Final Report

For more information and guidance on completion and submission of the report contact the Euphresco Call Secretariat (bgiovani@euphresco.net).

Project Title and Acronym
Current and Emerging Phytophthoras: research supporting risk assessment and risk management (CEP)

Project Duration:

Start date:	01/03/2012
End date:	31/03/2014

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2. Executive Summary

Project Summary

Current and Emerging Phytophthoras: research supporting risk assessment and risk management (CEP)

This EU-wide EUPHRESCO project brought together partners from nine countries in project meetings and workshops to exchange knowledge on diagnostic methodologies for *Phytophthora* species and facilitate the standardisation of protocols across Europe.

Validation work on in-field diagnostic tests highlighted the effectiveness of the use of lateral flow devices (LFDs) to identify plants infected by *Phytophthora* species. However, the in-field PCR and LAMP tests, which were at that point still undergoing development and validation, showed a much lower rate of detection compared to the results from laboratory isolation and identification.

Newly developed and validated real-time LAMP assays on the Genie II were demonstrated to be effective for detection of a range of *Phytophthora* species in different hosts and tissue types. The use of alkaline PEG extraction greatly increased the potential for use of LAMP-based testing outside the laboratory in a range of scenarios. This has led to the development of reagents in a kit format to increase accessibility and convenience for a range of potential end-users. Since the end of this project, the *P. ramorum* and *P. kernoviae* LAMP assays have been developed into kits, and the potential now exists for the other assays described in this report to be made available in the same way. Novel LAMP assays were developed and validated for use in detection of *P. austocedrae*, *P. lateralis* and *P. pseudosyringae* in the field. A generic LAMP assay for detection of *Phytophthora* species was also developed for use in any future outbreaks of *Phytophthora* species of quarantine importance.

Analyses of the genotypes of 65 isolates of *P. ramorum* confirmed the existence of a group of isolates unique to the UK which continue to diversify as the pathogen spreads in the natural environment. No geographical or host specificity was detected for these groups although continuing diversification is a sign of growth and spread of the disease. In contrast, genotypic variation within a small number of isolates sourced from Portugal was very limited except for a single isolate whose genotype suggested that this isolate may have moved in trade between Spain and Portugal. This illustrates the capability of these analyses in detecting pathways of spread for the rarer genotypes and can be useful in calculation dispersal distances and potential rates of spread of pathogens.

Clear distinctions were seen between UK and New Zealand isolates of *P. kernoviae* with no intermixing of genotypes between countries indicating no direct connection between the two populations and no evidence for NZ being the source of introduction of *P. kernoviae* to the UK. Genetic diversity was greater within the New Zealand population of *P. kernoviae* compared to that found in the UK suggesting

that the population in the UK is more recent in its introduction.

Extensive research and analysis from the UK on risk assessment and disease management in natural outbreaks have been shared with national representatives from nine EU countries, completing objectives for knowledge transfer and facilitating capabilities for response to any future outbreaks of *Phytophthora* species across Europe. An improvement in efficacy, versatility and provenance of diagnostic methodologies as outputs from this project could significantly enhance the development of improved surveillance strategies and hence aid timely risk analysis for current outbreaks as well as deliver generic approaches for future outbreaks caused by *Phytophthora* species. In addition, outputs from genotyping using microsatellite analysis, 454 and Illumina sequencing of *P. ramorum* isolates could inform risk assessments for epidemic development and assist in identification of pathways of disease entry and spread.

3. Main Report

Introduction

There has been a recent emergence of new phytophthora problems relevant to ornamental plants, forestry and plants in the wider environment. This includes the introduction of new non-native species (e.g. *P. ramorum*, *P. kernoviae*, *P. lateralis* and *P. austrocedra*e) and the emergence of new hybrids (e.g. alder phytophthora and hybrids of *P. hedraiandra* and *P. cactorum*). There is also a number of new species emerging in non-European countries (e.g. *P. pinifolia* in Chile) and there are likely to be many unknown species (not yet described) that could be a threat to Europe. In order to future-proof the UK and Europe from future and current epidemics caused by *Phytophthora* species there is a need to improve current diagnostic and risk assessment methodologies.

There has been considerable progress in the development of detection methods for non-native *Phytophthora* species such as *P. ramorum* and *P. kernoviae* since they were first recorded causing outbreaks in the UK in 2002 and 2003 respectively. Since then, detection methods such as isolation, baiting, lateral flow devices (LFDs), PCR, TaqMan and PD+ (a commercial service involving molecular detection of pathogen DNA from LFDs) have been developed and validated for use in diagnosing disease outbreaks, predominantly in *Rhododendron* species. The increasing host range of both *P. ramorum* and *P. kernoviae* over time has demanded greater versatility and breadth of performance from the existing diagnostic approaches. Originally validated on a limited range of hosts, these methods now need to be effective for detection of pathogens in over 150 potential hosts for *P. ramorum* and over 35 for *P. kernoviae*, and from a range of substrates such as leaves, soil, leaf litter, water, compost, bark, wood chips, twigs and conifer needles. Since 2002, *P. ramorum* has caused disease outbreaks across England and Wales, Scotland, Ireland, Northern Ireland and countries across Europe. There are now a significant number of research groups working on risk assessment and management of a number of *Phytophthora* species affecting the wider environment, with considerable activity being devoted to the development of reliable diagnostic protocols for disease detection. In addition, new technologies and approaches such as LAMP (Loop mediated isothermal AMPlification) assays now offer further improvements in detection and diagnosis, however these need to be evaluated for wider use. Genotyping approaches such as microsatellite analysis, and use of illumina and 454 sequencing also offer the opportunity to analyse populations and track spread to improve and educate risk analyses.

This project aimed to draw these activities and technologies together to consolidate work in validation of both current and emerging diagnostic approaches for use across current hosts and substrates for *P. ramorum* and *P. kernoviae*. This would help to ensure that the existing technologies can be reliably extended to cover this increasing range of potentially infected host species and contaminated substrates. In addition, work aimed to identify which methods/methodologies have generic application for use in detection of other phytophthoras such as those detailed above (particular emphasis given to *P. kernoviae* and *P. lateralis*).

Work Plan and Objectives

1. Project management

The project was led and co-ordinated by Fera and involved scientists from twelve laboratories in nine different countries across Europe. The project work plan (Table 1) was developed and agreed at the project kick-off meeting held at Fera (Sand Hutton, UK) between 1 and 2nd March 2012. Given the range of current or emerging phytophthora problems, there was a need to optimise resources by ensuring effective co-ordination of phytophthora research, sharing of information and avoidance of duplication across Europe. This was addressed through joint international meetings, appropriate joint outputs and inclusion of appropriate additional work in a wider European context.

