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Characterization of Hessian fly from Israel

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For those who need reminded to “Always keep fighting”

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ABSTRACT

Johnson, Alisha J. Ph.D., Purdue University, May 2015. Characterization of Hessian fly from Israel. Major Professor: Richard H. Shukle.

Mayetiola destructor Say, the Hessian fly, is a gall midge and a member of the Dipteran family Cecidomyiidae. It is a common pest of wheat found throughout all of the major wheat growing areas of the world and poses a serious economic threat to the United States (US), particularly in the Southeast winter wheat region. Damage to wheat is done solely by feeding first and second in-star larvae. Hessian fly (Hf) infestations result in a loss in grain yield by the stunting and/or killing of seedling wheat plants in the winter and by causing breakage at the nodes of the plant in the spring. Feeding begins as the larvae settles at the base of the plant and establishes a feeding site by creating a layer nutritive tissue. Control of Hf in the US is primarily performed through avoidance by planting after the bulk emergence of the fly and through planting resistant wheat cultivars which contain a Hf-specific *R* gene.

In Israel, Hf is found throughout the primary agricultural region but is not considered an economic threat. No cultural practices are used to control the insect, and Hf resistant wheat cultivars are not deployed in commercial agriculture. Native grasses and wild wheat progenitors that can serve as alternative hosts are readily available in non-cultivated areas. The sampling Hf in Israel will provide information from a

Mediterranean population which is as close to Fertile Crescent, the center of origin to both Hf and the domestication of wheat, as can be currently sampled. This will allow the examination of population structure in Israel, of differential expression of effector proteins, of virulence to Hf *R* genes when resistant wheat cultivars are not deployed, and of the genetic inheritance of avirulence genes (*Avr*) in virulent Hf. This will allow advancement in the understanding of the Hf-wheat interaction that can be used to create more effective and long-lasting control of Hf in US.

Samples of a dipteran pest of wheat from multiple locations in the agricultural area of Israel were tested to confirm identity, describe local populations and suggest the use of deploying resistance (*R*) genes in wheat cultivars for control of Hf. Morphological evaluation of adults and a free-choice oviposition preference test documenting that females overwhelmingly preferred to oviposit on wheat instead of barley supported the identification of the Israeli samples as Hf. Using the cytochrome c oxidase subunit I (*coxI*), the Barcoding Region, nine haplotypes were revealed. These results supported the identification of Hf as all nine haplotypes fell within a single clade that was significantly separated from other gall midge species including M*ayetiola hordei*. A greenhouse culture was established for one of the sampling locations, Magen, and it was evaluated for virulence to 19 different *R* genes. .Magen was significantly virulent to 11 of the 19 *R* genes tested, and complementation analysis documented that, for four of the *R* genes tested, the Israeli Hf shared loci for virulence with Hf from the US. Levels of Hf infestation at seven Israeli fields were at least at the 5–8% level, which historically has indicated a significant yield loss. Microsatellite genotyping of the five Hf collections

from Israel revealed two mixed populations in Israel that are distinctly separate from the single population in Syria.

Evidence is emerging that some proteins secreted by gall forming plant-parasites act as effectors responsible for systemic changes in the host plant, such as galling and nutrient tissue formation. A large number of secreted salivary gland proteins (SSGPs), the putative effectors responsible for the physiological changes elicited in susceptible seedling wheat by Hf larvae, have been documented. However, how the genes encoding these candidate effectors might respond under field conditions is unknown. Microarray analysis was performed to investigate variation in SSGP transcript abundance among field collections from different geographic regions (southeastern US, central US, and the Mediterranean). Results revealed significant variation in SSGP transcript abundance among the field collections studied. The field collections separated into three distinct groups that corresponded to the wheat classes grown in the different geographic regions as well as to recently described Hessian fly populations. These data support previous reports correlating Hessian fly population structure with micropopulation differences due to agro-ecosystem parameters such as cultivation of regionally adapted wheat varieties, deployment of resistance genes, and variation in climatic conditions.

Hf larvae produce a large number of secreted salivary effector proteins involved in effector triggered immunity that elicit systemic changes in susceptible wheat as well as trigger the defense response in resistant wheat. One of the avirulence effectors responsible for the interaction between Hf larvae and resistance gene *H13* in wheat has recently been cloned and characterized using Hf populations from the US. Within the US, virulence is a sex-linked, recessive trait and was shown to be associated with three

independent insertions that resulted in a loss of expression of the avirulence gene. Genetic crosses testing for the inheritance of virulence to *H13* in Hf from Israel revealed that it is controlled by a sex-linked, recessive trait at a single loci. Additionally, no complementation occurred between crosses of virulent US and virulent Israeli Hf, supporting the hypothesis that virulence resides at the same locus in both populations. However, no insertions were identified in the coding region nor upstream or downstream of the coding region. Further, no single nucleotide polymorphisms or frame shifts corresponding to virulence were identified. These data suggest the molecular basis of virulence in the Israeli population to resistance gene *H13* in wheat is not the same as in the US.

As the most effective form of Hf control employs the planting of resistant wheat cultivars containing one or more *H* genes, frequent Hf sampling is required to monitor the level of virulence present in locally adapted populations. A novel assay for detecting virulence in the field was created by sampling Hf males using sticky traps baited with Hf sex pheromone and the molecular marker for virulence to *H13*. The Hf gene that controls virulence in Hf to resistance gene *H13* in wheat has recently been cloned and characterized, and diagnostic molecular markers for the alleles controlling avirulence and virulence are now available. Utilizing two separate PCR reactions, the six alleles for avirulence and virulence can be scored based on band size on a 2% agarose gel. The results support the most recent survey of virulence to *H13* as scored through the testing of live insects infesting *H13* wheat in the greenhouse. Throughout the southeast, all three avirulence alleles can be identified while the most frequently identified allele for virulence corresponded to a 5kb insertion into exon 1 of *vH13*. In South Carolina, the

PCR assay is sensitive enough to detect the spread of virulence into two counties previously documented as 100% susceptible to *H13*.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Introduction

Mayetiola destructor (Say), is an invasive pest of wheat (*Triticum aestivum* L.) in North America. Colloquially known as the Hessian fly (Hf), it can be found throughout most wheat growing areas of the world, barring Japan and Australia (Yokoyama, 2011; Botha *et al.*, 2005). The term Hf is a derogative coined during Colonial America (Pauley, 2002). In the late 1700s, Hf established residence in Long Island, New York decimating fields that had once been home to Hessian mercenaries during the Revolutionary War. Hf rapidly spread throughout New England, and the early American farmers chose the name for its' negative connotation. Today, Hf has become the most common insect pest of wheat in North America causing millions of dollars in damage through reduced grain yield, particularly in the eastern soft-winter-wheat region (Buntin, 1999; Ratcliffe *et al.*, 2000).

Hf is a gall midge and a member of the Cecidomyiidae, the sixth largest family of Dipterans (Ratcliffe and Hatchett, 1997). Adults emerge as small, black midges that can survive three to four days (Harris *et al*., 2003). Females mate to a single male fly soon after emergence and begin laying eggs on the upper surface of the wheat leaf within an hour of mating (Harris and Rose, 1991). Within three to five days, depending upon temperature, the eggs hatch. The newly emerged larvae crawl down the leaf blade to

enter the whorl of the plant. In fall infestations, larvae establish a feeding site near the crown of seedling wheat plants while in spring infestations Hf larvae lodge at the nodes during culm elongation (Buntin, 1999). There are two feeding in-star stages with a molt at day six that precedes the second in-star (Stuart *et al.*, 2012). At day 12, the gut begins to shrivel and turns a dark green color as feeding ceases. The cuticle of the third in-star larvae hardens and turns a dark brown color at day 21. Due to its resemblance in shape and color to a seed from the flax plant, the larvae is referred to as a flaxseed. At this stage, Hf can delay their development and enter diapause. This diapause can last up to two years, but usually, lasts only until spring or the following fall (Wellso, 1991). If allowed to continue through development, adults will emerge around day 30. These small insects (3mm) are weak fliers and are not found higher than a few inches above the wheat canopy (Anderson *et al.*, 2012; Withers *et al.*, 1997). Thus, Hf will not spread rapidly without human intervention. This comes via the transportation of flaxseed which accomplishes dispersal at much greater distances than the Hf can move unaided (Morton *et al.*, 2011). Generally, in the United States (US), 1-2 generation of Hf occur; however, 6-8 generations can occur in the warmer, wetter Southeast (Buntin and Chapin, 1990).

Unlike other Cecidomyids, Hf does not create a typical gall. There is no enlarged swelling, but a layer of nutritive tissue forms at the feeding site. This tissue layer is composed of cells that provide a sink for the redirection of the plant's resources to directly feed the developing larva (Rohfritsch, 1987). In the compatible interaction, larvae are able to survive on and stunt the susceptible plant. A newly hatched larva settles near the base of the plant between leaf blades. Small mandibular stylets puncture the abaxial surface of the leaf tissue, salivary gland effectors are injected into the plant, and

the cell wall ruptures within hours (Harris *et al.*, 2006; 2010). After two days of larval attack, cells adjacent to the puncture site show increased vacuolation, disruption to the nucleus, and cytoplasmic degradation (Harris *et al.*, 2006). As feeding progresses, a layer of nutritive cells forms around the larva creating a depression in the leaf as additional epidermal cells are transformed into nutritive cells (Harris *et al*., 2006). The nuclear and cytoplasmic contents of these cells will begin to leak out of through the ruptured cell walls making nutrients available for the larva to ingest. The virulent larva will rapidly increase in size taking on a translucent color with a green-tinged gut and multiple white fat bodies parallel to the gut**.**

In the incompatible interaction, the plant is able to defend itself from Hf attack. An avirulent larva creates multiple puncture sites along the abaxial surface of the leaf blade but cannot establish a permanent feeding site. The larva will writhe between the leaf surfaces and become disoriented from the epidermal groove (Subramanyam *et al.*, 2006). Near puncture sites, cells walls thicken and Golgi-ER production increases (Harris *et al.*, 2012). Unable to induce the changes in the wheat epidermal cells required to produce nutritive cells, avirulent larvae will shrivel and die within five to six days leaving the plant to resume its normal growth and development. Due to their red-colored appearance, dead avirulent larvae are termed “dead reds”.

Hf’s damage to wheat is due solely to larval feeding. This damage is irreversible after four days (Byers and Gallun, 1972). During fall infestations, damage to seedlings is exhibited by stunted, dark blue-green plants, and heavy infestations can kill young plants. Larval infestation in the spring prevents normal elongation of internodes and transportation of nutrients to developing grain as well as weakening of the stem from

lodging at the nodes (Buntin, 1999). Three methods of control are commonly practiced: avoidance, destruction of infested plants, and resistant wheat cultivars. As irreversible damage to wheat occurs within a short window of time, systemic pesticide treatments are not applicable due to the cost of continuous applications during the months of Hf emergence. Seed treatments of pesticides have been somewhat effective in some regions of the US that only experience 1-2 generations of Hf per year (Reisig *et al.*, 2013).

The simplest method of control, avoidance, uses the principle of planting after the "fly-free date". This date uses historical information from past weather patterns to predict when the fly will emerge such that seedling plants will not be available for oviposition. Without the preferred host present in abundance, Hf populations will be greatly reduced in size the following season. The removal and destruction of volunteer wheat and infested straw helps prevent carryover from one year to the next. The use of resistant wheat cultivars to provide genetic protection of wheat from the fly is by far the most commonly practiced control strategy (Buntin and Chapin, 1990). This practice dates back to 1782 when "Underhill" wheat, a yellow bearded cultivar stolen from a British ship, was found to survive Hf attack and subsequently replaced all previous susceptible cultivars in New England (Gallun, 1977; Pauly, 2002).

Recently, Hf's relationship with wheat has been reexamined using the Effector Triggered Immunity (ETI) model (Stuart *et al.*, 2012; Harris *et al.*, 2015). The model expands on the gene-for-gene model used to describe plant-insect interactions by including the insect's response to overcoming the host plant's resistance adaptations. Effectors are small molecules or proteins that are injected or secreted into the plant to alter the host tissue's structure and function (Hogenhout *et al.*, 2009). In response to these

effectors, the plants evolved resistance (*R*) genes that encode proteins that recognize the invader and stimulate the defense responses required for resistance (Chisholm *et al.,* 2006). In turn, insects react to this immune response by altering avirulence (*Avr*) proteins to avoid detection or by evolving new effector proteins to accomplish the same action without triggering the plant's immune response (Dangl *et al.*, 2013; Ashfield *et al.*, 2014). It is well documented that Hf has a gene-for-gene relationship with wheat, and the inheritance of virulence to several *H* genes, the Hf resistance genes in wheat, has been documented. (Hatchett and Gallun, 1970; Gallun, 1977; Formousoh *et al.*, 1996; Zantoko and Shukle, 1997). However, the Hf effectors responsible for nutritive tissue formation and for the adaptations responsible for virulence are still being identified.

Many fluid feeding insects secrete salivary substances into host plants and Hf has adapted special mouthpart structures for injection into wheat tissues, several studies have focused on identifying putative effectors from Hf salivary secretions (Miles, 1999; Hatchett *et al.*, 1990). Hundreds of secreted salivary gland proteins (SSGPs) were detected in cDNA libraries and through whole genome sequencing. All SSGPs share three features: primary expression in salivary gland tissues during the first in-star stage, an N-terminal secretion signal, and small size (<10kDA) (Chen *et al.*, 2004). Many members of the same family have also been found to reside in clusters in the same region on a chromosome and that these regions are near mapped *Avr* genes (Chen et al., 2006). Analysis of the salivary gland transcriptome revealed that most transcripts were for protein synthesis and house-keeping functions and that these proteins did not accumulate in salivary glands indicating secretion outside of the insect shortly after synthesis (Chen *et al.*, 2008). SSGPs were also found to occur in tandem arrays where many regions

including promoters, secretion signals, 5' and 3' untranslated regions, and introns were more highly conserved than the protein coding regions (Chen *et al.*, 2010). In total, this evidence suggests that SSGPs are the putative effectors responsible for the Hf's ETI response. The unusual conservation in non-coding regions coupled with the diversity found within the coding region of related SSGPs indicates that strong positive selection is occurring, and positive selection has been described as the driving force in plant defense responses and pathogen effector genes (Michelmore and Meyers, 1998; Bishop *et al.*, 2000).

Sequencing of the Hf genome detected that a significant portion of the genome (7%) is comprised of SSGPs (Zhao *et al.*, 2015). These genes have little to no homology in other insect genomes. While the majority of SSGPs occur within tandem repeat arrays, many were also unique and dispersed outside of the short chromosomal regions where the arrays occur. With such a large portion of the genome devoted to SSGPs, this gives additional evidence that these genes serve as putative effectors.

One family of SSGPs was also further characterized in Zhao *et al.* (2015). SSGP-71 is the largest family of effectors identified in arthropods. Most of the members of this family encode a secretion signal, a cyclin-like F box domain, and a series of leucine-rich repeats as well as share homology to E3-ligases found in plants. Virulence to *H6* was been mapped to a scaffold which only contains genes from the SSGP-71 family, and loss of expression in virulent larvae has identified *Mdes009086-RA* as the candidate *Avr* gene. Likewise, mapping of virulence to *H9* identified a null allele of another member of SSGP-71 (*Mdes015365-RA*) corresponding to virulence to *H9*.

The first Hf *Avr* gene to be cloned and characterized is *vH13* (Aggarwal *et al.*, 2014). Virulence to *H13* is a sex-linked, recessive trait (Zantoko and Shukle, 1997). It resides between markers 124 and 134 on chromosome X2 (Rider *et al.*, 2002; Lobo *et al.*, 2006). *vH13* is an SSGP; it is a small protein (116 amino acids) with an N-terminus secretion signal that is interrupted by an intron. *vH13* does not share homology to any other genes in the NCBI database nor in the Hf genome. As Hf males are hemizygous for the X chromosomes, a single copy of a mutant allele inherited from the mother is all that is required to obtain virulence (Harris *et al.*, 2015). This has allowed for the identification of multiple *vH13* alleles from several southeastern US Hf field populations. Three alleles have been described for the incompatible reaction with *H13*, the avirulent phenotype. They vary in sequence only in copy number of an imperfect repeat, 12 amino acids in the second exon that can be repeated one to three times. Three alleles corresponding to the virulent phenotype have also been identified. All are insertions that lead to a loss in function of *vH13*: 1) a 4.7kb inserted at the end of exon 1, 2) a 254bp insertion at the intron-exon boundary, and 3) a 461bp insertion within exon 2. While three *Avr* genes have been identified, there are still many more that remain elusive as 35 *H* genes have been described in wheat (Liu et al., 2005; Li et al., 2013).

To convey resistance to Hf in the US, *H* genes have been incorporated into most commercial cultivars. Combined with planting after the fly-free date, the majority of the US can avoid significant yield loss to Hf. However, the repeated deployment of resistant cultivars has created a selection pressure in the field that has led to the creation of locally adapted populations which are virulent to one or more *H* genes (Lidell and Schuster, 1990; Smiley *et al.*, 2004; Watson, 2005; Chen *et al.*, 2009a; Cambron *et al.*, 2010). In

the southeast, multiple generations of Hf occur within a single growing season, and successive deployment of multiple *H* genes has become routine (Buntin and Chapin, 1990; Buntin *et al.*, 1992). *H* genes have a 6-8 year window of effectiveness in the field, and resistance to all four of the commonly deployed *H* genes is common (Gould, 1986; Buntin *et al.*, 1992; Ratcliffe *et al.*, 2000; Cambron *et al.*, 2010). Therefore, it is necessary to continue learning the biology of Hf effectors and how wheat responds to Hf attack.

Exposure to *H* genes can also happen naturally outside of commercial agriculture. Few of the *H* genes arose originally in common wheat (*T. aestivum*). When given the choice, Hf selects wheat to other grass species (Gagné *et al.*, 1991; Chen *et al.*, 2009b). However, Hf can survive on 17 genera of host plants within Triticeae and have been found to live on these alternative hosts when wheat is not readily available (Jones, 1938, 1939; Zeiss *et al.*, 1993; Harris *et al.*, 2001, 2003). The use of Hf sex pheromone baited traps have also identified Hf living inside conservation areas far from commercial wheat fields with only native wild grasses available (Anderson *et al.*, 2012). As many *H* genes were obtained in the genome from hybridizations with other grasses species like rye (*Secale cereale* L.), barley *Hordeum vulgare* L.), emmer (*Triticum dicoccum*), durum (*Tritcum durum*), and goat grass (*Aegilops tauschii*), screening of these grasses can identify new sources of Hf resistance (Liu *et al.*, 2005). However, the use of the *H* gene commercially can be immaterial when populations have developed virulence from exposure when the field population survived on the alternative host from which the *H* gene was adapted.

