Miljø- og
Fødevareministeriet
Miljøstyrelsen

Genetisk baseret varsling af kartoffelskimmel angreb med bio-markører

bioMark

Bekæmpelsesmiddel-
forskning nr. 175

August 2018

Udgiver: Miljøstyrelsen

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ISBN: 978-87-93710-58-0

Indhold

Forord

Kartoffelskimmel (*Phytophthora infestans*) er en af de mest alvorlige plantesygdomme globalt. Det anslås at tab pga. kartoffelskimmel årligt løber op i mellem 6-7 milliarder kroner (Llorente et al. 2010). I Danmark anses kartoffelskimmel for at være den vigtigste skadegører i kartofler. Svampen forekommer hvert år og oftest med kraftige angreb. For at begrænse tab i udbytte og kvalitet sprøjter de fleste landmænd alle koventionelt dyrkede spise- og stivelses kartofler gentagne gange i sæsonen. Behandlingshyppigheden er i forhold til andre afgrøder meget højt og lå i 2011 på 7 (Miljøstyrelsen 2012). Der er udviklet et klimabaseret beslutningsstøttesystem (*Skimmelstyring*), som kan udpege perioder med forventet angreb af kartoffelskimmel og dermed det potentielle behov for bekæmpelse. Modellen til beregning af infektionstrykket er bla. udviklet i projektet REFUKA (Miljøstyrelsens bekæmpelsesmiddelprogram 2004-2007) og er nu ved at blive implementeret til anvendelse i praksis som web-baseret beslutningsstøtte (GUDP projektet *Skimmelstyring*, 2012-2015). Der er ikke sket en egentlig udvikling af den biologiske model siden REFUKA undersøgelserne for 5-8 år siden (sporangieproduktion, sporangiespreding og infektionsforhold). Vi ved i dag meget lidt om skimmelsvampens populationsstruktur i Danmark og de ændringer som evt. kan være forekommet og konsekvenserne for modellens beregningsgrundlag. Foreløbige undersøgelser 2011-2012 har vist forekomst af genotyper (13-A2), som man ikke troede forekom i Danmark, og som i bl.a. England har givet problemer med bl.a. fungicidresistens overfor metalaxyl og øget aggressivitet i visse sortstyper. Den øgede forekomst i UK af genotypen 13-A2 har betydet en mere begrænset anbefaling i anvendelsen af metalaxyl og omlægning til andre midler med kurative egenskaber. Et andet problem er *Skimmelstyrings* beregning af den anbefalede fungiciddosis. Modellen kan forudsige potentielle infektionsperioder, men den aktuelle dosis afhænger meget af den faktiske forekomst af sporer. I dag anvendes det landsdækkende moniteringsnetværk (konsulenternes konstaterede angreb i mark eller område) som beregningsgrundlag, men der er behov for en øget præcisering og dermed større sikkerhed i dosisberegningerne.

Formålet med projektet var at tilvejebringe den biologiske baggrund der er nødvendig for at kunne foretage en tidlig risikovurdering af angreb af kartoffelskimmel. I projektet blev den populationsgenetiske baggrund for angreb af kartoffelskimmel klarlagt, herunder antal og fordeling af genotyper samt graden af rekombination. For at kunne foretage en risikovurdering i forbindelse med kartoffelskimmelangreb er det vigtigt at kunne koble genotyper med fænotypiske egenskaber (traits) hos svampen som betinger virulens og fungicidresistens. Vi har derfor i dette projekt anvendt svampens resistens overfor fungicidet metalaxyl som eksempel på en egenskab der kan karakteriseres med genetiske markører (bio-markører). Ved at monitorere populationer af kartoffelskimmel på et givent tidspunkt for disse biomarkører kan man sammen med klima data forudse risiko for angreb af høj-virulente og fungicidresistente typer med stor sikkerhed, og dermed anvende (eller undgå) fungicid behandlinger med specifikke midler mere hensigtsmæssigt.

Projektet er financieret af Miljøstyrelsen (j. nr. MST-667-00155) og udført som et samarbejde mellem Københavns Universitet, Aarhus Universitet og Videncenter for Landbrug. Følgende har deltaget i projektet:

Søren Rosendahl, Professor, Biologisk Institut, Københavns Universitet:
Projektleder og ansvarlig for populationsgenetiske undersøgelser
Rasmus Kjøller, Lektor, Biologisk Institut, Københavns Universitet
Ansvarlig for aktivitet vedr. hurtig detektion af *P. infestans* i felten.

Melanie Montes, PhD studerende, Biologisk Institut, Københavns Universitet
Har udført laboratoriearbejdet og stået for databehandling samt udarbejdelse af manuskripter
Bent J Nielsen, Lektor, Institut for Agroøkologi Aarhus Universitet
Ansvarlig for aktivitet vedr. test af fungicidresistens og matingtyper samt koordinering af isolatindsamling.
Lars Bødker, Landskonsulent Videncentret for Landbrug , (nu SEGES)
Har sammen med Bent J. Nielsen koordineret prøvetagning i forbindelse med kartoffelskimmelangreb.

Konklusion og sammenfatning

Kartoffelskimmel er en af de mest alvorlige plantesygdomme globalt. Bekæmpelse af kartoffel-kartoffelskimmel (patogenet *Phytophthora infestans*) er stærkt afhængig af brugen af kemisk bekæmpelse. I projektet blev fungicidet metalaxyl-M (Mefenoxam) anvendt som eksempel på et middel, som har spillet en vigtig rolle i at bekæmpe sygdommen, og hvor resistens overfor fungicidet hos visse isolater har givet anledning til en stigende bekymring. Den genetiske baggrund for metalaxyl-resistens antyder muligheden for at forbinde resistente fænotyper til specifikke populationsgenetiske markører. For at gøre dette, kræves det, at den populationsgenetiske struktur samt graden af klonal og seksuel reproduktion i populationen er kendt. Dynamikken i metalaxyl-M resistens i danske populationer af *P. infestans* og den populationsgenetiske struktur blev karakteriseret i løbet af 2013 vækstsæsonen ved hjælp af simple sequence repeats (SSR) genotyper og enkelt nukleotid polymorfismer (SNP)-baseret mitokondrie haplotyp-bestemmelse af over 80 isolater. Begge parringstyper A1 og A2 var til stede i de fleste områder, men test for rekombination viste, at klonal reproduktion dominerede i danske populationer. Genotyperne var ikke knyttet til mitokondrie-haplotype, og der var ingen differentiering mellem haplotyper, men snarere mellem markerne. Resistente fænotyper var knyttet til specifikke SSR alleler, hvilket demonstrerer potentialet for et mere præcis SNP-baseret markørsystem til at forudsige resistens overfor metalaxyl-M.

I projektet brugte vi en EvaGreen-baseret-analyse, som er følsom nok til at detektere enkelt *P. infestans*-sporangier, og en Burkard multi-vial cyklon sampler til indsamling af luftbårne sporer, og teste dette system under markforhold i sommeren 2014 i Danmark. Ved at sammenligne resultaterne af vores qPCR assay med infektion tryk målinger beregnet ved beslutningsstøttesystemet (DSS), der anvendes i regionen (www.skimmelstyring.dk), og de faktiske observerede udbrud i omkringliggende områder, kunne vi se, hvordan information om tilstedeværelsen af smitte potentielt kunne påvirke, hvordan beslutninger til at sprøjte i regionen kunne træffes. Den EvaGreen-baserede qPCR-analyse viste sig følsom nok til at opdage lave niveauer af sporangier i felten. Sammen med sine lave omkostninger i forhold til TaqMan qPCR, og EvaGreens evne til at fungere godt under hurtig PCR (15 sekund cykler anvendes her), gør det til en ideel metode at anvende i beslutningsstøttesystemer, hvor hurtige resultater er en nødvendighed.

Konkluderende, afslørede den genetiske struktur, at kartoffelskimmel er domineret af klonal reproduktion, hvilket gør det muligt at identificere enkelt genetiske markører knyttet til Metalaxyl-resistens. Den populationsgenetiske struktur af udbrud af kartoffel-kartoffelskimmel, afslører yderligere, at stokastiske processer er af stor betydning i de indledende faser af udbruddet, samt at populationen var domineret af resistente genotyper mod slutningen af sæsonen. Disse oplysninger kombineret med demonstration af detektionsgrænserne i marken, viser potentialet i at udvikle genetisk baserede varslingssystemer til kartoffelskimmel. Fungicidresistens blev anvendt i nærværende projekt som et eksempel på et fænotypisk træk, som har betydning for angrebenes voldsomhed, men andre træk som virulens og spiringsegenskaber bør indgå i et fremtidigt detektionssystem. Et beslutningsstøttesystem med sådanne oplysninger vil kunne danne grundlag for en reduktion i fungicidanvendelse ved at begrænse fungicidbehandling til angreb med høj-virulente fungicidfølsomme genotyper.

Summary and Conclusion

Potato blight is one of the most serious plant diseases globally. Control of the potato late blight pathogen, *Phytophthora infestans*, relies heavily on chemicals. In this project, the fungicide metalaxyl-M (Mefenoxam) is used as an example of a fungicide that has played an important role in controlling the disease, but where insensitivity to the fungicide in certain isolates is a major concern. A genetic basis for resistance to metalaxyl suggests the possibility for linking resistance phenotypes to specific population genetic markers, but in order to do this, the population genetic structure and mode of reproduction in a population must first be well described. The dynamics of metalaxyl-M resistance in the Danish population of *P. infestans* was characterized over the course of the 2013 growing season, as was the population genetic structure, using simple sequence repeat (SSR) genotypes and single nucleotide polymorphism (SNP)-based mitochondrial haplotyping of over 80 isolates. Both mating types A1 and A2 were present in most fields, but tests for recombination showed that clonal reproduction dominates in Danish populations. Genotype was not linked to haplotype and no differentiation was observed at the haplotype level, but rather among fields. Resistance phenotypes were linked to specific SSR alleles, demonstrating the potential for a more precise SNP-based marker system for predicting resistance to metalaxyl-M.

We used a EvaGreen-based assay which was sensitive enough to detect single *P. infestans* sporangia, and a Burkard multi-vial cyclone sampler as an alternative to sticky tape for collecting airborne sporangia, and test these under field conditions during the summer growing season of 2014 in Denmark with background noise from other spores. We also compare the results of our qPCR assay with the infection pressure measurements calculated by the decision support system (DSS) used in the region (www.skimmelstyring.dk) and the actual observed outbreaks in surrounding fields, in order to see how this information on the presence of inoculum could potentially influence how decisions to spray in this region are made in the future. The EvaGreen-based qPCR assay proved sensitive enough to detect low levels of sporangia in the field. Along with its low cost compared to TaqMan qPCR, EvaGreen's ability to function well under fast cycling PCR, such as the 15 second cycles used here, makes it an ideal candidate for use in DSSs, where quick results are crucial.

In conclussion, the revealed population genetic structure dominated by clonal reproduction, made it possible to identify single genetic markers linked to Metalaxyl resistance. The population genetic structure of the outbreaks of poptato late blight, further reveal that stochastic processes are of major importance in the initial phases of the outbreak, but the population became dominated by resistant genotypes towards the end of the season. This information combined with the demonstration of detection limits in the field, advocate for delelopment of a genetically based warning system for potato late blight. Fungicide resistance was used in the present project as an example of a phenotypic trait which impacts disease severety, but other traits such as virulence and germination should be included in a future detection system. A decision support system with such information, will provide the basis for a reduction in fungicide use, by limiting fungicde treatment to attacs with highly virulent sensitive genotypes.

1. General introduction

Late blight caused by *Phytophthora infestans* is one of the most serious plant diseases worldwide. In Europe it is estimated to cost more than €1,000,000,000 annually in yield losses and control (Haverkort et al., 2008). In Denmark late blight is considered to be the main pathogen of potatoes. The fungus occurs every year and usually with serious outbreaks. To limit losses in yield and quality, conventional farmers repeatedly apply fungicides during the season in crops of both starch- and ware potatoes. In 2011 the Treatment frequency index (TFI) was 7, which is very high relative to other crops (Miljøstyrelsen 2012). A climate-based decision support system (Mold Control) has been developed, which can identify periods of expected attack of late blight and thus the potential need for control. The model for calculating the infection pressure is developed in the project REFUKA (Environmental Protection Agency's pesticide program 2004-2007) and is now being implemented into applications such as web-based decision support (GUDP project Mold Control, 2012-2015). There has not been a significant development of the biological model since REFUKA studies 5-8 years ago (sporangia production, sporangia dispersal and infection conditions). Today we know very little about the population structure of late blight in Denmark and the changes that may have occurred in recent years, and the consequences such changes may have for the model calculation. Preliminary studies 2011-2012 have shown that the presence of genotype (13-A2), which was not thought to occur in Denmark, and which particularly in England has caused problems in relation to fungicide resistance towards metalaxyl and increased aggressiveness in certain types of potato varieties. The increased incidence in the UK by genotype 13-A2 has resulted in a more restricted recommendation on the use of metalaxyl and where possible a substitution with other agents. Another problem is how the Mold Control is calculating the recommended fungicide doses. The model can predict potential infection periods, but the actual infection dose depends very much on the actual incidence of spores. Today Mold Control is used nationwide in Denmark as a monitoring network as a standard, but there is a need for greater clarification and thus greater certainty in dose calculations.

The purpose of this project was to explore ways to find specific genetic markers that correlate with virulence of late blight. Detection achieved with such markers will make it possible, not just to warn for potential late blight risks, but also to make a risk assessment of the potential damage. A prerequisite to make such a warning system is that the genetic markers are linked to fungal properties. To clarify this, it is necessary to characterize the genetic structure of late blight epidemics, including knowledge on the ability of the fungus to recombine and its dispersal. Furthermore, knowledge is required of the genetic diversity behind late blight epidemics. The population genetic structure and mode of reproduction of *Phytophthora infestans* in Denmark is

not known. A high genotypic diversity in Nordic countries compared to the rest of Europe has been interpreted as a result of sexual reproduction (Brurberg et al., 2011). If sexual recombination is occurring frequently, there should be no linkage between the locus for resistance and other loci unless the genes and markers are physically linked on the same chromosomes. On the other hand a clonal structure would result in a linkage between the genetic markers and the virulence traits of interest. In this project the resistance to Metalaxyl is used as an example of such a trait.

The project has three objectives:

1. To characterize the genetic structure of the potato blight in Denmark
2. To elucidate the connection between specific genetic markers and fungal properties – here fungicide resistance
3. To clarify the detection limit using genetic detection of airborne spores.

The first two objectives are met in chapter 2 and the third objective in chapter 3.