Table 1. Details of CEP Project work plan

Task	Partners involved	Completion date
1. Project management	**Fera**	
- Set up share site		April 2012
2. Improved Diagnostics The objective of this activity was to improve the surveillance and monitoring of current and emerging *Phytophthora* species. This will be done by evaluating current assays and methods, validating extraction methods, and where necessary developing new diagnostic tools.	**Fera**	
a) Direct isolation	**PPS**	
- Forward methodologies	All	May 2012
- Amalgamate methodologies	PPS	June 2012
- Distribute methodologies.	PPS	June 2012
- Evaluate methodologies and report back to group	All	October 2012
- Summarise findings	PPS	February 2013
b) DNA extraction and sample preparation (including soil and plant material). Procedures currently being used or under development for DNA extraction shared amongst all partners.	**UPV/INIA**	
- Forward methodologies		
- Amalgamate methodologies	All	May 2012
- Distribute methodologies.	UPV	June 2012
- Evaluate methodologies and report back to group	UPV	June 2012
- Summarise findings	All	October 2012
	UPV/INIA	February 2013
c) PCR assay. Procedures currently being used or under development for amplification, purification and detection of the PCR product will be shared	**Fera**	

amongst all partners. - Share current primers - PCR amplification and product purification will be conducted preferably at each participating laboratory using the protocols provided and evaluated. Results will be reported back to group. - Methods will be compared (if considered necessary) - Summarise findings	 All All All Fera	 May 2012 October 2012 Nov 2012 Dec 2012
d) Baiting - Forward methodologies - Amalgamate methodologies - Distribute methodologies. - Evaluate methodologies and report back to group - Summarise findings	**ILVO** All ILVO ILVO All ILVO	 May 2012 June 2012 June 2012 October 2012 February 2013
e) Investigating unknown phytophthoras - Forward generic diagnostic methods - Collate generic diagnostic methods - Evaluate methods and report back to group	**Fera/FR**	 May 2012 June 2012 October 2012
f) Lateral Flow Device (LFD). Serological tests are currently being used on different hosts to detect *Phytophthora* species. Currently, there is no shared information on how results from LFDs vary with host tissue. - Share data/experience - PD Plus. Confirm host range and share results.	**Fera** AFBI, DAFM, Fera Fera	 May 2013 May 2013
g) Loop-mediated isothermal amplification (LAMP) has been developed by Fera for use in the development of rapid on-site molecular diagnostic tests for *Phytophthora* species. However, no assay currently exists for *P. lateralis*. Also, the *P. kernoviae* assay has not yet been validated. - Validate for Pk - Develop for Pl - Comparison test for *P. ramorum*. - Summarise findings	**Fera** Fera ARC, FR, PPS ILVO, UPV/INIA Fera	 May 2013 May 2013 May 2013 July 2013
h) Macroarray diagnostics have been developed by CRA for use in the development of rapid molecular diagnostic tests for fungal and *Phytophthora* species. However, no assay currently exists for *P. lateralis* and *P. kernoviae.* - Development oligo probe for Pl, Pk *P. ramorum* and *P. hedraiandra*	**CRA** CRA	 Sept 2012

- Validation and report back to group	CRA plus other labs as required	Sept 2012
3. Genotyping. Participants will share DNA sequence (or where necessary, DNA or isolates) for standardised phylogenetic analysis	**FR, Fera**	
- Draft material transfer agreement - Provision of isolates - Genotyping - Collate data and produce report	FR, Fera ARC, Fera, FR SASA, ILVO, Fera, AFBI, DAFM	May 2012 On-going On-going Nov 2013
4. Risk assessment	**AFBI**	
a) *P. lateralis*	**AFBI**	
- Refine host list - Report from AFBI ongoing work - Report from ongoing hybridisation work (C. Brazier)	AFBI, FR, SASA AFBI FR	Nov 2013 Nov 2013 Nov 2013
b) General	**Fera**	
- Share information and data from epidemiological studies	**Fera**, All	March 2013
- Share information and approaches from site studies	**SASA**, All	October 2012
- Share protocols for bioassays (pathogenicity testing)	AFBI, FR, ILVO, UPV/INIA, **INRB**	Dec 2012
- Identify gaps in knowledge with respect to host range	**DAFM**, All	October 2012
- Report on analysis of genotyping data to inform risk	**Fera**	Nov 2013
- Share data from modelling studies	**Fera**, All	Nov 2013
- Collation of awareness of emerging phytophthoras	**DAFM**	Nov 2013
- Collate data and produce report	Fera, SASA, INRB, DAFM	February 2014
5. Disease management	**FR**	
- Share information and effectiveness of known management strategies	All	March 2014

NB. Partners indicated in bold co-ordinated the Task

This report includes research work funded by Defra under project PH0443 which provided the UK government support for Fera to participate in and lead this EUPHRESCO project. The Fera research addressed a number of objectives to progress detection and validation of diagnostic methodologies of current and emerging phytophthoras (listed below) in support the EUPHRESO CEP work plan. Fera was the only organisation to receive 'real pot' (RP) funding for this project and, unless otherwise stated, time given by other partners to this project was supported by 'virtual pot' funding from their national research programmes.

Fera specific objectives

1. Validate existing methodologies (isolation, baiting, LFD, DNA extraction, PD+, PCR, TaqMan) for use in detection of *P. ramorum* and *P. kernoviae* in a range of hosts and substrates. Ring tests or inter-laboratory comparisons with collaborator organisations
2. Development of LAMP assay and validation of diagnostics methods for *P. kernoviae, P. ramorum, P. lateralis* and other key *Phytophthora* species of quarantine importance including development of generic diagnostic protocols for use in the detection of other *Phytophthora* species
3. Undertake analysis of spatial relationships of currently identified genotypes of isolates of *P. ramorum* across the UK and Europe and carry out additional targeted analyses of further isolates to investigate pathways of spread and distance of dispersal in order to inform and update risk analyses
4. Explore potential for development of genotyping approaches for *P. kernoviae* (may be clonal) and *P. lateralis*

2. Improved diagnostics

(i) Sharing of protocols and expertise

One of the main aims of CEP was to draw together activities from the main research groups to consolidate work in validation of both current and emerging diagnostic approaches. These would be used across current hosts and substrates for *P. ramorum* and *P. kernoviae* to ensure that the existing technologies could be relied upon to cover the increasing range of infected host species and contaminated substrates. In addition, the proposed work would identify which methods/methodologies had generic application for use in detection of other phytophthoras (including *P. kernoviae* and *P. lateralis* in particular) or for detection of other quarantine fungal pathogens in difficult substrates such as tree host material.

To help with this objective, diagnostic protocols routinely used, or recently developed, were requested from CEP partners. Between April 2012 and February 2013, 15 SOPs from 6 participating labs were shared across the consortium. The SOPs relate to methods from the detection and identification of *P. ramorum* and *P. kernoviae* from environmental samples. Table 2 lists the SOPs provided by each country grouped by type of diagnostic test; all protocols were made available to the partners via the project share site.

