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Title: Integrated management of lettuce downy mildew

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1. Industry Summary

Bremia lactucae, the causal agent of Lettuce Downy Mildew (LDM), was investigated in conjunction with AHDB project (CP 184) due to its impact on the lettuce industry and which focused on a range of aerial oomycetes including *Bremia*. The research aimed to understand the diversity of the pathogen population and characteristics affecting disease control. In addition, an in-field detection assay for aerial sporangia of *B. lactucae* was validated.

Molecular markers, specifically simple sequence repeat (SSR) markers, were developed and used to analyse the genetic diversity of *B. lactucae* collected from LDM outbreaks in the UK. Ten SSR markers identified more than 50 multi-locus genotypes (MLGs) originating from 29 disease outbreaks across a wide geographical area of the UK from 2019 to 2022. The population was genetically diverse and consisted of a mix of multiple distinct pathogen genotypes and minor genetic variants of each genotype. The presence of genetically identical, or almost identical, MLGs across years and/or sample location (county), indicated that the pathogen can survive as a genetic clone from one season to the next and these clones are distributed widely from, for example, Kent to Dorset and Lancashire to Fife.

The 52 UK *B. lactucae* samples sampled from 2019-2022 had a diverse range of virulence profiles. The differentials R4T57 D (*Dm4*) and cv Colorado (*Dm18*) were frequently overcome and generally ineffective against the *B. lactucae* population in the UK. A potential shift in virulence was observed, as the differentials cv. Dandie, cv. Balesta and cv. Bartoli were resistant until 2021 but were overcome by 20.6%, 32.3% and 47.1% of samples respectively, collected after this date. Many samples did not match the designated virulence profiles set by the International Bremia Evaluation Board (IBEB, 2023). There was no consistent association between virulence and the genetic diversity determined by SSRs.

DNA of *B. lactucae* was detectable in air samples collected from a commercial field site in 2021 before disease symptoms were observed, using both the LAMP and qPCR assays. A dispersal gradient was demonstrated, with better detection of the pathogen in samples collected from spore samplers located closer to the centre of the epidemic. However, *B. lactucae* sporangia were detected up to 100m from a disease source, demonstrating sporangial dispersal up to at least this distance.

This knowledge benefits the lettuce growing industry as follows:

- Information on the pathogen population helps to understand inoculum survival over winter, disease spread and the genetic diversity that drives changes in the phenotype.
- Knowledge of the virulence profiles of the UK *B. lactucae* population (the R genes in lettuce which are effective and not effective) helps determine cultivar choice.
- The development and validation of tests which are able to detect and quantify airborne inoculum of *B. lactucae* aid in disease prediction.
- Collectively, the study informs integrated crop protection practices for LDM.
- An industry consortium has formed to continue the *B. lactucae* virulence monitoring in 2023.

2. Introduction

Lettuce is a leafy vegetable crop grown for fresh consumption. In the UK, approximately 4000 ha of land was used to grow 95 thousand tonnes of lettuce worth £187 million (DEFRA, 2019). However, the industry is adversely affected by lettuce downy mildew (LDM), a foliar disease caused by *Bremia lactucae,* that reduces the quality, yield and therefore marketability of lettuce crops. LDM is more prevalent in cooler climates and is estimated to cause over £15 million in annual crop losses in the UK (AHDB, 2019).

B. lactucae is an obligate biotroph, which means a living host is required to complete its life cycle. This dependency on a living host makes it challenging to maintain *B. lactucae* for research. The main transmission of *B. lactucae* inoculum is through aerially dispersed sporangia. There is ongoing debate in the literature regarding the primary source of inoculum between lettuce growing seasons as to whether it originates as sporangia from other *Lactuca* spp. hosts (Lebeda et al., 2002; Thines et al., 2010) and/ or overwintering sexual oospores (Morgan, 1978, 1983). This pathogen is genetically complex with both asexual and sexual reproduction, the latter requiring co-infection by two mating types *B1* and *B2* (Michelmore & Ingram, 1980), although some strains have been reported as self-compatible (Michelmore & Ingram, 1982). The pathogen can also exist as a heterokaryon meaning a single hyphal strand contains multiple genetically diverse nuclei. In other words, *B. lactucae* has a range of means of generating genetic diversity and shuffling of genetic material. This genetic complexity poses challenges, as a high level of pathogen diversity in the presence of selection pressure (for example from host resistance genes or fungicides) increases the potential for adaptation against management practices.

The rapid emergence of *B. lactucae* strains capable of overcoming individual *Dm* resistance genes has led to a continuous search for novel sources of resistance (Parra et al., 2016). Prior studies have

documented the existence of strains with reduced sensitivity to fungicide active ingredients or the ability to overcome host resistance genes, underscoring the ongoing risk associated with evolution in *B. lactucae* (Brown et al., 2004; Cobelli et al., 1998; Crute et al., 1987; Lebeda & Zinkernagel, 2003b; Schettini et al., 1991; Trimboli & Nieuwenhuis, 2011). Consequently, the effectiveness of management methods is inherently influenced by the specific pathogen population being targeted.

A lack of effective host resistance and ineffective chemical control measures can result in challenges in managing LDM, and even crop failure. Responsible stewardship of disease management methods is therefore important. Understanding how diverse the *B. lactucae* population is both genetically and for disease traits could aid management decisions. Additionally, early warning of the presence of the pathogen in-field can prevent further spread and aid in targeted chemical control application with LDM management (Crandall et al., 2018).

To analyse the genetic diversity of the *B. lactucae* population, a method to identify variation at the appropriate level was required. Simple sequence repeat (SSR) markers, which are repeats of a short DNA motif, were chosen for this purpose (Vieira et al., 2016). SSR markers are generally neutral segments of the genome, theoretically not affected by selection pressure. These markers differ in motif repeat length due to imperfect DNA replication, which provides an appropriate degree of genetic polymorphism (Ellegren, 2004). SSR markers are defined as codominant, meaning the presence of multiple alleles at a single locus (position in the genome) can be detected. Despite the availability of a whole genome sequence of *B. lactucae* (Fletcher et al., 2019) there has been little development of SSR markers to monitor populations. Since *B. lactucae* often exists as a diploid organism with two sets of chromosomes, but can also be heterokaryotic, there is a high chance of detecting more than one allele at any particular locus/marker.

A set of SSR markers can provide a multi-locus genotype, essentially a genetic fingerprint for each sample tested, that is amplified using PCR primers specific to a single SSR locus. Multiplex amplification of multiple loci means that even with limited amounts of pathogen DNA a multi-locus genotype (MLG) can be obtained, which in a pathogen that is difficult to culture is beneficial (Grünwald et al., 2017). This type of SSR marker system is also used in criminal forensics and has worked effectively with similar polyploid oomycete pathogens for population diversity studies such as the Fight Against Blight project with *Phytophthora infestans* which causes late potato blight (Lees et al., 2006). Though SSR markers are neutral, they may be located near genes of interest which may result in an association with certain phenotypic traits (Zhan et al., 2005). In addition to this there are many software and multivariate analysis tools available for SSR data on polyploids that have mixed reproductive systems (Dufresne et al., 2014; Jombart et al., 2010; Kamvar et al., 2014, 2015).

In addition to genetic and phenotypic monitoring of *B. lactucae,* another approach to management is monitoring the presence of *B. lactucae* inoculum. As *B. lactucae* can take as little as 4 days to complete its asexual cycle (Verhoeff, 1960), which produces aerial sporangia, early detection of the pathogen in-field can aid in prompt management and mitigate further spread. Aerial sampling for *B. lactucae* has been carried out previously either with time consuming manual methods such as counting sporangia captured on silica grease impaction tape (Carisse & Philion, 2002; Fall et al., 2015) and more recently qPCR has been used on aerial samples (Dhar et al., 2020; Kunjeti et al., 2016). Although a qPCR assay to detect *B. lactucae* sporangia currently exists, qPCR can be sensitive to contamination, and utilises expensive thermocyclers, making them less practical for widespread use. Loop-mediated isothermal amplification (LAMP) assays have a high tolerance for cruder samples (Notomi et al., 2015; Wong et al., 2018), and being isothermal does not require thermocyclers (Wong et al., 2018). LAMP assays have been adapted for in-field detection devices or less sophisticated heating apparatus. Therefore, a real-time LAMP assay that amplified *B. lactucae* DNA was developed prior to this PhD study through an associated project funded by the AHDB (project CP184) which this project has tested via aerial sampling under commercial field conditions.

