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Metabarcoding analysis of *Phytophthora* diversity in spore traps and implications for disease forecasting in the *Phytophthora ramorum* management zone

PHC2019/08 - Project Final Report

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Forest Research

The James Hutton Institute

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Research Team: Carolyn Riddell developed the project and carried out the laboratory work. Sarah Green developed the project and aided analysis, Toni Clarke carried out statistical data analysis and Peter Cock processed the raw sequencing data to assign species identities within the samples.

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Content

1 Summary

Surveillance and monitoring of airborne pathogens is a key tool in the management of healthy forests and controlling disease outbreaks. Wind dispersed spores are challenging to track and forecast since spread to uninfected areas is typically only identified once a pathogen has established and several trees begin to decline or die. A Scottish Forestry-funded project was carried out in autumn 2019 to validate different spore-trapping techniques for monitoring airborne *Phytophthora ramorum (P. ramorum)* inoculum using a species-specific qPCR assay in relation to climatic variables (Frederickson-Matika *et al.* 2020). The aim of the project was to provide evidence to support the implementation of a large-scale network of wind vane traps, which would allow forest managers to make more timely disease management decisions and control *P. ramorum* more effectively.

Our Plant Health Centre-funded project aimed to add further value to the Scottish Forestry project by investigating the suitability of DNA metabarcoding for screening spore trap samples for *P. ramorum* and other *Phytophthora* species, seeking early data for aerially dispersed *Phytophthora* species that may become problematic in UK forests. Our results provide evidence that metabarcoding can detect *P. ramorum* at rates similar to the species-specific *P. ramorum* qPCR used in the Scottish Forestry project and that *P. ramorum* capture and oomycete species diversity varies according to spore trap type. We also tested a lineage-specific qPCR assay to monitor EU1 and EU2 lineage composition of *P. ramorum*. Our findings showed that the lineage-specific quantitative PCR (qPCR) resulted in a high number of false negatives and would not be suited to monitoring the lineage composition of spore trap DNA samples likely because the concentration of target DNA is too low. Our study highlights the value of both monitoring *P. ramorum* dispersal and detecting other *Phytophthora* species, in order to predict and understand changes in disease severity in UK tree host species. We demonstrated that use of both rain and wind-borne inoculum capture methods might be advantageous due to the variety of weather conditions under which inoculum can disperse from an infected stand. Although there was no evidence of the EU1 lineage being present in our study site, lineage-specific qPCR assays can allow rapid testing of the prevalence of different *P. ramorum* lineages in bark and mycelial samples, thus allowing early detection of new lineage incursions.

2 Background

Since 2009, the oomycete pathogen *P. ramorum* has been spreading through the UK's commercial larch population leading to large-scale tree death and losses by felling to slow the pathogen's spread. Two *P. ramorum* lineages exist in the UK: EU1, causing damage in England and Wales and EU2 subsequently found causing damage in Northern Ireland and southwest Scotland (King *et al.* 2015). EU2 grows faster and is more aggressive than EU1 when colonising larch bark, which may cause the trees to die more rapidly (Franceschini *et al.* 2014). Pathogenicity may come at the cost of sporulation as EU2 produces smaller sporangia and sporulates at lower levels on larch needles than EU1 (Harris *et al.* 2021). *Phytophthora ramorum* is a heterothallic species, requiring two compatible mating types for sexual DNA recombination but all isolates tested from UK host species have the same A1 mating type, which prevents out-crossing between the lineages (Brasier & Kirk 2004; van Poucke *et al.* 2012) However, the EU1 and EU2 lineages are now established in larch stands <10km apart in South-West Scotland and if their ranges begin to overlap, there is a potential risk of somatic DNA recombination occurring between the lineages when they come into physical contact with one another (King *et al.* 2015).

Regular monitoring of rain and wind-dispersed inoculum is an essential tool in forecasting and managing severe disease outbreaks caused by pathogens such as *P. ramorum*. Monitoring can also reveal more about the dispersal distance, seasonality and environmental factors

required for spore release in the early stages of a new disease outbreak. Successful monitoring schemes rely on development of methodologies specific to the targeted disease. A Scottish Forestry-funded project has been researching the most effective means to capture windborne *P. ramorum* inoculum to improve monitoring and forecasting of *P. ramorum* outbreaks, comparing the performance of wind vane traps against two other well-established trapping techniques to identify the most suitable and efficient method for capturing *P.ramorum* spores (Frederickson-Matika *et al* 2020). The trapping techniques varied by dispersal vector (wind vs rain), how wind-borne spores are collected (passive vs forced air flow onto a spore tape trapping medium), the temporal resolution provided (daily vs weekly) and set-up and maintenance costs. A qPCR assay with specificity to *P. ramorum* was used to quantify inoculum levels after DNA extraction from the spore tapes or rainwater.

This Plant Health Centre-funded project aims to add further value to the Scottish Forestry-funded project by:

2.1 Testing whether DNA metabarcoding is a suitable alternative to qPCR

Metabarcoding provides the potential advantage of detecting a range of airborne Phytophthoras, in addition to *P. ramorum*. Previous studies have proven the suitability of metabarcoding for assessing *Phytophthora* species diversity in soil samples (Riddell *et al.* 2019a) and insect traps (Tremblay *et al.* 2019) by sequencing a stretch of genomic DNA that is universal to all pathogens but still unique (a barcode) to each species, circumventing the need to develop species-specific assays. The technique has recently been applied to spore-traps to compare the fungal composition of deciduous and coniferous forests (Redondo *et al.* 2020). Metabarcoding has successfully revealed the presence and diversity of *Phytophthora* species in plant nursery water and root samples and highlighted potential effects of nursery practice on the spread of these diseases (see https://www.forestresearch.gov.uk/research/global-threats-from-phytophthora-spp/).

Metabarcoding of spore trap DNA samples would allow simultaneous monitoring of current *Phytophthora* pathogens as well as surveillance for *Phytophthora* species that are increasing in prevalence and may become problematic for our forest tree species in the future (for example *P. pseudosyringae, P. foliorum* and *P. kernoviae* in addition to *P. ramorum*).

2.2 Comparing the sensitivity of metabarcoding with qPCR for detection of P. ramorum

Quantitative PCR is often held up as the gold standard in DNA detection and quantification, making it an ideal tool for diagnostics and disease monitoring. Therefore, it is important to test the limitations of the nested PCR approach used to create metabarcode libraries and whether the technique can detect *P. ramorum* DNA down to the lower levels of *P. ramorum* found by qPCR in the Scottish Forestry-funded project.