A project progress and review meeting was held at the DAFM Laboratory Complex, Backweston, Celbridge, Co. Kildare (Ireland) on 20-21 March 2013 with a workshop at the Agri-Food and Biotechnology Institute (AFBI), Belfast on the 22nd. This project meeting included a full review of all protocols listed in Table 2.

Table 2. Protocols shared between project partners

Test type	SOP Name	Title	Originator
Direct isolation			
	SOP 3	Protocol for Euphresco_CEP_plating	ILVO (BE)
	SOP 1	Protocol for Euphresco_CEP_tissue maceration and DNA extraction	ILVO (BE)
	SOP 5	IT-CRA / IT-MIPAAF SOPs	CRA/MIPAAF (IT)
	SOP 7	Detection of *Phytophthora ramorum* and *Phytophthora kernoviae* on Woody Hosts	DAFF (IE)
	SOP 8	NL *Morphological identification of Phytophthora* ramorum	PPS (NL)
	SOP 10	NL Isolation of fungi from plant material	PPS (NL)
	SOP 12	Fera SOP - direct isolation *Phytophthora ramorum* and *Phytophthora kernoviae* testing	Fera (UK)
	SOP 14	Diagnostic protocol for *Phytophthora kernoviae* (Draft).	Fera (UK)
	SOP 15	Pt Recognition of Disease Symptoms, Isolation and Identification of *Phytophthora* species	INRB (PT)
DNA extraction			
	SOP 5	IT-CRA / IT-MIPAAF SOPs	CRA/MIPAAF (IT)
	SOP 11	NL baiting test_Phytophthora_sp	ILVO (BE)
	SOP 13	Fera SOP - TaqMan SOD testing	Fera
Conventional PCR assay			
	SOP 5	IT-CRA / IT-MIPAAF SOPs (conventional PCR; *P. ramorum*)	CRA/MIPAAF (IT)
	SOP 6	Molecular detection of *Phytophthora* NPPO (NL)	PPS (NL)
	SOP 7	Detection of *Phytophthora ramorum* and *Phytophthora kernoviae* on Woody Hosts	DAFF (IE)
	SOP 14	(Draft). Diagnostic protocol for *Phytophthora kernoviae*	Fera (UK)
Real-time PCR assay			
	SOP 2	Protocol for Euphresco_CEP_PCR	ILVO (BE)
	SOP 6	Molecular detection of *Phytophthora* NPPO (NL)	PPS (NL)
	SOP 7	Detection of Phytophthora ramorum and Phytophthora kernoviae on Woody Hosts	DAFF (IE)
	SOP13	Fera SOP 13- TaqMan *Phytophthora ramorum* and *Phytophthora kernoviae*	Fera (UK)

		testing	
	SOP 14	(Draft). Diagnostic protocol for *Phytophthora kernoviae*	Fera (UK)
Baiting	SOP 4	Protocol for Euphresco_CEP_baiting	ILVO (BE)
	SOP 5	IT-CRA / IT-MIPAAF SOPs	CRA/MIPAAF (IT)
	SOP 11	NL baiting test_Phytophthora_sp	PPS (NL)
LFD			
LAMP		Not available at time Not available at time	Fera Fera
Microarray			
		Not available at time	CRA/MIPAAF (IT)
Mating type test			
	SOP 9	NL Mating type test_Phytopthora_spp	PPS (NL)

(ii) Validation of protocols for direct isolation in the laboratory (all partners)

A workshop on the use of different direct isolation techniques was held at AFBI (Northern Ireland) as part of the Year 2 meeting; this allowed all participants to trial the different methodologies. Following the meeting, a review of the differences between the protocols provided by six different countries was carried out by Dr Johan Meffert (NL – PPS) and Dr James Choiseul (IE – DAFF) (Table 3). A number of organisations amended their protocols as a result of this exercise. In general, the main change was to use a more selective media during isolation.

Table 3. Summary of Analysis methods for *Phytophthora* isolation

Partner	Visual pre-assess-ment	Substrate	No. sub-samples taken	Deconta minate	Pre-treatment	Decontamination method	De-contaminati on time	Media	Size of pieces	No pieces plated	Incubation duration	Incubation Temp (°C)	Incubation Conditions
FERA	Y	Leaf	4	Y		Wash in water; rinse one or more times until clean	15 mins	P5ARP-H		>4	>5d	16-22	**Bench away from sunlight**
		Aerial parts				Washing in water then rinse twice							
		Dry leaf, aerial material			Soak in water for 24hrs	Rinse twice							
		Roots and dirty material				Rise in tap water until clean							
		Tree bark, oil			Bait uninfected leaves in Petri's mineral for 3-5 d	Rinse twice		P5ARP-H					
PPSN	Y			Y		Washing in water	10-60 secs	CA, SNA, WA	3x3 mm	36	>3d	22	**Dark**
						50% ethanol then rinse	10 secs						
Estonia	Y			Y		70% ethanol then rinse		P5ARP-H		5	3-5d	20-25	
ILVO	Y	Leaves		Y		0.5%NaOH then rinse	30 secs	P5ARP + 5ppm pimaricin		NS	5d	20	**Dark**
DAFM	Y	Leaves, roots, stem all spp.	5-10	Y		Washing in water	No time limit	P5ARP-H	10x10 mm	5-10	5-7d	Room Temperature	**Bench**
		Dry leaf, aerial material			Soak in water for 24hrs	Washing in water	No time limit	P5ARP-H	10x10 mm	5-10	5-7d	Room Temperature	**Bench**
EPPO ('06)	Y	Plant material		Y	Inc. for -5d in sealed humid box	Soak in Alcohol solution then rinse with sterile water	2-5 mins	P5ARP-H (preferred)	2x2 mm	>4		20-25	**Bench or Incubator**
				Y	Inc. for -5d in sealed humid box	Soak in sodium hypochlorite (0.5%) solution; rinse with sterile water	2-5 mins	P5ARP-H (preferred)	2x2 mm	>4		20-25	**Bench or Incubator**
				Y		Others							
		Water		N	Baiting for 3-7d			P5ARP-H or CPA	2x2 mm	>4		20-25	**Bench or Incubator**
		Soil		N	Bait uninfected leaves in Petri's mineral, sterile water for 3-7 d	As per plant material							

(iii) Validation of tests for use on field samples (Fera)

Two managed gardens, one known to be infected with both *P. ramorum* (Pr) and *P. kernoviae* (Pk), and one infected by *P. ramorum* only, were used as test sites to validate methodologies for detection of *P. ramorum* and *P. kernoviae* in the field. Molecular biologists from Fera visited the garden in October 2012 and set up a comparative test involving a visual inspection conducted by the local PHSI officer and in-field tests using LFD, PCR (SmartCycler) and LAMP (Genie) (*P. kernoviae* only) for confirmation of infection by either *P. ramorum* or *P. kernoviae*. All samples were taken back to the laboratory and confirmatory diagnoses carried out by isolation from the plant material.

