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Studies on the genetic diversity of primary and secondary infections of *Plasmopara viticola* using RAPD/PCR

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Summary

In 1998 and 1999 isolates from primary and secondary infections of *Plasmopara viticola* (downy mildew) were sampled from several grapevine varieties. Fungal isolates were taken from the same vineyard located in Württemberg/ Germany. Using the random amplified polymorphic DNA (RAPD) technique as an initial screening for genetic variation within *P. viticola* isolates, different molecular fingerprint patterns were obtained. Out of 48 isolates of primary infections 27 (1998) and 29 (1999) different genotypes were found. In contrast, variability of secondary infections was lower: about 9 in 1998 and 14 in 1999 different genotypes out of 48 isolates. The high genotype diversity in primary infections and the low level of genotype diversity in secondary infections strongly suggests environmentally directed selection.

K e y w o r d s : downy mildew, grapevine, RAPD/PCR, fingerprint pattern.

Introduction

Plasmopara viticola propagates asexually in summer by forming sporangia and zoospores, while in autumn sexual propagation starts and diploid oospores are produced in host tissues (EMMET *et al.* 1992; VERCESCI *et al.* 1999). Primary infection occurs when water-borne sporangia or zoospores, produced by germinating oospores, are splashed onto wet vine foliage. Then motile zoospores move to stomata and penetrate stomatal apertures via a germ tube. After an incubation period sporangiophores and sporangia are produced and grow out through stomatal pores in warm humid nights. Secondary infection follows wind or water-splash dispersal of these sporangia (ESPINO and NESBITT 1982). Dependent on weather conditions several circles of secondary infections can take place during one season.

RAPD markers were used to obtain more information about the biology and epidemiology of *P. viticola*. DNA markers based on PCR are a powerful tool to study the genetics of fungal plant pathogens (EDEL 1998). Moreover, random amplified polymorphic DNA (RAPD) has been successfully used in various genetic studies (WELSH and MCCLELLAND 1990; CASTAGNONE-SERENO *et al.* 1994; DELYE *et al.* 1997; ANNAMALAI 1999). RAPD is a type of genetic marker based on DNA amplification with single or multiple arbitrary primers requiring no knowledge of the target DNA sequence. Thus, amplification products are produced that often show size polymorphism within species. The reproducibility within one laboratory is usually satisfactory (TOMMERUP *et al.* 1995). Furthermore, DÉLYE *et al.* (1995) have demonstrated that even two different DNA polymerases or three different thermocyclers show similar RAPD profiles of *Uncinula necator*. Therefore, the RAPD/PCR technique seemed to be suitable to estimate the genetic diversity of a *P. viticola* population on a small scale.

The aim of this study was to compare the genetic variability of isolates deriving from primary infections to that of isolates deriving from secondary infections.

Material and Methods

In June 1998 and 1999 48 isolates of *P. viticola* were sampled from primary infections found on *V. vinifera* cvs Müller-Thurgau, Traminer, Samtrot and Weißburgunder located in Wildeck (Baden-Württemberg, Germany). Temperature was recorded by an Adcon device 2.5 m above ground level. Using the addVANTAGE program (Adcon, Austria) we determined the exact time of oospore germination of primary infection and possible infection with *P. viticola*. To ensure that each primary infection corresponded to one infecting zoospore, only single, circular oil spots were taken. Each vine showing a primary infection was marked in order to sample secondary infections at the same location. Finally, at the end of the vegetation period in September/October 48 isolates from secondary infections were sampled. Samples were taken from the same grapevines in June and October, respectively. To obtain sufficient fungal material for DNA extraction, leaves containing oil spots were incubated for 48 h at 20 °C and almost 100 % r.h. Thereby *P. viticola* formed sufficient sporangia at the leaf surface which were harvested for DNA extraction. If too little sporangia were formed, *P. viticola* was propagated on detached leaves of the susceptible cv. Trollinger until sufficient material for DNA extraction was available.

DNA extraction and amplification was performed as described by SEIDEL *et al.* (1998). Genomic DNA was extracted from 0.5 mg sporangia according to the method of ZHU *et al.* (1993) using benzyl chloride for chemical cell wall disintegration. PCR was performed using a ready-to-go RAPD

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analysis kit from Pharmacia (Uppsala, Sweden). 25 pmol of primer, 30 ng of template DNA and distilled water were added to give a final volume of 25 µl. Random primers were obtained from Pharmacia and Roth (Karlsruhe, Germany). After screening of 26 different decamer primers, the primers Pha6 [5' CCCGTCAGCA 3'] (Pharmacia) and Roth 170-06 [5' GGACTCCACG 3'] (Roth) were used subsequently because they revealed a high level of polymorphism between isolates of *P. viticola*. Thus clear and reproducible RAPD-patterns were obtained. Samples were placed in a thermocycler (Progene, Techne, Cambridge, U.K.) and subjected to the following cycle profile: 1 cycle at 95 °C for 5 min followed by 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. 5 µl of the reaction mixture was analysed by electrophoresis on 7.5 % PAA-TBE gels. Gels were stained with silver (BUDOWLE and ALLEN 1990) and documented with ImageMaster VDS, Pharmacia. Data were used to construct phenograms, using a program based on UPGMA (Image Master 1D software, Pharmacia). Analysis was repeated at least twice from extraction to electrophoresis of amplification products.