In Israel, the Negev region is a semi-arid desert where vegetables, grains, and fruits are grown commercially. Israel can produce 100-200,000 million tons (MT) of wheat per year; however, more is require to meet the demand of human and animal consumption (Shachar 2010, 2011). New ideas to increase wheat yield have renewed interest in Hf in Israel, a common pest of wheat in Syria and North Africa (Naber *et al.*, 2003). Hf was first identified in northern Israel in the winter of 1938 where heavy infestations devastated wheat production (Duvdevany, 1939). Today, Hf is frequently found in the agricultural areas of the northern Negev and the southern Coastal Plain where wheat is grown commercially (Rivnay, 1962; Avidov and Harpaz, 1969).

Population genetics studies of Hf have revealed that Hf in Israel is much different genetically than Hf from the US. Of the seven *12S* haplotypes of Hf found throughout the world, only a single haplotype was identified in Israel, and this haplotype was not found in Syria, Kazakhstan, Morocco, Spain, New Zealand, or the US (Johnson *et al.*, 2004). Analysis of a nuclear marker, intron 1 from the ortholog of a *Drosophila white* gene, found that two Hf populations from Israel were significantly divergent from all other regions under study including Syria and the US; however, some gene flow occurred between Syria and Israel as *wint1* alleles were shared in common and a population reconstruction incorporating both mitochondrial and nuclear data pooled Syria and Israel into a single population in contrast to three others (Kazakhstan, Spain and Morocco, and North America) (Johnson *et al.*, 2011). By utilizing 18 microsatellite loci, the Syria-Israeli population previously reported was found to be divided by geography as Hf from Israel clustered independent of all sampling locations in the Old World (Syria, Kazakhstan, Spain, and Morocco) as well as in the US (Morton and Schemerhorn, 2013).

Resistant cultivars are not commercially deployed for the control of Hf in Israel. However, the chance that Hf has been naturally exposed to *H* genes in the field is great as Hf from Syria was found to be resistant to a wide array of *H* genes despite a lack of commercial exposure to resistant wheat cultivars (El Bouhssini *et al.*, 2008). Located to the southwest of the Fertile Crescent, Israel is near the center of origin for the domestication of wheat (Gepts, 2002). Studies of wheat cultivars in Israel have found that there is considerable genetic diversity in wheat from mixed cultivar planting, inter-regional seed exchange, and natural cross breeding between local and introduced wheat varieties (Poiarkova and Blum, 1983). For hundreds of years, regionally adapted cultivars were developed in Israel by sowing different wheat species and Middle Eastern land races into the same fields (Blum *et al.*, 1989; Simms and Russell, 1997). An assessment of wheat fields in the early 1980s indicated that 22 *T. durum* cultivars from five different local landrace groups, six *T. aestivum* cultivars, and two *Triticum compactum* cultivars were present throughout Israel (Poiarkova and Blum, 1983). Current agricultural practices have decreased that diversity as *T. aestivum* cultivars from North Africa are in common widespread use (Atzmon and Scwarzback, 2004). However, many alternative Hf hosts are also widely available outside of commercial fields (Kislev *et al.*, 1995).

Galilee and the Jordan River Valley are the described center of origin for wild southern emmer, one of the progenitors of *T. durum* (Nevo and Beiles, 1989). Israeli durum landraces were very different from any other areas of the Fertile Crescent due to the high diversity in local emmer landraces and the ability of durum to hybridize with these readily available genetically diverse emmers (Peng *et al.*, 2000; Ozkan *et al.*, 2011). Indeed, before widespread cultivation of locally adapted durum disappeared in Israel, the

diversity of local cultivars in the Negev is estimated to have exceeded the entirety of diversity in the world (Ozbeck *et al.*, 2011). As several *H* genes have been introduced into common wheat from durum, it is unknown what impact to virulence this has had on Hf in Israel.

These differences between Hf populations in the US and in Israel as well as the differences in exposure to different selection pressures from wild grasses and commercial cultivars give a compelling case for studying Hf in Israel. Therefore, the goal of this dissertation is to characterize Hf from Israel by 1) confirming the identity of Hf field collections through the use of morphological and genetic markers, 2) assessing population structure in field collections through the use microsatellite markers, 3) evaluating virulence to different *H* genes, 4) studying expression data of different SSGP families in comparison to different field collections in the US, 5) examining the inheritance of *vH13* through the use of genetic crosses, and 6) using molecular techniques to document the molecular basis of virulence and avirulence alleles to *H13* in Israel. A separate study to testing the use of pheromone traps and molecular markers to document the frequency of virulence/avirulence alleles to resistance gene *H13* in field populations of Hf from the southeastern US will also planned. These studies will have a great impact on how we view Hf's interactions with wheat.

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CHAPTER TWO: BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF HESSIAN FLY (DIPTERA: CECIDOMYIIDAE) FROM ISRAEL

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CHAPTER 2. BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF HESSIAN FLY (DIPTERA: CECIDOMYIIDAE) FROM ISRAEL

2.1 Abstract

Samples of a dipteran pest of wheat were tested to confirm identity, describe local populations and suggest the use of deploying resistance (*R*) genes in wheat cultivars for control of *Mayetiola destructor*, Hessian fly (HF). Morphological evaluation of adults and a free-choice oviposition preference test documenting that females overwhelmingly preferred to oviposit on wheat instead of barley supported they were HF. Using the cytochrome c oxidase subunit I (*coxI*), the Barcoding Region, nine haplotypes were revealed. Two were found only in the Israeli collections and averaged 3% sequence divergence compared to the other seven haplotypes found in the United States, Israel and Syria. In evaluations of virulence, the Israeli HF in culture was virulent to 11 of the 19 (*R*) genes tested, and complementation analysis documented that, for four of the *R* genes tested, the Israeli HF shared loci for virulence with HF from the United States. Levels of HF infestation at seven Israeli fields were at least at the 5–8% level, which historically has indicated a significant yield loss. Microsatellite genotyping of the five HF collections from Israel revealed mixed populations in Israel that are distinctly separate from the single population in Syria.

2.2 Introduction

The Negev is a semi-arid desert region located in the southern portion of Israel. The primary agricultural use for this area is the growing of vegetables, grains, and fruit. On average 100,000–120,000 million tons (MT) of wheat can be produced per year; however, Israel is currently in the midst of a multi-year drought, which has decreased wheat production to under 100,000 MT per year (Shachar, 2010). Israel is not self-sustaining in wheat production, and wheat imports are needed to meet the demand for both human and animal consumption (1.7 MT) (Shachar, 2011).

One way to increase wheat yield is through the control of wheat pests. The Hessian fly (HF), *Mayetiola destructor* (Say) [Diptera: Cecidomyiidae], is a common threat in most wheat- growing areas of the world (Ratcliffe & Hatchett, 1997). HF is believed to be endemic to the Fertile Crescent and to have coevolved with the wheat genus *Triticum* (Harlan & Zohary, 1966; Lev-Yadun *et al.*, 2000; Zohary & Hopf, 2000; Stukenbrock *et al.*, 2006). It is the main destructive pest of wheat in the southeastern United States and has caused significant economic loss in terms of reduced grain yield in that region (Buntin, 1999; Ratcliffe *et al.*, 2000). HF was first reported in northern Israel in the winter of 1938 to 1939 when heavy infestations were found in fields of wheat (Duvdevany, 1939). Today, it is known to occur in the agricultural areas of the northern Negev and the southern Coastal Plain (Rivnay, 1962; Avidov & Harpaz, 1969). Whether the insect is endemic to the Coastal Plain and northern Negev or was introduced from some other location before it was first reported in 1939 is unknown. Though it is not officially classified as a pest in Israel, HF has been a significant pest of wheat across North Africa since the early 1900s.

Adults are short lived (3–4 days) and do not feed. Females will mate and lay their eggs on the adaxial surface of a leaf blade within hours of emergence. After 3–5 days (depending on temperature), the eggs hatch, and the neonate larvae crawl down the leaf blade and enter the whorl of the plant. A feeding site that includes formation of a nutritive cell layer to provide nutrient-rich cytoplasm for the larva to feed on (Rohfritsch, 1987; Harris *et al.*, 2006) is established near the crown tissue in seedling plants or at infested nodes in jointing plants.

While HF is a gall midge, no true gall (i.e. outgrowth or swelling) is formed in the plant. The larvae feed for approximately 12 days through both the first and early second instars. Feeding stops by the middle of the second instar before molting to the third nstar, hich is contained within a puparium formed from the cuticle of the second instar. Third instars will either diapause to overwinter or complete their development to adulthood, depending on temperature and rainfall. In North America, there are commonly two generations per year; however, colder northern regions may see one generation while warmer southern regions may see six to eight (Buntin & Chapin, 1990; Lidell & Schuster, 1990). In Israel, there are usually two generations per year, although in the past couple of years, due to mild winters, three generations were observed.

All damage to wheat is due to feeding by the larvae. In seedling plants, larval feeding irreversibly stunts infested primary shoots or tillers and prevents them from heading, resulting in yield loss (Byers & Gallun, 1972). In older, jointing plants, the redirection of nutrients from the plant to the insect decreases seed yield and results in lodging at infested nodes that makes harvesting difficult (Buntin, 1999).

Currently, the best control for HF is the use of resistant wheat cultivars (Chen *et al.*, 2009). A HF is considered virulent if the larvae are capable of surviving and stunting the plant, while resistance in wheat is expressed as larval antibiosis within the first instar, leaving no lasting effects on the plant (Ratcliffe & Hatchett, 1997). Resistance has been found in common and durum wheat cultivars, wild wheat relatives, rye and Baroness barley. To date, 33 resistance (*R*) genes (*H1–H32* and *Hdic*) have been identified in various progenitors of wheat, as well as *Triticum durum* and *T. aestivum* cultivars (Ratcliffe & Hatchett, 1997; Martin-Sanchez *et al.*, 2003; Williams *et al.*, 2003; Liu *et al.*, 2005; Sardesai *et al.*, 2005). Unfortunately, the deployment of resistant cultivars places a selection pressure on HF populations. This leads to the appearance of genotypes (biotypes) that can overcome resistance. In the field, *R* genes have a 6–8 year window of effectiveness (Hatchett *et al.*, 1987; Ratcliffe *et al*., 2000). Since adult HFs are weak fliers (Harris *et al.*, 2003), primary dispersal is done through human transportation of puparia in infested straw.

Previous studies on local varieties of Negev wheat cultivars indicated there is considerable genetic diversity in wheat within this area due to mixed cultivar planting, inter-regional seed exchange, and natural cross-breeding between local and introduced varieties (Poiarkova & Blum, 1983). Additionally, wild wheat (emmer, *T. turgidum* ssp. *dicoccoides*) is endemic to the Galilee and, to a lesser extent, the Jerusalem area (Nevo & Beiles, 1989).

Initial population studies with both mitochondrial and nuclear markers identified a population of HF from the northern Negev as possibly ancestral to what is found in the United States (Johnson *et al.*, 2004, 2011). The combination of increased genetic

diversity in the host plant and the isolation of potentially ancient populations of HF in Israel could have implications for documenting the ancestry of HF in the Fertile Crescent region of the Middle East, as well as further defining the wheat/HF interactions in regards to the emergence of genotypes of the pest that can overcome genes for resistance in wheat.

The objectives of the present study were: (i) to confirm the identity of HF from Israel using morphological characters, DNA barcoding, and oviposition preference on wheat; (ii) to evaluate virulence in the Israeli HF to different R genes in wheat; (iii) to determine field infestation levels; and (iv) to assess population structure using microsatellite markers with multiple collections from different locations within Israel.

2.3 Materials and Methods

Sample sites and collection of HF

HF was sampled in Israel from five sites: three in the northern Negev (Kibbutz Magen, Kibbutz Ruhama, and Gilat) and two from the southern Coastal Plain (Kibbutz Yad Mordechai and Kibbutz Zikim) (Figure 2.1). Collections were made by randomly harvesting plants from three to five different areas within an infested field. Collected samples of infested wheat plants were shipped FedEx under APHIS permit number P526P-09-00335 to the USDA-ARS Crop Production and Pest Control Research Unit in West Lafayette, IN, USA. Infested plants were placed in plastic boxes (26 × 39 cm) to allow for adult emergence. Boxes were maintained at 18°C, and the infested plant material was misted occasionally to maintain humidity and enhance adult eclosion. As adults emerged, representative samples were preserved in 100% ethanol at 20°C for later

extraction of DNA and evaluation with the cytochrome c oxidase I (*coxI*) barcoding sequence and microsatellite markers.

Initially, collections of HF from Magen, Ruhama, and Gilat were successfully brought into culture. However, the Gilat and Ruhama collections were not sustainable, and only the Magen collection was successfully cultured under the environmental chamber and greenhouse conditions by the protocols described by Foster *et al.* (1988) and Black *et al.* (1990) for further laboratory testing. HF samples preserved in 100% ethanol from Lattakia, Syria, as well as a sample of Barley stem gall midge (BM) (*Mayetiola hordei* (Keiffer) [Diptera: Cecidomyiidae]) were kindly provided by Dr Mustapha El-Bouhssini, Senior Entomologist, International Center for Agricultural Research in the Dry Areas, Aleppo, Syria.

Morphological evaluation and oviposition preference

Adults were initially identified as HF by comparing morphological characters described by Gagné *et al.* (1991) to differentiate it from the BM, a congener found in the Mediterranean basin that closely resembles HF. HF puparia were examined under an Olympus SZX16 stereo microscope for distribution of spicules and attachment of the plant's cell wall to the puparia. Adult females were inspected at the 6th–8th abdominal tergites using measurements and descriptions as described in Gagné *et al.* (1991). In HF, the 6th tergite is wider (0.458 mm), the 7th tergite flares out anteriorly and the 8th tergite is wedge-shaped. In BM, the 6th tergite is narrower (0.417 mm) and the 8th tergite is rectangular. Adult males were inspected for the long gonostyli and deeply separated and parallel hypoproctal lobes associated to HF.

When given a choice between oviposition on wheat or barley, HF females significantly prefer to oviposit on wheat while BM prefers barley (Gagné *et al.*, 1991). To further support the identity of HF from Israel, a barley-wheat free choice oviposition test was performed using the Magen culture. The barley cultivars, 'Baroness', 'Harrison' and 'Radiant', and the wheat cultivars, 'Iris', 'Seneca', Monon', 'Magnum', and 'Caldwell', were seeded in flats with two replicates separated spatially. Wheat was seeded in randomized rows at the ends and in the middle of each flat, and the barley cultivars were seeded in randomized rows between the rows of wheat in each flat. Flats were placed in environmental chambers at 18°C with a 16 h photoperiod for germination. When the seedlings had reached the 1.5 leaf stage, each flat was caged with netting and 150 gravid females from the Magen culture were allowed to oviposit in a free-choice manner on the plants in each flat. Before hatch, eggs were counted on 20 randomly selected wheat plants from each row and from 20 randomly selected barley plants from each row to evaluate oviposition preference of the females.

Though very similar in appearance to HF, the BM creates a gall at its feeding site at the base of the whorl that adheres to the cell wall of the plant and makes removal difficult. Conversely, HF does not create a visible gall at its feeding site, stunts susceptible wheat and is easily removed from the plant. Further, HF infestation of barley is either asymptomatic or results in mild stunting.

Eggs hatched in approximately 4–5 days and the netting was removed. Plants were sampled at 14 days post-hatch to evaluate for stunting and/or lack of galling at the feeding site and to confirm the presence of larvae within the leaf sheath. Galling at the base of the infested whorl of barley plants would indicate the BM, while stunting of

wheat plants would indicate HF. Infested barley plants were scored for lack of a gall at the feeding site and being either asymptomatic or displaying mild stunting, as well as ease of removal of puparia from the plant. Statistical testing for significance between the mean numbers of eggs laid on wheat compared to barley was performed by a Mann-Whitney test within the program R (R Development Core Team; http://www.R-project.org) (Hornik, 2011).

DNA barcoding using coxI

DNA from individual flies was isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). Ten individuals from each of the Magen, Ruhama, Gilat, Zikim and Yad Mordechai, Israel collections, as well as the Lattakia, Syria and Dallas County, Alabama collections were selected for barcoding analysis. Hebert's *coxI* barcoding primers LCO1490 and HCO2198 were used to amplify an approximately 700 base pair (bp) sequence (Hebert *et al.*, 2003, 2004; Smith *et al.*, 2005; Ratnasingham & Hebert, 2007). Each 25 µl reaction contained 5 µl of 5 × GoTaq polymerase reaction buffer (Promega, Madison, WI, USA), 3 mmoles $MgCl_2$, 10 pmoles each primer, 0.2 mmoles each dNTP (Promega dNTP mix), 2.5 units of GoTaq polymerase (Promega). Polymerase Chain Reactions (PCR) cycling was with a DNA Engine Dyad PTC-220 and PTC-221 (BioRad, Hercules, CA, USA) under the following conditions: denaturing at 95°C for 2 min; 35 cycles of denaturing at 95°C for 1 min, annealing at 50°C for 30 s, extension at 72°C for 1 min; final extension at 72°C for 10 min. In order to obtain the longest sequence, *cox1* fragments were cloned using the pCR®4-TOPO® vector into electrocompetent TOP10 cells (Invitrogen, Grand Island, NY, USA). Three clones per

individual were sequenced through the Purdue Genomics High Throughput Center. A consensus sequence was made for the *coxI* sequence for each individual, and all *coxI* sequences were aligned using ClustalW2 (Chenna *et al.*, 2003). Arlequin 3.11 (Excoffier *et al.*, 2005) was used to calculate FST. PAUP* (Swofford, 2003) and Treeview (Page, 1996) were used to create the phylogenetic reconstruction using the distance neighbor-joining model F84 and parsimony algorithms. Two gall midge species were used as outgroups in the reconstruction, *M. hordei* (JN638248.1-full length *coxI*) and *Rabdophaga rigidae* (AB244544.1-partial length *coxI*). *R. rigidae* (Osten Sacken), the willow beaked gall midge, is from the same tribe as HF, Oligotrophini. TCS was used to calculate the networking relationships of coxI barcodes (Clement *et al.*, 2000).

Evaluation of virulence

The response of the Magen collection to different R genes in wheat was conducted with wheat lines carrying a different *R* gene seeded in flats (two replicates) in the manner described for the virulence flat test methodology developed by Chen *et al.* (2009). Nineteen lines carrying the following single *R* genes or gene combination were seeded in each flat: *H3*, *H5*, *H6*, *H7H8*, *H9*, *H10*, *H11*, *H12*, *H13*, *H14*, *H16*, *H17*, *H18*, *H21*, *H22*, *H23*, *H24*, *H25*, *H31* and *H32*. These were lines in which sufficient seed was available for virulence testing and represented 19 of the 33 named HF *R* genes in wheat. The susceptible wheat cultivar 'Newton' (carrying no *R* gene) was also seeded in 'check' rows at the ends and in the middle of each flat to check for uniformity of infestation throughout the flat. Fifteen to 20 seeds of each line were seeded in randomized half-rows

in each flat. Flats were then placed in controlled environmental chambers at 18°C with a 16 h photoperiod for seed germination.

