Eur J Plant Pathol (2010) 127:501–508
DOI 10.1007/s10658-010-9615-z

Microsatellite based population structure of *Plasmopara viticola* at single vine scale

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Accepted: 22 March 2010 / Published online: 25 April 2010

Abstract The genetic structure of a *Plasmopara viticola* population was characterized on five single vines, one for each cultivar Regent, Merlot, Isabella, Müller-Thurgau and Solaris, using four neutral specific polymorphic microsatellite markers. Five-hundred and seventy samples were collected at four dates in the period between the 10th of July and the 23rd of August 2006. On average over all five cultivars, 67% of the genotypes present on the single selected vines derived from primary infections and caused 37% of the lesions genotyped. Fifty-three percent of these genotypes occurred only once on the vine throughout the survey period, while 14% were able to asexually reproduce on the selected single vine throughout the survey period, causing 23% of the lesions. Thirty-three percent of the genotypes on the single vine derived from other vines, 28% from vines of other cultivars in the other rows, and 5% from vines of the same cultivar in the same row. New primary infections appear all along the sampling dates. The overwhelmingly quantitative role of primary infections at vineyard scale was known, however here we observed the phenomenon also at the single vine scale and the reduced contribution of secondary lesions to the populations present on more resistant cultivars compared to the susceptible cultivars. As the sampling extended almost to defoliation, the results are judged to be representative of a typical *P. viticola* epidemic.

Keywords Downy mildew · Grapevines · SSR markers · Population genetics · Primary infections · Secondary infections

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Introduction

Plasmopara viticola (Berk. and Curt.) Berl. and de Toni, the causal agent of grapevine downy mildew, is considered one of the most important grapevine pathogens worldwide. The diploid heterothallic (Wong et al. 2001) obligate biotroph oomycete attacks leaves, fruits and young shoots, resulting in plant defoliation, production of low quality or entirely destroyed grapes, and weakening, dwarfing and killing of young shoots.

Recent population genetic studies have shown that primary oosporic infections contribute to the epidemic throughout the season (May to late October, depending on the region), with most genotypes having a limited ability to spread

asexually (Gobbin et al. 2003b; Rumbou and Gessler 2004, 2006; Koopman et al. 2007). A highly variable but consistently relevant proportion of the lesions appearing up to the stage of one–two lesions per leaf are caused by new genotypes. Only a few of these genotypes will be able to produce secondary lesions. Gobbin et al. (2005) distinguished five possible secondary dispersal patterns: (i) clonal multiplication at distances shorter than 1 m from the putative primary lesion (close to the source), (ii) clonal multiplication close to the source followed by plot-scaled dispersal, (iii) multicluster plot-scaled dispersal without previous clonal multiplication close to the source, (iv) random plot-scaled dispersion without previous clonal multiplication close to the source, and (v) minor clonal multiplication and dispersal.

The most frequent pattern of an epidemic is composed of a random distribution of genetically different genotypes (resulting from primary infections) throughout the vineyard and some spatially localized clustered lesions (secondary infections) derived from a single genotype (type ii) (Gobbin et al. 2003b, 2005; Rumbou and Gessler 2004, 2006).

Starting from published data of European (Gobbin et al. 2003b, 2005, Rumbou and Gessler 2004, 2006), Australian (Hug 2005) and American *P. viticola* populations (Eugster 2003), Gessler et al. (2006) focused on the few cases were data of samplings at small scale were available and concluded that secondary infections were spatially localized in most cases (at leaf, branch or vine scale) and that the average number of lesions formed by a single genotype was higher compared to the plot scale, indicating that under those conditions secondary infections were the cause of damage.

Our objective was to investigate and quantify, at the single vine plant scale, the amount of primary and secondary infections and to determine the genetic structure of *P. viticola* populations along one season to gain more information on the role of primary and secondary infections at small scale. This was done through performing an intense, and where possible complete, sampling on vines of five grapevine cultivars, chosen for their popularity and different degrees of susceptibility to *P. viticola* in order to investigate a larger panel of possible scenarios of population structure on single vines.

