POPULATION GENETICS OF *PHYTOPHTHORA INFESTANS* IN THE UNITED STATES

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POPULATION GENETICS OF *PHYTOPHTHORA INFESTANS* IN THE UNITED STATES

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Late blight disease caused by *Phytophthora infestans* continues to pose a great challenge for potato and tomato growers around the world. Despite the numerous efforts conducted to alleviate the losses caused by this disease, the pathogen's diversity has been a key factor in disease control failures. Continuous monitoring of pathogen traits such as fungicide sensitivity and host preference are essential for selecting the best disease mitigation method. The overall objective of this study was to understand the *P. infestans* population in the USA. More specifically, my research objectives were: i) To characterize the phenotypes of the most recent and most prevalent strains of *P. infestans* in the US; ii) To investigate a set of rare and diverse genotypes detected in the northeastern US in 2010 and 2011; iii) To study the phenotypic diversity (mating type, host preference, sensitivity to mefenoxam, the effect of temperature on release of zoospores and the effect of temperature on mycelial growth) of a diverse panel of *P. infestans* from the US, Mexico and the Netherlands and determine the genetic relatedness among them; iv) To conducted a genome-wide association study to identify genetic markers associated with important phenotypic traits; and v) To investigate the characteristics of mefenoxam acquired resistance. Differences in mating type, mefenoxam sensitivity, pathogenicity on potato and tomato, and zoospore release at different temperatures were identified between the recent genotypes of *P. infestans* in the US (US-8, US-22, US-23,

and US-24). The genetic characteristics of the rare and diverse genotypes detected in the northeastern US were consistent with a recombinant population. The phenotypic analyses conducted on strains from the US, Mexico and the Netherlands, revealed a broad range of phenotypic responses. Eleven association hits for mating type in *P. infestans* where found with a $P < 1e\text{-}5$. The phenomenon of acquired resistance was not unique to certain strains of *P. infestans*; originally sensitive genotypes US-22, US-23, and US-24 were all able to acquire resistance after a single transfer through mefenoxam containing media. Thirty-two genes were found to be significantly differentially expressed in response to mefenoxam in all originally sensitive genotypes studied.

BIOGRAPHICAL SKETCH

Giovanna Danies Turano was born and grew up in Barranquilla, Colombia. She graduated from Karl C. Parrish School in 2004. Hereafter she enrolled in the undergraduate Biology and Microbiology programs at Universidad de los Andes in Bogotá, Colombia. She received her BSc. Biology and BSc. Microbiology degrees in 2009. Subsequently, Giovanna started her MSc. degree on Biological Sciences majoring in Plant Pathology from Universidad de los Andes in Bogotá, Colombia under guidance of Dr. Silvia Restrepo. After completion of her MSc. degree in 2011, Giovanna was offered a job as a research scholar at Cornell University in the Department of Plant Pathology and Plant-Microbe Biology. In fall of 2011, Giovanna was accepted at Cornell University in the Department of Plant Pathology and Plant-Microbe Biology as a Ph.D. She pursued her Ph.D. in the laboratory of Dr. William E. Fry, majoring in plant pathology and having minors in population biology and genomics.

To my family, professors, and friends for making this experience one of the most memorable in my life.

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I would like to thank my parents and my brother for always supporting me and encouraging me to always give the best of me. Thank you, for warming up the cold winters with your infinite love. Marcelo, thank you for making me the happiest person in the world and for making every stressful moment disappear when you are around. Te amo infinito!

Bill thank you for guiding me to successfully accomplish my career goals. Thank you for inspiring me, for being an outstanding mentor, and friend. I am grateful to Chip, Jason, and Nik for your valuable advise throughout my PhD. Silvia thank you for directing me towards this career path. In the process I gained an amazing friend, whose advice and company through these four years have been invaluable.

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CHAPTER 1

Introduction

1.1 Late blight disease: historic and modern economic importance

Late blight has been a major threat to global food security ever since the Irish potato famine of the mid-19th century. This disease is a continuing problem on potato and tomato crops worldwide. Around 365 million tons of potatoes are produced worldwide, making it the fifth largest crop in the world (Food and Agriculture Organization of the United Nations 2012). Under suitable environmental conditions the disease can spread rapidly and can cause complete crop loss. The extent of economic damage due to late blight depends on several factors including climate, variety of potato or tomato grown, use of crop protection agents, and general cultural practices.

Globally, late blight costs billions of dollars annually. Management mostly involves the use of fungicides and cultural procedures designed to reduce the introduction, survival, or infection rate of the causal organism, *Phytophthora infestans*. The worldwide cost of potato late blight alone exceeds $5 billion per year, including $1 billion spent on fungicides (Judelson, USAblight). On tomatoes, the disease can be and has been equally devastating. The most recent example occurred in 2009 in the northeastern United States when infected tomato transplants were distributed via large national retail stores who obtained transplants from a national supplier (Fry et al. 2013). The subsequent pandemic in the mid-Atlantic and northeastern regions eliminated tomato plants in many organic farms and home gardens.

1.2 Biology of *Phytophthora infestans*

1.2.1 Taxonomic classification

Late blight disease is caused by the oomycete pathogen *Phytophthora infestans*. Despite the resemblance between oomycetes and fungi (growth via filamentous hyphal tips, nutrition via absorption, and reproduction via spores), oomycetes are evolutionarily distinct from fungi. Oomycetes are more closely related to brown algae than to true fungi. In contrast to fungi, oomycetes have cell walls composed of β-1-3, and β-1-6 glucans, produce biflagellated zoospores, produce antheridia and oogonia as gametangia, and are diploid in their vegetative state. Taxonomically, oomycetes belong to the phylum Oomycota, which are part of the kingdom Protista. It is believed that oomycetes may have evolved from the encapsulation of a photosynthetic eukaryote, with the plastid being lost secondarily in oomycetes (Fry 2008).

1.2.2 Life cycle

Phytophthora infestans is a hemibiotrophic pathogen having an initial biotrophic infection phase during which the pathogen spreads within the host tissue, followed by a necrotrophic phase during which host cell death is induced (Dodds and Rathjen 2010). Leaves, stems, tubers (in the case of potatoes) and fruits (in the case of tomatoes) are all susceptible (Figure 1.1). *Phytophthora infestans* has a polycyclic life cycle, that is, it has multiple cycles of infection during a single epidemic. Lesions on infected leaves produce sporangia, some of which are dispersed to other parts of the same leaves, other leaves, or other plants. When these sporangia settle on new healthy tissue, some can cause new infections. These new infections result in new lesions, new sporangia, and then additional infections. Therefore, the

asexual life cycle of *P. infestans* can be completed rapidly with production of massive numbers of sporangia that are readily dispersed.

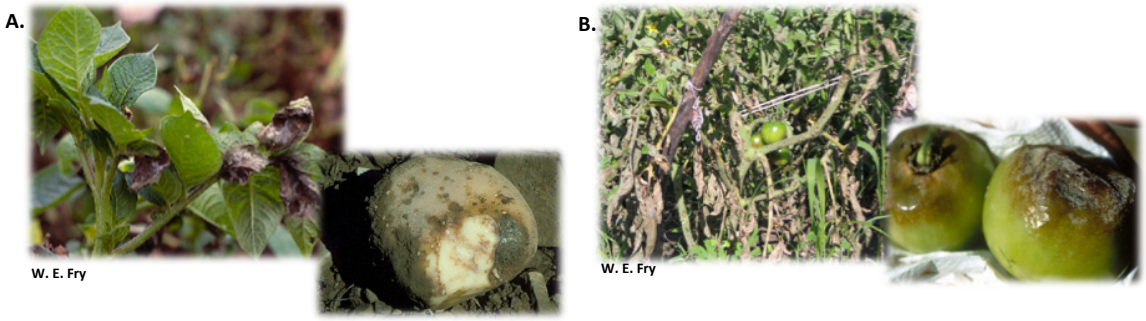


Figure 1.1 Typical late blight symptoms on **A.** potatoes and **B.** tomatoes.

Phytophthora infestans reproduces both sexually and asexually (Figure 1.2). Sexual reproduction serves both as a source of variation and survival, whereas asexual reproduction serves as a mechanism for dispersion and rapid population growth. Sporangia, the asexual spores, are readily dislodged, particularly in response to changes in relative humidity, and can be aerially dispersed to other plant tissues (Erwin and Ribeiro 1996). Sporangia germinate either by formation of a germ tube that eventually grows to form a mycelium (direct germination) or by differentiation of the cytoplasm within the sporangium into discrete zoospores that are then released through an exit pore (indirect germination). Direct germination is favored at temperatures at and above the optimum for mycelial growth (20-25ºC). On the other hand, indirect germination is favored at temperatures below 15ºC. When sporangia are deposited on a surface and exposed to free moisture and cool temperatures, they may germinate indirectly by releasing between 8 and 12 biflagellated zoospores, each capable of initiating an infection. Thus, low temperatures (10-15ºC) are more likely to lead to successful infections than are high temperatures (22-26ºC).

Phytophthora infestans is heterothallic, requiring both mating types (designated as A1 and A2) for sexual reproduction to occur. When individuals of opposite mating types are present in the same field and infect the same leaf, hormones are produced that move from one thallus to the other, stimulating the production of oogonia and antheridia (female and male gametangia, respectively). Each thallus is bisexual; thus, although infrequent in practice, selfing can occur within a single isolate in culture (Judelson 1996; Judelson, 1997a; Shattock et al., 1986). Therefore, sex determination is not linked to mating type (Fry 2008, Judelson 1997b). When antheridia and oogonia meet, fertilization leads to the production of a sexual spore called oospore. The oospore serves both as a survival structure and as a source of variation via sexual recombination. As survival structures, oospores have been demonstrated to persist for up to four years in the soil (Mayton et al. 2000, Turkensteen et al. 2000). They survive very low temperatures well, but not higher temperatures, being unable to survive 2 h at 46ºC or 12 h at 40ºC (Fay and Fry 1997). As sources of variation, oospores produced from a mating between an A1 and an A2 strain are typically hybrids (Judelson 1997c, Shattock et al. 1986).

In locations with primarily asexual reproduction, the contribution of an occasional oospore to the population structure is likely to be minimal, because most progeny appear to be dramatically less fit than either parent (Mayton et al. 2000). With sexual reproduction limited or absent, mycelia in infected tubers may be the main mechanism for overwintering.

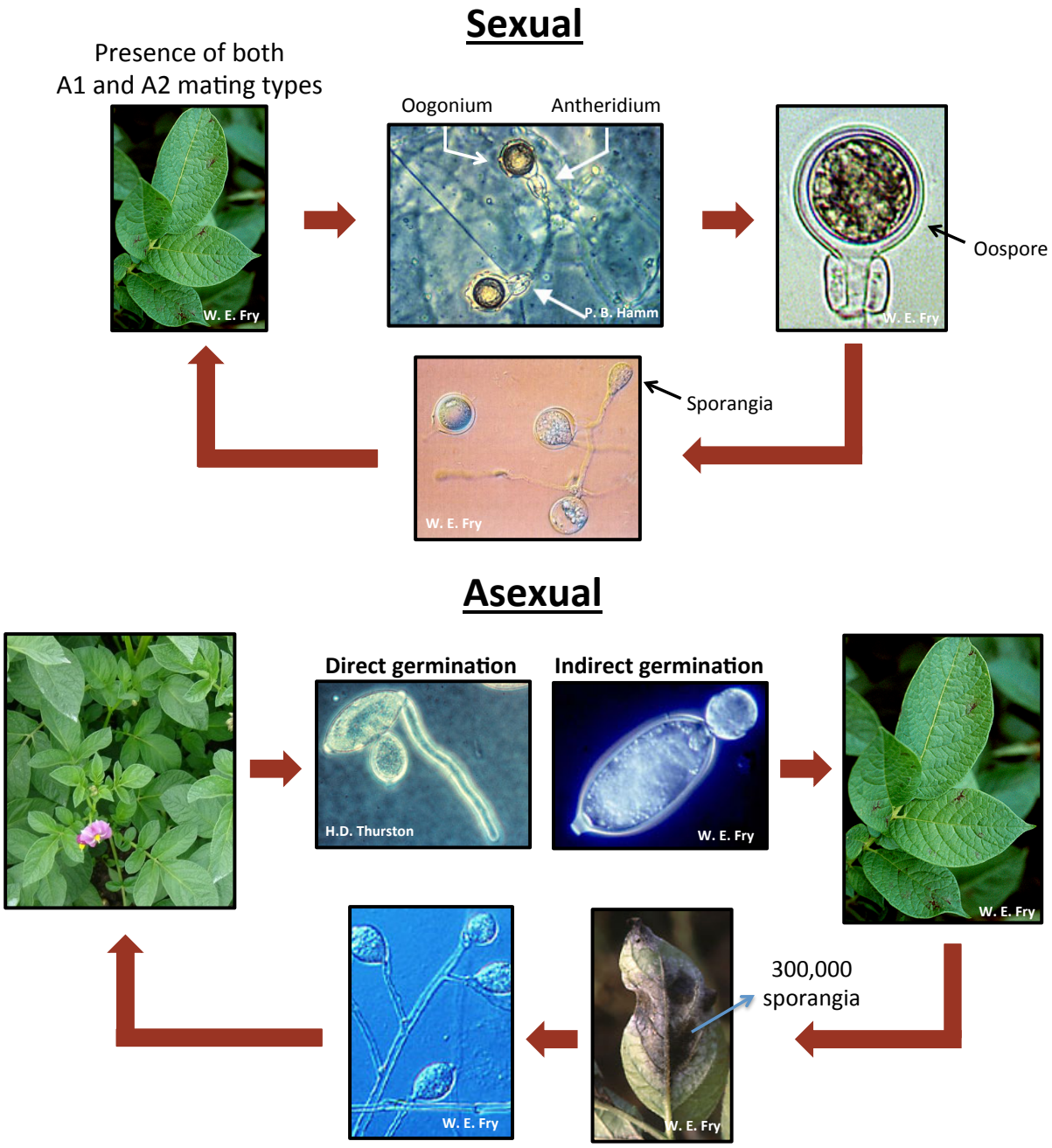


Figure 1.2 Life cycle of *Phytophthora infestans*.

1.3 Markers used to describe clonal lineages of *Phytophthora infestans*

A clonal lineage is a descendant from a single individual, and variation within a lineage arises

by mutation or mitotic recombination (Goodwin 1997, Grünwald et al. 2012). Clonal lineages are typically quite distinct and commonly defined using a set of genetic and phenotypic markers (Figure 1.3). The most common markers used to determine the genotype of a *P. infestans* isolate include: i) two allozymes, glucose-6-phosphate isomerase and peptidase; ii) a restriction fragment length polymorphism (RFLP) assay using a moderately repetitive DNA probe, RG57 (Goodwin et al. 1994b), which provides information on more than 25 different loci; iii) mitochondrial haplotyping, historically done using restriction enzyme digestion of PCR products for RFLP analysis that allowed the identification of four different haplotypes (Griffith and Shaw 1998), and more recently, five mitochondrial loci that allow identification of at least 36 different mitochondrial haplotypes in *P. infestans* (Martin et al. 2012); iv) twelve microsatellite loci that have been demonstrated to reveal polymorphisms among *P. infestans* isolates (Li et al. 2013); and v) the determination of the isolate's mating type.

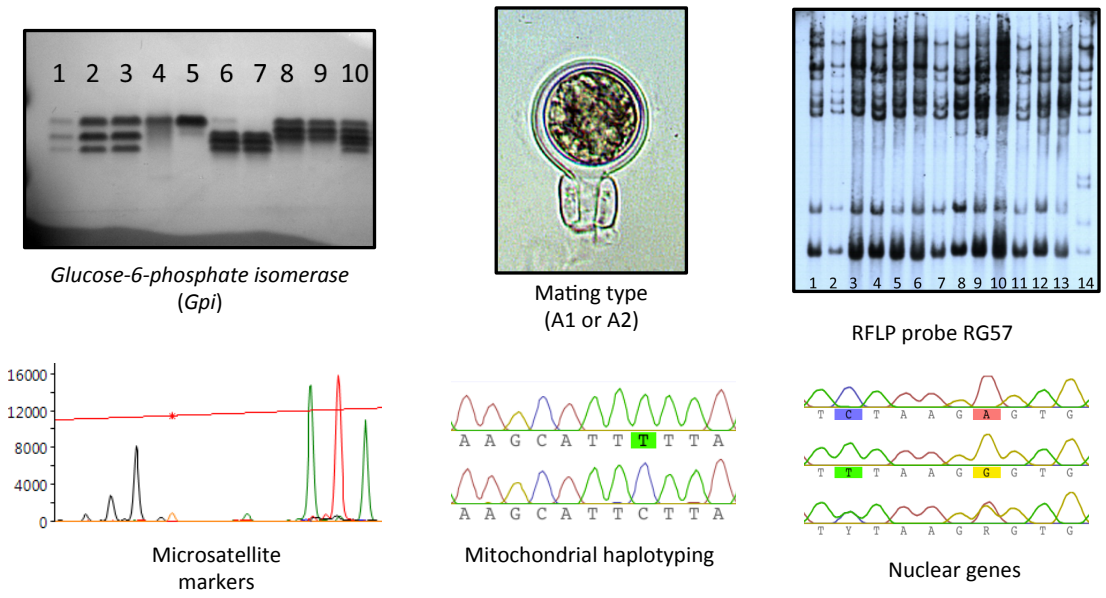


Figure 1.3 Genetic and phenotypic markers used to define clonal lineages of *Phytophthora infestans*.

1.4 Population genetics of *Phytophthora infestans* worldwide

Epidemics that cause significant crop loss involve millions of infection events involving an entire population of the pathogen and their host plants. For the establishment of an adequate pest management strategy, the determination of methods to control the entire pathogen population is therefore required. It is crucial to know whether growers are facing one or multiple populations, how these differ from one another, if they are recombining through sexual reproduction, and if the pathogen is restricted to a specific host. The efficiency of control of the pathogen depends on knowledge of its diversity, geographical distribution, and population subdivision. In general, if the pathogen has high genetic variation, the likelihood of overcoming host resistance or adapting to fungicides is higher (McDonald and McDermott 1993).

In the case of *P. infestans*, migrations have had a major impact on the population dynamics of this plant pathogen (Figure 1.4). Sporangia may be dispersed in water, aerially, or through infected plant parts (probably the most likely avenue for long-distance dispersal). *Phytophthora infestans* was first described in the mid 1840s (Berkeley 1846). The most famous epidemic occurred in Europe in 1845 and lead to the potato famine in Ireland. Until the early 1980's, Mexico was the only place in the world where the two mating types, A1 and A2, could be found and sexual reproduction occurred (Grünwald and Flier 2005).

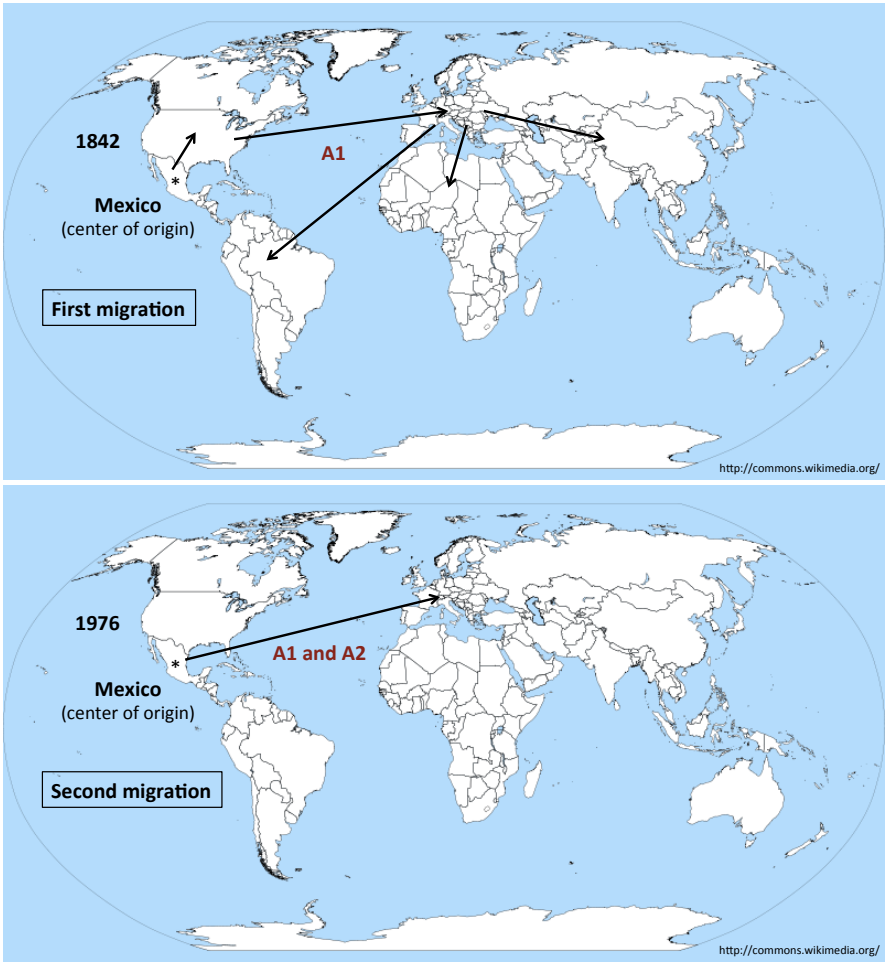


Figure 1.4 Migration events out of Mexico that have played a major role on the population dynamics of *Phytophthora infestans* worldwide.

1.4.1 *Phytophthora infestans* in Mexico

Mexico has been considered the center of origin of *P. infestans* due to the high genetic diversity of the pathogen in this region, the presence of the two mating types (A1 and A2), and the presence of two close relatives, *Phytophthora ipomoeae* and *Phytophthora miriabilis*, which are endemic to central Mexico (Goss et al. 2014). The populations of *P. infestans* in the central highlands of Mexico are reproducing sexually given that the two mating types occur in

a 1:1 ratio (Goodwin et al. 1992, Grünwald et al. 2001), oospores are formed ubiquitously on leaves, stems, and tubers (Fernandez-Pavia et al. 2002, Flier et al. 2001, Gallegly and Galindo 1958), and neutral genetic markers show that the isolates in this region are highly diverse (Grünwald et al. 2001, Tooley et al. 1985).

Evidence suggests that at least two different migration events out of Mexico occurred since the 1840's (Goodwin 1997). The first global migration of *P. infestans* out of Mexico resulted in founder events because the pathogen was being introduced into previously unoccupied territories. The first migration event occurred before 1842 from central Mexico into the United States (Stevens 1933). The pathogen then probably migrated from the United States into Europe in 1845 (Bourke 1964) and from Europe it spread to almost all potato growing areas around the world (Cox and Large 1960). Populations founded in this initial migration experienced a severe genetic bottleneck that greatly reduced their level of genetic variation. Probably only a few genotypes (which did not include the A2 mating type) were introduced into the United States from Mexico and only a limited number of these were transported subsequently from the United States into Europe.

A second migration event occurred after the summer of 1976 when 25,000 tons of potatoes were shipped from Mexico to Europe. In the early 1980's the A2 mating type was first discovered in Europe, the pathogen's diversity increased, and new genotypes of greater fitness rapidly replaced the old clonal lineages.

1.4.2 *Phytophthora infestans* in Europe

Phytophthora infestans populations in Europe have undergone at least two migration events. The first one took place in the mid-19th century introducing isolates from the United States

into Europe and the second one in the late 1970's introducing isolates from Mexico into Europe. Analyses of herbarium specimens collected between 1845 and 1896 suggested that populations in Europe were dominated by a single genotype designated HERB-1 (Yoshida et al. 2014). This lineage apparently persisted for 50 years until it was replaced by the closely related US-1 clonal lineage (Yoshida et al. 2014). From Europe, US-1 was spread panglobally (Goodwin 1997). The second migration introduced both A1 and A2 mating type individuals. The introduction of the A2 mating type into Europe enabled sexual reproduction of the pathogen and displaced the older A1 population that had been dominant in most parts of the world. The first reports of the A2 mating type in Europe came from Switzerland (Hohl and Iselin 1984), followed by reports from many other European countries, such as Germany, England, Netherlands, Scotland, and Sweden (Daggett et al. 1993, Frinking et al. 1987, Kadir and Umaerus 1987, Malcolmson 1985, Tantius et al. 1986).

Despite the presence of the A2 mating type throughout Europe, its frequency has varied significantly. Low frequencies of the A2 mating type have been observed in Ireland (Cooke et al. 2006, Griffin et al. 2002), France (Lebreton et al. 1998), and the United Kingdom (Cooke et al. 2003, Day et al. 2004, Shattock et al. 1990), whereas higher frequencies of the A2 mating type have been observed in the Netherlands (Zwankhuizen et al. 2000), Poland (Kapsa 2001, Sujkowski et al. 1994), Hungary (Bakonyi et al. 2002), Norway (Hermansen et al. 2000), and Finland (Hermansen et al. 2000, Lehtinen et al. 2007). The distribution of the different mating types can provide information on the likelihood of sexual reproduction and the subsequent formation of oospores. An even distribution of the A1 and A2 mating types as is observed in the Nordic countries, increases the chances for sexual reproduction to occur and is also the expected result from a sexual population (Lehtinen et al.

2008, Yuen and Andersson 2013). Additional lines of evidence that provide support for sexual recombination in the Nordic region of Europe include field observations consistent with a soil-borne source of inoculum such as those reported by Andersson et al. (1998) in Sweden, Lehtinen and Hannukkala (2004) and Hannukkala et al. (2007) in Finland, Evenhuis et al. (2007) in the Netherlands, and Bødker et al. (2006) in Denmark. A limited number of studies have surveyed individual fields for the presence of oospores in leaf tissue (Andersson et al. 1998, Dahlberg et al. 2002, Drenth et al. 1993, Hanson and Shattock 1998, Hermansen et al. 2002, Hjelm 2003, Lehtinen and Hannukkala 2004). In a study conducted by Lehtinen and Hannukkala (2004), oospores were observed in stems collected from fields with histories of late blight as well as early infections.

Interestingly, population genetic analyses conducted in Western Europe have shown a dominance of asexual clones and numerous rare genotypes (Cooke et al. 2003, Cooke et al. 2012, Cooke et al. 2006, Day et al. 2004, Knapova and Gisi 2002). Since 2005, an A2 lineage designated as 13_A2 has increased in frequency from approximately 12 to 70% in Great Britain over three seasons (Cooke et al. 2008). The 13_A2 genotype was first recorded in the Netherlands in 2004 (Cooke et al. 2007). Isolates of the 13_A2 lineage are highly aggressive on cultivated potatoes, they outcompete other aggressive lineages in the field, they have overcome previously effective forms of plant host resistance, and they seem to be resistant to the systemic fungicide metalaxyl (Cooke et al. 2012, Detourne et al. 2007, Shaw et al. 2007).

1.4.3 *Phytophthora infestans* in Africa and Asia

It is believed that *P. infestans* was first introduced in Africa in 1941 (Nattrass and Ryan 1951). In northern Africa (Morocco and Egypt) both A1 and A2 mating types have been

reported (Baka 1997, El-Kornay 1994, Hammi et al. 2001, Hammi et al. 2002, Sedegui et al. 2000, Shaat 2002). In Morocco, some diversity has been documented and both mating types have been found within the same field, creating the potential for sexual reproduction (Hammi et al. 2001, Sedegui et al. 2000). Further south in Africa, it seems that the A1 mating type predominates (Fry et al. 2009) and despite the presence of distinct genotypes (Forbes et al. 1998, Goodwin et al. 1994a, Pule et al. 2008, Schiessendopper and Molnar 2002), the US-1 clonal lineage seems to prevail. Metalaxyl-resistant individuals of *P. infestans* are often present in Africa (Fontem et al. 2005, Hammi et al. 2002, Kankwatsa et al. 2003, McLeod et al. 2001, Mukalazi et al. 2001, Olanya et al. 2001, Sedegui et al. 2000).

As is the case in southern Africa, the US-1 clonal lineage still seems to persist in several countries in Asia, including Japan, Nepal, Taiwan, and Thailand (Guo et al. 2008, Kato and Naito 2001, Koh et al. 1994, Le et al. 2008, Nishimura et al. 1999). However, other lineages of both the A1 and the A2 mating type have been reported in Bangladesh (Forbes 2004), China (Zhang et al. 1996), India (Singh et al. 1994), Indonesia (Nishimura et al. 1999), Israel (Cohen 2002, Grinberger et al. 1989), Japan (Akino et al. 2008), Korea (Park et al. 2008), Nepal (Shrestha et al. 1998), Pakistan (Ahmad and Mirza 1995), Siberia (Elansky et al. 2001), and Thailand (Nishimura et al. 1999).

1.4.4 *Phytophthora infestans* in South America

The Andes are considered to be the center of origin of potato (Ames and Spooner 2008) and thus they have been proposed as the center of origin of *P. infestans* (Abad and Abad 1997, Gomez-Alpizar et al. 2007). However, the absence of sexual reproduction, and presence of clonality in South America are in conflict with this idea (Fry et al. 2009). Both mating types

are found in southern South America (Argentina, Brazil, Paraguay, and Uruguay) (Forbes 2004). Yet, no evidence of sexual recombination in field populations has been documented. In Bolivia, the vast majority of isolates collected are of the A2 mating type, whereas in Chile, Colombia, Ecuador, Peru, and Venezuela, *P. infestans* isolates of the A1 mating type seem to predominate (Forbes 2004). Despite the presence of both mating types in South America, the high degree of host specificity seems to play an important role in impeding sexual reproduction (Oliva et al. 2002, Perez et al. 2001).

1.4.5 *Phytophthora infestans* in North America

Late blight was first detected in the northeastern United States in the early 1840's (Stevens 1933). Data suggest that *P. infestans* was first introduced into the United States from Mexico (Fry et al. 1993, Goodwin et al. 1994a, Stevens 1933), probably through potato tubers that were imported by curious botanists or agronomists who visited Mexico during the 19th century (Goodwin 1997). This first migration event introduced the US-1 clonal lineage (A1 mating type) as well as other genotypes that appear to be related to the US-1 clonal lineage (Goodwin 1997, Goodwin et al. 1994b). The second important migration seems to have taken place from northwestern Mexico in the late 1970's when the US-6 clonal lineage (A1 mating type) was introduced. In contrast to the US-1 genotype, which mainly infected potato crops, the US-6 clonal lineage was able to infect both potatoes and tomatoes. In the early 1990s, two new genotypes, US-7 and US-8, were detected in the United States and Canada. These genotypes were probably initially introduced into the United States through infected tomato fruits that were imported from northwestern Mexico (Goodwin 1997). In contrast to the previous genotypes of *P. infestans* found in the United States and Canada, US-7 and US-8

were of the A2 mating type and were highly resistant to the fungicide metalaxyl (Goodwin et al. 1996). The US-8 clonal lineage, which mainly infects potatoes and is a very efficient colonizer of potato tubers, rapidly spread throughout the United States. During 1994 and 1995, this genotype was detected in 23 different states (Fry and Goodwin 1997). On tomatoes, populations of *P. infestans* seem to be erratic, with strains detected in one year not necessarily being the same as those from the previous year. Lineages that have been prevalent on tomatoes include US-6, US-7, US-11, and US-17 (Fry et al. 2009).

The widespread occurrence of both A1 and A2 mating type strains in the United States and Canada increased the probability of sexual recombination in these countries. Yet, most populations in a single field have been monomorphic for mating type and thus sexual recombination is expected to be rare (Goodwin et al. 1995, Goodwin et al. 1998, Peters et al. 1998). However, studies conducted by Goodwin et al. (1995) and Gavino et al. (2000) in the 1990s have suggested that some populations in British Columbia and in the Columbia Basin of Oregon and Washington might have contained recombinant genotypes. One of these recombinant genotypes is believed to be lineage US-11 (Gavino et al. 2000).

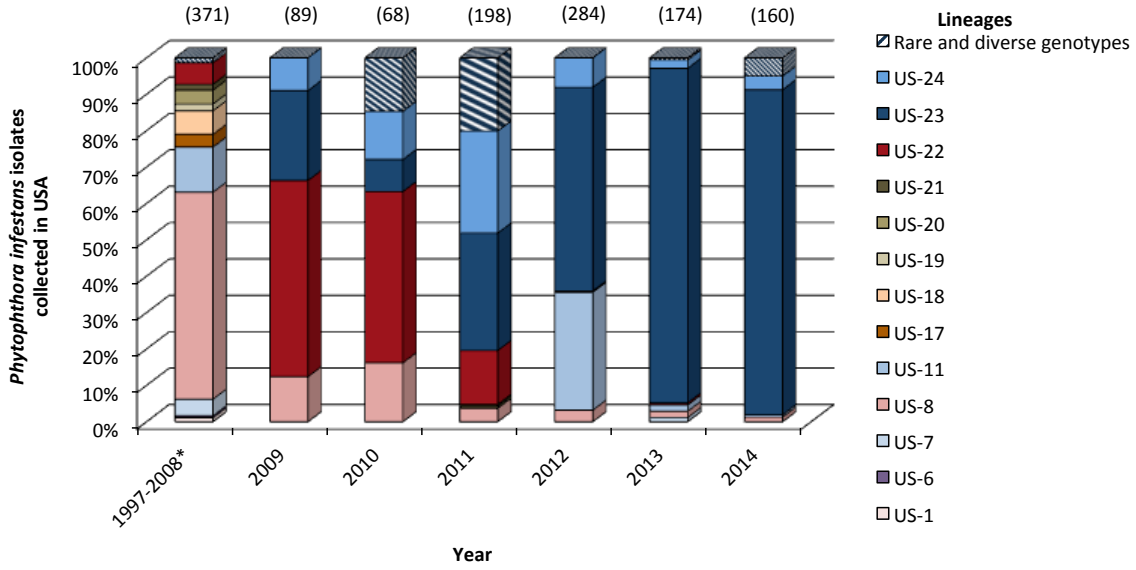
In 2009, North America was again reminded of how devastating late blight could be. A late blight pandemic began in mid-June over much of the northeastern United States. A single genotype of *P. infestans*, designated as US-22, was dispersed via infected tomato transplants that were shipped to garden centers in large retail stores throughout the Northeast (Figure 1.5). Both employees in the garden centers as well as home gardeners were unaware of the disease and did not recognize its symptoms. Consequently, infected transplants were planted in home gardens throughout the Northeast (Fry et al. 2013). In addition, the environmental conditions during the summer of 2009 were favorable for the development of

the disease. Nights were cool (optimal for indirect germination), days were warm (stimulating mycelia growth), and rain occurred relentlessly during the start of the pandemic, making it challenging for growers to apply fungicides. Unfortunately, knowledge regarding the sensitivity of US-22 to mefenoxam (formerly metalaxyl) and its host preference did not become available until after the epidemic was well established (see **Chapter 2** and Danies et al. (2012)). In contrast to US-8, which had been the prevalent genotype until then, US-22 was sensitive to mefenoxam and was able to infect both potatoes and tomatoes.

In addition to US-8 and US-22, two other genotypes of *P. infestans* were present in the United States in 2009. These genotypes were designated as lineage US-23 and lineage US-24. Lineage US-23 was present in the East (Pennsylvania) and in the South, whereas lineage US-24 was reported in the upper Midwestern United States (Fry et al. 2013). Results of phenotypic analyses of these two lineages are included in **Chapter 2**. From 2010 until today, lineage US-23 has prevailed. This lineage has shown to be a great pathogen in both potato and tomato crops (Danies et al. 2012). Fortunately, US-23 is sensitive to mefenoxam, thus allowing the use of this highly efficient fungicide for management purposes.

Interestingly, in 2011, clonal lineage US-11 was detected on tomatoes in California. This lineage had been prevalent in the mid-1990s and had not been reported in recent years (Hu et al. 2012). Additionally, several new and rare genotypes were detected in the East during the summers of 2010 and 2011 (see **Chapter 3**). Most of these genotypes were found in a region that centered around central New York State. The genetic characteristics of this population of *P.* infestans are consistent with a recombinant population. The ratio of A1 to A2 mating types among these genotypes was close to the 1:1 ratio expected for sexual recombination. Fortunately, these strains have not been detected since 2011. However, the

fact that this population existed indicates that sexual recombination in the United States is possible and may happen again.



*Fry lab; Hu et al. 2012 Plant Disease; and Wangsomboondee et al. 2002 Ecology and Population Biology.

Figure 1.5 Genotypes of *Phytophthora infestans* in the United States from 1997 to 2014 (Fry et al. 2015).

1.5 Implications of having a simple population structure

Because of the fact that in any given year only a limited number of clonal lineages has been reported in the United States, it seems reasonable to characterize these for epidemiologically important phenotypic traits, such as fungicide sensitivity and host preference. Knowledge about prevalent strains of *P. infestans* aids disease control. For example, if isolates causing an outbreak on potato are known to be nonaggressive on tomato, then fungicide sprays on nearby tomato fields can be reduced. These phenotypic analyses can take weeks or months, but if genotyping through molecular techniques assigns strains to a lineage of known phenotype,

results can be obtained within one day.

In countries where *P. infestans* is able to survive mainly as clonal lineages, disease control measures are directed towards pathogen-free planting material and towards the reduction of sporangia that may come from other infected plant material, such as potato cull piles, and tubers or seeds that remain on the soil from the previous cropping season. On the other hand, in countries where *P. infestans* regularly reproduces sexually, disease management strategies become more challenging. An immediate consequence of the continuous sexual reproduction of the pathogen is the presence of an initial source of inoculum in the soil that restricts the frequency with which potatoes can be grown.

1.6 Finding genetic markers associated with phenotypic traits of agronomic importance

As mentioned earlier, phenotypic analysis may take weeks to months, thus making it challenging to obtain results in time for management decisions. This is usually the case when we are faced with a new genotype of the pathogen that has not been previously characterized. The 2009 late blight pandemic in the northeastern United States clearly exemplifies this; information regarding the fungicide sensitivity of the strain causing the 2009 pandemic became available until after the epidemic was well established, and thus farmers were not aware that this new genotype, US-22, was sensitive to the highly effective fungicide mefenoxam.

Hence, an understanding of the genetic basis of complex traits that can be used for late blight management is critical to rapidly predict the pathogen's phenotype. Informed management decisions have tangible economic and environmental benefits by leading to lower on-farm expenses, reduced fungicide applications, more effective disease suppression,

and increased sustainable production. Despite the scientific efforts to identify the genetic basis behind these epidemiologically important pathogen traits, not much is known presently. New technologies and analysis methods are driving plant pathology and plant disease management from data-poor to a data-rich environment. Next-generation sequencing of plant pathogens is revolutionizing the field as newly abundant data enable and facilitate the discovery and use of millions of single nucleotide polymorphisms (SNPs) in diverse genomes. Genotyping by sequencing (GBS) is a next-generation sequencing protocol that has allowed SNP discovery and genotyping of a variety of organisms, including *P. infestans*.

As a first step in determining the genetic basis of some important phenotypic traits in *P. infestans*, I systematically assessed five traits (mating type, host preference, sensitivity to mefenoxam, the effect of temperature on release of zoospores, and the effect of temperature on mycelial growth) of a diverse panel of *P. infestans*: The panel consisted of i) the dominant clones in the US from the 1990s to 2013, ii) a recombinant population detected in northeastern US in 2010 and 2011 (Danies et al. 2014), and iii) a natural sexual population from Mexico (**Chapter 4**). The phenotypic analyses revealed a broad range of phenotypic responses. Additionally, I conducted a genome-wide association study to identify genetic markers associated with mating type for these isolates. Eleven SNP markers gave association hits for mating type with a $P < 1e\text{-}5$.

There may not be a 'silver bullet' against late blight. The pathogen's diversity is a key factor in disease control failures. Continuous monitoring of pathogen traits such as fungicide sensitivity, host preference, and the effect of temperature on growth are essential to selecting the best disease mitigation method.

1.7 Evolutionary potential of *Phytophthora infestans*

Why has *P. infestans* prevailed for more than a century? To better answer this question, I will analyze the evolutionary potential of *P. infestans* according to the framework proposed by McDonald and Linde (2002) to predict how mutation, population size, migration, mode of reproduction, and selection govern this pathogen's evolution. The interactions between these evolutionary processes in *P. infestans* have allowed the pathogen to rapidly adapt to control strategies such as genetically resistant cultivars, making management of late blight disease a constant challenge (Fry W. E. 2008).

The genome of *P. infestans* has approximately 240 megabases, several times larger than that of related species within the genus *Phytophthora* (Haas et al. 2009). This expansion results from the proliferation of repetitive DNA that accounts for approximately 74% of the genome (Haas et al. 2009). *Phytophthora infestans*' genome is organized in an unusual way, having blocks of gene-dense regions where repeat content is relatively low and the order of the genes is conserved, separated by gene-sparse regions in which gene order is not conserved, and repeat content is high (Haas et al. 2009). The gene-sparse regions are enriched in genes that code for secreted proteins implicated in pathogenesis, show copy number variation, and have high non-synonymous to synonymous substitution rates (Raffaele et al. 2010).

1.7.1 Mutation

The *P. infestans* genome contains a strikingly rich and diverse population of mobile (transposable) elements (Haas et al. 2009). These mobile elements have the potential for creating or reversing mutations, leading to changes in the DNA sequence of individual genes.

Consistent with a model of repeat-driven expansion of the *P. infestans* genome, Haas et al. (2009) reported that the vast majority of repeat elements in the genome are highly similar to their consensus sequences in their respective transposon family, indicating a high rate of recent transposon activity.

When mutations are coupled with directional selection, virulent or pesticide-resistant strains have the potential to increase in frequency rapidly (McDonald and Linde 2002). This is particularly advantageous in an organism such as *P. infestans*, given that this pathogen can produce an extremely large number of sporangia within individual plants. These large populations of sporangia make it more likely that new mutants with higher fitness will emerge within a host, are able to multiply within the infected host, and spread to new, uninfected hosts before the mutation is lost through genetic drift (see below) or chance (McDonald and Linde 2002).

Like many other pathogens, *P. infestans* secretes proteins that are able to change the host's physiology and facilitate colonization. These secreted proteins implicated in pathogenesis are most commonly known as effector proteins. The genome of *P. infestans* revealed large families of genes encoding for these secreted effector proteins. Haas et al. (2009) found approximately 60% more predicted RXLR genes in *P. infestans*, than in *Phytophthora sojae* and *Phytophthora ramorum*. Furthermore, approximately half of these RXLR effectors were unique to *P. infestans* (Haas et al. 2009). Many of these effector proteins are known to interact with plant resistant (R) proteins. When an R protein recognizes the presence of an effector, the plant mounts a defense response that is usually accompanied by localized cell death to restrict the growth of the pathogen (Jones and Dangl 2006). Hence, to be successful, the pathogen needs to mutate or eliminate the effector gene that is being

recognized by the plant, or acquire a new effector to suppress the immune response. Effector genes are localized in the highly dynamic and expanded gene-sparse regions of the *P. infestans* genome and therefore have the potential to evolve rapidly (Raffaele et al. 2010). This genome plasticity has certainly played a crucial role in the rapid adaptability of *P. infestans* to host plants and highlights its evolutionary potential (Haas et al. 2009).

