Genetic Diversity of *Phytophthora ramorum* in Nursery Trade and Managed Environment in Scotland[1]

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Abstract

Scottish *Phytophthora ramorum* isolates (228) collected between 2002 and 2011 from almost every outbreak site in Scotland were genotyped using seven microsatellite markers as described by Vercauteren et al. (2010). Thirty multilocus genotypes were identified within the Scottish population, with 51 percent of the isolates belonging to the main European genotype EU1MG1 and 13 unique detected genotypes. Ten of those genotypes were site specific, often represented by single isolates. Three *P. ramorum* isolates, all from the same location, belonged to the new EU2 European lineage.

The number of genotypes found in the managed environment was higher than the number found in the horticultural trade (25 vs. 11), probably due to the fact that outbreaks in nurseries are usually detected earlier and are quickly eradicated. Evidence of locally-evolved genotypes was found in some outbreak sites.

Keywords: *Phytophthora ramorum*, genetic diversity, nursery, managed environment

Introduction

Although *Phytophthora ramorum* was first discovered in 1993 on *Rhododendron* and *Viburnum* spp. in nurseries in Germany and The Netherlands (Werres et al. 2001), it only raised the attention of a wider range of scientists when it became apparent that it was responsible for sudden oak death on oaks (*Quercus* spp.) and tanoaks (*Notholithocarpus densiflorus* Hook. & Arn.) Manos, Cannon & S.H. Oh) (Rizzo et al. 2002) in North America. In contrast, in Europe the pathogen caused only limited ecological damage, despite being present in most European countries and having a remarkably wide host range. Findings outside the nursery trade were limited, the main host being *Rhododendron ponticum* L. Even in the United Kingdom, as the worst affected European country, only 28 trees were infected with the disease by March 2008 (Tracy 2009). However, since 2009 it has been found on Japanese larch (*Larix kaempferi* (Lam.) Carrière) and, to lesser extent, also on other *Larix* spp. in the United Kingdom. By 2010, an estimated 1900 ha of larch plantations were showing symptoms of the disease, triggering the invention of the term sudden larch death (Brasier and Webber 2010).

Phytophthora ramorum has so far only been found in North America and Europe. Early AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeats) studies (Ivors et al. 2004, 2006) showed different populations on both continents, but only limited genetic variation within these populations. More recently, additional new SSR markers revealed more variation within the United States population of *P. ramorum* (Mascheretti et al. 2008; Prospero et al. 2004, 2007) and within the European population (Vercauteren et al. 2010). The latter were able to distinguish 30 multilocus genotypes within the Belgian population using four previously described and three newly identified markers.

As of early 2012, three distinct genetic lineages had been reported: the EU1 lineage present in Europe and a small number of nurseries along the United States west coast, and the NA1 and NA2

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lineages currently restricted to North America (Goss et al. 2009, Grünwald et al. 2009, Ivors et al. 2006, Martin 2008). The dominant NA1 lineage is found in forestry and nursery environments; whereas, NA2 has been recovered only from nurseries and from a few waterways. Recently, a new *P. ramorum* lineage, designated EU2, has been reported (Van Poucke et al. 2012), with the known geographical origin of those isolates currently limited to Northern Ireland and western Scotland.

Phytophthora ramorum is a heterothallic species requiring two mating types (A1 and A2) for sexual recombination. All NA1 and NA2 isolates so far have been found to be of the A2 mating type; whereas, the EU1 and EU2 isolates, with the exception of three Belgian isolates, belong to the A1 mating type (Brasier and Kirk 2004, Van Poucke et al. 2012, Werres and Kaminski 2005). However, there has been no evidence of natural sexual reproduction in population studies (Ivors et al. 2006, Vercauteren et al. 2010), and in *vitro* mating studies with *P. ramorum* have revealed slow and reduced production of gametangia (Brasier and Kirk 2004) and progeny showing reduced fitness (Boutet et al. 2010).

