A GENOMICS BASED APPROACH TO MANAGING DOWNY MILDEW OF LIMA BEAN

by

Terence Tariro Mhora

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant and Soil Sciences

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To all who read this, anything is possible, just put your mind to it, work hard and love hard.

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ABSTRACT

Lima bean is the cornerstone of the vegetable processing industry in the Mid-Atlantic region (MAR). An overall nationwide decline in lima bean production has allowed the MAR to occupy this niche market area and increase its production and revenue. However, *Phytophthora phaseoli,* the causal agent of downy mildew remains a scourge for lima bean producers, causing significant periodic losses. New races of *P. phaseoli* have worsened the situation for farmers, rendering resistant cultivars susceptible and increasing their chemical input costs as they attempt to limit yield losses. This dissertation seeks to address this issue through two main objectives: first, through the development of genetic resources that will enable more targeted breeding for crop resistance to *P. phaseoli* and second, by examining the phenotypes and the population structure of the pathogen in grower fields. The latter objective provides a better understanding of the pathogens resistance to mefenoxam and its clonal structure. The overall goal of this work is to provide a pathway to more efficient disease control strategies through a better understanding of the pathogen and the host, leading to improved lima bean production in the MAR.

Chapter 1

LITERATURE REVIEW

Lima bean (*Phaseolus lunatus*), the second most cultivated member of the Phaseolus genus occupies a significant proportion of the acreage used for vegetable processing crops in the Mid-Atlantic Region (New Jersey, Pennsylvania, Delaware, Maryland, and Virginia). Over 8,000 hectares (ha) of lima bean are planted annually in the Delmarva region alone (Ernest and Johnson, 2013). This area of production makes up approximately 60% of the nationwide (CA, DE, IL, MD, OR, WA, and WI states) crop reserved for canning and freezing (Tolomeo et al., 2013). As a result, the production of other processed vegetables is largely dependent on the more profitable production of lima bean, which is the cornerstone of the Delaware vegetable processing industry (Davidson et al., 2008). Additionally, the region is one of the few areas producing the crop, providing a unique niche market that the local processors occupy (Donofrio et al., 2013; Kee et al., 2004). The three main commercial classes of lima bean used for processing are the small-seeded green baby lima, the small-seeded speckled lima, and the large-seeded Fordhook lima. These lines can be traced back to at least two sites of domestication, the Andean and Mesoamerican regions (Quat Ng, 2013). The Fordhooks occupy only 10% of the total area planted to lima beans as of 2012 and are currently at a yield increase plateau compared to the baby lima's, which are showing year to year yield improvements (Tolomeo et al., 2013).

The Fordhook type is mainly grown for dry bean production in California, where it originated in the early 1900's. Yields in the east have been traditionally low, owing to poor climate adaptability and attack by the oomycete pathogen *Phytophthora phaseoli* (Ernest and Johnson, 2011). There has been a resurgence of interest in this type in the Mid-Atlantic Region (MAR) due to increased consumer demand. This makes it imperative to produce disease resistant cultivars capable of high yields in this region. Resistance to *P. phaseoli* was bred into Fordhook lines released by the USDA in the 1970s and 80s. These cultivars are however no longer commercially available due to the emergence of new physiological races of *P. phaseoli*, these being defined as subdivisions of a pathogen species distinguished from its other members by cultivar specialization for pathogenicity (Davidson et al., 2008; Ernest and Johnson, 2011; Kirk et al., 2001). Concentrated Fordhook (CFH) and Fordhook 242 (FH242) are the newer commercially available cultivars of the Fordhook type, but are also susceptible to the new physiological races (hereafter referred to as "race"). These Fordhook cultivars are also heat and drought intolerant and take longer to harvest in the MAR climate, prompting the need for improved cultivars.

Downy mildew of lima bean caused by *P. phaseoli* was first reported infecting lima bean in 1889 by Thaxter. This disease is characterized by white cottony mycelia with red boarders on pods while it also infects flower racemes, shoot tips, leaves and petioles (Fig 1; Davidson et al., 2008). In 1903, the pathogen had spread from Connecticut to Delaware and was observed on lima bean by Smith (Wester, 1970). The major pathogen race at that time was race A, which prompted a breeding exercise that yielded a resistant lima bean cultivar, Thaxter (Wester, 1970). Before the

commercial release of this cultivar in 1958, a different race of the pathogen had been discovered. Within nine years, more races emerged, with race D appearing in the 1970's and dominating until the emergence of race E (in 1995) and race F (in 2000) (Evans et al., 2002). The emergence of these two new races was synonymous with huge losses, which peaked at 40% of farm yields, an estimated $3M loss in 2000 (Davidson et al., 2008). Differential race screening is still the most commonly used method of determining the race of *P. phaseoli* in the field, where race F is currently predominant (Davidson et al., 2008). Races A to D are no longer observed in the field while there are currently no cultivars resistant to both races E and F (Kunjeti et al., 2012).

Figure 1 White cottony mycelia on shoot tips, pods and racemes characteristic of *Phytophthora phaseoli* (Mulrooney, 2011)

Background and Significance

Improving crop productivity requires in depth knowledge of both the crop and its pathogens, enabling growers to make adaptations that will be beneficial. Such adaptations include decisions on cultivars to be grown in a season based on information such as the pathogens present in a field; the choice of chemical control and timing of its use in disease management among others. To use these adaptations, it is necessary to understand the history, phenotype and the genetic structure of both host and pathogen. In the MAR, resistance to *P. phaseoli*, heat and drought tolerance are some of the more desired features in commercial cultivars. The ability to predict disease onset and to use preventative fungicide sprays is now more efficient, but the presence of fungicide resistant isolates and the possibility of a new race emerging are still great and there is need to address this (Personal communication, Thomas Evans).

There are currently 13,991 accessions of lima bean held in 75 institutions, providing a large reservoir of genetic resources for potentially improving the crop (Quat Ng, 2013). Genetic bottlenecks brought about through domestication and the reliance of a small genepool as breeding germplasm can result in a plateau in genetic gains, with agronomic catastrophes more likely due to factors such as breakdown of resistance or the emergence of a new pathogen. Early records show the first cultivar released by the USDA breeding program to have been the baby Fordhook in 1939 (Stavely, 1991). The USDA initiated the first breeding program using basic, large scale intensive phenotypic screening and cultivar selection against many conditions such as high disease pressure and elevated temperature (Allard, 1954; Ernest et al.,

2006; Kendrick and Allard, 1955; Wester, 1970). Similar methods were used to develop Thaxter, the first downy mildew resistant cultivar released in 1959 after experiencing severe losses to the disease (Wester, 1970). The emergence of new races of *P. phaseoli* prompted the breeding of downy mildew resistant cultivars using resistance found in other accessions (Wester, 1970).

Recent breeding approaches have utilized technology such as the RAPD, AFLP, ISSR markers and DNA sequencing to obtain markers that have been subsequently used in breeding schemes (Andueza-Noh et al., 2013; Castińeiras et al., 2007; Martínez-Castillo et al., 2008; Nienhuis et al., 1995). The use of high throughput marker technology has recently been used to test for domestication scenarios of lima bean and is yet to be applied to breeding efforts (Chaćon-Sánchez and Martinez-Castillo, 2017). An objective of this dissertation is to describe the genetic diversity of cultivars in the US and compare them to members of a diversity panel (256 accessions) that has been sourced from in and around the areas of lima bean domestication. The goal of this objective is to produce knowledge which will allow informed breeding choices to be made but also to produce genetic resources in the form of molecular markers which will be used to develop more efficient and better adapted cultivars containing the traits required in this region. Phenotypes related to productivity and to disease resistance to *P. phaseoli* and *P. capsici* have also been collected for the diversity panel.

Lima Bean (*Phaseolus lunatus*)

A member of the Fabaceae family, lima bean is one of the five domesticated species of Phaseolus and is the second most important among them (Gepts, 2001; Penha et al., 2017). Lima bean is the most distant of these other *Phaseolus* species and is the only one among them that does not constitute a syngameon (Gepts, 2001; Maquet et al., 1999). The closest members of the Phaseolineae to *P. lunatus* are *P. augusti*, *P. bolivianus*, and *P. pachyrrhizoides*. This membership is based on AFLP, seed storage protein allozymes and morphology (Fig 2; Maquet et al., 1999; Caicedo et al., 1999). These close allies of lima bean are important for conservation purposes as they are a potential source of genetic diversity.

Archaeological evidence suggests that lima bean originated from Guatemala and spread throughout the Americas via trade routes, giving rise to what was formerly called the Hopi (northern branch), the Carib or West Indies branch and the Inca or southern branch (Mackie, 1943). The use of more recent genetic analysis methods has shown lima bean to have undergone at least three domestication events, with genepools from central-western Mexico (MI), Guatemala – Costa Rica (MII) and the Andean region (AI) (Chaćon-Sánchez and Martinez-Castillo, 2017; Motta-Aldana et al., 2010; Martínez-Castillo et al., 2008). A test of the domestication scenarios of lima bean using genome-wide SNP markers suggests the possibility of a fourth gene pool from central Colombia (AII), a result which had been suggested in 1999 (Chaćon-Sánchez and Martinez-Castillo, 2017; Caicedo et al., 1999). Lima bean has historically been an important crop in the US, with evidence of extensive cultivation beginning in pre-Columbian times (Mackie, 1943). The crop was adopted by post-Columbian

settlers who selected and maintained their own landraces, giving rise to early cultivars such as the Henderson Bush, the first credited baby lima bean cultivar (Mackie, 1943). These early landraces and cultivars are what became sources of germplasm to the USDA and contributed to the diversity panel we have assembled (Personal communication, Emmalea Ernest).

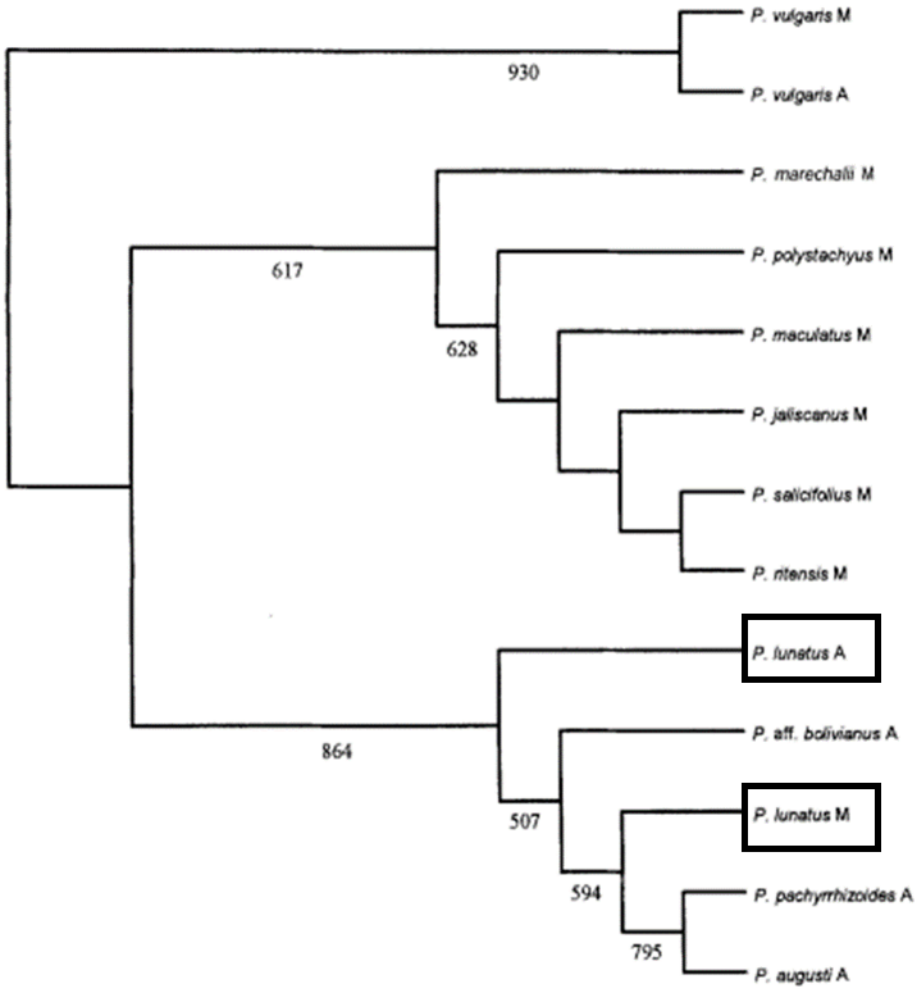

Figure 2 A cladogram of *Phaseolus lunatus* and its allied wild species. The letters after each species name are abbreviations for Mesoamerican (M) and Andean (A) (Maquet et al., 1999).

Lima bean is mostly self-compatible and has a mixed reproductive system, where self-fertilization is dominant due to the timing of pollen grain and stigma maturation as well as the proximity of the sexual structures within the floral bud (Webster et al., 1979). Outcrossing rates of between 0.2 – 48% have been reported, with more recent reports of 38.1% natural outcrossing occurring in Brazilian germplasm (Penha et al., 2017). Outcrossing in nature is due to insect pollination, with bees being the main pollinators of lima bean. This legume has a unique taste due to linamarin, a cyanogenic glucoside that imparts the unique taste of lima bean. This glucoside is found in safe concentrations in most lima bean cultivars and landraces except for some from the Caribbean, where excessive amounts of the compound are above the safety limits recommended by the Food and Drug Administration (FDA) of the USA.

Diseases of Lima Bean

Lima bean is susceptible to many diseases which threaten yields and in the MAR, the most damaging are downy mildew (*P. phaseoli*), pod blight (*P. capsici*), root knot nematode (RKN from *Meloidogyne* spp.) and white mold (*Sclerotinia sclerotiorum*). Below ground diseases of lima bean that have been of importance to the region include Rhizoctonia root rot (*Rhizoctonia solani*) and Pythium root rot or damping off (*Pythium* spp.), which are controlled by rotation with non-legume crops and by seed treatments (Kee et al., 1997). Fusarium root rot caused by *Fusarium solani* f. sp. *phaseoli* is another below ground disease that causes losses in Alabama (Sikora et al., 2014). Above ground diseases that have been important in the MAR

include anthracnose (*Colletotrichum lindemuthianum*) and gray mold (Botrytis), whereas other regions have reported powdery mildew (*Erysiphe polygoni*), common bacterial blight (*Xanthomonas phaseoli*) and halo blight (*Pseudomonas syringae* pv. *phaseolicola*). Viral diseases can also infect lima bean, although they are uncommon and difficult to identify in the field due to similarities with abiotic stresses and their variable symptoms which can differ with cultivar, age of plants, virus strain, and environmental conditions (Sikora et al., 2014). The legume is also susceptible to insect damage, with cutworms, Mexican bean beetle (*Epilachna varivestis* Mulsant), tarnished plant bugs, mites, aphids, leafhoppers, lygus bugs, corn earworms and fall armyworms being the more common pests. Podworm damage (corn earworms and fall armyworms) has been severe in some years, requiring timely treatment with methomyl (Kee et al., 1997).

Plant Pathogenic Oomycetes

The major pathogens of lima bean are the oomycetes and the fungi. The most damaging pathogen of lima bean is arguably *Phytophthora phaseoli*, an oomycete which has altered the geographic location of the hub of the lima bean industry on the east coast of the United States. This organism is an oomycete and its genus name, *Phytophthora*, means “plant destroyer” an apt description for this genus of plant pathogens. The genus *Phytophthora* has left its mark in human history, having been the cause of the Irish potato famine, a catastrophe caused by *Phytophthora infestans*, the causal organism of potato late blight (Fry and Goodwin, 1997). Oomycetes, also known as water molds were once grouped with fungi and are more closely related to

brown algae (heterokonts) in the Stramenopiles, a major eukaryotic kingdom (Kamoun, 2003). Oomycetes are a diverse group of eukaryotic microorganisms that include both saprophytes and pathogens of plants, insects, crustaceans, fish, vertebrate animals and various microorganisms. The World Phytophthora Genetic Resource Collection (WPC) consists of over 8,000 isolates that are distributed among over 140 identified species of Phytophthora to date, with most of the genetic data for these isolates contained in Phytophthora-ID (Grunwald et al., 2011; www.phytophthora.ucr.edu/).

History of *Phytophthora phaseoli*

The lima bean pathogen, *Phytophthora phaseoli* Thaxt is a homothallic oomycete in the kingdom Stramenopila within Phytophthora Clade 1c (Fig 3; Blair et al., 2012). The pathogen was first identified infecting lima bean in Hamden, Connecticut in 1889 by Thaxter (Thaxter 1889). The disease progressively moved south and by 1903, Smith had identified the oomycete as being "injurious" in Delaware and Maryland, with yield losses of 25 to 90% being experienced in Frederick County, Maryland in 1905 (Wester 1961; Wester and Jorgensen 1959). There are six known races of *P. phaseoli*, races A through F. The development of new races has been shown to be driven by resistant cultivars, with the first observation being the advent of race B on the breeding line U. S. No. 355 in October of 1958 (Wester and Jorgensen, 1969). Thaxter, a new cultivar, had been found resistant to as many isolates of *P. phaseoli* as were available and a year before its release in 1959, was found to be susceptible to the new race (B), which did not become destructive

until 1967 (Wester and Jorgensen, 1959; Zaumeyer W. J and Wester R. E., 1968). Race C appeared in 1970, the same year as the release of Dover, the replacement of Thaxter, which had resistance to both races A and B (Wester, 1970a; Wester, 1970b; Wester, 1970c). Race D appeared in 1976 and was followed by races E (1995) and F (2000) (Sherf and Macnab, 1986; Evans et al. 2002; Evans et al., 2007). Race F is now the most prevalent race in grower fields (Davidson et al., 2008; Mhora et al., 2016).

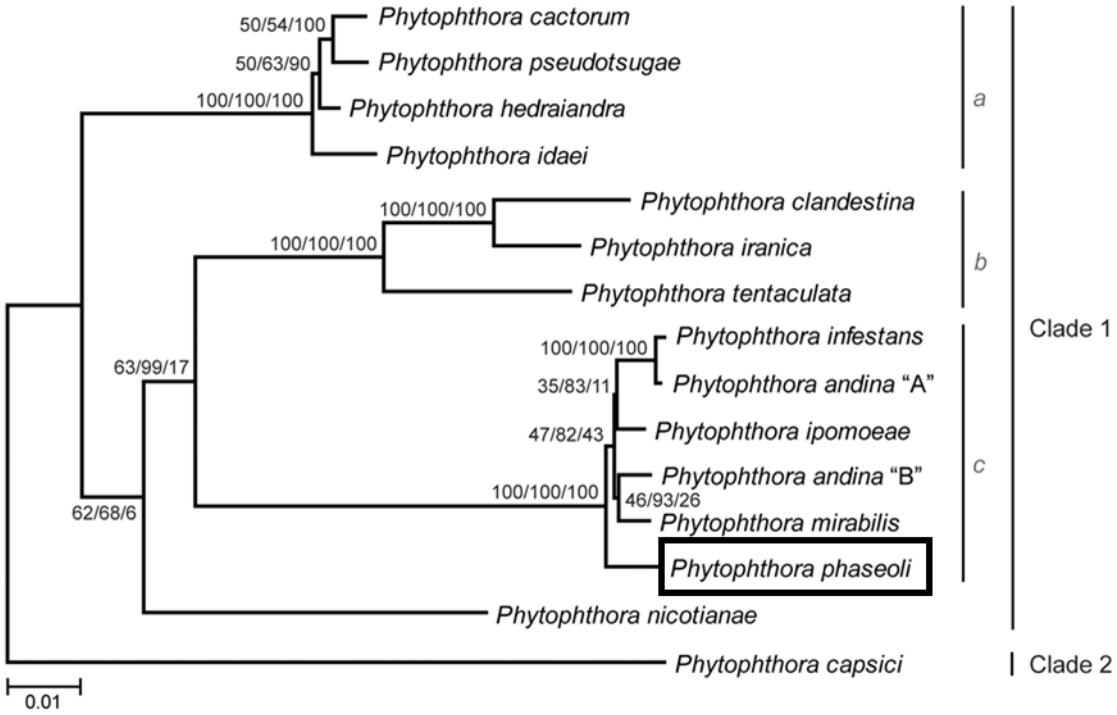

Figure 3 Phylogeny of Phytophthora Clade 1 based on mitochondrial and nuclear loci. Maximum likelihood (ML) branch lengths are shown. Numbers on nodes represent bootstrap support values for ML (left); Bayesian Posterior Probabilities as percentages (middle) and Maximum Parsimony (right) (Blair et al., 2012).

Biology of *Phytophthora phaseoli*

This organism is characterized by fungal-like growth and infects and colonizes flower racemes, shoot tips and pods of lima bean, producing white downy mycelia and sporangia on shoot tips and pods. There is a characteristic reddish-brown margin around the mycelia of infected lima bean pods (Fig 1.), a diagnostic feature of *P. phaseoli* infection (Evans et al., 2007). The organism has oval or elliptical semipapillate sporangia which have a truncate base (Fig 4a, c). Sporangia measure between 20-24µm and 3-50µm, are non-proliferating and caducous, with short pedicels (5-20 µm long) (Fig 4a, b). *Phytophthora phaseoli* does not form chlamydospores or hyphal swellings (Fig 4c). Sexual structures are formed in this homothallic oomycete, with flattened spherical shaped amphigynous antheridia and subspherical oogonia with a diameter that ranges between 16.2-35.6 µm (Fig 4d) (Erwin and Ribeiro, 1996). The sexual structures for sexual reproduction (antheridia and oogonia) appear in the same hypha, forming oospores which overwinter on infected debris and cause disease in the following planting seasons (Fig 5) (Wester and Goth, 1965). It has been shown that a rotation or a fallow period of at least two years will reduce chances of infection in a new crop (Personal communication, Evans; Sikora et al., 2014). In the asexual phase of the lifecycle, infection is due to water borne or airborne sporangia, with the lifecycle from germination to sporulation taking an average seven days (Fig 6). The sporangia of *P. phaseoli* are readily released into the air without the aid of moving water, where slight drying and a decrease in ambient

humidity may favor aerial release, an observation that has also only been made in *P. infestans* (Duniway, 1983).

Control of Downy Mildew

The major control of downy mildew in the MAR is currently Ridomil Gold® Copper, a fungicide whose active compound, mefenoxam has both curative and preventative properties against *P. phaseoli*. Management of downy mildew on lima bean using chemical control (Bordeaux mix) was first suggested a few years after a scientific description of the pathogen (Sturgis, 1897). Exactly 50 years later, a six-year study showed that dusting with copper fungicides gave control, although care was needed during processing to avoid copper phytotoxicity on harvested pods. Spraying of copper fungicides or Bordeaux mix resulted in phytotoxicity characterized by damaged foliage and discolored pods (Cunningham, 1947; Heuberger and Crossan, 1959). A fungicide research group established at the University of Delaware conducted trials beginning 1946 and between then and 1959, demonstrated the effectiveness of maneb sprays, the efficacy which was validated by a local lima bean processor (Heuberger and Crossan, 1959). The effectiveness of resistant cultivars over the years resulted in reduced need for chemical control, resulting in maneb being deregistered in 1989. The appearance of race E resulted in the need to reintroduce chemical control when large losses became apparent in 1998 (Evans et al., 2007). Chemical control of downy mildew can be implemented as a preventative spray where application is informed by a risk model. Chemical options currently available for preventative applications are Omega (Fluaznam), Headline (Pyraclostrobin), Forum

(Dimethomorph), Ranman (Cyazofamid), phosphonate fungicides (e.g., Prophyt) and Ridomil Gold Copper. When downy mildew is observed in the field, Ridomil Gold Copper and Potassium phosphite salts can be used to manage the disease (2016 Mid-Atlantic Commercial Vegetable Production Recommendations).

There is an increasing concern of *P. phaseoli* gaining resistance to chemical control, with mefenoxam, the industry standard being the highest risk as it has a single mode of action (MOA) (Evans et al., 2007; McDonald and Linde, 2002a; Parra and Ristaino, 2001). The MOA of mefenoxam is the selective inhibition of ribosomal RNA synthesis (Parra and Ristaino, 2001). Most of the available chemicals to control many plant diseases target only a single MOA, making them high risk chemicals since the pathogen need only change this one target. Misuse of mefenoxam, and indeed these other chemicals results in plant pathogens such as *P. capsici* acquiring resistance and causing unexpected losses and expenses as companies produce a wider variety of more expensive chemicals differing in their MOA (Davey et al., 2008; Fry et al., 2009; Parra and Ristaino., 2001). Fungicide use guidelines have been formulated to mitigate fungicide resistance (Wyenandt et al., 2009).

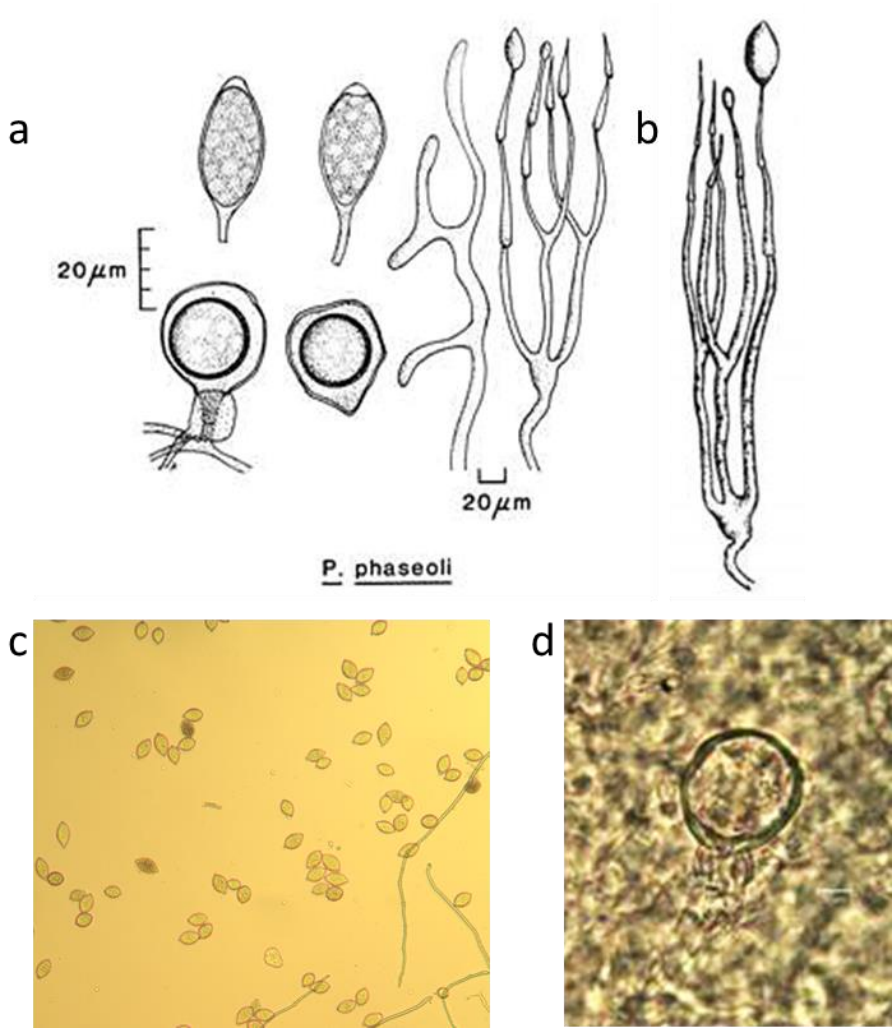

Figure 4 Morphology of *Phytophthora phaseoli*. (a) Upper row, ovoid to elliptical sporangia with short pedicels. Lower row, Subspherical, globose oogonia with amphigynous antheridia. Right, Mycelium and sympodially branched sporangiophores. (Erwin and Ribeiro, 1996); (b) compound sympodial sporangiophore (Blackwell, 1949). (c) caducous sporangia with short pedicels and aceptate, hyaline hyphae (Courtesy Terence Mhora). (d) Oogonium of isolate Phyp15 with attached amphigynous antheridia (Courtesy Nancy Gregory, University of Delaware).

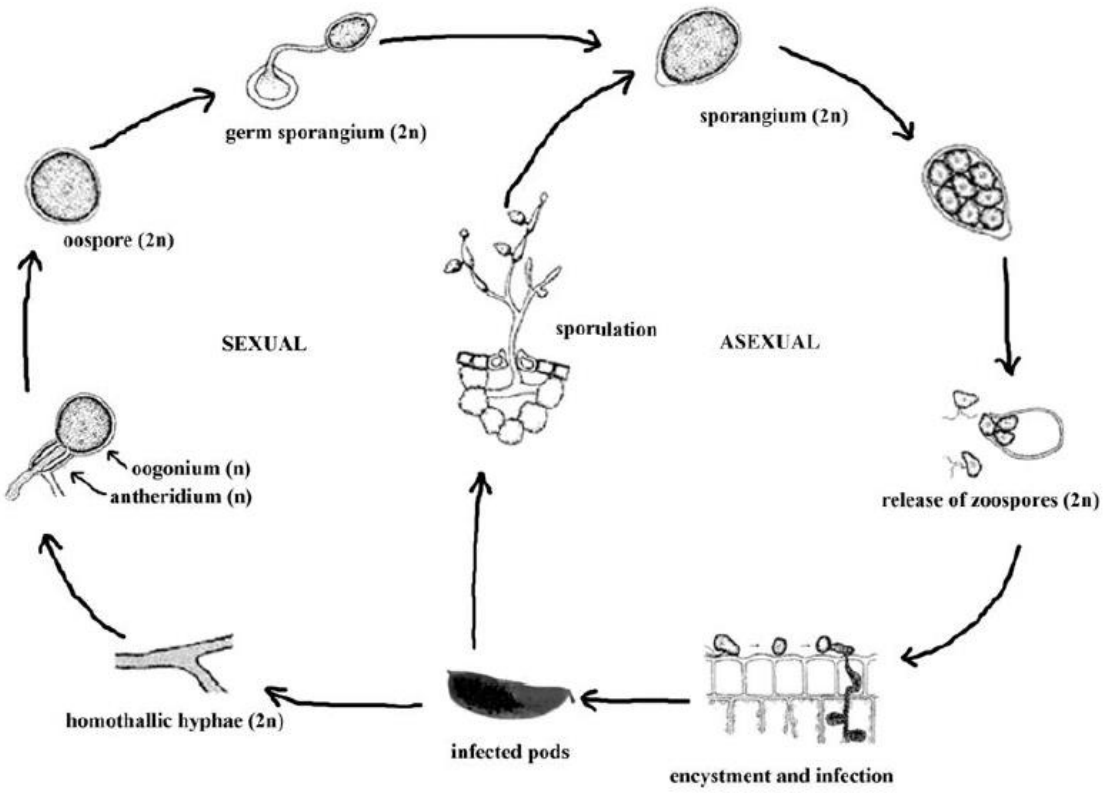

Figure 5 Lifecycle of *Phytophthora phaseoli* showing the sexual (overwintering) and the asexual (disease) phases. (modified from Agrios, 1978 and Judelson, 1997)

Utilization of Genetic Diversity in Disease Management

Sources of genetic diversity and traits which are often overlooked when developing commercial cultivars originate from wild accessions sourced from areas of domestication and from landraces. It is suggested that these yield stabilizing traits are a result of resilience in the face of biotic and abiotic stresses, a hallmark of low input agriculture (Jackson et al., 2007). The International Treaty on Plant Genetic Resources is an example of initiatives that seek to conserve and sustainably use plant genetic resources for food and agriculture fairly and equitably. This organization provides

guidelines towards meeting its mandate through systems such as the Multilateral System of Access and Benefit-sharing, a method of sharing seeds internationally and funding seedbanks (www.fao.org/plant-treaty/en/). Genetic diversity is important in reducing the adverse effects of climate change, plant pests and diseases on yield, with heterogeneous cropping systems demonstrating this concept in a variety of crops and pathosystems which include lima bean and *E. varivestis* Mulsant; *Empoasca fabae* Harris on potatoes; and rice and *Magnaporthe orzyzae,* among others (Cantelo and Sanford, 1984; Zhu et al., 2000). Heterosis has been demonstrated in many crops including lima bean, rice and maize, where gains have been observed in pest and disease resistance, flood and drought tolerance, yields and flowering time among other traits (Flint-Garcia et al., 2009; Mackill et al., 1993; Makumbi et al., 2011; Wester, 1960). Benefits to the environment have also been shown, with benefits from utilization of biodiversity having been shown to improve energy and nutrient fluxes, population recovery of some plant and insect species among other benefits (Crutsinger et al., 2006; Hughes et al., 2008). To ensure that the benefits of heterosis are realized, it is important to make informed decisions when selecting accessions or breeding lines for making crosses. A common caveat is the probability of hybrid incompatibility or undesirable linkages that occurs when making wide crosses (Mumba and Galwey, 1999). The phenomenon of hybrid incompatibility occurs between landraces from the Andean and Mesoamerican gene pools for both lima bean and common bean. Markers associated with hybrid incompatibility have been identified in common bean and similar markers together with in depth knowledge of the parents' genetic structure would be useful in a lima bean breeding program.