Material and methods

Experimental plot

The experimental vineyard was planted in May 2004 in Cugnasco (46° 10′ 0″ North, 8° 53′ 0″ East, Ticino, Southern Switzerland). The plot considered here consisted of five rows at a distance of 2 m, each row contained 15 plants of a cultivar at 0.8 m distance. Five *V. vinifera* and interspecific hybrid cultivars (Merlot, Müller-Thurgau and Solaris, Regent and Isabella) were selected for their popularity and different degrees of susceptibility to *P. viticola*. Vines were trained in a Guyot system. Pruning, canopy management, fertilization and weeds control were conducted as in the surrounding vine-growers' vineyards. Treatments with Slick® (Difeconazole, 250 g/l a. i., used concentration 0.0125%, Syngenta Agro AG, Switzerland) against powdery mildew were performed.

Disease assessment

Disease severity was assessed on all leaves of one randomly chosen shoot (previously labelled) per stock at four dates (5th, 19th and 30th of July and 25th of August). A modified (in order to discriminate low levels of severity) Horsfall-Barratt disease rating scale (logarithmic scale, compensates for the human error in estimating the amount of disease present, Horsfall and Barratt 1945) was implemented. In the modified scale, disease rating of 0 corresponds to 0% of foliage with disease symptoms, 1 = 0* to 1%, 2 = 1* to 3%, 3 = 3* to 6%, 4 = 6* to 12%, 5 = 12* to 25%, 6 = 25* to 50%, 7 = 50* to 75%, 8 = 75* to 88% and 9 = 88* to 94%, 10 = 94* to 100% (the asterisk indicates a value slightly exceeding the indicated value) was implemented. Ratings were back-transformed to percentages using the midpoint rule (Campbell and Madden 1990) and the obtained values were averaged by the total number of leaves of the selected shoots. Completely defoliated branches were assigned a value of 100% disease severity.

Sample collection and processing

Sampling was performed at six dates (26th of May, 9th of June, 10th, 19th and 30th of July and 23rd of August 2006). Sampling in the experimental plot

started when the first symptoms were observed on vines; subsequently samples were collected following the completion of incubation after important infection events determined by rain and temperature. This continued until downy mildew generated a mosaic pattern, which impeded the observation of well delimited lesions. Whenever possible, samples were collected the same day as the disease assessment. Collected lesions were assigned coordinates (row and plant number) to locate their exact position in the vineyard (Gobbin et al. 2003a).

Samples consisted of half a sporulating lesion (about 1 cm^2, including some healthy leaf tissue) excised with a cutter (Gobbin et al. 2003a). On one vine in the central position of the row (single selected vine), all well delimited lesions (but maximally 150 lesions per vine per sampling), having a diameter greater than 0.8 cm were collected on all sampling dates. On the other 14 vines of each cultivar, a maximum of four lesions per vine and sampling date were randomly collected (a representative number to monitor the epidemic according to Gessler et al. (2006), Gobbin et al. (2003a, b, c, 2005, 2006), Rumbou and Gessler (2004, 2006), Hug (2005), Eugster (2003)).

DNA extraction was performed as described in Gobbin et al. (2003a). Samples were analyzed using newly designed primers targeting the four polymorphic *P. viticola*-specific SSR loci, ISA, CES, BER and GOB (Table 1). The new primers were designed on sequences obtained from the cloned loci originally used by Gobbin et al. (2003a) using the software primer 3. Amplification was performed as described in Gobbin et al. (2003a) with a 56°C annealing temperature for all primers. PCR products were combined using 1–3 μl of each PCR product and sterile water to a final volume of 15 μl. Four microliters of the combined PCR products were mixed with 9 μl of HiDi formamide and 0.2 μl of GeneScan 500 LIZ size standard (Applied Biosystems, Foster City, CA). Samples were denaturated for 2 min at 94°C and cooled on ice. Fragments were separated on an ABI PRISM 3730xl (samples collected in 2005), 3100 and 3130 (samples collected in 2006) sequencer following the manufacturers' instructions. Fragments were analyzed with the program Genemapper v 4.0.