In Scotland, the history of *P. ramorum* can be split into two time periods. During the period from the first finding in April 2002 until the end of 2006, the disease was restricted to the ornamental nursery trade. Phytosanitary measures successfully reduced the number of outbreaks each year down to no outbreaks in 2006 (Schlenzig 2006). In the second period, from 2007 to the present, the disease was also found on ornamental plants in the managed environment, such as historic gardens, parks, and country estates, and the overall number of outbreaks increased dramatically, although the number of outbreaks in nurseries remained very low.

This paper reports the genotyping results of the Scottish *P. ramorum* population using the SSR markers described by Vercauteren et al 2010. It compares the population in the nursery trade with the population in the managed environment, and investigates the genetic diversity over the 10 years from the first outbreak until 2011.

Materials and Methods

Isolates and DNA Extraction

Samples of symptomatic material from host plants moving in trade or established plants in public gardens, parks, and country estates were collected by Scottish Government inspectors during official surveillance for the organism. The pathogen was isolated using semi-selective V8 medium as described by Jung et al. 1996. The government laboratory at Science and Advice for Scottish Agriculture had collected 228 isolates, comprising representative isolates from almost every outbreak site in Scotland from 2002 to December 2011. Depending on the size and duration of outbreak, 1 to 22 isolates were collected per site over the years. A total of 71 percent of the isolates had been isolated from *Rhododendron* spp., 17 percent from *Viburnum* spp., and the remainder from other hosts like *Magnolia* spp. or *Pieris* spp.

The Nucleo Spin Plant DNA extraction kit (Macherey & Nagel Inc.) was used to extract DNA. Mycelium from fungal cultures was transferred into 1.5 mL microcentrifuge tubes with 400 µL C1 extraction buffer (supplied with the kit) and was ground using micro-pestles with sterile sand as the grinding agent. Further extraction was performed in accordance with the manufacturer's instructions. The DNA from eight isolates from the Belgian study (Vercauteren et al. 2010) was included as reference.

SSR Genotyping

Seven SSR loci were genotyped that had previously shown variation among European *P. ramorum* isolates: 18, 64, 82a (Ivors et al. 2006), 82b, ILVOPrMS133, ILVOPrMS145a, and ILVOPrMS145c. All primer sequences and repeat motifs can be found in Vercauteren et al. 2010. Primers ILVOPrMS133 and ILVOPrMS145 were used without the M13reverse tag, but with an added 5'-GGGT-3' "pigtail" to the 5' end of the reverse primer to reduce stuttering. The resulting differences in allele sizes were taken into account for comparison with other European genotypes. Forward

primers were labelled with FAM (18, 64, ILVOPrMS133), NED (82), or VIC (ILVOPrMS145). All primers were combined in a multiplex PCR reaction. The reaction volume was 12 µl. Final PCR concentrations were 1x Type-it Microsatellite PCR Master Mix (Qiagen), 0.1 µM of primer pairs 82 and ILVOPrMS145 and 0.14 µM of primer pairs 18, 64 and ILVOPrMS133, and 10 ng template DNA. Amplification conditions were an initial denaturation at 95 ºC for 5 minutes, followed by 28 cycles of 30s at 95 ºC, 90s at 58 ºC, and 20s at 72 ºC followed by the final extension at 60 ºC for 30 min. Then 1 µl of each PCR product was mixed with 8.7 µl Hi-Di formamide loading buffer (Life Technologies) and 0.3 µl Gene Scan 500 LIZ size standard (Applied Biosystems), denatured for 5 min at 95 ºC, and run on a ABI 3130xl genetic analyzer (Applied Biosystems). Results were analyzed using Gene Mapper 4.0 (Applied Biosystems).

Where possible, genotypes were designated in accordance with a European SSR genotyping study conducted by the Institute for Agricultural and Fisheries Research (IVLO) in Belgium (2010 to 2011), in which over 1,300 isolates from throughout Europe had been genotyped (K. Heungens, IVLO, personal communication).

