OUTCROSSING AND DIVERSITY OF VEGETATIVE COMPATIBILITY TYPES IN POPULATIONS OF *EUTYPA LATA* FROM GRAPEVINES

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SUMMARY

The sources of inoculum of Eutypa dieback of grapevines, caused by *Eutypa lata*, are not fully understood. Ascospores are thought to be the main source, but in populations with low disease incidence conidia may be important. Earlier studies of this disease in Italy and Germany did not report sexual structures (perithecia) of *E. lata* on grapevines, leaving open the possibility that populations of this fungus are clonal in some areas. However, we found perithecia in 24% and 43% of symptomatic vines in vineyards in Italy and Germany, respectively. Spatial patterns of symptomatic vines in the German vineyard were significantly aggregated but the aggregation was not strong, as might be expected if the fungus dispersed by conidia or mycelium on pruning tools. In contrast, vines with stromata and perithecia were not significantly aggregated in space. Vegetative compatibility (vc) types segregated among ascospore progeny in all perithecia sampled from both vineyards, indicating that this fungus consistently outcrosses in these populations. Finally, we found a high diversity of vc types in both vineyards, where isolates from different vines (with one exception) had unique vc types. These results reject the hypothesis that clonal reproduction of *E. lata* is epidemiologically significant, and strongly suggests that ascospores of *E. lata* are an important source of inoculum. Furthermore, *E. lata*'s mating system is characterized by outcrossing in nature.

Key words: Eutypa dieback, epidemiology, fungal mating system, reproductive biology, *Vitis vinifera*.

INTRODUCTION

The reproductive biology and mating system of fungal plant pathogens in field populations are biologically important for two very different reasons (Milgroom, 1996). First, the epidemiological characteristics of inoculum produced by sexual or asexual reproduction can be markedly different with respect to dispersal, survival and/or abundance. Second, recombination during sexual reproduction is likely to result in greater genotypic diversity, potentially affecting variation in ecologically important traits such as pathotypes, aggressiveness or resistance to fungicides. Because of the importance of reproductive biology of pathogens many plant pathologists have addressed questions about sexual reproduction (or the lack of it) in a variety of plant pathogens (Anderson and Kohn, 1995; Milgroom, 1996; Brown, 1999). The most direct way to understand the reproductive biology of a pathogen is to study its biology in nature. A complementary approach is to describe the multilocus genetic structure of populations in order to make inferences about reproductive biology and mating systems (Milgroom, 1996; Brown, 1999). In this research, we combine studies of the biology, epidemiology and population structure to understand the reproductive biology of *Eutypa lata* (Pers.: Fr.) Tul. and C. Tul., the ascomycete that causes Eutypa dieback of grapevines.

The epidemiology of Eutypa dieback is not fully understood. The pathogen is known to infect the host through wounds, usually after pruning. Freshly made pruning wounds, more than one year old, exposing large surfaces of wood, are the most susceptible infection courts (Moller and Kasimatis, 1980; Petzoldt *et al.*, 1981; Chapuis *et al.*, 1998). Ascospores are implicated as the most important source of inoculum for this disease; in fact, germination of conidia did not occur (Moller and Kasimatis, 1978) or was observed rarely (Belarbi and Mur, 1983; Ju *et al.*, 1991), and the disease is more serious and may spread more quickly when perithecia are present in vineyards, compared to slower disease progress in the absence of perithecia (Munkvold *et al.*, 1993; Hughes *et al.*, 1998). Also, disease symptoms observed in vineyards in California appear to be spatially random, probably because of an influx of airborne ascospore inoculum (Munkvold *et al.*, 1993). In contrast, if conidia or mycelium, which are splash dispersed (Ju *et al.*, 1991) or spread by pruning tools, re-

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spectively, were significant sources of inoculum, spatial patterns would be expected to be highly aggregated because of short distance dispersal (Munkvold *et al.*, 1993).

