

PHYLOGEOGRAPHY, POPULATION STRUCTURE, AND MATING SYSTEM OF THE  
GRAPE POWDERY MILDEW FUNGUS, *ERYSIPHE NECATOR*

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PHYLOGEOGRAPHY, POPULATION STRUCTURE, AND MATING SYSTEM OF THE  
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To understand the invasion history of the grape powdery mildew fungus, *Erysiphe necator*, I investigated the evolutionary relationships between introduced populations of Europe, Australia and the western US and populations in the eastern US. Additionally, I tested the hypothesis that populations of *E. necator* in the eastern US are structured based on geography and *Vitis* host species. Multilocus sequencing analysis of three nuclear gene regions from 146 isolates of *E. necator* is consistent with the hypothesis that introduced populations are derived from two separate introductions from the eastern US. The invasion history of *E. necator* follows a pattern consistent with plant-mediated dispersal. *E. necator* shows geographic structure, but no genetic structure across *Vitis* host species, except with respect to *V. rotundifolia*. In ascomycetes, mating compatibility is regulated by the mating-type locus, *MATI*. I identified and sequenced genes at the *MATI* locus in *Erysiphe necator* and developed a PCR-based marker for determining mating type. I designed degenerate primers that amplify conserved regions of *MATI-1* and *MATI-2* in other powdery mildew fungi. The mating-type genes in *E. necator* are similar to those of other Leotiomycetes; however, the structure of the *MATI* locus in *E. necator*, like the *MATI-2* idiomorph of *Blumeria graminis*, is markedly different from other ascomycetes in that it is greatly expanded and may contain a large amount of repetitive DNA. Random mating and recombination in heterothallic fungi should result in high genotypic diversity, 1:1 mating-type ratios, and random associations of alleles at different loci, *i.e.*, linkage equilibrium. I sampled

isolates from vineyards in Burdett, NY and Winchester, VA. Isolates were genotyped for mating type and 11 SSR markers. After clone correction, mating-type ratios in the three populations did not deviate from 1:1. Genotypic diversity was high, but even with clone correction, I detected significant linkage disequilibrium in all populations. Vineyard populations were spatially structured, which likely results from short dispersal distances. Overall, these results suggest that selection for clonal genotypes and spatial genetic aggregation during the asexual phase of the epidemic contribute to persistent linkage disequilibrium even though populations undergo an annual sexual cycle.

## BIOGRAPHICAL SKETCH

Marin Talbot Brewer grew up in Mentor, Ohio. She attended the University of Cincinnati and received a Bachelor of Science degree in 1998. In 2003, Marin received a Master of Science degree in Plant, Soil, and Environmental Science from the University of Maine where she worked on the biological and cultural control of Rhizoctonia disease of potato. After completion of her Masters degree, she worked for three years as a Research Associate in the Department of Horticulture and Crop Science at The Ohio State University. Marin began her graduate studies in the Department of Plant Pathology and Plant-Microbe Biology at Cornell University in 2006.

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## CHAPTER 1

### PHYLOGEOGRAPHY AND POPULATION STRUCTURE OF THE GRAPE POWDERY MILDEW FUNGUS, *ERYSIPHE NECATOR*, FROM DIVERSE *VITIS* SPECIES \*

#### ***Abstract***

The grape powdery mildew fungus, *Erysiphe necator*, was introduced into Europe more than 160 years ago and is now distributed everywhere that grapes are grown. To understand the invasion history of this pathogen we investigated the evolutionary relationships between introduced populations of Europe, Australia and the western US and populations in the eastern US, where *E. necator* is thought to be native. Additionally, we tested the hypothesis that populations of *E. necator* in the eastern US are structured based on geography and *Vitis* host species. We sequenced three nuclear gene regions covering 1803 nucleotides from 146 isolates of *E. necator* collected from the eastern US, Europe, Australia, and the western US. Phylogeographic analyses show that the two genetic groups in Europe represent two separate introductions and that the genetic groups may be derived from eastern US ancestors. Populations from the western US and Europe share haplotypes, suggesting that the western US population was introduced from Europe. Populations in Australia are derived from European populations. Haplotype richness and nucleotide diversity were significantly greater in the eastern US populations than in the introduced populations. Populations within the eastern US are geographically differentiated;

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however, no structure was detected with respect to host habitat (i.e., wild or cultivated). Populations from muscadine grapes, *V. rotundifolia*, are genetically distinct from populations from other *Vitis* host species, yet no differentiation was detected among populations from other *Vitis* species. Multilocus sequencing analysis of the grape powdery mildew fungus is consistent with the hypothesis that populations in Europe, Australia and the western US are derived from two separate introductions and their ancestors were likely from native populations in the eastern US. The invasion history of *E. necator* follows a pattern consistent with plant-mediated dispersal; however, more exhaustive sampling is required to make more precise conclusions as to origin. *E. necator* shows no genetic structure across *Vitis* host species, except with respect to *V. rotundifolia*.

### ***Introduction***

Introduced pathogens have led to devastating epidemics in naïve host populations that lack evolved defences, as demonstrated by the plant pathogen *Cryphonectria parasitica*, the fungus that causes chestnut blight. Its introduction from Asia (Milgroom et al., 1996) practically eliminated the American chestnut (*Castanea dentata*) and markedly altered the species composition of forests throughout eastern North America. Source pathogen populations are expected to be more diverse than introduced populations because introduced populations have smaller effective population sizes due to losses in genetic diversity from population bottlenecks and genetic drift associated with small founder population sizes (Nei et al., 1975; Dlugosch and Parker, 2008). However, this pattern could be reversed if multiple divergent lineages from separate sources colonize an area (Petit et al., 2003; Genton et al., 2005). Where introductions are few, haplotypes in introduced populations should be a subset of those in the source

population (May et al., 2006; Pringle et al., 2009). Additionally, for sexually reproducing organisms, recombination from sexual reproduction may be more prevalent in source or native populations, whereas clonal reproduction may dominate in introduced or marginal populations since multiple mating types necessary for sexual reproduction may not be present (Goodwin et al., 1994; Eckert, 2001; Milgroom et al., 2008). However, lack of variation in introduced populations can make it difficult to detect recombination.

The focus of this research is the invasion history and population structure of the grape powdery mildew fungus, *Erysiphe necator* (formerly *Uncinula necator*), an obligate parasite of *Vitis* species that was introduced into Europe and, eventually, all other wine-producing regions of the world. Historical records support the hypothesis that the source of the introduction is eastern North America (Weltzien, 1978). Powdery mildew was described on grapes in North America in 1834, prior to its discovery in Europe in 1845 (Large, 1940). Eastern North America is the center of origin for many wild species of *Vitis* that have relatively high levels of resistance to many diseases and pests of grapevines, including powdery mildew (Lepik, 1970; Lenné and Wood, 1991). After its introduction to Europe, grape powdery mildew was observed throughout all wine-producing regions of the world, including California in 1859 (Smith, 1961) and Australia in 1866 (Emmett et al., 1990). *E. necator* most likely dispersed long distances by the movement of grapevines, which were frequently traded between continents in the mid-1800's and later. *E. necator* remains dormant as mycelium in dormant buds, or as sexual spores in cleistothecia in the bark of vines (Pearson and Gaertel, 1985; Pearson and Gadoury, 1987).

Population genetic studies on *E. necator* to date have been limited to introduced populations in Europe and Australia where two distinct, yet sympatric, genetic groups have been consistently found (Délye et al., 1997; Evans et al., 1997; Stummer et al., 2000; Miazzi et al.,

2003; Péros et al., 2005; Núñez et al., 2006; Montarry et al., 2008). The groups, designated as A and B (or groups I and III in earlier studies), were originally identified using anonymous markers assayed by RAPDs, ISSRs and AFLPs. Subsequent gene sequence analysis detected fixed nucleotide differences between groups at several nuclear loci, including 14  $\alpha$ -demethylase (*CYP51*) and the internal transcribed spacer (*ITS*) regions of ribosomal DNA (rDNA) (Délye et al., 1999), and beta-tubulin (*TUB2*) (Amrani and Corio-Costet, 2006). In India, a third genetic group was found, defined by RAPDs and a unique *ITS* sequence (Délye et al., 1997; Délye et al., 1999). Small differences in reproductive fitness (Montarry et al., 2008) and temporal variation have been found between groups A and B (Délye et al., 1999; Miazzi et al., 2003; Péros et al., 2005; Montarry et al., 2008) leading to the hypothesis that temporal variation between the groups may be maintaining the differentiation by preventing interbreeding (Montarry et al., 2009). Group A is genetically less diverse than group B, thus it has been suggested that it is clonal, whereas group B is sexual reproducing (Délye et al., 1997; Péros et al., 2005). Groups A and B produce viable sexual progeny (ascospores) in laboratory crosses (Stummer et al., 2000; Miazzi et al., 2003; Stummer and Scott, 2003), but recombinants have not been found in nature.

We had two major objectives for this study. First, to understand the evolutionary processes that led to the existence of groups A and B of *E. necator* in introduced populations, we tested the hypothesis that A and B were derived from separate introductions, as opposed to diverging after their introduction. To address this question, it was essential to study the population structure in eastern North America, the putative source population. Because no information was available on the population genetics of *E. necator* in North America, our major second objective was to describe the diversity and population structure in the eastern US. We tested the hypothesis that if the eastern US population was a potential source of introductions,

haplotypes found in introduced populations of Europe, Australia, and the western US would also be found in the eastern US. Moreover, we predicted that populations in the eastern US would have greater haplotype and nucleotide diversity than introduced populations. Finally, we tested the hypotheses that the population in the eastern US is structured by geography, *Vitis* host species, or host habitat (wild or cultivated *Vitis*).

## **Results**

**Genetic diversity in eastern US and introduced populations.** We obtained 146 isolates of *E. necator* from diverse wild and cultivated *Vitis* species collected from the eastern US (northeast, southeast and central) and from cultivated *V. vinifera* from the western US, Europe, and Australia (Table 1.1). We also collected isolates of powdery mildew (*E. necator* var. *ampelopsidis* (Braun and Takamatsu, 2000)) from *Parthenocissus quinquefolia*. We sequenced a total of 1803 nucleotides from three nuclear gene regions: the internal transcribed spacer and the intergenic spacer regions of nuclear rDNA (*ITS/IGS*), beta-tubulin (*TUB2*), and translation elongation factor 1- $\alpha$  (*EF1- $\alpha$* ). We were unable to amplify *IGS* from isolates sampled from *P. quinquefolia*.

However, *E. necator* var. *ampelopsidis* from *P. quinquefolia* is markedly divergent with 94.9%, 93.0%, and 91.7% similarity to the consensus sequence of isolates from *Vitis* spp. for *ITS*, *TUB2*, and *EF1- $\alpha$* , respectively. For comparison, the lowest sequence similarity within *E. necator* from *Vitis* spp. was 99.8%, 99.5%, and 99.4%. Among *E. necator* from *Vitis* spp. there were 37 segregating sites and 45 multilocus haplotypes (Table 1.2). *EF1- $\alpha$*  contained the most segregating sites, followed by *TUB2*, and *ITS/IGS*. All of the polymorphisms in *EF1- $\alpha$*  and *TUB2* were found in introns or as synonymous substitutions in coding regions.

**Table 1.1: *Vitis* host species, host habitats and geographic regions where *Erysiphe necator* was collected.**

| <i>Vitis</i> Host Species   | Wild | Cultivated | Host Regions  |
|---|------|------------|---|
| <i>V. vinifera</i> (European wine grape)                                      |      | 71         | southeast US, central US, northeast US, western US, Europe, Australia |
| Vinifera hybrids <sup>1</sup>   |      | 29         | southeast US, central US, northeast US                                |
| <i>V. labrusca</i> and <i>labrusca</i> hybrids <sup>2</sup> (e.g., ‘Concord’) | 7    | 9          | southeast US, northeast US  |
| <i>V. aestivalis</i>  | 13   |            | southeast US, central US, northeast US                                |
| <i>V. riparia</i>   | 12   |            | southeast US, central US, northeast US                                |
| <i>V. rotundifolia</i> (muscadine) <sup>3</sup>                               | 2    | 3          | southeast US  |

<sup>1</sup>vinifera hybrids refer to interspecific hybrids derived from crosses between the European wine grape, *V. vinifera*, and wild American *Vitis* species other than *V. labrusca*.

<sup>2</sup>*labrusca* hybrids (*i.e.* ‘Concord’ and ‘Niagara’; sometimes referred to as *V. labruscana*) refer to *V. vinifera* x *V. labrusca* hybrids that are derived primarily from *V. labrusca* because of repeated backcrossing.

<sup>3</sup>Sometimes referred to as *Muscadinia rotundifolia*.

Based on measurements of Tajima’s *D* (Tajima, 1989), *ITS/IGS* and *EF1- $\alpha$*  do not deviate from neutral evolution (Table 1.3). However, *TUB2* deviates significantly from neutrality in the eastern US population. Significant negative values for Tajima’s *D* can result from population bottlenecks followed by rapid population expansion or from selective sweeps acting on or near the loci under investigation. Since this effect is not detected across the entire genome, as would be expected with demographic effects, this is suggestive that the deviation from neutrality in *TUB2* is from selection.

**Table 1.2: Haplotypes and polymorphic sites among isolates of *Erysiphe necator* based on partial sequences of three gene regions.**

| Haplotype <sup>1</sup> | N  | Polymorphic Sites <sup>2</sup> |              |                 | Region (N) <sup>3</sup> | Host <i>Vitis</i> spp. (N) <sup>4</sup> |
|------------------------|----|--------------------------------|--------------|-----------------|-------------------------|---|
|                        |    | ITS / IGS                      | TUB2         | EF1- $\alpha$   |                         |   |
| 1 ( <i>aaa</i> )       | 16 | TGGTGG / CGGTC                 | TGCCTTTATCCC | CTCGACCATTTTCGC | SE (8), NE (8)          | vin (8), hyb (3), aes (2), rip (3)      |
| 2 ( <i>aab</i> )       | 4  | ..... / .....                  | .....        | .....C.....     | NE (4)                  | vin (2), hyb (1), rip (1)               |
| 3 ( <i>aac</i> )       | 1  | ..... / .....                  | .....        | .....T..        | NE (1)                  | hyb (1)                                 |
| 4 ( <i>aad</i> )       | 1  | ..... / .....                  | .....        | .....C....      | SE (1)                  | aes (1)                                 |
| 5 ( <i>aae</i> )       | 3  | ..... / .....                  | .....        | .....A.....     | NE (3)                  | hyb (2), lab (1)                        |
| 6 ( <i>aaf</i> )       | 4  | ..... / .....                  | .....        | .....A.G.....   | NE (4)                  | hyb (2), lab (1), rip (1)               |
| 7 ( <i>aag</i> )       | 1  | ..... / .....                  | .....        | TC.....         | NE (1)                  | rip (1)                                 |
| 8 ( <i>aah</i> )       | 7  | ..... / .....                  | .....        | TC...A.....A.   | SE (2), NE (5)          | hyb (3), lab (1), aes (1), rip (2)      |
| 9 ( <i>aai</i> )       | 6  | ..... / .....                  | .....        | .....T.....T    | SE (1), NE (5)          | vin (1), lab (1), aes (3), rip (1)      |
| 10 ( <i>aaj</i> )      | 6  | ..... / .....                  | .....        | .....T.....T    | NE (6)                  | hyb (5), lab (1)                        |
| 11 ( <i>aak</i> )      | 5  | ..... / .....                  | .....        | ...T.....T      | SE (3), C (1), NE (1)   | vin (2), hyb (2), rip (1)               |
| 12 ( <i>aal</i> )      | 2  | ..... / .....                  | .....        | .....C..T       | SE (1), NE (1)          | vin (2)                                 |
| 13 ( <i>aam</i> )      | 1  | ..... / .....                  | .....        | ...G.....C..T   | NE (1)                  | vin (1)                                 |
| 14 ( <i>aba</i> )      | 3  | ..... / .....                  | ..T.....     | .....           | SE (3)                  | vin (1), lab (1), aes (1)               |
| 15 ( <i>abb</i> )      | 1  | ..... / .....                  | ..T.....     | .....C.....     | SE (1)                  | lab (1)                                 |
| 16 ( <i>abl</i> )      | 1  | ..... / .....                  | ..T.....     | .....C..T       | C (1)                   | hyb (1)                                 |
| 17 ( <i>abn</i> )      | 1  | ..... / .....                  | ..T.....     | .....A.         | C (1)                   | rip (1)                                 |
| 18 ( <i>ach</i> )      | 1  | ..... / .....                  | .C.....      | TC...A.....A.   | C (1)                   | vin (1)                                 |
| 19 ( <i>acl</i> )      | 2  | ..... / .....                  | .C.....      | .....C..T       | C (2)                   | vin (1), hyb (1)                        |
| 20 ( <i>aco</i> )      | 1  | ..... / .....                  | .C.....      | ..A.....C..T    | C (1)                   | hyb (1)                                 |
| 21 ( <i>adb</i> )      | 1  | ..... / .....                  | C.....       | .....C.....     | NE (1)                  | vin (1)                                 |
| 22 ( <i>aeh</i> )      | 2  | ..... / .....                  | .....T..     | TC...A.....A.   | NE (2)                  | hyb (2)                                 |
| 23 ( <i>afn</i> )      | 2  | ..... / .....                  | .....T....   | .....A.         | C (2)                   | vin (2)                                 |
| 24 ( <i>agk</i> )      | 1  | ..... / .....                  | .....T.      | ...T.....T      | SE (1)                  | vin (1)                                 |
| 25 ( <i>baa</i> )      | 3  | ..... / ....T                  | .....        | .....           | SE (1), NE (2)          | lab (3)                                 |
| 26 ( <i>bab</i> )      | 2  | ..... / ....T                  | .....        | .....C.....     | NE (2)                  | vin (1), lab (1)                        |

**Table 1.2 (continued)**

|                   |    |                 |             |                         |                                     |
|-------------------|----|-----------------|-------------|-------------------------|-------------------------------------|
| 27 ( <i>bai</i> ) | 1  | ..... / .....T  | .....T      | NE (1)                  | lab (1)                             |
| 28 ( <i>bal</i> ) | 1  | ..... / .....T  | .....C..T   | NE (1)                  | vin (1)                             |
| 29 ( <i>bba</i> ) | 1  | ..... / .....T  | ..T.....    | NE (1)                  | hyb (1)                             |
| 30 ( <i>bbl</i> ) | 1  | ..... / .....T  | ..T.....    | NE (1)                  | hyb (1)                             |
| 31 ( <i>bha</i> ) | 2  | ..... / .....T  | ..T..A..... | NE (2)                  | lab (2)                             |
| 32 ( <i>bia</i> ) | 1  | ..... / .....T  | .....C..... | NE (1)                  | lab (1)                             |
| 33 ( <i>cba</i> ) | 23 | ..... / ...CT   | ..T.....    | SE (8), EU (13), AU (2) | vin (18), hyb (1), aes (2), rip (2) |
| 34 ( <i>dba</i> ) | 1  | ..... / ..CCT   | ..T.....    | SE (1)                  | rot (1)                             |
| 35 ( <i>dja</i> ) | 4  | ..... / ..CCT   | ..TG.....   | SE (4)                  | rot (4)                             |
| 36 ( <i>eba</i> ) | 1  | ....C. / .....  | ..T.....    | NE (1)                  | vin (1)                             |
| 37 ( <i>fae</i> ) | 1  | .T.... / .....  | .....A..... | SE (1)                  | hyb (1)                             |
| 38 ( <i>gac</i> ) | 1  | .....A / .....  | .....T..    | C (1)                   | hyb (1)                             |
| 39 ( <i>hac</i> ) | 1  | ..A... / .....  | .....T..    | SE (1)                  | aes (1)                             |
| 40 ( <i>ika</i> ) | 1  | ...A.. / .....T | ..T.C.....  | NE (1)                  | aes (1)                             |
| 41 ( <i>jai</i> ) | 18 | C..... / T..... | .....T      | W (12), EU (6)          | vin (18)                            |
| 42 ( <i>jak</i> ) | 1  | C..... / T..... | ...T.....T  | EU (1)                  | vin (1)                             |
| 43 ( <i>jli</i> ) | 3  | C..... / T..... | .....T      | W (1), EU (2)           | vin (3)                             |
| 44 ( <i>jll</i> ) | 2  | C..... / T..... | .....T      | EU (2)                  | vin (2)                             |
| 45 ( <i>kmi</i> ) | 4  | C..... / TA...  | .....C...   | AU (4)                  | vin (4)                             |

<sup>1</sup>Numbers refer to the multilocus haplotypes; letters in parentheses refer to the haplotypes for *ITS/IGS*, *TUB2*, and *EF1- $\alpha$* , respectively. Group A is represented by haplotype 33 and group B is represented by haplotypes 41-45.

