Persistence and Spatial Autocorrelation of Clones of *Erysiphe necator* Overwintering as Mycelium in Dormant Buds in an Isolated Vineyard in Northern Italy

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ABSTRACT

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The population structure of the grape powdery mildew fungus, *Erysiphe necator* (formerly *Uncinula necator*), has been hypothesized to vary from being clonal to highly diverse and recombining. We report here on the structure of an *E. necator* population sampled during a 4-year period from an isolated vineyard in northern Italy (Voghera, Pavia Province). We obtained 54 isolates of *E. necator* that overwintered asexually as mycelium in grapevine buds and caused severe symptoms on the emerging shoots, known as flag shoots. All isolates were genotyped for mating type, four multilocus polymerase chain reaction (PCR)-based markers (a total of 64 loci were scored), and two single-copy loci designed to identify genetic subgroups in *E. necator*. All isolates had the same mating type and single-locus alleles that correlate to isolates from flag shoots in other areas. Only 2 of the 64 loci scored from multilocus

markers were polymorphic; 46 of the 54 isolates had the same multilocus haplotype. Seven isolates had a second haplotype that was recovered over 3 years, and only a single isolate was found with a third haplotype. Both variant haplotypes differed from the main clonal haplotype by single loci. Spatial autocorrelation analyses showed that vines with flag shoots were not aggregated within years, but they were aggregated between consecutive years. These results demonstrate that this subpopulation of *E. necator* on flag shoots is composed of a single clonal lineage that has persisted for at least 4 years. We speculate that the lack of diversity in the flag shoot subpopulation in this vineyard is the result of restricted immigration from surrounding areas and genetic drift operating through founder effects and periodic bottlenecks. We propose a model that integrates epidemiology and population genetics to explain the variation observed in genetic structure of *E. necator* flag shoot subpopulations from different vineyards or viticultural regions.

Additional keywords: *Oidium tuckeri*, overwintering, population structure, *Vitis vinifera*.

Populations of plant pathogens may vary considerably in genotypic diversity and genetic structure within species depending on their history and location. For example, native populations in centers of diversity may be highly diverse and randomly mating, while founder populations may be less diverse and sometimes clonal (14,18,21). Populations of some plant pathogens can be characterized as metapopulations where local populations evolve independently and periodically experience extinction and recolonization (1,15,17). The diversity and genetic structure of local populations, therefore, may vary depending on population size and age, and the history of colonization.

The grape powdery mildew fungus, *Erysiphe necator* (formerly *Uncinula necator*), is an example of a plant pathogen whose populations vary in genotypic diversity and genetic structure. Populations in Europe vary from having little diversity and an apparently clonal structure to having high diversity, with some showing evidence of recombination (4,5,7,9,19,22,26). The overwintering modes of *E. necator*, either sexually or asexually, are potential determinants of genotypic diversity and population structure. *E. necator* can survive over winter asexually as mycelium, haustoria and conidia in dormant buds of grapevines, and in spring cause severe symptoms on the shoots developing from infected buds; these symptomatic shoots are known as flag shoots (25). *E. necator* can also survive over winter sexually as asco-

spores in cleistothecia, which serve as primary inoculum in the spring (2,3). The two types of overwintering and primary inoculum sources vary geographically. Ascospore inoculum is found in virtually all viticultural regions, whereas flag shoots are not found in some vineyards or in some regions, especially where winter temperatures are very low (4,22,25).

One of the complicating factors for understanding the population structure of *E. necator* is that genetically distinct subgroups have been identified (7,8,10,19,22,26,30), possibly representing independent evolutionary lineages or even cryptic species. In some European populations, two of the subgroups correlate roughly with overwintering mode. This correlation does not hold in all cases, however: some isolates collected from flag shoots had genotypes associated with ascospore overwintering, and some isolates collected later in the season, which were presumed to be derived from ascospores, had genotypes associated with flag shoots (4,7,8,22,26). Some of the early reports on genotypic diversity in *E. necator* suggested that subpopulations sampled from flag shoots, after overwintering asexually, were less diverse than subpopulations sampled later in the season, presumably from ascospores (7,8). This trend was demonstrated for relatively small samples from a few vineyards in France and Germany where genotypic diversity assessed using random amplified polymorphism DNA (RAPD) markers was low and only one mating type was found among flag shoot isolates (7,8). With more extensive sampling, Péros et al. (26) found 10 RAPD haplotypes among 72 flag shoot isolates in two locations in southern France; 49 isolates had the same haplotype and were found in multiple vineyards and in multiple years, demonstrating the persistence of clones from