Aims:

One of the main aims of this research is to characterise the contemporary *B. lactucae* population by monitoring LDM outbreaks in the UK and testing samples for both genetic diversity with a newly developed SSR assay and for phenotypic traits such as virulence that are relevant to disease control.

Another key aim is to validate an in-field LAMP diagnostic assay for the detection of *B. lactucae* DNA from airborne sporangia captured using aerial samplers.

The outcome of this research will provide valuable insights to support decision making processes, in crop breeding along with fungicide and resistance gene stewardship. To date there is very little known about the contemporary UK populations of this pathogen.

3. Materials and methods

3.1. Sourcing of *B. lactucae* material and maintenance of isolates

3.1.1. Sample sourcing

Samples of *B. lactucae* were collected from lettuce plants that were naturally infected with the pathogen. The majority of samples received were from industry stakeholders, who were contacted directly or indirectly through publications and newsletters.

Interested parties received sample packs, including sampling instructions and equipment along with pre-paid return envelopes. Instructions requested at least four *B. lactucae* lesions on leaves per outbreak. In addition, four lesions were to be pressed onto supplied FTA ® card classic card (Whatman®, Merck/Sigma Aldrich), which would store *B. lactucae* DNA. FTA cards provided added insurance in cases where the *B. lactucae* leaf material did not survive postage or was unsuccessful at infecting lettuce seedlings. Leaf samples were crucial for bulking inoculum to test sample phenotypes.

From 2019 to 2022, 298 samples were received, 148 leaf samples and 150 FTAs, originating from 46 outbreaks from 12 UK counties. Two reference isolates from Naktuinbouw (NAK) were also used for comparison with UK samples in the SSR assay development. Additional international DNA samples stored on FTA cards were obtained from contacts in industry to increase the DNA panel to analyse and to observe for allelic variation outside of the UK. Permission to release industry FTA card results has not yet been granted.

3.1.2. Sample maintenance

A sporangial suspension from each sample was collected by washing the sporangia off sporulating lesions using deionised water. Lesions that were not sporulating or had sparse sporulation were placed in dark and high humidity conditions to promote sporulation. Lesions that did not look recoverable were pressed into FTA cards.

As an obligate biotroph, *B. lactucae* obtains nutrients from living host tissues and requires a rotation of susceptible seedlings to maintain or bulk up biological material. In a Perspex box, susceptible seeds of cv Green Tower were sown on damp filter paper. To maintain high humidity and prevent the filter paper drying out, the Perspex box was then wrapped in a clear plastic bag and placed in a north facing greenhouse (approximate daytime temperature of 15°C). Seedlings were sprayed with the sporangial suspension of the target *B. lactucae* sample when they were 7-days old or 2 cm long.

After 7-14 days, depending on the level of sporulation, sporangia would be washed off seedlings to make sporangial suspensions either for storage, DNA extractions or for maintenance.

3.2. Assessing genotypic diversity of *B. lactucae* population

Development of an SSR assay to genotype *B. lactucae* UK samples benefited from contact with a leading research group in University of California, Davis, USA (C. Acharya & R. Michelmore, personal communication) and mining of the published genome sequence (Fletcher et al., 2019) at Hutton. Candidate markers were screened against relevant samples of *B. lactucae* to examine their amplification and genetic variability and this process culminated in the following PCR protocol with ten SSR markers.

Each multiplex SSR assay reaction comprised 6.25 µl Qiagen Type-IT Multiplex PCR Mix, 10 pairs of locus-specific PCR primers at locus-specific primer concentrations (0.02 µM for 1001, Marker 1 and Marker 5; 0.09 µM for Marker 4 and 1008; 0.2 µM for 1011; 0.22 µM for Marker 2; 0.38 µM for Marker 7 and 0.14 µM for Marker 10), *B. lactucae* DNA template (either 1 µl of DNA suspension (~4 ng/µl) or a 2mm FTA card disc) and HPLC grade deionised water to complete volume to 12.5 µl. Negative controls used only water instead of the DNA template.

PCR products were diluted to a 1 in 20 ratio with deionised water. Diluted PCR products were denatured using 10.2 µl of a mix of 6 µl GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems) in 1 ml of HiDi Formamide and run at Hutton on an Applied Biosystems 3730 capillary electrophoresis machine according to the manufacturer's instructions. Peaks and named alleles (Figure 1) were manually called in GeneMapper (v5.0) before data analysis using the following R packages installed with their dependencies: *poppr* (v2.9.4), *magrittr* (2.0.3), *ggplot2* (v3.4.2), *reshape2* (v1.4.4), *adgenet* (v 2.1.10), *ade4* (v1.7-22) and *ape* (v5.7-1).

3.3. Assessing *B. lactucae* virulence

The International Bremia Evaluation Board (IBEB) standardised virulence test was used (IBEB, 2023). Seven-day old lettuce seedlings of different cultivars/lines with various resistance genes were sprayed with sporangial suspensions of *B. lactucae* and then incubated at 90% RH and 15°C. After 10 days, seedlings were assessed for symptoms following the IBEB index and given a sextet score for their virulence profile. A batch of 35 isolates were tested on differential set C before set D was released and then 12 isolates were tested on set D. If isolates matched a designated IBEB race it was noted, designated races, such as Bl: 36 EU, are set by IBEB based on economic importance and abundance.

3.4. Aerial monitoring for sporangia of *B. lactucae*

A LAMP assay developed at Hutton (CP184) to detect *B. lactucae* was validated and was applied to DNA extracted from samples collected by aerial samplers placed in field trials and in commercial field settings. The LAMP assay was compared to a qPCR assay (Kunjeti et al., 2016) using the same samples.

3.4.1. Monitoring aerial dispersal of *B. lactucae* sporangia

To determine the dispersal gradient of *B. lactucae* sporangia, six aerial samplers (Grips-99M rotorod samplers) were placed at varying distances from an inoculum source. A lettuce field trial at Hutton was inoculated by introducing lettuce plants infected with *B. lactucae* which were placed in each field plot when they each had at least one sporulating lesion. The plants were irrigated regularly to encourage disease spread and netted to prevent rabbit and bird damage.

An aerial sampler was placed in the centre of the lettuce trial field (position 0 m), the other five samplers were placed in a line on the axis of the prevailing wind at distances relative to the central sampler, at 5 m, 30 m, 60 m and 90 m in a north-easterly direction, and -5 m in a south-westerly direction. Aerial samplers were programmed to sample twice per week, 2-3 days apart, with the rotorod spinning for 2 mins on and 2 mins off between 04:00-23:00.

The trials ran on the**;** 03/06/2021-18/08/2021, 19/08/2021-14/10/2021 and 28/07/2022-17/10/2022. DNA was extracted from perspex Rotorods using the YCL kit (Qiagen) following the manufacturer's instructions before amplification using the qPCR assay as described by Kunjeti et al., (2016) and the real-time LAMP assay. Each sample was run in duplicate, along with a serial dilution of genomic DNA as a positive control and a HPLC grade water negative DNA template.