After seedling plants had reached the 1.5 leaf stage, each flat was caged separately with netting, and 150 gravid females from the Magen culture were aspirated from plastic emergence boxes and released under the netting. Females were allowed to oviposit in a free-choice manner. Egg hatch was observed 4–5 days after oviposition at which time the netting was removed. Flats were maintained in growth chambers, and plants were evaluated at 14 days post-hatch for resistance or susceptibility. Resistant plants were not stunted, exhibited normal growth habit and, when dissected, contained dead 1st- instar larvae. Plants with no dead larvae (escapes from infestation) were discarded. Susceptible plants contained living larvae and exhibited stunting and a darker green color that is associated with infestation. The total number of resistant and susceptible plants from both flat replicates was recorded.

Since there was no documentation that HF *R* genes have ever been deployed in Israel (P.G. Weintraub, unpublished data), it was hypothesized that the Israeli HF should be equivalent to the Great Plains (GP) Biotype in the United States (avirulent to all *R* genes). Therefore, a ratio of resistant to susceptible plants of 1:0 is expected. Goodness of fit for the number of observed resistant plants to the number of expected resistant plants was tested by $\chi 2$ analysis where degrees of freedom (df) = 1.

Complementation analysis

Complementation assays to document if the Magen collection shared loci for virulence to *H3*, *H5*, *H6* and *H7H8* with HF from the United States were performed in

four-way differential pots with three to five plants of the wheat cultivars ‘Monon’ (carrying *H3*), ‘Magnum’ (carrying *H5*), ‘Caldwell’ (carrying *H6*) and ‘Seneca’ (carrying *H7H8*) seeded in separate quadrants. Biotype L HF (known to be virulent to *H3*, *H5*, *H6* and *H7H8*) and Magen adults were allowed to emerge in separate boxes. Reciprocal crosses were made between Magen females × Biotype L males and Biotype L females × Magen males. A single virgin female and one male were introduced into caged pots where mating and oviposition occurred. The caged pots were placed in a controlled environmental chamber at 18°C with a 16 h photoperiod and scored for virulence at 12 days post-hatch by dissecting each plant to locate developing larvae.

HF infestation levels in Israeli wheat fields

In 2008–2009, wheat plants (150–200 plants per field) were sampled from random locations near the edges and in the center of the five fields in the northern Negev and the southern Coastal Plain previously identified above (see fig. 1) to assess for potential yield loss. In 2010, infestation levels in fields at Kibbutz Alumim and at Kibbutz Be’eri in the northern Negev (fig. 1) were also documented to assess potential yield loss.

Microsatellite amplification and genotyping

Twenty-five microsatellite markers (Schemerhorn *et al.*, 2008, 2009) were selected from the available pool used with HF collections in the United States. These markers were selected for their location on autosomes and for the previously identified variability within United States populations at these loci (Morton et al., 2011). PCR was

performed according to the protocol in Schemerhorn *et al.* (2009), and polymorphisms were scored using a CEQ 8000 (Beckman-Coulter, Brea, CA, USA). Microsatellite analyses (FST, AMOVA, HWE, pairwise linkage disequilibrium and molecular diversity indices) were performed using Arlequin 3.11 (Excoffier *et al.*, 2005). Microchecker 2.2.3 (Van Oosterhout *et al.*, 2004) was used to check for genotyping errors that cause deviation from HWE, such as stuttering, large allele dropout, null alleles and typographical errors. In order to detect recent changes in effective population size, BOTTLENECK 1.2.02 was also performed (Cornuet & Luikart, 1997). Structure 2.3.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003, 2007; Hubisz *et al.*, 2009) was used to analyze the population structure comprised by the five Israeli collections using the microsatellite loci, and k was calculated using the method of Evanno *et al.* (2005).

2.4 Results

Morphological evaluation and ovipostion preference

Male and female adults from Israel were examined and confirmed to be HF by use of the morphological characters (Gagné *et al.*, 1991). These results documented that the putative HFs from Israel were morphologically in agreement with HF and not BM. In the barley-wheat free-choice test, female flies from the Magen culture oviposited on average 56 eggs per leaf on wheat plants, while in comparison only 11 eggs per leaf were laid on barley plants (Figure 2.2). The contrast between the mean number of eggs laid on wheat compared to barley was statistically significant ($P \leq 0.05$).

DNA barcoding

Nine haplotypes of the *coxI* barcode (haplotypes 1–9) for HF were identified (GeneBank: JN638239.1–JN638247.1). Gilat and Yad Mordechai contained only haplotypes 1 and 2 while Zikim contained 1, 2 and 4. Ruhama was composed of haplotypes 2 and 3. Magen contained only haplotype 4. Morocco included 5 and 8, two haplotypes that did not appear elsewhere. Alabama consisted of 6 and 7. Syria was the most diverse with haplotypes 2, 3, 6, 7 and 9. The genetic distances were calculated using F84 (Felsenstein, 1984). The distance between the outgroups and the nine haplotypes ranged from 9.37–11.19% (10.1% average) for *M. hordei* and 13.86–15.75% (15.1% average) for *R. rigidae*. The distances for the nine HF haplotypes fell into two groups: group 1 contained haplotypes 1 and 2, and group 2 contained haplotypes 3 through 9. Within group 1, the distance was 0.14%, while within group two the haplotypes ranged 0.14–1.34% (0.75% average). However, the distance between group 1 and group 2 was much greater, 2.90–4.11% (3.32% average).

In population pairwise F_{ST} (Table 2.1), all sample sites separated with less than 1% distance except for Zikim, Gilat, Ruhama and Yad Mordechai, which did not separate significantly from one another. A network containing all nine haplotypes could not be built with greater than 95% confidence. Dividing the haplotypes into clades corrected this problem. The networks for clades 1 and 2 were identical to the parsimony tree. The number of mutational steps for each haplotype is located on the branches of Figure 3.3A. Both a 50% majority rule distance neighbor-joining tree and a parsimony tree (Figure 3.3B) were constructed and found to be congruent. The tree reveals isolation of the *coxI*-1 and *coxI*-2 sequences from the other seven barcodes identified. There is a lineage

expansion of *coxI*-9 into two groups: one containing Syria, Morocco and Alabama samples and another containing Israeli and Syrian samples. These results are congruent with previous analyses (Naber *et al.*, 2000; Johnson *et al.*, 2004, 2011) using RFLP, mitochondrial and nuclear markers in regards to both isolation in Israel and the relationships between Syria, Morocco and the United States. AMOVA analysis revealed that there is more variance among populations (80.05%) than within populations (19.95%), which is consistent with previous data for mitochondrial loci (Johnson *et al.*, 2004, 2011).

Evaluation of virulence

The results for the two virulence test replicates were combined and tested for significance (Table 2.2). The Magen HF was hypothesized to be avirulent to all of the *R* genes tested since it was not believed to have undergone selection pressure from any of the *R* genes. Thus, a ratio of 1:0 was expected for avirulent to virulent phenotypes. However, the HF from the Magen culture was virulent (significantly divergent from the expected 1:0 ratio) to *H3*, *H5*, *H6*, *H7H8*, *H9*, *H10*, *H11*, *H13*, *H14*, *H16* and *H23*. Though a few virulent individuals were scored on lines carrying other *R* genes, virulence to *H12*, *H17*, *H18*, *H22*, *H24*, *H25*, *H31* and *H32*, the result was not significantly different from the expected 1:0 ratio of avirulence to virulence.

Complementation analysis

The wheat plants infested with the F_1 progeny from the complementation crosses showed the typical susceptible reaction to HF infestation. The F_1 individuals from both

the Magen female × L male and L female × Magen male were virulent to *H3*, *H5*, *H6* and *H7H8*, indicating no complementation occurred that would have resulted in an avirulent genotype to the *R* genes tested.

HF infestation levels in Israeli wheat fields

In the field at the Gilat Research Center, infestation was approximately 3–5% of the sampled plants and was patchy within the field. At the Magen location, 20% of plants were infested at the corner of the field and 5% in the middle of the field. A 75% infestation was found at Zikim, with the entire field being evenly infested. Sampling in fields at Alumim documented that infestation ranged from 17.6–32.7%, and at Be'eri infestation ranged from 5.2–20.3%.

Microsatellite genotyping

Twenty-five microsatellite markers were initially selected for use with the Israeli HF collections based on their autosomal location and variability in collections from the United States. Only eight (Hf14, Hf101, Hf102, Hf104, Hf109, Hf113, Hf114 and Hf164) were polymorphic with HF individuals from the Israeli collections (Table 2.3). AMOVA analysis of the micro- satellite markers revealed that there is more variance within populations (85.89%) than among populations (14.11%), which is consistent with previous data for nuclear loci (Johnson *et al.*, 2011). Wright's F_{ST} (Table 2.4) significantly reveals the separation of each of the following collections from all other collections: Syria, Gilat, and Ruhama. The collections of Magen, Yad Mordechai, and Zikim were not found to be significantly different.

No recent expansion or allele frequency change was detected, an indication that a bottleneck had not recently taken place. Pairwise linkage disequilibrium was not detected. Average gene diversity over all loci in all Israeli locations ranged between 0.332–0.376, while in Syria it was 0.604 (Table 2.3). Hardy-Weinberg equilibrium (HWE) was calculated with a Bonferroni correction for multiple tests using Arlequin with a significance of $P \leq 0.05$ (Table 2.3). Seven loci indicated a departure from HWE in some but not all populations. H14 was the only locus that was in HWE in all samples.

The Structure results indicate three populations (Figure 2.4). Syria (green) is clearly a separate population from every collection in Israel. Each Israeli sample location contains a mixture of two populations. Gilat and Ruhama contain individuals that are primarily from population 1 (red), Magen and Yad Mordechai contain a more proportionate distribution of both populations, while Zikim primarily contains population 2 (blue). Since each collection contains both populations, mixing has occurred among them.

2.5 Discussion

Confirmed identification of Hessian fly in Israel

Morphological evaluation of adults and puparia from field collections at the five sites in Israel supported their identity as HF. However, the intraspecific divergence within the *coxI* barcodes among individuals from all collections revealed two distinct lineages of HF. All nine *coxI* HF haplotypes clearly separated from the BM and *R. rigidae coxI* with a barcoding gap (intraspecific/interspecific variation) of 33% between *M. destructor* and *M. hordei*. The use of null nuclear markers distributed throughout the two HF autosomes

supported the population division between Syria and Israel, while dividing Israel into two intermixed populations. There is no direct correlation between the mitochondrial barcoding lineage and nuclear microsatellite populations; and, therefore, there is no support to effectively divide the two mitochondrial lineages of HF.

There is not enough evidence presented within this study to report the identification of a cryptic species of HF in Israel. If the lineage divergence revealed by barcoding is recent, the lower mutational rate within the nuclear genome is masking the beginning of speciation (McKeon *et al.*, 2010).However, the results do support the two previous studies (Johnson *et al.*, 2004, 2011) that revealed mitochondrial isolation in Israel and limited nuclear gene flow between Syria and Israel.

Influence of Israel on HF

Geographic barriers surround the entirety of Israel. The Mediterranean Ocean provides the western barrier, while the Jordan River and Dead Sea run the length of the eastern barrier. Rocky mountains in the north separate Israel from Syria and Lebanon. The vast, dry Negev Desert fills the southern borders. The majority of commercial agriculture is performed in reclaimed areas of the northern Negev.

Cultivation of food crops is directly influenced by war, migration of tribes and colonization (Aaronsohn, 1910). Since HF is primarily dispersed through human transportation of puparia, these political barriers can greatly influence gene flow. Israel lies within a much-disputed area of the Fertile Crescent. Many ancient civilizations have lived in this region, bringing with them different cultivars of wheat and cultivation practices. As political and religious hostilities arose in the region, agricultural trade was

frequently interrupted, which prompted the creation of locally adapted cultivars or landraces (Aaronsohn, 1910). Some of these landraces were so geographically specific that a difference of 10 km was substantial enough to prohibit widespread distribution (Aaronsohn, 1910). Until the last century, these landraces were the primary sources of wheat in Israel, as widespread commercial farming was not practiced. Given both geographic and political barriers to gene flow, the location and history of Israel may have contributed to the isolation of the Israeli-only *coxI* barcodes.

Using microsatellites, three populations are revealed among the six sampled locations. The Syrian population is completely separated from the Israeli populations and contains higher average gene diversity over all loci. Though some alleles are shared, there is a gene flow barrier between the two countries, as indicated by the high F_{ST} values. Further support from the barcoding analysis reveals that while some gene flow may have occurred (recently or in the distant past) with the sharing of mitochondrial haplotypes, the four *coxI* lineages outside of Israel are derived from a Syrian haplotype. As Syria was basal to the six alleles in clade two, this indicates that Syria is an important location in the initial distribution of HF from the Fertile Crescent, as supported by Naber *et al.* (2000).

Very few microsatellite loci are in HWE that could indicate that one or more of the five assumptions (nonrandom mating, mutation, gene flow, selection and genetic drift) are being violated. Migration may be the most direct reason for the differences in allele frequencies. HF adults are weak fliers, and dispersal over greater distances is generally due to human movement of wheat straw infested with HF puparia (Harris *et al.*, 2003). In addition, there are geopolitical barriers in agricultural regions of Israel that

restrict human movement and, therefore, the dispersal of HF resulting in isolation or preferred migration between particular locations.

The moderate levels of inbreeding and lower levels of average gene diversity over loci seen within each Israeli collection indicate isolation from Syria. While *R* genes in wheat are not used to control HF in Israel, seed treatments are sporadically used. The varied distribution of fields with HF control would create empty pockets of land where HF no longer exists, introducing isolation between locations within a single generation. Isolation in combination with low gene flow due to HF's lack of migration will contribute to inbreeding rates.

Influence of wheat cultivation on virulence of HF in Israel

The domestication of wheat occurred in the area north of the Fertile Crescent known today as Turkey and Transcaucasia (Gepts, 2002). In general, domestication influenced the genetic diversity inherent within populations through differing dispersal and cultivation practices. In modern times, commercial breeding practices focus on crossing two elite lines for desirable traits at the direct cost of genetic diversity. In situ conservation by subsistence farmers at or near the origin of domestication naturally retains the genetic diversity of wheat through the growing of local landraces and wild and heirloom cultivars (Gepts, 2002). These serve as reservoirs of diversity, which can be introgressed into elite lines to combat the loss by commercial breeding. For hundreds of years, local farmers in Israel have favoured regularly sowing different wheat species and regional Middle Eastern landraces in the same fields (Blum *et al.*, 1989; Simms & Russell, 1997). An assessment of wheat fields indicated that 22 *T. durum* (durum)

cultivars from five different local landrace groups, six *T. aestivum* (common wheat) cultivars, and two *T. compactum* cultivars were present across Israel (Poiarkova & Blum, 1983).

Over the years, the diversity of wheat cultivars in Israel has rapidly decreased as commercial farming replaced local, subsistence farming. Modernization began in the 1880s and focused on locally adapted varieties of durum; but, in the 1950s, common wheat cultivars from North Africa replaced them until the near disappearance of durum by the 1970s (Atzmon & Scwarzbach, 2004; Poiarkova & Blum, 1983).

The ancestor of modern durum, *T. dicoccoides* (wild southern emmer), is the result of a natural hybridization of *T. uratu* (wild einkorn wheat) and an extinct relative of *Aegilops speltoides* (a wild goat grass species), while common wheat, known to have arisen independently in many locations, is a hybrid of *T. dicoccon* (domesticated northern emmer) and *Ae. tauschii* (Taush's goat grass) (Salamini *et al.*, 2002; Dubcovsky & Dvorak, 2007). Before the disappearance of locally adapted durum landraces, it was estimated that the diversity of cultivars within the Negev region exceeded not only the diversity found in the entirety of the Middle East but also the world, suggesting that Israel served as the center of origin for wild southern emmer (Ozbeck *et al.*, 2007). The Israeli durum landraces are very different from those in other areas of the Fertile Crescent due to the high diversity found in the Jordan Valley and their ability to hybridize with wild emmer (Peng *et al.*, 2000; Ozkan *et al.*, 2011). These novel hybrids within Israel contain phenotypes with important ecological benefits as well as a high degree of plasticity to adapt successfully in their environment (Ahern *et al.*, 2009; Agrawal, 2001).

Despite the absence of commercially deployed resistant wheat cultivars in Israel, virulence in the Magen HF closely resembled that documented by Cambron *et al.* (2010) for HF from the southeastern United States, which consistently deploys *R* genes. Of the *R* genes that Israel is virulent to, three are from common wheat (*H3*, *H5*, *H7H8*), two from Taush's goat grass (*H13* and *H23*) and six from durum (*H9*, *H10*, *H11*, *H14* and *H16*) (Liu *et al.*, 2005). The Magen HF was avirulent to *H12* from common wheat, to *H22*, *H24* and *H32* from Taush's goat grass, to *H17*, *H18* and *H31* from durum, and to *H25* from rye (*Secale cereale*) (Liu *et al.*, 2005; Sardesai *et al.*, 2005). The combination of high genetic diversity in both wild emmer and durum landraces, as well as the proximity to the center of wheat domestication, may have exposed HF in Israel to these *R* genes long before HF's introduction into North America and direct selection pressure through deployment of *R* genes.

This comparison between virulence in HF from the southeastern United States and the Magen HF suggests two important hypotheses: (i) that HF genes controlling virulence to *R* genes in wheat have long resided in the genome within populations near the center of origin and (ii) that virulence to *R* genes in wheat is maintained within HF populations without direct selection pressure.

HF collections from locations in the Fertile Crescent (i.e. Israel and Syria) both display virulence to a wide array of *R* genes. Surprisingly, HF from Syria has been identified as the most virulent population with only *H25* and *H26* showing efficacy in protecting wheat (El Bouhssinni *et al.*, 2009). Understanding the mechanism of selection for virulent HF genotypes in the collections from Israel and Syria will require additional

study and could have significant implications for understanding how virulence emerges in HF populations.

Influence of rainfall and wheat availability on HF in Israel

In Israel, wheat is primarily planted in two climatic regions: the Coastal Plain (Zikim and Mordechai) and northern Negev (Gilat, Ruhama, and Magen). The microsatellite analysis weakly supports a population division between these two climatic regions; however, human dispersal and/or migration has mixed the two populations. The Coastal Plains receive more rainfall on average; however, the northern Negev receives a higher frequency of high intense rains in autumn (September to November). Commercial wheat is sown in November while local farmers plant in December when the rains have diminished (Sharon & Kutiel, 1986). The Negev remains dry for most of winter until the 'greening up' process begins in February when the rains return (Svoray & Karnieli, 2010).