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flag shoots. In contrast, studies of *E. necator* in Spain and Italy have shown greater genotypic diversity among flag shoot isolates than those in France and Germany. Miazzi et al. (19) found that all flag shoot isolates in southern Italy had unique RAPD haplotypes and mating types were in approximately 1:1 ratios. In Spain, as in southern Italy, Núñez et al. (22) found that all flag shoot isolates had unique amplified fragment length polymorphism (AFLP) haplotypes. Similarly, Cortesi et al. (5) found high genotypic diversity and 1:1 mating type ratios among flag shoot isolates from a single vineyard sampled over 4 years in Tuscany. Almost all flag shoot isolates had unique inter-simple sequence repeat (ISSR) haplotypes. Furthermore, Cortesi et al. (4) concluded that the flag shoot subpopulation was practically indistinguishable from an ascospore-derived population in diversity and structure. The genetic structure of *E. necator* populations sampled from flag shoots, therefore, varies considerably depending on geographic location.

In this study, we report an example of a clonal flag shoot subpopulation of *E. necator* from an isolated vineyard in the Po Valley in the Lombardy region in northern Italy. Our objectives were to estimate the extent of genotypic variation in a flag shoot subpopulation in a single vineyard over 4 years, and to test whether we would find the same multilocus haplotypes repeatedly in time and space. We found a markedly different structure than in a previous study of a flag shoot subpopulation in central Italy (5): this isolated subpopulation has almost no genotypic diversity and is composed of a single clone that persisted for at least 4 years.

MATERIALS AND METHODS

Study site. The site for this study was an isolated vineyard at an agricultural technical school in the city of Voghera, about 50 km south of Milan in the Province of Pavia. The vineyard was approximately 1.5 ha in size and was used for training viticulture students. The nearest vineyard to this study site was approximately 10 km away in the viticultural area of Oltrepo Pavese; the nearest grapevines in home gardens were more than 500 m away. The vineyard was planted in 1925 with *Vitis vinifera* cvs. Croatina and Barbera. Like all varieties of *V. vinifera*, these are susceptible to powdery mildew. Distances between rows and between vines were 2.5 and 1.2 m, respectively, with a total of 620 vines surveyed each year of the study. We chose this site to sample *E. necator* because of a history of finding flag shoots regularly for several years. This vineyard was managed organically and therefore control of powdery mildew relied on applications of sulfur.

Sampling *E. necator*. We sampled *E. necator* isolates from flag shoots using the same approach and methods as we did in Tuscany (5). Each year for 4 years (2001 to 2004) we searched

the entire vineyard carefully for flag shoots biweekly from budbreak until mid-June. All vines were checked visually each time and the location of each flag shoot was recorded. We attempted to isolate *E. necator* from every flag shoot by inoculating grape seedlings grown in individual plastic containers to prevent cross-contamination of isolates. When successful in isolating *E. necator*, we purified each culture by transferring one single chain of conidia to a new seedling. Isolates were maintained on seedlings for genotyping as described previously (5).

