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# Genetic structure of the fungal grapevine pathogen *Eutypa lata* from four continents

R. Travadon[a], K. Baumgartner[b]*, P. E. Rolshausen[c], W. D. Gubler[a], M. R. Sosnowski[d], P. Lecomte[e], F. Halleen[f] and J.-P. Péros[g]

[a]*Department of Plant Pathology, University of California, Davis, CA 95616;* [b]*USDA-ARS, University of California, One Shields Avenue, Davis, CA 95616;* [c]*Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA;* [d]*South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001, Australia;* [e]*INRA, UMR 1065 Santé et AgroEcologie du Vignoble, 33883 Villenave d'Ornon Cedex, France;* [f]*ARC Infruitec-Nietvoorbij, P. Bag X5026, Stellenbosch 7599, South Africa; and* [g]*INRA, UMR 1334 Amélioration Génétique et Adaptation des Plantes méditerranéennes et Tropicales, 34060 Montpellier Cedex, France*

The generalist ascomycete fungus *Eutypa lata* causes Eutypa dieback of grapevine (*Vitis vinifera*) worldwide. To decipher the cosmopolitan distribution of this fungus, the population genetic structure of 17 geographic samples was investigated from four continental regions (Australia, California, Europe and South Africa), based on analysis of 293 isolates genotyped with nine microsatellite markers. High levels of haplotypic richness ($R$ = 0·91–1) and absence of multilocus linkage disequilibrium among loci supported the preponderance of sexual reproduction in all regions examined. Nonetheless, the identification of identical multilocus haplotypes with identical vegetative compatibility groups, in some vineyards in California and South Africa, suggests that asexual dispersal of the fungus among neighbouring plants could be a rare means of disease spread. The greatest levels of allelic richness ($A$ = 4·89–4·97) and gene diversity ($H$ = 0·66–0·69) were found in Europe among geographic samples from coastal areas surrounding the Mediterranean Sea, whereas the lowest genetic diversity was found in South Africa and Australia ($A$ = 2·78–3·74; $H$ = 0·49–0·57). Samples from California, Australia and South Africa, which had lower genetic diversity than those of Europe, were also characterized by demographic disequilibrium and, thus, may represent founding populations of the pathogen. Low but significant levels of genetic differentiation among all samples ($D_{EST}$ = 0·12, $P$ = 0·001; $F_{ST}$ = 0·03, $P$ = 0·001) are consistent with historical gene flow preventing differentiation at continental scales. These findings suggest that global, human-mediated spread of the fungus may have resulted in its current global distribution.

*Keywords*: ascomycete, genetic bottlenecks, microsatellite markers, population genetic structure, *Vitis vinifera*

## Introduction

The world wine industry is based on cultivation of the European grapevine (*Vitis vinifera*), which is indigenous to Eurasia (This *et al.*, 2006). Global exchange of *V. vinifera* has been associated with several dramatic pest and pathogen epidemics. The most famous example is introduction of the insect *Daktulosphaira vitifoliae* (grape phylloxera) from its native range in eastern North America to the Old World in the 1800s, which subsequently caused devastating epidemics in most European grape-growing regions. Ironically, grape phylloxera were carried on North American *Vitis* spp. brought to Europe for the purpose of breeding grapes for resistance to powdery mildew (*Erysiphe necator*; Ascomycota, Erysiphaceae), which was also previously introduced to Europe from North America. Furthermore, the importation of eastern North American *Vitis* to Europe inadvertently introduced a second very destructive pathogen, downy mildew (*Plasmopara viticola*; Oomycota, Peronosporaceae). Phylloxera, powdery mildew and downy mildew are now found in all major grape-growing regions of the world.

The population genetic structure of several grapevine pathogens has recently been examined at a global scale, and such large-scale geographic investigations have provided insights into possible introductions. For instance, multilocus sequencing analysis revealed two separate introductions of the powdery mildew fungus *E. necator* to Europe from eastern North America, populations from the latter of which are characterized by the greatest haplotype richness and nucleotide diversity. Also, the identification of shared haplotypes between continental regions suggests that human-mediated dispersal of infected plant material from Europe was probably the cause of subsequent introductions of *E. necator* to both western North America and Australia (Brewer &

*E-mail: kbaumgartner@ucdavis.edu

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Milgroom, 2010). Microsatellite analysis of the fungus *Phaeomoniella chlamydospora* (Ascomycota, Herpotrichiellaceae), one of the causal agents of Petri disease and Esca, revealed higher genetic diversity in France than in Australia (Smetham *et al.*, 2010). Lower genetic diversity in the Australian population of *P. chlamydospora* suggests that a recent population bottleneck following its introduction caused loss of alleles through genetic drift associated with small population sizes. Low mitochondrial diversity in European populations of the downy mildew fungus *P. viticola*, thought to originate from North America, is consistent with a population bottleneck following recent introduction to Europe (Chen *et al.*, 2007) and high levels of nuclear genetic diversity suggest that there were multiple introductions of diverse genotypes to Europe (Gobbin *et al.*, 2006).

Another grapevine disease found worldwide is Eutypa dieback (syn. dying arm disease), a chronic infection of grapevine wood caused by the fungus *Eutypa lata* (Ascomycota, Diatrypaceae) (Carter, 1991). Upon landing on pruning wounds (i.e. infection courts), its wind-dispersed, sexual spores (ascospores) germinate. The mycelium colonizes the adjacent spurs and can spread to the cordon and the trunk, eventually breaking the cambial connection to the shoots (Carter, 1991). Mating occurs between haploid mycelia within the infected wood and the resulting zygote undergoes meiosis to form ascospores. Asexual spores (conidia) are produced *in vitro* and *in planta* (Carter, 1991), but are thought to have no role in infection (Péros *et al.*, 1997; Péros & Larignon, 1998).

