/00-13/07/00	2 (1+1)	103	93	0.97
Italy	Grinzane (Pedimont)	gri	24/05/00-13/07/00	2 (1+1)	62	51	0.93
Italy	Racca Guarente (Pedimont)	rac	24/05/00-13/07/00	2 (1+1)	77	66	0.96
Italy	Casarsa (Veneto)	cas	17/05/00-04/07/00	3 (1+2)	196	142	0.91
Italy	Codroipo (Veneto)	cod	17/05/00-04/07/00	3 (1+2)	222	188	0.95
Italy	Carpineta (Emilia-Romagna)	car	25/05/01-27/08.01	3 (3+0)	124	83	0.89
Germany	Erbach (Rhineland)	erb	18/05/00-01/08/00	22 (22+0)	287	192	0.89
Germany	Lorch (Rhineland)	lor	05/06/00-27/06/00	7 (7+0)	361	228	0.83
Greece	Lefkohora (Messini)	lef	13/05/00	1 (0+1)	32	29	0.96
Greece	Haikali (Ahaia)	hai	12/05/00	1 (0+1)	39	35	0.96
Greece	Rapsani (Larissa)	rap	31/05/00,27/10/01	2 (0+2)	135	111	0.92
Greece	Naoussa (Imathia)	nao	01/06/00,23/10/01	2 (0+2)	150	74	0.71
Greece	Damassi (Larissa)	dam	29/05/01-15/10/01	6 (3+3)	656	131	0.62
Greece	Messenikolas a (Karditsa)	me-a	23/05/00-27/06/00	4 (0+4)	278	88	0.63
Greece	Messenikolas b (Karditsa)	me-b	08/06/01-19/10/01	5 (0+5)	674	229	0.73
Greece	N. Aghialos (Magnissia)	agh	28/05/01-02/07/02	9 (9+0)	632	71	0.28
Greece	Preveza a (Preveza)	pr-a	07/06/00-15/07/00	3 (0+3)	140	87	0.85
Greece	Preveza b (Preveza)	pr-b	31/05/01-13/11/01	4 (0+4)	479	150	0.65
Greece	Lefkada (Lefkada)	lek	31/05/01-22/11/01	4 (0+4)	430	129	0.65
Greece	Kephalonia (Kephalonia)	kef	13/06/01-16/07/02	5 (5+0)	199	32	0.44
Greece	Zakynthos (Zakynthos)	zak	14/06/01-18/07/01	2 (0+2)	152	23	0.45
Total	**Europe**			**137**	**9229**	**4328**	

a: the first number in parenthesis refers to the number of exhaustive samplings (TSS), while the second refers to the number of partial samplings (PSS), in chronological order.

Microsatellite locus amplification and genotyping

For genotyping all the collected lesions, we used automated high throughput DNA extraction, PCR amplification of the four *P. viticola* specific microsatellite loci ISA, GOB, CES, and BER, sequencer based fragment analysis and automatic allele scoring. Every step was performed exactly as described in Gobbin *et al.,* (2003a). *P. viticola* genotypes were defined as a unique microsatellite allele pattern after PCR amplification with the four microsatellite markers ISA, GOB, CES, and BER. Samples presenting the same allele pattern were considered as clones (derived from the same oospore), while the ones presenting a different allele pattern were assumed to have derived from different oospores.

Genetic analysis

In this study, we considered exclusively the clone-corrected populations (oospore-derived or sexual). As a result, the impact of all genotypes was equalized. After microsatellite analysis, all the 137 independent samples (9229 lesions collected from 32 plots) were therefore clone-corrected. Afterward, the clone-corrected samples collected within the same plot (on average, four samples per plot) during one or two growing seasons were subjected to a pairwise test of differentiation applying the log-likelihood statistic F_{ST} for diploid populations (Goudet *et al.,* 1996) with the use of FSTAT software (FSTAT, version 2.9.3.1; Goudet, 1995; 32 groups of pairwise tests). Populations with no significant difference at the 5% nominal level were pooled together, as they all were partitions of the same population. Again, they were subjected to clone-correction (4328 site-specific genotypes collected from 32 plots were obtained). The samples showing significant differences were treated separately, even if collected from the same plot (me-a and me-b, pre and pr-b). Thirty-four populations, each constituted by different genotypes, were finally obtained and used for the genetic analysis. The numbers of different genotypes identified in each clone-corrected (oosporic) population are presented in Table 1.

The genetic diversity was studied separately for each of the 34 populations. At each locus, number of alleles, allele frequencies, observed (H_o) and expected heterozygosity (H_e) were assessed by FSTAT. Departures from the Hardy-Weinberg equilibrium were tested by the

Markov chain randomization method of Guo and Thompson (1992) with the use of GENEPOP
version 3.1d (Raymond and Rousset, 1995) by setting 1000 dememorization steps and 2000
batches with 1000 iterations per batch. Locus BER, which in most populations possessed 2-3
alleles, was tested for HWE by the complete enumeration method, as described by Louis and
Dempster (1987).

The occurrence of null alleles, as a consequence of deletions or mutations at primer
hybridization sites, is a possible problem associated with microsatellite 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. To account for this problem, the
presence of "null" (also called non-amplifying) alleles within populations was calculated with
the software Identity 1.0 (Wagner and Sefc, 1999).

Genotypic diversity was estimated using individual genotypes. Shannon's index (H), which
accounts for both abundance and evenness of the genotypes distribution, was calculated for
each population using the total number of individuals collected in each plot (Krebs, 1989).
Shannon's equitability (E_H) was then calculated by dividing H by H_{max} (here H_{max}= lnN, where
N is the number of individuals in each sample).

Population genetic structure was first examined by testing the null hypothesis that the
distribution of alleles is not significantly different across all 34 populations. Pairwise tests for
allelic differentiation was performed by using the overall loci G-statistic, and their
significance was evaluated after applying the sequential Bonferroni correction for multiple
tests. The degree of differentiation among the plots was quantified using Weir and
Cokerham's (1984) estimator (theta) of Wright's F_{ST}, as calculated by FSTAT. In order to
integrate the stepwise mutation model of microsatellites in our study of population
structure, we also calculated the standardized R_{ST} value (Slatkin, 1995) by means of SPAGEDI 1.1
(Hardy and Vekemans 2002).

Genetic distance matrixes were estimated using both measures based on variance in allele
frequencies (Shriver's Dsw) (Shriver *et al.,* 1995) and on variance due to number of repeats
(Goldstein's $\delta\mu^2$) (Goldstein and Pollock, 1997) by using the software POPULATIONS (Langella

2001). Neighbor-joining phylogenetic trees (NJ) were visualized by means of the software TREE EXPLORER, included in MEGA, version 3 (Kumar *et al.,* 2001).

Isolation by distance

We constructed a graph showing the relationship between pairwise F_{ST} and the geographical distance using the software ISOLDE available with the GENEPOP package (Rousset, 1997). Pairwise distances among the 34 collection sites were calculated by means of geographical maps of Europe, without correcting for Earth curvature. Significance in the isolation by distance relationship was tested statistically using a Mantel test (1000 permutations) available within the GENEPOP package. This test assesses whether the pairwise genetic distance matrix is correlated with the pairwise geographic distance matrix.

RESULTS

Genetic variation

Pairwise test of differentiation calculated by FSTAT showed that samples collected within the same plot were overwhelmingly non-significantly different (423 cases out of 455 pairwise tests). Exceptionally, the 2001 samples of both the Messenikolas and Preveza plots (me-b and pr-b samples, respectively), were statistically different from the respective 2000 collections (me-a and pr-a samples, respectively). Finally, out of 137 independent collections, we obtained 34 pooled and clone-corrected populations (Table 1), coming from 32 European plots: 21 from central Europe (cEu) and 11 from Greece (Gr). In cEu, 5233 lesions were collected and 3203 plot-specific genotypes were identified. In Gr 3996, lesions were sampled belonging to 1189 plot-specific genotypes. Globally, we sampled 9229 oilspots (4392 plot-specific genotypes, Table 1). Microsatellite analysis revealed different degrees of polymorphism among microsatellite markers of the 34 *P. viticola* populations (Table 2). Low allelic variation was observed at the loci ISA and BER, whereas the markers CES and GOB were very variable. Gene diversity ranged from a minimum of zero to a maximum of one. Three of the four markers employed showed incompleteness in the allele size distribution. In all 34 populations, as well as when all the samples were pooled together, the locus GOB and CES showed trimodal and bimodal frequency distribution, respectively, while ISA had a distribution gap of 20 bp.

Table 2. Summary of genetic variation within each of the 34 clone corrected *P. viticola* populations. N refers to the sample size and k to the number of alleles per SSR marker of the individuals studied. SSR markers are listed in the headings. Stars represent significant ("*": P<0.01) deviations from Hardy-Weinberg equilibrium.