2.3 Investigate the P. ramorum *lineage composition in South-West Scotland*

Further value can be gained from the Scottish Forestry-funded project by investigating the lineage composition of *P. ramorum* inoculum. The EU2 lineage predominates in South-West Scotland within a management zone for *P. ramorum* and to date has been contained to this area with a few sporadic cases in Northern Ireland (King *et al.* 2015). Conversely the EU1 lineage colonising larch in England and Wales is only found occasionally in the management zone. EU2's success over the EU1 lineage in the area could be explained by founding of larch disease exclusively by the EU2 lineage or by a competitive advantage of EU2 over EU1, such as more aggressive colonisation of larch bark (Harris *et al.* 2013, Webber *et al.* 2014). Two more lineages of *P. ramorum* have been introduced to North America (NA1 and NA2) as well as EU1 and all have had major impacts on species of oak (Rizzo *et al.* 2005). The possibility of the arrival of North American lineages into the UK adds further risk to our tree species. We

propose to use a new lineage-specific *P. ramorum* qPCR assay (Feau *et al* 2019) to analyse the lineage composition of the airborne inoculum captured in the spore traps.

3 Methods

Spore trap set-up and molecular methodology was undertaken by Forest Research and Forestry and Land Scotland staff, with their work funded by these organisations.

3.1 Site and preparation

An experimental spore-trapping site (grid ref NX4370-7457) was established at Lamachan in the *P. ramorum* management zone in Dumfries and Galloway to determine the capture efficiency of wind vane traps during the 2019 season. Since 2016, this site had been regularly assessed for *P. ramorum* incidence and severity as part of a PhD research programme and during 2018 fine shoot dieback had been widely recorded in the larch stand (Heather Dun, pers comm).

Having chosen the site, the adjacent area to the larch stand was cleared of several Sitka spruce trees that would otherwise have influenced wind flow over the site and deep surface brash that was unsuitable for stable trap placement and ease of access. The trapping was due to begin in April, but access issues to the area delayed site preparation and equipment installation was completed in early September 2019.

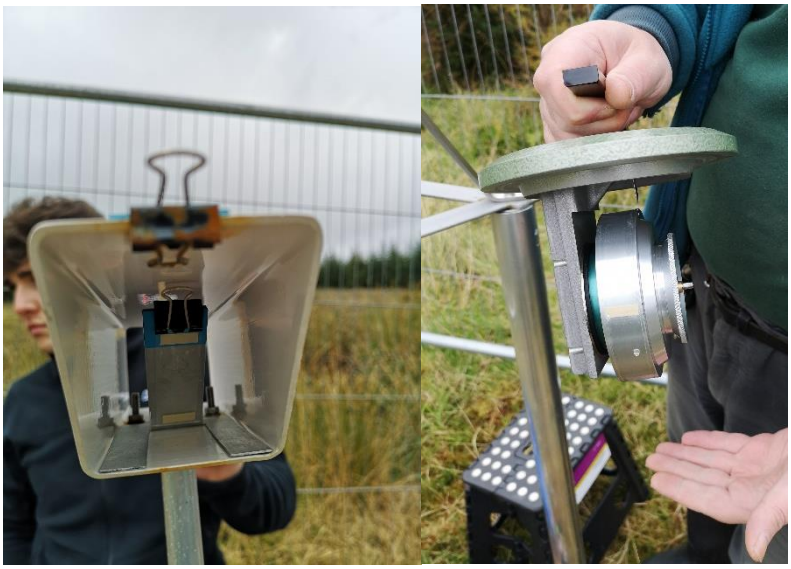
Three trapping sites were cleared at 1m (NX43680 74533), 9m (NX43685 74541) and 80m (NX43710 745602) distance from the stand in a NE direction, with Heras fencing to enclose and secure a wind vane trap, Burkard trap and rainfall trap at each position. At the 9 m distance a Vantage Pro2 (Weatherlink™) weather station recorded rainfall, wind speed, wind direction, temperature and relative humidity (Figure 1) at 10-minute intervals and data were downloaded at every visit.

Figure 1. Left to right: rainfall trap, weather station, wind vane trap and Burkard trap in situ at one of the three trapping locations

3.1.1 Wind vane and Burkard traps

Each wind vane trap contained 2 'catch' slides, one flat on the upper surface of the trap at 1.5m and one at a 45° angle inside the trap (Figure 2). Burkard traps were mounted so that the intake was also 1.5 m above and at 90° to the ground. The Burkard contained one long piece of tape (the equivalent of 7 slides) mounted around a clockwork revolving drum that was wound weekly (Figure 2). Trap flow rate was verified and battery exchanged weekly to maintain the recommended flow rate in the Burkards. However, loss of flow rate, due to loss of battery power, became a notable problem during weeks 9-11, possibly due to low temperatures. This issue could be dealt with in future with use of a larger capacity battery and, possibly, a solar panel to top-up power supply.

Figure 2 Left: Inside wind vane trap showing angled lower slide & upper horizontal slide held by bulldog clip. Right: Metal clockwork drum of Burkard with peripheral tape band as trapping surface.



The trapping surface for wind vanes and Burkard traps was a vaseline/paraffin wax mixture applied to a melinex tape support. To minimise background contamination, tapes were prepared in a laboratory that does not handle *P. ramorum* and were stored in slide mailers (wind vanes) or canisters (Burkards) until required. Some tapes were maintained in the freezer as negative controls and were processed with field samples to rule out background contamination. Traps were changed weekly on the same day at roughly the same time, from 17th September until 3rd December 2019.

After collection, each Burkard tape was cut into 7 pieces using aseptic techniques, carefully labelling the start and finish point for each day of the week. All tapes were stored at -20°C before DNA extraction.

3.1.2 Rainfall traps

Rainfall traps were made from 2l milk bottles, pre-sterilised in 10% bleach, with a funnel of 16 cm diameter as the surface area (Figure 1). In weeks 6, 7 and 8 two additional traps were set up within an infected tree showing needle symptoms on the edge of the focal stand of larch; one at the base of the tree next to the main stem and another 2m from the ground in the centre of the tree to measure the levels of inoculum washed off the tree during periods of rain. Traps were exchanged weekly. Rainwater volume was recorded before vacuum-filtration though a 3µm Millipore filter, and the filter was stored at -20°C.