*Eutypa lata* is reported from a total of 88 hosts scattered among four continents (Europe, North America, Australia and South Africa) (Carter *et al.*, 1983). It is a generalist pathogen, but is only reported consistently among continents from the cultivated host grapevine and certain species of stone fruits, namely *Prunus armeniaca* (apricot). The first report of the fungus in the world is of its anamorph *Libertella blepharis* from *Prunus* in Scotland (Smith, 1900). The anamorph was subsequently described from apricot in South Australia in 1933 where, later, the teleomorph *E. lata* was first reported (Carter, 1957). In North America, the first report is of the anamorph from apricot in California (English *et al.*, 1962). The pathogen was also diagnosed from apricot in South Africa (Matthee *et al.*, 1974). Reports from wild hosts are much less consistent from continent to continent, primarily due to the endemic nature of such hosts. However, in spite of the pathogen's broad host range, phylogenetic analyses of various nuclear loci (Rolshausen *et al.*, 2006; Trouillas & Gubler, 2010) and restriction fragment length polymorphism DNA (Lardner *et al.*, 2007) provide no evidence of host specificity. Further evidence that *E. lata* isolates from different hosts are not genetically differentiated comes from the ability to consistently amplify all nine of the *E. lata*-specific microsatellite loci (Baumgartner *et al.*, 2009) from a collection of isolates from grapevine, apricot, *Acer* (maple), *Salix* (willow), in addition to the neotypes of *E. lata* that originate from Europe, which are all from wild hosts (e.g. *Tilia*, *Lonicera*) (K. Baumgartner, USDA-Agricultural Research Service, Davis, CA, USA, unpublished observations).

Detailed studies on the genetic diversity of *E. lata* come from vineyards, where the epidemiology of Eutypa dieback is best studied and where the pathogen is found in high densities, in part due to the high availability of pruning wounds. In contrast, natural wounds on wild hosts are relatively scarce, thus limiting the density of infections. Regardless of the spatial scale of study, from one vineyard to multiple vineyards separated by hundreds of kilometres, *E. lata* populations are randomly mating (Péros *et al.*, 1997; Péros & Larignon, 1998; Lardner *et al.*, 2007). However, evidence of restricted gene flow among vineyards across continental Europe (Péros & Berger, 2003) suggests that mountain ranges may constitute physical barriers to spore dispersal. At larger spatial scales that are probably outside the range of spore dispersal, high levels of gene flow were revealed between some Australian and European vineyards, suggesting that human-mediated movement of infected plant material may help explain the geographic distribution of *E. lata* (Péros & Berger, 2003). As one step toward explaining the worldwide distribution of *E. lata*, this study describes the population genetic structure from grape-growing regions with a history of Eutypa dieback, using microsatellite markers (Baumgartner *et al.*, 2009). The main objectives were: (i) to assess the relative importance of sexual versus asexual reproduction in each region examined, (ii) to identify putative founder populations, and (iii) to examine the genetic relatedness of *E. lata* populations from four major wine-producing regions of the world (Australia, California, Europe and South Africa).

## Material and methods

### Fungal isolates

A total of 293 isolates representing 17 geographic samples were collected from vineyards in four continents (Table 1). Isolates from the same vineyard were considered a geographic sample. Australian samples were from three vineyards in South Australia, separated by distances of 80–135 km (Fig. 1a). North American samples were from four vineyards in California, separated by distances of 0·05–21 km (Fig. 1b). At several locations in South Africa and Europe, it was not possible to obtain sufficiently large numbers of isolates from a single vineyard because of the destructive sampling required to isolate *E. lata* from necrotic wood. Thus, the sampling scheme was modified to group isolates from separate vineyards into geographic samples. For example, French samples Fr3 and Fr4 were from 16 vineyards in the Aquitaine region and 15 vineyards in the Alsace region, respectively (Table 1). In South Africa, four geographic samples from a total of eight vineyards in the Western Cape Province, were separated by distances of 0·05–35 km (Fig. 1c). European samples were from a total of six wine-growing regions in France, Italy and Spain, separated by distances

Table 1 *Eutypa lata* samples from grapevines on four continental regions

| Geographic sample | Country | Region | $V^a$ | *D* max[b] | *Vitis vinifera* cultivar(s) | Year collected | $N^c$ |
|---|---|---|---|---|---|---|---|
| Aust1 | Australia | South Australia | 1 | 0·2 | Cabernet Sauvignon | 2004 | 10 |
| Aust2 | Australia | South Australia | 1 | 0·2 | Shiraz | 2003 | 12 |
| Aust3 | Australia | South Australia | 1 | 0·2 | Shiraz | 2003 | 12 |
| Cal1 | United States | California | 1 | 0·2 | Merlot | 2005 | 19 |
| Cal2 | United States | California | 1 | 0·2 | Cabernet Sauvignon | 2005 | 36 |
| Cal3 | United States | California | 1 | 0·2 | Merlot | 2003 | 18 |
| Cal4 | United States | California | 1 | 0·2 | Cabernet Sauvignon | 2003 | 27 |
| SAf1 | South Africa | Western Cape | 2 | 2 | Cabernet Sauvignon | 2009 | 15 |
| SAf2 | South Africa | Western Cape | 2 | 0·6 | Cabernet Sauvignon, Chenin blanc | 2003 | 24 |
| SAf3 | South Africa | Western Cape | 3 | 2 | Cabernet Sauvignon, Chenin blanc, Shiraz | 2003 | 12 |
| SAf4 | South Africa | Western Cape | 1 | 0·2 | Cabernet Sauvignon | 2009 | 12 |
| Fr1[d] | France | Languedoc-Roussillon | 1 | 0·2 | Chenanson, Gramon | 1994 | 16 |
| Fr2[e] | France | Charente | 1 | 0·2 | Ugni blanc | 1996 | 16 |
| Fr3[f] | France | Aquitaine | 16 | 80 | Cabernet Sauvignon | 1996 | 16 |
| Fr4[f] | France | Alsace | 15 | 30 | Auxerrois, Chasselas, Gewurztraminer, Pinot, Pinot blanc, Riesling | 1997 | 16 |
| It[f] | Italy | Veneto | 6 | 100 | Corvina, Ugni blanc | 1995, 1996 | 16 |
| Sp[f] | Spain | Catalonia | 2 | 30 | Cabernet Sauvignon, Chenin blanc | 1999 | 16 |

[a]Number of vineyards sampled.
[b]Maximal linear distance covered (km).
[c]Number of isolates.
[d]Isolates examined by Péros *et al.* (1997).
[e]Isolates examined by Péros & Larignon (1998).
[f]Isolates examined by Péros & Berger (2003).