A total of 91 leaf samples were collected from a range of host species including Rhododendron (32), Magnolia (17), Pieris (4), Viburnum (4) and other species (34). Results of the subsequent diagnostic tests are shown in Table 4.

Table 4. Number of samples positive for *P. ramorum* or *P. kernoviae* using five different diagnostic tests

	Positive Pr	Positive Pk	Other Phytophthora	Negative	Total Phytophthora positive samples
Visual assessment	34 (no species distinction)		-	57	34
LFD	28 (no species distinction)		-	63	28
Direct isolation	19	9 (+1 both)	3	59	32
In-field LAMP (Pk only)	-	6	-	85	6 (Pk)
In-field PCR	4	5	-	82	9

All but one of the LFD negatives were also negative by PCR and isolation. However, in 5 of the 29 Pr/Pk positives, LFDs failed to detect the pathogen present. Comparison between visual assessments and isolation results showed a high level of accuracy in symptom recognition by the (very experienced) PHSI inspector with 32 out of the 34 positives by visual symptoms confirmed as phytophthora by isolation. Although there was a reasonable correlation between LFD results and isolation, the results from in-field PCR or LAMP tests were disappointingly low compared to the isolation data. Results highlighted the need for more validation work to improve the detection efficiency of these in-field tests. This was addressed under Objective 3.

Testing work carried out at one of the two gardens identified two plant species not previously confirmed as natural hosts of *P. ramorum*. These were *Aronia*

melanocarpa and *Corylopsis pauciflora*. Both landowners were advised of all the results generated.

(iv) Development and validation of new LAMP assays for detection of *Phytophthora* (Fera)

Nucleic acid-based methods have been developed for various *Phytophthora* species, including PCR/real-time PCR for *P. ramorum*, *P. kernoviae* and *P. lateralis*. Methods based on loop-mediated isothermal amplification (LAMP) have also previously been developed for detection of *P. ramorum* and *P. kernoviae*. More recent use of LAMP for plant pathogen detection at Fera has concentrated on the use of the Genie II instrument (OptiGene, UK) for real-time fluorescence detection. Work on this objective focused on the characterisation and validation of LAMP methods and development of new assays for additional targets (*P. austrocedrae*, *P. pseudosyringae* and a generic *Phytophthora* spp. assay).

LAMP primers were based on multiple sequence alignments of target and non-target sequences downloaded from public databases. Alignments were constructed using Clustal V in MegAlign (DNA Star). Candidate primer sequences were selected on the basis of calculations of primer length, GC content and annealing temperature, and to minimise predicted secondary structure and dimer formation. Primer sets typically consist of two external primers (F3, B3), two internal primers (FIP, BIP) and two loop primers (F-loop, B-loop), targeting a total of eight primer binding sites, as shown in Figure 1.

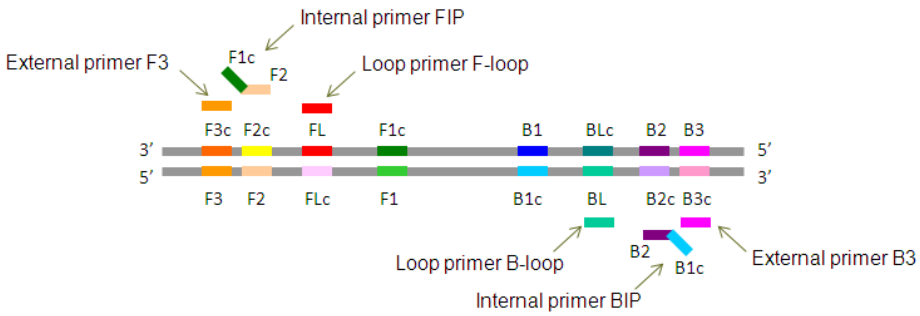

Figure 1. Schematic showing LAMP primer binding sites.

In the development of some of the assays described below, multiple versions were tested to optimise performance in terms of sensitivity and/or specificity. Factors which were taken into account in the process of assay design included the amplicon length (with a preference for shorter amplicons, where possible, to increase amplification efficiency and/or sensitivity), the inclusion/omission of loop primers, and the inclusion of 'stem' primers (Gandelman *et al*., 2011) designed in the centre of the amplicon between primer binding sites F1 and B1 which are reported to increase amplification speed and sensitivity.

LAMP was carried out with real-time fluorescence monitoring on the Genie II instrument using Isothermal Master Mix (OptiGene). Primer concentrations were

typically 200 nM for each external primer, 2 µM for each internal primer and 1 µM for each loop/stem primer.

Testing of the assays was carried out using conventionally extracted DNA prepared from cultures and infected plant material using a magnetic bead-based extraction method and the KingFisher mL platform (Thermo Scientific). A major driver for the use of LAMP is the potential to develop methods which can be used without laboratory equipment, so samples were also tested using a crude sample preparation method in which material is briefly shaken with alkaline polyethylene glycol (PEG) buffer (Chomczynski & Rymaszewski 2006) and diluted in water (typically 1 in 10) before testing.

(a) LAMP for detection of *P. kernoviae* (Fera)

LAMP primers for detection of *P. kernoviae* have already been developed (Tomlinson *et al*., 2010). These primers target the same ITS sequence as the TaqMan real-time PCR method in routine use at Fera (Hughes *et al*., 2011). Results from validation of this assay (see above) indicated that more development was required to improve sensitivity, Since the original development of this assay, use of the simplified sample processing method using alkaline PEG extraction and the Genie II for real-time LAMP has been developed at Fera for the detection of fungal plant pathogens in non-laboratory conditions. Work was therefore carried out to establish the application of this approach to the detection of *P. kernoviae*. Table 5 shows the results obtained by testing samples composed of different ratios of *P. kernoviae*-inoculated and healthy rhododendron leaf using (i) PEG extraction followed by LAMP and (ii) conventional magnetic bead-based extraction followed by real-time PCR. For comparison, results are also shown for replicate samples tested by *Phytophthora* spp. LFD.

Table 5. Results obtained testing rhododendron leaf samples containing different amounts of *P. kernoviae*-infected tissue by weight (inoculated tissue mixed with healthy tissue).

% infected tissue	PEG/*P. kernoviae* LAMP	*P. kernoviae* real-time PCR	*Phytophthora* spp. LFD
10	+	+*	+
0.1	-	-	-

*Ct value close to limit of detection.