3.2 DNA extraction

3.2.1 Tapes

Burkard tapes from each weekly collection were cut into 7 equal pieces, each representing one day, using aseptic techniques. Each piece of Burkard tape, and individual tapes from wind vane traps, each representing a 7-day exposure period, were bisected longitudinally. One half was stored at -20°C, while the other was cut into 4 pieces and DNA extracted using the MasterPure Yeast DNA Purification kit (Lucigen). The kit protocol was followed except that at step 1, the sample in 300µl kit reagent was shaken with microbeads on a MPBio. In the final step, DNA was resuspended in 30µl elution buffer. As Burkard trap DNA extractions each represented one day of sampling, the 2µl of DNA sample was pooled into a single tube by the week of collection (seven samples per pool, totalling 14µl) to make the test comparable with the other wind and rain traps where each DNA extraction represented a one-week sampling or exposure period. Pooling the Burkard samples in this way did run the risk of diluting *P. ramorum*-positive samples with samples that that did not contain *P. ramorum* DNA, but it was hoped that the nested approach in the metabarcoding would bring *P. ramorum* DNA up to detectable levels.

3.2.2 Rainwater filters

Filters were cut in half using aseptic techniques and DNA was extracted from a half filter, the other half being stored at -20°C. For some samples there were multiple filters and these were extracted separately. The same extraction protocol was followed as described above for tapes (see section 3.2.1), with the following modification. Due to the almost complete absorption of 300µl lysis reagent by the filter, extraction was in 800µl buffer (400µl kit lysis reagent plus 400µl Longmire buffer). After the MPBio step, 600µl eluate was removed for further processing. Finally, DNA was resuspended in 30µl elution buffer.

3.3 Lineage-specific qPCR assay

A total of 93 *Phytophthora ramorum*-positive samples were selected from the Scottish Forestry project and tested for the presence of EU1 and EU2 lineages using a qPCR assay developed by Feau *et al.* (2019), with primer modifications to improve the assay provided by personal communication with the authors. Samples were first tested with EU2 since it is the predominant lineage in South West Scotland and expected to appear most frequently.

To test the Burkard spore trap samples, the same DNA pool created for metabarcoding was used consisting of 7 daily DNA samples pooled in equal quantities (2µl per daily DNA sample into a single tube to make 14µl) by week of collection for direct comparison between the trapping methods. Each DNA sample used two technical replicates and 2ul of DNA as well as lineage-specific DNA standards from 1ng to 0.1pg to quantify the level of *P. ramorum* present. Although pooling the Burkard samples ran the risk of diluting *P. ramorum* signal out, there was a good chance that the lineage-qPCRs would be sensitive enough to detect *P. ramorum*.

3.4 Nested PCR and indexing of oomycete-positive samples

To amplify oomycete sequences, all 101 DNA samples from rain, wind and Burkard traps from the Scottish Forestry project were processed through a nested PCR that enriches for a 250bp region of the ribosomal RNA (rRNA) internal transcribed spacer (ITS1) gene (Scibetta *et al.* 2012). The same pools of Burkard DNA samples created for the lineage-specific qPCR were used for metabarcoding analysis. The nested PCR primers are designed to be specific to *Phytophthora* but will also amplify some closely related oomycetes such as downy mildews. Samples that produced a product in the nested PCR were oomycete-positive and were identified by gel electrophoresis on a 1% agarose gel. Ninety-one oomycete positive PCR reactions were prepared for sequencing following the protocols described for 16S

metagenomic sequencing library preparation (Illumina, 2013) and each uniquely indexed to assign reads back to each sample.

All DNA libraries were quantified, normalised and pooled for paired-end (2x250bp) sequencing on an Illumina flowcell using the MiSeq v.2 500bp standard kit at the James Hutton Institute, Dundee. Standard DNA control mixes containing four synthetic sequences at known concentrations were also run through metabarcoding, indexing and sequencing to calibrate the plate and check for cross-contamination between samples.

After quality control and de-multiplexing, FASTQ files for sample reads were exported for bioinformatic analysis. Sequence data were processed using the bioinformatics software 'THAPBI PICT' (https://doi.org/10.5281/zenodo.4529395). THAPBI PICT performed quality trimming and merged paired reads, removed primers and collated unique sequences with a minimum sample abundance of 50 reads. The unique sequences were matched to a species in the THAPBI PICT tool's curated ITS1 database using the default classifier, which requires perfect matches or at most a one base pair difference. Any sequences not matching the curated database, but which matched a broader set of sequences downloaded from NCBI database based on 2bp difference, were reported to genus only. If a sequence did not match anything within a 2bp difference, then it was marked as 'unknown'. For the purposes of this report, the most abundant unknown sequences were run through BLASTn (Altschul *et al.* 1990) in GenBank using default parameters to identify the closest genus in the GenBank nt database.

3.5 Statistical data analysis

3.5.1 Data cleaning

Prior to analysis, the date of sample collection was converted into a numeric 'days' variable with 17th July 2019 as timepoint '0' (zero) and all timepoints referred to as the number of days beyond this day (range 0-72 days [11 timepoints, weekly intervals]). A binary variable denoting the presence or absence of *P. ramorum* in each trap at each timepoint was created for each trap along with a unique trap identifier. The two rain traps that had been placed into the infected tree within the stand later in the trapping period were also removed as these were not part of the original experiment (reduced N of traps from 11 to 9).

3.5.2 Analysis of P. ramorum data

Analysis was conducted in R (version 4.0.2, R Core Team 2018), with graphics produced using ggplot2 in R (Wickham, 2016).

A generalized linear mixed-effect model was used to determine if the presence/absence of *P. ramorum* differed between rain and wind traps. A binomial model was fit with a logit link using the lme4 package (Bates *et al.* 2015). Trap type and transect position were fit as fixed effect categorical covariates. Trap ID was included as a random effect to account for repeated measures. Initially timepoint was included as a fixed effect covariate as time in days. However, this assumes that the relationship between *P. ramorum* detection and time is linear. Natural cubic splines were applied to days with 2, 3 and 4 degrees of freedom. These models were compared using Akaike's Information Criterion (AIC) values and splines with 2 degrees of freedom selected as the optimal model. The addition of an interaction between trap and time also improved model fit and was retained.