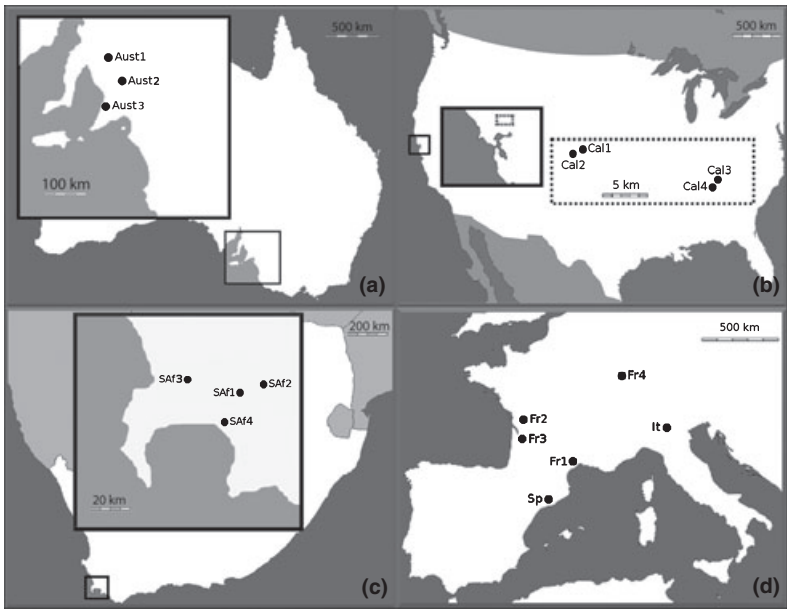


Figure 1 Sampling locations of *Eutypa lata* samples from (a) Australia, (b) California, (c) South Africa and (d) Europe. See Table 1 and Materials and Methods for details.

of 100–920 km (Fig. 1d). European samples are subsets of samples previously examined with RAPD markers (Péros *et al.*, 1997; Péros & Larignon, 1998; Péros & Berger, 2003). Each isolate was recovered from an individual vine, except in California, where up to five isolates were recovered per vine; hence the higher number of isolates per sample (Table 1).

### DNA extraction and microsatellite amplification

Monosporous isolates were recovered from perithecia and mass-hyphal isolates from necrotic wood on trunks or cordons. Cultures were incubated on potato dextrose agar (PDA; Difco) at 25°C in the dark for 14 days. DNA was extracted from aerial mycelia (DNeasy® Plant kit; QIAGEN). DNA extraction from European isolates was as previously described (Péros *et al.*, 1997). All 293 isolates were genotyped with nine microsatellite markers (*B03*, *C02*, *G09*, *F06*, *E07*, *F01*, *B11*, *F02* and *G01*) (Baumgartner *et al.*, 2009), with a PCR reaction mixture (20 μL final volume) containing 2 ng genomic DNA, 1× PCR buffer, 200 μM dNTP, 1·5 mM $MgCl_2$, 0·5 μM primer, and 0·25 U GoTaq® DNA Polymerase (Promega). Amplifications were carried out using one cycle of 94°C for 4 min, followed either by 35 cycles (*B03*, *C02*, *G09*, *F06*, *E07*, *F01*) or 40 cycles (*B11*, *F02*, *G01*) of: 94°C for 30 s, 53°C for 30 s (*B03*, *C02*, *G09*, *F06*, *E07*, *F01*) or 50°C for 45 s (*B11*, *F02*, *G01*), 72°C for 1 min, and 5 min of final extension at 72°C. Allele assignments were performed on an ABI PRISM® 3130xl sequencer (GENEMARKER v.1·75; SoftGenetics LLC).

### Genetic diversity

Multilocus haplotypes (MLHs) were constructed from alleles at all nine loci. The number of identical MLHs were determined within and across geographic samples using GENCLONE 2·0 (Arnaud-Haond & Belkhir, 2007). Isolates with identical MLHs were tested for vegetative compatibility by pairing their mycelia (Péros *et al.*, 1997). Paired isolates that merged into a single colony (i.e. vegetatively compatible isolates) were considered to be genetically identical clones, and those separated by a visible line of necrotic mycelia (i.e. vegetatively incompatible isolates) were considered genetically distinct. Two compatible isolates originating from two distinct sexual reproduction events can share the same MLH, and so the probability was estimated of an MLH occurring $n$ times as a consequence of distinct sexual reproduction events ($P_{sex}$), based on allele frequencies and the number of loci, using GENCLONE. Once clones were identified, haplotypic richness ($R$) was estimated as $R = (G - 1)/(N - 1)$, where $G$ is the number of unique haplotypes and $N$ is the sample size (Dorken & Eckert, 2001). This index varies from 0 in a monoclonal population to 1 when all samples have distinct MLHs. To prevent over-representation of alleles due to the presence of clones, clone-corrected datasets were used for further analysis.

Genetic diversity was estimated within each geographic sample by estimating the total number of alleles over the nine loci, number of private alleles (alleles unique to a particular sample), allelic richness ($A$) and private allelic richness ($R_{pa}$), and unbiased gene diversity ($H$) (Nei, 1978). $R_{pa}$ was adjusted for variable sample size, based on estimates for the geographic sample with the fewest MLHs (Aust1 with 10 MLHs), using HP-rare (Kalinowski, 2004). Estimates were made of $H$, unbiased with regard to sample size, and $A$, also adjusted to a common minimum sample size of 10 MLHs using rarefaction statistics, on average per locus, using FSTAT 2·9·3·2 (Goudet, 1995). Within each continental region, $H$ and $A$ averaged across loci and geographic samples were also estimated. For each comparison between continental regions, differences in $H$ and $A$ were assessed in two-sided permutation tests implemented in FSTAT using 10 000 random permutations of MLHs among groups. In addition to the rarefaction procedures used above to compare $H$ and $A$ among samples of unequal size, a resampling-without-replacement procedure was used, implemented in GENCLONE. Resampling 1000 times from the set of $N$ MLHs per continental region, the program generates all possible subsets of data of $n$ MLHs to estimate $H$ and $A$. This permutation procedure allows one to verify if an asymptote was reached when $n$ approaches $N$, and to compare allelic richness and gene diversity for a common, minimum sample size of 33 MLHs (Australia).

### Gametic linkage disequilibrium

For each geographic sample, the index of multilocus gametic disequilibrium $r_d$ was computed, which is based on the index of association $I_A$, but is independent of the number of loci. Departure from the null hypothesis of random association of alleles, consistent with random mating (no linkage disequilibrium; $r_d = 0$), was assessed by permuting alleles (1000 permutations) between MLHs independently per locus, using MULTILOCUS 1·3 (Agapow & Burt, 2001).

