Plant Pathology (2019) **68**, 878–888 Doi: 10.1111/ppa.13015

Improved detection and identification of the sudden oak death pathogen *Phytophthora ramorum* and the Port Orford cedar root pathogen *Phytophthora lateralis*

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Early detection provides the best way to prevent introduction and establishment of alien plant pathogens. Amplification of DNA by PCR has revolutionized the detection and monitoring of plant pathogens. Most of those assays rely on the amplification of a fraction of the genome of the targeted species. With the availability of whole genomes for a growing number of fungi and oomycetes it is becoming possible to compare genomes and discover regions that are unique to a target organism. This study has applied this pipeline to develop a set of hierarchical TaqMan real-time PCR detection assays targeting DNA of all four *Phytophthora ramorum* lineages, and a closely related species, *P. lateralis*. Nine assays were generated: three targeting DNA of all *P. ramorum* lineages, one for each lineage of *P. ramorum*, one for *P. lateralis* and one targeting DNA of *P. ramorum* and *P. lateralis*. These assays were very accurate and sensitive, ranging from 98.7% to 100% detection accuracy of 2–10 gene copies of the targeted taxa from pure cultures or inoculated tissues. This level of sensitivity is within the lowest theoretical limit of detection of DNA. It is expected that these assays will be useful because of their high level of specificity and the ease with which they can be multiplexed because of the inherent flexibility in primer and probe design afforded by their lack of conservation in non-target species.

Keywords: EU1 and EU2 lineages, NA1, NA2, sudden oak death, TaqMan probes, unique genes

Introduction

Human-mediated movement of plants and plant products is undoubtedly recognized as one of the major forces driving the spread of invasive plant pathogens, threatening the health and sustainability of ecosystems and causing serious economic and social costs (Chapman *et al.*, 2017). Members of the *Phytophthora* genus (Oomycete, Stramenopiles) are pathogens that commonly spread through the transport of plants and plant products via the plant-for-planting trade (Jung *et al.*, 2016). For example, the nursery and ornamental plant trade has played a key role in spreading *Phytophthora ramorum*, causing outbreaks of sudden oak death in North America and sudden larch death in the UK, and *Phytophthora lateralis*, the causal agent of Port Orford cedar root disease in western USA, UK and continental Europe (Brasier & Webber, 2010; Robin *et al.*, 2011; Grünwald *et al.*, 2012).

Phytophthora ramorum emerged in the early 1990s in Europe and on the West Coast of North America and has since expanded to a large number of hosts and ecosystems (Grünwald *et al.*, 2012). Four genetically distinct clonal lineages are currently recognized within *P. ramorum*. These lineages are believed to have diverged prior to the current outbreak and have probably emerged in their current range as a result of distinct migration events (Ivors *et al.*, 2006). Lineages NA1 and NA2 have an exclusive distribution in North America (Ivors *et al.*, 2006) while EU1 is found throughout

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Published online 22 April 2019

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Europe, in the Pacific Northwest of the USA and in British Columbia, Canada (Ivors *et al.*, 2006; Schenck *et al.*, 2018); EU2 has so far only been reported in the UK (Van Poucke *et al.*, 2012). Lineage frequency varies according to geography. Lineage NA2 is generally more abundant in Canada, while NA1 is most abundant in California and EU1 is most frequent in Europe. The EU1 lineage has been found on Douglas fir and grand fir in Oregon forests and its frequency has been increasing in Canada since 2013 (LeBoldus *et al.*, 2018; Shamoun *et al.*, 2018). As the lineages have different phenotypic characteristics, with NA2 and EU1 being more aggressive than NA1, lineage identity is important when conducting surveys and monitoring the pathogen (Elliott *et al.*, 2011). *Phytophthora lateralis* is closely related to *P. ramorum* and is an invasive pathogen causing mortality of Port Orford cedar (*Chamaecyparis lawsoniana*). Since its first report in the 1920s on nursery stock near Seattle, WA (USA), the pathogen continues to spread throughout the natural range of Port Orford cedar, making this tree species no longer an important part of the nursery trade and wood export market in North America. Outbreaks of *P. lateralis* have been recently reported in Europe, probably resulting from further nursery stock movement (Robin *et al.*, 2011; Green *et al.*, 2012).

As *P. ramorum* is a regulated pathogen in North America, Europe and Asia, a variety of molecular markers have been developed to improve its detection and monitoring, including conventional and nested PCR, real-time PCR (qPCR), molecular beacons and isothermal amplification (e.g. Cooke *et al.*, 2007; Miles *et al.*, 2015). Real-time PCR using TaqMan probes constitutes the most sensitive, specific and rapid method (Martinelli *et al.*, 2015). *Phytophthora ramorum*-specific TaqMan assays have been designed targeting several genes or genome regions including the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (rDNA) (Hayden *et al.*, 2006; Hughes *et al.*, 2006; Bilodeau *et al.*, 2007a), β-tubulin and elicitin (Hayden *et al.*, 2006; Bilodeau *et al.*, 2009) and mitochondrial genes, such as *coxI* (Tooley *et al.*, 2006) and the *atp9-nad9* gene region (Tooley *et al.*, 2006; Miles *et al.*, 2017). Discrimination among the four lineages has also been achieved through a variety of molecular markers including conventional Sanger sequencing of mitochondrial and nuclear DNA (Martin, 2008), AFLPs (Ivors *et al.*, 2004), PCR-RFLP (Martin, 2008; Elliott *et al.*, 2011; Van Poucke *et al.*, 2012), microsatellites (Ivors *et al.*, 2006; Gagnon *et al.*, 2017) and allele-specific oligonucleotide-PCR (ASP-PCR) (Bilodeau *et al.*, 2007b).

The approaches mentioned above use only a small number of conserved genes or genome regions and assay specificity is achieved by designing primer and probes that target polymorphic sites (as single nucleotide polymorphisms, SNPs) within these gene regions, which allows discrimination between the target lineages and taxa. However, targeting SNPs solely in conserved gene regions makes it difficult to find discriminant fixed SNPs to design assays for closely related species and increases the risk of obtaining false positives. For example, there are only nine SNPs between the ITS sequences of *P. ramorum* and *P. lateralis* over an 850 bp alignment. ITS-based assays targeting DNA of *P. ramorum* are well known to cross amplify with DNA of *P. lateralis*, particularly when used at high DNA concentrations (Hughes *et al.*, 2006; Bilodeau *et al.*, 2007a).