3.5.3 Final model for P. ramorum data

Phyt.ram ~ Wind_or_rain + Transect_pos + splines::ns(day, df = 2) * Wind_or_rain + (1 | ID)

Model fit and assumptions were tested using the DHARMa package (Hartig 2020). The Durbin-Watson test for autocorrelation was not significant, confirming there was no residual

temporal autocorrelation in the residuals. Estimated marginal means (predicted values) for each trap type and timepoint were calculated using the emmeans package (Lenth 2020).

3.5.4 Analysis of species diversity

Species diversity was analysed by counting the number of different species caught in each trap at each timepoint. Read counts in the 'Unknown' category were assigned a count of 1. This created a count variable potentially ranging from 0-10. However, in this dataset the count range was 0-5. A Poisson regression model was fitted with transect position, day, and trap type as fixed effects and trap ID as a random effect. All three trap types were compared using this method. The addition of natural splines for time in days did not improve model fit and the interaction of day and trap type was non-significant and not retained.

3.5.5 Final model for species diversity

Diversity ~ Trap + day + Transect_pos + (1|ID)

No residual temporal autocorrelation or overdispersion was detected in this model. Adjusted marginal means were extracted using the emmeans package and pairwise comparisons made with Tukey HSD correction for multiple comparisons.

4 Results and discussion

4.1 Testing whether DNA metabarcoding is a suitable alternative to qPCR:

4.1.1 Metabarcoding detection of P. ramorum

A total of 101 samples (39 rainwater; 31 Burkard; 30 wind vane) from the Scottish Forestry Project were tested using the nested PCR (Scibetta *et al.* 2012). Electrophoresis of the PCR products identified 91 samples, including 37 rain trap, 28 Burkard and 26 wind vane samples that were positive for the presence of oomycetes and were subsequently indexed for metabarcoding. After sequencing and processing sample data through the THABIPICT pipeline, 89 samples successfully returned sequences while two libraries failed to return any data (Table 1). The number of reads per sample varied from 3417 to greater than 20,000.

Table 1. Table listing the frequency of detection of each species, genus or 'unknown sequence' that yielded reads in each trap type over the eleven-week spore trapping period, from a total of 27 Burkard, 25 wind vane and 37 rain trap metabarcode libraries

	Trap type		
Closest sequence match	Burkard	Wind vane	Rain trap
***Phytophthora* species:**			
P. ramorum	7	8	23
P. foliorum	0	2	0
P. obscura	0	1	0
P. plurivora	0	1	0
Downy mildew genera:			
Bremia spp.	16	6	15
Peronospora spp.	6	3	5
Hyaloperonospora spp.	4	0	3
Paraperonospora spp.	1	0	0
Plasmopara spp.	6	3	0
Sequences with no match to curated database:			
'Uncultured *Phytophthora*'	13	4	3
'Unknown species'	13	13	20

Phytophthora ramorum DNA was amplified in 38 of the 89 oomycete-positive samples yielding sequence reads: 23 rainwater, 8 wind vane and 7 Burkards (Table 1). In eighteen of these samples, *P. ramorum* was the only sequence present in the sample. Comparison of *P. ramorum* detection by metabarcoding versus the *P. ramorum* species-specific qPCR assay showed that metabarcoding detected 70% of the *P. ramorum* positive samples identified by qPCR as well as an additional two samples. There was no correlation between the *P. ramorum* DNA quantities in the species-specific qPCR from the Scottish Forestry project and read numbers in the corresponding metabarcode sample in our project. This is unsurprising as metabarcoding sequencing creates libraries derived from the product of two PCRs so it is likely that PCR biases and individual target abundances will affect the read numbers in the final library and make it difficult to accurately quantify each sequence in a sample.

Table 2. Number of samples in which *Phytophthora ramorum* was detected by metabarcoding in each of three trap types over the eleven-week trapping period in autumn 2019.

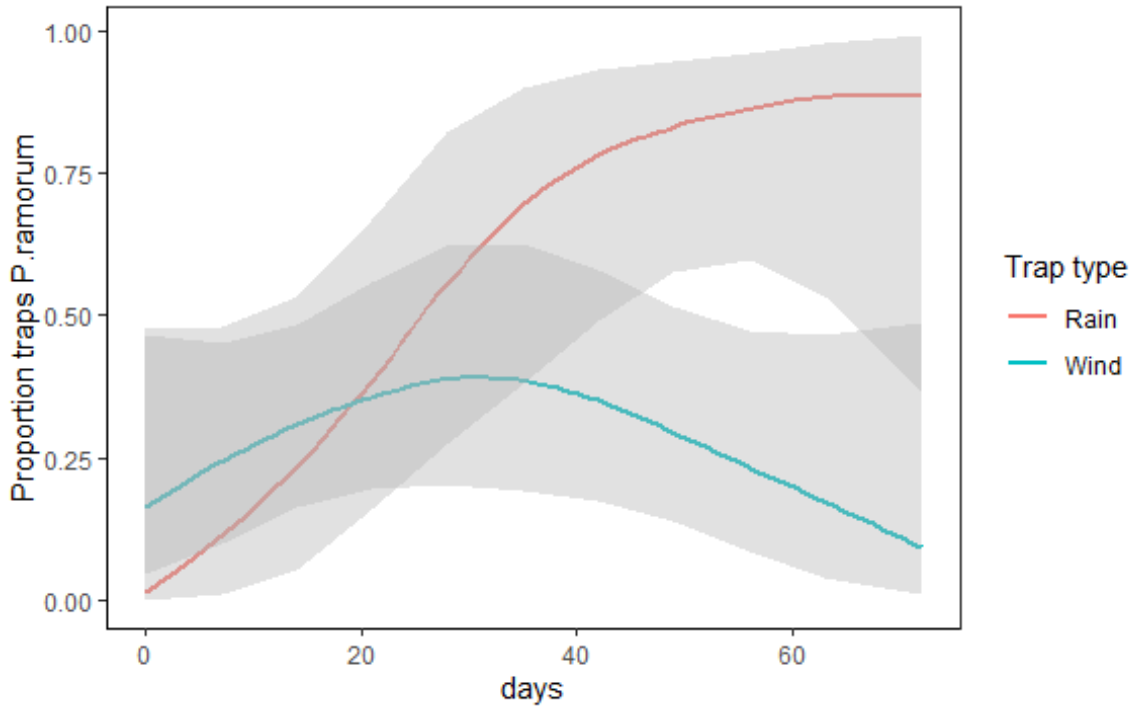
	Number of *P. ramorum* positive samples detected by the three trapping methods each week		
Week	Burkard (n=3)	Wind vane (n=6)	Rain trap (n=3)
17/9/201	-	1	-
24/9/2019	-	3	-
1/10/2019	-	1	1
8/10/2019	-	1	2
15/10/2019	3	1	1
22/10/2019	1	-	2
29/10/2019	-	-	2
5/11/2019	-	-	2
12/11/2019	2	-	2
19/11/2019	1	-	2
28/11/2019	-	-	2