The project was conducted as a collaboration between Copenhagen University, Aarhus University and Videncenter for Landbrug, now SEGES:

Søren Rosendahl, Professor, Department of Biology, Copenhagen University:
Head of project and responsible for population genetic studies
Rasmus Kjøller, Asociate professor, Department of Biology, Copenhagen University:
Responsible for fast detection of *P. infestans* in the field.
Melanie Montes, PhD studerende, Department of Biology, Copenhagen University:
Laboratory work, data processing and writing the manuscripts
Bent J Nielsen, Asociate professor, Department of Agroecology, Aarhus University
Responsible for fungicide resistence test, mating type test and coordination of sampling.
Lars Bødker, National consultant, SEGES
Together with Bent J Nielsen, coordination of sampling

2. Population genetics of *Phytophthora infestans* in Denmark reveals dominantly clonal populations and specific alleles linked to metalaxyl-M resistance

2.1 Introduction

The devastating plant pathogen *Phytophthora infestans* (Oomycota) has been causing late blight on tomato and potato in Europe since the 1840s. In Europe it is estimated to cost more than €1,000,000,000 annually in yield losses and control (Haverkort et al., 2008). Chemical fungicides are used heavily in attempts to control the disease, and in Denmark approximately 20 percent of fungicides used in potatoes 2012-2014 although they make up less than two percent of agricultural area (Miljøstyrelsen, 2012, 2013, 2014).

Despite a long history of chemical control of oomycete pathogens, there are surprisingly few cases of naturally occurring resistance to control chemicals reported in *Phytophthora infestans* (Randall et al., 2014). Phenylamide control chemicals, such as Metalaxyl, are the exception. Use of Metalaxyl began in the 1970s and field isolates insensitive to Metalaxyl-M (Mefenoxam) were soon reported (Davidse et al., 1981). However, it continues to be an effective means of controlling *P. infestans* and is still used in most regions worldwide.

The precise molecular mode of action for phenylamide resistance in *Phytophthora* has eluded researchers for decades, but it has been suggested that one or two loci, MEX1 and MEX2, with incomplete dominance are involved, along with several minor genes epistatic to these loci (Judelson & Roberts, 1999). A recent study by Randall et al. (2014) identified a single nucleotide polymorphism (SNP), RPA190, in a gene for the large subunit of RNA polymerase I that was strongly associated (86%) with insensitivity to Mefenoxam, and found that this allele was sufficient to confer insensitivity when transformed into sensitive isolates. A small subset of isolates from a single genotypic background showed insensitivity although they did not have this SNP, and it is possible that a second locus is involved in their case (Randall et al., 2014). If a single gene is indeed enough to confer insensitivity, it should be possible to link resistance phenotypes to specific genetic markers. If these same markers are used to determine population genetic structure, then one would also expect to see changes in population structure reflected in changes in resistance phenotype, and *vice versa*.

In Europe there is a history of clonal lineages made up of single haplotypes dominating the *P. infestans* population, beginning with the HERB-1 lineage (haplotype Ia) that caused the Irish Potato Famine (May & Ristaino, 2004; Yoshida et al., 2013), which was later displaced by the lineage US-1 of haplotype Ib (Gavino & Fry, 2002; May & Ristaino, 2004), and more recently the aggressive strain 13_A2 of haplotype Ia (Cooke et al., 2012; Li et al., 2012). Although more genetic diversity has been introduced into Europe with the displacement of the US-1 lineage, generally genotypes appear to belong to a single haplotype when tested (Li et al., 2012; Kildea et al., 2013). Studies that characterize both the genotype and haplotype of the same isolates in Nordic countries are lacking.

The population genetic structure and mode of reproduction of *Phytophthora infestans* in Denmark remains unclear. A high genotypic diversity in Nordic countries compared to the rest of Europe, uncovered by microsatellite analyses, was interpreted as a result of sexual reproduction occurring in the region (Brurberg et al., 2011; Sjöholm et al., 2013). In order for sexual reproduction of *P. infestans* to take place, two different mating types, A1 and A2, must be present. The A2 mating type was first detected outside of Mexico in Europe in 1984 (Hohl & Iselin, 1984), but in some parts of Europe clonal reproduction is still thought to dominate (Ireland, Cooke et al., 2012a; U.K., Cooke et al., 2012b; France, Montarry et al., 2010). In contrast, the lack of a dominating genotype and evidence pointing to soil borne inoculum (sexually produced oospores) in Scandinavia has led to the conclusion that sexual reproduction is occurring (Yuen & Andersson, 2012). If sexual recombination is occurring frequently, there should be no linkage disequilibrium between the locus for resistance and other loci unless the genes and markers are physically linked on the same chromosomes.

The objectives of the present study were: (1) to test for population genetic structure and reproductive mode in a more intensive sampling of the Danish population, including both within and among field variation, (2) to test the potential for using genetic markers to predict resistance phenotypes, and finally, (3) to implement a new SNP-based haplotype screening method and compare genotypic and haplotypic variation. We hypothesized that resistance phenotypes can be linked to specific alleles, and therefore looked at the population structure using Simple Sequence Repeat (SSR) genotypes. We predicted that genotypes are also linked to mitochondrial haplotypes. We took several samples from the same fields multiple times within a single growing season, in the limited area of Jutland, Denmark, and tested for Mefenoxam resistance. We employed the multiplex PCR method using twelve simple sequence repeat (SSR) markers developed by Li et al. (2013a), that has since been used to genotype populations in many different regions of the world, and developed a SNP-based method for determining haplotypes.

2.2 Materials and Methods.

2.2.1 Sampling

A total of 84 samples were collected from seven fields (Table 1; Figure 1). Sampling dates and locations of isolates are listed in supplementary table S1. Fields were sampled as soon as possible upon the first sign of infection. In 2013 we experienced a particularly dry summer season that led to later than normal first occurrences. The intention was to visit the same location up to four times throughout the season, though in many cases it was not possible to find late blight in the same place again due to desiccation of the fields or intense spraying of infected sites by the farmers. This meant that three fields were only sampled once, three were sampled twice, and only one of the fields was visited four times. The majority (49%) of samples therefore come from the latter field in Dronninglund.

Isolation was performed by taking a leaf with a lesion and placing it on a petri dish with a small amount of rye agar. Once the lesion started sporulating, sporangia were transferred to new petri dishes and re-isolated several times over the coming weeks to ensure that there was only one isolate per sample. Cultures were then stored on rye agar at 4˚C until ready for use.

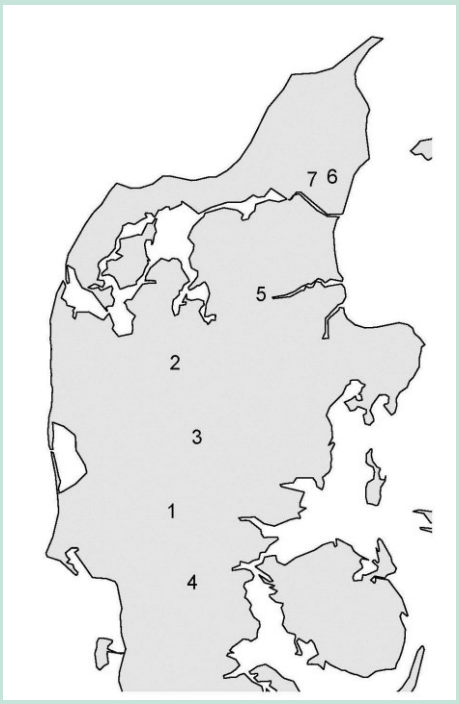
TABLE 1 FIELD LOCATIONS, POTATO CULTIVAR, NUMBER OF SAMPLING DATES, AND THE TOTAL NUMBER OF SAMPLES FROM EACH SITE FOR ALL SAMPLINGS

Field Nr.	Location	Potato Variety	No. of Sampling Dates	No. of samples
1	Bilund	Frieslander	1	4
2	Karup	Kardal	2	5
3	Ejstrupholm	Different ware potatoes	1	6
4	Rødding	Kuras	1	5
5	Hobro	Exelency	2	13
6	Dronninglund	Kuras	4	41
7	Vodskov	Kuras	2	10

2.2.2 Phenotypic characterization

The level of MFX Mefenoxam (Metalaxyl-M) resistance was determined using a floating leaf test (Syngenta). Fresh leaves were taken from four week old plants and 15 mm disks were placed in the wells of a plate containing a dilution series with six different concentrations of Metalaxyl-M: 0.001, 0.01, 0.1, 1, 10 and 100 ppm. Tests with no Metalaxyl-M (0) were also run, but on separate plates to account for the vapor effect of the chemicals. The leaf disks were inoculated with 20 µL of a solution containing $2x10^4$ sporangia/mL and observed after six days for signs of infection. There were four replicates performed for each isolate. The IC_{50}, i.e. the amount needed to inhibit growth by 50%, was calculated and isolates were classified into groups of sensitive ($IC_{50} < 1$) or resistant ($IC_{50} > 1$).

Mating type was determined by placing 0.5 x 0.5 cm plugs of the test isolate grown on rye agar on a 9 cm rye agar plate across from plugs of reference isolates 281_A2 or 299_A1 (Syngenta). Each test was run twice, once with each reference isolate. The isolates were allowed to grow together for ca. 10 days before being examined under the microscope for the presence of oospores. If an isolate produced oospores when grown with, for example, reference isolate 281_A2, then it was classified as having the opposite mating type, A1.



FIGUR 1 MAP OF FIELD LOCATIONS IN JUTLAND, DENMARK. 1, BILUND; 2, KARUP; 3, EJSTRUPHOLM; 4, RØDDING; 5, HOBRO; 6, DRONNINGLUND; 7, VODSKOV

2.2.3 DNA extraction

DNA was extracted from live culture isolates by cutting a plug from the agar, slicing off the top layer where most of the mycelia were found, and placing it directly in QuickExtract™ Plant DNA extraction solution (Epicentre) and following the manufacturer's protocol.

2.2.4 Mitochondrial haplotyping

The four reference mitochondrial haplotypes (Ia, Ib, IIa, IIb) genome sequenced by Avila-Adame et al. (2006) were analyzed in SNAP workbench 2.0 (Price & Carbone, 2005) to identify Single Nucleotide Polymorphisms (SNPs). Regions containing several informative SNPs which would allow us to distinguish between all four haplotypes were aligned in BioEdit v.7.2 (Hall, 1999). Primers were designed to amplify those regions using Primer3 (Koressaar, 2007; Untergrasser et al., 2012). Primer sequences and the annealing temperatures used in subsequent PCR are listed in table 2. PCR was performed in a volume of 25 µL consisting of 12.5 µL VWR Red Taq 2x Master Mix, 2 µL MgCl2, 0.75 µL of both forward and reverse primers, and 1 µL template DNA. PCR was conducted on a BioRad T100™ Thermocycler (Bio-Rad Laboratories, Inc.) and conditions were: 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 30 s, listed annealing temperature for 30 s^{-1} min, 72 °C for 1 min, and a final extension at 72 °C for 6 min. PCR products were sequenced (Macrogen Europe) and sequences were corrected and aligned to the reference sequences using MEGA v. 6.06 (Tamura et al., 2013).

2.2.5 SSR genotyping

Of the 12 SSR loci and primers described by Li et al. (2013a), only 10 were used in this experiment: Pi02/PinfSSR3, PinfSSR11, PinfSSR8, PinfSSR4, Pi04, Pi70, PinfSSR6, Pi63, PinfSSR2, and Pi4B. These 10 primers consistently produced no more than two peaks. Primers D13 and G11 resulted in either no peaks or too many in the majority of samples initially tested, and were therefore not included in the experiment. The dyes and primer concentrations used were kept the same as Li et al. (2013a). Reactions were tested first in uniplex and then run in two separate multiplex reactions under identical conditions. PCR was performed in a volume of 10 µL using VWR Red Taq 2x Master Mix, 2mM MgCl2, and varying concentrations of primers, following (Li et al., 2013a). Amplifications were run in a BioRad T100™ Thermocycler (Bio-Rad Laboratories, Inc.) with initial denaturation at 95˚C for 15 minutes, followed by 30 cycles of 95˚C for 20 s, 58˚C for 90 s and 72˚C for 60 s, and a final extension at 72˚C for 20 minutes. The products were then diluted 1:10 with 8.75 µL HiDi™ Formamide and 0.25 µL GeneScan™ 500 LIZ™ dye size standard (Applied Biosystems) and run on an Applied Biosystems 3130XL Genetic Analyzer (Applied Biosystems).

TABLE 2. MITOCHONDRIAL PRIMERS USED FOR HAPLOTYPING ISOLATES. PRIMER SEQUENCES, THE ANNEALING TEMPERATURE USED FOR PCR, EXPECTED PRODUCT SIZE, AND SNPS INCLUDED IN THE SEQUENCED REGIONS AND THE HAPLOTYPES THEY DISTINGUISH.

Primer	Sequence	Ann. Temp (°C)	Product Size (bp)	SNP	Haplotype
PinAB4c	fwd-TGCCTTAATAGGTGCAGTAACTTT	57	500	G/A	IA,IB/IIA,IIB
	rev- AACCGTAGCAGCAGCAGAAT			A/T	IA,IB/IIA,IIB
				G/A	IA,IB,IIA/IIB
				A/T	IB,IIA,IIB/IA
PinAB6c.2	fwd-CCTTTTGTATTTACTAACGCTTCTTT	58	391	T/G	IA,IB/IIA,IIB
	rev-GGAACTAATGCTATACTTGCACCA				
PinAB8b.1	fwd- CACAAAATCACCCTTACCATTT	56	444	A/C	IA,IB/IIA,IIB
	rev- TGTGCCCATGTAATTGTTGC				
PinAB4b	fwd-CGATATCTCGGTAAAACAGCTC	56	473	T/C	IA,IB/IIA,IIB
	rev- GGTGCATTAAGATCAACAGCA			T/C	IA,IB,IIA/IIB
				A/G	IA,IB,IIA/IIB
				A/G	IA,IB,IIA/IIB

2.2.6 Clonal diversity and allelic richness

Multilocus gentoypes (MLGs) were identified using GeneMapper v. 5.0 (Applied Biosystems, 2005). Calculations of allele frequencies and identification of private alleles, as well as calculation of the Shannon information index for diversity, allelic richness (Ar), and unbiased Heterozygosity (Hnb) for each population were carried out using GenAlEx v.6.5 (Peakall & Smouse, 2006, 2012). A matrix of Nei's (1978) genetic distance between populations was also generated using GenAlEx. Genotypic richness was calculated as R= (M-1)/(N-1), where M is the number of unique MLGs and N the number of samples for each population (Dorken & Eckert, 2001). Isolates sharing the same alleles at all 10 loci were identified using GenClone 2.0 (Arnaud-Haond & Belkhir, 2007). Computation of pair-wise genetic distances between the identified MLGs was carried out in GenClone 2.0 (Arnaud-Haond & Belkhir, 2007) to determine how many unique MLGs could have resulted from scoring errors or somatic mutations of the same clonal lineage. In cases where the same MLG was sampled more than once, the probability that the nth occurrence of a given MLG was derived multiple times from sexual recombination was estimated as Psex n re-encountered.