Validation data for this assay have been compiled in line with EPPO Standard PM 7/98 and will be submitted to the EPPO validation database. The limit of detection of the *P. kernoviae* LAMP assay was approximately 17 pg DNA, and no cross reactivity with other *Phytophthora* species was predicted or observed. Repeatability and reproducibility were assessed as follows: 17 out of 18 replicate reactions at the limit of detection of the assay gave positive results when tested by the same operator using the same equipment on the same day (94.4% repeatability at the limit of detection), and 6/6 replicate reactions at the limit of detection gave positive results when tested by a different operator using a different instrument on a different day (100% reproducibility at the limit of detection).

(b) LAMP for detection of *P. ramorum* (Fera)

LAMP primers for detection of *P. ramorum* have been published previously (Tomlinson *et al*., 2007) which target the same region as the real-time PCR assay in routine use at Fera (Hughes *et al*., 2006). However, these primers were found to give low fluorescent signals when run on the Genie II instrument. In order to align *P. ramorum* detection with other testing methods which use this instrument, we investigated the development of alternative primer sets. The primary theoretical concern in the design of *P. ramorum* primers targeting the ITS region is the similarity of the *P. ramorum* sequence to *P. lateralis*, from which *P. ramorum* differs by less than 10 bases distributed across the ITS1-5.8S-ITS2 regions (note that differences in host species/tissue for the two pathogens may make this of less practical concern). An alternative primer set targeting ITS2 rather than ITS1 was highly sensitive to *P. ramorum* DNA and compared well with real-time PCR in initial testing, but was found to cross react with *P. lateralis* DNA when tested at or above 1 ng DNA per reaction.

As an alternative to the ITS-based LAMP assays, *P. ramorum* primers were designed to target the *Ypt* gene, which exhibits more interspecific variation than the rRNA genes (Schena & Cooke 2006). These primers were used in combination with an alternative isothermal master mix from OptiGene to compensate for the reduction in amplification speed which was attributed to the change from a multi-copy target (ITS) to a single copy target (*Ypt*). No cross reactivity was observed with *P. lateralis* DNA, even at high concentrations (Figure 2), and the assay was suitable for use with PEG extraction (Table 6).

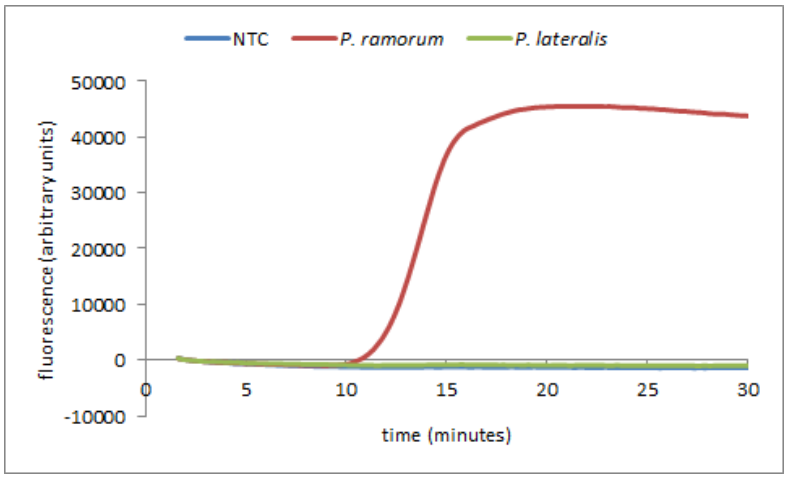

Figure 2**.** Real-time LAMP results obtained using Ypt primers for *P. ramorum* on the Genie II. (NTC: no-template control).

Table 6. Results obtained testing rhododendron leaf samples containing different amounts *of P. ramorum* infected tissue by weight (inoculated tissue mixed with healthy tissue).

% infected tissue	PEG/*P. ramorum* LAMP	*P. ramorum* real-time PCR*	*Phytophthora* spp. LFD
10	+	+	+
0.1	+ (3/6 samples)	+	-

* using primers 114F and 190R

Validation data for this assay have been compiled in line with EPPO Standard PM 7/98 and will be submitted to the EPPO validation database. The limit of detection using this assay was approximately 40 pg DNA when testing extracts from culture. No cross reactivity with non-target species (including *P. lateralis*) was predicted or observed. Repeatability and reproducibility were assessed as follows: 18 out of 18 replicate reactions at the limit of detection of the assay gave positive results when tested by the same operator using the same equipment on the same day (100% repeatability at the limit of detection), and 6/6 replicate reactions at the limit of detection gave positive results when tested by a different operator using a different instrument on a different day (100% reproducibility at the limit of detection).

Update on subsequent developments arising from this project

The results described above were obtained using samples of naturally and artificially infected rhododendron; it was observed that results were less robust using the *Ypt* primers when testing naturally infected larch wood samples provided by Forestry Commission Plant Health Officers. A subsequent Defra Horizon Scanning and Technology Implementation (HSTI) project was carried out at Fera in 2014/15 to modify and validate the method using ITSv2 primers for testing larch wood samples.

(b) LAMP for detection of *P. lateralis* (Fera)

Primers were developed for detection of *P. lateralis* which target the ITS2 region and the *Ypt* gene. The ITS primers were found to cross react with *P. ramorum* DNA, but no cross reactivity was observed for the *Ypt* primers. In an attempt to increase the speed of amplification while targeting the *Ypt* gene, a primer set was designed to include 'stem' primers (Gandelman *et al*., 2011). Optimal sensitivity and speed of amplification were observed for primer set *Ypt* v2 + stem primer 2 (Figure 3).

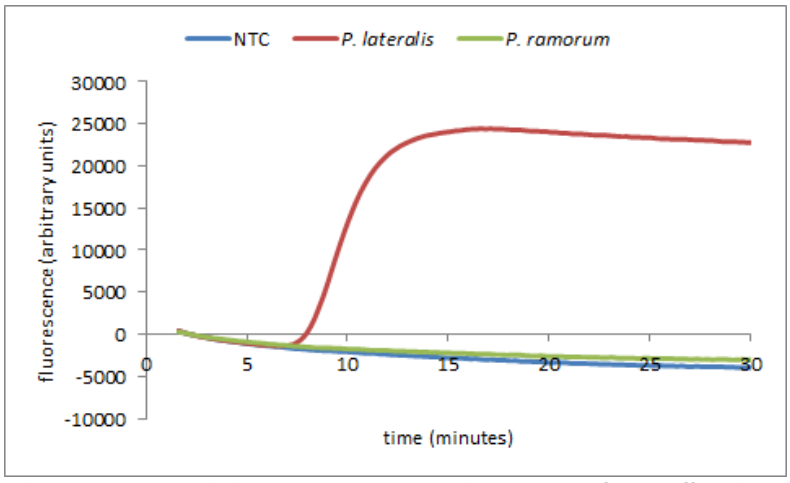

Figure 3. Real-time LAMP results for *P. lateralis* detection using Ypt primers *on* the Genie II. NTC: no-template control.

