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Clonal expansion of the Belgian *Phytophthora ramorum* populations based on new microsatellite markers

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Abstract

Co-existence of both mating types A1 and A2 within the EU1 lineage of *Phytophthora ramorum* has only been observed in Belgium, which begs the question whether sexual reproduction is occurring. A collection of 411 Belgian *P. ramorum* isolates was established during a 7-year survey. Our main objectives were genetic characterization of this population to test for sexual reproduction, determination of population structure, evolution and spread, and evaluation of the effectiveness and impact of control measures. Novel, polymorphic simple sequence repeat (SSR) markers were developed after screening 149 candidate loci. Eighty isolates of *P. ramorum*, broadly representing the Belgian population, were analyzed using four previously described and three newly identified polymorphic microsatellite loci as well as amplified fragment length polymorphisms. SSR analysis was most informative and was used to screen the entire Belgian population. Thirty multilocus genotypes were identified, but 68% of the isolates belonged to the main genotype EU1MG1. Although accumulated mutation events were detected, the overall level of genetic diversity within the Belgian isolates of *P. ramorum* appears to be limited, indicating a relatively recent clonal expansion. Based on our SSR analysis there is no evidence of sexual recombination in the Belgian population of *P. ramorum*. Metalaxyl use decreased the genetic diversity of *P. ramorum* until 2005, when the majority of the isolates had become resistant. Most genotypes were site-specific and despite systematic removal of symptomatic and neighbouring plants, some genotypes were detected over a period of several years at a single site, sometimes discontinuously, indicating (latent) survival of the pathogen at those sites.

Keywords: metalaxyl resistance, nurseries, oomycetes, population genetics, Sudden Oak Death, universal labelling

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Introduction

Phytophthora ramorum is a diploid oomycete plant pathogen that was first detected in 1993, causing leaf blight and twig dieback on *Rhododendron* and *Viburnum* nursery plants in the Netherlands and Germany (Werres *et al.* 2001). The same pathogen is also responsible for causing extensive mortality on oaks (mainly *Quercus agrifolia*) and tanoaks (*Lithocarpus densiflorus*) in California, killing over 200 000 trees in the Big Sur area alone (Meentemeyer *et al.* 2008). Since then, the disease, also known as Sudden Oak Death, has been found in North America on various native host plants in the forests in California and Oregon and in nursery stock in over 20 states in the USA and in Canada (Grünwald *et al.* 2008b). In Europe, *P. ramorum* has mainly been detected in ornamental nurseries, especially on *Rhododendron*,

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Viburnum and *Camellia*. It has been found in a considerable number of EU countries including Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Ireland, Italy, the Netherlands, Latvia, Lithuania, Luxembourg, Norway, Poland, Portugal, Slovenia, Spain, Switzerland, Sweden and the UK (Werres *et al.* 2001; Moralejo & Werres 2002; De Merlier *et al.* 2003; Lane *et al.* 2003; Herrero *et al.* 2006; Husson *et al.* 2007; Lilja & Rytkonen 2007). A limited number of trees have been infected by *P. ramorum* in Europe (Brasier *et al.* 2004; Tracy 2009). In 2003, the first *P. ramorum*-infected *Quercus falcata* tree was confirmed in the UK, along with several *Quercus rubra* trees in the Netherlands (Brasier *et al.* 2004). Genera or species in the family Fagaceae with susceptible bark (especially *Quercus* and *Fagus*) are considered most at risk of developing potentially lethal stem cankers (Brasier *et al.* 2002; Moralejo *et al.* 2009). However, the total number of European trees that are known to have died from *P. ramorum* is less than 50, and almost all of these were in the immediate proximity of infected *Rhododendron ponticum* plants (Brasier *et al.* 2004; Tracy 2009).

Three clonal lineages of *P. ramorum* have been described, with isolates of the EU1 lineage mainly present in Europe, and isolates of the NA1 and NA2 lineages only present in North America (Ivors *et al.* 2006; Martin 2008; Grünwald *et al.* 2009). *P. ramorum* is heterothallic, having two opposite mating types A1 and A2. With the exception of three Belgian isolates collected in 2002 and 2003, all EU1 isolates are of A1 mating type (Werres & De Merlier 2003; Grünwald *et al.* 2009). All NA1 and NA2 isolates are of A2 mating type, while EU1 isolates detected in North American nurseries are A1 (Hansen *et al.* 2003; Prospero *et al.* 2007). The presence of both mating types at a single site might lead to genetic recombination and an increase in the pathogens fitness and host range. The formation of sexual resting spores could also increase the long-term survival potential of *P. ramorum* (Grünwald *et al.* 2008b). As the EU1 A2 findings were limited and have been eradicated, the risk for recombination is currently largest in North America, where both mating types have been found in coexistence although sexual reproduction has to date not occurred (Grünwald *et al.* 2008a; Goss *et al.* 2009b; Prospero *et al.* 2009). However, no information is currently available regarding sexual recombination in Europe in situations where isolates of both mating types coexisted.

Based on the dramatic impact of *P. ramorum* on certain tree species in the US and the potential risk for damage in Europe, the European Community established emergency phytosanitary measures to prevent the further introduction and spread of the pathogen in 2002 (Commission Decisions 2002/757/EC, 2004/426/EC, and 2007/201/EC). Measures include controls on the import and trade of susceptible plants into and within the EU, including requirements for plant passports as well as surveys on specific species of susceptible host plants. In Belgium, these EU emergency phytosanitary measures were implemented by the National Plant Protection Service (FAVV), which conducted annual (before 2007) to biannual (after 2006) inspections of all commercial sites with host plants (>300 sites, with less than 50 nurseries specialized in *Rhododendron* or *Viburnum* production), as well as 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 (FAVV, personal communication). The pathogen has not yet been detected in forests. The EU-mandated eradication measures have been put in effect at all sites with infected plants. This includes destruction of all host plants within a 2-m radius of the original finding and extra controls during a 3-month quarantine period for all remaining host plants in a 10-m radius. Eradication measures seemed to be quite effective outdoors and at most nursery sites, as *P. ramorum* symptoms were detected during more than 2 years at only six sites.

Information on population structure over time is a basis for assessing pathogen evolution and spread. It can also help determine whether sexual recombination has taken place, aid in the evaluation of the efficacy of emergency phytosanitary measures, and determine the effect of fungicide treatments on population structure. A fungicide of specific interest is metalaxyl, as it is commonly used for control of *Phytophthora* pathogens in nursery environments and is well known to impose a strong selection for resistance on a *Phytophthora* population (Grünwald & Flier 2005; Grünwald *et al.* 2006). Resistance to metalaxyl in *Phytophthora infestans* is thought to be controlled by a single chromosomal gene, subject to further regulation by several additional loci (Gisi & Cohen 1996; Fabritius *et al.* 1997; Lee *et al.* 1999). Since metalaxyl was used regularly in Belgium, populations of *P. ramorum* might be structured by use of this fungicide. Studies on the genetic diversity of the European *P. ramorum* population based on amplified fragment length polymorphisms (AFLP) indicated that the European population consists of mainly unique, closely related AFLP genotypes (Ivors *et al.* 2004). According to Ivors *et al.* (2006), genotypic diversity using simple sequence repeat (SSR) was even more limited than with AFLP. Three primer pairs (18, 64 and 82) were informative and distinguished seven multilocus genotypes (MGs) within a selection of European isolates, with 86% of the isolates belonging to one main genotype. Microsatellite-based genotyping of the North

American populations of *P. ramorum* was facilitated by the identification of additional polymorphic SSR markers by Prospero *et al.* (2004, 2007). They found that the 2001–2004 *P. ramorum* forest population in Oregon is also characterized by low levels of genetic variation and has all the characteristics of an introduced organism. All isolates with the A2 mating type belonged to the same clonal lineage and no sexual recombinants were found. In contrast to the forest population, the nursery population of *P. ramorum* was not dominated by a single MG. There was low gene flow and significant differentiation between nursery and forest populations. In California, Mascheretti *et al.* (2008) found three widely distributed MGs of *P. ramorum* in the native forests and 32 less-broadly distributed MGs that may have evolved locally at new infestation sites. More recently, Goss *et al.* (2009b) studied the entire US nursery population. They found three clonal lineages with the NA1 lineage being distributed over much of the continental US. Two eastward migration pathways were revealed in the clustering of NA1 isolates into two groups, originating either from the Pacific Northwest or California. Populations remain clonal, despite occasional presence of both mating types, and appear to be structured by migration, rapid mutation and genetic drift.

Simple sequence repeat markers were developed mostly for the US populations of *P. ramorum*. The number of SSR primer pairs identified by Ivors *et al.* (2006) that are polymorphic for the EU1 population are limited. The transferability of the SSR primers from Prospero *et al.* (2004, 2007), which are very informative for the NA1 lineage, has not been described for the EU1 lineage. To be able to determine the microsatellite-based genetic diversity of EU1 isolates, additional polymorphic microsatellite markers were needed.