Population	number of alleles (k)				heterozygosity (expected/observed)			
	N	k_{ISA}	k_{GOB}	k_{CES}	k_{BER}	ISA	GOB	CES	BER
ayy	178	4	33	7	2	0.61/0.61	0.92/0.91	0.64/0.69	0.20/0.13
bla	726	5	54	11	3	0.57/0.61	0.92/0.90	0.56/0.53	0.22/0.19*
bom	82	5	27	7	2	0.67/0.85	0.88/0.95	0.78/0.88	0.36/0.27
per	216	4	38	9	2	0.54/0.55	0.92/0.93	0.69/0.65	0.12/0.09
vic	228	4	34	9	2	0.59/0.54	0.91/0.95	0.70/0.62	0.22/0.16
sta	258	4	28	6	2	0.56/0.50	0.81/0.85	0.69/0.66	0.23/0.22
bia	382	4	47	9	2	0.60/0.64	0.93/0.94	0.74/0.64	0.09/0.07
cum	386	5	61	13	2	0.58/0.59	0.95/0.91	0.69/0.68	0.06/0.05
mom	380	4	54	14	2	0.52/0.59	0.93/0.94	0.70/0.67	0.12/0.09
nac	928	4	60	16	3	0.51/0.54	0.91/0.87	0.74/0.73*	0.19/0.12*
sdo	244	4	31	12	2	0.48/0.55	0.93/0.89	0.63/0.69	0.15/0.11
smi	182	4	34	11	2	0.43/0.40	0.94/0.87	0.66/0.63	0.19/0.10*
alb	130	4	36	9	2	0.52/0.48	0.96/0.97	0.68/0.60	0.25/0.17
can	186	5	37	11	2	0.56/0.60	0.90/0.92	0.70/0.75	0.08/0.06
gri	102	4	30	10	2	0.55/0.57	0.93/0.84	0.76/0.71	0.11/0.08
rac	132	4	43	10	2	0.55/0.61	0.94/0.88	0.71/0.71	0.07/0.05
cas	284	4	46	13	2	0.46/0.51	0.95/0.92	0.72/0.65*	0.11/0.06*
cod	376	4	53	9	2	0.45/0.45	0.95/0.95	0.70/0.68	0.16/0.13
car	166	4	46	12	2	0.52/0.54	0.94/0.93	0.67/0.66	0.07/0.05
erb	384	4	46	16	2	0.47/0.51	0.94/0.92*	0.76/0.53*	0.21/0.11*
lor	456	5	33	12	3	0.52/0.52	0.92/0.89*	0.70/0.47*	0.21/0.16*
lef	56	3	27	10	1	0.26/0.30	0.96/0.93	0.84/0.88	0/0
hai	68	3	26	7	2	0.40/0.45	0.96/0.94	0.81/0.88	0.33/0.35
rap	216	4	46	12	3	0.44/0.44	0.97/0.91	0.83/0.87	0.33/0.31
nao	146	3	43	11	3	0.42/0.41	0.97/0.96	0.81/0.75	0.22/0.16*
dam[a]	224	4	38	11	3	0.26/0.24	0.94/0.89	0.80/0.81	0.29/0.35
me-a	176	4	41	9	3	0.50/0.53	0.96/0.91	0.72/0.69	0.27/0.19
me-b[b]	454	4	58	17	4	0.40/0.42	0.97/0.93	0.75/0.70	0.38/0.28*
agh	138	4	41	11	3	0.44/0.47	0.97/0.91	0.81/0.67	0.25/0.17
pr-a	144	3	37	14	2	0.52/0.44	0.95/0.93	0.80/0.85	0.13/0.08
pr-b[a]	280	3	48	15	3	0.40/0.35	0.94/0.92	0.85/0.89	0.07/0.04
lek[a]	242	3	51	14	3	0.35/0.25*	0.97/0.88	0.84/0.85	0.03/0.02
kef	64	2	21	11	2	0.27/0.32	0.93/0.97	0.85/0.94	0.22/0.13
zak	42	2	17	8	2	0.41/0.45	0.93/1.00	0.84/0.91	0.09/0.10
total	**9369**	**6**	**101**	**26**	**6**	**0.48/0.50**	**0.93/0.92**	**0.74/0.72**	**0.18/0.14**

nc: non calculated ; *: not in HWE; a: in HWE the pooled first three samples

b: in HWE the pooled first four samples

Locus ISA showed low allelic variation (k_{ISA}= 6) and medium gene diversity (total av. H_{obs} = 0.50; Table 2). It reached its lowest variability in the populations of Kephalonia (kef) and Zakynthos (zak) islands, where two alleles were found, while, in cEu, it always exhibited from four to five alleles. The allele ISA-118.3, although it existed in all central European plots, in Greece, it was present only in five out of the 13 populations (rap, dam, me-a, me-b and agh, all from Thessaly region). The allele that showed the highest frequency (p) was ISA-144.1 in the Damassi population ($P_{ISA-144.1}$= 0.86). Two alleles, ISA-140.2 and ISA-144.1, were shared among all 34 populations, while there was no private allele. Locus ISA had higher average gene diversity in cEu (av. H_o = 0.54) than in Gr (av. H_o= 0.39).

Locus GOB had the highest variability (av. k_{GOB} in cEu and Gr is 42 and 38, respectively) and the highest gene diversity of all loci in all plots (av. H_{obs}= 0.92) showing 101 alleles in total., Its minimal variation was observed in some Greek islands (21 alleles in Kephalonia, 17 alleles in Zakynthos), where the incidence of the disease was also very low. The allele GOB-210.0, which showed the highest frequency among all populations (av. $p_{210.0}$= 0.36), differed remarkably in frequency between cEu (av. $p_{GOB-210.0}$= 0.2) and Gr (av. $p_{GOB-210.0}$= 0.05). Only two alleles (GOB-385.7 and GOB-389.8) were shared among the 34 populations. Fourteen alleles were private (av. P=0.008) and belonged to seven plots in cEu (bla: GOB-215.6, bom: GOB-217.6, bia: GOB-249.4, GOB-251.3, erb: GOB-257.0, GOB-420.0, cod: GOB-341.0, nac: GOB-422.0, GOB-434.3, mom: GOB-432.4) and to one plot in Gr (me-a: GOB-231.7, me-b: GOB-235.7, GOB-337.0 and GOB-243.7).

 Locus CES revealed from 6 to 17 alleles, according to the populations, and high total allelic variation (k_{CES}= 26). As in the case of locus GOB, the allele with the highest frequency (bla, $P_{CES-143.0}$= 0.62) varied in frequency between cEu and Gr (av. $p_{CES-143.0}$ are in cEu and Gr, 0.47 and 0.30, respectively). The 34 populations shared five CES alleles (CES-143.0, CES-163.6, CES-175.4, CES-177.7, CES-179.5) and only the population bla possessed one private allele ($P_{CES-199.0}$= 0.001). In contrast to locus ISA, locus CES was more variable in Greece (av. H_{obs} in cEu= 0.70, av. H_o in Gr= 0.81).

Similarly to locus ISA, locus BER had six alleles but its diversity was very low (overall av. H_{obs}= 0.14). Locus BER reached, though, exceptionally high values in one French (bom) and three

Greek populations (hai, rap, and me-b). The locus showed its highest variability (four alleles) in me-b; it constantly had 2-3 alleles in each population of cEu and was monomorphic in one population in Peloponessus (lef). One allele (BER-180.9) was common in all plots. Three out of the 6 alleles were private and diagnostic in the following populations: lor (BER-177.1), me-b (BER-183.6) and bla (BER-187.5).

Hardy-Weinberg expectations were followed in 25 populations out of 34 at all loci (Table 2). The remaining nine populations were not in HWE for at least one locus. In the two German populations (erb and lor) only locus ISA was in HWE. Populations smi, bla, nao and me-b showed significant deviations from HWE expectations at locus BER; nac and cas were not in HWE at loci CES and BER and lek was not in HWE at locus ISA. Particular situations were manifested in some Greek plots, where individual samples followed HWE, while the same samples pooled together deviated from it. The me-b, pr-b and lek populations included late autumn samples as well. The pooled spring/summer samples of these populations and the autumn populations separately, followed HWE expectations (exception: locus BER in me-b). In contrast, when the autumn sample was pooled together with the spring/summer ones a highly significant deviation from HWE was observed (P= 0.000). In the case of Damassi, the pooled first 3 samples (29/05/01- 29/06/01) followed HWE expectations, while the pooled six 2001 collections, (including the samples from 12/07 to 15/10) did not (results not shown).

A low probability of a null allele occurrence at the loci ISA, GOB and CES for any population was estimated: 97 times out of 102 the probability was less than 0.05, ensuring a correct genotyping of the samples for those markers (Table 3). For the loci ISA, GOB and CES, respectively, the highest null-allele frequency estimations were recorded in lek ($P_{ISA-null}$= 7%), in gri and lek ($P_{GOB-null}$= 4%) and in both German populations ($P_{CES-null}$= 13%). The locus BER showed in 30 population from 1% to 8% probability of having null alleles. All negative values for allele frequencies were considered as equal to "zero". The presence of undetected null alleles may have generated an apparent heterozygote excess for the loci CES and BER (in 21 and 31 populations, respectively), but only occasionally led to deviations from HWE (twice for CES and eight times for BER).

