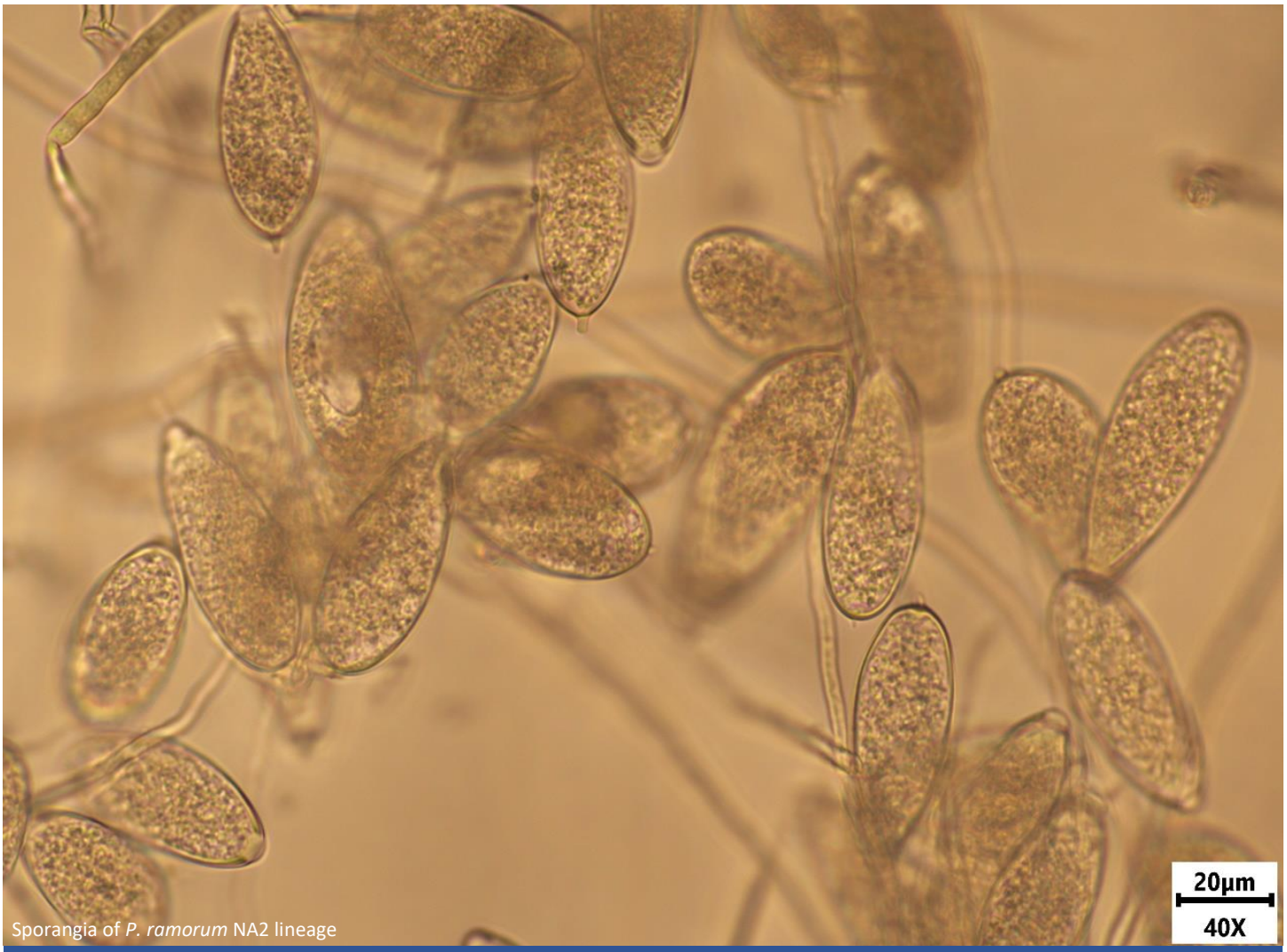

Sporangia of *P. ramorum* NA2 lineage

Validation of methods for the detection of *Phytophthora ramorum* lineages

EURL Work Programme 2021
Sub-activity 1.4

European Union Reference Laboratory (EURL) for Pests on Plants, on Fungi and Oomycetes

French Agency for Food, Environmental and Occupational Health and Safety (ANSES), Plant Health Laboratory – Mycology Unit, Nancy, France

anses

Validation of methods for the detection of *Phytophthora ramorum* lineages

Sub-activity 1.4. Production of methods. Producing improved or modified methods on plant pests, and participating in validation studies of methods.

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Table of contents

1. Introduction

Phytophthora ramorum is a destructive oomycete that belongs to the order Peronosporales. It is the causal agent of the sudden oak death, sudden larch death and ramorum blight (Werres *et al.* 2001, Grünwald *et al.* 2008). This pathogen has a wide host range including *Fagus sylvatica, Larix kaempferi*, *L. decidua*, *L.* x *eurolepis, Lithocarpus densiflorus, Quercus agrifolia*, *Q. kelloggii*, *Q. parvula* var. *shrevei*, and *Ubellularia californiica*. Ornamentals plants such as *Rhododendron* spp. and *Viburnum* spp., have also been reported as hosts of this pathogen.

The infection of *P. ramorum* is mainly aerial, affecting the foliage of the plants and causing leaf decay and wilt. Its dispersal occurs by deciduous sporangia present on the leaf surface, which are blown away by wind, fog and rain splashes (Grünwald *et al.* 2012, Davidson *et al.* 2015). Moreover, *P. ramorum* causes stem cankers, trunk cankers and bleeding cankers on the affected trees, causing necrosis of the phloem tissues. Clogged vessels are unable to transport nutrients and water, and the tree crown declines (Grünwald *et al.* 2008).

Until 2021, there were four clonal lineages of *P. ramorum* present mainly in Europe and North America, named after its first detection on these territories: EU1, EU2, NA1 and NA2 (Grünwald *et al.* 2009). EU1 is widely found in Europe, as well as in Canada (British Columbia) and the United States (California, Oregon and Washington State). To date, NA1 and NA2 lineages have been only reported in North America. EU2 lineage has only been found in the United Kingdom (Northern Ireland and Scotland). In 2021, Jung *et al.* reported the existence of eight Asian *P. ramorum* lineages that are suggested as the ancestors of the EU and NA lineages.

Phytophthora ramorum is a heterothallic species, i.e. two different mating types need to meet in order to reproduce sexually through the formation of oogonia and antheridia. In *P. ramorum*, there are two known mating types (A1 and A2). Both mating types are found in North America and Europe, but in different proportions. Lineages EU1 and EU2 mainly found in Europe comprise mating type A1, while mating type A2 is found in Canada and the United States in the NA1 and NA2 lineages (Werres & De Merlier 2003). Although some isolates of the A2 mating type were found in Belgium (Boutet *et al.* 2010) and isolates of the A1 mating type were found in North America (Hansen *et al.* 2003), no evidence of sexual reproduction of *P. ramorum* has been detected in natural conditions.

Quarantine measures and regulations are in place to prevent the spread and introduction of *P. ramorum* to new areas. Nowadays non-European Union lineages of *P. ramorum* are included in the new “Plant Health Law”, Regulation (EU) 2016/2031, in the EU plant pest list Annex II of Commission Implementing Regulation (EU) 2019/2072 of 28 November 2019.

The European and Mediterranean Plant Protection Organization (EPPO) has published a diagnostic guide for the detection and identification of *P. ramorum sensu lato*, PM 7/66 (1) (EPPO 2006). However, this protocol does not include any method for the detection and differentiation of the four *P. ramorum* clonal lineages. More recently, some methods for the differentiation of the four lineages using different approaches have been published: microsatellites (Gagnon *et al.* 2017), restriction fragment length polymorphism (PCR-RFLP) (Kroon *et al.* 2004, Elliott *et al.* 2009, King *et al.* 2015) and real-time PCR (Gagnon *et al.* 2014, Feau *et al.* 2019).

2. Aim and output of the project

The main goal of this project is to validate a molecular diagnostic method for the differentiation of the existing *P. ramorum* lineages and to assess its performance based on criteria such as sensitivity, specificity, repeatability, as well as its robustness and transferability. It is of particular importance to mention that the evaluation of the molecular assays will include strains belonging to the recently described lineages from Asia, as well as strains of the existing lineages of *P. ramorum*. The results obtained in the current project will provide useful information and clues regarding the need for the development of new diagnostics methods able to separate North American and European from Asian lineages.

This project aims to gather information on the performance of the methods used to discriminate *P. ramorum* lineages, as well as its possible weaknesses and strengths.

3. Selection of the method

3.1. Literature review

Table 1 lists available molecular assays for the detection of *P. ramorum sensu lato* or its different genotypes. It also summarizes the advantages and disadvantages of each protocol.

Table 1 Comparative listing of the *P. ramorum* detection protocols available in the literature

Method	Advantages	Drawbacks
① Feau N, Ojeda DI, Beauseigle S, Bilodeau GJ, Brar A, Cervantes-Arango S, Dale AL, Dhillon B, Hammett C, Herath P, Shamoun SF, Tsui CKM, Tanguay P, Hamelin RC. 2019. Improved detection and identification of the sudden oak death pathogen *Phytophthora ramorum* and the Port Oxford cedar root pathogen *Phytophthora lateralis*. *Plant Pathology* 68: 878 – 888.	• High specificity – use of whole genome sequencing and comparison approach to find very specific genomic regions able to discriminate all four lineages. • Use of individual hydrolysis probes (one per lineage) – adds flexibility in the test (no need to include them all, according to the target). • Test discriminates species closely related, like *Phytophthora lateralis*, *P. foliorum* and *P. hibernalis*, which avoids false positives. • Very sensitive and accurate test – able to detect 2 to 10 copies of the target gene from pure cultures or infected plant tissue, with a detection accuracy between 98.7% and 100%.	• Assay based on the use of hydrolysis probes, hence is more expensive, especially because this assay involves four different primer pairs and probes, one for each *P. ramorum* lineage. • Assay has not been tested in multiplex, i.e., using all four probes in the same PCR tube, which also increments the cost as several PCR reactions are needed to elucidate the *P. ramorum* lineage.
② Gagnon MC, Feau N, Dale AL, Dhillon B, Hamelin RC, Brasier CM, Grünwald NJ, Brière SC, Bilodeau GJ. 2017. Development and validation of polymorphic microsatellite loci for the NA2 lineage of *Phytophthora ramorum* from whole genome sequence data. *Plant Disease* 101: 666 – 673.	• High specificity by using whole genome sequencing to find specific regions of the NA2 lineage. • Microsatellite-based detection are easy and quick to implement and a cost-effective.	• Test performed only on 6 - 12 isolates per lineage, and thus, it needs to be tested and further optimized to find the best combination of markers for lineage differentiation.
③ Franceschini S, Webber JF, Sancisi-Frey S, Brasier CM. 2014. Gene x environment tests discriminate the new EU2 evolutionary lineage of *Phytophthora ramorum* and indicate that it is adaptively different. *Forest Pathology* 44: 219 – 232.	• Distinction of the four *P. ramorum* lineages.	• Needs *P. ramorum* in pure culture to perform the tests, it was not developed to be performed directly on plant material. • Culture-based methods are time consuming. • Less sensitive and accurate than existing molecular methods.