The increase in genomic resources makes it possible to mine entire genomes of pathogens and their close relatives to identify genes or genomic regions of greater discriminatory power that can be translated in real-time PCR assays of high accuracy. This study used a pipeline for genome-enhanced detection and identification (GEDI) that identifies unique genes or genome regions in the target species by comparing available genomes of the target and non-target species (Feau *et al.*, 2018). Here, this genome comparison pipeline has been used to identify genes and genome regions that are only found in the targeted taxa at the desired hierarchical level (*P. ramorum* and *P. lateralis* and each of the four lineages of *P. ramorum*) and real-time PCR assays have been designed that discriminate among species and lineages.

Materials and methods

In silico assay development

The GEDI pipeline described by Feau *et al.* (2018) was applied to identify genes and genome regions that were only found at three hierarchical levels: (i) group: genes conserved in the sister species *P. ramorum* and *P. lateralis* but absent in other phytophthoras; (ii) species: genes conserved in all lineages of *P. ramorum* but absent in other phytophthoras (including *P. lateralis*) and genes present in the North American lineage of *P. lateralis* but absent from all other phytophthoras (including *P. ramorum*); and (iii) lineages: unique genes found in each of the currently recognized lineages of *P. ramorum* (NA1, NA2, EU1 and EU2).

Candidate genes targeting *P. ramorum* and *P. lateralis* were obtained by Feau *et al.* (2018) as follows: the genomes of eight *Phytophthora* species were compared (including species of the same phylogenetic clade as *P. ramorum* and *P. lateralis* and species with well-annotated genomes; Table S1) and clusters of homologous genes (orthologues and paralogues) generated to identify candidate clusters uniquely present in both *P. ramorum* and *P. lateralis* (group level) and each of *P. ramorum* and *P. lateralis* (species level) but absent in the other taxa (Feau *et al.*, 2018). The same approach was repeated in this study on *P. ramorum* lineages using the genomes of 107 *P. ramorum* isolates from all four lineages from North America and Europe (38 strains of NA1, 17 strains of NA2, 46 strains of EU1 and 6 strains of EU2; SRA accession PRJNA427329).

Primers and probes were designed as described by Feau *et al.* (2018), manually inspected and further modified if required to improve amplification yield and avoid selecting genome locations that comprise polymorphisms within the targeted taxa. Modifications involved moving the primer location or changing its size if, according to the sequences available, a more suitable location was encountered in the gene region.

In vitro screening of real-time PCR assays

Specificity screening by conventional PCR

A first round of screening was conducted to eliminate candidate primer pairs that proved to be nonspecific (i.e. generating a PCR

product with DNA from a non-target taxon and/or not producing the expected PCR product with DNA from the target taxa). Candidate primer pairs for group (*P. ramorum* + *P. lateralis*) and species (*P. ramorum* or *P. lateralis*) assays were first screened using a panel of 47 *Phytophthora* species distributed among nine phylogenetic clades, as described by Feau *et al.* (2018) (Table S2). Primer pairs targeting DNA from *P. ramorum* lineages were screened for specificity using a panel of 17 individuals from the four lineages. Total genomic DNA was extracted from pure cultures by the CTAB (cetyl trimethylammonium bromide) method (Möller *et al.*, 1992). DNA was eluted in TE (Tris-EDTA) buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8) and used as template in conventional PCR and real-time PCR. PCRs were performed in a total volume of 25 μL containing a final concentration of 1× buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 μM each primer and 2.2 ng DNA template. All PCRs were performed with 0.2 U Platinum *Taq* DNA polymerase (Invitrogen) using the following conditions: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; and a final extension step of 72 °C for 10 min.

Design and testing of the TaqMan probes

A TaqMan probe was designed for each primer pair for group and species assays that passed the first screen and tested on the panel of *Phytophthora* species used in the previous screening, expanded to include individuals from the four *P. ramorum* lineages and four *Pythium* species. For the four *P. ramorum* lineages, TaqMan probes and primers were tested on a DNA panel of nine NA1, six NA2, three EU1 and four EU2 individuals and two close relatives of *P. ramorum* within clade 8: *P. lateralis* and *P. hibernalis* (Table S2). All qPCRs were performed individually (monoplex) with a final concentration of 1× QuantiTect Multiplex PCR No ROX Master Mix (QIAGEN) and 0.4 μM of each primer in a 10 μL reaction volume with the following conditions: 95 °C, 15 min enzyme activation step, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. All TaqMan probes were labelled with fluorescein (6-FAM) at the 5′ end and with the quencher Iowa Black FQ (ZEN-IBFQ) (Integrated DNA Technologies) and used at a final concentration of 200 nM. Each sample was run in duplicate using 2.2 ng DNA template.

Efficiency and limit of detection

The efficiency of the real-time PCR and the limit of detection for each selected assay were determined using a series of 8–10 dilutions (1/5 dilution with a starting concentration of 2.2 ng) of DNA from the targeted taxa. The performance of each dilution was evaluated with the same PCR conditions as those described above and all reactions were run in triplicate. PCR efficiency was calculated with the formula $E = (10^{-1/\text{slope}} - 1) \times 100$, where E is the amplification efficiency and the slope is derived from the plot of the logarithm of template concentration and C_t (cycle threshold) value. The limit of detection for each assay was assigned to the greatest dilution of the 8–10 dilution series for which each of the three replicates had a C_t value <40.0. The DNA concentration of this dilution was translated to the equivalent number of genome copies as described by Lamarche *et al.* (2015).