The first objective of this study was to determine the structure of the Belgian EU1 population based on newly developed and existing microsatellite markers and compare it with structure based on AFLP analysis. The second objective was to analyze the Belgian population, which is unique in that it also contains three isolates of A2 mating type, for indications of genetic recombination. The third and last objective was to relate the data on genetic diversity with those of isolate geographical origin, isolation year, host and fungicide resistance, and use this information to evaluate the success of the eradication efforts.

Materials and methods

Phytophthora ramorum isolates

Starting in 2002, the Belgian Plant Protection Service (FAVV) conducted the EU-mandated annual to biannual inspections of host plants at nurseries, in public parks, and in forests for symptoms of *Phytophthora ramorum*. Diagnosis of *P. ramorum* from symptomatic plant material was done via plating onto semi-selective pimaricin–ampicillin–rifampicin–pentachloronitrobenzene agar medium (Jeffers & Martin 1986) and morphological identification (EPPO 2006) at the Flemish Institute for Agricultural and Fisheries Research (ILVO) or via real-time polymerase chain reaction (PCR) (EPPO 2006; Hayden *et al.* 2009) at the Walloon Agricultural Research Centre (CRA-W), for which each laboratory is ISO17025 accredited and has the appropriate biosafety infrastructure. After isolation, cultures were stored on V8 and corn meal agar (BBL^{TM}) at 4 °C and transferred annually onto fresh medium. All (411) Belgian isolates collected between 2002 and 2008 were used in this study. The majority (370 isolates) originated in Flanders, where most of the ornamental nurseries are located. The isolates were collected from plants in nurseries (400), public parks (6) and private gardens (5). Reference strains from the EU1 clonal lineage consisting of different MGs based on Ivors *et al.* (2006) were obtained from the laboratories where they had originally been isolated (Table 1).

Metalaxyl resistance and growth rate

All *P. ramorum* isolates of Flemish origin were evaluated for resistance to metalaxyl. Based on Heungens *et al.* (2006), isolates were considered resistant to metalaxyl if no growth was observed from a mycelial plug on V8 agar amended with 1 μg/mL metalaxyl and 10 μg/mL rifampicin, while normal colony growth (within the 95% confidence interval of the average growth rate of all isolates) was observed on metalaxyl-free medium. Plates were incubated at 20 °C in the dark. Growth rate was determined on V8 agar amended with 10 μg/mL rifampicin by measuring the colony diameter 7 and 13 days post inoculation (at 20 °C in the dark).

AFLP analysis

A selection of 80 isolates was used for AFLP analysis following a stratified sample based on isolation year, host and site. Isolates were grown in 20 mL sterile Potato Dextrose Broth (ForMedium^{TM}) on a rotary shaker (150 rpm) at room temperature for 7–10 days. Mycelium was placed onto Whatman grade 1 qualitative filter paper and washed with sterile distilled water. Mycelium was transferred into 1.5 mL Eppendorf tubes and frozen in liquid nitrogen. Genomic DNA was isolated from 100 mg mycelium using the Puregene DNA isolation kit (Gentra) and eluted in 50 μL DHS buffer. DNA samples were stored at −20 °C.

Table 1 *Phytophthora ramorum* isolates used in the primary and secondary screening of candidate microsatellite markers polymorphic for the EU1 clonal lineage

Isolate code	Isolation year	Host plant	Origin	SSR genotype (Ivors *et al.* 2006)	SSR genotype* (ILVO)	AFLP genotype (ILVO)
PRI483[KI]	2000	*Rhododendron* sp.	CA, USA	US1	—	—
PR/D/04/284	2004	*Rhododendron 'Cosmopolitan'*	Belgium	EU1	EU1MG20	VII
1574[PG]	—	*Rhododendron* sp.	UK	EU6	EU1MG1	I
BBA 15/01-11a[SW]	2001	*Viburnum* sp.	Germany	EU2	†	I
BBA 15/01-14[SW]	2001	*Viburnum* sp.	Germany	EU7	‡	I
2N386[NS]	2002	*Viburnum x bodnantense*	France	EU5	EU1MG5	I
2N389[NS]	2002	*Viburnum tinus*	France	EU3	EU1MG5	VIII
1599[PG]	—	*Rhododendron* 'Cheer'	England	EU4	EU1MG2	I
PR/D/03/335	2003	*Rhododendron* sp.	Belgium	EU1	EU1MG13	VI
PR/D/06/008	2006	*Rhododendron* sp.	Belgium	EU1	EU1MG24	I

*The polymorphisms with marker 64 for isolate 2N389 and with marker 82 for isolate 2N386 and 1574 could not be replicated in this study.

KI, Kelly Ivors; NS, Nathalie Schenck; SW, Sabine Werres; PG, Patricia Giltrap.

PRI483 = Pr52 = CBS110537 = ATCC MYA-2436; 1574 = CSL 2022689; BBA 15/01-11a = PRI 514 = PRI 679 = PR-01-023; 1599 = CSL 2023125.

†, polymorphic for locus MS18 (218/266) and MS82a (127/133); ‡, polymorphic for locus MS82a (127/133).

Amplified fragment length polymorphisms reactions were performed as described by Ivors *et al.* (2004). Following ligation, DNA was first amplified by PCR using nonselective *Eco*RI (E00) (5′-GACTGCGTACCAATTC-3′) and *Mse*I (M00) (5′-GATGAGTCCTGAGTAA-3′) primers. For the selective PCR, five primer pairs were used in the analysis: (E00-AC) + (M00-AC), (E00-TA) + (M00-AG), (E00-TC) + (M00-AG), (E00-GC) + (M00-AC) and (E00-GG) + (M00-CC). The products were sized on an ABI3130 Genetic Analyzer (Applied Biosystems) using Rox 500 as the size marker. Electropherograms were scored manually. To test reproducibility, complete analysis (including independent DNA extractions) of 15 single spore isolates was repeated three times with two primer pairs. Some variability was observed. Consequently, the AFLP reactions of the 80 isolates were repeated three times and only fragments (ranging in size from 70 to 600 bp) that could be scored unambiguously in all replicates per isolate were scored. Such fragments were scored for presence or absence in all isolates.

DNA extraction for microsatellite analysis

For the 80 isolates that were also used for AFLP analysis, DNA was extracted as described above. The genomic DNA of all other isolates was isolated with a fast DNA extraction method (Kris Van Poucke, personal communication). Mycelial plugs of an actively growing culture (7–10 days) on V8 agar were covered with ultrapure water in a Petri dish and heated at 750 W in a microwave oven for 25 s. The agar was removed and the mycelium was transferred into a 1.5 mL microtube on ice, containing 10 μL ultra-pure water. After pulverization in liquid nitrogen, the samples were centrifuged for 3 min at 20 817 g. The supernatant was used in the PCR reaction.

Identification of new polymorphic SSR markers

The first set of candidate microsatellite markers were PCR amplified with the 23 primer pairs designed by Ivors *et al.* (2006) that were polymorphic in their primary test set of eight NA1 and EU1 isolates, but which they did not test on their complete set of isolates. The second set of candidate markers were amplified with the 10 primer pairs described by Prospero *et al.* (2004, 2007), which showed polymorphism within the NA1 population but had not yet been screened for polymorphism within EU1 isolates. The third and last set of candidate microsatellite markers consisted of 71 dinucleotide, 27 trinucleotide, 11 tetranucleotide, 1 pentanucleotide and 6 hexanucleotide repeat loci (SSR) that were selected by screening the genome of *P. ramorum* (available online at http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.download.html) (Tyler *et al.* 2006) for repeats. Primers flanking these new loci were designed using the program PRIMER3 with the following criteria: min. T_m = 60 °C, GC content ≥50%, primer size 18–21 bp, PCR product size between 100 and 450 bp, and GC clamp = 1. To avoid the costs of genotyping with individual fluorescently labelled primers, we used universal fluorescent labelling (Shimizu *et al.* 2002). In this three-primer method, detection is based on the addition of a M13reverse (5′-CAGGAAACAGCTAT GACC-3′) tag (with no homology with the target

genome) at the 5′-end of the forward primer. This tag provides a complementary sequence to the universal fluorescent primer (HEX or FAM-labelled M13), generating fluorescent products that are sized by capillary electrophoresis. Efficiency of this method was tested and optimized by comparing three universally labelled primers with FAM-labelled primers for microsatellite loci 18, 64 and 82a-b.

Final PCR concentrations were 25 ng template DNA, 200 μM dNTPs, 1 U FastStart *Taq* DNA polymerase (Roche Applied Science), 2.0 mM $MgCl_2$ and 0.1 μM of each primer. PCR amplifications were performed in 20 μL reaction volumes in a GeneAmp® PCR System 9700 (Applied Biosystems) with an initial denaturation at 95 °C for 4 min followed by five cycles of 20 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C; 20 cycles of 20 s at 95 °C, 30 s at 60 °C minus 0.5 °C per cycle, and 1 min at 72 °C; 10 cycles of 20 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C; and a final extension at 72 °C for 30 min. In some cases, different primer pairs were combined in a multiplex PCR reaction. Five μL PCR products were first scored for successful amplification on 1.5% agarose gels, and subsequently sized on an ABI 3130 sequencer (Applied Biosystems) using Rox 500 as size standard. Results were analyzed using Genemapper (Applied Biosystems).