Genotyping. Each isolate was genotyped using methods described in our previous studies (4,5). To determine mating type, we paired each isolate on grape seedlings with mating-type testers from Tuscany to determine whether they would form cleistothecia. *E. necator* is heterothallic and typically forms cleistothecia only with testers of one mating type or the other (13,20). Other markers were obtained from polymerase chain reaction (PCR)-based methods. Conidia and mycelium were harvested from mildew colonies cultured for 10 to 15 days on grape leaves using a cyclone separator and DNA was prepared as described previously (11). We used four PCR-based markers described by Stummer et al. (30). PCR was conducted using microsatellite primers (CAC)₅ and (GACA)₄, the primers for the core sequence of M13 minisatellite DNA (29) and the intron-splice junction primer, R1 (31); for convenience, we refer to these four markers collectively as inter-simple sequence repeats (ISSRs). The repeatability of these markers was demonstrated previously by showing that the same ISSR haplotypes were obtained from DNA extracted from isolates cultured at different times (5). In this study, we used the same isolate on each gel as a positive control, and we used water instead of template DNA in PCR reactions as negative controls. Only bands that were repeatable between replicate genotyping were scored.

We also genotyped all isolates using two markers that were designed to identify genetic subgroups within *E. necator*. One marker is based on a single-nucleotide polymorphism in the *CYP51* gene (10); the other is an anonymous sequence-characterized amplified region (SCAR) developed from an RAPD marker (16). Detailed methods for using these markers were described previously (4), and reference isolates from Tuscany were used for comparison.

Spatial autocorrelation analyses. We used spatial autocorrelation analyses as described by Cortesi et al. (5) to test for aggregation of flag shoots within and between years. Within each year we tested the null hypothesis that the mean distance between flag shoots was not different from the mean distance when flag shoots were assigned to vines at random. We also tested for spatial autocorrelation between years based on the null hypothesis that the mean distance between flag shoots in one year and flag

TABLE 1. Incidence and spatial autocorrelation of vines with flag shoots caused by *Erysiphe necator* in an isolated vineyard in Voghera (province of Pavia, Italy)

Year	No. flag shoots	No. vines with flag shoots ^a	Spatial autocorrelation within years ^b			Spatial autocorrelation between years ^c			No. vines with flag shoots in current and previous years	Binomial probability ^d
			Obs. distance	Random distance	P	Obs. distance	Random distance	P		
2001	20	20	22.9	24.9	0.171	— ^e	—	—	—	—
2002	26	21	24.7	24.8	0.486	22.9	26.1	0.027	8	0.0063
2003	24	6	16.8	24.8	0.064	21.1	26.1	0.020	4	0.0004
2004	3	2	42.2	24.5	0.856	22.7	21.2	0.627	1	0.0192
All	73	36	22.6	24.9	0.086	—	—	—	—	—

^a Total number of vines examined each year is 620.

^b Spatial autocorrelation within years tests the null hypothesis that the mean distance between vines with flag shoots was not different from the mean distance when flag shoots are assigned to vines at random; P values are based on 1,000 randomizations (5).

^c Spatial autocorrelation between years tests the null hypothesis that the mean distance between vines with flag shoot in 1 year and vines with flag shoots in the previous year was not different from the mean distance when flag shoots are assigned to vines at random; P values are based on 1,000 randomizations (5).

^d Probability of sampling the observed number of vines with flag shoots in two consecutive years if flag shoots are independent between years = $[N!/(N-x)!] p^x(1-p)^{N-x}$, where N is the number of flag shoots in season j, x is the number of vines with flag shoots in both season j and in the previous season, j-1, and p is the probability of that a flag shoot in season j occurs on a vine that had a flag shoot in season j-1. P was estimated as the proportion of vines with flag shoots in the previous season j-1, e.g., for 2002, p = 20/620.

^e Analysis is not applicable.

shoots in the previous year was not different from the mean distance when flag shoots were assigned to vines at random. *P* values were estimated from 1,000 randomizations (Cortesi et al. [5] provides details on significance testing).

RESULTS

Flag shoot survey and sampling. In total, we observed 73 flag shoots over the 4 years of this study. Nine vines had more than one flag shoot in a single year, including one vine with 10 flag

Fig. 1. Map of an isolated vineyard in Voghera, Italy showing locations of grape powdery mildew flag shoot symptoms over 4 years (2001 to 2004). Number of years when flag shoots were found is shown for each vine; dots indicate no flag shoots found during the 4 years.

shoots; seven vines had flag shoots for two consecutive years, and three vines had flag shoots for three consecutive years. The binomial probabilities of finding flag shoots on the same vine in consecutive years if they occurred independently between years are very low for all years (Table 1). In total, 36 out of 620 vines had flag shoots in 4 years (Fig. 1), from which we obtained 54 isolates of *E. necator*.