Genotype distribution

All the following definitions refer to the single vine of each cultivar: Two groups of infections were defined: 1) primary infections (genotypes observed first on the single selected vine and therefore assumed to be oosporic infections) and 2) secondary infections (genotypes observed prior on any vines of the same or different cultivar). Primary infections consist of 1a) genotypes which do not generate secondary progeny on the same single selected vine (single genotypes), and 1b) genotypes which generate secondary progeny on the same single selected vine (clonal genotypes). Secondary infections consist of: 2a) immigrants originating from vines of a different cultivar, 2b) immigrants originating from the same cultivar as the considered single selected vine, or 2c) lesions derived from genotypes which generated secondary progeny on the same single vine (Fig. 1)

Results

Disease progress

Disease severity assessment was started on the 5th of July; before this day lesions were observed sporadically on few vines (estimated severity at 26th of May and 9th of June less than 0.5%).

Table 1 Sequences of five new designed primers of the four variable loci ISA, CES, BER and GOB (Gobbin et al. 2003a). Annealing temperature is 56°C for all primers

Locus	Primer sequence (5′–3′)[a]	fpl[b]
ISA	$ISANew_f$: GGC ATG GAC GTT GAC TCA C ISA_r: Gobbin et al. 2003a	ROX
CES	CES_f: Gobbin et al. 2003a $CESNew_r$: CAT CAG AAT GTT TGT GTG TG	NED
BER	$BERNew_f$: CAA GCA ATG CAA TGG TCT TC $BERNew_r$: GGC ATC ACT CTC TAC CTG CTC	HEX
GOB	GOB_f: Gobbin et al. 2003a $GOBNew_r$: ATC GCA CAG CTT AAT GCA TAT C	FAM