Validation with inoculated plant material

The real-time PCR assays were further tested on DNA from six plant species inoculated with the four *P. ramorum* lineages (five individuals per lineage) and eight plant species inoculated with *P. lateralis* (three isolates; Table S3). These plants were selected as representative of horticultural (*Camellia* sp., *Vitis* sp.), tree species (*Acer macrophyllum*, *Arbutus menziesii*, *Thuja occidentalis*) and understorey forest vegetation (*Rhododendron* sp., *Gaultheria shallon*) of British Columbia (Canada). *Phytophthora ramorum* isolates were inoculated onto detached leaves of potted *Camellia* sp., *A. macrophyllum*, *Rhododendron* sp., *G. shallon*, *A. menziesii* and *Vitis* sp. *Phytophthora lateralis* isolates were inoculated on these six hosts and also *Pieris* sp. and *T. occidentalis*. Leaves were surface-sterilized in 0.525% w/v sodium hypochlorite, rinsed in sterile dH_2O and dried briefly on paper towels, then wounded by making three 1 mm wounds next to the midrib with a sterile tool before inoculation. Inoculum plugs 7 mm in diameter were taken from actively growing cultures of *P. ramorum* and placed mycelium side down on the lower leaf surface. A plug of 15% V8 agar (V8A) was placed on control leaves. Samples were harvested 10 days after inoculations. DNA was extracted from 50 mg of ground, infected tissues (after removing the mycelium plug) using the CTAB protocol of Doyle & Doyle (1987). DNA was eluted in 100 μL TE buffer, incubated at 37 °C for 1 h with 5 μL RNAase (10 mg mL^{-1}) and then stored at −20 °C until utilization as template in real-time PCR.

Results of these detections were then benchmarked with those obtained with the ITS, β-tubulin and elicitin real-time PCR assays that are used in the Canadian Food Inspection Agency Plant Pathology Research Laboratory for detecting *P. ramorum* (Bilodeau *et al.*, 2007a). These assays were tested on the same DNA by using the qPCR protocol described above. Each qPCR assay was tested separately on all DNA samples with two technical replicates. For each inoculated sample, a real-time PCR assay targeting the RuBisCO plant gene (ribulose-1,5-bisphosphate carboxylase/oxygenase), was used as a positive control of the PCR reagents and the DNA extraction (Bilodeau *et al.*, 2009).

Bayesian interpretation

To estimate the accuracy of each assay in detecting DNA from the target taxon, C_t values obtained in the previous experiments with DNA from pure cultures and infected plant material were interpreted using the naïve Bayes classifier described by Bergeron *et al.* (2019). Briefly, the classifier is trained on C_t values obtained from collections of 'positive' (i.e. DNA from target taxa) and 'negative' (DNA from non-target taxa) samples to provide a probability for an unknown sample of being positive and negative (Bergeron *et al.*, 2019). For each assay, 10 000 training sets were built by randomly picking 79 C_t values taken (either from positive or negative samples) from the set of 158 C_t values obtained with the qPCR tests made on DNA from cultures and from infected plant material. C_t values from the 79 samples left were then used as a testing set and reassigned by the naïve Bayes classifier as being either positive or negative. True positives were DNA samples of the species targeted by the assay identified as being positive with the classifier. True negatives were DNA samples of non-targeted species identified as being negative by the classifier. A false positive is a DNA sample from non-targeted taxa that is positive with the classifier and a false negative is a DNA sample from the targeted taxa that returned a negative identification with the classifier. Accuracy of the detection assay was defined as the rate of true positive and true negative identification over all the identifications done. This approach was applied only to the group and species-level assays, i.e. targeting DNA from *P. ramorum* (Pram-C62, Pram-C1040, Pram-C1162, ITS, β-tubulin and Elicitin), *P. lateralis* (Plat-C19) or both species together (Pramlat-C11). For the lineage assays,

the small number of true positive samples experimentally tested (18 for PramEU1-C358 to 32 for PramEU2-C268) prevented using the naïve Bayes classifier.

Results

Identification of candidate genes and screening by conventional PCR

After filtering for false positives, a total of 37 candidate clusters of homologous genes were identified that were present in the genomes of all four lineages of *P. ramorum* and absent in those of other *Phytophthora* spp. used for comparisons (Feau *et al.*, 2018). A subset was selected consisting of 28 primer pairs located on different scaffolds in *P. ramorum* that were tested in a first round of *in vitro* screening, to eliminate those that generated amplicons with DNA from non-targeted species and/or those that failed to generate an amplicon with DNA from the targeted species. Five of those 28 primer pairs (17.9%) were specific to *P. ramorum*, including those targeting the multicopy candidate gene Pram-C62 (Fig. 1). One hundred and eighty unique candidate clusters were identified for *P. lateralis*; six were retained for their specificity out of the 16 tested, resulting in a success rate twice as high as with *P. ramorum* (37.5%). At the group level (i.e. *P. ramorum* + *P. lateralis*), five candidate clusters out of nine predicted and tested were retained. A low number of unique candidate gene clusters were found within the lineages of *P. ramorum* (17 for EU2 to five in NA1), probably due to low levels of divergence between these lineages. Two to six candidate clusters were tested for specificity, resulting in two candidates retained for both NA1 and EU1, and three candidates for NA2 and EU2 (Fig. 1). Overall, 28.6% of the candidate clusters tested passed the specificity test; this success rate rose to 55.8% when considering *P. ramorum* lineages only.

Design and testing of the TaqMan probes

TaqMan probes were designed for the 16 candidate gene clusters retained for *P. ramorum* (five clusters), *P. lateralis* (six clusters) and *P. ramorum* + *P. lateralis* (five clusters) and tested for specificity in real-time PCR (Fig. 2). Non-specific detection with *P. lateralis* DNA was observed with the Pram-C998 and Pram-158 assays targeting DNA from *P. ramorum*, resulting in their rejection. Although Pram-

