Genetic Diversity of *Phytophthora ramorum* in Belgium[1]

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Abstract

Phytophthora ramorum is thought to be an introduced pathogen in North America and in Europe based on the presence of only three clonal lineages. The North American lineages (NA1 and NA2) are responsible for infections in North American forests and nurseries, while the European lineage (EU1) is responsible for infections in Europe, mostly in nurseries. There have also been a few isolated findings of the EU1 lineage in North American nurseries. *P. ramorum* is heterothallic, with two opposite mating types, A1 and A2. The A1 mating type was originally only found in Europe, with the exception of three A2 isolates of the EU1 lineage collected in Belgium in 2002 and 2003. Although there is no evidence for sexual reproduction in nature, the presence of both mating types at a single site might lead to genetic recombination.

To verify the hypothesis of asexual reproduction in nature, and to verify the hypothesis that *P. ramorum* was recently introduced in Belgium and therefore possesses only a limited amount of genetic diversity, the Belgian *P. ramorum* population was screened with AFLP and SSLP markers. Use of the AFLP method with five primer combinations on a selected number of isolates (80) revealed 13 polymorphic fragments. These markers identified eight isolates that differed from the main genotype by one to three polymorphisms. Use of SSLP with existing microsatellite markers revealed a limited number of polymorphisms in the EU1 population. Additional microsatellite markers were then sought. A total of 146 candidate polymorphic microsatellites were prescreened using 10 isolates belonging to different EU1 genotypes. This resulted in two new primer pairs that amplify a total of three polymorphic loci, of which one was very useful. Seven markers (four existing and three new) were used to screen all 411 Belgian isolates. In total, 30 genotypes were identified, but 68 percent of the isolates belong to the main genotype EU1MG1. Although indications of accumulated mutation events were present, the overall level of genetic diversity within these isolates of *P. ramorum* appears to be limited, indicating a relatively recent clonal dispersion of the pathogen. Most of the genotypes were site-specific and some of them were detected over a period of several years at a single site, sometimes discontinuously. This indicated (latent) survival of the pathogen at those sites and led to questions about the efficiency of the eradication measures. No marker recombination was observed, indicating that no sexual recombination was found in nature.

Introduction

Since 2002, European Union (EU) emergency phytosanitary measures have been taken to prevent the introduction and spread of *Phytophthora ramorum* in Europe. In Belgium, these measures are implemented by the National Plant Protection Service (FAVV), which conducts annual inspections at all nursery and retail sites that house

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potential host plants. They also conduct surveys at parks and forests for symptoms of *P. ramorum*. Since 2002, *P. ramorum* has been detected in approximately 60 nurseries, and on new *Rhododendron* plantings in two public parks and five private gardens. Quarantine measures were implemented at all sites and the isolates have been stored.

All isolates belong to the A1 mating type, with the exception of three A2 isolates of the EU1 lineage collected in 2002 and 2003. Although there is no evidence of sexual reproduction in nature, the presence of both mating types at a single site might lead to genetic recombination.

Molecular marker systems, such as AFLP and microsatellite polymorphisms (SSLP), have been used to determine the genetic diversity within *P. ramorum* populations and to verify the presence of sexual recombination events (Ivors and others 2004, 2006, Mascheretti and others 2008, Prospero and others 2004, 2007).

However, the microsatellite markers that are currently available show few polymorphisms in the EU1 clonal lineage. The first objective of our research was thus to identify novel, polymorphic microsatellite loci in the EU1 lineage. We then used the newly developed and existing microsatellite markers, as well as AFLP, to determine the genetic diversity of the Belgian EU1 population. Indications of genetic recombination in this population were verified with the microsatellite markers. Another objective of the research was to relate the data on genetic diversity with those of isolate geographical origin, isolation year, and fungicide resistance, and to use this information to evaluate the success of the eradication efforts.