3.4.2. Monitoring for aerial sporangia at commercial field sites

Aerial sampling for sporangia of *B. lactucae* was carried out in commercial crops between 07/07/2020-09/10/2020, 23/05/2021-27/09/2021, and 08/07/2022-29/09/2022. Commercial sites in Cambridgeshire, England (courtesy of G's Growers Ltd), and Fife, Scotland (courtesy of Kettle Produce Ltd) were sampled using Rotorod samplers (Burkard, Rickmansworth, UK), or Grips-99M (Aerobiology Research Laboratories, Nepean, Canada), in which two petroleum-jelly (Vaseline®) coated Perspex rotorods, to which sporangia adhere, were spun by a motor. Aerial samplers were transferred periodically to different lettuce fields in the same region due to the short cultivation times of the lettuce crop. The sampling program and timings for the aerial samplers were the same as in the Hutton field trial. Each site had two samplers which were placed 50 m apart and adjacent to the

lettuce crop in line with the prevailing wind direction. DNA was extracted using the YCL kit (Qiagen) following the manufacturer's instructions before amplifying with the qPCR assay as described in (Kunjeti et al., 2016) and real-time LAMP assay (in thesis preparation). Each sample was run in duplicate, along with a serial dilution of genomic DNA as a positive control and negative DNA template using HPLC grade water.

4. Results

4.1. Genotypic diversity of *B. lactucae* samples collected from the UK

From 2019 to 2022, 298 *B. lactucae* samples were received, 148 leaf samples and 150 lesions pressed onto FTA cards, from 46 outbreaks across 12 UK counties. Not all live samples were recoverable, and on some FTAs the DNA collected was of low quantity, therefore there is a discrepancy between the number of samples received and those processed and successfully genotyped.

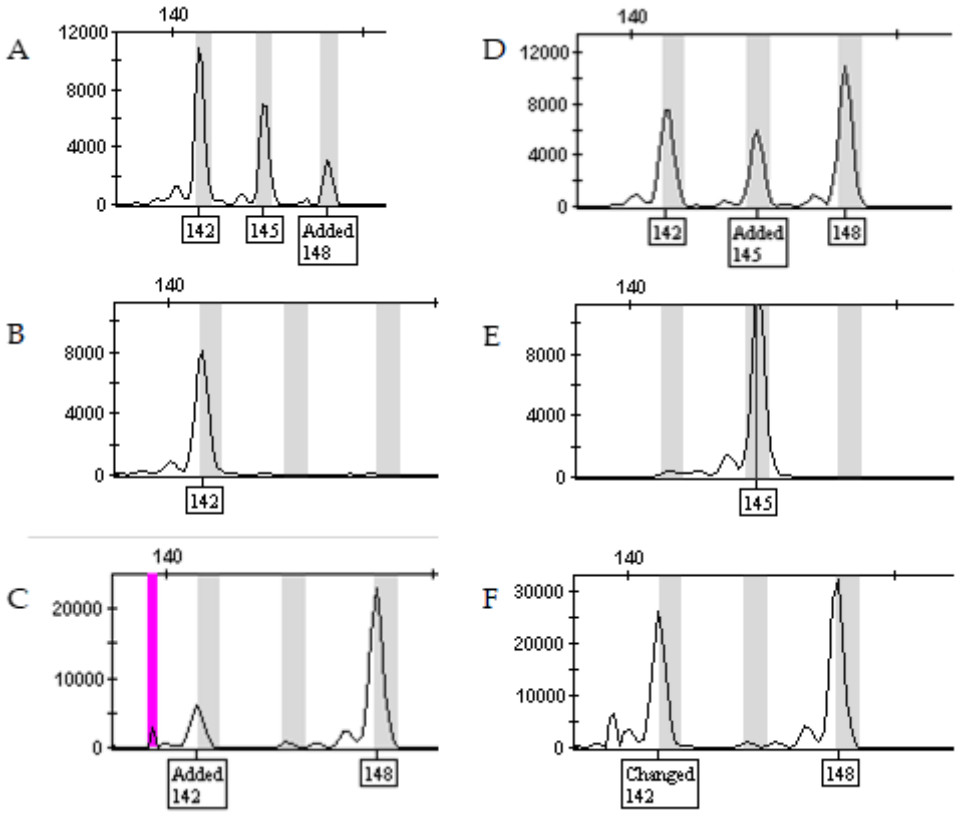

Figure 1, Examples of allele variation at SSR Marker 1. Multiple allele combinations and differences in peak height ratios were observed. Peak height variation may be attributed to nuclear sorting in heterokaryotic hyphae of B. lactucae. *A & D Triploid alleles, B & E single alleles, C & F di-alleles with differing peak heights.*

The 10 SSR loci had one to four alleles at each marker locus with changes in the number, size and ratio of peaks being observed across most markers. Marker 1 for example, generated four combinations (Figure 1), two of which (142/145/148 in A and D) and (142/148 in C and F) generated alleles that varied only in peak height ratio. Samples with unclear SSR alleles were re-tested yielding a total of 292 genotyped samples. Data from samples that were re-genotyped were kept to help detect any evidence of allele sorting and to prevent any bias in selection of which data to remove. Data was filtered to remove the 57 samples with missing alleles at two or more markers. In total 168 DNA samples were genotyped successfully with repeats bringing the total number of genotyping reactions to 254 (Table 1).

Sample numbers varied from year to year depending, partly on the suitability of weather conditions for LDM infection and spread. Fewest samples were supplied in 2020 due to Covid-19 which restricted travel and caused postal delays which affected the viability of live samples. In nineteen of the sampled disease outbreaks the lettuce cultivars were identified, three of the outbreaks referred to the lettuce morphotype instead of cultivar, e.g. iceberg, and two outbreaks referred to cultivar mixes. Most samples came from commercial crops with a few from allotments.

Table 1, Genetic diversity analysis of 254 genotype reads of UK B. lactucae *samples collected from 2019-2020 and genotyped at 10 SSR loci. Names of each index and their descriptions are shown in table footnotes.*

Year	N	MLG	eMLG[a]	SE[b]	H[c]	G[d]	lambda corrected[e]	E.5[f]	Hexp[g]
2019	59	33	17.20	1.82	3.05	12.30	0.94	0.560	0.509
2020	26	17	17.00	0.00	2.63	10.90	0.95	0.770	0.459
2021	94	54	20.10	1.87	3.67	26.60	0.97	0.671	0.469
2022	75	43	18.90	1.84	3.36	15.30	0.95	0.517	0.357
Total	254	135	22.10	1.79	4.47	49.40	0.98	0.559	0.479

[a] eMLG is the expected number of MLGs at the lowest common sample size (n=26), ≥ 10 based on rarefaction [b] SE is standard error for rarefaction analysis used to create eMLGs [c] H is Shannon-Wiener Index of MLG diversity (Shannon, 2001) the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations, is sensitive to number of different MLGs and evenness. [d] G is Stoddart and Taylor's Index of MLG diversity (Stoddart & Taylor, 1988), the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations. [e] lambda corrected is Simpson's Index (Simpson, 1949) of diversity, corrected to population size, values from 0-1, with 0 being no diversity, and 1 all genotypes are different. [f] E.5 is Evenness (Grünwald et al., 2003), values from 0-1, with 0 being complete unevenness. [G] Hexp is Nei's unbiased gene diversity (Nei, 1978), i.e. expected heterozygosity, values from 0-1, with 0 not heterozygous/ no diversity.

With 135 MLGs identified across the 254 UK genotype reads (Table 1) the population of *B. lactucae* may be considered genetically diverse. It should, however, be noted that this is the first survey to estimate the genetic diversity of *B. lactucae* using SSR markers and thus no benchmark exists for this pathogen. Despite fluctuations in the number of MLGs observed each year, once the data was corrected for sample size, the expected MLGs (eMLGs) and most other measures of diversity were similar across the years (Simpson, 1949) and there was no clear trend for change in overall genetic diversity across the years 2019 to 2022 (Table1).