2.2.7 Spatial analyses of clonal structure

To test for sampling bias on the genotypic diversity, the spatial structure of the population was analyzed by using the UTM coordinates as distance measures. Edge effect (Ee) and aggregation index (Ac) were estimated using GenClone 2.0 (Arnaud-Haond & Belkhir, 2007). The Edge effect analysis tests whether the sampling design leads to an overestimation of diversity due to larger

clones on the periphery of the sampling area appearing as unique or rare MLGs, by testing whether apparent unique or rare MLGs are more often found on the edge of the sampling areas (Arnaud-Haond et al., 2007). The aggregation index tests for spatial aggregation of clonal lineages, where an Ac of 0 indicates completely intermixed MLGs and an Ac of 1 indicates that all the nearest neighbors share the same MLG (Arnaud-Haond et al., 2007). The statistical significance for both analyses was tested against the null hypothesis of random distribution using 1000 permutations.

2.2.8 Population structure analysis

Calculation of F_{IS} was carried out in GeneClone 2.0 (Arnaud-Haond & Belkhir 2007), based on the unbiased heterozygosity Hnb for small sample sizes, as described by Nei (1978). The F_{IS} can range from 1 to -1, where a positive F_{IS} is indicative of a lack of heterozygous individuals and a negative FIS of an excess of heterozygotes, compared to what is expected in Hardy-Weinberg equilibrium. In a population exhibiting complete panmixia, F_{IS} is expected to be zero (Wright, 1951).

A pairwise test for linkage disequilibrium between loci was carried out using GenePop v. 4.3 (Rousset, 2008) and a Bonferroni correction to account for multiple testing. The Markov Chain parameters were set as a dememorization of 10,000, 100 batches, and 5,000 iterations per batch.

The data was clone corrected by removing identical genotypes from the same field to ensure that the same clonal individual was not being counted several times in the following analyses.

The index of association (I_A) was calculated to test for multilocus linkage disequilibrium using the program MultiLocus v. 1.2 (Agapow & Burt, 2000) and significance was determined using an F-Test. The index of association is a measure of the observed versus expected variance in a population, $I_A = [V_O/V_E] - 1$. In a situation of total panmixia, where populations are freely recombining, $I_A = 0$. A value significantly larger than 0 indicates an increasing degree of linkage between loci and therefore possible clonality. The I_A was run for all the isolates together, and then for the individual mitochondrial haplotypes to eliminate the possibility that any linkage seen is only an artifact of the mitochondrial haplotypes acting as separate subpopulations.
Population differentiation analysis was also run using Multilocus v. 1.2, which calculates Weir's (1996) formulation of Wright's F_{ST}, Θ, for diploid populations while maintaining the linkage disequilibrium or deviations from Hardy Weinberg equilibrium found in the data set during its randomizations (Agapow & Burt, 2000). The populations were first defined as the different fields from which isolates were collected and in a second run as the different mitochondrial haplotypes.
A principle coordinate analysis (PCA) was performed using PC-ORD v. 5.0 (Grandin, 2006), in which SSR alleles were treated as quantitative characters assuming that differences in the number of repeats correlates with genetic distance between alleles. A Mantel test for association between two distance matrices was run to test for correlation between MLGs and mitochondrial haplotypes using the Monte Carlo test method with 1000 randomizations.

2.2.9 Odds ratios

The odds ratio (OR) express the odds of showing resistance ("diseased") given the presence of a certain allele ("exposed"). The odds ratio was calculated using Microsoft Excel for each allele with 25 or more observations by treating resistant isolates as one group, and sensitive isolates as the control using the formular: $OR = R_E{*}S_N / R_N{*}S_E$, where R_E are resistant with the allele, S_N are sensitive without the allele, R_N are resistant without the allele and S_E sensitive with the alelle. An odds ratio above one suggests that there is an increased likelihood of resistance when that allele is present. A Chi square test was run to test which alleles showed a significantly higher odds ratio, which could not be explained by chance alone ($P<0.01$). Loci displaying alleles with significant ORs were located on the T30-4 *P. infestans* reference genome (Haas et al., 2009) using the FungiDB online database (Stajich et al., 2012).

2.3 Results.

2.3.1 Multilocus genotypes

For 81 samples, eight or more SSR loci were successfully genotyped. Resampling of different subsets of these loci among the 84 isolates using the GENCLONE software shows that the number of MLGs detected begins to reach an asymptote between eight to ten loci, suggesting that clones could be reliably identified (supplementary figure S1). A total of 35 unique SSR genotypes were identified, consisting of 36 different alleles (supplementary table S2). In seven cases, the genetic distance between two MLGs was uncharacteristically low, suggesting that they could have resulted from scoring errors or somatic mutations. The probability that two individuals with the same genotype resulted from independent instances of sexual recombination was very low, with values lower than $1.66 *10^{-4}$ (table 4), indicating that the repeated MLGs are true clonal lineages. No MLG was shared between fields. The most common MLGwas found in Dronninglund and makes up 25 % of the samples, though this is probably an effect of the larger sample size from that field.
Allele frequencies are listed in supplementary table S3. Six private alleles were found. The Nei's (1978) genetic distances between fields are listed in supplementary table S4. Diversity measures varied greatly for each population and are listed in table 3. The mean Shannon's Information index for the total population gave a diversity of 0.666. No edge effect was seen (E_e = -0.06, p = 0.679), indicating that the observed genotypic diversity was not an effect of sampling design. A significant aggregation was found (Ac = 0.378, p<0.0001), indicating that MLGs were strongly aggregated in space and not intermixed.

TABLE 3 NUMBER OF COPIES (N) AND THE PROBABILITY THAT THE NTH OCCURRENCE OF A GIVEN MULTILOCUS GENOTYPE (MLG) WAS DERIVED MULTIPLE TIMES FROM SEXUAL RECOMBINATION (PSEX N RE-ENCOUNTERED) FOR EACH MLG FOUND MORE THAN ONCE IN ANALYSIS OF 79 *PHYTOPHTHORA INFESTANS* ISOLATES

	n	P_{sex} n Re-encounter
MLG3	2	1,66E-4
MLG4	4	8,60E-12
MLG6	4	3,07E-13
MLG7	2	5,32E-06
MLG8	2	4,48E-05
MLG10	4	6,79E-11
MLG17	6	3,50E-18
MLG23	5	5,98E-18
MLG26	21	5,16E-72
MLG30	2	3,29E-05
MLG32	3	2,78E-09

2.3.2 Haplotypes

The total distribution of mating types for all fields was 45.1% A1 and 54.9% A2. The mating type of each isolate is listed in supplementary table S1. In most fields, both mating types were present. However, in field one all isolates belonged to A1 and in field four all belonged to A2. This could be a result of small sample size in these locations, or due to the fact that these fields were visited only

once in the beginning of the season. In fields five and six, only one mating type was present during the first sampling and the second mating type appeared later in the season.
The mitochondrial haplotypes for isolates are listed in supplementary table S1. Haplotype Ia was the most common at 69 %. The remaining isolates were made up of haplotype IIa (17.9 %) and haplotype Ib (13.1 %) (table 6). Haplotype IIb was not present in the sampled isolates. No additional SNPs representing previously unknown haplotypes were found. The Mantel test showed no significant correlation between MLGs and haplotypes ($r = 0.042$; $p = 0.288$) and no clustering of haplotypes was observed in the principle coordinate analysis of MLGs (figure 2). This suggests a lack of linkage between nuclear genotype and mitochondrial haplotype.

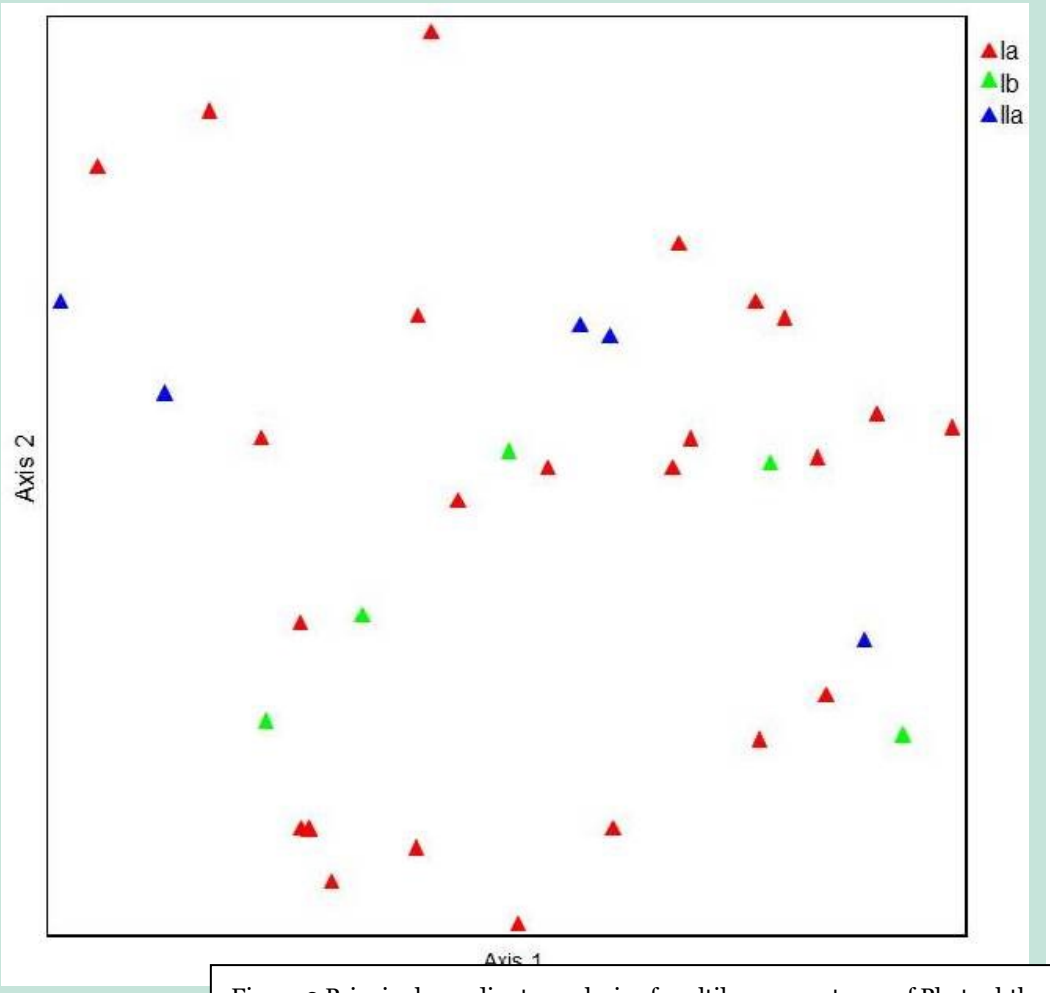
TABLE 4 MULTILOCUS GENOTYPIC DIVERSITY MEASURES OF *PHYTOPHTHORA INFESTANS* FROM SEVEN DIFFERENT FIELDS IN DENMARK, COLLECTED IN 2013

Field No.	n	R[a]	A_r[b]	H_{nb}[c]	Shannons[d]
1	4	0	1.800	0.471	0.555
2	5	1	2.600	0.516	0.758
3	6	0.75	2.900	0.515	0.811
4	5	0.25	2.100	0.387	0.540
5	13	0.45	2.600	0.355	0.586
6	41	0.24	2.900	0.462	0.750
7	10	0.56	2.300	0.455	0.662

a- Genotypic Richness Index. R = (M-1)/(N-1) as defined by Dorken and Eckert (2001). b- Allelic Richness- mean number of alleles per locus. c- Unbiased heterozygosity- the average probability across loci to draw at random different alleles in the same population. d- Shannon Information Index

2.3.3 Recombination

A number of analyses pointed towards a lack of recombination in the population as a whole. The FIS for each locus is listed in table 5. All but two loci exhibited a negative F_{IS} value. The pairwise test for linkage disequilibrium was significant at the $p < 0.001$ level for all loci pairs except for two loci, SSR8 and Pi70, for which the null hypothesis of free recombination could not be rejected when paired against all other loci (Table S5). Locus SSR8 had the most missing data, and locus Pi70 displayed a very low level of variation, which would account for the lack of statistical significance. The index of association for all isolates was significantly higher than zero at $IA = 0.418$ ($p < 0.001$) suggesting no recombination. The IA was also significant at the level of individual haplotypes (table 6). Calculations of theta showed that there was differentiation at the field level ($\Theta = 0.0268$; $p<0.01$), but not between haplotypes ($\Theta = -0.004$; $p=0.38$). The distinct mitochondrial haplotypes are not acting as separate populations, and evidence points towards dominating clonal reproduction in the Danish population as a whole.

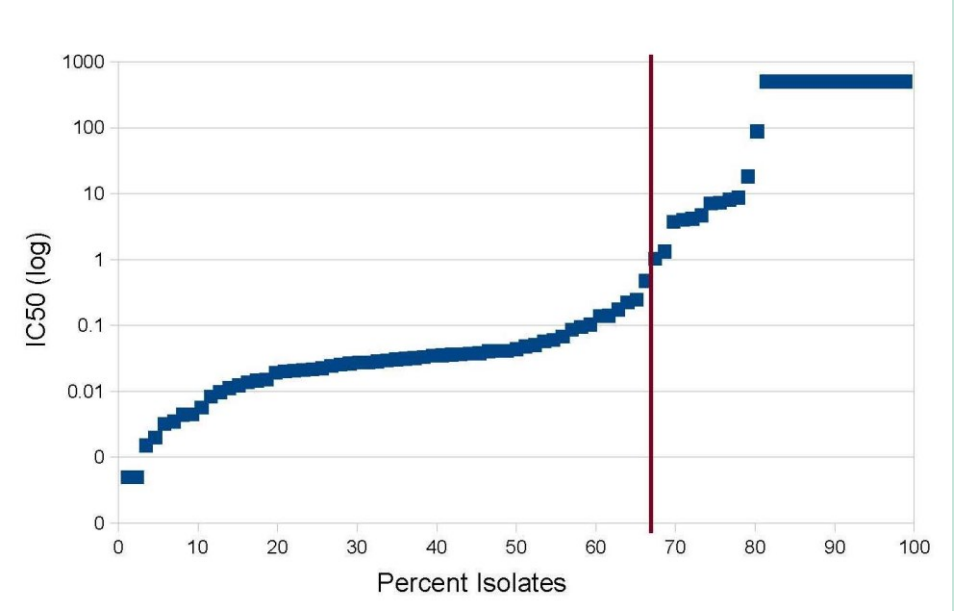


FIGUR 2 PRINCIPAL
INFESTANS LABELLE
VISIBLE CLUSTERIN
CORRELATION FOUN
0 042, P = 0 288), S

Figure 2 Principal coordinate analysis of multilocus genotypes of Phytophthora infestans labelled with their corresponding mitochondrial haplotype. There is no visible clustering of the three haplotypes (Ia, Ib and IIa) present, and no correlation found between genotype and haplotype using the Mantel test (r = 0 042, P = 0 288), suggesting that they are not linked.