### Individual-based clustering analysis

Tests were carried out for the presence of population structure (i.e. more than one genetic cluster), possibly hidden by *a priori* grouping of MLHs into geographic populations, using a Bayesian method of assignment in STRUCTURE 2·2 (Pritchard *et al.*, 2000). This method uses a Markov Chain Monte Carlo algorithm to assign MLHs to a genetic cluster, assuming Hardy–Weinberg equilibrium and minimizing linkage disequilibrium among loci within clusters. The likelihood of the posterior probability distributions was computed for each number of clusters $K$ from 1 to 20. Each $K$ was simulated 10 times, with a run length of 300 000 iterations after the specified burn-in (300 000 iterations), using the admixture model of genetic ancestry and the correlated model of allele frequency. The number of clusters was estimated as $\Delta K$, which is based on the second order rate of change of the

likelihood of posterior probability of the data L($K$) between successive $K$ values (Evanno *et al.*, 2005).

### Genetic differentiation among populations

The null hypothesis of no genetic differentiation among geographic samples was tested by estimating $D_{EST}$ (Jost, 2008), a measure of genetic differentiation independent of the gene diversity defined by Nei (1978). Indeed, traditional measures of genetic differentiation, such as $G_{ST}$ and $F_{ST}$, necessarily approach zero when gene diversity is high (Jost, 2008). Also, $G_{ST}$ and $F_{ST}$ are not appropriate for markers with more than two alleles and do not reflect genetic divergence between populations; $D_{EST}$ is independent of the number of alleles and better reflects genetic differentiation between populations (Gerlach *et al.*, 2010). $D_{EST}$ was calculated using the DEMEtics package (Gerlach *et al.*, 2010) in R (R Development Core Team, 2010). Two datasets were used to estimate the overall $D_{EST}$ value: one with all nine loci and the other excluding the highly variable locus *B11* (Table 2). This latter dataset was used to estimate pair-wise $D_{EST}$ values between geographic samples. Geographic samples were considered significantly differentiated when $D_{EST}$ was larger than 95% of the values obtained by bootstrapping (1000 random allocations of MLHs over geographic samples). $P$ values were adjusted for multiple comparisons according to the method of Benjamini & Hochberg (1995). An estimate was also made of $\theta$ (Weir & Cockerham, 1984), an unbiased estimator of $F_{ST}$, using FSTAT. $\theta$ is an appropriate indicator of migration rates for populations consistent with the finite island model (Gerlach *et al.*, 2010). Genetic differentiation among geographic samples was also examined through principal coordinates analysis (PCoA), based on Cavalli-Sforza and Edwards' chord distance (Cavalli-Sforza & Edwards, 1967). Chord distance makes no assumptions of constant population size or uniform mutation rates among loci. Using two datasets (including or excluding locus *B11*), the chord distance matrix was built using POPULATIONS 1·2·30 (http://www.bioinformatics.org/~tryphon/populations/) and PCoA was conducted in GENALEX 6 (Peakall & Smouse, 2006).

### Recent demographic history

A test to detect evidence of recent population size fluctuations was performed with BOTTLENECK (Piry *et al.*, 1999). For each geographic sample and for each locus, an estimate was made of gene diversity as expected under the assumption of mutation-drift equilibrium (expected gene diversity) from the number of alleles and sample size, and compared to gene diversity calculated directly from the allele frequencies (observed gene diversity). In a population that experienced a recent reduction in effective size, due for instance to recent establishment of a few founders, gene diversity is higher than expected at an equilibrium between the creation of alleles by mutation and their loss by genetic drift. In such populations, rare alleles are lost rapidly, and thus allelic diversity declines faster than gene diversity (Cornuet & Luikart, 1996). Gene diversity was estimated under two mutation models: the infinite allele model (IAM) and the two-phase model (TPM). In the IAM, each mutation creates a novel allele at a constant rate. TPM is an 'offshoot' of the stepwise mutation model (SMM; each mutation creates a novel allele by adding or deleting a consensus unit of the microsatellite) developed to account for a proportion of larger mutation events (addition or deletion of several units). Both mutation models were tested because the loci used in this study are imperfect microsatellite loci (i.e. they harbour interrupted repeats of the consensus unit) and such loci theoretically tend toward the IAM (Cornuet & Luikart, 1996). These loci are less likely to mutate than perfect repeat microsatellites and, thus, are more likely to retain a longer-lasting bottleneck signature. The proportion of alleles attributed to the SMM under TPM was 70%, allowing 30% of multiple-step mutations, with a

Table 2 Frequency of the most common alleles (above 0·05), gene diversity ($h$), number of alleles and Jost's index of genetic differentiation ($D_{EST}$) per microsatellite locus across the 17 *Eutypa lata* geographic samples

| Microsatellite | Allele size (bp) | Allele frequency | $h$ (gene diversity)[a] | Number of alleles | $D_{EST}$[b] |
|---|---|---|---|---|---|
| B03 | 201 | 0·08 | 0·24 | 8 | 0·03 |
| | 222 | 0·85 | | | |
| C02 | 247 | 0·59 | 0·52 | 8 | 0·03 |
| | 253 | 0·38 | | | |
| G09 | 219 | 0·31 | 0·59 | 9 | 0·03 |
| | 223 | 0·05 | | | |
| | 227 | 0·56 | | | |
| F06 | 148 | 0·70 | 0·38 | 5 | 0·09** |
| | 152 | 0·28 | | | |
| E07 | 186 | 0·07 | 0·65 | 8 | 0·16*** |
| | 198 | 0·09 | | | |
| | 201 | 0·16 | | | |
| | 204 | 0·56 | | | |
| | 210 | 0·07 | | | |
| F01 | 219 | 0·24 | 0·79 | 14 | 0·32*** |
| | 227 | 0·08 | | | |
| | 235 | 0·20 | | | |
| | 239 | 0·06 | | | |
| | 243 | 0·21 | | | |
| | 247 | 0·14 | | | |
| B11 | 241 | 0·12 | 0·85 | 26 | 0·31** |
| | 256 | 0·31 | | | |
| | 268 | 0·07 | | | |
| | 277 | 0·06 | | | |
| | 289 | 0·05 | | | |
| F02 | 226 | 0·47 | 0·60 | 6 | 0·07 |
| | 229 | 0·07 | | | |
| | 232 | 0·41 | | | |
| G01 | 223 | 0·40 | 0·52 | 8 | 0·03 |
| | 239 | 0·57 | | | |

[a]Unbiased gene diversity (Nei, 1978).
[b]Index of genetic differentiation (Jost, 2008). Values significantly different from values obtained after 1000 permutations of genotypes over geographic samples are denoted by **($P$ < 0·01) and ***($P$ = 0·001).

variance of 10 among multiple steps (default parameters). Estimations were based on 5000 replications. The Wilcoxon sign-rank test was performed to determine whether a geographic sample exhibits a significant number of loci with gene diversity excess.

