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PERMANENT GENETIC RESOURCES NOTE

Microsatellite markers for the grapevine pathogen, *Eutypa lata*

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Abstract

We isolated and characterized nine polymorphic microsatellite markers for *Eutypa lata*, a fungal pathogen responsible for Eutypa dieback of grapevine, in populations from two California vineyards (24 isolates per vineyard). Allele frequency ranged from two to 11 alleles per locus and haploid gene diversity ranged from 0.33 to 0.83. All samples comprised unique haplotypes. Our results suggest that there is sufficient allelic polymorphism to estimate fine-scale spatial structure, and to identify possible sources of inoculum from habitats outside of vineyards.

Keywords: Ascomycota, Diatrypaceae, Eutypa dieback, *Eutypa lata*, plant pathogen, *Vitis vinifera*

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The means of spread of Eutypa dieback from vine-to-vine within vineyards is likely due to dispersal of *Eutypa lata* sexual spores (ascospores), and not asexual spores (conidia), based on evidence of distributions of vegetative compatibility groups, reproductive structures (perithecia), and symptomatic grapevines (Munkvold *et al*. 1993; Cortesi & Milgroom 2001). 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 (Peros & Berger 2003), forest trees (Rolshausen *et al*. 2006), or apricot orchards (DeScenzo *et al*. 1999). To evaluate the relatedness of *E. lata* populations from vineyards, forests, and apricot orchards, we isolated and characterized nine *E. lata*-specific microsatellite markers.

Genomic DNA was extracted from an isolate of *E. lata* from Switzerland [isolate 208.87; Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands], purified (GENECLEAN III Kit, MP Biomedicals), digested with *Taq* αI (New England BioLabs), and enriched for both a trinucleotide, CAC_{10}, and a tetranucleotide mixture ($AAAC_6$, $AAAG_6$, $AAAT_8$, $AGAT_8$; Integrated DNA Technologies). Digested DNA was ligated to linker oligonucleotides 20B (5′-GCGGTTCCCGGTCGAGTTGG-3′) and 22B (5′-pCGCCAACTCGACCGGGAACCGC-3′) (Kretzer *et al*. 2000), and the resulting linker-ligated DNA was used as template for pre-enrichment using polymerase chain reaction (PCR) (GeneAmp PCR System 9700, Applied Biosystems), following cycling conditions described in Glenn & Schable (2005). Pre-enrichment PCR products were purified (MinElute PCR Purification Kit, QIAGEN) and hybridized with the 3′-biotinylated microsatellite tri- and tetranucleotides listed above, using the following cycling parameters: 95 °C for 5 min, 0.3 °C decrease per second from 95 to 70 °C, 70 °C for 30 min, 0.3 °C decrease per second from 70 to 65 °C, 65 °C for 1 h, 0.3 °C decrease per second from 65 to 60 °C, 60 °C for 4 h, 0.3 °C decrease per second from 60 to 55 °C, 55 °C for 30 min, 0.3 °C decrease per second from 55 to 50 °C, 50 °C for 10 min, fast decrease to 43 °C, and a final hold at 43 °C. Linker-ligated restriction fragments enriched with microsatellites were captured onto Streptavidin Dynabeads M-280 (Dynal Biotech). 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), and screened for positive inserts. Ninety-six positive colonies were amplified from fragments enriched for CAC_{10} and 96 for the tetranucleotide mixture, and sequenced (BigDye Terminator version

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Table 1 Characterization of nine polymorphic microsatellite loci from *Eutypa lata*. N_A, number of alleles; *H*, haploid allele diversity