2.3.4 Metalaxyl-M resistance

The test for Metalaxyl-M resistance revealed a pattern where sensitive isolates were extremely sensitive and resistant isolates were extremely resistant, with few intermediate phenotypes (figure 3). In total 66.3 % of isolates were sensitive (S; IC_{50} < 1.0) and 33.7 % were resistant (R; IC_{50} > 1.00). A large portion of resistant isolates were not affected by even the largest amounts of Metalaxyl-M tested in this study, and therefore have an IC_{50} > 500 (58.6 % of resistant isolates; 19.7 % of total). The Metalaxyl-M resistance and IC_{50} for each isolate are listed in supplementary table S1.



FIGUR 3 DISTRIBUTION OF THE LOG (HALF MAXIMAL INHIBITORY CONCENTRATION OF METALAXYL-M) (LOG IC_{50}) OF ISOLATES OF *PHYTOPHTHORA INFESTANS*. SENSITIVE PHENOTYPES (IC_{50} < 1) MAKE UP 66,3% OF ISOLATES.

The odds ratio for having resistance given the presence of a certain allele is displayed in figure 4 for the most common alleles. Approximately half of the alleles have odds ratios near or above one, but the Chi Square test was used to determine which of these represented a significant difference from an equal distribution and were not simply due to chance. Two of the alleles whose presence indicate the highest probability of a phenotype of resistance are allele 285 (OR= 5.14) from locus SSR4 and allele 195 (OR= 9.12) from locus Pi70. Alleles 166 and 170, both from the Pi04 locus, also show significant odds ratios, higher than three. These specific alleles were linked to a phenotype of high resistance to Metalaxyl-M. These three loci were subsequently located on the reference genome and none were found on the same supercontig as the gene containing the RPA190 SNP that confers Metalaxyl resistance (Table S6). Although the chromosomes of *P. infestans* have yet to be indentified, this search allowed us to confirm that the alleles are not in very close physical proximity to genes known to operate in resistance.

TABLE 5 MEASURES OF HETEROZYGOSITY FOR 10 LOCI ACROSS ALL POPULATIONS OF *PHYTOPHTHORA INFESTANS*

Locus	H_{NB}	H_{OBS}	F_{IS}
SSR4	0.75	0.88	**-0.18**
SSR8	0.51	0.71	**-0.40**
SSR2	0.42	0.53	**-0.27**
Pi63	0.49	0.58	**-0.20**
Pi02	0.51	0.63	**-0.25**
SSR11	0.44	0.42	0.07
Pi04	0.65	0.52	0.20
Pi70	0.16	0.17	**-0.03**
SSR6	0.49	0.64	**-0.32**
Pi4B	0.57	0.77	**-0.36**

[a]H_{NB}, the unbiased expected heterozygosity for small sample sizes. [b]H_{OBS}, the observed heterozygosity. [c]F_{IS}, inbreeding coefficient of an individual, I, relative to the subpopulation, S. The F_{IS} statistic is based on H_{NB} to account for small sample size. Negative F_{IS} values, in bold, are indicative of clonal reproduction.

2.4 Discussion.

The presence of both A1 and A2 mating types in the majority of fields sampled here suggest the possibility for sexual reproduction and recombination. However, the presence of different mating types and even the production of oospores alone is not enough to determine whether sexually produced offspring are successfully causing infections or successful in causing outbreaks from one year to the next. In the oomycete *Aphanomyces euteiches*, strong evidence exists for dominantly clonal reproduction despite the presence of oospores (Grünwald & Hoheisel, 2006).

Several studies have suggested that high genotypic diversity in *P. infestans* can be used as an indicator for sexual reproduction, and is due to a high level of sexual recombination (Brurberg et al., 2011; Sjöholm et al., 2013). In this study, diversity measures varied greatly from field to field (Table 3), but in general were slightly lower than what has been previously reported for Denmark (Brurberg et al., 2011; Sjöholm et al., 2013). In several cases, however, the highest levels of diversity are found in regions that have only one mating type present and no possibility of sexual reproduction (Delgado et. al, 2013; Li et. al, 2013b; Barquero et al., 2005). High genotypic diversity should not be synonymized with high rates of recombination; instead it is important to run tests for linkage disequilibrium to determine whether or not sexual recombination is occurring in a population. In the current study this was calculated as the index of association (Agapow & Burt, 2000). We found that the I_A was significantly higher than zero (I_A=0.4, P<0.001), allowing us to reject the hypothesis of a freely recombining population and suggesting linkage between loci and probable clonality.

In addition, F_{IS} values were negative for all but two of the loci (table 5). A negative F_{IS} is expected in clonal diploid populations, where mutation events at each locus lead to an accumulation of heterozygosity over time (Balloux et al., 2003). In parasites with acyclic life cycles, it is common to see extremely low F_{IS} values, approaching -1, while even small amounts of sexual reproduction will cause the F_{IS} to shift rapidly back towards 0 (Prugnolle & Meeûs, 2008). The observed variance in F_{IS} values between loci and generally low F_{IS} values overall could be indicative of very low levels of cryptic sex in a dominantly clonal population (Balloux et al., 2003). While personal observations suggest that some early infections may be caused by soil-borne oospores, it appears that clonal isolates are still dominating in this region. Brurberg et al. (2011) conducted a similar SSR analysis in the Nordic countries of Denmark, Finland, Norway, and Sweden, and concluded that the populations were sexual based on the level of diversity and Fst values. This could be due to the

different sampling strategy employed, sampling only once from each field. When taking multiple samples from the same field in the current study we found that the same genotype appeared several times, but still many different genotypes were found within single fields.

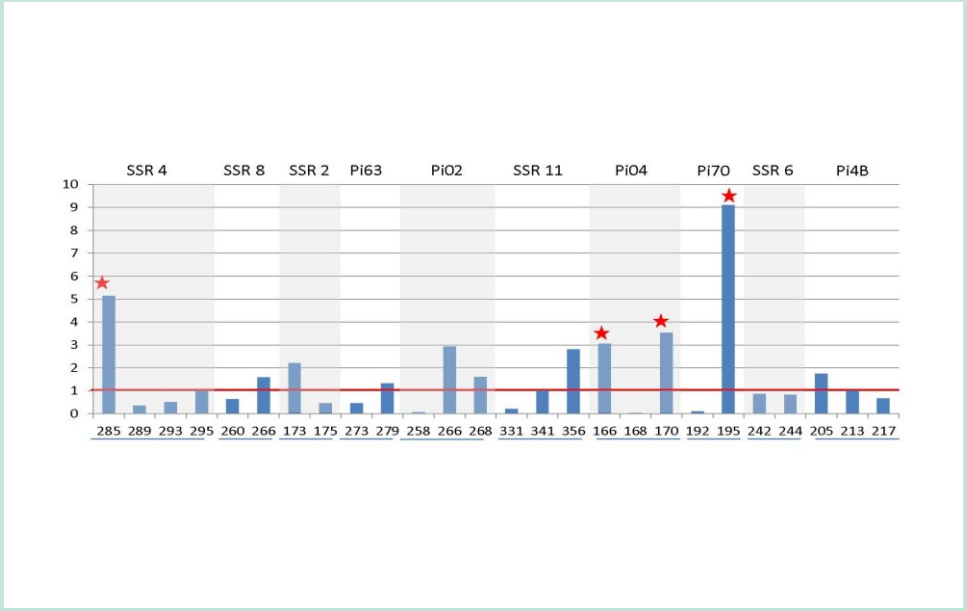
TABLE 6 INDEX OF ASSOCIATION (I_A) FOR INDIVIDUAL HAPLOTYPES AND FOR THE TOTAL POPULATION OF ISOLATES OF *PHYTOPHTHORA INFESTANS*, DETERMINED USING 10 SSR LOCI

	N[a]	I_A	*p*-value
Haplotype Ia	58 (69.0)	0.41	0.001
Haplotype Ib	11 (13.1)	0.51	<0.001
Haplotype IIa	15 (17.9)	0.15	0.007
Total	84	0.41	<0.001

[a]Values in parentheses are the percentage of the total population

The I_A analysis was conducted on clone corrected data, so the pattern of clonality observed was not the result of counting the same individual more than once. The results show that by sampling only once from each field a large part of the genotypic diversity remains unsampled. A later study of the same Nordic countries found that the IA was much higher in Denmark at the individual field level, although not for the total population (Sjöholm et al., 2013). In about 50% of fields tested in that study, the I_A was significantly different from zero (Sjöholm et al., 2013).

Genetic variation is not necessarily a result of recombination alone and can also be generated through other mechanisms in asexually reproducing organisms. It has been shown that there is genetic variation in neutral RAPD and AFLP markers of asexually produced progeny of *Phytophthora infestans*, and while this variation could be explained by random mutation, the high levels of phenotypic variability in virulence of the isolates suggests that other mechanisms such as gene conversion could also play a role (Abu-El Samen et al., 2003). In the closely related *Phytophthora sojae*, mitotic gene conversion has been observed as a frequent occurrence that is likely widespread throughout the genome in certain genetic hybrids (Chamnanpunt et al., 2001). Somatic mutations in microsatellite loci have been observed in a number of plant species (Lian et al., 2004; Marriage et al., 2009; O'Connell & Ritland, 2004), and it has been suggested that such mitotic mutations in microsatellite loci could play a significant role in clonal species (Lian et al., 2004; Marriage et al., 2009). If one considers that it only takes four to five days for *P. infestans* to complete its asexual life cycle and that it therefore goes through numerous generations over the course of a single growing season, the possibility that the diversity we see may be due to somatic mutations should not be discounted.



FIGUR 4 ODDS RATIOS FOR THE MOST COMMON ALLELES (OBSERVED >25 TIMES) AT DIFFERENT LOCI, SHOWING THE ODDS OF RESISTANCE TO METALAXYL-M GIVEN THE PRESENCE OF THE ALLELE. THE HORIZONTAL LINE (BOLD OR RED) DENOTES AN ODDS RATIO OF 1, ABOVE WHICH THERE ARE INCREASED ODDS OF RESISTANCE TO METALAXYL-M. ODDS RATIOS DEVIATING SIGNIFICANTLY (P<0.05) FROM A RANDOM DISTRIBUTION ARE ANNOTATED WITH AN ASTERISK (*).

The question remains why one should find higher genotypic diversity in Nordic countries than in the rest of Europe. Use of Metalaxyl is relatively low in Scandinavia compared to other regions (Lehtinen et al., 2008) and it has been observed that diversity increases with a decrease or lack of Metalaxyl use in both *Phytophthora infestans* (Grünwald et al., 2006) and in *Phytophthora ramorum* (Vercauteren et al., 2010). Studies that have measured diversity in *P. infestans* over the past couple of decades have varied greatly in the markers used and the sampling strategies employed, which makes a meta-analysis of diversity challenging. Perhaps the efforts made through networks like EuroBlight (www.euroblight.net) to sample using the same SSR markers throughout Europe, will allow us to answer whether diversity is indeed higher in Nordic countries, and how it correlates to factors such as mode of reproduction, weather patterns, and management practices.

However, as this study shows, it is also crucial to make sure the sampling strategy used captures all of the diversity present.

Three out of the four known mitochondrial haplotypes for *Phytophthora infestans* were found in our samples. Haplotype Ia was the most common with a lower frequency of IIa, as is true for many other regions in Europe (Cooke et al., 2012; Day et al., 2004; Flier et al. 2007; Knapova & Gisi 2002). Unexpectedly, we found multiple nuclear genotypes with the mitochondrial haplotype Ib. The clonal lineage US-1 with haplotype Ib dominated Europe in the mid-1970s, but was thereafter displaced by lineages of the other haplotypes. Haplotype Ib was last detected on potato in Great Britain in 1986 (Day & Shattock, 1997) and reports of its presence in the rest of Europe since the 1980s have also been rare (Flier et al., 2007; Knapova & Gisi, 2002; Lebreton & Andrivon, 1998). However, it seems to have been recently re-introduced in the form of a single genotype, 6_A1, in Britain, Ireland and the Netherlands (Kildea et al., 2013). In contrast, we found several different genotypes with the Ib haplotype. The RFLP method used by Kildea et al. (2013) to distinguish haplotype is based on the presence of a single mutation (Griffith & Shaw, 1998), which makes it difficult to relate exactly to our study and one cannot be certain whether it indeed represents a haplotype identical to that of US-1, or whether this mutation could have arisen again independently (Kildea et al., 2013). In the current study we used 10 SNPs from four different regions of the mitochondrial genome to determine haplotype. It is unlikely that such a large number of identical mutations would evolve independently again. The more simple explanation is that haplotype Ib survived longer in Denmark or that these haplotypes were reintroduced more than once via seed potatoes.

The four mitochondrial haplotypes are separated by a large number of mutations (170 polymorphic sites) (Avila-Adame et al., 2006), which suggests an ancient divergence. As such, one would perhaps expect the distinct haplotypes to act as different populations with three different distributions of genetic distance and three different variances. This would lead to obvious deviations from the expected variance of a single recombining population, and an I_A significantly different from zero. To account for this we also ran the test for IA within the isolates of individual haplotypes and still got significant results (table 6), so we could discount the possibility that the linkage observed was simply due to the presence of three very distinct haplotypes. When we then tested for population differentiation, we found that there was indeed no differentiation between the three haplotypes (Θ=-0.004; P=0.38). A principal coordinate analysis showed that haplotypes did not group together based on MLGs (figure 2), and a Mantel test further confirmed that there was no significant correlation between MLG and haplotype. This lack of linkage observed between MLGs and mitochondrial haplotype is quite surprising. Even in a sexually recombining population, one would expect progeny to group together by haplotype, if the mitochondria are uniparentally inherited. The observed pattern could be explained by rare instances of sexual reproduction followed by longer periods of clonality, but the exact mechanisms through which certain haplotypes are passed on over others needs to be further examined.