[a] f: forward primer, r: reverse primer

[b] Forward primer labelling

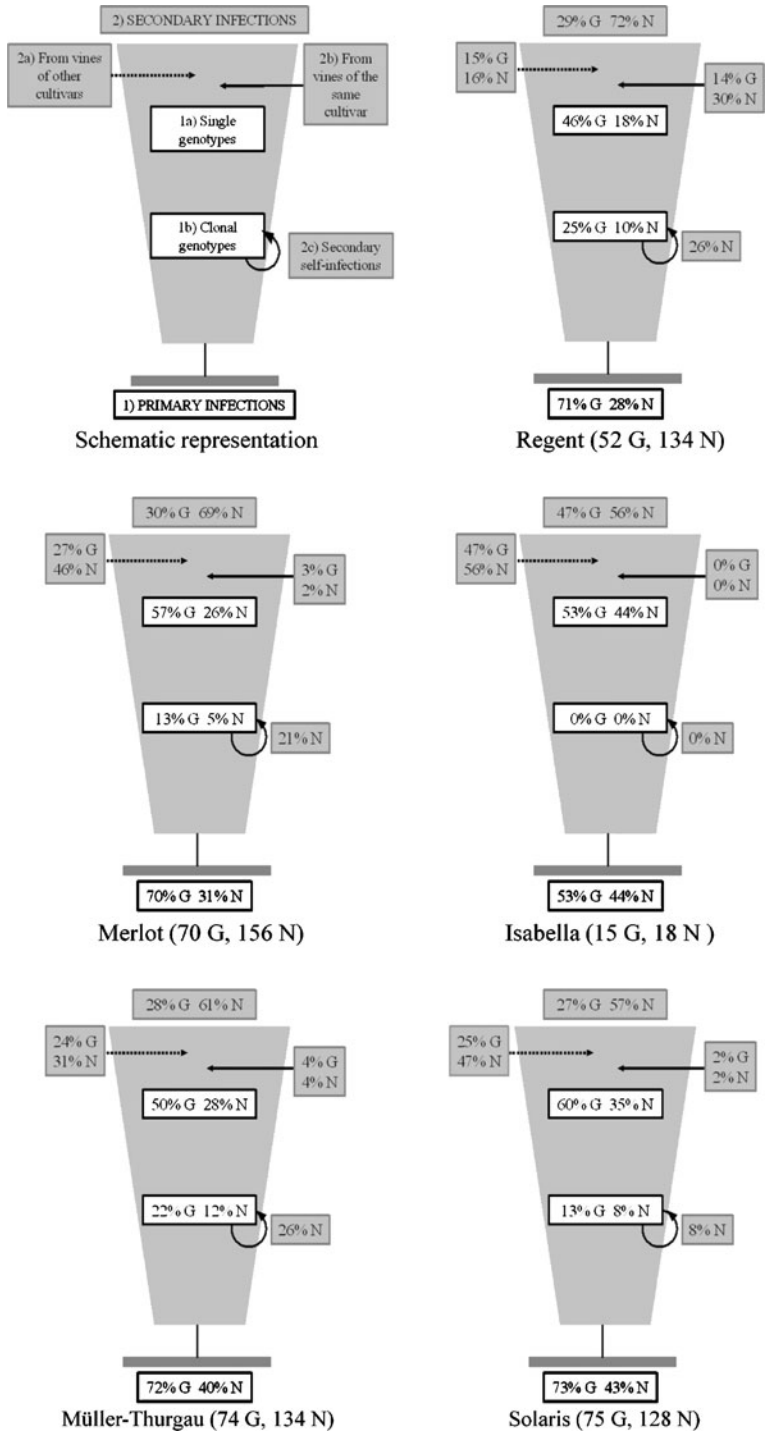

Fig. 1 Percentual distribution of genotypes (G) and lesions (N) at vine scale for each single selected vine of cultivars Regent, Merlot, Isabella, Müller-Thurgau and Solaris. All sampling dates were pooled. %N: percentage of lesions, %G: percentage of genotypes

The highest average disease severity values were observed at 25th of August 2006 for Merlot (70%) and Müller-Thurgau (69%), the lowest for Solaris (17%) and Isabella (24%), and intermediate for Regent (33%). The steepest disease severity increase on Müller-Thurgau occurred between the 19th and the 30th of July (from 9 to 27%); and between the 30th of July and the 25th of August on vines of the cultivars

Merlot (from 13 to 70%), Regent (from 1 to 33%), Solaris (from 1 to 17%) and Isabella (from 6 to 24%; Fig. 2).

Genotype distribution

Over the whole experimental plot, 857 samples were successfully genotyped of which 570 were on the five single vines. Sixty seven percent of the genotypes present on the five single selected vines were genotypes not identified beforehand and were therefore assumed to be primary oospore-derived infections (1); on average they caused 37% of the lesions genotyped. Fifty-three percent of these genotypes occurred only once throughout the survey period (1a), while 14% were able to asexually reproduce on the same single selected vine throughout the survey period (1b clonal genotypes), causing 23% of the lesions. Thirty-three percent of the genotypes on the single selected vines were immigrants derived from other vines, 28% from vines of a different cultivar (2b), and 5% from vines of the same cultivar (2a) (Fig. 1).

Considering the single selected vine as an isolated vine, therefore excluding all immigrants, 51, 60, 100, 60 and 83% of the lesions present on the single selected vine of cultivars Regent, Merlot, Isabella, Müller-Thurgau and Solaris, respectively were primary infections; and 49, 40, 0, 40 and 17% were secondary infections derived asexually from lesions present previously on the particular vine (data not shown).

For particular cultivars a notable difference in the average values was noted. Forty-seven percent of the lesions observed on the single selected vine of cultivar Isabella were caused by immigrants from other cultivars (2b, Fig. 1). A low percentage of secondary self-infection (0 and 8%) was observed on Isabella and Solaris compared with Regent, Müller-Thurgau and Merlot (26, 26 and 21%). Regent presented a high (30%) number of immigrant from the same cultivar (2a) compared with the others, which ranged between 0 and 4%. By contrast, immigrants from other cultivars (2b) appeared at a lower % on Regent than on the other cultivars (Fig. 1).