For each microsatellite locus, interlineage polymorphism between an EU1 (PR/D/04/284) and a NA1 isolate (PRI483) was assessed first, as the chance of interlineage polymorphism was expected to be bigger than that of intra-lineage polymorphism. Interlineage polymorphic loci were then analyzed further with a panel of 8 EU1 isolates that belong to separate MG groups based on the study of Ivors *et al.* (2006) or that showed polymorphism in preliminary screening. Loci that were polymorphic in these selected isolates were analyzed in all isolates (Table 1).

To test the reproducibility of our microsatellite sizing, analysis of the 26 isolates (within the 80 isolates that were also used for AFLP) that did not belong to the main EU1MG1 genotype was replicated.

To investigate the influence of culture storage on allele sizes, microsatellites of five isolates were analyzed in independent DNA extractions conducted in 2002–2004 as well as in 2008 from annually transferred cultures of these isolates. For isolates 556 and 557, we also used the DNA extracted by Ivors *et al.* (2006) as well as DNA that was recently extracted from these isolates.

DNA sequence determination of polymorphic microsatellite loci

The DNA sequence of the polymorphic microsatellite loci ILVOPrMS133 and ILVOPrMS145a–c was determined as follows. PCR amplification of the loci was performed as described above, but using 50 μL reaction volumes. The entire PCR product of each microsatellite locus from isolates BBA 9/95 (=CSL2266 = CBS 101.553 = PD 20019539 = PRI 233 = PRI 653; EU1MG1; isolated in Germany in 1995 on *Rhododendron catawbiense*) and Pr218 (isolated in Sonoma Co., CA on *Rhamnus cathartica*; NA1) were separated on a 2% Nusieve 3:1 Agarose gel (Lonza Bioscience). DNA in excised fragments was extracted with the Nucleospin ExtractII kit (Macherey-Nagel) and cloned using the TOPO TA cloning kit (Invitrogen). Primer pairs 82 and ILVOPrMS145 amplified two and three loci, respectively. The alleles that matched the different loci were determined by first aligning the primers to the available genome sequence data (BLASTn, Altschul *et al.* 1990). Nested PCR with scaffold-specific primers and the labelled primers allowed determination of the two alleles at each locus. Genotyping of *P. ramorum* progeny (X. Boutet, unpublished data) was used to determine linkage between the alleles. All novel SSR loci were confirmed by sequence analysis and analyzed for expected repeat motif variation.

Statistical and population genetic analyses

All isolates were genotyped using the new polymorphic microsatellite loci (82b, ILVOPrMS145a and ILVOPrMS145c) as well as the four previously described microsatellite loci (18, 64, 82a and ILVOPrMS133).

Polymorphic information content and expected heterozygosity values (Nei 1978) for each locus were calculated using the Microsatellite toolkit (Park 2001).

To assess the possible evolutionary relationships among the EU1 *P. ramorum* MGs, a matrix of genotype distances was calculated using the method of Bruvo *et al.* (2004), taking the repeat number into account. A minimum spanning network was calculated from the matrix using MINSPNET (Excoffier & Smouse 1994). The network was visualized using GRAPHVIZ (http://www.graphviz.org).

The *P. ramorum* populations were analyzed by year (2002–2008) and nursery site for all sites where more than five isolates were detected. Genotypic diversity was measured by richness, diversity and evenness. Genotypic richness (NG_{Exp}) was estimated using rarefaction curves (Grünwald *et al.* 2003), giving the expected number of genotypes in a population of $n = 28$ (i.e. the smallest population observed). Genotypic diversity was calculated using the Stoddart and Taylor's index $G = 1/\Sigma_i\, p_i^2$, where p_i = the observed frequency of the ith genotype (Stoddart & Taylor 1988). The index $E_5 = (1/\lambda - 1)/(e^{H'} - 1)$, where λ corresponds to Simpson's index (Simpson 1949) and H' to Shannon-Wiener's index (Shannon & Weaver 1949), was used to

quantify the genotypic evenness in the population. Confidence intervals of 90% for G and E_5 values were calculated from 1000 bootstrap samples of the original distribution in R (R Development Core Team 2008). Gene diversity was characterized by allelic richness and was calculated in microsatellite analyzer (Dieringer & Schlotterer 2003) and corrected for sample size with the rarefaction method of ElMousadik & Petit (1996). Genetic variation within and among populations was compared using Wright's F-statistics as implemented in microsatellite analyzer (Weir & Cockerham 1984). Statistical significance of the F_{ST} estimates was tested with 10 000 randomization replicates per comparison. Minimum spanning networks were constructed for all nurseries where more than four MGs were detected. To support the hypothesis of clonal reproduction, we analyzed patterns of linkage disequilibria between loci in the population using GENEPOP 3.3 (Raymond & Rousset 1995). Differences in radial growth rates between metalaxyl-resistant and metalaxyl-sensitive isolates were tested for significance using analysis of variance using STATISTICA version 8.0 (StatSoft, Inc., Tulsa, OK, USA). The Levene test in STATISTICA and the F-test in MSExcel were used for testing differences in variance of growth rate between the years.

Results

Phytophthora ramorum isolates, metalaxyl resistance, and growth rate characteristics

The 411 isolates of *Phytophthora ramorum* included in this study were obtained from either *Rhododendron* (90.5%) or *Viburnum* (9.5%) plants and represent the complete isolate collection from the Belgian surveys of 2002–2008. For each identified *P. ramorum* infection, the legally defined sanitary measures for eradication were taken by the Belgian plant protection service. At 61.6% of all nurseries where *P. ramorum* has been detected, epidemics were contained to a single year. At four *Rhododendron* nursery sites (labelled A through D) *P. ramorum* was detected in more than three out of the 7 years studied. At seven other nurseries where at least five *P. ramorum* isolates were obtained, these were collected during 3 years (nurseries E and F), 2 years (nurseries G, H, I, J) or a single year (nursery K).

Metalaxyl resistance and growth rate characteristics of each year are shown in Table 2. The percentage of metalaxyl-resistant isolates increased in the period 2002–2005, and decreased again in 2006–2008. Averaged over all years, metalaxyl-resistant isolates had a significantly higher ($P = 0.0084$) radial growth rate (4.45 mm/day) compared with metalaxyl-sensitive isolates (4.18 mm/day).

Identification of new polymorphic microsatellite loci using the candidate primer pairs

Out of the 23 incompletely tested primer pairs from the study of Ivors *et al.* (2006), only one primer pair (ILVOPrMS10) was polymorphic within the subset of nine European test isolates and none of the primer pairs described by Prospero *et al.* (2004, 2007) were polymorphic within the panel of our eight EU1 test isolates (second screening only). Of the 116 primer pairs we designed, 109 (94.0%) produced a PCR amplicon. Of

Table 2 Average yearly characteristics of *Phytophthora ramorum* isolates collected from 2002 to 2008 in Belgium

Year of isolation	Number of isolates	*Viburnum* (%)*	EU1MG1† (%)	Metalaxyl resistant (%)‡	Growth rate (mm/day) (mean ± SD)§	Allelic richness¶	NG_{Exp}	G	E_5
2002	28	42.9	67.9	32.1	3.95 ± 0.67	2.63	7	2.09 (1.47–2.94)	0.48 (0.39–0.59)
2003	49	12.5	75.5	12.2	3.90 ± 0.66	2.56	7	1.73 (1.31–2.20)	0.36 (0.32–0.41)
2004	111	3.6	81.1	52.3	3.87 ± 0.66	2.44	5	1.51 (1.29–1.74)	0.35 (0.32–0.39)
2005	55	4.7	80.0	92.7	3.92 ± 0.43	2.38	5	1.54 (1.22–1.88)	0.43 (0.36–0.51)
2006	37	10.0	64.9	86.5	4.19 ± 0.26	2.77	7	2.25 (1.58–3.08)	0.49 (0.39–0.61)
2007	39	7.9	56.4	75.7	4.16 ± 0.19	2.74	8	2.88 (1.98–4.02)	0.51 (0.41–0.63)
2008	92	7.7	50.0	44.1	3.98 ± 0.33	2.75	8	3.47 (2.71–4.45)	0.51 (0.43–0.60)
Total	411	9.5	68.6	55.6	3.96 ± 0.52	2.61	7	2.08 (1.88–2.31)	0.32 (0.30–0.34)

*Percentage of isolates recovered from *Viburnum* is relative to the total number of isolates detected with known host plant species (% *Rhododendron* = 100 - % *Viburnum*).

†Percentage of isolates belonging to multilocus genotype group EU1MG1

‡Percentage of isolates where colony growth was observed on metalaxyl-containing medium (1 μg/mL).

§Growth rate was determined between 7 and 13 days post inoculation on the nonmetalaxyl-containing control plates.

¶Allelic richness is averaged across all SSR loci considered.

