Identifying Sources of Inoculum for Eutypa Dieback Infections in Vineyards

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The means of spread of Eutypa dieback from vine-to-vine within vineyards is likely due to dispersal of sexual spores (ascospores) of the causal fungus, Eutypa lata, based on evidence of distributions of vegetative compatibility groups, reproductive structures (perithecia), and symptoms (2, 6). Asexual spores (conidia) are produced in nature, but are not infectious. Ascospores are released from perithecia following rain and are wind-dispersed. They infect grapevine vascular tissue by colonizing susceptible wounds (e.g., pruning wounds, freeze-damaged tissue).

Although it seems clear that ascospores initiate infections of vines, the origin of ascospores that initiate the first infections in a healthy vineyard is not clear. Possible sources include distant vineyards (8), forest trees (10), or apricot orchards (3). To evaluate the relatedness of E. lata populations from vineyards, forests, and apricot orchards, we isolated and characterized nine E. lata-specific microsatellite markers. As numerous Eutypa species infect grapevines, forest trees, and apricots, we also evaluated our markers for E. armeniacae, E. laevata, E. leptoplaca, and E. petrakii var. petrakii. These closely related species are not distinguishable from E. lata in culture, and it is, therefore, critical to ensure that markers will not inadvertently amplify isolates of different species.

Materials and Methods

Genomic DNA was extracted from an isolate of E. lata from Switzerland [isolate 208.87; Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands], purified (GENECLEAN III Kit, MP Biomedicals, Solon, OH), digested with TaqαI (New England BioLabs, Ipswich, MA), and enriched for both a trinucleotide, CAC10, and a tetranucleotide mixture (AAAC6, AAAG6, AAAT8, AGAT8; Integrated DNA Technologies, Coralville, IL). Digested DNA was ligated to linker oligonucleotides 20B (5'-GCG GTT CCC GGT CGA GTT GG-3') and 22B (5'-pCGC CAA CTC GAC CGG GAA CCG C-3') (5), and the resulting linker-ligated DNA was used as template for pre-enrichment using the polymerase chain reaction (PCR) (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) (4). Linker-ligated restriction fragments enriched with microsatellites were captured onto Streptavidin Dynabeads M-280 (Dynal Biotech, Oslo, Norway). Captured DNA fragments were eluted from the Dynabeads, amplified by PCR, purified, then rehybridized with the biotinylated oligonucleotides in a repeated (serial) enrichment reaction. PCR products from the second enrichment were purified, cloned (TOPO TA 2.1 Cloning Kit, Invitrogen, Carlsbad, CA), and screened for positive inserts. Ninety-six positive colonies were amplified from fragments enriched for CAC10 and 96 for the tetranucleotide mixture, and sequenced (BigDye Terminator version 3.1 Cycle Sequencing Kit, ABI 3100, Applied Biosystems). Sequences were screened for microsatellite repeats and 24 primer pairs were designed (Primer3 v.0.4.0.0; 11).

The 24 primer pairs were first used to screen for allelic variation with genomic DNA from a small subset of haploid isolates of E. lata: two from California (isolates CS2 and CS16, (9) and one from Italy (isolate MD1, Instituto di Pathologia Vegetale, Milan, Italy). PCR was performed in multiplex or simplex reactions, using a ‘touchdown’ protocol (1). PCR fragment sizes were analyzed with the ABI 3100 (Applied Biosystems) and sized with the ROX-500 size standard after excluding the 250 bp standard (GeneScan v. 3.7, Applied Biosystems).
Of the 24 primer pairs, nine gave consistent PCR amplicons for all three isolates of *E. lata*. The nine primer pairs were then used to screen for polymorphisms on genomic DNA from a set of 48 isolates representing two populations (24 isolates per population), which were collected from 24 symptomatic grapevines in each of two northern California vineyards located 50 km apart. Genepop on the web (available at http://genepop.curtin.edu.au/) was used to calculate the number of alleles and to test for linkage disequilibrium (LD) within populations, using the Markov chain parameters. Genalex 6 (7) was used to estimate the haploid gene diversity. In addition, the nine primer pairs were tested on the following related taxa (CBS identification numbers in parentheses): *E. armeniaceae* (622.84), *E. laevata* (291.87), *E. leptoplaca* (286.87), and *E. petrakii* var. *petrakii* (245.87).

**Results and Discussion**

All nine primer pairs were found to be polymorphic. The loci examined revealed high allelic variation, having the total number of alleles ranging from two to 11 alleles per locus. Haploid gene diversity ranged from 0.33 to 0.83 per locus (mean for Population 1 = 0.53, Population 2 = 0.54). Genotypic diversity was high within the two populations, with each sample comprising a unique genotype, which is consistent with ascospore infection and not conidia infection. Significant pairwise linkage disequilibrium (LD) was detected in two loci (B11 and G09 across both populations; \( P = 0.046 \)). The absence of LD at eight of the nine loci and our finding of high genotypic diversity suggests that recombination via outcrossing comprises a significant contribution to the population structure of *E. lata*. The nine primer combinations gave negative and/or consistent amplicons of the closely-related species, *E. armeniaceae*, *E. laevata*, *E. leptoplaca*, and *E. petrakii* var. *petrakii*. These nine polymorphic microsatellite loci, therefore, appear be a suitable tool for studies of fine-scale, spatial population structure and distribution, for estimating dispersal distances, and for identification of siblings. In addition, it should be possible to identify the possible sources of initial source infections from local and distant forests, vineyards, and apricot orchards.

**References**