In the southeast United States, Hessian fly cannot be controlled through the use of fly-free date planting techniques. Warm temperatures coupled with significant rainfall signals the end of HF aestivation, and this leads to multiple fall broods if wheat is planted too early or volunteer wheat is readily available. Coastal areas in Georgia usually have four broods per year: two fall, one winter, and one spring (Buntin & Chapin, 1990). As the northern Negev and southern Georgia share latitudinal coordinates, it is highly likely that multiple broods occur in both winter and spring every growing year.

In order to increase the chances of multiple broods per season, there must be readily available sources of wheat for HF. The different planting times between

commercial and local farming is equivalent to one life cycle of HF. A warm, wet December could trigger aestivation from HF in commercial fields and lead to a second winter brood in subsistence fields. Prolonged droughts have increased the number of abandoned and untilled silage fields, which in the United States serve as safe havens for diapausing HF (Atzmon & Schwarzbach, 2004). In addition, volunteer wheat is often found as weedy roadside borders since transportation through the ages has readily scatters seeds (Cook, 1913). Wild emmer found in rocky, uncultivated areas can also serve as a host for HF.

A mixture of two populations was also detected in the southeastern United States (Morton *et al.*, 2011). No bottleneck was detected, but the availability of the host plant in silage fields before the fly free dates played an important role in increasing the number of broods per year. Evolutionary differences from mutations can accumulate faster within isolated areas where more broods per year occur, leading to increased genetic drift (Masel, 2011). Local and spatial factors provided limited influence over the large area of the southeastern United States; however, genetic drift within the small geographic region under study could provide a potential explanation for the separation of the Israeli populations from Syria where there are fewer broods per year.

Influence of HF on Israel

Yield loss from HF infestations of wheat is considered to become significant when fall infestations exceed 5–8% of the plants in a field and when spring infestations exceed 13–20% (Buntin, 1999). These estimates were initially made for the southeastern United States, but they should also be applicable to Israel. Infestation levels for fall

infestations in six of the seven fields surveyed substantially exceeded the infestation levels for significant yield losses, and the 3–5% spotty infestation levels in the field at the Gilat location was equal to a significant yield loss at some locations within the field. Estimation of virulence and yield loss within fields in Israel suggests that the use of resistant cultivars would greatly reduce losses due to HF infestations. Historically, there has been no program to introgress HF *R* genes into wheat lines adapted to Israel. The seed treatment insecticides Cruiser (Syngenta) and Gaucho (Bayer) are used with wheat for control of HF and other insect pests in Israel; however, application of these seed treatments introduces a significant additional cost into wheat production. Additionally, these seed treatment will not protect the crop from spring infestations. Thus, introgression of HF *R* genes into wheat lines adapted to agronomic conditions in Israel is a control strategy worthy of consideration. The current study has documented the *R* genes *H12*, *H17*, *H18*, *H25* and *H32* provided effective resistance toward the Magen HF and should be effective in protection of wheat in Israel.

2.6 Conclusions

Hessian fly has been positively identified as a wheat pest in Israel. It occurs at a level of infestation that significantly impacts yield loss. The use of wheat cultivars that contain at least one of the *R* genes for *H12*, *H17*, *H18*, *H22*, *H24*, *H25*, *H31* and *H32* are suggested for immediate use to control HF and increase crop yield. While levels of differentiation in the *coxI* barcoding region are well within species tolerances, isolation of HF in Israel has occurred. Additional research is required to positively identify if the

mitochondrial and nuclear evidence reported here can support Israeli HF as a cryptic species.

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2.9 Tables

Table 2.1. Wright's F_{ST} for *coxI* from Hessian fly. Significant values are in bold, and $p \leq 0.05$.

	Alabama	Morocco	Gilat	Ruhama	Mordechai	Magen	Zikim	Syria
Alabama	*							
Morocco	**0.802**	*						
Gilat	**0.984**	**0.974**	*					
Ruhama	**0.858**	**0.833**	0.01	*				
Mordechai	**0.981**	**0.972**	0.065	**0.086**	*			
Magen	**0.967**	**0.933**	**0.992**	**0.863**	**0.99**	*		
Zikim	**0.869**	**0.847**	-0.031	-0.084	-0.02	**0.873**	*	
Syria	**0.37**	**0.271**	**0.759**	**0.591**	**0.77**	**0.323**	**0.638**	*

Table 2.2. Virulence analysis of Hessian fly from Israel using 20 different lines of wheat. χ^2 values were calculated using the program R with $p \leq 0.05$.

Gene	Line ID	#R	#S	χ^2	*p* value
H3	MONON	1	32	31.030	**<0.0001**
H5	MAGNUM	0	36	36.000	**<0.0001**
H6	CALDWELL	0	32	32.000	**<0.0001**
H7H8	SENECA	1	31	30.031	**<0.0001**
H9	IRIS	21	18	8.307	**0.0039**
H10	JOY	19	16	7.314	**0.0068**
H11	KAREN	2	31	29.121	**<0.0001**
H12	LOLA	22	3	0.360	0.548
H13	MOLLY	18	21	11.307	**0.0008**
H14	921676A3-5	11	27	19.184	**<0.0001**
H16	921682A4-6	8	30	23.684	**<0.0001**
H17	921680D1-7	37	5	0.595	0.4405
H18	MARQUILLO	25	2	0.148	0.7005
H22	KSWGRC01	32	12	3.273	0.0704
H23	KSWGRC03	0	34	34.000	**<0.0001**
H24	KSWGRC6	27	11	3.184	0.0744
H25	KSWGRC20	42	0	0.000	1.0000
H31	P921696A1-15-2-1	23	10	3.030	0.0817
H32	SYNTHETIC	29	1	0.033	0.8559
no gene	NEWTON	0	40	0.000	1.0000

Table 2.3. Microsatellite statistics listed by locus for each population. Abbreviations are as follows: n=sample size, N_A=number of alleles per locus, H_o=observed heterozygosity, H_e=expected heterozygosity, HWE-p=p-value for Hardy Weinberg equilibrium where $p \leq 0.05$, and F_{IS}=inbreeding coefficient.

	H101	**H113**	**H14**	**H104**	**H164**	**H102**	**H114**	**H109**	**F_{IS} over all loci**	**Average gene diversity over all loci**
Gilat									0.041	0.336+/-0.200
n	48	48	48	48	48	48	48	48		
N_A	2	3	2	3	3	3	3	2		
H_o	0.375	0.667	0.500	0.104	0.375	0.333	0.299	0.000		
H_e	0.504	0.598	0.486	0.101	0.410	0.284	0.270	0.040		
HWE-p	0.089	0.640	1.000	1.000	**0.025**	0.645	0.364	**0.011**		
F_{IS}	0.259	-0.106	-0.035	-0.035	0.085	-0.176	0.151	1.000		
Ruhama									0.155	0.332+/-0.218
n	46	47	44	48	46	43	48	44		
N_A	3	5	3	3	2	3	3	2		
H_o	0.261	0.404	0.341	0.083	0.478	0.023	0.292	0.023		
H_e	0.441	0.445	0.410	0.120	0.500	0.069	0.255	0.023		
HWE-p	**0.000**	**0.006**	**0.001**	0.154	1.000	**0.011**	0.632	1.000		
F_{IS}	0.405	0.181	0.092	0.305	0.038	0.664	-0.150	0.000		
Magen									0.075	0.364+/-0.218
n	48	48	48	48	48	48	48	48		

N_A	3	10	5	5	3	4	5	2		
H_o	0.521	0.563	0.542	0.167	0.208	0.063	0.479	0.042		
H_e	0.499	0.624	0.503	0.228	0.260	0.140	0.512	0.041		
HWE-p	0.889	**0.009**	**0.001**	0.153	0.400	**0.000**	0.400	1.000		
F_{IS}	-0.012	0.082	-0.079	0.142	0.199	0.557	0.062	-0.011		
Mordechai									0.241	0.341+/-0.217
n	46	46	46	46	46	46	46	46		
N_A	3	5	4	6	3	4	6	2		
H_o	0.565	0.348	0.435	0.218	0.174	0.065	0.435	0.022		
H_e	0.511	0.634	0.494	0.361	0.384	0.085	0.459	0.064		
HWE-p	**0.010**	**0.000**	**0.028**	**0.000**	**0.000**	**0.022**	0.543	**0.033**		
F_{IS}	-0.077	0.436	0.121	0.311	0.574	0.237	0.053	0.662		
Zikim									0.164	0.376+/-0.221
n	25	25	25	25	25	25	25	25		
N_A	2	7	3	3	2	3	5	1		
H_o	0.440	0.560	0.280	0.280	0.800	0.280	0.600	-		
H_e	0.458	0.811	0.528	0.287	0.078	0.313	0.529	-		
HWE-p	1.000	**0.005**	**0.002**	0.481	1.000	**0.031**	0.074	-		
F_{IS}	0.093	0.312	0.389	-0.008	-0.022	0.158	-0.107			
Syria									0.206	0.604+/-.0342
n	46	44	46	43	43	45	45	46		
N_A	5	8	3	4	7	2	5	3		
H_o	0.391	0.455	0.543	0.465	0.628	0.444	0.622	0.435		

H_e	0.736	0.835	0.636	0.678	0.585	0.433	0.677	0.424
HWE-p	**0.000**	**0.000**	**0.017**	**0.015**	**0.008**	1.000	0.409	0.490
F_{IS}	0.500	0.462	0.146	0.302	-0.074	-0.057	0.058	-0.025

Table 2.4. Wright's F_{ST} scores are located below the diagonal. Bolded numbers are significant differences ($p \leq 0.05$). Distance (km) between locations is listed above the diagonal. It is roughly 475 km from the Negev region of Israel to Lattakia, Syria.

	Gilat	Ruhama	Mordechai	Magen	Zikim
Gilat		7.2	31.5	21.5	18.2
Ruhama	**0.085**		28.1	27.6	12..5
Mordechai	**0.014**	**0.111**		34.6	32.5
Magen	**0.019**	**0.072**	0.005		17.7
Zikim	**0.029**	**0.136**	0.013	**0.019**	
Syria	**0.237**	**0.299**	**0.253**	**0.228**	**0.165**

2.10 Figures

Figure 2.1. Sample Locations. This map displays the Israeli collection sites. Stars indicate the Hessian fly sample locations of Kibbutz Yad Mordechai, Kibbutz Ruhama, Kibbutz Zikim, Gilat, and Kibbutz Magen. The locations where infestation levels were sampled, Kibbutz Be'eri and Kibbutz Alumim, are also shown. Country borders are in yellow while the Palestinian territories of the West Bank and Gaza Strip are in red.

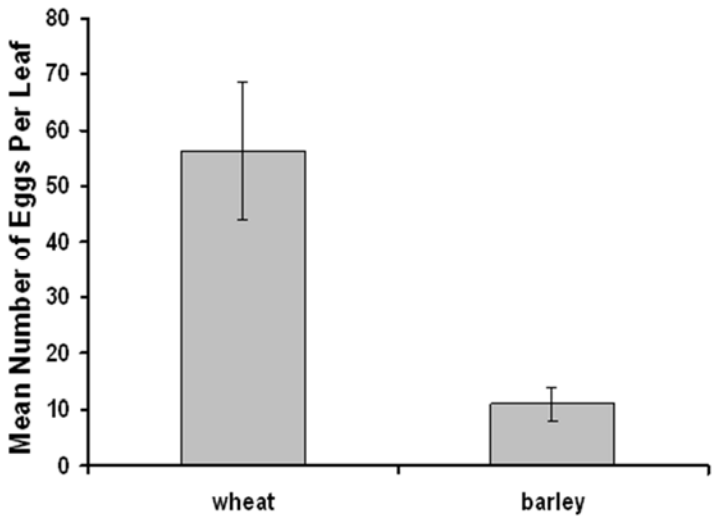

Figure 2.2. Wheat-barley oviposition preference. Using a Mann-Whitney test, the mean number of eggs per leaf laid by Hessian fly on wheat (56) was found to be significant to the number of eggs found on barley (11). The bars on the columns indicate standard error.

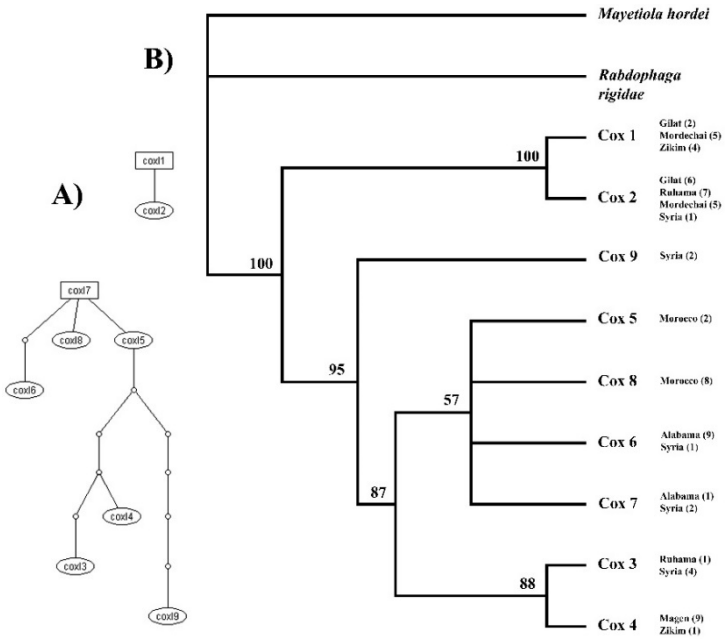

Figure 2.3. Network and Parsimony phylogenetic reconstruction of *coxI* isolates. A) The unconnected networks for clades 1 and 2. Each line represents a mutational step. B) The parsimony tree displays bootstrap values (n reps=10,000) at the nodes. The *coxI* sequence from *Mayetiola hordei* and *Rabdophaga rigidae* were used as outgroups. Sites where the isolates occurred are located beside the branch, and the number of individuals found per location is in parenthesis.

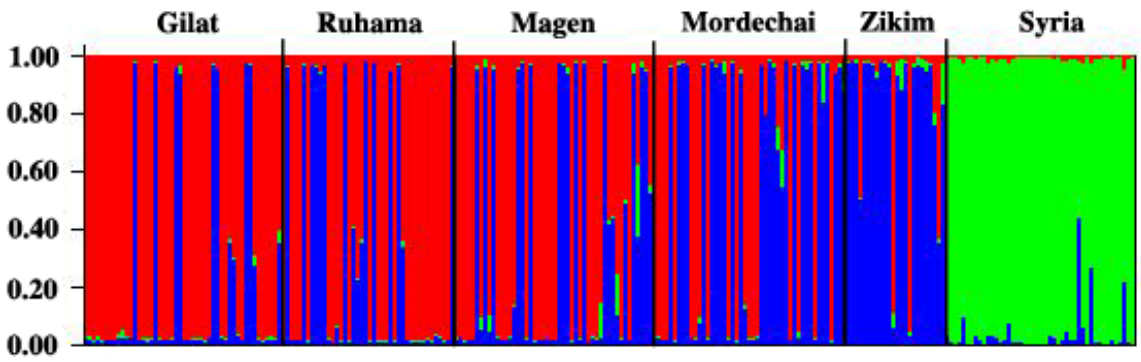

Figure 2.4. Structure diagram. Using microsatellite markers, three populations of Hessian fly were defined. Syria is composed of a single population (green) while the five Israeli locations are split into two mixed populations (red and blue).

CHAPTER THREE: DIFFERENTIAL EXPRESSION OF CANDIDATE SALIVARY EFFECTOR PROTEINS IN FIELD COLLECTIONS OF HESSIAN FLY, *MAYETIOLA DESTRUCTOR*

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CHAPTER 3. DIFFERENTICAL EXPRESSION OF CANDIDATE SALIVARY EFFECTOR PROTEINS IN FIELD COLLECTION OF HESSIAN FLY, *MAYETIOLA DESTRUCTOR*

3.1 Abstract

Evidence is emerging that some proteins secreted by gall forming plant-parasites act as effectors responsible for systemic changes in the host plant, such as galling and nutrient tissue formation. A large number of secreted salivary gland proteins (SSGPs) that are the putative effectors responsible for the physiological changes elicited in susceptible seedling wheat by Hessian fly, *Mayetiola destructor* (Say), larvae have been documented. However, how the genes encoding these candidate effectors might respond under field conditions is unknown. The goal of this study was to use microarray analysis to investigate variation in SSGP transcript abundance among field collections from different geographic regions (southeastern United States, central United States, and the Middle East). Results revealed significant variation in SSGP transcript abundance among the field collections studied. The field collections separated into three distinct groups that corresponded to the wheat classes grown in the different geographic regions as well as to recently described Hessian fly populations. These data support previous reports correlating Hessian fly population structure with micropopulation differences due to agro-ecosystem parameters such as cultivation of regionally adapted wheat varieties, deployment of resistance genes, and variation in climatic conditions.

3.2 Introduction

Proteins and other molecules secreted by the salivary glands of phytophagous insects have been proposed to act as 'effectors' that, when injected into their hosts, facilitate and enhance penetration by mouthpart stylets, initiate digestion of host-cell contents for ingestion, and suppress host defense responses resulting in the modification and manipulation of host processes in a manner advantageous to the pest (Hori, 1992; Alfano, 2009; Hogenhout *et al*., 2009; Hogenhout & Bos, 2011; DeLay *et al*., 2012). It has recently been hypothesized that, during gall formation, the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), uses an effector-based strategy that is similar to biotrophic plant pathogens (Stuart *et al*., 2012). This gall midge is a recurrent pest of wheat, *Triticum aestivum* L., in many of the wheat production areas worldwide and is the most important insect pest of wheat in the southeastern United States (Ratcliffe & Hatchett, 1997; Cambron *et al*., 2010).

All damage to seedling wheat by Hessian fly is due to feeding by 1st-instar larvae. Hatchling larvae enter the whorl, and upon settling near the base of the plant, rapidly induce changes that include the formation of a nutritive tissue that nourishes the developing larvae, a rapid increase in host-cell permeability, and stunting of the plant (Harris *et al*., 2006; Saltzman *et al*., 2008; Williams *et al*., 2011). Further, even if infesting larvae are removed from the seedling plant, normal growth cannot be restored (Byers & Gallun, 1972).

There are three methods to control Hessian fly damage to wheat in the field: avoidance, seed treatment with a systemic insecticide, and deploying genetically resistant wheat. Planting after the historically titled 'fly-free' date is the basis of avoidance;

however, in most of the warmer southeastern United States, an effective fly-free date cannot be relied upon to prevent damage to wheat, as temperatures do not remain consistently cold enough to prevent Hessian fly adult emergence. Seed treatment is generally effective for only 2 – 3 weeks post germination. Therefore, the most successful method of control is the deployment of genetically resistant wheat. Thirty-five different Hessian fly resistance (*R*) genes in wheat have been identified and characterized (Liu *et al*., 2005; Sardesai *et al*., 2005; Li *et al*., 2013; McDonald *et al*., 2014). This resistance is expressed as antibiosis of 1st-instar larvae and is controlled by single genes that are dominant or semi-dominant (Gallun, 1977; Harris *et al*., 2003; Williams *et al.*, 2003). In the insect, virulence to *R* genes is controlled by non-allelic recessive genes at single loci and operates on a gene-for-gene basis with resistance (Hatchett & Gallun, 1970; Formusoh *et al*., 1996; Zantoko & Shukle, 1997).