Previous research on the population genetics of *E. lata* showed that population structures of *E. lata* in vineyards in France were consistent with expectations of random mating (Péros *et al.*, 1997; Péros and Larignon, 1998). These studies are in agreement with epidemiological data, concluding that ascospores were the most likely source of primary inoculum (Péros *et al.*, 1997). Inferences about random mating were made from analysis of gametic disequilibrium of RAPD markers, and from the high diversity of vegetative compatibility (vc) types. Although the simplest explanation for these data is that *E. lata* is reproducing sexually, since perithecia were observed in these populations, a thorough study of the mating system of *E. lata* has still not been done. Péros and Berger (1999) found diverse RAPD genotypes among ascospores, but could not show conclusively that selfing did not occur because they pooled ascospores from multiple perithecia in the same stromata. Furthermore, as pointed out by these authors (Péros *et al.*, 1997; Péros and Larignon, 1998; Péros and Berger, 1999), other populations of *E. lata* may behave differently, with some populations reproducing sexually while others reproduce clonally, as found in *Cryphonectria parasitica* (Liu *et al.*, 1996; Milgroom and Cortesi, 1999). This last point could be particularly important for populations of *E. lata* in Italy and Germany, because, in contrast to France (Dubos, 1994) or California (Munkvold *et al.*, 1993), most vineyards are characterized by low disease incidence and slow epidemic development (Minervini *et al.*, 1996; H.H. Kassemeyer, personal communication). Perithecia of *E. lata* have been reported to occur on grapevines in several countries (Carter, 1991). To our knowledge, however, there are no published reports of perithecia for this fungus on grapevines in Italy or Germany. If perithecia are not present, then the epidemiology of this disease could be quite different.

Our overall objective was to study the reproductive biology of *E. lata* in two vineyards, one in Italy and one in Germany. Our specific objectives were: (*i*) to estimate the incidence of stromata and perithecia of *E. lata*; (*ii*) to analyze spatial patterns of dieback symptoms, stromata and perithecia in one vineyard to make inferences about types of inoculum; (*iii*) to describe the mating system of the fungus in nature by determining if perithecia result from outcrossing or selfing; and (*iv*) to estimate the genotypic diversity of *E. lata* populations, based on vegetative compatibility types. A preliminary report of this work was published recently (Cortesi *et al.*, 2000).

MATERIALS AND METHODS

Study areas. We studied the reproductive biology of *E. lata* in two vineyards of *Vitis vinifera* which were known to have high disease incidence (Minervini *et al.*, 1996; H.H. Kassemeyer, personal communication). These vineyards are not necessarily representative of these two countries because of high disease incidence, however, a large number of vines from the same vineyard is needed to find clones of *E. lata* if conidia are a source of inoculum. One vineyard, located in San Benedetto di Lugana (Verona) in Italy, had 31 year-old vines of cv. 'Trebbiano' grafted on Kober5BB rootstock, and were capovolto-trained. The other vineyard, located in Dottingen near Freiburg, Germany, had 26 year-old vines of cv. 'Chasselas' grafted on '125AA' rootstock, and were Guyot-trained. We collected 50 vines showing typical foliar symptoms (Carter, 1988) from San Benedetto in September 1996. In November 1996 we examined 984 vines in Dottingen for the presence of severe dwarfing of the internodes of the shoots or dieback symptoms typical of this disease (Carter, 1988). The location of each symptomatic vine was recorded and a total of 93 vines were collected for observation in the laboratory (Fig. 1).

Incidence of stromata and perithecia of *E. lata*. Vines collected from San Benedetto and Dottingen were stripped of bark and examined under a dissecting microscope for the presence of stromata and perithecia. Identification as *E. lata* was based on stromatal morphology (Carter, 1988); ascospore morphology (Carter and Talbot, 1974) was also observed when perithecia were sampled to determine whether they were the result of outcrossing or selfing (see below).