Method	Advantages	Drawbacks
④ Gagnon MC, Bergeron MJ, Hamelin RC, Grünwald NJ, Bilodeau GJ. 2014. Real-time PCR assay to distinguish *Phytophthora ramorum* lineages using the cellulose binding elicitor lectin (CBEL) locus. *Canadian Journal of Plant Pathology* 36: 367 – 376.	• Use of real-time PCR by SYBR Green, based on allele-specific oligonucleotide-PCR (ASO-PCR), which is more sensitive and cheaper than hydrolysis probes. • Allows the differentiation of the EU2 lineage, based on the previous method developed by Bilodeau *et al*. (2007) which allowed the differentiation of the other three lineages of *P. ramorum*.	• The need of using two different primer pairs, each one designed to detect one allele of the genotype variant of each *P. ramorum* lineage, makes the number of PCR reactions per sample to increase. In total, eight PCRs are required to identify the lineage, which increases the overall cost of the test. • In general, real-time PCR assays using SYBR Green are less specific compared to hydrolysis probes. However, this can be compensated with the study of melting curves and Ct values obtained with SYBR Green assays.
⑤ Van Poucke K, Franceschini S, Webber JF, Vercauteren A, Turner JA, McCracken AR, Heungens K, Brasier CM. 2012. Discovery of a fourth evolutionary lineage of *Phytophthora ramorum*: EU2. Fungal Biology 116: 1178 – 1191.	• Microsatellite-based detection are easy and quick to implement and cost-effective. • The use of multi-locus sequence analysis makes this method very affordable to any laboratory (it does not require specific equipment or real-time machines).	• Time consuming due to the need to sequence PCR products, carry out sequence analysis and phylogenetic reconstructions. • It needs to perform the PCR and analysis of all four lineages at the same time to identify the North American lineages.
⑥ Vercauteren A, Larsen M, Goss E, Grünwald NJ, Maes M, Heungens K. 2011. Identification of new polymorphic microsatellite markers in the NA1 and NA2 lineages of *Phytophthora ramorum*. *Mycologia* 103: 1245 – 1249.	• Microsatellite-based detection are easy and quick to implement and a cost-effective.	• Only differentiates North American lineages, European lineages were not included in the test.
⑦ Elliott M, Sumampong G, Varga A, Shamoun SF, James D, Masri S, Brière SC, Grünwald NJ. 2009. PCR-RFLP markers identify three lineages of the North American and European populations of *Phytophthora ramorum*. *Forest Pathology* 39: 266 – 278.	• PCR-RFLP is a rapid and simple method to implement and do not require specialised laboratory equipment. • Assay based on the COXI (cytochrome c oxidase subunit I) gene which showed more differences between lineages than the ITS region.	• This assay showed cross reactivity between NA1 lineage of *P. ramorum* and *P. foliorum*. • This assay does not include the EU2 because this lineage was described in 2012. • RFLPs require high quality DNA, and it might be difficult to implement on infected plant tissues.

Method	Advantages	Drawbacks
⑧ Bilodeau GJ, Lévesque CA, de Cock AWAM, Brière SC, Hamelin RC. 2007. Differentiation of European and North American genotypes of *Phytophthora ramorum* by real-time polymerase chain reaction primer extension. *Canadian Journal of Plant Pathology* 29: 408 – 420.	• Use of real-time PCR by SYBR Green, based on allele-specific oligonucleotide-PCR (ASO-PCR), which is sensitive and is cheaper than the use of hydrolysis probes. • The method can be performed on both, pure cultures and infected plant material. • It uses two loci: CBEL and β-tubulin.	• This assay does not include the EU2 because this lineage was described in 2012. • In general, real-time PCR assays using SYBR Green are less specific compared to hydrolysis probes. This fact can somewhat be compensated with the study of melting curves and Ct values obtained with SYBR Green assays.
⑨ Ivors K, Garbelotto M, Vries IDE, Ruyter-Spira C, Hekkert BTe, Rosenzweig N, Bonants P. 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.	• Microsatellite-based detection are easy and quick to implement and a cost-effective. • Differentiation of NA1, NA2 and EU1 lineages.	• This assay does not include the EU2 because this lineage was described in 2012.
⑩ Ivors KL, Hayden KJ, Bonants PJM, Rizzo DM, Garbelotto M. 2004. AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycological Research* 108: 378 – 392.	• AFLP and phylogenies are used in combination, using three loci (ITS, COXII and nad5).	• AFLP results are sometimes difficult to reproduce (inconsistency issues). • This assay does not include the EU2 because this lineage was described in 2012. • Time consuming due to the inclusion of sequencing of PCR products.
⑪ Kroon LPNM, Verstappen ECP, Kow LFF, Flier WG, Bonants PJM. 2004. A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. *Phytopathology* 94: 613 – 620.	• PCR-RFLP is a rapid and simple method to implement and do not require specialized lab equipment. • Differentiation of NA1, NA2 and EU1 lineages.	• This assay does not include the EU2 because this lineage was described in 2012. • RFLPs require high quality DNA, and it might be difficult to implement on infected plant tissues.

3.2. Outcome of the comparison and selection of the method for validation.

In 2019, Feau *et al.* developed an improved real-time PCR method for the detection and identification of *P. ramorum* and its four lineages (NA1, NA2, EU1 and EU2) using individual hydrolysis probes (one per lineage). According to the authors, this method was accurate and specific to detect and identify the four existing *P. ramorum* lineages. It was validated using artificially contaminated plant material.

Real-time PCR is one of the main techniques used in diagnostic laboratories due to its high sensitivity and accuracy in comparison with other methods. This in combination with the use of whole genome sequencing to find very specific and suitable regions able to differentiate all *P. ramorum* lineages makes the assays developed by Feau *et al.* (2019) the best candidate for validation. Moreover, this method was also developed using infected plant material, which might speed up the detection and identification of *P. ramorum* lineages, as it does not require isolation of the pathogen from infected tissue.

4. Materials and methods

4.1. Collection of *Phytophthora* spp. strains and preparation of monohyphal strains

Different strains of the four existing lineages of *P. ramorum* and other closely related *Phytophthora* species were obtained from different sources, geographic origins and hosts (Table 2). It is important to highlight that strains from the recently described Asian lineages by Jung *et al*. (2021) were included. The strains used in this study covered much of the known genetic diversity of *P. ramorum* worldwide.

Table 2. List of *Phytophthora* spp. strains used for the validation of the method for the detection of *P. ramorum* lineages.

Species	Strain code	Origin	Year of isolation	Host	Organ/source	Owner
Phytophthora ramorum lineage EU1	EURL ANSES-F044	France	2017	*Larix* sp.	Branch	ANSES LSV
	EURL ANSES-F045	France	2017	*Larix* sp.	Branch	ANSES LSV
	EURL ANSES-F046	France	2017	*Larix* sp.	Needles	ANSES LSV
	EURL ANSES-F047	France	2018	*Larix* sp.	Wood	ANSES LSV
	EURL ANSES-F048	France	2018	*Larix* sp.	Wood	ANSES LSV
	EURL ANSES-F049	France	2018	*Larix* sp.	Wood	ANSES LSV
	EURL ANSES-F050	France	2018	*Larix* sp.	Wood	ANSES LSV
	EURL ANSES-F051	France	2018	*Larix* sp.	Wood	ANSES LSV
	EURL ANSES-F052	France	2020	*Rhododendron* sp.	Leaves	ANSES LSV
	03-0107	Canada	2020	*Rhododendron* sp.	-	G. Bilodeau
	EURL ANSES-F060	Ireland	2005	*Rhododendron* sp.	-	R. O'Hanlon
	EURL ANSES-F061	Ireland	2011	*Fagus sylvatica*	-	R. O'Hanlon
	EURL ANSES-F064	Ireland	2014	*Fagus sylvatica*	Stem canker	R. O'Hanlon
Phytophthora ramorum lineage EU2	EURL ANSES-F062	UK - NI	2012	*Larix* sp.	-	R. O'Hanlon
	EURL ANSES-F063	UK - NI	2012	*Larix* sp.	-	R. O'Hanlon
	P2111	UK - NI	2007	*Quercus rubra*	Bark	J. Webber
	P2460	UK - NI	2010	*Larix kaempferi*	Bark	J. Webber
	P2566	UK - NI	2011	*Rhododendron ponticum*	Shoots	J. Webber
	P2586	UK - SC	2011	*Larix kaempferi*	Leaves	J. Webber
	S52817	UK - SC	2018	*Picea sitchensis*	Shoots	J. Webber
	P4995	-	-	-	-	G. Bilodeau

Species	Strain code	Origin	Year of isolation	Host	Organ/source	Owner
Phytophthora ramorum lineage NA1	P5010	-	-	-	-	G. Bilodeau
	PR-09-167	USA	-	*Kalmia latifolia*	-	G. Bilodeau
	P5009	-	-	-	-	G. Bilodeau
	PR-09-175	USA	-	*Camellia japonica*	-	G. Bilodeau
	PR-11-010	USA	-	*Camellia japonica*	-	G. Bilodeau
	PR-11-001	USA	-	*Lithocarpus densiflorus*	-	G. Bilodeau
	EURL ANSES-F037	USA	2005	-	-	N. Grünwald
	EURL ANSES-F038	USA	2019	*Lithocarpus densiflorus*	-	N. Grünwald
	EURL ANSES-F039	USA	2019	*Lithocarpus densiflorus*	-	N. Grünwald
Phytophthora ramorum lineage NA2	PR-05-16845	Canada	-	*Ardisia* sp.	-	G. Bilodeau
	PR-10-4389a	Canada	-	*Rhododendron* sp.	-	G. Bilodeau
	PR-04-38813	Canada	-	*Viburnum tinus*	-	G. Bilodeau
	PR-06-0012	Canada	-	-	Soil	G. Bilodeau
	PR-06-4942	Canada	-	*Distyllium myricoides*	-	G. Bilodeau
	PR-04-20470	Canada	-	*Rhododendron* sp.	-	G. Bilodeau
	EURL ANSES-F040	USA	2004	*Rhododendron* sp.	Leaves	N. Grünwald
	EURL ANSES-F041	USA	2019	*Rhododendron* sp.	Leaves	N. Grünwald
	EURL ANSES-F042	USA	2019	*Rhododendron* sp.	Leaves	N. Grünwald
Phytophthora ramorum lineage IC1	VN57	Vietnam	2021	Laurosilva forest	Stream	T. Jung
	VN313	Vietnam	2021	Laurosilva forest	Stream	T. Jung
	VN831	Vietnam	2021	Laurosilva forest	Leaves *Rh.*	T. Jung
	VN1015	Vietnam	2021	Laurosilva forest	Leaves	T. Jung
Phytophthora ramorum lineage IC2	VN142	Vietnam	2021	Laurosilva forest	Stream	T. Jung
	VN150	Vietnam	2021	Laurosilva forest	Stream	T. Jung
	VN169	Vietnam	2021	Laurosilva forest	Stream	T. Jung
	VN314	Vietnam	2021	Laurosilva forest	Stream	T. Jung
Phytophthora ramorum lineage IC3	VN88	Vietnam	2021	Laurosilva forest	Stream	T. Jung
Phytophthora ramorum lineage IC4	VN851	Vietnam	2021	Laurosilva forest	Leaves	T. Jung
Phytophthora ramorum lineage IC5	VN863	Vietnam	2021	Laurosilva forest	Leaves	T. Jung
Phytophthora ramorum lineage NP1	JP236	Japan	2021	Laurosilva forest	Leaves	T. Jung
	JP716	Japan	2021	Laurosilva forest	Leaves	T. Jung
	JP916	Japan	2021	Laurosilva forest	Leaves	T. Jung
	JP1202	Japan	2021	Laurosilva forest	Leaves	T. Jung
Phytophthora ramorum lineage NP2	JP387	Japan	2021	Laurosilva forest	Stream	T. Jung
	JP462	Japan	2021	Laurosilva forest	Stream	T. Jung
Phytophthora ramorum lineage NP3	JP975	Japan	2021	Laurosilva forest	Leaves	T. Jung
Phytophthora lateralis	EURL ANSES-F053	France	1998	*Chamaecyparis. lawsoniana*	-	ANSES LSV
	EURL ANSES-F054	USA	2011	*C. lawsoniana*	Wood	ANSES LSV
	EURL ANSES-F055	France	2011	*C. lawsoniana*	Wood	ANSES LSV
	EURL ANSES-F056	Scotland	2011	*C. lawsoniana*	Wood	ANSES LSV
	EURL ANSES-F057	Netherlands	2011	*C. lawsoniana*	Wood	ANSES LSV
	EURL ANSES-F058	France	2012	*C. lawsoniana*	Branch	ANSES LSV
	EURL ANSES-F059	Ireland	2012	*C. lawsoniana*	-	R. O'Hanlon
Phytophthora foliorum	CBS 121655	USA	-	-	-	CBS
Phytophthora hibernalis	CBS 270.31	USA	-	-	-	CBS
Phytophthora syringae	PH14-227	Ireland	2014	*Viburnum* sp.	-	R. O'Hanlon
Phytophthora kernoviae	PF12-106	Ireland	2011	*Rhododendron* sp.	-	R. O'Hanlon
	PR12-513	Ireland	2011	*Rhododendron* sp.	-	R. O'Hanlon
	PR12-518	Ireland	2011	*Rhododendron* sp.	-	R. O'Hanlon
	PR12-524	Ireland	2011	*Rhododendron* sp.	-	R. O'Hanlon
Phytophthora pseudosyringae	PR12W-033	UK	2012	*Fagus sylvatica*	-	R. O'Hanlon
Phytophthora gonapodyides	PR13-377	Ireland	2014	-	Footwash	R. O'Hanlon
Phytophthora ×cambivora	PR13-379	Ireland	2014	*Fagus sylvatica*	-	R. O'Hanlon

All cultures used in this project were previously purified using the hyphal-tip technique. For this, *Phytophthora* spp. were subcultured on water agar (WA) and incubated at 22°C in the dark until mycelial growth appeared on surface of the medium. Hyphal tips were excised using a fine needle under the stereomicroscope and transferred to potato dextrose agar (PDA, Difco) culture medium.