Another example of *P. infestans*' ability to quickly adapt to adverse environments is the observation of individuals belonging to 'sensitive' clonal lineages becoming tolerant of the fungicide mefenoxam upon a single passage through mefenoxam-containing medium (see **Chapter 5** or Childers et al. (2014)). Although we only tested three different 'sensitive' lineages of *P. infestans*, we suspect that the ability to acquire mefenoxam-resistance may be a general characteristic of mefenoxam-sensitive isolates of *P. infestans*. Given the speed and consistency of acquired resistance, an epigenetic mechanism seems likely.

1.7.2 Population size

The disease cycle of *P. infestans* can be remarkably rapid with penetration, colonization, sporulation, and dispersal occurring in less than five days (Fry and Goodwin 1997). Each individual late blight lesion can produce as many as 300,000 sporangia per day (Legard et al. 1995) and multiple lesions may occur on a single leaflet. Because large populations produce a higher number of mutations than small populations, the chances of a favorable mutation occurring in large populations is greater. The greater number of favorable mutations that will remain in the population will result in the larger population being more genetically diverse over time than the smaller population. In addition, small populations are more at risk of losing particular genes due to genetic drift, the random change in allele frequency due to chance.

Because of the impact of population size on the evolutionary potential of the pathogen, any disease management program that keeps pathogen population sizes small contributes to its control by limiting the genetic diversity in the pathogen population (McDonald and Linde 2002). *Phytophthora infestans* may undergo regular significant reductions in population size as a result of crop rotations or annual climatic extremes (in countries where winters are severe). These reductions in population size may eliminate the majority of individuals, making the pathogen less diverse and slower to adapt than populations that maintain a large size year round (McDonald and Linde 2002).

1.7.3 Migration

Migration is a process in which particular alleles or complete genotypes (individuals) in the case of clonally reproducing individuals, are exchanged among geographically separated populations (McDonald and Linde 2002). Migrations have played an important role in the life history of *P. infestans.* One important consequence of this process has been the introduction of the A2 mating type into places where only the A1 mating type had been detected previously. The presence of the two mating types has allowed the occurrence of sexual reproduction outside of Mexico, where *P. infestans* is believed to have originated. Sexual reproduction creates new combinations of alleles and results in the production of oospores, which are resistant to adverse environmental conditions. Another important consequence of migration has been the introduction of mefenoxam-resistant strains in places where only sensitive isolates occurred before.

The fact that *P. infestans* is able to infect potato tubers has allowed this pathogen to move great distances, introducing genes as well as whole genotypes into new populations. As

a result of intercontinental travel and commerce, humans have allowed the pathogen to move beyond its natural dispersal limits (Fry et al. 1993, Fry et al. 2013, Goodwin et al. 1994a, Goodwin et al. 1996, McDonald and Linde 2002). It is because of this that quarantine measures or any disease management tactic that limits the movement of individuals among populations would limit the spread of *P. infestans* (McDonald and Linde 2002).

1.7.4 Mode of reproduction

Plant pathogens may reproduce sexually through meiosis or asexually through mitosis and some may display both modes of reproduction. The mode of reproduction affects how gene diversity is distributed within and among individuals in a population (McDonald and Linde 2002). Pathogens that are able to reproduce both sexually and asexually possess a greater evolutionary potential than those that can reproduce solely by either sexual or asexual reproduction. As mentioned before, the sexual cycle serves both as a source of variation and a strategy for survival. Sexual reproduction may limit the usefulness of some disease control strategies given that a recombining pathogen population has the potential of creating new combinations of virulence alleles as rapidly as breeders can introduce resistance genes (McDonald and Linde 2002). The asexual cycle on the other hand serves as a mechanism for dispersal and rapid population growth that facilitates the rapid dispersion of newly generated mutants that possess an increased fitness under the conditions the pathogen is growing.

1.7.5 Selection

Selection is the main force that drives changes in allele frequencies. Pathogen populations exposed to major gene resistance, or complete resistance conditioned by a single gene, will

face stronger selective pressure and therefore evolve more quickly than populations that are exposed to the weaker selection imposed by incomplete resistance, or quantitative resistance conditioned by multiple genes of partial effect (McDonald and Linde 2002, Poland et al. 2009). This selection pressure together with the genetic plasticity of *P. infestans*, particularly associated with genes involved in pathogenesis, is recognized as one of the main causes for failure of *R* genes in cultivated potatoes as a disease management strategy (Wastie 1991). Furthermore, Abu-El Samen et al. (2003) showed that *P. infestans* has the ability to continuously change in virulence even during asexual reproduction.

1.8 Risk posed by *Phytophthora infestans* for overcoming control methods

Phytophthora infestans has a high evolutionary potential and thus poses a great risk of overcoming major resistance genes and evolving to counteract other control methods, such as fungicide application (McDonald and Linde 2002). The pathogen's genome plasticity, with the presence of transposable elements and repeat-rich regions, fosters the emergence of a high number of mutations. This increases the likelihood that mutations overcoming resistance genes or pesticide activity will be present in the pathogen population. The generation of such beneficial mutations coupled with strong directional selection greatly increases the chances that advantageous mutations will persist in the population. The multiple mechanisms used by *P. infestans* to move between hosts have allowed the transmission of virulent and/or fungicide-resistant genotypes across large geographical areas.

A mixed reproductive system (sexual and asexual) gives *P. infestans* a high evolutionary potential. The sexual cycle creates new combinations of alleles through recombination. These recombined genotypes are then exposed to different crop environments,

which may include new resistance genes or pesticide products containing new active ingredients. Those genotypes (combinations of alleles) that are the fittest will be held together through asexual reproduction and may increase to high frequencies in selected clones (McDonald and Linde 2002).

Based on predictions by McDonald and Linde (2002) regarding the relative risk of plant pathogens for overcoming resistance, *P. infestans* possesses the highest possible 'risk value'. Understanding the evolutionary potential of pathogens will prove useful to optimize the management of resistance genes and pesticides to maximize their usefulness and minimize the losses that result from reduced efficacy of these control methods (McDonald and Linde 2002).

1.9 Objectives and scope of this dissertation

The overall objective of this work was to understand the population genetics of *P. infestans* in the United States. I initially investigated epidemiologically important phenotypic characteristics of the recently emerged clonal lineages (US-22, US-23, and US-24) in the United States (**Chapter 2**). Subsequently, I investigated a set of rare and diverse genotypes detected in a region that centered around central New York State in 2010 and 2011 (**Chapter 3**). Next, I systematically assessed five traits (mating type, host preference, sensitivity to mefenoxam, the effect of temperature on release of zoospores, and the effect of temperature on mycelial growth) of a diverse panel of *P. infestans*: The panel consisted of i) the dominant clones in the US from the 1990s to 2013, ii) a recombinant population detected in northeastern US in 2010 and 2011 (Danies et al. 2014), and iii) a natural sexual population from Mexico (**Chapter 4**). For these isolates I conducted a genome-wide association study to

identify genetic markers associated with mating type. Finally, in **Chapter 5**, I investigate the characteristics of mefenoxam-acquired resistance. More specifically, I addressed the following three questions: 1) Is the phenomenon of acquired resistance unique to certain strains of *P. infestans*? 2) How fast does acquired resistance to mefenoxam occur? and 3) What are the gene expression difference between initially sensitive isolates and their derivatives with acquired mefenoxam resistance?

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CHAPTER 2*

Phenotypic characterization of recent clonal lineages of *Phytophthora infestans* in the United States

2.1 Abstract

Phytophthora infestans, the causal agent of late blight disease, has been reported in the United States and Canada since the mid XIX century. Due to the lack of, or very limited, sexual reproduction, the populations of *P. infestans* in the United States are primarily reproducing asexually and thus show a simple genetic structure. The emergence of new clonal lineages of *P. infestans* (US-22, US-23 and US-24) responsible for the late blight epidemics in the northeastern region of the United States in the summers of 2009 and 2010 stimulated an investigation into phenotypic traits associated with these genotypes. Mating type, differences in sensitivity to mefenoxam, differences in pathogenicity on potato and tomato, and differences in rate of germination were studied for clonal lineages US-8, US-22, US-23 and US-24. Both A1 and A2 mating types were detected. Lineages US-22, US-23 and US-24 were generally sensitive to mefenoxam while US-8 was resistant. US-8 and US-24 were primarily pathogenic on potato while US-22 and US-23 were pathogenic on both potato and tomato. Indirect germination was favored at lower temperatures (5 and 10ºC) whereas direct germination, though uncommon, was favored at higher temperatures (20 and 25ºC). Sporangia of US-24 released zoospores more rapidly than did sporangia of US-22 and US-23.

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The association of characteristic phenotypic traits with genotype enables the prediction of phenotypic traits from rapid genotypic analyses for improved disease management.

2.2 Key words

Phytophthora infestans, clonal lineage, sensitivity to mefenoxam, pathogenicity on potato and tomato, germination rate, late blight

2.3 Introduction

Phytophthora infestans, the causal agent of late blight disease, has been reported in the United States since the 1840s (Stevens 1933). Since its introduction, it is hypothesized that the pathogen population has been primarily reproducing clonally, and new clonal lineages have emerged mainly by mitotic recombination, mutation, or migration events (Goodwin S. B. et al. 1994). Due to the simple population structure of *P. infestans* in the United States, it has been possible to group individuals into clonal lineages based on neutral markers such as mating type, mitochondrial haplotype, nuclear DNA fingerprint patterns and allozyme genotype (Goodwin S. B. et al. 1994). More recently, this approach has been complemented with the use of microsatellite markers (Myers et al. 2010, Small et al. 2012).

Isolates within lineages are recognized to be more similar to each other than to isolates in a different clonal lineage for characteristics such as metalaxyl or mefenoxam resistance and host preference (Goodwin S. B. et al. 1995a, Goodwin S. B. et al. 1996, Lambert and Currier 1997, Legard et al. 1995, Miller et al. 1998). Several studies have characterized previously dominant clonal lineages and demonstrated the existence of considerable, predictable variation among different clonal lineages in epidemiologically important phenotypic traits.

Some examples include, phenylamide fungicide resistance in clonal lineages US-7 and US-8 (Goodwin S. B. et al. 1998), and that US-8 is pathogenic mainly on potatoes while US-7, US-11 and US-17 are pathogenic on both potatoes and tomatoes (Goodwin S. B. et al. 1998, Legard et al. 1995).

Environmental variables such as temperature and relative humidity have a significant impact on the development of late blight. Early studies conducted by Melhus (1915) and Crosier (1934) indicated that indirect germination was predominant at temperatures below 20˚C. In a study by Mizubuti and Fry (1998) differential effects of temperature on sporangia germination were reported; the optimal temperature for indirect germination was lower for lineages US-7 and US-8 than it was for US-1. Clonal lineages US-7 and US-8 germinated indirectly at temperatures below 15˚C. The differential effects of temperature on sporangia germination may differentially influence the efficacy of sporangia to initiate infection, and are therefore important for disease epidemiology and disease management.

The re-emergence of late blight in Northeastern United States and Canada in the summers of 2009 and 2010 revealed the existence of new clonal lineages of *P. infestans* (Hu et al. 2012). Both potatoes and tomatoes were infected by the new clonal lineages. Knowledge regarding the sensitivity of the new genotypes to the fungicide mefenoxam, their host preference, and other epidemiological characteristics could provide information important to disease management decisions. The objective of this study was to investigate epidemiologically important phenotypic characteristics of the recently emerged clonal lineages in the United States and Canada. Sensitivity to the systemic fungicide mefenoxam, pathogenicity on potato and tomato and the effect of temperature on sporangium germination were investigated for clonal lineages US-8, US-22, US-23 and US-24.

2.4 Materials and Methods

2.4.1 Isolates and isolation

Isolates were obtained from throughout the United States and from select provinces in Canada during 2009, 2010 and 2011. Sampling of *P. infestans* isolates is limited to those places where the pathogen occurs, and cannot be predicted from one season to another. For this reason a predetermined sampling strategy could not be employed. Isolates collected for this study were those reported by extension personnel and plant disease diagnostic labs in our network. Isolates were maintained on pea agar (Jaime-Garcia et al. 2000), rye B agar (Caten and Jinks 1968) and on tomato and/or potato leaflets (depending on the isolate) at 15ºC. When the source of the isolates was sporulating lesions, a small block of media (rye B and pea agar) with antibiotics (ampicillin (100 µg ml-1), rifampicin (125 µg ml-1), and pentachloronitrobenzene (25 µg ml-1), was placed in contact with sporangia, which was then transferred to Petri plates of agar medium. From the plates, colonies were selected and transferred again onto rye B and pea agar media.

2.4.2 Mating type

Mating type was determined by pairing an unknown isolate with a known isolate of *P. infestans*, either A1 mating type (US970001 US-17 genotype) or A2 mating type (US040009, US-8 genotype), on rye B or pea agar media. Petri plates were kept at 20°C for 10-14 days. The hyphal interface of the two colonies was investigated microscopically using 125X magnification. Isolates that formed oospores at the interface with the known A1 isolate were designated A2 and those that formed oospores with the known A2 isolate were designated A1.

The known isolates (A1 and A2) were paired as positive controls, while negative controls consisted of pairing the known isolates with themselves (same mating type).

2.4.3 Mitochondrial haplotype

Mitochondrial DNA haplotype was determined using PCR-RFLP analysis, as described by Griffith and Shaw (1998), with two primer pairs (F1-R1 and F2-R2). Reference US-1 and US-8 isolates were included as positive controls.

2.4.4 Glucose-6-phosphate isomerase

Mycelia and/or sporangia obtained from cultures grown on rye B or pea agar or from infected leaflets were used to determine *glucose-6-phosphate isomerase* (GPI) allozyme genotypes. Analyses were carried out using cellulose acetate electrophoresis as described by Goodwin S. B. et al. (1995b). One or more reference isolates representing US-1 (SA960008), US-8 (US040009), and/or US-17 (US970001) were included in all GPI analyses.

2.4.5 DNA extraction and RFLP analysis with probe RG-57

DNA extractions and subsequent RFLP analysis with the RG-57 DNA probe were performed using a method modified from Goodwin S. B. A. et al. (1992). Southern blot analysis was conducted using the Amersham gene images AlkPhos direct labeling and detection system (GE Healthcare) according to the manufacturer's instructions. The US-1 (SA960008) reference isolate was used in RG-57 analyses. Presence or absence of all fingerprint fragments was scored visually.

2.4.6 Multiplex microsatellite marker analysis

Simple sequence repeats (SSRs) were analyzed for all isolates, using protocols developed previously (Lees et al. 2006) and described in the Protocol section March 2008 by the Eucablight Network (www.eucablight.org). Three separate multiplex reactions were conducted using three panels of primers (Table 2.1). Master mixes were prepared. Amounts per single reaction are shown in Table 2.2.

Table 2.1 *Phytophthora infestans* microsatellite primer information.

Primer	Primer sequence	Cooke allele size range (bp) *US lineages size range (bp)*[a]	Label	Annealing tempereature	Repeat	Reference
D13F D13RLong	F: TGCCCCCTGCTCACTC R: GCTCGAATTCATTTTACAGACTTG	106-166 *106-147*	6-FAM	56	$(CT)_{27}$	(16) (modified)
Pi02F Pi02R	F: CAGCCTCCGTGCAAGA R: AAGGTGCGCGAAGACC	142-168 *154-166*	PET	58	$(TG)_{11}$	(16)
Pi4BFcap Pi4BR	F: AAAATAAAGCCTTTGGTTCA R: GCAAGCGAGGTTTGTAGATT	203-225 *213-226*	NED	58	$(TC)_{34}$	(14)
PiG11Fcap PiG11R	F: TGCTATTTATCAAGCGTGGG R: TACAATCTGCAGCCGTAAGA	138-212 *127-159*	VIC	56 or 58	$(TC)_{26}$	(14)
Pi56Fcap Pi56RLong	F: AACTATCTATCGGCGTGCAT R: CAGGCCGCAATTGTAAGA	252-255 *255-257*	PET	58	$(AT)_{10}$	(16)
Pi04Fcap Pi04R	F: AGCGGCTTACCGATGG R: CAGCGGCTGTTTCGAC	160-172 *166-170*	NED	58	$(GT)_{6}$	(16)
Pi63F Pi63Rlong	F: ATGACGAAGATGAAAGTGAGG R: CGTATTTTCCTGTTTATCTAACACC	272-281 *269-279*	6-FAM	58	$(GAG)_{8}$	(16)
Pi33Fcap Pi33R	F: TGCCGACGACAAGGAA R: CGGTCTGCTGCTGCTC	203-209 *203-206*	VIC	58	$(CAG)_{5}$	(16)
Pi16Fcap Pi16R	F: CACAGCACGCGGAATC R: ACGCCGAGTGTCCTGA	173-177 *173-177*	NED	58	$(GA)_{7}$	(16)
Pi70F Pi70R	F: ATGAAAATACGTCAATGCTCG R: CGTTGGATATTTCTATTTCTTCG	189-195 *187-193*	6-FAM	58	$(AAG)_{8}$	(16)
Pi89Fcap Pi89R	F: GAGAACGCACAATGTAAGGC R: ACATAAATACACGCTGAACGG	177-211 *177-181*	6-FAM	58	$(AT)_{9}$	(16)

[a] Size ranges.

PCR amplification was conducted with a standardized set of thermocycling conditions: initial activation step of 95°C for 10 min, followed by 28 cycles of 95°C for 20 sec, 58°C for 25 sec, 72°C for 60 sec and a final extension step of 72°C for 20 min. Post PCR processing was conducted by dispensing 9.5 µl of size standards in high-deionized formamide mix, 0.05 µl of Genescan-500 LIZ size standard (ABI, PN 4322682) and 9.45 µl of Hi-Di™

Formamide (ABI, PN 4311320), and 0.5 µl each of the PCR products from panel 1-3 reactions into each well of a 96-well ABI 3730xl plate. PCR products were analyzed on an ABI 3730xl capillary system with POP-7™ Polymer (ABI, PN 4335615). PCR amplicons were compared to a set of size standards and alleles were scored accordingly (Lees et al. 2006).

Table 2.2 Polymerase chain reaction (PCR) reactions for simple sequence repeat analysis for *Phytophthora infestans* isolates[a].

	Volume (µl)		
Reagents	**Plate 1**	**Plate 2**	**Plate 3**
HPLC grade water[b]	7.95	6.79	8.375
10X Immolase PCR buffer	1.2	1.2	1.25
10 mM dNTPs	0.125	0.187	0.125
50 mM $MgCl_2$	0.375	0.42	0.375
10 µM primers[c]			
Pi02	0.125	…	…
Pi89	0.275	…	…
Pi4B	0.475	…	…
PiG11	…	0.3	…
Pi04	…	0.125	…
Pi70	…	0.375	…
Pi56	…	0.3	…
Pi63	…	0.3	…
Pi16	…	…	0.188
Pi33	…	…	0.1
D13	…	…	0.35
Immolase Taq (5u μl^{-1})	0.1	0.1	0.1
Template DNA (~ 1 ng μl^{-1})	1	1	1

[a] Multiplex PCR reactions were conducted using three panels of primers based on a protocol developed by the Eucablight Network (www.eucablight.org) as described in the Protocol section March 2008. Reagents used from Bioline.
[b] HPLC = high-performance liquid chromatography.
[c] Forward and reverse primers.

2.4.7 Mefenoxam sensitivity assay

Mefenoxam sensitivity of isolates was assessed as described previously by Therrien et al. (1993), except that mefenoxam was used in place of metalaxyl. Isolates were grown on V8 agar or pea agar amended with Ridomil Gold EC, of which 49% was the active ingredient

(mefenoxam) (Syngenta, Greensboro, NC), such that the final concentrations of the active ingredient were 0, 5, or 100 ug ml^{-1}. Mycelial plugs (8 mm diameter) were obtained from actively growing cultures, transferred to the test plates and incubated for approximately 10 to 12 days, or until growth on the control mefenoxam plate (0 ug ml^{-1}) was approximately 80% of the diameter of the petri plate. Assessment of mefenoxam sensitivity was determined on the basis of radial growth of cultures grown on plates amended with mefenoxam (5 or 100 µg ml^{-1}) compared to non-amended controls. Growth on mefenoxam-amended plates, 5 and 100 µg ml^{-1}, was represented as a proportion of the growth on the non-amended control plates.

Effects of mefenoxam concentration, lineage and year of collection on colony growth were analyzed using JMP 9.0.2 (SAS Institute, Cary, NC, USA). Standard least square analysis was used, where replications were considered random terms, while mefenoxam concentration, lineage and year of collection were considered as fixed effects. To determine if means of colony growth on mefenoxam amended plates, for each lineage, differed between years, a Tukey-Kramer HSD test with $\alpha = 0.05$ was performed.

2.4.8 Inoculum production

A sporangial suspension was used for inoculation of potato and tomato leaflets. Sporangia were washed from sporulating lesions on tomato or potato leaflets, which had been maintained in water-agar moist-chambers at 15°C with a 16-h light period for 8 days prior to inoculation. The sporangial suspension was adjusted to 4,000 sporangia ml^{-1} using a haemocytometer and maintained at 4°C for 2 h before being applied, by pipetting 20 µl on a leaflet. Leaflets were obtained from four to five weeks old potato cultivar ‘Yukon Gold’ and tomato cultivar ‘Rutgers’ plants grown under greenhouse conditions.

2.4.9 Potato-tomato pathogenicity

In order to determine differences in host preference for isolates, each isolate was inoculated onto both potato Yukon Gold and tomato Rutgers leaflets. Eight US-8, thirty-four US-22, seven US-23 and nine US-24 isolates were used for this study (Supplementary Table 2.1). Plants were grown in the greenhouse (ca 25°C daytime and 20°C nighttime) and when four to five weeks old, recently matured leaflets were harvested. Inoculations were carried out in 150 mm Petri plates containing 75 ml of water agar (1.5%) in the smaller half – which served as the lid (top). Leaflets were placed (abaxial side up) on the base of the moist chamber. Each moist chamber contained five potato or five tomato leaflets, abaxial side up. All five leaflets were inoculated with 20 µL of a sporangial suspension (described above) of the same isolate, deposited on one side of the main vein of the leaflet. After the leaflets were inoculated, the petri plate was sealed with parafilm and incubated at 15°C with a 16-h light period. The experiment was conducted at least twice for each isolate.

Lesion size and number of sporangia per lesion were measured at six days after inoculation. Lesion areas were estimated by taking two perpendicular measurements (length and width) starting from the widest diameter, using a ruler. Subsequently, the number of sporangia produced on each lesion was determined. Individual lesions were excised and placed into 14-ml disposable polypropylene culture tubes with 3 ml of preservative solution (0.04 M copper sulfate, 0.2 M sodium acetate, acetic acid, pH 5.4) (Spielman et al. 1991). The tubes were then vortexed for 10 seconds to dislodge and suspend sporangia, and aliquots counted with a haemocytometer. Haemocytometer counts were repeated at least twice. The total number of sporangia per lesion was then calculated by averaging all the independent counts. The assay was conducted at a standardized temperature of 15°C. Lesion area and

sporulation was compared between potato and tomato for each clonal lineage separately. The statistical significance of differences in mean lesion size and sporangia production between hosts, for each clonal lineage, was determined using a Student's t test with $\alpha = 0.05$.

2.4.10 Temperature effect on sporangium germination

The effect of temperature on sporangium germination was assessed. Three 400-µL droplets of 1.5% water agar were dispensed into three independent circular silicone-molds placed on top of a glass microscope slide. A 20 µl droplet of a suspension consisting of 4,000 sporangia ml^{-1} was deposited on each water agar droplet. Three isolates for each lineage (US-8, US-22, US-23 and US-24) were used for this study (Supplementary Table 2.1). Inoculated slides were placed in incubators at 10, 15, 20, and 25°C with no light. After 16 h, total germination (direct, indirect and no-germination) was determined by microscopic observation of each water agar droplet. For each isolate three replicates were conducted at each temperature, and the experiment was repeated at least twice. In a second experiment, germination was assessed at 5 and 10ºC.

Effects of temperature and lineage on germination (indirect or direct) were analyzed using JMP 9.0.2 (SAS Institute, Cary, NC, USA). Standard least square analysis was used, where replications were considered random terms, while temperature and lineage were considered as fixed effects. To determine if mean germination differed between temperatures, for each lineage, a Tukey-Kramer HSD test with $\alpha = 0.05$ was performed.

2.4.11 Effect of temperature on the rate of sporangium germination

Sporangia were observed at 30, 90, 120, 240, and 960 minutes after incubation at 15 and 4°C. Inoculation was performed as described above. For the 15°C assay, a single slide with three

independent repetitions (three circular water agar droplets that had each been inoculated with 20 µl of the same sporangial suspension) was assessed for the five time points studied, for each isolate. Total germination (direct, indirect and no-germination) was counted at the first 30-minute assessment and thereafter only indirect germination (sporangia that had released their zoospores) was recorded. This was possible since slides could be incubated and assessed at 15°C for the duration of the assay. For the 4°C assay, independent measurements of total germination were carried out for each respective time point. That is, a different slide with three circular water agar droplets that had each been inoculated with 20 µl of the same sporangial suspension was assessed for each of the five time points studied. This was due to the difficulty of maintaining slides at 4°C while assessing germination microscopically. Three isolates were included for each of the four clonal lineages studied (US-8, US-22, US-23, and US-24) (Supplementary Table 2.1). Percentage of total germination that was indirect was calculated for each of the time points considered. The experiment was conducted at least twice for each isolate.

Effects of time and lineage on indirect germination were analyzed using JMP 9.0.2 (SAS Institute, Cary, NC, USA). A standard least square analysis was used, where replications were considered random terms, while time and lineage were considered as fixed effects. To determine if mean germination, within a lineage, differed between time periods studied, a Tukey-Kramer HSD test with $\alpha = 0.05$ was performed. To study differences in the rate at which indirect germination occurred over time (from 0 to 240 min after exposure of sporangia to 4 or 15ºC) data were transformed to achieve linearity. Data were adequately described by a negative exponential model (Madden et al. 2007) for all lineages studied. Slopes representing the rate of indirect germination over time were compared using a t test

with $\alpha = 0.05$.

2.5 Results

2.5.1 Isolates and isolation

In 2009, 2010 and 2011, approximately 350 samples were received and processed. In 2009 approximately 71 samples were received from 6 states (FL, ME, NJ, NY, TN, and VA) within the United States and 57 of these were isolated into culture and characterized. In 2010, approximately 81 samples were received from 14 states within the United States (CT, HI, ID, KY, LA, MA, MD, ME, MT, NH, NY, PA, WA, and WI) and one province of Canada (ON). Among these, 69 were successfully isolated into culture and characterized. In 2011 approximately 204 samples were received from 17 states within the United States (CA, CT, DE, FL, ID, ME, MN, ND, NH, NY, OH, OR, PA, RI, VA, WA, and WI) and one province of Canada (ON). Among these, 116 were successfully isolated and characterized. Isolation was successful on both pea and rye B agar, with rapid growth observed on pea agar.

Clonal lineages were defined according to mating type, mitochondrial haplotype, *Gpi* genotype, RG-57 DNA fingerprint profile (Table 2.3), and microsatellite genotype (Table 2.4). Clonal lineages US-8 and US-22 were A2 mating type, whereas clonal lineages US-23 and US-24 were A1 mating type. All four clonal lineages were determined to have the Ia mitochondrial haplotype. Each lineage was described by a unique genotype at the locus for *glucose-6-phosphate isomerase*. US-8 was 100/111/122; US-22 was 100/122; US-23 was 100/100 and US-24 was 100/111 (Table 2.3). Each lineage showed a unique RFLP fingerprint as determined by RG57, with 10 bands being polymorphic for these four lineages (Table 2.3). Alleles at eleven microsatellite loci successfully distinguished the four clonal lineages studied

(Table 2.4). Furthermore, for the isolates studied, two variants were identified within US-22 based on microsatellite results. These variants differed from the typical US-22 at markers Pi89 (Var1 - eight isolates) and D13 (Var2 - one isolate). Similarly one variant was identified within US-24 at marker Pi02 (Table 2.4).

Table 2.3 Summary of multilocus genotypes of four *Phytophthora infestans* lineages in the United States and Canada[a].

Clonal lineage	Mating type	Mitochondrial haplotype	GPI	RG57																									
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	24a	25
US-8	A2	Ia	100/111/122	1	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	1
US-22	A2	Ia	100/122	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
US-23	A1	Ia	100/100	1	1	0	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	0	1	1	0	0	1	1	1
US-24	A1	Ia	100/111	1	0	1	0	1	0	1	0	0	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	1

[a] MT = mating type, Hap = mitochondrial haplotype, and GPI = glucose-g-phosphate isomerase.

Table 2.4 Observed allele sizes for four clonal lineages of *Phytophthora infestans* amplified with 11 microsatellite markers.

	Size (bp) for each lineage (isolate identity)						
SSR marker[a]	US-8 (US100028)	US-22 (US090042)	US-22 var1 (US100001)	US-22 var2 (US110002)	US-23 (BL2009P4)	US-24 (ND822P)	US-24 var 1 (US110159)
Pi02	…	…	…	…	162	…	162
Pi02	162	162	162	162	164	162	164
Pi02	164	164	164	164	166	164	166
Pi4B	213	213	213	213	213	217	217
Pi4B	226	213	213	213	217	226	226
Pi89	179	177	177	177	179	177	177
Pi89	179	179	181	179	179	179	179
Pi04	166	166	166	166	170	166	166
Pi04	170	170	170	170	170	170	170
Pi56	255	255	255	255	253	255	255
Pi56	255	255	255	255	255	255	255
Pi70	190	190	190	190	190	190	190
Pi70	190	193	193	193	190	193	193
PiG11	155	131	131	131	140	155	155
PiG11	155	155	155	155	155	155	155
Pi63	279	279	279	279	270	279	279
Pi63	279	279	279	279	279	279	279
D13	106	Null	Null	147	134	106	106
D13	110	Null	Null	147	134	110	110
Pi16	173	177	177	177	177	173	173
Pi16	177	177	177	177	177	177	177
Pi33	203	203	203	203	203	203	203
Pi33	203	206	206	206	206	206	206

[a] SSR = simple-sequence repeat.

The predominant clonal lineages sampled were US-8, US-22, US-23, and US-24. Although US-8 is a well-characterized lineage it was included in the study for comparative purposes. In 2009, only US-8 and US-22 were identified, with a total of 11 US-8 and 43 US-22 isolates. In 2010 and 2011 a total of 11 and 3 US-8, 33 and 25 US-22, 6 and 61 US-23 and 9 and 50 US-24 isolates were collected, for each year respectively. The summary data for host of origin for all three years for each clonal lineage were as follows (P=potato; T=tomato); for US-8, 25 (P) and 0 (T); for US-22, 15 (P) and 86 (T); for US-23, 13 (P) and 54 (T); and for US-24, 57 (P) and 2 (T).

2.5.2 Sensitivity to mefenoxam

Sensitivity to mefenoxam was assessed for 206 isolates (Supplementary Table 2.1). In general, US-8 was resistant, US-22 and US-23 were sensitive, and US-24 was generally sensitive (Figure 2.1). Eight, ninety-seven, ninety-four and seventy-five percent of the isolates belonging to clonal lineages US-8, -22, -23 and -24 respectively, grew less than forty percent relative to the control (0 μg ml^{-1}) on mefenoxam amended plates (5 and 100 μg ml^{-1}). Differences in mefenoxam sensitivity between years, for each clonal lineage, were analyzed using a standard least square model. For US-8, US-22 and US-23 mefenoxam sensitivity did not vary significantly among the years studied (US-8, $P = 0.36$; US-22, $P = 0.36$ and US-23, $P = 0.39$) (Figure 2.2). However, for US-24, isolates collected in 2011 were less sensitive to mefenoxam than those collected in 2010 ($P = 0.005$) (Figure 2.2).

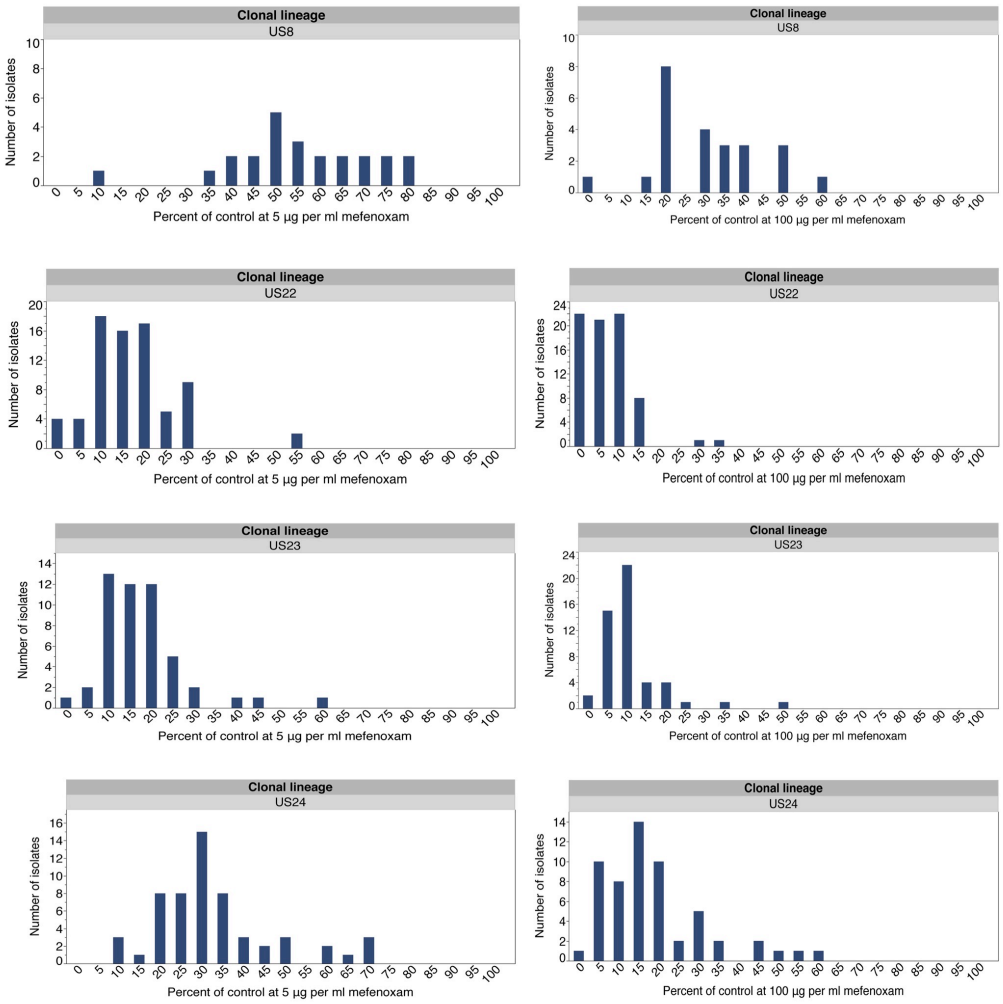

Figure 2.1 Response of *Phytophthora infestans* isolates to mefenoxam. Relative growth (as percentage of control) at 5 µg ml^{-1} (left) and 100 µg ml^{-1} (right) relative to control (0 µg ml^{-1}). Sample sizes for each lineage are US-8, n = 24; US-22, n = 75; US-23, n = 50; US-24, n = 57, for a total sample size of 206 isolates. US-8 has been described as resistant given that 92% of the isolates grew more than 40% relative to the control (0 µg ml^{-1}) on mefenoxam amended plates (5 and 100 µg ml^{-1}), US-22 and US-23 have been described as sensitive given that 97 and 94% of the isolates respectively grew less than 40% relative to the control, and US-24 has been described as generally sensitive given that 75% of the isolates grew less than 40% relative to the control.

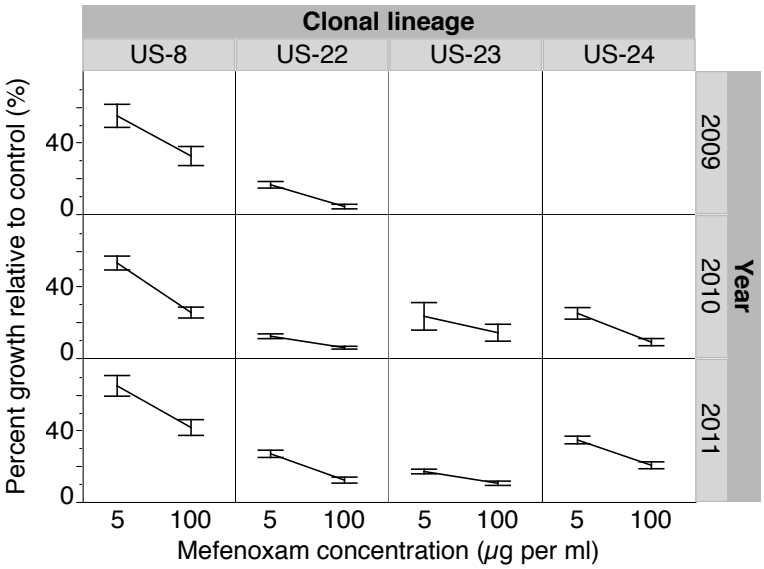

Figure 2.2 Sensitivity to mefenoxam of four *Phytophthora infestans* clonal lineages in three consecutive years. Data represent the relative (radial) growth of isolates on medium containing mefenoxam at 5 and 100 μg ml^{-1} relative to growth with no mefenoxam. The number of isolates in the clonal lineages for 2009, 2010 and 2011 were respectively: US-8, n = 10, 11, and 3; US-22, n = 25, 32, and 18; US-23, n = 0, 6, and 44; US-24, n = 0, 9, and 48, respectively, for a total sample size of 206 isolates. Error bars represent one standard error from the mean.

Due to the diversity in microsatellite genotype for the US-22 lineage (one dominant genotype and two variants), we tested whether there was diversity in mefenoxam sensitivity associated with either variant. US-22 variants at Pi89 (Var1 – eight isolates) were only collected in 2010 whereas diversity at marker D13 (Var2 – one isolate) was only found in 2011. US-22 Var1 did not differ significantly in its sensitivity to mefenoxam from the typical US-22 isolates collected and analyzed from 2010 (P = 0.75). Similarly, Var2, which was

represented by a single isolate, did not differ significantly in its sensitivity to mefenoxam relative to the typical US-22 isolates analyzed from 2011 ($P = 0.54$).

2.5.3 Potato-tomato pathogenicity

The relative pathogenicity of the four clonal lineages on potato and tomato was assessed by measuring lesion size and sporulation at six days post inoculation (dpi). Lesion area ranged from 1.4 cm^2 (US-24, on tomato) to 7.9 cm^2 (US-8, potato) (Figure 2.3). US-8 and US-24 produced larger lesions on potato than on tomato ($P < 0.0001$). While both US-22 and US-23 had slightly larger mean lesion areas on tomato in comparison to potato, these differences were not significant ($P = 0.17$ and $P = 0.25$, respectively). For each of the clonal lineages studied, sporulation differed significantly between hosts ($P < 0.0001$) (Figure 2.3). Mean sporulation ranged from 341 sporangia ml^{-1} (US-24 on tomato) to 16,308 sporangia ml^{-1} (US-23 on tomato). US-8 and US-24 sporulated more profusely on potato than on tomato, with a mean sporulation of 7,244 and 2,950 sporangia ml^{-1} on potato leaflets, respectively, versus 1,158 and 341 sporangia ml^{-1} on tomato, respectively. In contrast, lineages US-22 and US-23 sporulated more profusely on tomato leaflets with a mean sporulation of 2,598 and 5,476 sporangia ml^{-1} on potato leaflets, respectively, versus 11,669 and 16,308 sporangia ml^{-1} on tomato, respectively. When comparing among lineages, we found that US-22 and US-23 are pathogenic to both potato and tomato (Figure 2.3). However, it seems that US-23 might be even more aggressive than US-22 on both potatoes and tomatoes. US-24 is pathogenic mainly on potatoes, and not at all aggressive to tomato. These pathogenicity characteristics are similar to those of US-8 (Figure 2.3).

To determine whether Var1 isolates (eight isolates total) within the US-22 lineage

differed in host preference when compared to the typical US-22 isolates, these were analyzed as separate groups. For lesion area, no significant interaction was observed between the two US-22 genotypes and hosts (P = 0.47). For both genotypes, there may have been a trend toward larger lesion area on tomato than potato (P = 0.09). However, Var1 isolates produced smaller lesions on both potato and tomato than did the predominant US-22 isolates (P = 0.04). For Var1 isolates lesion areas on potato and tomato were 4.98 and 5.40 cm^2, respectively, and for typical US-22 isolates lesion areas on potato and tomato were 5.55 and 6.58 cm^2, respectively. For mean sporulation, the interaction between pathogen genotype (US-22 Var1 and the typical US-22 genotype) and host was significant (P = 0.01). This was due to reduced sporulation of Var1 on tomato compared to the typical US-22 (P = 0.002). Sporulation on potato and tomato was 2,577 and 8,234 sporangia ml^{-1} respectively, for Var1, and 2,602 and 12,345 sporangia ml^{-1} respectively, for the typical US-22 isolates.