Spatial pattern analyses. Spatial patterns of symptoms in the Dottingen vineyard were tested for nonrandom aggregations using matrix comparison analyses (Milgroom *et al.*, 1991; Cortesi *et al.*, 2000). Vines were spaced 1.4 m apart in 12 rows, with an average of 82 vines per row. To determine whether symptomatic vines were significantly aggregated within vineyards, we tested the null hypothesis that the average distance between vines with symptoms was not different from the average distance between vines when locations were assigned at random. Significance testing was done by randomization (Milgroom *et al.*, 1991). The same number of symptomatic vines was randomly reassigned to loca-

tions within each vineyard. The average distance between symptomatic vines was calculated for each randomization to generate a distribution of average distances under the null hypothesis of no spatial aggregation. Randomization was conducted 1000 times for each test. The observed average distance was compared to the null distribution to determine the proportion of the null distribution with values less than the observed distance. This proportion was used as an estimate of the *P*-value for significance testing. This same analysis was also conducted for vines with stromata and vines with perithecia.

Isolation of *E. lata* from wood and perithecia. *E. lata* was isolated from mycelium in diseased wood and from stromata underneath the exfoliating bark, taking care to avoid perithecia in the stromata. The trunk of each vine was cut in three section and surface sterilized by submersion in 90% ethanol for 30 sec, followed by flaming. Isolations from wood were made by removing a thin slice of the outer wood from the upper horizontal surface with a sterile scalpel; wood chips (approx. 10 x 5 x 1 mm) were sliced along the margin of the brown necrotic wood sectors between healthy and diseased wood. Twelve chips, six for each of two 9 cm diameter Petri plates, were placed onto 2% malt extract agar (Difco Co., Detroit, MI) amended with 50 mg l^{-1} rose bengal and 50 mg l^{-1} tetracycline. Isolations were also done on 3.6% potato dextrose agar (Difco Co., Detroit, MI) amended with 50 mg l^{-1} tetracycline and 4 mg l^{-1} captan (PDAtc). Wood chips were incubated at room temperature (approx 22°C) for 3-4 days before mycelia with morphology resembling *E. lata* were subcultured onto 2% water agar (Difco Co., Detroit, MI). After 2-3 days of incubation single hyphal tips were transferred to PDA. Isolates were grown on PDA at 22°C, 12 hours photoperiod, for approx 4 weeks, until they produced cirri. Isolates from wood were classified as *E. lata* based on mycelial and conidial morphology (Carter and Talbot, 1974), and were stored in PDAtc slants at 5°C in the culture collection at the University of Milan.

Ascospores were sampled from perithecia to determine whether each perithecium was the result of outcrossing or selfing. Single perithecia were dissected from stromata with sterile scalpel and forceps, after which they were pierced with a sterile needle. A droplet of ascospores was collected on the needle and rinsed off by dipping it into 0.5 ml sterile water in microcentrifuge tubes. The ascospore suspension was then spread onto 2% water agar and allowed to germinate for 2 days at 22°C. Twenty to 30 single germinated ascospore progeny from each perithecium were transferred onto PDAtc. Mycelium surrounding several sampled perithecia were transferred onto PDAtc and grown for 2-3 days in order to obtain single hyphal-tip isolates, as described above; these isolates were considered maternal isolates of the ascospore progeny from their respective perithecia. Ascospore progeny from San Benedetto and from Dottingen were labeled MD and FRI, respectively, followed by the number of the vine on which the stroma was found and by M for the maternal isolates.