<sup>2</sup>Only segregating sites are listed, which correspond to the following nucleotide positions in the referenced GenBank accessions: *ITS* (GQ255473; total length 591 nucleotides): 48, 84, 86, 170, 420, 462; *IGS* (GQ255476; 347 nt): 108, 206, 211, 216, 223; *TUB2* (GQ255475; 442 nt): 24, 37, 79, 82, 128, 183, 207, 288, 316, 344, 356, 368; *EF1- $\alpha$*  (GQ255471; 423 nt): 2, 9, 25, 33, 102, 189, 210, 227, 228, 231, 336, 381, 384, 420.

<sup>3</sup>regions are: SE = southeast US, C = central US, NE = northeast US, W = western US, EU = Europe, AU = Australia.

<sup>4</sup>hosts are: vin = *V. vinifera*, hyb = *V. vinifera* hybrids, lab = *V. labrusca* and *V. labrusca* hybrids, aes = *V. aestivalis*, rip = *V. riparia*, rot = *V. rotundifolia*. *N* = the number of isolates from each host with the designated haplotype.

**Table 1.3: Haplotype richness, sequence diversity and neutrality estimates for populations of *Erysiphe necator*.**

| Locus                          | Population <sup>1</sup> | Haplotype richness ( $h_R$ ) <sup>2</sup> | Watterson's $\theta$ ( $\theta_w$ ) | $\pi$                         | Tajima's $D$ ( $P$ -value) |
|--------------------------------|-------------------------|---|-------------------------------------|-------------------------------|----------------------------|
| <i>ITS/IGS</i>                 | Eastern US              | <b>6</b> (9) <sup>3</sup>                 | 0.00123                             | 0.00085                       | -1.167 (0.108)             |
|                                | Introduced              | <b>3</b>                                  | 0.00123                             | 0.00217                       | 1.901 (0.975)              |
|                                |                         | <b><math>P = 0.013</math></b>             | $P = 0.485$                         | $P = 1.000$                   |                            |
| <i>TUB2</i>                    | Eastern US              | <b>7</b> (11)                             | <b>0.00314</b>                      | 0.00159                       | <b>-1.598 (0.013)</b>      |
|                                | Introduced              | <b>4</b>                                  | <b>0.00157</b>                      | 0.00192                       | 0.478 (0.727)              |
|                                |                         | <b><math>P = 0.049</math></b>             | <b><math>P = 0.050</math></b>       | $P = 0.833$                   |                            |
| <i>EF1-<math>\alpha</math></i> | Eastern US              | <b>11</b> (15)                            | <b>0.00601</b>                      | <b>0.00472</b>                | -0.680 (0.270)             |
|                                | Introduced              | <b>4</b>                                  | <b>0.00164</b>                      | <b>0.00142</b>                | -0.281 (0.411)             |
|                                |                         | <b><math>P &lt; 0.001</math></b>          | <b><math>P = 0.001</math></b>       | <b><math>P = 0.001</math></b> |                            |
| Combined                       | Eastern US              | <b>21</b> (40)                            | <b>0.00282</b>                      | 0.00194                       | -                          |
|                                | Introduced              | <b>6</b>                                  | <b>0.00141</b>                      | 0.00193                       | -                          |
|                                |                         | <b><math>P &lt; 0.001</math></b>          | <b><math>P = 0.001</math></b>       | $P = 0.497$                   |                            |

<sup>1</sup>The introduced population comprises Europe, Australia and the western US; the eastern US comprises northeast, southeast and central US (Table 1.1).

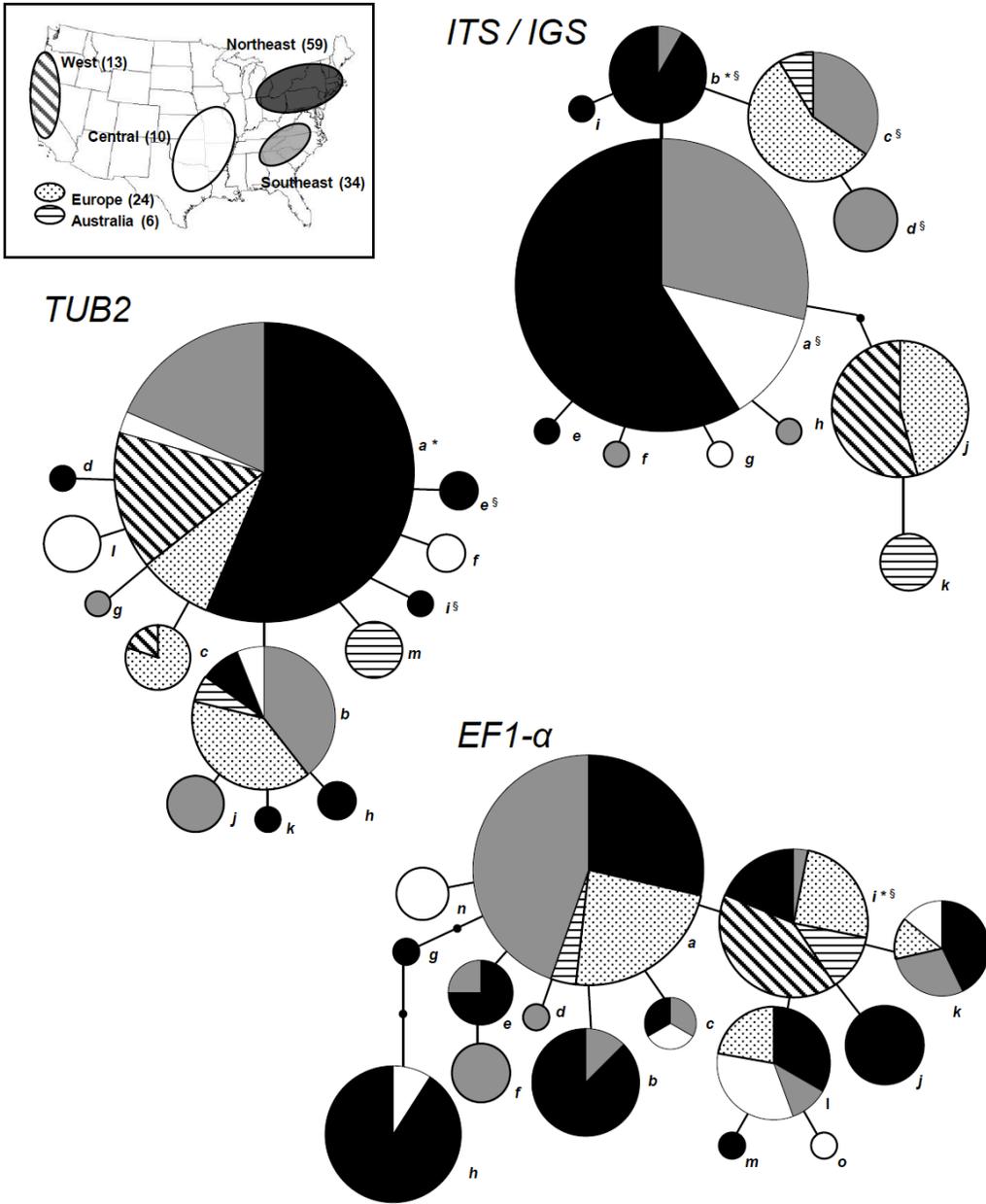
<sup>2</sup>To account for sample size differences in eastern US ( $N = 103$ ) and introduced populations ( $N = 43$ ), we used rarefaction analysis [64] for haplotype richness,  $\theta_w$ , and  $\pi$  in the native population. Diversity estimates where the eastern US population is significantly more diverse than the introduced populations ( $P < 0.05$ ) are in bold.

<sup>3</sup>Numbers in parentheses are the number of observed haplotypes among the 103 isolates from the eastern US population without correction by rarefaction analysis.

We estimated several population genetic parameters in the eastern US and introduced populations including: haplotype richness ( $h_R$ ), Watterson's theta ( $\theta_w$ ), and pairwise nucleotide diversity ( $\pi$ ). Haplotype richness is significantly greater in the eastern US than in the introduced population for each locus and the multilocus haplotypes (Table 1.3), even when adjustments are made for differences in sample size (see Methods). Additionally, there is greater nucleotide polymorphism ( $\theta_w$ ) in the eastern US population for *TUB2*, *EF1- $\alpha$*  and for all three loci combined. There is greater pairwise nucleotide diversity ( $\pi$ ) for *EF1- $\alpha$*  in the eastern US population. However,  $\pi$  is greater in the introduced population for *ITS/IGS*.

**Phylogeography.** To determine evolutionary relationships among isolates, we constructed networks for the three gene regions and multilocus haplotypes (Figures 1.1 and 1.2). Ancestral haplotypes identified based on rooting probability (Castelloe and Templeton, 1994; Templeton et al., 1995) and maximum parsimony using *E. necator* var. *ampelopsidis* as an outgroup were from the eastern US in all cases. Based on maximum parsimony the outgroup haplotypes would be at least 23 mutational steps from the putative ancestors for *ITS/IGS*, 29 steps for *TUB2*, 35 steps for *EF1- $\alpha$* , and at least 87 steps for the combined multilocus haplotype. Because of this degree of divergence TCS did not place the outgroup in the same network. The internal position of haplotypes from eastern North America is particularly noticeable for the multilocus network (Figure 1.2), whereas all haplotypes of isolates from introduced populations (represented by striped and stippled patterns) are at or near the tips of the network. Isolates from the western US have the same haplotypes (nos. 41 and 43) as isolates in group B from Europe (Table 1.2, Figures 1.1 and 1.2), which suggests that populations in the western US were introduced from Europe. In addition to the 13 isolates from the western US reported here, 17 isolates from California and one from Oregon had the same *IGS* sequence that is found only in group B (data not shown). We did not sequence additional loci for these isolates from the western US because all were like group B for *IGS*.

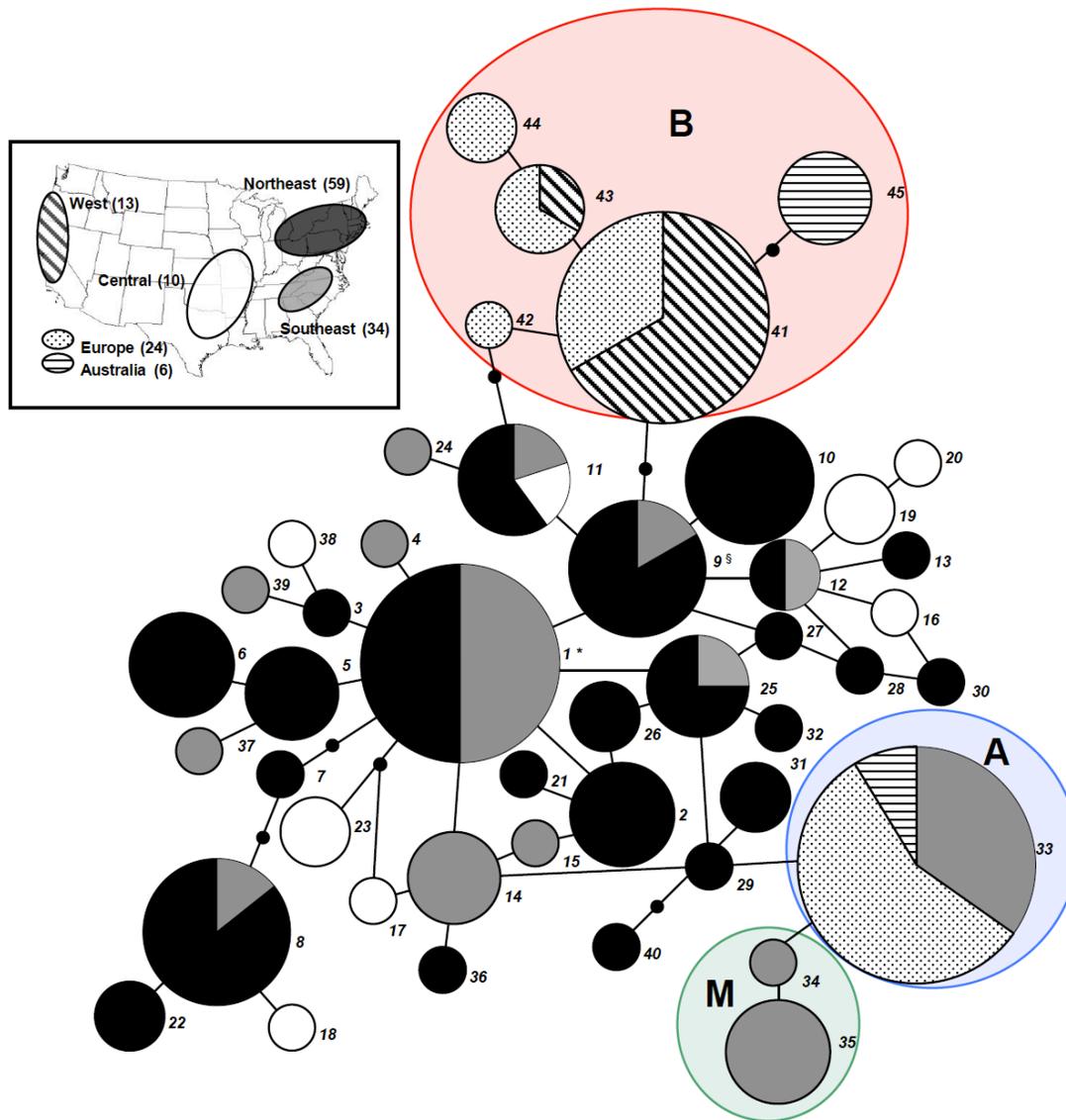
The majority of haplotypes for the individual loci, and especially for the multilocus network, are represented by individuals from the eastern US populations, demonstrating that populations in the eastern US are more diverse than in Europe, Australia and the western US. The sample size from the eastern US population is larger; however, this difference is accounted for in the comparisons of diversity estimates. Although isolates from the central US are



**Figure 1.1.** Haplotype networks of *ITS/IGS*, *TUB2* and *EF1- $\alpha$* . Networks constructed in TCS 1.21. Each haplotype is represented as a circle proportional in size to the number of isolates in each haplotype. Inferred intermediate haplotypes are represented by a small solid dot. Each line segment represents a single mutation. The letters defining haplotypes in Table 1.2 are shown to the right of each node. Geographic origins of isolates in each haplotype are proportionally represented in pie charts by different patterns shown in the key in the centre of the figure. The ancestral haplotypes determined by root probability are indicated by asterisks (\*), whereas those determined by maximum parsimony using *E. necator* var. *ampelopsidis* as the outgroup are indicated by §.

represented by diverse haplotypes, they are mostly derived haplotypes at or near the tips of the multilocus network, which suggests that this region is peripheral to the centre of diversity. All isolates obtained from *V. rotundifolia* belonged to two multilocus haplotypes (nos. 34 and 35; labelled 'M' in Figure 1.2) at the tips, derived from group A (haplotype no. 33) and not shared by isolates from any other host species.

The haplotype networks show that genetic groups A and B from introduced populations are distinct from each other at all loci and are derived from North American ancestors (Figures 1.1 and 1.2). Because of these differences, and the internal position of North American haplotypes, groups A and B almost certainly represent two separate introductions instead of diverging after introduction. We found no genetic variation among group A isolates, and this same multilocus haplotype (no. 33) was common in the southeastern US in isolates from diverse wild and cultivated host species, including *V. vinifera*, *vinifera* hybrids, *V. aestivalis*, and *V. riparia* (Table 1.2). In contrast, we found five multilocus haplotypes (nos. 41-45) forming a discrete lineage in group B; none of these multilocus haplotypes was found in eastern North America. However, group B does not differ from the eastern US population at all loci (Figure 1.1; Table 1.2). It differs for *ITS/IGS*, yet shares several haplotypes for *EF1- $\alpha$*  and *TUB2* with the eastern US population.



**Figure 1.2.** Multilocus haplotype network for *Erysiphe necator*. Network constructed in TCS 1.21. Each haplotype is represented as a circle proportional in size to the number of isolates in each haplotype. Inferred intermediate haplotypes are represented by a small solid dot. Each line segment represents a single mutation. The numbers defining multilocus haplotypes in Table 1.2 are shown to the right of each node. Geographic origins of isolates in each haplotype are proportionally represented in pie charts by different patterns shown in the key in the upper left. The haplotypes that include group B isolates are enclosed in a magenta ellipse and marked with a ‘B’; the haplotype that includes group A isolates is enclosed in a green ellipse and marked with an ‘A’; and the haplotypes that include isolates from muscadine grapes (*V. rotundifolia*) are enclosed in a blue ellipse and are marked with an ‘M’. The ancestral haplotype determined by root probability is indicated by an asterisk (\*), whereas that determined by maximum parsimony using *E. necator* var. *ampelopsidis* as an outgroup is indicated by §.

**Population differentiation.** Differentiation was estimated between the eastern US and introduced populations, and among geographic regions, *Vitis* host habitats, and *Vitis* host species in eastern US. Eastern US and introduced populations are significantly differentiated (Table 1.4). Within the eastern US, we detected significant differentiation among geographic regions (southeast US, northeast US, and central US). Geographic differentiation within the eastern US was detected when isolates from all hosts were included in the analysis. Because many hosts are more abundant in particular geographic regions (for example, *V. riparia* in the northeast US or *V. rotundifolia* in the southeast US), we conducted tests of geographic subdivision on isolates collected only from *V. vinifera* and vinifera hybrid hosts, which are found abundantly among the three regions, to avoid confounding host and geography. Geographic subdivision was still evident when the analyses only included isolates from *V. vinifera* and vinifera hybrid hosts (Table 1.4).

There was no differentiation between populations from wild and cultivated host habitats or among host species within geographic regions (Table 1.4), except when isolates from *V. rotundifolia* were included in the analyses.

**Table 1.4: Population structure of *Erysiphe necator* by geographic region and host species.**

| Populations compared (sample sizes are in parentheses)   | $S_{nn}^1$                             | $H_{ST}^1$                             |
|--|--|--|
| Eastern US (103) vs. introduced (43) populations   | <b>0.9182</b><br>( <b>&lt; 0.001</b> ) | <b>0.0627</b><br>( <b>&lt; 0.001</b> ) |
| Within the eastern US  |  |  |
| by geographic region   | <b>0.6856</b>                          | <b>0.0280</b>                          |
| northeast (59) vs. southeast (34) vs. central (10)   | ( <b>&lt; 0.001</b> )                  | ( <b>&lt; 0.001</b> )                  |
| by geographic region on <i>Vitis vinifera</i> and vinifera hybrid hosts  | <b>0.6384</b>                          | <b>0.0396</b>                          |
| northeast (33) vs. southeast (15) vs. central (9)  | ( <b>&lt; 0.001</b> )                  | ( <b>0.001</b> )                       |
| by host agro-ecological habitat <sup>2</sup>   | 0.5733                                 | 0.0002                                 |
| cultivated (59) vs. wild (34) hosts  | (0.133)                                | (0.407)                                |
| by host species within the northeast US  | 0.3532                                 | 0.0051                                 |
| <i>V. vinifera</i> (13) vs. vinifera hybrid (20) vs. <i>V. labrusca</i> (10) vs. <i>V. riparia</i> (8)                                 | (0.064)                                | (0.333)                                |
| by host species within the southeast US including <i>V. rotundifolia</i> <sup>3</sup>  | <b>0.3529</b>                          | <b>0.0857</b>                          |
| <i>V. rotundifolia</i> (5) vs. <i>V. vinifera</i> (11) vs. vinifera hybrid (4) vs. <i>V. labrusca</i> (3) vs. <i>V. aestivalis</i> (9) | ( <b>0.008</b> )                       | ( <b>0.007</b> )                       |
| by host species within the southeast US excluding <i>V. rotundifolia</i>   | 0.2552                                 | -0.0179                                |
| <i>V. vinifera</i> (11) vs. vinifera hybrid (4) vs. <i>V. labrusca</i> (3) vs. <i>V. aestivalis</i> (9)                                | (0.712)                                | (0.709)                                |

<sup>1</sup>The nearest neighbour statistic ( $S_{nn}$ ) measures the proportion of times the most similar sequence ('nearest neighbour') is from the same population.  $H_{ST}$  is a measure of population subdivision that estimates  $F_{ST}$  among haplotypes. Significant differentiation between or among populations ( $\alpha = 0.05$ ) is shown in bold.  $P$ -values are in parentheses.

<sup>2</sup>Isolates from the central US were excluded because all but one were from cultivated species.

<sup>3</sup>A single isolate from *V. riparia* was excluded from the southeast population.