Results and Discussion

In 1998 and 1999 a population of 48 isolates deriving from primary infections and 48 isolates from secondary infections were screened with RAPD/PCR using different primers for DNA amplification. Samples from primary infections revealed a high genetic variability according to their different fingerprint patterns (Fig. 1). In 1998 27 different isolates of primary infections were observed, while isolates deriving from secondary infections showed less genetic variability (Fig. 2): Nine groups out of 48 isolates tested exhibited different fingerprint patterns. In 1999 similar results were obtained. The phenogram of primary infections revealed 29 distinct groups (Fig. 3). In contrast, the phenogram of secondary infections revealed 14 distinct groups. Moreover, the genotype of three isolates (Wi 52, Wi 53 and Wi 54) was much more abundant.

Although the isolates had been sampled on a small scale, they exhibited different fingerprint patterns indicating a high genetic variability. Moreover, even on one leaf different oil spots were found that showed different fingerprint patterns (data not shown).

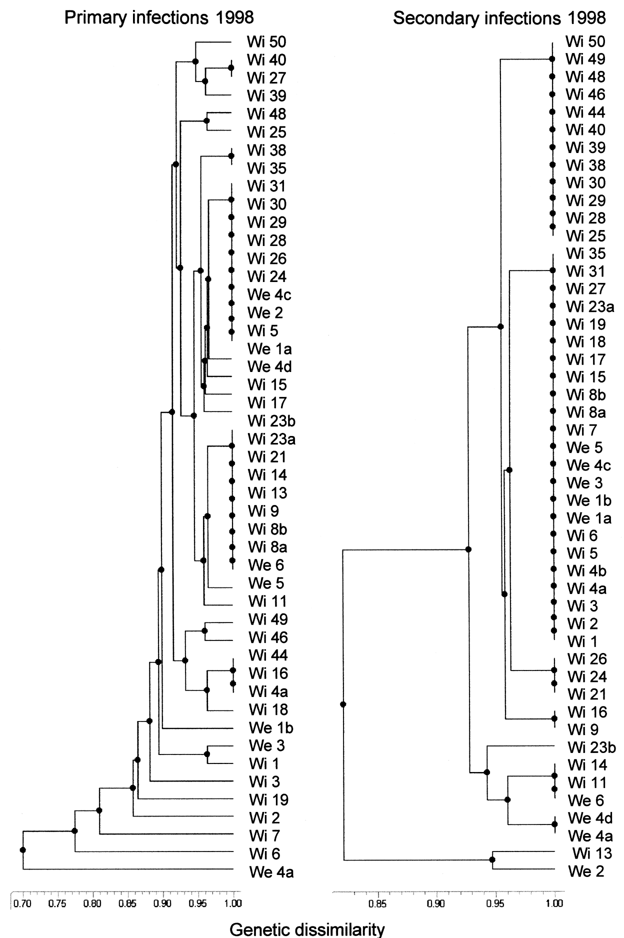

Fig. 2: Phenograms of primary and secondary infections of *P. viticola* generated from cluster analysis of RAPD data using UPGMA in 1998.

We cannot totally exclude that single oil spots correspond to infections of different zoopores. However, this does not seem to be of great importance, since the experiments were repeated in different years. Furthermore it was shown that different primers reveal similar results.

High genetic variability has also been described for other populations of sexually reproducing fungi; *e.g.*, in *Gremmeniella abietina* a surprisingly high rate of variation can be found even on one single host tree (WANG *et al.* 1997). However, *Plasmopara halstedii*, which causes downy mildew in sunflower, did not indicate genetic variability