DNA extracts from naturally infected wood samples were tested using TaqMan real-time PCR and LAMP using primer set Ypt v2 + stem primer 2. TaqMan Ct values were in the range 24 – 35 cycles; positive LAMP results were obtained for samples which gave TaqMan Ct values <28 cycles, with time to positive (Tp) values in the range 11 – 19 minutes. These results therefore confirm that the assays will work in difficult substrates such as wood which often contains potential inhibitors to the assay.

(d) LAMP for detection of *P. austrocedrae* (Fera)

Primers for detection of *P. austrocedrae* were developed in 2014 during a Defra-funded HSTI project. This assay targets ITS2. PEG extraction from infected juniper stems was successful (Figure 4). No cross reactivity was observed for extracts from non-target *Phytophthora* species including *P. syringae*, and a limit of detection of <1 pg DNA was established. This method could therefore now be used for comparative testing on a larger scale or trial deployment, as required.

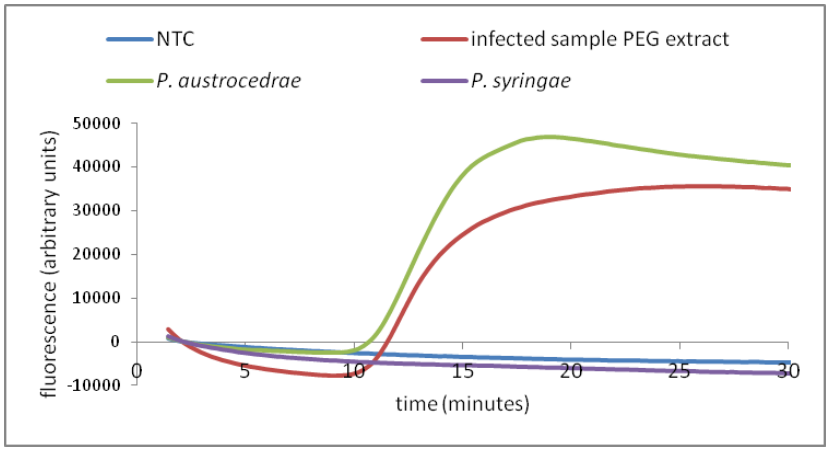

Figure 4. Real-time LAMP results for *P. austrocedrae* detection on the Genie II. NTC: no-template control.

(e) LAMP for detection of *P. pseudosyringae* (Fera)

Primers for detection of *P. pseudosyringae* were developed which target the *Ypt* gene. No cross reactivity was observed with extracts from non-target *Phytophthora* species including *P. ilicis* and *P. nemorosa* (Table 7).

Table 7**.** Real-time LAMP results for *P. pseudosyringae* detection on the Genie II.

	Tp value (min:sec)	Ta (°C)
P. pseudosyringae	13:00	90.37
P. ilicis	-	-
P. nemorosa	-	-
No template control	-	-

Tp – Time to positive; Ta – Anneal peak

PEG extraction was found to be suitable for DNA extraction from vaccinium (stem and leaf), as determined by COX LAMP, but *P. pseudosyringae*-infected samples have yet to be tested. However, this assay could now be used for comparative testing of infected material, with the potential for trial deployment if required.

(f) Generic LAMP assay for detection of *Phytophthora* spp. (Fera)

Primers were designed for generic detection of *Phytophthora* spp. which target the 5.8S region of the ribosomal RNA genes. This assay is intended to be used as a positive control for non-species specific detection of any *Phytophthora* spp. in infected material. The assay is not completely specific to the genus *Phytophthora*, and is likely

also to amplify some, but not all, oomycetes species outside the target genus (e.g. *Peronospora* spp. and *Pythium* spp.). The assay could therefore potentially be used to test symptomatic material for which species-specific LAMP assays have tested negative, serving as an internal control (for example, to demonstrate that amplifiable DNA has been extracted, in a similar manner to the use of the COX LAMP assay for detection of plant DNA) or playing a role in the initial detection of new/emerging *Phytophthora* spp. in the field.

Amplification was observed for extracts from 13/13 *Phytophthora* spp. (Table 8). A limit of detection of <4 pg DNA was established for this assay.

Table 8. Real-time LAMP results for *Phytophthora* spp. control assay.

Species	Time to positive (min:sec)	Anneal peak (°C)
P. syringae	9:00	86.04
P. pseudosyringae	8:00	86.23
P. insolita	8:15	86.19
P. cryptogea	7:15	86.39
P. palmivora	9:15	86.13
P. cambivora	7:30	86.42
P. gonapodyides	7:30	86.32
P. citrophthora	7:15	86.29
P. infestans	7:30	86.25
P. cinnamomi	7:30	86.08
P. ramorum	7:15	86.14
P. kernoviae	8:00	86.09
P. megasperma	8:15	85.89

Use of this assay in conjunction with PEG extraction for *in planta* detection was demonstrated by testing rhododendron leaf inoculated with *P. ramorum* (Table 9).

Table 9**.** Results obtained following testing of rhododendron leaf samples containing different amounts of *P. ramorum*-infected tissue by weight (inoculated tissue mixed with healthy tissue) using the *Phytophthora* spp. LAMP assay and *Phytophthora* LFDs.

% infected tissue	LAMP	LFD
10	+	+
0.1	+ (2/3 samples)	-
0	-	-

3. Analysis of genotypes of isolates of *P. ramorum*, P. kernoviae and P. lateralis in the UK and in Europe (UK-Fera & BE-ILVO)

(i) *P. ramorum*

Collaboration with experts in Belgium to genotype the UK population of *P. ramorum* in Defra project PH0602 showed that the make-up of the population of *P. ramorum* in the UK was very different to the rest of Europe, where the populations in each country were all very similar. A total of 31 different genotypes were identified within the *P. ramorum* population tested and of these, 9 genotypes (EU1MG42 to EU1MG49 and EU1MG70) were unique to the UK. The number and frequency of the novel genotypes have become more prevalent as the national outbreak has progressed. Three genotypes (EU1MG1, EU1MG42 and EU1MG43) represented 68% of the population and the novel genotype EU1MG43 dominated the UK population in terms of outbreaks in the natural environment. A strong association was confirmed between genotype and location, with EU1MG42 and EU1MG43 causing outbreaks primarily in the natural environment and EU1MG1 most commonly found affected plants on nurseries. However, there was no host specificity detected for any of the genotypes. Analysis of a second set of samples comprising eight isolates all from larch showed that five belonged to *P. ramorum* genotypes EU1MG1, EU1MG43 and EU1MG58. The remaining three isolates were identified as belonging to a new EU lineage (EU2). These results were published by Van Poucke *et a*l. (2012).