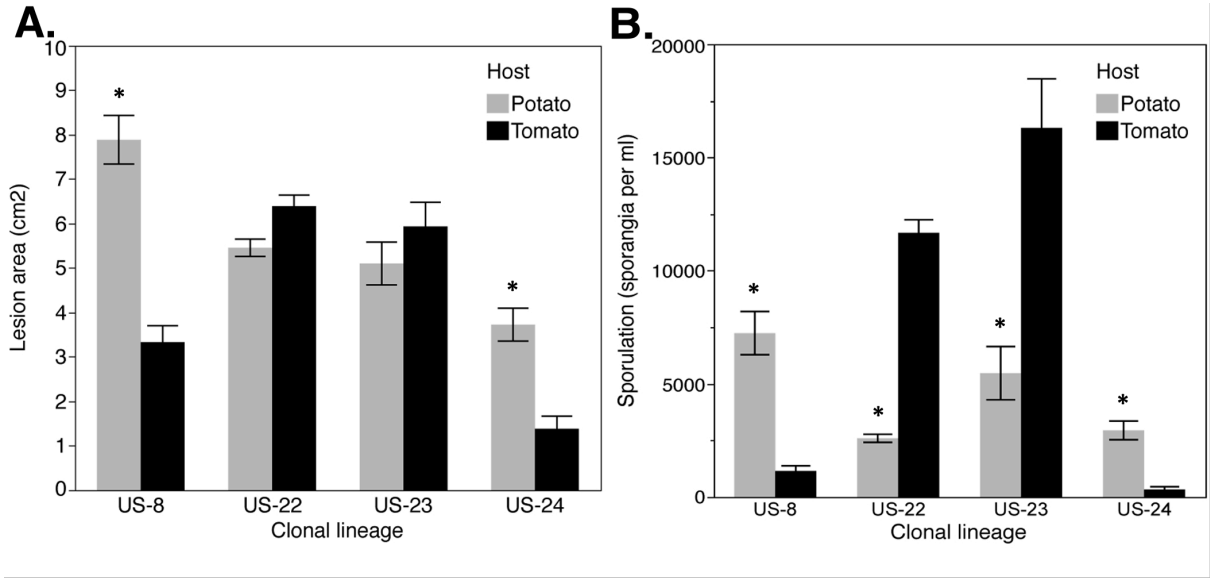

Figure 2.3 Pathogenicity on potato and tomato for isolates within four clonal lineages of *Phytophthora infestans*. **A,** Lesion areas produced on potato and tomato. **B,** Sporangia produced per infection site on potato and tomato. Clonal lineages US-8, US-22, US-23 and US-24 include 8, 34, 7 and 9 isolates, respectively. Bars represent mean lesion area for **A** and mean sporulation (per infection site) for **B** for all isolates within each clonal lineage. Error bars represent one standard error from the mean. The asterisk (*****) indicates a significant difference within a clonal lineage for either sporulation or lesion size for potato versus tomato. Lesion areas and sporulation were measured at 6 days post inoculation.

2.5.4 Temperature effect on total sporangial germination

The influence of temperature on sporangial germination was tested. In one experiment, germination was evaluated after 16 h at 10, 15, 20 and 25ºC (Figure 2.4) and in a second experiment germination was assessed after 16 h at 5 and 10ºC. Indirect germination was highest at 5 and 10ºC, with germination decreasing as temperature increased to 20ºC. There were no significant differences in germination between temperatures 20 and 25ºC. Direct germination was not commonly observed, but when it occurred it was mostly observed at higher temperatures (20 and 25ºC) (Figure 2.4). In these two experiments, US-8 and US-23 showed greater direct germination in comparison with US-22 and US-24 ($P < 0.01$). The isolates in US-22 Var1 did not differ significantly from the typical US-22 genotype in either indirect or direct germination at any temperature. Differences in indirect germination between US-8 and US-24 at 10ºC were significant ($P < 0.0262$). Overall, US-8 displayed the lowest indirect germination at 10ºC.

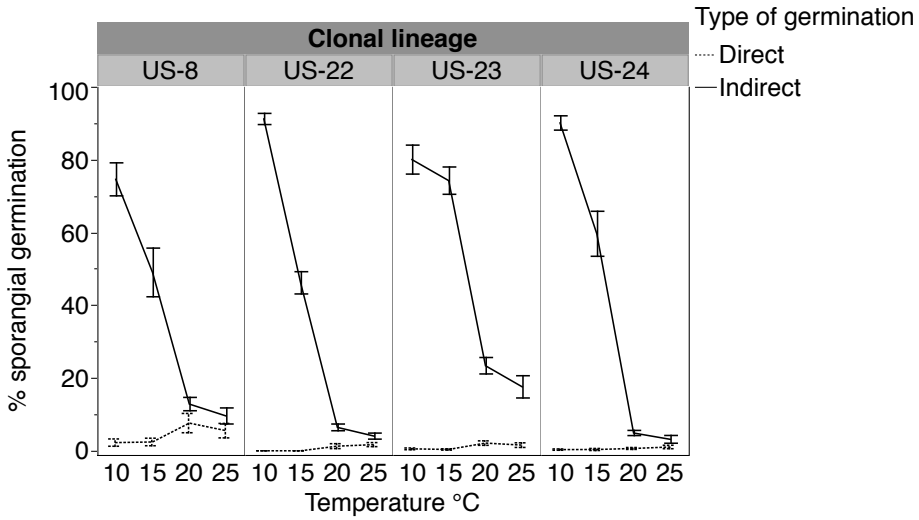

Figure 2.4 Proportion of sporangia that had germinated within 16 h at 10, 15, 20 and 25ºC. Data points indicate the mean percentage of germination of three isolates in three independent experiments. Error bars represent one standard error from the mean.

2.5.5 Effect of temperature on the rate of sporangial germination

The rate at which sporangia released zoospores was affected strongly by temperature and there were some large differences among clonal lineages (Figure 2.5). Germination was much faster at 4°C than at 15°C. For example, within 90 min more than 30% of the isolates had released zoospores at 4°C, whereas at 15°C less than 20% of the isolates had released their zoospores. Sporangia of US-24 released zoospores more rapidly than did sporangia of the other lineages at either 4 or 15°C (Figure 2.5). For example, within 30 min at 4°C, more than 75% of the US-24 sporangia had liberated zoospores, whereas zoospore release from the other lineages ranged from about 20% to about 35% (Figure 2.5A). At 15°C, zoospore release was slower for all lineages, but still US-24 released zoospores from more sporangia than did the other lineages ($P < 0.05$). After 30 min at 15°C, 13% of the US-24 sporangia had released

zoospores but only 4% of the US-23 sporangia had released zoospores (Figure 2.5B).

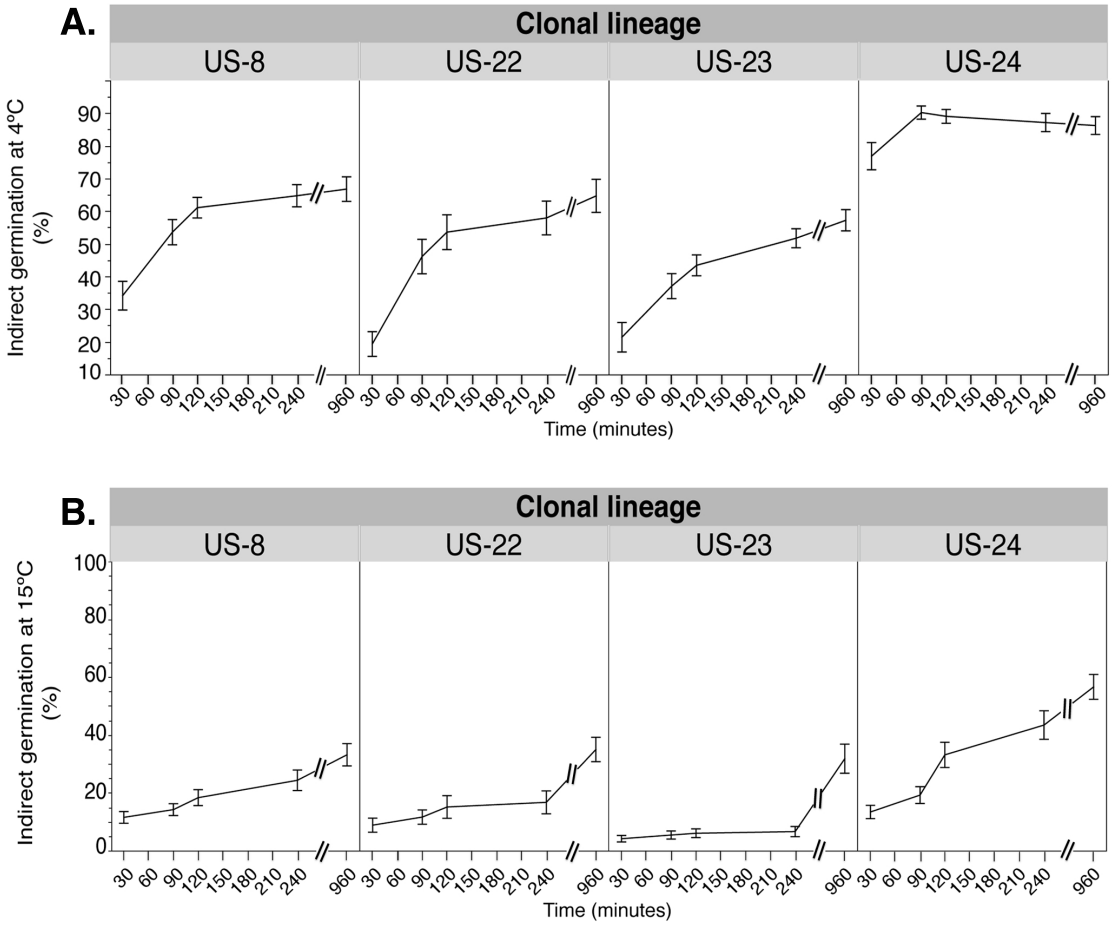

Figure 2.5 Proportion of sporangia that had germinated at 30, 90, 120, 240 and 960 min at **A,** 4ºC or **B,** 15ºC. Data points are the means obtained for three isolates in three independent experiments. Error bars represent one standard error from the mean.

2.6 Discussion

Several important phenotypic characteristics of the four dominant clonal lineages of *P. infestans* identified in samples from the 2009, 2010 and 2011 epidemics in the United States were determined. These lineages were initially defined on the basis of mating type, mitochondrial haplotype, *Gpi* genotype, and RG-57 DNA fingerprint profile, but subsequently

refined by the addition of microsatellite markers. The microsatellite markers identified some genotypic diversity within some clonal lineages.

The ability to categorize individuals into clonal lineages has been helpful for the study of migration patterns and population biology of *P. infestans* (Goodwin S. B. et al. 1998). Once the phenotype is known, it is possible to make lineage-specific management recommendations. This study reports phenotypes for sensitivity to mefenoxam, host preference and germination at different temperatures for the recently important clonal lineages in the United States (US-22, US-23 and US-24).

Sensitivity to mefenoxam was tested in an *in vitro* assay comparing three different concentrations of the active ingredient (0, 5 and 100 μg ml^{-1}). The recently described clonal lineages (US-22, US-23 and US-24) were generally sensitive to mefenoxam (Figure 2.1). US-8, which has been dominant on potatoes in the United States since the mid-1990s (Fry and Goodwin 1997), and is now being displaced by novel clonal lineages, has maintained stable resistance to mefenoxam. Notably, US-24 showed an increase in the mean colony growth, on mefenoxam-amended plates, for isolates from 2011 in comparison with isolates from 2010, indicating a potential decrease in sensitivity to mefenoxam. As far as we know, there has not been an increased use of mefenoxam, so we do not know of a factor to explain the decreased sensitivity. This decrease in sensitivity might be the result of a new better-fit subpopulation of the pathogen, although a deeper analysis must be performed to confirm this hypothesis. Knowledge regarding the sensitivity of the lineages to mefenoxam is important in terms of selecting the most appropriate fungicide.

Host preference was studied on potato (Yukon Gold) and tomato (Rutgers). Clonal lineages US-8 and US-24 showed a preference for potato as a host and were not at all

aggressive on tomato. In contrast, US-22 and US-23, showed a preference for tomato although they were also pathogenic on potato. The original host, from which these samples were collected, reflected the same findings. In the years studied, 100% of US-8, 15% of US-22, 19% of US-23 and 97% of US-24 isolates were collected from potato. Differences observed among clonal lineages on Rutgers and Yukon gold are consistent with the fact that US24 and US8 were typically reported on potatoes and not on tomatoes, whereas US22 and US23 have been typically reported on both potatoes and tomatoes.

The lineage-host combination determined the type of growth observed. US-8 and US-24 caused large necrotic lesions and profuse sporulation on potato, while limited necrosis and very little sporulation was observed on tomato. Clonal lineages US-22 and US-23 caused necrotic lesions on potato and profuse biotrophic growth on tomato (Figure 2.6). This biotrophic growth might have resulted in an underestimation of lesion area given that it was difficult to define the boundaries of the lesions.

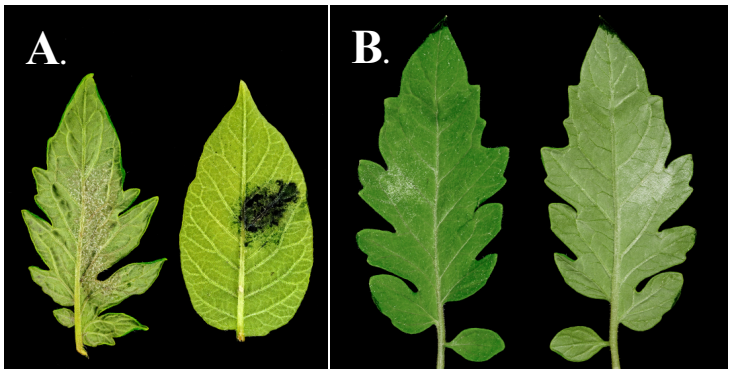

Figure 2.6 Biotrophic growth was observed on tomato leaflets six days after inoculation and incubation at 15°C. **A,** Growth of an isolate of *Phytophthora infestans* belonging to clonal lineage US-22 on tomato (left) and on potato (right). **B,** Growth of an isolate of *P. infestans* belonging clonal lineage US-23 on the adaxial side of a tomato leaflet (left) and on the abaxial side of the same leaflet (right).

Eight members of the US-22 lineage (Var1) differed from others by one allele (Supplementary Table 2.1). This variant is apparently less fit on tomato. It was interesting to note that this SSR variation is likely to be a neutral change that allows tracking of a sub-clone. It is intriguing that the US-22 variant2 changes from null to 147bp at the D13 locus. The chance of a back mutation from null to amplification seems slim. However, the SSR analysis was performed at least twice for the variant individuals and is unlikely to be an error. We presently do not have a satisfactory model to explain this situation.

It has been known for nearly a century that late blight is highly dependent on environmental conditions (Melhus 1915, Sato 1994). When sporangia are deposited on a surface and then exposed to free moisture and cool temperatures, they may germinate indirectly by releasing between eight to twelve bi-flagellated zoospores, each capable of initiating an infection (Erwin and Ribeiro 1996). The effect of temperature on sporangial germination has been extensively studied during the early 1900s by Melhus (1915) and Crosier (1934) and in the late 1900s by Mizubuti and Fry (1998). Both Crosier (1934) and Melhus (1915), found that the optimal temperature for indirect germination was approximately 15ºC. Mizibuti and Fry (1998) found similar results for isolates within clonal lineage US-1, suggesting that the isolates studied by Melhus and Crosier at the beginning of the 1900s may have belonged to the US-1 clonal lineage. In contrast to US-1, Mizubuti and Fry (1998) found that isolates belonging to US-7 and US-8 lineages, germinated indirectly better at lower temperatures (approximately 10ºC). These reports are in accordance with the results of the present study, where isolates belonging to clonal lineages US-8, US-22, US-23 and US-24 released zoospores better at 5 and 10ºC than they did at higher temperatures. Similar to these previous studies, indirect germination at 20ºC was reduced to less than half

the maximum germination recorded. Direct germination was not commonly observed, but when it occurred it was mostly observed at higher temperatures (20 and 25ºC). These results are consistent with those reported by Melhus (1915) and Crosier (1934).

Indirect germination rate was also influenced by temperature. Lower temperatures (4ºC in comparison to 15ºC) resulted in increased indirect germination rate and increased total germination. Differences in rate of indirect germination between clonal lineages were observed, with the most striking difference observed for clonal lineage US-24, where indirect germination was dramatically faster than that of any of the other three clonal lineages studied. As suggested by Mizubuti and Fry (1998), rapid germination might enable the pathogen to overcome environmental limitations. The release of zoospores at lower temperatures could play an important role in disease development under conditions where low temperatures and short wet periods are frequent. Among the clonal lineages studied, US-24 might possess a competitive advantage under conditions of short, cool infectious periods. The differences in germination rate and response to temperature will be incorporated in the late blight decision support model.

If sexual reproduction continues to remain limited in the United States and Canada, populations of the pathogen in these regions will remain relatively simple and perhaps stable. An understanding of phenotypic traits associated with unique clonal lineages of *P. infestans*, has direct implications for potato and tomato late blight management. With the availability of rapid genotype analyses and rapid communication, in-season disease management may be adjusted based on the results of rapid genotypic analyses.

2.7 Acknowledgements

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2.9 Supplemental Material

Supplementary Table 2.1 *Phytophthora infestans* isolates used in this study.

Clonal lineage	Isolate	Location County/State abbreviation or Country	Original host	Year	Collector
US-8	US090010 [M]	Genesee/NY	Potato	2009	Don Sweet [1]
	US090015 [M]	Franklin/NY	Potato	2009	CPDDC[2]
	US090016 [M]	Steuben/NY	Potato	2009	CPDDC[2]
	US090024 [M]	Wyoming/NY	Potato	2009	James McCormick[3]
	US090025 [M]	Wyoming/NY	Potato	2009	James McCormick[3]
	US090027 [M]	Wyoming/NY	Potato	2009	James McCormick[3]
	US090028 [M]	Wyoming/NY	Potato	2009	James McCormick[3]
	US090034 [M]	Wayne/NY	Potato	2009	Amara Camp[4]
	US090046 [M]	Tompkins/NY	Potato	2009	William E. Fry[4]
	US090047 [M]	Tompkins/NY	Potato	2009	William E. Fry[4]
	US100021 [MP]	Erie/PA	Potato	2010	Andrew Muza[5]
	US100024 [MPG]	Shelburne/ON Canada	Potato	2010	Eugenia Banks[6]
	US100025 [MP]	Shelburne/ON Canada	Potato	2010	Eugenia Banks[6]
	US100026 [M]	Shelburne/ON Canada	Potato	2010	Eugenia Banks[6]
	US100027 [MPG]	Shelburne/ON Canada	Potato	2010	Eugenia Banks[6]
	US100028 [MP]	Shelburne/ON Canada	Potato	2010	Eugenia Banks[6]
	US100031 [MPG]	Shelburne/ON Canada	Potato	2010	Eugenia Banks[6]
	US100036 [M]	Yates/NY	Potato	2010	Carol MacNeil[4]
	US100037 [MP]	Yates/NY	Potato	2010	Carol MacNeil[4]
	US100048 [MP]	Wayne/NY	Potato	2010	Carol MacNeil[4]
	US100068 [M]	Bonners Ferry/ID	Potato	2010	Niklaus Grunwald[7]
	US110063 [M]	Erie/PA	Potato	2011	Andrew Muza[5]
	US110097 [M]	Bonners Ferry/ID	Potato	2011	Niklaus Grunwald[7]
	US110098 [M]	Bonners Ferry/ID	Potato	2011	Niklaus Grunwald[7]
US-22	US080004 [P]	Suffolk/NY	Tomato	2008	Margaret McGrath[4]
	US090002 [M]	Tompkins/NY	Tomato	2009	Keith Perry[4]
	US090003 [M]	Chenango/NY	Tomato	2009	Steve Markarian[8]
	US090004 [M]	Suffolk/NY	Tomato	2009	Margaret McGrath[4]
	US090005 [M]	Suffolk/NY	Tomato	2009	Margaret McGrath[4]
	US090007 [M]	Sullivan/NY	Tomato	2009	Teresa Rusinek[9]
	US090009 [M]	Tompkins/NY	Tomato	2009	CPDDC[2]
	US090011 [M]	Onondaga/NY	Tomato	2009	CPDDC[2]
	US090012 [P]	Westchester/NY	Tomato	2009	CPDDC[2]
	US090017 [MP]	Essex/NY	Tomato	2009	CPDDC[2]
	US090018 [M]	Clinton/NY	Tomato	2009	Fran Behan[10]
	US090021 [M]	Tioga/NY	Potato	2009	CPDDC[2]
	US090022 [M]	Otsego/NY	Tomato	2009	CPDDC[2]
	US090030 [M]	Onondaga/NY	Tomato	2009	CPDDC[2]
	US090031 [M]	Orange/NY	Tomato	2009	CPDDC[2]
	US090038 [PG]	Tompkins/NY	Potato	2009	CPDDC[2]

US090039 M	Oneida/NY	Potato	2009	CPDDC[2]
US090040 MP	Chautuaqua/NY	Tomato	2009	CPDDC[2]
US090041 M	NJ	Tomato	2009	CPDDC[2]
US090042 M	Chautuaqua/NY	Tomato	2009	CPDDC[2]
US090043 M*	Oswego/NY	Tomato	2009	CPDDC[2]
US090044 P	Tompkins/NY	Tomato	2009	CPDDC[2]
US090048 P	Lincoln/ME	Potato	2009	Rob Johanson[11]
US090049 M	Lincoln/ME	Potato	2009	Rob Johanson[11]
US090050 M	Montgomery/VA	Tomato	2009	Dave Schmale[12]
US090051 M	Montgomery/VA	Tomato	2009	Dave Schmale[12]
US090053 M	Montgomery/VA	Potato	2009	Dave Schmale[12]
US090054 M	FL	Tomato	2009	Ryan Donahoo[13]
US090055 M	FL	Tomato	2009	Ryan Donahoo[13]
US090056 M	TN	Tomato	2009	Steve Bost[14]
US100009 MP	Suffolk/NY	Potato	2010	Margaret McGrath[4]
US100012 MP	Suffolk/NY	Potato	2010	Margaret McGrath[4]
US100013 M	Chenango/NY	Tomato	2010	CPDDC[2]
US100014 MP	Livingston/NY	Tomato	2010	Christine Smart[4]
US100015 MP	Marquette/WI	Potato	2010	Amanda Gevens[15]
US100017 M	Broome/NY	Tomato	2010	Linda Dabulewicz[16]
US100018 MP	Hampshire/MA	Tomato	2010	Rob Wick[17]
US100020 MP	Clarion/PA	Tomato	2010	PSPDC[18]
US100035 MP	Tioga/NY	Tomato	2010	Tom Zitter[4]
US100038 MP	Chenango/NY	Potato	2010	CPDDC[2]
US100040 MP	ME	Tomato	2010	CPDDC[2]
US100041 MP	Tompkins/NY	Tomato	2010	Cliff Kraft[4]
US100042 MP	PA	Tomato	2010	PSPDC[18]
US100043 MP*	Suffolk/NY	Tomato	2010	Margaret McGrath[4]
US100044 MP	Madison/NY	Tomato	2010	CPDDC[2]
US100045 MP	NH	Tomato	2010	Cheryl Smith[19]
US100046 MP	PA	Tomato	2010	PSPDC[18]
US100047 MP	PA	Tomato	2010	PSPDC[18]
US100049 MP	Hillsboro/NH	Tomato	2010	Cheryl Smith[19]
US100050 MP	Hartford/CT	Tomato	2010	Joan Allen[20]
US100051 MP	Hartford/CT	Tomato	2010	Joan Allen[20]
US100052 MP	Hartford County CT	Tomato	2010	Joan Allen[20]
US100053 MPG	Rockingham/NH	Tomato	2010	Cheryl Smith[19]
US100056 MP	St Lawrence/NY	Tomato	2010	CPDDC[2]
US110023 M*	Knox/ME	Tomato	2011	Bruce Watt[21]
US110024 M	Lincoln/ME	Tomato	2011	Bruce Watt[21]
US110025 M	Waldo/ME	Tomato	2011	Bruce Watt[21]
US110026 M	Knox/ME	Tomato	2011	Paul Meinersmann[22]
US110048 M	Knox/ME	Tomato	2011	Bruce Watt[21]
US110049 M	Waldo/ME	Tomato	2011	Bruce Watt[21]
US110050 M	Knox/ME	Tomato	2011	Bruce Watt[21]
US110051 M*	Kennebec/ME	Tomato	2011	Bruce Watt[21]
US110052 M*	Knox/ME	Tomato	2011	Bruce Watt[21]
US110053 M	Columbia/NY	Tomato	2011	Chuck Bornt[4]

	US110055 [M]	Sommerset/ME	Tomato	2011	Bruce Watt[21]
	US110056 [M]	Penobscot/ME	Tomato	2011	Bruce Watt[21]
	US110070 [M]	Chenango County NY	Tomato	2011	Rebecca Hargrave[4]
	US110088 [M]	St. Lawrence County NY	Tomato	2011	Paul Hetzler[4]
	US110096 [M]	Penobscot/ME	Tomato	2011	Bruce Watt[21]
	US110111 [M]	Montgomery/NY	Tomato	2011	Jesse Walter[23]
	US110140 [M]	Madison/NY	Tomato	2011	Gina Bisco[24]
US-22[x]	US100001 [MP]	Lafayette Parish/LA	Tomato	2010	Don Ferrin[25]
	US100003 [MP]	Boone/KY	Tomato	2010	Kenneth W. Seebold Jr.[26]
	US100004 [M]	Clark/KY	Tomato	2010	Kenneth W. Seebold Jr.[26]
	US100005 [M]	Fayette/KY	Tomato	2010	Kenneth W. Seebold Jr.[26]
	US100006 [MP]	Boone/KY	Tomato	2010	Kenneth W. Seebold Jr.[26]
	US100007 [MP]	Boone/KY	Tomato	2010	Kenneth W. Seebold Jr.[26]
	US100010 [MP]	Suffolk/NY	Tomato	2010	Margaret McGrath[4]
	US100011 [MPG]	Suffolk/NY	Tomato	2010	Margaret McGrath[4]
US-22[y]	US110002 [M]	Waldo/ME	Tomato	2011	Bruce Watt[21]
US-23	BL2009 P4 [PG]	-	-	2009	Ken Deahl[27]
	US100002 [M*]	St. Mary's/MD	Tomato	2010	Benjamin Beale[28]
	US100008 [MP*]	New Haven/CT	Tomato	2010	Sharon Douglas[29]
	US100016 [MPG]	Waukesha/WI	Tomato	2010	Amanda Gevens[15]
	US100054 [MP]	Grafton/NH	Tomato	2010	Cheryl Smith[19]
	US100055 [MPG]	Grafton/NH	Tomato	2010	Cheryl Smith[19]
	US100064 [MP]	St. Mary's/MD	Tomato	2010	Benjamin Beale[28]
	US110001 [MP]	Hartford/CT	Tomato	2011	Sharon Douglas[29]
	US110005 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110006 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110007 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110008 [M]	Suffolk/NY	Potato	2011	Margaret McGrath[4]
	US110009 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110010 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110011 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110012 [M]	Suffolk/NY	Potato	2011	Margaret McGrath[4]
	US110013 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110014 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110015 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110016 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110018 [M*]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
	US110019 [M]	Cambria/PA	Potato	2011	Beth Gugino[30]
	US110020 [M]	Greene/VA	Potato	2011	Mary Ann Hansen[31]
	US110027 [M]	Northumberland/PA	Tomato	2011	Beth Gugino[30]
	US110029 [M]	Washington/RI	Tomato	2011	Heather Faubert[32]
	US110041 [M]	Waukesha/WI	Tomato	2011	Amanda Gevens[15]
	US110042 [M]	Aroostook/ME	Potato	2011	Steve Johnson[21]
	US110043 [M*]	Aroostook/ME	Tomato	2011	Steve Johnson[21]
	US110044 [M]	Aroostook/ME	Potato	2011	Steve Johnson[21]
	US110045 [M*]	Aroostook/ME	Tomato	2011	Steve Johnson[21]

US110047 [M]	Grafton/NH	Tomato	2011	Cheryl Smith[19]
US110059 [M]	Clinton/NY	Tomato	2011	Jolene Wallace[4]
US110062 [M*]	Aroostook/ME	Potato	2011	Steve Johnson[21]
US110067 [M]	New Haven/CT	Tomato	2011	Sharon Douglas[29]
US110068 [M]	Hartford/CT	Tomato	2011	UCPDC[33]
US110076 [M]	Snyder/PA	Tomato	2011	Beth Gugino[30]
US110077 [M]	Montour/PA	Tomato	2011	Beth Gugino[30]
US110080 [M]	Cambria/PA	Potato	2011	Beth Gugino[30]
US110081 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
US110087 [M]	Berks/PA	Tomato	2011	Robert E. Leiby[34]
US110089 [M*]	Aroostook/ME	Potato	2011	Steve Johnson[21]
US110090 [M]	Victoria/NB, Canada	Potato	2011	Steve Johnson[21]
US110091 [M*]	Aroostook/ME	Potato	2011	Steve Johnson[21]
US110095 [M*]	New Haven/CT	Tomato	2011	Sharon Douglas[29]
US110107 [M]	Centre/PA	Tomato	2011	Sharon Douglas[29]
US110108 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
US110109 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
US110110 [M]	Suffolk/NY	Tomato	2011	Margaret McGrath[4]
US110136 [M]	Lehigh/PA	Tomato	2011	Robert E. Leiby[34]
US110137 [M]	Bedford/PA	Tomato	2011	Beth Gugino[30]
US-24 ND822i [P]	-	-	-	Ken Deahl[27]
US100057 [MPG]	MT	Potato	2010	Susie Siemsen[35]
US100058 [MP]	MT	Potato	2010	Susie Siemsen[35]
US100059 [MPG]	MT	Potato	2010	Susie Siemsen[35]
US100060 [MP]	MT	Potato	2010	Susie Siemsen[35]
US100061 [MP]	MT	Potato	2010	Susie Siemsen[35]
US100062 [M*]	MT	Potato	2010	Susie Siemsen[35]
US100063 [MPG]	MT	Potato	2010	Susie Siemsen[35]
US100066 [MP]	MT	Tomato	2010	Susie Siemsen[35]
US100067 [MP]	Othello/WA	Potato	2010	Dennis Johnson[36]
US110003 [M]	ND	Potato	2011	Gary Secor[37]
US110004 [M*]	ND	Potato	2011	Gary Secor[37]
US110021 [M*]	Adams/MN	Potato	2011	Gary Secor[37]
US110022 [M]	Caribou/ME	Potato	2011	Steve Johnson[21]
US110030 [M]	ND	Potato	2011	Gary Secor[37]
US110031 [M]	ND	Potato	2011	Gary Secor[37]
US110032 [M]	ND	Potato	2011	Gary Secor[37]
US110033 [M]	ND	Potato	2011	Gary Secor[37]
US110034 [M]	ND	Potato	2011	Gary Secor[37]
US110035 [M]	ND	Potato	2011	Gary Secor[37]
US110036 [M]	ND	Potato	2011	Gary Secor[37]
US110037 [M]	ND	Potato	2011	Gary Secor[37]
US110038 [M]	ND	Potato	2011	Gary Secor[37]
US110046 [M]	Aroostook/ME	Potato	2011	Steve Johnson[21]
US110099 [M]	WA	Potato	2011	Niklaus Grunwald[7]
US110100 [M]	George/WA	Potato	2011	Niklaus Grunwald[7]
US110101 [M]	WA	Potato	2011	Niklaus Grunwald[7]

	US110104 [M]	Adams/WA	Potato	2011	Niklaus Grunwald[7]
	US110105 [M]	Adams/WA	Potato	2011	Niklaus Grunwald[7]
	US110113 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110115 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110116 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110117 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110118 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110119 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110120 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110121 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110122 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110126 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110127 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110129 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110130 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110131 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110132 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110133 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110134 [M]	Tompkins/NY	Potato	2011	William Fry[4]
	US110147 [M]	Linn/OR	Tomato	2011	Niklaus Grunwald[7]
	US110149 [M]	ND	Potato	2011	Gary Secor[37]
	US110150 [M]	Clay/MN	Potato	2011	Gary Secor[37]
	US110151 [M]	Clay/MN	Potato	2011	Gary Secor[37]
	US110152 [M]	Yuma/CO	Potato	2011	Gary Secor[37]
	US110153 [M]	Walsh/ND	Potato	2011	Gary Secor[37]
	US110154 [M]	Walsh/ND	Potato	2011	Gary Secor[37]
	US110155 [M]	Walsh/ND	Potato	2011	Gary Secor[37]
	US110156 [M*]	Walsh/ND	Potato	2011	Gary Secor[37]
	US110157 [M]	Grand Forks/ND	Potato	2011	Gary Secor[37]
	US110158 [M]	Grand Forks/ND	Potato	2011	Gary Secor[37]
US-24[z]	US110159 [M]	Cass/ND	Potato	2011	Gary Secor[37]

[M] Isolates used for the sensitivity to mefenoxam assay
[P] Isolates used for the potato-tomato pathogenicity assay
[G] Isolates used for the germination assays
[x] US-22 Variant at marker Pi89 (Var1)
[y] US-22 Variant at marker D13 (Var2)
[z] US-24 Variant at marker Pi02
* Microsatellite data missing.
[1] Scottsville, NY
[2] CPDDC: Cornell Plant Disease Diagnostic Clinic
[3] Bliss, NY
[4] Cornell University
[5] Penn State
[6] Guelph, ON, Canada
[7] USDA-ARS Corvallis, OR
[8] New York State Agriculture and Markets
[9] Kingston, NY
[10] Plattsburgh, NY
[11] Dresden, ME

[12] Blacksburg, VA
[13] University of Florida
[14] University of Tennessee
[15] University of Wisconsin-Madison
[16] Harpursville, NY
[17] University of Massachusetts Amherst
[18] PSPDC: Penn State Plant Disease Clinic
[19] University of New Hampshire
[20] University of Connecticut
[21] University of Maine
[22] Warren, ME
[23] St. Johnsville, NY
[24] Chittenango, NY
[25] Baton Rouge, LA
[26] University of Kentucky
[27] USDA-ARS Beltsville, MD
[28] University of Maryland
[29] New Haven, CT
[30] Penn State University
[31] Virginia Tech University
[32] University of Rhode Island
[33] UCPDC: University of Connecticut Plant Disease Clinic
[34] Harrisburg, PA
[35]Montana State University
[36] Washington State University
[37] North Dakota State University

CHAPTER 3*

An ephemeral sexual population of *Phytophthora infestans* in the northeastern United States and Canada

3.1 Abstract

Phytophthora infestans, the causal agent of late blight disease, has been reported in North America since the mid-nineteenth century. In the United States the lack of or very limited sexual reproduction has resulted in largely clonal populations of *P. infestans*. In 2010 and 2011, but not in 2012 or 2013, 20 rare and diverse genotypes of *P. infestans* were detected in a region that centered around central New York State. The ratio of A1 to A2 mating types among these genotypes was close to the 50:50 ratio expected for sexual recombination. These genotypes were diverse at the *glucose-6-phosphate isomerase* locus, differed in their microsatellite profiles, showed different banding patterns in a restriction fragment length polymorphism assay using a moderately repetitive and highly polymorphic probe (RG57), were polymorphic for four different nuclear genes and differed in their sensitivity to the systemic fungicide mefenoxam. The null hypothesis of linkage equilibrium was not rejected, which suggests the population could be sexual. These new genotypes were monomorphic in their mitochondrial haplotype that was the same as US-22. Through parentage exclusion testing using microsatellite data and sequences of four nuclear genes, recent dominant lineages US-8, US-11, US-23, and US-24 were excluded as possible parents for these

* Danies G, Myers K, Mideros MF, Restrepo S, Martin FN, Cooke DEL, Smart CD, Ristaino JB, Seaman AJ, Gugino BK, Grünwald NJ, Fry WE. 2014. An ephemeral sexual population of *Phytophthora infestans* in the Northeastern United States and Canada. PLoS ONE 9: doi: 10.1371/journal.pone.0116354.

genotypes. Further analyses indicated that US-22 could not be eliminated as a possible parent for 14 of the 20 genotypes. We conclude that US-22 could be a parent of some, but not all, of the new genotypes found in 2010 and 2011. There were at least two other parents for this population and the genotypic characteristics of the other parents were identified.

3.2 Key words

Phytophthora infestans, population genetics, sexual reproduction, clonal lineages, potato late blight

3.3 Introduction

Phytophthora infestans, a plant pathogenic oomycete, is a major constraint to potato and tomato production globally (Fry W. E. 2008). This pathogen reproduces both sexually and asexually, the former only when both mating types, A1 and A2, occur in the same location. Sexual reproduction is apparently uncommon worldwide with the exception of some regions such as central Mexico and northern Europe (Fry W. E. 2008, Grünwald and Flier 2005, Grünwald et al. 2001). Sexual reproduction results in the production of spores (oospores) (Flier et al. 2001) resistant to environmental extremes, which serve as a source of primary inoculum when present in the soil (Fernandez-Pavia et al. 2004). Oospores are able to survive in a dormant state for several years until conditions are optimal for the spores to germinate and cause infection (Drenth et al. 1995). In the United States there is no evidence for continual sexual reproduction even though both mating types exist. This apparent lack of widespread sexual reproduction has resulted in clonal populations of *P. infestans*. A clonal lineage is a descendant from a single individual and variation within a lineage arises by

mutation or mitotic recombination (Goodwin S. B. 1997, Grünwald et al. 2012). The diverse clonal lineages of *P. infestans* present in the United States have likely been introduced via migrations (Goodwin S. B. et al. 1995b, Goodwin S. B. et al. 1994b).

Historically, clonal lineages of *P. infestans* have been defined based on mating type, mitochondrial haplotype, nuclear DNA fingerprint patterns, and allozyme genotype (Goodwin S. B. et al. 1994b). More recently, microsatellite markers have been utilized to characterize USA populations (Myers et al. 2010, Small et al. 2012). Individuals within a lineage have the same multi-locus genotype (MLG) with only minor variation. Clonal lineages in the United States have typically been quite distinct, commonly defined using a subset of the markers identified above. Individual isolates within a clone also share phenotypic traits such as fungicide sensitivity, host preference, and aggressiveness (Cooke et al. 2012, Danies et al. 2013, Goodwin S. B. et al. 1995b, 1996, Lambert and Currier 1997, Legard et al. 1995, Miller J. S. et al. 1998). However, mutations at pathogenicity loci occur rapidly (Goodwin S. B. et al. 1995b).

Twenty-four diverse genotypes or clonal lineages of *P. infestans* have been detected and have been dominant in the past 40 years in the United States (Fry W. E. et al. 2013). Several lineages of *P. infestans* may coexist in the United States in any particular year but epidemic populations have typically been composed of one or rarely a few lineages. In the summers of 2010 and 2011 (but not in 2012 or 2013), greater diversity was detected in west-central New York State than had been observed in the entire United States in the previous ten years.

The primary objective of this study was to determine whether the diverse population of *P. infestans* detected around west-central New York State in 2010 and 2011 was a result of

sexual reproduction. Genetic markers with different mutation rates (nuclear and mitochondrial genes) were used to address the following two working hypotheses: (1) These rare and diverse genotypes of *P. infestans* are the outcome of one or more recombination events; and (2) if so, one of the currently dominant clonal lineages in the USA might be a parental genotype. The hypothesis that these genotypes might represent a sexual population was not rejected. Parental exclusion tests failed to eliminate lineage US-22 as a potential parent for most of the NYS-2010/11 population. Other parents must be involved, and the genotypic characteristics of these other parents are inferred.

3.4 Materials and Methods

3.4.1 Isolates

Isolates used in this study were those obtained from samples submitted by persons with extension responsibilities. Samples were infected tomato or potato leaflets showing typical late blight symptoms. The samples were submitted voluntarily by the owners of the affected plants. The domestic permit for submission of samples was APHIS permit P526P-13-03974 (or its predecessor) and the international permit for submission of samples was APHIS permit P526P-14-00763 (or its predecessor). In total there were 59 isolates including 20 USA reference isolates and 39 isolates detected in an area that centered around west-central New York State in 2010 and 2011. The population detected in and around New York State in 2010 and 2011 is referred to as the NYS-2010/11 population and the individuals are referred to as GDT-01 through GDT-20 (Supplementary Table 3.1). Cultures were maintained and DNA was extracted as previously described (Danies et al. 2013). Different sets of genetic markers, an allozyme test using the glucose-6-phosphate isomerase, a restriction fragment length

polymorphism assay using a moderately repetitive DNA probe RG57, mitochondrial haplotyping, 12 microsatellite loci, and four nuclear gene sequences as well as the mating type of each isolate were used to determine the isolate's genotype.

3.4.2 Initial diversity assays

3.4.2.1 Mating type

Mating type was determined by pairing an unknown isolate with a known isolate of *P. infestans,* either A1 mating type (US970001 US-17 genotype) or A2 mating type (US040009, US-8 genotype), on rye B (Caten and Jinks 1968) or pea (Jaime-Garcia et al. 2000) agar media. Petri plates were kept at 20°C for 10-14 days. The hyphal interface of the two colonies was investigated microscopically using 125X magnification. Isolates that formed oospores at the interface with the known A1 isolate were designated A2 and those that formed oospores with the known A2 isolate were designated A1. The known isolates (A1 and A2) were paired as positive controls, while negative controls consisted of pairing the known isolates with themselves (same mating type).

3.4.2.2 Glucose-6-phosphate isomerase

Mycelia and/or sporangia obtained from cultures grown on rye B, pea agar or from infected leaflets were used to determine *glucose-6-phosphate isomerase* (*GPI*) allozyme genotypes. Analyses were carried out using cellulose acetate electrophoresis as previously described (Goodwin S. B. et al. 1995a). At least one reference isolate representing US-1 (SA960008), US-8 (US040009), and/or US-17 (US970001) was included in all *GPI* analyses.

3.4.2.3 DNA extaction and RFLP analysis with probe RG57

DNA extractions and subsequent restriction fragment length polymorphism (RFLP) analysis with the RG57 DNA probe were performed using a method modified from Goodwin S. B. A. et al. (1992). Southern blot analysis was conducted using the Amersham gene images AlkPhos direct labeling and detection system (GE Healthcare) according to the manufacturer's instructions. The US-1 (SA960008) reference isolate was used in RG57 analyses. Presence or absence of known fingerprint fragments was scored visually.

3.4.2.4 Mefenoxam sensitivity assay

Mefenoxam sensitivity of isolates was assessed as described previously by Therrien et al. (1993), except that mefenoxam was used in place of metalaxyl. Isolates were grown on pea agar amended with Ridomil Gold SL (Syngenta, Greensboro, NC) such that the final concentrations of the active ingredient (mefenoxam) were 0, 5, or 100 μg ml^{-1}. Mycelial plugs (8 mm diameter) were obtained from actively growing cultures, transferred to the test plates and incubated for approximately 10 to 12 days, or until growth on the control mefenoxam plate (0 μg ml^{-1}) was approximately 75 to 90% of the diameter of the petri plate. Assessment of mefenoxam sensitivity was determined on the basis of radial growth of cultures grown on plates amended with mefenoxam (5 or 100 μg ml^{-1}) compared to non-amended controls. Growth on mefenoxam-amended plates, 5 and 100 μg ml^{-1}, was represented as a proportion of the growth on the non-amended control plates.

3.4.2.5 Mitochondrial haplotyping

Mitochondrial haplotype was determined following the protocol reported by Martin et al. (2012). This protocol was designed to determine the smallest number of loci needed to

classify mitochondrial haplotypes for *P. infestans*. In total five loci were sequenced. The first locus included the *rpl5-rns* region that includes the downstream part of P3 and most of P5. The second locus included regions *rns-cox2*, as well as *orf79*. The third locus included regions *cox1-nad9,* including *atp-9* and the downstream half of the P4 region. The fourth locus included *nad3-nad5*. The fifth locus included regions *nad6-nad4L* as well as the upstream half of the P6 region. This procedure has allowed the identification of at least 36 different mitochondrial haplotypes in *P. infestans*. The first step of this mitochondrial haplotyping protocol was to sequence the second and fifth loci for all isolates. This would allow the discrimination of at least 27 haplotypes. Based on the results obtained from sequencing loci two and five, it was only necessary to sequence locus three in all isolates to distinguish between haplotypes.