Vegetative compatibility (vc) tests. In most ascomycetes, vegetative incompatibility is controlled by multiple vegetative (or heterokaryon) incompatibility (*vic*) loci such that any two isolates are compatible only if they have the same alleles in all *vic* loci (Leslie, 1993; Cortesi and Milgroom, 1998). Testing for vegetative incompatibility in *E. lata* was done on both mycelial isolates and ascospore progeny using similar techniques as those used for *C. parasitica* (Cortesi *et al.*, 1996). Tests were done by pairing isolates on Czapeck agar (3.5% w/v Czapeck-dox broth and 1.5% w/v Bacto agar, Difco Laboratories, Detroit, MI) amended with 50 mg l^{-1} bromophenol blue, which was added to enhance the visibility of the incompatible reaction, as done with *C. parasitica* (Powell, 1995). Six pairs of isolates were tested per 9-cm-diameter Petri plate as described for *C. parasitica* (Cortesi *et al.*, 1996). Each isolate was paired with itself as a control for compatibility. Paired isolates were incubated in the dark for 2 weeks at 24°C. Incompatibility was determined by a barrage between colonies of paired isolates and/or by a dark brown line between colonies visible from the bottom of the plate.

Vegetative compatibility tests were carried out on ascospore isolates from within the same perithecium, and on all field isolates. Twelve ascospore progeny (with few exceptions) from each perithecium were randomly selected and paired in all combinations against each other within the same perithecium. Progeny from a perithecium were determined to be the result of outcrossing if any of the progeny were vegetatively incompatible with sibling ascospores (Milgroom *et al.*, 1993). For a sample of 11 perithecia from 6 stromata, we also paired 19-30 ascospore progeny with their respective maternal isolates.

Mycelial field isolates were tested for vegetative incompatibility in two stages. First, all isolates from the same vines were paired in all combinations to determine the diversity within vines. Second, one isolate from each vc type within each vine was paired in all combinations with all other vc types from all other vines, both within and between vineyards.

RESULTS

Incidence of stromata and perithecia of *E. lata.* In San Benedetto, 12 of the 50 vines had stromata of *E. lata*; all 12 stromata contained perithecia. Similarly, 46 of the 93 vines examined from Dottingen had stromata of *E. lata*, however, only 40 of these had perithecia. The estimated incidences of stromata bearing perithecia on symptomatic vines in these two vineyards were 24% and 43%, respectively.

Spatial pattern analyses. Vines showing symptoms of Eutypa dieback were significantly aggregated in the vineyard in Dottingen (Table 1, Fig. 1). On average, symptomatic vines were closer together than expected if symptoms were assigned to vines at random in the vineyard. However, aggregations were not strong, as the mean distance between symptomatic vines was < 10% smaller than the distance expected under the null hypothesis of randomness (Table 1). Symptomatic vines with stromata were not significantly aggregated (P = 0.17), even though the mean distances were comparable to the distance between symptomatic vines; vines with perithecia were marginally aggregated (P = 0.052) (Table 1). This difference in significance is most likely because of lower statistical power from smaller numbers of vines analyzed with stromata and perithecia than with symptoms.

Tests for outcrossing or selfing. Vegetative compatibility types segregated among ascospore progeny in all perithecia examined (N = 7 in San Benedetto, N = 20 in Dottingen, Table 2). Almost every ascospore progeny tested was vegetatively incompatible with every other ascospore progeny from the same perithecium. In addition, when ascospore progeny were tested against maternal isolates in a subset of 11 perithecia, few were compatible. These results demonstrate that outcrossing occurred in natural populations and that the diversity of vc types that results from outcrossing is high.