It is equally important to understand pathogen population dynamics to have a more effective disease or pest management strategy (Cooke and Lees, 2004). Considering the effect of host diversity and how this may affect evolution of the pathogen provides insights into better management. Changes in lima bean pathogen populations has also been observed, with the most well documented case in lima bean being that of the evolution of different races of *P. phaseoli*. Pathogen evolution risk models are recommended to inform breeding strategies, these models based on evolutionary characteristics of the pathogen. Mode of reproduction is a significant component of the model (Fig 6; McDonald and Linde, 2002a). The homothallic nature of *P. phaseoli* does not exclude it from the high-risk pool as it produces oospores, a sexually derived overwintering structure that can also be a product of outcrossing in some homothallic oomycetes to (Francis and St Clair, 1993). The two-speed genome, a genome characteristic shown in *P. infestans*, *P. sojae* and *P. ramorum* was also demonstrated in *Phytophthora phaseoli*, a characteristic defined by a rapidly evolving gene sparse region and a gene dense region where the rates of gene evolution are markedly less (Haas et al., 2009; Raffaelle et al., 2010). The gene sparse region contains plant induced genes and is rapidly evolving, containing a high repeat content, high rates of structural polymorphisms, mutations and genes involved in epigenetic processes (Raffaele et al., 2009). The high mutational rate of *P. phaseoli*, the presence of active transposable elements, aerial dispersion, efficient directional selection, presence of overwintering oospores and the possibility of outcrossing, makes the organism a high risk of evolution (McDonald and Linde 2002a).

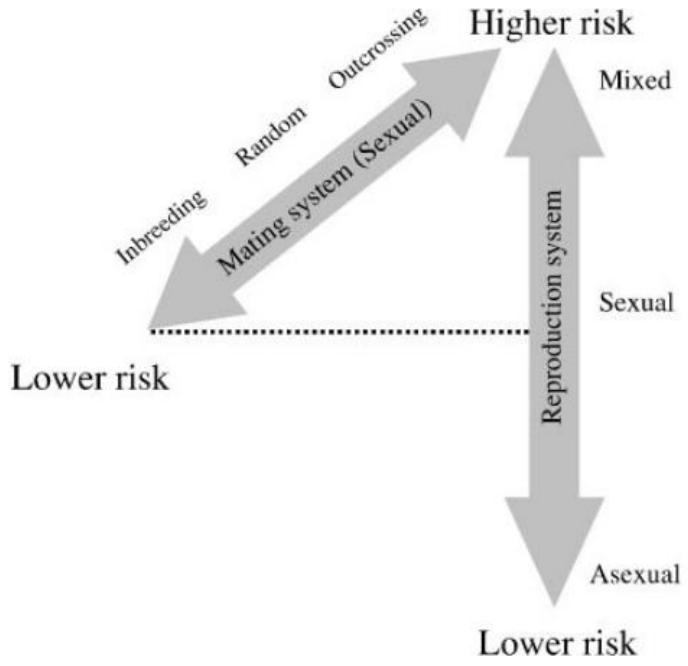

Figure 6 The interaction between pathogen mating and reproductive systems in determining the evolutionary potential of pathogen populations.

The population structure of *P. phaseoli* is not well known, with previous research suggesting a clonal population (Davidson et al., 2008). Despite a lack of some of this knowledge, the high-risk status of *P. phaseoli* has been observed. Directional selection of the pathogen has resulted in the evolution of races within a few years of deploying a new resistance gene, showing the ineffectiveness of single major gene resistance (MGR) and the need to use major resistance gene pyramiding and possibly quantitative resistance and MGR mixtures and multilines as methods of control (McDonald and Linde, 2002b). Similarly, the use of chemical control on this pathogen is a high risk, meaning that added precaution and strict observance of FRAC guidelines must be followed to prevent more incidences of resistance to mefenoxam.

Previous studies on the population structure of *P. phaseoli* had short comings in sample collection strategies and the ultimate sample size which was not adequate to compare between field and within field populations (Davidson et al., 2008; Pule et al., 2013). There is a need to revisit microbiological techniques and to devise optimal conditions for isolation, isolate maintenance and storage as the organism is slow growing and susceptible to bacterial contamination. The most recent research on optimum media was conducted in the 1950's (Hyre and Cox, 1953). The choice of molecular markers used was also not ideal as the markers obtained were unable to resolve the population structure of the isolates (Davidson et al., 2008).

Methods of Assessing Genetic Diversity

Morphology, amplified fragment length polymorphisms (AFLPs) and allozymes are the only methods that have been used to describe populations of *P. phaseoli* (Davidson et al., 2008). Lima bean has however been better studied, with a variety of genotyping methods and analyses being used. Lima bean has been described using morphology, seed protein characterization, allozyme patterns, random amplified polymorphic DNA (RAPD) markers, AFLP and next generation sequencing (NGS) techniques (DeBouck et al., 1989; Nienhuis et al., 1995; Caicedo et al., 1999; Chacón-Sánchez et al., 2017). Not all genetic markers are ideal and the advent of SNP genotyping has allowed the characteristics of ideal markers to be attained, where markers should be selectively neutral; polymorphic; specific to single loci; have low potential for homoplasy; independent; codominant, be repeatable and unambiguously scored (Milgroom, 2015). Cost and ease of development are also important in defining

an ideal marker. Genotyping-by-sequencing is a recent technique that is highly adaptable and can be used for almost any organism. It has been shown to be a superior genotyping technique as it fulfils the hallmarks of generating ideal markers (Milgroom, 2015). Genotyping-by-Sequencing reduces the genome using restriction enzymes, followed by multiplexing, size selection, PCR, more size selection and then sequencing of the remaining loci using NGS (Manching et al., 2017). Data analysis is a challenge which is touted as the biggest barrier in the use of GBS, more so for organisms which have no sequenced reference genome, resulting in the discovery of fewer, albeit high quality SNPs (Torkamaneh et al., 2016). There are three basic steps to analyzing GBS data, these being raw data processing; read alignment to a reference genome or de novo assembly of the sequence tags; and variant discovery and annotation (Kagale et al., 2016). There are various approaches and pipelines for analyzing this form of data, with some that use and validate de novo references to cater for organisms which do not have reference genomes (Bradbury et al., 2007; Catchen et al., 2013; Elshire et al., 2011; Manching et al., 2017; Mhora et al., 2016). It is equally important when using this data in non-reference organisms to ensure that controls are incorporated into the experiment, allowing filters to be designed based not just on quality scores, but on the biology of the organism and parameters which exclude repeat or chimera sequences (Browning and Browning, 2011; Catchen et al., 2013, Edgar et al., 2011; Kagale et al., 2016). These filters are important as it has been shown that the most significant source of genotyping error is due to repeat regions of the genome and from genotyping errors. It is equally important to validate and be aware of the software one chooses to analyze their data as there are known faults in certain software (Torkamaneh et al., 2016).

Genotyping-by-sequencing is suitable for population studies, germplasm characterization, breeding and trait mapping in genome wide association studies (GWAS). This technique has the advantage of being able to detect genomic regions that harbor sequence variants responsible for complex traits and to capture regulatory regions that control expression of genes responsible for agronomically important phenotypes (Elshire et al., 2011; Manching et al., 2017; Mhora et al., 2016; Van Tassell et al., 2008). Additional traits such as heat tolerance and drought resistance are also part of the long-term plans of the Delmarva region and these techniques could facilitate the generation of broader knowledge and resources such as genetic markers to accelerate the breeding process in lima bean (Ernest and Johnson, 2011; Semagn et al., 2014).

Project Hypotheses and Objectives

This background information, coupled with the needs of the MAR in terms of lima bean improvement and enhanced disease control lead us to the below hypotheses, which will be tested using an interdisciplinary approach. Information will be generated on both host and pathogen, providing advice and genetic resources for plant breeders, diagnosticians and growers, who will use it all to ultimately improve lima bean production in the MAR.

Hypothesis 1: The population structure of *P. phaseoli* in the MAR is characterized by its race structure.

Hypothesis 2: There is resistance to mefenoxam in *P. phaseoli* isolates from the MAR

Hypothesis 3: The genetic diversity of commercial lima bean cultivars in the MAR is narrow and can be improved by external sources contained in our diversity panel

Objectives

1- Collect, isolate and phenotype (resistance to mefenoxam, morphology and race) populations of *P. phaseoli* from grower fields in the MAR

2- Optimize GBS in a pilot study using a limited collection of *P. phaseoli*, an organism without a reference genome and to develop diagnostic markers

3- Optimize a non-reference GBS approach in lima bean using an F_2 population segregating for resistance to race F

4- Identify and validate loci responsible for conferring resistance to *P. phaseoli* and develop markers for marker assisted selection

5- Characterize the genetic diversity of a diversity panel of lima bean accessions from different geographic locations and compare it to existing local cultivars

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Chapter 2

AN ASSESSMENT OF MEFENOXAM INSENSITIVITY IN *PHYTOPHTHORA PHASEOLI* IDENTIFIES RISKS TO PROFITABLE LIMA BEAN PRODUCTION IN DELAWARE

Introduction

Lima bean is the most important vegetable crop for the state of Delaware, the largest producer for the frozen vegetable production industry in the United States. Lima bean yields can be severely affected by disease-causing pathogens, one of which is *Phytophthora phaseoli*, the most historically important pathogen to lima bean production in the eastern United States. The lima bean pathogen, *P. phaseoli* is a homothallic oomycete in the kingdom Stramenopila. This organism is characterized by fungal-like growth and infects and colonizes flowers, racemes, shoot tips and pods of lima bean in the field, producing white downy mycelia and sporangia on infected tissue. Infection by *P. phaseoli* is also characterized by a reddish-brown margin around the lesion on infected lima bean pods, a diagnostic feature of *P. phaseoli* infection (Evans et al., 2007).

Downy mildew was first identified in Connecticut where it decimated entire crops (Thaxter, 1889), forcing producers south to New York, New Jersey and ultimately to Delaware, as attempts were made to evade the pathogen. In 1903, the

pathogen had spread from Connecticut to Delaware, where Smith observed the pathogen causing “injurious damage” on lima bean (Wester, 1970). The only observed physiological race at that time was race A, which prompted a breeding exercise that yielded the resistant lima bean cultivar, Thaxter (Wester, 1970). Physiological races are distinguished by their specialization for pathogenicity on different cultivars of the same species and are hereafter referred to as race (Kirk et al., 2001). Before the commercial release of Thaxter in 1958, race B of the pathogen had been discovered, the first time a different race had been observed. Within nine years, race C emerged and was followed by race D, which dominated during the 1970s and 1980s until the emergence of race E (in 1995) and race F (in 2000) (Evans et al., 2002). The emergence of these two new races were concurrent with huge losses, which peaked at 40% of farm yields, an estimated $3M loss in 2000 (Davidson et al., 2008, Kee et al., 1997). These new races E and F were also heat tolerant, capable of infecting the crop later in the season and at higher temperatures (Evans et al., 2002). Differential cultivar susceptibility is still the only method of identifying and characterizing the race structure of populations of *P. phaseoli.* Race F is still the only race currently observed in the field (Davidson et al., 2008). There are no commercial cultivars resistant to both races E and F nor are there any horticulturally acceptable cultivars in use that carry race F resistance, leaving mefenoxam application as the most effective form of control after onset of the disease (Evans, Personal communication, Kunjeti et al., 2012). Copper and labelled phosphonate applications can be used as a preventative spray when the forecast predicts conducive conditions for the pathogen (Evans et al., 2002).

It is imperative to monitor plant pathogen populations as this allows informed decisions to be made by growers, vastly improving existing disease control strategies by informing cultivar and fungicide choices. Improvements in lima bean cultivar development have not only resulted in more choices for growers but also in molecular resources which breeders are using to accelerate cultivar improvement programs (Mhora et al., 2016). The ability to characterize and identify pathogen populations in grower fields using rapid methods will result in the optimal selection of an effective fungicide (with an appropriate mode of action) and/ or cultivar choice by appropriately deploying or rotating fungicides and R genes. The availability and use of fungicides with different modes of action (MOA) in conjunction with fungicide use guidelines provides alternatives that growers can effectively use when the pathogen populations in their specific locations or fields have been evaluated for fungicide insensitivity (Wyenandt et al., 2009). These steps are integral in ensuring the longevity of a MOA in the field (van den Bosch et al., 2018).

Mefenoxam is the most widely used chemical control method for downy mildew in lima bean and is also used in combination with copper in curative applications (Davidson et al., 2008). There has been over reliance of this and other chemicals, resulting in plant pathogen populations such as *P. capsici* developing resistance (Davey et al., 2008; Fry et al., 2009). Resistance to Mefenoxam has also been observed in *P. phaseoli* populations, lending further cause to effectively characterize pathogen populations for their insensitivity to the product and for their race structure (Davidson et al., 2008). Phenotypic characterization is a commonly used method to assess fungicide insensitivity (Dekker, 1988) and is the first step in

identifying resistance mechanisms and developing more rapid and sensitive molecular detection tools (Zulak et al., 2018).

Allozymes and amplified fragment length polymorphism (AFLP) have been traditionally used to resolve populations of *Phytophthora* by showing their origin, stratification and migration patterns among other population characteristics (Goodwin et al., 1995; Ivors et al., 2004). These methods were however unsuccessful in describing populations of *P. phaseoli*. Assessment of the Glucose-6-phosphate isomerase (*Gpi*) and Peptidase (*Pep*) loci using the method of Goodwin et al., (1995) on isolates of *P. phaseoli* revealed that these loci were monomorphic (Davidson, 2002). The AFLP data was effective in differentiating *P. phaseoli* from *P. infestans* and *P. capsici* but was unable to differentiate the organism based on characteristics such as mefenoxam sensitivity, race, geographic location and migration patterns among others, leading to assumptions that the 180 isolates collected from the MAR at that time were a single clonal population (Davidson, 2002; Davidson, 2008). The allozyme analysis was repeated in the Fry Lab at Cornell University, Ithaca, New York, using some of the older isolates used by Davidson et al., (2008) as a control and a subsample of the new isolates collected in the 2013 and 2014 field season. These analyses on four duplicate isolates showed the absence of discernible polymorphism at this locus (Fig. 7), consistent with a lack of mutations at this locus over time.

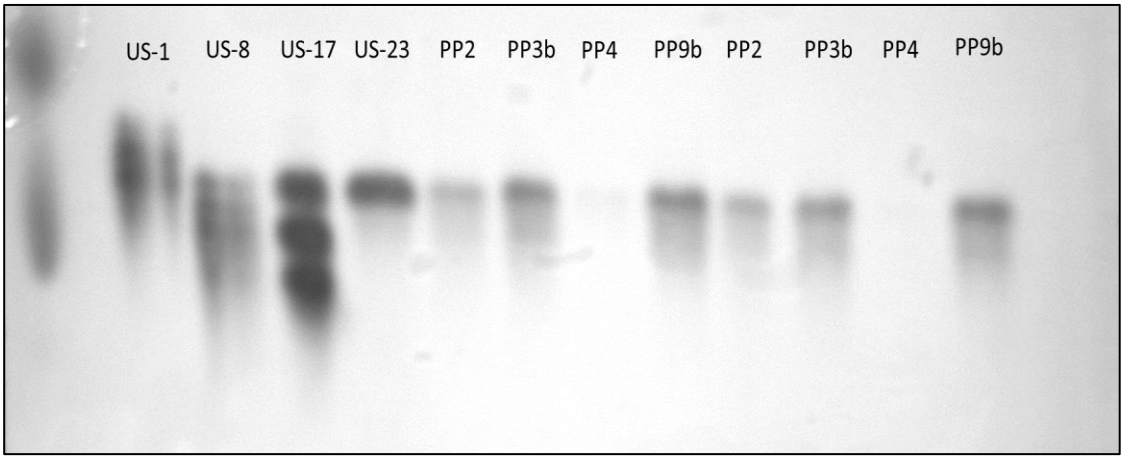

Figure 7 *Gpi* analysis of races C (PhyP2); D (PhyP4); E (PhyP31) and F (PhyP37) of *Phytophthora phaseoli*. Races E and F were isolates collected from the field in the 2014 season.

High throughput and more intensive molecular techniques have since been developed, Genotyping-by-Sequencing (GBS) being one such example. Genotyping-by-sequencing is a reduced representation sequencing technique that reduces genome complexity using restriction enzymes followed by multiplexing, rounds of size selection on the DNA, PCR and sequencing of the selected loci using next generation sequencing (Manching et al., 2017). This method is highly adaptable and can be used for population studies, germplasm characterization, breeding and trait mapping in a range of organisms (Elshire et al., 2011). This method also has the advantage of capturing regulatory regions that control the expression of genes responsible for important phenotypes and are often located in non-coding genomic DNA (Elshire et al., 2011). Techniques such as GBS make it possible to analyze a pathogen's population structure. The combination of population genetic information and pathogen phenotype data can allow the design of molecular markers which can be used to rapidly characterize populations and phenotypes. Inferences derived from using

molecular markers can drive timely production decisions such as cultivar and chemical choices, resulting in enhanced pathogen management.

In this study, a description of the assessment of mefenoxam sensitivity in isolates of *P. phaseoli* from the MAR was made. The potential of GBS as a tool for describing the population structure of diverse isolates of *P. phaseoli* was also conducted in a pilot study which used 11 historic isolates comprised of races C, D, E and F. This combined information allows better information to growers on any incidences of mefenoxam insensitivity and the presence of new clonal lineages. These two factors are important in the management of downy mildew of lima bean as mefenoxam is the most efficient and cost effective curative control available on the market. The emergence of mefenoxam insensitive populations would cause losses due to lack of production or the transition to more expensive fungicides with a different MOA. Surveillance of the clonal structure of *P. phaseoli* is also critical as the emergence of a new clonal lineage may have implications such as different phenotypes (Hansen et al., 2016; Saville et al., 2015) which may require different management strategies to be employed. The homothallic nature of *P. phaseoli* means that mutation and selection for more fit individuals is key in the acquisition of a new phenotype, while migration is critical in ensuring the spread of these more fit individuals (Hansen et al., 2016; Danies et al., 2014). Some examples of phenotypes that appear with new clonal lineages include host shifts, breaking of host resistance, increased virulence and fungicide resistance (Saville et al., 2015). The success of investigating the population structure of pathogens using GBS to help formulate management guidelines has been demonstrated in other species of *Phytophthora*,

where sources of inoculum (Tabima et al., 2017) and the sexual behavior of a pathogen was successfully monitored (Carlson et al., 2017). A successful pilot study using GBS on a small population will allow a broader investigation on the population structure to of *P. phaseoli* to follow, permitting in-depth assessments such as the detection of population shifts to become possible.

Materials and Methods

Isolate Collection, Media Formulation and Mefenoxam Sensitivity Assays

There are eleven isolates of *Phytophthora phaseoli* in our collection that have been previously characterized using allozymes, AFLPs and phenotyping (Davidson, 2002; Davidson et al., 2008). These isolates were obtained from infected lima bean tissue that was submitted to the University of Delaware's plant diagnostic clinic from different grower fields located in the New Castle (2 isolates), Kent (5 isolates) and Sussex (4 isolates) counties between years 2000 and 2006 (Table 1). Isolates are maintained in the UD Plant & Soil Sciences Microbial Culture Collection.

There were an additional 93 isolates collected between the years 2013 and 2017 (details of isolates in link), bringing the total number of isolates in the collection to 104. These additional isolates were mainly collected from Sussex county Delaware, with others being collected from Kent and New Castle counties in Delaware, Cecil County in Maryland and Salem County in New Jersey. Rye isolating agar was used to isolate the pathogen from infected plant material. All 104 isolates were screened for

mefenoxam insensitivity and a random sample (4 isolates) assayed at the *Gpi* locus (Fig 7). Growth rates of the pathogen were very slow, making isolation and maintenance of the new isolates difficult. Improvements in the media formulation were required to enhance isolate maintenance and to perform reliable phenotyping. Frozen lima bean agar (Calvert et al., 1960), pea agar (Kunjeti et al., 2012) and oatmeal agar (18 g oatmeal agar, 3 g agar, [Becton, Dickinson and Company, NJ, USA], 500 ml deionized water) were amended with different combinations of either one, two or three of the micronutrients D-alanine (0.1%), dextrose (0.5%) and thiamine (0.06%). These trace elements have been shown to greatly improve growth of *P. phaseoli* on media (Hyre and Cox, 1953).

The slow growing nature of *P. phaseoli* and the presence of currently uncharacterized biofilms containing bacteria (Evans, personal communication) which can outcompete the oomycete isolates makes it difficult to grow from single zoospores. A range of methods including flooding sporangia with either water or Chen-Zentmeyer solution (Chen and Zentmyer, 1969) at multiple temperatures was unsuccessful at obtaining motile or viable zoospores for culture.

To assess the heterogeneity of composite field isolates, single sporangial culture techniques were ultimately used to subsample composite isolates. Composite isolates are defined in this study as being cultures of *P. phaseoli* which have been mass transferred from infected field samples and not from single sporangia or zoospores. Single sporangia were obtained by filtering sporangial suspensions using sterile nylon mesh filters (Membrane Solutions, WA, USA) with a pore size of 60 µm

to exclude mycelia and media debris while allowing sporangia to pass through (maximum diameter of *P. phaseoli* sporangia is ~50 µm). Filtered sporangia were spread on water agar, potato dextrose agar (25% of the Becton, Dickinson and Company instructions) and lima bean agar. Sporangia that formed germination tubes were labelled after a day and transferred to individual plates after signs that the isolates were actively growing and had not originated from multiple sporangia or mycelia.

Field isolates and single sporangial isolates derived from these field isolates were tested for mefenoxam sensitivity using Ridomil Gold SL (45.3% mefenoxam) over two years by independent researchers. Mefenoxam sensitivity assays were conducted using the method described by Parra and Ristaino (2001), who had modified a similar method of assessing metalaxyl sensitivity by Therrien et al., (1993). Both methods described an isolate as being either sensitive, intermediately sensitive or insensitive when it was grown in media containing different doses of mefenoxam and compared to its growth in medium containing no mefenoxam (control). Three concentrations (5, 50 and 100 ppm) were initially tested during mefenoxam sensitivity assays on all the new isolates (N = 93). It was found that there was no significant difference in the relative amount of growth in sensitive isolates using either 5, 50 or 100 ppm concentrations, leading to use of only 5 ppm and 100 ppm in assessing mefenoxam sensitivity in the historical and single sporangial isolates.

The different categories of sensitivity are described as: sensitive, where growth of less than 40% of the control in 5 ppm and 100 ppm of mefenoxam is observed;

intermediately sensitive where growth of over 40% of the control at 5 ppm but less than 40% of the control in 100 ppm of mefenoxam is observed and as insensitive where growth of over 40% of the control in both 5 ppm and 100 ppm of mefenoxam is observed. An isolate was only assigned a mefenoxam sensitivity rating of insensitive or intermediately sensitive when the same isolate had the same result over the two years of testing, with the only exception being when an isolate was only tested in one year.

To ensure that the activity of mefenoxam was not reduced over the four weeks of isolate testing, Petri dishes containing lima bean agar amended with dextrose (0.5%) and different concentrations of mefenoxam (5, 50 and 100 ppm) were incubated together with isolates that were being screened. Mefenoxam sensitive isolates of *P. capsici* were then grown on these incubated plates to assess if they would be inhibited at comparable rates to isolates grown in the same media that was freshly made. This was done to confirm that isolate growth was not affected by changes in mefenoxam activity as it is not known how stable mefenoxam was in our experimental setup described in Davidson et al., (2008). Comparison of growth was made using average colony diameter which was measured as the difference between the diameter of the agar plug and the longest diameter (d1) and its perpendicular measurement (d2) of the colony. The area occupied by the colony was also calculated by multiplying the two measured diameters (d1 × d2). Analysis of variance followed by the Tukey-Kramer HSD method was used to compare all treatments to each other.

Genotyping of Isolates and Data Analysis

Isolates were grown in frozen lima bean broth, made using 65 g of frozen lima beans (ACME brand, Delaware, USA) that were covered with deionized water, thawed in a microwave oven, blended, filtered with reagent grade cheesecloth and made up to a volume of 650 ml with deionized water. Lima bean broth was aliquoted into 50 ml flasks and autoclaved. Each isolate was grown in autoclaved broth over ten days at 100 rpm on an orbital shaker, harvested and the mycelial tissue lyophilized for 36 hours. DNA was extracted from the tissue of each isolate using the Wizard Promega DNA extraction kit (Promega, Wisconsin, USA). To ensure high quality DNA, quality checks were conducted, these being quantification on the NanoDrop and with the PicoGreen (Invitrogen, Massachusetts, USA) fluorimetric method followed by gel electrophoresis. High quality DNA was used in GBS of the isolates. The restriction enzyme pairs of *Sbf1* and *Csp6* were used in the GBS protocol described in Manching et al., (2017). Samples were size-selected for 300 bp fragments on the Blue Pippin (Sage Science Inc., Massachusetts, USA) after the multiplexing step to ensure accurate and repeatable genomic sampling. Size selection was carried out at the University of Delaware Sequencing and Genotyping Center (UDSGC). The GBS library was sequenced using 101 bp single end Illumina sequencing at the UDSGC and the raw sequence data was processed using the non-reference pipeline of RedRep, a custom bioinformatics pipeline described in Manching et al., (2017).

Processed GBS data were analyzed using a custom R script in which the data were filtered for completeness and quality using the vcfR Package (Knaus and Grunwald, 2017). Genotype data were filtered by excluding SNPs with a read depth

outside the range of 5 – 2500; more than 15% missing data per SNP and more than 15% missing data per individual. Previous research suggested that isolates of *P. phaseoli* found in Delaware belonged to a single clonal lineage (Davidson, 2002, Davidson et al., 2008). To test this hypothesis, the I_A was calculated in Poppr, an R package for the genetic analysis of populations, to determine the clonal nature of the historic isolates by assessing their rates of linkage disequilibrium (Goss et al., 2014; Kamvar et al., 2014; Kamvar et al., 2015).The index of association (I_A) is a model free approach which uses permutation to assess if loci are linked over all loci in a data set.

Results

Media Formulation and Mefenoxam Sensitivity Assays

When different isolates were tested on the amended media, it was found that growth rates and the amounts of growth were different among isolates. Frozen lima bean agar amended with 0.5% dextrose was found to result in significantly higher growth rates of *P. phaseoli* isolates than other media formulations, with an average diameter of 7.26 cm ± 0.19 cm on the fourth week of measurement (Fig 8). This growth surpassed the growth in pea agar (Peasy) (3.23 ± 0.42 cm) and in non-amended lima bean agar (4.74 ± 0.09 cm) which were previously recommended (Kunjeti et al., 2012). Frozen lima bean agar amended with dextrose was subsequently used for growth and mefenoxam sensitivity evaluations. The diameters of every composite isolate were normally distributed and ranged between 35 and 76 mm on the fourth week of incubation for each composite field isolate. There were five fields that each

had at least five isolates collected from that location and on the same date. Isolates from these fields were chosen to show the range and variability of composite isolate growth from a similar location before exposure to varying concentrations of mefenoxam over a four-week period (Fig 9). The fields were numbered 1 – 5, with field 1 (N = 21), field 2 (N = 10), field 3 (N = 10) and field 5 (N = 5) from Sussex county in Delaware, while field 4 (N = 6) was in Salem county, New Jersey. When media with no mefenoxam was considered, the field from Salem County in New Jersey (field 4) had isolates with the overall highest absolute growth rate and largest amount of colony growth among the sampled fields, whereas field 3 in Sussex County was similar to both New Jersey and Delaware field isolates (Fig 9).

Single zoosporangial isolates of *P. phaseoli* were never obtained. All combinations of temperature and suspension solution in either the flooded plates or sporangial suspensions did not result in the release of visibly motile or viable zoospores. This was in contrast to a control experiment on *P. capsici* isolates, where zoospores were clearly motile and it was possible to obtain single zoosporangial isolates from these zoospores in all treatments, with the Chen-Zentmyer solution yielding the greatest number of released zoospores. Use of the nylon mesh filter to obtain single sporangial isolates from composite field samples of *P. phaseoli* was however successful. There were between two and seven single sporangial isolates obtained for each composite field isolate and these were also tested for mefenoxam sensitivity alongside the composite field isolates.

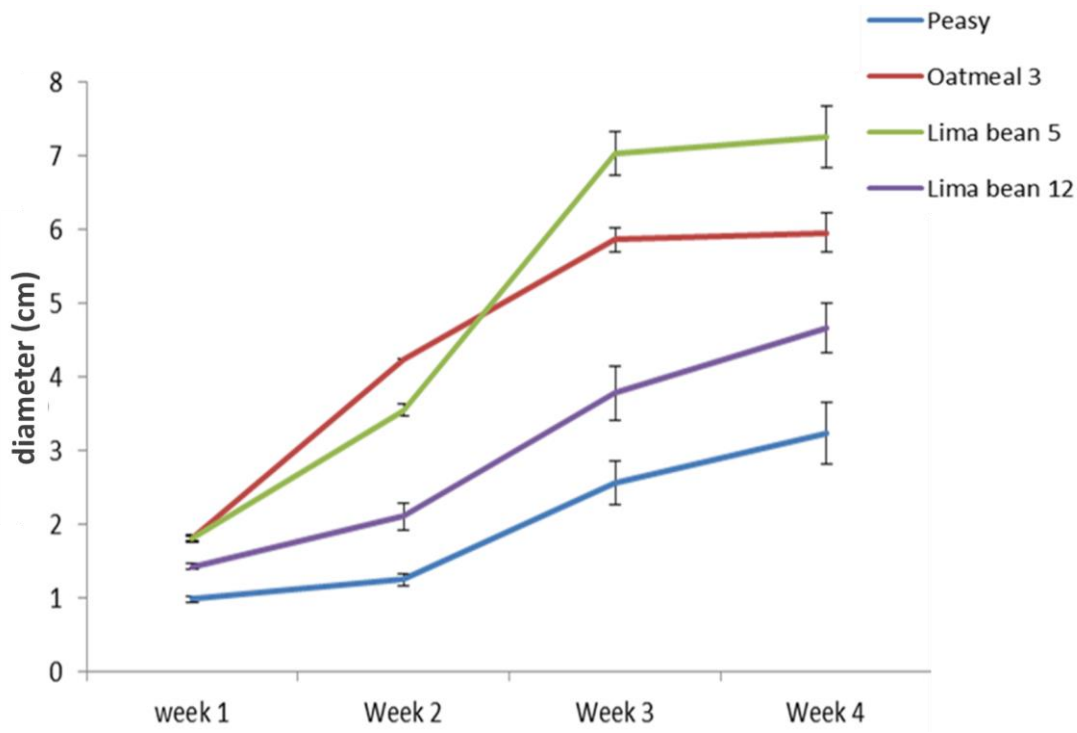

Figure 8 A subset of different culture media used to determine the best substrates for allowing the greatest amount of growth in an isolate of *Phytophthora phaseoli*. Lima bean 5 was lima bean agar amended with 0.5% dextrose, Lima bean 12 was plain frozen lima bean agar; Oatmeal 3 was oatmeal agar with 0.5% dextrose. Peasy was pea agar with no amendments.

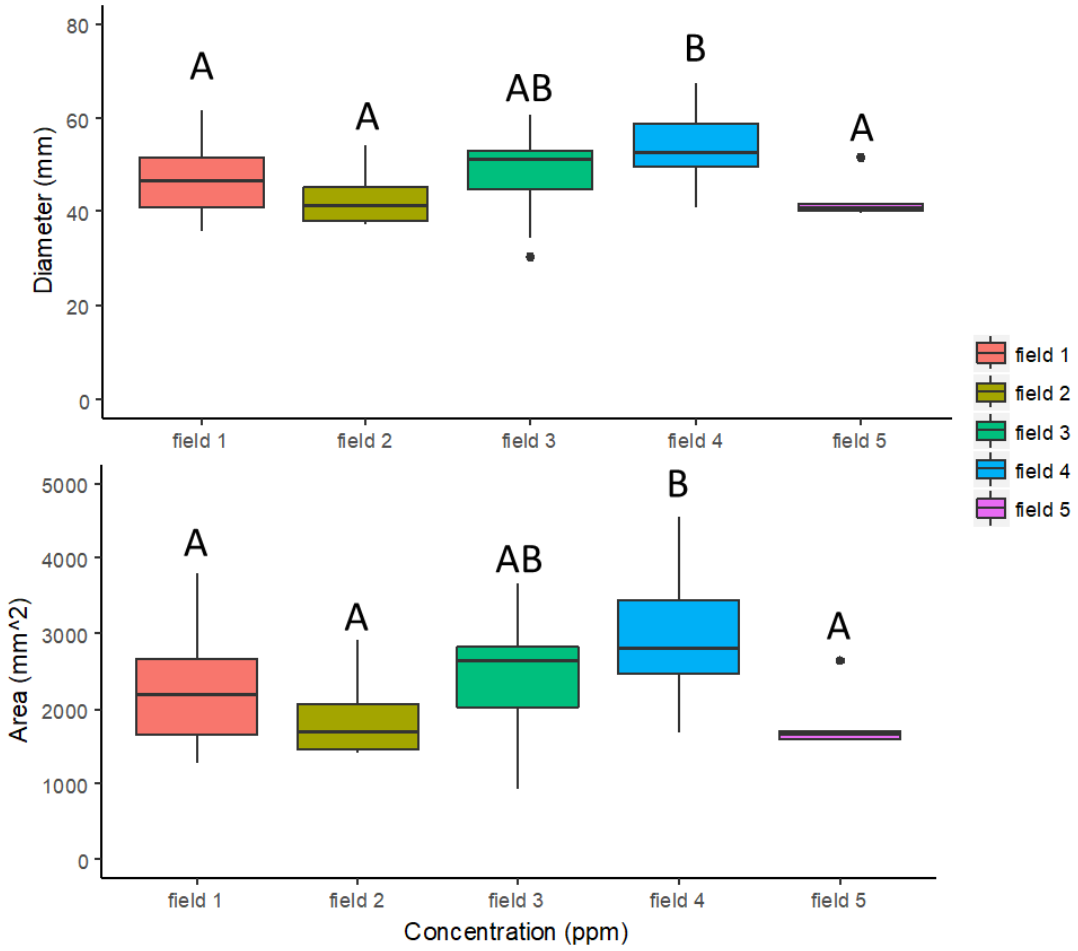

Figure 9 The absolute amount of growth in frozen lima bean agar with no mefenoxam added to it and grouped by collection field. Both diameter (top) and area (bottom) of isolates were used to compare the different fields. Letters above each boxplot denote field isolates that are significantly different using the Tukey-Kramer HSD.