Results

All seven SSR markers were polymorphic in the Scottish population and distinguished 30 multilocus genotypes amongst the 228 Scottish isolates. The European main genotype (EU1MG1) was by far the most common genotype, with 51 percent of isolates found at 44 outbreak sites (fig. 1). It was consistently the most frequently found genotype throughout the 10 years of this study. Also relatively widespread were EU1MG5 (12 outbreak sites), EU1MG 18 (six outbreak sites), and EU1MG44 (four outbreak sites). EU1MG13 and EU1MG2, common genotypes on the European continent (K. Heungens, ILVO, personal communication), were found only on one occasion and not at all, respectively. A comparison with the ILVO study revealed that 13 of the detected genotypes were new to Europe (including the rest of the United Kingdom) and unique to Scotland (K. Heungens, IVLO, personal communication). Ten of those genotypes were site specific, often represented by single isolates. Almost 20 percent of isolates have a deletion of three repeats in marker 133. This mutation is unique to the United Kingdom and not found anywhere else in Europe (K. Heungens, IVLO, personal communication).

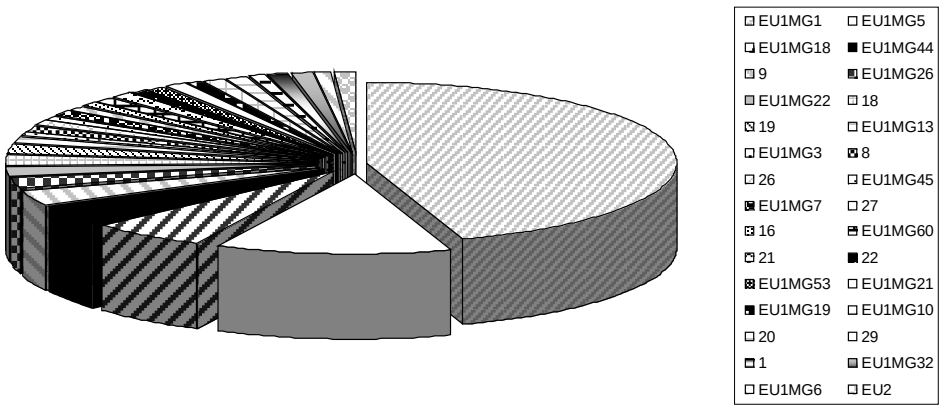

Figure 1—Multilocus genotypes found in Scotland from 2002 to 2011 according to the number of outbreak sites where they were present.

A total of 225 Scottish isolates belonged to the EU1 lineage. Three isolates did not match the EU1 or any of the other genetic lineages known at the time of the study. All three isolates were displaying the same SSR profile and have been collected in 2011 from the same garden location in the southwest of Scotland. The same deviating SSR profile had been found in three other isolates from Northern Ireland. Further analysis revealed that they belong to the new EU2 genetic lineage (Van Poucke et al. 2012; K. Heungens, IVLO, personal communication).

Six genotypes (68 percent of isolates) were found in both settings (horticultural trade and managed environment), and 25 genotypes were found at 39 sites in the managed environment compared to 11 genotypes found in 25 nurseries and garden centers (figs. 2 and 3). Five genotypes (2 percent of isolates) were present in nurseries and garden centers only. Nineteen genotypes (30 percent of isolates) were present only in the managed environment. The diversity of genotypes was therefore higher in the managed environment than it was in the horticultural trade.

Of the 13 outbreak sites from which at least five isolates had been collected, only two sites were infected by single genotypes. The number of genotypes in the other sites varied considerably, independently from the extent of the outbreak and the number of isolates collected. For example, from one garden outbreak site on the west coast of Scotland, 21 isolates had been collected over 4 years, all but one belonging to the same genotype. A park in Glasgow on the other hand yielded six different genotypes amongst only 11 isolates collected over 3 years.