## Results

### Genetic diversity within samples

A total of 92 alleles were found across the nine microsatellite loci. Number of alleles per locus ranged from five for locus *F06* to 26 for locus *B11* (Table 2). Based on all loci, there were 276 MLHs from a total of 293 isolates. Further delineation of isolates with identical MLHs by vegetative compatibility tests and previous RAPD analysis, the latter in geographic sample 'It' (Péros & Berger, 2003), identified 288 unique individuals. Haplotypic richness *R* ranged from 0·91 to 1 within each geographic sample (Table 3). Two compatible isolates with identical MLHs in Cal1 were probably the result of clonal spread, as the probability of encountering this MLH a second time in the sample, assuming random mating, was low ($P_{sex}$ = 0·05). Similarly, two pairs of compatible isolates with identical MLHs in SAf2 probably resulted from clonal spread ($P_{sex}$ < 0·0049). Pairs of compatible isolates with identical MLHs were identified from different geographic samples within South Africa (SAf2-SAf3, and SAf2-SAf4; $P_{sex}$ < 0·028). In geographic samples Cal2 and Cal3, three and two isolates from a single grapevine, respectively, had identical MLHs, but were vegetatively incompatible.

Genetic diversity quantified as both allelic richness *A* and gene diversity *H* averaged across geographic samples was significantly higher in Europe than in South Africa (*A* = 4·15 vs. 3·11; *P* = 0·004; *H* = 0·61 vs. 0·53; *P* = 0·014) (Table 3). Similarly, allelic richness was significantly higher in Europe than in Australia (*A* = 4·15 vs. 3·21; *P* = 0·026). All other pair-wise comparisons between continental regions did not reveal any significant

Table 3 Indices of genetic diversity within *Eutypa lata* geographic samples

| Continental region/ Geographic sample | $N$[a] | MLH[b] | $R$[c] | Alleles | $A$[d] | Private alleles | $R_{pa}$[e] | $H$[f] | $r_d$[g] | $P$ value |
|---|---|---|---|---|---|---|---|---|---|---|
| Australia | | | | | | | | | | |
| Aust1 | 10 | 10 | 1 | 25 | 2·78 | 0 | 0 | 0·52 | 0·016 | 0·287 |
| Aust2 | 12 | 11 (12) | 1 | 28 | 3·11 | 0 | 0 | 0·52 | 0·010 | 0·349 |
| Aust3 | 12 | 12 | 1 | 34 | 3·74 | 3 | 0·33 | 0·57 | 0·037 | 0·083 |
| Average[h] | | | | | 3·21[B] | | | 0·54[AB] | | |
| California | | | | | | | | | | |
| Cal1 | 19 | 18 (18) | 0·94 | 31 | 3·29 | 1 | 0·1 | 0·56 | –0·019 | 0·923 |
| Cal2 | 36 | 34 (36) | 1 | 43 | 3·76 | 1 | 0·07 | 0·56 | –0·006 | 0·74 |
| Cal3 | 18 | 17 (18) | 1 | 38 | 3·96 | 0 | 0·08 | 0·62 | –0·002 | 0·507 |
| Cal4 | 27 | 26 (27) | 1 | 38 | 3·67 | 1 | 0·07 | 0·57 | –0·009 | 0·761 |
| Average | | | | | 3·67[AB] | | | 0·57[AB] | | |
| South Africa | | | | | | | | | | |
| SAf1 | 15 | 14 (15) | 1 | 30 | 3·27 | 0 | 0·02 | 0·57 | –0·001 | 0·472 |
| SAf2 | 24 | 20 (22) | 0·91 | 35 | 3·50 | 0 | 0 | 0·51 | –0·006 | 0·647 |
| SAf3 | 12 | 10 (12) | 1 | 26 | 2·89 | 0 | 0 | 0·56 | 0·207 | <0·001 |
| SAf4 | 12 | 11 (12) | 1 | 25 | 2·78 | 0 | 0 | 0·49 | 0·110 | 0·001 |
| Average | | | | | 3·11[B] | | | 0·53[B] | | |
| Europe | | | | | | | | | | |
| Fr1 | 16 | 16 | 1 | 48 | 4·97 | 8 | 0·82 | 0·69 | 0·036 | 0·048 |
| Fr2 | 16 | 16 | 1 | 37 | 3·90 | 1 | 0·11 | 0·61 | 0·001 | 0·463 |
| Fr3 | 16 | 16 | 1 | 34 | 3·57 | 1 | 0·11 | 0·54 | –0·002 | 0·529 |
| Fr4 | 16 | 16 | 1 | 35 | 3·66 | 2 | 0·22 | 0·55 | –0·038 | 0·98 |
| It | 16 | 13 (16) | 1 | 45 | 4·89 | 6 | 0·67 | 0·66 | 0·004 | 0·434 |
| Sp | 16 | 16 | 1 | 37 | 3·90 | 2 | 0·22 | 0·59 | –0·009 | 0·667 |
| Average | | | | | 4·15[A] | | | 0·61[A] | | |

[a]Number of isolates.

[b]Number of multilocus haplotypes (MLHs) based on nine microsatellite loci. Number of MLHs corrected either after vegetative compatibility tests or based on previously published RAPD profiles (Péros & Berger, 2003) are indicated in parentheses.

[c]Haplotypic richness (Dorken & Eckert, 2001).

[d]Allelic richness estimated in a sample of 10 MLHs, the smallest sample size in the dataset (Aust1).

[e]Private allelic richness (Kalinowski, 2004) estimated in a sample of 10 MLHs, the smallest sample size in the dataset (Aust1).

[f]Unbiased gene diversity (Nei, 1978).