## *Discussion*

**Patterns of introduction and invasion.** The multilocus haplotype network (Figure 1.2) demonstrates that the eastern US population is ancestral to the introduced populations (Castelloe and Templeton, 1994; Templeton et al., 1995). Therefore, our results are consistent with the hypothesis that *E. necator* was introduced into Europe from eastern North America (Weltzien, 1978) because populations in Italy and France are derived from North American ancestors. Additionally, populations in California share haplotypes with populations from Europe, suggesting the possibility that they could have been introduced from Europe; the reverse direction of introduction is less likely given the historical records of trade in grapevines and that grape powdery mildew was first observed in Europe in 1845, but not in California until 1859. However, we cannot make any firm conclusions because we do not know which genetic group, A or B, was introduced into Europe first. Populations in the eastern US are more genotypically diverse than those in Europe, Australia and the western US. Several of the haplotypes for individual loci in the introduced populations were found in eastern North America, which is expected when comparing source and introduced populations. An alternative hypothesis, that *E. necator* was introduced into Europe from Japan has been suggested (Salmon, 1900), but there is no evidence to support this claim and we were unable to obtain samples from Japan.

At least two haplotypes of powdery mildew, progenitors of groups A and B, were introduced and successfully invaded Europe and Australia. If there had been a single introduction, individuals in the introduced populations would represent a monophyletic group; the single introduction hypothesis can be rejected based on the relationships of group A and B haplotypes in the multilocus network (Figure 1.2). The introductions, and successful invasions, may have occurred at separate times or multiple, distinct haplotypes may have been introduced

during a single event. Previous studies in French and Australian populations of *E. necator*, based on anonymous markers, also found greater diversity in group B than in group A (Stummer et al., 2000; Péros et al., 2005) and it has been hypothesized that group A is predominantly asexual, while group B undergoes sexual recombination. Most studies have shown that group A consists of a single mating type (Délye et al., 1997; Stummer et al., 2000; Péros et al., 2005). We expected to find both genetic groups from the introduced populations in the eastern US population. However, we found isolates in the southeastern US with the same multilocus haplotype as that in group A, but we did not find any with the same haplotype as those in group B. Group B haplotypes may have diverged by genetic drift from the original founders since the first introductions into Europe more than 160 years ago. Sexual reproduction and recombination in group B, coupled with selection for new haplotypes on a different host species and in environmental conditions in Europe, could also have led to divergence. Alternatively, our sample size in eastern North America may not have been large enough to include haplotypes that are less common in the eastern US population, which by chance could have been introduced into Europe. An alternative explanation is that the unique alleles specific to genetic group B came from an entirely different source, which we did not sample for this study.

Genotypic diversity was significantly greater in the eastern US population than in introduced populations, although measures of gene diversity were not always greater (Table 1.3). In fact, pairwise nucleotide diversity ( $\pi$ ) was significantly greater for *ITS/IGS* in the introduced population. One explanation for this finding is that the occurrence of two distinct genetic groups in the introduced populations results in high gene diversity because of fixed nucleotide differences between lineages, but low genotypic diversity because there is little or no variation within groups. This discrepancy is similar to finding high gene diversity combined with low

genotypic diversity in clonal diploid populations with fixed heterozygosity (Balloux et al., 2003; Goyeau et al., 2007). Multiple introductions of distinct lineages from different sources into new ranges can result in greater diversity than expected during an invasion (Petit et al., 2003; Genton et al., 2005). Additionally, gene diversity was overestimated in Europe and Australia because our samples were not random, but rather were artificially constructed with roughly equal numbers of isolates from the two genetic groups, whereas group B is typically found at a greater frequency than group A in populations in Europe (Délye et al., 1997; Miazzi et al., 2003; Péros et al., 2005; Núñez et al., 2006; Montarry et al., 2008). Moreover, the lack of diversity within groups A and B validated our strategy of sequencing relatively small samples from Europe and Australia where extensive sampling only found these two discrete groups (Délye et al., 1997; Evans et al., 1997; Stummer et al., 2000; Miazzi et al., 2003; Péros et al., 2005; Núñez et al., 2006; Montarry et al., 2008). Haplotypic (or allelic) richness is one of the best measures for reductions in diversity associated with population bottlenecks because rare haplotypes are often lost during founder events even if overall gene diversity is not largely affected (Nei et al., 1975).

Our results are consistent with historical records of the movement of grapevines and plant-mediated introductions of *E. necator* into Europe, California, and Australia. After grape powdery mildew spread throughout Europe by the mid-1850s, additional vines were imported from eastern North America as sources of resistance. Unfortunately, this resulted in the introduction of additional grape pests and diseases into Europe, including the phylloxera aphid and downy mildew (Downie, 2002; Gobbin et al., 2006). Additional importations of grapevines from eastern North America for resistance to these pests/diseases may have led also to additional introductions *E. necator*. Secondary introductions of *E. necator* from Europe into California and Australia are also consistent with historical records of the movement of grapevines. During the

1850's and 1860's large collections of *V. vinifera* were brought to California from Europe (Pinney, 1989). Powdery mildew was first described in California in 1859 and in Australia in 1866 (Smith, 1961; Emmett et al., 1990), so it is likely that it was introduced on vines imported at this time. Two of the four group B multilocus haplotypes found in Europe are also found in the western US. It is not clear why both genetic groups were introduced into Australia, but only group B is present in California. It is possible that group A is present in California, but at such a low frequency that we did not sample it. Nevertheless, it is surprising that since its introduction over 150 years ago, additional genotypes of *E. necator* have not been successfully introduced by the movement of vines from the eastern US to Europe, Australia or the western US.

**Absence of host specificity among *Vitis* host species, except *V. rotundifolia*.** With the exception of specialization on muscadine grapes, *V. rotundifolia*, we found no genetic differentiation among populations from *Vitis* host species. This was not unexpected. The best-studied powdery mildew fungus, *Blumeria graminis*, shows specialization among host genera rather than among species within a genus (Wyand and Brown, 2003; Inuma et al., 2007). Similarly, *E. necator* demonstrates host specialization at the level of host genus. Gadoury and Pearson (Gadoury and Pearson, 1991) showed that *E. necator* var. *ampelopsidis* sampled from *P. quinquefolia* was only rarely pathogenic on *Vitis* species. Multilocus sequencing of *E. necator* var. *ampelopsidis*, as in the formae speciales of *B. graminis*, showed that this type of marked host specialization correlates to marked genetic divergence from *E. necator* on *Vitis*. Alternatively, there may be population divergence among *Vitis* hosts, but we are not able to detect it with the conserved genes used in this study. Other fungi show specialization at the level of host species. For example, microsatellite markers, which are more polymorphic than

multilocus sequences, allowing for better detection of differentiation, showed specialization of *Microbotryum violaceum* at the level of host species (Bucheli et al., 2001). The lack of specialization could be also explained by recent colonization of *Vitis* hosts by *E. necator* or recent diversification of *Vitis* species in North America. In closely related species or populations undergoing speciation, genetic divergence may only be evident at one or a few loci involved in adaptation and reproductive isolation (Dopman et al., 2005).

We found that *E. necator* populations from muscadines are genetically distinct from populations on other *Vitis* species. Although, the haplotypes of muscadine isolates differ from those from other *Vitis* species by one to two mutations there is a strong phenotypic difference that is a potential isolating mechanism. Another study demonstrated marked host specialization to muscadine in laboratory inoculations, but not among other *Vitis* species (Frenkel et al., 2010). Although isolates from muscadines could infect other *Vitis* species in the lab, we did not find haplotypes from the muscadine lineage from other *Vitis* species in the field even when they were sympatric with muscadines. Populations of *E. necator* from muscadine and other *Vitis* species could be in the early stages of speciation resulting from host specialization. Alternatively, muscadine isolates may have alleles that evade recognition by host defences in a gene-for-gene interaction. Resistance to powdery mildew controlled by a single, but complex, genetic locus has been demonstrated in muscadines and they are a source of resistance in breeding programs (Barker et al., 2005). It is important to test any new resistant cultivars derived from muscadines with diverse powdery mildew populations from the regions where muscadines are endemic to ensure that the resistance would be durable.

Population differentiation of *E. necator* was not detected between wild and cultivated hosts. In some cases, crop domestication can lead to the divergence of pathogen populations on

wild relatives and crop plants (Couch et al., 2005). Moreover, management strategies or high-density cultivation of crop plants can lead to population differentiation between pathogens from natural ecosystems and agricultural ecosystems (Crouch et al., 2009). The lack of population structure in *E. necator* indicates that gene flow is presently occurring or has occurred historically between the powdery mildew populations from wild and cultivated hosts.

**Conclusions.** Our results are consistent with the hypotheses that populations of the grape powdery mildew fungus, *E. necator*, in Europe are derived from two separate introductions and that their ancestors were likely from native populations in the eastern US. Multilocus sequencing analysis and historical records are also consistent the hypothesis that the initial introductions into Europe were followed by secondary introductions from Europe into the western US and Australia and were likely the result of plant-mediated dispersal in the grapevines that were frequently traded between continents during the time of introductions. Within the eastern US, populations of *E. necator* do not demonstrate divergence based on host habitat or *Vitis* host species, with the exception of specialization to muscadine grapes, *V. rotundifolia*.

### ***Materials and methods***

**Grape powdery mildew pathosystem.** Powdery mildew fungi are haploid ascomycetes that are obligate parasites of plants that produce colonies of superficial hyphae and asexual spores (conidia). *E. necator* infects *Vitis* species and other members of the Vitaceae. *E. necator* can also reproduce sexually if individuals of both of the two mating types are present (Gadoury and Pearson, 1991).

Diverse wild *Vitis* species are found throughout eastern North America (Galet, 1979),

with many of the species demonstrating at least some susceptibility to powdery mildew (Staudt, 1997). We sampled *E. necator* from cultivated grapes and from four of the most common wild species: *V. riparia*, *V. aestivalis*, *V. labrusca*, and *V. rotundifolia* (Table 1.1). *V. riparia* is common in colder regions of central and northeastern North America. Both *V. aestivalis* and *V. labrusca* are distributed throughout the northeastern US and the higher elevations in the southeastern US. The muscadine grape, *V. rotundifolia*, which is endemic to and widely distributed throughout the southeastern US, has considerable resistance to powdery mildew (Olmo, 1986), and is genetically and morphologically distinct from other *Vitis* spp., such that it is sometimes considered to be in a separate genus, *Muscadinia* (Olmo, 1995).

Cultivated varieties grown throughout eastern North America are also diverse. Interspecific hybrids derived from crosses between the European wine grape, *V. vinifera*, and wild American *Vitis* species are common as cultivated vines (Pearson and Gadoury, 1992). Cultivated labrusca hybrids (*i.e.* ‘Concord’ and ‘Niagara’; sometimes referred to as *V. labruscana*) were derived mostly from *V. labrusca* and are grown in colder climates of eastern North America. In contrast to the diversity of hosts in eastern North America, most other major wine-producing regions are dominated by *V. vinifera*, which is native to Eurasia (Zohary and Spiegel-Roy, 1975) and highly susceptible to *E. necator* (Pearson and Goheen, 1988).

**Sampling, isolate maintenance and DNA extraction.** We sampled *E. necator* from the eastern US (northeast, southeast, central) and western US. Most of our sampling from wild host species in the southeast was limited to higher elevations because we were not able to find mildew on wild species other than *V. rotundifolia* at lower elevations of the coastal plain. We speculate that this was due to high temperatures and drought that were not conducive to mildew prior to our

sampling in 2008. Samples from the eastern US were collected to maximize the diversity of host species and host habitats. Samples from France, Italy and Australia were obtained from collaborators who generously sent genomic DNA from *E. necator* isolates collected from cultivated *V. vinifera* and previously identified as genetic group A or B. In this respect, samples from France, Italy and Australia do not represent random samples but they do reflect the diversity found in each country. Populations of *E. necator* in Europe and Australia have been extensively sampled across broad geographic regions (Délye et al., 1997; Evans et al., 1997; Stummer et al., 2000; Miazzi et al., 2003; Péros et al., 2005; Núñez et al., 2006). Among these studies, a total of approximately 1000 *E. necator* isolates were genotyped with various markers, and each study demonstrated that populations are structured into two genetic lineages designated as groups A and B. We reasoned that additional sampling was not necessary in Europe and Australia for this study because little genetic diversity had been found within the two lineages despite extensive sampling from different cultivars, years, and times of year. We consider that our sampling represents the diversity of isolates in Europe and Australia since the isolates came from both genetic groups across different regions of France, Italy and Australia (we were not able to obtain DNA from other locations with published reports of previous genotyping). In fact, the DNAs we sequenced from Australia were identified as having distinct genotypes (Stummer et al., 2000), representative of the total genetic diversity found there previously. *E. necator* was only sampled from *V. vinifera* in Europe and Australia because this is the dominant species present in these regions. Therefore, we did not sample from wild species outside of the eastern US.

Isolates of powdery mildew from *Parthenocissus quinquefolia*, also in the Vitaceae, were collected in Ithaca, NY, USA for comparison with powdery mildew from *Vitis* species. Powdery

mildew from *P. quinquefolia* is considered variety *ampelopsidis* of *E. necator* (Braun and Takamatsu, 2000). Isolates from *Parthenocissus* species exhibit host specialization, although some can infect *V. vinifera* but with greatly reduced growth compared to isolates from *V. vinifera* (Gadoury and Pearson, 1991).

Mildew isolates were maintained as described by Evans *et al.* (1996) on young leaves of *V. vinifera* 'Cabernet Sauvignon' grown in a greenhouse. Leaves were surface-sterilized in 0.6% sodium hypochlorite for 1.5 min, rinsed twice with sterile distilled water and air dried in a sterile laminar flow hood. Leaves were kept in Petri dishes containing 20 ml of 2% water agar. Colonies of *E. necator* were initially isolated by touching a mildew colony from an infected leaf to a surface-sterilized leaf. Asexual spores (conidia) from the resulting colonies were transferred 6-12 days later with a sterile pipette tip to another surface-sterilized leaf at least once to rid the colonies of contaminants prior to DNA extraction. Isolates were maintained by transferring to new leaves approximately once per month.

For DNA extraction, conidia and hyphae were collected from colonies 2-3 weeks after inoculation by touching a 1-cm<sup>2</sup> piece of office tape (Scotch Tape, 3M) to the colony multiple times until the tape was covered in fungal tissue. The tape was placed in a 1.5 mL microcentrifuge tube with 100 µL of 5% chelex (Walsh *et al.*, 1991; Hirata and Takamatsu, 1996), vortexed for 30 sec and incubated at 95 °C for 20 min. The solution was vortexed again for 5 sec centrifuged briefly, and the supernatant was removed and used as the DNA template for PCR.

**Multilocus sequencing, sequence alignment, and haplotype network construction.** Three nuclear loci were PCR-amplified and sequenced from each isolate. The gene regions we

sequenced included: *ITS/IGS*, *TUB2*, and *EF1- $\alpha$* . *ITS* (Délye et al., 1999) and *TUB2* (Amrani and Corio-Costet, 2006) had been identified previously in *E. necator*, whereas *IGS* and *EF1- $\alpha$*  were identified in *E. necator* in this study. For *ITS*, we developed primers ITSEnF: 5'-AAGGATCATTACAGAGCGAGAGG-3' and ITSEnR: 5'-GGATGACCGGACAAAGGTG-3'. For *TUB2*, we designed primers Bt2c: 5'-CAGACTGGCCAATGCGTA-3' and Bt2d: 5'-AGTTCAGCACCTCGGTGTA-3' based on the published sequence (GenBank accession no. AY074934) (Amrani and Corio-Costet, 2006). We identified the *IGS* region in *E. necator* with the conserved ascomycete primers IGS-12a and NS1R (Carbone and Kohn, 1999), then developed primer IGSEn1: 5'-TTTCGGGGGAAAGCCACCA-3' to pair with NS1R for improved PCR amplification. *EF1- $\alpha$*  was identified in *E. necator* by designing degenerate primers to conserved regions of *EF1- $\alpha$*  in *Sclerotinia sclerotiorum* (GenBank accession no. DQ471086) and *Botrytis cinerea* (GenBank accession no. DQ471045). We then developed primers EF1-5: 5'-ATAGCGACGATGAGCTGCTT-3' and EF1-6: 5'-TCGAAAAGGTTTGTTCAGCA-3' for improved PCR amplification. The PCR reactions for *ITS*, *IGS*, and *TUB2* were carried out in a total volume of 25  $\mu$ L. Reaction components included 2.5  $\mu$ L of 10X PCR buffer (Takara Bio, Inc.), 2.5  $\mu$ L dNTPs, 1.25  $\mu$ L of 10  $\mu$ M forward and reverse primers, 0.75 U ExTaq (Takara Bio, Inc.), and 1  $\mu$ L DNA template. Cycling conditions included an initial denaturation at 95  $^{\circ}$ C for 2 min followed by 35 cycles with a denaturation step at 94.5  $^{\circ}$ C for 1 min, annealing at 56  $^{\circ}$ C for 1 min, extension at 72  $^{\circ}$ C for 1 min, followed by a final extension at 72  $^{\circ}$ C for 5 min. PCR products were purified with QIAquick spin columns (QIAGEN). The PCR reaction for *EF1- $\alpha$*  was carried out in a total volume of 50  $\mu$ L with all components added at 2X the volumes used in the reactions for the other loci. Thermal cycling was carried out as described for the other loci. The *EF1- $\alpha$*  PCR products were purified by

electrophoresis in a 1% agarose gel, excision of the band and purification with the QIAEX II Gel Extraction Kit (QIAGEN). All DNA fragments were sequenced at the Cornell University Life Sciences Core Laboratories Centre using the Applied Biosystems Automated 3730 DNA Analyzer with Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase ABI. All gene regions were sequenced in both directions in at least one isolate. Sequences of *EF1- $\alpha$* , *ITS*, *TUB2* and *IGS* for haplotype *I* (Table 1.2) are deposited in GenBank with accession numbers GQ255471, GQ255473, GQ255475, and GQ255476, respectively. Sequences of *EF1- $\alpha$* , *ITS* and *TUB2* and from *E. necator* var. *ampelopsidis* isolates from *P. quinquefolia* are deposited under accession numbers GQ255474, GQ255470 and GQ255472, respectively.

Sequences were aligned and manually edited in SeqMan (DNASTAR, Inc). Haplotype networks were constructed for each locus and for combined multilocus sequences by statistical parsimony with the program TCS 1.21 (Templeton et al., 1992; Clement et al., 2000). Haplotype networks are preferable for intraspecific analyses because they allow for the coexistence of ancestral and derived haplotypes and account for recombination (Posada and Crandall, 2001). Alternative, most parsimonious networks are accounted for by this method by loops in the network. The networks were assembled based on an absolute distance matrix between haplotypes, i.e., the number of mutations separating each haplotype, with a parsimony probability of 95%. The ancestral haplotype for each network was predicted based on rooting probability, which assesses the frequency of a particular haplotype and the number of linkages (Castelloe and Templeton, 1994). We also predicted the ancestral haplotype by maximum parsimony using *E. necator* var. *ampelopsidis* as an outgroup. Outgroup haplotypes could not be incorporated into the network with TCS due to high divergence.

**Estimates of diversity and tests of neutrality.** To compare diversity between source and introduced populations we estimated several population genetic parameters. They include: haplotype richness ( $h_R$ ), the total number of haplotypes; Watterson's theta ( $\theta_w$ ), which is a measure of nucleotide polymorphism equivalent to  $2N_e\mu$  (in a haploid population) and an estimate of the effective population size (Watterson, 1975); and  $\pi$ , the pairwise nucleotide diversity (Nei, 1987). Each parameter was estimated for the eastern US and introduced populations separately using DnaSP v5 (Librado and Rozas, 2009). To avoid the bias in diversity estimates caused by differences in sample sizes, we used bootstrapping to conduct rarefaction analysis (Grünwald et al., 2003). For the eastern US population, we sampled a smaller number of individuals, with replacement, equal to the sample size of the introduced population and estimated  $h_R$ ,  $\theta_w$ , and  $\pi$ . This was repeated 1000 times and the median estimates for each parameter were recorded. We conducted a one-tailed test to determine if eastern US populations were more diverse than introduced populations.  $P$ -values were estimated as the proportion of the null distribution that was less than the observed diversity estimate for the introduced population.

Tajima's D (Tajima, 1989) was calculated by using DnaSP v5 to test for departure from an equilibrium neutral model of evolution. Significant departures from neutrality were determined by permutation tests with 1000 replications.