NG_{Exp}, number of expected multilocus genotypes in a sample of $n = 28$ (i.e. smallest population compared); G, Stoddart and Taylor's index (90% confidence interval); E_5, index of evenness (90% confidence interval).

these, 85 (73.3%) generated polymorphic fragments between the EU1 and the NA1 isolates. Two (1.7%) of these primer pairs (ILVOPrMS133 and ILVOPrMS145) (Table 3) produced polymorphic fragments within the nine EU1 isolates.

Characterization of the novel SSR loci polymorphic for the EU1 clonal lineage

Sequence analysis revealed that the new primer pairs that amplify polymorphic fragments for the EU1 clonal lineage do indeed amplify microsatellite-containing loci (Table 4). Differences in size among the amplicons were due to a different number of repeat units in the microsatellites. Primer pair ILVOPrMS10 amplifies the same locus as locus 64 and was not used for further genotyping.

Sequencing and BLASTn of locus ILVOPrMS133 revealed that it was identical to GenBank accession nos DQ641493 and DQ641494. These GenBank accessions correspond to microsatellite marker PrMS43 (Prospero *et al.* 2007). We identified the polymorphism of locus ILVOPrMS133 but not of locus PrMS43 because the EU1 isolate of the first screening, which is the only one revealing the polymorphism among the test isolates for this locus, was not used for primers PrMS43. Interlineage polymorphism had already been demonstrated for PrMS43, so only the second level screening was performed.

Primer pair 82 and ILVOPrMS145 produced amplicons corresponding to two or three loci on separate scaffolds of the genome (Table 4). Four amplicons were generated with primer pair 82, which is one more than reported in Ivors *et al.* (2006). This indicates that at least two loci are amplified by this primer pair. Aligning the primers to the genome of *P. ramorum* reveals three matching sequences that are located on three different scaffolds (284, 341 and 703). The progeny data showed linkage between alleles 137 and 235, and 111 and 127. Primer 82_I was designed specific to scaffold 341; amplification gave a 4.6-kb band. Nested PCR resulted in 127/137 for EU1 and 109/113 for NA1. Hence, the 127 and 137 bp fragments represent the two heterozygous alleles of the microsatellite locus 82a from scaffold 341 and the 111 and 235 bp fragments represent the two heterozygous alleles of the microsatellite locus 82b (scaffolds 284 and 703).

Primer pair ILVOPrMS145 generated five bands in the EU1 isolates and four in the NA1 isolate, indicating that there were at least three loci involved. Aligning the primer pairs to the genome identified two scaffolds (77 and 2199), each with a single match. Primer pairs specific to each scaffold were designed (ILVOPrMS145_I:

Table 3 Target loci, primer sequences, and repeat motifs of 11 primer pairs used to amplify the EU1-polymorphic loci of *Phytophthora ramorum*

Primer pair name	Primer sequence (5′–3′)*	Locus amplified	Repeat motif†
18‡	F: [FAM] TGCCATCACAACACAAATCC R: TGTGCTATCTTTCCTGAACGG	l8	(AC)39
64[c]	F: [FAM] GCGCTAAGAAAGACACTCCG R: CAACATGTAGCCATTGCAGG	64	(CT)16
82‡	F: [FAM] CCACGTCATTGGGTGACTTC R: CGTACAAGTCACGACTCCCC	82a,b	(GT)14
ILVOPrMS133§	F: CAGGAAACAGCTATGACCAATATGCAAAAAGGCAGGAG R: CCGCGTAACCTAGTCTGCTC	ILVOPrMS133	(GACA)77
ILVOPrMS145§	F: CAGGAAACAGCTATGACCTGGCAGTGTTCTTCAACAGC R: ATTCCCGTGAACAGCGTATC	ILVOPrMS145a,b,c	(AGCGAC)15
82_I	F: GGCCTAGTGTGCCAGCTTAC R: CGTACAAGTCACGACTCCCC	82a	—
ILVOPrMS145_I	F: TCCCGATTATCAACACCACA R: ATTCCCGTGAACAGCGTATC	ILVOPrMS145b	—
ILVOPrMS145_II	F: CTAATATGCCGCTGGTGGAT R: ATTCCCGTGAACAGCGTATC	ILVOPrMS145a,c	—
ILVOPrMS145_III	F: CTAATATGCCGCTGGTGGAT R: TCCATCACGCGTATTGTTGT	ILVOPrMS145a	—
ILVOPrMS145_IV	F: TCCCGATTATCAACACCACA R: GTAAGTGGCGGTCCTAACCA	ILVOPrMS145b,c	—

*Forward (F) and reverse (R) primers. Use of a fluorophore (FAM) is specified within primer sequence.
†Repeat motif and the number of repeats in the 7× draft genome sequence of *Phytophthora ramorum* isolate Pr102.
‡Described by Ivors *et al.* (2006).
§Forward primer includes tag sequence (5′-CAGGAAACAGCTATGACC-3′) for universal labelling.

Table 4 Allele sizes and frequencies of the eight microsatellite loci used in this study, including GenBank accession nos of allele sequences for EU1MG1 isolate BBA 9/95 (this study) or EU1MG1 isolate RH/122/98 (Ivors *et al.* 2006)

Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†,‡	Freq.	GenBank
18				*64*				*82a*				*82b*			
218	220	50.0	DQ103275§	352	350	50.0	DQ103292§	127	127	50.0	DQ103305§	110	ND[e]	50.0	
260		1.7		398	394	39.7	DQ103294§	133		1.7		227		1.7	
262	264	48.3	DQ103278§	400		8.6		138	137	48.3	GQ223248¶	237		3.4	
				402		1.7						241	235	31.0	GQ223249¶
												243		6.9	
												245		5.2	
												247		1.7	
Length*	Length†	Freq.	GenBank	Length*	Length†,‡	Freq.	GenBank	Length*	Length†,‡	Freq.	GenBank	Length*	Length†,‡	Freq.	GenBank
ILVOPrMS133				*ILVOPrMS145a*				*ILVOPrMS145b*				*ILVOPrMS145c*			
155	ND	46.6		186	187	50.0	GQ223234¶	196	199	100	GQ223240¶	202	205	50.0	GQ223243¶
163	165	50.0	GQ223238¶	212		6.9						243		1.7	
167		1.7		217	223	39.7	GQ223241¶					249		6.9	
171		1.7		222		3.4						254		3.4	
												260		12.1	
												265	277	20.7	GQ223242¶
												270		5.2	

*Size of microsatellite amplicon based on results from capillary electrophoresis (using an ABI3130, Applied Biosystems) and Rox 500 as size standard. Differences between fragment sizes based on DNA sequencing vs. capillary electrophoresis were proportional to fragment size ($R^2 > 0.999$), and were also observed by Pasqualotto *et al.* (2007).
†Size of microsatellite amplicon based on results from actual sequence analysis.
‡Sequence data for alleles at locus PrMS82b (111/207) and loci ILVOPrMS145a–c (187/199, 187/223, and 187/265) were also obtained for NA1 isolate Pr218 (GenBank accession nos GQ223250, GQ223244, GQ223245, GQ223246, and GQ223247).
§Sequence data of EU1MG1 isolate RH/122/98, obtained by Ivors et al. (2006).
¶Sequence data of EU1MG1 isolate BBA 9/95.
ND, not determined.

scaffold 77; ILVOPrMS145_II: scaffold 2199). After nested PCR, the first primer pair resulted in the identification of one locus on scaffold 77, which was homozygous in the EU1MG1 isolate (199/199) and heterozygous in the NA1 isolate (187/199) and is labelled ILVOPrMS145b. No polymorphisms were detected at this locus within the EU1 population. Primer pair ILVOPrMS145_II amplified four fragments in the EU1 isolate and three fragments in the NA1 isolate. Genotyping of *P. ramorum* progeny (X. Boutet unpublished data) and analysis of the sequence at the ends of scaffolds 77 and 2199 indicated that the loci were probably in close proximity. Primers ILVOPrMS145_III and ILVOPrMS145_IV were designed to amplify the sequence of the neighbouring loci and determine the matching alleles. After a nested PCR with the nonspecific primer ILVOPrMS145, ILVOPrMS145_III amplified two alleles (187/223) whereas ILVOPrMS145_IV amplified three alleles (199/205/277) in the EU1MG1 isolate. Knowing that 199/199 is the homozygous locus (ILVOPrMS145b), we determined that in the EU1MG1 isolate, 187/223 (ILVOPrMS145a) and 205/277 (ILVOPrMS145c) were the allele sizes of the second and third locus, while in NA1 the allele sizes of these loci were 187/223 and 187/265, respectively. All primer pairs used to characterize the microsatellite loci are shown in Table 3.

Comparison of AFLP and microsatellite marker systems and SSR reliability

A total of 222 AFLP fragments were generated with the five primer combinations that could be scored reliably. Of these, 13 fragments (5.9%) were polymorphic among the Belgian *P. ramorum* isolates and 46 fragments (20.7%) were polymorphic between the Belgian and the American isolates. The microsatellite analysis distinguished 10 genotypes, with 58 isolates belonging to the main genotype. AFLP identified only seven genotypes, with 72 isolates belonging to the most frequent genotype. The microsatellite analysis identified eight isolates that did not belong to the main microsatellite-based genotype EU1MG1 but still grouped with the most frequent AFLP-based genotype. The AFLP method identified four isolates that did not group with the most frequent AFLP-based genotype but belonged to the main microsatellite-based genotype EU1MG1.