Mefenoxam sensitivity assays on both new and historical isolates showed that there were isolates of *P. phaseoli* in the MAR that are intermediately sensitive and insensitive to mefenoxam (Fig 10). Among the recently collected isolates that were intermediately insensitive, all came from two fields in the Milton area of Sussex County in Delaware (table in link). Isolate PhyP12, one of the historic isolates

collected in 2003 was insensitive to mefenoxam, a similar observation to isolates collected from the same location (Dominiak, 2002). Similarly, single sporangial isolates made independently from isolates from the same field were also consistently insensitive over two years of independent testing. There was an insensitive single sporangial isolate which at 5 ppm of mefenoxam, grew at comparable rates to when it was grown in the control medium, demonstrating the existence of isolates which are highly adapted to mefenoxam (Fig 10).

A comparison of the amount of growth in all the isolates in the collection was made after four weeks, using measurements of both area and diameter of isolates grown in Petri plates containing frozen lima bean medium containing no mefenoxam. Sensitive isolates had more amounts of growth and a broader range of measurements when compared to the insensitive isolates, which had a lower and narrower range of growth (Fig 11). To investigate if isolate variability arising from different collection time and field location could be influencing the difference in growth between sensitive and intermediately insensitive isolates, field 1 was independently assessed. Field 1 was chosen as most of the intermediately sensitive isolates were observed in this field. It was found that intermediately sensitive isolates in field 1 also had statistically less growth (28.4 – 34.9 mm) when compared to the sensitive isolates (31.3 – 55.8 mm) from the same field, a similar observation made when all the isolates were analyzed together. This observation suggested a growth rate penalty in isolates that are insensitive to mefenoxam when grown in frozen lima bean agar with no mefenoxam added to it.

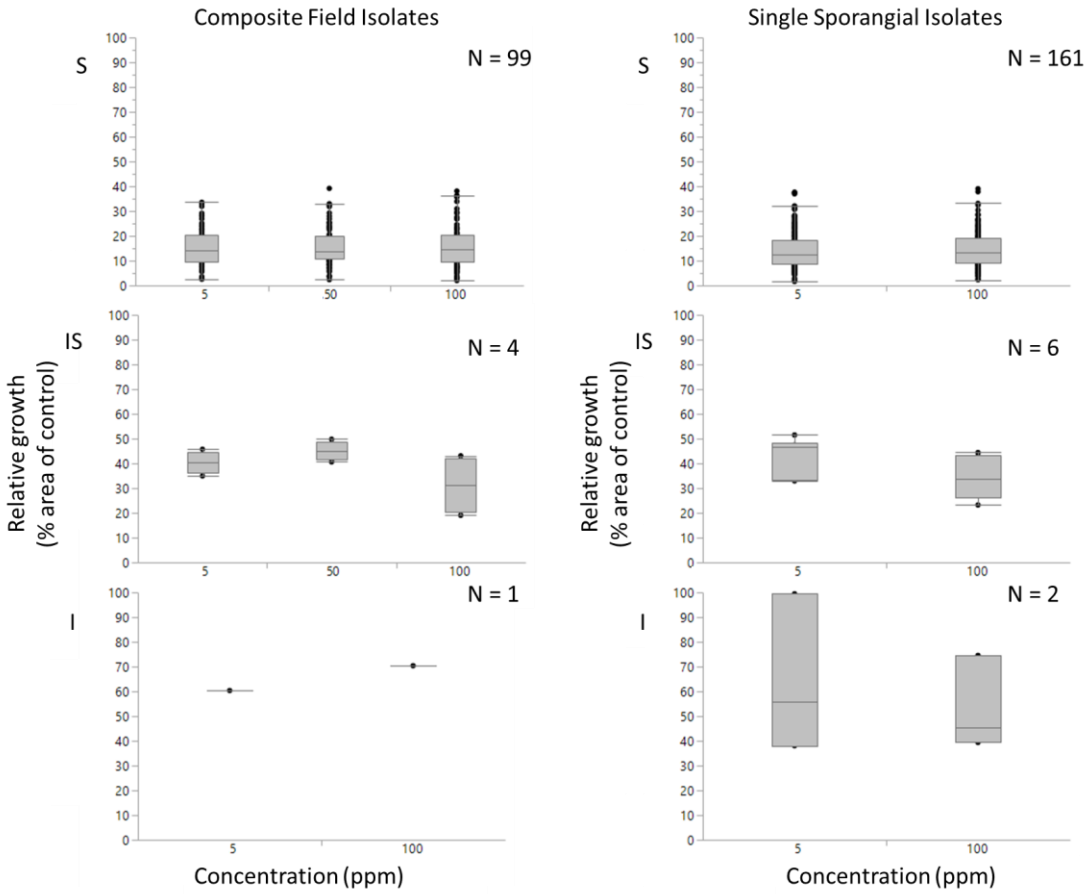

Figure 10 Distributions of the relative growth for each different sensitivity group for both composite and single sporangial isolates of *Phytophthora phaseoli*. Mefenoxam sensitivity assays were conducted by independent researchers over two years, with a subsample being assessed in 2016 and the entire collection being assessed in 2017 - 2018. Letters to the left of each graph denote: **S** – sensitive, for graphs on the top row; **IR** – intermediately sensitive, for graphs in the middle row; **I** – insensitive, for graphs in the lower row. The N for each group is shown in the top right-hand corner of each graph.

Single sporangial isolates had statistically confirmed reductions in growth when compared to their counterpart composite isolates, demonstrating an advantage in *P. phaseoli* of growing as mixed populations (Fig 12). The amount of growth in

composite isolates were compared to single sporangial isolates from the same field and from the same sensitivity group to limit variance from different field location and from mefenoxam resistance. The results within the other fields were similar and so only data for field 1 are presented (Fig 12). Composite isolates grown in non-amended media had statistically more growth that single sporangial isolates in both sensitive and insensitive groups. This demonstrates that mixed isolate populations complement each other and grow better than single sporangial isolates on frozen lima bean agar. We had insufficient data to calculate accurate EC50 values as we only used either three or two concentrations to determine mefenoxam sensitivity. Mefenoxam sensitivity assays using ten concentrations ranging from 1 - 100 ppm are currently being conducted on representative isolates to determine their EC50 values.

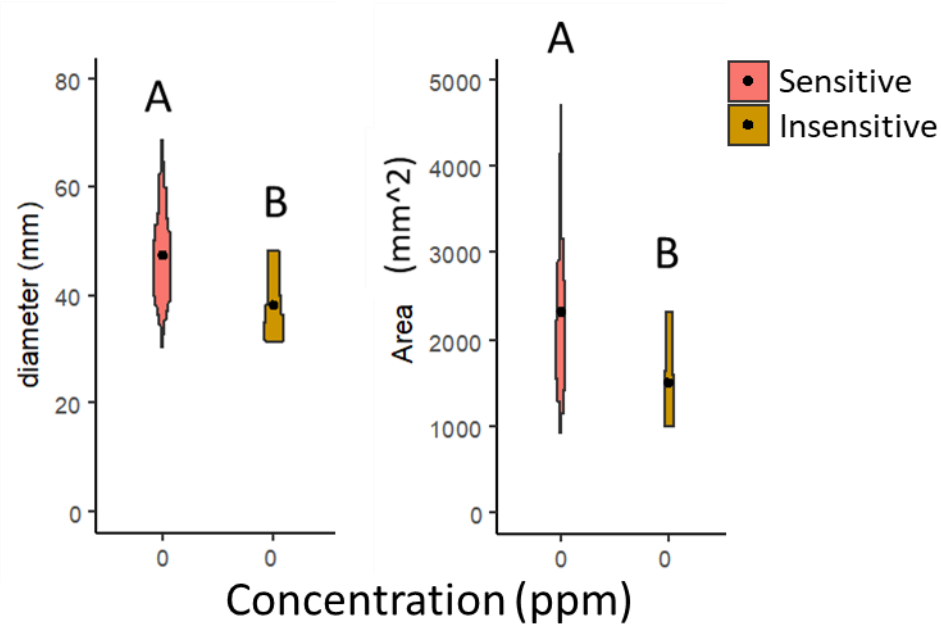

Figure 11 A comparison of the absolute growth rates of mefenoxam sensitive composite isolates (N = 99) of *Phytophthora phaseoli* compared to insensitive and intermediately sensitive isolates (labelled as insensitive; N = 5). Comparisons of growth rate were made using both colony diameter and the area which the mycelia occupied in a Petri plate. The absolute growth of these isolates was measured from isolates grown on the control frozen lima bean medium containing no mefenoxam. Letters above each violin plot denote significant differences determined using the Tukey-Kramer HSD.

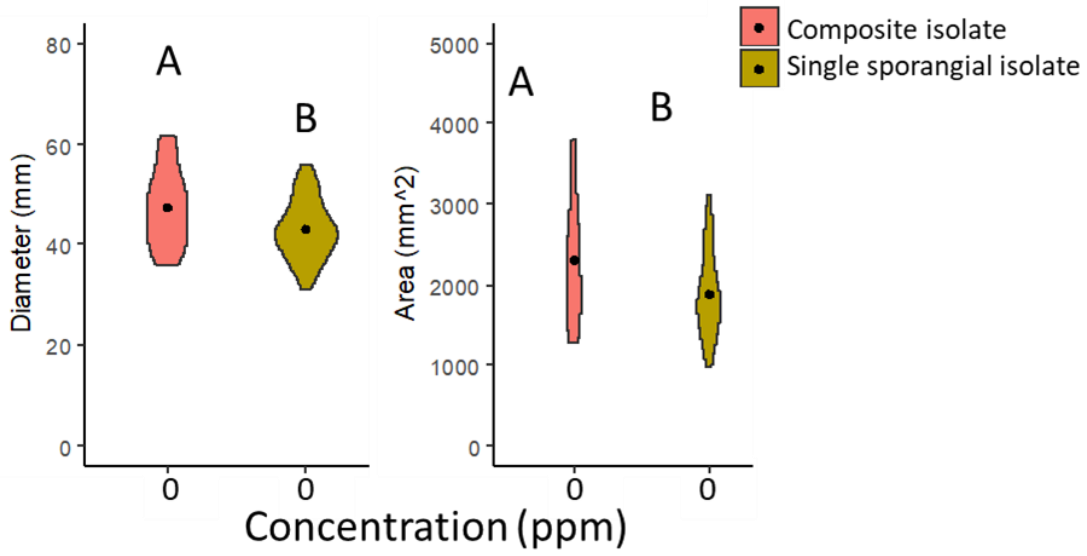

Figure 12 A comparison of the absolute growth rates of composite isolates (N = 20) of *Phytophthora phaseoli* compared to single sporangial isolates derived from them (N = 48). Comparisons of growth rate were made using both colony diameter and the area which the mycelia occupied in a Petri plate. The absolute growth of these isolates was measured from isolates grown on the control frozen lima bean medium containing no mefenoxam. Letters above each violin plot denote significant differences determined using the Tukey-Kramer HSD.

GBS Data Analysis

Genotyping-by-sequencing (GBS), a cost-effective method for genotyping multiple individuals across the whole genome was conducted on the 11 historic isolates in our collection which are comprised of races C, D, E and F (Table 1). The choice of enzyme pairs in the genome complexity reduction step of GBS is critical to maximize the number of adequately sequenced loci. The enzyme combination of

Sbf1/CviQ1 produced the highest number of loci and sequences compared to *PstI/CviQ1* (Table 2). The filtered data consisted of 1,577 SNP variants, 4.14% missing data within the entire filtered data set and 14.8% missing data being the most within an individual (Fig 13).

Table 1 Details of historical isolates of *Phytophthora phaseoli* used in the genotyping by sequencing pilot study

Isolate	Race	Collection site	Collection year	[a] Mefenoxam sensitivity	[b] Dry weight (g)	Colony Diameter ± SD
PhyP2	C	DE	2000	S	0.0605	70.3 ± 9.29
PhyP4	D	DE	2000	S	0.0607	69.3 ± 4.01
PhyP6	E	DE	2000	S	0.0838	58.7 ± 2.33
PhyP11	E	DE	2003	S	0.0778	46.7 ± 1.04
PhyP12	E	DE	2003	I	0.0943	54.8 ± 1.60
PhyP15	E	Newark	NA	S	0.0569	58.7 ± 1.63
PhyP18A	F	Sussex	2003	S	0.0677	54.2 ± 1.04
PhyP18B	F	Sussex	2003	S	0.0561	52.3 ± 0.29
PhyP19	F	Sussex	2003	S	0.0825	66.5 ± 1.32
PhyP22	E	Sussex	2003	S	0.0419	51.3 ± 1.15
PhyP25	F	Sussex	2006	S	0.0806	55.2 ± 5.96

[a] – S = sensitive, I = insensitive
[b] – Dry weight is the weight of mycelia from *Phytophthora phaseoli* cultures that have been grown in lima bean broth and lyophilized for 48 hours.

Table 2 Preliminary GBS study done using 11 isolates. The library had a total of 50,604,123 reads after QC

	Sbf1/CviQ1	*Pst1/CviQ1*
Total no. of reads	47,198,893	9,421,795
Read count after QC	42,223,059	8,381,064
Percent reads retained	89.46	88.95

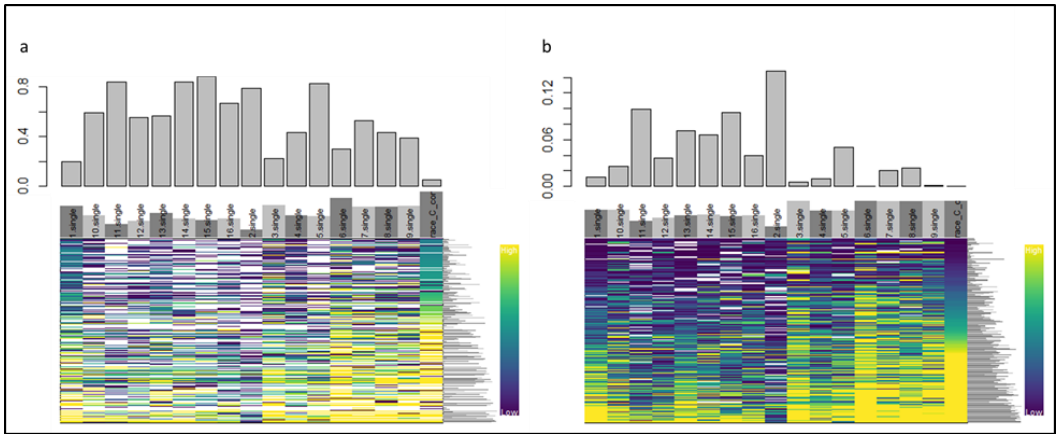

Figure 13 The quality of the data (a) before and (b) after filtering. White spaces in the heatmaps indicate missing data whereas the scale is from purple (low read depth) to yellow (high read depth). The histograms above the heatmaps show the proportion of missing data per individual.

An assessment of the possibility of random mating in these 11 historic isolates was conducted by testing linkage disequilibrium in a pilot study to test whether GBS is a useful method for future isolate testing on larger populations. The index of association (I_A) was calculated for the overall population and for groups of isolates from races E (N = 5) and F (N = 4). The null hypothesis of no linkage between markers was rejected using both the I_A (P = 0.000 1 for all tests; Fig 14) and the

standardized index of association, suggesting that the entire historic population originated from a single clonal lineage.

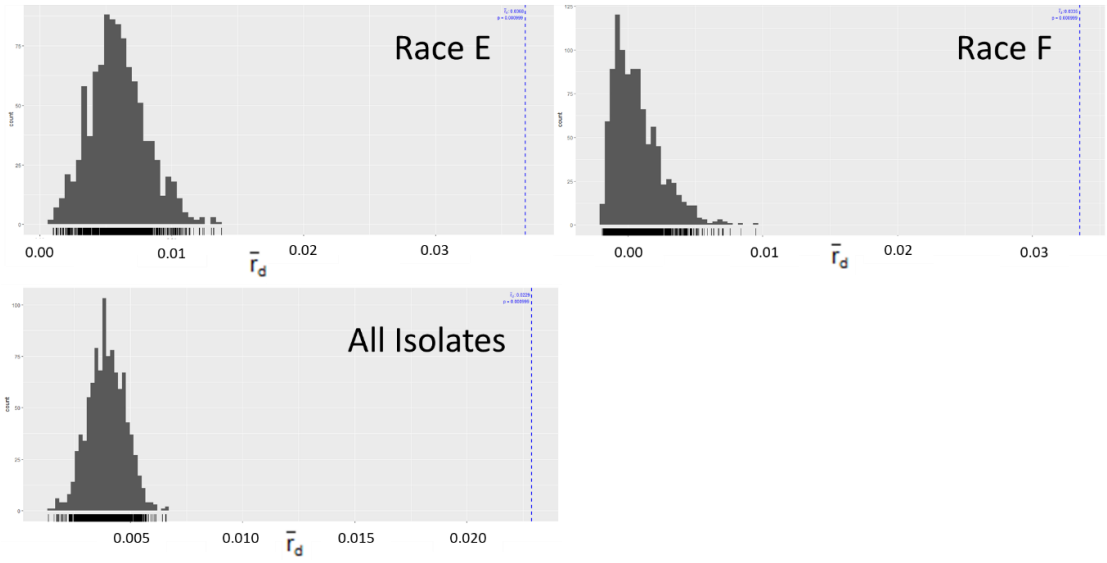

Figure 14 Index of association (I_A) for races E (N = 5) and F (N = 4) and for all isolates in the historic collection. Each I_A is calculated after 1,000 replicates to determine significance between the observed value of I_A (blue dashed line) versus the hypothesis of no linkage among markers (grey bars).

Discussion

Amendment of culture media using either dextrose, or combinations of dextrose, thiamine and/or D-alanine as previously described by Hyre and Cox (1953) showed a marked improvement in growth of isolates of *P. phaseoli.* Isolates took between three and four weeks to grow to a diameter of approximately 8 cm in the

amended medium that resulted in the most growth at a consistent temperature of 20 C. Lima bean agar amended with 0.5% dextrose proved to be an optimum simple medium to prepare while maintaining growth rates similar to media with additional thiamine and D-alanine. The isolates however did not grow as rapidly as observed with other species of *Phytophthora*, where diameter growth rates of 7 cm in one to two weeks were observed in *P. infestans* (Danies et al., 2015; Erwin and Ribeiro 1996). Differential growth rates had previously been observed in *P. infestans* and resulted in final measurements being taken at differing time points as determined by the isolates rate of 80% coverage of the petri dish (Childers et al., 2015). We opted to measure at uniform time points, once every seven days and then a 28-day final time point, where maximum growth had been observed during testing of media formulations. This uniform measuring allowed a comparison to be made among isolates at similar time points.

The method of Therrien et al, (1993) has been shown to be useful in categorizing isolates of *Phytophthora* for metalaxyl and mefenoxam sensitivity since 1993 (Abeysekara et al., 2018; Parra and Ristaino, 2001). Mefenoxam sensitivity assays demonstrated the overall efficacy of mefenoxam in the Mid – Atlantic region. Some single sporangial isolates showed differences in mefenoxam sensitivity when compared to the composite sample from which they were obtained. These insensitive or intermediately sensitive single sporangial isolates obtained from sensitive field isolates are from field populations which potentially contain individuals with mixed resistance to mefenoxam. Mixed populations are well documented, with the emergence of insensitive individuals having been demonstrated in just a single passage

through a non-lethal dose of mefenoxam in *P. infestans* whereas up to 12 were necessary for other genera in the *Peronosporales* (Bruin and Edgington, 1981; Childers et al., 2015). These observations demonstrate the emergence phase, which is the process of gaining fungicide insensitive mutants in the field. This stage is then followed by the selection phase, where resistance can be sustained if selective pressure is maintained through continued use of the fungicide. Conversely, the selection phase can result in a loss of these resistant individuals if the MOA is changed or the selective pressure is released (Mikaberidze et al., 2017). These two stages are then followed by the adjustment phase in which the frequency of insensitive pathogen individuals affects fungicide efficacy and forces growers to change their MOA (van den Bosch et al., 2018).

These data demonstrate that the emergence phase in Delaware has begun and it is imperative to delay further emergence while slowing down or preventing the selection phase of insensitive isolates. The data suggests that populations of *P. phaseoli* in Delaware have type 1 partial resistance, which is characterized by a decrease in the maximum fungicide effect (Mikaberidze et al., 2017). It will be important to focus future research on confirming this type of resistance in local populations to ensure that complete or type 2 partial resistance is prevented and that appropriate measures such as change of MOA or adjustment of dosage are taken to reduce or eliminate these insensitive populations (Mikaberidze et al., 2017; van den Bosch et al., 2018).

Acquisitions of mefenoxam resistance have occasionally been associated with isolates exhibiting different forms of growth penalties which are characterized by features such as reduced *in vitro* growth (Bruin and Edgington, 1981). We were able to find a similar growth penalty in isolates from Delaware, where insensitive and intermediate isolates had statistically decreased growth on unamended medium. More recent studies have shown that the opposite can also be true, with insensitive isolates of *P. capsici*, and *P. nicotianae* showing similar levels of growth in media when compared to sensitive isolates (Café-Filho and Ristaino, 2008; Hu et al., 2008). The next experiments should include *in vivo* tests of the slow growing insensitive isolates of *P. phaseoli* and an assessment of their sporulation to confirm *in vitro* observations and to assess fitness. This data will provide an additional layer of information when determining strategies to delay, reduce or prevent the emergence of insensitive mutants. Fungicide regimes which delay the time to emergence of resistant mutants are ideal as a management strategy and have been proven to be effective (Mikaberidze et al., 2017). Resistance to mefenoxam and its associated reduction in growth has been shown to be transient, although the effect can be permanent, as demonstrated in isolates of *P. capsici* (Bruin and Edgington, 1981; Childers et al., 2015).

Mefenoxam insensitivity was found to be concentrated in growing areas around the Milton area of Sussex county (detailed description of isolates from field 1 is found in the link), which is known to have the highest density of fields planted with lima bean (Evans, Personal Communication). Mefenoxam sensitivity assays showed that four composite isolates or single sporangial isolates derived from them were consistently insensitive over the two years that independent assays were conducted on

the isolates. Milton is a risk due to its near central location to the rest of the vegetable industry in Delaware and the ability of the organism to travel long distances via wind dissemination, making mefenoxam insensitivity there an important factor. The closest organism to *P. phaseoli* with dissemination data is *P. infestans*, which has been observed to travel distances of between 60 to a 100 km within a growing season (Goodwin et al., 1998; Weste, 1983). More recent models have shown the pathogen to travel between 10 – 20 km within a three-hour period, with UV, humidity and temperature additionally playing a role in the viability of sporangia (Aylor et al., 2001; Erwin and Ribeiro, 1996;). These statistics reiterate the urgency needed to prevent the spread of insensitive populations of *P. phaseoli*, as the entire Delaware industry is at risk.

A set of EST markers (PCSSR19 and PCN3 described in Abeysekara et al., 2018) routinely used in the lab to detect mefenoxam sensitivity in *P. capsici* were not successful in predicting the mefenoxam sensitivity phenotypes in these isolates of *P. phaseoli*. These two EST markers are used in combination and have been shown to have a 79% predictive ability in isolates of *P. capsici*. It may be that the mechanism of mefenoxam insensitivity in *P. phaseoli* is different to that of *P. capsici* or that the genomic region linked to mefenoxam insensitivity in *P. capsici* is absent in *P. phaseoli*. This phenomenon has been confirmed in *P. infestans*, where the apparently independent evolution of insensitivity to mefenoxam in different populations is due to different loci (Parra and Ristaino, 2001).

A pilot study of the population structure of *P. phaseoli* used a more comprehensive genotyping strategy. The eleven isolates used in this pilot GBS study were essentially a snapshot of the temporal sampling conducted on the organism over the past 40 years. These eleven isolates were maintained as active cultures by mass transfer every four weeks. Actively maintaining hundreds of cultures this way is however risky as losses due to contamination, equipment failure, sample mix up among other causes can result in the loss of an entire collection. Long-term storage methods that were previously used on isolates of *P. phaseoli* were not effective as recovery rates were low after as little as three years using both cryogenic and seed-based storage. It is prudent to rejuvenate isolates every two years to ensure higher rates of recovery when using either method, preferably with seed storage. This current isolate collection has been sampled and preserved as lyophilized and frozen (-80 °C) mycelial tissue as it is possible that active cultures may be lost in both long term storage and as active cultures. Isolate sampling conducted in the first years was not equally intensive due to logistic challenges in the face of sporadic infections, often resulting in farmers spraying before an intensive sampling exercise could be conducted. Future sampling will require coordinated and timely information channels and the availability of an ad hoc team to conduct sampling using more intensive per field sampling techniques such as those described by Pule et al, (2013).

Validation of SNP markers when using GBS is critical in ground-truthing data and numerous methods which include the use of segregating populations and parent-hybrid trios have been used to ensure call accuracy of GBS data (Carlson et al., 2017; Manching et al., 2017). The absence of a reference genome and the homothallic nature

of *P. phaseoli* made it difficult to use most of these previously mentioned methods in this experiment. Technical and biological replicates alone were used as validation in this experiment. Technical replicates were genotyped and the percentage differences of the genotype calls was assessed for each of isolates PhyP2 (4.8%), PhyP6 (18.7%) and PhyP15 (20.0%); whereas both technical and biological replicates were assessed for PhyP4 (39.5%). These differences in call rate at 582 SNP locations over all replicated isolates suggested high levels of genotyping error, likely due to the differential sequencing of genomic repeats, as the read depths of the affected SNPs was greater than 12, a read depth where the probability of erroneous calling of heterozygotes is extremely low (Manching et al., 2017). Genome repeats in the genus *Phytophthora* are well documented, with *P. infestans*, a sister species to *P. phaseoli* in clade 1C containing approximately 74% of repetitive DNA within its genome (Haas et al., 2009). The use of a low read depth filter (5X) was unlikely to have increased the likelihood over what is expected for failure to observe heterozygotes in this case, as only between 2.02 - 29.7% of the affected SNPs among the replicates had a read depth of less than 12X among all individuals and across all affected SNPs. The fact that 12.7% of the SNPs for PhyP2 and approximately 50% of the SNPs for each of PhyP4, 6 and 15 were unique among each isolate group suggests that these sites may indeed be heterozygous and/ or belong to genomic repeat regions. Kompetitive Allele Specific (KASP) assays may be designed to determine the validity of this assumption of heterozygosity or genome repetition using a subset of these affected loci.

Little is known about the population structure of *P. phaseoli*, with previous results indicating a clonal population after AFLP and allozyme analysis (Davidson et

al., 2008). The pathogen is homothallic, a strategy which is primarily used by pathogens to produce hardy environmentally stable spores and to escape from the effects of deleterious mutations (Wilson et al., 2015). There is a small likelihood of gene flow among homothallic populations due to the presence of sexual structures which make outcrossing between two individuals possible (Francis and St Clair, 1993). The GBS data from the pilot study was used to calculate the index of association (I_A), a measure used to test for linkage and a means to support the hypothesis of sexual or clonal reproduction (Goss et al., 2014, Tabima et al., 2017).

The I_A showed that there was high linkage among the markers in the total population and similarly in all populations stratified by race (Fig 14), supporting the hypothesis of either a clonal or selfing mode of reproduction in *P. phaseoli*. The likelihood of clonality was also supported by low heterozygosity, high inbreeding coefficients and correspondingly low measures of genetic distance among these isolates. More importantly, the observation of a single clonal lineage over an extended period means that either undetectable levels of migration or no migration from external sources is occurring. It is important to be able to observe the emergence of a new clonal lineage as these have often been found to have novel phenotypes in *P. infestans*, requiring different management approaches to be taken (Saville et al., 2015). The correlation of a new clonal lineage and new phenotype is not always true (Knaus et al., 2016), making phenotyping a necessity when new lineages are observed.

It is imperative that measures are taken to ensure the safe and efficient management of resources to prevent severe downy mildew epidemics using guidelines

such as those proposed for managing late blight caused by *P. infestans* (McDonald and Linde, 2002). Improving the detection of mefenoxam insensitivity and describing it better will allow more timely and informed management decisions to be made, ultimately slowing down the emergence phase and slowing down or preventing the selection phase from occurring. The pilot study using GBS on the historic isolates of *P. phaseoli* lays a foundation to achieve these goals as the emergence of new clonal lineages can be monitored. Progress in improving lima bean cultivars for resistance to *P. phaseoli* means a higher potential for the emergence of new races. A description of the populations of *P. phaseoli* using lessons from this pilot study may also allow the surveillance of race structure to be conducted, improving the decision-making process for growers. Overall, this work provides a platform to continue understanding downy mildew management dynamics by describing current challenges within pathogen populations and the direction which needs to be taken to prevent yield losses due to *P. phaseoli*.

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Chapter 3

GENOTYPING-BY-SEQUENCING TO PREDICT RESISTANCE TO LIMA BEAN DOWNY MILDEW IN A DIVERSITY PANEL

Introduction

Lima bean is an integral crop of the United States' (U.S.) mid-Atlantic region (MAR) where Delaware produces over 60% of the nation's crop for canning and freezing (Tolomeo et al., 2013). The cultivated lima bean is thought to have reached the east coast of the U.S. via a number of trade routes operated by the Native Americans in pre-Columbian times (Mackie, 1943). Lima bean is native to tropical Central and South America, with the Andes thought to be the crop's general center of origin (Fofana et al., 1999; Serrano-Serrano, 2010). Biochemical and DNA-based assays have demonstrated that lima bean has been domesticated at least twice giving rise to the Andean gene pool, characterized by large seeds and the Mesoamerican gene pools (MI and MII), characterized by small seeds (DeBouck et al., 1989; Fofana et al., 2001; Gutiérrez Salgado et al., 1995; Nienhuis et al., 1995; Serrano-Serrano et al., 2012).

Lima bean grown for the vegetable processing industry is most productive in cool, humid weather, which is favorable for pollination and pod set (Kee et al., 1997), but also conducive for the onset of plant diseases. Downy mildew of lima bean, caused by *Phytophthora phaseoli* (Thaxt), is one such disease, first discovered in Hamden,

Connecticut in 1889 by Thaxter (Connecticut Experiment Station Report, 1905). The pathogen progressively moved south and by 1903 Smith had identified the oomycete being "injurious" in Delaware and Maryland with yield losses of 25-90% being experienced in Frederick County, Maryland in 1905. Breeding for resistance to *P. phaseoli* was initiated in 1946 by USDA researchers who found resistant accessions of lima bean from different parts of the world (Wester and Jorgensen, 1959, Wester, 1961). Recurrent adaptation of the pathogen to genetic resistance deployed against it over the years resulted in epidemics in the 1950's and 60's (Zaumeyer and Wester, 1968), with the most recent adaptation of the pathogen to an R gene occurring in 2000, when an estimated production loss of 40%, equal to a farm value loss of $3,000,000 was experienced in Delaware (Evans et al, 2007). There are six documented physiological races of *P. phaseoli* (races A through F); race A was the initial race discovered by Thaxter in 1889, while race F is currently predominant in the field and has been found in grower fields since the year 2000 (Davidson et al., 2008).

Breeding for resistance has been a major form of control against downy mildew. A lima bean breeding program was initiated at the University of Delaware in 2004 to develop new cultivars for the MAR, including cultivars with improved resistance. Fungicides play a major role in disease management, their efficiency is improved by concurrent use of disease risk prediction models (Evans et al., 2007). However, mefenoxam-resistant isolates of *P. phaseoli* have been detected (T. Evans, personal communication), elevating the importance of genetic resistance which is easier to deploy, cheaper and environmentally friendlier than chemical control. Traditional breeding and selection methods currently in use are generally dependent

on favorable weather conditions and multiple crop seasons to develop new cultivars. Marker-assisted selection has been used to speed up selection for traits such as drought resistance, seed quality and growth habit in other legumes (Kumar et al., 2011; Schneider et al., 1997; Torres et al., 2010). Most of these crops however have had substantial research conducted on them, resulting in valuable resources such as sequenced genomes, linkage maps and characterized genes, none of which are available for lima bean. The advent of high-throughput, affordable sequencing technology allows such resources to begin being generated for lima bean.

Genetic analysis of populations using reduced representation sequencing approaches, such as restriction site associated DNA (RAD) tagging and genotyping-by-sequencing (GBS), subsample the genome at homologous locations across individuals, allowing the identification of single nucleotide polymorphisms (SNPs) evenly throughout the genome (Catchen et al., 2013; Elshire et al., 2010; Poland et al., 2012). GBS in particular has numerous advantages that include more cost-efficiency and less labor than other available methods (Poland et al., 2012). The challenge with GBS is in working with organisms that lack a reference genome. While the use of de novo assembly of short sequence reads via software such as STACKS allows circumvention of the need for a reference genome (Catchen et al., 2013), sequence validation is complicated and caveats such as incorporating sequencing artifacts and errors into genotype data must be considered. The availability of statistical methods and resources such as segregating populations with known phenotypes are useful in such instances, allowing the validation of sequence data from GBS analysis (Elshire et al., 2010; Nielsen et al., 2011). Loci identified through methods that utilize GBS can

be converted into marker assays such as Kompetitive Allele Specific PCR (KASP) assays, a uniplex genotyping technology that utilizes allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation (http://www.lgcgroup.com/; Semagn et al., 2013). This genotyping technology allows markers that have been identified through sequencing to be tested on different individuals at lower costs and with higher throughput (Hiremath et al., 2012). In this paper, we will discuss how GBS was utilized to obtain markers for race F resistance in lima bean. These markers were then validated and used to predict resistance to race F in a recently acquired diverse panel of lima bean accessions, the first time these methods have been used in lima bean breeding.