**Population structure.** Differentiation among geographic regions, host habitats, and host species in eastern North America was estimated on combined multilocus sequences. For these analyses *labrusca* hybrids were grouped with *V. labrusca* rather than the *vinifera* hybrid group since they are most similar to *V. labrusca* (National Grape Registry <http://ngr.ucdavis.edu>). The nearest

neighbour statistic ( $S_{nn}$ ) measures how often the most similar sequence or sequences ('nearest neighbour') is from the same designated population (Hudson, 2000). This statistic was selected for analyses because it has high power with small sample sizes.  $S_{nn}$  estimates the proportion of nearest neighbours that are from the same population versus from a different population. With two populations, for example, a value close to 1 suggests that the two populations are highly differentiated, because almost every sequence would be most similar to other sequences from the same population, whereas a value of 0.5 would be expected if populations are not genetically structured because the closest sequences would be most similar to those from either population with equal probability. We also estimated differentiation with  $H_{ST}$ , a powerful measure of population subdivision that estimates  $F_{ST}$  among haplotypes (Hudson et al., 1992). Both  $S_{nn}$  and  $H_{ST}$  were calculated by using DnaSP v5.  $P$ -values were estimated by permutation tests with 1000 replications.

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## CHAPTER 2

# IDENTIFICATION AND STRUCTURE OF THE MATING-TYPE LOCUS AND DEVELOPMENT OF PCR-BASED MARKERS FOR MATING TYPE IN POWDERY MILDEW FUNGI\*

### ***Abstract***

In ascomycetes, mating compatibility is regulated by the mating-type locus, *MATI*. The objectives of this study were to identify and sequence genes at the *MATI* locus in the powdery mildew fungus, *Erysiphe necator*, to develop a PCR-based marker for determining mating type in *E. necator*, and to develop degenerate primers for amplification by PCR of conserved regions of mating-type idiomorphs in other powdery mildew fungi. We identified *MATI-2-1* of the *MATI-2* idiomorph in *E. necator* based on the homologous sequence in the genome of *B. graminis* f. sp. *hordei* and we found *MATI-1-1* and *MATI-1-3* of the *MATI-1* idiomorph from transcriptome sequences of *E. necator*. We developed and applied a reliable PCR-based multiplex marker to confirm that genotype correlated with mating phenotype, which was determined by pairing with mating-type tester isolates. Additionally, we used the marker to genotype populations of *E. necator* from different *Vitis* spp. from throughout the USA. We found both mating types were present in all populations and mating type ratios did not deviate from 1:1. The mating-type genes in *E. necator* are similar to those of other Leotiomycetes;

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however, the structure of the *MATI* locus in *E. necator*, like the *MATI-2* idiomorph of *Blumeria graminis*, is markedly different from other ascomycetes in that it is greatly expanded and may contain a large amount of repetitive DNA. As a result, we were unable to amplify and sequence either idiomorph in its entirety. We designed degenerate primers that amplify conserved regions of *MATI-1* and *MATI-2* in *E. necator*, *Podosphaera xanthii*, *Microsphaera syringae*, and *Blumeria graminis*, representing the major clades of the Erysiphales. These degenerate primers or sequences obtained in this study from these species can be used to identify and sequence *MATI* genes or design mating-type markers in other powdery mildew fungi as well.

### ***Introduction***

In fungi, the sexual cycle — from the recognition of a potential mate to the development of sexual structures — is regulated by genes at mating-type loci. Depending on the fungal species, mating compatibility and sexual reproduction may be controlled by a single locus with two alternative mating types or by two loci with two or more specificities at each locus (Kronstad and Staben, 1997; Debuchy et al., 2010). An understanding of the genetic basis of sexual reproduction, facilitated by markers for studying mating in nature, is essential to our knowledge of fungal mating systems, and would contribute to studies of evolution, genetics, and epidemiology.

Self-incompatible (heterothallic) fungi in the phylum Ascomycota contain a single mating-type locus (*MATI*) with one of two possible specificities. For two heterothallic individuals to be compatible and mate successfully, each must possess a different mating specificity at the *MATI* locus. In most filamentous ascomycetes the mating types are named *MATI-1* and *MATI-2* (Turgeon and Yoder, 2000). The alternative sequences at the *MATI* locus

are called idiomorphs rather than alleles because their nucleotide sequences are dissimilar and do not appear to share a common ancestry (Metzenberg and Glass, 1990). The regions flanking the idiomorphs are homologous for isolates of both mating types and the gene order in the flanking regions is conserved among most ascomycetes (Debuchy and Turgeon, 2006). The idiomorphs *MATI-1* and *MATI-2* are characterized by conserved motifs in their encoded proteins. *MATI-1* is characterized by the gene *MATI-1-1* that encodes a protein containing an alpha 1 ( $\alpha$ 1) box, whereas *MATI-2* is characterised by the gene *MATI-2-1* that encodes a protein with a high mobility group (HMG) domain (Turgeon and Yoder, 2000). A recent study found amino acid sequence conservation and structural similarities between the  $\alpha$ 1 box of *MATI-1-1* and the HMG domain of *MATI-2-1*, which suggests that they may have originated from a common ancestor (Martin et al., 2010). Idiomorphs may contain genes in addition to *MATI-1-1* or *MATI-2-1*; for example, some species of ascomycetes have *MATI-1* idiomorphs with *MATI-1-3*, which is also characterized as having an HMG domain, although it belongs to a different phylogenetic grouping and is not as conserved as in *MATI-2-1* (Debuchy and Turgeon, 2006).

The powdery mildew fungi (Erysiphales), most of which regularly reproduce sexually, are biotrophic plant pathogens. Several of these fungi have contributed to our understanding of development, epidemiology, genetics, fungicide resistance, and host-pathogen interactions (Brown, 2002; Bushnell, 2002; Both et al., 2005). However, only one mating-type idiomorph has been identified in the Erysiphales. The *MATI* locus was recently described from the genome sequence of a *MATI-2* isolate of *Blumeria graminis* f. sp. *hordei* (Spanu et al., 2010). The nucleotide sequence of *MATI-2-1* was clearly homologous to *MATI-2-1* of other ascomycetes, yet the locus is not closely flanked by genes that flank *MATI* in other ascomycetes. In *B. graminis* f. sp. *hordei*, nearly two-thirds of the genome is composed of transposable elements,

including the region surrounding *MATI-2-1*. A thorough description of the *MATI* locus requires comparison of sequences from both mating types to determine where idiomorphs end and homologous flanking regions begin. However, the *MATI-1* idiomorph has not been identified yet in *B. graminis* f. sp. *hordei*, because genome sequence is only available for a *MATI-2* isolate, and therefore the extent of expansion in the idiomorphs has not been determined. Identification of mating-type genes in powdery mildew fungi would be useful for comparative studies on the structure and evolution of the *MATI* locus within the Erysiphales, and among other ascomycetes, especially given the anomalies likely caused by transposable elements in *MATI-2* in *B. graminis* f. sp. *hordei*.

The overall aim of this research was to identify the mating-type genes in both idiomorphs and describe the *MATI* locus of the grape powdery mildew fungus, *Erysiphe necator* (syn. *Uncinula necator*). *E. necator* is heterothallic (Gadoury and Pearson, 1991; Miazzi et al., 1997) and survives overwinter as ascospores in cleistothecia in many temperate regions (Pearson and Gadoury, 1987; Cortesi et al., 1997). The distribution of mating types has been determined in several populations based on phenotype by crossing field isolates in the laboratory with tester isolates of the two mating types (Gadoury and Pearson, 1991; Délye et al., 1997; Stummer and Scott, 2003; Cortesi et al., 2005; Cortesi et al., 2008). Mating-type ratios are not significantly different from 1:1 in some populations (Miazzi et al., 1997; Stummer et al., 2000; Cortesi et al., 2005), as expected under random mating, but deviate from 1:1 in others (Délye et al., 1997; Cortesi et al., 2008). In Europe, deviations from 1:1 ratios occur in populations in which *E. necator* overwinter asexually as mycelium in dormant buds, which give rise to symptoms known as flagshoots. For example, ratios are generally skewed in favor of one mating type among isolates collected from flagshoots in Europe (Délye et al., 1997), and in one population sampled

from flagshoots in a vineyard in northern Italy, all isolates had the same mating type and were clonal (Cortesi et al., 2008). Because the identity of mating types of tester isolates may vary among studies, it is difficult to compare mating-type data between studies. In North America, both mating types of *E. necator* were detected among isolates from diverse locations in the northeastern USA (Gadoury and Pearson, 1991) but nothing is known about mating type in other regions. In this study our objectives were 1) to identify genes and sequence the idiomorphs at the *MATI* locus in *E. necator*, 2) to develop a PCR-based marker for mating type in *E. necator*, 3) to determine if mating types are present in 1:1 ratios, which are expected under random mating, in *E. necator* populations from different regions of the USA, and 4) to design degenerate primers to amplify conserved regions of both idiomorphs by PCR from other powdery mildew fungi.

### ***Materials and methods***

**Crosses to determine mating-type phenotype in *E. necator*.** We determined the mating-type phenotype of 10 isolates of *E. necator* from the eastern USA and 10 isolates from Reggio Emilia, Italy for comparison to molecular genotyping by PCR (described below). We also compared mating-type phenotypes of isolates from Italy (Voghera,  $N = 12$  and Montalcino,  $N = 28$ ), which were reported previously (Cortesi et al., 2005; Cortesi et al., 2008) to molecular genotypes. All isolates were cultured and maintained as described previously (Cortesi et al., 2004; Brewer and Milgroom, 2010) and were derived from single-conidial chains to ensure that they were of single genotypes. The mating-type phenotype of each isolate from Italy was determined by pairing with tester isolates of each mating type on seedlings as described previously (Cortesi et al., 2004). The phenotypes of isolates from the eastern USA were determined similarly, but by using

sexually compatible tester isolates from the eastern USA on detached leaves prepared as described in Brewer and Milgroom (2010). As a control, all isolates were also paired with themselves. There were three replicates of each pairing. Detached leaves or seedlings were monitored for the presence of cleistothecia. For cleistothecia produced on detached leaves, the presence of ascospores was assessed by squashing 10 to 20 cleistothecia per cross on a glass slide and visually inspecting with a compound light microscope. The viability of ascospores from cleistothecia in Montalcino and Reggio Emilia produced on seedlings was assessed with fluorescein diacetate as described previously (Nadel, 1989; Cortesi et al., 1995). For each location, isolates of the same mating type were arbitrarily designated as MAT<sup>+</sup> or MAT<sup>-</sup>.

**Identification of mating-type genes.** Two sexually compatible isolates, designated G14 and G19, from Geneva, New York, USA, were used for identification and sequencing of the mating-type genes in *E. necator*. DNA from these isolates was extracted as described by Evans et al. (1996).

We used the nucleotide sequence of *MAT1-2-1* of *B. graminis* f. sp. *hordei* ([www.BluGen.org](http://www.BluGen.org)) as a starting point to find *MAT1-2* in *E. necator*. Based on this sequence, we designed primers in the HMG domain to amplify *MAT1-2* by PCR from genomic DNA of *E. necator*. The PCR reaction was carried out in a total volume of 25  $\mu$ L. Reaction components were: 2.5  $\mu$ L of 10X PCR buffer, 2.5  $\mu$ L dNTPs (2.5 mM each), 1.25  $\mu$ L each of 10  $\mu$ M forward and reverse primers, 0.75 U ExTaq (Takara Bio USA, Madison, WI), and 1  $\mu$ L (20-300 ng) DNA template. Thermal cycling included three cycles at low annealing temperature (42  $^{\circ}$ C) to ensure amplification of the HMG domain in *E. necator* with the *B. graminis* primers. Cycling conditions were: initial denaturation at 95  $^{\circ}$ C for 2 min, followed by 3 cycles of 95  $^{\circ}$ C for 1 min, 42  $^{\circ}$ C for 1

min, 72 °C for 30 s, followed by 35 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 30 s, followed by a final extension at 72 °C for 5 min.

PCR products were purified by electrophoresis in a 1% agarose gel. The fragment of the predicted length was excised and purified with the QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA), cloned into the pGEM-T Easy Vector System (Promega, Madison, WI) following the manufacturer's instructions, and sequenced at the Cornell University Life Sciences Core Laboratories Center using the Applied Biosystems Automated 3730 DNA Analyzer with Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Sequences containing the HMG domain in *E. necator* were identified by their high similarity to sequences from *B. graminis* f. sp. *hordei* based on alignment by ClustalW in MegAlign (DNASTAR, Madison, WI).. We used several rounds of TAIL-PCR (Liu and Whittier, 1995), which combines primers to known sequence, e.g., starting in the HMG domain, with random primers, to extend the sequence outwards from a known starting sequence. Sequences of the HMG domain and those obtained by TAIL-PCR were aligned in Seqman (DNASTAR). To make sure we sequenced the correct region and that we were not amplifying sequence unique to a single isolate, we designed primers to each new sequence obtained by TAIL-PCR and performed PCR with that new primer and a primer to a region of known sequence using template DNA from multiple isolates. All regions were sequenced at least twice.

We used sequences from a normalized cDNA (transcriptome) database of *E. necator* isolate G14 as a starting point for finding *MATI-1*. We chose to sequence the transcriptome of this isolate, in part, because it was sexually compatible with isolates that contained *MATI-2* and we could not amplify conserved regions of *MATI-2* by PCR from it. RNA was isolated from mycelium and conidia from powdery mildew colonies 17 days post-inoculation with the RNeasy

Plant Mini Kit (QIAGEN) and provided to BioS&T (Montreal, QC, Canada) for preparation of uncloned normalized cDNA. Pyrosequencing by a 454-FLX (454 Life Sciences, Branford, CT, USA) was conducted at the Cornell University Life Sciences Core Laboratory Center. The default parameters in MIRA software (Chevreux et al., 2004) were used to assemble the reads into contigs *de novo*. We obtained 82 Mb of total sequence, which assembled into 32,405 total contigs with an average length of 591 bp and 9.1 reads per contig. We used tBLASTn to search the *E. necator* transcriptome database for sequences with high similarity to amino acid sequences of *MAT1-1-1*, *MAT1-1-3*, and *MAT1-1-4* in *Pyrenopeziza brassicae* (GenBank accession nos. CAA06844, CAA06846, CAA06845), *MAT1-1-1* and *MAT1-1-3* in *Rhynchosporium secalis* (CAD71141, CAD71142), and *MAT1-1-1* in *Sclerotinia sclerotiorum* (AAZ83721) and *Botryotinia fuckeliana* (EDN21841), and *MAT1-1-2* in *Neurospora crassa* (AAC37477), *Cryphonectria parasitica* (AAK83345), *Magnaporthe grisea* (BAC65088), and *Gibberella zeae* (AAG42811). Additionally, we searched for sequences with similarity to putative proteins of unknown function near the *MAT1* locus of *S. sclerotiorum* (EDO01528, EDO01530) and *B. fuckeliana* (EDN21840). TAIL-PCR was used to extend sequences of *MAT1-1* in *E. necator*, starting from sequences identified from the transcriptome, and sequencing was carried out as described above for *MAT1-2*.

**Development of a multiplex PCR-based mating-type marker for *E. necator*.** We designed primers to amplify sequence of the  $\alpha 1$  box of *MAT1-1-1* and the HMG domain of *MAT1-2-1* in *E. necator* by multiplex PCR (Table 2.1). The resulting fragments were predicted to be 408 bp for *MAT1-1* and 232 bp for *MAT1-2*. The PCR reaction was carried out in a total volume of 25  $\mu$ L. Reaction components included: 2.5  $\mu$ L of 10X PCR buffer (Takara), 2.5  $\mu$ L dNTPs (2.5

mM each), 1.0  $\mu$ L each of 10- $\mu$ M primers En $\alpha$ F2, En $\alpha$ R3, EnHMGF1 and EnHMGR1, 0.75 U ExTaq (Takara), and 1  $\mu$ L (20-300 ng) DNA template. Cycling conditions included an initial denaturation at 95 °C for 2 min followed by 35 cycles with a denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Ten microliters of each PCR product with loading dye was analyzed by electrophoresis through a 1% (w/v) agarose/TBE gel.

**Table 2.1: Primers for a multiplex PCR-based marker in *E. necator* and for amplification of conserved regions of the *MAT1* locus in other powdery mildew fungi.**

| Primer type   | Primer name     | Primer sequence (5' → 3')     | Size of PCR product (bp)     |
|---|-----------------|-------------------------------|------------------------------|
| <i>E. necator</i> -specific primers                                   | En $\alpha$ F2  | AAAGATGCACCTCTCGATGAA         | 408                          |
|   | En $\alpha$ R3  | AAGTTATAGAAGACATCGCAGTCA      |                              |
|   | EnHMGF1         | AAAAGTAAACATGCCGAAACA         | 232                          |
|   | EnHMGR1         | CCGTGGTCTGTAACGATAACC         |                              |
| Powdery mildew degenerate primers                                     | pm $\alpha$ dF2 | AARYCRGTNAATTCATGGAT          | 217-220/<br>190 <sup>a</sup> |
|   | pm $\alpha$ dR1 | ACTRTRTYACGAATCTTGAGTATG      |                              |
|   | pm $\alpha$ dR2 | GAGTATGCRGCAGNRATRATTGTCCACTT |                              |
|   | pmHMGdF         | CCTCCSAAYTCTTGGATTTTATAKCG    | 277-281 <sup>a</sup>         |
|   | pmHMGdR         | CGTTTAACTTCRGAAGRYTTCCGTGG    |                              |
| <i>Blumeria</i> -specific primers for the $\alpha$ 1 box <sup>b</sup> | Bgt $\alpha$ F1 | TTGAGTATGTGGCTTCAGG           | 161                          |
|   | Bgt $\alpha$ R1 | CCACTTGACCTTAAAAGGATC         |                              |

<sup>a</sup> PCR product sizes vary among powdery mildew species due to variation in intron size. The PCR products produced by pm $\alpha$ dF2 and pm $\alpha$ dR1 were 217 to 220 bp, whereas the PCR product produced by pm $\alpha$ dF2 and pm $\alpha$ dR2 in *B. graminis* f. sp. *tritici* was 190 bp. The PCR products produced by pmHMGdF and pmHMGdR were 277 to 281 bp.

<sup>b</sup> These primers were used to amplify by PCR the  $\alpha$ 1 box of *B. graminis* f. sp. *hordei*.

To test the mating-type marker, we performed multiplex PCR on DNA from 10 isolates from the eastern USA and 50 isolates from Italy that were assayed for mating-type phenotype in crosses. Genomic DNA from isolates from the USA and Reggio Emilia was prepared as described previously (Brewer and Milgroom, 2010). Briefly, conidia and hyphae were collected from 2-3 week-old colonies on detached leaves by touching a 1-cm<sup>2</sup> piece of office tape (Scotch Tape, 3M) to the colony multiple times until the tape was covered in fungal tissue. The tape was placed in a 1.5 mL microcentrifuge tube with 100 µL of 5% Chelex (Walsh et al., 1991; Hirata and Takamatsu, 1996), vortexed for 30 s and incubated at 95 °C for 20 min. The solution was vortexed again, centrifuged briefly, and the supernatant was removed and used as the DNA template for PCR. DNA from isolates from Montalcino and Voghera was prepared by Cortesi et al. (2005; 2008), as described in Evans et al. (1996).

**Analysis of mating-type distributions in populations of *E. necator* in the USA.** We used the PCR-based mating-type marker described above to identify mating types in populations from the USA. We performed PCR on DNA purified from 115 isolates characterized in a previous study by multilocus sequencing (Brewer and Milgroom, 2010). Isolates were collected from diverse *Vitis* species across the southeastern, northeastern, central, and western USA. Mating-type distributions were tested for deviation from the expected ratios of 1:1 using chi-square goodness-of-fit tests.

**Development of *MATI* primers for other powdery mildews.** Degenerate primers were designed for amplification of conserved regions of *MATI-1* and *MATI-2* in other powdery mildews. For *MATI-1*, we designed primers to the conserved sequences of the  $\alpha$ 1 box of

MAT1-1-1 in *E. necator*, *S. sclerotiorum*, *B. fuckeliana*, *P. brassicae*, and *R. secalis*. For MAT1-2, we designed primers to the conserved sequences of the HMG domain of MAT1-2-1 in *E. necator* and *B. graminis*. The primer pairs are pmHMGdF with pmHMGdR for MAT1-2 and pm $\alpha$ dF2 with pm $\alpha$ dR2 or pm $\alpha$ dR1 for MAT1-1 (Table 2.1). PCR, cloning and sequencing of the HMG domain regions of MAT1-2-1 and  $\alpha$ 1 box regions of MAT1-1-1 in other powdery mildew isolates was carried out as described above for identification of the HMG domain region of MAT1-2-1 in *E. necator*. The primers were tested on DNA isolated from *E. necator*, *B. graminis* f. sp. *hordei*, *B. graminis* f. sp. *tritici*, *Podosphaera xanthii*, and *Microsphaera syringae* (syn. *Erysiphe syringae*), which were chosen to span the major clades of the Erysiphales (Saenz and Taylor, 1999).