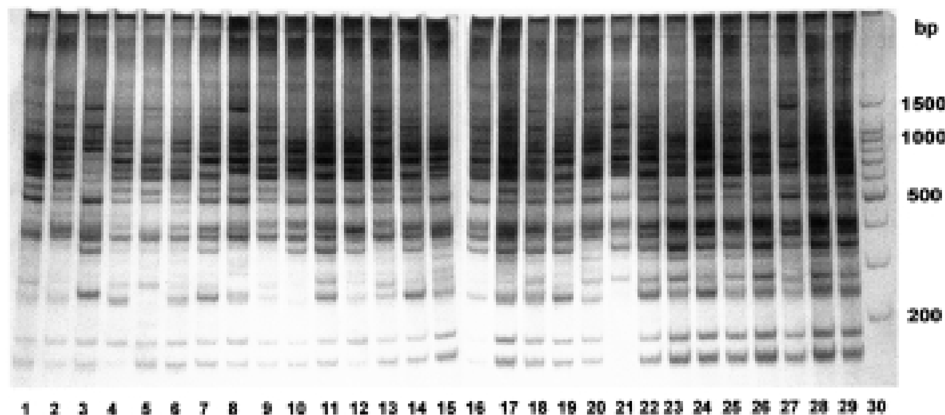

Fig. 1: Genetic polymorphism between different isolates of *P. viticola* deriving from primary infections revealed by primer Roth 170-06 (lanes 1 to 29). Lane 30 shows the size marker (100-bp DNA ladder, PROMEGA).

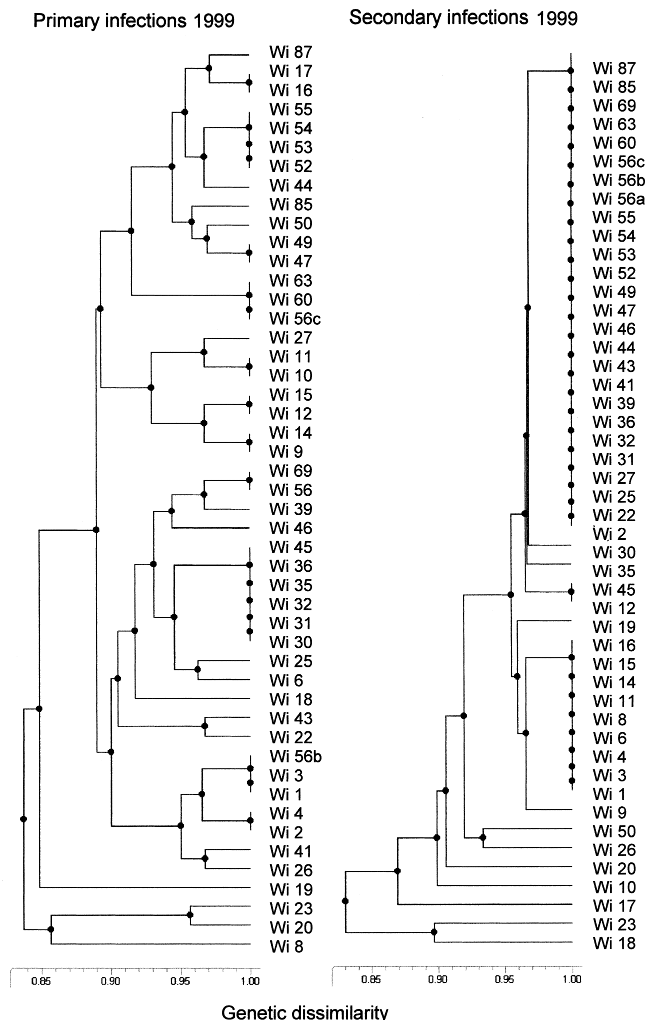

Fig. 3: Phenograms of primary and secondary infections of *P. viticola* generated from cluster analysis of RAPD data using UPGMA in 1999.

(Roeckel-Drevet *et al.* 1997). When sampling isolates of *P. viticola* from secondary infections in October 1998 and 1999 we frequently found a low rate of genotype variability. In contrast, Kump *et al.* (1998) described a high genetic variability of secondary infections in *P. viticola*, explained by numerous new primary infections in later stages of the epidemic.

Comparing variability of secondary infections of *P. viticola* to that of primary infections, the genetic variability was strongly reduced. It seems that during several cycles of secondary infections selection of the fittest isolates occurs. Selection is generally recognised to be a strong tool in shaping pathogen population structures (Huang *et al.* 1995). When the limited dispersal and highly asexual reproduction of a fungal population is coupled with localised adaptive differentiation, the potential for selection of isolates adapted to very specific environmental conditions can be very high; *e.g.*, Ennos and McConnell (1995) found that small scale environmental variation had a larger effect on the relative performance of *Crumenulopsis sororia* than differences in the environment over longer periods.

The new characteristics of these isolates would be diluted after sexual reproduction with other less fit genotypes. Then, during the next asexual reproductive phase, again selection of the fittest isolates might occur. They will eventually dominate within the population between circles of sexual reproduction. Therefore, the most successful asexual isolates are likely to be the most frequent parents of most of the next season's isolates (Duncan *et al.* 1998).

The high genetic variability of primary infections may be due to genetic recombination during genesis of oospores. Furthermore oospores which are able to persist in the soil for at least 5 years (Hill, pers. comm.) might skip one or several vegetation periods thus contributing to the high genetic variability in spring.

The high level of genotype diversity in primary infections and the low level of genotype diversity in secondary infections strongly suggests that environmentally directed selection takes place.

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