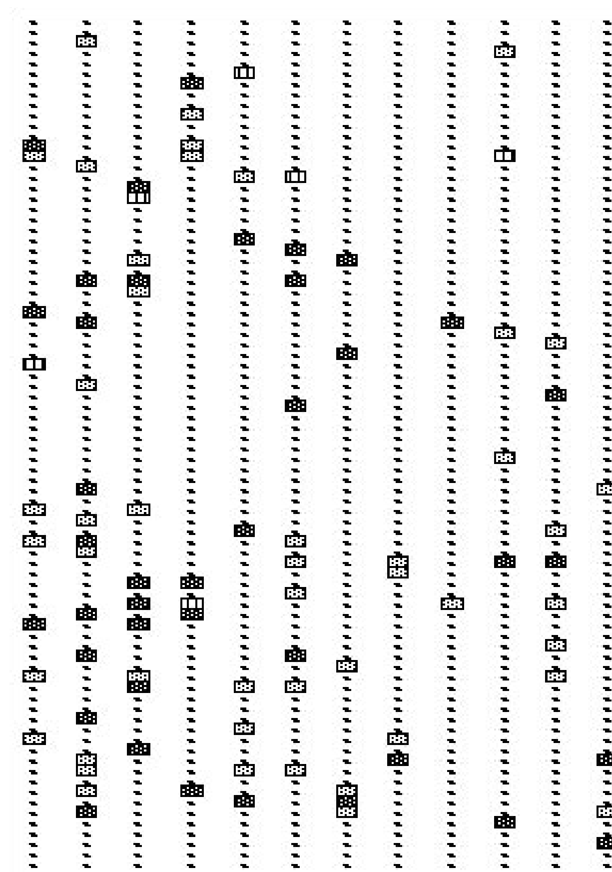

Fig. 1. Spatial locations of grapevines showing symptoms of Eutypa dieback in a vineyard in Dottingen, Germany. The locations of vines with symptoms of Eutypa dieback (gray boxes), vines with stromata and perithecia of *E. lata* (black boxes), and vines with stromata but without perithecia (ruled boxes) are indicated.

Diversity of vc types among field isolates. We obtained a total of 52 isolates from 32 vines in San Benedetto and 96 isolates from 48 vines in Dottingen. Two or more isolates were obtained from 14 and 29 vines in San Benedetto and Dottingen, respectively.

Table 1. Spatial analyses of vines with symptoms, stromata and perithecia of *E. lata* in Dottingen, Germany.

	N	Observed mean distance	Expected mean distance	P
Symptomatic vines	93	36.9	39.8	0.041
Vines with stromata	46	37.2	39.7	0.170
Vines with stromata containing perithecia	40	35.2	39.7	0.052

More isolates were obtained from wood than from stromata: 49 and 86 isolates were obtained from wood, while 3 and 10 were obtained from stromata in San Benedetto and Dottingen, respectively.

The diversity of vc types was high in both vineyards. We found a total of 34 vc types among 32 vines in San Benedetto, and 59 vc types among 48 vines in Dottingen. Multiple isolates collected from the same vine were more often of the same vc type than of different types (Fig. 2). However, more than one vc type was found in 3 and 10 vines in San Benedetto and Dottingen, respectively, indicating that multiple infections could be detected on these vines. Isolates from different vines had different vc types, with the exception of one pair of isolates in San Benedetto. No isolate from San Benedetto was compatible with any isolate from Dottingen.

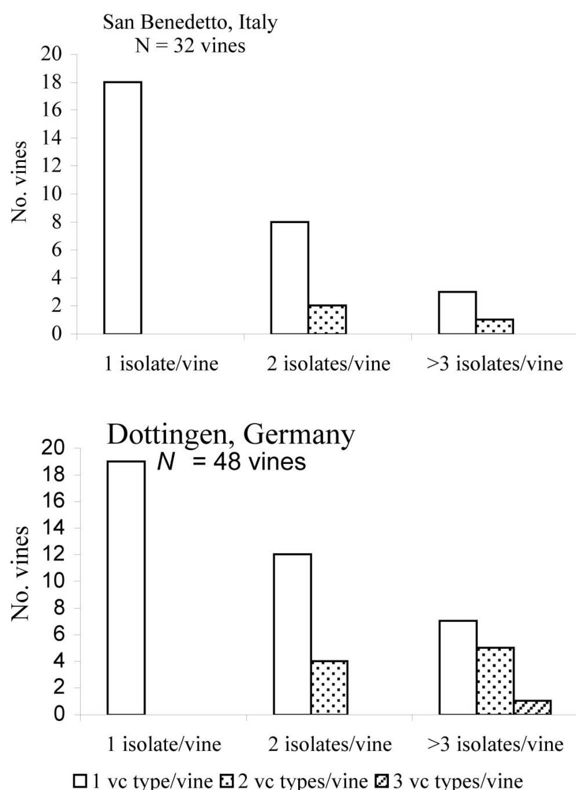

Fig. 2. Distribution of the number of vc types of *E. lata* found per vine as a function of the number of isolates obtained.