Rainfall traps appeared to yield the most *P. ramorum* positives (Table 2) although statistically these traps did not capture significantly more *P. ramorum* than wind-deposition trapping methods. However, there was a significant interaction between time and trap type (p=0.02). The ANOVA table for the GLMM is shown in Table 3. Analysis combined both Burkard and wind vane as 'wind deposition' trapping methods and compared these with the rainfall traps owing to small sample sizes. The interaction between trap type and time in terms of *P. ramorum* detection is only significant in weeks 8-10 (05/11/2019-19/11/2019) as the effect of trap type differs according to timepoint – more rain traps detected *P. ramorum*, but only in later time points (Figure 3).

Table 3. ANOVA table showing analysis for effect of trap type (wind or rain) and whether trap type interacted with time. Rain traps were compared with Burkard and wind vanes as a single wind-based trap group owing to low sample numbers.

	Chi sq	Df	Pr(>Chisq)
Wind_or_rain	3.45	1	0.06
splines::ns(day, df = 2)	3.04	2	0.22
Transect_pos	3.76	2	0.15
Wind_or_rain:splines::ns(day, df = 2)	7.55	2	0.02

Figure 3. The adjusted marginal means for each trap type by time. On the y axis are the proportion of traps which detected *Phytophthora ramorum* and the time in days is shown on the x-axis. The grey shading represents the 95% confidence intervals around the estimated marginal means. Only weeks 8-10 (days 49-63) have non-overlapping confidence intervals which highlights the time period over which rain traps captured significantly more inoculum than Burkhard and wine vane traps. Red line represents rainwater; blue line represents both Burkard and wind vane traps.



This result agreed with the qPCR data from the Scottish Forestry project (Frederickson-Matika *et al.* 2020) and indicates the value of the rainfall trap for monitoring short-distance inoculum dispersal. The two additional rain traps, placed within an infected tree in the focal stand in weeks 6, 7 and 8 of the trapping period, consistently captured *P. ramorum* DNA. These traps acted as positive controls and yielded very high quantities of target DNA in the qPCR assays conducted in the Scottish Forestry project. Therefore, any larger scale roll out of disease monitoring would benefit from incorporating rainfall traps to provide data on *P. ramorum* inoculum prevalence.

Wind vane and Burkard traps performed similarly in terms of *P. ramorum* inoculum capture as detected by metabarcoding. However, analysis of Burkard samples by qPCR in the Scottish Forestry-funded project yielded a much higher frequency of *P. ramorum* detections, identifying 55 Burkard trap samples containing the pathogen compared with the 38 detections reported by metabarcoding in this project. One possible explanation for the lower frequency of detection of *P. ramorum* by metabarcoding in the Burkard trap samples is that daily DNA extracts were pooled on a weekly basis for metabarcoding analysis, thus the individual samples in the pool were diluted. In the Scottish Forestry project qPCR was performed on daily DNA extracts to investigate daily patterns in inoculum capture in relation to climate variables. Metabarcode analysis of individual daily Burkard DNA samples might yield a higher frequency of *P. ramorum* detection. However, this was not done in this project due to the number of samples involved and associated costs. If metabarcoding is to be used for *Phytophthora* surveillance in Burkard traps in future, a single DNA extraction of an entire week's tape should form the basis of the analysis to prevent sample dilution and maximise the chance of detecting *P. ramorum*.

There was a notably lower frequency of *P. ramorum* detected using wind vane traps in 2019 in the Scottish Forestry project (Frederickson *et al.* 2020) compared to a pilot wind vane

trapping study conducted over a similar time period in the same region of SW Scotland in 2018 (Green *et al.* 2019). Based on weather data collected during the Scottish Forestry study in 2019, the low rate of *P. ramorum* detection could be explained by the fact that the prevalent wind direction was opposite to that expected, resulting in the spore traps being 'upwind' of the focal stand for the majority of the trapping period. A planned re-run of the trapping experiment in 2021 will place traps on different sides of the infected stand, rather than along a single transect, in order to explore wind direction as a factor in inoculum dispersal, as well as potentially trapping across a wider range of distances to test distance of inoculum dispersal from an infected stand.

The wind vane traps are being considered for a wider network of *P. ramorum* monitoring by the forest industry as they are considerably cheaper and easier to install and manage than Burkard traps which actively draw in air and are battery operated. The lower trapping position on the wind vanes might be more optimal for *P. ramorum* capture, as our study yielded six *P. ramorum* detections compared with two detections on the upper position.

The frequency of *P. ramorum* detection in all trap samples by metabarcoding was similar at the first and second trapping positions (1m and 9m from the infected stand, respectively), with 13/27 and 12/29 positive samples, respectively, but fewer detections of *P. ramorum* occurred at the third trapping position (80m from the infected stand and with 7/29 positive samples).