The salivary glands of Hessian fly larvae express hundreds of transcripts that are specific to the Hessian fly and do not show homology to any known genes (Chen *et al*., 2010). Identified through an expressed sequence tag (EST) study, SSGPs are hypothesized to be effectors that reprogram the biochemical and physiological pathways of susceptible wheat to benefit the infesting larvae (Chen *et al*., 2004; Liu *et al*., 2007; Zhu *et al*., 2008). SSGPs are identified by three attributes: small size (50-200 amino acids), a secretion signal at the amino terminus, and localized expression in the salivary glands (Chen *et al*., 2007). SSGPs are categorized into families that are defined as related proteins that share secretion signal peptides (Chen *et al*., 2006). The genes encoding these small SSGPs are commonly identified in multi-genic clusters created by gene duplication and diversification with conserved intergenic regions and highly diversified

coding regions (Chen *et al*., 2010). This unusual conservation is a unique feature of SSGPs, suggesting rapid evolution in response to selection pressures (Chen *et al.,* 2010).

If SSGPs are the effectors in the wheat-Hessian fly interaction, then investigating their expression in the context of field populations is important to understanding the underlying biology of the Hessian fly. To date, no data are available on the expression of SSGP transcripts in field collections from different geographic regions. There is population data using microsatellite markers that document the structure of Hessian fly populations (Morton *et al.*, 2011; Morton & Schemerhorn, 2013). Therefore, the focus of the present study was to compare transcript abundance from four previously studied SSGP families in Hessian fly from different geographic regions (Liu *et al*., 2004; Chen *et al*., 2008; Chen *et al*., 2010). We hypothesized that the SSGPs, acting as effectors, should vary in transcript abundance among field collections of Hessian fly from different geographic regions (southeastern United States, Central United States, and the Middle East) due to biological and ecological parameters associated with the collection sites. Significant variation in SSGP transcript abundance among the field collections was observed. SSGP transcript abundance separated field collections into groups that corresponded with the major wheat classes grown in the geographic regions as well as previously described Hessian fly populations.

3.3 Results

Relative abundance among field collections of transcripts encoding SSGPs

To document the abundance of SSGP transcripts across different geographic regions, we carried out a microarray experiment. The Affymetrix microarray was

composed of 444 probe sets dedicated to SSGP sequences identified from an EST project (Chen *et al*., 2004; 2008). Redundancies in alleles and gene copy number can make analyses of SSGP transcript abundance difficult; therefore, duplicates were removed from the analysis, and four previously described families (*vide supra*) were selected for evaluation.

Within each SSGP family under study, heat maps for transcript fold-change (Figure 3.1) were used to visualize the expression of the transcripts for the six field collections relative to the laboratory Biotype GP that has the lowest frequency of virulent genotypes of any Hessian fly biotype and is thought to represent a nascent state with respect to selection pressure from exposure to *R* genes in wheat (Harris and Rose, 1998). Significant $\log_2$ fold-changes in transcript abundance ≥2-fold with significance at $p<0.05$ in the field collections relative to Biotype GP are documented on the heat maps with an asterisk (*). Families 1 and 11 had the fewest transcripts showing significant variation in abundance relative to Biotype GP, while Families 2 and 4 had the greatest (19 significant fold-changes in Family 2 and 14 significant fold-changes in Family 4). Greater decreases in transcript abundance relative to Biotype GP occurred than increases across the four families. Twenty-five genes showed decreased relative transcript abundance, and 13 showed increased abundance. Within the United States, Alabama and Georgia had the greatest number of SSGP transcripts showing significant variation in abundance relative to Biotype GP. In Texas, Colorado, and Kansas, most SSGPs were expressed in levels relative to Biotype GP with few significant fold-changes. Israel also had significant variation in the relative abundance of SSGP transcripts in Families 2, 4, and 11. The complete data sets with GenBank accession numbers and *p* values for significance in

variation of transcript abundance for the SSGP genes are given in Appendix Tables 3.1-3.4.

Within the three geographic regions field collections were made from, the fold-change patterns within each of the SSGP families showed similar trends. In particular, the fold-change patterns for collections from Kansas and Colorado were extremely similar for transcripts across the four SSGP families (Figure 3.1). The southeastern collections also showed similar trends although fold-changes in Georgia were not often as statistically significant as in Alabama (Figure 3.1). Fold-change patterns for transcript abundance in Texas, while not as close as between Kansas and Colorado, were similar to Kansas and Colorado across many transcripts within the four SSGP families. Fold-change patterns for transcripts across the four families in Israel showed three significant variations in transcript abundance (*MDEST789*, *L4H12*, and *MDEST685*) that were distinct from geographic locations within the United States. Fold-change patterns of transcripts within a family were also fairly consistent across geographic regions; however, small differences were present that could correspond to transcripts that might be suitable for further exploration relative to differences in agro-ecosystem parameters.

Hessian fly field collections from all three geographic locations were found to be significantly different ($p<0.05$) in gene expression rates measured as $\log_2$ fold-changes when grouped according to the three different wheat classes cultivated at the geographic locations. The non-metric multidimensional scaling (NMDS) ordination plot (Figure 3.2) shows the grouping of these field collections. The first axis (NMDS1) separated field collections from the southeastern soft-red-winter wheat geographic locations (Georgia and Alabama) from field collections from the Central hard-red-winter wheat locations

(Kansas, Colorado and Texas). The second axis (NMDS2) separated the Middle Eastern (Israel) field collection from hard-red-spring wheat from the collections made in the United States from soft-red and hard-red winter wheat.

Relative transcript abundance from the microarray analysis was further supported by quantitative Real-Time PCR (qRT-PCR) for three SSGP sequences within each family. Significant $\log_2$ fold-changes ≥2-fold in comparison to Biotype GP are listed beside the microarray values (Table 3.1). Significance was scored at $p<0.05$ and is indicated by grey highlighted boxes. The abundance trends identified on the microarray (equivalent, decreased, and increased) are similar to those found with qRT-PCR.

Phylogenetic analyses

Phylogenetic trees were constructed to show the evolutionary relationships within each SSGP family. As members of each family share identical or highly similar secretion signals as well as 5' and 3' noncoding regions, diversity is often found within the coding sequence. SSGPs that share high sequence identity are commonly found in arrays of tandem repeats; thus, phylogenies may reveal SSGPs with increased copy number.

For Family 1, the phylogenetic tree shows two clades (Appendix Figure 3.1). While the general trend for Family 1 showed an increase in transcript abundance, only two SSGP sequences showed a significant increase in transcript abundance (*S12A11* and *G8F2*). Additionally, SSGP sequence *MDEST798* showed a significant decrease in transcript abundance in the collections from Alabama and Georgia. The three SSGP sequences *S12A11, G8F2* and *MDEST798* were located within the first clade.

In Family 2, there was a single large clade containing most of the genes in this family and three smaller clades (Figure 3.3). While, in general, the trend for Family 2 showed lower abundance in comparison to Biotype GP, the fourth clade contained a unique branch. Transcript abundance for SSGP sequences *S20B4*, *S3E10*, and *S8D5* were significantly increased in the collections from Israel, Alabama, and Georgia while transcript abundance for SSGP sequences *S18E7* and *S12G8* were significantly increased only in the collections from Alabama and Georgia. Although not statistically significant, the collection from Texas also showed a trend toward an increase in transcript abundance for *S20B4*, *S3E10*, and *S8D5*, while Colorado and Kansas were equivalent to the Biotype GP reference. BLAST revealed all of the five sequences were located on scaffold X1Random.8 at the same location in the Hessian fly genome. Two additional SSGP sequences showed an increase in transcript abundance outside of clade four (*S14F7* in Alabama and Georgia and *MDEST789* in Israel).

Family 4 (Appendix Figure 3.2) also showed two clades; however, the SSGP sequences showing significant changes in transcript abundance were dispersed throughout the tree. Two SSGP sequences where transcript abundance varied significantly relative to that in Biotype GP (*MDEST685* and *MDEST1048*) grouped together in Family 11 (Appendix Figure 3.3). However, no other correlations between phylogenetic groups and transcript abundance within families were documented in the current study.

3.4 Discussion

The microarray analysis revealed significant differential expression of SSGP transcripts, the candidate effectors in the Hessian fly-wheat interaction, among field collections from different geographic regions relative to SSGP transcript abundance of the Biotype GP reference. The field collections under study and the Biotype GP reference were reared on the same variety of susceptible wheat (cv. Newton, carrying no genes for resistance). Thus, the variations in expression documented are not due to different wheat genotypes. Therefore, the documented variation in expression of SSGP genes is associated with genetic adaptations that accumulated over time from environmental and agro-ecosystem selection pressures (Morton *et al*., 2011, Morton & Schemerhorn, 2013). These selection pressures could influence population structure and evolution in the field and influence the expression of effectors.

In the current study, SSGP transcript abundance in the six field collections fell into three geographic groups based on similarity of SSGP expression and wheat class grown in the geographic region: (1) southeastern United States; (2) Central United States; and (3) the Middle East. These groupings were also in agreement with a previously published population survey that revealed the worldwide structure of Hessian fly populations using microsatellite markers (Morton & Schemerhorn, 2013). Alabama and Georgia are located in the southeastern United States where soft-red-winter wheat varieties are grown, and multiple *R* genes (*H*3, *H*5, *H*6, *H*7*H*8, *H*9, and *H*13) have been deployed in adapted wheat varieties (Cambron *et al*., 2010). Kansas, Colorado, and Texas are in the Central United States, where hard-red-winter wheat cultivars are primarily grown, and *R* genes have not been deployed to the same extent as in the

southeastern United States (Garcés-Carrera *et al*., 2014). In Israel *R* genes are not commercially deployed and hard-red-spring wheat is predominantly grown (Johnson *et* al., 2012). Our microarray analysis of SSGP sequences further support the findings that Hessian fly populations across multiple locations within the United States have low levels of local adaptation that are due to the sharing of agro-ecosystem pressures over large geographic areas (Black *et al.*, 1990; Morton *et al*. 2008). These local adaptations result in micropopulations that vary within the larger overall population.

The equivalency in abundance of transcripts encoding SSGPs between Kansas and Biotype GP is not surprising. The laboratory Biotype GP reference used in the present study was derived from a field collection made in Ellis County, Kansas and maintained under greenhouse conditions since 1986 (Harris & Rose, 1989). The microarray analysis indicates that the diversity in SSGP transcript abundance in the laboratory Biotype GP reference and in the current field collection from Ellis County, Kansas are essentially identical. While deployment of *R* genes *H*3 and *H*6 has occurred in recent years, their usage is neither consistent nor widespread in Kansas. This indicates that field conditions over the last 25 years have resulted in little significant variation between the current Ellis County collection and the reference Biotype GP from agro-ecosystem pressures. The similarity in abundance of SSGP transcripts between Kit Carson County, Colorado and Ellis County, Kansas, located 200 linear miles apart, could also be associated with similarity in environmental and agro-ecosystem selection pressures between the field collection sites in eastern Colorado and central Kansas.

In the Central United States additional agro-ecosystem parameters that can affect Hessian fly populations are: a low number of generations per year and the lack of

successive deployment of multiple *R* genes over time. Generally, there are two generations per year of Hessian fly (fall and spring) that can be controlled by planting after the 'fly-free' date. Therefore, the deployment of *R* genes for control of Hessian fly has not been as extensive as in the southeastern United States. With a limited number of generations per year and dispersed local deployment of resistant cultivars, the number of virulent Hessian fly in the field is slow to accumulate and perpetuates the repeated use of a resistant cultivar (Gould, 1986). Recently, low levels of virulence have been identified in Kansas (Chen *et al*., 2009). A new survey from Texas shows that virulence in the field is increasing as the repeated, annual deployment of multiple *R* genes increases (Garcés-Carrera *et al*., 2014). However, neither shows the widespread high proportion of virulence seen in the southeastern United States (Cambron *et al*., 2010).

In the southeastern United States, climate, availability of alternative hosts, and the successive deployment of *R* genes can affect the biology of Hessian fly. The growing of wheat for forage and the presence of alternative host plants increases the availability of host plants during the warm, wet growing season before the "fly-free date' leading to multiple generations (6-8) (Buntin & Raymer, 1989a; Buntin & Chapin, 1990; Buntin *et al*., 1992; Flanders *et al*., 2014). Together, these factors negate the avoidance practice, as host plants are always readily available for each generation and aids in populations rapidly overcoming resistant wheat cultivars.

The greatest variation in expression among all four of the SSGP families under study occurred in collections from Alabama and Georgia. Successive deployment of wheat cultivars carrying *R* genes has resulted in a decline in *R* gene efficacy, an increase in field populations of Hessian fly that can overcome formerly resistant wheat, and the

highest proportion of local adaptation to *R* genes in the United States (Cambron *et al*., 2010; Ratcliffe, 2013). Between 1986 and 2000, deployment of the *R* genes *H3, H5, H6,* and the gene combination *H7H8* led to the evolution of Hessian fly from being moderately virulent to *H3* to 100% virulent to all four of the deployed genes (Buntin & Raymer, 1989b; Alabama Cooperative Extension System, 2013). This successive deployment of *R* genes could also be a factor influencing SSGP expression in field populations from the Southeast.

Populations near the center of origin for a species can but do not always show the most significant genetic diversity (Harlan, 1974). The Israeli field collection did not show the greatest variation in relative abundance of SSGP transcripts nor the greatest virulence to known *R* genes (Johnson *et al*., 2012). Hessian fly is thought to have coevolved with the genus *Triticum* in the Fertile Crescent, and high frequencies of Hessian fly virulence to the identified *R* genes have been documented in field collections of the fly from Syria (Ratcliffe & Hatchett, 1997; El Bouhssini *et al*., 2009). Climatic differences in temperature and moisture that drive the generational cycle have impacted Hessian fly population structure in Israel (Johnson *et al*., 2010). In Israel >95% of the wheat currently grown is hard-red-spring and has replaced cultivation of local land races and Durum wheat [*T. turgidum* L. ssp. durum (Desf.)]. Since *R* genes for Hessian fly resistance have not been deployed in Israel, indigenous wild wheat as well as alternative grass hosts could be the sources of *R* gene exposure for Israeli Hessian fly populations. Additionally, lack of migration resulting in low gene flow and isolation separates the Israeli populations from neighboring populations such as those in Syria. Thus, the class of wheat cultivated coupled with very different environmental conditions, isolation and

low gene flow, and sporadic exposure to *R* genes could be factors resulting in the differentiation of SSGP expression between the Israeli collection and those made in the southeastern and Central United States.

Fitness costs associated with virulence and adaptive responses should play an important role in plant-parasite coevolution (Montarry *et al*., 2010). Reproductive fitness costs have been associated with Hessian fly virulence to resistance genes *H9* and *H13* in wheat (Zhang *et al.*, 2011). Most of the decreases in relative abundance of transcripts encoding SSGPs were found in the Southeast and this could be associated with fitness costs associated with these SSGPs. However, a clearer understanding of the significance of the differential expression of SSGPs reported here requires knowledge of the role of the SSGPs during interactions with both susceptible and resistant wheat, respectively. Currently, this knowledge is lacking, and this is a hindrance to fully understanding the diversity in expression of SSGPs among Hessian fly populations documented here.

Differential expression of SSGPs could also be attributed to variation in copy number of tandem repeats. Within Family 2, one branch in a clade of related SSGP sequences showed similarity in relative abundance significantly greater than in Biotype GP for flies from Alabama and Georgia (*S3E10*, *S20B4*, *S8D5*, *S18E7*, and *S12G8*) as well as Israel (*S3E10*, *S20B4*, and *S8D5*). In Colorado and Kansas, the relative abundance of these transcripts was equivalent to that in Biotype GP. However, while the collections from Texas were not significantly different from Biotype GP, they did show a trend toward an increase. While BLAST results to the Hessian fly genome sequence positioned all five sequences (*S18E7, S20B4, S3E10, S8D5*, and *S12G8*) on the same scaffold (X1Random.8), problems with the assembly of the Hessian fly genome sequence

often position SSGP sequences at a single locus due to sequence similarity. Further, no sequenced BAC clones were available to resolve whether these five sequences occupy the same location. However, an analysis of the alignments for these five transcripts suggests that variation among the transcripts is greater than would be expected for alleles and could represent tandem repeats that have diverged over time (i.e. paralogs). Future sequencing of BAC clones in this region of the genome should resolve this question.

3.5 Conclusion

A microarray-based study documented significant variation in transcript abundance within a set of four SSGP families among Hessian fly field collections from three distinct geographic regions by the wheat class predominantly grown in the regions. These data support findings from previous studies indicating that ecological and agro-ecosystem dynamics within the three geographic regions exert different selection pressures associated with the different geographic regions and influence Hessian fly population structure.

3.6 Acknowledgements

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3.7 Experimental Procedures

Field collection of insect material

Hessian fly field collections of fall infestations were made from five localities in the United States (Pike County, Georgia; Limestone County, Alabama; Brazos County, Texas; Ellis County, Kansas; and Kit Carson County, Colorado) and one locality in the Middle East (Northern Negev, Magen, Israel). Within the United States, the collection localities represented the southeastern and central geographic regions. The Magen, Israel collection is from the Middle East where Hessian fly and the genus *Triticum* are proposed to have coevolved (Ratcliffe & Hatchett, 1997). The laboratory Great Plains Biotype (GP) that is defined as having a low frequency of virulence to the known *R* genes (Harris & Rose, 1989) was used as a reference biotype for comparison of transcript abundance.

Field collections were made by randomly harvesting approximately 500 infested plants from three to five different areas within infested field (Johnson *et al*., 2012). Collections of fly from the different areas within a field were pooled and treated as one sample. Field collections underwent one cycle of increase in the greenhouse under conditions documented to retain genetic diversity (Foster *et al*. 1977; Black *et al*. 1990). Adults were allowed to emerge, mate, and oviposit under mesh tents on flats of Cultivar 'Newton', that carries no Hessian fly resistance genes. When infesting larvae reached the 3rd-instar within puparia, the flats were sifted to remove soil, and the infested plant material was placed into cold storage at 4^{o}C. Under these conditions larvae retain their viability for up to a year. Infested plant material was removed from cold storage to allow adult emergence to infest Newton wheat in pots for SSGP expression studies.