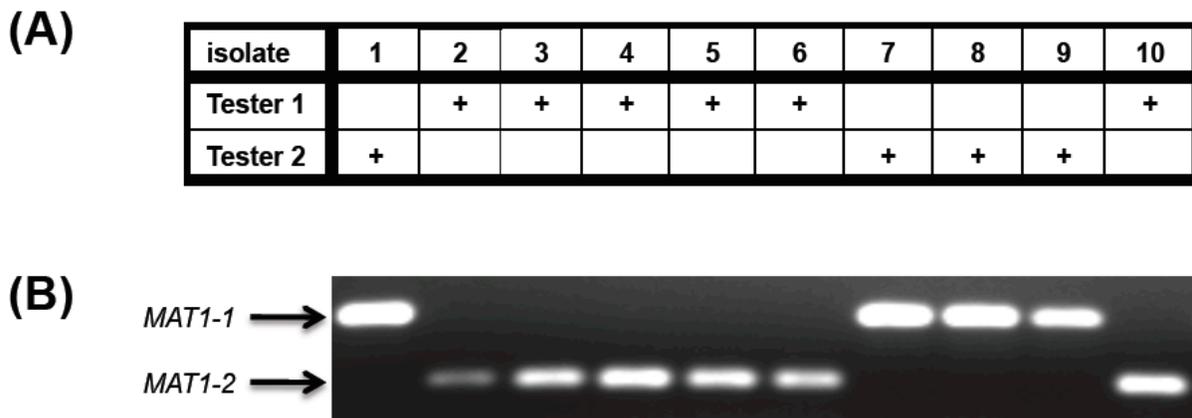
We modified methods to obtain the  $\alpha$ 1 box region of MAT1-1 from *B. graminis* because of difficulties amplifying this sequence with the degenerate primers pm $\alpha$ dF2 and pm $\alpha$ dR1 (see Results). To amplify the  $\alpha$ 1 box region from *B. graminis* f. sp. *tritici*, we used the primer combination pm $\alpha$ dF2/pm $\alpha$ dR2 (Table 2.1); subsequent cloning and sequencing was performed as described above for other powdery mildew species. To amplify the  $\alpha$ 1 box of *B. graminis* f. sp. *hordei* we used primers Bgt $\alpha$ F1/Bgt $\alpha$ R1 (Table 2.1) based on the sequence from *B. graminis* f. sp. *tritici*. This PCR was carried out in a total volume of 28  $\mu$ L. Reaction components included: 25  $\mu$ L Taq Polymerase Master Mix (Platinum PCR Supermix, Invitrogen), 1.0  $\mu$ L each of 10  $\mu$ M primers, and 1  $\mu$ L (100 ng) DNA template. Cycling conditions included an initial denaturation step at 94 °C for 2 min followed by 3 cycles with a denaturation step at 94 °C for 30 s, annealing at 52 °C for 15 s, and extension at 72 °C for 1 min. Additional rounds of touch-down PCR were conducted under the same cycling conditions for 3 cycles each except with annealing temperatures of 48 °C, 44 °C, and 42 °C, followed by 31 cycles with annealing at 52 °C

followed by a final extension at 72 °C for 3 min. The resulting fragment size was 161 bp.

Products were reamplified to produce ample product for sequencing, purified using a QIAquick PCR Purification Kit (QIAGEN, West Sussex, UK) and sequenced in both directions by GATC-Biotech, (London, UK).

## Results

**Mating-type phenotypes.** All isolates of *E. necator* readily formed cleistothecia when crossed with one or the other of the two mating-type tester isolates (Figure 2.1A; Table 2.2). None formed cleistothecia when paired with both mating-type testers or when paired with themselves. Ascospores were produced within the cleistothecia of compatible crosses. Based on these results, we assigned isolates arbitrarily to mating types MAT+ and MAT-.



**Figure 2.1.** Correlation of mating-type phenotype with multiplex PCR amplification of mating-type genes in *E. necator*. (A) Ten isolates of *E. necator* were crossed with two tester isolates of opposite mating types. Pairs of isolates that formed cleistothecia are marked with ‘+’ on the grid. (B) PCR products amplified with primers specific to the  $\alpha$ 1 box region of *MAT1-1-1* (408 bp) or the HMG domain region of *MAT1-2-1* (232 bp) of *E. necator* in a multiplex PCR reaction. The sequences of primers for multiplex PCR (En $\alpha$ F2, En $\alpha$ R3, EnHMGF1 and EnHMGR1) are shown in Table 2.1.

**Table 2.2: Mating-type phenotype and genotype of *E. necator* isolates from Italy**

| Location      | Mating-type phenotype <sup>a</sup> | Mating-type genotype <sup>b</sup> |               |
|---------------|------------------------------------|-----------------------------------|---------------|
|               |                                    | <i>MAT1-1</i>                     | <i>MAT1-2</i> |
| Reggio Emilia | MAT+                               | 4                                 | 0             |
|               | MAT-                               | 0                                 | 6             |
| Voghera       | MAT+                               | 12                                | 0             |
|               | MAT-                               | 0                                 | 0             |
| Montalcino    | MAT+                               | 9                                 | 0             |
|               | MAT-                               | 0                                 | 19            |

<sup>a</sup>Mating-type phenotype was determined by pairing all isolates with tester isolates of each mating type. Phenotype data from Voghera and Montalcino were reported previously (Cortesi et al., 2005; Cortesi et al., 2008).

<sup>b</sup>Mating-type genotype was determined by the multiplex PCR-based marker described in this study using the *E. necator*-specific primers shown in Table 2.1.

### Identification of *MAT1* genes.

We identified *MAT1-2-1* in *E. necator* based on sequence similarity to *MAT1-2-1* in the *B. graminis* f. sp. *hordei* genome sequence ([www.BluGen.org](http://www.BluGen.org)). Because we used primers designed from the HMG domain of *B. graminis* and several cycles of low annealing temperature in the PCR, we amplified multiple PCR products from genomic DNA of *E. necator* that resulted in smears and distinct bands on an agarose gel; therefore, we cloned fragments of the expected length (approximately 275 bp) for sequencing. The nucleotide sequence from one of the clones was highly similar (70 %) to the HMG-coding region in *B. graminis* (Figure 2.2A and B) and the translated amino acid sequence aligned well (80 % similarity to *B. graminis*) with the HMG domain of *MAT1-2-1* in other species (Figure 2.2A).

### (A) MAT1-2-1 HMG domain

*E. necator* RNQKRPPNSWILYRKSKHAETVSKNPNI PNCKISTLIARMWARESNEVRERYKALAERAKYQHTVDNPGYRYRPRKSSEVKRR  
*B. graminis* ATQKRPPNSWILYRKCKHAETVSONPGLTNNQISTLISAMWAAESDEVRSAYKALADRVKQOHAADNPGYRYKPRKPSEVKRR  
*P. brassicae* NVIARPPNCFILFRQAMHAAVVAANPGVHNNVISRLISGMWRESPEEIEHYKALAEAKARHLHLYPNYRFTPRKSSDKRR  
*R. secalis* NVVARPPNCFILFRQHLHPMVVRDNPGLHNNVISTMISKMWHGAPSEIREQYKELAAEAKRQHKLLYPDYHYEPRKSSEKRR  
*S. sclerotiorum* PKIIPRANEWILYRADNHIPIKKAYPGITNNEISSI LAGMWAATPERRLKYYKIRADLLKEAHKKAQYPTKYAPRKPSEKRR

### (B) MAT1-2-1 ORF

*E. necator* MSNLVYRCHNVVEAMLSSKDYDFSNYPR-----KIATISQIWATISLQITESNNIVALSLKSFCECLSPDQCKMILYNYKYITNKDGMF  
*B. graminis* MPDIFLTSDHCF TENKLSMSSTTSQGNRGTIGRRALANIFQMWASISMQITEYNNIIIVIPSKMFSLWTAQDQIYLLSQYSAITNENGIF

*E. necator* AADQIVPRVFIGPIEHFRSKRLLYGSECIPTILSDFNDHLPEPPSASTSLNSKQPSFWNQEETHISSLNNREPS**RNRQKRPPNSWILYR**  
*B. graminis* VADSTILPRVFLGPISHFQSKNLHYVPGYIPTLLQTSNNHQR-IHISKAINGICNSNSQHSKFSLLNSKISQQKSS**FATQKRPPNSWILYR**

*E. necator* **KSKHAETVSKNPNI PNCKISTLIARMWARESNEVRERYKALAERAKYQHTVDNPGYRYRPRKSSEVKRRQKKKLVODTDSILSLELPIGH**  
*B. graminis* **KGKHAETVSONPGLTNNQISTLISAMWAAESDEVRSAYKALADRVKQOHAADNPGYRYKPRKPSEVKRRQKKKVAQDSNYTQFTQLNPAP**

*E. necator* HCX---DNYIFDXKIKKAEVLSRSRISKXPFLPEER-----PENLGIYSSRGLNFFHLASAI PVNSSEIDFQOYFEEV  
*B. graminis* SLIPTEIQRTLSTNETTTDEQIHGSQQTVDEIPRSSSPYIDPSRSGIDAQITP-PADRSSPMDFHTDEIDEDQWEMES

### (C) MAT1-1-1 $\alpha$ 1 box

*E. necator* ARTNKPVNSWIGFRTFY--KKIFPGMPQKEASIHKALWEQDPFKIKWTII SAAYSKIRDI-VTK  
*P. brassicae* VLPKKSLSNSWMAFRSFY--QNLFPHLQKEASIIYLTALWKRDPFKAKWTII AAAYSKIRNT-VGK  
*R. secalis* VLPKKSLSNSWMAFRSFY--LRIFSHLQKEASIIYLTTLWKGDPFKAKWTII AAAYSKIRNT-VGK  
*B. fuckeliana* EAPRRSVNSYILFRTFY MILTVLGNIPQKYKSAILSVLWGRDPFHAKWSIIARAYTLMRDAGVRR  
*S. sclerotiorum* EAPRRSVNSYILFRTFY MILNVLGNIPQKYKSAILSVLWGRDPFHAKWSIIARAYTLMRDTNVR

### (D) MAT1-1-3 HMG domain

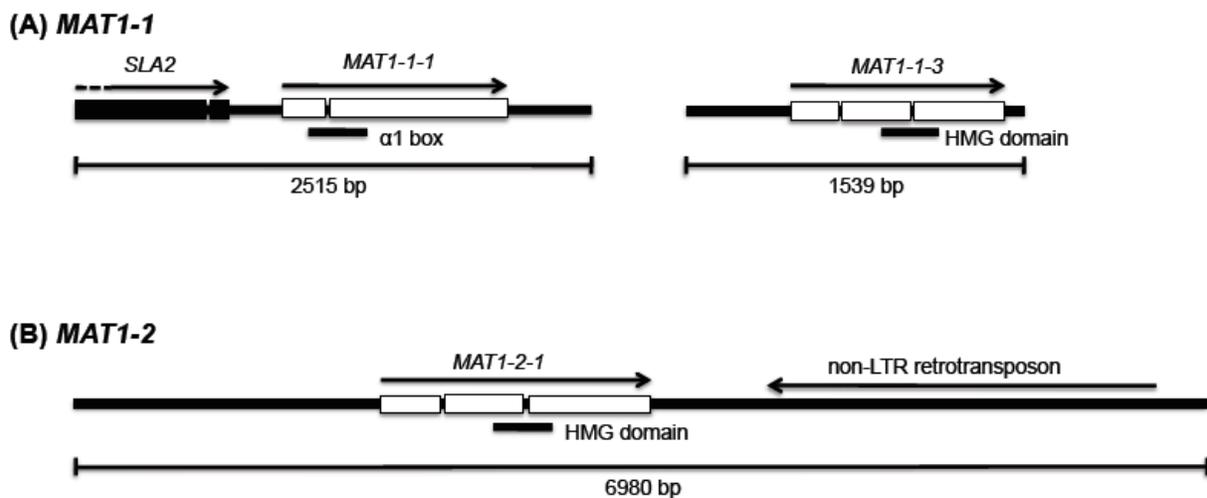
*E. necator* HHIRKPRNAWIIFRQEMMVKFRAQIGHLHTSEQSKVISKMWKK-TPRHTKE-FYTTLAHKEKADLLAQYP  
*P. brassicae* NEVPKQNSWVLYLKDSYGQVKLENPGMKTQISGIVATNWL-AGTKVKEYYKDLAQKLEGHAAFYG  
*R. secalis* NANPKPPNSWILYLNERYKQVKENKLLKTSQISVITAKWKN-NEILKAE--YKALATVAKQHHEALCG  
*C. parasitica* RRVPRRNSWILYRSEKSKLLHTERPGLKAVDISLVSEMWAF-EEVEVKQ-YYTHLAEIEARQHREKYP  
*Y. lipolytica* PKVLKPRNSYILFRNATSRCSQSIDF--NSAFVSRVSSYIINGSGIENPIVRRWFVKYMGFFEAALYHEVDYP

**Figure 2.2.** Alignments of putative amino acid sequences in *E. necator* to mating-type genes in other species of ascomycetes: **(A)** MAT1-2-1 HMG domain of *E. necator* (GenBank accession no. HQ244438), *B. graminis* f. sp. *hordei* ([www.BluGen.org](http://www.BluGen.org)), *Pyrenopeziza brassicae* (CAA06843), *Rhynchosporium secalis* (CAD62166), and *Sclerotinia sclerotiorum* (AAZ83720); **(B)** MAT1-2-1 proteins of *E. necator* (HQ244438) and *B. graminis* f. sp. *hordei* ([www.BluGen.org](http://www.BluGen.org)) with the HMG domain in bold type; **(C)** MAT1-1-1  $\alpha$ 1 box of *E. necator* (HQ244436), *P. brassicae* (CAA06844), *R. secalis* (CAD71141), *Botryotinia fuckeliana* (EDN21841), and *S. sclerotiorum* (AAZ83721); and **(D)** MAT1-1-3 HMG domain of *E. necator* (HQ244436), *P. brassicae* (CAA06846), *R. secalis* (CAD71142), *Cryphonectria parasitica* (AAK83344), and *Yarrowia lipolytica* (CAA07613). Amino acids shaded in dark gray (■) are identical to *E. necator*, whereas amino acids shaded in light grey (□) are identical among other species in the alignment.

After multiple rounds of TAIL-PCR extending in both directions from the HMG domain, we obtained 6980 nucleotides of sequence from the *MAT1-2* idiomorph of *E. necator* (GenBank accession no. HQ244438), including the entire sequence of *MAT1-2-1* (Figure 2.3B). The open reading frame (ORF) of *MAT1-2-1*, inferred by sequence similarity to other ascomycetes, is 1113 bp with three exons of 221 bp, 356 bp and 425 bp, separated by 63-bp and 48-bp introns (Figure 2.3B). The encoded protein is predicted to be 333 amino acids in length (Figure 2.2B). The start codon and intron positions for *E. necator* were predicted based on alignment with *B. graminis* and other Leotiomycetes. There is no sequence similarity between the non-coding regions bordering *MAT1-2-1* in *E. necator* and *B. graminis* f. sp. *hordei*. Downstream from *MAT1-2-1* in *E. necator* (Figure 2.3B) is sequence highly similar (E-value = 1e-46) to the TE1b retrotransposon (GenBank accession no. EU098096) of *B. graminis*, a non-long terminal repeat transposable element. We attempted to amplify short sequences at both ends of the 6980 bp *MAT1-2* sequence in isolates of both mating types, but only amplified sequence in isolates with the *MAT1-2* mating phenotype. This indicated that we did not sequence the regions flanking the idiomorph because by definition flanking regions are homologous and should amplify in isolates of both mating types.

We identified *MAT1-1-1* and *MAT1-1-3* in the transcriptome of *E. necator* isolate G14 by tBLASTn with amino acid sequences from *S. sclerotiorum*, *P. brassicae*, *R. secalis*, and *B. fuckeliana*. We did not identify *MAT1-1-2*, *MAT1-1-4* or the other *MAT1*-associated genes present in *S. sclerotiorum* and *B. fuckeliana*. The inferred amino acid sequences have similarities to the  $\alpha$ 1 box region of MAT1-1-1 (61 % similarity to *P. brassicae*) and the HMG domain region of MAT1-1-3 (29 % similarity to *R. secalis*) from other ascomycetes (Figure 2.2C and D). After multiple rounds of TAIL-PCR we obtained 2515 bp of sequence containing *MAT1-1-1* and

adjacent regions (Figure 2.3A). The predicted ORF of *MAT1-1-1* (GenBank accession no. HQ244436) is 916 bp with two exons of 133 bp and 734 bp separated by a 49-bp intron. The encoded protein is predicted to be 288 amino acids in length. The 2515-bp sequence includes 700 bp of the 3' region of the cytoskeleton assembly control protein (*SLA2*) 432 bp upstream of *MAT1-1-1*; *SLA2* is typically found near the *MAT1* locus in many ascomycetes (Debuchy and Turgeon, 2006). Using TAIL-PCR beginning in the *MAT1-1-3* homolog, we also obtained 1539 bp of sequence that contains the full length of *MAT1-1-3* (GenBank accession no. HQ244437) and adjacent regions (Figure 2.3A). The predicted ORF is 971 bp, contains three exons of 155 bp, 269 bp, and 452 bp, separated by 50-bp and 45-bp introns. The encoded protein is predicted to be 291 amino acids in length.



**Figure 2.3.** Sequenced regions of the mating-type (*MAT1*) locus of *E. necator*. **(A)** *MAT1-1* putative open reading frames *MAT1-1-1* (GenBank accession no. HQ244436) and *MAT1-1-3* (HQ244437) are on non-contiguous sequences that could not be linked by PCR amplification. Arrows above ORFs indicate the directions of transcription. The cytoskeleton assembly control protein (*SLA2*) is 432 bp upstream of *MAT1-1-1*. Locations of conserved sequences, the  $\alpha 1$  box in *MAT1-1-1* and HMG in *MAT1-1-3*, are shown with bars below the genes. **(B)** *MAT1-2* putative open reading frame *MAT1-2-1* (HQ244438) and downstream sequences homologous to the TE1b retrotransposon (EU098096) of *B. graminis*, a non-long terminal repeat transposable element. Arrows above ORFs indicate the directions of transcription. Location of the conserved sequence of HMG in *MAT1-2-1* is shown with a bar below the gene.

In other Leotiomycetes *MATI-1-1* and *MATI-1-3* are usually separated by 1.5 to 2.0 kb of sequence (Singh et al., 1999; Foster and Fitt, 2004); however, despite repeated attempts in all possible orientations, we were unable to amplify the gap between *MATI-1-1* and *MATI-1-3* by PCR. Similarly, although we could amplify *SLA2* in both *MATI-1* and *MATI-2* isolates, we were unable to amplify any sequence using primers to *SLA2* and primers to either end of the 6980-bp sequence containing *MATI-2-1*, even though *SLA2* is only 432 bp from *MATI-1-1* and we could easily amplify this region using primers to *SLA2* and *MATI-1-1* (data not shown). We identified DNA lyase (*APN2*; HQ293023) in the *E. necator* transcriptome because it is often located adjacent to *MATI* on the opposite side of *SLA2* in many ascomycetes (Debuchy and Turgeon, 2006). However, we were unable to PCR-amplify between *APN2* and *SLA2*, *MATI-1-1*, *MATI-1-3* or *MATI-2-1*. Despite multiple attempts, we were not successful in obtaining additional nucleotide sequences from *MATI-1* or *MATI-2* with additional rounds of TAIL-PCR.

**PCR-based *MATI* marker for *E. necator*.** The primers En $\alpha$ F2 and En $\alpha$ R3, designed from *MATI-1-1*, and primers EnHMGF1 and EnHMGR1, designed from *MATI-2-1* in *E. necator* (Table 2.1) used in a multiplex PCR consistently identified mating types *MATI-1* and *MATI-2*, respectively. The resulting PCR products for *MATI-1* and *MATI-2* correlated perfectly to mating-type phenotypes for the isolates in our test panel (Figure 2.1). All isolates that produced cleistothecia with tester 2 corresponded with PCR amplification of *MATI-1*, and all isolates that produced cleistothecia when crossed with tester 1 corresponded with PCR amplification of *MATI-2*. PCR amplification of *MATI-1-3* sequences also correlated perfectly to mating-type phenotype and amplification of *MATI-1-1* (data not shown). The PCR products, 408 bp for *MATI-1* and 232 bp for *MATI-2*, are easily distinguishable on a 1% agarose gel. Genotypes of

this marker also correlated perfectly with mating-type phenotypes in samples from three Italian vineyards (Table 2.2).

**Mating-type distributions in populations of *E. necator* in the USA.** Both mating types were present in populations of *E. necator* in each of the regions sampled in the USA (Table 2.3).