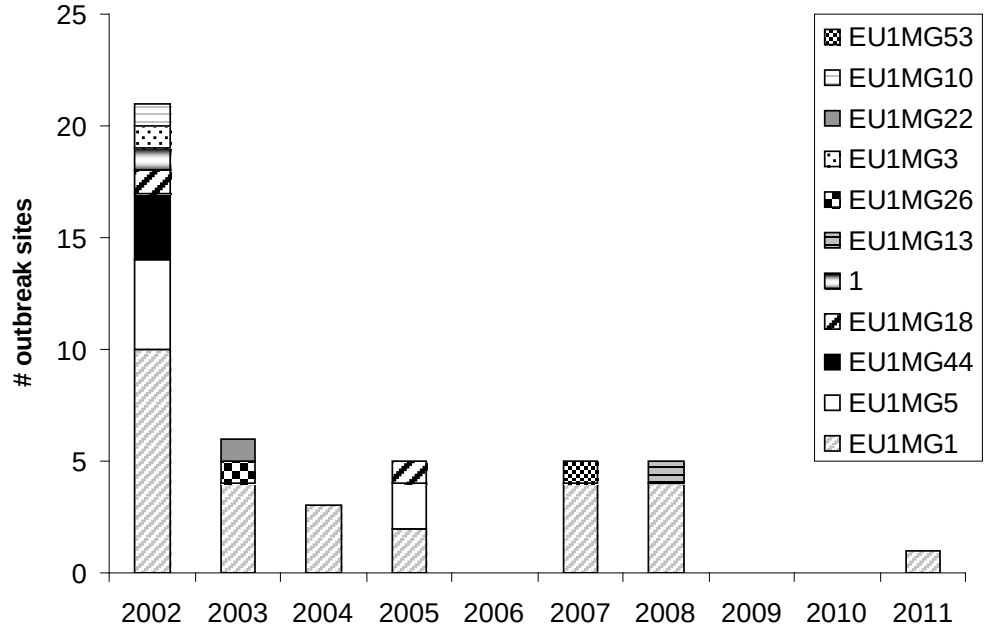

Figure 2—Genotypes detected in nurseries and garden centers. Genotypes unique to Scotland are indicated by simple numbers. Genotypes found elsewhere in Europe are named in accordance to European genotyping study as numbers with prefix “EU1MG” (K. Heungens, Institute for Agricultural and Fisheries Research, personal communication).

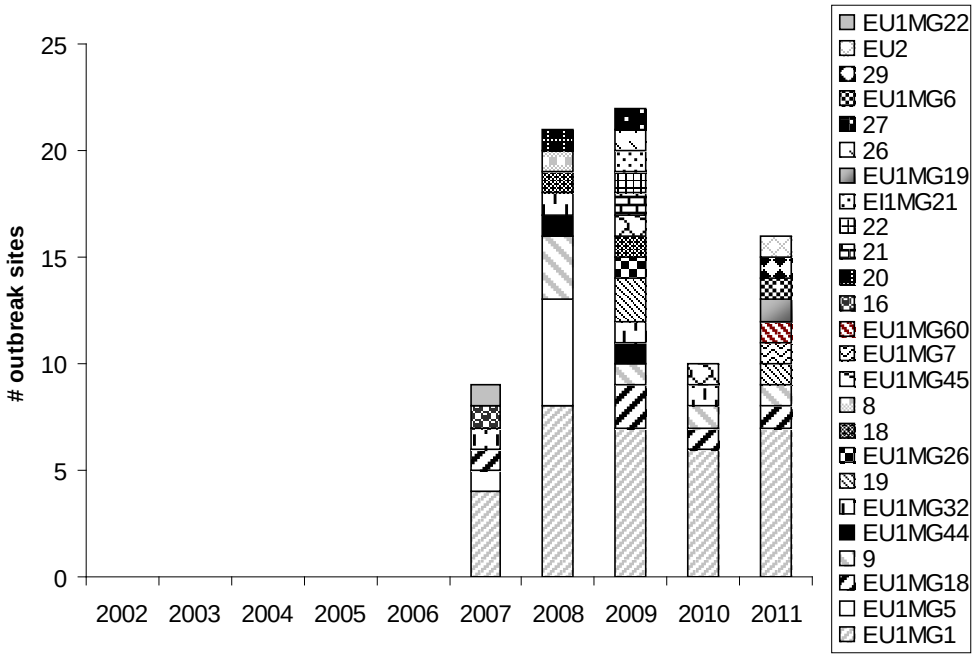

Figure 3—Genotypes detected in the managed environment. Genotypes unique to Scotland are indicated by simple numbers. Genotypes found elsewhere in Europe are named in accordance to European genotyping study as numbers with prefix "EU1MG" (K. Heungens, Institute for Agricultural and Fisheries Research, personal communication).