The measurement of the genotypic diversity of the populations we studied revealed a difference between the Gr and cEu populations; the Shannon Equitability had an average

value of 0.68 and 0.85, respectively (Table 1). Furthermore, the minimum value in Gr (agh: 0.28) was much lower than the one exceptionally low in cEu (bom: 0.39).

Table 3. Null allele frequency estimation within each of the 34 clone-corrected *P. viticola* populations.

Population	Null allele frequency estimation				Population	Null allele frequency estimation			
	ISA	GOB	CES	BER		ISA	GOB	CES	BER
ayy	0	0	0	0.05	cod	0	0	0.01	0.07
bla	0	0.01	0.01	0.03	car	0	0	0	0.02
bom	0	0	0	0.06	erb	0	0.01	0.13	0.08
per	0	0	0.02	0.03	lor	0	0.01	0.13	0.04
vic	0.03	0	0.05	0.04	lef	0	0	0	0
sta	0.04	0	0.01	0.01	hai	0	0	0	0
bia	0	0	0.06	0.01	rap	0	0.02	0	0.02
cum	0	0	0	0.01	nao	0	0	0.03	0.05
mom	0	0	0.01	0.02	dam	0.02	0.02	0	0
nac	0	0.01	0.01	0.06	me-a	0	0.02	0.01	0.06
sdo	0	0.02	0	0.03	me-b	0	0.02	0.03	0.07
smi	0.02	0.03	0.02	0.07	agh	0	0.02	0.07	0.06
alb	0.03	0	0.05	0.06	pr-a	0.05	0.01	0	0.04
can	0	0	0	0.01	pr-b	0.03	0.01	0	0.02
gri	0	0.04	0.03	0.03	le4	0.07	0.04	0	0.02
rac	0	0.02	0	0.03	kef	0	0	0	0.08
cas	0	0	0.05	0.03	zak	0	0	0	0

Population differentiation and genetic structure

Fixation indices are measures of population differentiation that incorporate information on both the frequency and identity of the alleles. In this study, F_{ST} ranged from a low value of 0 (estimates lower than zero were treated as zero) to 0.137 (comparison: bla/dam), with an average of 0.03. ($0.01 \leq F_{ST} \leq 0.05$ means low genetic differentiation, according to Wright, 1978). R_{ST} values, which are the equivalent of F_{ST} under a stepwise mutation model, were slightly higher than F_{ST} estimates, ranging from 0 to 0.214 (pairwise comparison: bla/pr-a), with an average value of 0.046 (Table 4). F_{ST} estimates within cEu populations were lower (av. F_{ST}= 0.018) than within Gr populations (av. F_{ST}= 0.027). The maximum value obtained in cEu was F_{ST}= 0.082 (bla/smi) and in Gr it was F_{ST}= 0.078 (dam/pr-a). The average F_{ST} value

between cEu and Gr populations was higher (F_{ST}= 0.041). A similar trend was exhibited by R_{ST}: within cEu av. R_{ST} (0.012) was lower than within Gr populations (av. R_{ST}= 0.028), while among Gr and cEu populations, an av. R_{ST}= 0.046 was found.

Pairwise tests of population differentiation calculated by FSTAT (for F_{ST} estimates) revealed 524 (93.4%) significant and 37 non-significant differentiations among the _P. viticola_ populations after Bonferroni correction for multiple tests (p= 0.000089; 561 pairwise comparisons) (Table 4). Ten non-significant tests (ns) were obtained when testing population differentiation within Gr (out of 78 pairwise tests), 25 within cEu (out of 210 tests), while only 2 ns (out of 273 tests) were obtained when testing Gr with cEu. This result is in accordance with the fact that the F_{ST} and R_{ST} values are higher when comparing the Greek with the central European populations. Car (11 ns), rac (7 ns), lef (4 ns) and hai (3 ns) were the four most undifferentiated populations (two among cEu and two among Gr populations, respectively). Nine populations (26%), including all three French, both German populations, one Swiss (sta) and three Greek (dam, pr-a, pr-b), always showed significant differences among all comparisons.

Table 4. Estimates of pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) averaged over loci. "*" corresponds to significance at the 5% nominal level after standard Bonferroni corrections.