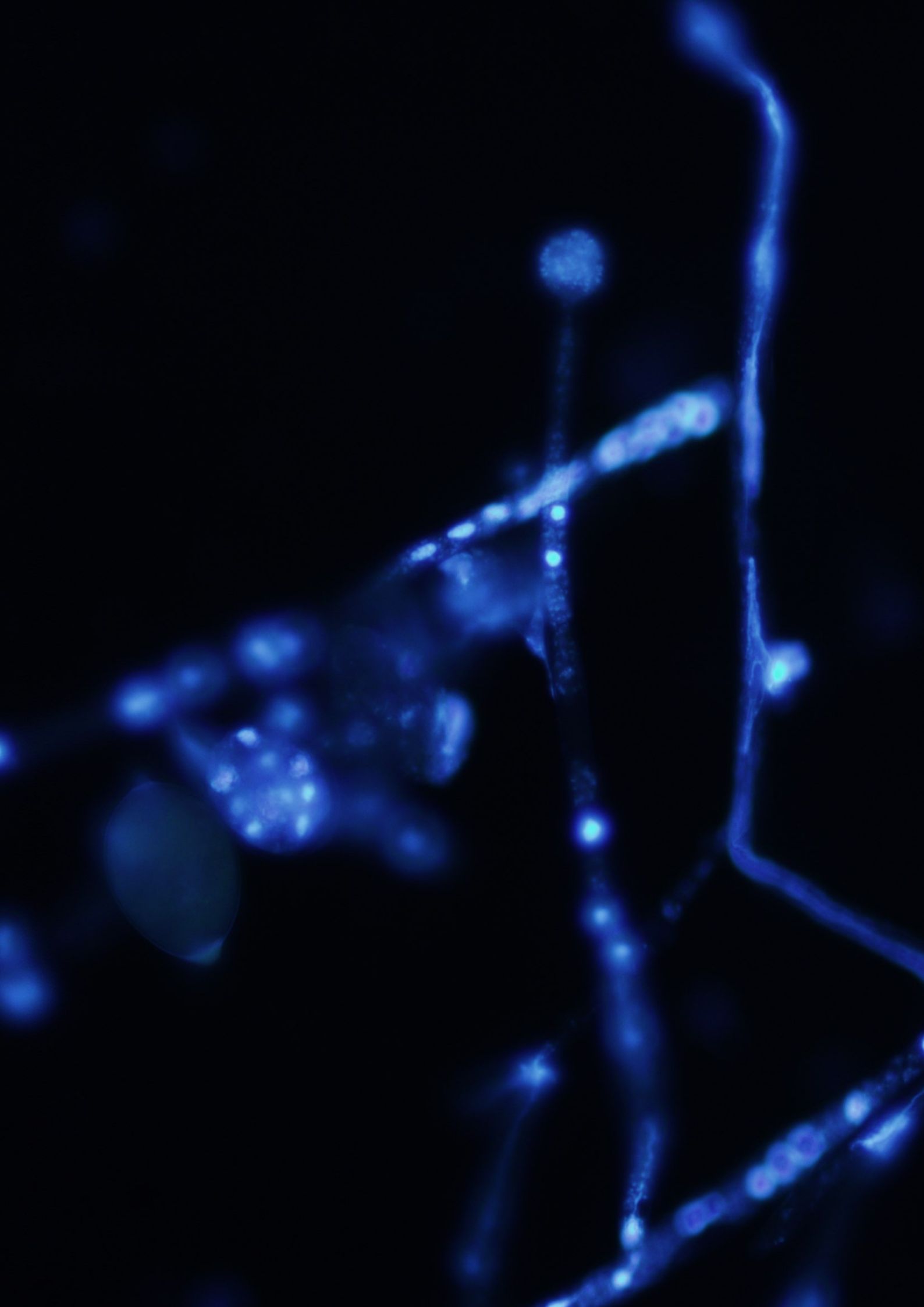
We did find that there was a significant level of differentiation in MLGs between fields (Θ = 0.0268; P<0.01). Sjöholm et al. (2013) also found weak differentiation at the field level in Nordic countries. This suggests that the initial inoculum, whether from a seed potato or from oospores, is playing a bigger role than long distance spread from one field to the next. The aggregation index based on spatial data was also significant (Ac = 0.378, p<0.0001), pointing towards clones originating from a single point.

It has been suggested that resistance to Metalaxyl in *Phytophthora infestans* is controlled by only one or two major loci in close proximity along with a host of minor genes. One such locus has recently been identified in the large subunit of RNA polymerase I (Randall et al., 2014). In our own isolates we see a pattern of resistance that matches this hypothesis, where most isolates fall into the two extremes of highly sensitive with an IC_{50} below 1.0, or highly resistant with and IC_{50} above 500 (figure 3). If resistance was controlled by a large number of different genes, we would expect to see a range of intermediate phenotypes, unless the population is strictly clonal.

Odds ratios revealed that specific alleles were strongly linked to Metalaxyl-M insensitivity in the populations sampled. Although there were a few samples with the same genotype whose level of resistance differed, we were able to determine that certain alleles are much more likely to occur in Metalaxyl-M resistant individuals. The loci in question are not located on the same supercontigs as the RPA190 SNP (table S6). This pattern would be unexpected in a sexually reproducing population where recombination would likely disrupt such a linkage, but is not surprising in light of the linkage disequilibrium found between nearly all loci (table S5). This suggests that resistance is to some extent linked to genotype and shows the potential for developing a more refined genetic marker system using more slowly evolving markers such as SNPs as predictors for Metalaxyl resistance.

In light of the lack of recombination evident in the populations from this study, it is not surprising to find that certain alleles are linked to a higher occurrence of resistance to Metalaxyl-M. What holds more interest is rather the absence of linkage between the multilocus genotypes and the mitochondrial haplotypes observed. More studies that simultaneously look at SSR genotype and mitochondrial haplotype of the same individuals will illuminate whether this relationship, or lack thereof, holds true in other populations around the world. The mechanism behind this pattern warrants further investigation.

FIGURE ON NEXT PAGE: MYCELIUM OF *PHYTOPHTHORA INFESTANS* STAINED WITH DAPI THAT REACT WITH DNA AND STAIN THE NUCLEI. NUCLEI IN THE HYPHAE CAN BE SEEN AS FLOURESCENT 'PEARLS ON STRINGS'. IN THE MIDDLE LEFT SECTION OF THE IMAGE, A ROUND SPORANGIUM WITH SEVERAL NUCLEI IS SEEN.



3. Development of a EvaGreen-based quantitative real-time PCR assay for airborne sporangia of *Phytophthora infestans* sampled under field conditions

3.1 Introduction

The oomycete *Phytophthora infestans* is the pathogen responsible for late blight on potatoes and tomatoes. *P. infestans* is spread through the dispersal of three types of spores: sexually produced oospores, asexually produced multinucleate sporangia, which are predominant, and uninucleate zoospores formed by cytoplasmic cleavage of the sporangia which are in turn released from the sporangia (Judelson and Blanco, 2005). Zoospores require liquid, such as a water film, to spread via swimming. Sporangia, however, can be dispersed by both water and wind, and can therefore travel larger distances of many kilometers, up to 20 km in three hours at wind speeds of 1-2 m s^{-1} (Aylor et al., 2001). At the height of the growing season, and when weather conditions are optimal, *P. infestans* can complete its asexual life cycle within 5 days, therefore releasing thousands of sporangia and leading to rapid spread of the disease. Potato late blight therefore leads to significant losses in yield globally each year (Haverkort et al., 2008).

An important tool in combating the disease is the use of fungicides, and large amounts are sprayed on potatoes during the growing season each year. In order to decrease the amount and optimize the timing of applications, a number of forecasting schemes, or so-called decision-support systems (DSSs) have been implemented around the world (ex. BlightPro DSS; http://newa.cornell.edu/index.php?page=potato-late-blight-dss), which predict the times of highest risk for infection based primarily on weather conditions (i.e. temperature, humidity, precipitation, UV light intensity). While these DSSs have reduced the amount of fungicides used compared to a fixed-interval spray program, they often still lead to unnecessary spraying, because they are based on the assumption that sporangia are always present in the fields, and that inoculum is not limiting (Taylor et al., 2003). This may not always be the case, and therefore a number of recent studies have focused on the use of airborne inoculum concentrations as a complement to existing DSSs (Aylor et al., 2011, Fall et al., 2015a, Fall et al., 2015b, Skelsey et al., 2009).

Integrating the detection and quantification of airborne inoculum into DSSs has already seen some success for other plant pathogens, such as Botrytis Leaf Blight of onions (Carisse et al., 2009) and Sclerotina rot of carrots (Parker et al., 2014), but the current systems are based on labor and time intensive microscopy and culturing on selective media respectively. The development of molecular methods to detect species or strains using quantitative real-time PCR (qPCR) offers an alternative to these systems, which potentially would greatly speed up the detection and reporting process (Carisse et al., 2009, Parker et al., 2014). In *P. infestans* such a qPCR assay for the quantification of sporangia was recently developed and tested on four different lineages of potatoes grown and inoculated in growth chambers (Fall et al., 2015b). The assay was developed using special primers with TaqMan probes, and sporangia were collected using a rotating-arm spore sampler (Fall et al., 2015b). SYBR Green based qPCR is an alternative method that has proven to generally be as efficient as TaqMan qPCR, but more cost effective as it does not require the synthesis of fluorescence-labeled probes (Arikawa et al., 2008, Karsai et al., 2002). Previously SYBR Green-based qPCR has successfully been used to detect and monitor *P. infestans* in planta (Llorente et al., 2010). In the current study we explore the possibility of an alternative qPCR assay based on EvaGreen technology and the use of the Burkard multi-vial cyclone (http://www.burkardscientific.co.uk/) to sample. EvaGreen is a third generation dsDNA binding dye that is thought to have some additional advantages over SYBR Green, such as less PCR inhibition, which allows it to be used at higher concentrations, and less non-specific binding and binding to single-stranded DNA (Mao et al., 2007).

FIGUR 5 THE LOCATION OF THE SPORE TRAP IS IN THE STRIP BETWEEN THE FIELD PLOTS INDICATED BY THE STAR. THE TWO DANIELLA TRIAL PLOTS WHOSE INFECTION WAS MEASURED IN THIS STUDY ARE DEMARCATED BY BOXES: NORTHWEST IN BLUE AND SOUTHEAST IN RED. THE PREDOMINATE WIND DIRECTION IN DENMARK IS WESTWARD, BUT THE SAMPLER WAS ALWAYS ORIENTED TOWARDS THE CURRENT WIND DIRECTIONS.

The Burkard multi-vial cyclone sampler offers the advantages that (1) the sample is collected directly into an eppendorf tube which can be easily used in subsequent molecular analyses, and (2) a high level of automation means it can be programmed to sample for a set time and then switch to a new tube, allowing it to run for up to a week without human interaction (West and Kimber, 2015). It is currently used for the detection of spores in the Brassica Alert network (http://www.syngenta-crop.co.uk/brassica-alert). A previous study using this sampler for the detection of fungal spores found that PCR detection was less successful in the presence of noise from other background airborne particles such as dust, pollen, and non-target spores, but that DNA purification was helpful in that regard (Williams et al., 2001). As such background noise is expected when running a sampler in the field, we tested different extraction procedures for their PCR sensitivity when *P. infestans* sporangia were added to a background mix of airborne particles.

In this pilot study we attempted to: (1) optimize a particular EvaGreen-based assay to be sensitive enough to detect single P. infestans sporangia, (2) test the sensitivity of detection with or without background noise from other spores and dust, (3) test the Burkard multi-vial cyclone sampler as a means of quantifying P.

infestans under field conditions during the summer growing season of 2014 in Denmark, and (4) compare our field qPCR assay with the infection pressure measurements calculated by the DSS used in the region, Blight Management (BM; www.skimmelstyring.dk), and the actual observed outbreaks in surrounding fields, in order to see how this information on the presence of inoculum could potentially influence how decisions to spray in this region are made in the future. To achieve this, a single Burkard spore trap was placed between fields of susceptible potato varieties, of which those trial plots untreated by fungicides were of particular interest in the current study (figure 5), and were inspected throughout the season for the development of infection. Spreader rows between the trial plots were artificially inoculated with a sporangial suspension on July 1st, 2014. The spore trap was allowed to run for 24 hours a day throughout the 2014 growing season, and an EvaGreen-based qPCR using mitochondrial primers was subsequently used to quantify the airborne sporangia following DNA extraction.

3.2 Materials and Methods

3.2.1 Primer Design

Primers were designed to amplify a 151 base pair segment of the NAD4 region of the mitochondria (Table). These primers bind to sequences that are unique to *P. infestans*, but based on close similarity according to BLAST (via www.fungidb.org), could potentially bind with three other oomycete species: *Phytophthora parasitica*, *Pythium ultimum,* and *Pythium arrhenomanes*. However, these species are not expected to be largely present in a potato crop dominated area, and/or are not expected to be highly present in an airborne form as they are restricted to soil or water (Meng et al., 2014**)**.

Primer name	Sequence	Product size
P2F5 (forward)	5k-CGTCAATAGGTTGTCCTAAAGC-3k	151
P2R6 (reverse)	5k-GAACCTGATGTTGTTGGTGTTG-3k	

3.2.2 DNA extraction

Two different kits were tested for PCR detection of single spores, with and without background noise from other airborne particles. Sporangia were harvested from pure culture isolates grown on pea agar at 15 ˚C in the dark. Sporangia were harvested by covering the surface with water and gently scraping off the mycelia, shaking the solution for 2 minutes, and then filtering through gauze. Sporangia were then hand-picked using a capillary under the microscope and transferred in varying quantities, from **1-15** sporangia, to eppendorf tubes for subsequent DNA extraction.

In order to mimic the background noise of other spores and airborne particles that are collected by the spore sampler, the spore sampler was allowed to run at Copenhagen University for six hours per day for several weeks. To our knowledge, there are no nearby potato fields, and these collections were undertaken at a time of year where there are not expected to be any *Phytophthora infestans* spores in the air. These tubes then had varying amounts of *P. infestans* sporangia added to them with a capillary, again from **1-15** sporangia, before going through the same extraction protocols.

FIGUR 6 THE BURKARD MULTI-VIAL CYCLONE SAMPLER . THE VIALS (EPPENDORF TUBES) ARE VISIBLE.

The first protocol tested was the QuickExtract Plant DNA extraction solution (Epicentre), which we have successfully used in the past to extract from *P. infestans* mycelia. Extraction was carried out according to manufacturer's instructions and required less than 10 minutes per sample. The second protocol tested was the DNeasy Plant mini-kit (Quiagen), with an extended 1 hour initial lysis at 65˚C and two 10 minute elutions at 65˚C using warm elution buffer to maximize DNA yield. In both cases blanks without any added *P. infestans* sporangia were included as negative controls. The same PCR mix was used to test both extractions, as follows: 1 µL of each the forward and reverse primers (10 µM), 5 µL 5x HotFire Pol Blend Master Mix (Solis Biodyne), 1 µL DNA template, and 12 µL water for a final volume of 20 µL. The two types of extracts were subjected to the same PCR conditions, as follows: 95 ºC for 15 minutes, followed by 25 cycles of 95 ºC for 15 s, 55 ºC for 15 s, and 72 ºC for 15 s, and a final extension at 72 ºC for 5 min. The PCR products were run on a 1.5% agarose gel for 20 min at 120V.

3.2.3 Field collections

Potato trials were carried out at Aarhus University Flakkebjerg (AU) as part of a larger project testing a variety of spraying regimes (Nielsen, 2015). Potatoes were planted in sandy clay loam (JB 5-6), with a randomized complete block design of four replicates in the starch varieties Folva, Kuras and Dianella. Plot size was 36 m². Of interest in this study were the Daniella plots untreated by any fungicides (figure 5). The potatoes were planted on 1 May, 2014 and emerged on the 1 June. The weather in July was dry and the trial site was irrigated by boom six times (25 mm water) from mid-June to the end of July. The late blight trials were artificially inoculated on 1 July by spraying of a sporangial suspension of *P. infestans* (1000 sporangia/ml) from a mixture of different isolates over spreader rows between the blocks. The percent of late blight infection in each field plot was recorded approximately once per week form July 8-October 1, 2014 (13 times/plot). A visual assessment was made of the percentage of green leaf area attacked by late blight, following the EPPO guidelines PP 1/002 (4) (revised in 2009).