Mating-type ratios did not differ ( $P > 0.05$ ) from 1:1 in each region, but was marginally significant when all populations were combined.

**Table 2.3: Mating type distributions in populations of *E. necator* from different regions of the USA.**

| Region    | <i>MAT1-1</i> | <i>MAT1-2</i> | $\chi^2$ <sup>a</sup> |
|-----------|---------------|---------------|-----------------------|
| Southeast | 12            | 22            | 2.94                  |
| Central   | 8             | 2             | 3.60                  |
| Northeast | 22            | 36            | 3.38                  |
| West      | 5             | 8             | 0.69                  |
| Total     | 47            | 68            | 3.83                  |

<sup>a</sup> $P > 0.05$  when  $\chi^2 < 3.84$ .

**Identification of *MAT1* in other powdery mildews.** With the degenerate primer pairs pm $\alpha$ F2/pm $\alpha$ R1 and pmHMGdF/pmHMGdR (Table 2.1) we amplified and sequenced regions of the  $\alpha$ 1 box of MAT1-1-1 and the HMG domain of MAT1-2-1, respectively, in *E. necator*, *B. graminis* f. sp. *hordei* (HMG only; [www.BluGen.org](http://www.BluGen.org)), *B. graminis* f. sp. *tritici* (HGM only; GenBank accession no. HQ171899), *Podosphaera xanthii* (HQ171903, HQ171900), and *Microsphaera syringae* (HQ1711904, HQ171901) (Figure 2. 4). The fragment sizes were approximately 278 bp and 220 bp for sequences from the HMG domain and  $\alpha$ 1 box,

## (A) MAT1-1-1 $\alpha$ 1 box

### nucleotide sequence

```
En TGGTITTAGGAGTGAGTCTTTTAACTTTTAAACAGAAATCGTGACTAATGTGAATAAGCATTITATAAAAATATTTCTGGAAATGCCACAAAAGGAGCTTCAATTCATCTCAAGCTTTATGGG
Ms TGGTITTAGGAGTACGTCFTT--TAAGTTATATATTTTATTTGTAACCTAACGTCGAGAAGCGTTTATAAAAATATTTCCCAAGAAATCCACAGAAAGGAGCTTCTGTTCATCTCAAGCATTATGGAA
Px TGGCTTCAGAGATGATGTCATG--CTCTTTTATCATGATGCAAAAGCTAAACAGATCAAGCATTITATAAAAATATTTCCCAAAAATGCCAGAAAAGGATCCACCTTCTAACAAAATTTGGAA
Bgh GGTATGCCAAG--CGATATTTCCAGATTAATCATAACTAATAGTAGACAGCTTCTATAAAAATGATTCCTAAATATGCCGCAAAAAGATGCATCCTCAATCTTACAAATCCTATGGAA
Bgt TGGCTTCAGGAGTATGCCAAG--CAATATTTCCAGATTAATCATAACTGACGGTAGACAGCTTCTATAAAAATGATTCCTAAATATGCCGCAAAAAGATGCATCCTCAATCTTACAAATCCTATGGAA

En ACAGAGCCCTTTAAAATTAAGTGGACAAATATCTCTGCAAG
Ms GAAGAGCCCTTTAAAATCTAAGTGGACAAATCATCTCTGCAAG
Px CCGTGAATCCGTTCAAGGCTAAGTGGTCTATAAATCTCTGCAAG
Bgh CAAG
Bgt CAAGGATCCCTTTAAAGTCT
```

### amino acid sequence

```
En GFRIFYRNFIFGMPQREASVHLKALWEDPFFKIKWTIISA
Ms GFRIFYRNFIFGMPQREASVHLKALWEDPFFKIKWTIISA
Px GFRIFYRNFIFGMPQREASVHLKALWEDPFFKIKWTIISA
Bgh AFYRNFVFNMPQKDASSILLILWNE
Bgt GFRIFYRNFVFNMPQKDASSILLILWNRDFFK
```

## (B) MAT1-2-1 HMG domain

### nucleotide sequence

```
En TAAAAGTAAACATGCCGAAACAGTTTCAAGAAACCCAAATTTACCTAATTTGTAATAATTCGTTAGTGCAGTCCC--TTGCCCTTC--AAATATTTTAAATGACAA--TAAACAGCTACCTTGATAGCTCGTG
Ms AAAAAGCAACATGCCGAAACAGTTTACAGAAATCCGAATTTACCTAATTTGTAATAATTCGTTAGTGCAGTCCC--TTATCTTATAT--AAGTATTTTAAATGACAA--TGAATAGCCACTCAATCGCTCGTG
Px GAAGGCTAAGCATGGAAGAAAGTCTCTCCGAATCTTGGCTGACTAACCAAAATCTGTAGCGTTATAGACTAATTTGAATGTATTTCTAAATGTGTCTTCTG--TAGCAACCTTAACTCTACAA
Bgh TAAAAGTAAACATGCCGAAACAGTTTCTCAAAAATCCAGGCTTACCAATAACCAAAATATGTAAGTTGAAGAAA--TAAGTATAACCAAAAGAAAAC--TAA--GACCC--TG--TAGCCACACTGATCAGCGGGA
Bgt TCAACATAAGCAACCCAAACCTGCTCTCAAAAATCCAGGCTTACCAACCAAAATATGTAAGTTGAAGAAA--TAATGTGACCCGAAGAAATAC--TAA--GACCT--TG--TAGCCACACTGATCAGCGGGA

En TGTGGCTCGAGAAATCAAAATGAAGTACGTGAAAGATATAAGGCATAGCAGAAACGTGCCAAATACCG--CATACTGTGTGATAACCTGGTTATCGTTACAGA
Ms TGTGGCTCGAGAGTCAAAAAGAGTTAGGAAAGATATAAGCATTAGCAGAAATCTGCTAAAATCCAAACATACTGTGTGATAATCTGGTTATCGTTACAGA
Px TGTGGCTACAGACCAAAAAGAGTTGATGCGTTCAAAATCTGGCAGACCGTGTAAAATCCAAACATGCAACATGCAAGTTGAAAATCCAGTTATCGTTACAGG
Bgh TGTGGCTCGCAGAGTCCGAAAGTGGTTCGAAAGTGCATATAAAGCTCTAGCAGATCGTGTCAAGCAACCAACATCGCGCTGACAAACCCCGGCTATCGCTATAAA
Bgt TGTGGCTCGCAGAGTCCGAAAGTGAAGTGAATATAAAGCTAAGGCAGATCTTATCAAGCAACCAACATCGCGCTGACAAACCCCGGCTATCGCTATAAA
```

### amino acid sequence

```
En KSFHAEIVSINPNLPNCKISTLIARMWAESNEVREYKALAEERAYQHTVDNPGYRYR
Ms KSFHAEIVLQNPPLNPNCKISTLIARMWAESNEVREYKALAEERAYQHTVDNPGYRYR
Px KAFHAEIVSINPNLNNQISTLISIMWAESNEVREYKALAEERAYQHTVDNPGYRYR
Bgh KSFHAEIVSINPNLNNQISTLISIMWAESNEVREYKALAEERAYQHTVDNPGYRYR
Bgt QRFHAEIVSINPNLNNQISTLISIMWAESNEVREYKAFADLIQQHAAADNPGYRYR
```

**Figure 2.4.** Alignments of nucleotide and amino acid sequences of conserved regions of mating-type genes amplified by PCR with degenerate primers shown in Table 2.1. **(A)** the MAT1-1-1  $\alpha$ 1 box from *E. necator* (*En*; GenBank accession no. HQ244436), *Microsphaera syringae* (*Ms*; HQ1711904) *Podosphaera xanthii* (*Px*; HQ171903), *B. graminis* f. sp. *hordei* (*Bgh*; JF357622) and *Blumeria graminis* f. sp. *tritici* (*Bgt*; HQ171902). Nucleotide and amino acid sequences of *MAT1-1* in *Bgt* are shorter at the 3' ends because a different primer was used (see text). The sequences in *Bgh* are shorter at the 3' and 5' ends because primers Bgt $\alpha$ F1 and Bgt $\alpha$ R1 based on sequence from *Bgt* were used. **(B)** the MAT1-2-1 HMG from *E. necator* (HQ244438), *M. syringae* (HQ171901), *P. xanthii* (HQ171900), *B. graminis* f. sp. *hordei* (*Bgh*; [www.BluGen.org](http://www.BluGen.org)), and *B. graminis* f. sp. *tritici* (HQ171899). Amino acids and nucleotides shaded in dark gray (■) are identical among three or more sequences in the alignment, whereas amino acids and nucleotides shaded in light grey (□) are identical between two sequences. There is no shading in intron sequences, which are italicized. Primer sequences have been removed.

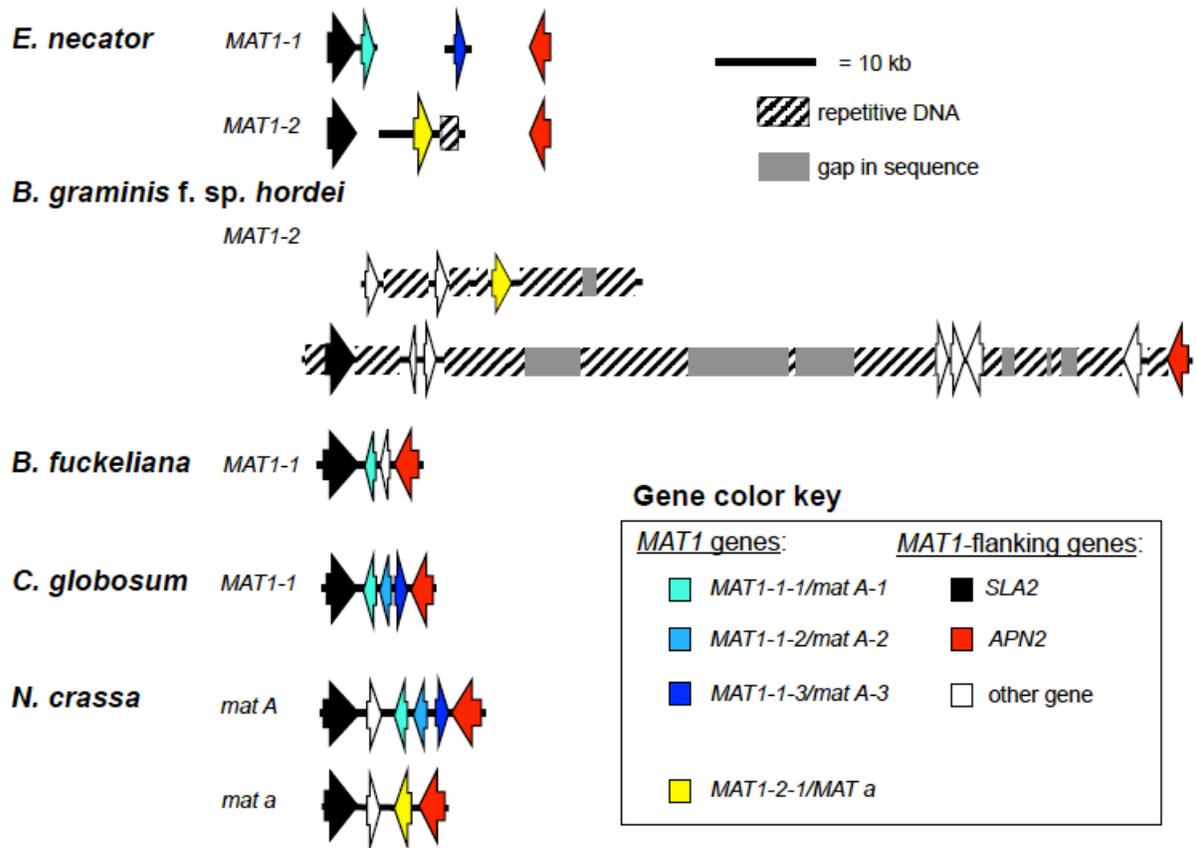
respectively. Fragment sizes varied slightly among species based on differences in intron lengths (Table 2.1; Figure 2.4). Amplification of the HMG box region of *MAT1-2-1* using degenerate primers yielded fragments that were distinct when visualized on an agarose gel, with ample product to sequence directly from fragments excised from the gel. Amplification of the  $\alpha 1$  box region of *MAT1-1-1* using the degenerate primers usually resulted in less distinct and less abundant fragments, which required cloning to produce enough template for sequencing. PCR amplification of the  $\alpha 1$  box region from *B. graminis* was not successful with primers pm $\alpha$ F2/pm $\alpha$ R1; however, we did amplify a 190-bp fragment from this region from *B. graminis* f. sp. *tritici* using the primers pm $\alpha$ F2/pm $\alpha$ R2 (GenBank accession no. HQ171902). By designing PCR primers Bgt $\alpha$ F1/Bgt $\alpha$ R1 (Table 2.1) to the  $\alpha 1$  box region from *B. graminis* f. sp. *tritici*, we amplified a 161-bp fragment from *B. graminis* f. sp. *hordei* (JF357622) (Figure 2.4). Gene trees constructed from nucleotide sequences of the  $\alpha 1$  box and HMG domain regions from the powdery mildews in this study (not shown) had the same topology among genera as did the gene tree of the internal transcribed spacer region of rDNA (*ITS*) (Saenz and Taylor, 1999).

## ***Discussion***

**The *MAT1* locus in *E. necator* contains homologous genes to other ascomycetes, but is markedly different in structure.** We identified the mating-type genes *MAT1-2-1* of the *MAT1-2* idiomorph and *MAT1-1-1* and *MAT1-1-3* of the *MAT1-1* idiomorph in *E. necator*; no other mating-type genes were found. For *MAT1-2*, this was not surprising because *MAT1-2-1* is the only gene found in *MAT1-2* idiomorphs in *B. graminis* f. sp. *hordei*, *R. secalis*, and *P. brassicae* (Singh et al., 1999; Foster and Fitt, 2004; Spanu et al., 2010), which are the only other heterothallic Leotiomycetes with characterized *MAT1-2* idiomorphs. For *MAT1-1*, on the other

hand, both *MATI-1-1* and *MATI-1-3* were detected in *R. secalis* and *P. brassicae*, and *MATI-1-4* was also detected in *P. brassicae*; only *MATI-1-1* was detected in the genome sequence of the heterothallic Leotiomycete *B. fuckeliana*. Therefore, the number and identity of genes in the *MATI-1* idiomorph are variable among the Leotiomycetes; *MATI-1-1*, however, is always present. Additional mating-type genes could be present in the genome of *E. necator*, but are not in close proximity to *MATI-2-1* or were not expressed under the conditions in which the *MATI-1* isolate of *E. necator* was growing prior to RNA isolation for transcriptome sequencing. Our discovery of *MATI* genes in *E. necator* underscores the importance of genome and transcriptome sequences in identifying loci and developing markers for addressing questions about the genetics and biology of fungi.

Although we identified mating-type genes in *E. necator* by their sequence similarity to homologs in other ascomycetes, the structure of the *MATI* locus appears to be markedly different (Figure 2.5). We sequenced 6980 bp of the *MATI-2* idiomorph and did not reach flanking sequence common to both mating types, which was confirmed by lack of PCR amplification of end sequences in *MATI-1* isolates. Therefore, we estimate that the *MATI-2* idiomorph is at least 7 kb in length. In other Leotiomycetes, the *MATI-2* idiomorph is approximately 3 kb (Singh et al., 1999; Foster and Fitt, 2004), and at most 5 kb in other ascomycetes (Debuchy and Turgeon, 2006). The size of the *MATI-2* idiomorph in *B. graminis* f. sp. *hordei* is not yet known because sequence information from a *MATI-1* isolate, essential for determining where the homologous sequence flanking the idiomorphs ends and where the non-homologous idiomorph sequences begin, is not yet available. However, the *MATI* locus in *B. graminis* f. sp. *hordei* is not closely flanked by genes that flank the *MATI* locus in other ascomycetes (Spanu et al., 2010). *MATI* loci in most sequenced ascomycetes, including all other Leotiomycetes studied to date, are flanked by



**Figure 2.5.** Organization of the mating-type locus (*MAT1*) in the powdery mildew fungi *E. necator* (this study) and *B. graminis* f. sp. *hordei* (Spanu et al., 2010) is different from other ascomycetes. *Botryotinia fuckeliana*, *Neurospora crassa* and *Chaetomium globosum* show the typical structure of the *MAT1* locus, which is flanked by the cytoskeleton assembly protein (*SLA2*, black) and DNA lyase (*APN2*, orange), and ranges in size from 1.3 to 5.7 kb for *MAT1-1* (or *mat A*) and 1.2 to 4.5 kb for *MAT1-2* (or *mat a*) (Debuchy and Turgeon, 2006). In *E. necator*, the *MAT1-2* idiomorph is at least 7 kb based on the fact that we did not obtain sequence common to both mating types adjacent to *MAT1-2-1* in the flanking regions. In *E. necator*, *SLA2* is upstream of *MAT1-1-1* (light blue), but not close enough to PCR-amplify sequence between *SLA2* and sequences in the *MAT1-2* idiomorph even though we could amplify portions of *SLA2* alone in *MAT1-2* isolates. We also failed to amplify sequence between *MAT1-1-1* and *MAT1-1-3* (dark blue), or between either idiomorph sequence and *APN2*. Therefore, the genes associated with *MAT1* in *E. necator* are shown on separate contigs. In *B. graminis* f. sp. *hordei*, the overall distance between *SLA2* and *APN2* is 110 kb and *MAT1-2-1* (yellow) is on a separate supercontig. Arrows represent open reading frames and the direction represents the directions of transcription.

*SLA2* on one side and *APN2* on the other (Debuchy and Turgeon, 2006). In *E. necator*, we found *SLA2* upstream of *MATI-1-1*, but we were unable to PCR-amplify sequence between *SLA2* and sequences in the *MATI-2* idiomorph even though we could amplify portions of *SLA2* alone in *MATI-2* isolates. We also failed to amplify sequence between *MATI-1-1* and *MATI-1-3*, between either idiomorph sequence and *APN2*, or between *SLA2* and *APN2*. These results suggest that, aside from *MATI-1-1* and *SLA2*, *MATI* genes and those in the flanking region are too far apart to amplify by PCR and/or that the genes are completely unlinked. Approximately two-thirds of the *B. graminis* f. sp. *hordei* genome is transposable elements (Spanu et al., 2010), so it is probable that the *E. necator* genome also contains an abundance of transposable elements, or other repetitive DNA. The presence of repetitive DNA could explain why we had difficulty obtaining additional sequence with TAIL-PCR. We also attempted to use inverse PCR (Ochman et al., 1988) to obtain additional sequence, but were not successful (unpublished data). We found a retroelement in *E. necator* downstream of *MATI-2-1*. There is a transposon downstream from *MATI-2-1* in *B. graminis* f. sp. *hordei* also; however it is not a homolog of TE1b, as found in *E. necator*. In the future, bacterial artificial chromosome (BAC) or fosmid libraries of *E. necator* may be needed for sequencing both idiomorphs in their entirety.

As more genome sequences of powdery mildew fungi become available it will be interesting to see if the anomalous structure of the *MATI* locus is common. Repetitive elements have been found to accumulate near reproductive genes and are hypothesized to play a role in their evolution in fungi (Hood, 2002). They are not purged by recombination because the idiomorphs are non-recombining regions. Additionally, genes involved in repeat-induced point mutation (RIP), a mechanism that inactivates repetitive DNA in some fungi (Cambareri et al., 1989; Braumann et al., 2008), are not found among the five powdery mildew genomes and/or

transcriptomes sequenced (Spanu et al., 2010), so repetitive sequences can accumulate.

**PCR-based multiplex *MAT1* markers for *E. necator* were developed.** We developed a simple, robust, and reliable multiplex PCR-based marker for determining mating type in *E. necator*. By following the protocol described in the Materials and methods, and using PCR primers in Table 2.1, the mating type of *E. necator* isolates can be determined relatively quickly and easily. However, we used DNA extracted from young colonies grown in laboratory conditions on surface-sterilized leaves. The performance of this marker on DNA extracted from lesions of *E. necator* taken directly from the field (Montarry et al., 2009) needs to be investigated. We verified that the mating-type genotype generated by this marker correlated perfectly with mating-type phenotypes of 60 isolates from Italy and the eastern US. Mating-type data based on this PCR method can be used to choose isolates for making crosses, estimate mating-type ratios for making inferences about sexual reproduction within populations, and study epidemiology without having to pair isolates with tester isolates to determine their mating type by making crosses. Also, the mating type can now be designated and determined by sequences of homologous idiomorphs (Turgeon and Yoder, 2000), rather than by using arbitrary nomenclature (e.g., MAT+ and MAT-), which had been used in the past because the idiomorphs were not known. In some studies, mating types were arbitrarily designated *MAT1-1* and *MAT1-2* without knowledge of the idiomorph sequences (Miazzi et al., 1997; Miazzi et al., 2003). This designation needs to be tested and changed if, by chance, these names were applied to the wrong mating types. With a marker for *E. necator* that identifies the mating-type idiomorph, it is now possible to make comparisons accurately among studies by different researchers without sharing tester isolates.