	ayy	bla	bom	per	vic	sta	bia	cum	mom	nac	sdo	smi	alb	can	gri	rac	cas	cod	car	erb	lor	lef	hai	rap	nao	dam	me-a	me-b	agh	pr-a	pr-b	le4	kef	zak
ayy		0.01	0	0	0	0	0	0	0	0	0.03	0.03	0.03	0	0	0	0	0.03	0	0	0	0.06	0.06	0.04	0.05	0.02	0.03	0.02	0.01	0.16	0.11	0.01	0.08	0.09
bla	0.02*		0	0	0	0	0.01	0.03	0	0.01	0.07	0.07	0.07	0.01	0.02	0.03	0.01	0.07	0.02	0.03	0.02	0.11	0.11	0.08	0.09	0.05	0.07	0.06	0.05	0.21	0.17	0.05	0.14	0.15
bom	0.03*	0.07*		0	0	0	0	0.02	0	0	0.06	0.06	0.06	0	0	0.02	0	0.05	0.02	0.02	0.01	0.08	0.08	0.08	0.09	0.06	0.06	0.06	0.03	0.21	0.15	0.03	0.1	0.1
per	0.02*	0.04*	0.03*		0	0	0	0.01	0	0	0.05	0.04	0.05	0	0	0.01	0	0.04	0.01	0.01	0	0.08	0.08	0.05	0.07	0.03	0.04	0.04	0.03	0.19	0.13	0.03	0.1	0.11
vic	0.02*	0.04*	0.01*	0*		0	0	0.01	0	0	0.04	0.04	0.04	0	0	0.01	0	0.04	0.01	0.01	0	0.07	0.07	0.05	0.06	0.03	0.04	0.03	0.02	0.18	0.13	0.02	0.09	0.1
sta	0.02*	0.05*	0.03*	0.02*	0.01*		0	0.01	0	0	0.04	0.03	0.03	0	0	0.01	0	0.03	0.01	0.01	0	0.06	0.05	0.04	0.05	0.02	0.03	0.03	0.02	0.15	0.11	0.02	0.07	0.08
bia	0.03*	0.06*	0.03*	0.01*	0.01*	0.02*		0	0	0	0.03	0.03	0.03	0	0	0	0	0.03	0	0	0	0.06	0.05	0.03	0.04	0.01	0.02	0.02	0.01	0.15	0.1	0.01	0.07	0.09
cum	0.02*	0.05*	0.03*	0*	0.01*	0.02*	0*		0.02	0	0.01	0.01	0.01	0	0	0	0	0.01	0	0	0	0.03	0.03	0.01	0.02	0	0	0	0	0.11	0.07	0.01	0.04	0.06
mom	0.03*	0.06*	0.03*	0*	0*	0.02*	0.01*	0.01*		0	0.05	0.05	0.05	0	0	0.01	0	0.04	0.01	0.02	0.01	0.09	0.08	0.06	0.07	0.03	0.05	0.04	0.03	0.19	0.14	0.03	0.11	0.12
nac	0.02*	0.06*	0.02*	0.01*	0.01*	0.02*	0.01*	0.01*	0.01*		0.03	0.02	0.02	0	0	0	0	0.02	0	0	0	0.04	0.04	0.02	0.03	0.01	0.02	0.01	0.01	0.13	0.08	0.01	0.06	0.07
sdo	0.03*	0.07*	0.04*	0.01*	0.02*	0.03*	0.02*	0.01*	0.01*	0.01*		0	0	0.02	0.02	0	0.03	0	0.01	0.02	0.02	0.01	0.01	0	0	0.01	0	0	0.01	0.06	0.04	0.02	0.02	0.03
smi	0.04*	0.08*	0.03*	0.02*	0.02*	0.03*	0.02*	0.01*	0.01*	0.01*	0		0	0.02	0.02	0	0.02	0	0.01	0.01	0.02	0	0	0	0	0	0	0	0.01	0.06	0.04	0.01	0.01	0.02
alb	0.02*	0.04*	0.03*	0.01*	0.01*	0.02*	0.01*	0.01	0.01*	0.01*	0.01*	0.01*		0.02	0.02	0	0.03	0	0.01	0.01	0.02	0	0.01	0	0	0.01	0	0	0.01	0.07	0.04	0.01	0.01	0.03
can	0.01*	0.04*	0.03*	0*	0*	0.01*	0.01*	0	0.01*	0.01*	0.01*	0.02*	0.01*		0	0	0	0.02	0	0	0	0.04	0.04	0.02	0.03	0.01	0.01	0.01	0.01	0.13	0.09	0.01	0.06	0.07
gri	0.02*	0.06*	0.03*	0*	0.01*	0.02*	0	0	0.01*	0*	0.01*	0.02*	0.01	0.01		0	0	0.01	0	0	0	0.04	0.04	0.02	0.03	0.01	0.02	0.01	0	0.15	0.09	0	0.06	0.07
rac	0.02*	0.05*	0.03*	0*	0*	0.02*	0*	0	0	0	0.01	0.01*	0	0	0		0	0	0	0	0	0.02	0.02	0.01	0.01	0	0	0	0	0.11	0.07	0	0.04	0.05
cas	0.04*	0.07*	0.03*	0.01*	0.01*	0.03*	0.01*	0.01*	0*	0.01*	0.01*	0.01*	0.02*	0.01*	0.01*	0.01*		0.02	0	0	0	0.05	0.05	0.03	0.04	0.01	0.02	0.01	0.01	0.14	0.1	0.01	0.07	0.08
cod	0.04*	0.07*	0.03*	0.01*	0.01*	0.02*	0.01*	0.01*	0*	0.01*	0.01*	0.01*	0.01*	0.01*	0.01*	0.01*	0*		0.01	0.01	0.02	0	0	0	0	0	0	0	0.01	0.06	0.04	0.01	0.01	0.02
car	0.03*	0.05*	0.03*	0	0.01	0.02*	0*	0	0	0.01*	0.01*	0.01	0	0	0.01	0	0	0		0	0	0.03	0.03	0.01	0.02	0	0.01	0	0.01	0.12	0.08	0.01	0.05	0.06
erb	0.03*	0.07*	0.03*	0.02*	0.02*	0.03*	0.02*	0.02*	0.02*	0.01*	0.02*	0.01*	0.02*	0.02*	0.01*	0.02*	0.02*	0.02*	0.02*		0	0.03	0.03	0.01	0.02	0	0.01	0	0	0.13	0.07	0	0.05	0.06
lor	0.02*	0.06*	0.03*	0.02*	0.02*	0.02*	0.02*	0.02*	0.02*	0.01*	0.02*	0.02*	0.02*	0.02*	0.01*	0.02*	0.03*	0.03*	0.02*	0.01*		0.04	0.04	0.02	0.03	0.01	0.01	0.01	0	0.13	0.09	0.01	0.06	0.07
lef	0.09*	0.12*	0.07*	0.03*	0.04*	0.07*	0.03*	0.03*	0.03*	0.03*	0.04*	0.03*	0.03*	0.05*	0.02*	0.03*	0.02*	0.02*	0.03	0.03*	0.05*		0	0.01	0	0.04	0.01	0.03	0.02	0.02	0	0.02	0	0
hai	0.06*	0.11*	0.03*	0.04*	0.04*	0.06*	0.04*	0.04*	0.04*	0.03*	0.04*	0.03*	0.02	0.05*	0.03*	0.04*	0.04*	0.03*	0.04*	0.02*	0.03*	0.03*		0.01	0	0.04	0.01	0.03	0.02	0.03	0	0.02	0	0
rap	0.06*	0.1*	0.03*	0.03*	0.03*	0.05*	0.03*	0.03*	0.03*	0.02*	0.03*	0.02*	0.02*	0.04*	0.02*	0.03*	0.02*	0.02*	0.03*	0.01*	0.03*	0.02*	0.01*		0	0.01	0	0		0.09	0.04	0.01	0.02	0.04
nao	0.04*	0.09*	0.04*	0.03*	0.03*	0.05*	0.02*	0.02*	0.03*	0.02*	0.02*	0.02*	0.01*	0.03*	0.01*	0.02*	0.02*	0.02*	0.02*	0.01*	0.02*	0.02*	0.01	0		0.02	0	0.01	0.01	0.06	0.02	0.01	0	0.02
dam	0.09*	0.14*	0.06*	0.05*	0.06*	0.08*	0.05*	0.06*	0.05*	0.04*	0.05*	0.03*	0.04*	0.07*	0.05*	0.05*	0.04*	0.04*	0.04*	0.03*	0.05*	0.03*	0.03*	0.01*	0.02*		0	0	0	0.16	0.08	0.01	0.07	0.1
me-a	0.05*	0.07*	0.04*	0.02*	0.02*	0.05*	0.02*	0.02*	0.02*	0.02*	0.02*	0.02*	0.01*	0.03*	0.01*	0.02*	0.02*	0.02*	0.02*	0.02*	0.03*	0.03*	0.01	0.01*	0.01*	0.03*		0	0	0.1	0.05	0.01	0.03	0.05
me-b	0.06*	0.09*	0.04*	0.03*	0.03*	0.05*	0.03*	0.03*	0.03*	0.03*	0.02*	0.02*	0.01*	0.04*	0.02*	0.03*	0.02*	0.02*	0.03*	0.02*	0.03*	0.03*	0.01*	0	0.01*	0.01*	0.01*		0.01	0.13	0.08	0.01	0.05	0.08
agh	0.05*	0.1*	0.03*	0.03*	0.03*	0.05*	0.03*	0.03*	0.03*	0.02*	0.03*	0.02*	0.02*	0.04*	0.02*	0.03*	0.02*	0.02*	0.03*	0.01*	0.03*	0.02	0.01	0.01*	0*	0.03*	0.02*	0.01*		0.12	0.05	0	0.04	0.05
pr-a	0.06*	0.08*	0.06*	0.05*	0.05*	0.07*	0.05*	0.05*	0.05*	0.05*	0.07*	0.07*	0.04*	0.05*	0.04*	0.04*	0.06*	0.05*	0.05*	0.05*	0.06*	0.04*	0.03*	0.05*	0.04*	0.08*	0.04*	0.06*	0.04*		0.01	0.11	0.01	0
pr-b	0.09*	0.13*	0.07*	0.06*	0.07*	0.08*	0.05*	0.06*	0.06*	0.05*	0.07*	0.07*	0.05*	0.06*	0.04*	0.05*	0.06*	0.05*	0.06*	0.05*	0.06*	0.03*	0.03*	0.04*	0.03*	0.06*	0.05*	0.06*	0.04*	0.02*		0.05	0	0
le4	0.06*	0.1*	0.05*	0.03*	0.04*	0.05*	0.03*	0.03*	0.03*	0.02*	0.03*	0.03*	0.03*	0.03*	0.02*	0.02*	0.02*	0.02*	0.02*	0.02*	0.03*	0.01	0.02*	0.02*	0.01*	0.04*	0.03*	0.03*	0.01*	0.04*	0.02*		0.03	0.04
kef	0.07*	0.11*	0.05*	0.04*	0.04*	0.06*	0.04*	0.04*	0.04*	0.02*	0.03*	0.02*	0.03*	0.04*	0.03*	0.03*	0.02*	0.03*	0.04*	0.02*	0.03*	0.02	0.01*	0.01*	0.01*	0.03*	0.03*	0.02*	0.01*	0.05*	0.03*	0.01*		0
zak	0.06*	0.1*	0.06*	0.05*	0.06*	0.07*	0.05*	0.05*	0.05*	0.04*	0.05*	0.05*	0.05*	0.05*	0.03*	0.05*	0.05*	0.05*	0.05*	0.03*	0.03*	0.03	0.03*	0.04*	0.02*	0.07*	0.04*	0.05*	0.03*	0.05*	0.03*	0.02*	0.01	

Shriver's genetic distance Dsw was highest between Greek populations (mainly dam, pr-a, pr-b and zak) and French populations (bla, ayy and bom) and lowest between populations sampled from regions close to the Southern part of the Alps. The Neighbor-Joining phenogram based on Shriver's Dsw, placed populations in a relationship that map closely onto their geographical locations (Fig. 2a). Two major branches were distinguished (cEu and Gr clusters), which were subdivided into the French/Swiss (excluding the southern Swiss populations: bia, cum, mom), South of Alps and German clusters and the Greek East/mainland and West clusters, respectively. The German populations (lor, erb), the French/Swiss populations (bla, ayy, sta, bom, vic, per) and the populations sampled South of the Alps (sdo, smi, cas, cod, car, mom, rac, cum, bia, gri, nac) were clearly clustered. Only alb and can, although collected from the same region (Piedmont, Italy), were clustered within geographically heterogeneous branches. The Greek East/mainland branch included me-a, dam, me-b, rap, agh, while the West branch included pr-a, pr-b, hai, lef, lek, kef and zak. The only Greek population not fitting to any branch was nao, which is located in Northern Greece. In addition, the tree managed to differentiate the district of Thessaly (me-a, me-b, rap, dam) from the other regions and to group pr-a and pr-b - which are neighbor plots - in a branch opposite to the island cluster (lek, kef, zak).