The *K* clustering analysis is an unbiased analysis of genetic similarity amongst MLGs that was then studied in relation to the sample location and/or year of sampling. A total of 7 genetic clusters were identified and in many cases samples from the same outbreak, district and year were placed in the same cluster. This suggests that samples from the same location were genetically identical or nearly identical and represent a localised epidemic from a nearby source of primary inoculum (data not shown). Bruvo's genetic distance is a measure of the genetic differences between genotypes in organisms with a possible range of chromosome complements, termed ploidy (Bruvo et al., 2004). Representative samples from each *K* cluster group were selected and their Bruvo distances used to construct a phylogenetic tree to visualise the genotypic relatedness within and between the *K* cluster groups (Figure 2).

The tree showed a complex mix of degrees of relatedness with some groups of genetically identical or nearly identical samples and others with greater Bruvo distances of up to 0.25 from other samples. Many samples with values of 0 to around 0.05 were from the same outbreak; the three samples from the 2021_Bl1 outbreak in Cambridgeshire, for example, all grouped into *K* cluster 3, forming a single grouping in the tree that is representative of a genetic clonal lineage (Figure 2). Two samples from an outbreak in West Sussex (2019 Bl16 G&E) differed by less than 0.05 and probably represent minor variants of a clone. One *K* cluster group of interest, group 5, also formed a single group in the tree despite comprising samples from multiple counties and years: Norfolk 2019, West Sussex 2020, and Suffolk 2020 (Figure 2). This group 5 also has samples from Cambridgeshire from 2019, 2020 and 2021, suggesting a single clonal lineage that survived overwinter. In some cases, samples from the same outbreak were genetically quite distinct. For example, 2021 Bl10F 2 and 2021 Bl10H were both from the same Lincolnshire outbreak but in distinct parts of the Bruvo distance tree and in *K* clusters 5 and 4, respectively. In several *K* cluster groups (2, 4, 6, and 7) the clusters based on the Bruvo distance tree did not correspond to the *K* clustering. *K* cluster group 2, for example, comprised samples from 2021 and 2022 in Fife, 2019 Surrey, and some 2022 Lancashire samples that were in three different clades of the tree.

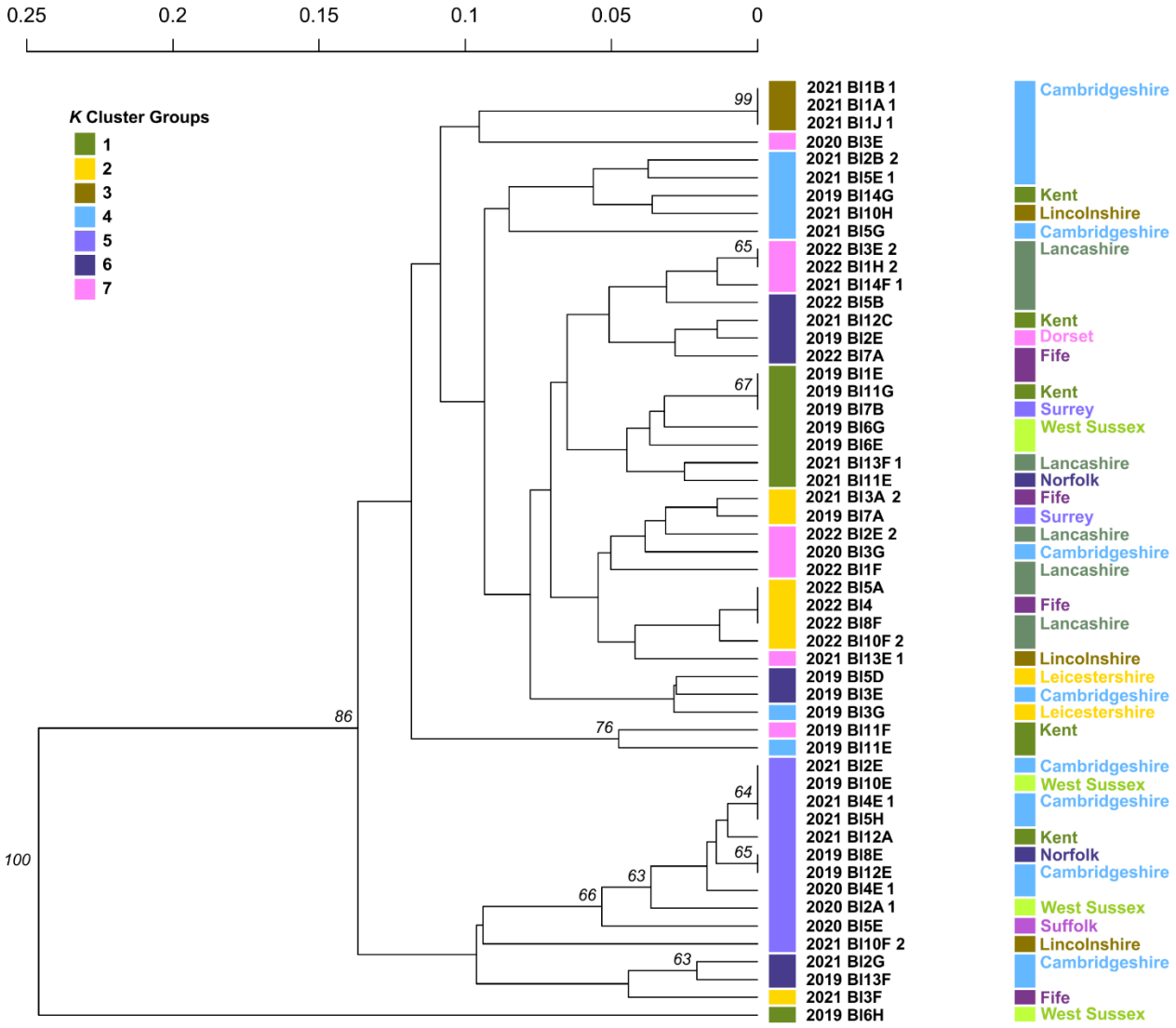

Figure 2, Phylogenetic tree constructed using UPGMA algorithm on Bruvo's distances between genotypes from representative samples of each K cluster group. MLL filters were applied to account for genotyping error. Node labels are percentages of 999 trees tested. Colours next to sample names are the K cluster groups, and colours to the right are associated with county (names included). The scale bar above the tree shows the Bruvo distance of nodes in the tree. As clusters do not perfectly correspond to dendrogram clusters it indicates a level of panmixia (sexual reproduction) and gene flow from other sources.

In addition, samples with three and four alleles at a single locus were observed and this was more common with certain SSR markers, particularly Marker 1 (Figure 1) and 7 (data not shown) than others. Peak height ratios also varied which adds an additional layer of genetic complexity (see discussion).

To summarise, a complex picture is drawn of a mix of genetically distinct clonal populations and sub-populations of their minor genetic variants, each of which may either be restricted to local spread, on the scale of outbreak or county, or sampled across a wider geographic scale and over more than one season. The population structure comprises clones that are often locally distributed but sometimes more geographically widespread. A single outbreak is generally caused by a single

pathogen clone but more complex outbreaks with more than one clone were recorded. Some clonal populations were widespread and found across more than one season indicating the existence of an overwintering inoculum survival mechanism.

4.2. Virulence profiles of *B. lactucae* samples collected in the UK

The virulence profile of 52 *B. lactucae* samples originating from 26 outbreaks across 11 counties of the UK was determined using the IBEB method. The IBEB differential set consists of lettuce cultivars and clones each containing different known host resistance (R) genes. Most of the UK *B. lactucae* samples assessed did not have a virulence profile that corresponded exactly to a designated IBEB race. All samples could overcome the universally susceptible differential cv. Green Towers, indicating that the testing was robust. Samples 2019_Bl2A and 2019_Bl2B from Dorset shared the same virulence profile as the designated IBEB race Bl:35 EU. Notably, virulence profiles of *B. lactucae* samples from the same outbreak tended to be similar, but not identical.