PDA plates were incubated until hyphal tips formed small colonies, and then transferred to a new PDA plate to obtain monohyphal pure cultures.

4.2. Genomic DNA extraction

A sterile disc of cellophane was placed on the PDA medium. Then, a plug of actively growing mycelium of *Phytophthora* spp. was transferred to the center of the cellophane. Plates were incubated in the dark at 22°C until the mycelium had covered the cellophane. Afterwards, the mycelium was collected for genomic DNA extraction (gDNA).

Mycelium was transferred to Lysing Matrix A tubes (MP Biomedicals) and ground for one min at 6.5 revolutions/s using the FastPrep 24 homogeniser (MP Biomedicals). Genomic DNA extractions were performed using the DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. Final DNA elutions were made using 100 µL of elution buffer provided in the DNA extraction kit. The DNA was quantified using a Nanodrop and stored at -20°C until the PCR tests were performed.

4.3. Real-time PCR reaction conditions and preparation of plasmidic DNA for positive reaction control

Feau *et al.* (2019) developed four independent real-time PCR assays using hydrolysis probes for the detection of the EU1, EU2, NA1 and NA2 lineages of *P. ramorum,* respectively. The oligonucleotides (primers and associated hydrolysis probes) are described in Table 3.

Table 3: Characteristics of the *P. ramorum*-specific oligonucleotides described by Feau *et al.* 2019

P. ramorum target	Code	Sequence (5'-3')
NA1 lineage gene C399 « Gag-pol fusion protein ».	PramNA1-399-F2	GCATGTCGTCCATGTCAATC
	PramNA1-399-R1	AATCGACGAAACGTTGGAAG
	PramNA1-399-P2	FAM-CATCATGCGCTGGAAAGTCG-BHQ1
NA2 lineage gene C356 « hypothetical protein ».	PramNA2-356-F	TATGGCAGTGCGAATGTTG
	PramNA2-356-R	GTCGTTGGCGTAGAAATCAA
	PramNA2-356-P	FAM-TTTACGCTATCGTCTGCTGCGAC-BHQ1
EU1 lineage gene C358 « hypothetical protein »	PramEU1-358-F	GTCGGCCTTAAGAAGTCGTC
	PramEU1-358-R	ATCCCGAATAGGGCTAGAGG
	PramEU1-358-P	FAM-CTTGTGCACCACCACAAGAATCC-BHQ1
EU2 lineage gene C268 « hypothetical protein ».	PramEU2-268-F	GCCACCACAAATACAAGCAC
	PramEU2-268-R	TGTGCTACTCGACTGGGTCT
	PramEU2-268-P	FAM-ATTTGAGGCCGAGCCATTAGTGA-BHQ1

Table 4 shows the PCR mixture and concentration of reagents for each lineage-specific assay, while Table 5 shows the PCR conditions used for all four independent reactions.

The qPCR master mix used by the EURL throughout the validation experiments was the *Core Kit no ROX* (Eurogentec), and the reactions were run using a Rotor-Gene Q (Qiagen).

Table 4. Composition of the reaction mixture for the lineage-specific real time PCR according to Feau *et al*. (2019)

Reagent	Final concentration in the mix
Molecular grade water	to 20 µL
Polymerase Buffer*	1 x
HotGold Star DNA Polymerase*	0.025 U μL^{-1}
$MgCl_2$*	5 mM
dNTPs*	0.20 mM each
Forward primer	0.40 µM
Reverse primer	0.40 µM
Hydrolysis probe	0.20 µM

* provided individually in the Core Kit no ROX (Eurogentec)

Table 5. Conditions for the lineage-specific real time PCR according to Feau *et al*. (2019)

Step	Temperature	Duration	N cycles
Initial denaturation	95 °C	10 min	1
denaturation	95°C	15 sec	40
Hybridization/polymerisation	60°C	1 min	

For each of the four assays, a synthetic plasmidic DNA containing the respective target region was prepared and used as a standard positive control. Genomic DNA from the following reference strains was used:

- *P. ramorum* lineage EU1: EURL ANSES-F 047, ex *Larix* sp., France.
- *P. ramorum* lineage EU2: EURL ANSES-F 062, ex *Larix* sp., UK.
- *P. ramorum* lineage NA1: EURL ANSES-F 037, host unknown, USA.
- *P. ramorum* lineage NA2: EURL ANSES-F 040, ex *Rhododendron* sp., USA.

4.4. Assessment of performance values

4.4.1. Analytical sensitivity

Standard curves and limit of detection (LOD) were calculated using serial dilutions of the target DNA for each of the four assays. Plasmidic DNA (pDNA) serial dilutions were prepared for each *P. ramorum* lineage, in a background of tris-EDTA buffer (TE, 1x) on the one hand, and in a background of DNA extracted from healthy Rhododendron leaves at 1 ng μL^{-1} (1:1 ratio) on the other hand. The final concentrations of the target plasmidic DNA were as follows (in plasmidic copies [pc] per µL):

- *P. ramorum* lineage EU1: 2.5 $x10^6$ pc μL^{-1} to 2.5 $x10^{-1}$ pc μL^{-1}.
- *P. ramorum* lineage EU2: 2.4 $x10^6$ pc μL^{-1} to 2.4 $x10^{-1}$ pc μL^{-1}.
- *P. ramorum* lineage NA1: 2.3 $x10^6$ pc μL^{-1} to 2.3 $x10^{-1}$ pc μL^{-1}.
- *P. ramorum* lineage NA2: 2.4 $x10^6$ pc μL^{-1} to 2.4 $x10^{-1}$ pc μL^{-1}.

4.4.2. Inclusivity and specificity

A panel of genomic DNAs extracted from 57 *Phytophthora ramorum* strains from different countries, hosts and mating types was tested using the four real-time PCR assays to determine their inclusivity, i.e. their ability to detect all the strains or variants of the target lineage of *P. ramorum*. Other 17 strains representing eight different non-target species of *Phytophthora* spp. were added to the panel.

The sample panel also included strains of the new Asian *P. ramorum* lineages described by Jung *et al*. (2021), which are believed to represent parental lines of the NA and EU lineages. There were five lineages originating from Vietnam (IC1 - IC5) and three lineages originating from Japan (NP1 - NP3). So far, there are no lineage-specific detection methods for these Asian lines, and it was not known whether any of the existing methods were able to detect them.

Genomic DNA was standardized at a working concentration of 1 ng μL^{-1} for each of the *P. ramorum* strains. In addition, the four real-time PCRs assays were performed using non-standardized DNA concentration obtained from 57 strains of *P. ramorum* (i.e. undiluted solution of DNA obtained from the extraction). The reason for using a high concentration of DNA was to assess the performance of the tests when concentrated DNA is used as template. Two replicates per sample were amplified in each assay.

Lastly, the four real-time PCR assays were also run using a matrix of DNA extracted from healthy *Rhododendron* sp. leaves (0.1 ng μL^{-1}) and artificially spiked with 1 ng μL^{-1} of genomic DNA extracted from *Phytophthora* spp. (*P. ramorum* n=57, other *Phytophthora* species n=17).

4.4.3. Assessment of the ability of the four assays to be used in other labs.

The **repeatability** (intra-assay variation) of each lineage-specific real-time PCR assay was evaluated in a single run with 10 replicates of different plasmid DNA concentrations set at 10x LOD, 100x LOD, 1000x LOD. A 1 ng μL^{-1} gDNA solution of the target lineage of *P. ramorum* was diluted in a background of DNA of Rhododendron sp. Moreover, 1 ng μL^{-1} DNA extracts from non-target species (the genetically related *P. hibernalis*, *P. foliorum*, and *P. lateralis*) were included.

The **reproducibility** (inter-assay variation) was not assessed *per se* during this study. Robustness and transferability experiments actually included several runs on different real-time PCR equipment, performed by different operators each time. This allowed the assessment of the reproducibility of the assays.

To examine the **robustness** of each lineage-specific real-time PCR assay, i.e., its ability to withstand experimental variations without compromising sensitivity and specificity, several reaction parameters were deliberately modified. To verify the effect on sensitivity, the robustness of the four real-time PCR assays was challenged with a variation in the final PCR reaction volume (10 or 30 µL) and slight variations in the hybridization temperature (58°C and 62 °C). The assays were carried out with 10 replicates of different plasmid DNA concentrations set at 10x LOD, 100x LOD, as well as two 1 ng μL^{-1} gDNA solutions of the target lineage of *P. ramorum* diluted in Rhododendron DNA.

We also examined the **transferability** of each lineage-specific real-time PCR assay, i.e. its ability to withstand reagents or thermocycler changes without compromising sensitivity and specificity. Two

commercial real-time PCR master mixes (Master Mix Eurogentec and Takara Premix ExTaq (probe qPCR) were compared to the reference Core Kit no ROX (Eurogentec), and two types of equipment were also used (Qiagen Rotor-Gene and Lightcycler 480, Roche). Sensitivity was compared using 10 replicates of different pDNA concentrations set at 10x LOD, 100x LOD, as well as two 1 ng μL^{-1} solution of gDNA from one strain of the target lineage of *P. ramorum*, diluted in Rhododendron DNA. Genomic DNA extracts (1 ng μL^{-1}) from non-target species (closely related *P. hibernalis*, *P. foliorum*, and *P. lateralis*) were also included in the experiments to assess the effects of using different reagents on the specificity of the assay. The change of critical reagents, as well as qPCR platforms may have effect on either sensitivity or specificity, or both (Ioos *et al*. 2019).

4.4.4. Test of spiked plant sample DNAs

To test the specificity of each assay when using infected host tissue, DNA extracts from healthy Rhododendron leaves were spiked individually with DNA from strains belonging to the different lineages of *P. ramorum*. The use of artificially inoculated Rhododendron leaves was not an option in this study, because neither the pathogenicity of the Asian lineages nor the pathogenicity of closely related species is known on *Rhododendron* spp.

For the sake of homogeneity of the experimental set up, DNA from healthy Rhododendron leaves at 1 ng μL^{-1} was spiked with 0.1 ng μL^{-1} DNA extracts of European, North American, and Asian lineages of *P. ramorum* and other *Phytophthora* species such as *P.* × *cambivora, P. lateralis, P. foliorum, P. hibernalis, P. pseudosyringae, P. gonapodyides,* and *P kernoviae*.