The $\delta\mu^2$ estimator, despite that it is especially developed for microsatellite data, produced a NJ tree that was mainly unresolved (Fig. 2b). Its main feature was the highlighting of the divergence between Greek and French populations; nevertheless, it failed to distinguish the German branch and clearly separated populations that are very close geographically, as mom from cum and cod from cas. Furthermore, Greek populations were mixed among Italian and South Swiss populations. It wasn't able to resolve genetic structuring and phylogeographical relationship as well as the Dsw-based phylogram.
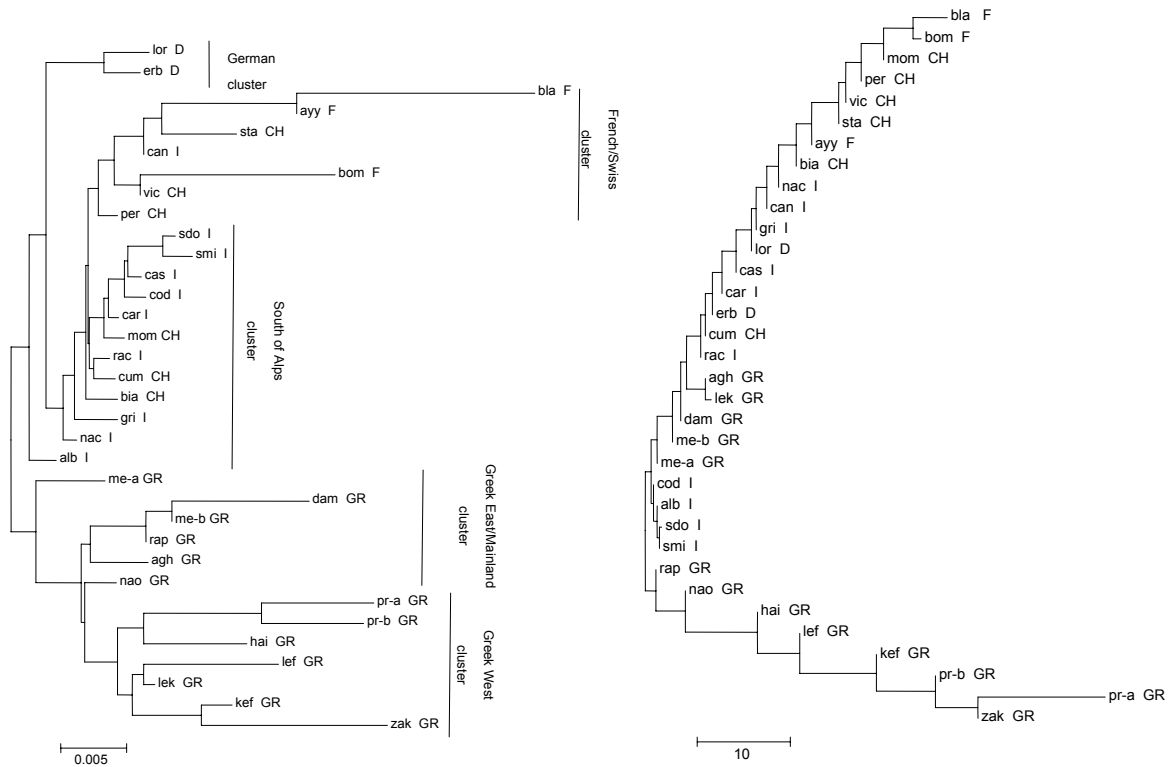
Figure 2. Unrooted neighbor-joining trees based on Shriver's genetic distance Dsw (2a, left) and on Goldstein's $\delta\mu^2$ (2b, right) among 34 *P. viticola* populations. Country of origin of populations is given after population names.

Isolation by distance

There was a highly significant positive relationship (P= 0.000) between pairwise F_{ST} and geographical distance of geographical distance. Displaying the same distance variables, R_{ST} exhibited both a similar positive trend and p-value (P= 0.000). Linear correlation among geographical distance, or its logarithm (data not shown), and any diversity estimator was generally low. The linear regression between Fst and geographical distance resulted in a slope of $3 \cdot 10^{-5}$ and an y-intercept of 0.01 (R^2= 0.38; Fig. 3).

The significance of the Mantel test for isolation by distance remained unchanged when we removed all 13 Greek populations from the data set (squares in Fig. 3, slope: $6*10^{-5}$, y-intercept: $2*10^{-4}$; R^2= 0.46). Depriving the cEu from French and German populations, significance was no

longer assessed (R^2= 0.00; P= 0.25). The test performed exclusively on the Greek populations was no longer significant (P= 0.25). In Greece, populations separated by 100-200 Km (straight line), did exhibit either a moderate genetic distance (dam/pr-a: F_{ST}= 0.08, dist: 140 Km) or a low genetic distance (agh/lek: F_{ST}= 0.01, dist: 150 Km). In central Europe, a distance eight times longer than the dam/pr-a comparison is required for reaching genetic distance of the same magnitude (bla/smi: F_{ST}= 0.082, dist: 1050 km). Exceptionally, in central Europe, only two French populations (bla and bom), which are separated by approx. 35 Km, showed an F_{ST} value of 0.070. The maximal genetic distance of F_{ST}= 0.137 is achieved by comparing bla with dam, which are separated by 1500 Km.
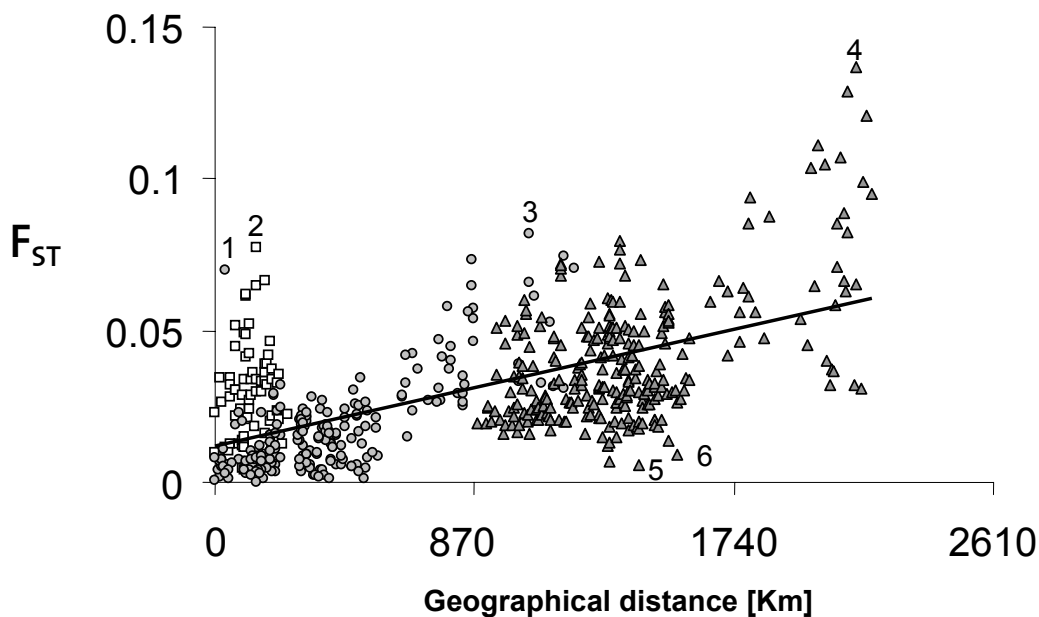
Figure 3. Genetic distance F_{ST} plotted against the geographical distance (km). The solid line represents the best fit linear regression (y-intercept = 0.01; slope= 3E-5; R2= 0.38; p= 0.000; 1200 permutations). Squares, dots and triangles refer to pairwise comparisons within Greek populations, within central European populations and among Greek and central European populations, respectively. Numbers above symbols refer to the following pairwise comparisons: 1: bla/bom; 2: dam/pr-a; 3: bla/smi, 4: bla/dam, 5: erb/nao and 6: erb/rap.

DISCUSSION

The most important outcome of this study is the assessment of a huge diversity within and among *P. viticola* populations, both in gene and genotypic level. Two of our markers (GOB and CES) exhibited very high allelic variation and heterozygosity (H_{obs} values at locus GOB attained 1). On the genotypic level different individuals constituted, on average 77%, of each population. Genetic diversity within and between populations reached high values with F_{ST} coming up to 0.15. 155 pairwise comparisons out of 273 (57%) showed an $F_{ST} \geq 0.03$. Only 14% of the within cEu pairwise F_{ST} reached 0.03, while, within Gr, a more pronounced percentage was found (44%). The high diversity revealed provides evidence about the key role of sexual recombination of *P. viticola* and/or presumably a high mutation rate at microsatellite markers (mostly GOB and CES).

Four lines of evidence, i.e. the results of the pairwise test of differentiation, of the phylogenetic relationship among populations, of the observed pattern of isolation by distance and, finally, of the achievement of HWE expectations, consistently revealed high substructure among *P. viticola* populations. Those outcomes constitute evidence that the heterogeneity of allele frequency distributions we observed is the result of low gene flow on the spatial scale separating most of the sampled populations.