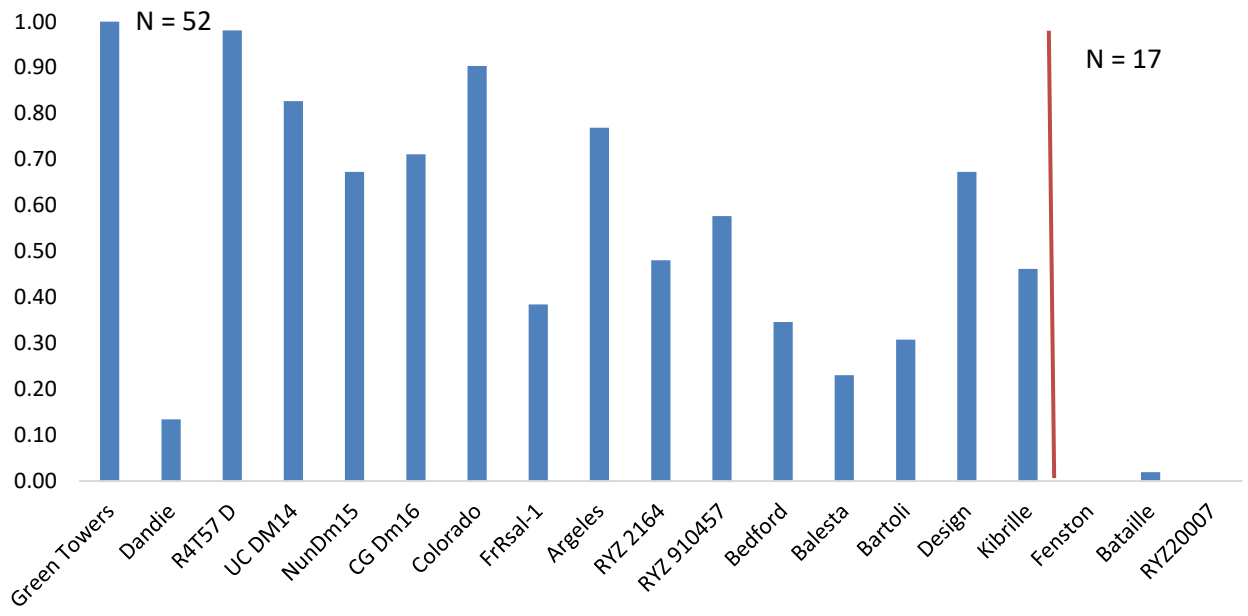

Figure 3 Proportion of B. lactucae samples that could overcome each differential line. Right of the red line are the 3 new differentials added to set-C to make set-D, which were only tested against 17 B. lactucae samples.

Of the *B. lactucae* samples tested, ≥90% could overcome the differential lines cv. R4T57 D (*Dm*4) and cv. Colorado (*Dm*18). The differential lines in set C that had resistance to most samples were cv. Dandie (*Dm*3), cv. Balesta (R54) and cv. Bartoli (R55) with only 13, 23 and 31% of samples overcoming their resistance, respectively (Figure 3).

Interestingly, only samples that were collected in 2021 and 2022 could overcome cv. Dandie, cv. Balesta and cv. Bartoli and this may reflect a shift in the population. Most samples with virulence against these three lines were collected from Cambridgeshire and Lancashire. All differential lines

from set C were overcome by at least one *B. lactucae* sample tested. Only one differential line from set D (cv. Bataille) was overcome from a single sample collected from Fife in 2022 though fewer *B. lactucae* samples were tested with this set (Figure 3). A general overview of host resistance is shown (Table 2) in which the proportion of samples that successfully infected differential lines is grouped by year and county.

4.2.1. Association between SSR genotype and virulence

The relationship between genetic fingerprint (by *K* cluster group analysis) and virulence profile was examined for cases in which both data sets were available (n=16). For the cases where direct comparison between DNA sample and virulence test could be made, samples from the same outbreak were used.

Some similarity in virulence profile of samples linked with *K* cluster group 5 was observed. A sample from Norfolk 2019 (2019_Bl8A) and one from West Sussex 2019 (2019_Bl10A), for example, had identical virulence profiles (Table 3). This identical virulence profile was similar to other samples within the cluster, such as 2020 West Sussex sample (2020_Bl2A) and two 2020 Cambridgeshire samples (2020_Bl4E, 2020_Bl4G). Some similarities were observed between samples from three outbreaks from Cambridgeshire in which four differentials were overcome in each sample, but no identical virulence profiles were observed across outbreaks (Table 2). Similarly, virulence profiles associated with cluster group 1, comprising Lancashire samples from 2022, Fife samples from 2021 and 2022, and Surrey samples from 2019, showed that virulence profiles were only similar when samples were sourced from the same outbreak (data not shown). However, there was commonality in that all live samples could overcome Dm4 and Dm14 in group 5.

To summarise, there is no strong empirical evidence from this study of a direct association between genotype and virulence profile beyond the generalities that samples from the same outbreak tend to have genotypes and virulence profiles with more similarity than to those from other outbreaks.

Table 2. Proportion of differential lines overcome by B. lactucae samples collection by UK county compared to two non-UK reference races. n = number of samples tested. Only differential set-C is displayed here. Colour scale ranges from red in which all samples can overcome the differential to green where no samples could overcome the differential. Each differential line contains a different set of resistance genes or factors (DM or R genes).

Year	County	N.	Green Towers 0	Dandie Dm3	R4T57 D Dm4	UC DM14 Dm14	NunDm15 Dm15	CG Dm16 Dm16	Colorado Dm18	FrRsal-1 Rsal-1	Argeles R38	RYZ 2164 Dm24/38	RYZ 910457 R52	Bedford R53	Balesta R54	Bartoli R55	Design R56	Kibrille Dm11,R57
2019		15	1.00	0.00	1.00	0.67	0.80	0.93	1.00	0.47	0.80	0.40	0.93	0.53	0.00	0.00	0.87	0.60
	Cambridgeshire	4	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.75	0.00	0.00	0.75	0.75
	Dorset	2	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00
	Fife	2	1.00	0.00	1.00	0.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	1.00
	Kent	2	1.00	0.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.50	0.00
	Leicestershire	1	1.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00
	Norfolk	1	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00
	Surrey	2	1.00	0.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.00	0.50	0.50	0.00	0.00	1.00	0.50
	West Sussex	1	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00
2020		3	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00
	Cambridgeshire	2	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00
	West Sussex	1	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
2021		22	1.00	0.14	1.00	0.95	0.64	0.82	0.86	0.23	0.91	0.64	0.41	0.05	0.55	0.41	0.45	0.50
	Cambridgeshire	8	1.00	0.38	1.00	1.00	0.13	1.00	1.00	0.00	1.00	0.25	0.13	0.13	0.88	0.38	0.25	0.63
	Dundee	1	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00	0.00
	Fife	4	1.00	0.00	1.00	1.00	1.00	1.00	0.50	0.75	0.50	0.50	0.50	0.00	0.00	0.00	0.00	0.25
	Lancashire	5	1.00	0.00	1.00	0.80	1.00	0.20	1.00	0.20	1.00	1.00	1.00	0.00	0.20	1.00	0.80	0.20
	Lincolnshire	4	1.00	0.00	1.00	1.00	0.75	1.00	0.75	0.00	1.00	1.00	0.25	0.00	1.00	0.00	0.75	1.00
2022		12	1.00	0.33	0.92	1.00	0.75	0.17	0.83	0.67	0.42	0.42	0.67	0.75	0.00	0.58	0.83	0.33
	Fife	3	1.00	0.00	1.00	1.00	0.67	0.33	0.67	0.67	0.00	0.33	0.67	1.00	0.00	0.00	0.67	0.00
	Lancashire	9	1.00	0.44	0.89	1.00	0.78	0.11	0.89	0.67	0.56	0.44	0.67	0.67	0.00	0.78	0.89	0.44
Total		57	1.00	0.13	0.98	0.83	0.67	0.71	0.90	0.38	0.77	0.48	0.60	0.35	0.23	0.31	0.67	0.46
	Reference1	1	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
	Reference2	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	1.00	1.00

Table 3, Virulence profiles of samples of B. lactucae collected from outbreaks that had samples in K cluster group 5 tested using IBEB Differential Set C. + indicates that the differential line was overcome. Outbreaks are separated by dotted lines.