**Both mating types are present in populations of *E. necator* in the USA.** We found that both mating types are present across regions of the USA and that mating-type ratios do not differ from 1:1, as expected under random mating. This is consistent with cleistothecia being common throughout the USA and studies suggesting that ascospores are an important source of inoculum (Pearson and Gadoury, 1987). Deviation from 1:1 ratios have been found in introduced ranges of *E. necator*, especially in populations in which the fungus overwinters asexually in dormant buds and causes flagshoot symptoms the next season (Délye et al., 1997; Cortesi et al., 2008). Some populations that overwinter asexually are subjected to severe population bottlenecks that can lead to reduced diversity, and at its extreme, to complete clonality (Cortesi et al., 2008). However, both mating types are found in some flagshoot populations (Miazzi et al., 1997; Cortesi et al., 2004). We found both mating types among the three isolates in this study collected from a single vineyard in Madera County, California, where *E. necator* is mainly propagated asexually (W.D. Gubler, personal communication). More detailed studies of populations in the USA are necessary to determine if distributions vary based on overwintering mode or other biological differences among populations.

**Identification of conserved regions of the *MAT1* locus in other powdery mildew fungi.** We designed primers that amplify *MAT1-1* and *MAT1-2* across several genera of the Erysiphales. Short conserved sequences in both idiomorphs of *B. graminis* f. sp. *hordei*, *B. graminis* f. sp. *tritici*, *M. syringae*, and *P. xanthii* are now available (Figure 2.4). Multiplex PCR-based markers for these species could be developed and tested from these sequences, as we described here for *E. necator*. Additionally, these conserved regions could provide a starting point for sequencing

and characterizing the mating-type genes of these species. The degenerate primers can be used to identify and sequence mating-type genes or design mating-type markers in other powdery mildew fungi. These primers amplified conserved regions of both idiomorphs across several genera of the Erysiphales, which spanned the major clades of the order (Saenz and Taylor, 1999); therefore, we expect that they will work for most other powdery mildew species.

Although *B. graminis* f.sp. *tritici* and *B. graminis* f. sp. *hordei* are classified as *formae speciales* of the same species, they are divergent at the highly conserved HMG domain of *MAT1-2-1* and  $\alpha 1$  box of *MAT1-1-1*, even at the amino acid level (Figure 2.4). The degenerate primers worked best on DNA extracted from young mildew colonies growing under laboratory conditions on surface-sterilized leaves. We were not able to amplify *MAT1-1* of *B. graminis* f. sp. *hordei* with the degenerate primers from DNA extracted from field-collected isolates or from mixed plant and fungal DNA samples; however, we could amplify *MAT1-2* from these materials. *MAT1-1* in *B. graminis* f. sp. *hordei* was amplified with primers based on the *MAT1-1* sequence from *B. graminis* f.sp. *tritici*. This suggests that even if researchers have difficulty amplifying *MAT1-1* from a powdery mildew of interest with the degenerate primers, primers designed based on sequence from a closely related species could lead to successful amplification by PCR. These degenerate primers are important for exploring *MAT1* sequences in other powdery mildew species, and now studies characterizing the mating-type loci of powdery mildew fungi can be conducted.

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## CHAPTER 3

### LINKAGE DISEQUILIBRIUM AND SPATIAL AGGREGATION OF GENOTYPES OF THE GRAPE POWDERY MILDEW FUNGUS, *ERYSIPHE NECATOR*, WITHIN VINEYARDS

#### ***Abstract***

Random mating and recombination in heterothallic fungi should result in high genotypic diversity, 1:1 mating-type ratios (in ascomycetes), and random associations of alleles at different loci, *i.e.*, linkage equilibrium. To test for random mating in populations of the grape powdery mildew fungus, *Erysiphe necator*, we sampled isolates from vineyards of *Vitis vinifera* cv. Chardonnay in Burdett, NY (NY09) and Winchester, VA (VA09) at the end of the epidemic in the fall of 2009. We also sampled isolates from the same Winchester, VA vineyard in the spring of 2010 (VA10) at the onset of the epidemic, which was initiated by sexually produced inoculum (ascospores). Isolates were genotyped for mating type and 11 SSR markers. In the spring sample (VA10), nearly every isolate had a unique genotype; two genotypes were represented by two isolates each. In contrast, fall populations were less diverse. VA09 had 12 genotypes with two or more isolates; NY09 had three genotypes with two or more isolates, but one was represented by 20 isolates. After clone correction, mating-type ratios in the three populations did not deviate from 1:1. However, even with clone correction, we detected significant linkage disequilibrium in all populations, including VA10. Mantel tests detected positive correlations between genetic and geographic distances within vineyards. We also detected spatial autocorrelation in distance classes up to 24 m and 3 m in VA09 and NY09, respectively; *i.e.*, isolates within these distances were genetically more similar than expected at random. Spatial autocorrelation most likely

results from short dispersal distances. Overall, these results suggest that selection for clonal genotypes and spatial genetic aggregation during the asexual phase of the epidemic contribute to persistent linkage disequilibrium even though populations undergo an annual sexual cycle.

### ***Introduction***

Recombination resulting from sexual reproduction leads to new genotypes that permit organisms to better adapt to changing conditions and purges genomes of the accumulation of deleterious mutations (Muller, 1964). Many hypotheses have been presented on the advantages of sexual reproduction over asexual reproduction in the evolution of reproductive modes (Kondrashov, 1993). However, there are also costs to sex, such as breaking up favorable allele combinations and increasing the chances of parasite transmission (Burnett, 2003). An enormous diversity of mating systems and reproductive modes are found among fungi (Elliot, 1994), yet it is not evident why such diverse reproductive modes are maintained. A mixed mode of reproduction involving regular sexual reproduction combined with many cycles of asexual reproduction is common among plant pathogenic fungi, bringing with it the advantages conferred by sexual reproduction while simultaneously allowing beneficial combinations to persist and increase through asexual reproduction. Populations with mixed reproductive modes may produce diverse genotypes from sexual reproduction, but the fittest genotypes can increase in frequency rapidly because of clonal reproduction. This type of rapid evolution in which pathogen clones emerge rapidly in a selective sweep has been termed an “epidemic” model (Maynard Smith et al., 1993). Among plant pathogens, mixed modes of reproduction have been hypothesized to lead to more rapid evolution to overcome host resistance or develop resistance to fungicides (McDonald and Linde, 2002).

In a randomly mating, or panmictic, population all individuals are potential mates. Under random mating, we expect to find both mating types in equal proportions. Additionally, recombination during meiosis should result in high genotypic diversity and random associations of alleles at different loci, which is referred to as linkage (or gametic) equilibrium. Nonrandom mating occurs due to inbreeding, where selfing, or homothallism, is the most extreme form. Assortative mating is less extreme, but also a form of inbreeding where individuals mate with others that are similar to themselves, and is likely to occur among host specialized individuals or in spatially structured populations. Populations with limited gene flow, or dispersal, are not randomly mating and will experience an isolation-by-distance (IBD) population genetic structure (Wright, 1943). IBD can occur on a continental scale, where populations become more distinct as geographic distances increase, or on a smaller scale, such as within a vineyard, if dispersal is limited. In this case, patches of similar genotypes, or genetic neighborhoods, may arise. IBD on either scale can cause LD because the relative isolation of patches or populations will limit gene flow and result in nonrandom associations of alleles across the entire population, but not necessarily within a neighborhood.

The grape powdery mildew fungus, *Erysiphe necator*, has a mixed mode of reproduction with numerous cycles of asexual reproduction throughout the growing season and regular sexual reproduction annually at the end of the epidemic. It is a heterothallic fungus (Gadoury and Pearson, 1991; Miazzi et al., 1997) and thus, requires two individuals, each of a different mating type, *MATI-1* or *MATI-2*, for sexual reproduction. The products of sexual reproduction are the ascospores, which are produced in cleistothecia. Ascospores are an important source of inoculum in many regions (Pearson and Gadoury, 1987; Cortesi et al., 1997) and the only source of inoculum in regions with cold winters. In milder climates, *E. necator* can also overwinter as

dormant mycelium inside of buds, which in the spring give rise to shoots covered with mycelium and conidia. In Europe and Australia, where *E. necator* has been introduced, populations are structured into two distinct genetic groups that appear to be reproductively isolated (Délye et al., 1997; Stummer et al., 2000; Péros et al., 2005; Núñez et al., 2006; Montarry et al., 2008) and are, therefore, not randomly mating. Recent studies of the population genetics of *E. necator* in the eastern USA showed that genetic diversity is much greater there than in introduced populations in Europe, Australia or the west coast of the USA, and it is the likely source for the introductions to these new areas (Brewer and Milgroom, 2010; Frenkel et al., in press).

*E. necator* can be dispersed on infected plant material, as ascospores or conidia. Conidia appear to have a steep dispersal gradient, dispersing only on the order of several meters (Cortesi et al., 2004). However, little is known about the geographic range of ascospore dispersal in *E. necator*. It is generally assumed that spores are dispersed by extreme wind (Grove, 2004) and/or rain (Gadoury and Pearson, 1990), but whether or not gene flow is limited or long-distance has not been thoroughly investigated. Recent studies have shown large-scale geographic structure within eastern North America (Brewer and Milgroom, 2010; Frenkel et al., in press), which suggests that long-distance dispersal is not prevalent. Both of these studies also showed evidence of recombination, and Frenkel et al. (in press) found lack of linkage disequilibrium in relatively small samples analyzed across broad geographic areas. In this study, we were interested in determining if local populations of *E. necator* within single vineyards in the eastern USA are randomly mating because we wanted to understand if random mating was occurring on a local scale. Additionally, the genetic differentiation detected over large geographic scales (Brewer and Milgroom, 2010; Frenkel et al., in press) would not be a contributing factor in vineyard-sized populations. The objectives of this study were: (1) to determine if local populations of *E.*

*necator* are randomly mating, (2) to determine if genotypes of *E. necator* are spatially aggregated within vineyards, and (3) to determine if there is temporal and/or geographic divergence between populations that may contribute to LD.

### ***Materials and methods***

**Sampling.** Isolates of *E. necator* were collected from two vineyards of *V. vinifera* cv. Chardonnay in the fall of 2009 at the end of the epidemic: a commercial vineyard in the Finger Lakes Region in Burdett, NY on Sept. 21, 2009 (NY09) and a research vineyard at the Virginia Polytechnic Institute and State University, Agricultural Experiment Station in Winchester, VA on Oct. 12, 2009 (VA09). For NY09, we sampled 100 leaves with powdery mildew colonies from 10 rows at 3-m intervals (10 isolates per row). The vineyard in Virginia differed in shape and size, therefore, the same spatial sampling scheme could not be conducted at both vineyards. For VA09, we sampled 100 leaves with powdery mildew colonies from 3 rows at 3-m intervals (33 or 34 isolates per row). This research vineyard contained several plots of vines treated with different fungicides; however, some vines within each plot were not sprayed. VA09 isolates were collected from vines that had not been sprayed. Rows were approximately 3 m apart in both vineyards. We recorded the location of leaf samples within each vineyard for NY09 and VA09.

To understand the diversity of genotypes derived from sexual reproduction prior to asexual reproduction, we collected isolates at the onset of the epidemic that were assumed to be ascospore-derived colonies on June 12, 2010 from the Winchester, VA vineyard (VA10). Isolates were sampled the same as described for VA09 except that the location of each sample was not recorded. Despite intensive efforts, we could not sample isolates from the NY vineyard (or any other vineyards nearby) because disease incidence was too low early in the season due to

fungicide applications. By the time colonies were present it was too late in the season for them to be representative of the overwintering sexual population because of asexual reproduction and selection for fungicide-resistant genotypes.

**Isolation of *E. necator* and DNA extraction.** Mildew colonies were isolated on surface-sterilized leaves of *V. vinifera* cv. Cabernet Sauvignon as described previously (Evans et al., 1996; Brewer and Milgroom, 2010). Young leaves from vines in a greenhouse were surface sterilized in 0.6% sodium hypochlorite for 1.5 min, rinsed twice with sterile distilled water and air dried in a sterile laminar flow hood. Leaves were kept in Petri dishes containing 20 ml of 2% water agar. Isolates were obtained by touching a single, isolated mildew colony from a leaf collected in the field to a surface-sterilized leaf. Single conidial chains were transferred to another surface-sterilized leaf after 2 to 3 weeks with a sterile pipet tip. Abundant conidia from colonies arising from single-conidial chains were transferred to another surface-sterilized leaf and DNA was extracted 2 to 4 weeks later.

Genomic DNA was prepared as described previously (Brewer and Milgroom, 2010). Briefly, conidia and hyphae were collected from colonies by touching a 1-cm<sup>2</sup> piece of office tape (Scotch Tape, 3M) to the colony multiple times until the tape was covered in fungal tissue. The tape was placed in a 1.5 mL microcentrifuge tube with 100  $\mu$ L of 5% chelex (Walsh et al., 1991; Hirata and Takamatsu, 1996), vortexed for 30 sec and incubated at 95 °C for 20 min. The solution was vortexed again for 5 sec, centrifuged briefly, and the supernatant was removed and used as the DNA template for PCR.

**Identification of mating-type.** The mating type of each isolate was determined by using a PCR-

based marker (Brewer et al., 2011). The fragments of the PCR were predicted to be 408 bp for *MATI-1* and 232 bp for *MATI-2*. The PCR reaction was conducted in a total volume of 10  $\mu$ L. Reaction components included 1  $\mu$ L of 10X PCR buffer (Takara), 1  $\mu$ L dNTPs (2.5 mM each), 0.5  $\mu$ L each of primers En $\alpha$ F2, En $\alpha$ R3, EnHMGF1 and EnHMGR1 (10  $\mu$ M), 0.3 U ExTaq (Takara), and 1  $\mu$ L (20-300 ng) DNA template. Cycling conditions included an initial denaturation at 95  $^{\circ}$ C for 2 min followed by 35 cycles with a denaturation step at 95  $^{\circ}$ C for 30 s, annealing at 55  $^{\circ}$ C for 30 s, extension at 72  $^{\circ}$ C for 30 s, followed by a final extension at 72  $^{\circ}$ C for 5 min. Ten microliters of each PCR product with loading dye was analyzed by electrophoresis through a 1% (w/v) agarose/TBE gel.

**Multilocus genotyping.** The genotype of each isolate was determined by using 11 EST-SSRs (Frenkel et al., in press). The microsatellite markers used included *EnMS1 – EnMS7* and *EnMS9 – EnMS11*, and a new marker first described here, *EnMS12*. *EnMS12* was identified and developed in the same manner as the other EST-SSRs described in Frenkel et al. (in press). The PCR primers for *EnMS12* are *EnMS12F*: 5'– CACGACGTTGTAAAACGACCGCTCGTGCATCTTTATTGA – 3', which includes M13-specific sequence at the 5' end for multiplexing with fluorescent dyes as described previously (Schuelke, 2000), and *EnMS12R*: 5'– CGTGAAGCCCAAAGATAAGC – 3'. PCR and fragment analysis were carried out as described previously (Frenkel et al., in press). Briefly, PCR for all primer pairs was carried out in a total volume of 12.5  $\mu$ L. Reactions included 1.25  $\mu$ L of 10X PCR buffer (Takara), 1.25  $\mu$ L of 2.5 mM dNTPs, 0.2  $\mu$ L of 10  $\mu$ M forward primer, 0.4  $\mu$ L of 10  $\mu$ M reverse primer, 0.5  $\mu$ L of 10  $\mu$ M 5'-dye-labelled M13 primer (FAM, VIC, or NED; Applied Biosystems), 0.375 U ExTaq (Takara), and 1  $\mu$ L DNA template (20-300 ng).

Cycling conditions included an initial denaturation at 95 °C for 2 min, followed by 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. For fragment analysis, PCR products from two or three reactions, each with a different fluorescent dye (FAM, VIC or NED), were pooled and one microliter of the pooled reactions was added to 8.5 µL of HIDI formamide (Applied Biosystems), 0.3 µL ddH<sub>2</sub>O and 0.2 µL of GeneScan 500 LIZ size standard (Applied Biosystems) and heated for 5 min at 95 °C. Fragment analyses were conducted at the Cornell University Life Sciences Core Laboratories Center using an Applied Biosystems 3730xl DNA Analyzer. Allele sizes were analyzed using the GeneMapper Software v3.0 (Applied Biosystems).

The new EST-SSR marker, *EnMS12*, produced reproducible fragment sizes and showed consistent results for the populations in this study. The contig that was identified in *E. necator* isolate G14 contained the microsatellite repeat motif (CTT)<sub>8</sub>. The range of allele sizes including the M13-specific sequence was 188 to 200 bp. Four alleles with sizes of 188 bp, 191 bp, 197 bp, and 200 bp were observed among the three populations.

**Analyses for random mating.** Data were clone-corrected for some analyses to eliminate the effects of asexual reproduction on measures of sexual reproduction. The same multilocus genotypes can arise by recombination depending on allele frequencies.  $p_{\text{sex}}$  is a way of testing the likelihood of the same multilocus genotype arising by distinct sexual reproductive events for the given allele frequencies (Parks and Werth, 1993), as implemented in the program GenClone v.1.0 (Arnaud-Haond and Belkhir, 2007; Arnaud-Haond et al., 2007).

Mating-type distributions of total and clone-corrected samples were tested for deviation

from expected ratios of 1:1 using chi-square goodness-of-fit tests. Genotypic diversity ( $G$ ), the probability that two individuals taken at random have unique multilocus genotypes, was estimated for each population.  $G$  is estimated as  $[N/(N-1)](1-\sum p_i^2)$ , where  $p_i$  is the frequency of the  $i$ th multilocus genotype and  $N$  is the sample size in for each population. Multilocus linkage disequilibrium was determined for each total and clone-corrected sample by using the index of association ( $I_A$ ) (Brown et al., 1980; Maynard Smith et al., 1993) and  $\bar{r}_d$ , estimated with MultiLocus v.1.3b (Agapow and Burt, 2001).  $I_A$  is the observed variance of the number of loci for which all pairs of individuals differ divided by the expected variance under linkage equilibrium minus one, where a value not significantly different from zero indicates that the loci in the population are in linkage equilibrium.  $P$  - values were estimated by 1000 random permutations of the data. The magnitude of  $I_A$  can increase with the number of loci studied; however,  $\bar{r}_d$  is standardized, so comparisons can be made among populations that vary by the number of loci analyzed. Significant pairwise linkage disequilibrium was calculated with GENEPOP v.4.0 (Rousset, 2008).

**Spatial genetic analyses.** Mantel tests for matrix correspondence (Mantel, 1967; Smouse et al., 1986) were conducted in GenAlEx v.6.4 to determine if a significant relationship existed between genetic and geographic distances of isolates within the NY09 and VA09 vineyard populations of *E. necator*. Genetic distances between isolates were calculated as the sum of differences between genotypes where the same state at a locus yields a value of 0 and a different state yields a value of 1. A Mantel test provides a correlation coefficient ( $R_{xy}$ ) for the two data matrices of geographic and genetic distances of isolates, with a range from  $-1$  to  $+1$  with  $R_{xy} = 0$  indicative of no correlation.  $P$ -values were determined by comparing the frequency distribution

of 1000 random permutations with the original data.

To determine if patches of similar genotypes of *E. necator* existed within vineyards, spatial autocorrelation based on the methods of Smouse and Peakall (1999) was performed in GenAlEx v.6.4 on NY09 and VA09. The autocorrelation coefficient,  $r$ , is a measure of the genetic similarity of pairs of isolates within specified distance classes. If  $r$  is greater or less than the 95% confidence interval about the null hypothesis of no spatial genetic structure, determined by the distribution of 1000 random permutations of the data, then there is local spatial genetic structure within the distance class.

**Population structure analyses.** Nei's genetic identity ( $I$ ) (Nei, 1972) and genetic differentiation ( $\phi_{PT}$ , an analog of  $F_{ST}$  measured via AMOVA) among NY09, VA09, and VA10 populations (Peakall et al., 1995) were determined within GenAlEx v.6.4 on clone-corrected data. Significant differentiation between populations was determined by comparing observed values to the distribution of 1000 randomizations of the data.