[g]Index of multilocus linkage disequilibrium (Agapow & Burt, 2001) and associated *P* value.

[h]Allelic richness and gene diversity were averaged across loci and across geographic samples per continental region. Values followed by the same letter do not significantly differ (assessed after 10 000 random permutations of MLHs over regions; *P* > 0·05).

differences in genetic diversity measured as *A* and *H*. For a common, minimum sample size of 33 MLHs (sample size in Australia), the resampling procedure further revealed that allelic richness and gene diversity were highest in Europe (Fig. 2). Notably in each region, gene diversity quickly reached a plateau for sample sizes of approximately 10 MLHs. When examined at the within-geographic-sample scale, the highest values of *A* and *H* were found in European samples Fr1 and It, whereas the lowest values were found in geographic samples in Australia (Aust1) and South Africa (SAf4). Private allelic richness $R_{pa}$ was typically higher in European geographic samples (Table 3).

### Gametic linkage disequilibrium

Based on the multilocus linkage disequilibrium statistic $r_d$, the null hypothesis of random recombination across loci was not rejected for 14 of 17 geographic samples ($r_d$ = −0·038 to 0·037, $P > 0·05$; Table 3), suggesting that all MLHs resulted from sexual reproduction. Significant multilocus linkage disequilibrium was found in three geographic samples: SAf3, SAf4 and Fr1 ($P < 0·05$).

### Bayesian clustering analysis

The Bayesian method of assignment of MLHs did not detect any population subdivision. The likelihood of the posterior probability distributions, implemented by STRUCTURE, increased until $K = 20$. Computation of $\Delta K$ did not support $K \geq 2$. The lack of population structure is also evidenced by the fact that at $K > 1$, under the admixture model, the proportions of genetic ancestry of each MLH assigned to each cluster were symmetric; most MLHs were admixed. Therefore, a single genetic cluster contained all MLHs.

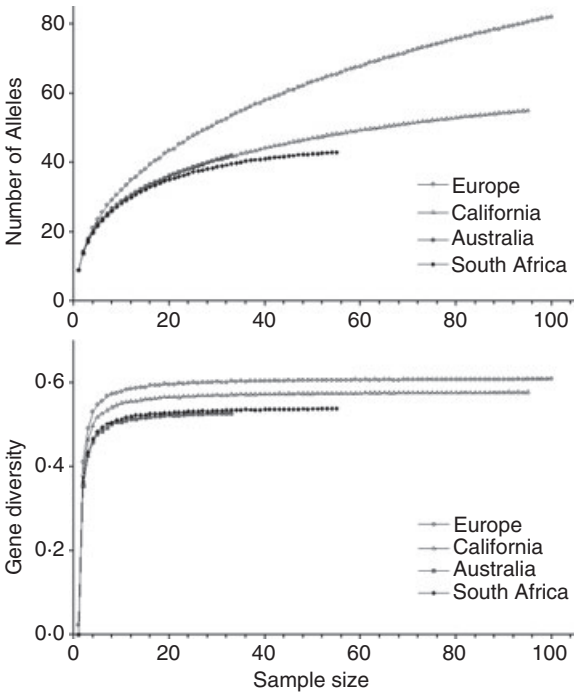


Figure 2 Number of alleles over the nine microsatellite loci (top) and unbiased gene diversity (bottom) estimated for different sample sizes in four continental regions in *Eutypa lata*. For each sample size *n*, both measures were estimated as the average of 1000 subsamples of size *n* from *N* multilocus haplotypes.

### Population differentiation

Overall genetic differentiation among the 17 geographic samples measured as the harmonic mean of $D_{EST}$ over loci was 0·12 ($P = 0·001$). Five loci (*B03*, *C02*, *F02*, *G01* and *G09*) did not reveal significant genetic differentiation among samples ($D_{EST}$ = 0·03–0·07; $P > 0·05$; Table 2). Loci *F06*, *E07*, *B11* and *F01* had significant contributions to genetic differentiation among all geographic samples ($D_{EST}$ = 0·09, 0·16, 0·31 and 0·32, respectively; $P < 0·005$). Without locus *B11*, which had a disproportionately large number of alleles relative to the other loci (Table 2), the harmonic mean of $D_{EST}$ over loci was 0·09 ($P = 0·001$) and $D_{EST}$ values and significance for all other loci remained unchanged. All pair-wise comparisons involving geographic samples SAf3 and SAf4 revealed significant $D_{EST}$ values ($D_{EST}$ = 0·08–0·22; $P < 0·05$), except for SAf1 vs. SAf4 ($D_{EST}$ = 0·03; $P = 0·26$) and SAf3 vs. It ($D_{EST}$ = 0·08; $P = 0·27$; Table 4). Geographic sample It was genetically differentiated from a majority of samples ($D_{EST}$ = 0·12–0·21; $P < 0·05$), except from Sp, SAf3, Fr1, Fr2 and Aust3 ($D_{EST}$ = 0·05–0·14; $P > 0·05$). Samples from France (Fr1, Fr2, Fr3 and Fr4) were not genetically differentiated from a majority of geographic samples from elsewhere (Table 4). Including or excluding locus *B11*, the overall genetic differentiation $\theta$ among the 17 geographic samples was 0·03 ($P = 0·001$).

Based on the results of PCoA on the chord distance matrix among geographic samples (Fig. 3), the grouping of most geographic samples from the four continental regions revealed high genetic similarity among these samples. Two geographic samples from South Africa (SAf3 and SAf4) and two from Europe (Fr1 and It), which are separated from the majority of geographic samples by the first two principal coordinates (explaining a total of 45% of genetic variation), probably represent genetically divergent pools of *E. lata*. Excluding locus *B11* to generate the chord distance matrix, the positioning of all geographic samples along the first two principal coordinates was similar (data not shown).

### Recent demographic history

Comparisons between observed and expected gene diversity under mutation-drift equilibrium revealed significant excess in observed gene diversity for a majority of loci in geographic samples from Australia (Aust1 and Aust3), California (Cal1 and Cal3) and South Africa (SAf1, SAf3 and SAf4). These findings were consistent under both mutation models IAM and TPM, and suggest recent

bottlenecks in these geographic samples (Table 5). No geographic samples from Europe deviated significantly from mutation-drift equilibrium under both mutation models.