		IBEB Differential Line																
		Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille
	R genes		Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38	Dm24/38	R52	R53	R54	R55	R56	Dm11,R57
	Grid Position		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
County	Sextet Value		1	2	4	8	16	32	1	2	4	8	16	32	1	2	4
Norfolk	2019_Bl8A	+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-
Cambridgeshire	2019_Bl9A	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
West Sussex	2019_Bl10A	+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-
Cambridgeshire	2019_Bl12A	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+
Cambridgeshire	2019_Bl12B	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+
West Sussex	2020_Bl2A	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-
Cambridgeshire	2020_Bl4E	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-
Cambridgeshire	2020_Bl4G	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
Cambridgeshire	2021_Bl2B	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+
Cambridgeshire	2021_Bl2D	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+
Cambridgeshire	2021_Bl2H	+	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-

4.3. Aerial sampling for sporangia

4.3.1. Aerial dispersal of *B. lactucae* sporangia

DNA of *B. lactucae* was detected in samples collected 100m from an inoculum source (disease symptoms) using both the LAMP and the qPCR assays (Figure 4**Error! Reference source not found.**). The aerial samplers located within in a 5 m radius of the trial had a higher incidence of detection for *B. lactucae* DNA using both diagnostic assays compared to samples collected from more distant samplers. In general, as the mean percentage of sporulating leaf area increased, there was a higher incidence of detection using the LAMP assay.

4.3.2. Aerial sampling at commercial field sites

Sampling in commercial fields in 2021 ran from 23rd of May to 23rd September. LDM outbreaks were reported at both commercial sampling sites in 2021. Weather conditions were generally conducive to disease throughout the growing period at both sites (data not shown). Contamination with inhibiting material, such as soil dust and insects, were detected in samples collected from the English field site.

A field at the Scottish site in 2021 showed LDM symptoms approximately 50m from one of the aerial samplers on the 16th of August (Figure 5). *B. lactucae* DNA was detected in samples collected prior to the reported LDM incidence with both diagnostic assays at this site. The qPCR assay detected *B. lactucae* DNA in samples collected on the 29th of July, a fortnight earlier than samples tested with the LAMP assay. However, the first qPCR positive with a DNA concentration of >0.5 pg/µl, indicative of a strongly positive result, was 8th of August, 8 days before LDM symptoms were recorded in the field.

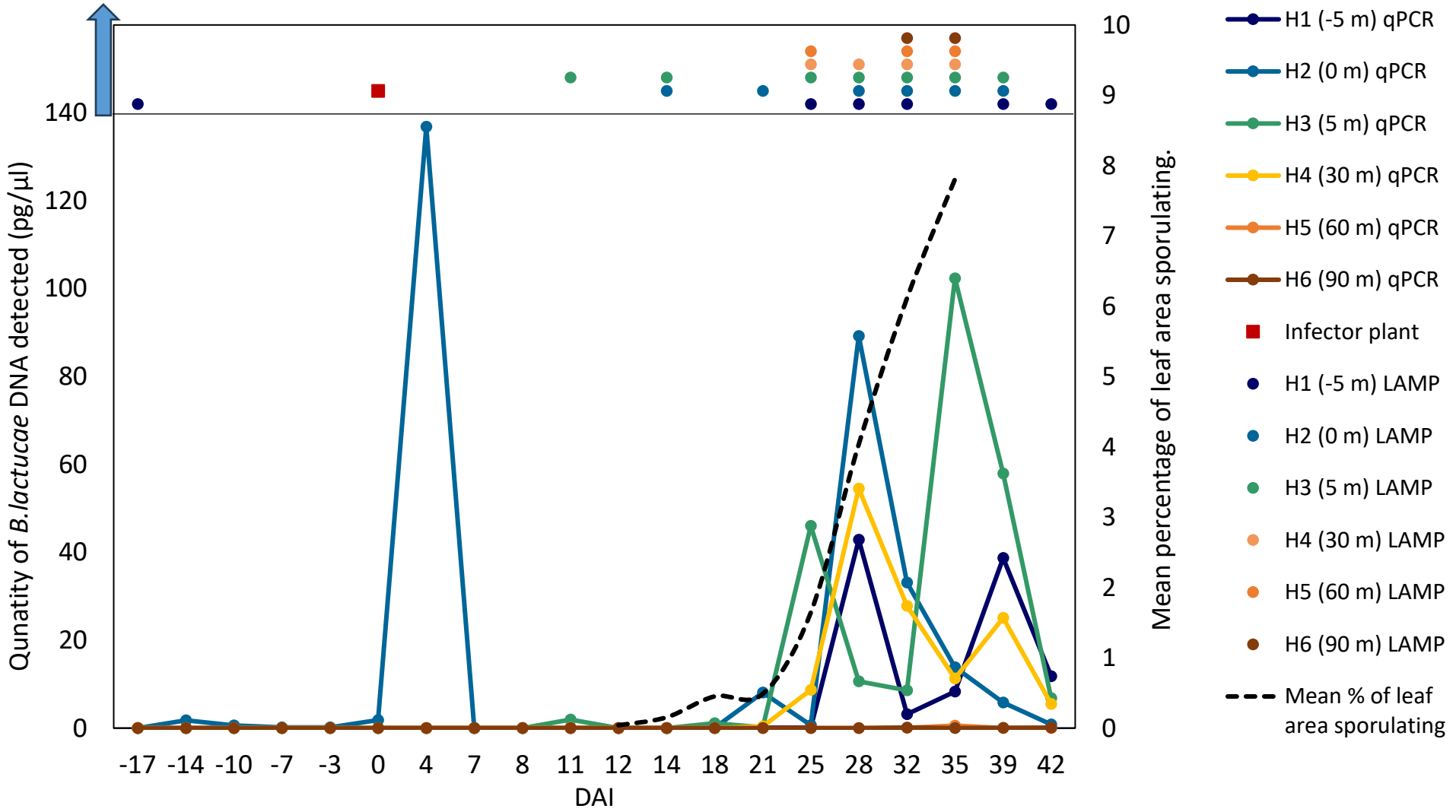

Figure 4, Detection of B. lactucae DNA in samples collected at different distances from an inoculum source. Aerial samples were collected between 23/08/2021–21/10/2021, quantity of DNA as measured using qPCR assay (lower figure) was between ~0.02-136 pg/µl. Mean percentage of leaf area sporulating was taken from living susceptible plants (~420). Results from LAMP assay are shown as positive/negative results represented by dots. Arrow shows increasing distance from inoculum source where H2 = 0m from inoculated plant. Results from aerial samplers are represented in the same colour for both assays. Assessments of disease were carried out at approximately 3-4 day intervals. Arrow shows the increasing distance between aerial samplers. Infector plant is both the position (in line with date placed out) and H2 (0 m) aerial sampler for proximity. DAI is days after inoculation.