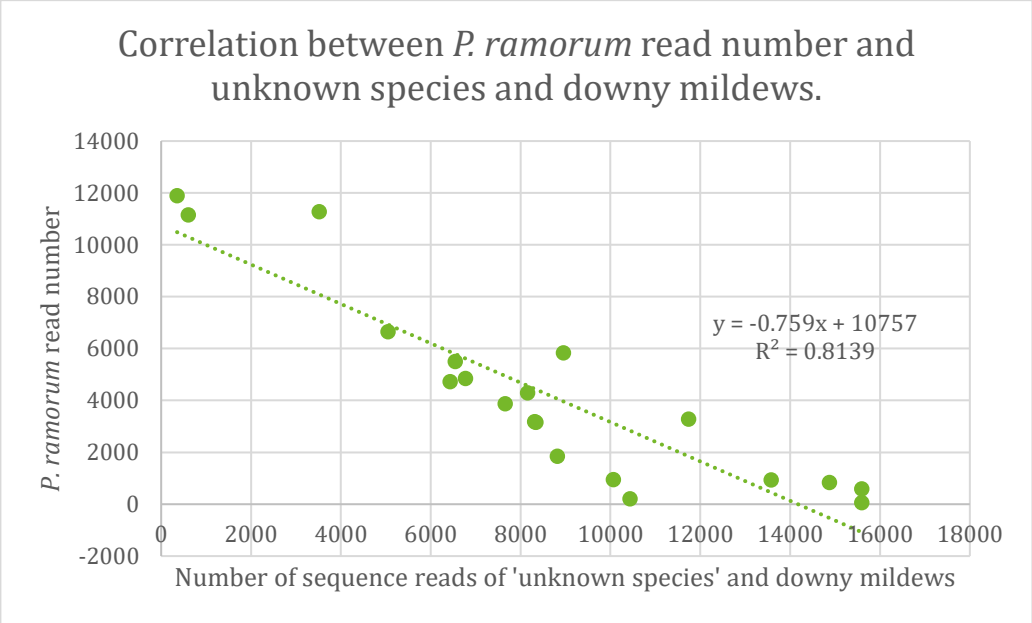
4.2 Metabarcoding detection of other species

DNA which matched other *Phytophthora* species was detected in just two samples collected from the same wind vane trap in week 8 (early November of the trapping period), at the position furthest from the *P. ramorum* outbreak. The lower wind vane trap sample contained a mix of *P. foliorum, P. plurivora* and *P. obscura* whilst the upper trap sample only contained *P. foliorum*. Both *P. obscura* and *P. foliorum* have previously been detected from rain trap samples in Perthshire (Riddell *et al.* 2019b). A close relation of *P. ramorum, P. foliorum* was first discovered in the US on Azalea (Donahoo *et al.* 2006) but was only discovered in the UK in 2016 in north-west Scotland on rhododendron (Schlenzig, *et al.* 2016). The wider distribution and UK range of *P. foliorum* is not known although its spores are aerially dispersed. *P. obscura* produces non-caducous (non-aerially dispersing) spores and has previously been isolated from soil samples beneath *Aesculus hippocastanum* and *Pieris*. This species is a close relation of *P. syringae and P. austrocedri* and has previously been detected in the US and Germany (Grünwald *et al.* 2012). *P. plurivora* is a soilborne *Phytophthora* commonly found throughout Europe, the UK and Canada (Jung & Burgess 2009) and is pathogenic on a broad range of hosts including several forest tree species. It is surprising to find two soilborne *Phytophthora*s on the wind vane traps. Aerial movement of these soil-dwelling *Phytophthoras* could occur by water splash of spores from the ground during heavy rain, or via vectors such as birds and insects, with the latter warranting further investigation. No reads for other *Phytophthora* species were detected in either of these samples. Interestingly, the lower position on the wind vane trap appeared better placed to capture inoculum from a wider variety of *Phytophthora* species, again strengthening the evidence for this position to be used should wind vanes be rolled out more widely for surveillance by the forestry industry.

Downy mildew genera (e.g., *Bremia*, *Peronospora*) were in high abundance, appearing in 78% of the samples and occurring most frequently at the beginning of the sampling period in September and early October. Downy mildews are obligate biotrophs on a large variety of herbaceous hosts and the decline in detection of these sequences in the traps as the experiment progressed would tie in with dieback of host foliage in the autumn. In 31 samples downy mildews were the only genera identified (14 Burkard samples, 11 rainwater samples and 6 wind vane samples) and similar high abundance has been found in other spore trap studies conducted in central Scotland at a similar time of year (Riddell *et al.* 2019b). However, co-occurrence of downy mildews with *P. ramorum* in a sample was relatively uncommon,

represented by just over 20% of samples and there was a negative correlation in read numbers between the two groups (Figure 4). Therefore, a high abundance of non-*Phytophthora* DNA in a sample tended to dominate the sequencing results, possibly masking *Phytophthora* if present at much lower levels. Another explanation for the negative correlation in reads between *P. ramorum* and downy mildews is that there is little overlap in the timing of their sporulation and the sequencing reflects oomycete diversity in those weeks. However, the Scottish Forestry qPCR analysis clearly indicates that *P. ramorum* was detectable in the initial six weeks of the trapping period, suggesting that non-*Phytophthora* inoculum could impact the times of year when metabarcoding could be utilised for *P. ramorum* detection in spore traps.

Figure 4. Graph showing the negative correlation between sequence read abundance of *Phytophthora ramorum* and non-*Phytophthora* (downy mildew genera) across spore trap samples



There were a high number of sequences discarded by the pipeline as 'unknown species' because they differed by more than 1 DNA base pair to the curated database sequence. Of the 89 samples that returned sequence reads, 51 samples contained sequences of 'unknown' species, and in 33 of these samples 'unknown species' were the only sequences found in the sample. Two particular 'unknown species' occurred in 22 samples in all three trap types. Manual BLAST analysis in the NCBI database identified these two unique sequences as 'uncultured *Phytophthora*', originally found in another metabarcoding project of holm oak soils in Spain (Catala *et al.* 2017). However, BLAST also showed that the closest matching *Phytophthora* species had 84% similarity to the unknown sequence, so the identity of the sequence is extremely uncertain. Three samples solely contained these uncultured *Phytophthoras*, which are also frequently found in public gardens and woodlands (Riddell *et al.* 2019a). The next 15 most frequent unknown species were run through BLASTn and all returned with closest matches to downy mildews.

4.3 Analysis of species diversity

Species diversity seen across the samples correlated significantly with the type of trap (p=0.04) (Table 4). Both Burkard and rain traps collected higher species diversity overall than the wind vane traps (Table 5). Species diversity varied significantly through the course of the collection period (p=0.01) with diversity across all traps decreasing over time, reflecting the initial high frequency of downy mildew genera at the beginning of the collection period which then tailed off around week 6 (Figure 5).