Materials and Methods

Plant material. An F_2 population consisting of 216 individuals was created by self-hybridizing a single individual from a cross between B2C (resistant to race F of *P. phaseoli*; Evans et al., 2007) and Bridgeton (susceptible to race F of *P. phaseoli*). Based on pedigree records, B2C is expected to be at least 80% similar to Bridgeton (Stavely, 1991; Thomas and Fisher, 1980). This F_2 population was utilized to develop DNA markers for resistance to race F of downy mildew through Bulked segregant analysis (Michelmore et al., 1991) using GBS (BSA-GBS). A geographically diverse collection of lima bean was used to validate the predictive ability of BSA-GBS derived DNA markers for resistance to race F of downy mildew. This lima bean collection, hereafter referred to as the diversity panel, consisted of accessions sourced from CIAT, the USDA, some seed companies, University of California lima bean

breeding program and the University of Delaware lima bean breeding program (Supplementary Table S1 in Mhora et al., 2016). Accessions in the diversity panel originated mainly from the Andean and Mesoamerican regions, the Caribbean Islands and cultivars developed in the U.S. Accessions in the panel also included some landraces from Africa (Nigeria and Zimbabwe), Asia (China and India) and Europe (France). Each individual of the F_2 population (B2C x Bridgeton) and the diversity panel were grown in four inch pots. The first trifoliate leaves were collected, uniquely labelled, frozen and lyophilized in preparation for DNA extraction.

Inoculum preparation, inoculation and disease evaluation. Inoculum was maintained and multiplied on Concentrated Fordhook lima bean which is susceptible to all known races of *P. phaseoli*. Initial inoculum in the form of sporangia and mycelia was obtained from two-week-old cultures of the *P. phaseoli* race F isolate, PhyP18, grown on frozen lima bean agar. The isolate was obtained from the University of Delaware's plant pathology culture collection. Sporangia of PhyP18 used as inoculum were obtained by weekly inoculations of progressively increasing numbers of four-day-old Concentrated Fordhook seedlings using a slurry derived from blending previously infected seedling hypocotyls in distilled water. Inoculated plants were maintained in a digitally controlled dew chamber (Percival 128 Scientific, Inc., Perry, IA) set at 20°C, relative humidity (RH) of 94% ± 1%, and 24 h light with an intensity of 50 μmol m-2s-1 of photosynthetic active radiation (PAR). Inoculated seedlings showed signs and symptoms of downy mildew after approximately seven days, upon which they were used as inoculum. This process was repeated until enough inoculum was available to inoculate the F_2 population planted in the field using

sporangia suspended in distilled water and quantified to 1 x 103 sporangia/ml using a haemocytometer.

Seedlings from the F_2 population derived from B2C x Bridgeton were transplanted to a research field in Georgetown, Delaware, in a completely randomized design with all plants in a single row, and susceptible border rows on either side. One inch irrigation was provided weekly using a traveling linear system as required until the flat pod stage when lima bean is most susceptible to downy mildew. Inoculations with race F of *P. phaseoli* were performed at the flat pod stage through two inoculations conducted at ten day intervals in September 2008. Inoculations were conducted using a backpack low-pressure manual pump sprayer (SOLO backpack sprayer, model 425, SOLO Incorporated, VA) in the early evening when temperatures were cooler. After inoculation, plants were misted intermittently each night using Micro-Bird misting nozzles (Rain Bird Corp., Glendora, CA) from 6 PM to 6 AM to increase humidity and a leaf wetness duration of at least 12 hours. Individual plants were evaluated for signs and symptoms of downy mildew weekly for a month. Each plant was evaluated for evidence of signs (white fluffy mycelium on shoot tips, pins and flat pods) and symptoms (red line surrounding signs on pods) of downy mildew. When necessary, confirmation of infection was made by evaluating samples microscopically for sporangia of *P. phaseoli*. Plants that did not develop signs or symptoms of downy mildew were considered resistant. Adjacent susceptible controls (Fordhook) were heavily infected.

Bulked segregant analysis using GBS in lima bean. DNA was extracted from young first trifoliate leaf tissue of each individual from the population using the Qiagen Dneasy 96 plant kit (Qiagen, Valencia, CA). DNA was checked for purity using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA), quantified using Picogreen (Thermo Fisher Scientific, MA) and standardized to 25 ng/µl. BSA was performed by making separate bulks of the resistant (140 individuals) and susceptible (61 individuals) plants using 200 ng of total DNA per individual. Only 201 of the 216 F_2 progeny were used in BSA due to 15 progeny not surviving to the point of inoculation. Individual DNA bulks were then processed through a custom GBS protocol. Two independently formed replicates of the resistant and susceptible bulks and each of the parents were digested with six different pairs of restriction enzymes: *PstI/ CviQI; PstI/ MseI; SbfI/ CviQI; SbfI/ MseI; NgoMVI/ CviQI* and *NgoMVI/ MseI*. Restricted DNA samples were ligated with barcoded adaptors and then pooled. Proportionate DNA (ng) from the resistant and susceptible bulks was mixed to match the ratio of resistant and susceptible individuals (140:61). Pooled DNA was size-selected to 300 bp (±30) using the BluePippin (Sage Science, Beverley, MA). The size-selected DNA was PCR amplified, quantified and sequenced using 101 bp single end sequencing on the Illumina HiSeq at the Delaware Biotechnology Institute (DBI), (Manching et al., 2017).

Genotype data analysis. The resulting sequence data were processed and analyzed using a custom REDuced REPresentation (redrep) bioinformatics pipeline that wraps open-source tools (primary components: fastqc (Andrews, 2010); BWA-MEM (Li, 2013); GATK (McKenna et al., 2010, DePristo et al., 2011) and custom

scripts for processing GBS data (https://github.com/UD-CBCB/RedRep). The redrep pipeline for reference-free analysis consists of sequence read quality control (redrep-qc), de novo reference assembly (redrep-cluster), mapping of reads onto the reference (redrep-refmap) and variant calling (redrep-SNPcall). Raw GBS sequence data were processed using redrep-qc to group reads by barcode ID and trim barcode and adaptor sequences from the reads. The next steps in QC were to filter out reads lacking the expected restriction site overhang sequences, short reads (<96 bp) and reads with a mean Phred quality score <30. Reduced representation reads from Bridgeton were used to construct the de novo reference using redrep-cluster due to its higher read depth and quality compared to B2C. Redrep-cluster is a cluster-based approach that iteratively utilizes the USEARCH cluster small_mem algorithm defining each cluster by one sequence known as the centroid, or representative sequence (Edgar et al., 2010). The effect of different clustering identity thresholds of 0.98id (98%), 0.99id (99%) and 1.0id (100%) on the redrep reference were compared. Identity thresholds are a parameter in which clusters are based on a percentage similarity to a centroid (representative sequence for which a cluster is based). Sequence reads from the entire library were then mapped against each of the redrep reference genomes and variant identification were performed.

Filters for read depth (<12), Phred scaled quality scores (<30) and missing genotype data per SNP locus were applied to the variant call data. Further filters were also applied to discard all markers that were not homozygous for the susceptible reference allele in Bridgeton, homozygous for the reference allele in B2C or heterozygous in both parents, leaving 146 loci. Statistical analyses to identify loci

significantly segregating with the race F resistance phenotype were conducted on the post filter SNPs. The Fisher's exact test ($p < 0.01$) was applied to the data that were not filtered, resulting in 12 loci which were significantly associated with the race F resistance phenotype. These 12 loci were advanced for KASP assay development.

KASP assay design, F_2 population genotyping and mapping of the race F resistance locus. KASP primers were designed (Supplementary Table S2 in Mhora et al., 2016) around the SNPs using Primer3 primer design software (Korressar and Remm, 2007; Untergrasser et al., 2012). Primers were synthesized by Integrated DNA Technologies (Iowa, USA). PCR conditions were optimized for each KASP assay primer and visualized on a 2% agarose gel (Fig. 15) to ensure optimal thermocycling conditions for each primer before running KASP assays on the ABI 7900HT fast qPCR instrument (Thermo Fisher Scientific, MA). KASP assays were conducted on the ABI 7900HT fast qPCR instrument using the high Rox master mix according to manufacturer's recommendations (http://www.lgcgroup.com/products/kasp-genotyping-chemistry/#.VZwa7RtViko). SNP assays were performed using each of the pass filter SNPs on each individual of the biparental F_2 progeny and scored as being homozygous reference (Bridgeton allele), homozygous non reference (B2C allele) or heterozygous. The genotype for each of the F_2 progeny was scored using the "allelic discrimination" assay module on the Sequence Detection Systems Software (SDS) version 2.3 and then manually to validate the software's genotype calling. Marker data obtained through KASP assay genotyping were analyzed using the R packages R/qtl version 2.0-4 and Onemap. Onemap was used to determine the recombination fraction between all pairs of markers using two-point tests. Once

recombination fractions had been calculated and tested, markers were arranged into linkage groups and mapping was carried out using the Kosambi mapping function and the seriation algorithm of Onemap to order markers to estimate the inter-marker distances and to plot a linkage map for the race F resistance locus (Margarido et al., 2013). Interval mapping to determine the effect of each locus on race F resistance and to estimate the location of the race F locus was done using Haley-Knott regression in R/qtl (Broman and Sen, 2009; Margarido et al., 2013).

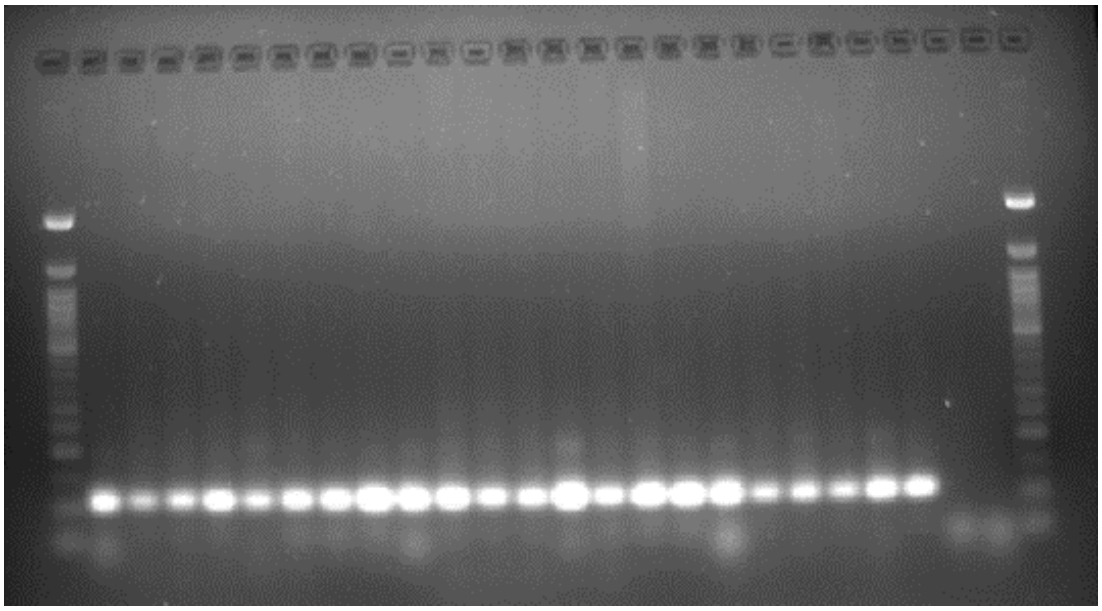

Figure 15 An optimized PCR reaction of randomly selected F_2 progeny using primers designed for KASP assay for the SNP on nucleotide 22 of locus 20_1930486_22. Lanes 1 and 26 are the 50bp DNA ladder; lanes 2-21 are randomly selected F_2 progeny; lane 22 is Bridgeton; lane 23 is B2C and lanes 24-25 are water blanks.

Utility and validation of race F resistance molecular markers in a diversity panel. In order to ascertain the utility of markers obtained using BSA-GBS as a race F resistance prediction tool, 256 diverse accessions of lima bean were genotyped using the KASP assays developed in this study. Six possible multilocus genotypes (MLGs) were identified after genotyping each accession of the diversity panel. Between three and five individuals from each haplotype group were selected, based on how many individuals were available per MLG. In this case, a haplotype is defined as a group of alleles which are found together. A randomized complete block design with three replicates per accession was used (only three seeds per accession were planted due to limited seed availability). Each seed was planted in a 4 inch pot containing redi earth. The pots were arranged in a randomized complete block design, watered and allowed to germinate on seedling heat mats. At germination, seed coats were carefully removed from each germinating seed and the emerging hypocotyls were inoculated using the previously described method. Each accession was individually scored after five days in the dew chamber as being either resistant or susceptible. In order to ascertain if any of the accessions that were being screened contained the "slow mildewing" trait that is also desirable, a rating scale between 0-4 was used to rate infections (0- no infection; 1- <50% plant covered with mycelia; 2->50%; 3- <50% with wilted seedling; 4- >50% with wilted seedling). Disease rating data were analyzed using ANOVA. The Dunnett's post-hoc analysis was then used to compare each MLG to the susceptible control Concentrated Fordhook in order to ascertain which MLGs were significantly different from the control.

Results

Bulked segregant analysis (BSA) using genotyping-by-sequencing (GBS) for a non-reference organism. Disease evaluations were conducted on the inoculated F_2 population planted in the University of Delaware's Research and Education Center, Georgetown research field after approximately seven days. One hundred and forty resistant progeny and 61 susceptible progeny were observed, with 15 progeny failing to survive to the point of inoculation (Supplementary Table S3 in Mhora et al., 2016). The chi-square test showed that segregation of the race F resistance phenotype followed a 3:1 segregation ratio indicating a dominant resistance phenotype ($\chi 2 = 3.066$, 2 df., $p < 0.05$). A multiplexed library of different paired enzyme treatments on the BSA samples was used to identify SNPs associated with race F resistance. A total of 47M raw sequence reads (Genebank Accession: PRJNA322045) were analyzed using the redrep non-ref pipeline pathway. Following redrep-qc, 12.5% of the sequences were filtered. Among the six paired enzyme treatments used for GBS, sequences from *PstI* and *CviQI* represented 47% of the total post-QC data. Consequently, this enzyme treatment yielded the greatest number of loci with sufficient read depth for further analysis.

We used three clustering settings in order to determine the threshold that would best result in the retention of polymorphic loci. Genetic expectations were then used as the basis to filter out non-conforming loci and determine an optimal threshold setting (Fig. 16). The 1.0 ID (100% identity) threshold was ultimately used to construct the de novo reference.

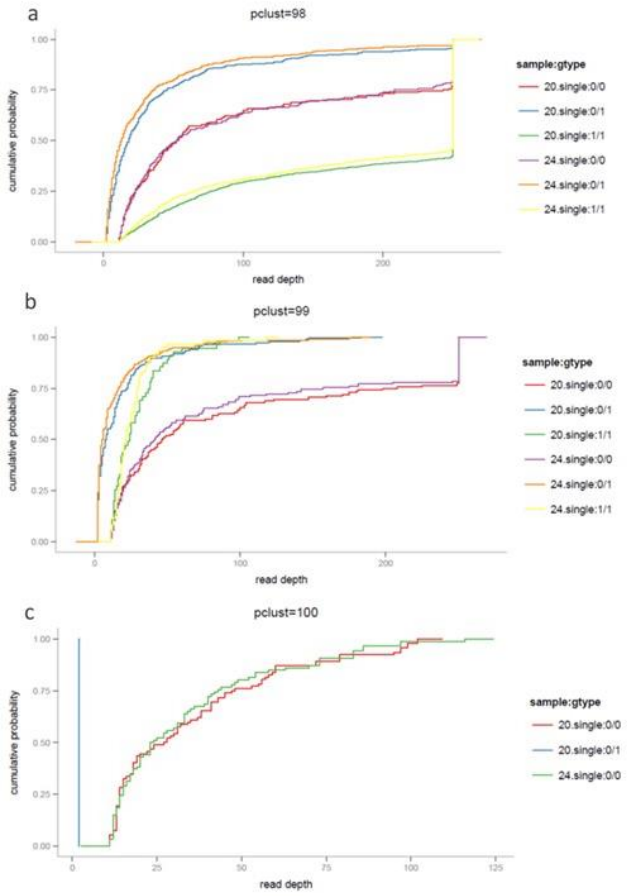

Figure 16 A comparison of the empirical cumulative distribution function of the allelic states of Bridgeton (susceptible) samples (processed using the *PstI/CviQI* enzyme pair) when Redrep_SNPcall is used to find variants using the de novo references assembled at (a) 0.98id, (b) 0.99id and (c) 1.00id. Sample 20 and 24 are replicates of one of the parents of the segregating population, Bridgeton. The allelic states are represented by the following nomenclature: 0/0 – Reference homozygote (BR); 1/1 – Alternative homozygote (B2C); 0/1 – Heterozygote

Development of molecular markers associated with race F resistance and resistance locus mapping. BSA-GBS associated SNPs were converted into KASP assays (Supplementary table S2 in Mhora et al., 2016). Conventional PCR showed that eight of the twelve assays amplified. These were used in KASP assays to genotype the

individual F_2 progeny. Seven of these eight markers mapped onto a single locus spanning 9.83 cM, which co-segregated with resistance to race F of downy mildew. The flanking markers most strongly associated with resistance (20_1930486_22 and 24_923176_25) were 3.28 cM and 1.60 cM away from the inferred location of the resistance gene (Fig. 17A and 17B).

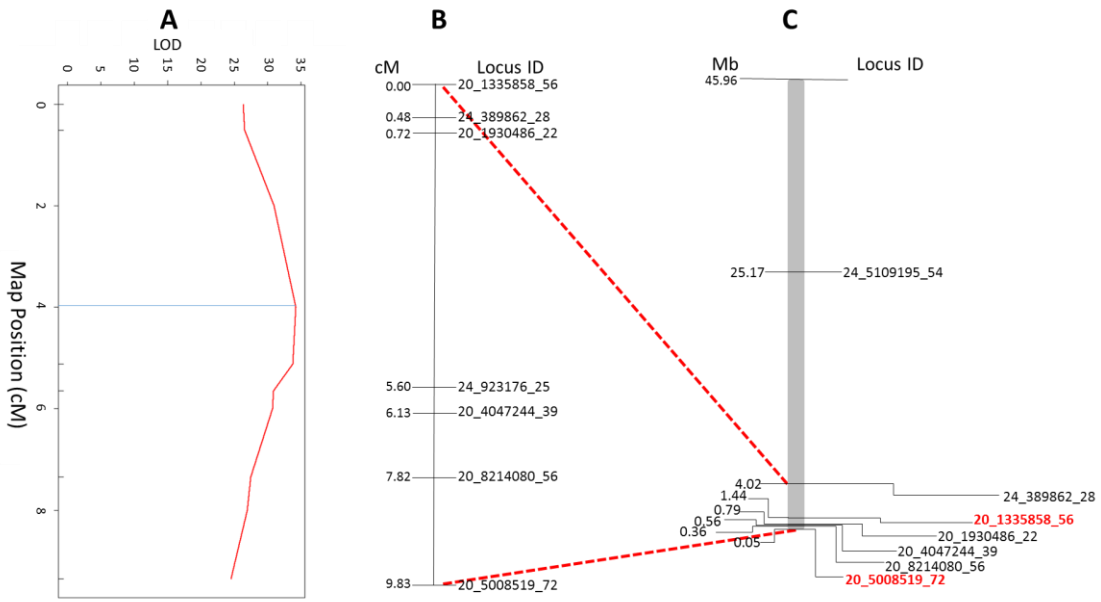

Figure 17 The genetic map of the race F resistance locus in comparison to the physical map of *Phaseolus vulgaris* chromosome 4. The likelihood map in Panel A shows the peak LOD score at 4 cM, indicative of the calculated position of the race F resistance locus with respect to the markers (blue dotted line). Panel B is a linkage map of the race F resistance locus showing the order and distances in centiMorgans of the six linked SNP loci. Panel C is the physical map location in megabases of each of the lima bean markers for resistance to race F of downy mildew on chromosome 4 of *P. vulgaris*. The markers in red font are those required for accurate prediction of race F resistance. The red dotted lines indicate where the markers on the lima bean genetic map span chromosome 4 of *P. vulgaris*.

Validation of the predictive accuracy of molecular markers on a lima bean diversity panel. All eight functional KASP assays were used to genotype the diversity panel consisting of 256 cultivars, landraces and wild germplasm (Supplementary Table S1 in Mhora et al., 2016). Among the U.S. cultivars were accessions whose race F resistance phenotype was known from previously conducted field inoculations (Maffei 15 – resistant; Cypress, C-elite and Concentrated Fordhook – susceptible; Ernest et al., 2011). Resistance to downy mildew in these accessions with known response to race F was accurately predicted using three of the seven KASP assays (20_1335858_56, 20_5008519_72 and 20_1930486_22). The diversity panel contained six of the nine theoretically possible haplotypes for these markers, and the B2C-resistance haplotype was rare (found in four of 256 accessions) in the collection (Supplementary table S3 in Mhora et al., 2016).

Inoculations with race F were performed on a subset of accessions selected based on their haplotypes to further assess the predictability of the 3-marker haplotype. All of the tested accessions that were homozygous for the B2C haplotype were resistant whereas 85% of the accessions heterozygous or homozygous for the Bridgeton (BR) allele were susceptible (Fig. 18). The B2C-B2C-B2C haplotype (highlighted by the blue box, Fig. 18A) was precise in predicting resistance to race F, however only two of these markers, after a complete screening of the diversity panel, are necessary for accurate prediction of race F resistance. Four accessions out of the 256 in the diversity panel were characterized by this haplotype including Maffei 15 (M15), Sussex, PI200919 (Cojutepeque, El Salvador) and PI200924 (Jalopa, Guatemala). Screening results using PhyP18 on accessions containing the other

haplotypes showed that only two of the markers were necessary to accurately predict resistance (20_1335858_56 and 20_5008519_72). However, there were resistant individuals that contained alternative haplotypes including BR-B2C-B2C for PI 310627 (Chaparota from Guatemala), BR-BR-B2C for PI 256816 (Frijol tierno from Ecuador) and BR-BR-BR for PI 256405 (Chilipuca from El Salvador).

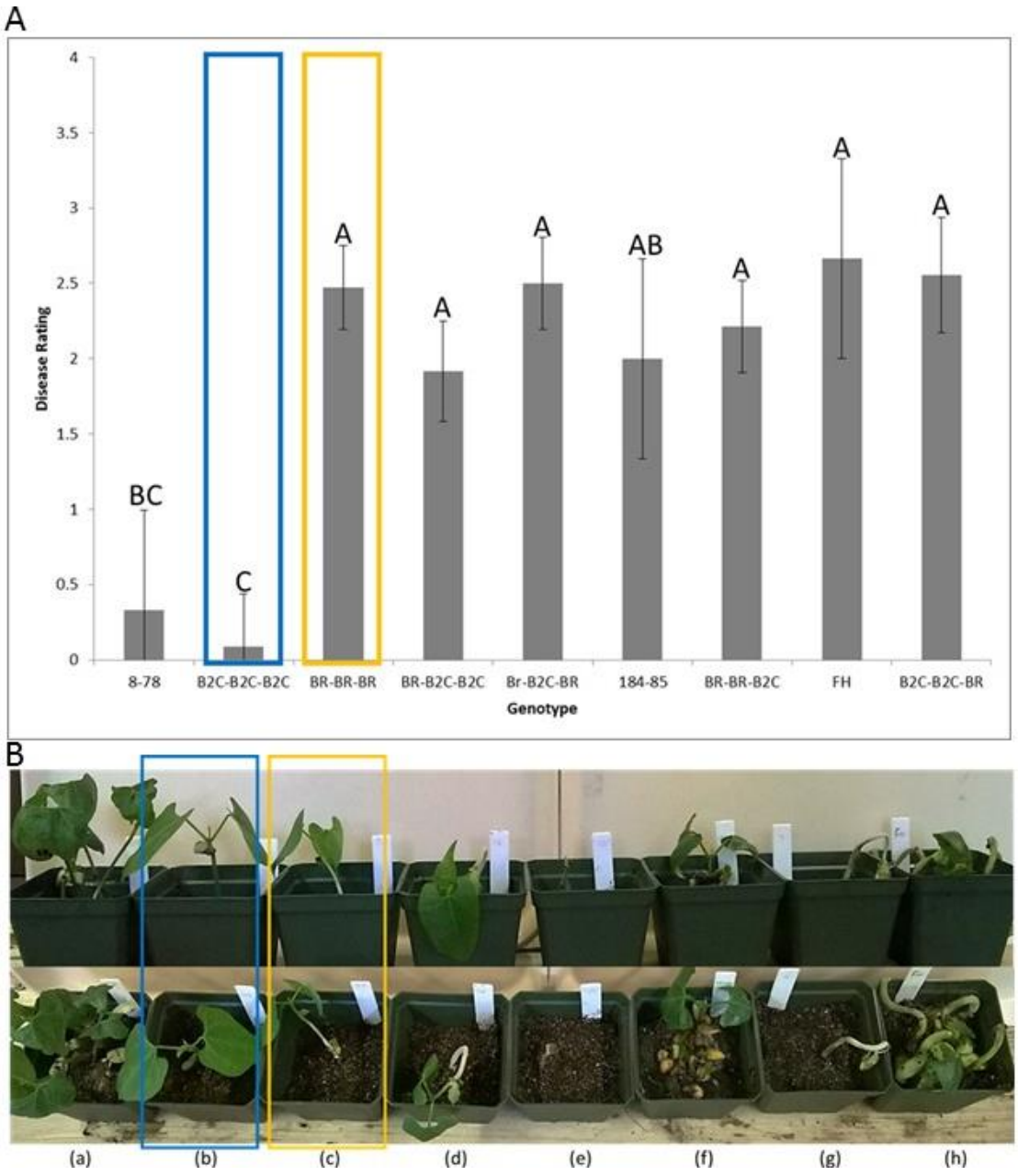
A
Disease Rating
4
3.5
3
2.5
2
1.5
1
0.5
0
BC
C
A
A
A
AB
A
A
A
8-78
B2C-B2C-B2C
BR-BR-BR
BR-B2C-B2C
Br-B2C-BR
184-85
BR-BR-B2C
FH
B2C-B2C-BR
Genotype
B
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Figure 18 Disease ratings and images of accessions consisting of the different marker haplotypes after inoculation with Phyp18. Haplotypes are made up of SNP variants of the markers 20_1335858_56, 20_5008519_72 and 20_1930486_22. Panel A shows the ratings of infection by *Phytophthora phaseoli* using a 0-4 rating scale on diversity panel accessions grouped by haplotype. The cultivars 8-78 (race F resistant), 184-85 (race F susceptible) and Fordhook (universally susceptible) are used as controls. The blue pane shows the B2C-B2C-B2C haplotype which is accurately predicting race F resistance. The orange panel is the anomaly, where the BR-BR-BR haplotype, overall predicted as susceptible has one resistant accession (PI 347779). Panel B shows how different accessions in different haplotype groups reacted phenotypically to race F inoculation relative to each other. The pots are photographed from a sideways and aerial position from pot (a) – (h). (a) 8-78 from the USA; (b) Accession PI 200919 from El Salvador with haplotype B2C-B2C-B2C; (c) Accession PI 347779 (Hopi 13) from the USA with haplotype BR-BR-BR but containing slow mildewing traits, thus being able to withstand severe infection; (d)1102-26 from The Dominican Republic with haplotype BR-B2C-B2C; I Accession PI 347819 (Willow leaf white) from the USA with the haplotype BR-B2C-BR; (f) 184-85 from the USA with a BR-BR-BR haplotype; (g) Accession 1102-3A from Haiti with haplotype BR-BR-B2C and (h) Accession PI 549479 (concentrated Fordhook) from the USA with the haplotype BR-BR-BR.

Collinearity of lima bean race F resistance map with *P. vulgaris*. All eight markers were mapped to the genome of a closely related species, *P. vulgaris* (Bonifácio et al., 2008; Schmutz et al., 2014). Seven of the eight KASP markers were found to span 24.85 Mb on chromosome 4 of common bean (Fig. 17C). The genome space between those markers found on the lima bean linkage map (six of the seven markers spanning 4 Mb locus on chromosome 4 of *P. vulgaris*) consisted of an R (Resistance) gene dense region containing 110 locus tags (http://www.ncbi.nlm.nih.gov/genome/proteins/380?genome_assembly_id=48590&gi=593705291), with 38 annotated as being R gene related (Phytozome; https://phytozome.jgi.doe.gov/; Goodstein et al. 2012). The seventh marker on the

lima bean linkage map maps to chromosome 7 of *P. vulgaris*. Furthermore, among the markers that were collinear on common bean and were accurate in predicting race F resistance (20_1335858_56 and 20_5008519_72 which are 1.39 Mb apart), we identified 21 locus tags containing 8 classes of resistance proteins in this region of chromosome 4 of *P. vulgaris* (Table 3; Li et al. 2012).

Table 3 Genome region between the markers accurately predicting race F resistance are collinear with a resistance gene-dense region in *Phaseolus vulgaris*.

Resistance protein class	Number of appearances
CC-NBS-LRR class	10
TIR-NBS-LRR class	8
NB-ARC domain	41
LRR and NB-ARC domain	21
RPP13	48
HOPZ-ACTIVATED RESISTANCE	18
RGA	32
Putative disease resistance	13

Discussion

Epidemics produced by race F of *P. phaseoli* are a threat to Delaware's status of leading lima bean sales to the vegetable processing industry in the MAR and indeed to the entire U.S. The utility of GBS in lima bean using a non-reference approach was optimized and demonstrated an efficient and high throughput method for genotyping lima bean and effectively characterizing traits of interest. The use of KASP assays developed from BSA-GBS markers allowed the mapping of a race F resistance locus and the KASP assays effectively identified downy mildew resistant lima bean

accessions from genetically diverse plant material. The most important outcome of this method was in the ability of the markers found using the reference free redrep pipeline to identify race F resistant lima bean accessions from our diversity panel. The validation of this method opens avenues for further research, allowing for dissection of the genetic architecture of traits of importance in lima bean such as disease resistance, heat tolerance, growth habit and cyanide content.

The diversity panel is a resource that is not only useful for validating the approach we used to identify race F resistance loci, but can also be useful in the identification of other important traits, including alternative race F resistance loci through phenotyping the entire diversity panel. Likely forms of alternative race F resistance were indeed observed, as three accessions in the diversity panel were found to be resistant to race F in our phenotype screen, but did not contain the predictive haplotype for resistance. This indicates that additional race F resistance loci may be present in other genome locations. These may be in the form of as yet unknown resistance genes, or quantitative trait loci (QTL). The former could lead to Resistance gene pyramiding, a proven method of increasing durability of resistance in commercial cultivars towards pathogens in a number of crops (Pederson and Leath, 1988). Pyramiding has been demonstrated to increase efficacy against infection and increase durability of resistance using both major resistance genes and genes conferring partial resistance (Li et al., 1999; Fukuoka et al., 2015; Huang et al, 1997). Alternative forms of resistance, such as QTL, can allow the deployment of multiple resistance genes against *P. phaseoli* in one cultivar, slowing down pathogen adaptation and reducing yield losses due to downy mildew. QTL confer quantitative resistance

but can contribute to durable resistance and confer broad-spectrum or non-race-specific resistance, providing an additive effect to overall resistance (Fukuoka et al., 2015). The “slow mildewing” trait observed in the accession PI 347779 (Hopi 13 from Arizona USA with BR-BR-BR haplotype; highlighted by the orange boarder in Fig 18B) is likely to be the effect of QTL, which can be in the form of defeated major genes such as the Xa4T gene in rice, contributing a residual resistance effect against pathogens (Li et al., 1999). Slow mildewing has been found to be durable in wheat against Blumeria graminis f. sp. Tritici due to its quantitative nature and also as an effective means of minimizing disease rates in the field through reduced sporulation and secondary inoculum production (Shaner, 1973). Rigorous phenotypic screening of the diversity panel, coupled with the GBS approach taken here, allows the possibility of either resistance pyramiding by targeting these multiple traits and introgressing them into one cultivar, or discovering the loci underlying the slow mildewing phenotype, either of which could lead to more durable resistance. We are currently using the methodology from this study to identify the QTL responsible for the slow mildewing trait for the subsequent development of improved commercial cultivars.

The loci discovered in this research were mapped to the genome of sister species *Phaseolus vulgaris* (common bean) and found to be collinear to the lima bean race F resistance map. The approximately 4 Mb region spanned by six of the seven loci from the lima bean linkage map consisted of an R (resistance) gene-enriched region. Additionally, Phytozome was used to conduct an analysis of the *P. vulgaris* genome in the approximate region between the loci accurately predicting race F resistance (1.39 Mb), showing the presence of 172 locus tags, with 21 of these being

associated with resistance proteins such as coiled-coil-nucleotide-binding-site-leucine-rich-repeats (CC-NBS-LRR)-and resistance related transporters (Table 3; Goodstein et al., 2012). Eight classes of these resistance related proteins were found to reside in these 21 locus tags (Table 3). This observation using the syntenic relationship between the two species of bean will allow a more targeted approach to identifying the causal R gene/s for downy mildew resistance. Indeed, collinearity between species has facilitated identification of markers linked to resistance genes, as in the case of the wheat resistance gene, Sr39, and rice (Niu et al., 2011).

Overall, this work establishes a platform which will allow for future dissection of the genetic architecture of other important traits of lima bean, including important but more complex traits, like slow mildewing and abiotic stresses, such as heat tolerance, which are important concerns to regional growers (T.A. Evans, personal communication). The development and validation of our method using a tractable genetic experimental population opens up the possibility of in-depth studies of diverse accessions of lima bean using these DNA library preparations and customized bioinformatic pipelines.