Genotyping. We found little genetic variation among isolates for the three types of markers (mating type, ISSRs, and single locus markers). All isolates had the same mating type, *MAT*-, as determined by mating type testers from Tuscany (4). There was very little variation in ISSR haplotypes among the 54 isolates. ISSR markers were polymorphic at only 2 of the 64 loci scored (Fig. 2); only three multilocus haplotypes were found. The most common haplotype comprised 46 of the 54 isolates and was found in all 4 years. The other two haplotypes differed from the dominant haplotype at one locus each. One of these variant haplotypes was found in 7 isolates, on 5 vines, sampled in 3 of the 4 years; the other was found in a single isolate in 2003.

No polymorphism was found among the 54 isolates for *CYP51* and *SCAR* markers, both of which putatively identify genetic subgroups of *E. necator*. Alleles at these loci were the same as those for group I (also called group A by some authors), which were previously associated with flag shoot populations (10,16).

Spatial autocorrelation analyses. We observed no significant spatial autocorrelation of vines with flag shoots in any of the 4 years, or when all 36 vines with flag shoots were analyzed together (Table 1). The mean distance between vines with flag shoots in 2003 was approximately 8 m less than the mean distance when flag shoots were randomly assigned to vines (*P* = 0.064), although the sample size for this year was small (*N* = 6). In contrast, there was significant spatial autocorrelation between years when comparing 2002 to 2001, and comparing 2003 to 2002 (Table 1). Spatial autocorrelation between years was not caused solely by the occurrence of flag shoots on the same vines in consecutive years. When analyses were repeated after removing vines with flag shoots in consecutive years, we still found significant autocorrelation (data not shown).

Fig. 2. Inter-simple sequence repeats genotyping of *Erysiphe necator* from an isolated vineyard in Voghera, Italy, using polymerase chain reaction primers A, (CAC)₅ and B, (GACA)₄ visualized in 1.5% agarose gels stained with ethidium bromide. Two polymorphic bands shown for (CAC)₅ in A are marked with arrows. Lanes with size markers are labeled “M” (top band 1,650 bp); lanes 14 and 13 in A and B, respectively, are negative controls.

DISCUSSION

The subpopulation of *E. necator* sampled from flag shoots in an isolated vineyard in northern Italy had almost no genetic variation for mating type, ISSR markers or for *CYP51* and SCAR markers. The little variation we did find is consistent with mutation in a clonal population comprising a single lineage; no evidence of recombination was found. These results contrast markedly with our previous study of a flag shoot subpopulation in a large viticultural area in Tuscany where mating types were in 1:1 ratios and almost every isolate had a unique ISSR haplotype (4,5). In the present study, we found only three ISSR haplotypes among 54 isolates, compared with 77 haplotypes among 79 isolates from Tuscany. We conclude, therefore, that the subpopulation of *E. necator* sampled from flag shoots in Voghera comprises a single clonal lineage, with minor variation caused by mutation at two loci.

The *CYP51* and SCAR markers developed to identify genetic subgroups or lineages in *E. necator* (10,16) were monomorphic in the flag shoot subpopulation in this study. In some populations, groups I and III identified by these markers correlated to isolates sampled from flag shoots and those sampled later in the season, respectively, although this correlation was not perfect (7,8,19,30). In this study, we found only the alleles correlated with group I, typically associated with flag shoots, whereas in Tuscany we previously found alleles characteristic of both groups among flag shoot isolates (4).

Although the structure and diversity of *E. necator* flag shoot populations varies markedly along a continuum from clonal, such as in Voghera (present study) or southern France (26) at one extreme, to highly diverse, as in Italy and Spain (5,19,22), the causes of such variation have not yet been explained. Here we present a model that integrates the epidemiology and population genetics of flag shoot subpopulations in an attempt to explain the variation observed among different regions.