## Discussion

Analysis of the population genetic structure of *E. lata* from vineyards on all four continents from which it is known, supports the importance of sexual recombination in all samples examined. Surprisingly, low levels of genetic differentiation were found among samples from different continents. These findings do not support the hypothesis that continental populations of *E. lata* evolved in complete allopatry for numerous successive generations, which would have resulted in genetic differentiation among geographically isolated populations. Instead, it seems more likely that long-distance, human-mediated dispersal of the fungus resulted in the genetic homogeneity observed at continental scales. Hence, historical gene flow may have prevented genetic differentiation at a global scale. Also, populations of *E. lata* in the New World and the southern hemisphere may result from recent establishment by a few founders, which is supported by their lower genetic diversity and the absence of demographic equilibrium, relative to European populations.

The fine-scale sampling scheme in some regions revealed new aspects of the infection process. The identification of closely related isolates (i.e. those with identical MLHs and different vegetative compatibility groups) from the same plant may represent infections by sibling ascospores (i.e. those originating from the same ascus). The finding of multiple isolates with distinct MLHs from the same plant suggests that multiple infections through different pruning wounds and possibly in different years are sometimes collectively responsible for the decline of a single plant. Most surprisingly, isolates were identified with identical vegetative compatibility groups and identical MLHs, unlikely to occur several times in the sample ($P_{sex} < 0{\cdot}05$), within the same vineyard in California and South Africa. This is the first evidence of asexual spread of Eutypa dieback, presumably via conidia, albeit at short distances (among neighbouring plants). These findings corroborate the only study documenting successful germination of conidia produced in culture (Ju *et al.*, 1991). Nonetheless, the findings of high levels of haplotypic richness and absence of linkage disequilibrium among microsatellite loci confirm the much greater importance of random mating in *E. lata* populations and of ascospores

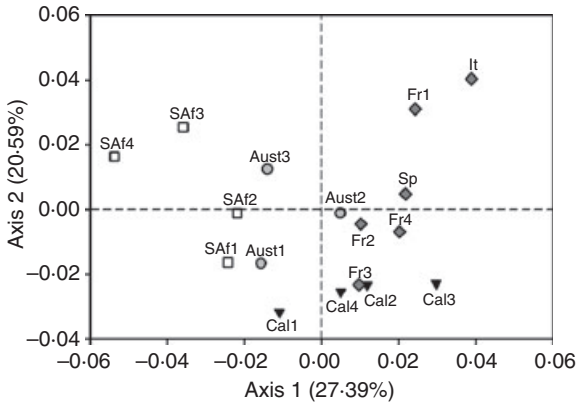


Figure 3 Principal coordinates analysis (PCoA) of 17 geographic samples of *Eutypa lata*. Analysis was based on Cavalli-Sforza & Edwards (1967) chord distance matrix among the geographic samples. Black triangles, dark grey diamonds, grey circles and white squares represent geographic samples from California, Europe, Australia and South Africa, respectively. Percent variation explained by each axis (principal coordinate) is shown in parentheses.

Table 4 Pair-wise genetic differentiation measured as $D_{EST}$ (Jost, 2008) among 17 *Eutypa lata* geographic samples from four continental regions

| | Aust1 | Aust2 | Aust3 | Cal1 | Cal2 | Cal3 | Cal4 | SAf1 | SAf2 | SAf3 | SAf4 | Fr1 | Fr2 | Fr3 | Fr4 | It | Sp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aust1 | – | | | | | | | | | | | | | | | | |
| Aust2 | 0·05 | – | | | | | | | | | | | | | | | |
| Aust3 | 0·05 | 0·02 | – | | | | | | | | | | | | | | |
| Cal1 | 0·07 | 0·10 | 0·08* | – | | | | | | | | | | | | | |
| Cal2 | 0·06 | 0·09* | 0·11* | 0·03 | – | | | | | | | | | | | | |
| Cal3 | 0·12* | 0·15* | 0·20* | 0·12* | 0·10* | – | | | | | | | | | | | |
| Cal4 | 0·06 | 0·06 | 0·08 | 0·02 | 0·01 | 0·07* | – | | | | | | | | | | |
| SAf1 | 0·10* | 0·13* | 0·10* | 0·03 | 0·05 | 0·12* | 0·06 | – | | | | | | | | | |
| SAf2 | 0·07 | 0·11* | 0·11* | 0·06 | 0·07* | 0·17* | 0·04 | 0·07 | – | | | | | | | | |
| SAf3 | 0·18* | 0·16* | 0·13* | 0·16* | 0·16* | 0·22* | 0·12* | 0·11* | 0·13* | – | | | | | | | |
| SAf4 | 0·14* | 0·12* | 0·14* | 0·09* | 0·13* | 0·17* | 0·12* | 0·03 | 0·13* | 0·07 | – | | | | | | |
| Fr1 | 0·09 | 0·09 | 0·11 | 0·08 | 0·06 | 0·07 | 0·05 | 0·06 | 0·08 | 0·15* | 0·13* | – | | | | | |
| Fr2 | 0·06 | 0·06 | 0·05 | 0·07 | 0·05 | 0·07 | 0·03 | 0·04 | 0·08 | 0·14* | 0·12* | 0·01 | – | | | | |
| Fr3 | 0·04 | 0·04 | 0·04 | 0·04 | 0·04 | 0·11* | 0·00 | 0·07* | 0·05 | 0·14* | 0·14* | 0·07 | 0·04 | – | | | |
| Fr4 | 0·05 | 0·05 | 0·07 | 0·09* | 0·03 | 0·10* | 0·03 | 0·09 | 0·11* | 0·18* | 0·16* | 0·06 | 0·01 | 0·03 | – | | |
| It | 0·21* | 0·14* | 0·14 | 0·20* | 0·17* | 0·18* | 0·12* | 0·21* | 0·14* | 0·08 | 0·20* | 0·09 | 0·13 | 0·15* | 0·16* | – | |
| Sp | 0·09* | 0·06 | 0·10 | 0·10* | 0·08* | 0·08* | 0·05 | 0·09 | 0·09* | 0·11* | 0·11* | 0·01 | 0·02 | 0·05 | 0·05 | 0·05 | – |

Significant pair-wise genetic differentiation adjusted for multiple comparisons are denoted by *($P < 0{\cdot}05$).