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Chapter 4

INROADS TO IMPROVING LIMA BEAN PRODUCTION IN THE US BY ASSESSING THE GENETIC DIVERSITY OF EXISTING CULTIVARS COMPARED TO A DIVERSITY PANEL

Introduction

Lima bean (*Phaseolus lunatus* L) is the cornerstone of Delaware's agricultural industry, supporting all otherwise non-profitable vegetable processing crops that are also grown in the region. Delaware is currently the leading producer of lima bean in the United States (US). The origin of lima bean is thought to be in central America, with the legume being divided into two main groups, the Mesoamerican and Andean genepools (Debouck et al., 1989; Maquet et al., 1999; Martinez Castillo, 2006). The Mesoamerican genepool is thought to have been domesticated twice, giving rise to the MI and MII genepools (Andueza-Noh et al., 2013). The Mesoamerican genepools are characterized by smaller beans with an average seed weight of 45 g/100 seeds whereas the Andean genepool is characterized by larger seeds with an average seed weight of about 87 g/100 seeds (Chacón-Sánchez and Martínez-Castillo, 2017).

Lima bean arrived in the US during pre-Columbian times along trade routes, where native Americans further selected the crop for adaptation to their specific climatic zones (Landon, 2008; Mackie, 1943). These pre-Columbian landraces served as a source for some of the early modern cultivars, with the original Henderson

cultivar being discovered in these local resources and becoming the first baby lima cultivar to be extensively produced in California (Mackie, 1943; Nienhuis et al, 1995). Signs of some of these adaptive selection practices are also observed in the Fordhook cultivars of the US, which have been bred to thrive in hot dry climates (Wester, 1961). This is opposite to their larger seeded counterparts in the Andean genepool which are adapted to narrow environmental conditions that are characterized by long, cool, dry summers (Nienhuis et al., 1995). Recent efforts to breed for drought, heat and disease tolerance in these Fordhook cultivars (Dreer et al., 1946) resulted in crosses being made between these larger Fordhooks and the Mesoamerican Hopi varieties (Wester, 1961). These crosses resulted in the modern Fordhook type cultivars which are genetically distant from both the major genepools (Nienhuis et al, 1995).

To improve our current cultivars with a focus on the immediate needs of the Delaware industry (Ernest et al., 2017), it is important to understand the available genetic resources at our disposal. Extensive phenotyping and genotyping of lima bean accessions will avail the necessary resources needed to allow informed breeding strategies to be used. Breeding strategies vary, and detailed knowledge of resources and the nature of the target traits allows optimization of these approaches which can be sped up and made more accurate using methods such as marker assisted selection (MAS) (Beebe et al., 2013). Breeding strategies such as advanced backcrossing and recurrent selection are also possible strategies that can be adopted in lima bean, with the method of choice dependent on the nature of the trait of interest (Beebe et al., 2013; Hallauer and Darrah, 1985; Tanksley and Nelson, 1996). Advanced backcrossing is an ideal and tested method that is used to expedite cultivar

development by combining quantitative trait loci (QTL) discovery and variety development by using advanced backcross progeny rather than balanced populations for QTL analysis (Tanksley and Nelson, 1996).

The pedigree of most commercial lima bean cultivars in the US can be traced back to a few individuals (Nienhuis and Sass, accessed 22 August 2018). This can lead to a reduction in the crops overall genetic variation and increase the likelihood of crop failure due to biotic and abiotic challenges in the face of changing climate (Tanksley and Nelson, 1996). The current needs of the industry are for downy mildew resistant, drought and heat tolerant cultivars (Ernest et al., 2017). Rising temperatures have seen yields reduced as flowers abscise in extreme heat, while new physiological races of the causal agent of downy mildew are more heat tolerant (Sirait et al., 1994; Ernest et al., 2017; Evans et al., 2007). The Fordhook cultivars are now a risk to production in Delaware, as they are all particularly susceptible to races E and F of downy mildew (Evans et al., 2007). The emergence of new diseases such as *P. capsici* also magnifies the threats that continue to build up against the industry. It is against these current and impending challenges that a solution is sought to improve lima bean cultivars.

Genetic diversity has long been a source of adaptation to adverse climatic conditions as proven in the classical examples of rice and wheat, where traits sourced from diverse collections stored in seed banks saved millions of people from starvation (Athwal, 1971; O'Toole and De Datta 1986; Septiningsih et al., 2009). Lima bean is no exception and is known to have a wide ecological range, allowing various forms of the crop to grow in conditions which include temperate, humid and semi-arid tropical

climates (Baudoin, 2004). There are currently 13,991 accessions of lima bean held in 75 institutions, providing a large reservoir of genetic resources for potentially improving the crop (Quat Ng, 2013). The utilization of genetic diversity has a history in lima bean improvement in the US, with disease resistance and drought tolerance being among some of the traits incorporated into current cultivars (Staveley, 1991). The ability to organize and understand the genetic composition of available resources will permit flexible approaches to lima bean improvement such as (1) expedited introgression of traits from similar parents, leading to advantages such as fewer rounds of backcrossing by avoiding distant donors in breeding crosses (Nienhuis et al., 1995); (2) wide genetic crosses to understand and improve adaptability (Lopes et al., 2015); genomic prediction and selection (Yu et al., 2016). Understanding the genetic composition of available resources will also be important to make more informed selections of accessions from seedbanks as these are prone to duplications and misclassification (Knüpffer et al., 1997).

A diversity panel consisting of cultivars and landraces sampled from the geographic locations native to the MI, MII and Andean genepools was collected. This diversity panel also contained cultivars and landraces from the US, the Caribbean islands and a few accessions collected from a few other countries around the world. To improve lima bean production in the US, an understanding of the available genetic diversity in the current breeding material is needed. These lima bean resources also need to be organized into related groups which will allow improved breeding tools and decisions to be made. It is important that genetic diversity is incorporated into the breeding program as it has been demonstrated that high levels of genetic variation can

increase individual vigor and the potential of populations to respond to selection (Kalinowski, 2004).

To achieve this, it is important to have an efficient and scalable method to characterize genetic diversity. Among the available methods for molecular genetic characterization, genotyping-by-sequencing (GBS) is currently one of the more cost-effective strategies and can be used in multiplexed libraries (Voss-Fels and Snowdon, 2016). In addition to cost effectiveness, GBS also meets most requirements of an ideal genotyping technique such as minimizing ascertainment bias, high repeatability and the ability to detect polymorphism and codominance (Milgroom, 2015). There are some limitations to GBS that include reproducibility among libraries and genotyping accuracy, all which can be consequence of either one or combinations of factors that include genome complexity, library preparation and DNA quality. A study to address some of these issues was conducted on *Zea mays* and demonstrated the ability of rounds of size selection to improve reproducibility among libraries (Manching et al., 2017). Previous experience with GBS in lima bean (Mhora et al., 2016) had shown that there was room to improve the enzyme combinations used in the protocol. An *in silico* digest approach to select optimal enzyme pairs and an additional round of size selection before sequencing were both implemented in further protocol optimization for lima bean libraries. The focus of this paper was to describe the genetic diversity of cultivars and landraces in the US, compare them to those from the crops center of diversity and to assess the potential of increasing local lima bean genetic diversity.

Materials and Methods

Plant Material

A diversity panel (DP) comprised of 263 members was collected from around the world, with most landraces and wild lima bean accessions originating from central and South America (Supplementary Table S1 in Mhora et al., 2016). Accessions of the diversity panel were obtained from the USDA's National Center for Genetic Resources Preservation (NCGRP), the International Center for Tropical Agriculture (CIAT), from collaborators in the Caribbean Islands (Puerto Rico and Haiti) and from both the California and University of Delaware's lima bean breeding programs.

Accessions from the diversity panel were assigned to their respective genepools primarily based on their geographic sampling location. These genepools were based on previous classifications derived from molecular data and its association to their geographic origins. There were 137 accessions of the DP that were from the United States (US). These US accessions were primarily grouped by the cardinal points of their sampling location (east for accessions on the same coast as Delaware and west those on the same coast as California) or as unknown if their sampling source was not provided (46 US-X accessions) (Table 6). These accessions were later classified using their genotype data in methods described below. There were 12 accessions in the DP which were collected in China, France, India, Nigeria and Zambia (Population X). The common bean accession G19833, the Andean reference for *P. vulgaris* (Schmutz et al., 2014), was used as the outgroup giving a total of 264 genotyped individuals. The 100 seed weight for each accession was weighed.

There were four replicated (two replicates per sample) genetic trios used as a control to assess the accuracy of GBS data. Trios were comprised of replicated parental lines and their replicated F_1 progeny. The genetic trio comprised of the two inbred parent lines Cypress and Jackson Wonder and their F_1 progeny (P1501702) was used to analyze genotyping accuracy between replicates and for expected genotypes in a trio as they had more complete genotyping data than the other trios. The remaining trios were made up of the following crosses; DP_203 × DP_46; DP_197 × DP_46 and DP_203 × DP_132. There were an additional 15 samples which were also duplicated for assessing genotyping accuracy, among these was G19833, the *P. vulgaris* outgroup.

Tissue Collection and DNA Extraction

Lima bean tissue for DNA extraction was collected from both young trifoliate leaves and from etiolated seedling stems. Before planting, all the seeds were scarified on the opposite side of the radicle, using a razor blade to make a small nick in the seed coat. Plants from which the first trifoliate leaves were obtained were grown in one-gallon pots containing Promix-HP growing medium (QC, Canada). Plants from which the etiolated tissue was obtained were grown in three-inch pots containing redi earth plug and seedling (Sungro, MA, USA), covered by large opaque plastic tubs and germinated on heating mats before being transferred to a dark growth chamber. Whole etiolated seedlings and trifoliate leaves from young greenhouse plants were separately grown and packaged in labelled sample envelopes and lyophilized for three days

(Labconco, MO, USA). Lyophilized tissue was weighed out into 96 well 1 µl U-bottom masterblocks (Greiner Bio-one, Austria) containing steel beads, frozen in liquid nitrogen and ground for 30 seconds in a Spex SamplePrep 2000 Geno/Grinder (Metuchen, NJ). The Dneasy 96 plant kit (Qiagen®) was immediately used to extract DNA from this ground tissue on a Biomek 2000 laboratory automation workstation (Beckman Coulter, IN, USA). The QIAgility automated liquid handling system was used to quantify the DNA using PicoGreen (P7589; Thermo Fisher Scientific, Inc., MA, USA), and then again to normalize the DNA to 12.5 ng/µl.

Genotyping-by-Sequencing (GBS)

Candidate enzyme pairs for GBS in lima bean were evaluated using an *in silico* digestion of the *P. vulgaris* reference genome (Schmutz et al., 2014), the closest sequenced species to *P. lunatus*. The enzyme pairs *MspI/ CviQI* and *Taq-α-I/CviQI* were selected due to the in-house availability of a 48-plex adapters set compatible with *CviQI* and their ability to evenly assay the *P. vulgaris* genome while capturing enough fragments to ensure a theoretical coverage of at least 12X for all the assayed loci (Table 4).

Table 4 *In silico* digestion results of five enzyme pairs on the genome of *P. vulgaris*

Enzyme pair	**Loci (64-384 bp)**
PstI/ CviQI	46,717
BsrI-A/ CviQI	60,168
MspI/ CviQI	**177,871**
MspI/ CviAII	223,327
TaqI/ CviQI	**381,424**
TaqI/ CviAII	525,957

Enzyme pairs in bold were used in GBS

The GBS protocol described by Manching et al (2017) was used for genotyping: RASP v2.0 adapters comprised of four TruSeq barcodes and 48 custom barcodes were used to construct two separate 192-plex libraries per enzyme combination. Among the 384 barcode assignments, 294 samples were used for this study, which comprised the 230 non replicated samples of the DP, the 30 samples comprised of 15 duplicate accessions, the 24 samples constituting the replicated trio sets, the 2 replicates of G19833 and 8 water blanks. The 90 excess barcodes were assigned to F_2 samples from the Cypress × Jackson Wonder cross for a separate study (samples with the notation P1501701_#). Libraries were sequenced (1x151 cycles) on an Illumina HiSeq 2500 at the University of Delaware's Sequencing and Genotyping Center at the Delaware Biotechnology Institute.

GBS Data Processing and Analysis

Libraries from the different enzyme combinations were processed separately, yielding two sets of data each comprised of 384 samples. Sequences from GBS were processed using RedRep, an open source computational pipeline for variant calling on GBS data (https://github.com/UD-CBCB/RedRep). RedRep scripts wrap commonly used software that includes FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) to deconvolute sequences by barcode; the CutAdapt package to remove adapters, trim low quality ends and filter out low quality reads (Martin, 2011); BWA-MEM (Li, 2013) to map reads to an unpublished lima bean reference genome assembly and the GATK HaplotypeCaller to identify sequence variants including single nucleotide polymorphisms (SNPs) and insertions or deletions (McKenna et al., 2010).

The two data sets (*vcf* files for *CviQ1*/*MspI* and *CviQ1*/*Taq-α-I*) were separately filtered for excessive missing data and low quality data using the same approach in VCFtools (Danecek et al., 2011). The first step of filtering involved excluding genotype data from a lima bean F_2 population that was sequenced for a different study. Each *vcf* file containing lima bean DP samples for this study (294 samples) was filtered for SNP loci with more than 50% missing data and with a minor allele count of four.

A copy of the trio data from this filtered dataset was made (trio data set) for each of the two enzyme combinations to ground truth the genotype data based on replicates and expected trio genotypes. A subset of the genotype data which was comprised of 286 samples (all F_1 progeny from trios were excluded) for each of the

enzyme combinations was also made and named the diversity panel dataset (DP data set). A filter for read depth (≥ 5) on each SNP locus was applied to all four datasets (two trio data sets and two DP data sets). The trio data sets from both the *CviQ1*/*MspI* and *CviQ1*/*Taq-α-I* libraries were merged after the filtering. Merging of datasets from both DP data sets was also done. There was in the end one set of data for the trio data set and one for the DP data set, each comprised of genotype data from both *CviQ1*/*MspI* and *CviQ1*/*Taq-α-I* libraries.

The –mendel function of VCFtools was used to report SNPs whose segregation pattern did not adhere to Mendelian laws of inheritance in the Cypress × Jackson Wonder trio and to flag these SNPs. All flagged SNPs from the trio analysis were catalogued. The SNP data in the DP data set was then filtered for all flagged SNPs catalogued in the trio data analysis. Further filters on the DP data set were applied, these being for individuals with more than 70% missing data and on loci with greater than 50% missing data. The replicated accessions were then analyzed to assess genotyping accuracy. Genotyping error was described as the total number of mismatched genotypes between replicates per SNP locus divided by the total number of SNP loci which have data in both replicate samples.

Population Genomic Analyses

To obtain some mean genetic diversity measures and inform subsequent analysis of the DP, the basic.stats function of Hierfstat was used (Goudet, 2005). These genetic diversity measures included observed heterozygosity (H_O), expected

heterozygosity (H_E) and the inbreeding coefficient (F_{IS}) observed heterozygosity was also assessed for each accession in the filtered data set. The DP data set was initially subset to exclude US accessions to allow observations to be made on how accessions collected from in and around the centers of origin were grouped and how these groups were related to germplasm, cultivars and accessions found in the US. This data set will be referred to as the AAOD (accessions from areas of domestication) data set. Analysis on the AAOD data set were conducted to observe how these accessions would cluster based on *a priori* knowledge and if the GBS data was sufficient to discriminate individuals from the different populations (Mesoamerican and Andean). This was critical to ensure that we could use the same model to determine the backgrounds of local lima bean accessions (local to the US). Bootstrapped phylogenetic approaches using neighbor joining (NJ) on a distance matrix calculated using the Prevosti's distance as implemented in poppr (Kamvar and Grunwald, 2018). The Prevosti's distance reflects the pairwise difference between individuals based on their allelic differences and was preferred as it makes no genetic assumptions like Nei's distance does, while also having the ability to handle missing data (Kamvar and Grunwald, 2018; Kamvar et al., 2015). The Prevosti's distance matrix does not have Euclidean properties and so principal component analysis (PCA) in Adegenet was used to cluster the groups of accessions on more than one plane as it similarly does not make any assumptions based on any evolutionary models (Jombart, 2008).

Discriminant analysis of principal components (DAPC) using the find.clusters method in Adegenet was used to assign each US accession a cluster label (Jombart, 2008). DAPC was preferred as it can be used to assign population groups without the

need for group priors while also allowing visualization using a multi dimensional scaling plot (Jombart et al., 2010). The *find.clusters* method of DAPC essentially uses a Bayesian approach to find an optimum number of genetic clusters (K-mean) from a successive range of K-means. DAPC is then used to perform PCA and discriminant analysis (DA), which maximizes on between group variability rather than within group variability (Jombart et al., 2010). After assigning each US accession to its respective population through a consensus of these analyses, F_{IS}, F_{ST} and H_O were computed to understand how the genomic structure and genetic diversity of local lima bean cultivars is impacted by breeding and selection in the East, West and overall US industry.

Stacks is a modular pipeline for processing and analyzing reduced representation data (Catchen et al., 2013). The *population* function of Stacks was used to calculate bootstrapped population genetic parameters such as heterozygosity over both SNP loci and within individuals of the DP. The *population* function was also used to estimate population differentiation, giving pairwise population F_{ST} values using a formula derived from Weir (Weir, 1996). The pairwise F_{ST} values between each population were plotted in R as a dendrogram to visualize distances among the different populations. The pairwise F_{ST} among the populations was also plotted for each locus to give insight on genomic regions that significantly characterize population differentiation.

Results

GBS Data Processing and Analysis

There were 225,032 variants (SNPs and indels) among 1,157 loci (i.e. read contigs spanning 598.78 million bases) in the *MspI/CviQI* library and 389,659 variants among 1,289 loci (601.24 million bases) in the *Taq-α-I/CviQI* libraries before any filtering or data manipulation. After filtering for minimum call rates and read depth, 13,977 (137 contigs spanning 473.18 million bases) and 5,172 (140 contigs spanning 462.53 million bases) variants remained in the two data sets respectively, with 132 variants shared between them.Therefore, with *CviQI* being a common denominator, *MspI* and *Taq-α-I* assayed distinct parts of the genome. The *MspI/CviQI* library performed better, yielding 72.91% of all the SNP loci in the final DP data set.

Trio data (Cypress,Jackson Wonder and their F_1 progeny) that had been independently processed using the same method as in the DP data set was found to have 30,282 SNP loci that had matching data and allowed a trio analysis to be conducted. Among the trio sets, only the Cypress × Jackson Wonder trio was used to assess genotyping accuracy as other trios had high levels of missing data in at least one of the three samples required for trio analysis. The overall genotyping error for all three replicates within the Cypress × Jackson Wonder trio was found to be highly consistent, with accuracy estimates of 96 - 98% per sample (Table 5). There were, 1,921 variant sites in the Cypress × Jackson Wonder trio did not adhere to the expected genotype in the F_1 or were inconsistently scored between replicates. The genomic regions from which some of the inconsistent SNPs in the trio were identified

were associated with repeat areas of the genome when blasted against the *P. vulgaris* reference genome.

Table 5 An assessment of genotyping accuracy in GBS of lima bean

	Accession	Bi-allelic SNPs (N ≥ 1)	Missing SNP data per replicate	Matching SNP data in both replicates	Mis-matched SNPs between replicates	Homo [b] SNPs	Genot-yping error	Proportion of missing SNPs in both samples	Proportion of het SNP loci [c]
Trio	Cypress	29965	12559	16663	743	16442	0.04	0.42	0.0133
	J. W.	30073	1754	27387	932	26884	0.03	0.06	0.0184
	F_1 [a]	29737	228	28799	710	28087	0.02	0.01	0.0247
Replicates after all filters [d]									
	DP_159	11,174	5,232	5,874	68	5,862	0.01	0.47	0.0020
	DP_160	17,681	1,756	15,857	68	15,817	0.00	0.10	0.0025
	DP_170	17,790	7,288	10,376	126	10,357	0.01	0.41	0.0018
	DP_171	16,723	7,810	8,830	83	8,803	0.01	0.47	0.0031
	DP_183	16,661	4,819	11,517	325	11,464	0.03	0.29	0.0046
	DP_186	17,129	6,854	10,181	94	10,169	0.01	0.40	0.0012
	DP_187	17,477	2,576	14,769	132	14,730	0.01	0.15	0.0026
	DP_188	17,246	4,260	12,942	44	12,927	0.00	0.25	0.0012
	DP_193	13,987	1,683	12,241	63	12,229	0.01	0.12	0.0010
	DP_202	17,591	1,703	15,742	146	15,671	0.01	0.10	0.0045
	DP_206	17,685	1,298	16,289	98	16,268	0.01	0.07	0.0013
	DP_210	14,366	4,492	9,776	98	9,719	0.01	0.31	0.0058
	DP_251	16,665	3,654	12,869	142	16,628	0.01	0.22	0.0029
	DP_255	12,307	5,725	6,501	81	6,477	0.01	0.47	0.0037
	G19833	6,015	2,375	3,530	110	3,507	0.03	0.39	0.0065
	J. W.	17,543	972	16,544	26	16,401	0.00	0.06	0.0086

[a] F_1 progeny of a cross between Cypress and Jackson Wonder; [b] homo = homozygous; [c] het = heterozygous; [d] Replicates were all duplicate samples

There were 19,149 SNP loci that met all the quality and completeness criteria in the DP data set. There were 24 samples of the diversity panel that were excluded from the analysis after filtering, due to failure to meet the lower threshold limit of 30% complete data over all retained loci (Table 6). The identity, origins and genepool classifications of the individuals which remained after filtering are listed in Table 11 of the Appendix. There were 17,997 SNP loci which remained after filtering out the catalogued SNPs identified in the trio data analysis, of which 1,034 were not captured in the independently filtered trio data set.

Table 6 Lima bean accessions in the diversity panel grouped by geographically assigned genepool

Gene pool	N	N (after QC[a] filters)
Andean	38	37
MI	10	9
MII	24	21
Carib	33	27
South-America	9	9
US-X	46	34
US-E	28	28
US-W	63	63
X[b]	12	11
Total	263	239

[a] quality control of GBS data based on trio analysis and locus and sample call rates; [b] an arbitrary genepool comprised of accessions collected in Africa, Asia and Europe; N = The number of individuals in a population.

The filtered diversity panel data was also validated for genotyping accuracy by assessing the genotyping error in the replicated samples (Table 5). The mean

genotyping error in the diversity panel data was 1.01%, and the 99% lower and upper confidence intervals for genotyping error among the replicated samples were 0.56% and 2.58%. A comparison between the dataset containing 19,149 SNP loci and that which had 17,997 SNP loci (filtered based on inconsistency and accuracy in the C-J trio) was made to assess the benefit of this filter using analysis of variance (ANOVA; $P = 0.002$), followed by post-hoc analysis (Kramer-Tukey HSD), which showed that additional filtering resulted in more accurate genotyping. These SNPs were ultimately used in all subsequent analyses of the genetic diversity of the lima bean diversity panel. There were between 43 and 6,541 non-reference genotypes (heterozygous or alternate to reference homozygous genotype) in the diversity panel dataset compared to between 288 and 330 non-reference SNP genotypes in the trio dataset.

Samples whose DNA was extracted from etiolated tissue had a generally more consistent genotyping output compared to samples from which DNA had been obtained from leaf tissue. The coefficient of variance (CV) of sequenced reads was lower in etiolated tissue (CV range of 0.78 - 1.29), resulting in more uniform read depth and data retention whereas samples whose DNA was extracted from leaf tissue (CV > 3) resulted in highly variable read depths and data loss through filtering of SNPs with very low read depths. This high variability in the percentage of SNP loci assayed between replicates was observed when samples were genotyped using DNA from leaf tissue in accessions such as Jackson Wonder (94%), Cypress (64%) and G19833 (61%). Samples which where genotyped from DNA extracted from etiolated tissue had a more even SNP output. Data from previous libraries using only leaf tissue and having higher CVs are not presented here.

The high similarity in seed shape, size and color, leaf morphology, disease resistance and genotype between the accessions DP_174 and DP_175 (genotype data 98.8% identical) suggested that these may be duplicate accessions in the gene bank. This percentage of mismatched genotypes falls between the 99% upper and lower confidence intervals (0.56 - 2.58%) of error rates between technical replicates. To assess the ability to detect duplicate samples using this genotype data, DP_173, an accession with similar disease resistance phenotypes and identical sampling location was compared to DP_174 and DP_175. The percentage of mismatched calls between DP_173 and both of DP_174 (90.46%) and DP_175 (87.99%) was outside the confidence intervals.

Heterozygosity of the diversity panel was assessed to infer outcrossing. There was a 99% chance that a randomly picked accession in the DP had a percentage heterozygosity of between 1.63 – 2.74%. The actual range of the percentage of heterozygous SNPs within a sample's genotype data was between 0.03 – 28.31% (Fig 19). Outlying accessions displaying high levels of heterozygosity have likely undergone outcrossing in what is an overall largely homozygous sample. Highly heterozygous accessions with at least 10% heterozygous loci in the DP originated from Brazil, the US and Peru (10 samples). It was interesting to note that the US accessions with the most heterozygosity were landraces collected from the Hopi reserves. This finding has implications on their agricultural practices and indicates sources for different forms of genetic diversity in already climate adapted accessions which US lima bean breeders can utilize. The next level of heterozygosity was for

accessions with heterozygous loci between 2.8 – 9.9% (51 accessions), which were mostly US cultivars or germplasm (57.3%), with the next largest group in this range of heterozygosity being accessions from Peru (21.3%).

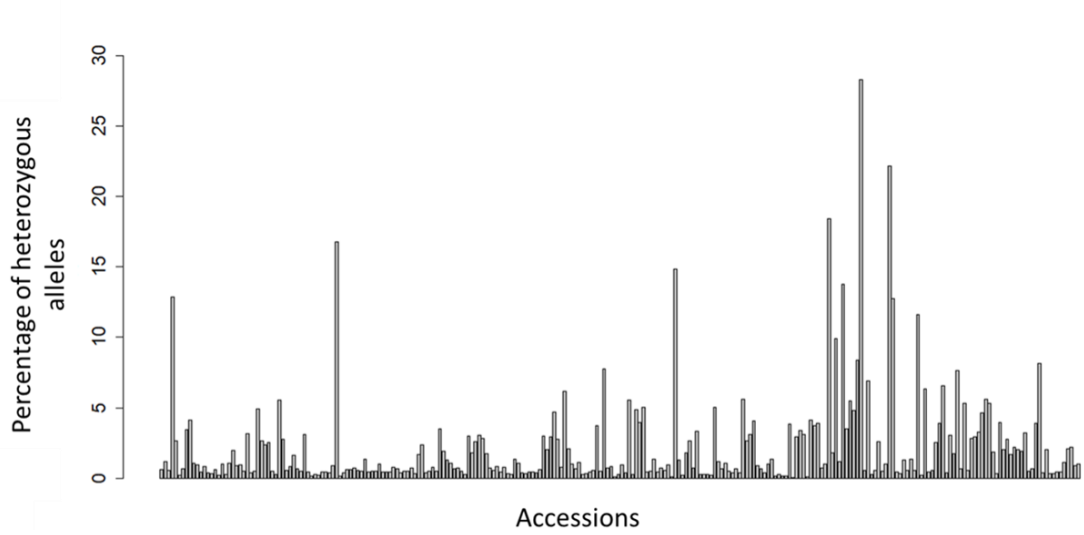

Figure 19 A distribution of the percentage of heterozygous SNP alleles contained in each individual of the diversity panel after filtering and merging the genotype data from the two *vcf* files described in the materials and methods section.

Population Genomic Analyses

The genetic structure of accessions in the DP that originated from the center of diversity was analyzed. This was done to compare their structure to that of the US group of accessions. To show the genetic grouping and distances between the different groups, principal components analysis (PCA) as implemented in Adegenet was chosen for its Euclidean properties which allow the data to be viewed using multiple dimensions. There were two major groups identified using PCA, these groups being

generally characterized by differing seed size (Fig 20). The Andean larger seeded accessions (AI - average 100 seed weight of 88.6 g) clustered separately from smaller seeded groups from the Andean (AII - average 100 seed weight of 36.3 g), the Mesoamerican region (MI - average 100 seed weight of 29.4 g and MII - average 100 seed weight of 45.1 g) and from the Caribbean Islands (Carib - average 100 seed weight of 34.5 g). There were outliers among the smaller seeded accessions, these outliers consisting of landraces sampled from the highland regions of Costa Rica and wild lima beans from the MII associated geographic location.

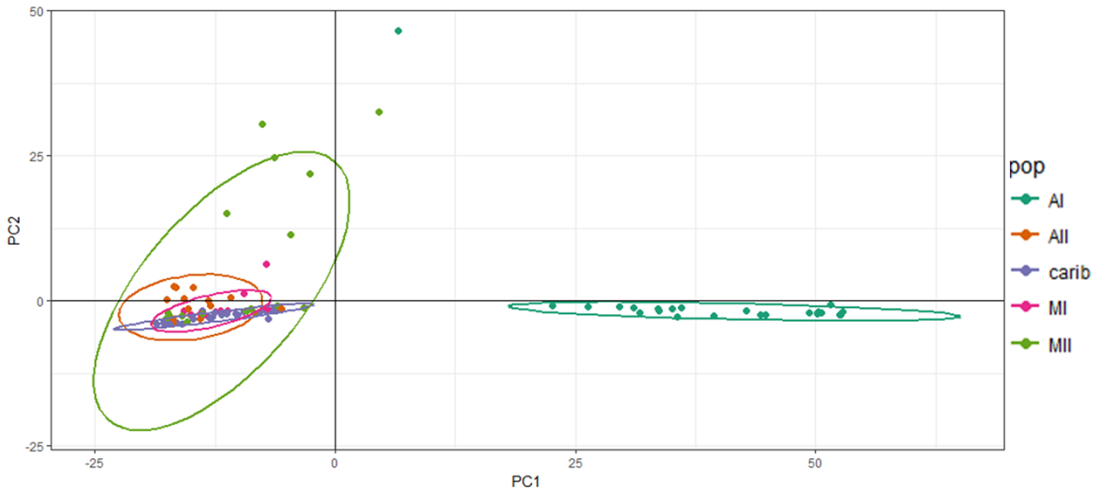

Figure 20 A principal component analysis (PCA) using 17,997 SNPs to describe the population structure of lima bean accessions originating from the central, Mesoamerican and Andean centers of domestication.

To reconstruct the phylogeny of these accessions without making any assumptions, the NJ tree building algorithm which can estimate evolutionary distances between samples and the assumption free absolute genetic distance method of Prevosti et al., (1975) was used. The phylogenetic reconstruction also showed a separation of

the two major genepools of the AAOD (Fig 21). The longer branch lengths between the MI and MII groups which were mostly grouped together and all the other accessions in both the Andean and minor Mesoamerican accessions suggested that they were more evolutionarily distant. The group of AII accessions along with a group of three Costa Rican accessions was more closely related to the AI group comprised of larger seeded lima bean (Fig 21). There was a group of AII accessions (0054, 0178 and 0197 in Fig 22A) and a group of three Costa Rican landraces (Fig 22C) that had similar shapes and size ranges that are typical of AI accessions in the DP (Fig 22B).

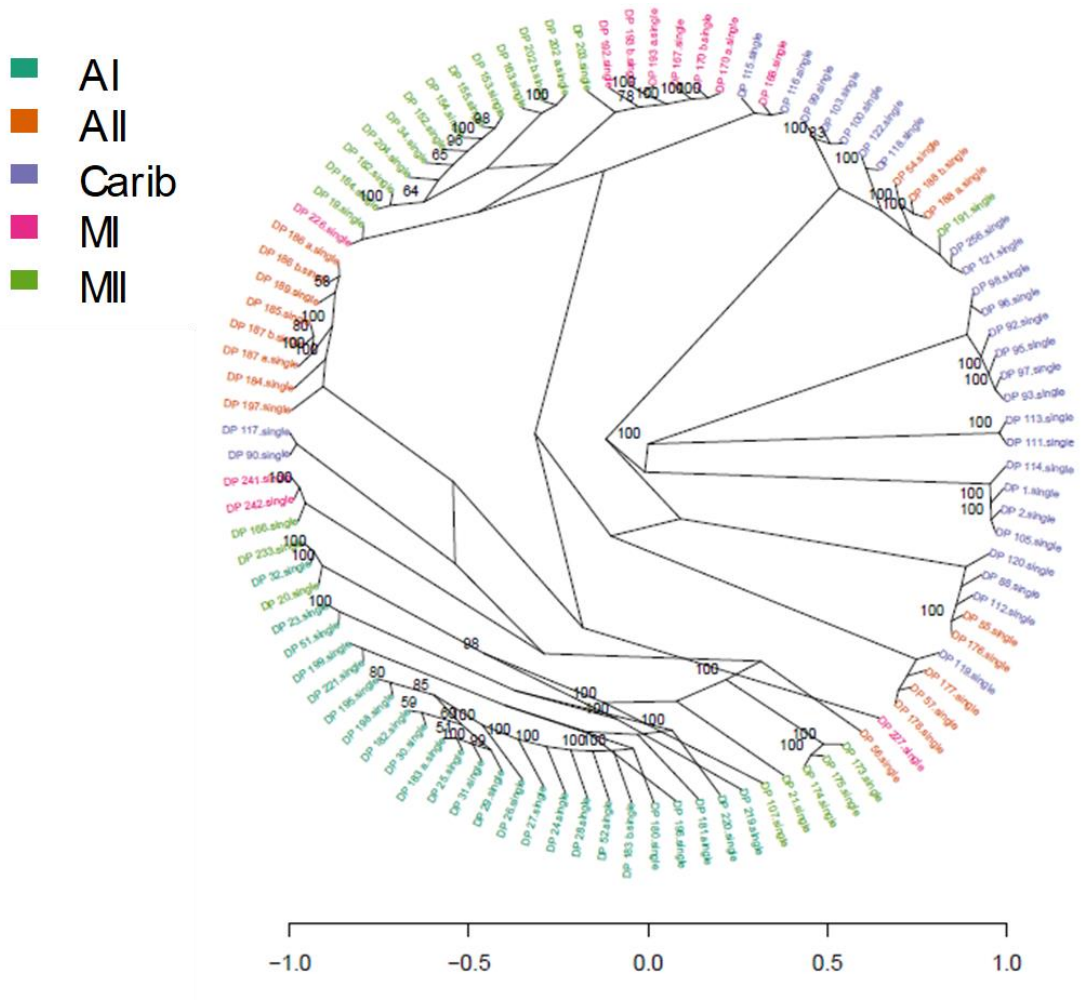

Figure 21 A Neighbor Joining phylogenetic tree showing the relationship among diversity panel accessions originating from lima beans different areas of domestication. The genetic distance matrix was calculated using the Prevosti's distance on 17,997 SNPs. Node labels represent bootstrap support (100 bootstraps).