First, the pairwise test of differentiation highlighted overwhelmingly significant genetic subdivisions (93.4% of pairwise tests) between populations collected at different locations. Significant genetic differentiation was assessed, even in cases when the Euclidean distances between the population pairs were relatively short, like the pairs cas/cod (25 km), cum/mom (40 km), me-b/rap (80 km) and bia/cum (22 Km). Genetic differentiation seems not to apply at distances of about 1 km (sdo/smi: non-significant). Apart from the situation of geographically close sites, non-significant differences were observed between kef and zak (island populations) and between rap and nao (Greek mainland populations). Four populations (car, rac, lef and hai) exceptionally showed consistent non-significant differentiation to several populations (four to nine). This is probably an artifact due to: the small population sizes, the absence of private alleles, the presence of European-wide frequent alleles and/or similar allele frequency distributions, which do not permit the

highlighting of genetic subdivision. The fact that each population is characterized by site-specific allele frequency distributions allowed us to assume that a low or occasionally absent gene flow takes place across infected vineyards already at modest distances (>1 Km) impeding the homogenization of allele frequencies distributions among sites.

Second, patterns of genetic divergences among cEu and Gr *P. viticola* populations, corresponding to the large geographical distances between them, were clearly recovered by the NJ tree based on genetic distance Dsw. Within the cEu group, the Swiss populations were grouped depending on their position in relation to the Alps. The West and North Swiss populations (per, vic and sta) were grouped with the French populations and the South Swiss (bia, cum, mom) with the North Italian populations. Regarding the Gr group, the two main branches were formed according to the placement of the plots on the East or West side of Tzumerka Mountain (2393m altitude); this is the most Southern part of the large mountain range of Pindos, which divides the country from North to South and constitutes a natural boundary for the pathogen's populations.

The divergence of *P. viticola* populations around the Alps suggests this major barrier prevents the gene flow between Southern and Northern viticultural areas. North of the Alps, we observed high genetic distances, consistent with the geographical distance, while major irresolution was found among populations placed south of the Alps. The absence of a clearly differentiated genetic substructuring may be an indication of a more relevant occurring gene flow among populations established South of the Alps than among other populations (which may happen thanks to the absence of geographical barriers, frequent human-mediated transport of infected vines and step-by-step migration among numerous patchy patterns of host areas).

Concerning Greece, the populations of each of the two main branches exhibit very high genetic distances among them in comparison to the populations of the cEu branches. This situation is caused by two unique features of the Gr: the presence of the sea and the Mediterranean climate effect. On one hand the sea minimizes the probability of migration events and their homogenizing effects. On the other hand, the specific climatic conditions (limited number of rain events during spring and early summer) permit a limited amount of primary infections and, consequently, a small effective oosporic population size. Later in the

season, during summer, long periods of heat and lack of humidity often cause bottleneck situations (as happened to dam population), diminishing even more the genotypic diversity (Rumbou and Gessler, 2003). As a result, populations already isolated by peculiar topography, further drift apart after climatic effects. The genetic distances estimated come to extreme values within the West cluster that includes the island populations (lek, kef, zak), suggesting that the sea may constitute a very effective barrier for *P. viticola* populations.

The phylogenetic tree based on the $\delta\mu^2$ estimator provided additional support for the divergence between Greek and French populations. Nevertheless, it failed in giving the high resolution of the tree based on Dsw. The $\delta\mu^2$ estimator of genetic distance has already been described as controversial to the Dsw and was judged as unreliable in the microsatellite analysis of elephant's phylogeographical relationships (Comstock *et al.,* 2002).

Third, the test for isolation by distance detected a positive association between geographic and genetic distances in *P. viticola* populations. Isolation by distance slopes departed from random expectations, indicating that the populations are spread in a geographical continuum. Even after removing all Greek populations, a significant isolation-by-distance in central Europe continued to be, suggesting that geographical distance plays a role in keeping populations separated. In contrast to the global positive correlation exhibited between genetic and geographical distance, Greek populations alone were not isolated by distance. For reasons discussed previously, neighbor populations are forced to diverge. As a result, we obtained F_{ST} values between Greek plots of 100-200 km distances that in cEu were only obtained between plots 1000 km away.

Fourth, the oosporic populations, independently from the sampling date or from the number of samplings which constituted them, in most cases respected the HWE principles. This evidence suggests that *P. viticola* oosporic populations constitute panmictic communities. Oosporic populations were found to be highly genetically diverse, which let us speculate a high rate of genetic recombination. Recombination prior to oosporic formation prevents the diversity purging effects of periodic selection, as recombination enables alleles to spread to many genotypes in the population, thereby preventing their extinction. Such a high recombination may be the counterbalancing element of the low gene flow assessed.

Our survey demonstrated that *P. viticola* populations are independent demographical and panmictic units. Four lines of evidence suggested a low gene flow, which results in genetic heterogeneity among European communities. In biological terms, natural occurring exchange of propagules (oospores or sporangia) is suspected to be low. This challenges the existing assumptions about the fast dispersal of the disease and can not explain the abrupt infestation of the European vineyards after the first report of its introduction. Alternative explanations may rely on multiple introduction events followed by human-mediated transport of infected material (rooted grape cuttings carrying soil that contains oospores) or on unreported pathogen introduction(s) prior to 1878. A study of the population in the centre of origin (North America) may give support to the hypotheses presented.

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P. viticola secondary sporangia. Picture by: HH Kassemeyer

General discussion
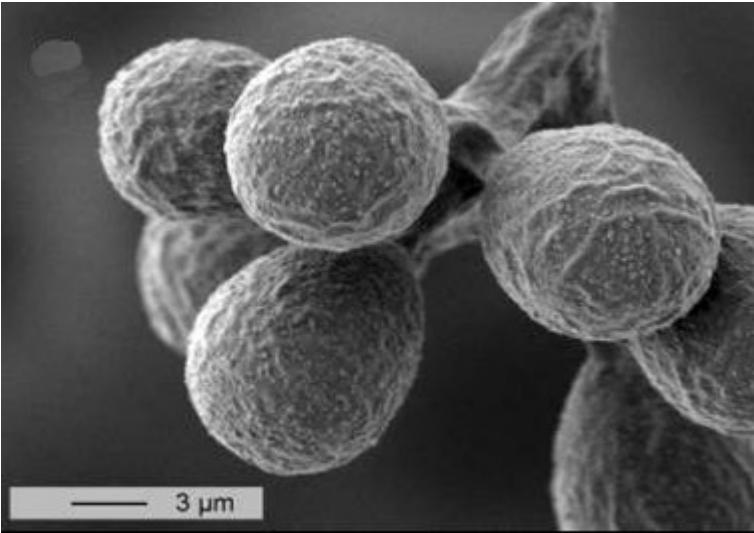
Interpretation key

Microsatellite analysis is a powerful method widely used in many scientific areas (paternity tests, criminology etc.) in order to define and identify genetic profiles of individuals and to allow distinctions among them. In our specific case it rigorously allowed the distinction among *P. viticola* genotypes, highlighting a huge variability among the thousands of individuals collected. Genomes of a primary lesion and the derived secondary lesion(s), as no sexual recombination occurs, are identical. Therefore, two lesions showing identical allele patterns (same genotype) were interpreted as clonal progeny, derived from the same primary lesion or oospore (single oospore-derived clonal lineage). The finding of two oil spots with identical genetic profiles was interpreted, according to the specific case, in the following three different manners. They may be: 1) both primary lesions generated by two zoospores produced by the same oospore, or 2) both secondary lesions derived from a common non-sampled "mother" (primary lesion), or 3) they can be "mother and son" (a primary lesion and a secondary lesion generated by the primary). Conversely, two oil spots showing different genetic profiles were interpreted as derived from independent oosporic infections (independent oospore-derived lineages).

In case of multiple identifications of the same genotype throughout the survey, we assumed the earliest identification of the genotype to be an oosporic-derived (primary) infection. Any subsequent identification of the same genotype was considered as a primary-derived (secondary) lesion. This assumption is probably correct for the epidemics where a total sampling strategy was applied, because if all oil spots were collected, than the earliest identification of every genotype corresponds to the primary lesion and any subsequent identification corresponds to its derived progeny (TSS, chapter 4, plots 10 to 18). When a partial sampling strategy was applied, this assumption may be far from the biological reality, because the earliest identification of a genotype, especially if sampling was performed late in the season or in cases of a high disease severity, may rather be a secondary lesion than a primary one (chapter 4, plots 1 to 9).

Redefining downy mildew primary and secondary cycles

For over 100 years it was widely speculated that the potential reproduction rate of the secondary phase largely outweighs the potential of oospores (Arens, 1929, Agrios, 1988). It was assumed that once the first primary lesions are sporulating the relative contribution of oospores to the progress of the epidemic is irrelevant. At the same time, it was assumed that secondary sporangia migration was largely responsible for disease spread from vineyard to vineyard, even across far distances, because of the historically rapid expansion of the pathogen all over Europe after its introduction. In this dissertation, we draw an opposite, more differentiated picture of the life cycle of *P. viticola* based on the genetic analysis of various pathogen populations collected at different times of the epidemics in vineyards across Europe. The new findings will be explained by following the *P. viticola* life cycle: we will first discuss the infectivity period of oospores, the occurrence of primary infections, the reproduction aptitude of genotypes (secondary cycle) and, finally, the migration or dispersion ability of the pathogen itself.