Discussion

Primary infections represent the most important contribution to the epidemics at the vine scale. Only a reduced number of genotypes, that varies depending from the resistance level of the grapevine cultivar, undergo secondary cycles re-infecting the vines on which they first appeared. An important contribution to the epidemics at single vines is made by asexually-derived genotypes coming from neighbouring vines; most of these derive from vines of different cultivars.

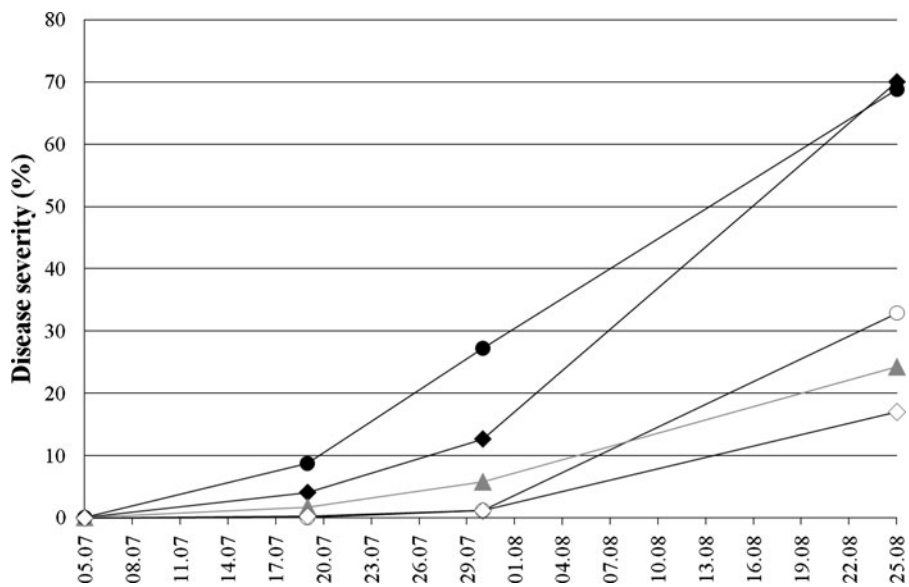

Fig. 2 *P. viticola* severity assessed on leaves the five cultivars Regent (white circle), Merlot (black rhombus), Isabella (grey triangle), Müller-Thurgau (black circle) and Solaris (white rhombus) throughout the period 5th July–25th August 2006 in Cugnasco

It is notable that no clonal reproduction is detected on Isabella and all immigrants originate from other cultivars. Therefore for an unknown reason, no genotype present on Isabella reproduces further. Isabella (interspecific hybrid (*V. labrusca* × *V. vinifera,* VIVC 2007)) is considered to be moderately resistant to *P. viticola*, however in the last years an increase in damage due to the pathogen has been observed in Ticino (Jermini and Gessler, personal communication). The leaves of Isabella are covered with hairs that repel water from the leaf surface, preventing the penetration of the host via germ tubes (Kortekamp and Zyprian 1999). Experiments performed by Kortekamp and Zyprian (1999) indicate the action of further defence mechanisms for *V. labrusca*, one parent of Isabella (VIVC 2007). These defence mechanisms could be responsible for the delay in heavy infection of vines of cultivar Isabella (steepest disease severity increase between the 25th of August and the 12th of September, data not shown) and for the relative low number of lesions.

A similar situation was observed for the highly resistant cultivar Solaris. Few lesions derived from asexual reproduction on single selected vine could be detected, but otherwise the percentage of primary infections was high and similar to that observed for the other cultivars. The vine architecture of Solaris is similar to Isabella, and is characterized by numerous large leaves; nevertheless leaves of Solaris are hairless. On Solaris, *P. viticola* induces a hypersensitive response, resulting in necrotic spots with no sporangiophores (Gindro et al. 2003, Pezet et al. 2004). In our experimental plot, sporulation on Solaris leaves was observed, albeit at a reduced level.