5. Results

5.1. Analytical sensitivity of each lineage-specific assay

The standard curve for each assay was built with three replicates for each target concentration level. The limit of detection (LOD) was then more precisely determined as the lowest concentration yielding 100% of positive results out of ten replicates. Table 6 shows the LOD assessed for each assay, as well as the parameters of the corresponding standard curve (Figure 1) in accordance with the diluent.

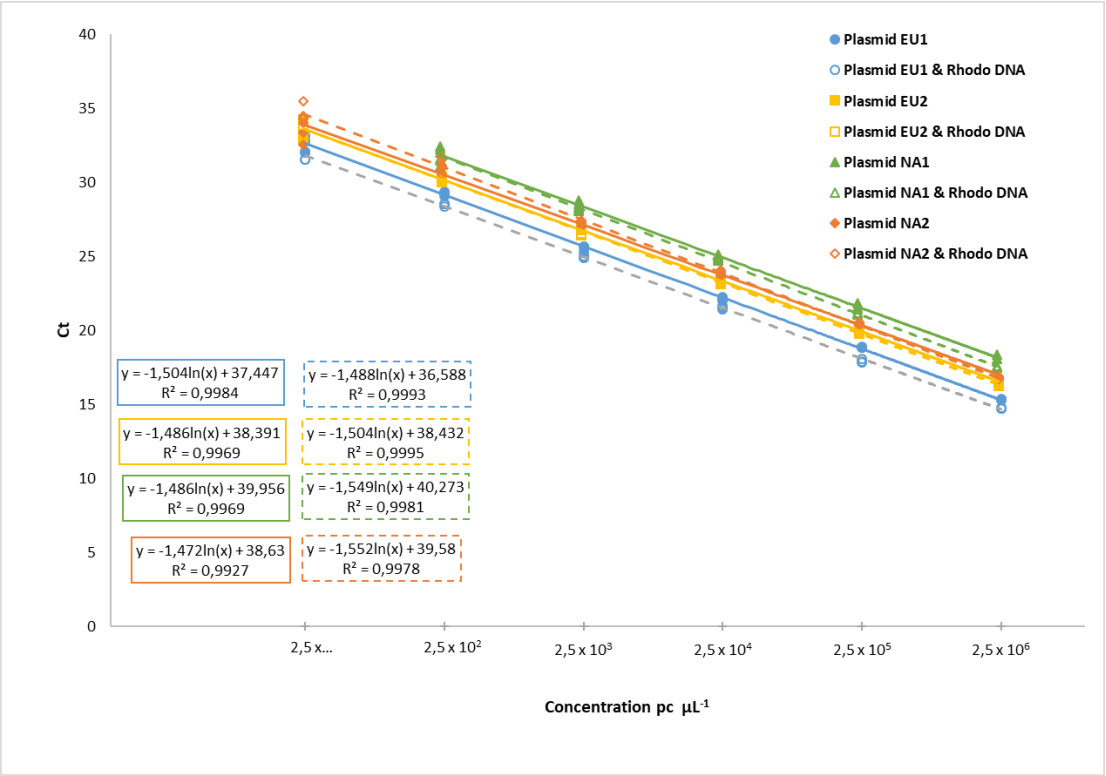

Figure 1: Standard curves for EU1, EU2, NA1 and NA2 lineage-specific assay using serial dilutions of plasmidic DNA, and two types of diluent.

Table 6: Limit of Detection and standard curve parameters for EU1, EU2, NA1 and NA2 lineage-specific assay using serial dilutions of plasmidic DNA, and two types of diluent

	P. ramorum EU1		*P. ramorum* EU2		*P. ramorum* NA1		*P. ramorum* NA2	
	pDNA 1x TE	pDNA + Rhodo	pDNA 1x TE	pDNA + Rhodo	pDNA 1x TE	pDNA + Rhodo	pDNA 1x TE	pDNA + Rhodo
LOD (cp/µL)	2.5×10^1	2.5×10^1	2.41×10^1	2.41×10^1	2.32×10^2	2.32×10^2	2.41×10^1	2.41×10^1
$Ct_{LOD\ 10\ reps}$ ± $SD_{10\ reps}$	33.50 ± 1.05	32.35 ± 0.53	34.05 ± 0.81	34.23 ± 1.37	33.28 ± 0.51	31.79 ± 0.29	34.06 ± 0.64	35.18 ± 1.78
R^2	0.996	0.982	0.997	0.991	0.997	0.987	0.993	0.978
E (%)	97.95	108.28	98.73	103.33	98.04	94.78	107.31	104.74

Results shows that the level of sensitivity is the same for all four assays. Positive results were consistently obtained with as little as 23-25 plasmidic copies per reaction tube. There was no observable effect of the diluent or background (either elution buffer or Rhododendron DNA) on the standard curve parameter whatsoever.

5.2. Inclusivity and specificity of each lineage-specific assay

Table 7 shows the results of each real-time PCR assay using DNA from different strains of *Phytophthora ramorum*, as well as DNA from non-target *Phytophthora* species. In addition, the experiment provided information about the specificity of each assay, and its ability not to cross-react with non-target lineages.

Table 7. Results of the specificity and inclusivity of the real-time PCR assays developed by Feau *et al.* (2019) for the detection of *Phytophthora ramorum* lineages on a panel of genomic DNA of *P. ramorum* and *Phytophthora* spp. adjusted to 1 ng µL^{-1} of DNA. Each DNA sample was tested in duplicate. "N": no amplification. Green figures are for the expected results, red figures are for cross-reactions.

Species	Lineage	Strain code	Ct values ± SD			
			qPCR EU1	qPCR EU2	qPCR NA1	qPCR NA2
Phytophthora ramorum	EU1	**EURL ANSES-F044**	22.75 ± 0.08	N	N	N
		EURL ANSES-F045	22.49 ± 0.01	N	N	N
		EURL ANSES-F046	22.28 ± 0.01	N	N	N
		EURL ANSES-F047	22.61 ± 0.03	N	N	N
		EURL ANSES-F048	22.51 ± 0.13	N	N	N
		EURL ANSES-F049	22.73 ± 0.57	N	N	N
		EURL ANSES-F050	22.96 ± 0.09	N	N	N
		EURL ANSES-F051	22.62 ± 0.09	N	N	N
		EURL ANSES-F052	23.33 ± 0.62	N	N	N
		03-0107	23.03 ± 0.23	N	N	N
		EURL ANSES-F060	22.37 ± 0.25	N	N	N
		EURL ANSES-F061	21.60 ± 0.02	N	N	N
		EURL ANSES-F064	22.05 ± 0.06	N	N	N
	EU2	**EURL ANSES-F062**	N	21.93 ± 0.13	N	N
		EURL ANSES-F063	N	21.97 ± 0.00	N	N
		P2111	N	24.83 ± 0.36	N	N
		P2460	N	**31.67 ± 0.53**	N	N
		P2566	N	22.32 ± 0.15	N	N
		P2586	N	23.06 ± 0.03	N	N
		S52817	N	22.00 ± 0.31	N	N
	NA1	**P4995**	N	N	21.80 ± 0.42	N
		P5010	N	N	21.55 ± 0.06	N
		PR-09-167	N	N	21.48 ± 0.23	N
		P5009	N	N	21.43 ± 0.07	N
		PR-09-175	N	N	21.61 ± 0.05	N
		PR-11-010	N	N	21.15 ± 0.04	N
		PR-11-001	N	N	21.31 ± 0.13	N
		EURL ANSES-F037	N	N	22.55 ± 0.02	N
		EURL ANSES-F038	N	N	21.80 ± 0.10	N
		EURL ANSES-F039	N	N	21.65 ± 0.31	N
	NA2	**PR-05-16845**	N	N	N	21.38 ± 0.11
		PR-10-4389a	N	N	N	22.59 ± 0.02
		PR-04-38813	N	N	N	21.19 ± 0.09
		PR-06-0012	N	N	N	21.39 ± 0.10
		PR-06-4942	N	N	N	21.88 ± 0.15
		PR-04-20470	N	N	N	21.06 ± 0.21
		EURL ANSES-F040	N	N	N	21.47 ± 0.30
		EURL ANSES-F041	N	N	N	21.95 ± 0.17
		EURL ANSES-F042	N	N	N	21.99 ± 0.07
Phytophthora lateralis	-	**EURL ANSES-F053**	N	N	N	N
	-	**EURL ANSES-F054**	N	N	N	N
	-	**EURL ANSES-F055**	N	N	N	N
	-	**EURL ANSES-F056**	N	N	N	N
	-	**EURL ANSES-F057**	N	N	N	N
	-	**EURL ANSES-F058**	N	N	N	N
	-	**EURL ANSES-F059**	N	N	N	N
Phytophthora foliorum	-	**CBS 121655**	N	N	N	N
Phytophthora hibernalis	-	**CBS 270.31**	N	N	N	N
Phytophthora syringae	-	**PH14-227**	N	N	N	N
Phytophthora pseudosyringae	-	**PR12W-033**	N	N	N	N
Phytophthora gonapodyides	-	**PR13-377**	N	N	N	N
Phytophthora × cambivora	-	**PR13-379**	N	N	31.34 ± 0.18	N
Phytophthora kernoviae	-	**PF12-106**	N	N	N	N
	-	**PR12-513**	N	N	N	N
	-	**PR12-518**	N	N	N	N
	-	**PR12-524**	N	N	N	N
Phytophthora ramorum	IC1	**VN57**	N	22.16 ± 0.23	19.55 ± 0.10	N
		VN313	N	22.04 ± 0.21	19.66 ± 0.13	N
		VN831	N	23.10 ± 0.02	20.41 ± 0.14	N
		VN1015	N	21.80 ± 0.17	19.84 ± 0.09	N
	IC2	**VN142**	N	21.03 ± 0.13	19.63 ± 0.10	N
		VN150	N	21.08 ± 0.10	19.51 ± 0.04	N
		VN169	N	20.96 ± 0.13	19.65 ± 0.29	N
		VN314	N	21.16 ± 0.20	19.88 ± 0.04	N
	IC3	**VN88**	N	22.12 ± 0.08	19.74 ± 0.26	N
	IC4	**VN851b**	N	21.37 ± 0.13	21.22 ± 0.17	20.48 ± 0.02
		VN863	N	22.30 ± 0.02	19.58 ± 0.07	19.88 ± 0.05
	NP1	**JP236**	N	N	N	N
		JP716	N	N	N	N
		JP916	N	N	N	N
		JP1202	N	N	N	N
	NP2	**JP387**	21.54 ± 0.06	N	20.12 ± 0.16	N
		JP462	21.25 ± 0.04	N	20.38 ± 0.01	N
	NP3	**JP975**	21.27 ± 0.22	N	19.53 ± 0.03	N

Outcome:

Each of the four lineage-specific real-time assays of Feau *et al*. (2019) proved to be fully inclusive. DNA from all the strains of each lineage yielded positive results with the respective primers/probe combination, regardless of origin or host. With the exception of one strain of *P.* × *cambivora* from Ireland, which DNA consistently yielded an unexpected late Ct value; no other *Phytophthora* species produced positive results.

However, with the notable exception of NP1 lineage from Japan, cross-reaction with DNA of all the other Asian lineages of *P. ramorum* was observed for all four assays. These results indicate that further detection protocols targeting Asian populations should be developed.

Table 8 shows the specificity of each *P. ramorum* lineage-specific assay when a high concentration of DNA from a non-target lineage is used. As previously mentioned, high concentrations of DNA of non-target taxon may affect the specificity of the assay. Results show that even in these more challenging conditions, the level specificity of all four assays was the same as the one observed with diluted DNA templates (1 ng μL^{-1}).