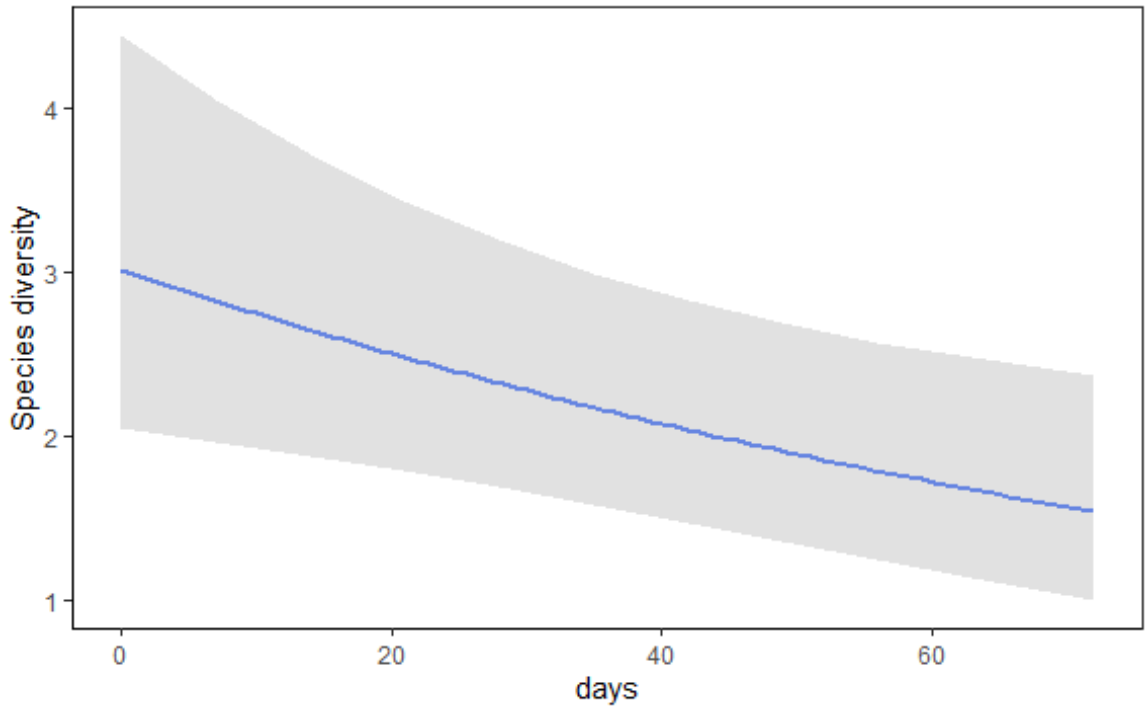
Table 4. ANOVA table - Species diversity

	Chisq	Df	Pr(>Chisq)
Trap	6.24	2	0.04
day	6.54	1	0.01
Transect_pos	0.24	2	0.89

Table5. Estimated marginal means for species diversity. Rate represents the adjusted mean diversity in each group (range 1-5, represents number of unique species detected in sample).

Trap	rate	SE	df	asymp.LCL	asymp.UCL
Rain_Funnel	2.15	0.26	Inf	1.68	2.73
Wind_Burkard	2.16	0.28	Inf	1.68	2.79
Wind Vane	1.34	0.23	Inf	0.97	1.87

Figure 5. The predicted values for species diversity on the y axis and time in days on the x axis. Grey shading represents 95% confidence intervals.



4.4 *Comparing the sensitivity of metabarcoding with qPCR for detection of P. ramorum*

Samples that were positive for *P. ramorum* in the Scottish Forestry qPCR that were missed by our metabarcoding analysis tended to have a high read number of downy mildew or unknown species in the sample (as discussed in section 4.2). It has previously been determined that the lowest minimum detection threshold for metabarcoding is one attogram (ag) (Cooke D., pers comm), which is five orders of magnitude greater in terms of sensitivity compared to the species-specific qPCR primers in the Scottish Forestry project where detection goes down to 100fg (Schena *et al.* 2012). This shows that the nested PCR in the metabarcoding protocol has

the potential to detect much lower levels of *P. ramorum* DNA than the species-specific qPCR assay. However, our results suggest a high abundance of other non-*Phytophthora* sequences in the sample may reduce this sensitivity and detection rate as they come to dominate the amplicon pool during the PCRs. Therefore, despite the improved sensitivity of the metabarcoding assay, high abundance of non-*Phytophthora* in a sample at certain times of year may prevent detection of *P. ramorum* at low levels, for example, at the beginning and end of the *P. ramorum* sporulation period. Development of a more *Phytophthora*-specific barcode is desirable to target this genus and exclude downy mildews from the sequencing, although this has proved difficult to date.

4.5 Testing the lineage composition of airborne inoculum

Out of 93 spore-trap DNA samples found to contain *P. ramorum* DNA using the species-specific qPCR assay in the Scottish Forestry project, only 13 were found to contain detectable levels of EU2 DNA using the lineage-specific qPCR. These included the two additional rain traps placed within infected trees in the focal stand in weeks 6, 7 and 8 of the spore trapping period (Table 6). No sample was found to contain DNA of the EU1 lineage.

*Table 6. DNA quantities detected for the thirteen samples that were positive for the presence of Phytophthora ramorum DNA using the EU2-specific assay compared with DNA quantities detected for the same samples using the P. ramorum species-specific assay. Columns indicate trap type, distance relative to the infected stand ('0' for the two rain traps placed within an infected tree in the stand) and mean DNA quantities detected in each assay. Samples marked with * had amplification in a single well of the two technical replicates.*

Trap type	Distance (m) of trap relative to stand	Week of spore trapping	Average Ct value for the EU2-lineage assay	EU2-lineage DNA (ng)	*P. ramorum* DNA detected (ng)
Wind - lower*	9	24/09/2019	38.8	0.0002	0.000021
Wind - upper	9	24/09/2019	38.3	0.00035	0.00028
Wind - lower*	9	15/10/2019	38.6	0.0006	0.000023
Rain*	9	15/10/2019	38.3	0.0007	0.000007
Rain	0	08/10/2019	37.3	0.001	0.0008
Rain	80	22/10/2019	30.35	0.03	0.0004
Rain	5	22/10/2019	28.75	0.09	0.2435
Rain*	0	29/10/2019	37.6	0.0002	0.0003
Rain	4	29/10/2019	23.8	2.6	6.475
Rain	5	29/10/2019	22.15	8.1	19.825
Rain	4	05/11/2019	28	0.1625	0.3
Rain	5	05/11/2019	27.2	0.27	0.7
Rain*	9	19/11/2019	38.8	0.0001	0.00004