Discussion

All genotyping of European *P. ramorum* isolates so far has shown the limited diversity of a near clonal population (Ivors et al. 2006, Vercauteren et al. 2010), and the Scottish population is no exception. However, there are differences from the population in the rest of Europe. About half of all Scottish isolates belong to genotype EU1MG1. This is lower than on the European continent, where the percentage is 64 percent (K. Heungens, IVLO, personal communication). A few relatively common genotypes in Europe are very rare or absent in Scotland. On the other hand, 13 genotypes found in Scotland are not present in the rest of Europe. The Belgian study of the European *P. ramorum* population identified 66 genotypes amongst about 1,300 isolates (K. Heungens, IVLO, personal communication). However, approximately a sixth of the number of isolates resulted in the finding of 30 genotypes in Scotland. Taking the lower number of isolates into account, the genetic diversity appears higher in Scotland. The diversity of a population can be an indication of its age or a sign of lively exchange with other populations. The latter is unlikely in Scotland, situated on an island on the northern fringe of Europe and with limited horticultural trade. However, there are a number of historic gardens with rhododendron collections, and the area has a past tradition of plant hunting (and exchange). The apparently higher diversity might also be a consequence of the high sample density. The Scottish isolate collection encompassed isolates from almost every outbreak site in Scotland, with the exception of two or three minor sites. Moreover, depending on size and duration, multiple isolates were collected from each site. This nearly "complete" sampling increases the likelihood of finding rare genotypes, specifically the ones represented only by single isolates, which are easily missed when a smaller share of the population is sampled.

The limited distribution of the new EU2 lineage and the fact that, despite 10 years of surveys, it was only found in one site, suggests that it has only recently been introduced to Scotland. The origin of this new lineage and how it spread between Northern Ireland and Scotland is unclear and requires further research. The distance between the outbreak site in Scotland and the Northern Irish coast is

approximately 80 km. Rizzo et al. (2005) consider 3 km as the upper limit for natural dispersal, making human mediated spread more likely.

Long-distance dispersal of *P. ramorum* has been linked to the movement of infected plants in the horticultural trade, meaning a higher likelihood of the introduction of new genotypes into these businesses. Therefore, it might be expected that they have a higher genetic diversity than sites in the managed environment. That this is not the case in Scotland is probably thanks to the official eradication measures introduced immediately after the first findings of this disease. According to Vercauteren et al. (2010), "The human-induced bottleneck due to eradication efforts can lead to extinction of the least abundant genotypes." Many of the rare genotypes in nurseries appear to have been eradicated by phytosanitary measures. For example, Scottish genotypes 1, 6, and 15 found in 2002 were never detected again, neither in nurseries nor in the environment. Although rare genotypes are more susceptible to eradication and extinction, in some cases they can become the major genotype of a site, building a very specific local population. Scottish genotype 2, for example, is only present in one historic garden, but in this garden it has been found in 20 out of 21 isolates collected over 3 years.

The managed environment on the other hand shows a high degree of genotype diversity all throughout the years, with a high number of rare genotypes especially in the later years. Outbreaks in the environment are much harder to eradicate since the complete removal of infected plants often is not possible, giving rare genotypes a better chance of survival.

In general, there are two possible explanations when a variety of genotypes is found at the same outbreak site: they either are separate introductions of the pathogen or they have developed locally at the site. In this study, unique or very rare genotypes were found on some sites which were showing just one minor difference in the SSR profile to the main genotype at the same site (data not shown). The fact that these rare genotypes have not been found anywhere else can be taken as evidence that they developed locally within the site. Vercauteren et al. (2010) also found some evidence of local evolution of new genotypes in their study. Outbreaks in the semi-natural environment are often discovered later than outbreaks in the closely monitored plant trade and are much more difficult to eradicate. Often they last for years, providing more time for new genotypes to develop.

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