Table 8. Specificity and inclusivity assessment of the real-time PCR assays developed by Feau *et al.* (2019) for the detection of *Phytophthora ramorum* lineages. A panel of genomic DNA of *P. ramorum* prepared at a high DNA concentration was used. Each DNA sample was assessed in duplicate. « * »: only 1 replicate/2 amplified, « n.a. »: not assessed, "N": no amplification. Red figures are for cross-reactions.

Species	Code	DNA Concentration (ng/µL)	*P. ramorum* qPCR (Ct_{moyen} ± SD)			
			EU1	EU2	NA1	NA2
***P. ramorum* EU1**	EURL ANSES-F 044	5.10	n.a.	N	N	N
	EURL ANSES-F 045	7.00	n.a.	N	N	N
	EURL ANSES-F 046	8.40	n.a.	N	N	N
	EURL ANSES-F 047	11.10	n.a.	N	N	N
	EURL ANSES-F 048	15.50	n.a.	N	N	N
	EURL ANSES-F 049	11.80	n.a.	N	N	N
	EURL ANSES-F 050	5.50	n.a.	N	N	N
	EURL ANSES-F 051	19.70	n.a.	N	N	N
	EURL ANSES-F 052	12.70	n.a.	N	N	N
	03-0107	12.80	n.a.	N	N	N
	EURL ANSES-F 060	21.80	n.a.	N	N	N
	EURL ANSES-F 061	23.50	n.a.	N	N	N
	EURL ANSES-F 064	11.40	n.a.	N	N	N
***P. ramorum* EU2**	EURL ANSES-F 062	27.00	N	n.a.	N	N
	EURL ANSES-F 063	25.50	N	n.a.	N	N
	P2111	3.70	N	n.a.	N	N
	P2460	12.20	N	n.a.	N	N
	P2566	12.40	N	n.a.	N	N
	P2586	4.90	N	n.a.	N	N
	S52817	14.00	N	n.a.	N	N
***P. ramorum* NA1**	P4995	7.20	N	N	n.a.	N
	P5010	13.70	36.50*	N	n.a.	N
	PR-09-167	17.60	N	N	n.a.	N
	P5009	66.30	N	N	n.a.	N
	PR-09-175	5.70	N	N	n.a.	N
	PR-11-010	12.50	N	N	n.a.	N
	PR-11-001	8.60	N	N	n.a.	N
	EURL ANSES-F 037	24.90	N	N	n.a.	N
	EURL ANSES-F 038	5.40	N	N	n.a.	N
	EURL ANSES-F 039	14.60	N	N	n.a.	N
***P. ramorum* NA2**	PR-05-16845	74.50	N	N	N	n.a.
	PR-10-4389a	30.30	N	N	N	n.a.
	PR-04-38813	58.90	N	N	N	n.a.
	PR-06-0012	48.00	N	N	N	n.a.
	PR-06-4942	52.20	N	N	N	n.a.
	PR-04-20470	60.30	N	N	N	n.a.
	EURL ANSES-F 040	39.40	N	N	N	n.a.
	EURL ANSES-F 041	31.90	N	N	N	n.a.
	EURL ANSES-F 042	25.60	N	N	N	n.a.
***P. ramorum* IC1**	VN57	40.20	N	19.40 ± 0.11	15.19 ± 0.08	N
	VN313	46.10	N	17.53 ± 0.05	14.11 ± 0.12	N
	VN831	88.00	N	27.56 ± 2.26	28.34 ± 0.05	N
	VN1015	36.00	N	18.70 ± 0.20	14.98 ± 0.11	N

***P. ramorum* IC2**	VN142	97.10	N	17.81 ± 0.37	14.36 ± 0.01	N
	VN150	66.10	N	17.21 ± 0.01	14.33 ± 0.07	N
	VN169	37.20	N	17.56 ± 0.02	14.73 ± 0.04	N
	VN314	40.10	N	18.20 ± 0.09	15.42 ± 0.01	N
***P. ramorum* IC3**	VN88	49.70	N	21.44 ± 0.04	18.63 ± 1.20	N
***P. ramorum* IC4**	VN851b	35.50	34.98*	26.59 ± 0.21	30.76 ± 0.94	20.48 ± 0.02
***P. ramorum* IC5**	VN863	40.00	N	21.66 ± 0.40	18.59 ± 0.07	16.52 ± 0.30
***P. ramorum* NP1**	JP236	83.00	N	N	N	N
	JP716	47.80	N	N	N	N
	JP916	35.90	N	N	N	N
	JP1202	58.80	N	N	N	N
***P. ramorum* NP2**	JP387	60.20	15.30 ± 0.07	N	N	N
	JP462	38.40	17.18 ± 0.09	N	N	N
***P. ramorum* NP3**	JP975	72.50	21.25 ± 0.07	N	N	N

The last experiment to assess the specificity of the lineage-specific assays was done with DNA extracts mimicking infected plant samples. The results presented in Table 9 are in line with those observed for DNA from pure cultures.

Table 9: Specificity and inclusivity assessment of the real-time PCR methods developed by Feau *et al.* (2019) for the detection of *Phytophthora ramorum* lineages. A panel of genomic DNA from *Phytophthora* spp. mixed with DNA from Rhododendron leaves was used. Each DNA sample was assessed in duplicate. « * »: only 1 replicate/2 amplified, "N": no amplification. Green figures are for expected results, red figures are for cross-reactions.

Species	Code	*P. ramorum* qPCR (Ct ± SD)			
		EU1	EU2	NA1	NA2
***P. ramorum* EU1**	EURL ANSES-F 044	25.44 ± 0.07	N	N	N
	EURL ANSES-F 045	25.13 ± 0.08	N	N	N
	EURL ANSES-F 046	25.34 ± 0.08	N	N	N
	EURL ANSES-F 047	25.34 ± 0.05	N	N	N
	EURL ANSES-F 048	25.24 ± 0.10	N	N	N
	EURL ANSES-F 049	25.32 ± 0.09	N	N	N
	EURL ANSES-F 050	25.54 ± 0.02	N	N	N
	EURL ANSES-F 051	25.28 ± 0.08	N	N	N
	EURL ANSES-F 052	25.52 ± 0.03	N	N	N
	03-0107	25.36 ± 0.07	N	N	N
	EURL ANSES-F 060	24.98 ± 0.10	N	N	N
	EURL ANSES-F 061	24.64 ± 0.08	N	N	N
	EURL ANSES-F 064	24.93 ± 0.06	N	N	N
***P. ramorum* EU2**	EURL ANSES-F 062	N	24.41 ± 0.18	N	N
	EURL ANSES-F 063	N	25.39 ± 0.05	N	N
	P2111	N	26.61 ± 0.03	N	N
	P2460	N	34.33 ± 0.82	N	N
	P2566	N	25.68 ± 0.11	N	N
	P2586	N	26.65 ± 0.33	N	N
	S52817	N	25.56 ± 0.18	N	N
***P. ramorum* NA1**	P4995	N	N	25.04 ± 0.01	N
	P5010	N	N	25.34 ± 0.21	N
	PR-09-167	N	N	26.68 ± 0.80	N
	P5009	N	N	29.98 ± 3.18	N
	PR-09-175	N	N	25.46 ± 0.02	N
	PR-11-010	N	N	24.87 ± 0.10	N
	PR-11-001	N	N	25.10 ± 0.01	N
	EURL ANSES-F 037	N	N	26.22 ± 0.09	N
	EURL ANSES-F 038	N	N	25.56 ± 0.12	N
	EURL ANSES-F 039	N	N	25.53 ± 0.23	N
***P. ramorum* NA2**	PR-05-16845	N	N	N	24.85 ± 0.02
	PR-10-4389a	N	N	N	26.47 ± 0.11
	PR-04-38813	N	N	N	25.30 ± 0.06
	PR-06-0012	N	N	N	25.32 ± 0.15
	PR-06-4942	N	N	N	25.98 ± 0.06
	PR-04-20470	N	N	N	25.49 ± 0.05
	EURL ANSES-F 040	N	N	N	25.46 ± 0.15
	EURL ANSES-F 041	N	N	N	25.73 ± 0.27
	EURL ANSES-F 042	N	N	N	26.10 ± 0.02
***P. ramorum* IC1**	VN57	N	26.16 ± 0.20	23.53 ± 0.13	N
	VN313	N	25.69 ± 0.10	23.34 ± 0.09	N
	VN831	N	27.00 ± 0.30	24.35 ± 0.09	N
	VN1015	N	25.84 ± 0.23	23.29 ± 0.06	N
***P. ramorum* IC2**	VN142	N	24.83 ± 0.09	23.76 ± 0.16	N
	VN150	N	24.62 ± 0.13	23.72 ± 0.29	N
	VN169	N	24.83 ± 0.11	23.38 ± 0.18	N
	VN314	N	24.61 ± 0.24	23.55 ± 0.06	N
***P. ramorum* IC3**	VN88	N	25.66 ± 0.07	23.45 ± 0.14	N
***P. ramorum* IC4**	VN851b	N	24.51 ± 0.04	24.97 ± 0.39	24.46 ± 0.48
***P. ramorum* IC5**	VN863	N	25.64 ± 0.43	23.53 ± 0.20	23.97 ± 0.20
***P. ramorum* NP1**	JP236	N	N	N	N
	JP716	N	N	N	N
	JP916	N	N	N	N
	JP1202	N	N	N	N
***P. ramorum* NP2**	JP387	24.93 ± 0.05	N	24.26 ± 0.30	N
	JP462	24.86 ± 0.11	N	24.27 ± 0.07	N
***P. ramorum* NP3**	JP975	24.64 ± 0.02	N	23.48 ± 0.01	N
Phytophthora lateralis	EURL ANSES-F 053	N	N	N	N
	EURL ANSES-F 054	N	N	N	N

	EURL ANSES-F 055	N	N	N	N
	EURL ANSES-F 056	N	N	N	N
	EURL ANSES-F 057	N	N	N	N
	EURL ANSES-F 058	N	N	N	N
	EURL ANSES-F 059	N	N	N	N
Phytophthora foliorum	CBS 121655	N	N	N	N
Phytophthora hibernalis	CBS 270.31	N	N	N	N
Phytophthora syringae	PH14-227	N	N	N	N
Phytophthora pseudosyringae	PR12W-033	N	N	N	N
Phytophthora gonapodyides	PR13-377	N	N	N	N
Phytophthora × cambivora	PR13-379	N	N	34.01 ± 0.07[a]	N
Phytophthora kernoviae	PF12-106	N	N	N	N
	PF12-513	N	N	N	N
	PF12-518	N	N	N	N
	PF12-524	N	N	N	N

5.3. Repeatability of the lineage-specific assays.

Tables 10 shows the results of repeatability for each of the four lineage-specific assays. It illustrates the high level of repeatability of the assays. Low coefficients of variation and the absence of cross-reaction with closely related species confirm that the sensitivity and the specificity are not affected by repeating the tests.