Allele sizes in the SSR analysis were not influenced by culture storage: no differences were observed between analyses of old cultures from 2002 to 2004 and the ones transferred every year on new medium. We also obtained the same fingerprint patterns with the DNA used by Ivors *et al.* (2006) as with freshly extracted DNA from the corresponding isolates. As the SSR analysis identified more polymorphisms and was very reproducible, all further genotyping was performed using the microsatellite markers.

Genetic diversity of the Belgian P. ramorum *population*

Microsatellite MGs were determined for the 411 Belgian isolates using the three previously described and the two new primer pairs, representing a total of eight microsatellite loci. Seven of these loci were polymorphic, resulting in a total of 30 MGs (Table S1, Supporting information). The number of alleles per locus varied from two to seven (Table S1, Supporting information). The identity of all polymorphic alleles was confirmed. The most informative microsatellite loci were ILVOPrMS145c and 82b, with seven alleles each. The expected heterozygosity values for the seven loci ranged from 0.502 to 0.604 and the polymorphic information content values ranged from 0.377 to 0.528.

In each year, the Belgian population was dominated by genotype EU1MG1 (68% of all isolates) (Table 5). This genotype is at the centre of the network (Fig. 1). Other genotypes that were detected during multiple years at more than one site are EU1MG5 (7.3%), EU1MG4 (4.1%), EU1MG7 (3.4%), EU1MG18 (3.2%), EU1MG13 (1.7%), EU1MG2 (1.2%) and EU1MG10 (1.0%). They are located close to the centre of the network. Fifteen genotypes were represented by a single isolate and are mostly found at the margin of the network. Genotypes at the margins of the network were in most cases site-specific (Fig. 1; Table 6). Some rare genotypes (such as EU1MG9, EU1MG12, EU1MG13, EU1MG22) are recovered over a period of several years at the same site, but not necessarily in each year (EU1MG9) (Table 6). Site-specific divergence is demonstrated at site D. EU1MG23 and EU1MG24 cluster together with EU1MG9, the genotype they probably evolved from based on additional mutations at loci 82b and 64, respectively (Figs 1 and 2; Table S1, Supporting information). The minimum spanning networks for nursery sites A, D and K also indicate local evolution of new MGs from one or a few founder genotypes (Fig. 2).

In most populations from the 11 *Rhododendron* nurseries with multiple (>5 isolates) findings of *P. ramorum* (A, B, C, E, I, J and F), EU1MG1 is dominant and F_{ST} values between these populations were not significant ($P > 0.05$). However, four nurseries had a *P. ramorum* population that was significantly different (sites D, G, H and K) from the populations where EU1MG1 is dominant.

No indications of sexual or mitotic recombination were found. First, no homozygous allelic rearrangements were observed at the heterozygous loci. Second, the levels of heterozygosity are very high (Table S1, Supporting information). Third, linkage disequilibrium tests showed linkage between 5 out of 21 pairs of loci

Table 5 Isolate distribution and average characteristics per year for each multilocus genotype (MG) detected between 2002 and 2008 in Belgium

	Isolates				Year						
MG	Number	% of total	Host distribution (% *Viburnum*)	Metalaxyl-resistant isolates (%)	2002	2003	2004	2005	2006	2007	2008
EU1MG1	282	68.61	9.33	55.8	19	37	90	44	24	22	46
EU1MG2	5	1.22	80.00	0.0	2	1	1		1		
EU1MG3	1	0.24	0.00	0.0	1						
EU1MG4	17	4.14	6.25	100.0	2	1	2	1	1	4	6
EU1MG5	30	7.30	11.54	50.0	2	2	6	5	3	3	9
EU1MG6	4	0.97	25.00	0.0	1						3
EU1MG7	14	3.41	0.00	33.3	1	1	1				11
EU1MG8	1	0.24	0.00	100.0							1
EU1MG9	6	1.46	0.00	100.0		1		1		3	1
EU1MG10	4	0.97	0.00	25.0		2		1			1
EU1MG11	1	0.24	0.00	0.0		1					
EU1MG12	3	0.73	0.00	0.0		1	2				
EU1MG13	7	1.70	0.00	42.9		1		2	1	2	1
EU1MG14	1	0.24	0.00	0.0		1					
EU1MG15	1	0.24	0.00	0.0			1				
EU1MG16	1	0.24	100.00	0.0			1				
EU1MG17	1	0.24	0.00	100.0			1				
EU1MG18	13	3.16	7.69	46.2			4			1	8
EU1MG19	2	0.49	0.00	50.0			1				1
EU1MG20	1	0.24	0.00	100.0			1				
EU1MG21	1	0.24	0.00	100.0				1			
EU1MG22	3	0.73	50.00	66.7					1	2	
EU1MG23	2	0.49	0.00	100.0					2		
EU1MG24	4	0.97	0.00	100.0					4		
EU1MG25	1	0.24	0.00	100.0						1	
EU1MG26	1	0.24	0.00	100.0						1	
EU1MG27	1	0.24	0.00	0.0							1
EU1MG28	1	0.24	0.00	0.0							1
EU1MG29	1	0.24	0.00	0.0							1
EU1MG30	1	0.24	0.00	100.0							1
Total	411	100.00	9.51	55.6	28	49	111	55	37	39	92

($P < 0.05$) and all loci display strong negative F_{IS} values (<−0.667) and F_{ST} values close to zero (<0.0110).

All genotypes detected on *Viburnum* host plants were also detected on *Rhododendron*, but not always at a comparable frequency. For example, 80% of all EU1MG2 isolates were recovered from *Viburnum*, at four different sites, while only 9.5% of all isolates were derived from *Viburnum* (Table 5). Other MGs detected on *Viburnum* were EU1MG1, EU1MG4, EU1MG5, EU1MG6, EU1MG18 and EU1MG22. Of all frequently observed MGs (>5 isolates), EU1MG7 and EU1MG13 were only detected on *Rhododendron* host plants. The genetic differentiation between populations on *Viburnum* and *Rhododendron* was not significant ($F_{ST} = 0.000597$).

The percentage of metalaxyl-resistant isolates increased 40% each year between 2003 and 2005. This was correlated with a decrease of allelic richness and a decrease of genotypic richness (NG_{Exp}), diversity (G) and evenness (E_5), which increased again each year after 2005 (Table 2). Genetic differentiation between the metalaxyl-resistant and metalaxyl-sensitive population was observed ($F_{ST} = 0.000982$; $P = 0.00010$) as well as between the populations from 2002 to 2005 vs. 2006 to 2008 ($F_{ST} = 0.002446$; $P = 0.00010$). The decrease of genetic diversity was also reflected in a significant ($P < 0.05$) decrease of the growth rate variance from 2004 to 2006 and an upward trend in average growth rate from 2003 to 2007.

Discussion

Identification and characterization of EU1-polymorphic microsatellite loci

As the previously identified markers by Ivors *et al.* (2006) and Prospero *et al.* (2004, 2007) reveal only a

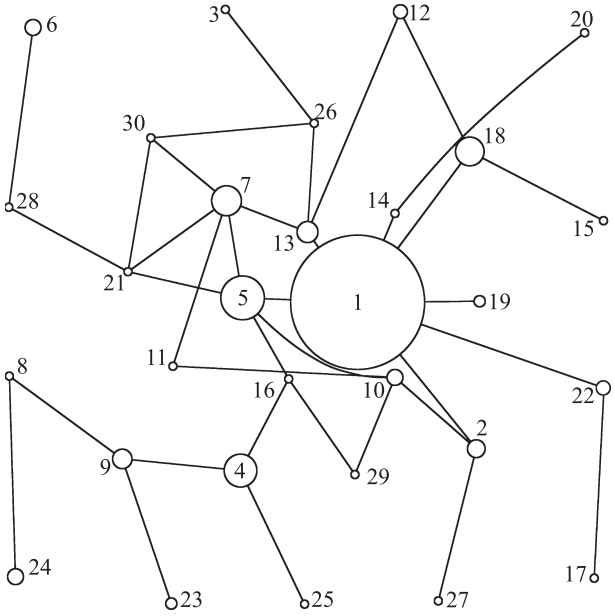

Fig. 1 Minimum spanning network of all Belgian multilocus genotypes (30 MGs). Branch sizes are proportional to genetic distance and surface areas of the circles are proportional to the numbers of isolates detected. The numbers refer to the multilocus genotypes (Table S1, Supporting information).

limited number of polymorphisms in the EU1 lineage, extra microsatellite markers needed to be developed. Finding polymorphic microsatellite markers in a clonal population characterized by low genetic diversity is challenging, even when the complete genome sequence is available (Dutech *et al.* 2007). A universally labelled fluorescent primer technique (Shimizu *et al.* 2002) made it affordable to test a large set of candidate loci. Screening of all 149 candidate primer pairs resulted in the identification of three primer pairs (ILVOPrMS10, ILVOPrMS133, ILVOPrMS145) that were polymorphic within the subset of the nine EU1 test isolates. We also identified an extra polymorphic locus that is amplified by primer pair 82 (Ivors *et al.* 2006). Using this primer pair, we observed four alleles, corresponding to two heterozygous loci 82a (127/137) and 82b (111/235), instead of the described three alleles (111/127/137) at a single locus 82. Primer pairs ILVOPrMS145 and 82, each of which amplify at least one hypervariable microsatellite locus, were most informative in our population of EU1 isolates. The applicability of the new markers was also tested in the NA1 lineage. Loci ILVOPrMS145a,c and 82b were polymorphic within four NA1 isolates tested (data not shown). These markers should thus also provide additional resolution during genotyping of NA1 isolates.