RNA extraction

Twenty seedlings of Newton wheat were grown in a 10 cm pot containing a sterile mixture of soil and potting mix. When plants reached the 1.5 leaf stage, they were infested with five gravid females by confining them under a plastic cup covering the pot. Four-day old 1st-instar larvae were released from the plants by dissecting the crown with forceps in deionized water. Preliminary analyses have documented that abundance of transcripts encoding SSGPs generally peaks in 1st-instar larvae four days after egg hatch (Shukle, unpublished results). Infestations were carried out with each of the six field collections in triplicate to produce three biological replicates (collections of larvae) for transcript abundance studies. Total RNA was extracted from the collected larvae using the RNAqueous-4PCR kit (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Extracted RNA was frozen at -80°C until further analysis. The RNA samples were used to carry out the microarray hybridization as well as the qRT-PCR analysis.

Microarray hybridizations

A custom microarray (Affymetrix, Santa Clara, CA) containing probes for 444 previously identified Hessian fly SSGP sequences was used in the current study. Microarray processing and hybridization were carried out in the Integrated Gene Expression Facility at Kansas State University following the procedures described in Liu *et al*. (2007). The Ovation RNA Amplification System V2 kit (NuGEN Technologies, San Carlos, CA) was used to convert 50 ng of RNA to anti-sense cDNA that was used for hybridization. The Minelute PCR purification kit (Qiagen, Valencia, CA) was used to

isolate single-stranded cDNA, which were quantified using a Nanodrop-ND-1000 spectrophotometer (Thermoscientific, Waltham, MA). The purified cDNA (3.75 μg) was fragmented and labeled using a FL-Ovation cDNA Biotin module V2 kit (NuGEN Technologies). Labeled fragments were checked for integrity by running the fragmented cDNA through a RNA nano-chip in Agilent Bioanalyzer (Santa Clara, CA). The hybridization mixture was prepared following the protocol included in the FL-Ovation cDNA Biotin module V2 kit and was then injected into the microarrays. After 18 hours of incubation in a GeneChip oven, standard protocol was followed to wash the microarrays, and they were stained with streptavidin phycoerythrin in a GeneChip fluidic station 450 (Affymetrix, Santa Clara, CA). The GeneChip scanner 3000-7G (Affymetrix) was used to scan the microarrays, and GeneChip operating software version 1.4 generated the initial image (.dat) and scaled image (.cel) files.

Microarray analyses

The microarray data from .cel files were analyzed using R (R Development Core Team, 2013) and Bioconductor (Gentleman *et al.*, 2004). The .cel files were imported into R using Affy-software, and microarray data were corrected for technical variation using the RMA procedure (Irrizary *et al*., 2003; Gautier *et al*., 2004). A total of 444 probes sets were assayed on 20 microarrays that were hybridized with DNA from the six Hessian fly field collections plus Biotype GP as the reference.

Differentially expressed genes were identified in the six field collections using hypothesis testing based on a probe-wise modified two-sample *t* test; therefore, 444 hypotheses tests were simultaneously performed for each of the six field collections with

Biotype GP as the reference (Efron, 2010). As a two-sample *t* test is an unreliable estimation of noise variance resulting from the limited number of biological replicates in the microarray data, a modified two-sample *t* test that has better statistical properties for testing differential expression of probes in microarrays was used (Smyth, 2004). Using the modified two-sample *t* test, *p* values were necessary for the differential correction for multiple comparisons to control the number of false positives (the probes that are falsely declared as showing differential signals). False discovery rate (FDR) has greater statistical power than family-wise error rate (FWER) procedures and has optimal properties for simultaneous hypotheses tests in analysis of microarrays where only a small fraction of transcripts are differentially expressed (Efron, 2010). The *p* values from the modified two-sample *t* test were adjusted using Benjamini-Hochberg's (1995) procedure for controlling FDR and obtaining adjusted *p* values. These *p* values can be directly compared with the standard cut-off of 0.05. For each field collection, the transcripts with abundance level changes having calculated adjusted *p* values less than 0.05 were considered to be differentially expressed.

Validation of microarray results by quantitative real-time PCR (qRT-PCR)

To validate the fold-change data observed in the microarray analysis for SSGP transcript abundance in the field collections relative to Biotype GP, three genes from each of the four SSGP families were selected for qRT-PCR analyses. These genes were selected on the basis of equivalent expression across all populations, decreased expression, and increased expression. One µg of DNase-treated RNA was used as template for synthesis of first strand cDNA with random hexamers using the Tetro cDNA

synthesis kit (Bioline, Taunton, MA) according to the manufacturer's instructions. As the Relative Standard Curve method was used, cDNA concentrations were quantified using a Nanodrop ND-1000 spectrophotometer and diluted to 10 ng/µl.

The software Primer Express version 3.0 (Applied Biosystems, Foster City, CA) was used to design gene-specific qRT-PCR primers that would amplify a 50-75 bp fragment between 58-62°C (Appendix Table 3.5). The qRT-PCR was performed on a LightCycler 480 (Roche Diagnostics, Indianapolis, IN) with SensiFAST SYBR no-ROX chemistry (Bioline). The total qRT-PCR volume of 20 µl contained 10 µl 2x SensiFAST SYBR No-ROX mix, 10 µM of a forward and a reverse gene-specific primer, and 40 ng of cDNA template per reaction. No-template samples were included in each PCR plate as negative controls. PCR parameters were as follows: 95°C for 2 min; 40 cycles of 95°C for 5 sec, 55°C for 10 sec, and 72°C for 20 sec. To determine the specificity of the reaction, a melt curve analysis was carried out following qRT-PCR, confirming amplification of a single product. The reactions were set up in triplicate for each of the three biological replicates in a 384-well plate. 18S ribosomal RNA (NCBI Accession No. KC177284.1) was used as an internal reference for transcript normalization. Transcript abundance data were calculated according to the Relative Standard Curve method (ABI User Bulletin #2, http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Relative expression values (REVs) were presented as $\log_2$ fold-change relative to transcript abundance in Biotype GP.

Significant differences in mean REVs associated with transcript abundance in the field collections relative to the Biotype GP reference were identified using a Dunnett

multiple comparisons test (Dunnett, 1955; 1964). Differences were considered significant at $p<0.05$.

Phylogenetic analyses

Clustal X, version 2.1 was used to create an alignment file for the nucleotide sequences (Larkin *et al.*, 2007). The best-fit model of nucleotide substitution was calculated using jModelTest2 (Guindon & Gascuel, 2003; Darriba et al., 2012). Bayesian maximum likelihood trees were constructed under the GTR+I+G model using MrBayes 3.2.1, and the analyses were computed in excess of 1 million generations until the split frequency deviation was less than or equal to 0.01. TreeView 1.6.6 was used to display the phylogenetic trees (Page, 1996Ronquist *et al.*, 2012). All trees were rooted with a lipase-like SSGP outgroup from the Asian rice gall midge *Orseolia oryzae* (Wood-Mason) (GeneBank Accession No: FJ196713) that is a homolog of a lipase-like SSGP for Hessian fly and encodes a protein with a secretion signal (Shukle *et al.*, 2009).

Ordination and analysis of Hessian fly field collections by wheat classes

A non-metric multidimensional scaling (NMDS) approach was used to group the Hessian fly field collections based on variation in wheat classes (i.e. soft-red-winter, hard-red-winter, and hard-red-spring) as a function of the 104 gene expression results within each collection from the microarray analysis. Gene expression data was standardized and a Euclidian distance matrix was calculated as a proximity matrix. To test the statistical significance of the field collection groupings, a permutational multivariate analysis of variance using the same proximity matrix (*vide supra*) was

conducted using the function ‘adonis’ from the R Package ‘vegan’ 2.0.1 (Oksanen *et al*., 2013). The statistical significance was calculated after 99999 permutations.

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3.9 Tables

Table 3.1. Validation of secreted salivary gland protein (SSGP) transcript abundance from the microarray analysis by quantitative real-time PCR (qRT-PCR). Three SSGP sequences (expressed sequence tags, ESTs) were selected from each family; one where transcript abundance was equivalent to that in Biotype GP, one where transcript abundance decreased in one or more field collections, and one where transcript abundance increased in one or more field collections. Significant fold changes ($p<0.5$) are highlighted in gray.

Family 1					**Family 2**			
EST	Location	Log_2 fold change microarray	Log_2 fold change qRT-PCR		EST	Location	Log_2 fold change microarray	Log_2 fold change qRT-PCR
MDEST700	Israel	0.077733468	0.379		*L7D5*	Israel	-0.426608955	0.0123
	Alabama	0.022047536	0.308			Alabama	-1.833044979	-0.174
	Georgia	0.042207697	0.355			Georgia	-0.532575579	-0.159
	Texas	-0.017914909	0.053			Texas	-0.555516459	-0.067
	Colorado	0.060313076	0.361			Colorado	0.052065782	-0.113
	Kansas	-0.032557243	-0.070			Kansas	0.055497373	0.094
MDEST798	Israel	0.382834123	0.428		*MDEST689*	Israel	0.358062704	0.430
	Alabama	-1.120323992	-0.326			Alabama	-2.606239269	-1.587
	Georgia	-2.117407727	-5.904			Georgia	-5.383489305	-2.966
	Texas	-0.224268014	0.493			Texas	-0.260119533	0.333
	Colorado	0.147308884	0.023			Colorado	0.327699189	0.439
	Kansas	0.721278658	0.525			Kansas	0.734141467	0.316

G8F2	Israel	0.036373589	0.971		*S20B4*	Israel	4.812618901	5.514
	Alabama	1.328591004	2.448			Alabama	3.764416323	5.298
	Georgia	0.147813982	1.266			Georgia	5.957124208	6.561
	Texas	-0.020539627	0.467			Texas	2.485906705	2.726
	Colorado	0.198159697	0.654			Colorado	0.865846488	-0.091
	Kansas	-0.024036948	1.026			Kansas	0.01447477	0.438
Family 4					**Family 11**			
MDEST817	Israel	-0.309475207	0.014		*SSGP-11C1*	Israel	0.319793191	0.059
	Alabama	-0.076787128	-0.48			Alabama	0.211247182	0.135
	Georgia	-0.006210489	-0.551			Georgia	0.172950997	0.061
	Texas	-0.085317029	-0.512			Texas	0.175970821	-0.414
	Colorado	-0.127456972	-0.461			Colorado	0.416042074	0.052
	Kansas	-0.193863967	-0.618			Kansas	0.444192339	0.111
MDEST747	Israel	-0.287153977	0.161		*MDEST1048*	Israel	-1.337873533	-1.526
	Alabama	-2.724266649	-0.621			Alabama	-1.743631184	-0.967
	Georgia	-2.955720298	-2.213			Georgia	-2.406430858	-1.331
	Texas	-1.393828877	-0.573			Texas	-0.764068986	-0.551
	Colorado	-0.118984453	-0.306			Colorado	0.009765357	0.115
	Kansas	0.733232293	0.258			Kansas	-0.000425817	0.172
S8A3	Israel	1.486031054	2.290		*MDEST685*	Israel	1.227504498	6.039
	Alabama	0.634000592	1.000			Alabama	0.042517634	2.051
	Georgia	2.513540395	2.139			Georgia	-0.111158045	0.931
	Texas	0.943275829	0.759			Texas	0.118463963	0.836
	Colorado	0.210749525	0.169			Colorado	0.139080735	1.124
	Kansas	-0.773248978	-0.943			Kansas	0.234566853	1.347

3.10 Figures

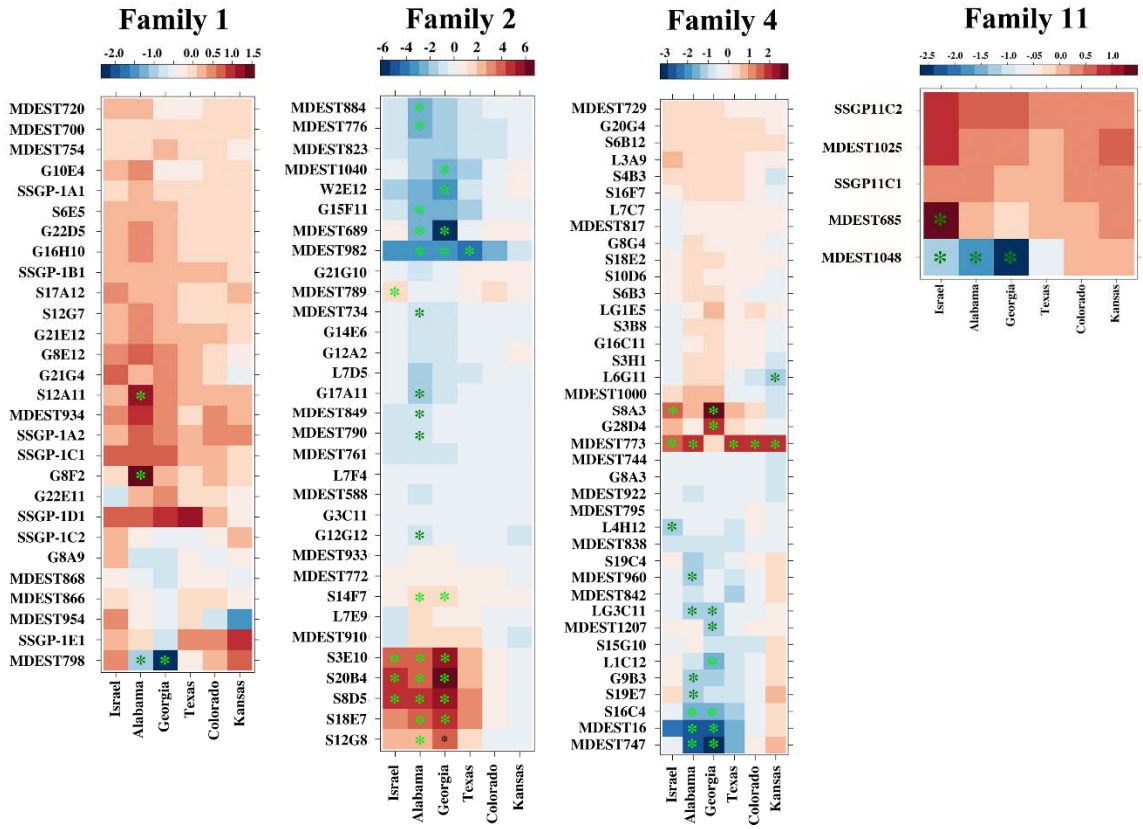

Figure 3.1. Heat map visualizing probe signal intensities for Hessian fly, *Mayetiola destructor* (Say), secreted salivary gland protein (SSGP) transcripts in Families 1, 2, 4, and 11. Fold-changes are normalized $\log_2$ signal intensities for probes in Hessian fly field collections relative to Biotype GP, respectively. Log_2 changes ≥2-fold with significance at $p \leq 0.05$ are indicated by *. Scale shows color code for $\log_2$ fold-changes. Positive fold-changes are indicated by red with darker tones indicating larger fold-change. Negative fold-changes are indicated by blue with darker tones indicating larger

fold-change. Heat maps were drawn using R/Bioconductor. GenBank accession numbers for SSGP transcripts are given in Table S2-S5.

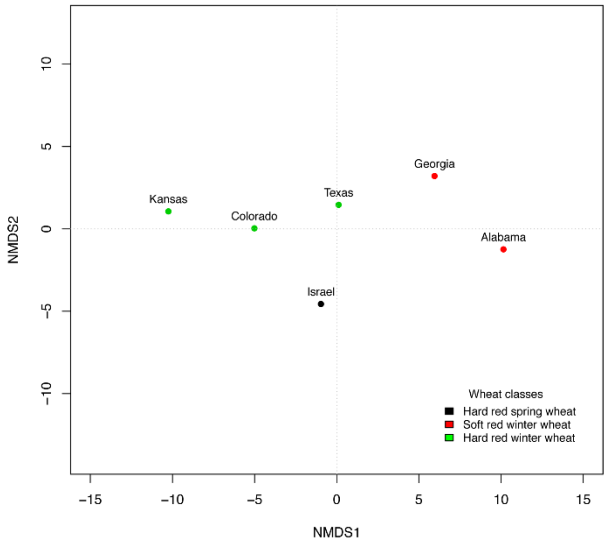

Figure 3.2. Ordination plot using non-metric multidimensional scaling (NMDS). The plot depicts the relationships between Hessian fly, *Mayetiola destructo*r (Say), field collections from the three different geographic locations (southeastern United States – Georgia and Alabama; Central United States – Kansas, Colorado, and Texas; Middle East – Israel) as grouped by the three different wheat classes (i.e. soft-red-winter, hard-red-winter, hard-red-spring) predominantly grown at these locations. The 104 secreted salivary gland protein (SSGP) gene expression data from the microarray results for the Hessian fly collections correlated to the three different wheat classes in the analysis.

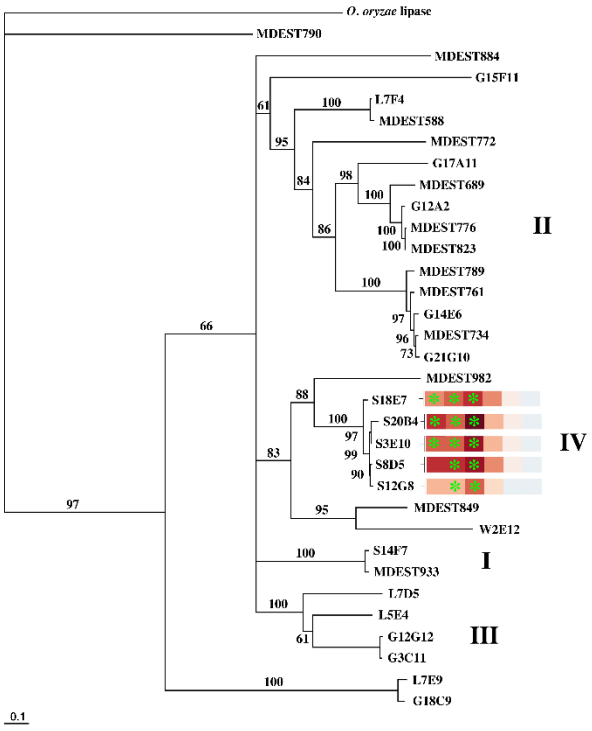

Figure 3.3. Bayesian phylogenetic tree of secreted salivary gland protein (SSGP) transcripts in Family 2. The phylogenetic reconstruction is rooted using the secreted salivary lipase-like gene from the Asian rice gall midge, *Orseolia oryzae* (Wood-Mason) as an outgroup, posterior probability values are located at the nodes, and clades are indicated by Roman Numerals. While the most significant variations in transcript abundance are located within Family 2, there is only a single branch that shows five related genes (*S20B4, S3E10, S18E7, S12G8*, and *S8D5*) with similar transcript abundance patterns as shown in the heat map insert for SSGP transcript probe intensities. These genes have a high degree of nucleotide similarity and as such may represent alleles or paralogs.