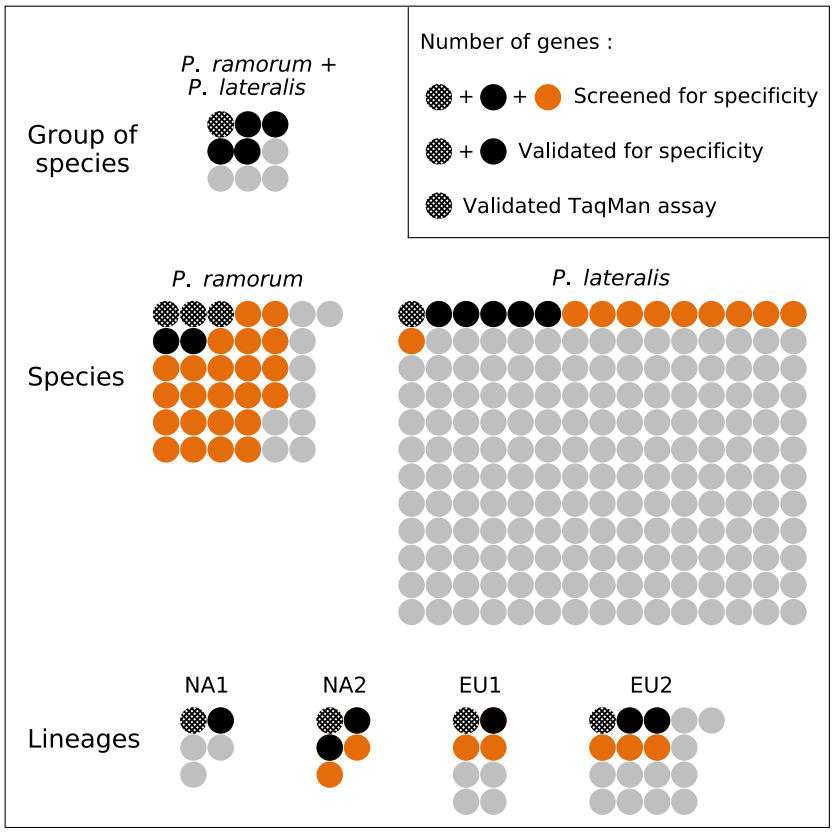

Figure 1 PCR screening of candidate gene clusters for specificity. For each group of species and lineage, the dot matrix represents the number of candidate genes predicted by the GEDI pipeline (all circles), the proportion of candidates tested for specificity (orange + black + dotted black), the number of candidates that passed the specificity test (black + dotted black) and those selected and validated as the final assays panel (dotted black).

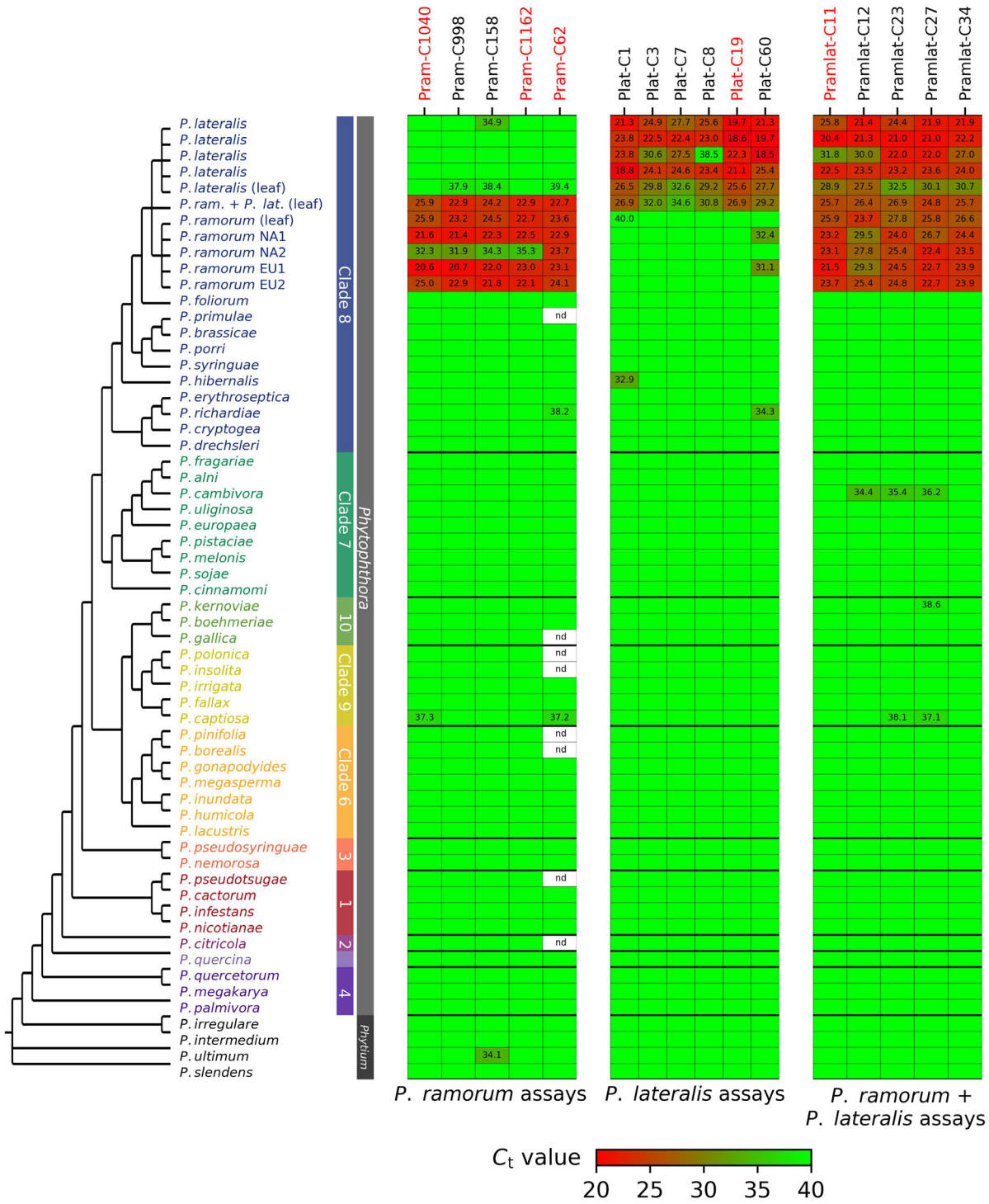

Figure 2 Specificity of the real-time PCR assays targeting *Phytophthora ramorum* or *P. lateralis* and the group-specific assays targeting both species. The NJ phylogenetic tree represents evolutionary relationships among *Phytophthora* and *Pythium* species inferred from an alignment of β-tubulin sequences. Only C_t values below 40.0 are represented. Real-time PCR assays highlighted in red are those that were selected and validated as the final assays panel; DNA sample × primers/probe combinations not tested are noted 'nd'. Samples labelled 'leaf' were obtained from inoculated *Rhododendron* leaves.