Table 10: Assessment of the repeatability of the four lineage-specific assays of Feau *et al*. (2019)

Assay / concentration	Proportion pos. results	Mean Ct (10 rep.)	SD	C.V. (%)
***P. ramorum* EU1**				
10x EU1 LOD	10/10	29.24	0.20	0.67
100x EU1 LOD	10/10	25.98	0.12	0.46
1000x EU1 LOD	10/10	22.50	0.20	0.88
P. ramorum EU1 (EURL ANSES-F 052) + Rhod. DNA	10/10	25.19	0.14	0.56
P. ramorum EU1 (03-0107) + Rhod. DNA	10/10	25.61	0.15	0.60
P. lateralis	0/10	-	-	-
P. foliorum	0/10	-	-	-
P. hibernalis	0/10	-	-	-
***P. ramorum* EU2**				
10x EU2 LOD	10/10	30.86	0.28	0.90
100x EU2 LOD	10/10	27.37	0.94	3.43
1000x EU2 LOD	10/10	23.69	0.17	0.71
P. ramorum EU2 (EURL ANSES-F 062) + Rhod. DNA	10/10	26.30	0.14	0.53
P. ramorum EU2 (P2111) + Rhod. DNA	10/10	27.52	0.24	0.88
P. lateralis	0/10	-	-	-
P. foliorum	0/10	-	-	-
P. hibernalis	0/10	-	-	-
***P. ramorum* NA1**				
10x NA1 LOD	10/10	28.60	0.12	0.42
100x NA1 LOD	10/10	25.06	0.27	1.07
1000x NA1 LOD	10/10	21.64	0.11	0.50
P. ramorum NA1 (EURL ANSES-F 037) + Rhod. DNA	10/10	26.52	0.25	0.93
P. ramorum NA1 (P4995) + Rhod. DNA	10/10	25.70	0.21	0.80
P. lateralis	0/10	-	-	-
P. foliorum	0/10	-	-	-
P. hibernalis	0/10	-	-	-
***P. ramorum* NA2**				
10x NA2 LOD	10/10	30.80	0.30	0.99
100x NA2 LOD	10/10	27.58	0.13	0.46
1000x NA2 LOD	10/10	24.54	0.22	0.88
P. ramorum NA2 (PR-16845) + Rhod. DNA	10/10	24.58	0.20	0.80
P. ramorum NA2 (EURL-ANSES-F040) + Rhod. DNA	10/10	24.97	0.11	0.46
P. lateralis	0/10	-	-	-
P. foliorum	0/10	-	-	-
P. hibernalis	0/10	-	-	-

5.4. Robustness of the lineage-specific assays.

Table 11 reports the effects of slight modifications of the real-time reaction parameters on each of the four lineage-specific assays developed by Feau *et al*. (2019). It shows that for all four assays, that modifications in either the hybridization temperature or the reaction volume had an effect on the qualitative result (positive/negative). However, significant differences could sometimes be observed on quantitative values (mean Ct), but without obvious pattern (Figures 2a, 2b).

Overall, *P. ramorum* was still detected even at low DNA concentrations even when a higher hybridization temperature was used. In addition, no cross-reaction was observed with DNA from closely related species, even at a lower hybridization temperature (conditions of decreased stringency). Modifying the reaction volume (+/- 100%) had no effect on the sensitivity or specificity of the assays. All these results support the robustness of all four lineage-specific real-time PCR assays developed by Feau *et al*. (2019).

Table 11. Robustness of the four lineage-specific assays. Changes in the sensitivity (Mean Ct value) of the assays were assessed by making slight modifications in the hybridization temperature or the reaction volume.

Assay / concentration	Deviation of reaction volume						Deviation of hybridization temperature					
	10 µL			30 µL			58°C			62°C		
	Mean Ct	SD	C.V.(%)	Mean Ct	SD	C.V.(%)	Mean Ct	SD	C.V.(%)	Mean Ct	SD	C.V.(%)
***P. ramorum* EU1**												
10x EU1 LOD	28.62	0.28	0.97	30.82	0.32	1.02	30.11	0.50	1.66	30.23	0.32	1.06
100x EU1 LOD	25.01	0.27	1.07	27.79	0.13	0.47	27.11	0.22	0.82	26.91	0.15	0.55
P. ramorum EU1 EURL F052 + Rhod. DNA	24.22	0.12	0.48	25.99	0.12	0.45	25.28	0.12	0.48	25.85	0.12	0.46
P. ramorum F03-0107 + Rhod. DNA	24.06	0.04	0.18	25.81	0.04	0.16	25.02	0.06	0.26	25.59	0.12	0.48
***P. ramorum* EU2**												
10x EU2 LOD	29.48	1.09	3.71	30.88	0.41	1.33	30.32	0.24	0.81	30.49	0.37	0.90
100x EU2 LOD	25.71	0.20	0.78	27.39	0.28	1.01	26.88	0.19	0.72	23.37	0.94	3.43
P. *ramorum* EU2 EURL F062 + Rhod. DNA	23.80	0.25	1.04	25.25	0.12	0.47	25.16	0.09	0.35	26.30	0.14	0.53
P. ramorum EU2 P2111 + Rhod. DNA	25.94	0.17	0.66	27.25	0.10	0.37	27.49	0.17	0.26	27.52	0.14	0.53
***P. ramorum* NA1**												
10x NA1 LOD	27.39	0.29	1.07	30.22	0.22	0.74	28.54	0.29	1.01	28.60	0.12	0.42
100x NA1 LOD	23.78	0.58	2.43	26.90	0.18	0.67	25.92	0.98	3.77	25.06	0.27	1.07
P. ramorum NA1 EURL F037 + Rhod. DNA	24.58	0.17	0.68	26.59	0.15	0.56	25.76	0.11	0.44	26.52	0.25	0.93
P. ramorum NA1 P4995 + Rhod. DNA	23/51	0.19	0.80	25.38	0.20	0.79	24.38	0.13	0.53	25.70	0.21	0.80
***P. ramorum* NA2**												
10x NA2 LOD	29.92	0.51	1.72	30.99	0.35	1.13	31.13	0.36	1.16	30.77	0.25	0.81
100x NA2 LOD	26.13	0.61	2.34	28.05	0.19	0.66	27.83	0.23	0.82	27.59	0.13	0.48
P. ramorum NA2 PR05-16845 + Rhod. DNA	24.03	0.35	1.44	24.96	0.13	0.52	24.88	0.24	0.98	24.65	0.17	0.69
P. ramorum EURL F040 + Rhod. DNA	24.21	0.27	1.10	25.06	0.12	0.46	24.70	0.22	0.89	24.70	0.13	0.54

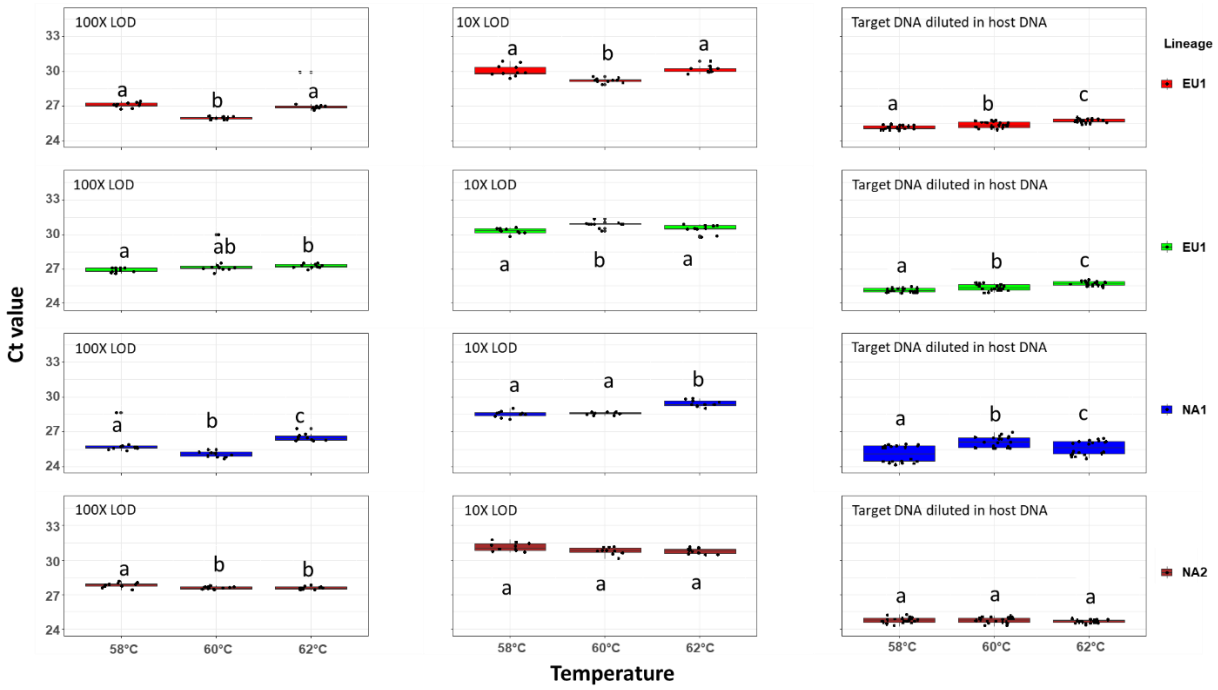

Figure 2a: Effect of temperature on the sensitivity (mean Ct value) for each of the four lineage-specific assays, with target DNA set at 10x LOD, 100x LOD or with gDNA. Effect of the temperature was assessed by Kruskal-Wallis test by ranks. Pairwise comparison of means were done using Wilcoxon rank sum exact test.

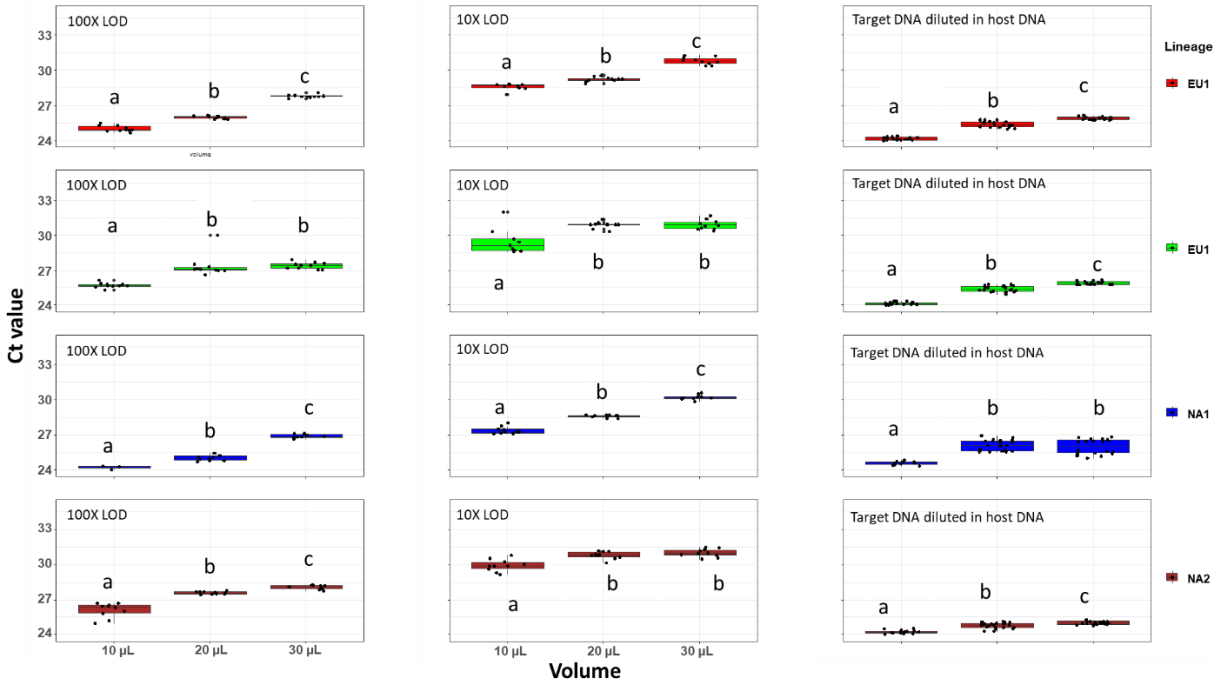

Figure 2b: Effect of the real-time PCR reaction volume on the sensitivity (mean Ct value) for each of the four lineage-specific assays, with target DNA set at 10x LOD, 100x LOD or with gDNA. Effect of the volume was assessed by Kruskal-Wallis test by ranks. Pairwise comparisons of means were done using Wilcoxon rank sum exact test.

5.5. Transferability of the lineage-specific assays

Table 12 illustrates the effects of change of critical reagents such as the type of commercial real-time PCR master mix on the mean Ct value, and for each of the assays. From a qualitative point of view, changing the master mix did not show any effect on the sensitivity or specificity of the assays. No cross reaction was observed with DNA from closely related *Phytophthora* species, and each target could be detected, even at very low concentrations, close to the limit of detection. However, significant differences between the different master mixes were observed for each of the lineage-specific assay, but without consistent pattern (Figure 3).