Flag shoots are the most likely source of inoculum for bud infection and the subsequent development of flag shoots the following year (5,32). This assertion is based on the following: (i) axillary buds are most susceptible on young shoots when 3 to 6 unfolded leaves are present (24,27,28); therefore, bud infection must occur during a short window of time early in the growing season; (ii) flag shoots produce copious amounts of inoculum at the time buds are susceptible to infection; and (iii) inoculum from flag shoots typically disperses short distances, giving rise to disease foci around flag shoots and spatial autocorrelation of flag shoots between years (5,32). We cannot completely rule out ascospore and/or immigrant sources of inoculum for bud infection, but in most years their abundance is much lower than inoculum from flag shoots at the time when buds are susceptible (3,4), making the probability of infection from these other sources relatively small.

Given the predominance of flag shoot inoculum for bud infection, a flag shoot subpopulation has the potential to persist asexually, with a relatively low genotypic diversity. However, ascospore and/or immigrant sources of inoculum may be important in some years, depending on environmental conditions for early ascospore release (3,4,12,23) and infection when buds are still susceptible to infection. Ascospore or immigrant sources of inoculum would increase genotypic diversity compared to asexual flag shoot inoculum (conidia) alone. Our previous findings from a flag shoot subpopulation in Tuscany (5) of high genotypic diversity, 1:1 mating type ratios and evidence for recombination are consistent with ascospores or immigrants being important inoculum sources, at least in some years.

The effects of genetic drift, exacerbated by bottlenecks and founder effects, can reconcile the findings of both high and low diversity in flag shoot subpopulations. The number of flag shoots in most commercial vineyards in Tuscany, for example, is

typically small because of pruning and the early application of fungicides to inhibit bud infection (6). Furthermore, the number of flag shoots can vary markedly between years in the same vineyard and sometimes flag shoots are extremely rare or not found at all (27,32), even when fungicides are not used (5). For example, we observed 20 to 26 flag shoots in 2001 to 2003 in this study, but only three in 2004 (Table 1). In more extreme cases, Rügner et al. (27) found no flag shoots in the same vineyard where their coworkers had found up to 78 in previous years; and Cortesi et al. (5) found 114 flag shoots in one year and only two in another year in the same vineyard. The genetic consequence of such bottlenecks is a marked reduction in genetic diversity. Complete population crashes might result in the local extinction of *E. necator* from dormant buds, in which case recolonization from other sources (ascospore and/or immigrant) is necessary to restore the flag shoot subpopulation. Here again, drift is likely to influence diversity because of the potential for founder effects if only a few haplotypes are responsible for recolonization. Therefore, the population dynamics of *E. necator* in infected buds potentially has a large effect on genetic variation because of genetic drift.

Finally, the variation observed among flag shoot subpopulations in different vineyards or regions depends on the size of the population and/or how much time has elapsed since the last bottleneck. Flag shoot subpopulations with one or a few clones are likely to be associated with recent bottlenecks and small populations where genetic drift has had the most effect. In contrast, more genetically diverse subpopulations are likely to be associated with early-season ascospore infection of buds, combined with large populations that have not recently experienced severe bottlenecks. This latter explanation may apply to the high diversity found in a flag shoot subpopulation in Tuscany (5): ascospore inoculum is present and conditions for infection occur soon after bud break in some years (2). Unfortunately, Cortesi et al. (5) did not genotype *E. necator* flag shoot isolates in Tuscany until 1997, 3 years after a bottleneck in 1994, nor did they sample in 2002, 1 year after the population crashed in 2001. Therefore, it is not possible to test this model from existing data in Tuscany or any other existing study of flag shoot subpopulations. A comprehensive test of this model would require monitoring vineyards for ascospore release, conditions for bud infection, and flag shoots in several regions, under diverse environmental conditions, over multiple years, in conjunction with genotyping flag shoot isolates to test whether genetic diversity decreases with population crashes (bottlenecks) and subsequently increases over time after the population is reestablished.

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