CHAPTER FOUR: GENETIC INHERITANCE AND MOLECULAR CHARACTERIZATION OF VIRULENCE IN AN ISRAELI POPULATION OF HESSIAN FLY (*MAYETIOLA DESTRUCTOR*) TO RESISTANCE GENE H13 IN WHEAT

CHAPTER 4. GENETIC INHERITANCE AND MOLECULAR CHARACTERIZATION OF VIRULENCE IN AN ISRAELI POPULATION OF HESSIAN FLY (*MAYETIOLA DESTRUCTOR*) TO RESISTANCE GENE H13 IN WHEAT

4.1 Abstract

It has been well documented that Hessian fly (Hf), *Mayetiola destructor* (Say), larvae produce a large number of secreted salivary effector proteins involved in effector triggered immunity that elicit systemic changes in susceptible wheat as well as trigger the defense response in resistant wheat. One of the avirulence effectors responsible for the interaction between Hf larvae and resistance gene *H13* in wheat has recently been cloned and characterized using Hf populations from the United States (US). Within the US, virulence was shown to be associated with three independent insertions that resulted in a loss of expression of the avirulence gene. The goal of the present study was to test the hypothesis that the inheritance and molecular basis of virulence in a genetically isolated Old World population is the same as in the United States (US). Genetic crosses testing for the inheritance of virulence to *H13* in Hf from Israel revealed that it is controlled by a sex-linked, recessive trait at a single loci. Additionally, no complementation occurred between crosses of virulent US and virulent Israeli Hf, supporting the hypothesis that virulence resides at the same locus in both populations. However, no insertions were identified in the coding region nor upstream or downstream of the coding region. Further, no single nucleotide polymorphisms or frame shifts corresponding to virulence were

identified. These data suggest the molecular basis of virulence in the Israeli population to resistance gene *H13* in wheat is not the same as in the US.

4.2 Introduction

Mayetiola destructor (Say) [Diptera: Cecidomyiidae], commonly known as the Hessian fly (Hf), is an invasive pest of wheat (*Triticum aestivum* L.) in North America. Hf has a significant impact on wheat production in the southeastern United States (US) where 6-8 generations of Hf can occur each year (Buntin and Chapin, 1990). The Midwest and the Pacific Northwest also experience periodic outbreaks; however, in these regions, Hf can still be controlled by planting after the "fly-free" date. This avoidance strategy uses previous knowledge of local weather patterns to calculate when to sow wheat such that seedling plants are not available during the bulk of the Hf fall emergence.

The majority of the Hf life cycle is spent living within the wheat plant (Stuart *et al.*, 2012). Adults are short-lived, poor fliers which do not regularly migrate long distances; human transport is the primary mode of dispersal (Morton *et al*., 2011). In fall infestations, females oviposit onto seedling plants. Upon hatching, larvae crawl down the leaf blade and establish a feeding site at the base of the whorl of the plant. In incompatible interactions, larvae cannot successfully establish a feeding site and die within four to six days (Subramanyam *et al.*, 2008). In compatible interactions, a layer of nutritive cell tissue forms at the feeding site where the larva will continue its growth and development through two feeding instars (Harris *et al.*, 2006). The cuticle sclerotizes and turns dark brown after 21 days. Due to the resemblance, these larvae are commonly referred to as a flaxseed. Third-instar larvae and pupae develop within the flaxseed, and

adults emerge around day 30 unless weather conditions indicate that an overwintering diapause is necessary.

For all wheat production regions world-wide, the best control strategy to prevent Hf damage is the deployment of resistant wheat cultivars. Thirty-five genes for Hf resistance in wheat (*H* genes) have been identified from *T. aestivum*, *Triticum durum* Desf, *Aegilops tauschii* Cross, and *Secale cereale* L., and all express resistance as larval antibiosis (Li *et al.*, 2013). Most are controlled by single-genes that are partially-to-completely dominant (Gallun, 1977; Harris *et al.*, 2003). Likewise, Hf has adapted its own genes, controlled by recessive alleles at different loci, to overcome plant resistance (Hatchett and Gallun, 1970; Formusoh *et al.*, 1996, Zantoko and Shukle, 1997). Therefore, it is necessary to geographically vary deployment of resistant wheat cultivars based on Hf population levels of virulence.

The repeated deployment of wheat resistance gene *H13* in some isolated areas of the southeastern US has created a selection pressure that has allowed levels of virulence to increase creating pockets of virulent Hf field populations (Ratcliffe *et al.*, 1994; Cambron *et al.*, 2010). Recently, the avirulence gene (*vH13*) has been identified and characterized in Hf from the US (Aggarwal *et al.*, 2014). Virulence to *H13* is a sex-linked, recessive trait (Zantoko and Shukle, 1997). It resides on the short arm of chromosome X2 between markers 124 and 134 (Rider *et al.*, 2002; Lobo *et al.*, 2006). *vH13*, like other putative effectors of Hf, is a small protein (116 amino acids) that contains a signal peptide on the amino terminus and does not correspond to any other known genes in the NCBI database (Aggarwal *et al.*, 2014). Three avirulence alleles corresponding to the incompatible interaction with *H13* have been identified; they vary in

sequence only by the copy number of the imperfect repeat (IR) that is located within the second exon. In the compatible interaction with *H13*, mutations leading to virulence were obtained through one of three possible insertions that disrupt the coding region and cause a loss of function (Aggarwal *et al.*, 2014). As Hf males are hemizygous for the X chromosomes, a single copy of a virulent allele conveys virulence while two copies are required in Hf females (Benatti *et al.*, 2010).

The Negev region of Israel (IS), a semi-arid desert that has been reclaimed for vegetable, grain, and fruit production, has been experiencing a multi-year drought. Novel ways to increase yield production have led to an interest in control of Hf through resistant wheat cultivars. Commercial wheat cultivars in Israel are not bred specifically for Hf resistance yet significant virulence to *H3*, *H5*, *H7H8*, *H9*, *H10*, *H11*, *H13*, *H14*, *H16*, and *H23* has been detected (Johnson *et al*., 2012). Unlike the US, Israel's locally adapted wheat varieties retain high genetic diversity from their wild wheat progenitors which may have naturally exposed Hf in Israel to some *H* genes (Poikara and Blum, 1983; Ahern *et al.*, 2009; Johnson *et al.*, 2012). Previous population genetic studies using both mitochondrial and nuclear markers including microsatellites have discovered that Hf in Israel is a possible ancestral lineage of Hf but not the most recent progenitor of Hf in the US, that there are two mixed populations of Hf within the field in Israel, and that gene flow is greatly restricted between Israel and Syria (Johnson *et al.*, 2004; 2011; 2012).

The goals of the present study were to test the following hypotheses: 1) The inheritance of virulence to resistance gene *H13* in Israel the same as in the US; 2) Virulence to *H13* resides at the same locus as previously described in the US; and 3) The

mechanism of virulence to *H13* in field populations isolated by great distance, time, local adaptions, and selection pressures are the same.

4.3 Results

Inheritance of virulence to H13 in Israel

A segregation analysis was performed to test the genetic control of virulence to *H13* in Israel (Figure 4.1). In the F_1 generation, all males displayed the virulent phenotype, and all females displayed the avirulent phenotype. This is consistent with the inheritance of a sex-linked trait in Hf where hemizygous males display the maternal phenotype and inherit a single copy of the gene. As Hf females are diploid for the sex chromosomes, their genotype will be heterozygous. Therefore, the phenotype of the F_1 females indicates that virulence is a recessive trait (♀*vv*, ♂*v*).

Knowing that virulence is a sex-linked, recessive trait, F_2 progeny are expected to sort 1:1 for virulence in both males and females. A chi-square test (X^2 test) of fitness was performed with 1 degree of freedom (Table 4.1). Neonate larvae were collected from 11 F_2 progeny pots resulting in 70 avirulent and 84 virulent larvae. With 1 degree of freedom, the observed results did not deviate from the theoretically expected 1:1 distribution. Virulence to *H13* resides at a single locus in Israel.

Complementation analysis between virulence in Israel and the United States to H13

To determine if the recessive mutations in the Israeli and US populations that controlled virulence to *H13* were at the same locus or at two different loci, a complementation analysis was performed. Reciprocal crosses between virulent female

and male Hf from the US and Israel were made (Figure 4.2). If complementation did not occur, the expected ratio of avirulent to virulent female progenies would be 0:1. No avirulent F_1 female progeny were identified in two-way differential pots. A total of 11 female progeny resulted from crosses between *USvir13* females by *ISvir13* males. A total of 9 female progeny resulted from crosses between *ISvir13* females by *USvir13* males. With 1 degree of freedom, the observed results do not deviate from the theoretically expected ratio (Table 4.1). These results support the hypothesis that the mutation that controls the phenotype of virulence to *H13* resides at the same locus in Israel and in the United States.

DNA sequence analysis of the Israeli alleles controlling virulence and avirulence to H13

The genotype with respect to virulence or avirulence in Israeli males was inferred based on the phenotype of female progeny resulting from crosses between the Israeli males and *USvir13* females. Using the same primers for which *vH13* was cloned and characterized in the US, no insertions in the coding region could be detected in avirulent nor virulent Israeli males. The 5' secretion signal (MKFVVAFMVLAICNQAFA) was intact as was the intron-exon boundary located between the glutamine and alanine residues within the secretion signal. Sequencing 452 bases upstream of the *ATG* and 197 bases downstream of the *TAA* did not reveal any insertions or deletions in Israeli males of either inferred genotype.

Analysis of the Israeli males revealed at least 30 SNPs within the coding region of the candidate *vH13* gene. Fifteen of the SNPs were transitions, and fifteen were transversions. However, none of these SNPs, in single or in combination, appeared to

correspond with the inferred virulent or avirulent Israeli male genotypes. Two alleles were found to be shared in common between avirulent and virulent Israeli males. Alleles from avirulent Israeli males displayed a greater gene diversity (0.9923 ± 0.0072) than those from virulent Israeli males (0.5771 ± 0.0783).

vH13 contains an imperfect repeat, a series of 12 amino acids near the end of the second exon, that can vary in number from a single copy to three copies (Aggarwal *et al.*, 2014). The IR was present in both avirulent and virulent P_1 males. Of the males under study, sequence analysis revealed that 1-2 copies of the IR were prevalent in Israel. While a few individuals had three copies of the IR, it was very rare allele.

A comparison of the avirulent US *vH13* alleles with the alleles from avirulent and virulent Israeli males was also conducted. An analysis of molecular variance (AMOVA) showed that more variation occurred within groups (76.47%) than among groups (23.53%). F_{ST} values revealed that the alleles from virulent Israeli males did not appear to significantly differ from the alleles in the US avirulent line (Table 4.2).

Transcriptomic analysis between Israeli and US alleles for virulence/avirulence to H13

To further study virulence to *vH13* in the Israeli population, lines avirulent (*ISavrH13*) and virulent (*ISvirH13*) to *H13* were selected from the bulk Israeli population. Using gene-specific primers, reverse transcription PCR (RT-PCR) of single larvae resulted in amplicons from both lines. No insertions, deletions, or SNPs corresponding to virulence or avirulence were detected. Genetic diversity in alleles from the *IsavirH13* line (0.5181 ± 0.0974) and from the *ISvirH13* line (0.6044 ± 0.0759) was more similar than in the DNA sequence analysis as fewer alleles were recovered.

AMOVA analysis of transcripts from avirulent Israeli larvae, virulent Israeli larvae, and avirulent US larvae indicates that there is more difference within the group (82.54%) than between groups (17.46%). F_{ST} supports the division between all three groups (Table 2). Of specific interest is the large value assigned to the difference between the US and Israeli alleles.

4.4 Discussion

Effector triggered immunity (ETI) is used to describe the coevolution of adaptations between host and pest to overcome the other's resistance mechanisms involved in the plant-pest interaction (Thompson and Burden, 1992; Brown and Tellier, 2011). ETI is an expanded model of the gene-for-gene interaction that focuses on the proteins (effectors) involved in the interaction (Chisholm *et al*., 2006, Dangl *et al.,* 2013). *Avr* genes are effectors that arose within the insect to colonize the host plant, and these effectors redirect resources from the host to the insect (Hogenhout *et al*., 2009). Hf serves as a model for ETI (Harris *et al*., 2015). In Hf, 5% of the genome is composed of putative effector genes, many with no known homology to other organisms (Zhao *et al*., 2015). These effectors are known as SSGPs (secreted salivary gland proteins), and each SSGP can be identified by the amino terminal secretion signal, expression in salivary gland tissues, and small size (Chen *et al.*, 2004). Families of SSGPs can be identified by the similarity of their secretion signal as well as the conservation of the non-coding intergenic regions found in tandem arrays of SSGPs (Chen *et al.*, 2010).

One family, SSGP-71, has been studied to great detail (Zhao *et al.*, 2015). It comprises the largest group of effectors in Hf and shares homology to E3 ubiquitin

ligases in plants. The candidate *Avr* genes for *H6* and *H9* fall within this family. This large reservoir of effectors contains many gene duplicates including alleles lacking function which could lead to virulence to wheat's *H* genes, the genes responsible for Hf resistance. When selection pressures are applied from cultivar-specific *H* genes, Hf can easily overcome resistance due to the null alleles of *Avr* genes at minor frequencies within the populations (Zhao *et al.*, 2015). Field populations have been shown to overcome resistance within 6-8 years of successive *H* gene deployment (Gould, 1986; Cambron *et al.*, 2010). Therefore, it is necessary for ongoing research to continue to characterize *Avr* genes in order to understand the molecular basis of the Hf's ETI response.

vH13 is a unique SSGP. It is a singleton with no known homology to other organisms, no identifiable domains, and no classification within the currently described SSGP families. Due to this exceptionality, there are no pools of related SSGPs from which null alleles can arise to provide virulence to *H13*. Each mutation leading to virulence must arise within the *vH13* gene. Through genetic crosses, virulence to *H13* in Israel was found to be a sex-linked, recessive trait at a single locus. This is congruent with the description of the trait in the US (Zantoko and Shukle, 1997). No complementation occurred between virulent US and virulent IS crosses signifying that the same locus, *vH13*, is associated to virulence to *H13*. Therefore, it is of great interest that the mutations which cause the loss of function in *vH13* in the US are absent in Israel.

At least three unrelated insertions leading to a loss of function of *vH13* have been detected within the southeastern US: 1) 4.7kb inserted at the end of exon 1, 2) a 254bp insertion at the intron-exon boundary, and 3) a 461bp insertion within exon 2 (Aggarwal

et al., 2014). Each insertion arose independently, and these insertions disrupt the reading frame creating an inactive protein that is not found to be expressed in virulent US larvae. However, in Israel, the *vH13* transcript is expressed in both the avirulent and virulent lines. As no simple mutations were identified, the mechanism for virulence in Israel must employ a different strategy to overcome *H13*.

Hf in Israel has been shown to be quite genetically divergent from populations in the US despite displaying a comparable phenotypic response to many *H* genes (Cambron *et al.*, 2010; Johnson *et al.*, 2012). Furthermore, population studies have discerned that Israel is also genetically distinct from Spain, Morocco, Kazakhstan, and Syria and that US populations have diverged significantly from their Old World predecessors (Johnson *et al.*, 2004; 2011; Morton and Schemerhorn, 2013).The large F_{ST} values observed between the *vH13* US and IS alleles lend further support to the divergence between the two sites. Isolation by time and distance will lead to the accumulation of divergent mutations within a species. The lack of gene flow may be why the three virulence alleles identified in the US were not identified in Israel.

In the US, a single population can span large geographic distances. Population structure can be correlated to differential SSGP expression corresponding to the wheat class planted within the geographic area (Johnson *et al.*, 2015). Currently, agricultural practices in Israel use cultivars of *T. aestivum* from North Africa, but, historically, locally adapted *T. durum* cultivars grew alongside the native wild wheats (Atzmon and Schwarzbach, 2004; Ozbeck *et al.*, 2007). Novel hybrids generated naturally in the field are important to generating new phenotypes with ecological benefits, like resistance to pests (Ahern *et al.*, 2009; Agrawal, 2001). As explained in the ETI model, as novel

adaptations occur in the host, novel mutations must also occur in the pest such that the plant cannot detect the insect (Chisholm, 2006; Dangl *et al.*, 2013). Thus, the mutations to *vH13* seen in the US may not be identified in Israel due to the differences in the Hf response to the cultivars of wheat planted both currently and historically.

Hf virulence naturally occurs in the field at significant levels in Israel. Unlike the US where selection pressures from select *H* genes are constant, resistant cultivars are not purposely deployed in Israeli agriculture. Yet, phenotypically, Israel displays a similar virulence profile to that found in the US. Located near the Fertile Crescent, Israel is known for great genetic diversity in wild and cultivated grasses, of which many can serve as hosts for Hf (Poiarkova and Blum, 1983; Harris *et al.*, 2015). Of the 35 described *H* genes, few originated in common wheat; many originated from other grass species including rye, barley, emmer, and goat grass before they were naturally hybridized into common wheat (Liu *et al.*, 2005). Therefore, Hf in Israel has encountered a diverse selection of *H* genes as novel hybrids and locally adapted agricultural cultivars perpetuated in the region. This leads to novel mutations within the local Hf population. As the Hf populations in the US and Israel have been shown to be divergent, it is logical to assume that novel mutations in *Avr* genes may not be shared between such distantly related populations. In the case of *vH13*, these novel mutations were not easily detected. Further research must be done to identify the mechanism of virulence to *H13* in Israel.

4.5 Conclusions

Genetic characterization of virulence to *H13* in Israel has been revealed to be a sex-linked, recessive trait at a single locus. Complementation analysis has documented

that the recessive mutations controlling virulence to *H13* in the US and in the Israeli population are at the same locus. However, unlike in the US, no insertions within the coding region or upstream or downstream of the coding region that could cause a loss of function mutation leading to virulence were identified. Additionally, a transcript was identified from individual larvae from both the avirulent and virulent Israeli lines, and no point mutations or frame shifts corresponding to virulence were identified. Therefore, the mechanism for virulence to *H13* in Israel is divergent from that in the US and further research is required to elucidate the molecular basis of virulence to H13 in the Israeli population.

4.6 Acknowledgements

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4.7 Experimental Procedures

Hessian fly cultivation and collection

Infested straw from Magen, Israel was collected in the fall of 2009 by Phyllis Weintraub of the Agricultural Research Organization, Volcani Center, Gilat, Israel (Johnson *et al.*, 2012). Flats of cultivar Newton, a highly susceptible wheat line that carries no genes for Hf resistance, were placed under a large mesh tent in the greenhouse,

and adults were allowed to emerge, mate, and oviposit (Black *et al.*, 1990). This large-scale greenhouse increase allows for random mating while increasing the population size and retaining the genetic diversity found within the sampled collection (Foster *et al.*, 1977). This field collection (Magen) has been maintained under these conditions with annual increases using a minimum of six flats of Newton. The *USvir13* Hf line, isolated originally from a field collection in Georgia, has been maintained in culture under the same greenhouse conditions for several years (Behura *et al*., 2004).