A third batch of 68 isolates was analysed within this project to examine genotype diversity of isolates found on rare tree hosts (14 isolates supplied by FR) and those isolated most recently by Fera (2011/12 onwards). Results detailed in Table 10 summarise the number of different genotypes identified in the batch of 68 isolates tested by ILVO in Belgium and Table 11 shows details of the hosts and source environment. As in the previous study the most common genotypes were EU1MG01 and EU1MG43, with EU1G01 more commonly associated with nursery plants and EU1MG43 more common found in the natural environment. The populations from 2011 and 2012 showed greater diversity than those tested previously and several new genotypes, which had evolved from EU1MG43, were found at low frequencies, all of the new genotypes were unique to the UK.

Table 10. Prevalence of genotypes of *P. ramorum* isolates (UK)

Genotype	Number	% isolates
EU1MG01	18	20.9
EU1MG05	7	8.1
EU1MG13	2	2.3
EU1MG32	1	1.2
EU1MG33	1	1.2
EU1MG42	2	2.3
EU1MG43	18	20.9
EU1MG44	2	2.3
EU1MG45	2	2.3
EU1MG46	5	5.8

EU1MG54	1	1.2
EU1MG58	1	1.2
EU1MG64	1	1.2
EU1MG77	1	1.2
EU1MG78	1	1.2
EU1MG79	1	1.2
EU1MG80	1	1.2
EU1MG81	1	1.2
EU1MG82	1	1.2
EU1MG83	1	1.2

The increased diversity of the UK population is clearly illustrated and shows that *P. ramorum* continues to evolve in the UK environment, producing further numbers of genotypes which are unique to the UK. Although the genotypes arising are clearly able to survive and spread in the natural environment, there is still no evidence of specialism for particular host species or environments or any indication of differences in aggressiveness between genotypes.

Table 11. Genotypes of isolates of *P. ramorum* isolated in 2011/12 or from rarer tree hosts

Isolate No.	Date	Source	Host	Genotype
FERA17	2011	W	Unknown	EU1MG13
FERA37	2011	N	Unknown	EU1MG43
FERA46	2012	W	Rhododendron	EU1MG43
FERA47	2012	W	Rhododendron	EU1MG82
FERA19	2011	G	Leucothoe fontanesiana	EU1MG43
FERA22	2011	G	Unknown	EU1MG43
FERA30	2011	G	Unknown	EU1MG43
FERA31	2011	G	Unknown	EU1MG44
FERA32	2011	G	Unknown	EU1MG44
FERA33	2011	G	Unknown	EU1MG46
FERA1	2011	N	Unknown	EU1MG01
FERA54	2012	G	Unknown	EU1MG42
FERA39	2011	W	Unknown	EU1MG05
FERA2	2011	N	Viburnum tinus	EU1MG01
FERA3	2011	G	Pieris	EU1MG43
FERA4	2011	G	Pieris formosa	EU1MG58
FERA5	2011	N	Rhododendron	EU1MG43
FERA6	2011	N	Rhododendron	EU1MG45
FERA7	2011	N	Viburnum tinus	EU1MG13
FERA8	2011	W	Pieris japonica	EU1MG01
FERA9	2011	G	Hedera	EU1MG43
FERA10	2011	N	Viburnum tinus	EU1MG01
FERA11	2011	N	Rhododendron	EU1MG43
FERA12	2011	W	Rhododendron	EU1MG43
FERA13	2011	G	Unknown	EU1MG43
FERA14	2011	W	Vaccinium myrtillus	EU1MG01
FERA15	2011	W	Rhododendron ponticum	EU1MG01
FERA16	2011	G	Pieris	EU1MG46
FERA18	2011	G	Pieris japonica	EU1MG01
FERA21	2011	W	Rhododendron	EU1MG01
FERA23	2011	W	Rhododendron	EU1MG05
FERA24	2011	W	Unknown	EU1MG01
FERA25	2011	N	Unknown	EU1MG01
FERA27	2011	N	Unknown	EU1MG01
FERA26	2011	G	Unknown	EU1MG01
FERA28	2011	G	Rhododendron ponticum	EU1MG80
FERA29	2011	W	Rhododendron	EU1MG64
FERA34	2011	W	Unknown	EU1MG05
FERA36	2011	W	Unknown	EU1MG05
FERA35	2011	W	Unknown	EU1MG01
FERA38	2011	W	Unknown	EU1MG45
FERA48	2012	W	Vaccinium myrtillus	EU1MG46
FERA40	2011	G	Unknown	EU1MG46
FERA41	2012	N	Viburnum tinus	EU1MG01
FERA42	2012	N	Rhododendron	EU1MG01
FERA43	2012	G	Rhododendron	EU1MG01
FERA44	2012	N	Rhododendron	EU1MG81
FERA45	2012	N	Rhododendron	EU1MG01

FERA49	2012	W	Vaccinium myrtillus	EU1MG46
FERA50	2012	N	Rhododendron	EU1MG43
FERA51	2012	N	Camellia (Ruby wedding)	EU1MG01
FERA52	2012	G	Pieris	EU1MG05
FERA53	2012	W	Rhododendron	EU1MG83
FERA56	?	?	Unknown	EU1MG43
FR17	2005	W	Fagus sylvatica	EU1MG78
FR7	2003	W	Castanea sativa	EU1MG43
FR13	2004	W	Nothofagus obliqua	EU1MG43
FR18	2005	W	Quercus petraea	EU1MG43
FR4	2003	W	Aesculus hippocastanum	EU1MG43
FR8	2004	W	Fagus sylvatica	EU1MG43
FR10	2004	W	Nothofagus obliqua	EU1MG77
FR19	2005	W	Quercus cerris	EU1MG79
FR1	2002	W	R. ponticum	EU1MG32
FR25	2007	W	Fagus sylvatica	EU1MG54
FR16	2005	W	Fagus sylvatica	EU1MG05
FR24	2007	W	Fagus sylvatica	EU1MG05
FR23	2007	W	Cinnamomum camphora	EU1MG33
FR27	2007	W	Cinnamomum camphora	EU1MG42

W = Woodland; N = Nursey; G = Garden

Through the EUPHRESCO collaboration, eleven isolates provided by colleagues in Portugal were prepared by Fera for testing in Belgium using the same methodology as that used for the UK isolates. Results from these analyses indicated a far less diverse population in Portugal compared to the UK with the most common genotypes (EU1MG1) found in nurseries predominating (Table 12). The finding of an isolate belonging to genotype EU1MG38 was of note as this genotype had previously only been found in Spain. This may be a co-incident evolution or could be an indication of transfer in trade between Spain and Portugal.