3.2.4 The Burkard Multi-Vial Cyclone Sampler

The Burkard Multi-Vial Cyclone Sampler (Burkard Manufacturing Co. Ltd.) is a dry cyclone sampler that vortexes in air at a rate of 16.6 L per minute into a chamber above a 1.5 mL eppendorf collection tube where particles settle as they separate from the incoming airflow (Figure 6). The sampler has a wind vane so that the intake valve is always facing the prevailing wind. The sampler holds 8 vials on a carousel which can be programmed to rotate at a set time interval to begin a new collection. The device is powered by solar panels and a backup battery (Figure 7). In the current study the sampler was placed on an open strip 24 meters wide, between two potato fields in order to get a general picture rather than have the results skewed by a single local outbreak very close to

the sampler. The fields west of the strip were of the variety Dianella (untreated plots; 30 meters NW of the spore sampler), and Folva and Kuras (only fungicide treated plots; 5 meters west of the spore sampler). The field east of the strip was of the variety Dianella (untreated plots; 20 meters SE of the spore sampler). It was left to run for 24 hours a day, seven days a week, from June 25th to October 3rd, 2014. Collection vials were programmed to switch at midnight each night. On Wednesday each week, tubes were collected from the sampler and stored at -20 ℃ for several weeks until ready for extraction with the DNeasy Plant mini-kit.

FIGUR 7 THE BURKARD MULTI-VIAL CYCLONE SAMPLER IN THE FIELD WITH THE COVER BEING REMOVED. PART OF THE SOLAR PANEL IS VISIBLE.

3.2.5 Set up of standard curve

The dilution series for the standard curve was made from sporangia harvested from a single pure culture isolate grown on pea agar medium in the dark at 15 ˚C. Sporangia were harvested after 3 weeks of growth by covering the surface with water and gently scraping off the mycelia, shaking the solution for 2 minutes, and then filtering through gauze. The concentration of sporangia in solution was measured using a hemocytometer. This volume with a known estimated number of sporangia was then allowed to sediment overnight at 15 ˚C in the dark. To ensure that all the sporangia had settled near the bottom of the tube, the solution was also spun down for 2 min at 20,000 × g, before the majority of the water was removed, leaving a concentrated spore solution of approximately 50-100 µL. It was confirmed microscopically that the water which was removed did not contain any of the sporangia.

Extraction was performed using a Qiagen DNeasy Plant mini-kit, with an extended 1 hour initial lysis at 65˚C and two 10 minute elutions at 65˚C using warm elution buffer to maximize DNA yield. DNA concentration after extraction was measured using a Qubit fluorimeter. This DNA extract made from approximately 703,000 spores was then diluted 8 times by a factor of 5, so that the lowest concentration in the dilution series corresponds to the DNA of approximately 9 spores.

3.2.6 qPCR

Quantitative real-time PCR (qPCR) was carried out using the 5x HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne), which eliminates the risk of false positives from primer dimers and non-specifically annealed primers through its 95˚C activation. A variety of different PCR mixes and cycling conditions were tested for the best standard curve. The ideal PCR mix was found to be 5 µL of Supermix, 1 µL of each the forward and reverse NAD primer (10 µM), 5 µL of template DNA, and 8 µL sterile water. The qPCR worked best with a 15 min incubation at 95˚C followed by 40 cycles of 95 ºC for 15 s, 55 ºC for 15 s, and 72 ºC for 15 s, after which the temperature was increased incrementally from 65 ºC to 95 ºC to test the melt curve. At least two replicates were run for each sample, and every run included negative controls with only the master mix. The number of sporangia per cubic meter air was calculated using the equation: (number of sporangia per collection tube × 1000 L m^{-3})/(16.6 L min^{-1} *× 60 min h^{-1} × 24 h).

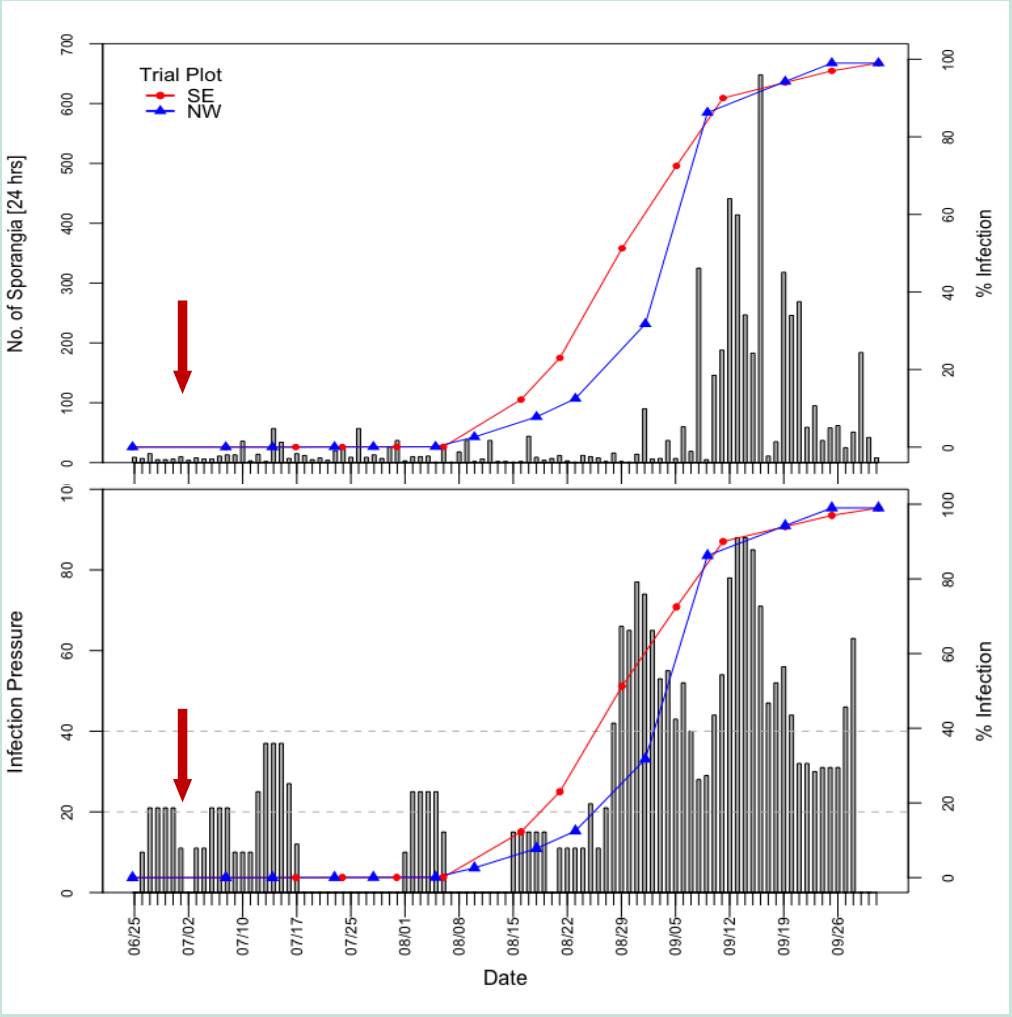
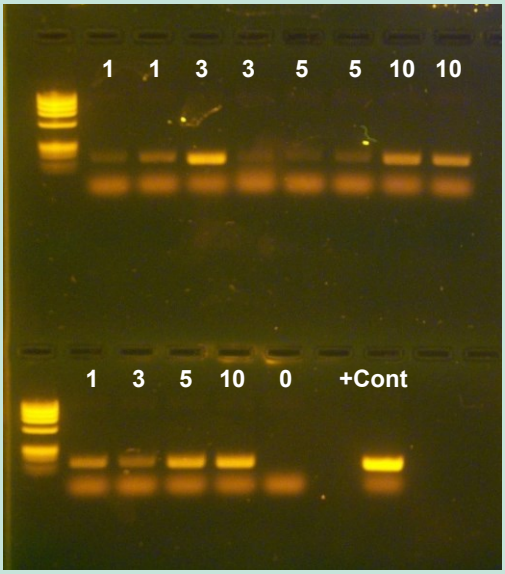


FIGURE 8. PREDICTORS OF INFECTION PLOTTED AGAINST OBSERVED INFECTION IN THE FIELD. THE RED AND BLUE LINES REPRESENT THE PERCENT OF INFECTED PLANTS OBSERVED IN TWO DANIELLA TRIAL PLOTS UNTREATED WITH FUNGICIDES, LOCATED SOUTH-EAST (SE) AND NORTH-WEST (NW) OF THE SPORE TRAP RESPECTIVELY. THE ARROWS INDICATE THE DAY SPREADER ROWS WERE ARTIFICIALLY INOCULATED BY SPORANGIA (JULY 1, 2014). (TOP): GREY BARS REPRESENT THE NUMBER OF SPORES COLLECTED BY THE SPORE TRAP IN 24 HOURS FOR EACH DAY, QUANTIFIED BY QPCR. (BOTTOM): GREY BARS REPRESENT THE INFECTION PRESSURE FOR EACH DAY, CALCULATED AS DESCRIBED IN EXPERIMENTAL PROCEDURES. VALUES ABOVE 20 INDICATE A 'MODERATE RISK' OF INFECTION AND ABOVE 40 ARE CONSIDERED 'HIGH RISK' (DEMARCATED BY DASHED HORIZONTAL LINES).

3.3 Results

A test of two different DNA extraction protocols revealed that both the QuickExtract and DNeasy kits were sufficient for detection of a single sporangium using PCR to amplify the NAD region of the mitochondria (Figure 8). Additionally, when *P. infestans* sporangia were added to tubes from the spore trap that had been run to collect other random spores and airborne particles, the PCR was not significantly inhibited by this background noise (figure 2). The DNeasy extraction kit was then used for subsequent qPCR.

The number of sporangia in the spore trap reflected the late blight incident (% infected plants) in the field (figure 8). The first symptoms were detected in the spreader rows on 8 July and in the untreated trial plots in mid-July. Due to dry weather in July and low infection pressure of late blight there was no disease development until the last part of July – start of August with a severe epidemic development in untreated plots in the last half of August and beginning of September. In mid-September almost all untreated plots of the susceptible variety Dianella were destroyed by late blight. Due to the unfavourable conditions for development of late blight, the start of the epidemic varied between the different trials. The weather conditions were very wet at the lifting of the potatoes in beginning of October and low to moderate attacks of tuber blight were seen in several plots.



FIGUR 9. TEST FOR DETECTION OF DNA USING NAD PRIMER AND NORMAL PCR ON SINGLE SPORES AFTER DNEASY KIT EXTRACTION. UPPER ROW: WITH BACKGROUND SPORES FROM SPORE TRAP; LOWER ROW: CONTROL (WITHOUT SPORE TRAP TUBES). THE NUMBER OF SPORANGIA ARE INDICATED ABOVE THE LANES.

EvaGreen-based qPCR was optimized before being run with the spore trap samples. The best standard curve was achieved when using 5 µL of template DNA, and had an average R2= 0.992 and E= 83.8% across all runs. The qPCR results between replicates were not identical, but were closely correlated ($R2=0.98$, $p<0.001$; figure 10). In 6.0% of samples, qPCR was not able to detect spores in one of the replicates although they were present in the other. Sporangia were detected throughout the entire season, but on most days only at low levels- less than one spore per cubic meter air (<23.9 sporangia in 24 hours). The largest peaks in sporangia occurred in late August- September (figure 8).

The infection pressure as calculated for the Danish DSS, Blight Management (www.skimmelstyring.dk), is shown for the nearby station in Dalmose (figure 8). A significant ($p < 0.001$), but weak ($R2= 0.277$) correlation was seen between infection pressure and number of airborne sporangia (figure 11).

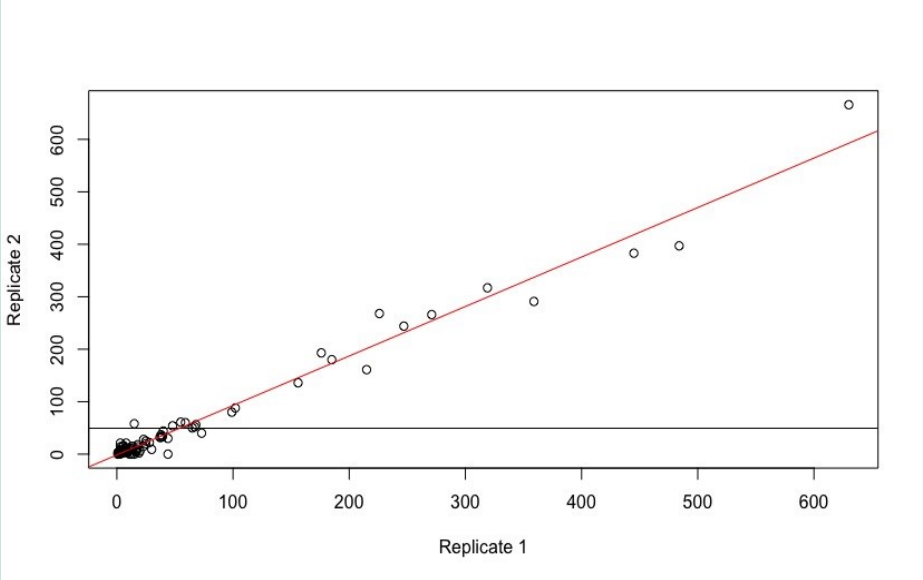


FIGURE 10. RELATIONSHIP BETWEEN THE TWO QPCR REPLICATES. A STRONG CORRELATION IS PRESENT (RED LINE; R^2=0.98, P <0.001). THE BLACK HORIZONTAL LINE IS THE MEAN VALUE FOR REPLICATE 2 AND REPRESENTS THE NULL MODEL OF NO RELATIONSHIP. THE VALUES ON THE AXES ARE THE QPCR MEASUREMENTS.

3.4 Discussion

3.4.1 EvaGreen-based qPCR results in comparison to the current DSS in Denmark

When we compare the results from our qPCR analysis with the information from the DSS based on meteorological data already in place, we find that infection pressure estimated from weather conditions is still a crucial, and in this case a more accurate, indicator for when infection will occur and spread (figure 8). In the current study we found that sporangia were present throughout the season at a low level, and even when fields were inoculated with spores (July 1st), a full epidemic didn't break out until weather conditions were suitable (late August, early September). Slight increases in the airborne spore concentration were observed around the time that disease first presented itself after inoculation (July 8 in spreader rows, July 17 in trial plots), but a real dramatic increase in the number of spores detected was not observed until September, when the plots already suffered from a full-blown epidemic. The first large peak of over 100 sporangia detected through qPCR did not occur until September 8th. By September 5th, late blight had already developed to 72 % in the SE Daniella trial plot, and similarly to 86% by September 9th in the NW Daniella trial plot. In the current study only a single spore trap was employed, and this perhaps was insufficient to get a real picture of the number of sporangia in the air. In Denmark particularly, infections are generally thought to be more focal and long distance spread from one field to the next limited (Montes et al., 2016), and therefore a higher density of samplers may be needed. In the end, the amount of samplers and the sensitivity of an inoculum detecting system needed depends on the threshold of sporangia needed to cause an infection. This in turn depends on a number of variables, on all sides of the disease triangle: the host, the pathogen, and the weather conditions. Studies show that the amount of *P. infestans* sporangia needed to cause an outbreak depends greatly on the potato cultivar in question; in susceptible cultivars, such as those included in this study, a single spore may be enough to start severe epidemic development under

favorable weather conditions, whereas in resistant cultivars it may require tens of thousands (Skelsey et al., 2009). As seen here, even when sporangia are sprayed near the fields, their presence is not enough to cause an infection when weather conditions are inhospitable as they were directly after inoculation in this case.