3.4.3 Multiplex microsatellite marker analysis

Twelve microsatellite loci previously demonstrated to reveal polymorphisms (Li et al. 2013) were genotyped for all isolates. Genotyping was conducted using the QIAGEN Type-it Microsatellite PCR Kit (QIAGEN, Cat. No. 206243). Amplifications were done as described by the manufacturer. PCR conditions were: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 90 s, and 72 °C for 20 s, and a final extension at 60 °C for 30 min. PCR products were analyzed on an ABI 3730xl capillary system with POP-7 Polymer (ABI, PN 4335615). PCR amplicons were compared with a set of size standards and alleles were scored accordingly (Li et al. 2013). At least one reference isolate representing US-8 (US100028), US-11 (US110028), US-22 (US090042), US-23 (BL2009P4) and/or US-24 (ND822Pi) was included in all microsatellite analyses.

3.4.4 Analyses using microsatellite data

The R package *Poppr 1.0.5* (Kamvar et al. 2014), which allows analysis of populations with mixed modes of reproduction (sexual and asexual) was used to analyze the microsatellite data. The 59 isolates were arbitrarily clustered into three groups according to their occurrence over time in the United States. Group one contained eight isolates including lineages of *P. infestans* that had been prevalent in the United States at one time, but not for the past 10 years (US-1, US-6, US-7, US-12, US-14, US-16, US-17, and US-19). Group two contained 12 isolates including lineages of *P. infestans* that were dominant over the past five years (US-8, US-11, US-22, US-23 and US-24) or that were first described during the past five years in the United States (US-20 and US-21). Group three contained the 39 NYS-2010/11 *P. infestans* isolates that are the focus of this study (GDT-01 to GDT-20).

To detect signs of linkage disequilibrium across the microsatellite loci, a standardized index of association that corrected for the number of loci ($\check{r}d$) (Agapow and Burt 2001) was calculated using clone corrected data.

Two different types of analyses were conducted to determine if the GDT isolates cluster as a single population and to observe if any of the previously known clonal lineages cluster with them. Population structure was inferred using the program *structure* 2.3 (Pritchard et al. 2000) by testing the number of population clusters (K) between 1 and 10 using the admixture model (Falush et al. 2003). A total of 10 independent runs of 100,000 iterations with burn-in period of 50,000 MCMC iterations were conducted. The results from *structure* were post-processed using *Structure Harvester* (Earl and Vonholdt 2012). The ΔK method according to Evanno et al. (2005) was used to evaluate the rate of change in the log probability of data between successive K values to infer the number of populations.

To visually assess between-population differentiation, and assess the contribution of individual alleles to population structuring, a discriminant analysis of principal components (DAPC) was done using the *adegenet* package for R (Jombart 2008). This multivariate method extracts information from genetic data and identifies genetic clusters using the *k*-means clustering algorithm based on the Bayesian Information Criterion (Jombart1 et al. 2011). This clustering algorithm finds a given number of groups that maximizes the variation between groups, and describes the relationships between these clusters.

3.4.5 Nuclear gene sequencing

Genes known to be polymorphic within *P. infestans* (Blair J. E. et al. 2012, Blair J. E. et al. 2008, Karlovsky and Prell 1991, Tyler et al. 2006) were chosen for sequencing (Table 3.1). PCR conditions for nuclear loci were the following, in a final volume of 20 µl: 1X PCR buffer with a final $MgSO_4$ concentration of 2 mM, 200 µM dNTPs, 0.5 µM of each primer, one unit of Platinium® *Taq* High Fidelity (Invitrogen), and ~10 ng template DNA. Thermal cycling protocols used an initial denaturation step at 94 ºC for two minutes; 35 cycles of 94 ºC for 30 sec, locus-specific annealing temperature for 30 seconds (Table 3.1), 68 ºC extension for 45 seconds; and a final extension at 68 ºC for 5 minutes.

PCR products were visualized on a 1% agarose gel to confirm amplicon size. An enzymatic purification protocol was used following the manufacturer's instructions (ExoSAP-IT, Affymetrix), and products were sequenced using BigDye terminator chemistry, and analyzed on an ABI 3730 instrument (Applied Biosystems) at the Cornell University Sequencing Core Facility. ABI trace files were analyzed using Geneious Pro v4.8.5 (). Sequence alignments were generated using MUSCLE (Edgar 2004) with default settings.

Bases with overlapping peaks in the electropherograms were considered heterozygous and coded according to IUPAC convention. Sequences were deposited to GenBank under the accession numbers [KM249146-KM249175].

Table 3.1 Nuclear loci sequenced, primers, and amplified conditions used in this study.

Locus	Primer name	Primer sequence (5' – 3)	T_a[a]	Reference
PITG_11126 (Conserved Hypothetical Protein)	PITG11126_F1	GGGGACTTCGCTGTTTGTTA	59.0 ºC	(Tyler et al. 2006)
	PITG11126_R1	ATGTTCATGTACGGCTGACG		
PUA (Conserved Hypothetical Protein)	PUA_F	AGGTCAAGTCCTCGCAGCAG	67.0 ºC	(Blair J. E. et al. 2012)
	PUA_R	AGGTCGTCRCCMAAGTG		
β –tubulin	Btub_F1	GCCAAGTTCTGGGAGGTCATC	58.4 ºC	(Blair J. E. et al. 2008)
	Btub_R1	CCTGGTACTGCTGGTACTCAG		
TRP1 (N-(5'-phosphoribosyl)anthranilate isomerase indole-3-glycerol-phosphate synthase)	TRP1_F3	GGGTAACATCCTGGAGGAGA	63.0 ºC	(Karlovsky and Prell 1991)
	TRP1_R3	TCGTACTTGACCACGTCTGC		

[a] Annealing temperature of primers for PCR.

3.4.6 Analyses using nuclear gene sequences

Haplotype phase of nuclear gene sequences with two or more heterozygous sites were determined by cloning PCR products for a subset of genotypes and sequencing inserts. Cloning of PCR products was done using the TOPO® TA Cloning® Kit for Subcloning, with One Shot® TOP10 chemically competent *Escherichia coli* cells (Invitrogen). Isolates cloned were SA960008 (US-1) and US100032 (GDT-15) for *PITG_11126*; SA960008 (US-1), Coffey7629 (US-6), US100019 (GDT-19) and US110040 (US-23) for *PUA*; US100033 (GDT-16) for *β-tubulin*; and SA960008 (US-1), US940494 (US-12), US050007 (US-11), and US110040 (US-23) for *TRP1*. Polyploids were identified (US-1, US-11, and US-23) and all sequences were included in our downstream analyses. Nucleotide diversity indices and the

polymorphic level for the four nuclear genes studied were calculated using DNAsp v. 5.10.01 (Librado and Rozas 2009). To test for neutral evolution, Tajima's D (Tajima 1989) was calculated using DnaSP v. 5.10.01.

Jmodeltest (Posada 2008) was used to estimate a nucleotide substitution model. Maximum likelihood (ML) gene trees were inferred using PhyML (Guindon and Gascuel 2003), implemented in the South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml/), using the substitution model selected by jmodeltest (K80 for PITG_11126, TN93 for TRP1, JC69 for β-tubulin and PUA). The transition/transversion ratio, proportion of invariable sites, and gamma distribution parameter were estimated from the data in PhyML using 6 rate categories. Data sets were bootstrapped using 1000 samples.

Gene trees were also inferred using MrBayes [70], implemented in CIPRES Science Gateway (Miller M. A. et al. 2010). The same nucleotide substitution model was used as for PhyML. One million MCMC generations were run, using a sample frequency of 500 generations and a burn-in of 25% of the total run. Two runs using four chains each (one cold and three heated chains) were performed. The default priors were used.

3.4.7 Parentage exclusion analysis

A visual parentage exclusion analysis (Jones and Arden 2003) was possible given that out of the 59 isolates (including USA reference isolates as well as GDT isolates) there were 37 unique *P. infestans* genotypes after clone correcting. This analysis is based on the fact that a particular offspring has two alleles for each autosomal marker corresponding to one from each of its progenitors. Based on this analysis, a genotype was excluded as a potential parent of one of the NYS-2010/11 isolates, if neither of the alleles present at a particular locus in the

candidate parent was present in the candidate progeny (a flow diagram with a chain of logic is shown on Supplementary Figure 3.1). Both microsatellite alleles and nuclear gene sequences were used for this analysis.

3.5 Results

In 2010 and 2011 we confirmed that there were individuals of *P. infestans* of opposite mating type from west-central New York and surrounding areas (Figure 3.1). We detected only A1 individuals in some counties, and only A2 individuals in other counties. However, both A1 and A2 individuals were reported from yet other counties. Thus, it was clear that this outbreak differed from most recent outbreaks in the United States in that there was a large region in which both mating types were admixed.

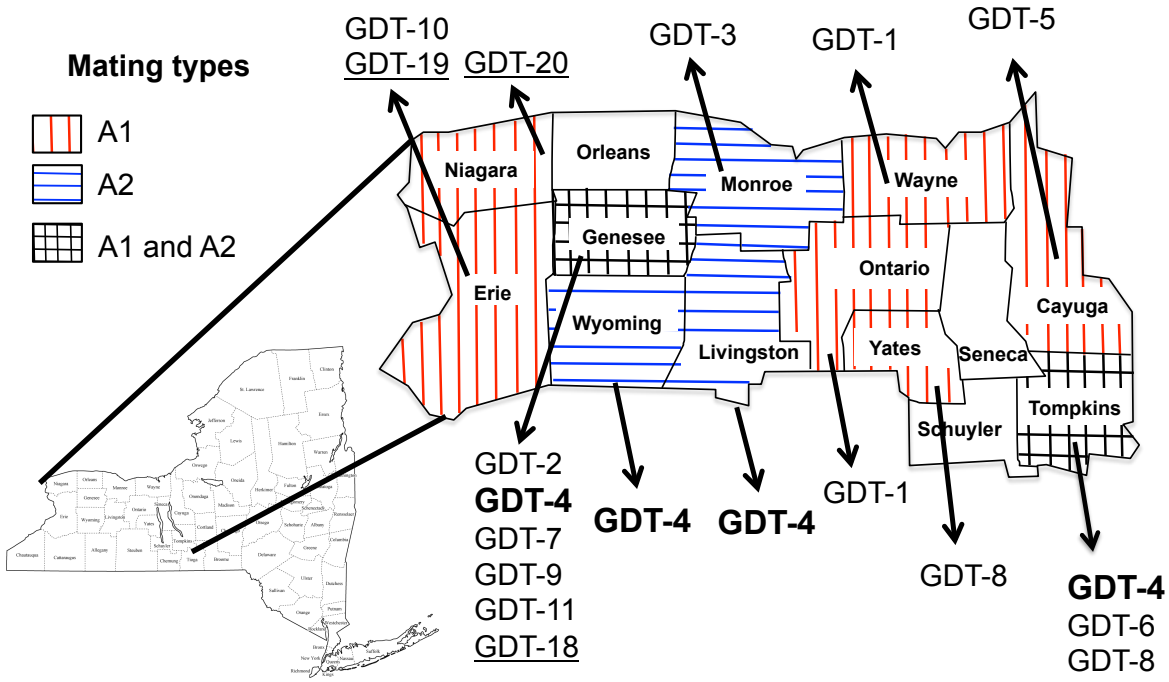

Figure 3.1 Spatial occurrence of the NYS-2010/11 population of *Phytophthora infestans* detected in western New York State. Genotypes that are underlined are those detected in 2010 all other genotypes were detected in 2011. Genotypes shown in bold are those that were found in several counties (GDT-01, GDT-04, and GDT-08). In New York State we detected only A1 individuals in six counties, and only A2 individuals in another three counties. However, both A1 and A2 individuals were reported from yet two other counties. Because of our limited sample size, we cannot conclude with certainty that both mating types were not present in counties where only a single mating type was detected.

Further analyses confirmed that these individuals constituted a diverse population. These genotypes displayed different banding patterns for the allozyme *glucose-6-phosphate isomerase* locus (Supplementary Table 3.2), showed different banding patterns at four loci in a restriction fragment length polymorphism assay using the moderately repetitive and highly polymorphic probe RG57 (Supplementary Table 3.2), differed in their microsatellite profiles (Supplementary Table 3.3), were polymorphic for four different nuclear genes (Supplementary Table 3.4 – Supplementary Table 3.7) and differed in their sensitivity to the systemic fungicide mefenoxam (Figure 3.2). However, they were monomorphic for their mitochondrial haplotype (Supplementary Table 3.2).

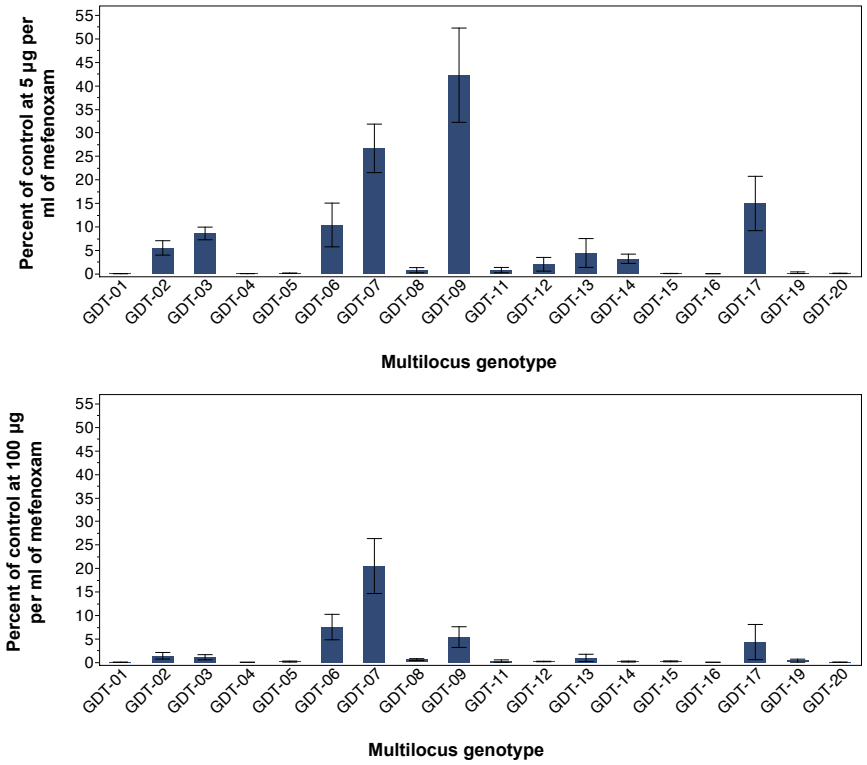

Figure 3.2 Response of *Phytophthora infestans* isolates to mefenoxam. Relative growth (as percentage of control) at 5 µg ml^{-1} (top) and 100 µg ml^{-1} (bottom) relative to control 0 µg ml^{-1} is presented. Sample sizes for each multilocus genotype can be found in Supplementary Table 3.1.

The 39 NYS-2010/11 isolates represented 20 distinct multi-locus genotypes (MLGs). Based on their genotypic profile these new and diverse genotypes were named GDT-01 to GDT-20. In addition to individuals from NY, there were individuals from Ohio, Pennsylvania and Ontario, Canada that also appeared to belong to this population (Supplementary Table 3.1). There were cases, where the same genotype was found in several New York state counties (Figure 3.1).

3.5.1 Population genetic analyses and recombination tests using microsatellite markers

In total, 50 microsatellite alleles were detected in the entire collection of genotypes. The number of alleles per locus ranged from two (for locus Pi04) to eight (for PiG11 and PinfSSR4) (Supplementary Table 3.8). Using the microsatellite loci, we tested the different groups against the null hypothesis of sexual reproduction by measuring the extent of linkage equilibrium across the microsatellite loci. When all isolates were analyzed together this null hypothesis was rejected (*P = 0.001*). This null hypothesis was rejected also for Group 1 (old isolates) alone (*P = 0.001*) and for Group 2 isolates (lineages that have been dominant in the United States over the past five years or those that have been detected for the first time during the past five years) alone (*P = 0.002*). However, for the NYS-2010/11 isolates (Group 3), we failed to reject the null hypothesis (*P=0.1490*). Thus this group seemed to have characteristics of a sexually reproducing population.

3.5.2 Genetic structure analysis

The population structure based on microsatellite data consisted of two distinct clusters (Supplementary Figure 3.2) and the ΔK analysis indicated that $K = 2$ was most likely the correct minimum number of clusters. Interestingly, US-22 clustered with the NYS-2010/11 population (Supplementary Figure 3.2). Supplementary Figure 3.2, shows the results for $K = 2$ to $K = 5$. At $K \geq 3$, population subdivision for the USA reference isolates was large relative to population subdivision for the GDT isolates and lineage US-22.

In general, the Discriminant Analysis of Principal Components (DAPC) is in agreement with the results obtained using *structure* (Figure 3.3). Based on the Bayesian Information Criterion, the number of clusters was eight (Supplementary Figure 3.3). Most of

the isolates within the NYS-2010/11 population (19 out of 20) clustered together with US-22. GDT-01 was grouped on a separate cluster along with lineage US-17, US-19, and US-21.

Figure 3. 3 Discriminant Analysis of Principal Components (DAPC) using 12 microsatellite loci. This scatterplot shows the first two principal components of the DAPC of *Phytophthora infestans* genotypes found in the United States. Groups are shown by different colors and inertia ellipses, while dots represent individual strains. Cluster 1 includes lineages US-6, US-7, US-11, US-12, and US-16; Cluster 2 includes isolates in lineage US-23; Cluster 3 includes lineages GDT-02, GDT-07, GDT-13, GDT-18, and GDT-20; Cluster 4 includes lineages GDT-03, GDT-04, GDT-08, GDT-08.1, GDT-14 and GDT-15; Cluster 5 includes lineages US-8, US-14, US-20, and US-24; Cluster 6 includes lineages GDT-05, GDT-06, GDT-09, GDT-10, GDT-11, GDT-12, GDT-16, GDT-17, and GDT-19; Cluster 7 includes lineages US-1; and Cluster 8 included lineages US-17, US-19, US-21 and GDT-01.

3.5.3 Population genetic analyses of nuclear loci

Nucleotide diversity was low for the four nuclear genes studied (Table 3.2). The nuclear gene *PUA* showed the highest number of segregating sites as well as the highest number of haplotypes (Table 3.2 and Supplementary Table 3.5). Tajima's D was not significant

indicating that all genes were evolving neutrally (0.51174, -0.75042, 1.01645, and -0.80032 with $p > 0.1$ for genes *PITG_11126*, *PUA*, *β-tubulin,* and *TRP1* respectively). Both negative and positive Tajima's D values were observed, yet these differences are minor and not distinguishable from noise. The four nuclear genes did not resolve the same topologies (Supplementary Figure 3.4). Clusters (defined according to the occurrence of *P. infestans* isolates over time in the United States) showed no clear grouping. Conflict among the gene trees is likely due to recombination among individuals. Even low levels of recombination leads to conflict among gene genealogies (Taylor et al. 2000).

Table 3.2 Summary statistics of loci *PITG_11126*, *PUA*, *β-tubulin* and *TRP1* from *Phytophthora infestans* in North America. The number of individuals per gene is different because for some genes more than one isolate per genotype was sequenced.

Locus	Number of individuals[a]	Length (bp)	Segregating sites	Genetic variability (Segregating sites/Length)	π[b]	θ Site[c]	Number of haplotypes	HD[d]
PITG_11126	40	776	6	0.00773	0.00190	0.00156	5	0.631
PUA	48	609	9	0.01478	0.00226	0.00320	9	0.520
β-tubulin	41	883	3	0.00340	0.00106	0.00068	4	0.517
TRP1	48	824	6	0.00728	0.00092	0.00142	8	0.435

[a] In some cases multiple isolates per multilocus genotype were sequenced.
[b] π = Nucleotide diversity (per site)
[c] θ site = Watterson's Theta (per site)
[d] HD = Haplotype diversity

3.5.4 Parentage analysis

If the NYS-2010/11 population represents a population that resulted from recent sexual reproduction, it would be interesting to determine the potential parents. We assumed that any of the lineages that have been dominant in the United States over the past five years or those that have been detected for the first time during the past five years (Group 2) could be

potential parents for the GDT isolates. Therefore we conducted a parentage exclusion test using microsatellite data. Based on this analysis, a potential parent of a candidate progeny was excluded if neither of the alleles present at a particular locus in that potential parent was present in the candidate progeny. For example, clonal lineage US-8 has alleles 108 and 112 for locus D13. Neither of these alleles was present in any of the NYS-2010/11 isolates. Thus, US-8 was excluded as a potential parent. Based on similar analyses it was possible to exclude lineages US-20, US-21, US-23, and US-24 as possible parents for the NYS-2010/11 isolates.

Lineages US-22 and US-11 remained as potential parents for at least some (but not all) of the NYS-2010/11 isolates. For example, US-22 was excluded as a parent for GDT-06, based on locus PinfSSR4; it was excluded as a potential parent for GDT-11 based on locus PinfSSR11 and it was excluded as a parent for GDT-12 based on two microsatellite loci PinfSSR4 and PinfSSR11 (Supplementary Table 3.9). Microsatellite data did not exclude US-22 as a parent for the other 17 GDT isolates. On the other hand, after parentage exclusion analysis using microsatellite data, clonal lineage US-11 remained as a potential parent only for seven of the twenty NYS-2010/11 isolates, GDT-03, GDT -04, GDT -06, GDT -09, GDT -10, GDT -14, and GDT -15 (Supplementary Table 3.10).

The diversity in the nuclear genes studied enabled further analysis of potential parentage of the NYS-2010/11 population. Using sequence analysis of the gene coding for the hypothetical protein PITG_11126, it was possible to exclude three additional GDT genotypes (GDT-04, GDT-05, and GDT-09) as progeny of US-22 (Supplementary Table 3.4). Analysis of the other three genes used in this study (*PUA*, *β-tubulin* and *TRP1*) provided data that were consistent with our hypothesis that US-22 is a possible parent for some of the GDT isolates (Supplementary Table 3.5 – Supplementary Table 3.7). Analysis of nuclear genes was also

applied to US-11 as a potential parent of the GDT isolates. Using sequence analysis of the genes coding for the hypothetical proteins *PITG_11126* and *PUA*, it was possible to exclude 14 GDT isolates as potential progeny of US-11. However, the six genotypes that remained as potential progeny of US-11 (GDT-02, GDT-08, GDT-12, GDT-13, GDT-18, and GDT-20) had been excluded as potential progeny of US-11 based on the microsatellite data.

If US-22 is one of the parents for at least some of the isolates within the NYS-2010/2011 population, it is possible to predict the banding pattern for the *glucose-6-phosphate isomerase*, the mating type and the microsatellite profile for the other putative parent(s). Assuming that US-22 is one of the parents, at least one of the other parent(s) must be A1 mating type. The other parent(s) must have an 111 allele for the *glucose-6-phosphate isomerase*, and must provide the following microsatellite alleles: an allele 110 for the D13 locus, an allele 189 for the Pi70 locus, an allele 225 for the Pi4B locus, an allele 258 for the Pi02 locus, alleles 284 and 288 for the PinfSSR4 locus and an allele 355 for the PinfSSR11 locus. These predictions are shown in Supplementary Table 3.11. In the same way, it is possible to predict the potential genotypes of loci *PITG_11126*, *PUA*, *β-tubulin* and *TRP1* of putative parent(s) for the GDT isolates when assuming that lineage US-22 is one of the parental genotypes for these isolates (Supplementary Table 3.12).

The mitochondrial haplotype data were interesting but not conclusive regarding parentage. Clonal lineage US-22 has the same mitochondrial haplotype (H20) as all of the GDT isolates.

3.6 Discussion

The genetic characteristics of the ephemeral population of *P. infestans* detected in the west-central region of New York State in 2010 and 2011 are consistent with a recombinant

population. Greater diversity was detected in that region during each of 2010 and 2011 than had been observed in the entire United States in the previous ten years. The sampling strategy or sampling intensity during 2010 and 2011 did not differ from other years. The number of samples received by our laboratory in 2010 and 2011 was 51 and 137, respectively. In 2012 and 2013 the number of samples received was 237 and 274, respectively. Thus, the diversity observed during 2010 and 2011 cannot be explained by an increase in the number of samples received and analyzed.

Our analyses of the NYS-2010/11 population are retrospective because they occurred after we became aware that these isolates were indeed unusual. It is challenging to obtain a truly random sample of *P. infestans*, given that late blight outbreaks occur sporadically and are typically clonal in the USA. Selection and/or drift may have played an important role prior to our analyses and may be an explanation for the disappearance of these genotypes after 2011. As a result we cannot regard these isolates as a comprehensive sample of a segregating population. However, their occurrence in a relatively small geographical area is unusual given the population structure of *P. infestans* in the USA. Only one other population with similar characteristics has been reported in the USA – in the Pacific Northwest in the late 1990s (Gavino et al. 2000). This population also had characteristics of a recombinant population (Gavino et al. 2000).

The NYS-2010/11 population displayed diverse banding patterns for the allozyme *glucose-6-phosphate isomerase*, showed different banding patterns in a restriction fragment length polymorphism assay using the moderately repetitive and highly polymorphic probe RG57, differed in their microsatellite profiles, were polymorphic for four different nuclear genes and differed in their sensitivity to the systemic fungicide mefenoxam. The ratio of

mating types among the NYS-2010/11 genotypes was close to the 50:50 ratio expected for sexual recombination.

The population structure of *P. infestans* in the USA is dramatically different from that in Sweden (Yuen and Andersson 2012) and central Mexico (Grünwald and Flier 2005) where sexual reproduction is a very common and significant factor in the ecology of *P. infestans*. The sexual population in Sweden is very recent (occurring in the latter part of the 20th century) and contributes to earlier and more devastating epidemics than before the occurrence of that population (Yuen and Andersson 2012). The sexual population in central Mexico is very old, because this location is the likely center of origin of *P. infestans* (Goss et al. 2014). It seems very likely that sexual recombination is still very rare and not a persistent contributor to the ecology of *P. infestan*s in the USA. However, the documentation now of two likely recombinant populations ((Gavino et al. 2000); and this study) indicate that sexual recombination in the USA is certainly possible and there is no reason to believe it will not happen again.

The genetic structure analyses done using *structure* and a discriminant analysis of principal components showed that the GDT isolates were grouped closely together and US-22 clustered within the GDT isolates. The index of association tested using microsatellite data failed to reject the hypothesis that the NYS-2010/11 individuals were a recombinant population. There are two possible scenarios that could explain this occurrence: 1) the recombination event(s) happened in or near west-central New York State or 2) the recombination event(s) took place somewhere outside New York State and the progeny from this event migrated as a cohort to central New York State, presumably on potato tubers or tomato transplants. We have no definite evidence of the location of the recombination

event(s), or if the oospore population still exists there. The fact that these strains have not been detected since 2011 is excellent news. They may have all died out. However, the fact that this population existed indicates that sexual recombination in the United States is a current possibility, and may happen again.

Our data suggest that clonal lineage US-22 could be a parent of some, but not all, of the new genotypes detected in 2010 and 2011. This is consistent with the fact that US-22 was the dominant lineage throughout the eastern United States in 2009 (Fry W. E. et al. 2013). In 2009 US-22 represented approximately 90% of the samples received and analyzed by our lab (Fry W. E. et al. 2013). At least two more parents are expected based on the nuclear gene sequences in the NYS-2010/11 population. This is because some isolates within the NYS-2010/11 population were homozygous for a site where US-22 was homozygous for a different nucleotide.

An additional hypothesis is that US-22 is a sibling of the GDT isolates, rather than a parent. Although we cannot definitively reject this hypothesis, at least two lines of reasoning support parentage rather than sib status for US-22 relative to the NYS-2010/11 population. First, US-22 was very widely distributed across the entire eastern part of the USA in 2009, so chronologically, US-22 was detected before the GDT isolates (Fry W. E. et al. 2013, Hu et al. 2012). Second, 18 of the 20 GDT isolates most similar to US-22 are heterozygous (341/355) at the microsatellite locus PinfSSR11 where US-22 is homozygous (341/341) (Supplementary Table 3.3). (The other two GDT isolates are 355/355 at this locus.) Thus, the more parsimonious explanation is that US-22 is a parent rather than an unusual sibling, and that the other parent is homozygous (355/355) at this locus.

It is interesting to note that for the microsatellite locus Pi70 we observed a high

frequency of the allele 189 within the NYS-2010/11 population. To date we have only found this allele in US-1 and a few Mexican isolates suggesting that a new introduction was also a contributor to this population.

A recombinant population with characteristics similar to the ones observed for the NYS-2010/11 population has been reported in the past in the United States. Gavino et al. (2000) reported a group of isolates that were collected in the Columbia basin of Oregon and Washington in 1993 that satisfied the expectations of sexual recombination. This population was ephemeral with the possible exception of US-11 that may have been one of the recombinants and has persisted to the present time as a successful clonal lineage in the United States (Fry W. E. et al. 2013). There are similarities between the Columbia basin population and the population described here (NYS-2010/11). In both cases opposite mating types were present in proximity; much greater neutral marker diversity was found than has been reported for most other epidemic populations of *P. infestans* in the United States and Canada, and several possible combinations of alleles occurred at many pairs of polymorphic loci.

The likelihood that many different migrations from diverse sources, or that many mutations caused the high degree of genotypic diversity found in the NYS-2010/11 population, seem very low. Migrations have been documented in the past in the United States and Canada but these have typically involved movement of single clonal lineages from known sources (Fry W. E. and Goodwin 1997, Goodwin S. B. et al. 1994a, Goodwin S. B. et al. 1994b). If the NYS-2010/11 isolates represent a migrant population it is reasonable to assume that these isolates arose at a single place and were then dispersed throughout central New York and surrounding areas, possibly via potato seed tubers or tomato transplants. It is unlikely that many diverse mutations occurred in such a short period of time in a single clonal

lineage (Gavino et al. 2000). In a previous study we investigated the diversity within lineages US-22 and US-23 (Danies et al. 2013) and found that mutations do happen but only in a few loci. It is thus unlikely that many isolates with many diverse mutations would have arisen exclusively in a restricted area of the United States. The mutations known to occur within the US-22 and US-23 lineages did not affect the conclusions derived from the parentage exclusion analysis. Because of the geographical location of the NYS-2010/11 isolates it is likely that they came from the same place. There are five or six states in the northern USA that produce seeds/tubers and all of these states have had late blight. The exact location of the actual plasmogamy events is unknown. Because of the geographically limited occurrence of these diverse GDT individuals, we hypothesize that recombination also occurred in a single location.

The diverse combination of markers used was essential to infer that the NYS-2010/11 population is probably recombinant and that US-22 is a likely parent. The banding patterns for the RFLP assay using the moderately repetitive, polymorphic probe (RG57) as well as the banding patterns for the allozyme *glucose-6-phosphate isomerase* allowed us to identify the diversity and uniqueness among the *P. infestans* isolates tested. The microsatellite profiles permitted us to further discriminate among the isolates, allowed us to eliminate certain genotypes as potential parents for the NYS-2010/11 isolates and further allowed the prediction of potential parental genotypes. The mitochondrial and nuclear genes studied revealed the relatedness among the NYS-2010/11 isolates. The nuclear gene sequences further allowed us to eliminate certain NYS-2010/11 genotypes as possible progeny of US-22 or US-11.

The parentage exclusion analysis left only US-22 as a potential parent from the current

dominant lineages. If we assume some mutations, then the US-8 lineage might survive the parentage exclusion analysis. For example it could be possible that a US-8 derivative could have been a parent for the NYS-2010/11 isolates if a mutation occurred that changed either allele (108 or 112) at the D13 locus to 110. However, this assumption seems unlikely since no derivatives of US-8 with a 110 allele at the D13 locus have been found in the United States. Alternatively, if US-8 had a third "null allele" at the D13 locus, it could have donated this allele to the GDT isolates. US-8 is known to have some loci with three alleles – i.e. at locusPinfSSR4 and at *GPI*. Consequently, it is not possible to absolutely eliminate the possibility that US-8 may have a third "null allele" at the D13 locus. Again, this scenario seems highly unlikely given that neither of the other two alleles is present in the GDT isolates.

Our best inference is that the NYS-2010/11 isolates represent a progeny that originated from at least two recombination events. The geographic location(s) of those recombination events remains unknown. The eventual impact of this recombination event cannot be predicted at this moment. The fact that individuals from this event were detected only in 2010 and 2011 and not in 2012 or 2013 suggests that these isolates were not as aggressive or as fit as subsequent dominant clonal lineages. However, the fact that there is now evidence for a second recombinant population of *P. infestans* detected in the USA indicates that sexual recombination is certainly possible, and there is no reason to believe that such populations will not occur in the future. Diligence in monitoring populations might enable the location of a recombination to be identified so that proper mitigation techniques could be applied.

3.7 Acknowledgements

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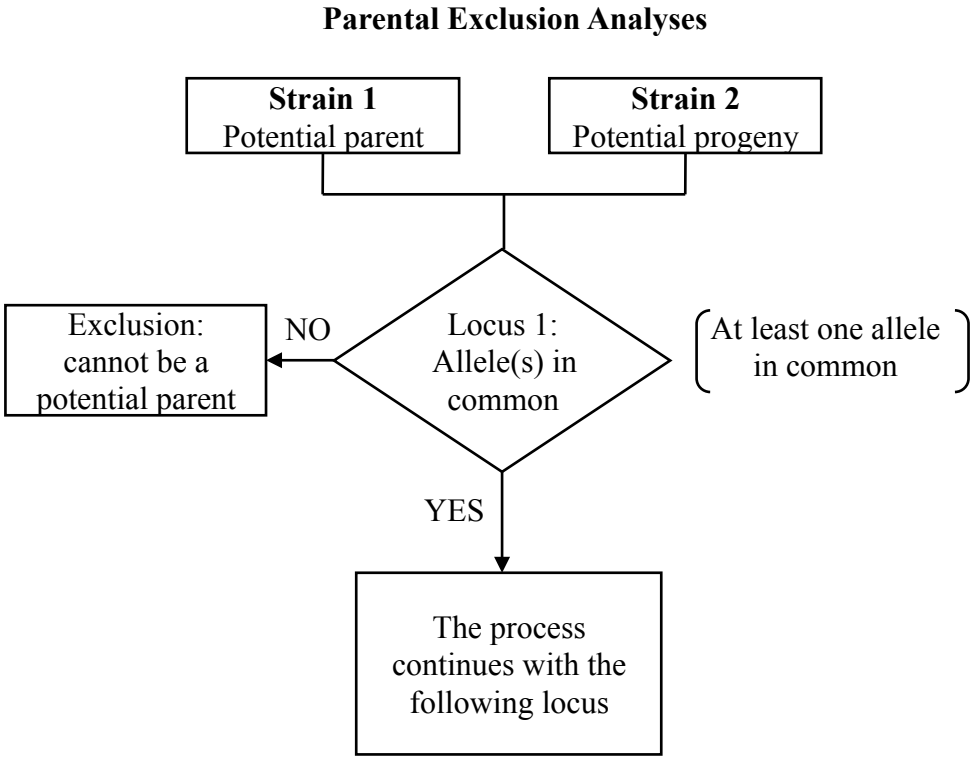
3.9 Supplemental Material



Supplementary Figure 3.1 Flow diagram showing how the parentage exclusion analyses were conducted. A visual parentage exclusion analyses was possible given that there were only 37 unique *Phytophthora infestans* genotypes.

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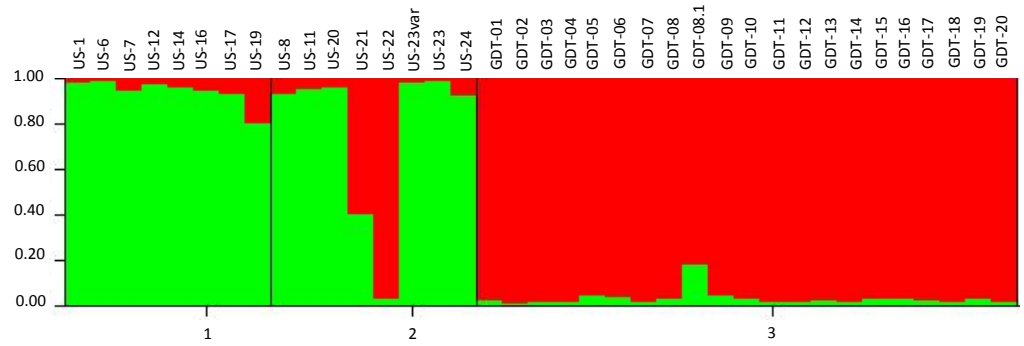
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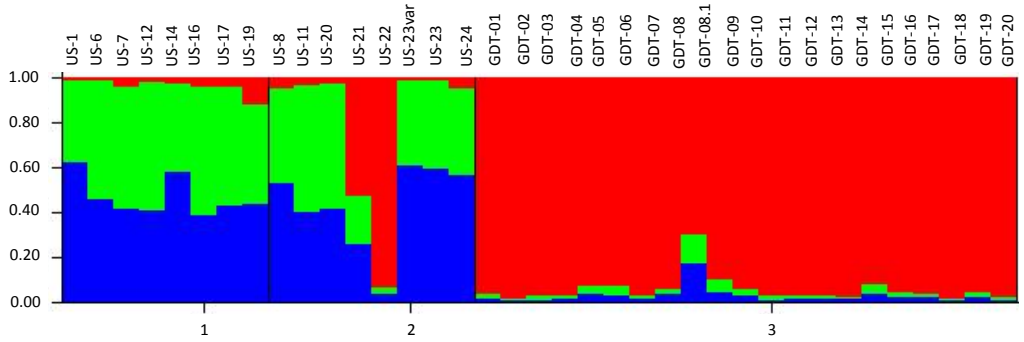
0.60
0.40
0.20
0.00
1
2
3

K=3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0.20
0.00
1
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3

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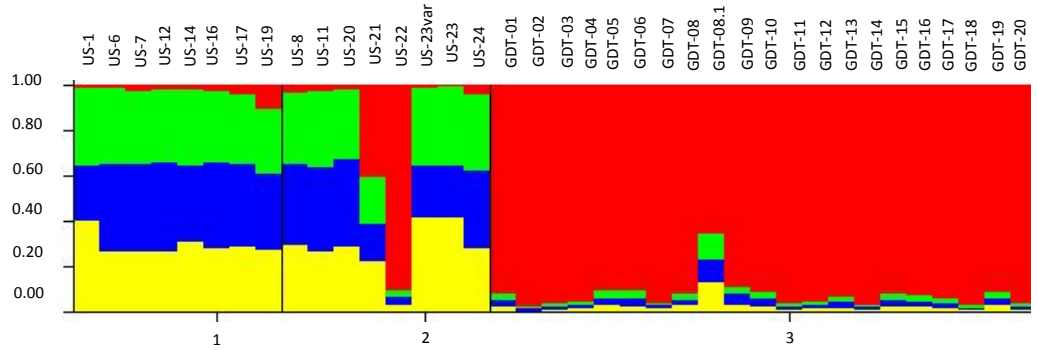
US-1 US-6 US-7 US-12 US-14 US-16 US-17 US-19 US-8 US-11 US-20 US-21 US-22 US-23var US-23 US-24 GDT-01 GDT-02 GDT-03 GDT-04 GDT-05 GDT-06 GDT-07 GDT-08 GDT-08.1 GDT-09 GDT-10 GDT-11 GDT-12 GDT-13 GDT-14 GDT-15 GDT-16 GDT-17 GDT-18 GDT-19 GDT-20
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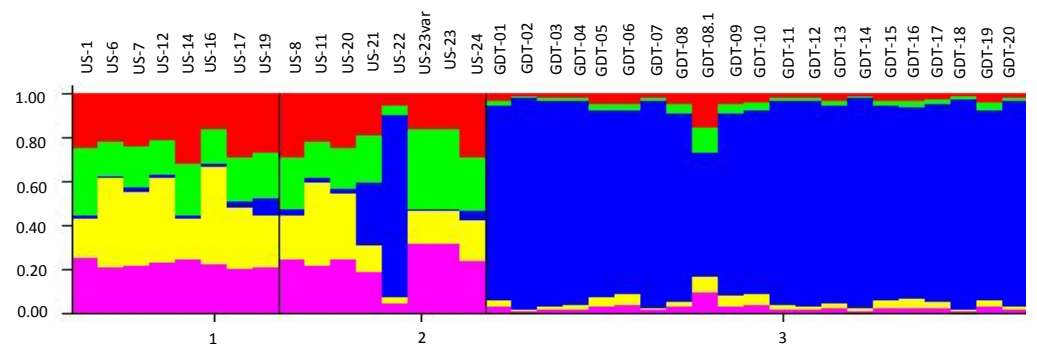
K=5


US-1 US-6 US-7 US-12 US-14 US-16 US-17 US-19 US-8 US-11 US-20 US-21 US-22 US-23var US-23 US-24 GDT-01 GDT-02 GDT-03 GDT-04 GDT-05 GDT-06 GDT-07 GDT-08 GDT-08.1 GDT-09 GDT-10 GDT-11 GDT-12 GDT-13 GDT-14 GDT-15 GDT-16 GDT-17 GDT-18 GDT-19 GDT-20
1.00
0.80
0.60
0.40
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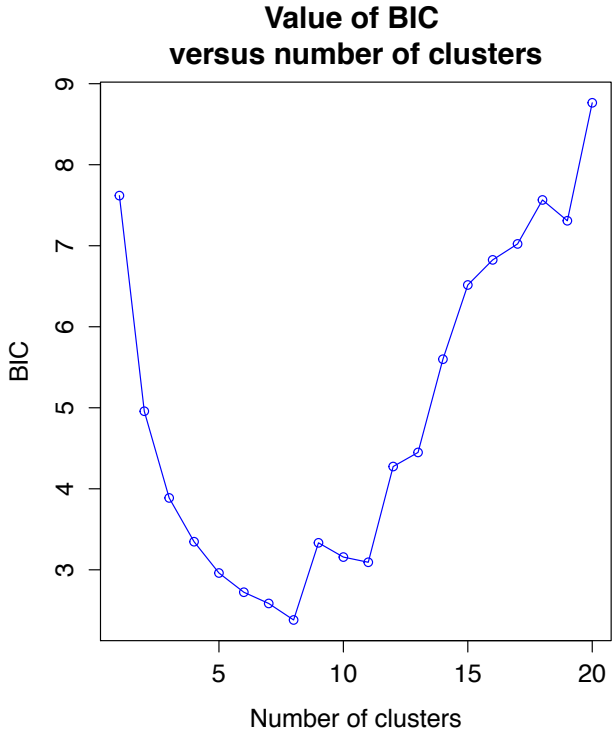
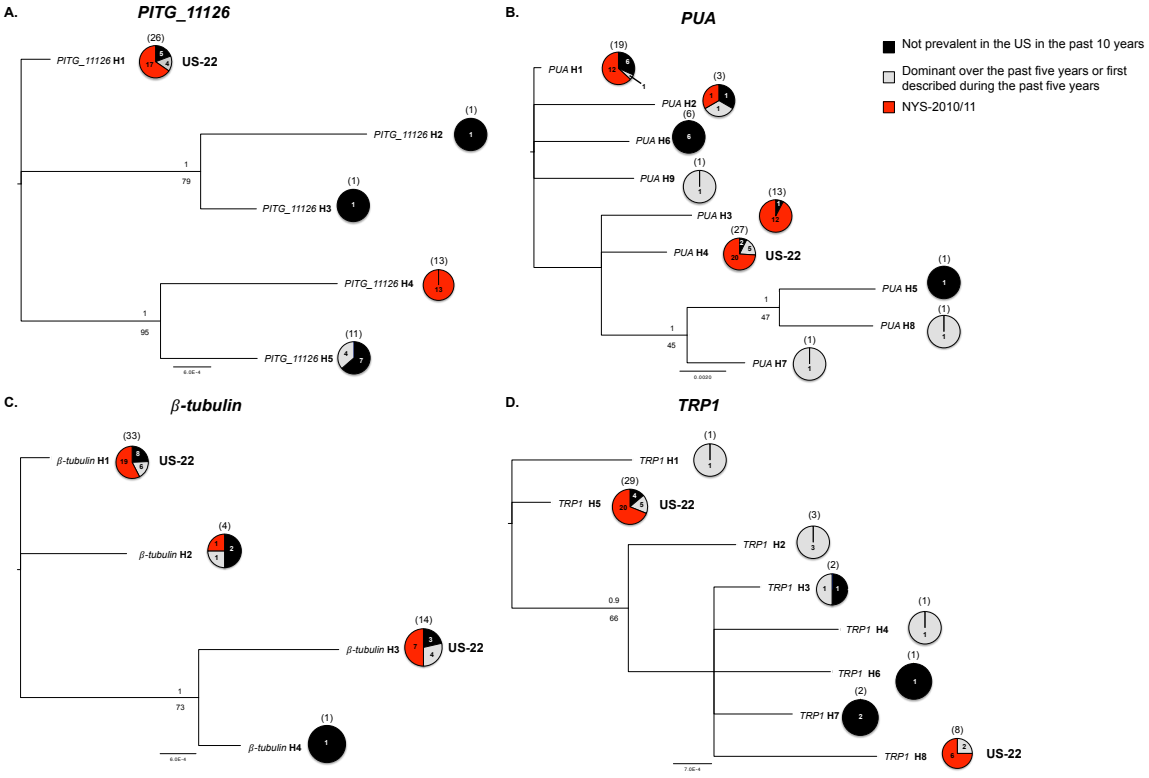
Supplementary Figure 3.2 STRUCTURE analysis of twelve microsatellite loci for *Phytophthora infestans* in the United States. Results for K=2 to K=5 are shown. Each color represents one population defined by STRUCTURE. Each isolate is represented by a vertical bar, and the length of each colored segment in each vertical bar represents the proportion contributed by the ancestral population. The number of inferred populations based on the ΔK method according to Evanno et al. [32] was two. Groups one, two, and three on the x-axis represent an arbitrary classification of isolates into groups according to their occurrence over time in the United States. Group one contained lineages of *P. infestans* that have not been prevalent in the United States for the past 10 years (US-1, US-6, US-7, US-12, US-14, US-16, US-17, and US-19). Group two contained lineages of *P. infestans* that have been dominant in the past five years (US-8, US-11, US-22, US-23 and US-24) or that have been first described during the past five years in the United States (US-20 and US-21). Group three contained the NYS-2010/11 *P. infestans* isolates that are the focus of this study (GDT-01 to GDT-20).