We had expected a higher rate of EU2-lineage detection in the samples since it is the dominant lineage in South West Scotland. However, we suggest that the lower sensitivity of the lineage-specific primer sets could explain the differences in detection rate between the lineage-specific and species-specific *P. ramorum* assays. When referring back to the literature detailing the

development of the assay and the detection limits for the individual primer sets, we see that EU2-specific and *P. ramorum* species-specific qPCR primers have detection limits of 141 and 100fg, respectively, while the EU1-specific primers can only detect down to 704fg (Schena *et al.* 2006, Feau *et al.* 2019). Data from the Scottish Forestry project, which used the species-specific primers, showed that often the *P. ramorum* concentration (regardless of lineage) rarely exceeded 300fg in the 2ul added to the qPCR (excluding positive control rainfall samples from within an infected tree), indicating that the EU1 primers would not be able to amplify EU1 DNA in the sample at such low levels. The EU1 primers also have a lower efficiency (88%) which is to say that they do not amplify DNA to detectable levels consistently across a broad range of DNA concentrations. While the EU2 primers perform better than the EU1 primers (94.5% efficiency), they are nonetheless less efficient than the species-specific qPCR primers (99% efficiency) which give the most reliable amplification (Schena *et al.* 2012, Feau *et al.* 2019).

To conclude, the lineage-specific primers appear to have limited use for the purposes of detection of *P. ramorum* in spore trap samples as we have shown that they yield too many false negatives in samples known to contain *P. ramorum*. These lineage-specific primers may not be useful for other environmental samples such as soil and water either when the target DNA concentrations are likely to be low. The lineage-specific assays would, however, be useful for lineage testing of samples where high target DNA concentrations are expected, such as when sampling bark directly from lesions or from isolate mycelia. This would allow monitoring of EU1 and EU2 prevalence and range progression in a locality where they may be coexisting.

5 Conclusions

Our project set out to add further value to a Scottish Forestry-funded project that was testing and identifying the best spore-trapping methods to capture and monitor airborne *P. ramorum* inoculum, using a species-specific *P. ramorum* qPCR assay.

We have shown that metabarcoding is a valuable tool to detect both *P. ramorum* and other *Phytophthora* species. Our results show that the technique detected 70% of those *P. ramorum* positive samples identified by the species-specific qPCR in the Scottish Forestry project as well as additional positives. The *P. ramorum* metabarcoding detection rate may have been affected by Burkard trap sample dilution; an effect which can be corrected in any future work. Although *Phytophthora* species diversity was very low in our study, it nonetheless provides confirmation of the value of metabarcoding in early detection of multiple species from a single spore trap collection. More broadly, oomycete diversity did differ significantly between the trap types, and this finding merits continued investigation as to whether *Phytophthora* diversity varies according to trap type. It is interesting that we detected two soilborne *Phytophthora* species in the wind vane traps. The explanation for this finding is unclear although other spore-trap metabarcoding studies in Scotland have also captured soilborne *Phytophthora*. In terms of the most successful traps for capturing *P. ramorum* inoculum¸ rainfall traps seem to provide more consistent detection in the latter part of the season and would be valuable to supplement the wind vane spore traps in a larger-scale roll out of spore trapping.

One potential limitation of the metabarcoding method used in our study is that the primers for the nested PCR can amplify other oomycetes, such as the downy mildews, in addition to *Phytophthora*. This could be problematic when surveying for *P. ramorum* when there are high levels of airborne downy mildew inoculum that might dominate the PCR and potentially mask *Phytophthora* at low levels in the sample. Therefore, although metabarcoding is many times more sensitive than the species-specific qPCR assay, non-*Phytophthora* species may inhibit

P. ramorum detection as evidenced by abundant species diversity at the start of the sampling period.

It would be beneficial to monitor the lineage composition of *P. ramorum* in the management zone since there is a potential risk of somatic recombination between lineages should they come into physical contact. Our results show that the lineage-specific *P. ramorum* qPCR is not suitable to detect the low levels of *P. ramorum* DNA in spore trap samples because many *P. ramorum* positive samples identified by the Scottish Forestry project were not detected by the lineage qPCR. However, the lineage qPCR assay would be a useful tool in monitoring co-occurrence of the EU1 and EU2 lineages in infected trees since higher yields of DNA are obtained from bark samples.

The main implications of our current findings in the future design of spore networks and studies are outlined below;

- Our study has generated insight into the potential of different methods aimed at detecting a broader range of pathogens and lineages while monitoring *P. ramorum* dispersal. These methods give added value to the task of sample collection and processing, potentially allowing for improved disease forecasting and understanding of the effectiveness of management operations such as felling.
- It is advantageous to target rainborne as well as windborne inoculum given that spore dispersal occurs under a variety of weather conditions, thus future monitoring should involve both wind vane and rainfall traps.
- Metabarcoding enabled the detection of *Phytophthora* species other than *P. ramorum* at our forest site, although the time of year over which the study was conducted may not have been optimal for detection of a broader range of species. The incorporation of metabarcoding-based surveillance for these species and their prevalence will provide early data on the potential threats to our trees from shifts in *Phytophthora* species prevalence under climate change, further accidental introductions of new *Phytophthora* species and, possibly, hybridisation events between *Phytophthora* species.
- Rapid lineage testing of samples of high target DNA content is possible using the qPCR assays tested here. This will allow for an early assessment of risk due to possible somatic recombination and changes in lineage prevalence given the potential for North American lineages to enter the UK.

6 Suggestions for further work

- Future spore-trapping studies should be extended into spring and summer as this is the period of most rapid lesion extension by *P. ramorum* (H. Dun *et al.* unpublished) and there is evidence for sporulation on asymptomatic larch needles during summer (Harris, 2015). Other *Phytophthora* species are also likely to be more active at this time.
- In our study, sample sizes were too small to draw statistically sound conclusions on the dispersal distance of *P. ramorum* inoculum or to make direct comparisons between wind vane and Burkard traps in terms of capturing *P. ramorum* inoculum. Future projects would need to increase sampling sizes and sampling distances to solve this issue.
- Ideally, another barcode should be sought that is more specific to *Phytophthora* since windborne oomycetes such as the downy mildews are in high abundance during the autumn sporulation period of *P. ramorum* and they can dominate the sequencing outputs using the current ITS1 primers. A second oomycete barcode, RPS10, is under development at Oregon State University, USA, although it is not suited to spore trapping studies as it targets a broader suite of genera and species. The best option to

avoid downy mildews in sequencing would be to develop another *Phytophthora*-specific barcode but this has not proved possible to date.

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