C62 cross-amplified slightly with DNA of *P. richardiae* and *P. capitosa* (C_t value of 38.2 and 37.2, respectively), this assay was retained because of its high sensitivity in detecting DNA from *P. ramorum* NA2 lineage (C_t of 23.7 versus 31.9–35.3 for the four other *P. ramorum* assays). For *P. lateralis*, all assays but Plat-C19 were rejected at

this step due to some nonspecific amplification with DNA of *P. ramorum* and *P. richardiae* (Plat-C60) and *P. hibernalis* (Plat-C1) and their lower performance in detecting DNA from the target species, particularly on infected plant material (Fig. 2). At the group level, only Pramlat-C11 was retained as this assay showed a better specificity than Pramlat-C12, Pramlat-C23 and Pramlat-C27 (which cross-reacted with *P. cambivora* and *P. capitosa* and/or *P. kernoviae*), and usually earlier C_t values than Pramlat-C34 with 8 out of the 11 *P. ramorum* and *P. lateralis* DNA samples tested (Fig. 2).

TaqMan probes were designed for the 10 *P. ramorum* lineage-specific genes retained in the previous screening step and tested on a DNA panel of 22 *P. ramorum*, one *P. lateralis* and one *P. hibernalis* individuals. Two of these assays were eliminated due to cross reactions with DNA from non-target lineages (Pram-NA2-355 with P2111 and Pram-EU1-352 with PR-10-4389a and P2111); Pram-NA1-835 was also rejected as this assay failed to detect DNA of the P5010 individual of the NA1 lineage (false negative; Fig. 3). All other assays were specific to their respective lineage, with the exception of Pram-NA1_399 that generated a late C_t value on P5010 (38.0) and P5009 (33.8) indicating that this assay could be subjected to lower specificity for some NA1 isolates (Fig. 3). The most sensitive assays (i.e. showing the lowest C_t on DNA from targeted individuals) were retained for the next steps for NA2 (NA2-353 and NA2-356) and EU2 (Pram_EU2_268; Fig. 3).

Assay efficiency and limit of detection

Ten assays were retained and tested for efficiency and sensitivity. For eight assays, efficiency was over 85% with values from 88% (*P. ramorum* EU1 lineage Pram-EU1-C358) to 113% (Pramlat-C11; Table 1), with an average of 99.0% ± 9.0. All assays reached the detection level of 2 to 10 DNA copies of the target DNA sample, corresponding to a range of 141–704 fg DNA (Table 1). The last assay, NA2-353, showed a PCR efficiency under 80% and was consequently discarded from the assay panel.

Assay performance with infected plant material

For the five assays targeting group and species (i.e. *P. ramorum*, *P. lateralis* and *P. lateralis* + *P. ramorum*) C_t values obtained with DNA from pure cultures were lower than those obtained with DNA extracted from infected plant material; this trend was statistically significant for all the assays, but Pram-C62 (Fig. S1). Neither false positive nor false negative was generated on this panel test with the group and species-level assays (Fig. 4a; Table S4). In contrast, specificity of the ITS assay targeting *P. ramorum* DNA (tested with the same PCR parameters as those used for the assays developed in this study) generated a high number of false positives with DNA from plant material inoculated with *P. lateralis* (Fig. 4a; Table S4).

True positives were detected for the *P. ramorum* lineage assays, with average C_t values ranging between 24.8 (PramEU1-C358) and 29.3 (PramNA1-C399) (under the experimental conditions here). However, few false positives were observed with the *P. ramorum* lineage assays, but usually with late C_t values (Fig. 4a). The assays targeting DNA of NA1 and NA2 cross-amplified with DNA from the same sample of *G. shallon* infected by the EU1 individual P5039 with C_t values of 36.8 and 33.3, respectively (Table S4). Similarly, the EU1 assay returned a C_t value of 35.9 on a DNA sample of *G. shallon* infected with the NA2 individual P5073 (Table S4).

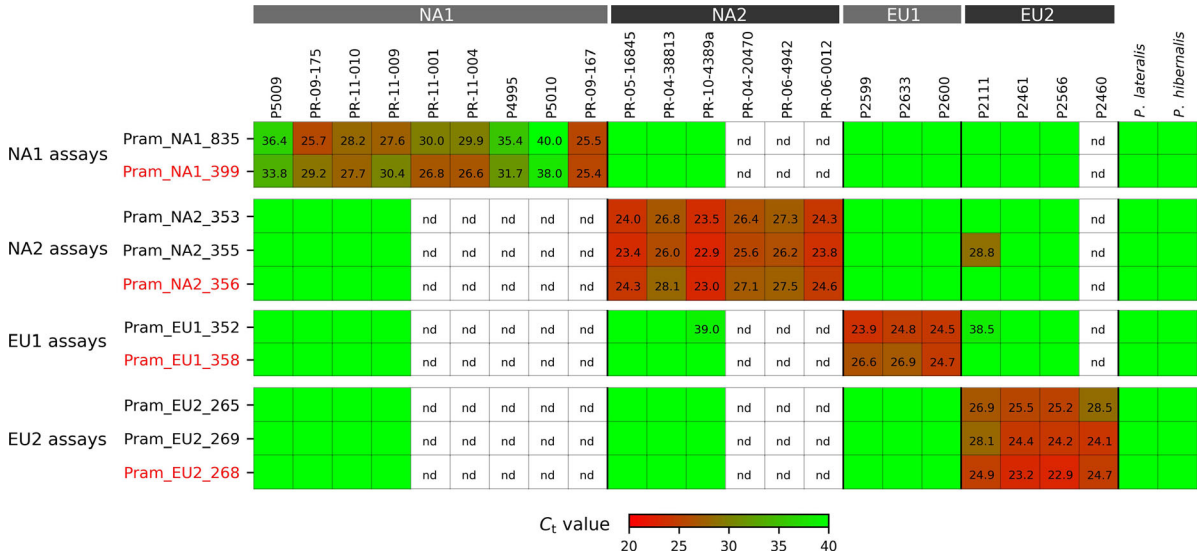

Figure 3 Specificity of the real-time PCR assays targeting the four *Phytophthora ramorum* lineages. Only C_t values below 40.0 are represented. Real-time PCR assays highlighted in red are those that were selected and validated as the final assays panel; DNA sample × primer/probe combinations not tested are noted 'nd'.