Supplementary Figure 3.3 Bayesian Information Criterion (BIC) values for increasing values of *K*. The BIC decreases until *K* = 8 clusters, after which BIC increases. *K* = 8 also matches the smallest BIC, thus 8 clusters were retained.



Supplementary Figure 3.4 MrBayes trees of haplotypes for each locus sequenced. Loci are **A,** *PITG_11126*, **B,** *PUA*, **C,** *β-tubulin*, and **D,** *TRP1* in *Phytophthora infestans*. Haplotypes shown for each branch tip, correspond to those in Tables S5, S6, S7 and S8. Bayesian posterior probabilities are shown above branches and bootstrap support values obtained by maximum likelihood are shown below branches. Values are not shown for branches that had less than 80% probability/support by both methods. Pie charts represent the number of isolates that contain a particular haplotype within each of the three clusters. Clusters were defined based on the occurrence of *P. infestans* isolates over time in the United States. Numbers within parentheses indicate the number of individuals that contain that haplotype.

Supplementary Table 3.1 *Phytophthora infestans* isolates used in this study.

Multilocus Genotype	Isolate	Location County/State abbreviation or Country/Geographic coordinates	Original host	Year	Collector
US-1	SA960008	Mpumalanga, South Africa/ 26ºS 30ºE	Potato	1996	Adele McLeod
US-6	Coffey7629	USA	-	-	Michael D. Coffey
US-7	Coffey7723	USA	-	-	Michael D. Coffey
US-8	US100028	Dufferin/ON Canada/ 44º05'N 80º12'W	Potato	2010	Eugenia Banks
	US110063	Erie/PA/ 42.10ºN 80.10ºW	Potato	2011	Andrew Musa
	US110102	Pasco/WA/ 46º14'19"N 119º6'31"W	Potato	2011	Niklaus Grünwald
US-11	US050007	Benton/OR/ 44º29'25"N 123º25'57"W	Tomato	2005	Melody Putnam
	US110028	Oneida/NY/ 43.24ºN 75.44ºW	Tomato	2011	Jerry Waskiewicz
US-12	US940494	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	1994	William Fry
US-14	Pi-001-01	MI/ 44ºN 85ºW	-	-	Ken Deahl
US-16	Coffey10112	OR/ 44ºN 120.5ºW	-	1994	Michael D. Coffey
US-17	US970001	Lee/FL/ 26.58ºN 81.92ºW	Tomato	1997	William Fry
US-19	NC09719	NC/ 35.5ºN 80ºW	Tomato	1997	Jean Ristaino
US-20	NC046	NC/ 35.5ºN 80ºW	Tomato	2004	Jean Ristaino
US-21	NC0719	NC/ 35.5ºN 80ºW	Tomato	2007	Jean Ristaino
US-22	US070001	Suffolk/NY/ 40.94ºN 72.68ºW	Tomato	2007	Margaret McGrath
	US090029	Orange/NY/	Tomato	2009	CPDDC

		41.40ºN 74.31ºW			
	US090042	Chautuaqua/NY/ 42.30ºN 79.41ºW	Tomato	2009	CPDDC
US-23var	BL2009P4	Blair/PA/ 40.47ºN 78.35ºW	Potato	2009	Ken Deahl
US-23	Pi431	-	-	2013	Dawn Tidd
	Pi432	-	-	2013	Dawn Tidd
	US110017	Suffolk/NY/ 40.94ºN 72.68ºW	Tomato	2011	Margaret McGrath
	US110040	Waukesha/WI/ 43.02ºN 88.31ºW	Tomato	2011	Amanda Gevens
	US110062	Aroostook/ME/ 46.65ºN 68.59ºW	Potato	2011	Steve Johnson
US-24	ND822Pi	ND/ 47ºN 100ºW	Tomato	2009	Gary Secor
	US110004	ND/ 47ºN 100ºW	Potato	2011	Gary Secor
	US110159	Cass/ND/ 46.93ºN 97.25ºW	Potato	2011	Gary Secor
GDT-01	US110057	Ontario/NY/ 42.85ºN 77.29ºW	Tomato	2011	Christine Smart
	US110058	Ontario/NY/ 42.85ºN 77.29ºW	Tomato	2011	Christine Smart
	US110066	Ontario/NY/ 42.85ºN 77.29ºW	Tomato	2011	Christine Smart
	US110073	Wayne/NY/ 43.28ºN 77.05ºW	Potato	2011	Christine Smart
	US110084	Ontario/NY/ 42.85ºN 77.29ºW	Tomato	2011	Christine Smart
	US110135	Ontario/NY/ 42.85ºN 77.29ºW	Nightshade	2011	Christine Smart
GDT-02	US110064	Genesee/NY/ 43.00ºN 78.19ºW	Potato	2011	Christine Smart
GDT-03	US110086	Monroe/NY/ 43.30ºN 77.69ºW	Potato	2011	Christine Smart
GDT-04	US110060	Wyoming/NY/ 42º50’N 78º5’W	Tomato	2011	Don Casiewicz
	US110065	Livingston/NY/ 42.73ºN 77.77ºW	Tomato	2011	Christine Smart
	US110074	Genesee/NY/ 43.00ºN 78.19ºW	Tomato	2011	Christine Smart

	US110075	Genesee/NY/ 43.00ºN 78.19ºW	Tomato	2011	Christine Smart
	US110078	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	2011	Keith Perry
	US110094	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	2011	Cliff Kraft
GDT-05	US110061	Cayuga/NY/ 42.94ºN 76.56ºW	Tomato	2011	Sharon Bachman
GDT-06	US110093	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	2011	Lisa Hahn
GDT-07	US110072	Genesee/NY/ 43.00ºN 78.19ºW	Potato	2011	Christine Smart
GDT-08	US110071	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	2011	Monica Roth
	US110114	Tompkins/NY/ 42.45ºN 76.47ºW	Potato	2011	William Fry
	US110128	Tompkins/NY/ 42.45ºN 76.47ºW	Potato	2011	William Fry
	US110141	Yates/NY/ 42.64ºN 77.10ºW	Tomato	2011	Abby Seaman
	US110142	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	2011	William Fry
GDT-08.1	US110069	Tompkins/NY/ 42.45ºN 76.47ºW	Tomato	2011	Tom Zitter
GDT-09	US110082	Genesee/NY/ 43.00ºN 78.19ºW	Tomato	2011	Christine Smart
	US110138	Genesee/NY/ 43.00ºN 78.19ºW	Tomato	2011	Christine Smart
	US110139	Genesee/NY/ 43.00ºN 78.19ºW	Tomato	2011	Christine Smart
GDT-10	US110083	Erie/NY/ 42.75N 78.78ºW	Tomato	2011	Sharon Bachman
GDT-11	US110085	Genesee/NY/ 43.00ºN 78.19ºW	Nightshade	2011	Christine Smart
GDT-12	US100029	Simcoe/ON Canada/ 44º1499ºN 79.8632ºW	Potato	2010	Eugenia Banks
GDT-13	US100023	Simcoe/ON Canada/ 43º35'N 79º44'W	Tomato	2010	Catarina Saude

GDT-14	US110054	Mercer/PA/ 41.31ºN 80.25ºW	Tomato	2011	Beth Gugino
	US110079	Venango/PA/ 41.40ºN 79.76ºW	Tomato	2011	Beth Gugino
	US110106	Indiana/PA/ 40.65ºN 79.09ºW	Tomato	2011	Beth Gugino
GDT-15	US100032	Oxford/ON Canada/ 43º14'N 80º36'W	Potato	2010	Eugenia Banks
GDT-16	US100033	Oxford/ON Canada/ 43º14'N 80º36'W	Tomato	2010	Eugenia Banks
GDT-17	US110092	Erie/OH/ 41.51ºN 82.61ºW	Tomato	2011	Sally Miller
GDT-18	US100022	Genesee/NY/ 43.00ºN 78.19ºW	Tomato	2010	Christine Smart
GDT-19	US100019	Erie/NY/ 42.75N 78.78ºW	Tomato	2010	Carol MacNeil
GDT-20	US100034	Niagara/NY/ 43.32ºN 78.79ºW	Tomato	2010	Christy Hoepting

CPDDC: Cornell Plant Disease Diagnostic Clinic

Supplementary Table 3.2 Mating type, and banding patterns for the allozyme *glucose-6-phosphate isomerase* and for a restriction fragment length polymorphism (RFLP) assay using the RG57 probe for the 20 unique NYS-2010/11 multilocus genotypes. Polymorphic sites for the RFLP assay using the RG57 probe are highlighted in grey. Seven of the 24 possible combinations are observed within the 20 NYS-2010/11 genotypes.

				RG57[e]																								
MLG[a]	**MT[b]**	**GPI[c]**	**MH[d]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**24a**	**25**
US-1	A1	86/100	H22	1	0	1	1	1	0	1	0	1	1	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
US-6	A1	100/100	-	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	0	0	1	0	1	1	0	0	1	0	1
US-7	A2	100/111	-	1	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
US-8	A2	100/111/122	H20	1	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	1
US-11	A1	100/100/111	H1	1	0	1	0	1	1	1	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
US-12	A1	100/111	-	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	1	0	1	1	0	0	1	0	1
US-14	A2	100/122	-	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	1
US-16	A1	100/111	-	1	0	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
US-17	A1	100/122	H28	1	0	1	0	0	0	1	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
US-19	A2	100/100	-	1	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
US-20	A2	100/100	-	1	0	1	0	1	0	1	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
US-21	A2	100/122	H20	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	1
US-22	A2	100/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
US-23[f]	A1	100/100	H25	1	1	0	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	1*	1	1	1*	0	1	1	1
US-24	A1	100/100	H13	1	0	1	0	1	0	1	0	0	1	0	0	1	1	0	1	0	0	0	1	1	0	1	1	0	1
GDT-01	A1	100/100	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	1	0	1
GDT-02	A2	111/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	1	0	1
GDT-03	A2	100/111/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	1	0	1
GDT-04	A2	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1

GDT-05	A1	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
GDT-06	A2	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
GDT-07	A2	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	**0**	0	**1**	**1**	1	1	**1**	0	1	0	1
GDT-08	A1	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	**1**	0	**1**	**1**	1	1	**0**	0	1	0	1
GDT-09	A2	100/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	**1**	0	**1**	**0**	1	1	**0**	0	1	0	1
GDT-10	A1	100/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	**1**	0	**1**	**1**	1	1	**1**	0	1	0	1
GDT-11	A1	111/111	H20	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
GDT-12	A1	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	0	0	1	0	1
GDT-13	A2	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	0	0	1	0	1
GDT-14	A1	111/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1
GDT-15	A2	111/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
GDT-16	A2	111/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1
GDT-17	A2	100/122	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	1	1	0	1	0	1
GDT-18	A2	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	**0**	0	**1**	**0**	1	1	**0**	0	1	0	1
GDT-19	A1	100/100	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0	1	0	1
GDT-20	A1	100/111	H20	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0	1	0	1

[a] Multilocus genotype
[b] Mating type
[c] Glucose-6-phosphate isomerase
[d] Mitochondrial haplotype
[e] Restriction Fragment Length Polymorphism (RFLP) bands using the RG57 probe
[f] Isolate BL2009 belonging to clonal lineage US-23 lacks band 17
[*] Bands 19 and 22 have not been described in previous reports for US-23 isolate. We decided to include them here because these two bands are seen on all US-23 isolates analyzed.

Supplementary Table 3.3 Microsatellite calls for multilocus genotypes (MLGs) of *Phytophthora infestans* used in this study. The first sixteen MLGs in the list are the reference isolates.

	D13*			PiG11			Pi04			PinfSSR2			Pi70			Pi4B			PinfSSR6			PinfSSR8			Pi02			Pi63			PinfSSR4			PinfSSR11	
MLG	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2
US-1	136	136	0	154	200	0	166	170	0	173	177	0	189	192	0	213	217	0	242	244	0	266	266	0	266	266	0	273	276	279	288	292	0	341	355
US-6	144	144	0	156	206	0	166	170	0	173	173	0	192	192	0	213	217	0	244	256	0	264	266	0	258	266	0	279	279	0	290	306	0	341	355
US-7	110	110	0	134	156	0	166	170	0	173	173	0	192	192	0	213	213	0	244	244	0	264	266	0	266	268	0	279	279	0	284	298	0	331	341
US-8	108	112	0	156	156	0	166	170	0	173	173	0	192	192	0	213	225	0	244	244	0	260	266	0	266	268	0	279	279	0	284	288	294	341	355
US-11	110	110	0	130	156	0	166	170	0	173	173	0	192	192	0	213	213	0	244	244	0	264	266	0	258	266	268	279	279	0	284	294	306	331	341
US-12	144	148	0	134	156	0	166	170	0	173	173	0	192	192	0	213	213	0	244	256	0	264	264	0	258	266	0	279	279	0	284	294	0	331	355
US-14	108	112	0	156	160	0	166	170	0	173	173	0	192	192	0	213	225	0	244	244	0	260	266	0	266	266	0	279	279	0	288	294	0	341	355
US-16	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	256	256	0	264	266	0	258	266	0	279	279	0	284	306	0	341	341
US-17	0	0	0	134	156	0	166	170	0	173	173	0	192	192	0	213	213	0	236	244	0	0	0	0	258	266	0	279	279	0	288	288	0	331	355
US-19	110	110	0	134	154	0	166	170	0	173	173	0	192	192	0	213	213	0	244	244	0	260	264	0	266	266	0	279	279	0	288	298	0	355	355
US-20	108	108	0	134	156	0	166	170	0	173	173	0	192	192	0	213	213	0	244	244	0	264	266	0	258	266	0	279	279	0	284	288	0	355	355
US-21	110	110	0	134	134	0	166	170	0	173	173	0	192	192	0	217	217	0	242	244	0	260	260	0	266	268	0	279	279	0	288	286	0	341	341
US-22	0	0	0	134	156	0	166	170	0	173	175	0	192	195	0	213	213	0	242	244	0	260	264	0	266	268	0	279	279	0	294	296	0	341	341
US-23var	136	136	0	142	156	206	170	170	0	173	175	0	192	192	0	213	217	0	244	244	0	260	266	0	266	268	270	273	279	0	288	294	296	331	341
US-23	136	136	0	142	156	206	170	170	0	173	175	0	192	192	0	213	217	0	244	244	0	260	266	0	266	268	270	273	279	0	288	294	0	331	341
US-24	108	112	0	156	156	0	166	170	0	173	173	0	192	195	0	217	225	0	244	244	0	260	266	0	266	268	0	279	279	0	284	288	296	341	355
GDT-01	110	110	0	134	134	0	166	166	0	173	173	0	192	192	0	213	213	0	242	244	0	260	260	0	258	268	0	279	279	0	288	296	0	341	355
GDT-02	0	0	0	134	156	0	166	166	0	173	175	0	189	195	0	213	225	0	242	242	0	260	260	0	258	266	0	279	279	0	284	294	0	341	355
GDT-03	110	110	0	134	156	0	166	170	0	173	173	0	189	192	195	213	213	0	242	244	0	260	264	0	258	266	268	279	279	0	288	294	0	341	355
GDT-04	110	110	0	134	156	0	166	170	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	258	266	268	279	279	0	284	294	296	341	355
GDT-05	0	0	0	156	156	0	166	170	0	173	173	0	192	195	0	213	213	0	242	244	0	260	264	0	258	266	0	279	279	0	288	296	0	341	355
GDT-06	110	110	0	156	156	0	166	170	0	173	175	0	192	192	0	213	213	0	242	244	0	260	264	0	258	266	0	279	279	0	284	284	0	341	355
GDT-07	0	0	0	156	156	0	166	166	0	173	175	0	192	195	0	213	225	0	242	244	0	260	260	0	258	266	0	279	279	0	288	294	0	341	355
GDT-08	0	0	0	134	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	266	258	0	279	279	0	288	294	0	341	355
GDT-08.1	0	0	0	134	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	266	268	0	279	279	0	288	292	294	341	355
GDT-09	110	110	0	156	156	0	166	170	0	173	173	0	189	192	0	213	213	0	244	244	0	260	264	0	266	266	0	279	279	0	288	294	0	341	355
GDT-10	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	294	0	341	355
GDT-11	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	242	242	0	260	264	0	258	266	0	279	279	0	288	294	0	355	355
GDT-12	110	110	0	156	156	0	166	170	0	173	173	0	189	192	0	213	225	0	242	244	0	260	260	0	258	266	0	279	279	0	284	288	0	355	355
GDT-13	0	0	0	134	156	0	166	166	0	173	173	0	192	195	0	213	225	0	242	244	0	260	260	0	258	266	0	279	279	0	284	294	0	341	355
GDT-14	110	110	0	156	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	258	266	268	279	279	0	288	294	296	341	355
GDT-15	110	110	0	156	156	0	166	170	0	173	175	0	189	192	0	213	213	0	244	244	0	260	264	0	258	268	0	279	279	0	284	294	0	341	355
GDT-16	0	0	0	156	156	0	166	166	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	296	0	341	355
GDT-17	110	110	0	134	156	0	166	170	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	296	0	341	355
GDT-18	0	0	0	156	156	0	166	166	0	173	175	0	192	195	0	213	225	0	242	242	0	260	260	0	266	268	0	279	279	0	288	296	0	341	355
GDT-19	0	0	0	156	156	0	166	170	0	173	173	0	192	195	0	213	225	0	242	244	0	260	260	0	266	266	0	279	279	0	288	294	0	341	355
GDT-20	0	0	0	156	156	0	166	166	0	173	175	0	192	192	0	213	225	0	242	242	0	260	260	0	258	268	0	279	279	0	284	294	0	341	355

* A weak 210 allele is observed for US-23 and a weak 230 allele is observed for some GDT isolates.

Supplementary Table 3.4 Polymorphic sites for a gene coding for a conserved hypothetical protein (*PITG_11126*) in 35 isolates of *Phytophthora infestans*. Isolates highlighted in yellow are those for which US-22 could not be a parent. Inferred haplotypes are identified with the letter H followed by a number. Total length of the sequence is indicated within parentheses.

	***PITG_11126* (776 bp)**					
	49	**167**	**460**	**549**	**554**	**725**
US-1	Y	G	R	T	G	Y
US-6	C	G	G	Y	R	C
US-7	C	G	G	C	A	C
US-8	C	G	G	Y	R	C
US-11	C	G	G	C	A	C
US-12	C	G	G	Y	R	C
US-14	C	G	G	Y	R	C
US-16	C	G	G	C	A	C
US-17	C	G	G	C	A	C
US-19	C	G	G	Y	R	C
US-20	C	G	G	C	A	C
US-21	C	G	G	T	G	C
US-22	**C**	**G**	**G**	**T**	**G**	**C**
US-23	-	-	-	-	-	-
US-24	C	G	G	Y	R	C
GDT-01	C	G	G	T	G	C
GDT-02	C	R	G	Y	R	C
GDT-03	C	G	G	T	G	C
GDT-04	C	A	G	C	A	C
GDT-05	C	A	G	C	A	C
GDT-06	C	G	G	T	G	C
GDT-07	C	G	G	T	G	C
GDT-08	C	R	G	Y	R	C
GDT-09	C	A	G	C	A	C
GDT-10	C	R	G	Y	R	C
GDT-11	C	R	G	Y	R	C
GDT-12	C	R	G	Y	R	C
GDT-13	C	R	G	Y	R	C
GDT-14	C	R	G	Y	R	C
GDT-15	C	R	G	Y	R	C
GDT-16	C	G	G	T	G	C
GDT-17	C	G	G	T	G	C
GDT-18	C	R	G	Y	R	C
GDT-19	C	G	G	T	G	C
GDT-20	C	R	G	Y	R	C
R = G/A	Y = C/T					
H1	C	G	G	T	G	C
H2	T	G	A	T	G	T
H3	T	G	G	T	G	C
H4	C	A	G	C	A	C
H5	C	G	G	C	A	C

Supplementary Table 3.5 Polymorphic sites for a gene coding for a conserved hypothetical protein (*PUA*) in 35 isolates of *Phytophthora infestans*. Inferred haplotypes are identified with the letter H followed by a number. Total length of the sequence is indicated within parentheses.

	***PUA* (609 bp)**								
	35	**82**	**187**	**381**	**389**	**532**	**555**	**556**	**586**
US-1	W	W	Y	G	A	G	Y	W	A
US-6	T	T	C	S	A	G	T	T	R
US-7	T	T	C	G	M	R	T	T	A
US-8	T	T	C	G	C	A	T	T	A
US-11	T	T	C	G	A	G	T	T	A
US-12	T	T	C	C	A	G	T	T	G
US-14	T	T	C	G	C	A	T	T	A
US-16	T	T	C	G	A	G	T	T	A
US-17	T	T	C	G	A	G	T	T	A
US-19	T	T	C	S	A	G	T	T	R
US-20	T	T	C	G	C	A	T	T	A
US-21	W	W	Y	S	M	R	T	T	A
US-22	**T**	**T**	**C**	**G**	**C**	**A**	**T**	**T**	**A**
US-23	W	W	Y	S	M	R	Y	T	A
US-24	T	T	C	G	C	A	T	T	A
GDT-01	T	T	C	G	M	R	T	T	A
GDT-02	T	T	C	G	M	R	T	T	A
GDT-03	T	T	C	G	M	R	T	T	A
GDT-04	T	T	C	G	C	A	T	T	A
GDT-05	T	T	C	G	M	R	T	T	A
GDT-06	T	T	C	G	C	A	T	T	A
GDT-07	T	T	C	G	M	R	T	T	A
GDT-08	T	T	C	G	M	R	T	T	A
GDT-09	T	T	C	G	M	R	T	T	A
GDT-10	T	T	C	G	C	A	T	T	A
GDT-11	T	T	C	G	C	A	T	T	A
GDT-12	T	T	C	G	M	R	T	T	A
GDT-13	T	T	C	G	M	R	T	T	A
GDT-14	T	T	C	G	C	A	T	T	A
GDT-15	T	T	C	G	C	A	T	T	A
GDT-16	T	T	C	G	C	A	T	T	A
GDT-17	T	T	C	G	C	A	T	T	A
GDT-18	T	T	C	G	M	R	T	T	A
GDT-19	T	T	C	G	M	R	T	T	A
GDT-20	T	T	C	G	M	R	T	T	A
M = C/A	R = G/A	S = C/G	W = A/T	Y = C/T					
H1	T	T	C	G	A	G	T	T	A
H2	T	T	C	C	A	G	T	T	G
H3	T	T	C	G	A	A	T	T	A
H4	T	T	C	G	C	A	T	T	A
H5	A	A	T	G	A	G	C	T	A
H6	T	T	C	G	A	G	T	A	A
H7	A	A	C	G	C	A	T	T	A
H8	A	A	T	C	A	A	T	T	A
H9	T	T	C	G	C	G	C	T	A

Supplementary Table 3.6 Polymorphic sites for the gene *ß-tubulin* in 35 isolates of *Phytophthora infestans*. Inferred haplotypes are identified with the letter H followed by a number. Total length of the sequence is indicated within parentheses.

	ß-tubulin (883 bp)		
	583	**808**	**829**
US-1	C	C	T
US-6	C	Y	T
US-7	Y	C	Y
US-8	Y	C	Y
US-11	Y	Y	Y
US-12	C	C	T
US-14	Y	C	Y
US-16	C	Y	T
US-17	C	C	T
US-19	Y	C	Y
US-20	C	Y	T
US-21	C	C	T
US-22	**Y**	**C**	**Y**
US-23	Y	C	Y
US-24	Y	C	Y
GDT-01	C	Y	T
GDT-02	C	C	T
GDT-03	C	C	T
GDT-04	T	C	Y
GDT-05	C	C	T
GDT-06	C	C	T
GDT-07	C	C	T
GDT-08	Y	C	Y
GDT-09	C	C	T
GDT-10	C	C	T
GDT-11	Y	C	Y
GDT-12	C	C	T
GDT-13	C	C	T
GDT-14	Y	C	Y
GDT-15	C	C	T
GDT-16	Y	C	Y
GDT-17	Y	C	Y
GDT-18	Y	C	Y
GDT-19	C	C	T
GDT-20	C	C	T
Y = C/T			
H1	C	C	T
H2	C	T	T
H3	T	C	C
H4	T	C	T

Supplementary Table 3.7 Polymorphic sites for the gene coding for the indole-3-glycerolphosphate synthase-*N*-(5'-phosphoribosyl)anthranilate isomerase (*TRP1*) in 35 isolates of *Phytophthora infestans*. Inferred haplotypes are identified with the letter H followed by a number. Total length of the sequence is indicated within parentheses.

	***TRP1* (824 bp)**					
	51	**78**	**132**	**563**	**614**	**714**
US-1	R	A	R	R	T	G
US-6	G	A	A	A	W	G
US-7	G	A	G	G	T	G
US-8	G	A	G	G	T	G
US-11	G	R	R	R	T	G
US-12	G	A	R	R	W	G
US-14	G	A	G	G	T	G
US-16	G	A	R	R	T	G
US-17	G	A	R	R	T	G
US-19	G	A	R	R	T	G
US-20	G	A	R	R	T	G
US-21	G	A	G	R	T	G
US-22	**G**	**A**	**R**	**G**	**T**	**G**
US-23	G	A	R	R	T	R
US-24	G	A	R	G	T	G
GDT-01	G	A	G	G	T	G
GDT-02	G	A	R	G	T	G
GDT-03	G	A	G	G	T	G
GDT-04	G	A	G	G	T	G
GDT-05	G	A	R	G	T	G
GDT-06	G	A	G	G	T	G
GDT-07	G	A	R	G	T	G
GDT-08	G	A	G	G	T	G
GDT-09	G	A	G	G	T	G
GDT-10	G	A	R	G	T	G
GDT-11	G	A	G	G	T	G
GDT-12	G	A	G	G	T	G
GDT-13	G	A	G	G	T	G
GDT-14	G	A	G	G	T	G
GDT-15	G	A	R	G	T	G
GDT-16	G	A	G	G	T	G
GDT-17	G	A	G	G	T	G
GDT-18	G	A	G	G	T	G
GDT-19	G	A	R	G	T	G
GDT-20	G	A	G	G	T	G
R = G/A	W = A/T					
H1	G	A	G	G	T	A
H2	G	A	G	A	T	G
H3	G	A	A	A	T	G
H4	G	G	A	A	T	G
H5	G	A	G	G	T	G
H6	A	A	A	A	T	G
H7	G	A	A	A	A	G
H8	G	A	A	G	T	G

Supplementary Table 3.8 Microsatellite allele names and sizes for the twelve-microsatellite loci used in this study. Allele sizes can differ slightly from one laboratory to another because of different equipment. In order to compare across locations, the community uses common standards to identify alleles. This sometimes results in an allele name being slightly different from the detected size. This keeps the allele names consistent with earlier publications [26].

D13	**Allele name**	**108**	**110**	**112**	**136**	**144**	**148**		
	Allele size	106	108	110	135	143	148		
PiG11	**Allele name**	**130**	**134**	**142**	**154**	**156**	**160**	**200**	**206**
	Allele size	130	134	142	155	157	161	200	206
Pi04	**Allele name**	**166**	**170**						
	Allele size	171	175						
SSR2	**Allele name**	**173**	**175**	**177**					
	Allele size	173	175	177					
Pi70	**Allele name**	**189**	**192**	**195**					
	Allele size	188	191	194					
Pi4B	**Allele name**	**213**	**217**	**225**					
	Allele size	216	220	228					
SSR6	**Allele name**	**236**	**242**	**244**	**256**				
	Allele size	238	244	246	258				
SSR8	**Allele name**	**260**	**264**	**266**					
	Allele size	262	266	268					
Pi02	**Allele name**	**258**	**266**	**268**	**270**				
	Allele size	260	268	270	272				
Pi63	**Allele name**	**273**	**276**	**279**					
	Allele size	272	275	280					
SSR4	**Allele name**	**284**	**288**	**290**	**292**	**294**	**296**	**298**	**306**
	Allele size	284	288	290	292	294	296	298	306
SSR11	**Allele name**	**331**	**341**	**355**					
	Allele size	331	341	356					

Supplementary Table 3.9 Microsatellite calls for multilocus genotypes (MLGs) of *Phytophthora infestans* used in this study. Isolates highlighted in grey are those for which US-22 could not be a parent. The alleles shown in red are those not present in US-22.

	D13			PiG11			Pi04			PinfSSR2			Pi70			Pi4B			PinfSSR6			PinfSSR8			Pi02			Pi63			PinfSSR4			PinfSSR11		
MLG	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
US-22	**0**	**0**	**0**	**134**	**156**	**0**	**166**	**170**	**0**	**173**	**175**	**0**	**192**	**195**	**0**	**213**	**213**	**0**	**242**	**244**	**0**	**260**	**264**	**0**	**266**	**268**	**0**	**279**	**279**	**0**	**294**	**296**	**0**	**341**	**341**	**0**
GDT-01	110	110	0	134	134	0	166	166	0	173	173	0	192	192	0	213	213	0	242	244	0	260	260	0	258	268	0	279	279	0	288	296	0	341	355	0
GDT-02	0	0	0	134	156	0	166	166	0	173	175	0	189	195	0	213	225	0	242	242	0	260	260	0	258	266	0	279	279	0	284	294	0	341	355	0
GDT-03	110	110	0	134	156	0	166	170	0	173	173	0	189	192	195	213	213	0	242	244	0	260	264	0	258	266	268	279	279	0	288	294	0	341	355	0
GDT-04	110	110	0	134	156	0	166	170	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	258	266	268	279	279	0	284	294	296	341	355	0
GDT-05	0	0	0	156	156	0	166	170	0	173	173	0	192	195	0	213	213	0	242	244	0	260	264	0	258	266	0	279	279	0	288	296	0	341	355	0
GDT-06	110	110	0	156	156	0	166	170	0	173	175	0	192	192	0	213	213	0	242	244	0	260	264	0	258	266	0	279	279	0	**284**	**284**	**0**	341	355	0
GDT-07	0	0	0	156	156	0	166	166	0	173	175	0	192	195	0	213	225	0	242	244	0	260	260	0	258	266	0	279	279	0	288	294	0	341	355	0
GDT-08	0	0	0	134	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	266	258	0	279	279	0	288	294	0	341	355	0
GDT-08.1	0	0	0	134	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	266	268	0	279	279	0	288	292	294	341	355	0
GDT-09	110	110	0	156	156	0	166	170	0	173	173	0	189	192	0	213	213	0	244	244	0	260	264	0	266	266	0	279	279	0	288	294	0	341	355	0
GDT-10	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	294	0	341	355	0
GDT-11	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	242	242	0	260	264	0	258	266	0	279	279	0	288	294	0	**355**	**355**	**0**
GDT-12	110	110	0	156	156	0	166	170	0	173	173	0	189	192	0	213	225	0	242	244	0	260	260	0	258	266	0	279	279	0	**284**	**288**	**0**	**355**	**355**	**0**
GDT-13	0	0	0	134	156	0	166	166	0	173	173	0	192	195	0	213	225	0	242	244	0	260	260	0	258	266	0	279	279	0	284	294	0	341	355	0
GDT-14	110	110	0	156	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	258	266	268	279	279	0	288	294	296	341	355	0
GDT-15	110	110	0	156	156	0	166	170	0	173	175	0	189	192	0	213	213	0	244	244	0	260	264	0	258	268	0	279	279	0	284	294	0	341	355	0
GDT-16	0	0	0	156	156	0	166	166	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	296	0	341	355	0
GDT-17	110	110	0	134	156	0	166	170	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	296	0	341	355	0
GDT-18	0	0	0	156	156	0	166	166	0	173	175	0	192	195	0	213	225	0	242	242	0	260	260	0	266	268	0	279	279	0	288	296	0	341	355	0
GDT-19	0	0	0	156	156	0	166	170	0	173	173	0	192	195	0	213	225	0	242	244	0	260	260	0	266	266	0	279	279	0	288	294	0	341	355	0
GDT-20	0	0	0	156	156	0	166	166	0	173	175	0	192	192	0	213	225	0	242	242	0	260	260	0	258	268	0	279	279	0	284	294	0	341	355	0

Supplementary Table 3.10 Microsatellite calls for multilocus genotypes (MLGs) of *Phytophthora infestans* used in this study. Isolates highlighted in grey are those for which US-11 could not be a parent. The alleles shown in red are those not present in US-11.

	D13			PiG11			Pi04			PinfSSR2			Pi70			Pi4B			PinfSSR6			PinfSSR8			Pi02			Pi63			PinfSSR4			PinfSSR11		
MLG	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
US-11	**110**	**110**	**0**	**130**	**156**	**0**	**166**	**170**	**0**	**173**	**173**	**0**	**192**	**192**	**0**	**213**	**213**	**0**	**244**	**244**	**0**	**264**	**266**	**0**	**258**	**266**	**268**	**279**	**279**	**0**	**284**	**294**	**306**	**331**	**341**	**0**
GDT-01	110	110	0	**134**	**134**	**0**	166	166	0	173	173	0	192	192	0	213	213	0	242	244	0	**260**	**260**	**0**	258	268	0	279	279	0	**288**	**296**	**0**	341	355	0
GDT-02	**0**	**0**	**0**	134	156	0	166	166	0	173	175	0	**189**	**195**	**0**	213	225	0	**242**	**242**	**0**	**260**	**260**	**0**	258	266	0	279	279	0	284	294	0	341	355	0
GDT-03	110	110	0	134	156	0	166	170	0	173	173	0	189	192	195	213	213	0	242	244	0	260	264	0	258	266	268	279	279	0	288	294	0	341	355	0
GDT-04	110	110	0	134	156	0	166	170	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	258	266	268	279	279	0	284	294	296	341	355	0
GDT-05	**0**	**0**	**0**	156	156	0	166	170	0	173	173	0	192	195	0	213	213	0	242	244	0	260	264	0	258	266	0	279	279	0	**288**	**296**	**0**	341	355	0
GDT-06	110	110	0	156	156	0	166	170	0	173	175	0	192	192	0	213	213	0	242	244	0	260	264	0	258	266	0	279	279	0	284	284	0	341	355	0
GDT-07	**0**	**0**	**0**	156	156	0	166	166	0	173	175	0	192	195	0	213	225	0	242	244	0	**260**	**260**	**0**	258	266	0	279	279	0	288	294	0	341	355	0
GDT-08	**0**	**0**	**0**	134	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	266	258	0	279	279	0	288	294	0	341	355	0
GDT-08.1	**0**	**0**	**0**	134	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	266	268	0	279	279	0	288	292	294	341	355	0
GDT-09	110	110	0	156	156	0	166	170	0	173	173	0	189	192	0	213	213	0	244	244	0	260	264	0	266	266	0	279	279	0	288	294	0	341	355	0
GDT-10	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	288	294	0	341	355	0
GDT-11	110	110	0	156	156	0	166	170	0	173	173	0	192	192	0	213	213	0	**242**	**242**	**0**	260	264	0	258	266	0	279	279	0	288	294	0	**355**	**355**	**0**
GDT-12	110	110	0	156	156	0	166	170	0	173	173	0	189	192	0	213	225	0	242	244	0	**260**	**260**	**0**	258	266	0	279	279	0	284	288	0	**355**	**355**	**0**
GDT-13	**0**	**0**	**0**	134	156	0	166	166	0	173	173	0	192	195	0	213	225	0	242	244	0	**260**	**260**	**0**	258	266	0	279	279	0	284	294	0	341	355	0
GDT-14	110	110	0	156	156	0	166	170	0	173	175	0	189	192	0	213	225	0	242	244	0	260	264	0	258	266	268	279	279	0	288	294	296	341	355	0
GDT-15	110	110	0	156	156	0	166	170	0	173	175	0	189	192	0	213	213	0	244	244	0	260	264	0	258	268	0	279	279	0	284	294	0	341	355	0
GDT-16	**0**	**0**	**0**	156	156	0	166	166	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	**288**	**296**	**0**	341	355	0
GDT-17	110	110	0	134	156	0	166	170	0	173	173	0	189	192	0	213	213	0	242	244	0	260	264	0	266	266	0	279	279	0	**288**	**296**	**0**	341	355	0
GDT-18	**0**	**0**	**0**	156	156	0	166	166	0	173	175	0	192	195	0	213	225	0	**242**	**242**	**0**	**260**	**260**	**0**	266	268	0	279	279	0	**288**	**296**	**0**	341	355	0
GDT-19	**0**	**0**	**0**	156	156	0	166	170	0	173	173	0	192	195	0	213	225	0	242	244	0	**260**	**260**	**0**	266	266	0	279	279	0	288	294	0	341	355	0
GDT-20	**0**	**0**	**0**	156	156	0	166	166	0	173	175	0	192	192	0	213	225	0	**242**	**242**	**0**	**260**	**260**	**0**	258	268	0	279	279	0	284	294	0	341	355	0

Supplementary Table 3.11 Possible genotypes for the allozyme *glucose-6-phosphate isomerase* and microsatellite loci of putative parents for the GDT isolates when assuming that lineage US-22 is one of the parental genotypes for these isolates. Clonal lineage US-22 could not be excluded as a potential parent for 17 of the 20 NYS-2010/11 isolates based on the banding patterns for the allozyme *glucose-6-phosphate isomerase* and microsatellite data. In red we show the alleles that the alternate parent or parents must possess to give rise to the genotypic profiles observed in the NYS-2010/11 isolates when assuming that lineage US-22 is one of the parental genotypes for these isolates.

Alleles present	GPI			Mating type	Microsatellite loci											
					D13	PiG11	Pi04	PinfSSR2	Pi70	Pi4B	PinfSSR6	PinfSSR8	Pi02	Pi63	PinfSSR4	PinfSSR11
US-22	100		122	A2	0	134/156	166/170	173/175	192/195	213	242/244	260/264	266/268	279	294/296	341
GDTs	100	111	122	A1/A2	0/110	134/156	166/170	173/175	189/192/195	213/225	242/244	260/264	258/266/268	279	284/288/294/296	341/355
Other parent(s)		**111**		**A1**	**110**	134/156	166/170	173/175	**189**	**225**	242/244	260/264	**258**	279	**284/288**	**355**

Supplementary Table 3.12 Possible genotypes of loci *PITG_11126*, *PUA*, β-*tubulin* and *TRP1* of putative parents for the GDT isolates when assuming that lineage US-22 is one of the parental genotypes for these isolates. In red we show the alleles that the alternate parent or parents must possess to give rise to the genotypic profiles observed in the NYS-2010/11 isolates when assuming that lineage US-22 is one of the parental genotypes for these isolates.