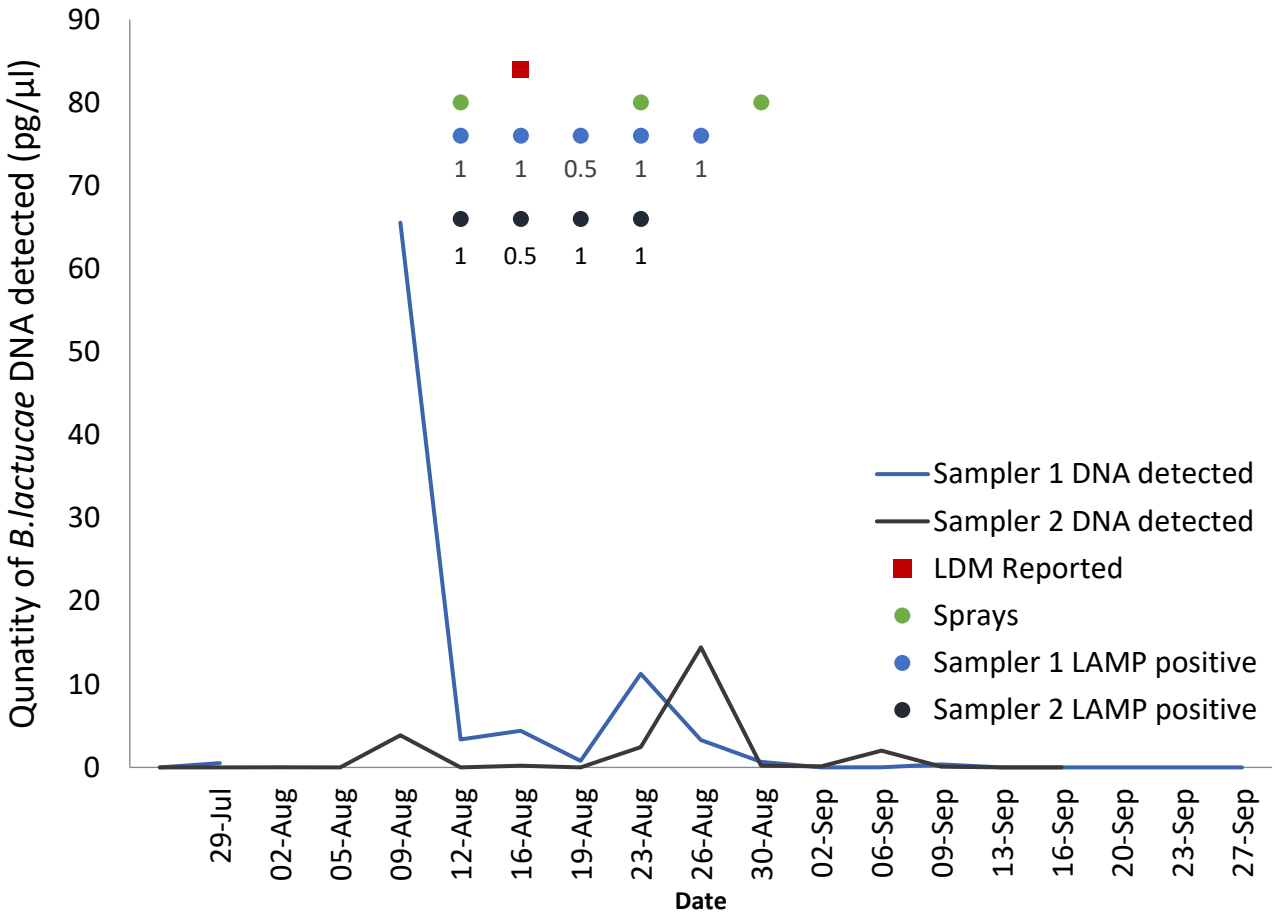

Figure 5, Detection of DNA of B. lactucae (pg/ µl) from aerial samples. Results from Scottish commercial sampling site over the 2021 growing season (29/07/21-27/09/21). Values ranged from 0.012pg/µl to >65pg/µl. Dates of fungicide applications are noted. There is missing data, 01/08/21-09/08/21 (sampler 1), and 20/09/21 (Sampler 2). Positive qPCR results are plotted as values, and positive LAMP results by binary positive/negative with the proportion of the assays (0.5 or 1.0) that tested positive when the sample was run in duplicate.

In 2022, sampling of commercial crops took place between the 08th of July 2022 and 29th of September 2022. At least one field at both commercial sites reported LDM symptoms. Samples obtained from the English commercial site were contaminated with windblown soil particles, which affected DNA amplification due to inhibition of the assay despite sample purification steps being included.

Two fields at the Scottish site reported LDM, on the 24th of August 2022 (field 2), and 22nd of September 2022 (field 3). In field 2, *B. lactucae* DNA was detected in samples collected on 18th and 22nd of August using the LAMP assay, six and two days respectively prior to the observed in-field LDM symptoms (Figure 6). Similarly, in field 3, samples retrieved on the 12th and 19th of September gave positive results when amplified using the LAMP assay (data not shown). In field 3 a single sample gave a positive result prior to the LDM observation using the qPCR assay (data not shown). The quantity of DNA detected at commercial sites was notably lower in 2021 than in 2022.

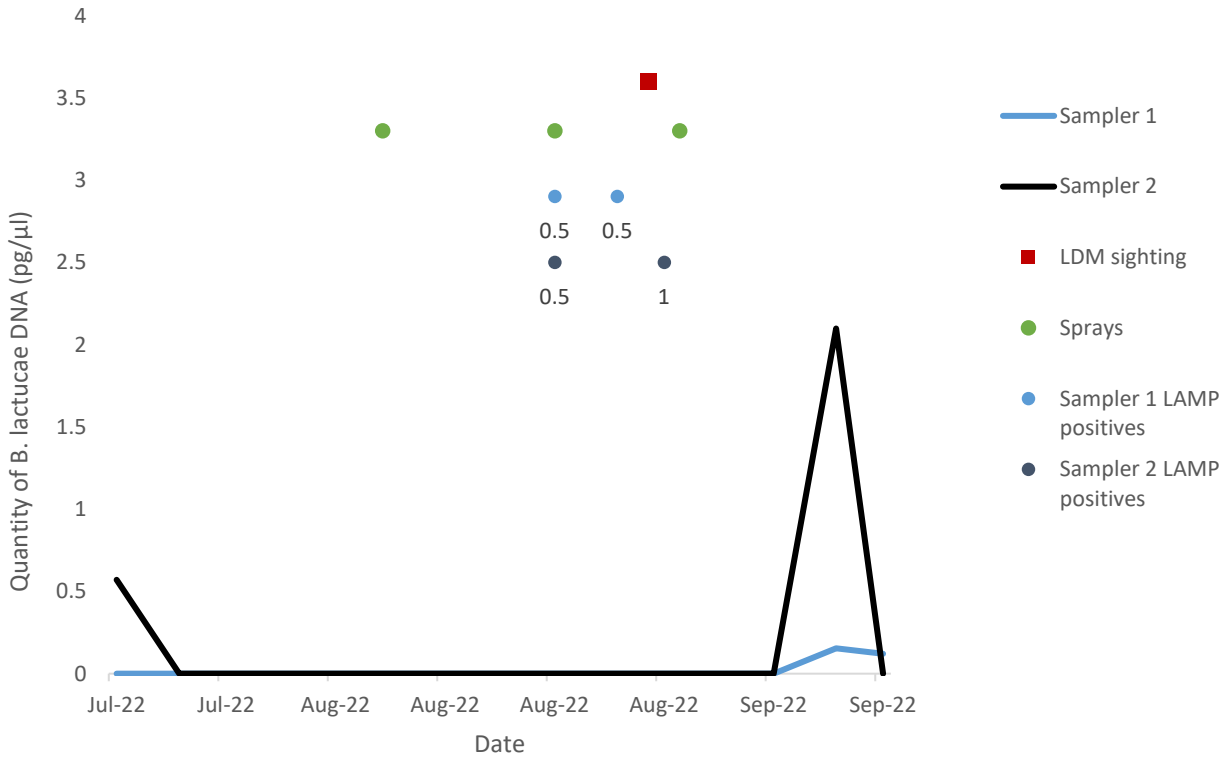

Figure 6, Detection of DNA of B. lactucae (pg/ µl). Results from Scottish field site, field two (21/07/2022-08/09/2022). Dates of fungicide applications are noted. Values range from 0.241 pg/µl to >2 pg/µl. Positive qPCR results are plotted as values, and positive LAMP results by binary positive/negative with the proportion of the assays (0.5 or 1.0) that tested positive when the sample was run in duplicate. Samplers 1 and 2 were 50 m apart in line with the prevailing wind direction.