The SSR analysis was highly reproducible in our laboratory: we consistently obtained the same allele sizing results. However, some differences were observed with

Table 6 Genetic diversity of Belgian *Phytophthora ramorum* isolates by nursery and collection year in nurseries with at least five findings

Nursery code	AR	Year	MG profile*
A	2,14	2002	**1**(1)
		2003	**1**(4); **11**(1); **14**(1)
		2004	**1**(19); **12**(2)
		2005	**1**(14)
		2006	**1**(2)
		2007	**1**(11); **13**(1); **25**(1)
		2008	**1**(2)
B	2,09	2004	**1**(41); **5**(2); **18**(2); **19**(1)
		2005	**1**(13); **13**(1)
		2006	**1**(15); **13**(1); **22**(1)
		2007	**1**(1); **22**(1)
		2008	**1**(7); **18**(1)
C	2,18	2004	**1**(6); **17**(1)
		2006	**1**(2)
		2007	**1**(2)
		2008	**1**(2); **18**(1)
D	2,59	2003	**5**(1); **7**(1); **9**(1); **10**(2)
		2004	**5**(1); **7**(1)
		2005	**1**(1); **9**(1)
		2006	**4**(1); **5**(1); **23**(2); **24**(4)
		2007	**1**(1); **4**(2); **9**(2)
		2008	**4**(4); **5**(3); **9**(1); **29**(1)
E	2,14	2003	**1**(1)
		2004	**1**(1); **4**(1)
		2005	**1**(1); **4**(1)
F	2,00	2003	**1**(14)
		2004	**1**(1)
		2005	**1**(1)
G	2,39	2007	**1**(1)
		2008	**1**(4); **6**(3); **18**(1); **19**(2); **28**(1)
H	2,22	2005	**1**(1); **5**(4); **21**(1)
		2006	**1**(1); **5**(2)
I	2,00	2003	**1**(3)
		2004	**1**(3)
J	2,14	2003	**1**(2); **13**(1)
		2004	**1**(3)
K	2,36	2008	**1**(11); **5**(2); **7**(11); **18**(2); **30**(1)

*Microsatellite profile for each nursery and isolation year. Numbers in bold refer to MG number in MG name (without EU1MG prefix). Numbers between brackets refer to the number of isolates.
AR, allelic richness (averaged across years and loci).

the results published by Ivors *et al.* (2006). First, primer pairs 18, 64 and 82 differentiated seven MGs in the European isolates in Ivors *et al.* (2006), but allele profiles for three out of the seven genotypes (EU3, EU5 and EU6) could not be replicated in this study. The second difference related to the different number of loci amplified with primer pair 82, which may be the result

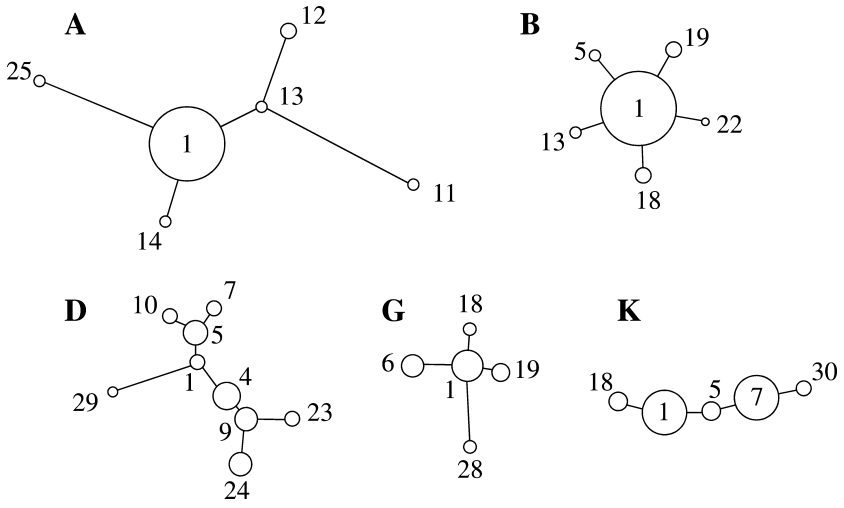

Fig. 2 Minimum spanning networks of *Phytophthora ramorum* populations from nurseries where more than four MGs were detected (nurseries A, B, D, G and K). Within each nursery, branch sizes are proportional to the genetic distance and surface area of the circles is proportional to the numbers of isolates detected. The numbers refer to the multilocus genotypes (Table S1, Supporting information).

of allelic dropout in Ivors *et al.* (2006). Interlaboratory reproducibility problems with microsatellite detection and sizing were also reported by Pasqualotto *et al.* (2007), and attributed to different machines, reagents and human error. Even though the method is reproducible and portable, standardization of the process with DNA of polymorphic reference isolates is recommended.

Although we could identify primer pairs that amplify two or three loci, this is not necessarily a general phenomenon. When two different amplicons are generated with one microsatellite primer pair, this usually indicates allele heterozygosity, not allele homozygosity at two loci. Considering the asexual reproduction of *Phytophthora ramorum*, the same rare allele could only be identified in a homozygous state if the same mutation would happen simultaneously or consecutively in both alleles. The two homozygous loci PrMS43a and PrMS43b described in Prospero *et al.* (2007) are thus more likely to represent the two heterozygous alleles of a single locus. This likelihood is further supported by this study, which identified two isolates with two separate genotypes that show this allele profile.

Comparison of AFLP and SSR

Our direct comparison using 80 isolates revealed more microsatellite-based diversity than AFLP-based diversity. Additionally, microsatellite markers are codominant and multiallelic, which makes them preferable for studying the population genetic structure of a diploid organism. Once sufficient polymorphic markers had been identified, the technical simplicity and limited cost of SSR made it our preferred technique. Other genetic diversity studies of *P. ramorum* have also shifted from AFLP (Ivors *et al.* 2004) to microsatellite analysis (Prospero *et al.* 2004, 2007, 2009; Ivors *et al.* 2006; Mascheretti *et al.* 2008; Goss *et al.* 2009b). In contrast to our work, Ivors *et al.* (2004, 2006) did not observe more diversity with SSR than with AFLP. Differences observed among the two studies might be the results of more stringent allele calling and replication for AFLP analysis as well as discovery of additional SSR loci in this study.

Genetic diversity of the Belgian P. ramorum *population*

Microsatellite analysis confirmed that all 411 Belgian isolates belong to the same lineage EU1. All differences detected between genotypes could be explained by a stepwise mutation model from a single microsatellite founder genotype EU1MG1, which is still dominant. The minimum-spanning network created with the MG data (Fig. 1) fits this pattern of a clonally expanding population. Although the overall level of genetic diversity within the EU1 population is small, microsatellite-based analyses using the previously described and newly developed primer pairs allowed the distinction of 30 genotypes. In contrast, the microsatellite markers from Ivors *et al.* (2006), would have only distinguished three previously sampled genotypes (97.1% MG EU1, 2.7% MG EU4 and 0.2% MG EU7) and two novel genotypes (0.29% MG EU8 and 1.46% MG EU9). The newly described polymorphic markers 82b and ILVOPrMS145c could, however, be subject to homoplasy given the rapid mutation rates for relatively young, clonal populations.

EU1MG1 is widespread in Belgium and probably evolved into new genotypes somewhere in Europe. Genotypes like EU1MG2, EU1MG5, EU1MG7, EU1MG13 and EU1MG18 are also common in Belgium and probably were derived first from EU1MG1 and spread through Europe via nursery shipments of *Rhodo-*

dendron and *Viburnum*. Rare genotypes are subject to genetic drift and can disappear from local populations. The human-induced bottleneck due to the eradication efforts can thus lead to extinction of the least abundant genotypes. A second hypothesis provided by Mascheretti *et al.* (2008) is that during the unfavourable season, populations of the pathogen crash to endemic levels and individual genotypes need to re-colonize infested sites at the beginning of the growth season. These seasonal variations offer an advantage to already established genotypes during the re-colonization process and may cause the extinction of rare genotypes through drift. A similar reduction of population size may occur during the winter months in nurseries.