	***PITG_11126* (776 bp)**						***PUA* (609 bp)**										***β-tubulin* (883 bp)**			***TRP1* (824 bp)**					
Alleles present \ Polymorphic site	49	167	460	549	554	725	35	82	187	381	382	389	532	555	556	586	583	808	829	51	78	132	563	614	714
US-22	C	G	G	T	G	C	T	T	C	G	G	C	A	T	T	A	Y	C	Y	G	A	R	G	T	G
GDTs	C	G/R/A	G	T/Y/C	G/R/A	C	T	T	C	G	G	C/M	A/R	T	T	T	C/Y/T	C/Y	T/Y	G	A	G/R	G	T	G
Other parent(s)	C	G/R/A	G	T/Y/C	G/R/A	C	T	T	C	G	G	C/M/A	G/R/A	T	T	T	C/Y/T	C/Y/T	C/Y/T	G	A	G/R/A	G	T	G

CHAPTER 4*

Enroute to GWAS: Exploring phenotypic diversity in *Phytophthora infestans*

4.1 Abstract

Phytophthora infestans, the causal agent of late blight of potatoes and tomatoes has been a major threat to global food security ever since the Irish famine of the 1800's. The use of resistant cultivars has proven to be challenging due to the absence of durable resistance genes. Furthermore, despite the usefulness of fungicides, the pathogen has proven capable of evolving resistance to certain highly effective fungicides. An understanding of the genetic basis of complex traits important to the pathogenicity or epidemiology of this organism would be of value in managing late blight because rapid analysis using molecular markers could inform the selection of the most effective mitigation tactics. As a first step in determining the genetic basis of some of these traits we systematically assessed five traits (mating type, pathogenicity on potato and tomato, sensitivity to mefenoxam, the effect of temperature on release of zoospores, and the effect of temperature on mycelial growth) of a diverse panel of *P. infestans*: The panel consisted of i) the dominant clones in the US from the 1990s to 2013, ii) a recombinant population detected in northeastern US in 2010 and 2011, iii) a natural sexual population from Mexico, and iv) an isolate from the Netherlands. For these isolates we initiated a genome-wide association study to identify genetic markers associated with mating type.

* Danies G, Romero-Navarro JA, Gonzalez-Garcia LN, Myers K, Bevels E, Bond M, Wu Y, and Fry WE. 2015. Genetic architecture of complex traits of *Phytophthora infestans* determined through genome-wide association mapping. *In preparation*.

4.2 Key words

Phytophthora infestans, mating type, pathogenicity on potato and tomato, sensitivity to mefenoxam, rate of indirect germination, effect of temperature on mycelial growth, genome-wide association study

4.3 Introduction

Late blight caused by the pathogen *Phytophthora infestans*, has been a major threat to global food security ever since the Irish famine of the 1800's. In the US, late blight is potentially important on nearly all of the approximately 1.1 million acres of potato production (Agricultural Statistics Board, NASS, USDA). The worldwide cost of potato late blight alone exceeds $5 billion per year, including $1 billion spent on fungicides (Judelson, USAblight). On tomatoes, the disease can be and has been equally devastating. The most recent example occurred in 2009 when infected tomato transplants were distributed via national large retail stores who obtained transplants from a national supplier. The subsequent pandemic in the mid-Atlantic and northeast regions eliminated tomato plants in many organic farms and home gardens.

Management of late blight mostly involves cultural procedures and fungicides designed to reduce the introduction, survival, or infection rate of *P. infestans*. Resistant cultivars are employed rarely in potato production due to the absence of durable resistance (*R*) genes. The use of fungicides has helped control late blight for more than a century, but the pathogen has proven capable of evolving resistance to certain highly effective fungicides. For example, the fungicide metalaxyl (now mefenoxam) was used with great success in the 1970s and 1980s, but resistant strains appeared that caused devastating losses (Fry and Goodwin 1997,

Goodwin et al. 1996). Interestingly, due to year-to-year population shifts, chemicals that lose effectiveness one year may regain value. For example, the 2009 late blight pandemic in the eastern US, was caused by a mefenoxam-sensitive lineage. The fact that mefenoxam was effective was not known widely until late in the season, too late to aid growers. This shows that knowledge of such traits can provide management opportunities.

Phenotypic analysis may take weeks to months, whereas certain molecular analyses of genotype can be accomplished in hours or days. Therefore, an understanding of the genetic basis of complex traits of value in managing late blight is critical to rapidly predict the pathogen's phenotype. Informed management decisions have tangible economic and environmental benefits by leading to lower on-farm expenses, reduced fungicide applications, more effective disease suppression and more sustainable production. Despite the scientific efforts and research to identify the genetic basis behind these important pathogen traits, not much is known today. Most of the research has focused on the pathogen's secreted host-translocated RXLR effectors that are able to change the host's physiology and facilitate colonization (Vleeshouwers et al. 2011).

Plant pathology and plant disease management are changing from a data-poor to a data-rich environment. Next-generation sequencing of plant pathogens is revolutionizing the field as newly abundant data enable and facilitate the discovery and use of millions of single nucleotide polymorphisms (SNPs) in diverse genomes. Genotyping by sequencing (GBS) is a next-generation sequencing protocol that has allowed the discovery and genotyping of SNPs in a variety of organisms including *P. infestans*. The discovery of SNPs associated with a trait of interest would allow the development of DNA-based assays for phenotypic traits of interest. In order to enable a genome-wide association study to identify SNP markers

associated with traits of interest, we needed precise phenotypic data on a large number of diverse isolates of *P. infestans*. We chose to assess mating type, pathogenicity on potato and tomato, sensitivity to mefenoxam, the effect of temperature on zoospore release from sporangia, and the effect of temperature on mycelial growth of a diverse panel of *P. infestans* isolates. The panel consisted of i) the dominant clones in the US from the 1990s to 2013, ii) a recombinant population detected in northeastern US in 2010 and 2011, iii) a natural sexual population from Mexico, and iv) an isolate from the Netherlands. For these isolates we initiated a genome-wide association study to identify genetic markers associated with mating type.

4.4 Materials and Methods

4.4.1 Isolates

The isolates used in this study included six US clonal lineages (US-7, US-8, US-11, US-22, US-23, and US-24), 18 isolates that seem to have characteristics of a sexually reproducing population collected in and around west-central New York State in 2010 and 2011(from now on referred to as the NYS-2010/11population) (Danies et al. 2014), one individual from the Netherlands, and 36 isolates collected in Central Mexico where sexual reproduction is ubiquitous. For one of the six US clonal lineages (US-23), six individuals that showed differences in their microsatellite profiles were included. Isolates were maintained on pea agar (Jaime-Garcia et al. 2000) with antibiotics (ampicillin (100 μg ml^{-1}), rifampicin (125 μg ml^{-1}), and pentachloronitrobenzene (25 μg ml^{-1}) and on tomato and/or potato leaflets (depending on the isolate) at 20ºC.

4.4.2 Phenotypic assays

4.4.2.1 Mating type

Mating type was determined by pairing an unknown isolate with a known isolate of *P. infestans,* either A1 mating type (US970001 US-17 genotype) or A2 mating type (US040009, US-8 genotype), on rye B (Caten and Jinks 1968) or pea (Jaime-Garcia et al. 2000) agar media. Negative controls consisted of pairing the unknown isolate with itself. Petri plates were kept at 20°C for 10-14 days. The hyphal interface of the two colonies was investigated microscopically using 125X magnification. Isolates that formed oospores at the interface with the known A1 isolate were designated A2 and those that formed oospores with the known A2 isolate were designated A1. The known isolates (A1 and A2) were paired as positive controls, while negative controls consisted of pairing the known isolates with themselves (same mating type).

Isolation of single zoospores was done for isolates that produced oospores in the presence of both the A1 and the A2 mating type testers as well as in the negative control (unknown isolate paired with itself). To do this, sporangia were washed with sterile distilled water from sporulating lesions on leaflets. The sporangial suspensions were adjusted to 8,000 sporangia per ml using a haemocytometer and maintained at 4ºC for 3 to 4 h to induce zoospore release. Subsequently, inoculations were carried out in 100 mm petri plates containing 20 ml of water agar (0.75%). Three independent petri plates were inoculated with 20, 40, or 100 μl of zoospore suspension of the same isolate. The zoospore suspension was spread using a sterile glass rod. Plates were then kept at 10ºC for 12 h to encourage zoospore germination and subsequently maintained at 15ºC for another 24 h. Individual zoospores were then picked using a sterile scalpel and placed onto pea agar medium. Colonies formed from

single zoospores were again tested for mating type as explained above.

4.4.2.2 Pathogenicity on potato and tomato

A sporangial suspension was used for inoculation of potato and tomato leaflets. Sporangia were washed from sporulating lesions on tomato or potato leaflets, which had been maintained in water-agar moist chambers at 20ºC for 6 to 8 days prior to inoculation. The sporangial suspension was adjusted to 10,000 sporangia per ml using a haemocytometer and maintained at 4ºC for 2 h.

In order to determine differences in pathogenicity on potato and tomato, each isolate was inoculated onto both potato 'Yukon Gold' and tomato 'Rutgers' leaflets. Plants were grown in the greenhouse (ca 25°C daytime and 20°C nighttime) and when four to five weeks old, recently matured leaflets were harvested. Inoculations were carried out in 150 mm petri plates containing 75 ml of water agar (1.5%) in the smaller half – which served as the lid (top). Leaflets were placed (abaxial side up) on the base of the moist chamber. Each moist chamber contained five potato or five tomato leaflets, abaxial side up. All five leaflets were inoculated with 20 μL of a sporangial suspension (described above) of the same isolate, deposited on one side of the main vein of the leaflet. After the leaflets were inoculated, the petri plate was sealed with parafilm and incubated at 15°C with a 16-h light period. Two days after inoculation, inoculum droplets were dried with Kimwipes, subsequently sealed with parafilm and incubated at 20ºC. The experiment was conducted at least twice for each isolate.

Lesion size and number of sporangia per lesion were measured at six days after inoculation. Lesion areas were estimated by taking two perpendicular measurements (length and width) starting from the widest diameter, using a ruler. Subsequently, the number of sporangia produced on each lesion was determined. Individual lesions were excised and

placed into 14-ml disposable polypropylene culture tubes with 3 ml of preservative solution (0.04 M copper sulfate, 0.2 M sodium acetate, acetic acid, pH 5.4) (Spielman et al. 1991). The tubes were then vortexed for 10 seconds to dislodge and suspend sporangia, and aliquots counted with a haemocytometer. Haemocytometer counts were repeated at least twice. The total number of sporangia per lesion was then calculated by averaging all the independent counts.

4.4.2.3 Mefenoxam sensitivity

Mefenoxam sensitivity of isolates was assessed as described previously by Therrien et al. (1993), except that mefenoxam was used in place of metalaxyl. Isolates were grown on pea agar amended with Ridomil Gold SL (Syngenta, Greensboro, NC) such that the final concentrations of the active ingredient (mefenoxam) were 0, 5, or 100 μg ml^{-1}. Mycelial plugs (8 mm diameter) were obtained from actively growing cultures, transferred to the test plates and incubated for approximately 10 to 12 days, or until growth on the control mefenoxam plate (0 μg ml^{-1}) was approximately 75 to 90% of the diameter of the petri plate. Assessment of mefenoxam sensitivity was determined on the basis of radial growth of cultures grown on plates amended with mefenoxam (5 or 100 μg ml^{-1}) compared to non-amended controls. Growth on mefenoxam-amended plates, 5 and 100 μg ml^{-1}, was represented as a proportion of the growth on the non-amended control plates.

4.4.2.4 Rate of indirect germination at 4ºC

Sporangia were observed at 30 and 120 minutes after incubation at 4°C. Inoculation was performed as described above for the pathogenicity on potato and tomato assay except that sporangial suspension was adjusted to 4,000 sporangia per ml and was immediately used to

conduct the rate of indirect germination experiments. Independent measurements of total germination were carried out for each respective time point. That is, a unique slide with three circular water agar droplets that had each been inoculated with 20 μl of the same sporangial suspension was assessed for each time point. This was due to the difficulty of maintaining slides at 4°C while assessing germination microscopically. Percentage of total germination that was indirect was calculated for each of the time points considered. The experiment was conducted at least twice for each isolate.

Effects of time and lineage on zoospore release (indirect germination) were analyzed using JMP Pro 11 (SAS Institute). A mixed effects model was conducted, where time, lineage, and their interaction were fixed effects, and trial and replicate nested within trial were considered random effects. Lineages that exhibited differences in mean indirect germination, within a time period (30 or 120 min), were identified using contrasts and Bonferroni corrected *P*-values.

4.4.2.5 Effect of temperature on mycelial growth

To determine the effect of temperature on mycelia growth, 1 cm diameter plugs of each lineage were placed in a 100 x 15 mm petri plate containing 10 ml of vacuum filtered pea broth. Each replicate consisted of four plates, each incubated at one of four different temperatures (10, 15, 20, and 25ºC) for eight days. Mycelia were subsequently dried using vacuum filtration, frozen at -80ºC, lyophilized, and placed in a drying chamber until it was weighed using a Sartorius A120S analytical balance. Two to six independent replicates were conducted for each isolate.

Effects of temperature and lineage on mycelial growth were analyzed using JMP Pro 11 (SAS Institute). A mixed effects model analysis was conducted, where temperature,

lineage, and their interaction were fixed effects, and replicate nested within lineage was considered a random effect. Because growth at 10ºC for all isolates was significantly less than growth at 15, 20, or 25ºC, we eliminated this temperature from our analyses to detect lineages that exhibited significant growth differences as a function of temperature at 15, 20, and 25ºC. Lineages that exhibited significant growth differences as a function of temperature at 15, 20, and 25ºC were identified using contrasts and Bonferroni corrected *P*-values. These lineages were further investigated using an LSMeans Differences Student's t test.

4.4.3 Genotyping and SNP discovery

Genomic DNA was isolated with a DNeasy® Plant Mini Kit (QIAGEN, Germany). Genotyping-by-Sequencing was performed as described by Elshire et al. (2011) at the Institute of Genomic Diversity (Cornell University). Briefly, genome complexity was reduced by digesting total genomic DNA from individual samples with the typeII restriction endonuclease *Ape*KI, which recognizes a degenerate 5 bp sequence (GCWGC, where W is A or T), and creates a 5' overhang (3 bp). Digested products were then ligated to adaptor pairs with enzyme-compatible overhangs; one adapter contained the barcode sequence and a binding site Illumina sequencing primer. Samples were then pooled, purified, and amplified with primers compatible to the adapter sequences. GBS library fragment-size distributions were checked on a BioAnalyzer (Agilent Technologies, Inc., USA). The PCR products were quantified and diluted for sequencing on the Illumina HiSeq 2500 (Illumina Inc., USA).

Samples sequenced in triplicates or duplicates (for five US-23 isolates, two Mexican isolates, and one isolate from Netherlands) served as technical replicates. Each of two 96-well plates, were multiplexed on a single Illumina flow cell lane. This comprised a total of 192

samples (including two blanks as controls). The GBS discovery pipeline for species with a reference genome, available in TASSEL (version 3.0.166 Date: April 17, 2014) (Bradbury et al. 2007), was used. Sequence reads were mapped against the *P. infestans* T30-4 draft genome sequence downloaded from the Broad Institute. After merging triplicates or duplicates we ended up with 66 *P. infestans* isolates and 570,192 SNPs. We subsequently filtered for proportion of missing data < 0.2 and for a minor allele frequency > 0.1. The resulting number of SNPs after filtering was 98,013. Filtered SNPs were used for analysis of population structure and marker trait associations. To estimate population structure in our panel, a Principal Coordinate Analysis (PCoA) was performed.

4.4.4 Genome-wide association analysis for mating type

Four statistical models comprising both general linear models (GLM) and mixed linear models (MLM) were used to calculate *P*-values for associating each marker with the trait of interest. We accounted for population structure to avoid spurious associations by TASSEL. The first five principal coordinates that together explained approximately 32% of the cumulative variance of all markers were included in the model as the Q-matrix. Kinship was calculated as described in Endelman and Jannink (2012) using the non-shrunk version. A kinship matrix (K-matrix) was computed in TASSEL using 5,007 randomly selected SNP markers. This matrix provides the estimated membership coefficients for each isolate in each of the subgroups. The K-matrix was used for population correction in the association models.

Results were compared to determine the best model for our analysis. The following models were tested: i) Naive model: GLM without any correction for population structure; ii) P-model: GLM with the Q-matrix as correction for population structure; iii) K-model: MLM

with K-matrix as correction for population structure, and iv) PK-model: MLM with Q-matrix and K-matrix as correction for population structure. The critical *P*-values for assessing the significance of SNPs associated with mating type were calculated with Bonferroni correction.

4.5 Results

4.5.1 Phenotypic assays

4.5.1.1 Mating type

Results for mating of each of the 66 isolates used are shown in Supplementary Table 1. In total there were 30 lineages of A1 mating type and 24 lineages of A2 mating type. For six of the 36 Mexican isolates assayed, oospores were observed in the negative controls (isolates paired against themselves) (Supplementary Table 4.1). Single-zoospore (uninucleate) isolates derived from 'self-fertile' strains paired against A1 and A2 mating type testers were also able to 'self-fertilize'. Pea agar plates (where isolates were routinely maintained) of these six Mexican isolates were further assessed for oospore production. On these plates isolates were not paired against themselves or against other isolates. Yet, production of oospores was again observed.

4.5.1.2 Pathogenicity on potato and tomato

Pathogenicity on potato and tomato was assessed for a subset of isolates within our panel (three isolates representing dominant clones in the US from the 1990s to 2013, ten isolates from a recombinant population detected in northeastern US in 2010 and 2011, seven isolates from a natural sexual population from Mexico, and one isolate form the Netherlands). Differences in pathogenicity on potato and tomato were observed among the 21 different *P*.

infestans genotypes studied. Clonal lineage US-24, 7 isolates from Mexico, and one isolate from the Netherlands, showed a strong preference for potatoes (Figure 4.1). In contrast, clonal lineage US-7, and all isolates from the NYS-2010/11 population seemed to grow equally well on both potatoes and tomatoes (Figure 4.1). Lesion size ranged from 14.74 cm^2 (MX-02, on potato) to 0.49 cm^2 (MX-04, on tomato). For all isolates studied lesion size was greater on potatoes than on tomatoes. For the seven Mexican isolates studied, lesion area on tomatoes was restricted to the place where the inoculum drop was deposited. Sporulation ranged from 53,389 sporangia per ml (MX-02, on potato) to 0 sporangia per ml (MX-01, MX-03, and MX-13, on tomato).

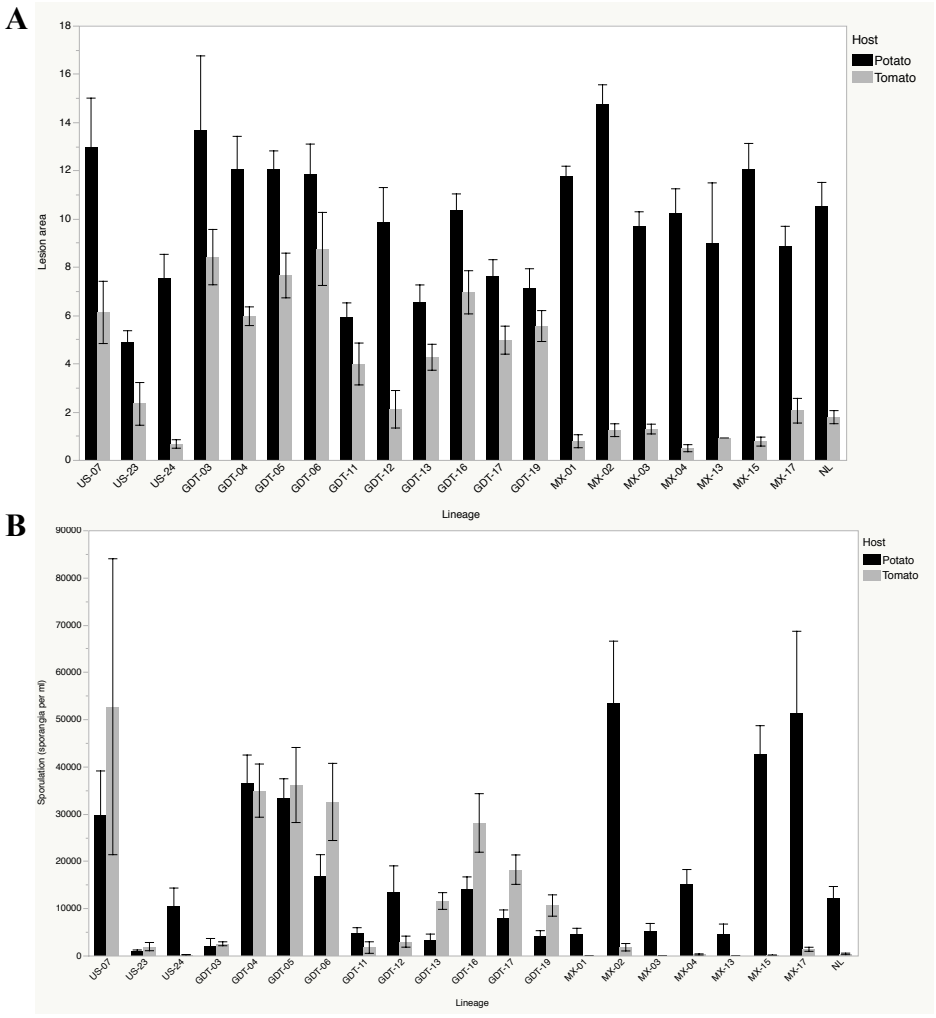


Figure 4.1 Pathogenicity on potato and tomato for isolates of *Phytophthora infestans* from the US, Mexico and the Netherlands. **A.** Lesion areas (cm^2) produced on potato and tomato. **B.** Sporangia produced per infection site on potato and tomato. There was one isolate per genotype. Error bars represent on standard error from the mean. Lesion areas and sporulation were measured 6 days post inoculation.

4.5.1.3 Mefenoxam sensitivity

Sensitivity to mefenoxam was assessed for 65 of the 66 isolates (isolate MX-36 was not included due to its slow growth rate). In general, the US standards used showed the response expected (Figure 4.2A and Supplementary Table 4.1). Isolates belonging to clonal lineages US-7 and US-11 were highly resistant; the isolate belonging to clonal lineage US-8 displayed an intermediate resistance, and isolates belonging to clonal lineages US-22, US-23, and US-24 where generally sensitive to mefenoxam. The NYS-2010/11 isolates were mostly sensitive to mefenoxam (Figure 4.2B and Supplementary Table 4.1). In contrast, isolates from Mexico showed a wide variety of response to mefenoxam (Figure 4.2C and Supplementary Table 4.1). Eight isolates were resistant to mefenoxam, nine displayed an intermediate phenotype, and 18 were sensitive. For two isolates, MX-23 and MX-27, mycelial growth seemed to be enhanced by the presence of mefenoxam.

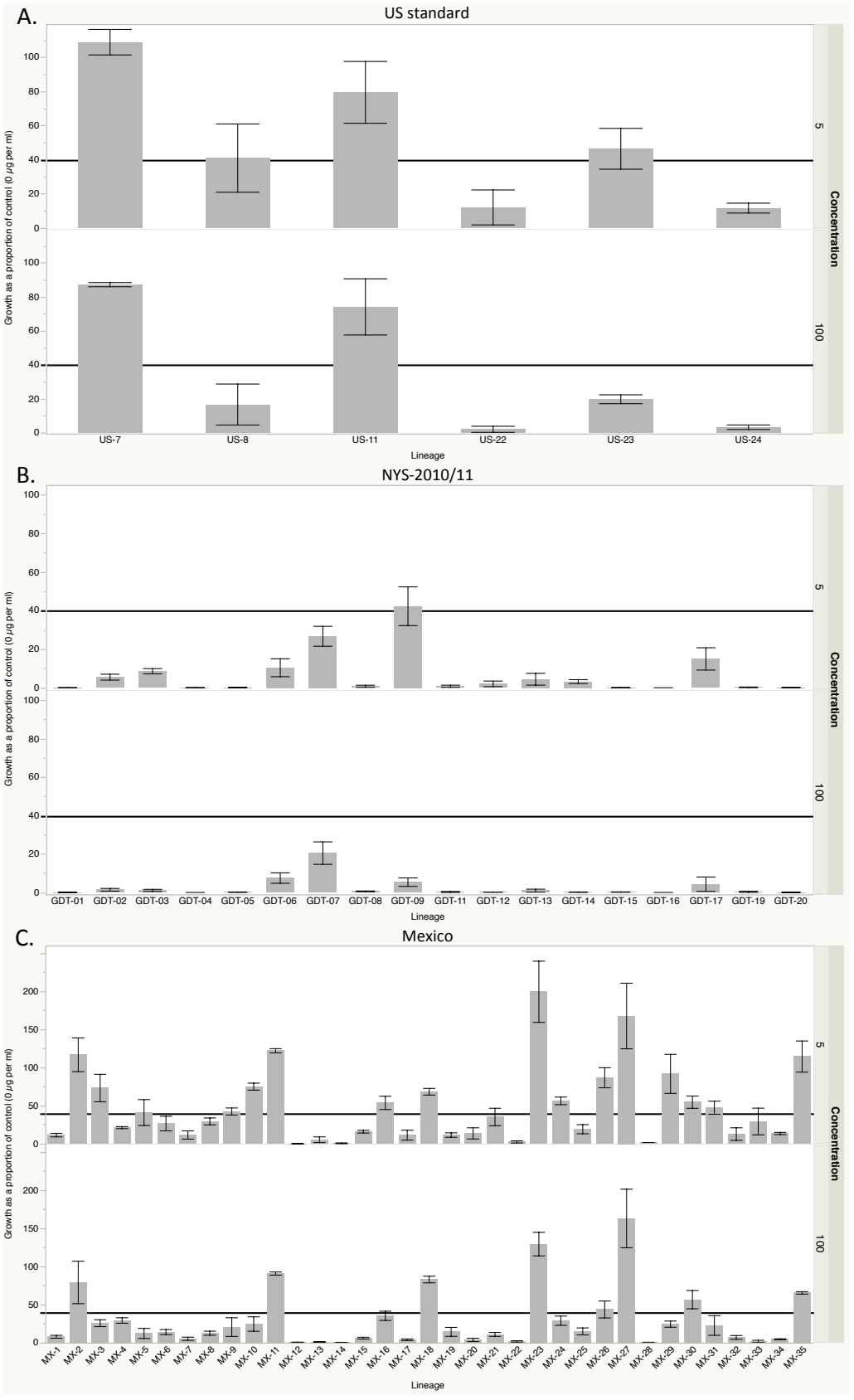

A.
US standard
Growth as a proportion of control (0 μg per ml)
Concentration
5
100
US-7
US-8
US-11
US-22
US-23
US-24
Lineage
B.
NYS-2010/11
GDT-01
GDT-02
GDT-03
GDT-04
GDT-05
GDT-06
GDT-07
GDT-08
GDT-09
GDT-11
GDT-12
GDT-13
GDT-14
GDT-15
GDT-16
GDT-17
GDT-19
GDT-20
C.
Mexico
MX-1
MX-2
MX-3
MX-4
MX-5
MX-6
MX-7
MX-8
MX-9
MX-10
MX-11
MX-12
MX-13
MX-14
MX-15
MX-16
MX-17
MX-18
MX-19
MX-20
MX-21
MX-22
MX-23
MX-24
MX-25
MX-26
MX-27
MX-28
MX-29
MX-30
MX-31
MX-32
MX-33
MX-34
MX-35

Figure 4.2 Response of *Phytophthora infestans* isolates to mefenoxam. **A.** Isolates belonging to six US clonal lineages that have been prevalent at one time or that are prevalent today in the US; **B.** Eighteen isolates that seem to have characteristics of a sexually reproducing population collected in and around west-central New York State in 2010 and 2011; and **C.** Thirty-five isolates collected in Central Mexico where sexual reproduction is ubiquitous. Relative growth (as percentage of control) at 5 μg ml^{-1} (top) and 100 μg ml^{-1} (bottom) relative to control (0 μg ml^{-1}). Isolates are described as resistant when growth is more than 40% relative to the control (0 μg ml^{-1}) on mefenoxam-amended plates (5 and 100 μg ml^{-1}), intermediate when growth is more than 40% relative to the control (0 μg ml^{-1}) on 5 μg ml^{-1} mefenoxam-amended plates but less than 40% relative to the control (0 μg ml^{-1}) on 100 μg ml^{-1} mefenoxam-amended plates, and sensitive when growth is less than 40% relative to the control on both 5 and 100 μg ml^{-1} mefenoxam amended plates. Error bars represent 1 standard error from the mean.

4.5.1.4 Rate of indirect germination at 4ºC

The rate of indirect germination was assessed for a subset of isolates within our panel (13 isolates belonging to the NYS-2010/11 population and 13 isolates from Mexico). Varied responses were observed among the isolates studied. Sporangia of MX-3 and MX-4 released zoospores more rapidly than did sporangia of the other isolates. For example, within 30 min at 4ºC, approximately 75% of the MX-3 and 52% of the MX-4 sporangia had liberated zoospores, whereas the average percentage of sporangia that had released zoospores for all isolates studied was approximately 20%. Within 30 min of incubation at 4ºC, zoospore release

was fastest for isolates MX-3, MX-4, MX-19, and MX 26. Zoospore release among these isolates did not differ significantly ($P > 0.05$). For 14 isolates zoospore release was significantly slower (within the first 30 min of incubation at 4ºC) than that observed for isolates MX-3, MX-4, MX-19, and MX-26. These isolates were GDT-5, GDT-6, GDT-7, GDT-12, GDT-15, GDT-16, MX-1, MX-2, MX-8, MX-16, MX-17, and MX-23.

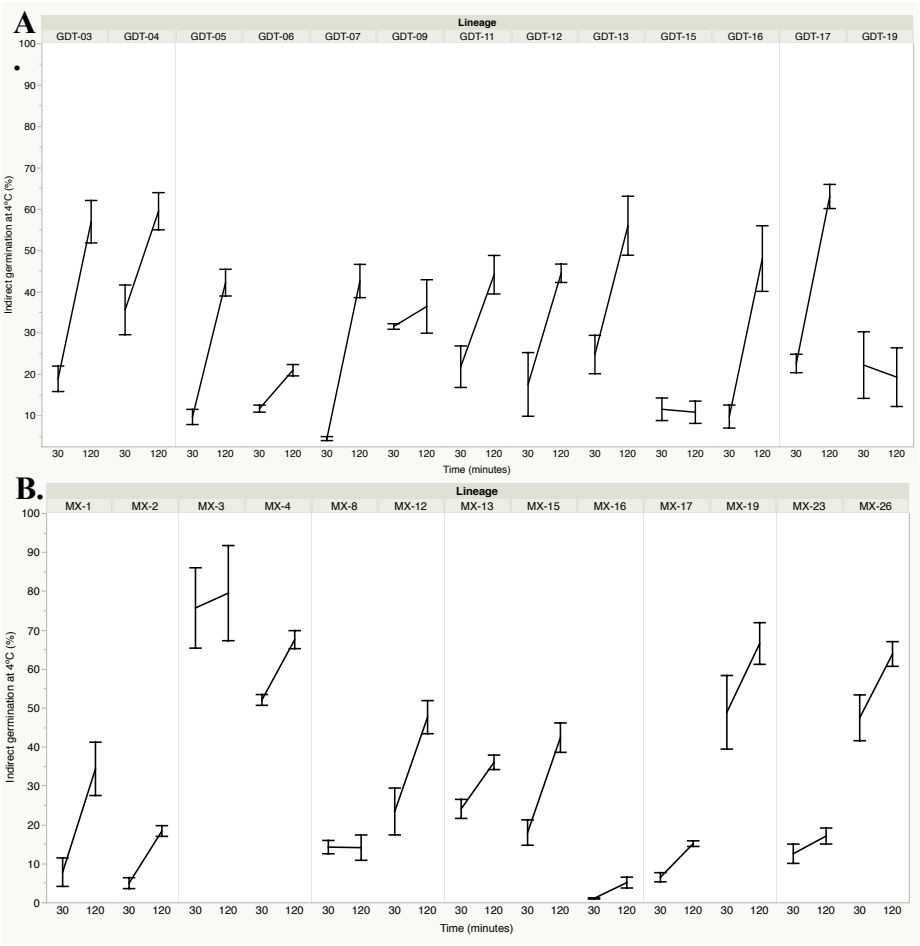


Figure 4.3 Proportion of sporangia that had germinated at 30 and 120 min at 4ºC. **A.** Thirteen isolates that seem to have characteristics of a sexually reproducing population collected in and around west-central New York State in 2010 and 2011; and **B.** Thirty-six isolates collected in Central Mexico where sexual reproduction is ubiquitous. Error bars represent one standard error from the mean.

4.5.1.5 Effect of temperature on mycelia growth

The effect of temperature on mycelia growth was assessed for 56 of the 66 isolates. Differences in mycelial growth in response to temperature were found ($P < 0.0001$). Mycelial growth at 10˚C was consistently less than growth at 15, 20 or 25ºC for all isolates studied (Figure 4.2). We thus excluded this temperature from our analyses and proceeded to investigate differences in mycelial growth at 15, 20, and 25ºC. Differences in response to temperature within an isolate were analyzed. *P* values were adjusted for multiple testing using a Bonferroni correction. Six out of the 56 isolates evaluated (Supplementary Table 4.2) showed significant differences between 15, 20 and 25ºC ($P < 0.0009$). Isolates MX-3 and MX-15 grew significantly less at 25ºC than at either 15 or 20ºC; Isolate MX-13 grew significantly better at 15ºC than at either 20 or 25ºC; Isolate MX-35 grew significantly less at 15ºC than at either 20 or 25ºC; and isolates MX-21 and MX-34 grew significantly better at 20ºC than at either 15 or 25ºC. With a less conservative *P*-value ($\alpha = 0.05$) 12 additional isolates (US-8, US-22, US-23, GDT-05, GDT-16, MX-1, MX-2, MX-5, MX-9, MX-18, MX-28, and MX-23) also showed differences in mycelial growth between temperatures (15, 20, and/or 25ºC) (data not shown). Average dry weights six days after incubation for all isolates assessed are shown in Supplementary Table 4.2 and Supplementary Figure 4.1.

4.5.2 Genotyping and SNP discovery

The total number of tags before merging was 2,285,465. Out of the total number of tags 1,163,220 (50.9%) were aligned to unique positions, 913,010 (39.9%) were aligned to multiple positions, and 209,235 (9.2%) could not be aligned to the reference genome. Triplicates or duplicates were merged resulting in 66 *P. infestans* isolates and 570,192 SNPs.

After filtering we ended up with 98,013 high quality SNP markers. Twenty-nine thousand five hundred SNP markers were located in regions containing genes. These were distributed within 7,783 genes. Thus, 43% of the annotated genes contained SNP markers. The number of SNPs within gene regions and the number of SNPs located in intergenic regions are found in Figure 4.4.

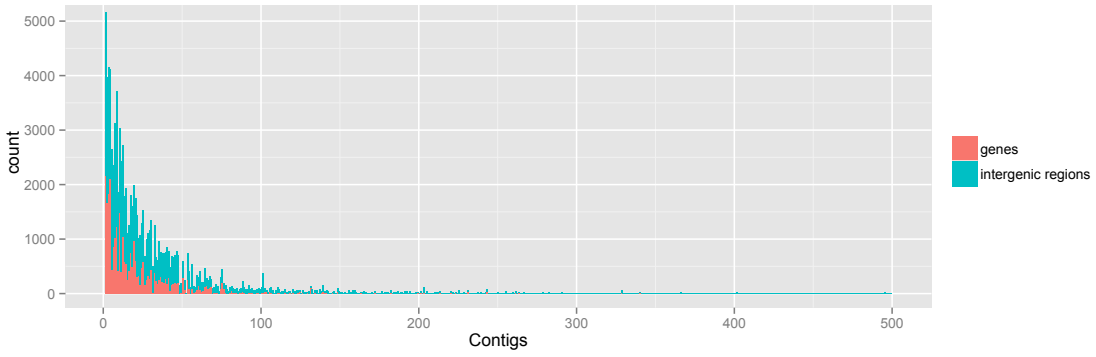


Figure 4.4 Number of SNP markers located within gene regions in red and SNP markers located in intergenic regions in blue. The first 500 scaffolds out of 4,921 are shown in this figure.

4.5.3 Population structure

A principal coordinate analysis was performed to provide spatial representation of the relative genetic distances among isolates of *P. infestans* included in this study (Figure 4.5). The first two principal coordinates explained 14.9 and 5.8 percent of the total variation, respectively. The first principal coordinate separated isolates belonging to the NYS-2010/11 population and clonal lineage US-22 from all other isolates. The Mexican isolates formed a single cluster that included clonal lineages US-7, US-8, US-11, and US-24. The second principal coordinate further separated isolates into three groups: 1) individuals belonging to clonal lineage US-23,

2) the isolate from the Netherlands, and 3) all other isolates.

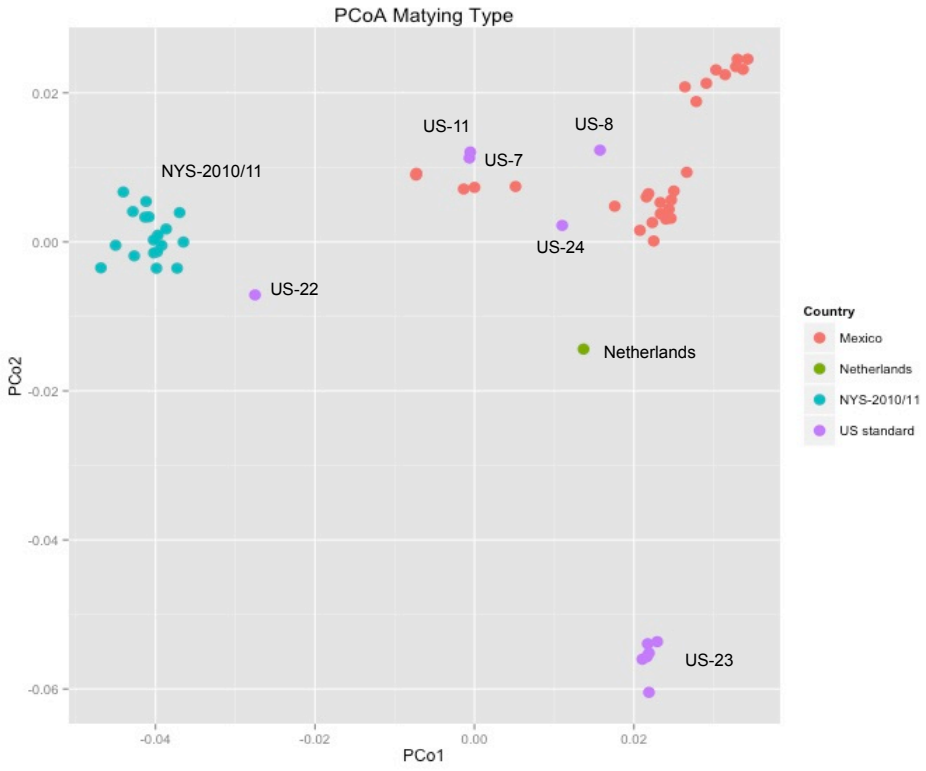


Figure 4.5 A two-dimensional plot of the Principal Coordinate Analysis (PCoA) of *Phytophthora infestans* isolates from Mexico, the Netherlands, and the US. The first and the second principal coordinates account for 14.9 and 5.8 of total variation, respectively.

4.5.4 Genome-wide association analysis for mating type

We tested four different models to detect associations between SNP markers and mating type. The quantile-quantile (QQ) plot of the observed *P*-values revealed a good overall fit with the mixed linear model (MLM), given that most of the *P*-values observed followed a uniform distribution, and only a few markers were in linkage disequilibrium with the causal polymorphism (Figure 4.6). The Q-matrix does not seem to affect the association results in either the GLM or the MLM.

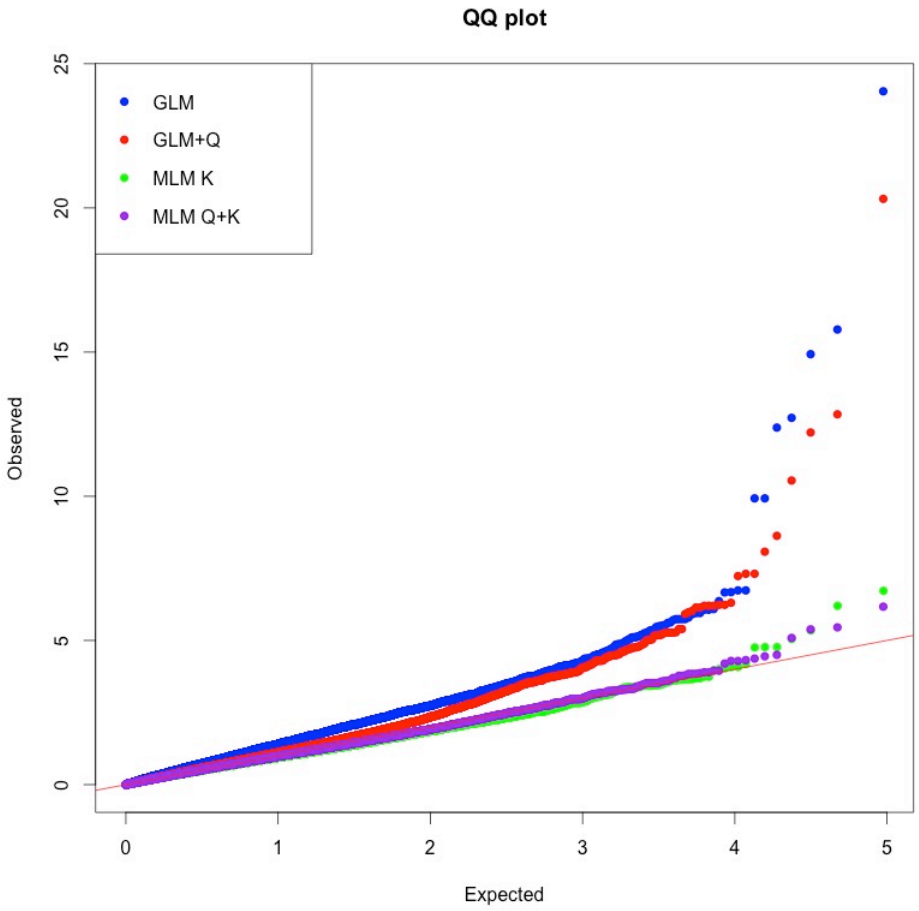


Figure 4.6 QQ-plot for the genome-wide association study of the mating type trait in *Phytophthora infestans*.

The eleven SNP markers with lowest *P*-value associated with mating type in *P. infestans* were located in scaffolds 1, 6, 8, 10, 23, 69 and 109 (Figure 4.7). The closest genes within a 1000 kb flanking region upstream and downstream of each significant SNP are shown in Table 4.1.