Material and Methods

All Belgian isolates collected during 2002 to 2008 were used in this study. A selection of 80 isolates was used for the comparison between AFLP and microsatellite analysis, using a stratified sample based on isolation year, host, and site. AFLP reactions were performed as described by Ivors and others (2004). For the microsatellite analysis, extra markers were needed to have a high-resolution screening. The first set of candidate microsatellite markers were 34 primer pairs designed by Ivors and others (2006) and Prospero and others (2004, 2007), which showed polymorphism within the NA1 population, but had not been screened yet for polymorphism within EU1 isolates. The second set of candidate microsatellite markers consisted of 71 dinucleotide, 27 trinucleotide, 11 tetranucleotide, one pentanucleotide, and six hexanucleotide repeat loci that were selected by screening the genome of *P. ramorum* (Tyler and others 2006) for repeats. To avoid the costs of genotyping with individual fluorescently labeled primers, universal fluorescent labeling was used (Shimizu and others 2002). For each microsatellite primer pair, polymorphism was assessed between an EU1 (PR/D/04/284) and an NA1 isolate (PRI483). This was the first selection criterion, because the chance for intra-lineage polymorphisms was expected to be small if no inter-lineage polymorphisms were present. Inter-lineage polymorphic loci were analyzed further with a panel of eight EU1 isolates that belong to separate MG groups based on the study of Ivors and others (2006) or showed polymorphism in preliminary screening. Loci that were polymorphic in these selected isolates were analyzed in all isolates.

All *P. ramorum* isolates were evaluated for resistance to metalaxyl, based on Heungens and others (2006). Growth rate was determined between seven and 13 days post inoculation on the non-metalaxyl containing control plates.

Results and Discussion

The 411 isolates of *P. ramorum* included in this study were obtained from *Rhododendron* (90.5 percent of the plants sampled) or *Viburnum* plants (9.5 percent). At 61.6 percent of all nurseries where *P. ramorum* has been detected, epidemics were contained to a single year.

Our direct comparison using 80 isolates revealed more microsatellite-based diversity than AFLP-based diversity, which contrasts with the work of Ivors and others (2004). Microsatellite analysis was also more appropriate than AFLP, owing to its co-dominant nature and better reproducibility. After identifying a sufficient number of polymorphic markers, we opted for microsatellite analysis' technical simplicity and limited cost. However, identification of polymorphic microsatellite markers in a clonal population with low genetic diversity is difficult, even when the complete genome sequence is available. Many microsatellites are polymorphic between EU1 and NA1 isolates, but not within the EU1 population. Due to the limited overall genetic diversity within the EU1 lineage, screening of all 149 candidate primer pairs resulted in the identification of only two new primer pairs.

Microsatellite alleles were determined for the 411 Belgian isolates using the three primer pairs previously described (Ivors and others 2006) and the two new primer pairs. As one of the primer pairs amplifies two loci and the other amplifies three loci, a total of eight microsatellite loci were studied, seven of which were polymorphic. Based on these markers, 30 microsatellite genotypes (MG) were identified. In each year, the Belgian population was dominated by genotype EU1MG1 (68 percent of all isolates). Site-specific divergence was demonstrated at several nursery sites. The persistence of rare genotypes, and the intermittent absence of a unique genotype in a specific site, was observed. This indicates latent survival of *P. ramorum* and failure of eradication at some nurseries.

No indications of sexual or mitotic recombination were found. Selection for more metalaxyl-resistant isolates was correlated with a decrease of allelic richness. An upward trend in average growth rate and a significant reduction in growth rate variance indicated selection for faster-growing isolates.

Belgian isolates of *P. ramorum* were the subject of this work, owing to the geographical origin of the European A2 isolates. However, the microsatellite loci identified in this work are excellent markers for genotyping the general EU population of *P. ramorum*. The newly identified microsatellite loci are also polymorphic within the NA1 population and are therefore very useful for higher-resolution genotyping of this population.

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