DISCUSSION

We found perithecia of *E. lata* on grapevines with dieback symptoms in Italy and Germany at fairly high incidences (24% and 43%, respectively). Although perithecia have been found in most places where Eutypa dieback occurs (Carter, 1991), they were not observed in the first report of this disease in Germany (Kassemeyer, 1987), or in the first report of this disease on grapevines in Italy in 1983 (Bisiach and Minervini, 1985). The lack of observation of perithecia initially was probably due to the limited number of vines showing symptoms or, alternatively, to the long period of time thought to be necessary for stromata to form (Carter, 1991). However, it is clear that stromata and perithecia of *E. lata* are present in Italy and Germany, and that this fungus may be reproducing sexually. Our finding of stromata and perithecia on living vines contrasts to previous reports of the necessity for dead vines for their development (Carter, 1991). Since the length of time it takes for *E. lata* to reproduce sexually is not known, a cautious approach to disease management might be to eliminate symptomatic vines to reduce the quantity of inoculum within the vineyard. The presence of perithecia and the lack of strong spatial aggregation of diseased vines are consistent with the hypothesis that this fungus reproduces by airborne ascospores in these two vineyards in Italy and Germany. These results are in agreement with previous studies showing slight aggregations of symptomatic vines when perithecia are present (Munkvold *et al.*, 1993). The hypothesis that *E. lata* reproduces clonally (asexually) in these vineyards can be rejected outright. All but two isolates in one vineyard had unique vc types when compared between vines. The high diversity of vc types found in San Benedetto and Dottingen is similar to that found in one vineyard in France (Péros *et al.*, 1997). If this pathogen disperses among vines asexually by conidia or mycelium spread by pruning tools, we would expect to see some evidence for clonality, *e.g.*, as found with pathogens like *Sclerotinia sclerotiorum* (Kohn, 1995). Therefore, our results do not support speculations on the role of conidia as a source of inoculum (Belarbi and Mur, 1983; Ju *et al.*, 1991; Munkvold *et al.*, 1993). Our findings also fail to support Munkvold's hypothesis (Munkvold *et al.*, 1993) that low disease incidence at the beginning of epidemics is caused in some vineyards by the dispersal of conidia, otherwise we would have expected to find the same vc type on different vines.

More than half of vines from which we obtained more than one isolate had only one vc type. The presence of only one vc type is probably the result of one fungal individual infecting and colonizing each vine. Conversely, the presence of multiple vc types from the same vines indicates that infection and colonization occurred more than once in these vines. The same vc type occurred on more than one vine only once in this study. This could have occurred by clonal spread, how-

ever, the same vc type can be produced from the same mating (Table 2), or arise independently from other recombination events. For example, in *C. parasitica*, vc types were shown to be unrelated to DNA fingerprint markers in some populations (Liu *et al.*, 1996), indicating independent origins of individuals of the same vc type.

The number of vc types observed can only be explained by a high degree of polymorphism at *vic* loci within these populations. For example, if vc type is controlled by multiple, independent *vic* loci and each locus has only two alleles, as in other ascomycetes (Leslie, 1993; Cortesi and Milgroom, 1998), then the maximum number of vc types is 2^k, where k is the number of polymorphic *vic* loci. In the two vineyards we studied, *E. lata* must have at least six polymorphic *vic* loci because we observed > 32 vc types in each. At least one additional *vic* locus must be polymorphic when both populations are considered together because we found more than 64 vc types in total. The actual number of polymorphic *vic* loci is probably underestimated because these are minimum estimates based on a relatively small number of isolates. Furthermore, the number of vc types observed among progeny from each perithecium

Table 2. Number of vegetative compatibility (vc) types segregating in ascospore progeny from each perithecium of *E. lata* in San Benedetto, Italy, and in Dottingen, Germany.