The type of defence mechanism may influence the selection of aggressive genotypes. Active defence mechanisms are more easily overcome. They include recognition of elicitors inducing hypersensitive response (Bellin et al. 2009; Godard et al. 2009; Boso and Kassemeyer 2008; Díez-Navajas et al. 2008; Kortekamp 2006; Kortekamp et al. 1998), accumulation of callose or phenolic compounds (lignins, coumarins, stilbenes and stilbene oligomers) (Godard et al. 2009; Kortekamp 2006; Richter et al. 2006; Kortekamp and Zyprian 2003; Aziz et al. 2003; Dai et al. 1995a; Calderon et al. 1994), increase of chitinase, glucanase (Aziz et al. 2003) and peroxidase activity (Godard et al. 2009; Kortekamp and Zyprian 2003). Passive defence mechanisms include structural characteristics of leaves and berries (Ficke et al. 2004; Kortekamp and Zyprian 1999; Kortekamp et al. 1999; Heintz and Blaich 1989) and preformed antifungal compounds (anthocyanins and other phenolic compounds, Kortekamp 2006; Dai et al. 1995a, b).

On Regent, the second most resistant cultivar used in the experiment, the highest percentage of lesions derived from asexual reproduction on single selected vine was observed. A large number of lesions was caused by genotypes found exclusively on vines of the same cultivar. As significant host-parasite interactions between *P. viticola* isolate and grapevine cultivars have been observed by Kast et al. (2000), this could be interpreted as a specific interaction with Regent-adapted genotypes.

Müller-Thurgau and Merlot had a high level of *P. viticola* primary infections, asexual reproduction on single selected vine, and from genotypes found previously on vines of other cultivars. On both susceptible cultivars, lesions were caused mostly by the most frequent genotypes observed in the whole plot.

The results are in agreement with previous experiments (Eugster 2003; Hug 2005) and with the finding that the most frequent pattern of *P. viticola* dispersal is characterized by some spatially localized clustered lesions derived from a single genotype (resulting from secondary infections) and a random distribution of genetically different genotypes (resulting from primary infections) (Gobbin et al. 2003b; 2005; Rumbou and Gessler 2004, 2006).

The results support the hypothesis that at the small scale secondary infections contribute substantially to damage, implying that generalized vineyard-wide epidemics are most probably the results of a large number of randomly distributed primary infections followed by uncontrolled secondary multiplication at the small scale (Gessler et al. 2006). Differences between cultivars were observed; an epidemic on resistant cultivars was mostly dependent on inoculum coming from surrounding vines, whereas on susceptible cultivars asexual reproduction on single selected vines caused on average one fifth of the number of lesions.

These results point out:

1) the importance of primary infections for the epidemics, and therefore their reduction through, for instance, the removal of the leaves from the vineyard in autumn (a sanitation measure

successfully practiced in some apple orchards against apple scab (*Venturia inaequalis*), where mechanization, antagonists and strategies (Beresford et al. 2008; Holb et al. 2006) are present. Ascospore viability in the field is for only one season (MacHardy et al. 2001). However oospores can survive and remain viable in soil for longer periods (Kennelly et al. 2007));

2) the reduction of secondary infections, through early treatments, to prevent the reproduction and subsequent spread at vine and plot scale of the most fit genotypes (Gobbin et al. 2003c; Jermini et al. 2003, 2006); and
3) the differences in epidemics between resistant and susceptible cultivars.

Acknowledgements This work was supported by SBF 03.0485-1 (EU-Project 501542 REPCO). The authors gratefully acknowledge Rocchina Abbas-Pennella for helping with the laboratory work, Michele Gusberti and Natasha Rosselli for helping with the collection of samples and disease assessment, and Roberto Rigoni and Mirto Ferretti for the maintenance work in the vineyard.

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