Table 12. Genotypes of isolates of *P. ramorum* sourced from Portugal

Isolate No.	Genotype
PT1	EU1MG1
PT10	EU1MG38
PT12	EU1MG3
PT2	EU1MG1
PT3	EU1MG1
PT4	EU1MG1
PT5	EU1MG1
PT6	EU1MG1
PT7	EU1MG5
PT8	EU1MG1
PT9	EU1MG1

(ii) *P. kernoviae* (Fera)

Microsatellite analysis of the genotypic diversity of *P. kernoviae* has previously indicated that the population in the UK was clonal (K. Hughes, Personal communication). However, a more recent Defra project (PH0601) involving collaboration between Fera and Exeter University reported that whole genome sequencing analyses of 19 *Phytophthora kernoviae* isolates, representative of the majority of outbreaks within the UK over the period 2004–2012, were able to differentiate the UK population. Analysis identified 3,016 candidate SNPs in the genome of *P. kernoviae* and the isolates investigated appeared to fall into two distinct genotype-groups, distinguishable from each other by about 1000 SNPs. The patterns of distribution of SNPs were consistent with recombination rather than purely clonal inheritance and reflected the homothallic nature of this fungus. The phylogenetic tree representing the whole genome sequence data, obtained from UK isolates as part of Defra project PH601, show the majority of isolates collected from Cornwall and Wales clustered together. This clustering indicated little genetic difference between isolates and possible local spread of the pathogen. A separate group of isolates from Ireland, Cheshire, Kent and Scotland were positioned some distance away on the phylogenetic tree, indicating these isolates were genetically diverse to the other group, possibly suggesting separate introductions from different sources.

Due to whole genome sequencing proving an effective method of distinguishing variation within UK *P. kernoviae* isolates, research was initiated in this project to analyse nine isolates of *P. kernoviae* collected by a Fera member of staff whilst in New Zealand (NZ) to investigate possible links between UK and NZ isolates; at the

time NZ was the only other country where *P. kernoviae* had been found in the natural environment. Comparison of the whole genome sequence data obtained from the UK and NZ isolates showed that they i fell into distinct clusters with all NZ isolates and all UK isolates grouping at separate ends of the tree. Several hundred putative SNPs separated NZ and UK isolates with the closest genetic relationship between NZ isolate and a UK isolate collected from Cheshire, followed by Southern Ireland and Kent. Analysis of the NZ isolates showed several hundred putative SNPs separating the isolates, this suggested a much greater degree of genetic diversity compared to the UK. When geographical location was investigated, only isolates which originated from the same region sat together on the same phylogenetic tree branch. Results do not support direct linkage between isolates in the UK and New Zealand and do not suggest that New Zealand was the source of the incursion into the UK.

(ii) *P. lateralis* (IE-DAFF)

Work on *P. lateralis* was progressed through the EUPHRESCO consortium, results from which were published in 2013 (Quinn *et al*., 2013). Approaches developed by collaborators at Exeter for analysis of *P. kernoviae* were used to sequence the genomes of four isolates of *P. lateralis* from two sites in Northern Ireland in 2011. The genomes of the four isolates analysed were almost identical but it was possible to distinguish between isolates based on several single-nucleotide polymorphisms (SNPs). These molecular markers could be used for tracking routes of spread in epidemiological studies.

4. Risk assessment (all partners)

Information pertaining to risk assessment and disease management has been shared across the consortium through direct communication at annual meetings and through exchange of documents. All documents relating to risks of Phytophthora pathogens to the UK (many of which were produced by members of this consortium from Fera, Forest Research and SASA) have since been collated into the UK Plant Health Risk Register https://secure.fera.defra.gov.uk/phiw/riskRegister/ which give access to Pest Risk Analyses, host lists, current status and recommended actions.

5. Disease management (all partners)

Updates on research projects were shared during meetings and the reports from work funded in the UK are available via the following links:

Defra:
http://randd.defra.gov.uk/Default.aspx?Location=None&Module=FilterSearchNewLook&Completed=0

Scottish Government:
https://cse.google.com/cse?cx=007197013444011456969:ll2jctu1uq8&start=0&q=Phytophthora%20more:Publications&oe=utf-8&sort=#gsc.tab=0&gsc.q=Phytophthora%20more%3APublications&gsc.page=1

Forestry Commission:
https://www.forestry.gov.uk/fr/INFD-5STC8A

DAFM (Ireland):
http://www.rohanlon.org/downloads/ROHanlonFinal.pdf

Conclusions and policy impacts

- This EU-wide EUPHRESCO project brought together partners from nine countries in project meetings and workshops to exchange knowledge on diagnostic methodologies for *Phytophthora* species and facilitate the standardisation of protocols across Europe.
- Validation work on in-field diagnostic tests highlighted the effectiveness of the use of lateral flow devices (LFDs) to identify plants infected by *Phytophthora* species. However, the in-field PCR and LAMP tests, which were at that point still undergoing development and validation, showed a much lower rate of detection compared to the results from laboratory isolation and identification.
- The newly developed and validated real-time LAMP assays on the Genie II were demonstrated to be effective for detection of a range of *Phytophthora* species in different hosts and tissue types. The use of alkaline PEG extraction greatly increased the potential for use of LAMP-based testing outside the laboratory in a range of scenarios. This has led to the development of reagents in a kit format to increase accessibility and convenience for a range of potential end-users.
- Since the end of this project, the *P. ramorum* and *P. kernoviae* LAMP assays have been developed into kits, and the potential now exists for the other assays described in this report to be made available in the same way.
- Novel LAMP assays were developed and validated for use in detection of *P. austocedrae*, *P. lateralis* and *P. pseudosyringae* in the field.
- A generic LAMP assay for detection of *Phytophthora* species was also developed for use in any future outbreaks of *Phytophthora* species of quarantine importance.
- Analyses of the genotypes of 65 isolates of *P. ramorum* confirmed the existence of a group of isolates unique to the UK which continue to diversify as the pathogen spreads in the natural environment. No geographical or host specificity was detected for these groups although continuing diversification is a sign of growth and spread of the disease.
- In contrast, genotypic variation within a small number of isolates sourced from Portugal was very limited except for a single isolate whose genotype suggested that this isolate may have moved in trade between Spain and Portugal. This illustrates the capability of these analyses in detecting pathways of spread for the rarer genotypes and can be useful in calculation dispersal distances and potential rates of spread of pathogens.
- Clear distinctions were seen between UK and New Zealand isolates of *P. kernoviae* with no intermixing of genotypes between countries indicating no direct connection between the two populations and no evidence for New Zealand being the source of introduction of *P. kernoviae* to the UK.

- Genetic diversity was greater within the New Zealand *P. kernoviae* population compared to that found in the UK suggesting that the population in the UK is more recent in its introduction.
- Extensive research and analysis from the UK on risk assessment and disease management in natural outbreaks have been shared with national representatives from nine EU countries, completing objectives for knowledge transfer and facilitating capabilities for response to any future outbreaks of *Phytophthora* species across Europe.

Acknowledgements

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