If a rare genotype is introduced into a nursery where no other genotype is present and if it can reach a population size that can overcome the human-induced and natural bottlenecks, unique populations can be formed, as is observed in sites D, H and K. Sites that have a large population of a dominant genotype may also receive more frequent phytosanitary surveys, and the resulting eradication efforts may then further decrease the chance of survival of rare genotypes. The formation of new populations that are different in composition from the founder population was also observed in California (Mascheretti *et al.* 2008) and was explained by rare long-range movement of the pathogen and local generation of new genotypes through stepwise mutation.

Most common genotypes were detected on both host species. However, three common genotypes were correlated with either *Viburnum* or *Rhododendron*. This was probably caused by the different nursery trade pathways of *Viburnum* and *Rhododendron* plants. The decrease in the number of findings on *Viburnum* after the first year is partially caused by changes in grower practices, where growers stopped producing the more sensitive cultivars such as *Viburnum x bodnantense* 'Dawn'.

Analysis of the Belgian P. ramorum *population for indications of sexual reproduction*

Microsatellite analysis of the Belgian isolates revealed no evidence for sexual reproduction, even though both mating types were present in Belgium in 2002 and 2003 and the mating system of *P. ramorum* is functional (X. Boutet, unpublished data). All MGs found in the Belgian population can have evolved by stepwise mutation from a clonally reproducing founder population, probably composed of a single genotype (EU1MG1). Furthermore, all loci display strong negative F_{IS} values, F_{ST} values close to zero, and significant linkage disequilibria at several loci, indicating that the population in purely clonal and most likely to be epidemic (de Meeus *et al.* 2006). The high levels of heterozygosity also indicate clonality (Birky 1996; Balloux *et al.* 2003). The two sets of chromosomes behave as two distinct haplotypes, which are independently accumulating mutations. Allelic gene copies at a locus can thus become highly divergent if mechanisms such as mitotic recombination are not active. It is possible that some chromosomal rearrangements were missed because of the low number of loci analyzed. However, the number of loci used was sufficient to demonstrate recombination in 76% of all progenies analyzed (X. Boutet, unpublished results). The exclusion of sexual reproduction in the *P. ramorum* populations in Europe and North America was also reported by Ivors *et al.* (2004, 2006), Mascheretti *et al.* (2008), Prospero *et al.* (2004, 2007, 2009) and Goss *et al.* (2009b). Goss *et al.* (2009a) provide evidence for ancient divergence in the genome of *P. ramorum* based on extensive analysis of nuclear genes, showing evidence for reproductive isolation. Clonal reproduction is very common in heterothallic *Phytophthora* species and both mating types can coexist in some areas without mating, even when sexual recombination is functional. This has been demonstrated for *P. cinnamomi* as well as for *Phytophthora infestans* (Mosa *et al.* 1993; Miller *et al.* 1997; Linde *et al.* 1999; Dobrowolski *et al.* 2003).

When comparing the sizes of the two EU1 alleles with those of the two NA1 alleles for each of the 109 microsatellites observed during the first screening, the divergence between the two alleles at the same locus within the same lineage is in most cases exceeding the divergence between the EU1 and NA1 lineage, the so-called Meselson effect (Welch & Meselson 2000). This information indicates that in the past, asexual reproduction was probably also the main way for *P. ramorum* to survive. This is in line with the evidence for ancient divergence and lack of recent sexuality in the genome of *P. ramorum* described by Goss *et al.* (2009a).

Effect of metalaxyl use and isolation year on the genetic diversity

The use of metalaxyl played a role in the current population structure of *P. ramorum*. When *P. ramorum* was first detected in 2002, metalaxyl was a very effective fungicide for control of *P. ramorum* (Heungens *et al.* 2006) and was frequently used in nurseries to control the disease. However, once resistance became widespread, the recommendation not to use the fungicide was made. The use of metalaxyl most probably resulted in a decrease of the genetic diversity because most genotypes were represented by a limited number of isolates, and therefore had a reduced chance of developing

resistance compared with the dominant genotype, even if resistance to this fungicide develops quickly (Dowley & O'Sullivan 1981). Selection was in favour of the resistant isolates until 2005, when metalaxyl was no longer effective and its use was limited. The percentage of sensitive isolates increased again, together with the allelic and genotypic diversity. Evolution in plant pathogen populations due to fungicide resistance is a common phenomenon and can have an important impact on management (Milgroom & Fry 1988; Staub 1991; Gisi & Cohen 1996; Grünwald *et al.* 2006). The sampling and analysis of the *P. ramorum* population also revealed this here. However, the extensive use of fungicides as a result of the *P. ramorum* control measures probably had an unnoticed but similar impact on the populations of other *Phytopthora* species present in the *Rhododendron* nurseries such as *Phytopthora citricola* and *Phytopthora cactorum*, and may therefore have broader implications. An upward trend in average growth rate and a significant reduction in growth rate variance from 2004 to 2006 indicate selection for faster-growing isolates. The increase in growth rate is correlated with the increase in metalaxyl resistance. Indeed, metalaxyl-resistant isolates had a significantly larger growth rate than the sensitive isolates. However, the decline in metalaxyl-resistant isolates in the absence of metalaxyl in some nurseries where resistance was quite general suggests that resistance has a cost. Similar results were observed in studies with *P. infestans* in Ireland (Dowley & O'Sullivan 1985) and the Netherlands (Davidse *et al.* 1989). Therefore, the increased growth rate of resistant isolates may be caused by the compensatory effects of additional mutations selected in the resistant background. The downward trend in growth rate in 2008 can be explained by the detection of slower-growing and metalaxyl-sensitive isolates of *P. ramorum* in nurseries where *P. ramorum* was previously not detected.

Evaluation of eradication efforts

The persistence of rare genotypes in specific sites demonstrates that eradication of *P. ramorum* in nurseries is not always successful. Latent survival of the pathogen seems to be the main reason. Indeed, direct evidence for latent survival was provided via the intermittent absence of a unique genotype at a specific site (EU1MG9). Survival in potting media has been suggested by Tjösvold *et al.* (2009) as a means for latent survival, complicating the eradication effort and questioning its potential. The unsuccessful eradication effort is in conflict with the situation in Oregon (Prospero *et al.* 2007): nursery genotypes in 2004 were not the same as those in 2003, making the authors conclude that the 2004 findings were from new introductions. Differences could be due to different plant production systems, different eradication measures or simply the difference in survey period in the two studies.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic logical alignment search tool (BLASTn). *Journal of Molecular Biology*, **215**, 403–410.

Balloux F, Lehmann L, de Meeus T (2003) The population genetics of clonal and partially clonal diploids. *Genetics*, **164**, 1635–1644.

Birky CW (1996) Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics*, **144**, 427–437.

Brasier CM, Rose J, Kirk SA, Webber JF (2002) Pathogenicity of *Phytophthora ramorum* isolates from North America and Europe to bark of European Fagaceae, American *Quercus rubra* and other forest trees. In: *Sudden Oak Death Science Symposium*. Monterey, USA.

Brasier CM, Denman S, Brown A, Webber JF (2004) Sudden oak death (*Phytophthora ramorum*) discovered on trees in Europe. *Mycological Research*, **108**, 1108–1110.

Bruvo R, Michiels NK, D'Souza TG, Schulenburg H (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology*, **13**, 2101–2106.

Davidse LC, Henken J, Vandalen A, Jespers ABK, Mantel BC (1989) 9 years of practical experience with phenylamide resistance in *Phytophthora infestans* in the Netherlands. *Netherlands Journal of Plant Pathology*, **95**, 197–213.

De Merlier D, Chandelier A, Cavelier M (2003) First Report of *Phytophthora ramorum* on *Viburnum bodnantense* in Belgium. *Plant Disease*, **87**, 203.

Dieringer D, Schlotterer C (2003) Microsatellite Analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, **3**, 167–169.

Dobrowolski MP, Tommerup IC, Shearer BL, O'Brien PA (2003) Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology*, **93**, 695–704.

Dowley LJ, O'Sullivan E (1981) Metalaxyl-resistant strains of *Phytophthora infestans* (Mont.) De Bary in Ireland. *Potato Research*, **24**, 417–421.

Dowley LJ, O'Sullivan E (1985) Monitoring metalaxyl resistance in populations of *Phytophthora infestans*. *Potato Research*, **28**, 531–533.

Dutech C, Enjalbert J, Fournier E *et al.* (2007) Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology*, **44**, 933–949.

ElMousadik A, Petit RJ (1996) High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L) Skeels] endemic to Morocco. *Theoretical and Applied Genetics*, **92**, 832–839.

EPPO (2006) *Phytophthora ramorum*. *EPPO Bulletin*, **36**, 145–155.

Excoffier L, Smouse PE (1994) Using allele frequencies and geographic subdivision to reconstruct gene trees within a species - molecular variance parsimony. *Genetics*, **136**, 343–359.

Fabritius AL, Shattock RC, Judelson HS (1997) Genetic analysis of metalaxyl insensitivity loci in *Phytophthora infestans* using linked DNA markers. *Phytopathology*, **87**, 1034–1040.