## ***Results***

**Genotyping and clonal composition of vineyard populations.** Ten of the eleven EST-SSR markers used for genotyping were used for analyses in each of the populations. The marker *EnMSI* was excluded from analyses of the NY09 population because we found 10 alleles, and therefore it was considered hypervariable and unreliable for interpreting alleles of the same size as being identical by descent (Frenkel et al., in press). However, only four alleles were detected at *EnMSI* for the VA09 and VA10 populations, so it was not excluded from these analyses. The marker *EnMS4*, however, was excluded from the VA09 and VA10 analyses due to inconsistent

amplification.

We obtained 62, 78, and 69 isolates for NY09, VA09, and VA10 rather than 100, which was the number of colonies collected, because original colonies were not viable, isolates were lost during single-chain transfers prior to DNA extraction or isolates were lost to contamination. Genotypic diversity ranged from 0.897 to 0.996. Based on the statistic  $p_{\text{sex}}$ , three of the high frequency genotypes (with 20, 12 and 6 isolates in each) had probabilities of 0.12, 0.12, and 0.09 that the second encounter of the respective genotype could have resulted from a distinct sexual reproductive event. However, all subsequent encounters of the same genotype produced  $p_{\text{sex}}$  values less than 0.05. All other genotypes represented more than once produced  $p_{\text{sex}}$  values less than 0.05. These estimates are based on allele frequencies and do not account for mating type so the probabilities are overestimates. Therefore, all genotypes that were represented more than once were considered to have arisen clonally. A large proportion of the isolates sampled from the vineyards in the fall (NY09 and VA09) were clones (Table 3.1). A single clone, with 20 of the 62 isolates sampled, dominated the NY09 population. We found 12 genotypes with two or more isolates in VA09; the four most common clones were represented by 15, 12, 7 and 6 isolates each; eight more clones had two isolates each. In the spring sample (VA10), we found 65 multilocus genotypes among 67 isolates, with two genotypes each with two isolates.

**Table 3.1: Clonal composition of vineyard populations of *E. necator* in Burdett, New York sampled in the fall of 2009 (NY09) and Winchester, Virginia sampled in the fall of 2009 (VA09) and the spring of 2010 (VA10).**

| Population | $N$ | $g^b$ | $G^c$ | Clonal fraction <sup>d</sup> | $N_2^e$ | Number of isolates in each clonal genotype |
|------------|-----|-------|-------|------------------------------|---------|--|
| NY09       | 62  | 40    | 0.897 | 0.36                         | 3       | 20, 3, 2                                   |
| VA09       | 78  | 34    | 0.923 | 0.56                         | 12      | 15, 12, 7, 6, 2, 2, 2, 2, 2, 2, 2, 2       |
| VA10       | 69  | 67    | 0.996 | 0.03                         | 2       | 2, 2                                       |

<sup>a</sup>Isolates were considered clones if multilocus haplotypes determined with 10 SSRs and the mating-type marker were represented more than once within a population.

<sup>b</sup>Number of multilocus genotypes

<sup>c</sup> $G$  is estimated as  $[N/(N-1)](1-\sum p_i^2)$ , where  $p_i$  is the frequency of the  $i$ th genotype and  $N$  is the sample size.

<sup>d</sup>Clonal fraction =  $1 - g/N$

<sup>e</sup>Number of genotypes represented by two or more isolates (clonal genotypes).

**Analyses of random mating.** Mating-type ratios did not differ significantly from 1:1 ( $P > 0.05$ ) except for the NY09 total sample, before clone correction (Table 3.2). The dominant clone in this population was a *MAT1-2* genotype, which skewed the mating-type ratio. After clone correction, the NY09 population did not deviate from 1:1.

Multilocus linkage disequilibrium ( $I_A$  and  $\bar{r}_d$ ) was significant within each population, even after clone correction ( $P < 0.001$ ). Values were higher in populations dominated by clones (NY09 and VA09). Multilocus linkage disequilibrium was reduced by clone correction in these populations; however, values remained significant. Out of the 45 pairs of loci in each population, there was significant ( $P \leq 0.01$ ) pairwise linkage disequilibrium between 6 (13 %), 20 (44 %), and 26 (58 %) pairs of loci in the clone-corrected samples of the NY09, VA09, and VA10 populations, respectively.

**Table 3.2: Mating-type distributions and multilocus linkage disequilibrium for vineyard populations of *E. necator* in Burdett, New York sampled in the fall of 2009 (NY09) and Winchester, Virginia sampled in the fall 2009 (VA09) and spring of 2010 (VA10).**

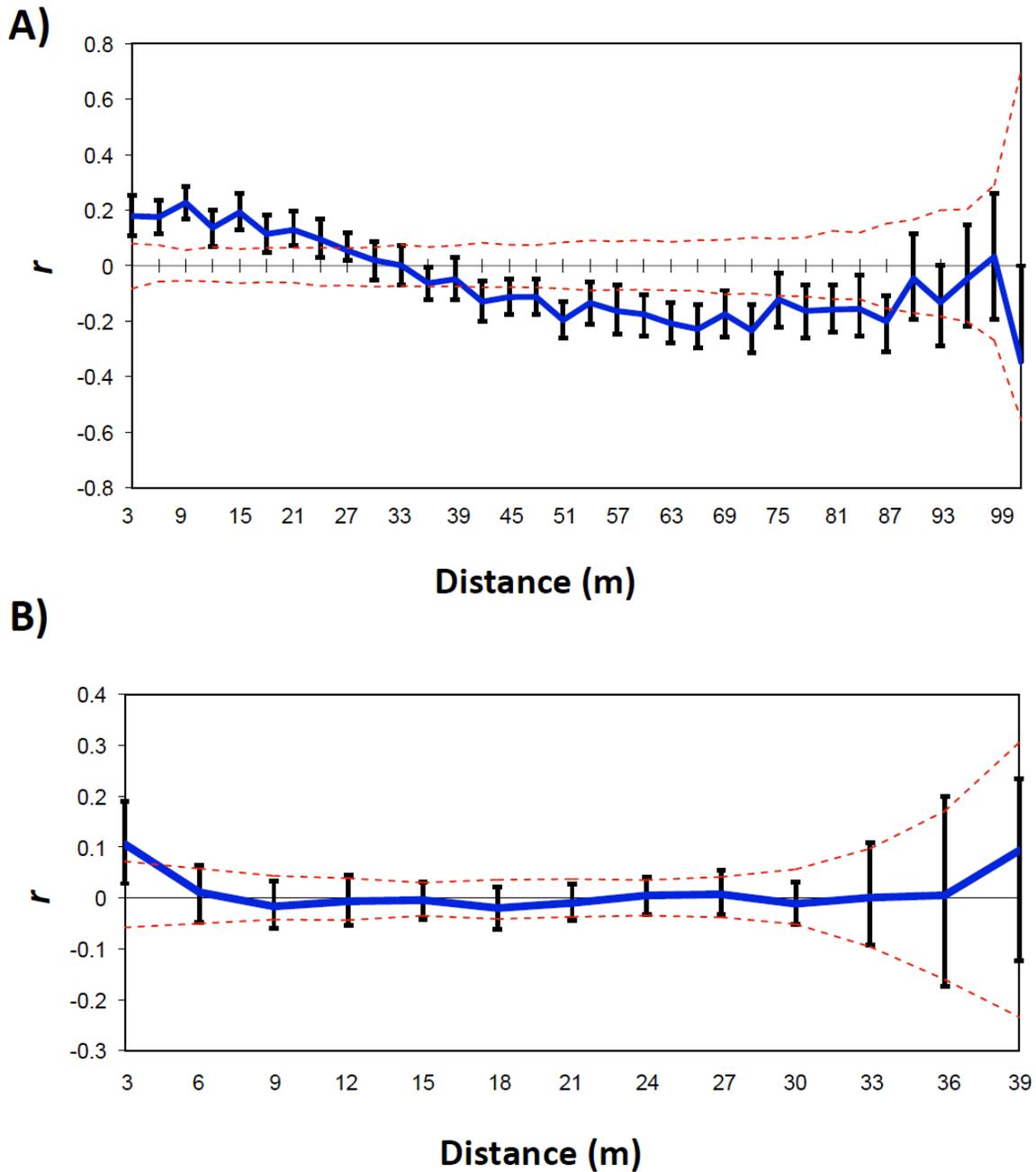
| Population <sup>a</sup> | <i>N</i> | Mating-type distributions |               |                      | Multilocus linkage disequilibrium <sup>c</sup> |             |
|-------------------------|----------|---------------------------|---------------|----------------------|--|-------------|
|                         |          | <i>MAT1-1</i>             | <i>MAT1-2</i> | $\chi^2$             | <i>I<sub>A</sub></i>                           | $\bar{r}_d$ |
| NY09                    |          |                           |               |                      |  |             |
| Total sample            | 62       | 17                        | 45            | 12.64** <sup>b</sup> | 1.49   | 0.17        |
| Clone-corrected sample  | 40       | 17                        | 23            | 0.90                 | 0.40   | 0.05        |
| VA09                    |          |                           |               |                      |  |             |
| Total sample            | 78       | 37                        | 41            | 0.20                 | 1.70   | 0.19        |
| Clone-corrected sample  | 34       | 17                        | 17            | 0.00                 | 0.97   | 0.12        |
| VA10                    |          |                           |               |                      |  |             |
| Total sample            | 69       | 28                        | 41            | 2.45                 | 0.60   | 0.07        |
| Clone-corrected sample  | 67       | 27                        | 40            | 2.52                 | 0.57   | 0.07        |

<sup>a</sup>Clone-corrected samples include a single representative of each multilocus genotype.

<sup>b</sup>Mating-type ratios deviate from 1:1 ( $P \leq 0.001$ ). P-values for all other samples are  $> 0.05$  ( $\chi^2 < 3.84$ ).

<sup>c</sup>Index of association,  $I_A$  and  $\bar{r}_d$ , were calculated with MultiLocus1.3b. All  $P$ -values are  $< 0.001$  and were determined by 1000 randomizations.

**Spatial autocorrelation of genotypes.** Spatial autocorrelation analyses indicated that spatial structure exists within vineyard populations of *E. necator*. Mantel tests showed correlations between genetic distance and geographic distance in NY09 ( $R_{XY} = 0.082$ ;  $P = 0.027$ ) and VA09 ( $R_{XY} = 0.282$ ;  $P = 0.001$ ). Spatial autocorrelation analyses demonstrated that isolates are more genetically similar to each other than expected at random in the smaller distance classes (Figure 3.1). In VA09, isolates in 24-m-distance classes or smaller are more similar to each other than expected at random ( $P \leq 0.05$ ); conversely, isolates from 40- to 87-m-distance classes are less similar to each other than expected at random (Figure 3.1A). In NY09, isolates in 3-m-distance classes are more similar than expected at random (Figure 3.1B).



**Fig. 3.1.** Spatial autocorrelation analysis of vineyard populations of *E. necator* shown as correlograms for **A)** Winchester, VA and **B)** Burdett, NY in Fall 2009. The autocorrelation coefficient  $r$  is a measure of the standardized pairwise genetic similarity of isolates within the corresponding distance class. 95 % confidence intervals for the null hypotheses of random spatial genetic structure based on 1000 permutations of the data are represented by dashed lines. Observed  $r$  values outside of the 95 % confidence interval indicate significant spatial structure for the corresponding distance class.

**Population structure.** The loci *EnMS1* and *EnMS4* were removed from analyses comparing the NY09 and VA09 or VA10 populations because they were only genotyped in one or the other population. Significant genetic differentiation,  $\phi_{PT}$ , was detected between the New York (NY09) and both Virginia (VA09 and VA10) populations (Table 3.3); yet, no differentiation was detected between the Virginia fall and spring populations (VA09 and VA10). The Virginia fall (VA09) and spring (VA10) populations were more similar to each other, as measured by Nei's genetic identity, than the New York (NY09) and Virginia (VA09 or VA10) populations.

**Table 3.3: Geographic and temporal genetic differentiation, measured by  $\phi_{PT}$  (above the diagonal) and Nei's genetic identity<sup>a</sup> (below the diagonal) between vineyard populations of *E. necator* in Burdett, New York sampled in the fall of 2009 (NY09) and Winchester, Virginia sampled in the fall of 2009 (VA09) and the spring of 2010 (VA10). *P*-values for  $\phi_{PT}$  are in parentheses.**

| Population | Population |                 |                 |
|------------|------------|-----------------|-----------------|
|            | VA09       | VA10            | NY09            |
| VA09       | -          | 0.01<br>(0.226) | 0.08<br>(0.001) |
| VA10       | 0.97       | -               | 0.10<br>(0.001) |
| NY09       | 0.86       | 0.83            | -               |

<sup>a</sup>All calculations performed in GenAlEx v.6.4 on clone-corrected data.

## ***Discussion***

We tested the hypothesis that populations of *E. necator* are randomly mating within vineyards. As predicted for random mating, mating-type ratios on clone-corrected data did not deviate significantly from 1:1 and genotypic diversity in the three populations was high. These results are consistent with the biology of *E. necator* because sexual reproduction occurs annually and ascospores are the source of primary inoculum each season, especially in cold climates where ascospores are the only form of overwintering inoculum (Pearson and Gadoury, 1987). In contrast to predictions under the hypothesis of random mating, however, significant linkage disequilibrium was detected in all three populations. LD is not expected among neutral markers in populations that undergo regular sexual reproduction because recombination breaks down LD. The linkage disequilibrium observed in these populations is most likely the result of asexual reproduction; *E. necator* typically has up to 10 or more asexual generations per year during the epidemic. Clones were prevalent in populations at the end of epidemics in both vineyards after multiple asexual generations, but were rare early in the epidemic in the one vineyard sampled at that time.

For pathogens with mixed modes of reproduction, i.e., both sexual and asexual generations occur on regular cycles, clone-correction is a way to infer the underlying genetic structure after sexual reproduction (Milgroom et al., 1992; Maynard Smith et al., 1993). For these populations, clone correction is valid as long as the probability of sampling the same genotype in randomly mating populations ( $p_{\text{sex}}$ ) is very small. In all populations of *E. necator* in this study,  $p_{\text{sex}}$  was small for genotypes represented more than once, indicating a significant contribution by clonal reproduction. Yet, not all fungi with a mixed mode of reproduction, including *Gibberella zeae*, show linkage disequilibrium within populations (Zeller et al., 2004).

For some fungal plant pathogens, clone correction reduces LD to insignificant levels, e.g., *Cryphonectria parasitica* (Milgroom et al., 1992) and *Mycosphaerella graminicola* (Zhan et al., 2003). Linkage disequilibrium after clone correction has been detected in other fungi with a mixed mode of reproduction (Brown and Wolfe, 1990; Bogacki et al., 2010; Dale et al., 2011). The fact that clone correction did not eliminate LD means that LD is caused by factors other than asexual reproduction alone.

LD may be caused by epistatic selection (Felsenstein, 1965) or hitch-hiking selection, admixture of genetically different populations or physical linkage among markers (Halliburton, 2004). Epistatic selection, which favors combinations of advantageous alleles, can cause linkage disequilibrium in randomly mating populations if selection is strong compared to the recombination breaking up associations among alleles. Epistatic selection has been suggested as a possible cause of linkage disequilibrium in the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*, where the use of resistant cultivars and selective fungicides favored combinations of avirulence and fungicide resistance alleles (Wolfe, 1984; Brown and Wolfe, 1990). However, other factors, such as random genetic drift followed by hitchhiking (clonal) selection associated with selection for avirulence or fungicide resistance alleles may have also played a role. In *E. necator*, however, we observed many pairs of loci in linkage disequilibrium, so it is unlikely that epistatic selection for combinations of favorable alleles is the main factor. During asexual generations, selection is acting on the entire genome, in which case, we observe LD because of hitchhiking selection. Variation in fitness among genotypes during the asexual phase potentially leads to an explosion of some of the fittest genotypes, as in the epidemic model (Maynard Smith et al., 1993). Frenkel et al. (2010) showed significant variation in lesion size and latent periods in *E. necator* in the eastern US. Additionally, variation in resistance to various

fungicides has been documented (Erickson and Wilcox, 1997; Wong and Wilcox, 2002; Miller and Gubler, 2004). Future studies would be needed to address whether dominant clones of *E. necator* at the end of the epidemic have greater fitness or fungicide resistance compared to the population at the beginning of the season. We predict that populations of *E. necator* fit an epidemic structure (Maynard Smith et al., 1993), which is characterized by a mixed mode of reproduction and linkage disequilibrium, in which a few highly fit genotypes reach high frequencies due to selective sweeps.

Another cause of LD is population admixture, e.g., sampling genetically different subpopulations and analyzing them as one population. Within a vineyard, limited dispersal of *E. necator* could be contributing to linkage disequilibrium because of admixture. If random mating occurs only within patches representing spatially restricted genetic neighborhoods, then population admixture will give the effect of inbreeding and lead to linkage disequilibrium across the entire population (Wright, 1943; Milgroom, 1996). We found spatial aggregation of genotypes in both vineyards at the end of the epidemics. Mantel correlations between physical and genetic distances were significant for both populations demonstrating some degree of isolation by distance or subdivision into genetic neighborhoods on a small scale. Spatial autocorrelation showed that isolates sampled from relatively short distances apart were more similar to each other than expected at random, indicating that dispersal distances are restricted; longer distance dispersal would lead to mixing of genotypes and no detectable spatial structure. In the VA09 population, aggregates of similar genotypes were 24 meters, whereas NY09 aggregates were 3 meters. These differences in patch sizes could be due to differences in vineyard sampling patterns. The VA vineyard was sampled in a rectangular-shaped pattern due to its limited size, whereas the NY vineyard was sampled earlier in a square pattern. Other

factors that could contribute to the differences in patch sizes are that the VA vineyard is a small research vineyard with few other grapevines nearby, whereas powdery mildew samples from the NY vineyard came from part of a large commercial vineyard in the Finger Lakes Region, which is a large grape production area. Additionally, the presence of a single dominant clone in NY versus several moderate-sized clones in VA may have caused the differences in patch sizes detected. We were not able to determine if autocorrelation was due to clonal genotypes or to isolates with unique genotypes that were genetically similar because we could not clone correct for spatial data.

We also detected population structure over a large geographic scale. Populations from the two vineyards were genetically differentiated indicating that gene flow is restricted. Fall and spring populations within a single vineyard (VA09 and VA10) were not differentiated suggesting that inoculum is relatively local and there is not a large influx of distant migrants at the onset of the epidemic. This would need to be tested further by sampling fall and spring vineyard populations along a geographic gradient. However, it is very difficult to find populations of *E. necator* within vineyards in the spring that are representative of the local population of ascospores. Many commercial vineyards are managed with fungicides early in the spring so that there are almost no colonies present at ascospore release preventing an analysis of all potential combinations in offspring. Research vineyards, which usually have non-sprayed control plots are an alternative; however, plots that have been inoculated with a single or a few isolates of *E. necator* could potentially affect population structure.

We are fairly confident that the linkage disequilibrium detected in this study is not due to physical linkage because we found that 13 % to 58 % of the pairs of loci were in disequilibrium in the three populations after clone correction. With the use of 10 different markers, it is

unlikely that linkage on the same chromosome is contributing to this level of pairwise disequilibrium. Ideally, sexual crosses would be constructed to determine if markers are genetically linked. However, despite repeated attempts we have not been able to obtain enough viable progeny from crosses of *E. necator* to evaluate recombination and segregation of alleles at these EST-SSR loci. We know of only a single cross that has been documented for *E. necator*; there were only 18 progeny and segregation of markers was abnormal (Stummer and Scott, 2003), likely because the cross was between isolates of the otherwise reproductively isolated genetic groups found in introduced populations. Of the 18 progeny obtained from this sexual cross and tested with 27 molecular markers, 13 progeny had parental genotypes and only five progeny were recombinants, with two recombinants of identical genotypes.

We hypothesize that a mixed reproductive mode with epidemic structure, combined with spatial aggregation of genotypes is likely contributing to linkage LD in *E. necator*. We speculate that LD persists in populations of *E. necator* because the increase in LD caused by clonal selection during the asexual phase is not completely broken down by a single annual generation of sexual reproduction. Linkage disequilibrium between unlinked loci can only decay by a maximum of 50% with recombination, so many of the alleles in the dominant clones can remain nonrandomly associated. Inbreeding, which is directly calculated as a decrease in observed relative to expected heterozygosity (Halliburton, 2004), is difficult to measure in haploid fungi. An alternative would be to genotype the diploid phase as the composite genotype of the progeny from each cleistothecium (Milgroom, 1995). We tried to genotype ascospores from single cleistothecia from both vineyards, but were not able to get reliable or consistent results even with whole genome amplification.

Modeling of recombination, clonal selection and limited dispersal in *E. necator* and other

plant pathogenic fungi may provide support for our hypothesis that LD can persist in sexual populations. Understanding how linkage disequilibrium affects the population structure and evolution of *E. necator* is important for understanding the biology of this devastating plant pathogen and determining whether or not association mapping can be done in this fungus.

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