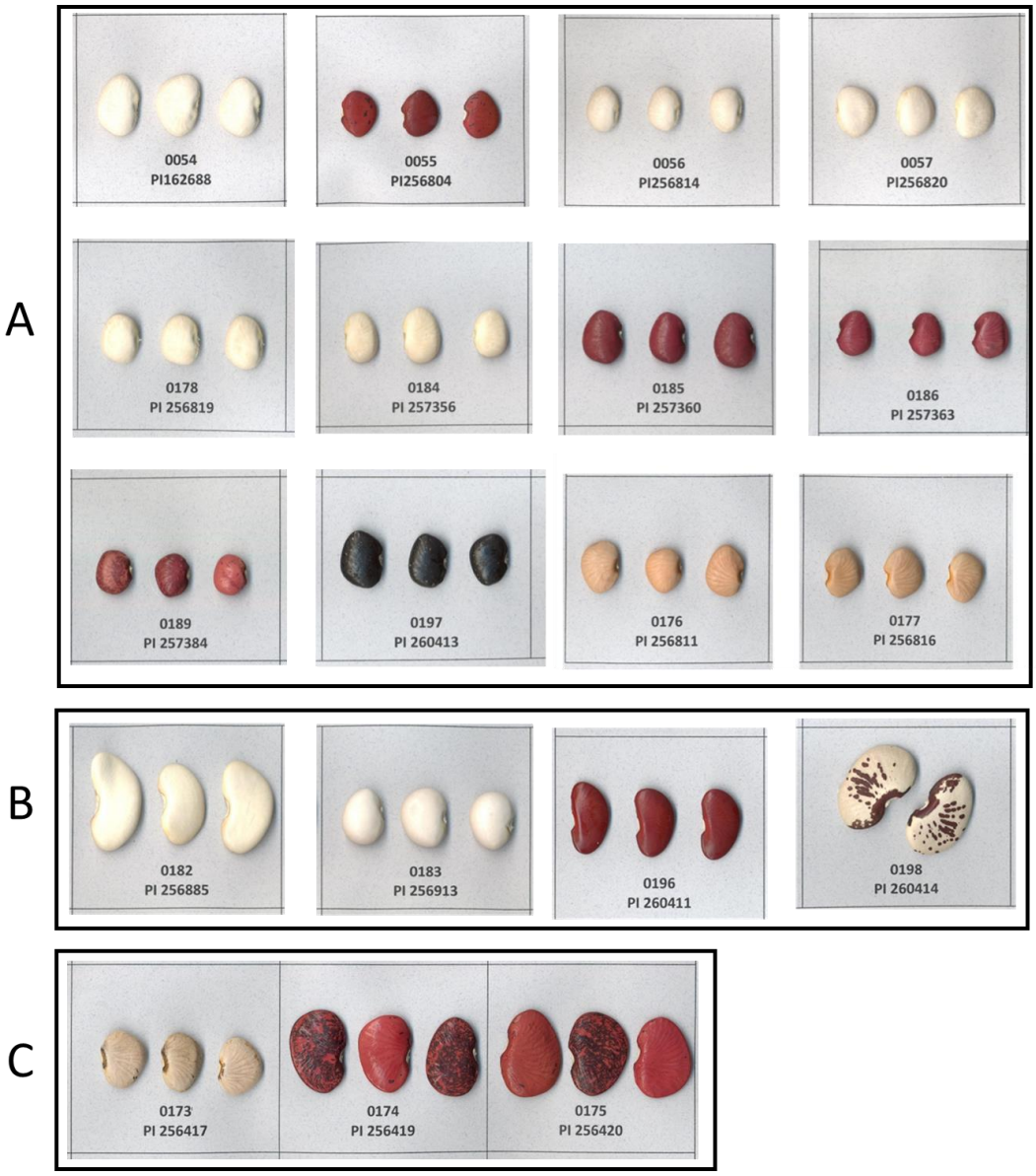

Figure 22 A comparison of some lima bean accessions from the AII group (22A) to four typical AI accessions (22B) and to three large seeded Costa Rican landraces (22C). Each accession is represented by three randomly selected seed samples. The accessions sample number is below the three seeds and below that sample number is the PI number assigned by the USDA – NCGRP. These images are courtesy of Emmalea Ernest.

There was poor resolution within the two major groups of DP accessions from the different areas of domestication for lima bean. To describe the genetic differences among these individuals, clustering was performed using the Bayesian Information criterion (BIC) statistic on accessions of the DP that excluded the US accessions. The US accessions were excluded from the initial analysis to establish the genetic structure of lima bean sourced from the primary areas of domestication. Clustering using BIC revealed an optimum of six groups within the DP, that being described as the least number of individuals that give the lowest BIC score (Fig 23A). The two major lima bean groups were separated along the y axis while lima bean was separated from the common bean outgroup along the x axis (Fig 23B). Andean accessions characterized by larger seeds formed two distinct groups (Fig 23B), while accessions with generally smaller seeds formed three groups among the Mesoamerican accessions (Fig 23B). The Andean common bean outgroup (*P. vulgaris*) was genetically distant from both lima bean groups but fell on the same position of the y axis as the Andean accessions in PCA, demonstrating genetic characteristics that are shared by beans from the Andean region. These shared traits are likely linked to selection for adaptation and not to seed size which is different between the two species of *Phaseolus*.

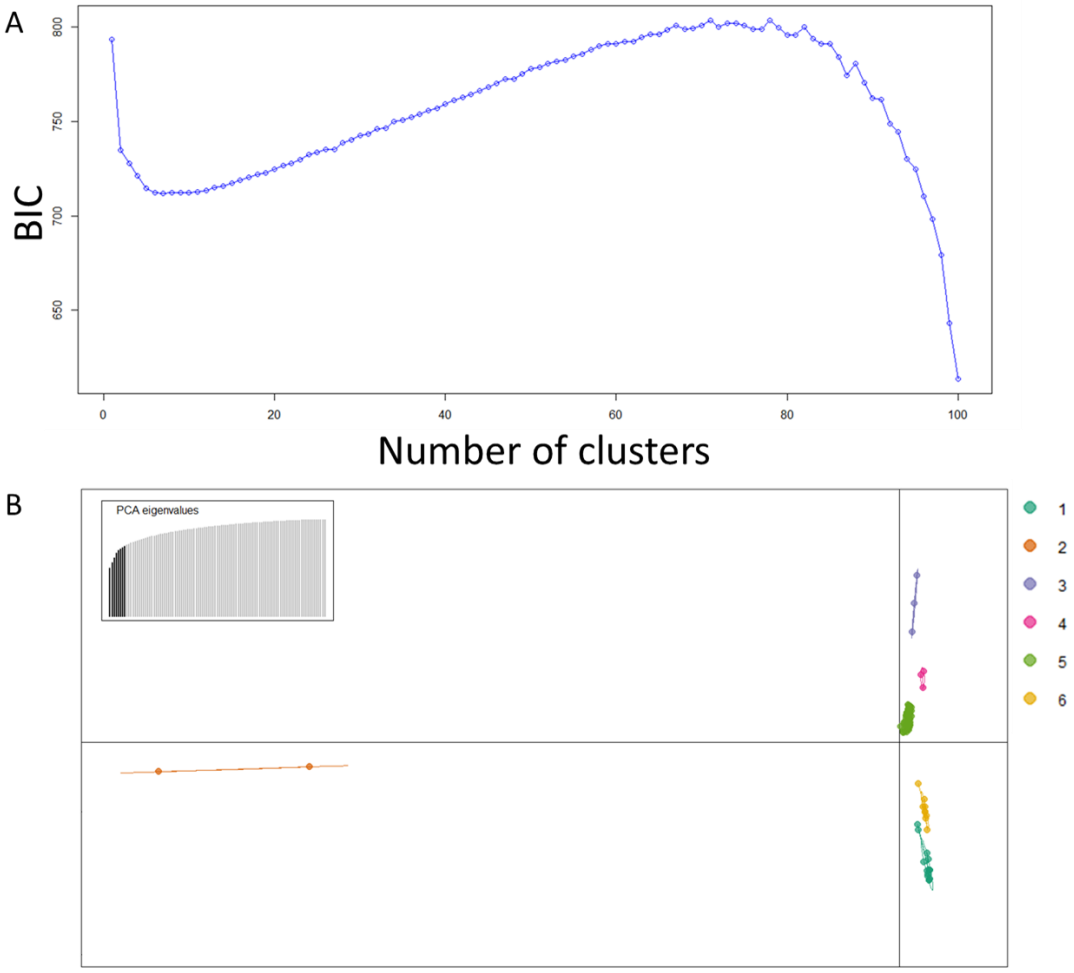

Figure 23 A clustering analysis using the Bayesian Information Criterion (BIC) statistic to determine the optimal number of populations contained in all the DP accessions that were sampled from the geographic locations associated with the MI, MII, Carib and Andean genepools. The BIC statistic demonstrated that six populations were optimal among these individuals (Fig 23A). In Fig 23B, populations 1 and 6 were from the Andean region, where beans were characterized by a larger seed size. These groups of Andean beans were separated by the y axis with populations 3, 4 and 5, which were comprised of smaller seeded Mesoamerican type beans. Population 2 was the common bean (*Phaseolus vulgaris*) outgroup and was separated from lima bean along the x axis of the clustering analysis.

The result of clustering the DP using BIC to inform grouping was also used to assign group numbers to US accessions, matching individuals to their closest DP group of lima beans sourced from primary areas of domestication. This method allowed US accessions to be assigned a group number such as US1 (for group 1 in Fig 23B), US3, US4 and US5. The clustering pattern of US accessions was compared to their Mesoamerican and Andean counterparts using PCA, where accessions were labelled by their BIC assignment (Fig 24). This was done to visualize the relationships and genetic distances between different groups of US accessions and the groups from the different areas of lima bean domestication. The genetically distant *P. vulgaris* sample was excluded from PCA analysis of all members of the DP to improve resolution of the clustering.

There were three distinct clusters among the Andean type cultivars and landraces of the DP using PCA. There were two clusters among these three that were groups of US accessions, these being groups US1 and US3. The first of these groups (US1) was comprised of larger seeded accessions typically comprised of both bush and vining Fordhook cultivars (Fig 25Bii.). Cultivar records show that most Fordhooks in the DP were derived from crosses between accessions with an AI origin and some smaller seeded Mesoamerican type cultivars such as Thorogreen (for their green color) or some of the Hopi landraces (for improved heat and disease tolerance) (Wester, 1961; Wester, 1967). The second US cluster was comprised of US3 accessions which were pole lima beans, characterized by long, heavy climbing vines and large pods and beans (Fig 25C). The US1 group formed a separate clade using NJ while US3 accessions were found in either the AI or US1 clade rather than as a

separate clade, an observation validated by the overlap of US3 accessions into either AI or US1 groups in the PCA (Fig 24). The US5 group was comprised of only one landrace (DP_63 called Christmas lima) which was grouped with the AI landraces. The AI and US1 groups of accessions were separated by PC2, with recorded breeding practices on US1 accessions being the likely source of variance along this axis (Fig 24).

A test to distinguish among the different groups of small seeded US accessions and their Mesoamerican and Andean sourced small seeded lima bean counterparts showed no distinct separation. The outliers among the different small seeded lima beans were comprised of a group of three Costa Rican landraces and four wild lima bean accessions. These results were similar to findings by Nienhuis et al., (1995) who used a similar collection from the US and genotyped it using random amplification polymorphic DNA (RAPD) markers. Overlaps between landraces from the Mesoamerican genepools were also similarly found by Chacón-Sánchez and Martínez-Castillo, (2017) who in addition used more wild accessions in which separate clusters were observed between the wild MI and MII accessions.

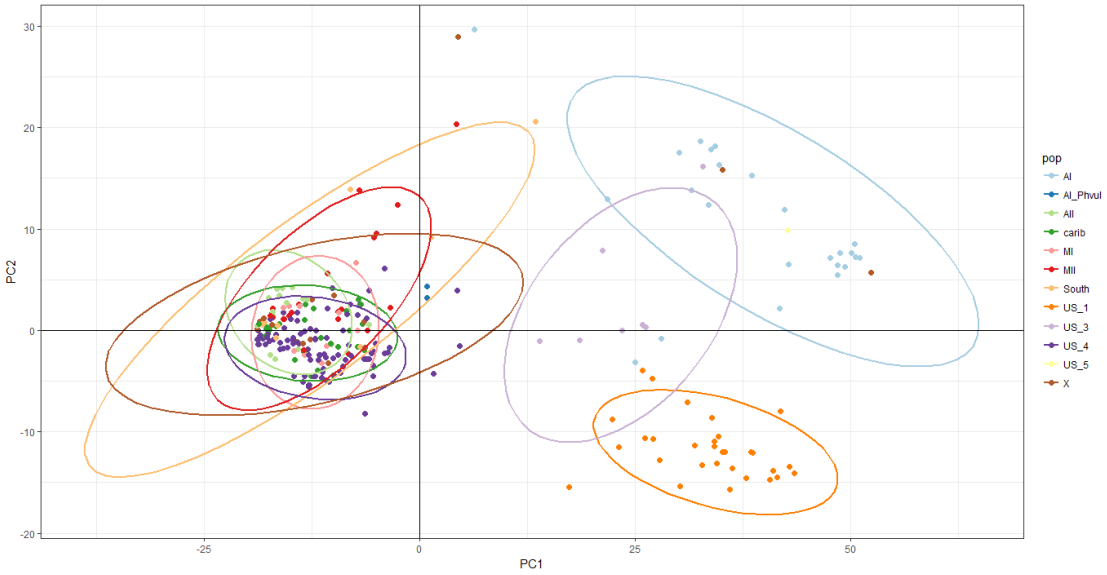

Figure 24 Principal components analysis (PCA) of the entire diversity panel using priors derived from Bayesian information criterion (BIC) to define groups. The x-axis (PC1) contributes 52.8% of the variance and while the y-axis (PC2) contributes 6% of the variance.

The populations from South America and from multiple locations (X) were more diverse as a group when the area they covered in PCA or the distribution of their members in the NJ phylogenetic trees was considered. This was due to their populations consisting of mixed genepools, a similar trend as that observed in the collective US sample when accessions within it are not parsed out by morphology or BIC assignment. The drivers and criteria for genetic selection in the US, AII and Carib accessions appeared to be similar when their F_{ST} was compared (Fig 26). These accessions were more closely related and less diverse in what is a sign of founder effects induced by artificial selection using a few similar sources.

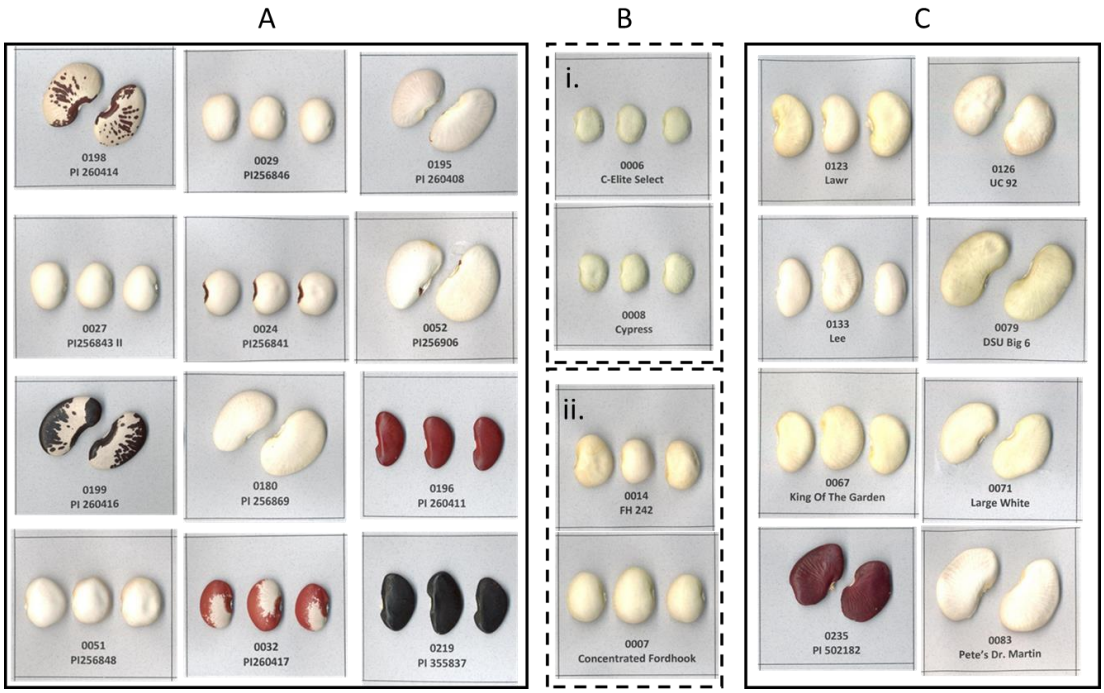

Figure 25 A comparison of lima bean accessions from the AI group (25A) to two of the most widely grown baby lima bean cultivars in Delaware (25Bi.), two of the most commonly grown Fordhook cultivars (25Bii.) and to the pole lima beans (25C). Each accession is represented by three randomly selected seed samples. The accessions sample number is below the three seeds and below that sample number is either the PI number assigned by the USDA – NCGRP or their common cultivar/ landrace name. These images are courtesy of Emmalea Ernest.

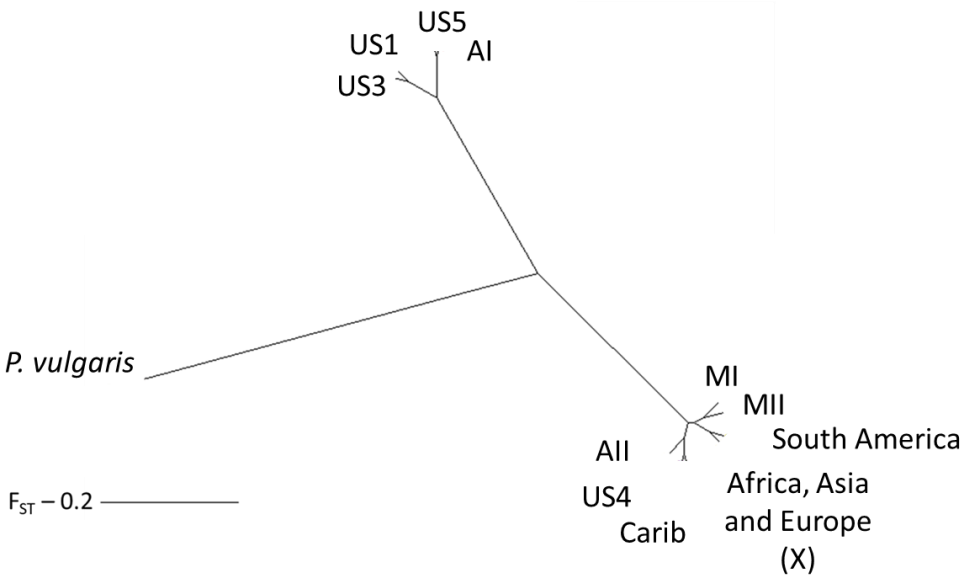

Figure 26 The genetic distance (Weir and Cockerham's F_{ST} with bootstrapping) between different populations of lima bean. The values of F_{ST} show greater genetic distances between the larger and smaller seeded cultivars and accessions whereas both lima bean groups are genetically distant from *Phaseolus vulgaris*.

The overall population indices indicated an expected reduction in genetic diversity in US landraces and cultivars, with higher inbreeding coefficients expected in cultivars which have undergone selection (Table 7). The US1 (F_{IS} = 0.13735) and US3 (F_{IS} = 0.10604) groups had lower inbreeding coefficients than the other groups, an indication of the recent outcrossing with different Mesoamerican type lima beans during breeding efforts for improvement of color, disease and drought tolerance. The

Caribbean and AII accessions also had relatively low inbreeding coefficients. The observed heterozygosity was lower than expected for all the genepools, evidence that the accessions in our diversity panel were not in Hardy-Weinberg equilibrium.

Table 7 Diversity indices of lima bean populations based on calculations using 17,997 SNPs

Population ID	N	Private alleles	H_O	H_E	F_{IS}
Overall US	131	134	0.02257	0.14495	0.36701
US_4	92	102	0.01601	0.05069	0.20119
carib	27	54	0.00947	0.02778	0.06253
US_1	30	19	0.03698	0.07557	0.13735
MII	22	593	0.01385	0.09509	0.30792
X	15	30	0.01028	0.14047	0.49300
South	9	32	0.05723	0.10714	0.14984
MI	11	153	0.00638	0.05369	0.14675
AII	17	64	0.00885	0.04414	0.11740
AI	24	416	0.05300	0.09976	0.26673
US_3	8	0	0.03746	0.07245	0.10604
US_5	1	0	0.02445	0.01223	0.00000
AI_Phvul	2	3192	0.02293	0.01392	-0.00219

N – Sample size; H_O - observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient; AI_Phulg – Andean landrace of *Phaseolus vulgaris*.

Private alleles are alleles found only in a single population among a larger collection of populations and are important in assessing the history and age of populations (Szpiech and Rosenberg, 2011). An assessment of the private alleles among populations of the DP showed an expected larger proportion of private alleles in the outgroup (*P. vulgaris*). Landraces from the Andean and MII genepools had the

highest number of private alleles, suggesting that the accessions collected from these geographic locations were more ancestral than the other genepools in which new mutations had not yet been established among the surveyed loci. There were more private alleles in the US4 group compared to the other US groups. This observation was likely due to the determinate nature of nearly half the cultivars in US4 and a tendency to breed for green seed coats, both chance mutations which are generally uncommon traits in lima bean (Mackie, 1943; Nienhuis et al., 1995). There were no private alleles among the US3 group and very few in US1, suggesting young populations which had not undergone and maintained many detectable mutations among the surveyed loci.

Discussion

There were 17,997 SNP loci that were retained for analysis after filtering, with high precision being observed in replicates which had upwards of 97% genotyping accuracy. This generally high level of precision was only slightly less than in a previous report where genotyping accuracy of at least 99.4% was achieved using the same protocol in *Zea mays* (Manching et al., 2017). An assessment of the choice of restriction enzyme combinations showed a significant effect, with the *MspI/CviQI* libraries producing a larger number of scorable SNPs which in addition had a higher and more evenly distributed read depth. This observation of differing genotyping output has also been observed in cassava, where enzyme choice showed significant effects on genotyping quality (Hamblin and Rabbi, 2014). We also observed that per sample variability in sequence and genotyping output was greatly reduced when DNA

extracted from etiolated tissue was used in GBS as compared to using DNA from young trifoliate leaf tissue.

The genetic distances among the Andean and Mesoamerican groups and between them and the larger seeded US accessions resulted in a similar genetic structure as found in a similar collection by Nienhuis et al., (1995). Climate data from FAO (Gommes et al, 2018) shows that temperature is similar throughout most of the geographical range of the Mesoamerican landraces but is significantly lower in the Andes. The genotypes resulting from crosses between the two major genepools while breeding for these larger seeded, heat and drought tolerant cultivars were probably the highest contributors to genetic variance (Fig 24).

A population of Mesoamerican type landraces found in Colombia, Ecuador, Peru and Argentina (AII) that were closely related using PCA and had shorter branch lengths connecting them in NJ analysis. These accessions were grouped with a few MII accessions which may be the source or close relative to what gave rise to these landraces. This pattern supports Mackie's (1943) hypothesis that lima bean may have travelled from the MII region to the Caribbean islands and to South America. A similar pattern in US cultivars and landraces was observed using NJ, where two groups of US4 cultivars are grouped with either MI or MII accessions which in addition have more private alleles, demonstrating the migration patterns of lima bean to the US from these more southern geographic locations.

There were seven outliers observed using both PCA and NJ methods and four of these accessions were wild lima bean (Fig 24). These accessions highlighted the kind of diversity we had not adequately captured in our panel and the potential to improve our cultivars by further investigating and utilizing them in collections comprised of larger sample sizes. Three of these accessions (DP_173 – DP_175 in Fig 22C) have disease resistance to both physiological races E and F of *Phytophthora phaseoli*, making them important resources to the Delaware breeding program. The landrace DP_173 has seed morphology and genotype data which is different from the very similar accessions DP_174 and DP_175 (98.9%). This observation demonstrates the risks of using non-genotyped accessions from seedbanks which are prone to replicating material they contain. The ability to genotype these accessions and to select unique accessions can assist breeding programs to focus on a few validated individuals and to save resources which would be used replicating experiments with the same material. The heterozygosity in these seemingly duplicated accessions was low, with only 0.29% of shared SNP loci being heterozygous. Heterozygosity in similar accessions may be important as sometimes traits are codominant, a property observed in lima bean cultivar selection practices where selfed progeny are observed having segregating phenotypes. This makes it equally important to also have phenotype data when making considerations for sampling accessions from a seedbank.

Principal coordinate analysis (PCA) visualized on three planes together with NJ showed overall similarities among landraces from the same geographic location (Fig 27). Despite the ability to observe groups of landraces linked by sampling location, it was not possible to distinguish between the potato and sieva races which

are previously described Mesoamerican subtypes of lima bean (Serrano-Serrano et al., 2010). Failure to clearly resolve these groups of landraces was likely a result of the sampling process, as some of these landraces were sourced from local markets. The sampling location of a landrace may not necessarily correlate with where it originated, likely causing this lack of resolution. An ethnobotany sampling approach such as the one used by Camacho-Pérez et al., (2018) would have made the sampling less random. Ethnobotanic approaches are associated with more detailed information on samples such as climatic adaptations, cultural practices and selection tendencies, based on statistics, interviews and *in situ* collections (Camacho-Pérez et al., 2018; Espinosa et al., 2012; Motta-Aldana et al., 2010).

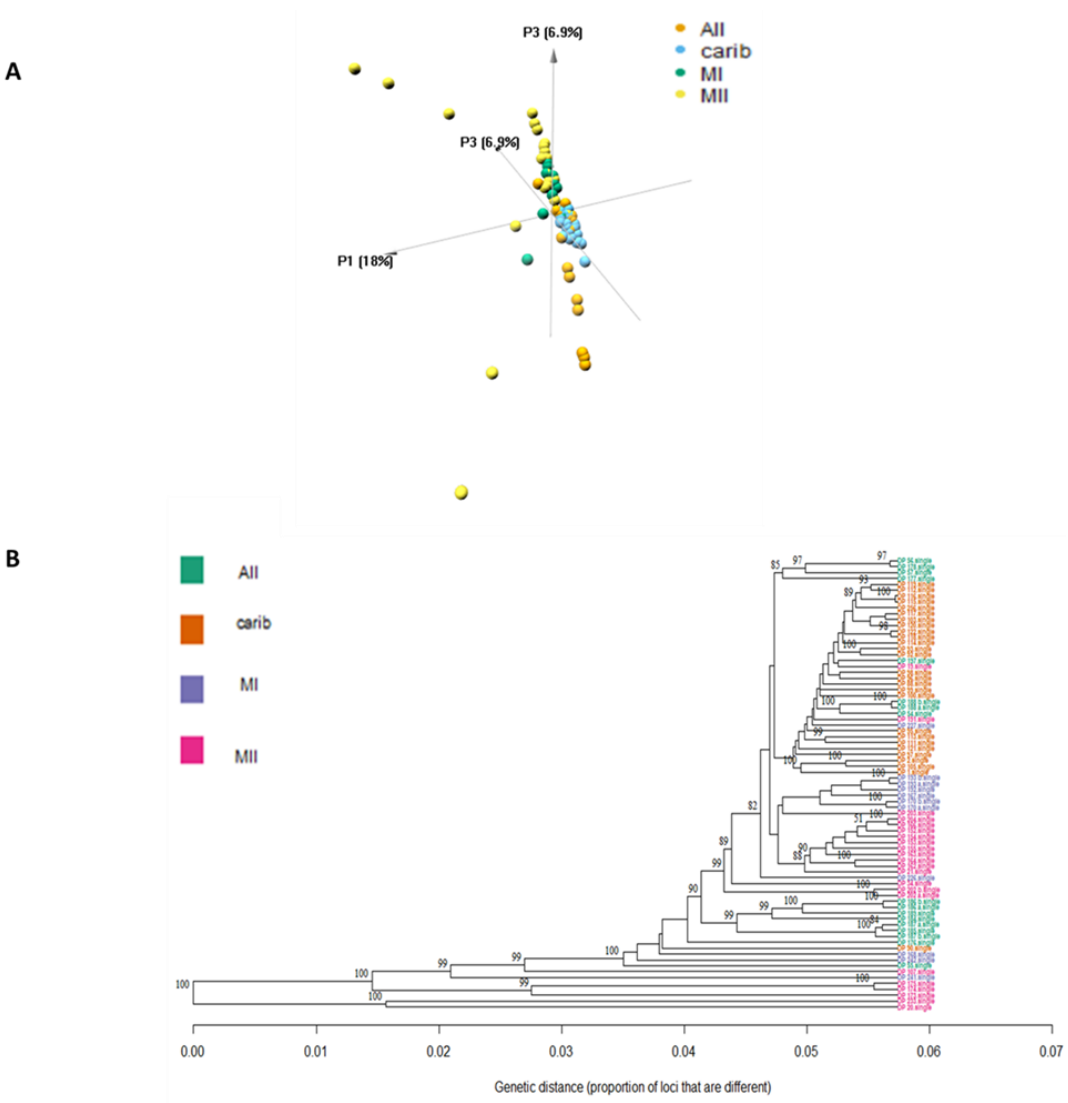

Figure 27 The structure of Mesoamerican landraces in our diversity panel. (A) a 3D multidimensional scaling (MDS) plot of all the Mesoamerican landraces in the diversity panel. (B) a phylogenetic approach to understand the structure of the Mesoamerican landraces. Numbers on the nodes represent bootstrap values used to support each node.

Grouping of landraces from the same sampling area suggests similarities in regional selection criteria, particularly in the MI and Carib landraces. An ethnobotany study revealed strict practices in maintaining cultivar purity of maize in Mesoamerica. The Milpa agricultural system where maize, beans and squash were grown together gives rise to the assumption that the purity of their lima bean landraces was similarly maintained (van Etten, 2006; Landon, 2008; Nigh and Diemont, 2013). There was evidence of directional selection among the MII landraces, demonstrated by the paucity of private alleles in most of this population but an abundance of private alleles (65.8%) among the wild accessions and in landraces found in high elevations of Costa Rica (Slatkin, 1985). The geographic terrain had a contributory factor as was the case in maize, with differences being observed between landraces from different climatic zones (van Etten, 2006). In addition, the lower genetic diversities and private alleles in the MI landraces compared to the MII landraces may point to the difference in Milpa cycles, where it was shown that shorter, more intensive cycles as seen in the MII associated geographical areas may have resulted in increased genetic diversity (Martinez-Castillo et al., 2006).

Isolation may be the reason why all the Carib landraces are clustered closely together. The low inbreeding coefficients and correspondingly low nucleotide diversity of these Caribbean landraces suggests a narrow genepool which is likely maintained by isolation and differentiated by genetic drift. Genetic drift has been demonstrated to act rapidly in smaller populations, resulting in the overall genetic diversity being approximately proportional to population size as observed in the Carib landraces (Torres-Florez et al., 2014). Another assumption about the Carib landraces

is that high levels of strictness and preservation of landrace purity may be the reason this group has a narrow genetic diversity but low inbreeding.

Overall heterozygosity of the DP was generally lower than in the most recent reports for lima bean (Chacón-Sánchez and Martínez-Castillo, 2017). There were however high levels of heterozygosity among some US cultivars, a surprise as they are mostly inbred. High levels of heterozygosity among lima bean cultivars in the US is however common, as improved cultivars have been selected out of segregating progeny from existing selfed cultivars. Confirmation of these claims is contained in the breeding records of some cultivars (Nienhuis and Sass, 2018) which are maintained by the Plant Variety Protection Database of the USDA (https://apps.ams.usda.gov/CMS/). Cultivars that arose from wide crosses between the two major genepools have expected excess residual heterozygosity which was not sufficiently reduced by backcrossing. These high levels of residual heterozygosity were observed in Fordhook cultivars in the DP, where the percentage of heterozygous loci within a sample was between 3.92 – 7.65%.

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The genetic diversity of cultivars and landraces in the US has been shown to be limited. Currently available resources have been characterized and have potential to substantially increase the genetic diversity in the US. We have identified unique genetic groups of Andean/ Mesoamerican hybrid cultivars and landraces that are a result of local selection and breeding efforts. These new groups demonstrate the ability to increase the genetic diversity of our cultivars while increasing their adaptability.

The identification of genetically distant landraces and wild type lima beans which have regionally important traits should result in improved cultivars with a higher potential of improved adaptability.