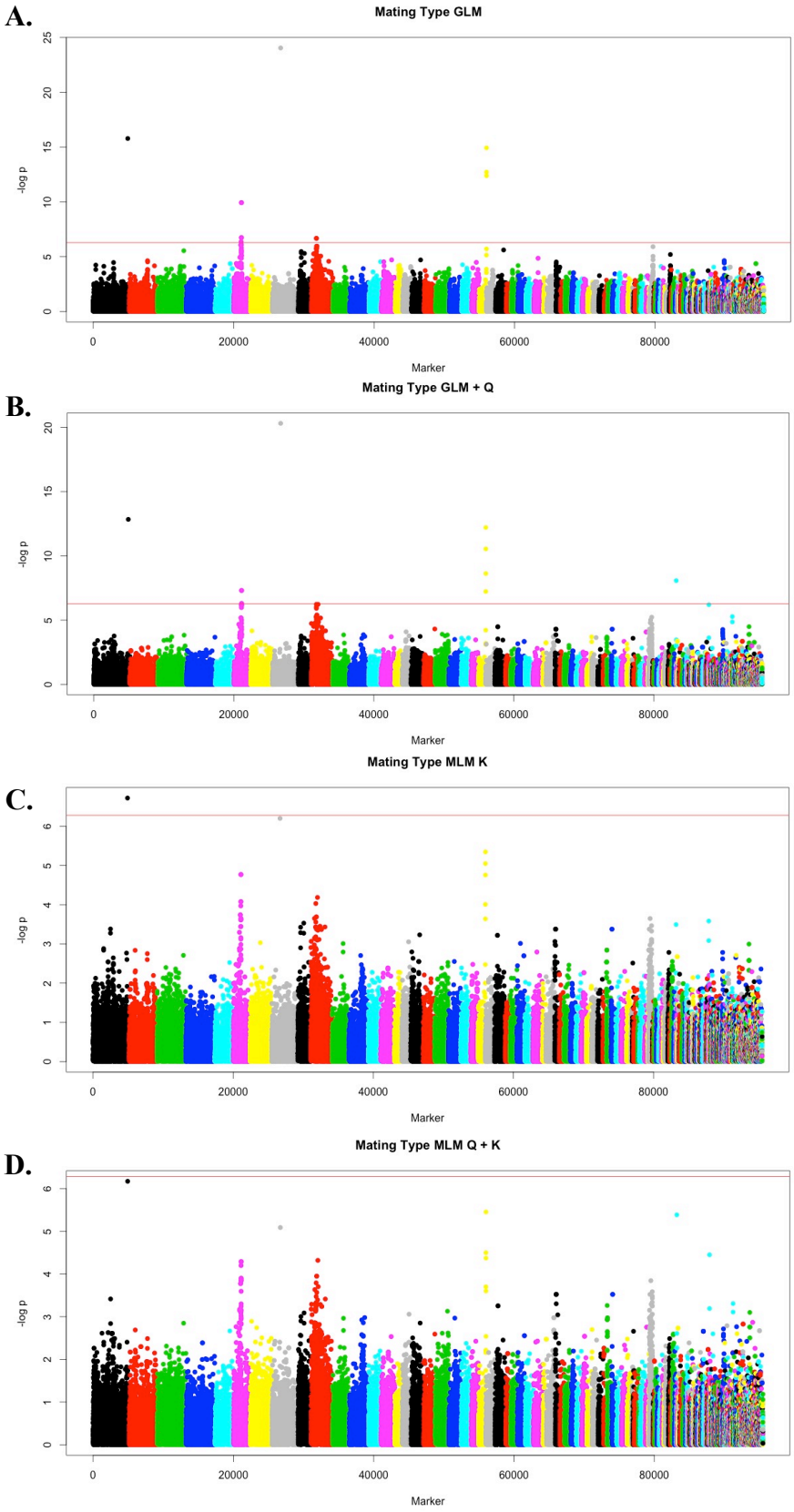

A.
Mating Type GLM
-log p
Marker
B.
Mating Type GLM + Q
-log p
Marker
C.
Mating Type MLM K
-log p
Marker
D.
Mating Type MLM Q + K
-log p
Marker

Figure 4.7 Manhattan plot of the genome-wide association study for the mating type trait in *Phytophthora infestans*. The *y-axis* represents the –log10 *P*-value from the association analysis of mating type. **A.** Naive model: GLM without any correction for population structure; **B.** P-model: GLM with PCs as correction for population structure; **C.** K-model: MLM with K-matrix as correction for population structure, and **D.** PK-model: MLM with PCs and K-matrix as correction for population structure. The critical *P*-values for assessing the significance of SNPs associated with mating type were calculated based on the Bonferroni correction indicated with the red horizontal reference line.

Table 4.1 Eleven lowest *P*-value association hits for mating type in *P. infestans*. Genes that are found within 1000 kb flanking region upstream and downstream of each significant SNP are shown.

SNP_id	Contig	Position	P-value	Genotype	Nearest Gene (distance (kb))[a]	Gene Name
S1_6344790	1	16344790	6.74E-07	T/C	PITG_01099 (520)	Elongator complex protein 3
					PITG_01100 (468)	Aquaporin, putative
S6_1726999	6	1726999	6.32E-05	A/G	PITG_04646 (0)	ATP-binding Cassette (ABC) Superfamily
S6_1745996	6	1745996	5.14E-05	T/C	Gypsy-26_PIT-I-int (0)	
S6_1746014	6	1746014	5.14E-05	A/C	Gypsy-26_PIT-I-int (0)	
S8_1236554	8	1236554	8.16E-06	T/G	GypsyPi-1c_I-int (0)	
S10_1127802	10	1127802	4.80E-05	A/C	PITG_06892 (25)	Conserved hypothetical protein
S23_2342979	23	2342979	3.16E-05	T/A	PITG_11524 (358)	Serine protease family S10, putative
					PITG_11525 (140)	Serine protease family S10, putative
S23_2351550	23	2351550	3.52E-06	G/A	PITG_11528 (488)	Aldose 1-epimerase, putative
S23_2353398	23	2353398	4.25E-05	A/G	NA	NA
S69_338163	69	338163	4.12E-06	T/C	PITG_18524 (374)	Hypothetical protein
S109_232610	109	232610	3.55E-05	C/T	NA	NA

[a] Distance from SNP marker. Distance equal to 0 means that the SNP of interest is included within the gene. NA: Information not available.

4.6 Discussion

Several important phenotypic characteristics of a diverse panel of *P. infestans* isolates were determined. These isolates were initially defined on the basis of mating type and microsatellite markers but subsequently refined by the addition of approximately 98,000 SNP markers obtained through genotyping-by-sequencing. The phenotypic traits studied were mating type, pathogenicity on potato and tomato, sensitivity to mefenoxam, the rate of indirect germination at 4ºC, and the effect of temperature on mycelial growth. Among this diverse panel of isolates we identified individuals of the A1 and the A2 mating type. Interestingly, six of the 36 Mexican isolates assayed seemed to be 'self-fertile'. There are two possible explanations for this: 1) the strain being assessed consisted of a mixture of two or more diverse genotypes of *P. infestans*, or 2) the isolate was able to 'self-fertilize' as has been previously reported (Goodwin and Drenth 1997, Savage et al. 1968, Smart et al. 1998). To test the first hypothesis, single-zoospore (uninucleate) isolates derived from 'self-fertile' strains were paired against A1 and A2 mating type testers. The derived single-zoospore isolates were also able to 'self-fertilize', thus a mixture of two or more genotypes cannot explain this phenomenon. To test for the second hypothesis, pea agar plates (where isolates were routinely maintained) were further assessed for oospore production. Production of oospores was again observed and thus the second hypothesis that these isolates are 'self-fertile' cannot be rejected.

Pathogenicity on potato and tomato differed among the isolates evaluated. All isolates tested were able to produce symptoms and sporulate on potato leaflets but only a subset of the isolates was capable of sporulating on tomato leaflets. In general, genotypes US-8 and US-24, as well as the isolates from Mexico and the isolate from the Netherlands showed a strong

preference for potato and were not at all aggressive on tomatoes. Genotypes US-7 and US-11 as well as the rare and diverse genotypes detected in the Northeast in 2010 and 2011 were pathogenic on both potato and tomato.

A broad range of responses to sensitivity to mefenoxam was observed among the panel of isolates studied. The most remarkable differences were observed within the Mexican isolates, where sensitivity ranged from extremely sensitive (no growth in the presence of mefenoxam) to highly resistant (where mycelial growth was enhanced by the presence of mefenoxam). Isolates from the NYS-2010/11 population were in general sensitive to mefenoxam.

The rate at which sporangia released zoospores differed between isolates. As had been previously reported by Danies et al. (2013), striking differences were observed in the percentage of sporangia capable of releasing zoospores within 30 minutes of incubation at 4ºC. For the panel of isolates included in this study, sporangia that had released zoospores within 30 minutes of incubation at 4ºC, ranged from approximately 2% to 80%.

Differences in mycelial growth were observed at different temperatures. For all isolates studied, growth at 10ºC seemed to be greatly stunted. The vast majority of isolates (50) were not significantly different from each other in terms of mycelial growth at 15, 20 or 25ºC. However, six isolates were significantly different from the others.

Up until today, the population structure of *P. infestans* in the US has been simple, composed mostly of a few clonal lineages (Fry et al. 2013). Therefore, in the US, it has been possible to phenotype individual clonal lineages (Danies et al. 2013), and provide information to farmers that would allow them to make informed management decisions. Yet, sexual reproduction events have been reported (Danies et al. 2014, Gavino et al. 2000), and it is just

a matter of time until sexual reproduction becomes ubiquitous. If this is to happen, the diversity of the pathogen would increase and consequently it would be challenging to obtain phenotypic data on time to be able to advise famers. Our ultimate goal is to develop a DNA-based assay that would allow the identification of phenotypic traits of interest. As a first step to accomplish this goal, it is important to understand the genetic basis behind such phenotypic traits.

In this study we initiated a genome-wide association study for the mating type trait in *P. infestans*. Eleven SNP markers gave association hits for mating type with a $P < 1e\text{-}5$. Further analyses are needed to confirm these associations. As far as we know none of these genes has been associated with the mating type trait in *P. infestans*. Association analyses for the other phenotypic traits investigated will be challenging. This is mainly due to the complexity of the genome, the low number of individuals and the difficulty in obtaining precise phenotypic data. Many of the phenotypic traits included in this study may be polygenic with small effect size. Therefore, increasing the sample size will improve the power to recover meaningful associations.

4.7 Acknowledgements

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4.9 Supplemental Material

Supplementary Table 4.1 Mating type and mefenoxam sensitivity of isolates used in this study.

Population	Lineage	Isolate	Mating type	Mefenoxam sensitivity
US standard	US-7	Coffey7723	A2	R
	US-8	US100048	A2	I
	US-11	US110160	A1	R
	US-22	US110002	A2	S
	US-23	US110059	A1	S
	US-23	2010_8106A	A1	S
	US-23	BL2009P4	A1	S
	US-23	US120096	A1	S
	US-23	US120143	A1	S
	US-23	US100016	A1	S
	US-24	US110157	A1	S
NYS-2010/11	GDT-01	US110084	A1	S
	GDT-02	US110064	A2	S
	GDT-03	US110086	A2	S
	GDT-04	US110074	A2	S
	GDT-05	US110061	A1	S
	GDT-06	US110093	A2	S
	GDT-07	US110072	A2	S
	GDT-08	US110071	A1	S
	GDT-09	US110082	A2	I
	GDT-11	US110085	A1	S
	GDT-12	US100029	A1	S
	GDT-13	US100023	A2	S
	GDT-14	US110054	A1	S
	GDT-15	US100032	A2	S
	GDT-16	US100033	A2	S
	GDT-17	US110092	A2	S
	GDT-19	US100019	A1	S
	GDT-20	US100034	A1	S
Mexico	MX-1	MX107	A1	S
	MX-2	MX73	A1	R
	MX-3	MX62	Selfing	I
	MX-4	MX84	A2	S
	MX-5	Tlax_713	A1	I
	MX-6	Tlax_739	A2	S
	MX-7	1949	A2	S
	MX-8	1633	A1	S
	MX-9	1632	A1	I
	MX-10	5707	A2	I

	MX-11	CH32	A1	R
	MX-12	1639	A1	S
	MX-13	1645	A2	S
	MX-14	1647	Selfing	S
	MX-15	1653	Selfing	S
	MX-16	1628	Selfing	R
	MX-17	1631	Selfing	S
	MX-18	F_02_17	A2	R
	MX-19	1970	A2	S
	MX-20	1655	A1	S
	MX-21	1657	A1	I
	MX-22	1662	A1	S
	MX-23	MX010006	A1	R
	MX-24	PUC_38	A1	I
	MX-25	MICH_7012	A1	S
	MX-26	MICH_7038	Selfing	R
	MX-27	3407	A2	R
	MX-28	MKH7045	A2	S
	MX-29	MX010046	A2	I
	MX-30	MX980046	A1	R
	MX-31	MX010007	A1	I
	MX-32	MX980137	A2	S
	MX-33	MX980221	NA	S
	MX-34	MX980352	A1	S
	MX-35	MX4683	A1	R
	MX-36	MXXX0051	A1	NA
Netherlands	NL	NL	A1	S

R: Resistant – growth on 5 and 100 μg per ml^{-1} of mefenoxam was greater than 40% relative to the control (0 μg per ml^{-1}).

I: Intermediate – growth on 5 μg per ml^{-1} was greater than 40% relative to the control (0 μg per ml^{-1}) but less than 40% relative to the control (0 μg per ml^{-1}) at 100μg per ml^{-1}.

S: Sensitive - growth on 5 and 100 μg per ml^{-1} of mefenoxam was less than 40% relative to the control (0 μg per ml^{-1}).

NA: Nota available

Supplementary Table 4.2 Average dry weight 6 days after incubation at 10, 15, 20 and 25ºC, respectively.

Population	Lineage	Temperature (ºC)	Average dry weight (g)
Us standard	US-7	10	0.00914
	US-7	15	0.04147
	US-7	20	0.04228
	US-7	25	0.03535
	US-8	10	0.00833
	US-8	15	0.05105
	US-8	20	0.05576
	US-8	25	0.03289
	US-11	10	0.00586
	US-11	15	0.03289
	US-11	20	0.04215
	US-11	25	0.04268
	US-22	10	0.00169
	US-22	15	0.03261
	US-22	20	0.06047
	US-22	25	0.04970
	US-23	10	0.00326
	US-23	15	0.03551
	US-23	20	0.04549
	US-23	25	0.02778
	US-24	10	0.01138
	US-24	15	0.04301
	US-24	20	0.05126
	US-24	25	0.03322
NYS-2010/11	GDT-01	10	0.00001
	GDT-01	15	0.00400
	GDT-01	20	0.00920
	GDT-01	25	0.00828
	GDT-02	10	0.00229
	GDT-02	15	0.03172
	GDT-02	20	0.04970
	GDT-02	25	0.03228
	GDT-03	10	0.00840
	GDT-03	15	0.02749
	GDT-03	20	0.03162
	GDT-03	25	0.02899
	GDT-04	10	0.00923
	GDT-04	15	0.04551
	GDT-04	20	0.05215

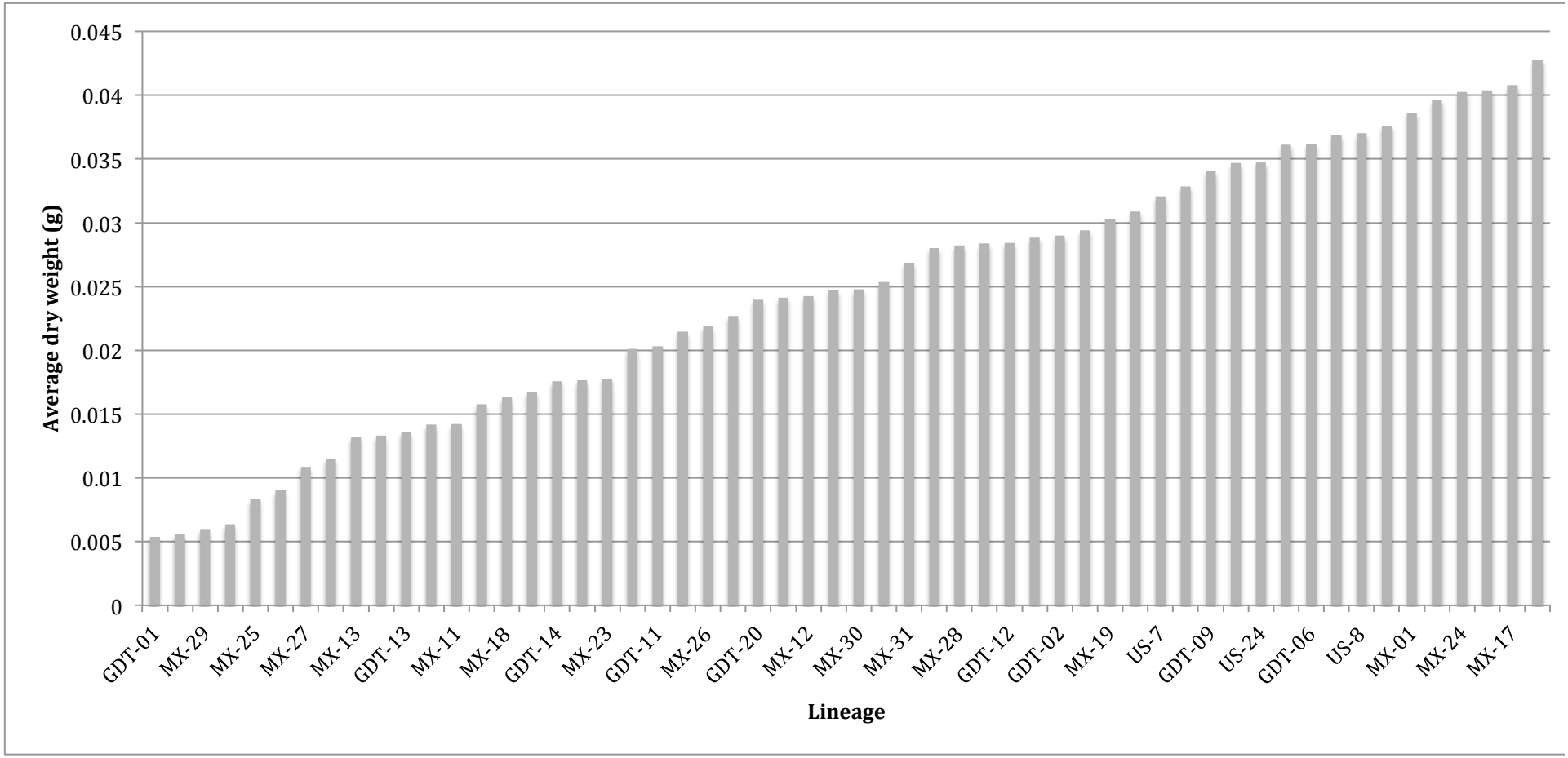
GDT-04	25	0.04349
GDT-05	10	0.00904
GDT-05	15	0.04429
GDT-05	20	0.06720
GDT-05	25	0.04101
GDT-06	10	0.00312
GDT-06	15	0.03455
GDT-06	20	0.05526
GDT-06	25	0.05175
GDT-07	10	0.00203
GDT-07	15	0.01369
GDT-07	20	0.02594
GDT-07	25	0.01508
GDT-08	10	0.00354
GDT-08	15	0.02446
GDT-08	20	0.03036
GDT-08	25	0.03242
GDT-09	10	0.00422
GDT-09	15	0.04500
GDT-09	20	0.04901
GDT-09	25	0.03795
GDT-11	10	0.00529
GDT-11	15	0.02690
GDT-11	20	0.02463
GDT-11	25	0.02437
GDT-12	10	0.00260
GDT-12	15	0.02811
GDT-12	20	0.04344
GDT-12	25	0.03959
GDT-13	10	0.00081
GDT-13	15	0.01505
GDT-13	20	0.02157
GDT-13	25	0.01961
GDT-14	10	0.00649
GDT-14	15	0.01918
GDT-14	20	0.02908
GDT-14	25	0.02233
GDT-15	10	0.00475
GDT-15	15	0.03208
GDT-15	20	0.04625
GDT-15	25	0.03230
GDT-16	10	0.00139
GDT-16	15	0.02154
GDT-16	20	0.04730
GDT-16	25	0.02854

	GDT-17	10	0.00787
	GDT-17	15	0.04135
	GDT-17	20	0.03963
	GDT-17	25	0.02883
	GDT-19	10	0.00972
	GDT-19	15	0.03908
	GDT-19	20	0.04958
	GDT-19	25	0.04036
	GDT-20	10	0.00230
	GDT-20	15	0.02421
	GDT-20	20	0.03330
	GDT-20	25	0.03607
Mexico	MX-01	10	0.01209
	MX-01	15	0.05973
	MX-01	20	0.05346
	MX-01	25	0.02914
	MX-02	10	0.00907
	MX-02	15	0.05568
	MX-02	20	0.05190
	MX-02	25	0.03087
	MX-03	10	0.02004
	MX-03	15	0.06870
	MX-03	20	0.05270
	MX-03	25	0.02961
	MX-04	10	0.00009
	MX-04	15	0.00770
	MX-04	20	0.01180
	MX-04	25	0.00579
	MX-05	10	0.00053
	MX-05	15	0.00596
	MX-05	20	0.02095
	MX-05	25	0.02578
	MX-06	10	0.00007
	MX-06	15	0.00472
	MX-06	20	0.00897
	MX-06	25	0.00877
	MX-07	10	0.00001
	MX-07	15	0.01981
	MX-07	20	0.03331
	MX-07	25	0.03275
	MX-08	10	0.00222
	MX-08	15	0.02369
	MX-08	20	0.02709
	MX-08	25	0.02740
	MX-09	10	0.00049

MX-09	15	0.01393
MX-09	20	0.03767
MX-09	25	0.01501
MX-11	10	0.00048
MX-11	15	0.01450
MX-11	20	0.02533
MX-11	25	0.01662
MX-12	10	0.00177
MX-12	15	0.02557
MX-12	20	0.04108
MX-12	25	0.02863
MX-13	10	0.00419
MX-13	15	0.03299
MX-13	20	0.01026
MX-13	25	0.00557
MX-14	10	0.01013
MX-14	15	0.05635
MX-14	20	0.04350
MX-14	25	0.05277
MX-15	10	0.00055
MX-15	15	0.01991
MX-15	20	0.01316
MX-15	25	0.00075
MX-16	10	0.00119
MX-16	15	0.01489
MX-16	20	0.02787
MX-16	25	0.01917
MX-17	10	0.00737
MX-17	15	0.05579
MX-17	20	0.05315
MX-17	25	0.04686
MX-18	10	0.00110
MX-18	15	0.01116
MX-18	20	0.03120
MX-18	25	0.02177
MX-19	10	0.00280
MX-19	15	0.03804
MX-19	20	0.04393
MX-19	25	0.03652
MX-20	10	0.00394
MX-20	15	0.02936
MX-20	20	0.04852
MX-20	25	0.03164
MX-21	10	0.00033
MX-21	15	0.01684

MX-21	20	0.03930
MX-21	25	0.01420
MX-23	10	0.00242
MX-23	15	0.02394
MX-23	20	0.03056
MX-23	25	0.01426
MX-24	10	0.00589
MX-24	15	0.03633
MX-24	20	0.05402
MX-24	25	0.06471
MX-25	10	0.00010
MX-25	15	0.00743
MX-25	20	0.01251
MX-25	25	0.01324
MX-26	10	0.00204
MX-26	15	0.02338
MX-26	20	0.03873
MX-26	25	0.02334
MX-27	10	0.00021
MX-27	15	0.00796
MX-27	20	0.01950
MX-27	25	0.01582
MX-28	10	0.00610
MX-28	15	0.03579
MX-28	20	0.04663
MX-28	25	0.02437
MX-29	10	0.00068
MX-29	15	0.00694
MX-29	20	0.01010
MX-29	25	0.00629
MX-30	10	0.00275
MX-30	15	0.02909
MX-30	20	0.04070
MX-30	25	0.02656
MX-31	10	0.00371
MX-31	15	0.03664
MX-31	20	0.05328
MX-31	25	0.02266
MX-33	10	0.00009
MX-33	15	0.01164
MX-33	20	0.02404
MX-33	25	0.01032
MX-34	10	0.00340
MX-34	15	0.02402
MX-34	20	0.05960

MX-34	25	0.01437
MX-35	10	0.00391
MX-35	15	0.02367
MX-35	20	0.05763
MX-35	25	0.04615



Supplementary Figure 4.1 Average dry weights for all isolates studied. Average dry weight corresponds to the average dry weight at 10, 15, 20, and 25ºC for each isolate, respectively.

CHAPTER 5*

Acquired resistance to mefenoxam in sensitive isolates of *Phytophthora infestans*

5.1 Abstract

The systemic fungicide mefenoxam has been important in the control of late blight disease caused by *Phytophthora infestans*. This phenylamide fungicide has a negative effect on the synthesis of ribosomal RNA, however, the genetic basis for inherited field resistance is still not completely clear. We recently observed that a sensitive isolate became tolerant after a single passage on mefenoxam-containing medium. Further analyses revealed that all sensitive isolates tested (in three diverse genotypes) acquired this resistance equally quickly. In contrast, isolates that were "resistant" to mefenoxam in the initial assessment (stably resistant) did not increase in resistance upon further exposure. However, there appeared to be a cost associated with acquired resistance in the initially sensitive isolates, in that isolates with acquired resistance grew more slowly on mefenoxam-free medium than did the same isolates that had never been exposed to mefenoxam. The acquired resistance of the sensitive isolates declined slightly with subsequent culturing on medium free of mefenoxam. To investigate the mechanism of acquired resistance, we employed strand-specific RNA sequencing. Many differentially expressed genes were genotype specific, but there was a set of genes differentially expressed in all genotypes. Among these were several genes (a phospholipase "Pi-PLD-like-3", two ATP binding cassette superfamily (ABC) transporters, and a mannitol

* Childers R, Danies G, Myers KL, Fei Z, Small IM, Fry W. 2014. Acquired resistance to mefenoxam in sensitive isolates of *Phytophthora infestans*. Phytopathology 105: 342-349.

dehydrogenase) which were up regulated and whose function might contribute to a resistance phenotype.

5.2 Key words

Phytophthora infestans, potato late blight. phenylamide, mefenoxam, acquired resistance, strand-specific RNA sequencing

5.3 Introduction

Phytophthora infestans is the causal agent of late blight of potatoes and tomatoes and a member of the Oomycota. The late blight disease is one of the most devastating of plant diseases and growers are very concerned about it. Effective management of the disease includes sanitation, host resistance (if available), and appropriate use of fungicides. The high efficacy, systemicity and oomycete specificity of phenylamide fungicides like mefenoxam resulted in their widespread usage soon after their commercial release during the late 1970s (Cohen and Coffey 1986). The phenylamides inhibit rRNA biosynthesis (polymerase complex I) in the target pathogens.

Unfortunately, resistance to mefenoxam appeared during the early 1980s (Davidse et al. 1983, Dowley and O'Sullivan 1981). Such resistance is inherited by progeny, and apparently controlled by one or a few dominant genes (Judelson and Roberts 1999, Lee et al. 1999). Recently a mutation in a subunit of RNA polymerase 1 was demonstrated to be responsible for resistance in a majority of insensitive isolates (Randall et al. 2014). Emergence of resistance was followed by a decrease in the usage of mefenoxam to control late blight (Dowley and O'Sullivan 1985). Interestingly, following the decline in use of

mefenoxam, sensitive populations of *P. infestans* have again been detected (Grünwald and Flier 2005). In very simple clonal populations consisting of a few clonal lineages that have been characterized phenotypically, it is possible to predict mefenoxam sensitivity based on genotypic analysis (Danies et al. 2013). Because genotypic analyses are typically much quicker than phenotypic analyses, genotypic data can be used to inform growers of the likely fungicide sensitivity of the lineages in their region (Danies et al. 2013, Fry et al. 2013).

The sensitivity or resistance of *P. infestans* to mefenoxam is commonly assessed *in vitro* by measuring the radial growth of the pathogen in response to diverse concentrations of the fungicide in amended media (Goodwin et al. 1996, Matuszak et al. 1994). Previously, sensitivity has been defined as at least a 60% reduction in radial growth of colonies grown in agar amended with 5 μg ml^{-1} mefenoxam as compared to colonies grown in mefenoxam-free medium (Danies et al. 2013, Goodwin et al. 1996, Matuszak et al. 1994). The recent predominance in the U.S. of clonal lineages (US-22, US-23 and US-24) that are sensitive to mefenoxam in such assays means that mefenoxam can once again be used to suppress late blight in the U.S. (Fry et al. 2013).

During the course of our *in vitro* assays to determine mefenoxam sensitivity of diverse isolates, we observed that one isolate appeared to become resistant after a single passage through mefenoxam-containing medium. Previous reports indicated that "in vitro" resistance appeared after repeated exposures to sub-lethal doses of mefenoxam (Bruin and Edgington 1981) (Staub et al. 1979), but the speed of adaptation, the generality and potential mechanisms have not been reported. For the purposes of this study, we have defined resistance as the ability of the isolate to grow at a rate greater than 40% of the control at both 5 and 100 μg ml^{-1} mefenoxam. Given the speed of this acquisition, a genetic basis for this

change in resistance seemed improbable, and therefore it seemed more likely that some physiological process had mediated this change. We hypothesized that "acquired resistance" to mefenoxam is a general characteristic of *P. infestans*, that it develops very rapidly upon exposure and that gene expression studies might reveal candidates to explain this phenomenon. Thus, the goals of our study were to confirm the acquired resistance to mefenoxam and to characterize that resistance in diverse genotypes of *P. infestans.* Upon confirmation of the phenomenon, we employed whole-transcriptome sequencing to investigate gene expression differences between initially sensitive isolates and their derivatives with acquired mefenoxam resistance.

5.4 Materials and Methods

5.4.1 Clonal lineages used

The isolates used in this study belonged to four clonal lineages. Different sets of genetic markers (described in Fry et al. 2013) were used to determine the isolate's genotype. The genetic marker used were: 12 microsatellite loci, a restriction fragment length polymorphism assay using a moderately repetitive DNA probe RG57, and an allozyme test using the glucose-6-phosphate isomerase. Furthermore, the mating type of each isolate was determined. With the exception of some minor variations within the microsatellite profiles, these individuals were identical within their assigned clonal lineage for all markers analyzed. Mutations are expected within clonal lineages, especially in rapidly evolving markers such as simple sequence repeats.

There was one isolate of US-8, two isolates of US-22, two isolates of US-23, and three isolates of clonal lineage US-24. In previous assays, isolates of the US-8 clonal lineage had

been identified as resistant (Danies et al. 2013, Goodwin et al. 1996). In contrast, isolates belonging to clonal lineages US-22, US-23 and US-24 had been identified as sensitive (Danies et al. 2013, Hu et al. 2012). For the purposes of this study, we define a sensitive strain as one that grows at less than 40% of the control at both 5 and 100 μg ml^{-1} mefenoxam. Given that isolates of clonal lineage US-8 had previously been found to be consistently and stably resistant to mefenoxam *in vitro*, this US-8 isolate was used as a positive control for mefenoxam resistance. All isolates were cultured on pea agar (Danies et al. 2013) and maintained at 20-22°C. Isolates belonging to clonal lineages US-22 and US-23 are pathogenic to both potato and tomato (Danies et al. 2013), whereas isolates belonging to clonal lineages US-8 and US-24 are pathogens primarily of potato.

5.4.2 Mefenoxam sensitivity assay

Mefenoxam sensitivity was assayed as radial growth on mefenoxam-amended medium, conducted as described previously by Therrien et al. (1993) with the exception that mefenoxam was substituted for metalaxyl. Isolates were grown on pea agar amended with Ridomil Gold SL (Syngenta, Greensboro, NC), which contains 49% mefenoxam as the active ingredient; the final concentrations of the active ingredient were 0, 5, or 100 μg ml^{-1}. Although the use of a dose range to calculate EC50 values could potentially give more insights in the dose response relationship, the technique of using discriminatory dosages (0, 5, or 100 μg ml^{-1} of mefenoxam) has been widely used for over 20 years and adequately serves the current purpose. Due to inherent variation in the rate of growth among isolates, a standard colony diameter on the control plates (0 μg ml^{-1}), rather than a standard incubation time was used to determine the period of incubation for each isolate. Therefore, for each sensitivity

assay, colony diameter on each treatment was measured when the growth of the isolate on the control plates (0 μg ml^{-1}) reached 60 to 70 mm. Subculturing from all isolates was carried out when growth on medium containing mefenoxam was at least 20 mm in diameter. All subculturing for each isolate was done on the same day. Growth on mefenoxam-amended plates at 5 and 100 μg ml^{-1} was presented as a percentage of the growth on the mefenoxam-free control plates.

5.4.3 Initial sensitivity and acquisition of resistance assays

For each isolate, initial sensitivity and occurrence of "acquired resistance" were assessed by determining the sensitivity of an isolate before and after it had been exposed to mefenoxam. To test for initial sensitivity, a subculture from each isolate (with no previous exposure to mefenoxam) was transferred to media containing 0, 5, and 100 μg ml^{-1} mefenoxam (Figure 5.1A). To test for acquired resistance, a subculture from each isolate with prior exposure to mefenoxam (5 or 100 μg ml^{-1}) was assessed for mefenoxam resistance by transferring mycelia to medium containing 0, 5 and 100 μg ml^{-1} mefenoxam and then comparing growth on mefenoxam-containing medium with that on mefenoxam-free medium (Figure 5.1A). To ensure that acquired resistance was not a result of spontaneous mutations, we conducted the experiment at least three times. In addition, subcultures that had never been exposed to mefenoxam were evaluated on media containing 0, 5, and 100 μg ml^{-1} mefenoxam at each transfer stage during the experiment.

Effects of previous mefenoxam exposure (0, 5, and 100 μg ml^{-1}), subsequent mefenoxam exposure (5 and 100 μg ml^{-1}), lineage, and their full factorial interactions, on colony growth were analyzed using JMP 10.0.0 (SAS Institute, Cary, NC, USA). Standard

least square analysis was used, replications were considered random terms, while mefenoxam exposures; lineage and interactions were considered as fixed effects. To determine if means of percent colony growth on mefenoxam-amended plates for each lineage differed between previous exposure concentrations, a Tukey's HSD test with $\alpha = 0.05$ was performed.

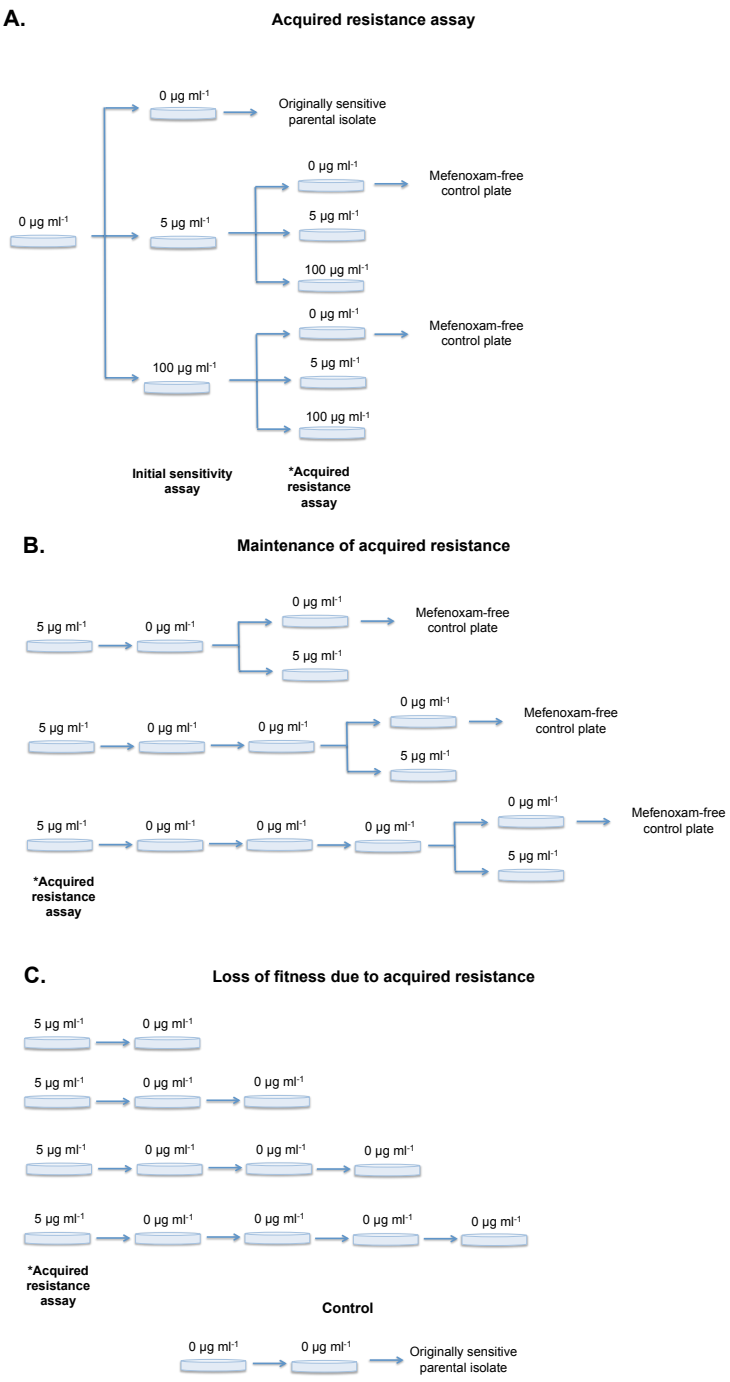


Figure 5.1 Experimental design and culturing sequence. **A,** Acquired resistance was determined by comparing the resistance to mefenoxam of isolates before and immediately after an exposure to mefenoxam. Initial sensitivity to mefenoxam was assessed by transferring isolates that had never been exposed to mefenoxam to 0, 5, and 100 μg ml^{-1} mefenoxam. Isolates that had been exposed to either 5 or 100 μg ml^{-1} mefenoxam were subsequently transferred again to 0, 5 and 100 μg ml^{-1} mefenoxam (*acquired resistance assay). **B,** Maintenance of acquired resistance was assessed by transferring isolates that had been exposed two times through mefenoxam-amended media through a series of one, two or three transfers on mefenoxam-free media. Maintenance of acquired resistance for each isolate was then assessed on mefenoxam-amended media. The same procedure was followed for isolates initially exposed to 100 μg ml^{-1} mefenoxam. **C,** To test for loss of fitness due to acquired resistance, isolates that had been exposed two times to mefenoxam-amended media were transferred one, two, three, or four times on mefenoxam-free media. Growth on mefenoxam-free media of initially sensitive isolates was used as control. This figure illustrates the protocol for isolates exposed to 5 μg ml^{-1} mefenoxam, but the same procedure was followed for isolates initially exposed to 100 μg ml^{-1} mefenoxam.

5.4.4 Maintenance of acquired resistance assays

After an isolate had acquired resistance the maintenance of that resistance was evaluated after repeated subculturing in the absence of mefenoxam (Figure 5.1B). Each isolate was assayed for mefenoxam sensitivity (described above) after one, two and three subcultures on mefenoxam-free medium.

After identifying the group of lineages that had demonstrated acquired resistance (US-

22, US-23, and US-24), effects of previous mefenoxam exposure (0, 5, or 100 μg ml^{-1}), number of transfers through mefenoxam-free medium (1, 2, or 3), subsequent mefenoxam exposure (5 or 100 μg ml^{-1}), and their full factorial interactions on colony growth were analyzed using JMP 10.0.0. Standard least square analysis was used, where isolates and replications were considered random terms, while previous mefenoxam exposure, number of mefenoxam-free transfers, subsequent mefenoxam exposure, and interactions were considered as fixed effects. To determine if relative growth on mefenoxam-amended plates changed after transfers through mefenoxam-free medium, a Tukey's HSD test with $\alpha = 0.05$ was performed.

5.4.5 Slower growth due to acquired resistance

After an isolate had acquired resistance, the growth rate of the isolate was evaluated by measuring colony growth in the absence of mefenoxam after one, two, three and four consecutive subcultures on mefenoxam-free media (Figure 5.1C). Effects of previous mefenoxam exposure (0, 5, or 100 μg ml^{-1}), number of mefenoxam-free transfers (1, 2, 3, or 4) and their interaction on colony growth were analyzed using JMP 10.0.0. Standard least-square analysis was used, where isolates and replications were considered random terms, while previous mefenoxam exposure, number of transfers through mefenoxam-free medium, and their interaction were considered as fixed effects. To determine if means of percent colony growth on mefenoxam-free media differed for each previous exposure concentration, a Tukey's HSD test with $\alpha = 0.05$ was performed.

5.4.6 Whole transcriptome-sequencing

Strand-specific RNA sequencing, following the method of Zhong et al. (2011) was used to examine gene expression differences between non-exposed isolates, and subcultures of the

same isolates after acquisition of resistance to mefenoxam. Isolates analyzed were one individual of US-8 as a stably resistant control, one individual of US-23, and two individuals of US-24. There were two treatments used for each isolate; in the first treatment, the isolate was cultured on pea agar without mefenoxam, and in the second treatment, the isolate was cultured on pea agar containing 100 μg ml^{-1} mefenoxam. These treatments were continued for three successive subcultures. Subsequently, those individuals that had been grown on mefenoxam-free medium were transferred to pea broth free of mefenoxam, and those individuals grown on pea medium with mefenoxam were transferred to pea broth with mefenoxam (100 μg ml^{-1}). For individuals growing in the presence of mefenoxam, the mycelia were harvested after 6 to 12 days. For individuals growing in the absence of mefenoxam, the mycelia were harvested after 4 to 8 days.

The experiment was conducted three times for three biological replications. Each biological replicate was started on a different date. Within each biological replicate there were two technical replicates. Total RNA was extracted using the RNeasy Plus Mini kit (QIAGEN). Twenty-four libraries (one per sample) were prepared following the method described in Zhong *et al.* (2011), and 20 ng of each library were multiplexed and run on an Ilumina HiSeq 2000 via 100-bp single-end read sequencing in a single lane at the Cornell University Sequencing Core Facility.

5.4.7 Bioinformatic and statistical analysis for the RNA-sequencing (RNA-seq)

RNA-seq reads were first aligned to ribosomal RNA and tRNA sequences using Bowtie (Langmead et al. 2009) allowing for two mismatches to remove any possible contaminations of these sequences. The resulting filtered reads were aligned to the draft genome of *P.*

infestans strain T30-4, available from the Broad Institute (*Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org) using TopHat (Trapnell et al. 2009) allowing one segment mismatch. Following alignments, raw counts for each gene were normalized to Reads Per Kilobase of exon model per million mapped reads (RPKM). The raw counts were then processed with the EdgeR package (Robinson et al. 2010) to examine genes that were differentially expressed (DE).