Table 12. Assessment of the change of qPCR master mix on the sensitivity and specificity of each of the lineage-specific assays of Feau *et al*. (2019)

Real-time PCR master mix	**Core kit no ROX (Eurogentec)**			**qPCR master mix (Eurogentec)**			**Premix ExTaq (Takara)**		
Assay / concentration	Mean Ct	SD	C.V. (%)	Mean Ct	SD	C.V. (%)	Mean Ct	SD	C.V. (%)
***P. ramorum* EU1**									
10x EU1 LOD	29.24	0.20	0.67	30.07	0.31	1.04	31.42	0.25	0.80
100x EU1 LOD	25.98	0.12	0.46	27.05	0.09	0.32	28.61	0.06	0.19
P. ramorum EU1 EURL F052 + rhod. DNA	25.19	0.14	0.56	26.25	0.19	0.72	29.12	0.23	0.79
P. ramorum F03-0107 = rhod. DNA	25.61	0.15	0.60	26.57	0.11	0.40	25.61	0.15	0.60
P. lateralis	-	-	-	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-	-	-	-
***P. ramorum* EU2**									
10x EU2 LOD	30.86	0.28	0.90	30.51	0.50	1.65	30.92	0.32	1.04
100x EU2 LOD	27.37	0.94	3.43	27.04	0.27	0.99	27.94	0.22	0.77
P. *ramorum* EU2 EURL F062 + rhod. DNA	26.30	0.14	0.53	26.66	0.22	0.83	26.85	0.21	0.77
P. ramorum EU2 P2111 + rhod. DNA	27.52	0.24	0.88	28.20	0.22	0.8	28.25	0.23	0.81
P. lateralis	-	-	-	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-	-	-	-
***P. ramorum* NA1**									
10x NA1 LOD	28.60	0.12	0.42	30.11	0.40	1.33	31.07	0.24	0.76
100x NA1 LOD	25.06	0.27	1.07	27.01	0.22	0.81	27.84	0.16	0.58
P. ramorum NA1 EURL F037 + rhod. DNA	26.52	0.25	0.93	27.73	0.35	1.25	27.74	0.19	0.69
P. ramorum NA1 P4995 + rhod. DNA	25.70	0.21	0.80	26.74	0.17	0.64	26.83	0.16	0.61
P. lateralis	-	-	-	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-	-	-	-
***P. ramorum* NA2**									
10x NA2 LOD	30.80	0.30	0.99	31.93	0.42	1.32	31.08	0.22	0.70
100x NA2 LOD	27.58	0.13	0.49	28.75	0.24	0.82	28.00	0.20	0.73
P. ramorum NA2 PR05-16845 + rhod. DNA	24.59	0.20	0.80	26.17	0.22	0.85	26.39	0.14	0.51
P. ramorum EURL F040 + rhod. DNA	24.97	0.11	0.46	26.26	0.14	0.54	26.49	0.22	0.85
P. lateralis	-	-	-	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-	-	-	-

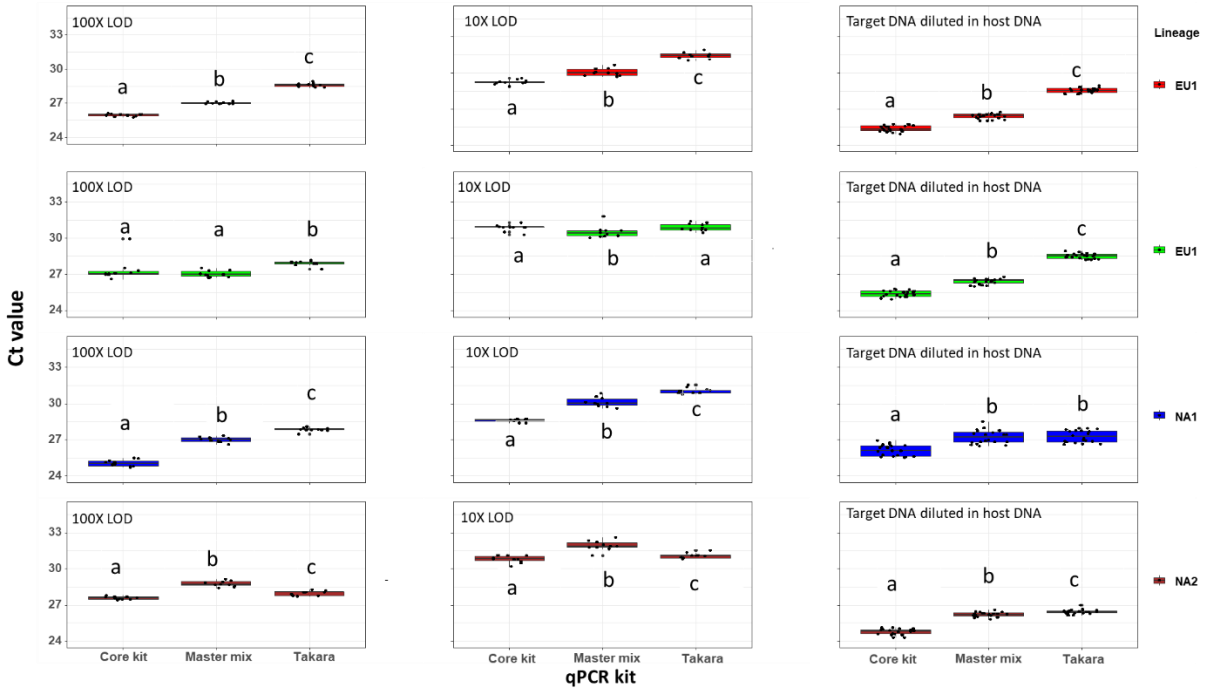

Figure 3: Effect of the change of qPCR master mix on the sensitivity of each lineage-specific assay (mean Ct value) with target DNA set at 10x LOD, 100x LOD or with gDNA. Effect of the qPCR kit was assessed by Kruskal-Wallis test by ranks. Pairwise comparisons of means were done using Wilcoxon rank sum exact test.

Table 13 summarizes the data generated with the panel of DNA for each lineage-specific assay, this time using two different qPCR platforms (block or rotor format). Although no qualitative effect on sensitivity or specificity was observed for EU1 and NA2 specific assays, a significant decrease of sensitivity generated false-negative results for EU2 and NA1 assays, when the level of the respective target was low, i.e. close to the Limit of Detection (10 and 100 times LOD) (Figure 4). This problem of sensitivity was also observed with EU1 and NA2 assays, with a consistent drift in mean Ct value (around four additional cycles) when switching from the Qiagen Rotor Gene Q (spinning rotor in a heating/cooling chamber) to the Roche Lightcycler (thermal block) platform.

Table 13. Assessment of the change of thermocycler platform on the sensitivity and specificity of each of the lineage-specific assay of Feau *et al*. (2019)

qPCR platform	**Qiagen RotorGene Q**			**Roche Lightcycler 480**		
Assay / concentration	Mean Ct	SD	C.V. (%)	Mean Ct	SD	C.V. (%)
***P. ramorum* EU1**						
10x EU1 LOD	29.24	0.20	0.67	33.85	0.70	2.06
100x EU1 LOD	25.98	0.12	0.46	30.86	0.06	0.19
P. ramorum EU1 EURL F052 + rhod. DNA	25.19	0.14	0.56	29.12	0.23	0.79
P. ramorum F03-0107 = rhod. DNA	25.61	0.15	0.60	29.48	0.16	0.54
P. lateralis	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-
***P. ramorum* EU2**						
10x EU2 LOD	30.86	0.28	0.90	No amplif.	n.a.	n.a.
100x EU2 LOD	27.37	0.94	3.43	No amplif.	n.a.	n.a.
P. *ramorum* EU2 EURL F062 + rhod. DNA	26.30	0.14	0.53	29.19*	0.07	0.22

P. ramorum EU2 P2111 + rhod. DNA	27.52	0.24	0.88	30.46**	0.20	0.65
P. lateralis	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-
***P. ramorum* NA1**						
10x NA1 LOD	28.60	0.12	0.42	No amplif.	n.a.	n.a.
100x NA1 LOD	25.06	0.27	1.07	No amplif.	n.a.	n.a.
P. ramorum NA1 EURL F037 + rhod. DNA	26.52	0.25	0.93	30.11*	0.12	0.40
P. ramorum NA1 P4995 + rhod. DNA	25.70	0.21	0.80		0.11	0.38
P. lateralis	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-
***P. ramorum* NA2**						
10x NA2 LOD	30.80	0.30	0.99	34.50	0.56	1.64
100x NA2 LOD	27.58	0.13	0.46	30.61	0.20	0.66
P. ramorum NA2 PR05-16845 + rhod. DNA	24.59	0.20	0.80	27.99	0.07	0.26
P. ramorum EURL F040 + rhod. DNA	24.97	0.11	0.46	28.03	0.07	0.24
P. lateralis	-	-	-	-	-	-
P. foliorum	-	-	-	-	-	-
P. hibernalis	-	-	-	-	-	-

* only 5/10 replicates amplified
** only 9/10 replicates amplified

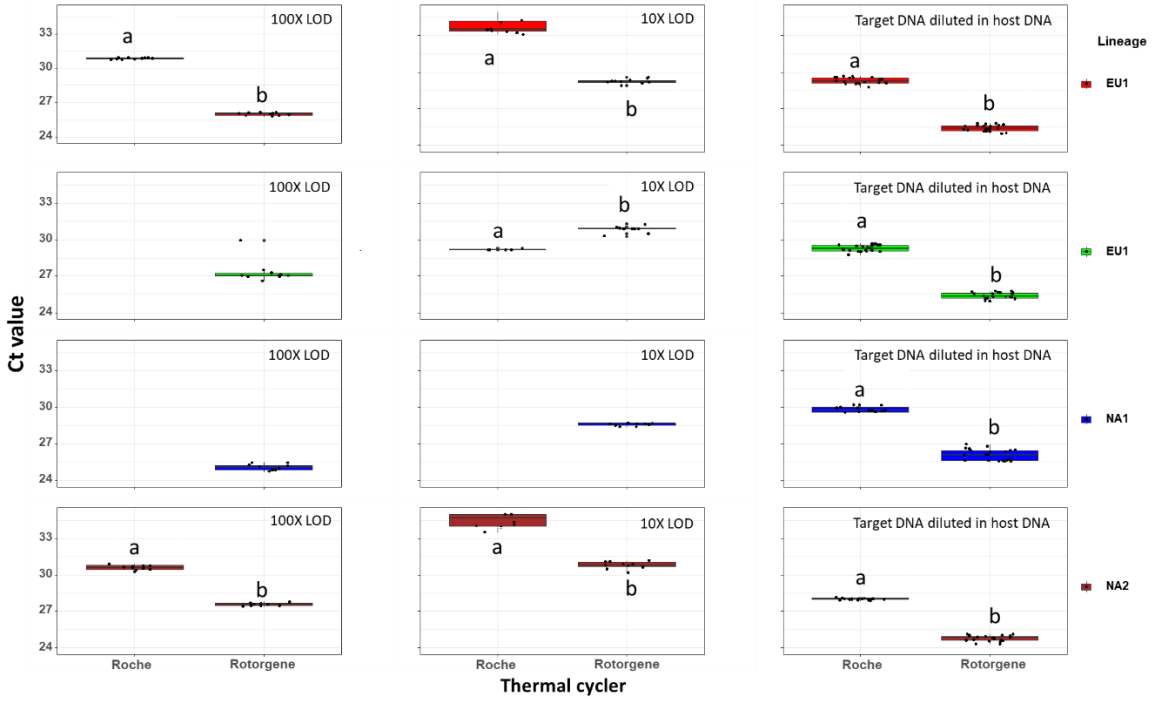

Figure 4: Effect of the change of qPCR platform (Roche=thermal block, RotorGene= spinning rotor) on the sensitivity of each lineage-specific assay (mean Ct value) with target DNA set at 10x LOD, 100x LOD or with gDNA. Effect of the thermal cycler was assessed Wilcoxon rank sum exact tests. At 10x LOD and 100x LOD, absence of box plot means that no Ct values could be generated.