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Chapter 5

DISCOVERY OF DIVERSE FORMS OF RESISTANCE TO DOWNY MILDEW OF LIMA BEAN IN DELAWARE

Introduction

Lima bean is a specialty crop grown primarily in the Mid-Atlantic Region (MAR) for the fresh and frozen vegetable market. This crop provides excellent health benefits to the human diet such as high levels of essential nutrients, dietary fiber and contributes to a low glycemic index (McIntosh and Miller, 2001; Trinidad et al., 2010). Delaware contributes 60% of the total production in the MAR as its climate is better suited to production of this commodity. This niche market has resulted in the region being able to support the local production of other vegetable processing crops such as peas, carrots and sweet corn which would otherwise have been too expensive to produce in this area.

Lima bean production is adversely affected by diseases that affect yield and quality of the harvested produce. Among the major pathogens causing these diseases of economic importance are *Phytophthora phaseoli*, causal agent of downy mildew and *P. capsici*, causal agent of pod rot. Downy mildew of lima bean has been a disease of economic importance in Delaware since the early 1900's. Breeding efforts to mitigate related losses in the US began in 1948 (Wester and Cetas, 1959). These breeding efforts were mainly targeted towards major gene resistance to regional

isolates of *P. phaseoli*. This resistance was however not durable and was readily defeated, with the first observations of a new race being made in 1958 (App, 1959). Adaptation by the pathogen also saw the emergence of two new races around the year 2000 (race E and race F). This occurrence prompted the need to effectively identify lima bean resistance to these new races and to develop a platform for stacking these traits into elite cultivars. Stacking/ pyramiding of resistance genes (R-genes) has been proved to result in durable field resistance, effectively slowing down pathogen adaptation, reducing farmer inputs and maximizing yields (Mundt, 2018; REX Consortium, 2016). There are however fewer R-gene pyramiding successes than expected and it is now known that more detailed information of the gene combinations and their interactions is required before pyramiding. It has also been found that simultaneous use of cultivars each with different R-genes is sometimes a better option than pyramiding (Lof and van der Werf, 2017; Mundt, 2018; Mundt, 2014).

To enable the implementation of these strategies, it is important to identify the necessary resistance genes. Identification of these genes enables the determination of which strategy is most efficient among overall pyramiding, pyramiding of optimal R-gene combinations and the use of multi lines with different R-genes or combinations thereof. The greatest diversity of R-genes would be expected in the centers of crop origin and diversity, with landraces and knowledge from local farmers providing the necessary resources to mitigate future losses (Mercer and Perales, 2010; Vavilov, 1987). The large and variable geographic span of the lima bean center of diversity coupled with the different agricultural practices in these regions allows wide genetic variability to be a possibility, an observation also made when comparing landraces

from different altitudes and communities (Eckert et al., 2008; Martínez-Castillo, 2006). Population structure is also affected by natural selection, a powerful force that can shift a population's genetic makeup through time. This force can increase average individual fitness of entire species populations in the face of a widespread disease while some adaptations will only provide an advantage under certain local conditions (Flanagan et al., 2017).

Marker assisted selection (MAS) can improve the efficiency and precision of the breeding process and introgression of traits (Collard and Mackill, 2008). Developing elite cultivars using MAS is still a long process due to the extended cycles of selection and the frequent inability to capture loci which contribute minor effects (Desta and Ortiz, 2014). Genomic selection (GS) has been proposed as a method that can compensate for the shortcomings of MAS as it has been demonstrated to be effective in animal breeding and more recently in plant improvement (Desta and Ortiz, 2014; Dwivedi et al., 2017). Genotyping-by-sequencing (GBS) has been proved as an effective method of conducting both MAS and GS (Mhora et al., 2016; Poland et al., 2012). Training populations whose phenotypes and genotypes are known and understood are required to develop markers and models for both MAS and GS, (Desta and Ortiz, 2014). A collection of lima bean landraces, cultivars and wild forms was collected from the center of lima bean diversity and from around the United States (US) to initiate the discovery of resistance to downy mildew. Phenotyping of this lima bean diversity panel was done to identify potential sources of resistance to *P. phaseoli* and to *P. capsici*, major pathogens in the region. The phenotyping was complemented with GBS of the accessions and genome-wide approaches to assess and identify loci

associated with resistance to these pathogens. Markers for genomic selection can be identified, allowing more rapid approaches toward identifying R-genes for developing the most effective control for lima bean diseases. The goal of this paper was to identify downy mildew resistant accessions, characterize them and identify loci associated with resistance to *P. phaseoli*.

Materials and Methods

Diversity Panel

There were 263 members of a diversity panel (DP) collected from around the world, with accessions originating mainly from Central and South America. Accessions were sourced from the USDAs National Center for Genetic Resources Preservation (NCGRP), the International Center for Tropical Agriculture (CIAT), from collaborators in the Caribbean Islands (Puerto Rico and Haiti) and from both the California and University of Delaware's lima bean breeding programs. There were six individuals of the diversity panel that were replicated with commercial versions sourced from seed companies. This replication was necessary as the seed company cultivars are routinely used for in-house race testing of isolates and may be different from seedbank copies. These replicated cultivars contain resistance to either race F (878 and Maffei 15) or race E (184-85, Bridgeton, c-elite and cypress) and not to both.

Experimental Plan and Field Layouts

The diversity panel consists of lima bean accessions which have variable photoperiod sensitivity and determinate or indeterminate phenotypes. These features make it difficult to time planting or infrastructure placement when running a field experiment with all the accessions at the same time. The amount of space and trellising equipment is also expensive and as such, it was necessary to select a subset of the diversity panel for replicated multiple site disease screening. The goal of the first year of screening was to identify this subset of accessions to carry for further testing. Phenotyping of the diversity panel was conducted at the University of Delaware farm on a plot measuring 61 m x 55 m. The field was divided into two sections for logistic and economic reasons, one section being for the bush type (determinate) accessions (35 accessions) and the other for vining (indeterminate) accessions that needed trellising (221 accessions; Fig 30). A random number generator was used to determine where each accession was planted in the field (Random.Org, Dublin, Ireland). Wooden labels were marked with each accessions' identifying number and inserted into the ground which had been marked using a push seeder. All the diversity panel seeds were scarified using a razor blade and hand planted according to the field map. Seeds were planted on the 27th of June 2016 at a seed density of 20 seeds per 152 cm (5 feet) for small seeded accessions whereas large seeded accessions were planted at 10 seeds per 152 cm. Rows containing the diversity panel were evenly spaced at 152 cm intervals and a row of c-elite (a commercial cultivar) was planted between them, acting as a positive control and a reservoir for disease. To ensure there was adequate space for the diversity panel accessions to grow, C-elite plants directly adjacent to the individuals of the diversity panel were removed to create a checker

board growth pattern (Fig 28). Trellising was done using 183 cm long oak wooden stakes (Nolts Produce Supplies, PA, USA) driven 30 cm into the ground on either side of an accession. Polypropylene twine was used to build a trellis around these stakes by tying a line at one-foot intervals along the height of the stakes and along the entire length of each row of the field, ensuring that the lima beans would be well supported. Three 30 cm high intervals of trellising were constructed along the length of the vining section (Fig 28, section A). Organic biodegradable twine was unable to hold the weight of the lima beans. Known cultivars described in the plant material section were planted as controls to confirm fidelity of the inoculum. Boarder rows consisting of *Zea mays* (maize) were grown around the field to maintain high levels of humidity around the edges of the field where lima beans were not adjacent to any other crops. Lima beans were maintained within the confines of their allotted space and were periodically trained onto the trellis as needed, preventing neighboring accessions from encroaching.

Figure 28 An aerial view of the field four weeks after planting. The red boarders section out the field into different divisions. Section A is composed of the vining habit and indeterminate accessions, whereas Section B is composed of the bush habit and determinate accessions (Image by Cassel, C, 2016).

A drip irrigation system was installed in the field to ensure an adequate water supply. Optimum pathogen conditions for *P. phaseoli* (race F) are high levels of leaf wetness, humidity and moderate to low temperatures (Evans et al., 2002). A misting system was installed to ensure that high leaf wetness and humidity were maintained and secondarily to cool down the field in the event of daytime temperatures exceeding 30°C after inoculation. The misting system consisted of 183 cm tall metal posts on which rubber tubes connected to a water source were fitted with Micro-Bird misting nozzles (Rain Bird Corp., Glendora, CA). Misters were installed at 3 m intervals as the radius of the spray was 3.1 m. Time and duration of misting (20 minutes every four hours from sunset to sunrise) was controlled by a battery-operated node controller mounted to a valve solenoid.

Inoculation

Inoculum for the field inoculations was prepared using previously described methods on hypocotyls of the susceptible cultivar Fordhook (Mhora et al., 2016). This protocol was scaled up to obtain enough sporangia to make a 20 L volume of inoculum with a concentration of 10-5 sporangia/ml. In the first week of August 2016, daily scouting of the field began to ensure that each accession was tagged and inoculated at its peak susceptibility stage as most individuals had different flowering times. All accessions and their adjacent C-elite controls were inoculated at the pin-pod and flat-pod stage of the accessions using a backpack low-pressure manual pump sprayer (SOLO backpack sprayer, model 425, SOLO Incorporated, VA). Inoculation was repeated four times due to the different flowering time of the different accessions, ensuring the highest probability of inducing infection. Experience has shown that multiple inoculations and an increase in disease pressure from adjacent susceptible controls limits the number of escapes even in the indeterminate, non-flowering accessions which are expected to show disease signs and symptoms on internodes, shoot tips and even on leaves in severe cases.

Disease Ratings

The field was rated for disease severity using a rating scale where a negative disease rating corresponded to complete resistance characterized by the absence of signs or symptoms and a positive rating corresponded to susceptibility at differing levels of disease intensity on any plant parts (Fig 29 b-h). Infected plant parts were collected and viewed under the microscope for the presence of sporangia which were within the morphometric range, a confirmation of the presence of the causal agent of downy mildew. Severity of infection was noted for each plant, with infection ranging from moderate to severe infection. Ratings were conducted after each week of inoculation and then again two weeks after the final inoculation. All accessions that had a resistant rating were rated again in the first week of October (three weeks after the last rating) to ensure that they had remained resistant throughout the growing season and under continued disease pressure.

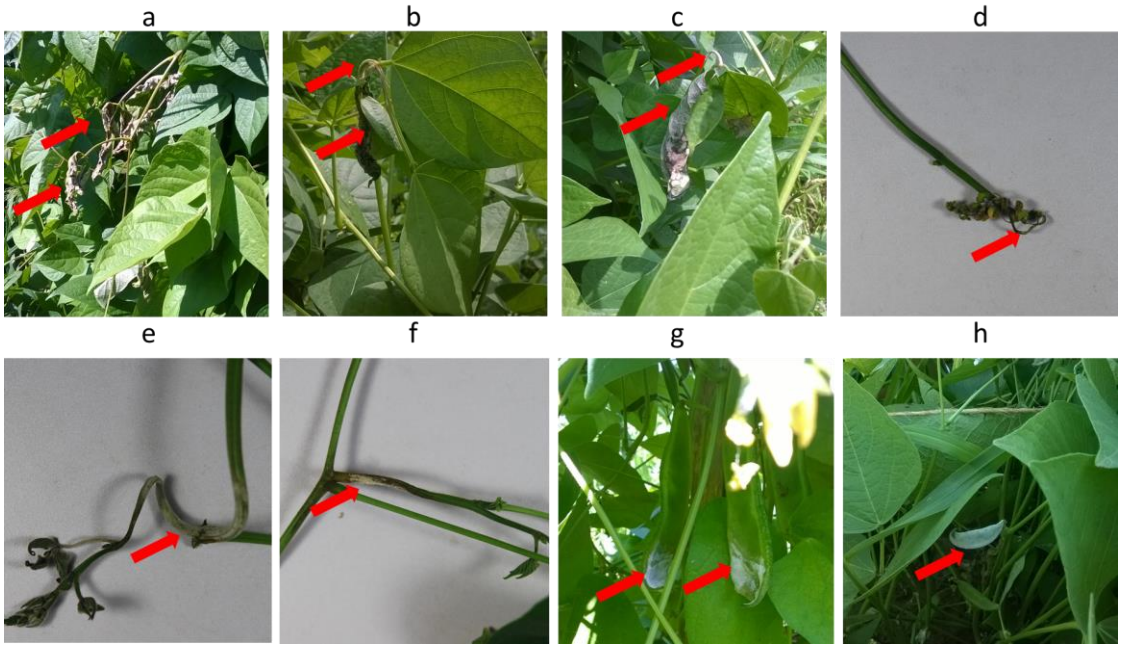

Figure 29 Signs and symptoms of downy mildew on different lima bean plant parts after inoculation with *Phytophthora phaseoli* sporangia. (29a) Hypersensitive response in plant tissue around downy mildew infections. Downy mildew signs and symptoms were also observed on petioles (Fig 29b and 29c), racemes (Fig 29d), emerging shoots (Fig 29e), internodes (Fig 29f) and pods (Figs 29g and 29h).

Dew Chamber Validation

All accessions evaluated for resistant to race F of *P. phaseoli* were validated for resistance using hypocotyl screens in the dew chamber. Scarified seeds (three per accession) were planted in four-inch pots containing Ready Earth plug and seedling mix (Sungro, MA, USA) and allowed to germinate. Inoculum was prepared as before and used to inoculate newly germinated hypocotyls with blended infected Fordhook seedling tissue. Pots were randomized and placed in a dew chamber set according to the method by Mhora et al., (2016). This experiment was repeated three times. Disease

ratings on each seedling was conducted using the same rating scale as in the field for each replicate at seven and nine-day intervals.

Selection Criteria and Disease Screening of a Diversity Panel Subset

There were 20 accessions of the diversity panel which were resistant in the field and 16 after dew chamber screening. Reciprocal accessions to the 20 (reciprocity being defined as accessions from the same geographic location and with similar morphology) were selected to be the negative controls of each accession. The cultivars used for physiological race determination (Bridgeton, cypress, 184-85, 878 and either c-elite or Maffei 15) and accessions thought to be resistant to *P. capsici* (21 - PI256405; 41 - PI 347826; 42 - PI 362772; 147 - PI 363057; 148 - PI 363054 and 234 - PI 477041) were also included in the list, bringing the number of individuals in the subset to 52 (Appendix, Table 11). Separate fields for *P. capsici*, race E of *P. phaseoli* (both in Newark, Delaware) and race F of *P. phaseoli* (Georgetown, Delaware) were planted in the same manner as described before. Different randomized complete block designs (RCBD), all with three replications were used to plant each field for each disease being screened. Confirmatory tests using dew chamber screens of all accessions of the subset were also carried out for both races E and F using RCBD. Confirmatory tests for *P. capsici* were carried out in a temperature controlled greenhouse using overhead misting and transparent plastic around the replicate blocks to maintain high humidity. Rating and confirmation of the disease was the same as previously described. Pod assays were also attempted to test the diversity panel for resistance to *P. capsici*. Pods that showed no signs of disease three weeks after

infection were collected for the first half of the diversity plot, surface sterilized using a 10% bleach (sodium hypochlorite) solution containing two drops of tween 20 for each 100mls of solution. Pods were then rinsed off in sterile distilled water before being placed into petri dishes containing moist, sterile filter paper. Three replicated plates per accession, each containing three pods per plate were inoculated with a cork bored agar plug of *P. capsici* (isolate P.C. #93) grown on V8 medium, lightly misted and sealed with parafilm. Negative control plates for the same accessions were not inoculated with *P. capsici* but were treated the same as inoculated plates.

Genotyping by Sequencing to Identify Markers for Genomic Selection

Genotyping, data processing and basic population summaries were performed as described in the previous chapter. Population structure of the phenotyped individuals was determined using PCA. The Fisher's exact test and discriminant analysis of principal components (DAPC) were both used to determine SNPs associated with resistance. Both these methods were used before and after correction for population structure. The Bonferroni and false discovery rate (FDR; Benjamini and Hochberg, 1995) methods were both used to correct for multiple testing.

Results

Disease Ratings

An accession was rated as resistant to downy mildew or pod blight when it showed no signs and/ or symptoms in all the field, dew chamber and greenhouse screening it was subjected to. There were 15 accessions that were resistant to race F, six to race E and three (PI 256417; PI 256419; PI 256420) that were resistant to both races E and F (Table 8). These accessions were found dispersed along central and South America, with the most accessions having been collected from Guatemala and Mexico (Fig 30).

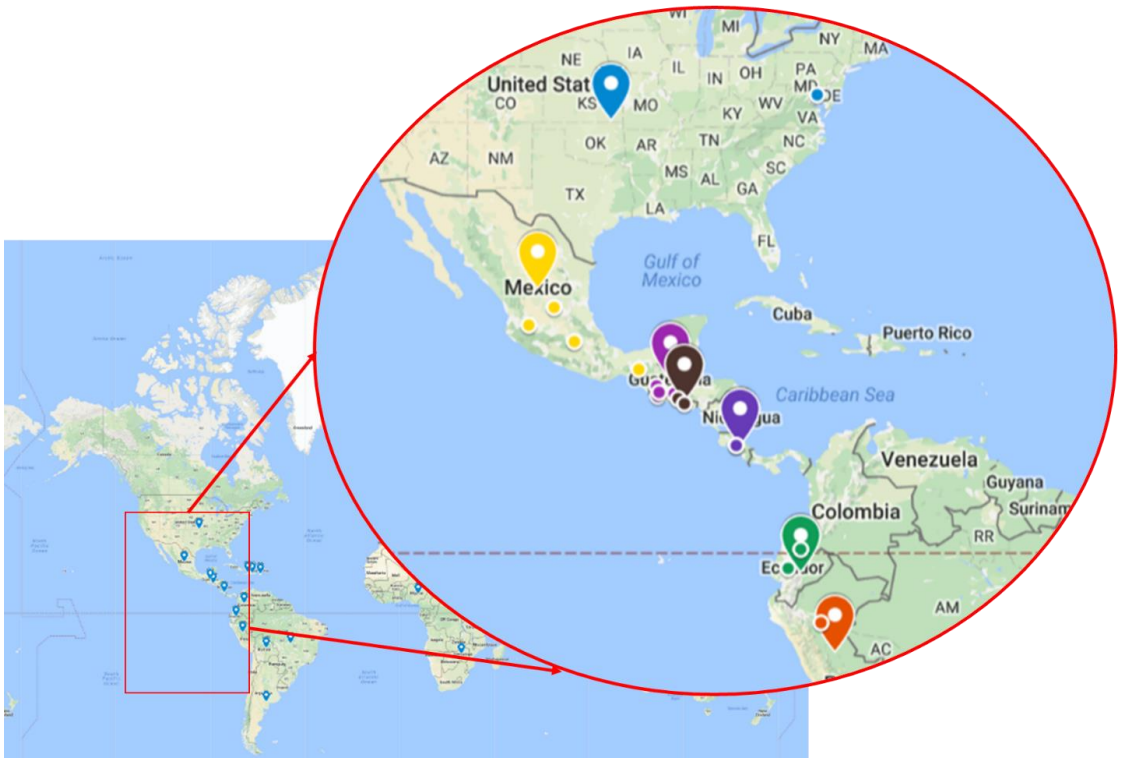

Figure 30 Geographical location of members of the diversity panel. Locations within the red box and magnified in the red circle are those locations where the resistant individuals originated. The large pins with different colors correspond to the different countries from which these accessions are from. The smaller points indicate the exact location where the accession was collected. The color of the large pins corresponds to that of the smaller dots representing exact locations within that country.

There were two accessions that were demonstrated to be important for breeding downy mildew resistant Fordhook cultivars in the MAR. These accessions are the large seeded Andean accessions from Ecuador, PI 355837 (race F resistance) and PI 355839 (resistance to race E). The accession PI 355839 is potentially resistant to both races, but we were unable to conduct greenhouse or dew chamber validations for race F resistance due to lack of seed. There were three accessions that were only resistant to race E in the field while there were two that reacted similarly for race F.

These accessions could either be escapes or contain weak resistance that could be useful in breeding for durable resistance. There were no accessions that were resistant to pod blight caused by *P. capsici*. The pod assay was not successful and was characterized by coenocytic, non-sporulating hyphae originating from the agar plug used as inoculum. Pods from controls and inoculated replicates of the pod assay showed signs and symptoms of downy mildew of lima bean, suggesting a latent infection by *P. phaseoli*.

Table 8 Ratings of all accessions which were overall resistant to either race E, F or both after field, dew chamber and greenhouse screening.

			Race E		Race F		
Accession	Origin	PI	Newark (F- 2017)	DC (2017)	Newark (F-2016)	Georgetown (F-2017)	DC (2016)
173	Costa Rica	PI 256417	R	R	R	R	R
174	Costa Rica	PI 256419	R	R	R	R	R
175	Costa Rica	PI 256420	R	R	R	R	R
17	USA	(PI 549521)	R	S	R	R	R
20	El Salvador	PI 256389	R	S	R	R	R
72	USA	PI 549512	S	S	R	R	R
108	Guatemala	PI 195342	S	S	R	R	R
164	El Salvador	PI 200919	S	S	R	R	R
166	Guatemala	PI 200924	S	S	R	R	R
167	Mexico	PI 201287	S	S	R	R	R
177	Ecuador	PI 256816	S	S	R	R	R
192	Mexico	PI 257548	R	S	R	R	R
203	Guatemala	PI 310627	S	S	R	R	R
219	Ecuador	PI 355837	S	R	R	R	R
226	Mexico	PI 433928	S	S	R	R	R
170	Mexico	PI 224713	R	R	R	R	S
220	Ecuador	PI 355839	R	R	R	R	NA
233	Guatemala	PI 451925	R	R	R	S	R

Genotyping by Sequencing to Identify Loci for Marker-Based Selection

Principal coordinate analysis, DAPC and phylogeny showed overall population structure existing within the subset individuals that were extensively phenotyped in both the field and the greenhouse (Fig 31). The population structure was a result of the presence of different genepools. There were two distinct groups that contained reciprocal accessions with regards to resistance to either race or overall resistance to downy mildew. The third cluster consisting of the Costa Rican landraces had no reciprocal accessions. A plot of the Eigen values showed that the first three principal components explained 47.84% of the population structure (PC1 – 23.31%; PC2 – 15.67%; PC3 – 8.86%) (Fig 31). These three principal components were excluded from the data set when correcting for population structure.

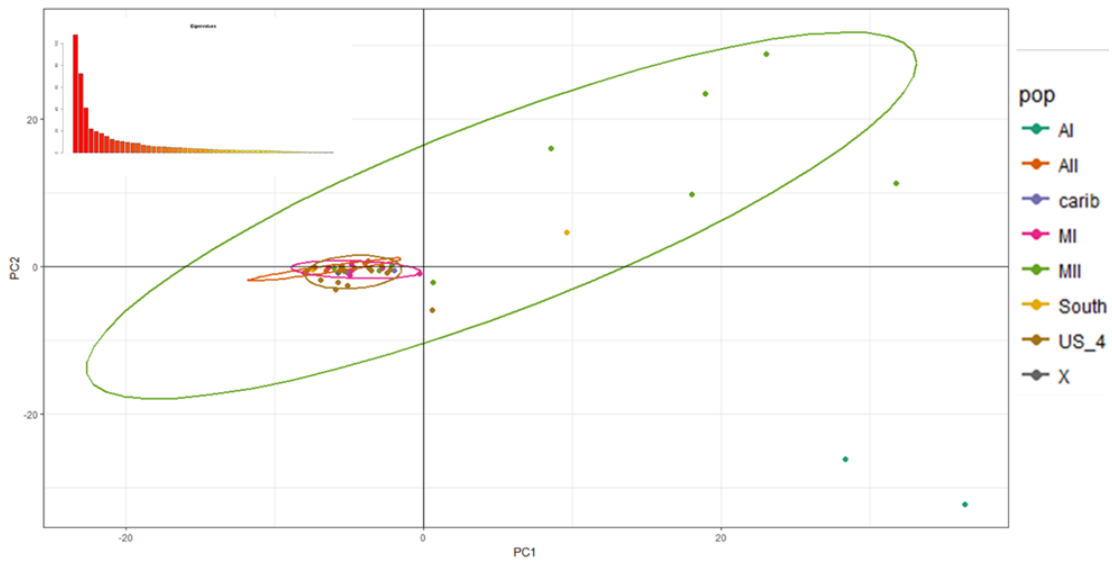

Figure 31 A PCA demonstrating the ability of the experimental design to eliminate the population structure of the phenotyped subset of accessions. The outliers were the wild accessions and the three Costa Rican landraces with dual resistance and the wild accessions in the MII genepool. The top left bar graph represents the eigen values for the PCA.

There were no SNPs found to be associated to resistance to any of the races of downy mildew after multiple test correction and after correction for population structure using PCA. Markers which were associated to resistance using the univariate methods were not the same as the ones identified using the multivariate methods. It was interesting to note that all the contigs from which the SNPs were found for both methods were the same. When compared to the contigs from which markers for race F resistance were identified (Mhora et al., 2016), it was found that all but one of the contigs was the same (Table 9).

Table 9 Contigs associated with resistance to downy mildew using both Fisher's test ($P < 0.01$) and DAPC. None of these Contigs were significant after correcting for multiple testing in the Fisher's test.

Race F	Race E	Race E and F
scaffold3121[a]	scaffold4009[b]	scaffold719
scaffold20197	scaffold14807[b]	scaffold940*
scaffold9180	scaffold20197	scaffold4009[b]
	scaffold9180	scaffold9180
		scaffold14807[b]
		scaffold19128
		scaffold20197
		scaffold23432
		scaffold3121[a]
		scaffold13072
		scaffold18682

*Contig that was not found to be associated to race F resistance in Mhora et al., 2016.; [a] race F resistance locus; [b] associated to race E resistance. The remaining loci are associated to both races.

Discussion

There were 15 accessions found to be resistant to race F of *P. phaseoli* based on both field and dew chamber experiments. There were six accessions found to be resistant to race F and three resistant to both races. These results are very important as resistance to both races is a much sought after trait which has yet to be achieved (Santamaria et al., 2018). Equally important was that two of these 18 resistant accessions were of Andean origin, the genepool closest to the Fordhook cultivars. This means it may become easier to introgress these resistance traits into the Fordhook lima beans which currently do not have resistance to either race E or F, potentially heralding a resurgence of the cultivars production in the MAR. Despite having race E resistance in one landrace and race F resistance in the other, there is a possibility of

pyramiding these two forms of resistance if they are not in repulsion, a previous observation in lima bean (Santamaria et al., 2018). This discovery presents an opportunity to test the effectiveness of simultaneous R-gene deployment compared to gene pyramiding in lima bean using established methods (Lof et al., 2017; Lof and van der Werf, 2017).

We observed accessions that were resistant in the field but not in the dew chambers which were set at optimal conditions for downy mildew. Resistance in the field can be dictated by environmental conditions, where sub-optimal conditions can result in failure of accessions with partial resistance to be develop signs and symptoms or be susceptible to a pathogen. Despite controlling for multiple variables in the field such as high inoculum pressure, moisture and leaf wetness, we could not optimally control temperature, insect pests or soil microbe composition. These are all factors which could have influenced our observations. Evidence of these influences have been demonstrated by the exposure of a plant host to microorganisms or chemical analogs that induce systemic acquired resistance (SAR) which can result in induced systemic resistance (ISR) and compromise field observations (Vallad and Goodman, 2004). These factors resulted in the limited use of pest control measures such as insecticides, exposing plants to stress from insect damage. Multiple sites and replication were used to limit these effects and to strengthen our data quality.

The advantage of using multiple sites and conditions was the identification of accessions such as PI 224713 which were resistant in two field sites but were found to be susceptible in the dew chamber. These observations are equally important as they

allow the identification of plants with weak or environmentally dependent resistance, which is often more durable (Brown, 2015). Genomic selection can be used in future to capture these loci with minor contributions by developing populations such as recombinant inbred lines (RILs) and near isogenic lines (NILs) which will facilitate easier trait discovery (Desta and Ortiz, 2014; Keurentjes et al., 2007). The identification of multiple forms of resistance allows a broad selection of resistance genes to be available for breeding. Resistance gene pyramiding has not always been experimentally effective (Mundt, 2014). The identification of multiple R-genes avails the option to test these pyramiding observations in lima bean while also comparing them to simultaneous R-gene use (Burdon et al., 2016; Mundt, 2014; REX Consortium, 2016). These discoveries from our diversity panel would later facilitate a more detailed understanding of resistance mechanisms in lima bean, allowing a suitable strategy to be employed when breeding for durable resistance such as that employed in wheat and barley for resistance to powdery mildew (Brown, 2015; Piffanelli et al., 2004; REX Consortium, 2016).

The resistant accessions were geographically dispersed along Central and South America, with the most number of resistant isolates coming from Guatemala and Mexico (Fig 30). Previously developed molecular markers for detecting resistance to race F of *P. phaseoli* identified four accessions, with all these also being identified as being resistant in the field and dew chamber disease screens. Sussex (Accession number 48 in our list) was however the exception of those accessions identified to be resistant using the new marker system. Sussex was partially susceptible in the field, with one replicate succumbing to infection in shoot tips but not on pods, flowers or

racemes. Sussex is historically known to be susceptible to race F, but based on marker data, previous dew chamber assays and field screens, it is hypothesized that the Sussex seed we have may be heterogeneous and segregating for resistance to race F of *P. phaseoli*.

The pod assay to determine resistance of the diversity panel to *P. capsici* was not successful. Coenocytic hyphae grew from inoculated plugs but the lack of sporangia did not allow us to positively identify the organism producing the hyphae using morphological methods. Furthermore, the presence of *P. phaseoli* which has similar hyphal structures made it difficult to determine the source of the mycelia in these mixed infections. Induced systemic resistance (ISR) could have been the main cause for lack of sporulation in these lab cultures as it is expected that this response would have been mounted after the field inoculations with *P. phaseoli* (Chester, 1933, Shoresh et al., 2010). This plant immune system is characterized by the mounting of various defense mechanisms such as the production of antimicrobial compounds (phytoalexins, siderophores, hydrogen cyanide, chitinases, etc) and physical defence mechanisms such as cell wall thickening (Wei et al., 1996). The effects of ISR could have resulted in *P. capsici* having reduced infectivity and failing to produce sporangia (Park et al., 2016). It will be important to continue searching for host resistance against pod blight as the pathogen has a broad host range and its inoculum persists in soil between growing seasons (French-Monar et al., 2007; Lamour et al., 2012).

We were unable to find any loci associated with resistance after correcting for multiple testing. The SNPs identified before correcting for multiple testing were on the

same loci as SNPs identified in DAPC. These matching loci were the same as those identified in a previous study that identified resistance loci to race F (Mhora et al., 2016). Identification of these loci will need to be conducted using experiments that are specific to detecting these causal genes (Keurentjes et al., 2007). The availability of a lima bean reference genome and crosses which have been made using some of these accessions we identified will improve our ability to identify these sources of resistance. It is known that resistance genes are frequently found clustered in linkage groups, an observation which has also been made in common bean (Crute and Pink, 1996). Resequencing of these loci coupled with a transcriptome study could well reveal the nature of downy mildew resistance as factors such as alternative splicing and recombination have sometimes been found to confound genomic data when it is solely used (Crute and Pink, 1996; Michelmore and Meyers, 1998). The ability of gaining diversity through recombination of these resistance loci has been proved, giving rise to the potential of increasing the diversity in these loci when crosses are made among the different accessions we have identified. The high rates of diversifying selection in the leucine rich repeat regions of R genes also makes it possible that the geographic spread of these accessions and the differential selective pressures in these regions may indeed be the cause of the diversity we suspect is in these accessions (Parniske et al., 1997).

The fixation indices between the different US landraces and the Costa Rican accessions with dual resistance shows the Costa Rican landraces to be equally distant from all the landrace groups and lima bean genepools (Fig 32F). The ability to make crosses between these groups has been proved by the University of Delaware's lima

bean breeding program, which is in the process of generating F_2 progeny to observe segregation patterns of resistance to downy mildew. The F_{ST} genome scans show how much diversity we potentially gain by making these crosses, with the most gains being made in crosses with the larger seeded US cultivars and landraces. The least gains in diversity would be made in narrower crosses (Fig 32C) where the average F_{ST} between the Andean and US3 population is 0.0914. The wider crosses between the Costa Rican landraces and the large seeded US accessions are yet to be made (the genetic distances between these groups is shown in Fig32D and 32E). These crosses are most important as there is a possibility of introgressing both race E and F resistance into the Fordhook cultivars which presently have no resistance to either of the new races of *P. phaseoli*.

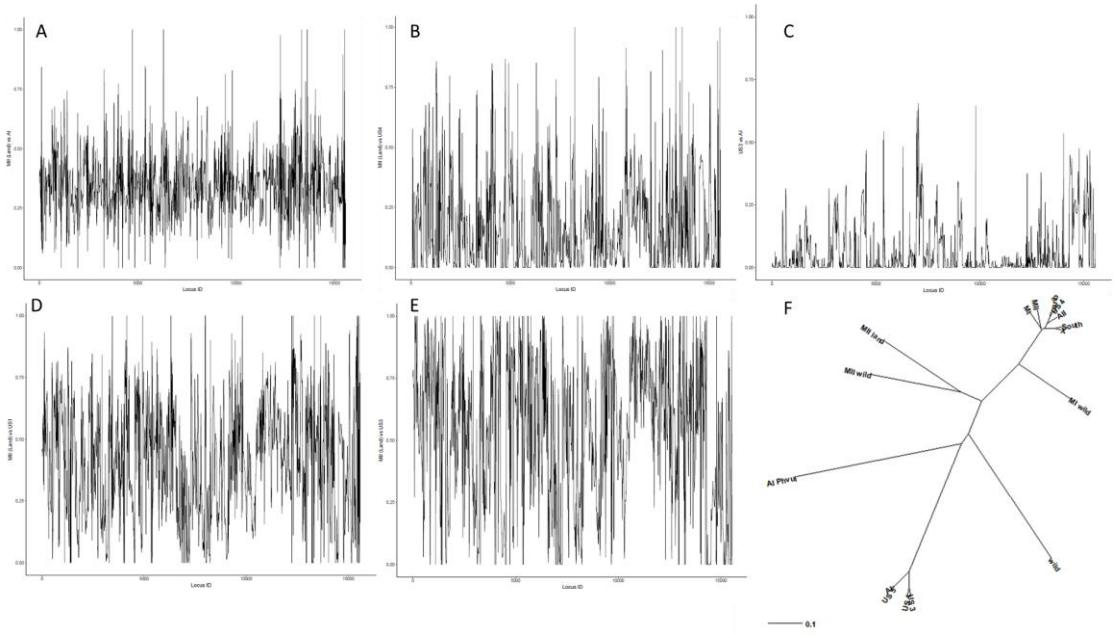

Figure 32 Genome scans of F_{ST} for the pairwise comparisons of (A) AI and the Costa Rican landraces (CRL); B US4 and CRL; (C) US1 and US3; (D) US1 and CRL; (E) US3 and CRL (F) the dendrogram shows the overall F_{ST} among all the genepools.