Gisi U, Cohen Y (1996) Resistance to phenylamide fungicides: a case study with *Phytophthora infestans* involving mating type and race structure. *Annual Review of Phytopathology*, **34**, 549–572.

Goss EM, Carbone I, Grunwald NJ (2009a) Ancient isolation and independent evolution of the three clonal lineages of the exotic sudden oak death pathogen *Phytophthora ramorum*. *Molecular Ecology*, **18**, 1161–1174.

Goss EM, Larsen M, Chastagner GA, Givens DR, Grünwald NJ (2009b) Population genetic analysis infers migration pathways of *Phytophthora ramorum* in US nurseries. *PLoS Pathogens*, **5**, e1000583, doi: 10.1371/journal.ppat.1000583.

Grünwald NJ, Flier WG (2005) Biology of *Phytophthora infestans* at its center of origin. *Annual Review of Phytopathology*, **43**, 171–190.

Grünwald NJ, Goodwin SB, Milgroom MG, Fry WE (2003) Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology*, **93**, 738–746.

Grünwald NJ, Sturbaum AK, Romero Montes G *et al.* (2006) Selection for fungicide resistance within a growing season in field populations of *Phytophthora infestans* at the center of origin. *Phytopathology*, **96**, 1397–1403.

Grünwald NJ, Goss EM, Larsen MM *et al.* (2008a) First report of the European lineage of *Phytophthora ramorum* on *Viburnum* and *Osmanthus* spp. in a California nursery. *Plant Disease*, **92**, 314.

Grünwald NJ, Goss EM, Press CM (2008b) *Phytophthora ramorum*: a pathogen with a remarkably wide host range causing sudden oak death on oaks and ramorum blight on woody ornamentals. *Molecular Plant Pathology*, **9**, 729–740.

Grünwald NJ, Goss EM, Ivors K *et al.* (2009) Standardizing the nomenclature for clonal lineages of the sudden oak death pathogen, *Phytophthora ramorum*. *Phytopathology*, **99**, 792–795.

Hansen EM, Reeser PW, Sutton W, Winton LM, Osterbauer N (2003) First report of A1 mating type of *Phytophthora ramorum* in North America. *Plant Disease*, **87**, 1267.

Hayden KJ, Ivors KL, Wilkinson C, Garbelotto M (2009) TaqMan chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods. *Phytopathology*, **96**, 846–854.

Herrero ML, Toppe B, Klemsdal SS, Stensvand A (2006) First report of *Phytophthora ramorum* in ornamental plants in Norway. *Plant Disease*, **90**, 1458.

Heungens K, De Dobbelaere I, Maes M (2006) Fungicide control of *Phytophthora ramorum* on Rhododendron. In: *Proceedings of the Sudden Oak Death Second Science Symposium: The state of our knowledge* (eds Frankel SJ, Shea PJ, Haverty MI), pp. 241–257. USDA Forest Service, Albany, CA.

Husson C, Delatour C, Frey P *et al.* (2007) First report of *Phytophthora ramorum* on ornamental plants in France. *Plant Disease*, **91**, 1359.

Ivors KL, Hayden KJ, Bonants PJ, Rizzo DM, Garbelotto M (2004) AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycological Research*, **108**, 378–392.

Ivors KL, Garbelotto M, Vries IDE *et al.* (2006) Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology*, **15**, 1493–1505.

Jeffers SN, Martin SB (1986) Comparison of 2 Media Selective for Phytophthora and Pythium Species. *Plant Disease*, **70**, 1038–1043.

Lane CR, Beales PA, Hughes KJD *et al.* (2003) First outbreak of *Phytophthora ramorum* in England, on *Viburnum tinus*. *Plant Pathology*, **52**, 414.

Lee TY, Mizubuti ESG, Fry WE (1999) Genetics of metalaxyl resistance in *Phytophthora infestans*. *Fungal Genetics and Biology*, **26**, 118–130.

Lilja A, Rytkonen A (2007) First report of *Phytophthora ramorum* and *P. inflata* in ornamental rhododendrons in Finland. *Plant Disease*, **91**, 1055.

Linde C, Drenth A, Wingfield MJ (1999) Gene and genotypic diversity of *Phytophthora cinnamomi* in South Africa and Australia revealed by DNA polymorphisms. *European Journal of Plant Pathology*, **105**, 667–680.

Martin FN (2008) Mitochondrial haplotype determination in the oomycete plant pathogen *Phytophthora ramorum*. *Current Genetics*, **54**, 23–34.

Mascheretti S, Croucher PJP, Vettraino A, Prospero S, Garbelotto M (2008) Reconstruction of the Sudden Oak Death epidemic in California through microsatellite analysis of the pathogen *Phytophthora ramorum*. *Molecular Ecology*, **17**, 2755–2768.

Meentemeyer RK, Rank NE, Shoemaker DA *et al.* (2008) Impact of sudden oak death on tree mortality in the Big Sur ecoregion of California. *Biological Invasions*, **10**, 1243–1255.

de Meeus T, Lehmann L, Balloux F (2006) Molecular epidemiology of clonal diploids: a quick overview and a short DIY (do it yourself) notice. *Infection Genetics and Evolution*, **6**, 163–170.

Milgroom MG, Fry WE (1988) A simulation analysis of the epidemiological principles for fungicide resistance management in pathogen populations. *Phytopathology*, **78**, 565–570.

Miller JS, Hamm PB, Johnson DA (1997) Characterization of the *Phytophthora infestans* population in the Columbia Basin of Oregon and Washington from 1992 to 1995. *Phytopathology*, **87**, 656–660.

Moralejo E, Werres S (2002) First Report of *Phytophthora ramorum* on *Rhododendron* sp. in Spain. *Plant Disease*, **86**, 1052.

Moralejo E, Garcia-Muñoz JA, Descals E (2009) Susceptibility of Iberian trees to *Phytophthora ramorum* and *P. cinnamomi*. *Plant Pathology*, **58**, 271–283.

Mosa AA, Kobayashi K, Ogoshi A, Kato M, Sato N (1993) Isoenzyme polymorphism and segregation in isolates of *Phytophthora infestans* from Japan. *Plant Pathology*, **42**, 26–34.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Park SDE (2001) *Trypanotolerance in West African cattle and the population genetic effects of selection*. PhD Thesis, University of Dublin, Dublin, Ireland.

Pasqualotto AC, Denning DW, Anderson MJ (2007) A cautionary tale: lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. *Journal of Clinical Microbiology*, **45**, 522–528.

Prospero S, Black JA, Winton LM (2004) Isolation and characterization of microsatellite markers in *Phytophthora ramorum*, the causal agent of sudden oak death. *Molecular Ecology Notes*, **4**, 672–674.

Prospero S, Hansen EM, Grünwald NJ, Winton LM (2007) Population dynamics of the sudden oak death pathogen *Phytophthora ramorum* in Oregon from 2001 to 2004. *Molecular Ecology*, **16**, 2958–2973.

Prospero S, Grünwald NJ, Winton LM, Hansen EM (2009) Migration patterns of the emerging plant pathogen *Phytophthora ramorum* on the west coast of the United States of America. *Phytopathology*, **99**, 739–749.

R Development Core Team (2008) *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Raymond M, Rousset F (1995) Genepop (Version-1.2) – population-genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Shannon CE, Weaver W (1949) *The Mathematical Theory of Communication*. University of Illinois Press, Urbana, Illinois.

Shimizu M, Kosaka N, Shimada T *et al.* (2002) Universal fluorescent labeling (UFL) method for automated microsatellite analysis. *DNA Research*, **9**, 173–178.

Simpson EH (1949) Measurement of diversity. *Nature*, **163**, 688.

Staub T (1991) Fungicide resistance: practical experience with antiresistance strategies and the role of integrated use. *Annual Review of Phytopatholgy*, **29**, 421–442.

Stoddart JA, Taylor JF (1988) Genotypic diversity - estimation and prediction in samples. *Genetics*, **118**, 705–711.

Tjösvold SA, Chambers DL, Fichtner EJ, Koike ST, Mori SR (2009) Disease risk of potting media infested with *Phytophthora ramorum* under nursery conditions. *Plant Disease*, **93**, 371–376.

Tracy DR (2009) *Phytophthora ramorum* and *Phytophthora kernoviae*: the woodland perspective. *EPPO Bulletin*, **39**, 161–167.

Tyler BM, Tripathy S, Zhang XM *et al.* (2006) *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science*, **313**, 1261–1266.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Welch DM, Meselson M (2000) Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. *Science*, **288**, 1211–1215.

Werres S, De Merlier D (2003) First detection of *Phytophthora ramorum* mating type A2 in Europe. *Plant Disease*, **87**, 1266.

Werres S, Marwitz R, Man in 't Veld W *et al.* (2001) *Phytophthora ramorum* sp nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research*, **105**, 1155–1165.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Allele profiles (in bp) at the seven polymorphic microsatellite loci[a] analyzed for the 30 multilocus genotypes (MG) of *Phytophthora ramorum* detected in Belgium from 2002 to 2008

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