Table 1 Real-time PCR assays developed for *Phytophthora ramorum* and *P. lateralis*.

Target species	Assay name	Target gene	Primer		Efficiency (%)	LOD (in number of gene copy)	Detection level (fg)	C_t value at LOD
			Name	Sequence (5′–3′)				
Lineage-specific								
P. ramorum NA1	PramNA1-C399	Gag-pol fusion protein	PramNA1-399-F2	GCATGTCGTCCATGTCAATC	91.16	10	704	38.27 ± 0.56
			PramNA1-399-P2	CATCATGCGCTGGAAAGTCG				
			PramNA1-399-R1	AATCGACGAAACGTTGGAAG				
P. ramorum NA2	PramNA2-C356	Hypothetical protein	PramNA2-356-F	TATGGCAGTGCGAATGTTG	94.97	10	704	34.62 ± 0.27
			PramNA2-356-R	GTCGTTGGCGTAGAAATCAA				
			PramNA2-356-P	TTTACGCTATCGTCTGCTGCGAC				
P. ramorum EU1	PramEU1-C358	Hypothetical protein	PramEU1-358-F	GTCGGCCTTAAGAAGTCGTC	87.97	10	704	35.79 ± 1.48
			PramEU1-358-R	ATCCCGAATAGGGCTAGAGG				
			PramEU1-358-P	CTTGTGCACCACCACAAGAATCC				
P. ramorum EU2	PramEU2-C268	Hypothetical protein	PramEU2-268-F	GCCACCACAAATACAAGCAC	94.49	2	140.8	37.85 ± 0.79
			PramEU2-268-R	TGTGCTACTCGACTGGGTCT				
			PramEU2-268-P	ATTTGAGGCCGAGCCATTAGTGA				
Species-specific								
P. ramorum	Pram-C1040	Hypothetical protein	Pram-C1040-F	TGAACGAAGACTTGGAGATGGAC	98.48	2	140.8	35.23 ± 0.43
			Pram-C1040-R	ACTTCACTTGATCGTCGGTTTCT				
			Pram-C1040-P	AATGGCTTGGAAGTGGGTTTGGGCGA				
	Pram-C1162	DIS3-like exonuclease 2	Pram-C1162-R	AGCGTGTCTATCTCGGCATC	90.12	10	704	36.26 ± 1.23
			Pram-C1162-F	GAGAAAACGCAGACACCCAAG				
			Pram-C1162-P	GACGGAGCAAGACTTGGACGCGATC				
	Pram-C62[a]	Hypothetical protein	Pram-C62-F	AACATGCTCGTGCTCAAGTG	110.06	10	704	35.47 ± 0.84
			Pram-C62-R	CGGTGTTCTGGCGTTCTAGT				
			Pram-C62-P	CAAGGGGACCGGAACCGTAT				
P. lateralis	Plat-C19[a]	Mucin-like protein	Plat-C19-R3	GTCACAGCATCAGGCACG	102.52	10	704	32.56 ± 0.12
			Plat-C19-F3	GTGCTGAGCGGTAGTAGCAG				
			Plat-C19-P	TGCGGGTGCTAGTGGCAGTA				
Group-specific								
P. ramorum + *P. lateralis*	Pramlat-C11[a]	Putative RxLR effector	Pramlat-C11-F	GAGCAAAACACTCTCGGCTT	113.00	10	704	32.97 ± 0.64
			Pramlat-C11-R	GTTGCTCCTCGCCAAAGTC				
			Pramlat-C11-P	GGTCGCAAACACCGCGAA				

LOD, limit of detection; F, forward primer; R, reverse primer; P, internal TaqMan probe. Isolates used for LOD: PramNA1-C399, Pram-C1040, Pram-C1162, Pram-C62 and Pramlat-C11: Pr-518 (lineage NA1); PramNA2-C356: Pr-11-1291 (NA2); PramEU1-C358: Pr-09-106 (EU1); PramEU2-C268: P2566 (EU2); Pramlat-C11: CBS168.42 (*P. lateralis*).
[a]Multicopy gene.