Overall, the discoveries made here provide the leverage necessary to utilize the new lima bean reference genome to begin breeding for lima bean with durable resistance while maintaining good agronomic traits. The potential discovery of more forms of resistance provides increased options in managing yield losses due to downy mildew. These discoveries present the breeding team with options that include pyramiding of resistance genes and/or breeding several cultivars bearing different R-genes. Both these strategies are proven for ensuring durable and effective plant disease management (REX Consortium, 2016). All these options provide additional layers to potential management strategies which can result in the expansion of Fordhook production, overall increase in crop yield and durability of disease resistance.

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Chapter 6

CONCLUSION AND FUTURE WORK

Conclusion

There is potential to increase lima bean profitability in the state of Delaware. The aim of this research was to increase profitability by managing plant disease related losses while reducing the use of chemical inputs. The ability to make timely and informed disease management decisions is critical as losses can be effectively avoided with minimal costs. The disease triangle is an important concept in plant pathology that encompasses knowledge of the host, the pathogen and the environment to understand plant disease. Understanding and targeting different areas of the disease triangle can lead to the achievement of these effective management strategies and its principles were demonstrated in this dissertation. The objectives that were accomplished overall aimed at describing the pathogen, delivering a pathogen monitoring system and providing the plant genetic resources that are necessary to accelerate and inform the plant breeding team when developing new cultivars.

The identification of mefenoxam insensitive isolates in the center of Delaware's lima bean production area was a major discovery in this study. It is a major concern as it threatens the most important control of downy mildew available to the industry. It is imperative to monitor and describe these pathogen populations, enabling targeted and effective management practices to be deployed. The GBS pilot

study demonstrated the ability of this new method to monitor the clonal structure of *P. phaseoli* while it also showed the potential of the approach to identify the race structure and fungicide resistance nature of the pathogen. This finding was important as it provides growers with the ability to employ informed disease management options such as changing the mode of action (MOA) of their fungicides, the cultivars they are growing or both. The absence of horticulturally acceptable cultivars that contain race F resistance is a major limitation, as this narrows the available options to growers. Cultivars such as M15 and 878 are resistant to race F, but are not agronomically attractive options, leaving them as a last resort. Resistance to races E and F were found in diverse accessions, providing the necessary resources for the breeding team to introgress these traits into elite cultivars.

Chemical control has been the major form of disease control in lima bean (Evans et al., 2007). Limiting the amount of fungicides use would be a financial advantage to growers while also reducing the carbon footprint of lima bean production. Nevertheless, fungicides provide an extra layer to enabling durable resistance (Rex Consortium, 2016). Reports of mefenoxam resistance in *Phytophthora phaseoli* however threatens use of the most effective and economically viable control method available. This necessitated a survey of local populations of the pathogen to ascertain their status with regards to mefenoxam insensitivity. It was found that isolates from Milton Delaware were insensitive or intermediately so. This puts the industry at risk as similar pathogens (*P. infestans*) can be disseminated up to 10 km within three hours (Aylor et al., 2001), enough distance to put most of the region at risk. This information is useful as surveillance will now be more targeted to confirm

this observation. Growers can also be advised to either leave their fields fallow for up to three years or alternatively plant the less acceptable race F resistant cultivars.

It is important to understand the pathogen structure affecting a cropping system as this allows crucial decisions to be made. The ability to identify the evolutionary potential of a pathogen allows adequate measures to be taken, avoiding breakdown of resistance genes or the over reliance of chemical control and its associated risks. The evolutionary potential of a plant pathogen is comprised of many factors which include their mode of reproduction, population size and dispersal mechanisms among others (McDonald and Linde, 2002). Sexual populations have been demonstrated to have a higher disease potential as recombination increases the potential effector combinations and subsequent risk of gaining new virulence genes (McDonald and Linde, 2002). The index of association (I_A) of both historic and more recent isolates from Delaware demonstrated that populations of *P. phaseoli* were from a single clonal lineage and that there were no new lineages or outcrossing. The high disease potential of *P. phaseoli* was however evident as observed by its rapid ability to overcome host resistance (Wester and Jorgenson, 1959; Wester, 1970a; Wester, 1970b). Similarity of *P. phaseoli*'s genome to that of *P. infestans* means that repeats and transposable elements in the effector containing region give it the potential to still recombine and adapt to host resistance, making it a high risk (McDonald and Linde, 2002; Raffaele et al., 2010). A population genetics approach could be used to identify a set of SNPs which could be developed into KASP markers, validated and used to test new isolates, providing growers with information on the race of the isolates in their fields and

allowing them to decide their cultivar choices. This potential was observed in the pilot study and a larger population size would confirm these findings.

The need for horticulturally acceptable race F resistant cultivars has already been emphasized, as these are an important tool in the management toolkit. The development of race F resistant cultivars can be improved by using marker assisted selection. We identified markers for a race F resistant locus, enabling us to provide an additional resource to accelerate the delivery of resistant cultivars (Mhora et al., 2016). A better understanding of available genetic resources in the form of landraces and wild forms from the centers of diversity and origin, together with our own local material will assist the breeding program to make more informative crosses and subsequently more rapid cultivar delivery. We characterized the diversity of a panel of accessions using both genotypic and phenotypic methods. The diversity panel was phenotyped for resistance to races E and F of *P. phaseoli* and to *P. capsici*, an emerging pathogen in the mid-Atlantic Region (MAR). There were 15 individuals found to be resistant to race F, six to race E and three to both races E and F. This was an important discovery as there are currently no cultivars that are resistant to both races (Santamaria et al., 2018). Crosses made using the three accessions bearing this dual resistance are at the F_1 stage and will be advanced to F_2 this coming fall (2018). The accessions bearing this dual resistance are from Costa Rica and are genetically distant from both the Andean and Mesoamerican genepools. These accessions seem to be hybrid Andean-Mesoamerican lima beans as they are equidistant (F_{ST}) from the major genepools and recent crosses already indicate that viable progeny are possible. This finding is important for the resuscitation of Fordhook production which was suffering from

disease susceptibility among other abiotic factors. The genotyping results also revealed an additional group of large seeded lima bean in the US, as the pole lima beans formed a separate group from the Fordhook type and the Andean accessions which were separated by their climatic adaptation. These findings demonstrate the potential of the available genetic resources and the ability to breed for adaptation by making informed wide crosses. These findings allow strategies for durable resistance to be bred into new lima bean cultivars. The identification of loci associated with downy mildew resistance and the potential discovery of multiple forms of resistance means that optimal strategies for utilizing these genes can be implemented. Overall, inroads to making maximum use of pathogen surveillance have been made. Growers can soon look forward to having information that can be acted upon appropriately using all possible resources needed.

Future Work

Surveillance of mefenoxam insensitivity will need to be continued, with a focus on the Milton grower fields. An understanding of the mechanism of resistance of these isolates to mefenoxam will be necessary as this will develop knowledge of resistance and provide a platform to develop markers for rapid detection of insensitive isolates. Mefenoxam insensitivity is known to be caused by a single amino acid change in the large subunit of RNA Polymerase I (RPA190) in *P. infestans* (Randall et al. 2014). Our proven ability to drive isolates to resistance in the lab will allow us to confirm if this gene is similarly affected in *P. phaseoli* and inform future experiments to gain a better understanding of the mechanism of insensitivity and develop

molecular markers for rapid detection of insensitive isolates in the field. Simultaneously, efforts will need to be undertaken to locally register additional fungicides with different MOA and add them to the Delaware extension factsheets (http://extension.udel.edu/factsheets/). An example of such a fungicide is ZORVEC™ ENICADE™ (oxathiopiproline), whose product label states that it can be used to control downy mildew of lima bean but is not registered for use in Delaware nor is it mentioned in the extension factsheets (https://www3.epa.gov).

The currently unpublished lima bean reference genome will assist acceleration of the lima bean breeding program and improve our understanding of the crop. We identified loci associated with resistance and bearing R-gene clusters. A better understanding of these loci is needed to understand the causal genes for downy mildew resistance. Resequencing these loci from the resistant and reciprocal susceptible accessions will allow a better understanding of the structure of these areas through locus comparisons. The crosses that have been made can also be advanced either to the F_2 stage or to RIL and NIL stages where loci contributing minor effects can be identified. The identification of these resistance loci can allow comparisons to be made between gene pyramiding and simultaneous use of resistance genes, allowing durable and effective resistance to downy mildew to be attained. Overall, the resources we have developed in this project position us to significantly improve lima bean production and disease management.

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Appendix A

SUPPLEMENTARY TABLES

Table 10 Identification, cultivar name, origin and assigned genepool for genotyped individuals of the diversity panel.

Accession	Common name	PI #	Ciat #	Material	Country	Gene Pool
DP_1	1102-10 (Beseba)	NA	G27529	landrace	Haiti	carib
DP_2	1102-6	NA	G27525	landrace	Haiti	carib
DP_3	184-85	NA		cultivar	US	US
DP_4	90-1	NA		breeding	US	US
DP_5	Bush Florida Butter	PI 549509		cultivar	US	US
DP_6	C-elite Select	NA		cultivar	US	US-W
DP_7	Concentrated Fordhook	PI 549479		cultivar	US	US-W
DP_8	Cypress	NA		cultivar	US	US
DP_9	DE0501102B	NA		breeding	US	US-E
DP_10	DE0504103A	NA		breeding	US	US-E
DP_11	Dixie Butterpea	PI 549462		cultivar	US	US-W
DP_12	Dover Tucker	PI 549456		cultivar	US	US-E
DP_13	FH 1072	PI 549519		breeding	US	US
DP_14	FH 242	PI 549464		cultivar	US	US-W
DP_15	Improved Kingston	NA		cultivar	US	US
DP_16	Jackson Wonder	PI 549467		cultivar	US	US-W
DP_17	Maffei 15	PI 549521		cultivar	US	US
DP_18	PA German Red Lima	NA		landrace	US	US-E
DP_19	Piloy	PI 189403	G25254	landrace	Guatemala	MII
DP_20	Chilipuca Negra Redonda	PI 256389	G25368	landrace	El Salvador	MII
DP_21	Chilipuca	PI 256405	G25383	landrace	El Salvador	MII
DP_22	Chilipuca	PI 256406	G25384	landrace	Salvador	MII
DP_23		PI 256838	G25433	landrace	Peru	Andean
DP_24		PI 256841	G25436	landrace	Peru	Andean

DP_25		PI 256842	G25437	landrace	Peru	Andean
DP_26		PI 256843 I	G25438	landrace	Peru	Andean
DP_27		PI 256843 II	G25438	landrace	Peru	Andean
DP_28		PI 256845	G25440	landrace	Peru	Andean
DP_29		PI 256846	G25441	landrace	Peru	Andean
DP_30		PI 256861	G25454	landrace	Peru	Andean
DP_31		PI 257377	G25517	landrace	Colombia	Andean
DP_32	PL-7	PI 260417		landrace	Bolivia	Andean
DP_33	Amarela	PI 299381	G25091	landrace	Brazil	South-America
DP_34	Pilgue	PI 310620	G25597	landrace	Guatemala	MII
DP_35	Hopi 13	PI 347779	G25624	landrace	US	US-W
DP_36	Hopi 15	PI 347781	G25626	landrace	US	US-W
DP_37	Hopi 155	PI 347784	G25629	landrace	US	US-W
DP_38	Hopi 2000	PI 347787	G25632	landrace	US	US-W
DP_39		PI 347792	G25637	landrace	US	US-W
DP_40	Willow Leaf, White	PI 347819	G25664	cultivar	US	US-W
DP_41		PI 347826	G25671	landrace	US	US-W
DP_42	BGH 277	PI 362772		landrace	Brazil	South-America
DP_43	BGH 277	PI 362772		landrace	Brazil	South-America
DP_44		PI 363030	G25106	landrace	Brazil	South-America
DP_45	Howur	PI 440807		cultivar	US	US-W
DP_46	Cave Dweller	PI 534918		landrace	US	US-W
DP_47	Sieva	PI 549469		cultivar	US	US-W
DP_48	Sussex	NA		breeding	US	US
DP_49	Thaxter	PI 549454		cultivar	US	US-E
DP_50	Virginia Butterbean	NA		landrace	US	US
DP_51		PI 256848	G25443	landrace	Peru	Andean
DP_52		PI 256906	G25493	landrace	Peru	Andean
DP_53	Hopi 50	PI 347782	G25627	landrace	US	US-W
DP_54	Manteca Ramas	PI 162688	G25235	landrace	Argentina	Andean
DP_55	Frijol Rojo	PI 256804	G25400	landrace	Colombia	Andean
DP_56		PI 256814	G25410	landrace	Ecuador	Andean
DP_57		PI 256820	G25415	landrace	Ecuador	Andean
DP_58	Westan	PI 347777	G25215	cultivar	US	US
DP_59	Hopi Red/ Pala Hatiqo	PI 476859		landrace	US	US-W
DP_60	Sprigg	PI 549516		cultivar	US	US-W
DP_61	Dover Bush	PI 549455		cultivar	US	US-E

DP_62	Bixby	PI 549460		cultivar	US	US-W
DP_63	Christmas Lima	PI 549461		cultivar	US	US-W
DP_64	Fordhook Bush det	PI 549465		cultivar	US	US-W
DP_65	Fordhook Bush ind	PI 549465		cultivar	US	US-W
DP_66	Henderson Bush	PI 549466		cultivar	US	US-W
DP_67	King of the Garden	PI 549468		cultivar	US	US-W
DP_68	Nemagreen	PI 549481		cultivar	US	US-W
DP_69	Florida Butter Speckled	PI 549487		cultivar	US	US
DP_70	Baby Potato	PI 549494		cultivar	US	US-E
DP_71	Large White	PI 549496		cultivar	US	US-E
DP_72	Maffei 76	PI 549512		cultivar	US	US
DP_73	Dompe 95	PI 549518		cultivar	US	US-W
DP_74	Benner Sussex	NA		landrace	US	US-E
DP_75	Big Momma	NA		landrace	US	US-E
DP_76	Coverdale	NA		landrace	US	US-E
DP_77	Dodd	NA		landrace	US	US-E
DP_78	Rohrer's Dr. Martin	NA		landrace	US	US
DP_79	DSU Big 6	NA		landrace	US	US
DP_80	Kuvilek	NA		landrace	US	US-E
DP_81	Jones	NA		landrace	US	US-E
DP_82	Moser	NA		landrace	US	US-E
DP_83	Pete's Dr. Martin	NA		landrace	US	US-E
DP_84	Susie	NA		landrace	US	US-E
DP_85	Zerbe	NA		landrace	US	US-E
DP_86	Lineberger - Warren	NA		landrace	US	US-E
DP_87	Lineberger - Layton	NA		landrace	US	US-E
DP_88	1102-13	NA	G27531	landrace	Puerto Rico	carib
DP_89	1102-14	NA		landrace	Puerto Rico	carib
DP_90	1102-17	NA	G27533	landrace	Dominican Republic	carib
DP_91	1102-18	NA	G27534	landrace	Dominican Republic	carib
DP_92	1102-19	NA	G27535	landrace	Dominican Republic	carib
DP_93	1102-20	NA	G27536	landrace	Dominican Republic	carib
DP_94	1102-22	NA	G27538	landrace	Dominican Republic	carib
DP_95	1102-23	NA	G27539	landrace	Dominican Republic	carib

DP_96	1102-24	NA	G27540	landrace	Dominican Republic	carib
DP_97	1102-25	NA	G27541	landrace	Dominican Republic	carib
DP_98	1102-4	NA	G27523	landrace	Haiti	carib
DP_99	1102-42	NA	G27558	landrace	Puerto Rico	carib
DP_100	1102-43	NA	G27559	landrace	Puerto Rico	carib
DP_101	1102-44	NA	G27560	landrace	Puerto Rico	carib
DP_102	1102-45	NA	G27561	landrace	Puerto Rico	carib
DP_103	1102-46A	NA	G27562	landrace	Puerto Rico	carib
DP_104	1102-5	NA	G27524	landrace	Haiti	carib
DP_105	1102-9	NA	G27528	landrace	Haiti	carib
DP_106	King Louie	NA		landrace	US	US-E
DP_107		PI 195339	G25256	landrace	Guatemala	MII
DP_108		PI 195342	G25259	landrace	Guatemala	MII
DP_109		PI 201478	G25291	landrace	Mexico	MI
DP_110		PI 249042	G32542	landrace	Nigeria	X
DP_111	1102-1	NA	G27519	landrace	Haiti	carib
DP_112	1102-11A	NA	G27530	landrace	Puerto Rico	carib
DP_113	1102-2	NA	G27520	landrace	Haiti	carib
DP_114	1102-21	NA	G27537	landrace	Dominican Republic	carib
DP_115	1102-26	NA	G27542	landrace	Dominican Republic	carib
DP_116	1102-31	NA	G27547	landrace	Dominican Republic	carib
DP_117	1102-3A	NA	G27521	landrace	Haiti	carib
DP_118	1102-41	NA	G27557	landrace	Puerto Rico	carib
DP_119	1102-48	NA	G27565	landrace	Puerto Rico	carib
DP_120	1102-49	NA	G27566	landrace	Puerto Rico	carib
DP_121	1102-7	NA	G27526	landrace	Haiti	carib
DP_122	1102-8	NA	G27527	landrace	Haiti	carib
DP_123	Lawr			landrace	US	US-E
DP_124	UC Beija Flor			cultivar	US	US
DP_125	Cariblanco Norte			cultivar	US	US

DP_126	UC 92			cultivar	US	US
DP_127	White Ventura	PI 549499		cultivar	US	US
DP_128	White Ventura 63	PI549502		cultivar	US	US
DP_129	White Ventura 65	PI549503		cultivar	US	US
DP_130		PI 347812	G25657	landrace	US	US-W
DP_131	Pat			cultivar	US	US
DP_132	Henderson			cultivar	US	US
DP_133	Lee			cultivar	US	US
DP_134	459-1			cultivar	US	US
DP_135	UC Haskell			cultivar	US	US
DP_136	UC Luna			cultivar	US	US
DP_137	Mezcla			cultivar	US	US
DP_138	Wilbur			cultivar	US	US
DP_139	Hopi 5989	PI 347786		landrace	US	US-W
DP_140	Worchester Indian Red Pole			landrace	US	US
DP_141	Violet's Multicolored Butterbean			landrace	US	US
DP_142	Alabama Blackeyed Butterbean			landrace	US	US
DP_143	Dixie Speckled Butterpea			landrace	US	US
DP_144	L 76	PI 347804	G25649	landrace	US	US-W
DP_145	L 136	PI 347829	G25674	landrace	US	US-W
DP_146		PI 399617	G26157	landrace	Brazil	South-America
DP_147		PI 363057	G26154	landrace	Brazil	South-America
DP_148		PI 363054	G26550	landrace	Brazil	South-America
DP_149		PI 362794	G25008	landrace	Brazil	South-America
DP_150		PI 363056	G25119	landrace	Brazil	South-America
DP_151		PI 347831	G25676	landrace	US	US-W
DP_152	Chilipuca Baya	PI 256383	C25362	landrace	El Salvador	MII
DP_153	Jaruma	PI 256387	G25366	landrace	El Salvador	MII
DP_154	Chilipuca	PI 256399	G25377	landrace	El Salvador	MII
DP_155	Chilipuca	PI 256403	G25381	landrace	El Salvador	MII
DP_156	Bins	PI 164155	G25237	landrace	India	X

DP_157	Val	PI 180324	G25241	landrace	India	X
DP_158	Bal	PI 180461	G25242	landrace	India	X
DP_159		PI 183343	G25181	landrace	India	X
DP_160		PI 188696	G25250	landrace	Nigeria	X
DP_161		PI 347834	G25679	breeding	US	US-W
DP_162		PI 197025	G25267	landrace	El Salvador	MII
DP_163		PI 200915	G25274	landrace	El Salvador	MII
DP_164		PI 200919	G25278	landrace	El Salvador	MII
DP_165		PI 200923	G25281	landrace	Salvador	MII
DP_166		PI 200924	G25282	landrace	Guatemala	MII
DP_167		PI 201287	G25288	landrace	Mexico	MI
DP_168		PI 202830	G25293	landrace	Mexico	MI
DP_169	Seven-year Bean	PI 221202	G25301	landrace	Zambia	X
DP_170	Pacle	PI 224713	G25303	landrace	Mexico	MI
DP_171		PI 249022	G25322	landrace	Nigeria	X
DP_172		PI 249034	G32534	landrace	Nigeria	X
DP_173		PI 256417	G25393	landrace	Costa Rica	MII
DP_174		PI 256419	G25395	landrace	Costa Rica	MII
DP_175		PI 256420		landrace	Costa Rica	MII
DP_176		PI 256811	G25407	landrace	Colombia	Andean
DP_177	Frijol Tierno	PI 256816	G25412	landrace	Ecuador	Andean
DP_178		PI 256819	G25414	landrace	Ecuador	Andean
DP_179		PI 256866	G25459	landrace	Peru	Andean
DP_180		PI 256869	G25462	landrace	Peru	Andean
DP_181		PI 256882		landrace	Peru	Andean
DP_182		PI 256885	G25477	landrace	Peru	Andean
DP_183		PI 256913	G25498	landrace	Peru	Andean
DP_184		PI 257356	G25505	landrace	Colombia	Andean
DP_185		PI 257360	G25509	landrace	Colombia	Andean
DP_186		PI 257363	G25512	landrace	Colombia	Andean
DP_187		PI 257365	G25514	landrace	Colombia	Andean
DP_188		PI 257381	G25521	landrace	Colombia	Andean
DP_189		PI 257384	G25523	landrace	Colombia	Andean
DP_190		PI 347836	G25681	landrace	US	US-W
DP_191	Mariguita	PI 257409	G25539	landrace	Costa Rica	MII
DP_192		PI 257548	G25551	landrace	Mexico	MI
DP_193		PI 257560	G25563	landrace	Mexico	MI

DP_194		PI 347839	G25684	landrace	US	US-W
DP_195		PI 260408	G25573	landrace	Peru	Andean
DP_196	Arvita	PI 260411	G25576	landrace	Peru	Andean
DP_197		PI 260413	G25577	landrace	Peru	Andean
DP_198		PI 260414	G25578	landrace	Bolivia	Andean
DP_199		PI 260416	G25580	landrace	Bolivia	Andean
DP_200	Fordhook 861 Charter	PI 549457		cultivar	US	US
DP_201	Purple Spray	PI 249040	G25340	landrace	Nigeria	X
DP_202	Ixtapacal	PI 310624	G25601	landrace	Guatemala	MII
DP_203	Chaparota	PI 310627	G25604	landrace	Guatemala	MII
DP_204	Frijol Curona	PI 311795	G25606	landrace	Guatemala	MII
DP_205	Pois du cap	PI 661750		landrace	France	X
DP_206	Hopi 14	PI 347780	G25625	landrace	US	US-W
DP_207	Hopi 5986	PI 347785	G25630	landrace	US	US-W
DP_208		PI 347790	G25635	landrace	US	US-W
DP_209	Sierra	PI 347798	G25643	cultivar	US	US-W
DP_210		PI 347799	G25644	landrace	US	US-W
DP_211		PI 347801	G25646	landrace	US	US-W
DP_212		PI 347807	G25652	landrace	US	US-W
DP_213		PI 347811	G25656	landrace	US	US-W
DP_214		PI 347813	G25658	landrace	US	US
DP_215		PI 347816	G25661	landrace	US	US
DP_216		PI 347818	G25663	landrace	US	US-W
DP_217		PI 347824	G25669	landrace	US	US-W
DP_218		PI 347842	G25687	landrace	US	US-W
DP_219	Torta pallara	PI 355837	G25690	landrace	Ecuador	Andean
DP_220	Torta pallara	PI 355839		wild	Ecuador	Andean
DP_221	Haba pallara	PI 355841	G25691	landrace	Ecuador	Andean
DP_222	Climbing Speckled	PI 638826		cultivar	US	US-E
DP_223		PI 427216		landrace	US	US-E
DP_224		PI 427217		landrace	US	US-E
DP_225		PI 427218		landrace	US	US-E
DP_226		PI 433928		landrace	Mexico	MI
DP_227	Bacalar claro	PI 438911		landrace	Mexico	MI
DP_228	Natiquo	PI 451715		landrace	US	US-W
DP_229	L 59	PI 451776		landrace	US	US-W
DP_230	L 79	PI 451777		landrace	US	US-W
DP_231	L 120A	PI 451780		landrace	US	US-W
DP_232	L 122A	PI 451782		landrace	US	US-W

DP_233		PI 451925		wild	Guatemala	MII
DP_234		PI 477041		landrace	US	US-W
DP_235		PI 502182		breeding	US	US-W
DP_236		PI 502183		breeding	US	US-W
DP_237	Cowey	PI 534913		landrace	US	US-W
DP_238	Ganymede	PI 534914		landrace	US	US-W
DP_239	Half & Half	PI 534915		landrace	US	US-W
DP_240	Red Calico	PI 534917		landrace	US	US
DP_241	NI 675	PI 535341		wild	Mexico	MI
DP_242	DGD 453	PI 535343	G25704	wild	Mexico	MI
DP_243	Triumph	PI 549473		cultivar	US	US-W
DP_244	Willow Leaf	PI 549474		cultivar	US	US-W
DP_245	Wasatch	PI 549482		cultivar	US	US-W
DP_246	Easy Shell	PI 549484		cultivar	US	US-W
DP_247	Buttergreen	PI 549485		cultivar	US	US-W
DP_248	Giant Florida Pole	PI 549493		cultivar	US	US-W
DP_249	Carpinteria	PI 549495		cultivar	US	US-E
DP_250	Giant Christmas	PI 549497		cultivar	US	US-E
DP_251	Mackie	PI 549507		cultivar	US	US-W
DP_252	F - 169	PI 549514		breeding	US	US
DP_253	Brown Crower Pole 118	PI 550301		cultivar	US	US
DP_254		PI 583558		landrace	China	X
DP_255	Black and Buff Bush	PI 347822		cultivar	US	US
DP_256	1102-6	NA	G27525	landrace	Haiti	carib
DP_257	878	NA	NA	cultivar	US	US
DP_258	185-85	NA	NA	cultivar	US	US
DP_259	bridgeton	NA	NA	cultivar	US	US
DP_260	c elite	NA	NA	cultivar	US	US
DP_261	M15	NA	NA	cultivar	US	US
DP_262	cypress	NA	NA	cultivar	US	US
DP_263	Jackson Wonder	NA	NA	cultivar	US	Andean
DP_264	G19833	NA	NA	landrace	Peru	US

Table 11 The overall features of all the accessions used in the field, dew chamber and greenhouse screens

Accession	Country	Type	Race F	Race E	Races E and F
Dp_100	Puerto Rico	Landrace	S	S	S
Dp_108	Guatemala	Landrace	R	S	S
Dp_117	Haiti	Landrace	S	S	S
Dp_12	US	Cultivar	S	S	S
Dp_145	US	Landrace	S	S	S
Dp_147	Brazil	Landrace	S	S	S
Dp_148	Brazil	Landrace	S	S	S
Dp_157	India	Landrace	S	S	S
Dp_164	El Salvador	Landrace	R	S	S
Dp_166	Guatemala	Landrace	R	S	S
Dp_167	Mexico	Landrace	R	S	S
Dp_168	Mexico	Landrace	S	S	S
Dp_17	US	Cultivar	R	S	S
DP_170	Mexico	LANDRACE	S	R	S
Dp_173	Costa Rica	Landrace	R	R	R
Dp_174	Costa Rica	Landrace	R	R	R
Dp_175	Costa Rica	Landrace	R	R	R
Dp_177	Ecuador	Landrace	R	S	S
Dp_19	Guatemala	Landrace	S	S	S
Dp_192	Mexico	Landrace	R	S	S
Dp_197	Peru	Landrace	S	S	S
Dp_20	El Salvador	Wild	R	S	S
Dp_203	Guatemala		R	S	S
Dp_21	El Salvador	Landrace	S	S	S
DP_210	US		S	S	S
Dp_218	US		S	S	S
Dp_219	Ecuador		R	S	S
Dp_220	Ecuador		S	R	S
Dp_226	Mexico		R	S	S
Dp_233	Guatemala	Wild	S	R	S
Dp_234	US		S	S	S
Dp_242	Mexico	Wild	S	S	S
Dp_252	US		S	S	S
Dp_253	US		S	S	S
Dp_257	US	Cultivar	R	S	S
Dp_258	US	Cultivar	S	R	S

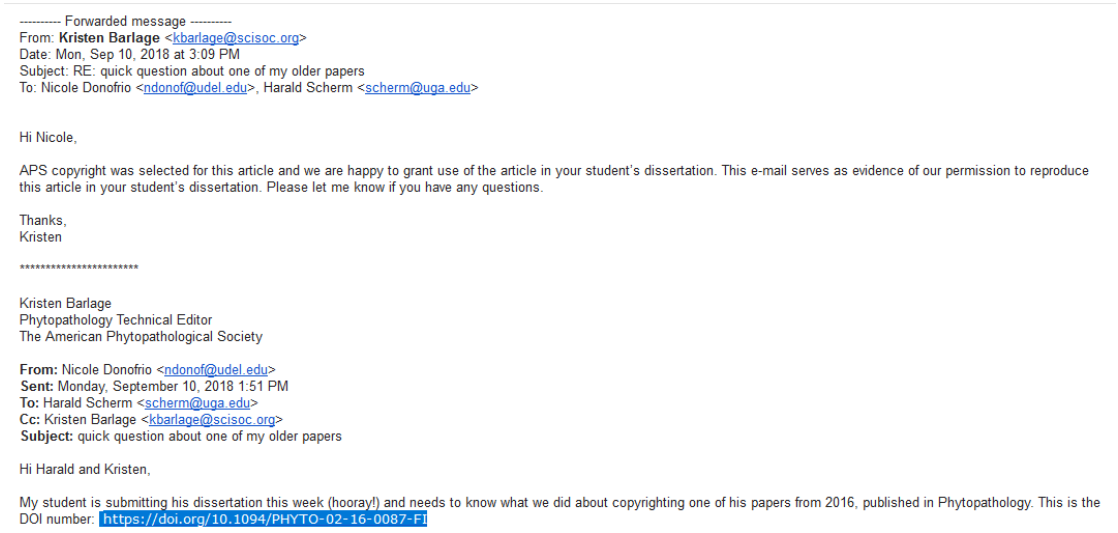
Accession	Country	Type	Race F	Race E	Races E and F
Dp_259	US		S	R	S
Dp_260	US	Cultivar	S	R	S
Dp_261	US	Cultivar	R	S	S
Dp_36	US	Landrace	S	S	S
Dp_41	US	Landrace	S	S	S
Dp_45	US	Cultivar	S	S	S
Dp_49	US	Cultivar	S	S	S
Dp_54	Argentina	Landrace	S	S	S
Dp_57	Ecuador	Landrace	S	S	S
Dp_61	US	Cultivar	S	S	S
Dp_69	US	Cultivar	S	S	S
Dp_72	US	Cultivar	R	S	S
Dp_8	US	Cultivar	S	R	S

Appendix B

PERMISSIONS

The image of the email conversation with the editor of Phytopathology giving rights to use chapter three of this dissertation is below. The reference for this chapter is:

Mhora, T. T., Ernest, E. G., Wisser, R. J., Evans, T. A., Patzoldt, M. E., Gregory, N. F., Westhafer, S. E., Polson, S. W. and Donofrio, N. M. 2016. Genotyping-by-Sequencing to Predict Resistance to Lima Bean Downy Mildew in a Diversity Panel. Phytopathology, 106 (10): 1152-1158. https://doi.org/10.1094/PHYTO-02-16-0087-FI



---------- Forwarded message ----------
From: **Kristen Barlage** <kbarlage@scisoc.org>
Date: Mon, Sep 10, 2018 at 3:09 PM
Subject: RE: quick question about one of my older papers
To: Nicole Donofrio <ndonof@udel.edu>, Harald Scherm <scherm@uga.edu>

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Thanks,
Kristen

****

Kristen Barlage
Phytopathology Technical Editor
The American Phytopathological Society

From: Nicole Donofrio <ndonof@udel.edu>
Sent: Monday, September 10, 2018 1:51 PM
To: Harald Scherm <scherm@uga.edu>
Cc: Kristen Barlage <kbarlage@scisoc.org>
Subject: quick question about one of my older papers

Hi Harald and Kristen,

My student is submitting his dissertation this week (hooray!) and needs to know what we did about copyrighting one of his papers from 2016, published in Phytopathology. This is the DOI number: https://doi.org/10.1094/PHYTO-02-16-0087-FI