We next searched for differences in gene expression that were common among isolates. To ensure consistency among replicates in the analysis of differential expression, tagwise dispersion estimates were used in all cases (Robinson et al. 2010). The default prior.df value (which moderates the weight placed on tagwise versus common dispersion estimates) of 10 was used for all analyses. EdgeR automatically controls for false positives by controlling the False Discovery Rate (FDR) following the method of Benjamini and Hochberg (1995).

Differential gene expression between an isolate with versus without acquired resistance was detected, and only genes with a FDR lower than 0.05 were retained. Then, differentially expressed genes that were common to the three originally sensitive isolates (one US-23 and two US-24 isolates) were identified (Table 5.1). We then explored the possibility that the same genes are differentially expressed also in the stably resistant US-8 (Table 5.1). Summary statistics were produced with JMP 10.0.0.

Table 5.1 Genes that were significantly differentially expressed in response to mefenoxam in four isolates of *Phytophthora infestans* (one US-8 isolate, one US-23 isolate and two US-24 isolates (US-24A and US-24B)). The data for the individual with acquired resistance were compared to the data for that individual without acquired resistance. The three biological replicates were used to calculate tag-wise gene dispersion estimates, favoring genes that behaved consistently across replicates. These estimates were used in a negative binomial model to estimate differential expression from the raw counts for each isolate. Annotations for genes that were shown to be differentially expressed in all individuals with acquired resistance in response to mefenoxam are shown below.

Gene[a]	Annotation[b]	Log_2FC[c]				FDR[d]				Log_2CPM[e]
		US-8	US-23	US-24A	US-24B	US-8	US-23	US-24A	US-24B	Average
PITG_00923	Phospholipase D, Pi-PLD-like-3	6.92	4.91	4.57	9.78	4.41E-08	2.66E-05	5.77E-04	1.14E-13	3.52
PITG_09160	Secreted RxLR effector peptide, putative	5.63	3.34	7.97	5.2	1.03E-04	1.83E-02	3.74E-04	1.28E-04	2.72
PITG_12458	Secreted RxLR effector peptide, putative	2.27	2.17	3.6	4.67	4.24E-03	7.48E-03	8.78E-07	1.53E-10	0.76
PITG_16256	Conserved hypothetical protein	3.02	2.19	2.88	5.04	1.39E-03	4.42E-02	3.83E-03	2.59E-09	1.44
PITG_09063	Conserved hypothetical protein	NS	3.23	2.85	3.23	NS	3.85E-03	1.79E-02	3.85E-03	1.82
PITG_07501	Crinkler (CRN) family protein	2.89	1.3	3.87	3.61	2.56E-09	1.12E-02	1.33E-17	3.96E-11	2.41
PITG_00147	Conserved hypothetical protein	NS	1.85	2.83	3.61	NS	1.16E-03	2.80E-07	1.41E-12	3.46
PITG_07468	Crinkler (CRN) family protein	2.81	1.13	3.8	3.36	2.76E-08	4.22E-02	3.49E-16	8.51E-10	2.23
PITG_07467	Crinkler (CRN) family protein	2.84	1.13	3.51	3.37	2.76E-08	4.60E-02	6.53E-14	1.15E-09	2.27

PITG_16991	Cell 12A endoglucanase	NS	1.65	1.95	4.04	NS	2.58E-02	1.03E-02	1.25E-10	3.11
PITG_05795	Conserved hypothetical protein*	2.56	2.19	2.14	2.68	7.23E-07	1.10E-04	5.71E-04	3.17E-07	2.80
PITG_22087	ATP-binding Cassette (ABC) Superfamily	1.53	1.94	2.16	2.61	4.46E-02	3.84E-03	1.78E-03	1.45E-05	4.99
PITG_16235	Secreted RxLR effector peptide, putative	3.12	2.65	1.88	1.83	4.55E-05	3.48E-04	1.55E-02	9.94E-03	2.84
PITG_16409	Secreted RxLR effector peptide, putative	3.59	2.51	1.65	2.11	2.73E-08	2.65E-04	1.77E-02	3.80E-04	3.27
PITG_08846	Mannitol dehydrogenase, putative	3.58	2.19	2.27	1.78	1.09E-07	2.39E-03	2.68E-03	1.42E-02	6.01
PITG_12664	Conserved hypothetical protein	1.62	1.79	1.76	2.52	1.14E-02	2.56E-03	1.22E-02	5.18E-06	1.92
PITG_11969	ATP-binding Cassette (ABC) Superfamily	1.34	2.19	1.78	1.76	1.59E-03	1.25E-08	1.34E-05	3.55E-06	6.78
PITG_02772	Conserved hypothetical protein	1.71	2.21	1.31	2.2	6.81E-05	5.27E-07	1.11E-02	2.19E-08	2.90
PITG_15627	Conserved hypothetical protein*	1.52	1.87	1.67	1.75	4.56E-02	6.02E-03	2.00E-02	8.01E-03	2.87
PITG_09065	Conserved hypothetical protein	NS	1.61	1.6	1.79	NS	2.00E-02	3.03E-02	5.01E-03	4.34
PITG_02748	Conserved hypothetical protein*	NS	1.28	1.85	1.49	NS	3.98E-02	2.55E-03	9.43E-03	2.66
PITG_10995	Conserved hypothetical protein*	NS	1.56	1.34	1.15	NS	3.71E-03	2.38E-02	3.90E-02	5.24
PITG_09097	Conserved hypothetical protein	NS	1.32	1.27	1.43	NS	7.39E-03	1.78E-02	1.56E-03	2.89

PITG_15998	Phospholipase A-2-activating protein, putative	NS	-0.92	-0.82	-0.89	NS	1.22E-02	3.92E-02	9.60E-03	5.32
PITG_16013	Conserved hypothetical protein*	NS	-1.01	-1.39	-1.09	NS	3.85E-02	3.16E-03	1.69E-02	3.28
PITG_16794	Di-N-acetylchitobiase, putative	-1.23	-1.24	-0.97	-1.34	7.92E-04	5.59E-04	1.43E-02	5.66E-05	3.61
PITG_10079	Conserved hypothetical protein*	NS	-1.54	-1.64	-1.02	NS	3.63E-04	5.21E-04	2.78E-02	2.90
PITG_16795	Conserved hypothetical protein	-2.17	-1.55	-1.51	-1.94	6.75E-04	2.32E-02	4.05E-02	1.73E-03	4.78
PITG_04948	Conserved hypothetical protein	-2.09	-2.41	-1.61	-1.81	6.80E-04	1.85E-03	4.11E-02	6.13E-03	1.66
PITG_07573	Conserved hypothetical protein*	NS	-2.06	-2.69	-2.61	NS	6.95E-03	1.01E-04	2.83E-05	3.59
PITG_09316	Secreted RxLR effector peptide, putative	NS	-2.07	-2.44	-2.86	NS	2.19E-02	2.35E-03	4.20E-05	1.96
PITG_08344	Conserved hypothetical protein	-4.09	-5.7	-6.28	-4.07	1.91E-03	2.03E-02	7.25E-03	9.52E-03	-0.04

[a]Accession number given to the transcript by the Broad institute (*Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org).
[b]Putative annotated functions of the specified genes.
[c]Log_2 of the fold change in response to mefenoxam exposure.
[d]False Discovery Rate.
[e]Average Log_2 Counts-Per-Million. EdgeR provides only a global average of Log_2 Counts-Per-Million for each gene.
[*]Conserved hypothetical proteins for which the closest annotated match has been listed in Table 5.2.

5.4.8 qRT-PCR to validate the RNA-seq results

To confirm the RNA-seq results, we performed a qRT-PCR for five genes that had a significant differential expression in response to mefenoxam in *P. infestans*. Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Bio-systems, Carlsbad, CA). Total transcript levels were determined by qRT-PCR using the SYBR® Green PCR Master Mix (Applied Bio-systems, Carlsbad, CA), following the manufacturer's protocol.

All genes were assayed in triplicate in 96-well plates and two biological replicates of each treatment were performed. Controls lacking reverse transcriptase and lacking template were included. Results were analyzed with the ABI PRISIM®7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) program and relative expression was calculated using REST 2009 Software (Pfaffl et al. 2002). The genes and primers were as follows: (i) PITG_11969 (ATP-binding cassette superfamily) (FW, GACGCCCAAGAGTAAAGATG; RV, CCGTTAATGCCCTTGAGTAG); (ii) PITG_00147 (Conserved Hypotyhetical Protein) (FW, CAGGAGCTTCAGCAACAG; RV, GCGAAGATGCGGAAGAC); (iii) PITG_00923 (Phospholipase D) (FW, TACCGTTCCCTACCTCATC; RV, GCCATCCCACTGACATTT); (iv) PITG_05795 (Conserved Hypothetical Protein) (FW, GTTGGAGAAGATGAAAGTCAATATG; RV, GTGGGTTGCGGTTCTTT); (v) PITG_22087 (ATP-binding cassette superfamily) (FW, CCTTCTCCAGCGTTTCTTC; RV, CAGAAGAGCATTCCCATACC); (vi) PITG_14461 (Actin-like protein) (FW, CGGTCTATATGGGCCAGAAAT; RV, GGGTCCACCTTCAGCATTT). PITG_14461(Actin-like protein) was used as a constitutively expressed endogenous control. RNA from isolates that had not been exposed to

mefenoxam was used as the calibrator.

5.5 Results

5.5.1 Acquired resistance

In agreement with previous studies, isolates belonging to lineage US-8 demonstrated preexisting resistance to mefenoxam (Figure 5.2). Substantial growth was observed for lineage US-8 growing on medium containing 5 and 100 μg ml^{-1} mefenoxam. For example, at a concentration of 5 μg ml^{-1} US-8 did not differ significantly in growth from its mefenoxam-free control ($P \approx 1.00$). Percent growth relative to the mefenoxam-free control for lineage US-8 was 94 and 65% at 5 and 100 μg ml^{-1}, respectively.

All isolates from clonal lineages US-22, US-23 and US-24 were largely sensitive to mefenoxam. At concentrations of 5 and 100 μg ml^{-1} these three lineages showed significantly reduced growth relative to mefenoxam-free controls as well as to US-8 ($P \leq 0.05$). Isolates from these three clonal lineages that had no previous exposure to mefenoxam had radial growth of 16-29% of the diameter of control plates when grown on 100 μg ml^{-1} mefenoxam (Figure 5.2).

Prior exposure to mefenoxam had a significant effect on subsequent colony growth in the presence of mefenoxam. A significant three-way interaction between prior exposure concentration of mefenoxam, lineage, and subsequent exposure concentration of mefenoxam was observed ($P \leq 0.0001$). All isolates of lineages US-22, US-23 and US-24 became resistant following exposure to mefenoxam at either 5 or 100 μg ml^{-1} ($P \leq 0.05$) (Figure 5.2). For example, without prior exposure to mefenoxam, the isolate of US-22 grew at 50% of the control on 5 μg ml^{-1} mefenoxam, and 29% of the control on 100 μg ml^{-1} mefenoxam. With

prior exposure to 5 µg ml^{-1} mefenoxam, this isolate grew at 85% of the control on 5 and 58% of the control on 100 µg ml^{-1} mefenoxam (Figure 5.2). Without prior exposure to mefenoxam the mean growth on 5 µg ml^{-1} mefenoxam for the two isolates of US-23 was 21% of the control on 5 µg ml^{-1} mefenoxam, and 16% of the control on 100 µg ml^{-1} mefenoxam. With prior exposure to 5 µg ml^{-1} mefenoxam, their mean growth was 79% of the control on 5 µg ml^{-1} mefenoxam and 52% of the control on 100 µg ml^{-1} mefenoxam. Without prior exposure to mefenoxam the mean growth on 5 µg ml^{-1} mefenoxam for the three isolates of US-24 was 42% of the control on 5 µg ml^{-1} mefenoxam and 23% of the control on 100 µg ml^{-1} mefenoxam. With prior exposure to 5 µg ml^{-1} mefenoxam their mean growth was 79% of the control on 5 µg ml^{-1} mefenoxam and 77% of the control on 100 µg ml^{-1} mefenoxam. For isolates belonging to sensitive lineages, increased resistance was also observed with prior exposure to 100 µg ml^{-1} mefenoxam (Figure 5.2). Levels of resistance did not increase following a second exposure to mefenoxam (data not presented).

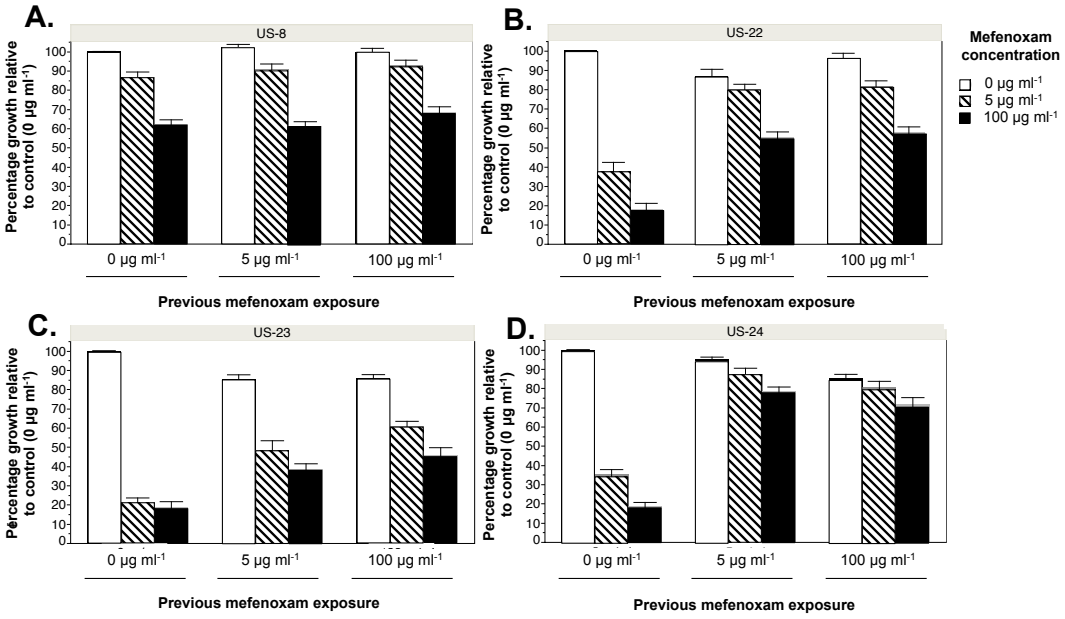


Figure 5.2 Response of four *Phytophthora infestans* lineages to mefenoxam at 0 (open bars), 5 (diagonal lines) and 100 (solid bars) μg ml^{-1}. A previous exposure of 0 μg ml^{-1} mefenoxam means that the isolate had not before been exposed to mefenoxam. A previous exposure of 5 μg ml^{-1} mefenoxam means that the isolate came from a medium containing 5 μg ml^{-1} mefenoxam and a previous exposure of 100 μg ml^{-1} mefenoxam means that the isolate came from a medium containing 100 μg ml^{-1} mefenoxam. US-8 (A) is stably resistant and US-22 (B), US-23(C) and US-24 (D) are regarded as sensitive. There was one isolate of US-8, one isolate of US-22, two isolates of US-23, and three isolates of US-24. Each error bar is constructed using one standard error from the mean.

5.5.2 Maintenance of acquired resistance

The number of transfers through mefenoxam-free media had a significant effect on the maintenance of acquired resistance. Isolates that had been transferred a single time to mefenoxam-free medium tended to grow more slowly in the presence of mefenoxam compared to isolates that had been maintained on mefenoxam-amended medium ($P = 0.11$ for an isolate on 5 μg ml^{-1} mefenoxam and $P = 0.07$ for an isolate on 100 μg ml^{-1} mefenoxam). However, after two successive transfers on mefenoxam-free medium, the previously resistant isolates began to lose their acquired resistance and grew significantly more slowly on mefenoxam-containing medium (5 or 100 μg ml^{-1}) than those consistently maintained on mefenoxam ($P \leq 0.05$).

5.5.3 Slower growth due to acquired resistance

Radial growth of isolates with acquired resistance was less on mefenoxam-free medium than was radial growth of their originally sensitive parental individuals ($P \leq 0.0001$). Mean relative

growth rates for isolates that had been exposed previously to 5 or 100 μg ml^{-1} mefenoxam were 91 and 88 percent, respectively. No significant two-way interaction between previous mefenoxam exposure (0, 5, and 100 μg ml^{-1}) and the number of transfers through mefenoxam-free media (0 μg ml^{-1}) was observed ($P = 0.41$). After a single transfer to mefenoxam-free medium isolates with prior exposure to mefenoxam (both 5 and 100 μg ml^{-1}) showed significantly reduced growth in comparison to isolates that had never been exposed to mefenoxam (0 μg ml^{-1}) ($P \leq 0.0001$). This reduced growth was maintained over three subsequent transfers on mefenoxam-free media. Reduced growth rate due to acquired resistance did not differ significantly between previous exposure to 5 or to 100 μg ml^{-1} mefenoxam ($P = 0.27$).

5.5.4 Whole transcriptome-sequencing

We obtained 177 million reads from sequencing the 24 distinct samples (four isolates, two treatments, three replications). After removal of reads aligning to ribosomal RNA, the number of reads per sample ranged from 5.5 to 9.5 million, of which 74 to 81% were aligned to the T30-4 draft genome to yield between 4.6 and 7.8 million raw counts per sample. Each library contained 14,273 to 15,492 expressed genes. When analyzed individually, isolates were found to have 535 to 1,152 genes differentially expressed with a False Discovery Rate (FDR) of less than 0.05 in response to mefenoxam.

Analysis of the raw counts using the EdgeR package revealed that differential expression clustered largely by clonal lineage when analyzed via Multi-Dimensional Scaling (Figure not shown). There was limited separation by treatment within these clusters, particularly within clonal lineage US-24.

Because of the phenotypic consistency of "acquired resistance" across all sensitive genotypes, we searched for genes that were differentially expressed in all sensitive genotypes in response to exposure to mefenoxam. This search revealed 32 candidate genes that were significantly differentially expressed in all three sensitive isolates with a FDR of less than 0.05 (Table 5.1). Of these 32 genes, nine were significantly downregulated and 23 were significantly upregulated. These genes included a phospholipase "Pi-PLD-like-3", two ATP binding cassette superfamily (ABC) transporters, one mannitol dehydrogenase, three CRN and five secreted RXLR effectors, and 17 conserved hypothetical proteins (Table 5.1), among others.

The genes that were differentially expressed in response to mefenoxam were also investigated in the stably resistant US-8 isolate. Among these 32 genes were 21 differentially expressed in common with the three sensitive isolates (Table 5.1).

There were 17 conserved hypothetical proteins represented among the 32 genes that were commonly differentially regulated upon exposure to mefenoxam. The similarities of some of these proteins to those of known or hypothesized function are indicated in Table 5.2. These similarities are based on amino acid sequence similarity to other proteins determined by protein-protein BLAST analysis. Among these conserved hypothetical proteins, one was similar to a TonB membrane receptor from *P. sojae*, one was similar to both Avr1b-1 from *P. sojae* and a glycosylphosphatidyl inositol (GPI) anchored protein from *P. infestans* (Table 5.2).

Table 5.2 Possible functions of differentially expressed genes annotated as "conserved hypothetical proteins". Possible functions were determined by using the protein-protein BLAST algorithm on NCBI. Sequence coverage, maximum identity and E-value are included as proxies for the level of similarity at the amino acid sequence level, between the annotated gene and the conserved hypothetical *Phytophthora infestans* gene.

Broad gene identifier	Annotation	NCBI reference or conserved domain	Organism	Annotation or Domain	Query Cover	Identity
PITG_05795	Conserved hypothetical protein	EGZ12418.1	*P. sojae*	TonB receptor activity	89%	65%
PITG_07573	Conserved hypothetical protein	XP_002904561.1	*P. infestans*	Predicted GPI-anchored protein	59%	98%
PITG_02748	Conserved hypothetical protein	RING[cd00162], PX[smart00312]	N/A	RING Zn finger, PhoX homologous domains	N/A	N/A
PITG_10079	Conserved hypothetical protein	RpsE[COG0098]	N/A	RpsERibosomal protein S5 domain	N/A	N/A
PITG_10995	Conserved hypothetical protein	FYVE[cd00065], DEP[cd04371], PTZ00303	N/A	FYVE Zn-binding, DEP, PTZ00303 Phosphatidyl inositol kinase (provisional) domains	N/A	N/A
PITG_15627	Conserved hypothetical protein	PRK12704	N/A	PRK12704 Phosphodiesterase (provisional) domain	N/A	N/A
PITG_16013	Conserved hypothetical protein	ATS1[COG5184]	N/A	ATS1 Alpha-tubulin suppressor and related RCC1 domain containing multi-domain	N/A	N/A

5.5.5 Validation of RNA-seq results using qRT-PCR

To validate the RNA-seq results, we analyzed the expression profile of five genes that were differentially expressed between isolates non-exposed and exposed to mefenoxam using qRT-PCR (Supplementary Figure 5.1). All of five genes showed the same significant differential expression profiles with both techniques.

5.6 Discussion

All individuals from all of the "sensitive" clonal lineages investigated became tolerant of mefenoxam upon exposure to mefenoxam after a single passage through mefenoxam-containing medium. Previous descriptions of such acquired resistance were detected after at least 4 to 12 passages through mefenoxam-containing medium (Bruin and Edgington 1981), (Staub et al. 1979). We found that repeated exposure had little impact on increasing this resistance. We suspect that the ability to acquire resistance may be a general characteristic of mefenoxam-sensitive isolates of *P. infestans*. Acquired resistance declined after two or three subcultures on medium free of mefenoxam, but we did not investigate if the original level of mefenoxam sensitivity could be reached with additional transfers. In previous studies, diverse isolates of *Phytophthora capsici* and *P. infestans* responded diversely after many subcultures on mefenoxam-free medium, with some isolates losing resistance and others retaining it (Staub et al. 1979).

We have demonstrated that sensitive lineages acquire resistance to mefenoxam if exposed to a non-lethal dose of mefenoxam. We suspect that acquisition of resistance is likely to be somewhat specific, but we have not systematically investigated other chemical or physical stresses to see if they also stimulate resistance to mefenoxam.

We also found that many sensitive isolates that had acquired resistance to mefenoxam seemed to be slightly retarded in growth in comparison to the parental isolates that had never been exposed to mefenoxam. Thus, it appeared that there is likely a cost associated with acquired resistance, which possibly could affect fitness. Again, this observation is consistent with previous reports (3).

Isolates of US-8 had similar patterns of growth in response to mefenoxam, even after previous exposure. This does not preclude the possibility that acquired resistance is conserved in *P. infestans*, as it may be that the ability to acquire resistance in US-8 isolates is retained, but masked or made unnecessary by the mechanism governing stable resistance to mefenoxam. The latter possibility is supported by the fact that US-8 also differentially expresses many of the genes that are differentially expressed in common among the sensitive isolates. The genetic basis for inherited field resistance to mefenoxam is still unclear. It is known that mefenoxam has a negative effect on the synthesis of RNA and specifically on ribosomal RNA (rRNA). Therefore, it likely involves the RNA polymerase I (RNApol1) as it transcribes rRNA. Randall et al. (Randall et al. 2014) identified and sequenced genes encoding RNApol1 subunits. They found that a small number of SNPs in the gene encoding the large subunit of RNApol1 was specific to insensitive isolates. Yet, Howard Judelson's group has sequenced this same region for a number of *P. infestans* isolates from the United States and found that these SNPs did not account for all cases of resistance (*personal communication*). Judelson's group (personal communication) has observed the SNP identified by Randall et al. (23) to be associated with resistant genotypes in isolates of US-8, yet this same SNP was sometimes found in the sensitive isolates. Therefore, it is likely that another gene or group of genes contribute(s) to stable mefenoxam resistance in some genotypes of *P.*

infestans.

Given the speed and consistency of acquired resistance, an epigenetic mechanism seemed likely. There exist a wide range of mechanisms that have been observed to confer fungicide or drug resistance in other systems, including efflux transport or direct detoxification of the active compounds (Judelson and Senthil 2006). Thus, we compared the transcriptome of isolates without acquired resistance to the transcriptome of isolates with acquired resistance using RNA-seq.

The tight clustering by clonal lineage observed in the multi-dimensional scaling analysis showed that most genes differentially expressed between non-exposed (sensitive) and exposed (with acquired resistance) were unique to each isolate. Therefore, most differences were due to isolate rather than due to exposure to mefenoxam. However, common to all sensitive isolates that had acquired resistance were 32 genes that were differentially expressed in each of these lineages (Table 5.1).

We further investigated some of the genes that were most highly differentially expressed upon acquisition of resistance. They include genes with putative functions that could potentially mediate acquired resistance to mefenoxam. Notable among these are two ATP-binding cassette superfamily proteins (ABC), which are part of a large family of transporters characterized by a highly conserved nucleotide-binding domain (Judelson and Senthil 2006). Most catalyze the ATP-dependent efflux of a broad spectrum of compounds from the cell (Judelson and Senthil 2006). These have been observed to mediate drug and multi-drug resistance in various organisms, including phytopathogenic fungi (Nakaune et al. 2002).

Another of the potential candidate genes is phospholipase D (PLD). These enzymes

cleave phosphatidyl inositol into inositol and phosphatidic acid. A previous study with *P. infestans* has identified 18 such genes, many more than in other Eukaryotes (Meijer et al. 2011). The same study also found that a few of those PLDs had extracellular activity, and posited that they might play a role in modifying host tissues during pathogenesis. Phosphatidic acid has been implicated as a signal in diverse contexts including secretion, vesicle trafficking, and modulation of receptor signaling (Wang et al. 2006), which might aid removal of mefenoxam from the cell or interfere with the activity of mefenoxam. Additionally, PLDs have been directly implicated in agonist-dependent cellular secretion. Thus, this PLD might function as one of the steps in a signaling pathway leading to the acquired resistance response perhaps via secretion of the molecule.

The conserved hypothetical protein showing similarity to a TonB-dependent receptor may play a role in mediating acquired resistance. This conserved hypothetical protein is similar in amino acid sequence to a TonB-dependent receptor protein found in *P. sojae* (Tyler et al. 2006). TonB proteins are highly conserved, and are anchored in the plasma membrane, projecting into the periplasmic space, where they often interact with receptors that are termed TonB-dependent receptors (Zhao et al. 1998). These receptors are often gated channels, and are primarily known for their role in mediating iron uptake through the use of siderophores. However, TonB and the receptors it interacts with have been implicated in efflux-mediated "intrinsic and acquired antibiotic resistance" in *Pseudomonas aeruginosa* (Zhao et al. 1998). This mechanism has been shown to influence but not entirely determine resistance (Zhao et al. 1998). It is possible that these two conserved hypothetical proteins might work in concert with ABC transporters to mediate efflux of mefenoxam.

The remaining candidate genes do not have previously documented roles in toxicant

resistance, but may be part of a stress response on the part of the pathogen – the stress being mefenoxam. Previous analyses of mannitol dehydrogenase *in vitro* show that it could be responsible for production of mannitol in the rust fungus, *Uromyces fabae* (Voegele et al. 2005). Polyols like mannitol have been shown to function as an osmoprotectant in various fungi (Clark et al. 2003, Shen et al. 1997a, b). Thus, one hypothesis is that mannitol dehydrogenase is produced by *P. infestans* as a response to toxicants – either in general or as a specific osmoprotectant response.

RXLR effectors, on the other hand, are known primarily for their role in promoting virulence on host plants. The RXLR translocation motif is required for translocation across the host cell membrane, where RXLR effectors are presumed to participate in suppressing Pathogen-Associated-Molecular-Pattern (PAMP) Triggered Immunity (PTI) (Birch et al. 2009, Whisson et al. 2007). The production of such specialized molecules in an *in vitro* test was unexpected, and a satisfying explanation for their induction awaits further investigation.

The identification of differentially regulated genes that are significantly expressed in common among the three originally sensitive isolates follows the assumption that these genotypes share a common mechanism for acquiring resistance. This assumption seems likely due to the similarity of the acquired resistance phenotypes among sensitive isolates. Also consistent with this hypothesis is the finding that the stably resistant US-8 isolate also differentially expressed many of the same genes that the sensitive isolates differentially express in common. However, because there was substantial diversity among isolates in the genes that were differentially expressed, we cannot rule out the possibility that different genotypes of *P. infestans* have different mechanisms responsible for their acquired resistance. Further work will be necessary to identify the precise mechanism(s) underlying acquired

resistance. As a next step, a gene-specific silencing method should be used to test the role of the candidate genes identified in this study.

The risk of this acquired resistance causing problems in field situations seems low. Previous studies (Bruin and Edgington 1981, Staub et al. 1979) found that isolates that had acquired resistance "in vitro" did not have high levels of resistance "in vivo". In our studies, isolates with acquired resistance had slower growth in culture, and so might not compete well in the field. These results are consistent with those of Bruin and Edgington (3). However, it is also possible that acquired resistance might operate in concert with the stable resistance as described by Randall et al (2014) to achieve an even greater level of resistance. It is also important for investigators to be aware that "sensitive" strains of *P. infestans* can rapidly acquire a resistance phenotype upon a single passage through mefenoxam-containing medium.

5.7 Acknowledgements

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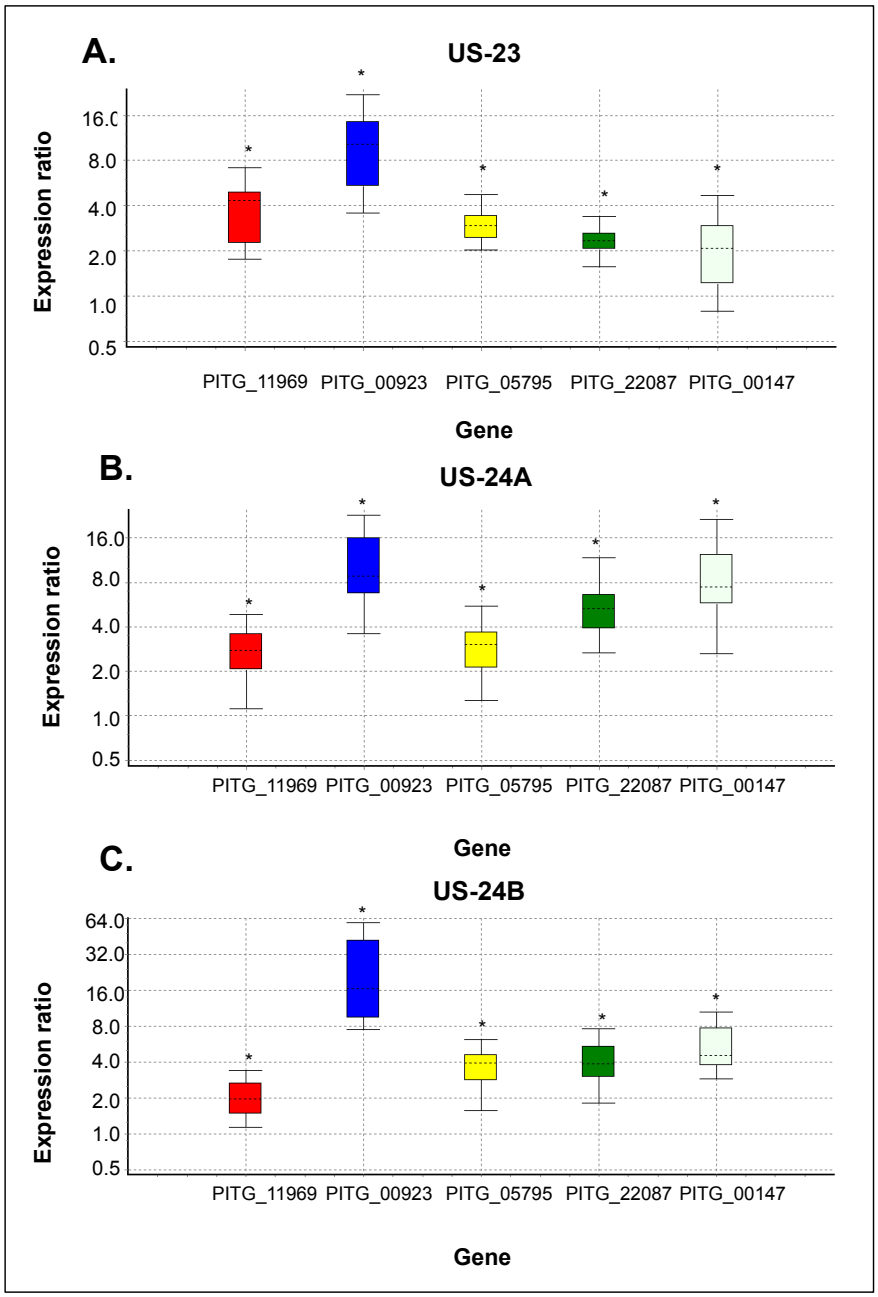
5.9 Supplemental Material



Supplementary Figure 5.2 qRT-PCR results for comparative analysis between non-exposed and mefenoxam-exposed isolates. Graph indicates relative expression for each gene. All genes were assayed in triplicate and two biological replicates of each treatment were performed. Each error bar is constructed using one standard error from the mean.

CHAPTER 6

Discussion

The overall objective of this study was to understand the population genetics of *Phytophthora infestans* in the United States. I have characterized phenotypes of the most recent and most prevalent strains of this pathogen in the US (US-8, US-22, US-23, and US-24), and growers throughout the Northeast have already used this information to make informed management decisions. For example, US-23 has been the dominant lineage in the US since 2011. Based on the data I have generated, growers have learned that if US-23 is in their fields, they must protect both potato and tomato crops. Furthermore, growers are now aware that they may use the fungicide mefenoxam to control late blight epidemics caused by this genotype. I have also investigated a novel set of rare and diverse genotypes of *P. infestans* detected in the Northeast in 2010 and 2011. The genetic characteristics of this population were consistent with a recombinant population. Greater diversity was detected in that region during each of 2010 and 2011 than had been observed in the entire United States in the previous ten years. The likelihood that many different migrations from diverse sources, or that many mutations caused the high degree of genotypic diversity found, seem low. Through parentage exclusion analyses using microsatellite markers and four nuclear gene sequences I found that clonal lineage US-22 could be a parent of some, but not all, of the new genotypes detected in 2010 and 2011. My best inference is that these isolates represent progeny that originated from at least two recombination events. The geographic location(s) of those recombination events

remains unknown. The eventual impact of this recombination event cannot be predicted at this moment. The fact that individuals from this event were detected only in 2010 and 2011 and not in 2012 or 2013 suggests that these isolates were not as aggressive or as fit as subsequent dominant clonal lineages. However, the fact that there is now evidence for a second recombinant population of *P. infestans* detected in the US indicates that sexual recombination is certainly possible, and there is no reason to believe that such populations will not occur in the future. Diligence in monitoring populations might enable the location of a recombination to be identified so that proper mitigation techniques could be applied.

The phylogenetic relationships of isolates of *P. infestans* in the US were further studied by using approximately 98,000 SNP markers obtained through genotyping-by-sequencing. Isolates of *P. infestans* from Mexico and one from the Netherlands were also included in this analysis. A maximum-likelihood (ML) phylogenetic tree was generated using RAxML (Stamatakis 2014) with 1,000 bootstrap replications. The Generalized time-reversible (categorical) (GTRCAT) nucleotide substitution model was used (Tavaré 1986).

In general, the dominant clones in the US from the 1990s to 2013 seem to be phylogenetically distinct (Figure 6.1). The topology of the tree supports previously reported hypotheses regarding the source as well as the parental-progeny relationships of certain US clonal lineages. For example, lineages US-7 and US-8 clusters within the Mexican isolates, supporting the hypothesis that these clonal lineages were introduced into the US from Mexico (Goodwin 1997). Clonal lineage US-7 (Coffey7723), has been proposed to be one of the parental genotypes for US-11 (Gavino et al. 2000). These lineages formed a monophyletic group with a bootstrap support value of 100. Furthermore, isolates within the NYS-2010/11 population formed a single cluster that includes clonal lineage US-22 (with a bootstrap value

of 100), which supports my hypothesis that this is one of the parental genotypes of some of the individuals of the NYS-2010/11 population (Danies et al. 2014). Clonal lineage US-8 is closely related to clonal lineage US-24, which has been previously shown using microsatellite markers (Fry et al. 2013). Isolates within clonal lineage US-23, are different from other clones that have been dominant in the US from the 1990s to 2013. Since 2011, US-23 has been the most prevalent clonal lineage in the US. The fact that it is very different to any of the other lineages probably suggests that an isolate or isolates from this lineage were introduced into the US from elsewhere. Individuals of the US-23 clonal lineage have been detected in Europe (Cooke D.E.L. *personal communication*). The similarity between US-23 and European isolates is further supported by the monophyletic group that includes these isolates and the isolate from the Netherlands.

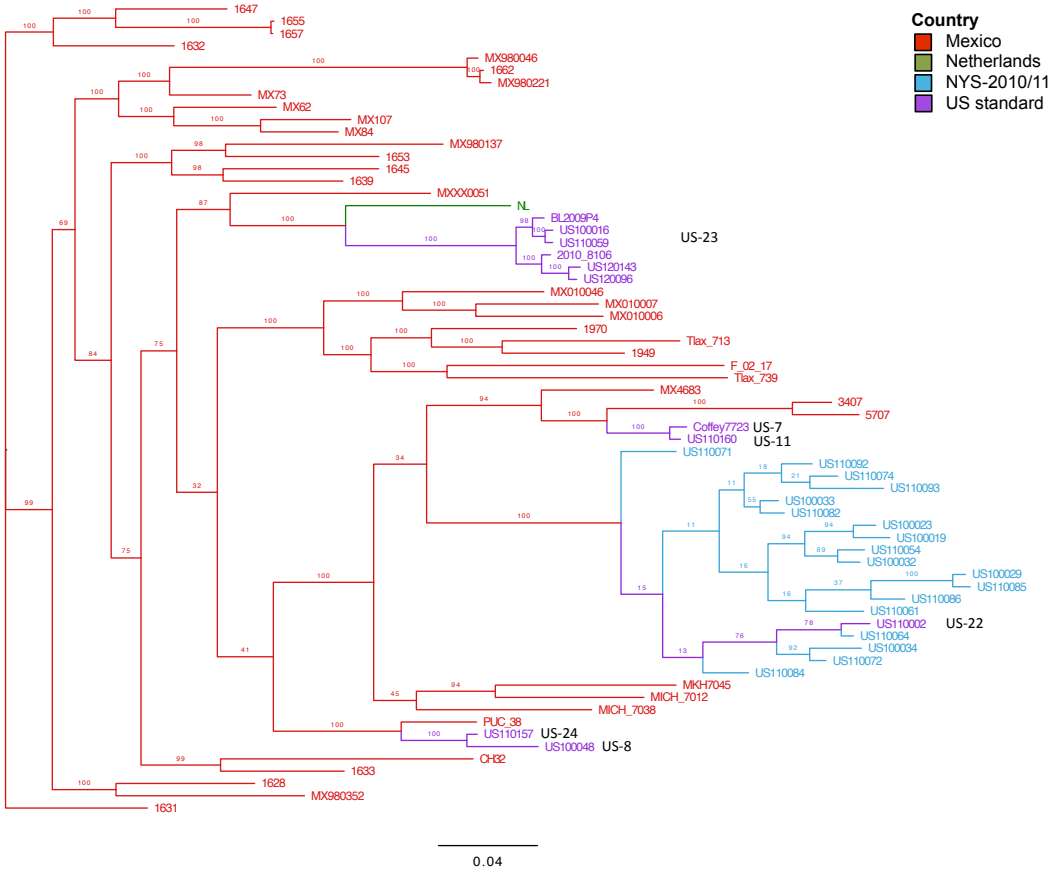


Figure 6.1 Maximum-likelihood phylogenetic tree of *Phytophthora infestans* generated by using RAxML with 1,000 bootstrap replications.

For the same panel of diverse *P. infestans* isolates used to construct the Maximum-likelihood phylogenetic tree, I systematically assessed five traits: mating type, pathogenicity on potato and tomato, sensitivity to mefenoxam, the effect of temperature on release of zoospores, and the effect of temperature on mycelial growth. Isolates of the A1 mating type, of the A2 mating type, and others that were self-fertile were identified. Genotypes US-8 and US-24, as well as the isolates from Mexico and the isolate from the Netherlands showed a strong preference for potato and were not at all aggressive on tomatoes. Genotypes US-7 and US-11 as well as the rare and diverse genotypes detected in the Northeast in 2010 and 2011 were pathogenic on both potato and tomato. A broad range of responses to sensitivity to mefenoxam was observed among the panel of isolates studied. The most remarkable differences were observed within the Mexican isolates, where sensitivity ranged from extremely sensitive (no growth in the presence of mefenoxam) to highly resistant (where mycelial growth was enhanced by the presence of mefenoxam). The rate at which sporangia released zoospores differed between isolates. Within 30 minutes of incubation at 4ºC, sporangia that had released zoospores ranged from approximately 2% to 80%. Differences in mycelial growth were observed at different temperatures. For all isolates studied, growth at 10ºC seemed to be greatly stunted. The vast majority of isolates (50 out of 56) did not show statistical differences in mycelial growth at 15, 20 or 25ºC. However, six isolates did show differences in growth in response to temperature.

Obtaining precise phenotypic data is challenging due to the high variance observed in

the assays conducted. Yet, the findings from this study show that real statistical differences within the phenotypic traits studied exist within our panel of *P. infestans* isolates. Undoubtedly, the phenotypic diversity present in *P. infestans* is a key factor contributing to the pathogen's success throughout time. Future efforts will be devoted to finalizing the phenotyping assays of the diverse panel of *P. infestans* isolates included in this study. The phenotypic data for diverse isolates of *P. infestans* along with the SNP markers generated through genotyping-by-sequencing hopefully will enable a genome-wide association study to find SNP markers associated with traits of interest. This will hopefully form the basis for future research that would lead us to the development of a specific DNA-based method to identify phenotypic traits of interest. An understanding of the genetic basis of complex traits important to the pathogenicity or epidemiology of *P. infestans* would be of value in managing late blight because rapid analysis using molecular markers could inform the selection of the most effective mitigation tactics.

Finally, I investigated the characteristics of mefenoxam-acquired resistance. I found that the phenomenon of acquired resistance was universal and was not unique to certain strains of *P. infestans.* Three originally sensitive genotypes (US-22, US-23, and US-24) were all able to acquire resistance to mefenoxam after a single exposure to sub-lethal concentrations of the fungicide. Thirty-two genes were found to be significantly differentially expressed in response to mefenoxam in all originally sensitive genotypes studied. However, because there was substantial diversity among isolates in the genes that were differentially expressed, we cannot rule out the possibility that different genotypes of *P. infestans* have different mechanisms responsible for their acquired resistance. Further work will be necessary to identify the precise mechanism(s) underlying acquired resistance. As a next step, a gene-

specific silencing method should be used to test the role of the candidate genes identified in this study.

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