The primary cycle

In 1987, Gehmann showed that oospore formation can be distributed during the season, explicitly from the appearance of the very first lesion until leaf fall. As a consequence, oospores produced in the same vegetative season entering the next winter, constitute a population characterized by variable physiological ages. This age-heterogeneity is probably reflected by oospores' ability to germinate under optimum conditions during the vegetative season subsequent to their formation. Jermini *et al.* (2003a) showed that samples of oospores produced the previous spring/summer/autumn, overwintered under natural conditions (vineyard) and, collected in May (therefore 8-11 months old), show a huge variance in germination time (from one up to 22 days) and in the number of macrosporangia produced (0-8 macrosporangia / (leaf disk * collection date)). Oospores collected from the vineyard at successive times, according to a weekly schedule, revealed progressively diminishing oospore germination ability with small variances, probably due to the progressive oosporic decay. Therefore Jermini *et al.* (2003a) supposed that the oospore field

populations that enter in winter are composed of oospores distributed in different age-physiological cohorts; with each cohort corresponding to different ripening levels and modulated by the climatic conditions and possibly by leaf effects. This heterogeneity should not be restricted to the year following the oospore production, but it may extend up to five years (Hill G, personal communication). This implies that an unknown proportion of oospores produced sexually in 2004 will germinate according to cohort-dynamic processes during the summers of 2005, 2006, 2007, 2008, 2009 and maybe even later.

Jermini's findings about macrosporangia formation are fully consistent with the population genetic findings described in the chapters 3 and 4. As we know that macrosporangia formation is the first step to primary infection appearance in vineyards, and that their germination occurs continuously, we should expect primary infections to occur for a prolonged period starting approximately from May. Besides the expected manifestation of the few early appearing primary infections (the genotypes that first triggered the epidemic), we consistently highlighted an input of new genotypes (primary lesions) at very different epidemic stages to all the epidemics surveyed. This trend occurred in all of the 18 epidemics surveyed in chapter 4, less markedly only in Gei and in Was. Even in August (on 01.08.00 in Erb, on 18.08.00 in Sta, on 27.08.00 in Car, on 28.08.00 in Vic) new genotypes were identified causing one or more lesions each. In Greek epidemics the same trend was confirmed (Rumbou and Gessler, 2003).

Over an average surveying period of 1.5 months of the 18 plots described in chapter 4, we found an oosporic infection density of 1.07 genotypes/m^2 (sd.:1.22) or 2.38 genotypes/vine (sd.:2.89). The highest density was found in Stäfa, where a total sampling (49 oil spots) of a single vine revealed 13 different genotypes on 23.07.01 (6.2 gen./m^2, data not shown). Another plot hosting uncontrolled epidemics, Cut, where 178 spots from 6 vines were sampled, showed 4.9 gen./m^2 (11.5 gen./vine, over three collection dates). Conversely, in an isolated and newly established mountain vineyard, Tes, only 0.04 gen./m^2 (0.05 gen./vine) were detected. Because our sampling and analysis capacity was often far from the effective number of oil spots that may have been collected, we speculate that the density of oosporic infections may be higher than 1.07±1.22 gen./m^2, reaching values shown in the Cut and Stäfa case studies. At vine and vineyard scale, oospore stocks may, therefore, play a much more relevant role in

epidemics than what is reported in the literature (Agrios, 1988; Blaise *et al.*, 1999; Lafon and Clerjeau, 1988). We propose a new paradigm: primary infections are very numerous and do not occur exclusively during a short period at the beginning of the epidemic.

The secondary cycle

Our research provided a wide-scale quantitative analysis of the proportion of *P. viticola* genotypes that undergoes secondary multiplication after successful primary infections. As a result, we did not detect the expected massive asexual reproduction. Instead, about 3/4 of the genotypes sampled (or about 1/3 of the lesions sampled), depending on the epidemic and sampling strategy, was constituted by genotypes that never gave rise to any asexual progeny or gave rise to progeny that were undetected or non-sampled by the observers. On one hand, a restricted genotype life-time may be an indication of a generalised low parasitic fitness of secondary sporangia. Some studies have shown many fungal species, such as the downy mildews, to be highly sensitive to environmental conditions. Rotem *et al.*, (1985), for example, have shown that solar radiation has an influence on spore survival, depending on the sensitivity of the species, and that long wavelength UV has a major influence on survival. Furthermore, inappropriate duration of leaf wetness or intolerance to temperatures that escape from the optimum may constitute other factors that lead to high mortality of lesions (or very restricted clonal multiplication) and even to disappearance of successful genotypes. On the other hand, an unobserved massive asexual multiplication may be an artefact of the grid sampling we applied. By collecting only 1-3 lesions per vine, we may have failed in detecting clones if the spatial spread of the secondary lesions was restricted to an area smaller than the resolution of the grid. If the clones are very numerous and highly aggregated, for instance on a few neighbouring leaves, a collection of three oil spots per vine will not be representative for the genetic diversity of the pathogen within the single vine.

Instead of finding the expected high number of clones generated by a small number of genotypes, we highlighted the opposite tendency: the majority of the genotypes that underwent asexual multiplication generated (apparently) only one to two descendants. A very small percentage of the total genotypes (3.1% ± 5.0%), were found to be dominating the

epidemic (the dominant genotype is defined as the genotype occurring as at least 10% of the total sample size). Within 12 of the 18 epidemics surveyed, we found one (or two) dominating genotype(s), but in 6 epidemics, no dominating genotype was identified. Overall, 17 dominant genotypes were identified among 2320 genotypes concurring in 18 epidemics. In Carpineta, for instance, the most frequent (not dominant) genotype *afevissa* caused only 7.3% of the total sample size, conversely, in Tesero, the most frequent and dominant genotype *evuzemca* was contributing 59% of the symptoms present in the plot. It was found that the 17 dominating genotypes emerged in 12 cases at the beginning of the epidemic (1st sampling), while, in three cases, they were identified within the first 20 days from the beginning of the epidemic. In the last two cases, both occurring in Cugnasco (cum and cut plots, located at 5 m distance from one another), two dominant genotypes first emerged in the donor plot (cut), and about one month after their first identification, emigrated to the acceptor plot (cum). The occurrence of a few genotypes dominating the epidemic could be due to a genetically derived fitness advantage of some highly competitive oospores in maturing early and starting early clonal multiplication. Early establishment and more time for asexual multiplication for those dominant genotypes can lead to high frequency of those genotypes in later assessments. Conversely, high frequency of clones may result because early appearing genotypes have more chances to undergo secondary cycles and, therefore, increase their number, not necessarily bound to any physiological fitness. Those hypotheses need to be statistically tested.

As a consequence of a genotype-specific aptitude in producing secondary lesions, an epidemic will be generated in a very variable proportion by a unique (or eventually two) genotypes, while the remaining proportion will be the result of the interaction of many genotypes. This evidence is well described by the Tesero case study (chapter 3); only two genotypes were responsible for 88% of the total lesions on 02.07.00 and one of them for 68% of the total lesions on 14.07.00, while 13 out of 15 genotypes did not produce any relevant damage (1-3 lesions each). The same trend was found by Rumbou and Gessler (2003) in Greek vineyards: the N. Aghialos case study, performed during the summers of 2001 and 2002, reveals that the two epidemics were essentially caused by one dominating genotype each year. In summer 2001, 70-90% of the sampled population, depending on the date, was

composed by the dominating genotype. In summer 2002, the dominant genotype covered 40-75% of the sample size. Rumbou and Gessler (2003) speculated that those genotypes were likely favoured to reproduce asexually and spread to the entire vineyard. The other genotypes (23 and 63, respectively) did not spread farther than to the immediately surrounding vines.

We conducted an aggregational analysis in order to assess an eventual spatial correlation among clones of the same genotypes that showed a particular reproductive success (MFG, most frequent genotypes). The clones of the MFGs were found to be highly aggregated, especially in Bommes, Cugnasco, Tesero and Geneva (Eugster *et al.,* 2003). The degree of aggregation was high at the beginning of the epidemic and decreased with the disease progress. The opposite result was obtained by analysing spatial distribution of MFG-, and second MFG-deprived populations. Those populations, composed essentially by single genotypes or genotypes found two or three times, overwhelmingly showed random distribution. This indicates that, if one (or a couple of) genotype(s) multiplies asexually, then all the clones may be dispersed in clusters and not randomly within the plot. Thus, downy mildew populations showing a clustered character may hide a single (or a couple) dominant genotypes, which are multiplying asexually. Conversely, a random pattern distribution may be interpreted as absence of a dominant genotype.