				Population 1 (n = 24)			Population 2 (n = 24)		
Locus	Motif	Primer sequence (5′– 3′) (fluorescent labels in italics)	GenBank Accession no.	N_A	*H*	Allele size range (bp)	N_A	*H*	Allele size range (bp)
B03	$(CAC)_n$	F: *NED*-CGAATAACTAACTACGCCCTACC R: GGGAGAAGAATGCGAGAATG	EU434720	2	0.38	201–222	3	0.35	201–234
B11	$(CAC)_n$	F: *VIC*-CGTCATGCGACATACGTACC R: GCACCATCAGCCAAAATATGA	EU434726	9	0.83	241–289	11	0.83	241–296
C02	$(CAC)_n$	F: *VIC*-ACGCTTCCACGGTTTAGAAG R: ACTCGTCCGCTACAACTCCA	EU434725	2	0.49	247–253	2	0.38	247–253
E07	$(CAC)_n$	F: *NED*-ACTCCATCAGCCACCTTCAC R: ATGTAAGCGCACAAAACGTG	EU434719	3	0.34	201–210	3	0.34	201–207
F01	$(AAAG)_n$	F: *VIC*-CCACCCTAATCAGCTTTGCTC R: TGGTGATAGCCGTGATGGTA	EU434724	4	0.70	219–247	5	0.78	219–247
F02	$(CAC)_n$	F: *NED*-CGGGATCGCTTAATCTACCA R: GAATTAGTTGCGGGCAAGG	EU434721	4	0.57	223–232	3	0.61	226–232
F06	$(AAAC)_n$	F: *6FAM*-TTACCTCGAGGAGAGCTTCG R: AACCCGCGTAGACTGCTATG	EU434718	2	0.33	148–152	2	0.49	148–152
G01	$(AGAT)_n$	F: *VIC*-ACCTTCTTGAAGCGGTTGAC R: TGGAATGTACGCCTTTCCTC	EU434723	2	0.50	223–239	2	0.44	223–239
G09	$(AAAG)_n$	F: *VIC*-AGAATCCTCACGGAAACCAC R: ACTTGAGGGAGGAGTGAAGC	EU434722	3	0.59	215–227	5	0.67	215–231

3.1 Cycle Sequencing Kit, ABI 3100, Applied Biosystems). Sequences were screened for microsatellite repeats and 24 primer pairs were designed (Primer 3 version 0.4.0; Rozen & Skaletsky 2000).

The 24 primer pairs were used to screen for allelic variation with genomic DNA from a small subset of haploid isolates of *E. lata* from California [isolates CS2 and CS16 (Rolshausen *et al*. 2008)]. PCR was performed in multiplex or simplex reactions using the following concentrations (25-μL reaction volume): 1× PCR Buffer (Go*Taq* Colorless Buffer, Promega), 1.25 U *Taq* (Go*Taq*, Promega), 0.2 μM fluorescent-labelled 5′ forward primer (Applied Biosystems), 1.0 μM reverse primer (Operon Biotechnologies), 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mg/mL BSA, 0.5 M betaine, and 10–100 pg of DNA template. PCR was performed using a 'touchdown' protocol (Bergemann *et al*. 2005). PCR fragment sizes were analysed with the ABI 3100 (Applied Biosystems) and sized with the ROX-500 size standard after excluding the 250-bp standard (GeneScan version 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 default Markov chain parameters. GenAlEx 6 (Peakall & Smouse 2006) was used to estimate the haploid gene diversity. All nine primer pairs were found to be polymorphic (Table 1). The loci examined had two to 11 alleles per locus. Haploid gene diversity ranged from 0.33 to 0.83 per locus (mean: Population 1 = 0.53; Population 2 = 0.54). Genotypic diversity was high within the two populations, with each sample comprising a unique genotype. No significant pairwise LD was detected in pairwise comparisons across all loci ($P > 0.01$). The absence of significant LD at nine loci, coupled with our finding of high genotypic diversity, suggests that recombination via outcrossing significantly contributed to the population structure of *E. lata*. These nine polymorphic microsatellite loci therefore appear to be a suitable tool for studies of fine-scale distribution of genotypes, spatial population structure and distribution, and for estimating dispersal distances. In addition, such a fool should enable the identification of the possible sources of inoculum from local and distant forests, vineyards, and apricot orchards.

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