Table 5 Tests for deviations from mutation-drift equilibrium in 17 *Eutypa lata* geographic samples. Ratio of loci presenting a gene diversity deficiency (*D*) or excess (*E*) under two mutation models (infinite allele model (IAM) and two-phase model (TPM)) are presented

| Geographic sample | *D*/*E* | |
|---|---|---|
| | IAM | TPM |
| Aust1 | 0/8** | 1/7** |
| Aust2 | 2/7* | 2/7 |
| Aust3 | 1/7* | 2/6* |
| Cal1 | 1/8** | 1/8** |
| Cal2 | 1/8* | 3/6 |
| Cal3 | 0/9** | 1/8** |
| Cal4 | 1/8** | 4/5 |
| SAf1 | 1/8** | 2/7* |
| SAf2 | 4/5 | 4/5 |
| SAf3 | 1/8** | 2/7* |
| SAf4 | 2/6* | 2/6* |
| Fr1 | 1/8** | 2/7 |
| Fr2 | 1/8* | 1/8 |
| Fr3 | 3/6 | 5/4 |
| Fr4 | 2/7 | 5/4 |
| It | 1/8** | 2/7 |
| Sp | 1/8* | 2/7 |

Ratios deviating from mutation-drift equilibrium according to Wilcoxon sign rank test are denoted by *($P < 0{\cdot}05$) or **($P < 0{\cdot}01$). Only polymorphic loci were considered.

as primary inoculum (Péros *et al.*, 1997; Péros & Larignon, 1998; Péros & Berger, 2003).

The highest levels of genetic diversity were found in Europe, in geographic samples from coastal areas surrounding the Mediterranean Sea (southeastern France (Fr1) and northern Italy (It)). There was no relationship between levels of genetic diversity within samples and the spatial area covered by each sample. For example, Fr1 originated from a single vineyard and was more genetically diverse than Fr3 or Fr4, each of which were collected from 15 and 16 vineyards in the Aquitaine and Alsace regions, respectively. Rather, highest genetic diversity in *E. lata* populations was found in the area where grape cultivation is the most ancient, relative to the other regions examined (Unwin, 1991). A pattern of geographic overlap between the most genetically diverse pathogen populations and areas of intensive crop cultivation has been reported for other pathogens of cultivated crops (Stukenbrock & McDonald, 2008). Centres of genetic diversity can, instead, correspond to geographic areas where multiple introductions of divergent pathogen genotypes have occurred (Petit *et al.*, 2003). Therefore, southeastern France and northern Italy may not be a centre of origin for *E. lata*, but instead a region where *E. lata* was regularly introduced from diverse source populations. Central Europe has proved to be a fruitful region for the discovery of new hosts of *E. lata* that express only mild symptoms, which may reflect a long co-evolutionary history of the fungus with these hosts in this region (Carter, 1991).

These results suggest that *E. lata* has recently become established in vineyards of Australia, California and South Africa. The loss of alleles, lower private allelic richness, and higher number of loci at mutation-drift disequilibrium in Australia, California and South Africa, relative to Europe, are typical signatures of genetic bottlenecks (Leberg, 2002). Such findings may reflect recent reductions in population size (Cornuet & Luikart, 1996) due to establishment by a few founders. Also, the low genetic diversity observed in samples from South Africa coupled with their genetic divergence from all other geographic samples are in agreement with the random fixation of a reduced number of alleles and provide additional support for a recent establishment of *E. lata* in South Africa. Loss of genetic diversity in a founder population relative to that of its source population is a function of its effective minimum (or founder) population size and of the growth rate of the population following introduction (Dlugosch & Parker, 2008). Therefore, higher measures of allelic richness and gene diversities in California than in Australia and South Africa may be due to a greater number of introductions of *E. lata* to this region. Furthermore, in *E. lata* the sexual fruiting bodies form several years after infection (Carter, 1991). Such a delay between introduction of a non-native organism and onset of sexual reproduction attenuates the expected loss of genetic diversity during a founder effect, as has been shown empirically for trees versus annual plants (Austerlitz *et al.*, 2000). Thus, it is possible that the relatively high measures of genetic diversity in putative introduction areas are due to multiple introductions from different sources over the course of several years, thereby building the founder population size before these diverse founders interbred.

Low genetic differentiation among geographic samples within continental regions may be due to local gene flow through ascospore dispersal. However, the Alps Mountains may constitute a physical barrier to gene flow among European *E. lata* populations, as the Italian sample examined was confirmed as genetically distinct from other European samples (Péros & Berger, 2003). Gene flow within a region is probably a consequence of a high density of vineyards and fragmented habitats with various alternative hosts. Although *E. lata* populations on alternative hosts might affect the pathogen genetic structure, high densities of *E. lata* occur only on the cultivated host grapevine and, to a lesser extent, apricot; pathogen populations among wild hosts are sparse (Carter, 1991). Moreover, individual isolates from multiple hosts have previously been examined with genetic markers and were shown to be genetically similar to those of grapevine (Rolshausen *et al.*, 2006; Lardner *et al.*, 2007). Finally, ongoing microsatellite analysis of populations from multiple hosts in California does not support the hypothesis of host specialization of *E. lata* (R. Travadon, University of California, Davis, CA, USA, unpublished observations). Therefore, considering that grapevine is the host with the highest pathogen population density, that wild host infections are scarce and that there is no evidence of genetic divergence among populations from different hosts, the findings from populations of *E. lata* on grapevine probably constitute an unbiased picture of the genetic structure of this generalist pathogen.

Significant, albeit low, genetic differentiation was revealed among geographic samples from four continents. Gene flow through passive spore dispersal between continents is extremely unlikely, as the distance between the continents seems outside the dispersal range of ascospores. In contrast, inadvertent importation of *Eutypa*-infected plant material is a more likely means of intercontinental dispersal of *E. lata*, and may be from historic movement of grapevine or some other host (before government regulatory agencies existed) or from current movement in spite of regulations on such activities. Although *E. lata* is not typically recovered from modern grapevine propagation material (e.g. one-year-old canes), introductions of *E. lata* in the past may have resulted from the trade of different forms of plant material (e.g. whole, mature plants). The genetic similarity among continents may, therefore, reflect intercontinental and historical gene flow that counterbalances local genetic drift. Assuming introductions did occur, regardless of their means, the effects of emigrant genotypes on local population structure and diversity were probably very gradual because *E. lata* spreads mainly through ascospore dispersal and ascospore production takes multiple years to occur on an infected plant (Carter, 1991).

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