Vineyard	Perithecium	*N*[1]	no. of vc types	no. progeny compatible with maternal isolate
San Benedetto				
	MD 29	12	11	–[2]
	MD 30 a[3]	12	12	1/29[4]
	MD 30 b	12	12	0/23
	MD 34	12	10	0/28
	MD 40	12	11	–
	MD 42	12	12	–
	MD 47	9	8	–
Dottingen				
	FRI 1 a	12	12	1/29
	FRI 1 b	12	12	0/19
	FRI 10	12	11	–
	FRI 17 a	12	8	0/29
	FRI 17 b	12	9	3/21
	FRI 23 a	12	9	0/30
	FRI 23 b	12	9	0/30
	FRI 33	12	11	–
	FRI 38 a	12	12	–
	FRI 38 b	12	11	–
	FRI 38 c	12	12	–
	FRI 39	12	11	–
	FRI 45	7	7	–
	FRI 57	7	7	–
	FRI 60	12	12	–
	FRI 62	12	11	–
	FRI 64	12	11	–
	FRI 69	12	12	–
	FRI 74 a	12	9	0/28
	FRI 74 b	12	11	2/25

[1] Number of ascospores tested per perithecium.
[2] No maternal isolate was tested.
[3] 'a' and 'b' indicate perithecia sampled from the same stroma.
[4] Number of ascospores compatible with maternal isolates/number of ascospore progeny tested.

(Table 2) indicates segregation at four or more *vic* loci (>8 vc types per perithecium) for most perithecia. The fact that we observed segregation at four or more *vic* loci in almost every perithecium probably indicates that more than six loci are polymorphic within each population. This degree of polymorphism at *vic* loci is adequate to detect outcrossing so that RAPDs or other molecular markers are not needed for determining the mating system of *E. lata* in nature. Furthermore, vc testing is simple and inexpensive relative to molecular markers and can be used to address questions in population biology (Leslie, 1993).

Evidence for sexual reproduction based on vc type diversity and spatial patterns are indirect ways to infer the reproductive biology of *E. lata*. Looking for sexual structures in these vineyards was an important step in studying *E. lata*'s biology (Milgroom, 1996), but it is even more relevant to determine its mating system directly, *i.e.*, whether *E. lata* perithecia are the result of outcrossing or selfing. All perithecia sampled in this study were the result of outcrossing (Table 2). Results of a previous study in France (Péros and Berger, 1999) agree with ours, but their study employed inadequate sampling (pooled samples and small sample sizes) so that reliable inferences could not be made. Few other studies have attempted to determine the mating system of ascomycetes in nature (Ennos and Swales, 1987; Milgroom *et al.*, 1993; Kohn, 1995), and yet mating systems need to be studied in nature because they may behave quite differently than in laboratory (Marra and Milgroom, 2001). The high diversity of vc types observed in ascospore progeny within perithecia corresponds to the high diversity in isolates obtained from vines. Therefore, all evidence supports the hypothesis that this fungus is sexually reproducing by outcrossing in these vineyards.

ACKNOWLEDGEMENTS

We would like to thank Dr. Hanns-Heinz Kassemeyer for his help in collecting diseased vines in Dottingen, Sabrina Lazzati for helping with vc testing, and Giacinta Minervini and Marco Bisiach for helpful discussions and suggestions. The research was supported, in part, by National Research Council of Italy (CNR), grant GENBIOFU no. 9903253.CT06.

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Received 31 January 2000
Accepted 9 February 2001

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