Spatial autocorrelation analysis was used to examine the directionality of aggregation within the vineyards. Clones of the MFG were particularly spatially autocorrelated along row direction (isotropic autocorrelation). In all the other directions examined, no relationship between spatial distance and semivariance could be assessed. Isotropism is easily explained by the regular host allocation along rows, which allow the disease to spread up and down the rows without discontinuity. Instead, a "jump" of about two metres is required to reach the nearest vine located to the adjacent row.

A logical outcome of the aggregational analysis suggests that the clones of a genotype are descendents of a primary infection previously issued within the same plot (autochthon origin) and not the consequence of secondary sporangia immigration (allochthon origin). The evidence for this assumption is based on following facts: 1a) this study revealed a strong aggregation of the clones of the same genotype (Was, Bom, Tes, Geneva…): according to

dispersion functions (Skogsmyr, 1994; Melé, 2001), we do not expect a huge quantity of secondary sporangia immigrating from far distances; 2a) the sporangia dispersion rate, even if roughly calculated, was found to be rather moderate, ranging around 1 to 2 m^2/day; 3a) generally, the chances of secondary multiplication followed by tens of successful infections are low, drastically reducing the potential number of secondary sporangia and, therefore, the chances of migration, and 4a) surrounding vineyards are constantly chemically treated according to precise schedules. This implies that vines are normally disease-free, providing low (or no) inoculum source. These four issues synergistically support the picture of a restricted pathogen's migration from vineyard to vineyard and consequently that *P. viticola* populations may be genetically separated communities.

Employing four statistical approaches, we tested the hypothesis of a restricted exchange of propagules among populations (low migration). Using 9229 samples from five European countries (chapter 5) we confirmed our supposition. 1b) The pairwise test of differentiation highlighted overwhelmingly significant genetic subdivisions between populations collected at different locations. Significant genetic subdivision was assessed even in cases when the Euclidean distances between the population pairs are relatively short (20-40 km). Genetic differentiation seemed not to apply at distances of about 1 km. 2b) The test for isolation by distance detected a positive association between geographic and genetic distances in *P. viticola* populations, indicating a disequilibrium between gene flow and genetic drift. Consequently, geographical distance contributes for keeping the populations genetically separated. Two major European mountain ranges and the presence of the sea are shown to be the main barriers that impede migration. 3b) The phylogenetical tree based on Shriver's Dm fully confirms the genetic subdivision assessed by the pairwise test of differentiation. 4b) The oosporic populations, independently from the sampling date or from the number of samplings that constituted it, in most cases, respected the HWE principles. If the HWE principles are respected, than, we can speculate that immigration events altering autochthon allele distribution do not occur.

This study disagrees, than, with the existing assumptions about the wide-range migration of the disease and therefore with the sudden infestation of the European vineyards after the first report of its introduction. As eight different approaches (1a-4a and 1b-4b) challenge the

commonly assumed historical speculations, we suggest alternative explanations. We speculate that *P. viticola* was introduced to Europe through several introduction events, which were then followed by human-mediated transport of infected material (rooted grape cuttings carrying soil that contains oospores). Introduction events may have occurred even earlier than reported by the literature. A study of the population in the centre of origin (North America) may give support to the hypotheses presented.

Implications for disease management strategies

The new knowledge emerging from this study has several consequences for disease controlling strategies. We may now explain why chemical treatment of the very first lesions plays a key role in the whole disease management strategy. Several agronomic engineers active in viticulture (Jermini M, Raynal M, personal communication) share the opinion that leaving the very first primary lesions untreated may lead to a practical difficulty in controlling the disease in later stages. If the very first primary lesions (genotypes) are normally quite rare (<1 lesion/vine) and have a 71% probability to not generating any secondary lesion, than this current opinion should not have arisen. Nevertheless, if we consider that a potentially aggressive genotype (dominant genotype) may hide among the 29% which survive, we may realize that leaving this particular genotype alive could lead to serious damage in later epidemiological periods. During immediate later stages, the eventual emergence of 1-3 genotypes apparently less frequent than the dominant one, were clearly shown to bring additional damage to the crop (Eugster *et al.*, 2003). If those few particularly successful early-appearing genotypes would be chemically annihilated, we may expect a significant reduction of the disease severity over time. The confirmation of the mentioned hypothesis is provided by the efficacy of a neglected downy mildew management strategy (minimal fungicide strategy, MFS, Jermini, 2003b). The MFS aims at controlling the disease based on 3-4 fungicide treatments only, instead of 7-9, and was proved to be extraordinarily effective for four years consecutively (1999-2002) in a humid and rainy South Swiss area (1600-2000 mm rain/year). A first treatment was performed at the apparition of the first symptoms, and this first treatment was followed by two or three additional fungicide

applications. The latter aim to delay the epidemic under a economic injury level of 5% severity at the *veraison* to avoid plant stress during the first ripening phase and, consequently, yield quality damages (Jermini *et al.*, 2003b). Such a strategy succeeded in keeping the severity level significantly low, in comparison to an untreated plot but, at the same time, it granted the same yield (exception: 1999), as a plot treated 7-9 times. Our recent genetic findings allow us to elucidate the success of this method. The effectiveness of this strategy may be based on the annihilation of the few really dangerous genotypes appearing early in the season, by means of the first 1-2 fungicide treatments. Later treatments just reduce the new primary lesions and their successive asexual progeny and keep disease severity below an economic injury level. Later appearing genotypes (primary infections followed by secondary cycles) should not cause relevant damage, as shown in chapter 4, and, therefore, they do not need to be treated intensively according to a two-week treatment schedule. Nevertheless the MFG fungicide application timing remains difficult because is not yet based on knowledge derived from a quantitative model or a fast/reliable method that allows the measurement of germinability of oospores.

A second highly relevant outcome of this research is based on the knowledge that the infection pressure is caused mostly by continuous input of oosporic (primary) infections. For disease management it appears reasonable to postulate that low amounts of overwintering inoculum should keep the infection pressure low during the next year. Control strategies may have to be designed to reduce oospore formation in the end of summer and the beginning of fall. Sanitation by physical removal of leaf debris year after year from the vineyards in late summer/fall would reduce the amount of overwintering oospores.

The new knowledge rising from this study has important consequences for disease forecasting models, too. It is surely not possible to predict the magnitude of progeny simply by counting the number of sporulating lesions, as the majority of models do, due to the apparently stochastic and genotype-specific asexual multiplications. It is now partially clear, why models based purely on secondary cycles (Blaise, 1999) or purely on subsequent oosporic infections (Hill, 1990) are subjected to unexpected failures. While the former take into account that exclusively a few early-appearing genotypes are fully responsible for massive clonal multiplication and ignores the continuous oosporic infections; the former ignore that

20-90% of the disease is caused by 1-2 dominant and asexually multiplicating genotypes. A new concept for an epidemiological model should be based on the integration of two different algorithms, each one dealing with a downy mildew cycle. The first algorithm should include a quantification of the oosporic pool, the dynamic of oospore maturation and calculate the major period of infectivity. It should model the bi-dimensional localization of the primary infections, which probably reflects the spatial pattern of oospore ripening. It should consider that oosporic lesions have a random distribution over the vineyard. The second algorithm should consider that, on average, only 30% of the primary lesions undergo asexual multiplication and that 1-2 early appearing genotype(s) may cause a variably high disease severity. It must consider that secondary lesions have a highly clustered pattern at vine level as soon as genotypes start an asexual cycle. More importantly, it must consider the step-by-step expansion of one (or two) dominant genotype(s), which first colonize a few vines and then they spread until they cover a variable percentage of the plot. Disease severity may become a function of vineyard sub-areas, as vines or simply x;y co-ordinates (at least in the initial epidemiological stages), and should not be calculated anymore as an average over the entire vineyard. Nevertheless, based on our present knowledge, this kind of spatial-dependent modeling seems an ambitious if not unrealistic task, mostly because of the unpredictability of random events.

Conclusions

The quantitative contribution of primary and secondary infection to an epidemic was surprisingly misconceived in the past. Employing molecular tools we were able to redefine the epidemiological cycle by quantifying the responsibility of oospores and sporangia to an epidemic. Several key-issues still remain unclear, as the influence of meteorological conditions to the degree of elicitation of the primary and secondary cycles or the ripening dynamic of oospores. The answer to these questions can not be given solely by means of DNA markers.

After the innovative findings of our work, new concepts in disease controlling strategies should be cogitated. By simple sanitation procedures, as eliminating the infected leaf debris from the plot in October, we may reduce the load of oospores and, consequently, the disease pressure. We may target fungicide applications to critical epidemic stages, within a relatively narrow window in May/June and afterwards just keep the disease under an economic injury level, without pretending the complete pathogen's annihilation. By reducing the environmental load of fungicides, we may contribute to escape from the vicious circle: "fungicide development / selection pressure / resistance development / new fungicide development…" and try to reach a more sustainable viticulture. Lots of solution-oriented thinking based on new knowledge is necessary, as well as practical validation of the exposed concepts. The way is still long…

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