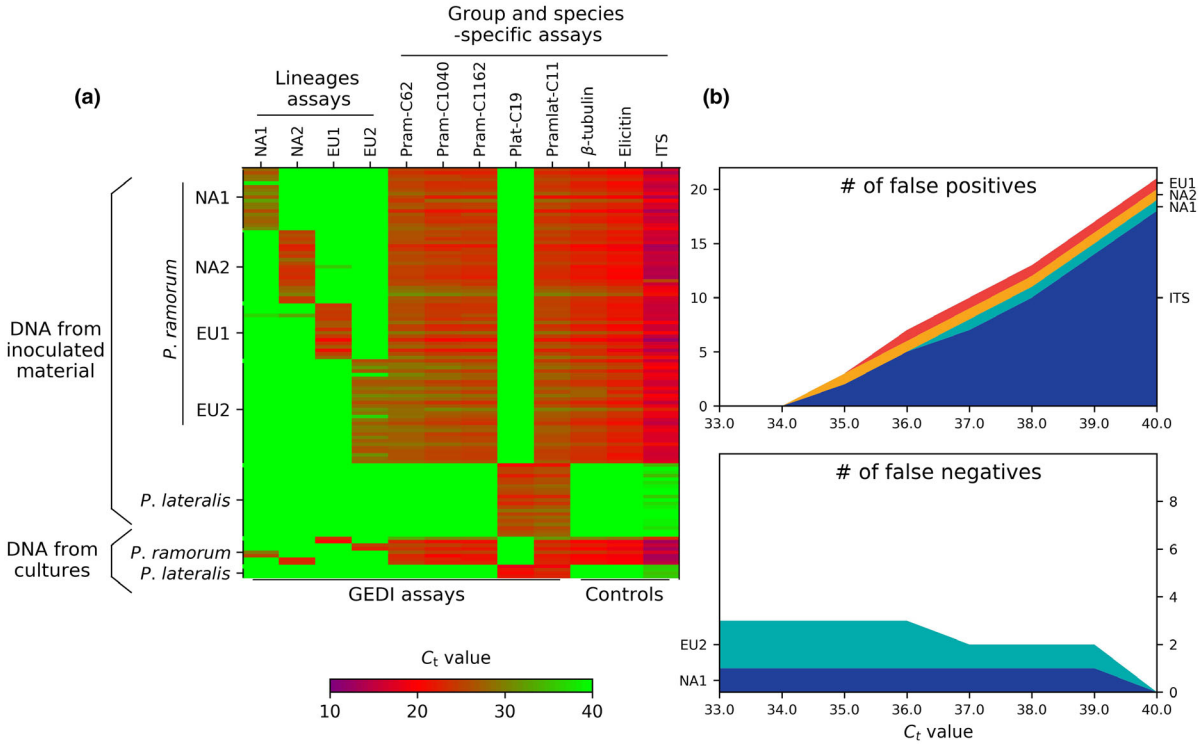

Figure 4 Real-time PCR assays testing on infected plant material. (a) C_t values (presented in Table S4) on DNA from inoculated material and cultures for the GEDI assays targeting *Phytophthora ramorum*, *P. lateralis* and *P. ramorum* + *P. lateralis* and three control assays (i.e. β-tubulin = Phy_ram482U_LNA_F/Phy_ram_653L_LNA_R, Elicitin = Prameli 102U_F/Prameli259L_R and ITS = ITSPrimer622U_F/ITSPrimer755L_R; (Bilodeau *et al.*, 2007a)). (b) Number of false positives (top) and false negatives (bottom) generated at different C_t cut-offs ranging from 33.0 to 40.0.

Using a C_t value cut-off of 35.0 (with the same qPCR experimental conditions) to declare a positive reaction would prevent the generation of the false positives observed with the NA1 and EU1 assays (Fig. 4b; Table S4), but might also have the adverse effect of affecting the detection of some true positives with Pram_NA1_399 (e.g. P5010; see Fig. 3). For the NA2 assay the cross-reaction with an EU1 isolate inoculated on *G. shallon* suggests that this cut-off should be brought down to 33.0, to reduce the risk of false positives with this lineage (Fig. 4b; Table S4). Only three false negatives were observed with the lineage assays: the NA1 assay on DNA from *Camellia* sp. infected by P5046 (C_t 39.2), and the EU2 assay on DNA from *Rhododendron* sp. samples infected with P2111 ($C_t \geq$ 40.0) and P2566 (C_t 36.6) (Fig. 4a; Table S4). Applying a C_t cut-off equal to or above 37.0 would prevent the generation of the second false negative obtained with the EU2 assay, as DNA from isolate P2561 infecting *Rhododendron* sp. resulted in a C_t of 36.6 (Fig. 4b; Table S4).

Accuracy of the assays

A naïve Bayes classifier was used, to avoid using an arbitrary C_t value threshold to declare a positive reaction. The classifier was trained on a set of C_t values obtained from positive and negative samples and assigned random samples of this training set as positive or negative to obtain an evaluation of the performance (TP and FP rates, accuracy) of each assay; this procedure was repeated 10 000 times, by generating each time a new random training set (from experimental C_t values obtained from infected tissues). For the five assays targeting DNA of *P. ramorum* and/or *P. lateralis* an accuracy in detection of 100% was obtained, whereas accuracy of these assays averaged 98.6% and 99.7% with arbitrary C_t cut-offs of 40.0 and 36.0, respectively (Fig. S2). Analysis of sensitivity and accuracy of the ITS and β-tubulin assays was increased by using the naïve Bayes classifier but remained slightly below the optimal value of 100% due to the presence of false negatives for the ITS assay and some false positives for the β-tubulin one (Fig. S2).

Discussion

Amplification of DNA by PCR has revolutionized the detection and monitoring of plant pathogens causing tree and crop diseases (Martinelli *et al.*, 2015). Most of the DNA-based detection assays developed to date rely on the amplification of a fraction of the genome of the targeted species, usually one to three genes. The most widely used genes or genome regions such as the internal

transcribed spacer of the ribosomal cluster (ITS rDNA), the intergenic spacer region (IGS) and the β-tubulin gene are usually conserved in all eukaryotes. This has been a useful feature because universal primers can be designed to obtain the DNA sequence of those genes and to design assays that target discriminant SNPs (Martinelli *et al.*, 2015). However, this approach has limitations when applied to taxa that diverged recently or to distinguish taxonomic subgroups such as clonal lineages within a species. Distinguishing lineages of plant pathogens such as *P. ramorum* can be critical because they have different phenotypic characteristics, including host range and aggressiveness, and some have opposite mating types (Elliott *et al.*, 2011). This is the case in Oregon, where the EU1 *P. ramorum* lineage was found for the first time in forest stands on grand fir (*Abies grandis*) and Douglas fir (*Pseudotsuga menzeii*) (LeBoldus *et al.*, 2018) and requires a management approach distinct from the previously established NA1 lineage.

With the increasing availability of whole genome sequences for some of the most important crop and tree pathogens, it becomes feasible to search for genome regions that are unique to a target organism or highly discriminant amongst closely related non-target species (Feau *et al.*, 2018). The genomes of more than 100 *P. ramorum* individuals have been resequenced from all four known lineages from Europe and North America and *de novo* genome assemblies generated of an additional six *Phytophthora* species, including close relatives, and a pipeline built that can be used to discover unique genome regions and develop DNA-based assays (Feau *et al.*, 2016, 2018). In the present study, this pipeline was applied to develop a set of real-time PCR detection assays targeting three hierarchical taxonomic levels, including *P. ramorum* and the sister species *P. lateralis* and all four currently recognized lineages of *P. ramorum*. This novel approach generated highly specific assays, as unique genome regions present only in the targeted taxa were identified. These assays were very accurate and sensitive, ranging from 98.7% to 100% detection of 2–10 gene copies of the targeted taxa. This level of sensitivity is within the lowest theoretical limit of detection of DNA (Bustin *et al.*, 2009).