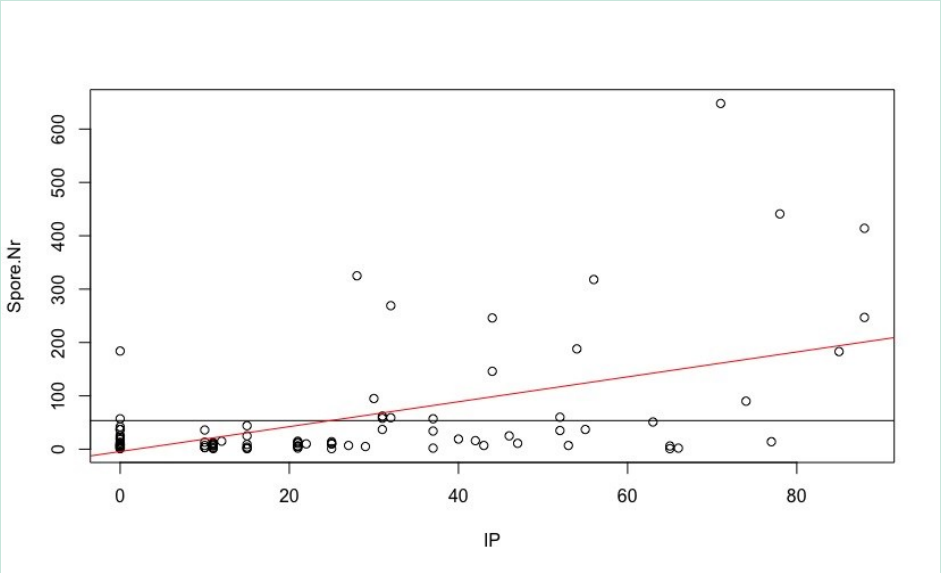


FIGURE 11. INFECTION PRESSURE (IP) AS AN INDICATOR FOR THE NUMBER OF AIRBORNE SPORANGIA DETECTED BY QPCR. IP WAS CALCULATED AT THE NEARBY STATION IN DALMOSE THROUGH THE BLIGHT MANAGEMENT PROGRAM AS DESCRIBED IN EXPERIMENTAL PROCEDURES. A WEAK POSITIVE CORRELATION BETWEEN IP AND THE NUMBER OF SPORANGIA PER 24 HOURS IS DETECTED (R^2=0.277; P < 0.001). THE BLACK HORIZONTAL LINE IS THE MEAN VALUE FOR NUMBER OF SPORANGIA AND REPRESENTS THE NULL MODEL OF NO RELATIONSHIP.

Conversely, had there been no spores present when the weather turned favorable for attack, late blight would not have developed. We found only a weak trend (figure 10; R^2=0.277; $p < 0.001$) between the infection pressure, which is calculated based on relative humidity and temperature, and the number of airborne sporangia detected using qPCR. Therefore, infection pressure alone should probably not be used as a proxy for the amount of airborne inocula present. Previously Iglesias et al. (2010) found a strong correlation between meteorological data and the concentration of airborne sporangia. However, Fall et al. (2015a) did not see the same pattern, and suggested that this may be due to sporangia being transported long-distance from other fields. In this case spores were present first, due partially to our artificial inoculation, enabling disease to spread rapidly as soon as the right conditions presented themselves. We conclude that a future DSS should consist of a combined approach incorporating both weather and inoculum data, perhaps as is already implemented in the Brassica Alert network (http://www.syngenta-crop.co.uk/brassica-alert). In this system, the molecular tests for airborne spore concentration are only carried out when the correct weather conditions for infection are detected. The current study suggests that it is not necessary to test for spore concentration throughout the season, but rather qPCR tests could be run only when infection pressure passes a certain threshold. Using the current system it would not be very labor intensive to have a spore trap running throughout the summer, and to quickly execute extraction and qPCR only when the correct weather conditions present themselves. Alternatively, the spore trap and qPCR could be run early in the season until sporangia are detected, or a peak in the sporangia indicates the first large attack, and inoculum could then be assumed to be present from that point forward, leading to spraying whenever the weather conditions are correct.

The clonal lineage of *P. infestans* also plays a role (Fall et al., 2015b), as they vary in virulence. It would therefore be of great advantage to not only be able to detect whether inoculum is present, but also to be able to molecularly determine the nature of the isolate releasing sporangia. In areas such as North America, where only a few dominant clonal lineages are present, it would be sufficient to be able to identify which clonal lineage is attacking, if the aggressiveness of these lineages has already been characterized, such as in Fall et al. (2015b). In areas such as Denmark, where the current study took place, it would be more appropriate to find markers linked to certain traits such as fungicide resistance or virulence, as the number of different clonal lineages is much higher and more variable from year to year (Montes et al., 2016), making it unfeasible to characterize each one in advance. In the current study a mitochondrial locus was amplified, but future studies should perhaps focus on the ability to amplify nuclear genes which could be used as such markers for important phenotypic traits.

3.4.2 Viability of different extraction protocols

It is crucial when evaluating a new qPCR assay to assess how well the artificially made standard curve reflects the real world. A key point is the extractability of the DNA and amount of inhibitors present under different conditions. Therefore, one of the first questions is which method to use for the extraction of one's DNA. In the current study we found that both QuickExtract and DNeasy extraction kits were able to detect small numbers (1-15) of spores in both pure and background spiked samples in a laboratory setting. Fall et al. (2015b) opted for a lossless DNA extraction without purification when creating their qPCR assay for *P. infestans,* and were able to confirm that the number of spores detected with the assay matched what was counted microscopically. One advantage of such a method is that the maximum amount of DNA remains, allowing for more sensitive detection of even a single spore. Although greenhouse and semi-field studies provide a wealth of information that can only be obtained in a controlled setting, it is important to keep in mind that when samplers are put out in the field they will collect more than just the spores of interest, and PCR inhibitors are likely to be present. In the current field collections, tubes often visibly contained other dark spores or soil dust, as well as small insects in some cases. When testing for PCR detection of the fungus *Penicillium roqueforti* using the same air sampler as the current study, it was found that in background air samples spiked with *P. roqueforti* spores, sensitivity was lowered compared to pure spore solutions and consistent results were only obtained using 1,000 or more spores (Williams et al., 2001). The sensitivity was greatly improved to 100 and sometimes a single spore, however, when a combination of DNA extraction and purification was implemented (Williams et al.2001). It is therefore important before testing an assay in the field to ensure that PCR detection is not inhibited using the spore treatment of choice, as was done in the current study. For qPCR trials, our field samples were extracted using the DNeasy kit, however for future implementation of spore trap sampling in DSSs where rapid analysis is crucial, the QuickExtract offers an attractive alternative as the whole protocol can be completed in under 10 minutes.

3.4.3 Comparing the Burkard Multi-vial Cyclone air sampler with other samplers

The current study employed a Burkard Multi-vial Cyclone air sampler, which has been successfully used in the past for sampling of *Sclerotinia sclerotiorum* ascospores (Parker et al., 2014), and is currently employed in the Brassica Alert Network (http://www.syngenta-crop.co.uk/brassica-alert). Alternatively, a rotating-arm spore sampler is often used for this purpose, including in studies of *P. infestans* (Fall et al., 2015a, 2015b). These rotating-arm samplers consist of greased rods onto which spores are impacted, and can therefore usually lead to some error from being improperly greased or covered in soil dust (Carisse et al., 2009). Indeed it has been calculated that the rod samplers have approximately 37% the efficiency of a seven day volumetric sampler (Aylor, 1993), but as this figure is known, it is possible to calculate it into models using Rotorod samplers (Fall et al., 2015a). The Burkard sampler possesses the extra advantage that particles are collected directly into eppendorf tubes, which eliminates the time needed to remove sporangia from rods and evaporate the grease. One downside to this method of

collection, however, is that when precipitation is high, water can also enter the sampler. This was the case on two occasions during the current study, where rain caused tubes to be filled one-fourth of the way with water. When sporangia are desiccated, they lack the ability to germinate (Judelson and Blanco, 2005), but if stored in water the number of sporangia may have multiplied giving an incorrect picture of the amount of sporangia in the air in the qPCR assay. It is unclear whether this was the case on the two days in question during the current study. On the first such day, the amount of sporangia detected was quite high (269), but followed two days with similarly high values (318, 246). In the second instance, only three sporangia were detected in the course of the 24 hours, so it seems that possible germination had no strong effect. The high level of automation using the Burkard sampler also makes it an attractive option, but it is thought that this type of sampler may have variable efficiency when it comes to collection (West and Kimber, 2015). Although there are definite advantages to not having to manually change the sampler each day, it is possible that spores left in the trap for several days before being stored in the freezer could suffer some degradation over time. However, no clear pattern of this was evident in the current samples, as the number of sporangia measured varied greatly throughout the week and did not decrease consistently with age within each week (data not presented).

The largest advantages of Rotorod samplers, and the reason they are likely more widespread, are their cost and the fact that they are lightweight (Aylor, 1993). This means that it is easier to install them at different heights over the canopy and also that a higher number can be employed for the same cost. Therefore, it is important to ask how many samplers are needed to get a good picture of what is happening in a field, and how they can best be placed. According to modeling carried out by Skelsey et al. (2009), the number of spores needed to cause yield loss varies between focal epidemics that are localized to one part of a field versus homogenous attacks. Having multiple samplers would therefore also be advantageous for determining the nature of an epidemic. For this pilot study, however, only a single sampler was employed. By placing the sampler between fields, and therefore not too close to the plant canopy, we avoided bias from a focal attack releasing spores very close to the sampler. Indeed it is sometimes recommended to place samplers high above the canopy, such as on rooftops, to buffer from this effect and to study the presence of inoculum over a regional scale (West and Kimber, 2015).

The EvaGreen-based qPCR assay proved sensitive enough to detect low levels of sporangia in the field. Along with its low cost compared to TaqMan qPCR, the dye's ability to function well under fast cycling PCR (Mao et al., 2007), such as the 15 second cycles used here, makes it an ideal candidate for use in DSSs, where quick results are crucial. However, the specificity and accuracy of the qPCR assay hasn't been validated microscopically. Of the species identified that could potentially also bind to the primers, *P. ultimum* is the only one that also attacks potatoes. It is, however, a soil pathogen, and isn't expected to disperse aerially unless it is bound to soil dust that is thrown into to air through plowing, for example (Francis and St. Clair, 1997). We also do not know if the qPCR discriminates between dead and living spores, and this could be tested more thoroughly under laboratory conditions.

4. Referencer

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Bilag 1: **Supplementary Table S1. List of all isolates tested, along with sampling date and location. Haplotype, mating type, resistance phenotype and genotype are listed where possible.**

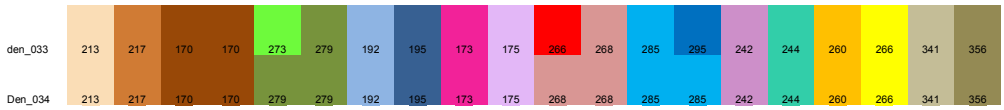
Isolate no.	Collection Date	Field No.	Haplotype	Mating Type	Resistance Class	IC50	Genotype
13_2_1	27_06_2013	2	Ib	a1	S	0.09486	den_030
13_2_2	27_06_2013	2	Ib	a1	S	0.04101	n/a
13_2_3	27_06_2013	2	Ib	a1	S	0.03625	den_030
13_2_4	27_06_2013	2	Ib	a1	S	0.0273	den_030
13_3_5	27_06_2013	3	Ia	a2	R	>500	den_004
13_3_6	27_06_2013	3	Ia	a2	R	4.656	den_011
13_3_8	27_06_2013	3	Ia	a1	R	>500	den_034
13_3_47	15_08_2013	3	Ia	a2	R	87.45	den_019
13_3_50	15_08_2013	3	Ia	a1	R	18.18	den_022
13_5_9	10_07_2013	5	Ia	a2	S	0.0045	n/a
13_5_10	10_07_2013	5	Ia	a2	S	0.00322	den_012
13_5_11	10_07_2013	5	Ib	a1	S	0.00443	den_025
13_5_12	10_07_2013	5	Ia	a2	S	0.00347	den_012
13_5_14	10_07_2013	5	Ia	a2	S	0.00151	den_024
13_5_16	10_07_2013	5	Ia	a2	S	0.0057	den_010
13_6_17	04_07_2013	6	Ia/Ib	a2	S	0.04365	den_007
13_6_18	04_07_2013	6	Ib	a2	R	7.111	den_007
13_6_21	04_07_2013	6	Ib	a2	S	0.05036	den_021
13_6_22	04_07_2013	6	Ib	a2	R	>500	den_007
13_6_24	04_07_2013	6	Ib	a2	S	0.01914	den_007
13_7_25	10_07_2013	7	IIa	a1	S	0.00199	den_032
13_7_26	10_07_2013	7	IIa	a1	S	0.02154	den_031
13_7_27	10_07_2013	7	IIa	a1	S	0.0146	den_031
13_7_28	10_07_2013	7	IIa	a1	S	0.0138	den_031
13_7_29	10_07_2013	7	IIa	a1	S	0.1409	den_031
13_7_30	10_07_2013	7	IIa	a1	S	0.0005	den_031
13_7_31	10_07_2013	7	IIa	a1	S	0.02247	den_031
13_7_32	10_07_2013	7	Ia	a1	S	0.0005	den_020
13_7_33	10_07_2013	7	Ia	a1	S	0.02057	den_023
13_7_64	03_09_2013	7	Ia	a2	S	0.2242	den_001
13_7_65	03_09_2013	7	Ia	a2	R	>500	n/a
13_7_66	03_09_2013	7	Ia	a1	R	>500	den_033
13_7_67	03_09_2013	7	Ia	a1	R	>500	den_033
13_8_34	10_07_2013	8	Ia	a2	S	0.03668	den_028
13_8_35	10_07_2013	8	Ia	a2	S	0.0286	den_028
13_8_36	10_07_2013	8	Ia	a2	S	0.03523	den_028