5. Discussion

This study is the first survey of *B. lactucae* genetic diversity in the UK using SSR markers. UK samples were observed to have considerable variation for genotype and virulence profile both between and within disease outbreaks. Despite this variation, there was evidence of clonal lineages or genetically similar groups that were not confined to one geographical region or year, such as *K* cluster group 5. Prior research has suggested that the European population tends to be diploid with high heterozygosity, and a frequent occurrence of sexual reproduction (Gustafsson et al., 1985; Michelmore & Wong, 2008). In contrast, isolates from Japan, Australia and Wisconsin (US) reportedly had genetic indicators of polyploid and heterokaryosis (Gustafsson et al., 1985; Michelmore & Wong, 2008). All the diversity indices (Shannon, 2001; Simpson, 1949; Stoddart & Taylor, 1988) calculated from our data on UK samples show high levels of diversity and expected heterozygosity, which is consistent with a degree of sexual reproduction (Table 1). Despite this, the observed difference in the number of MLGs identified (135) from the samples analysed (254) also suggests reproduction of clones, and thus asexual reproduction (Table 1).

There were samples with three and four alleles at a locus (Figure 1). As *B. lactucae* usually exists as a diploid organism with two sets of chromosomes one to two alleles are generally anticipated. The presence of more than two alleles in the UK samples suggests the presence of multiple genetically distinct nuclei in the hyphae causing a single lesion sample and this may be caused by heterokaryosis. Genome sequencing has demonstrated heterokaryosis in other *B. lactucae* populations of 31 samples collected from California, Arizona (n=3) and Europe (n=9), 18 had indications of heterokaryosis which did not correspond to location nor year (Fletcher et al., 2019). Therefore, the high genetic diversity observed in the UK *B. lactucae* population is probably a mix of both occasional sexual reproduction and/or the formation of heterokaryons. Data collected with UK samples further supports the theory of heterokaryosis in *B. lactucae* populations (Fletcher et al., 2019). Heterokaryosis is important and concerning as it can increase the pathogen fitness and ability to infect a wider range of cultivar lines (Fletcher et al., 2019). Selection of favourable alleles from a heterokaryon could explain the subtle variation in genotype, and similarly virulence, in a disease outbreak. Considering the UK is geographically separate from mainland Europe the reproductive behaviour the *B. lactucae* population may be different and as such, this study provides data to suggest that the UK population may undergo sexual and asexual reproduction and exist in heterokaryotic states.

Every differential line in set C was overcome by at least one UK sample of *B. lactucae* tested. As there is evidence that the ability to overcome resistance can be preserved in pathogen populations (Lebeda & Zinkernagel, 2003b, 2003a) this is problematic as virulence profiles can become more complex, increasing the challenge of managing LDM using cultivar resistance. Most UK samples did not have virulence profiles that exactly matched designated IBEB races, used by lettuce breeding companies to advertise *B. lactucae* resistance in lettuce varieties. In other words, a lettuce variety with a reported resistance to, for example, Bl: 16-37 EU races may still be infected by *B. lactucae.* This is not an unusual finding as many European samples also do not match the limited number of designated races and there is a high degree of variation in virulence. According to an IBEB press release (Plantum, 2021) there is a trend towards more localised outbreaks each caused by a specific race which they suggest is due to the introduction of a wider repertoire of resistance genes. Considering the virulence profiles of UK samples appeared to be more similar within an outbreak than between outbreaks this may also be the case in the UK. The proportion of UK samples that could overcome *Dm* genes varied from 0.13-0.98, demonstrating that some genes are of low value, such as *Dm4*, whereas others such as *Dm3* are still effective against most of the population.

The nature and range of genetic and virulence diversity of the UK samples collected over four seasons were complex. It was anticipated that associations between a pathogen sample's genotype and its virulence profile would be apparent. However, there was little evidence of strong associations between genotype and virulence profile in this dataset. The only associations between virulence profile and genotype were those of samples from the same outbreak that were generally similar in both genotype and virulence, often only differing by one or two alleles, or one or two of the differential lines overcome. This was the case with K cluster group 5, which had a specific genotypic pattern with minor genetic variants. Within this group samples 2020_Bl2A and 2020_Bl4E had identical virulence profiles and differed in genotype at three loci. However, in other outbreaks virulence and genotype did not match. K cluster group 3 samples 2021_Bl1A, 2023_Bl1B and 2021_Bl1J were essentially identical in MLG but differed in virulence phenotype at three differential lines from each other (data not shown).

Although there was evidence of clonality in the UK population, the lineages were more difficult to characterise and track than in other oomycete pathogens such as *Phytophthora infestans* (Li et al., 2013). This could be due to limited sampling or to the, above mentioned, genetic complexity of heterokaryosis. The majority of our sample collection was from commercial fields, but lettuce is also grown in allotments and gardens and a wild population of *B. lactucae* may occur there and on closely related wild populations of *Lactuca serriola*. Such hosts may have provided reservoirs of *B. lactucae* strains. An examination of these additional sources may have added detail to the population analysis. A good example of this is sample 2021_Bl4Cin Cambridgeshire which was able to overcome the resistance of *Dm3* (Table 3) in addition to having a different virulence profile to the commercial areas.

The objective of the LAMP assay development was to detect *B. lactucae* early in a disease outbreak or preferably prior to symptom development to inform prompt action, such as modification of fungicide spray programs to prevent further disease spread (Buja et al., 2021; Crandall et al., 2018). The real-time LAMP assay detected DNA of *B. lactucae* under commercial field environments where LDM was observed, but not all commercial environments that had observable LDM symptoms had positive results due to soil contaminants on aerial sampler samples from trials in England. The LAMP assay detected *B. lactucae* DNA two to six days before disease was reported in commercial fields in Scotland. Sampling was carried out twice per week, approximately 3-4 days apart and increased sampling frequency may have detected *B. lactucae* DNA more than six days ahead of symptom development.

The LAMP assay is often utilised as an alternative to conventional and quantitative PCR in part due to its high tolerance of crude DNA samples (Notomi et al., 2000; Wong et al., 2018). The LAMP

assay for detecting *B. lactucae,* though not a sensitive as the qPCR assay, was found to be less sensitive to inhibition from soil contamination. Spiking of commercial samples with high amounts of DNA demonstrated strong inhibition at some sites. Dilution of the DNA sample to reduce inhibition may have decreased inhibitors (Wang et al., 2017), however, this would not give accurate levels of *B. lactucae* DNA in the sample.

Our analysis did not suggest that agrochemical applications had a notable effect on pathogen detection however, soil did have a significant effect preventing fluorescent readings through discolouration, likely introducing PCR inhibitors (Watson & Blackwell, 2000) and overloading the adhesive surface preventing sporangial adhesion (Jackson & Bayliss, 2011; West & Kimber, 2015). Soil contamination of this level was unanticipated and initial and subsequent protocols were unable to resolve the issue.

LAMP assays can be made suitable for use in in-field detection devices or adapted for less sophisticated heating apparatus (e.g. water-baths) (Notomi et al., 2015; Wong et al., 2018). The LAMP assay for *B. lactucae* could be adapted to full in-field use utilising machinery, such as the Genie® II developed by Optigene (Optigene, 2018). A real-time LAMP assay was also utilised to study the epidemiology of *B. lactucae*, specifically the dispersal gradient which could give an indication of how wide an area can be affected from a single disease outbreak. We found that *B. lactucae* DNA was detected in samples collected from an aerial sampler 100m from an inoculum source. According to (Wu et al., 2001) sporangial dispersal was estimated to range between 80 m and 3000 m. As may be expected, consistent detection of *B. lactucae* DNA occurred directly adjacent to the inoculum source in the Hutton trial. Placement of aerial samplers would therefore possibly be more accurate for detecting sporangia if placed within rather than adjacent to a lettuce crop.

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