5.6. Test of artificially prepared plant / *Phytophthora* DNA extracts

Table 14 illustrates the results of each lineage-specific assay developed by Feau et al (2019), in the presence of plant DNA extract (Rhododendron leaves). The results are in line with the assessment of the specificity using pure *Phytophthora* DNA extracts (See §5.2).

Each of the four lineage-specific real-time assays developed by Feau *et al*. (2019) proved to be fully inclusive since DNA from all the strains of each lineage yielded positive results with the respective primers/probe combination, regardless of the origin or host, even in the presence of plant DNA. With the exception of one strain of *P.* × *cambivora* from Ireland whose DNA consistently yielded an unexpected late Ct value. No other *Phytophthora* species produced positive results.

However, with the notable exception of NP1 lineage from Japan, cross-reaction with DNA of all the other Asian lineages of *P. ramorum* was observed in all European and North-American lineage-specific assays.

Table 14. Results of the real-time PCR method developed by Feau *et al.* (2019) for the detection of *Phytophthora ramorum* lineages on a panel of genomic DNA of *P. ramorum* and other *Phytophthora* spp. mixed with DNA of healthy leaves of Rhododendron. Amplification of the 18S unit test to check the DNA quality and the absence of PCR inhibitors in the DNA samples. "N": no amplification. "*": amplification of only one out of the two replicates per sample; "[a]": sample considered negative as the mean Ct value obtained is higher than the threshold Ct. Green figures are for expected results, red figures are for cross-reactions.

Species	Lineage	Strain code	Ct values ± SD				
			qPCR EU1	qPCR EU2	qPCR NA1	qPCR NA2	18S Uni test
Phytophthora ramorum	EU1	EURL ANSES-F044	25.44 ± 0.07	N	N	N	11.07 ± 0.05
		EURL ANSES-F045	25.13 ± 0.08	N	N	N	11.05 ± 0.05
		EURL ANSES-F046	25.34 ± 0.08	N	N	N	11.44 ± 0.48
		EURL ANSES-F047	25.35 ± 0.05	N	N	N	11.10 ± 0.05
		EURL ANSES-F048	25.24 ± 0.10	N	N	N	11.10 ± 0.06
		EURL ANSES-F049	25.32 ± 0.09	N	N	N	11.09 ± 0.05
		EURL ANSES-F050	25.54 ± 0.02	N	N	N	11.14 ± 0.04
		EURL ANSES-F051	25.28 ± 0.08	N	N	N	11.01 ± 0.03
		EURL ANSES-F052	25.52 ± 0.03	N	N	N	10.95 ± 0.07
		03-0107	25.36 ± 0.07	N	N	N	10.96 ± 0.13
		EURL ANSES-F060	24.98 ± 0.10	N	N	N	11.11 ± 0.02
		EURL ANSES-F061	24.64 ± 0.08	N	N	N	11.18 ± 0.14
		EURL ANSES-F064	24.93 ± 0.06	N	N	N	11.40 ± 0.48
	EU2	EURL ANSES-F062	N	24.41 ± 0.18	N	N	10.97 ± 0.06
		EURL ANSES-F063	N	25.39 ± 0.05	N	N	11.11 ± 0.01
		P2111	N	26.61 ± 0.03	N	N	11.12 ± 0.05
		P2460	N	34.49*,[a]	N	N	11.12 ± 0.01
		P2566	N	25.68 ± 0.11	N	N	11.14 ± 0.09
		P2586	N	26.65 ± 0.33	N	N	11.15 ± 0.06
		S52817	N	25.56 ± 0.18	N	N	11.16 ± 0.07
	NA1	P4995	N	N	25.04 ± 0.01	N	11.18 ± 0.03
		P5010	N	N	25.34 ± 0.21	N	11.05 ± 0.04
		PR-09-167	N	N	26.68 ± 0.80	N	11.25 ± 0.08
		P5009	N	N	29.98 ± 3.18	N	11.24 ± 0.19
		PR-09-175	N	N	25.46 ± 0.02	N	11.11 ± 0.04
		PR-11-010	N	N	24.87 ± 0.10	N	11.04 ± 0.07
		PR-11-001	N	N	25.10 ± 0.01	N	11.08 ± 0.11
		EURL ANSES-F037	N	N	26.22 ± 0.09	N	11.13 ± 0.00
		EURL ANSES-F038	N	N	25.56 ± 0.12	N	11.00 ± 0.07
		EURL ANSES-F039	N	N	25.53 ± 0.23	N	11.16 ± 0.04
	NA2	PR-05-16845	N	N	N	24.85 ± 0.02	11.10 ± 0.04
		PR-10-4389a	N	N	N	26.47 ± 0.11	11.47 ± 0.42
		PR-04-38813	N	N	N	25.30 ± 0.06	10.97 ± 0.02
		PR-06-0012	N	N	N	25.32 ± 0.15	11.05 ± 0.01
		PR-06-4942	N	N	N	25.98 ± 0.06	11.16 ± 0.03
		PR-04-20470	N	N	N	25.49 ± 0.05	11.08 ± 0.00
		EURL ANSES-F040	N	N	N	25.46 ± 0.15	11.24 ± 0.07
		EURL ANSES-F041	N	N	N	25.73 ± 0.27	11.08 ± 0.01
		EURL ANSES-F042	N	N	N	26.10 ± 0.02	11.13 ± 0.08

Species	Lineage	Strain code	Ct values ± SD				
			qPCR EU1	qPCR EU2	qPCR NA1	qPCR NA2	18S Uni test
Phytophthora lateralis	-	**EURL ANSES-F053**	N	N	N	N	11.46 ± 0.45
	-	**EURL ANSES-F054**	N	N	N	N	11.08 ± 0.07
	-	**EURL ANSES-F055**	N	N	N	N	11.07 ± 0.02
	-	**EURL ANSES-F056**	N	N	N	N	11.15 ± 0.07
	-	**EURL ANSES-F057**	N	N	N	N	11.06 ± 0.04
	-	**EURL ANSES-F058**	N	N	N	N	11.11 ± 0.02
	-	**EURL ANSES-F059**	N	N	N	N	11.12 ± 0.01
Phytophthora foliorum	-	**CBS 121655**	N	N	N	N	11.22 ± 0.02
Phytophthora hibernalis	-	**CBS 270.31**	N	N	N	N	11.13 ± 0.02
Phytophthora syringae	-	**PH14-227**	N	N	N	N	11.05 ± 0.06
Phytophthora pseudosyringae	-	**PR12W-033**	N	N	N	N	11.02 ± 0.02
Phytopthora gonapodyides	-	**PR13-377**	N	N	N	N	10.95 ± 0.00
Phytophthora ×cambivora	-	**PR13-379**	N	N	34.01 ± 0.07[a]	N	11.06 ± 0.05
Phytophthora kernoviae	-	**PF12-106**	N	N	N	N	11.03 ± 0.03
	-	**PR12-513**	N	N	N	N	11.02 ± 0.02
	-	**PR12-518**	N	N	N	N	11.04 ± 0.01
	-	**PR12-524**	N	N	N	N	11.17 ± 0.06
Phytophthora ramorum	IC1	**VN57**	N	26.16 ± 0.20	23.53 ± 0.13	N	11.04 ± 0.06
		VN313	N	25.69 ± 0.10	23.34 ± 0.09	N	10.98 ± 0.06
		VN831	N	27.00 ± 0.30	24.35 ± 0.09	N	11.20 ± 0.04
		VN1015	N	25.84 ± 0.23	23.29 ± 0.06	N	11.09 ± 0.04
	IC2	**VN142**	N	24.83 ± 0.09	23.76 ± 0.16	N	11.13 ± 0.02
		VN150	N	24.62 ± 0.13	23.72 ± 0.29	N	10.97 ± 0.03
		VN169	N	24.83 ± 0.11	23.38 ± 0.18	N	11.11 ± 0.00
		VN314	N	24.61 ± 0.24	23.55 ± 0.06	N	11.05 ± 0.05
	IC3	**VN88**	N	25.66 ± 0.07	23.45 ± 0.14	N	11.08 ± 0.03
	IC4	**VN851b**	N	24.51 ± 0.04	24.97 ± 0.39	24.46 ± 0.48	10.97 ± 0.03
		VN863	N	25.64 ± 0.43	23.53 ± 0.20	23.97 ± 0.20	10.40 ± 0.40
	NP1	**JP236**	N	N	N	N	11.14 ± 0.09
		JP716	N	N	N	N	11.39 ± 0.28
		JP916	N	N	N	N	11.09 ± 0.02
		JP1202	N	N	N	N	11.33 ± 0.37
	NP2	**JP387**	24.93 ± 0.05	N	24.26 ± 0.30	N	11.14 ± 0.04
		JP462	24.86 ± 0.11	N	24.27 ± 0.07	N	11.06 ± 0.05
	NP3	**JP975**	24.64 ± 0.02	N	23.48 ± 0.01	N	11.69 ± 0.10

6. Conclusions

In the EURL view and after a thorough comparison of the assays published to date, the assays developed by Feau *et al*.(2019) appeared as the most promising for the identification / detection of each of the four major lineages (EU1, EU2, NA1 and NA2) of *P. ramorum*. Recently, Jung *et al*. (2021) described new lineages of *P. ramorum* in Asia, presumably the center of origin of this pathogen. The specificity of all existing assays targeting *P. ramorum sensu lato* was not completely known, since they were published before the new Asian lineages (IC1 to IC4 and NP1 to NP3) were formally reported.

This work was undertaken before a clarification on the regulation was made by the European Commission about the quarantine status of *P. ramorum*. In a letter issued on 07/02/2022, the DG SANTE stated, "*The Commission Implementing Regulation (EU) 2021/2285 amending the Commission Implementing Regulation (EU) 2019/2072 intends to clarify the pest status of* Phytophthora ramorum *isolates on basis of their geographical origins, i.e. EU isolates and non-EU isolates, without references to lineages. The non-EU isolates, which are obtained from plants, plant products and other objects originating from Third Countries, are considered as Union quarantine pests. The EU isolates, which are obtained from plants, plant products and other objects originating from EU Member States, are considered as Union regulated non-quarantine pests*."

Before this clarification was made, EURL considered that only non EU-lineages were considered as a quarantine organism, and therefore, a protocol enabling the distinction between the EU and NA lineages was needed. In this context, the validation of the assays developed by Feau *et al*. (2019) was undertaken by the EURL in the 2021-2022 work program. This report brings useful data about the performance criteria of all four assays, which can still be used as identification tools in case more information is required after the interception of non-EU strains of *P. ramorum*.

The major outcomes are:

- All four assays are sensitive and highly specific to their respective lineage. They do not cross react with DNA of closely related species. However, cross-reactions were observed for each of them with much of the strains belonging to Asian lineages of *P. ramorum*. Noteworthy exception, *P. ramorum* NP1 lineage, one of these described in Japan, is not detected by any of the four assays developed by Feau *et al*. (2019).
- All four assays proved to be repeatable, robust, and transferable. The assays performed well in terms of sensitivity and specificity. However, when changes of temperature or reagents were applied, a loss of sensitivity was observed in two of the assays (EU2 and NA1) when the qPCR platform was changed, and at a low level of target DNA (close to the LOD). The sensitivity was sometimes affected so that false-negative results were generated in some conditions. Therefore, caution should be taken before implementing this protocol in a lab, in order to determine the optimal LOD, which would be used as a Cut off value for negative samples.

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