13_8_37	10_07_2013	8	Ia	a2	S	0.03212	den_028
13_8_38	10_07_2013	8	Ia		S	0.05712	den_028
13_8_39	10_07_2013	8	Ia	a2	S	0.02447	den_028
13_8_40	10_07_2013	8	Ia	a2	S	0.02028	den_028
13_8_41	10_07_2013	8	Ia	a2	S	0.03111	den_028
13_8_42	10_07_2013	8	Ia	a2	S	0.02107	den_028
13_8_43	10_07_2013	8	Ia	a2	S	0.03458	den_028
13_8_44	10_07_2013	8	Ia	a2	S	0.1393	den_028
13_8_45	10_07_2013	8	Ia	a2	S	0.02579	den_028
13_8_46	10_07_2013	8	Ia	a2	S	0.02752	den_028
13_8_53	20_08_2013	8	Ia	a2	S	0.04134	den_028
13_8_55	20_08_2013	8	Ia	a2	S	0.03735	den_028
13_8_57	20_08_2013	8	IIa	a2	S	0.01233	den_006
13_8_58	20_08_2013	8	Ia	a2	S	0.02658	den_028
13_8_68	04_09_2013	8	Ia	a2	S	0.038	den_028
13_8_69	04_09_2013	8	Ia	a2	S	0.01497	den_028
13_8_70	04_09_2013	8	Ia	a2	S	0.03045	n/a
13_8_71	04_09_2013	8	IIa		S	0.0083	n/a
13_8_72	04_09_2013	8	IIa	a1	R	>500	den_016
13_8_74	04_09_2013	8	Ia	a2	S	0.0332	den_027
13_8_75	04_09_2013	8	Ia	a1	R	>500	n/a
13_8_76	04_09_2013	8	Ia	a1	S	0.00975	den_026
13_8_77	04_09_2013	8	Ia	a2	S	0.1024	den_028
13_8_78	04_09_2013	8	Ia	a2	S	0.08572	den_029
13_8_79	04_09_2013	8	IIa	a1	R	>500	den_015
13_8_80	04_09_2013	8	Ia	a1	R	4.157	den_026
13_8_81	04_09_2013	8	Ia	a2	S	0.06773	den_028
13_8_82	04_09_2013	8	Ia	a2	S	0.06009	den_028
13_8_94	25_09_2013	8	Ia	a2	R	>500	den_005
13_8_97	25_09_2013	8	IIa	a1	S	0.04785	den_014
13_8_99	25_09_2013	8	IIa	a1	R	8.678	den_014
13_8_100	25_09_2013	8	Ia	a2	R	>500	den_005
13_8_101	25_09_2013	8	Ia	a1	R	>500	den_017
13_8_102	25_09_2013	8	IIa	a1	R	>500	den_014
13_8_103	25_09_2013	8	IIa	a1	R	>500	den_014
13_8_104	25_09_2013	8	Ia	a2	R	>500	den_005
13_8_106	25_09_2013	8	Ia	a1	R	>500	den_005
13_8_107	25_09_2013	8	Ia	a1	R	>500	den_017
13_10_61	20_08_2013	10	Ia	a2	S	0.173	den_008
13_10_83	04_10_2013	10	Ia	a1	R	1.027	den_002
13_10_84	04_10_2013	10	Ia	a1	R	8.119	den_003
13_10_86	04_10_2013	10	Ia	a1	S	0.2448	den_009

13_10_88	04_10_2013	10	Ib	a2	S	0.02937	den_013
13_10_89	04_10_2013	10	Ia	a1	R	4.043	den_002
13_10_90	04_10_2013	10	Ia	a1	R	3.728	den_002
13_10_91	04_10_2013	10	Ia	a1	R	7.28	den_002
13_10_92	04_10_2013	10	Ia	a1	R	1.326	den_002
13_10_93	04_10_2013	10	Ia	a2	S	0.01121	den_018

Bilag 2: **Supplementary Table S2. All genotypes found with alleles for 8 or more loci. Only den_005, the 13_A2 “Blue 13” genotype, overlaps with genotypes listed by Li et al. (2013).side**

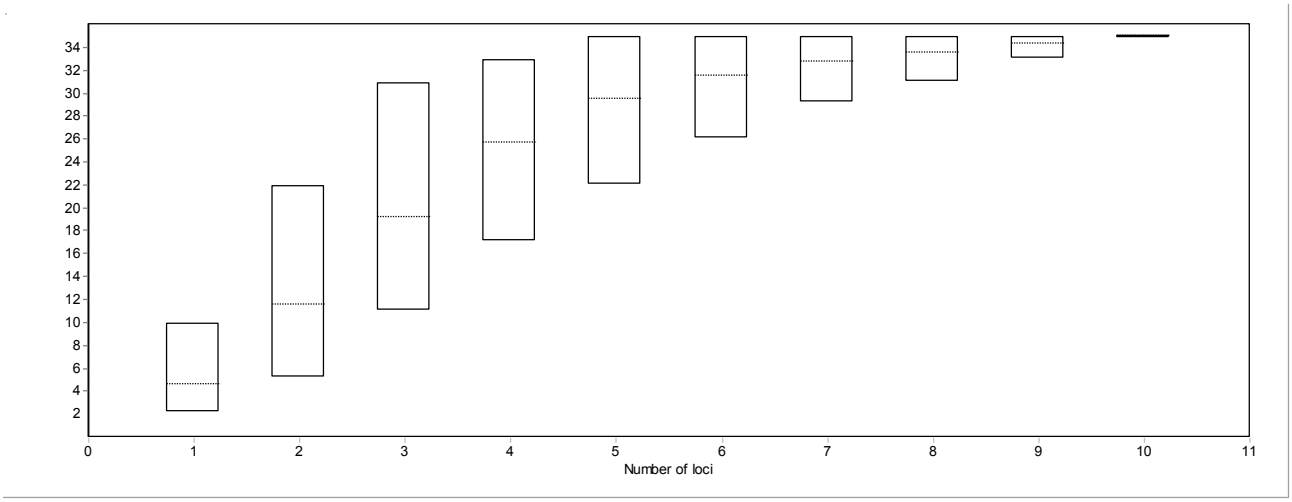
MLG	Pi4B		Pi04		Pi63		Pi70		SSR2		Pi02		SSR4		SSR6		SSR8		SSR11	
den_001	205	213	166	170	270	279	192	192	173	173	266	268	289	293	244	244			341	341
den_002	205	213	166	170	270	279	192	195	173	173	268	268	289	295	242	244	260	266	341	341
den_003	205	213	166	170	270	279	192	195	173	173	268	268	289	295	242	244	266	266	341	341
den_004	205	213	166	170	273	279	192	192	173	173	266	268	285	285	244	244	266	266	341	341
den_005*	205	213	166	170	273	279	192	192	173	173	266	268	285	295	240	244	260	266	341	341
den_006	205	213	166	170	273	279	192	192	173	175	268	272	289	295	242	244	260	266	341	356
den_007	205	213	166	170	279	279	192	192	173	175	268	268	285	295	242	244	266	266	341	341
den_008	205	213	168		270	279	192	195	173	175	268	268	289	295	242	244	260	266	341	356
den_009	205	213	168	168	279	279	192	192	173	175	258	268	295	295	244	244	260	260	341	341
den_010	205	213	168	172	273	273	192	192	173	175	258	268	291	295	244	244	260	266	331	341
den_011	205	217	166	170	273	279	192	192	173	175	258	268	289	295	242	244	266	266	341	356
den_012	205	217	166	170	276	279	192	192	173	173	266	268	285	295	244	244	260	266	341	356
den_013	213	213	166	170	273	273	192	192	173	175	266	268	289	295	242	244	260		356	356
den_014	213	213	166	170	279	279	192	192	173	173	266	268	285	293	244	244	260	266	341	356
den_015	213	213	166	170	279	279	192	192	173	173	266	268	289	293	244	244	260	266	341	356
den_016	213	213	166	170	279	279	192	192	173	173	268	268	285	293	244	244	260	266	341	356
den_017	213	213	166	170	279	279	192	192	173	173	268	268	291	295	242	242	260	266	331	341
den_018	213	213	166	170	279	279	192	192	173	175	268	268	295	295	242	244	260	260	341	341
den_019	213	213	168	168	273	279	192	192	175	175	268	268	287	289	244	244	260	266	341	341
den_020	213	213	168	168	279	279	192	192	173	173	268	268	289	293	244	244	260	260	331	331
den_021	213	213	168	168	279	279	192	195	173	175	266	268	285	295	244	244	260	266	331	356
den_022	213	213	168	168	279	279	192	195	173	175	268	268	285	295	240	244	266	266	341	356
den_023	213	213	168	170	279	279	192	192	173	173	268	268	289	293	244	244	260	260	331	331
den_024	213	217	166	170	273	279	192	192	173	173	258	268	285	299	242	244	260	260	341	341
den_025	213	217	166	170	273	279	192	192	173	173	266	268	289	293	242	244	260		341	356
den_026	213	217	166	170	279	279	192	192	173	173	266	268	285	285	242	244	266	266	341	341
den_027	213	217	168	168	273	279	192	192	173	175	258	268	289	293	242	244	260	266	341	341
den_028	213	217	168	168	273	279	192	192	173	175	258	268	289	295	242	244	260	266	341	341
den_029	213	217	168	168	273	279	192	192	173	175	258	270	289	293	242	244	260	266	341	341
den_030	213	217	168	168	273	279	192	192	173	175	266	268	293	295	240	244	260	266	331	341
den_031	213	217	168	168	279	279	192	192	173	173	268	268	289	293	244	244	260	260	331	341
den_032	213	217	168	168	279	279	192	192	173	173	268	268	289	293	244	244	260	266	331	341


den_033 213 217 170 170 273 279 192 195 173 175 266 268 285 295 242 244 260 266 341 356
Den_034 213 217 170 170 279 279 192 195 173 175 268 268 285 285 242 244 260 266 341 356

Bilag 3: **Supplementary Table S3. Allele frequency at each field. Alleles in bold print represent private alleles only found at one location. N= number of isolates with information for each locus.**

Locus	Allele/n	field2	field3	field5	field6	field7	field8	field10
ssr4	N	3	5	6	5	12	38	10
	285	0.000	0.500	0.250	0.500	0.083	0.171	0.000
	287	0.000	0.100	0.000	0.000	0.000	0.000	0.000
	289	0.000	0.200	0.083	0.000	0.417	0.329	0.400
	291	0.000	0.000	0.083	0.000	0.000	0.026	0.000
	293	0.500	0.000	0.083	0.000	0.417	0.105	0.000
	295	0.500	0.200	0.417	0.500	0.083	0.368	0.600
	299	0.000	0.000	0.083	0.000	0.000	0.000	0.000
ssr8	N	3	5	6	5	11	37	9
	260	0.500	0.200	0.583	0.100	0.864	0.486	0.667
	266	0.500	0.800	0.417	0.900	0.136	0.514	0.333
ssr2	N	4	5	6	5	13	41	10
	173	0.500	0.500	0.917	0.500	0.885	0.683	0.800
	175	0.500	0.500	0.083	0.500	0.115	0.317	0.200
Pi63	N	3	5	6	5	12	40	10
	270	0.000	0.000	0.000	0.000	0.042	0.000	0.350
	273	0.500	0.300	0.333	0.000	0.083	0.363	0.100
	276	0.000	0.000	0.167	0.000	0.000	0.000	0.000
	279	0.500	0.700	0.500	1.000	0.875	0.638	0.550
Pi02	N	3	5	6	5	13	40	10
	258	0.000	0.100	0.167	0.000	0.000	0.300	0.050
	266	0.500	0.100	0.333	0.100	0.154	0.138	0.050
	268	0.500	0.800	0.500	0.900	0.846	0.538	0.900
	270	0.000	0.000	0.000	0.000	0.000	0.013	0.000
	272	0.000	0.000	0.000	0.000	0.000	0.013	0.000
ssr11	N	3	5	6	5	13	40	10
	331	0.500	0.000	0.083	0.100	0.423	0.025	0.000
	341	0.500	0.700	0.583	0.800	0.500	0.875	0.850
	356	0.000	0.300	0.333	0.100	0.077	0.100	0.150
Pi04	N	2	5	6	5	13	41	10
	166	0.000	0.200	0.417	0.400	0.077	0.207	0.400
	168	1.000	0.400	0.083	0.200	0.654	0.585	0.200
	170	0.000	0.400	0.417	0.400	0.269	0.207	0.400
	172	0.000	0.000	0.083	0.000	0.000	0.000	0.000
Pi70	N	4	5	6	5	13	41	10
	192	1.000	0.800	1.000	0.900	0.923	1.000	0.650
	195	0.000	0.200	0.000	0.100	0.077	0.000	0.350
ssr6	N	4	5	6	5	13	41	10
	240	0.500	0.100	0.000	0.000	0.000	0.049	0.000
	242	0.000	0.200	0.167	0.400	0.077	0.402	0.450
	244	0.500	0.700	0.833	0.600	0.923	0.549	0.550
Pi4B	N	4	5	6	5	13	41	10
	205	0.000	0.200	0.333	0.400	0.038	0.061	0.400
	213	0.500	0.600	0.333	0.600	0.615	0.610	0.600
	217	0.500	0.200	0.333	0.000	0.346	0.329	0.000

Bilag 4: **Figure S1. Mean genetic diversity as a function of number of loci sampled. Loci were sampled 100 times.**



Genetisk baseret varsling af kartoffelskimmel angreb med bio-markører

I Danmark anses kartoffelskimmel (Phytophthora infestans) for at være den vigtigste skadegører i kartofler. Svampen forekommer hvert år og oftest med kraftige angreb. For at begrænse tab i udbytte og kvalitet sprøjter de fleste landmænd alle konventionelt dyrkede spise- og stivelses kartofler gentagne gange i sæsonen.

Formålet med projektet var at tilvejebringe den biologiske baggrund der er nødvendig for at kunne foretage en tidlig risikovurdering af angreb af kartoffelskimmel. I projektet blev den populationsgenetiske baggrund for angreb af kartoffelskimmel klarlagt, herunder antal og fordeling af genotyper samt graden af rekombination. For at kunne foretage en risikovurdering i forbindelse med kartoffelskimmelangreb er det vigtigt at kunne koble genotyper med fænotypiske egenskaber (traits) hos svampen som betinger virulens og fungicidresistens. Vi har derfor i dette projekt anvendt svampens resistens overfor fungicidet metalaxyl som eksempel på en egenskab der kan karakteriseres med genetiske markører (bio-markører). Ved at monitorere populationer af kartoffelskimmel på et givent tidspunkt for disse biomarkører kan man sammen med klimadata forudse risiko for angreb af høj-virulente og fungicidresistente typer med stor sikkerhed, og dermed anvende (eller undgå) fungicid behandlinger med specifikke midler mere hensigtsmæssigt.

Den genetiske struktur viser,at kartoffelskimmel er domineret af klonal reproduktion. Den populationsgenetiske struktur af udbrud af kartoffel kartoffelskimmel, afslører at stokastiske processer er af stor betydning i de indledende faser af udbruddet, samt at populationen er domineret af resistente genotyper hen imod slutningen af sæsonen.

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