By using the common approach of setting an arbitrary threshold C_t value to declare a positive reaction (C_t value cut-off of 36.0 in this study), all *P. ramorum* and *P. lateralis* assays yielded an accuracy of 100%, whereas false positives were obtained with the ITS and β-tubulin assays under the same real-time PCR conditions. A machine learning model using a naïve Bayes classifier was also developed, that trains on prior distributions of C_t values obtained on DNA from true positive and true negative samples to determine the probability that an unknown sample is a positive. Using this classifier, the accuracy of the less well performing assays (ITS and β-tubulin) was improved, indicating that alternate approaches to C_t value threshold like a simple machine learning model may help improve precision in declaring true positive samples. However, although relatively simple to implement, such an approach still requires some investment in testing DNA from true positive and false positive samples to build prior distributions of C_t values.

Mining *Phytophthora* genomic resources for the development of diagnostic and detection assays is promising; whole mitochondrial genomes of *Phytophthora* species have been compared to identify variable sequences within conserved regions where primers can be designed (Miles *et al.*, 2017). By targeting genome regions uniquely found in the targeted species, the present approach promises to reduce the likelihood of false positives, in particular those caused by close relatives. Indeed, previous assays aimed at amplifying the ITS region of *P. ramorum* have shown cross-reactivity with DNA of *P. lateralis* (Hayden *et al.*, 2006; Belbahri *et al.*, 2007; this study), especially at high DNA concentrations of the target and non-target species (Hughes *et al.*, 2006; Bilodeau *et al.*, 2007a). Similar nonspecific DNA amplifications potentially yielding false positives were reported for assays developed on other conserved genes such as β-tubulin and elicitin (Bilodeau *et al.*, 2009).

Given its high copy number, which translates into highly sensitive assays, with a limit of detection (LOD) ranging from 12.9 to 100 fg (Hayden *et al.*, 2006; Hughes *et al.*, 2006), the ITS region is the most commonly targeted gene for detection assay development. In-house testing using similar real-time PCR conditions and isolates (data not shown) yields LOD an order of magnitude lower for the ITS (Bilodeau *et al.*, 2007a) compared to mitochondrial genes (Tooley *et al.*, 2006), β-tubulin, elicitin or single copy genes (Bilodeau *et al.*, 2009) and the genome-derived assays from this study. There is always a compromise between sensitivity and specificity when developing detection assays and these factors must be balanced. The methods developed here achieved this balance by generating a rate of 100% accuracy for the group and species-specific assays. Highly sensitive multicopy (e.g. targeting the ITS) and specific (e.g. those reported here) assays could be combined, either in parallel in an array format or in a multiplex real-time PCR that would combine high sensitivity and specificity. One of the benefits of targeting unique regions of about 200–500 bp is that there is virtually no limitation in designing assays that can meet criteria of temperature conditions. This allows performing parallel runs in high-throughput formats (96 or 384) of the same samples with multiple assays in singleplex reactions targeting species and lineages, or to incorporate these assays in multiplex combinations.

One of the challenges of developing assays with primers and probes targeting conserved gene regions is the paucity of variable regions among closely related species. This limitation has been reported in *Pseudogymnoascus* (Shuey *et al.*, 2014) and *Phytophthora kernoviae* (Lamarche *et al.*, 2015), where the genes and genome regions commonly used for assay development did not possess sufficient variation to discriminate between the target species and its close relative. Discriminating

among lineages of *P. ramorum* was even more challenging as polymorphisms were rare in the ITS (Kroon *et al.*, 2004), β-tubulin and *CBEL* (Bilodeau *et al.*, 2007b) and the cytochrome c oxidase subunit 1 (*Cox 1*) gene (Kroon *et al.*, 2004). Currently, the discrimination of the *P. ramorum* lineages is based on the use of markers that require large amounts of high quality DNA and/or relatively longer protocols that are more complex to analyse (AFLPs, ISSR-PCR, microsatellites or Sanger sequencing), or on the combination of several allele-specific oligonucleotide-PCR assays (Gagnon *et al.*, 2017).

In conclusion, a set of novel highly accurate and sensitive real-time assays have been developed from unique genome regions at two hierarchical levels (species-specific and lineage-specific) for *P. ramorum*, as well as from its closely related species *P. lateralis*. These accurate and sensitive assays represent an improvement in the detection of *P. ramorum* and could either replace or complement assays developed from conserved gene regions.

Acknowledgements

This work was supported by Genome Canada's Large-Scale Applied Research Program (LSARP projects #2112 and 10106) and Genome Canada's Genomic Application Partnership Program (GAPP 6102) with additional funding from Genome B.C., Genome Quebec, Canadian Food Inspection Agency, Natural Resources Canada and FPInnovations. The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Distribution of C_t values obtained for the *Phytophthora ramorum* (Pram-C62, Pram-C1040 and Pram-C1162), *P. lateralis* (Plat-C19) and *P. ramorum* + *P. lateralis* (Pramlat-C11) assays on cultures and environmental samples. *t*-test comparison of means: ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Figure S2. Performance of the group and species-specific assays targeting *Phytophthora ramorum* and *P. lateralis*. Performance is expressed in term of false positive rate (top graph), false negative rate (middle) and accuracy (bottom) and was measured depending on two manual C_t cut-offs (36.0 and 40.0) and by using a naïve Bayes classifier.

Table S1. *Phytophthora* genome sequences used in this study.

Table S2. *Phytophthora* isolates tested in this study.

Table S3. Plant material inoculated with *Phytophthora ramorum* and *P. lateralis* and used as DNA template for the validation step.

Table S4. C_t values obtained with DNA templates from infected plant material for the nine TaqMan assays developed in this study and three controls.