A significant level of virulence to the wheat resistance gene *H13* was previously identified in Magen (Johnson *et al.*, 2012). Cultivar Molly carries this *H* gene and was used in this experiment to isolate lines of Magen Hf that were susceptible to *H13* (*ISavrH13*) and were resistant to *H13* (*ISvirH13*). Two-way differentials were created by planting both Newton and Molly seeds in the same four-inch pot; seeds were separated by distance and labeled. Plants were seeded and grown in the greenhouse until the 1-2 leaf stage. Females were allowed to mate and oviposit under a cup cage onto the two-way differentials. After infestation, pots were transferred into growth chambers (18ºC under a 16h photoperiod) until the formation of the flaxseed stage (day 21 post-hatch). For adult emergence, pots were removed from the chambers, covered with cup cages, and placed at room temperature.

For all generations, only pots where eggs were laid and hatched onto both varieties were used, and pots were scored for survival between days 6-8 post-hatch. This time point was chosen because dead, avirulent larvae (dead reds) can be easily identified on resistant plants due to their small size and shriveled, red appearance. The pot was labeled avirulent if the progeny survived only on Newton with dead reds found on Molly

or virulent if the progeny survived on both Newton and Molly. To create the $F_2 – F_7$ generations, avirulent males and a single avirulent female were mated onto differential pots as were virulent males and a single virulent female. Given the biology of *vH13* as previously described, heterozygous avirulent mothers can yield hemizygous virulent male offspring. Therefore, when virulence was detected in the avirulent line, all pots were discarded. The isolated lines are currently maintained on bulk increases of Newton (*ISavrH13*) and Molly (*ISvirH13*). Before use for subsequent experimentation, single females are allowed to mate and oviposit on two-way differentials to score for virulence as well as to create progeny pots of monogenic, virgin adults.

A segregation analysis was performed to test the genetic control of virulence to *H13* in Israel. Hf females contain a full complement of both autosomes and sex chromosomes (A1A2X1X2/A1A2X1X2) while males retain both autosomes and a single copy of the X chromosomes inherited from the mother (A1A2X1X2/A1A2OO). Putative crosses are displayed in Figure 4.1 with each outcome displayed in an avirulent to virulent ratio. The *ISvirH13* female mated to *ISavrH13* male was selected as the most informative cross. If the trait is autosomal, the F_1 generation would segregate 1:0, and the F_2 would segregate 3:1; the reciprocal cross would also yield the same ratios. If the trait is sex linked, the F_1 generation would segregate 1:0 for females and 0:1 for males while the F_2 would segregate 1:1 in both sexes. In the reciprocal cross, the ratios would be 1:0 in both sexes in the F_1, and in the F_2, females would be 1:0, and males would be 1:1. If the trait for virulence was in two, independent autosomal loci, the F_1 generation would segregate 1:0, and the F_2 would segregate 63:1. The reciprocal cross would yield the same ratios. If the trait for virulence was in two, independent sex-linked loci, the F_1

would segregate 1:0 in females and 0:1 in males while the F_2 both sexes would segregate 3:1. The reciprocal cross would yield 1:0 in the F_1, and females would be 1:0 and males would be 3:1 in the F_2.

Virgin *ISvirH13* females were mated to virgin *ISavrH13* males on two-way differentials. The subsequent female F_1 progeny were then crossed onto pots of Newton. For each progeny pot, leaves were removed from the plants and placed in a beaker of water the night before eggs were to hatch. A single neonate larva was placed onto the plant via a 2µl droplet of 0.001% NP40. At day six post-hatch, each plant was destructively sampled to score for virulence in the F_2. A X^2 test of fitness was performed to test the hypothesis that the observed values of the F_2 cross are the same as the expected theoretical distribution (1 avirulent: 1 virulent segregation in both sexes) with 1 degree of freedom.

To test the hypothesis that virulence to *H13* resides at the same locus in both Israel and the US, single virgin females (*USvirH13*) were mated to virgin *ISvirH13* males on two-way Newton and Molly differential pots. The reciprocal cross was made as well. Pots where eggs were not laid nor hatched onto both seed types were discarded from the experiment. Virulence was assessed at day six post-hatch by destructively sampling Newton and Molly plants. Plants were also checked for flaxseed at day 21 to ensure that larval development had continued. The remaining larvae were allowed to proceed through their development in order to score the sex of each individual cross. As males would only inherit their mother's genotype, only the results from female progeny were scored. A X^2 fitness test was performed to test the hypothesis that the observed values of the reciprocal crosses are the same as the expected theoretical distribution (0 avirulent: 1

virulent) if virulence resides at the same locus in Israel and the US with 1 degree of freedom.

In the US, insertions into the coding sequence of *vH13* are the cause of the lack of function mutations that lead to virulence thus the gene was isolated through PCR for cloning and sequencing. All collections were made through crosses prepared on two-way differentials, and all samples were flash frozen in liquid nitrogen and stored at -80°C until DNA or RNA isolation. Two collections were used to study *vH13* in Israel and are listed as follows.

1) *USvirH13* virgin females were mated to Israeli males of unknown virulence from the bulk population increase. The fathers were collected after mating for later extraction of DNA. The female progeny were scored for virulence to indicate the paternal genotype where avirulent progeny indicate an avirulent father and virulent progeny indicate a virulent father.

2) To collect individuals of known virulence, *ISavrH13* virgin females and males were crossed together as were *ISvirH13* virgin females and males. Five individual early second instar larvae were collected from more than ten individual crosses for both lines. Samples were stored for later RNA isolation. Adults were allowed to emerge from each progeny pot in order to score the sex; only males were selected for further analysis.

Molecular analyses

DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with a slight modification to the protocol for use with small tissue amounts. Individual flies were ground using small plastic pestles inside 1.5ml microcentrifuge tubes containing 25µl of lysis buffer. After grinding, additional buffer containing 20µl of

proteinase K was added to a final volume of 180µl. Tubes were placed overnight in a waterbath set to 56°C before resuming the Qiagen protocol. Elutions were performed with 100µl of the provided elution buffer. PCR was performed using 2µl of DNA template; -Mg 10X PCR Buffer, 50mM $MgCl_2$, 1U Platinum High Fidelity *Taq* (Life Technologies, Grand Island, NY); 10 mM PCR nucleotide mix (Promega, Madison, WI); and 10µM of both forward and reverse primers (Eurofins MWG Operon, Huntsville, AL) in a 25µl reaction volume. All PCRs were performed in a DNA Engine Dyad PTC-220 thermocycler (BioRad, Hercules, CA), and primers are listed in Table 4.3. The amplification cycle was as follows: 94°C/1min; 35 cycles of 94°C/30s, 55°C/30s, 68°C/30s; 68°C/10min. PCR reactions were purified by using the QIAquick gel extraction kit (Qiagen) prior to cloning.

RNA was isolated using the RNaqueous-4PCR kit (Life Technologies) with the previously described protocol modification for small tissue volumes. Elution was performed using 100µl of the provided buffer. All samples were *DNase* treated using the Turbo DNA-free kit (Life Technologies) for 30 min at 37°C before being concentrated to 20µl using the linear acrylamide and 5M ammonium acetate provided in the RNaqueous-4PCR kit. RNA samples were translated into gene specific cDNA using the SuperScript III one-step RT-PCR system with Platinum *Taq* high fidelity DNA polymerase (Life Technologies). A minimum of 20ng of template was used to perform each reaction with the *vH13F* and *R* gene specific primers. The cycle was performed as follows: 1 cycle of 55°C/30min, 94°C/2min; 40 cycles of 94°C/15s, 55°C/30s, 68°C/1min; and 1 cycle of 68°C/5min.

Cloning of all *vH13* bands were performed using the TOPO TA Cloning kit for sequencing with One Shot TOP10 cells (Life Technologies). Three clones per reaction containing the fragment of interest were selected for sequencing through the Purdue University Genomics Facility. All plasmid isolations were prepared using the Wizard *Plus* SV miniprep DNA purification system (Promega). Sequencing results were aligned using Bio Edit 7.2.5, and a consensus of the three individual clones was generated for each sample (Hall, 1999). Arlequin 3.11 was used to calculate AMOVA and F_{ST} (Excoffier et al., 2005).

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4.9 Tables

Table 4.1. X^2 goodness of fit test results for both the genetic crosses studying the genetic control of virulence to *H13* in Israel and complementation of virulence in Israel and the United States.

Segregation Analysis

Mating Female	Male	N	Phenotype Avirulent	Virulent	Exp. Ratio	X^2	*P*
ISvir13 F1	*ISavr13 F1*	154	70	84	1:1	1.273	3.841

Complementation Analysis

Mating Female	Male	N		Phenotype Avirulent	Virulent	Exp. Ratio	X^2	*P*
USvir13	*ISvir13*	11	8	0	19	0:1	0	1
ISvir13	*USvir13*	9	10	0	19	0:1	0	1

Table 4.2. F_{ST} values for the *vH13* alleles as calculated by Arlequin. Significant values ($p<0.05$) are highlighted in gray.

Alleles retrieved from DNA sequencing

	ISavr13	*ISvir13*	*USvir13*
ISavr13	0.000	-	-
ISvir13	0.21971	0.000	-
USvir13	0.06228	0.42677	0.000

Alleles retrieved from cDNA sequencing

	ISavr13	*ISvir13*	*USvir13*
ISavr13	0.000	-	-
ISvir13	0.06814	0.000	-
USvir13	0.25215	0.54899	0.000

Table 4.3. Primers used in the amplification of the *vH13* gene.

Name	Location	Sequence (5'-3')
vH13 F	4 AA 3' of the *ATG*	GGT TGC TTT TAT GGT TTT GG
vH13 R	9 AA 5' of the *TAA*	CTT CTC CTT CTT GGC TGT C
vH13 5'F	151 AA 5' of the *ATG*	GCA TCG CAA ACA AAA GCA AAA T
vH13 5'R	8 AA 3' of *ATG*	ATA AAA GCC ACA AAT TTC AT
vH13 3'F	9 AA 3' of the *TAA*	CAG CCA AGA AGG AGA AGA AAT
vH13 3'R	66 AA 3' of *TAA*	GCA ATT TTT AAG GAA CGA CGT GCA

4.10 Figures

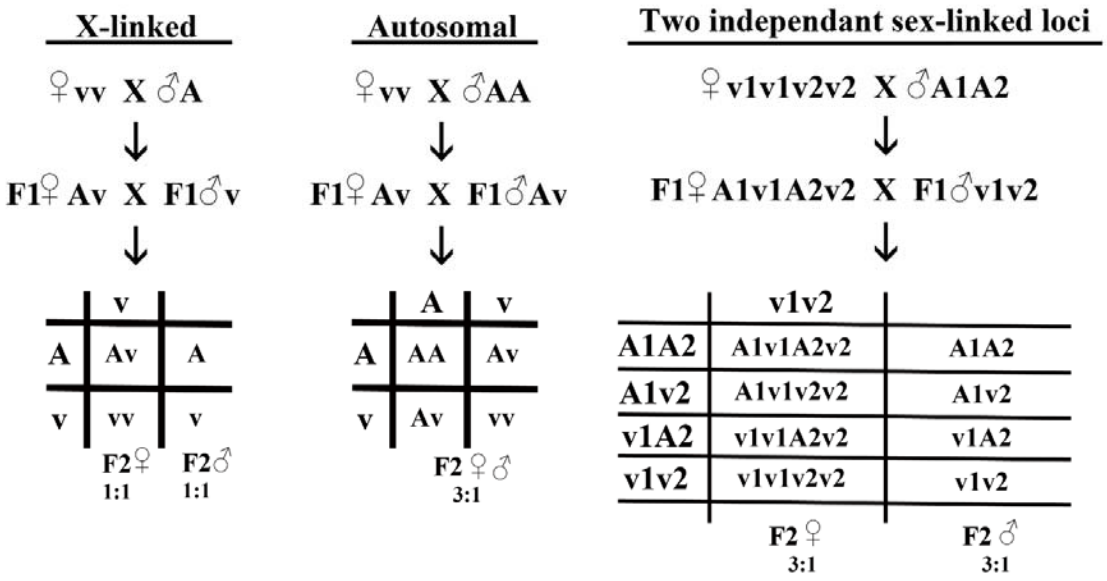

Figure 4.1. To test the genetic control of virulence to *H13* in Israel, virulent females were mated to avirulent males. The results of the putative crosses are as follows. If the trait is X-linked, the F_1 generation would segregate 1:0 for females and 0:1 for males while the F_2 would segregate 1:1 in both sexes. In the reciprocal cross, the ratios would be 1:0 in both sexes in the F_1, and in the F_2, females would be 1:0, and males would be 1:1. If the trait is autosomal, the F_1 generation would segregate 1:0, and the F_2 would segregate 3:1; the reciprocal cross would also yield the same ratios. If the trait for virulence was in two, independent autosomal loci (not shown), the F_1 generation would segregate 1:0, and the F_2 would segregate 63:1. The reciprocal cross would yield the same ratios. If the trait for virulence was in two, independent sex-linked loci, the F_1 would segregate 1:0 in females and 0:1 in males while the F_2 both sexes would segregate 3:1. The reciprocal cross would yield 1:0 in the F_1, and females would be 1:0 and males would be 3:1 in the F_2.

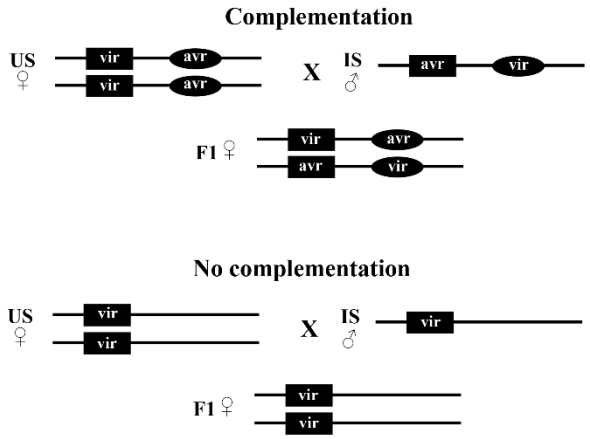

Figure 4.2. If complementation occurred between the US and IS populations, then F_1 females would display the avirulent phenotype. If no complementation occurred between the US and IS because virulence is controlled at the same locus in both populations, F_1 females would display the virulent phenotype.

CHAPTER FOUR: USE OF FEMALE PHEROMONE BAITED TRAPS AND MOLECULAR MARKERS TO ASSESS VIRULENCE IN FIELD POPULATIONS OF HESSIAN FLY (DIPTERA: CECIDOMYIIDAE) TO RESISTANCE GENE *H13* IN WHEAT

CHAPTER 5. USE OF FEMALE PHEROMONE BAITED TRAPS AND MOLECULAR MARKERS TO ASSESS VIRULENCE IN FIELD POPULATIONS OF HESSIAN FLY (DIPTERA: CECIDOMYIIDAE) TO RESISTANCE GENE *H13* IN WHEAT

5.1 Abstract

Mayetiola destructor (Say) is a serious pest of wheat in the southeastern United States. The Hessian fly (Hf) uses effector triggered immunity to overcome wheat's *H* genes, the resistance genes responsible for providing protection from the Hf. As the most effective form of Hf control employs the planting of resistant wheat cultivars containing one or more *H* genes, frequent Hf sampling is required to monitor the level of virulence present in locally adapted populations. Here, we present a novel assay for detecting virulence in the field. Hf males were collected in Alabama, North Carolina, and South Carolina using sticky traps baited with Hf sex pheromone. The Hf gene that controls virulence in Hf to resistance gene *H13* in wheat has recently been cloned and characterized, and diagnostic molecular markers for the alleles controlling avirulence and virulence are now available. Utilizing two separate PCR reactions, the six alleles for avirulence and virulence can be scored based on band size. Our results support the most recent survey of virulence to *H13* as scored through the testing of live insects infesting *H13* wheat in the greenhouse. Throughout the southeast, all three avirulence alleles can be identified while the most frequently identified allele for virulence corresponded to a 5kb insertion into exon 1 of *vH13*. In South Carolina, the PCR assay is sensitive enough

to detect the spread of virulence into two counties previously documented as 100% susceptible to *H13*.

5.2 Introduction

The Hessian fly, *Mayetiola destructor* (Say), is a gall midge found throughout the wheat (*Triticum aestivum* L.) producing areas of the United States (US). Females lay their eggs onto the adaxial surface of the leaf blade, and neonate larvae crawl down to the base of the whorl of the plant. In compatible interactions, a feeding site is established where a layer of nutritive cells forms to redirect the plant's nutrients to the virulent insect (Harris *et al.*, 2006; 2010). In the incompatible reaction, larvae die within 4-6 days as the avirulent insect cannot establish a feeding site (Subramanyam *et al.*, 2008). Damage to wheat plants occurs solely in the compatible interaction; the lack of nutritional resources for the plant leads to loss in grain yield and potential death of seedling plants (Byers and Gallun, 1972; Buntin, 1999).

The most effective method of Hf control is through the use of resistant wheat cultivars which provide protection from Hf though *H* genes that lead to the incompatible interaction (antibiosis) in avirulent larvae. To date, 35 *H* genes have been described (Liu *et al.*, 2005; Li *et al.*, 2013). The repeated deployment of resistant cultivars has created a selection pressure in the field that has led to the creation of locally adapted populations which are virulent to one or more *H* genes (Lidell and Schuster, 1990; Smiley *et al.*, 2004; Watson, 2005; Chen *et al.*, 2009; Cambron *et al.*, 2010). In the southeastern US where 6-8 generations of Hf can occur per year, planting wheat to avoid the bulk emergence of Hf is impossible (Buntin and Chapin, 1990). Thus, successive deployment

of multiple *H* genes has become routine and resistance to four or more *H* genes is common (Buntin *et al.*, 1992; Ratcliffe *et al.*, 2000; Cambron *et al.*, 2010).

Recently, the HF gene (*vH13*) controlling virulence to the wheat resistance gene *H13*, has been cloned and characterized (Aggarwal *et al.*, 2014). Virulence is expressed as a recessive, sex-linked trait (Zantoko and Shukle, 1997).Three alleles with large insertions were identified in virulent individuals: 1) a 461bp insertion within exon 2, 2) a 254bp insertion at the intron-exon boundary, and 3) a 4.7kb inserted at the end of exon 1. Three alleles were also identified in avirulent individuals; these alleles vary only in the copy number of an imperfect repeat (IR) which is 12 amino acids within exon 2 that can be repeated up to three times.

Seven components have been identified from Hf female ovipositor extracts of which a mixture of five of these components are necessary to attract males (McKay and Hatchett, 1984; Anderson *et al*., 2009). A synthetic pheromone blend of these five chemicals is commercially available and has been shown to reliably draw Hf males in the field to sticky traps (Anderson *et al.*, 2012). DNA, sufficient for PCR analysis, has also been shown to be recoverable from Hf males captured on sticky straps (Chen *et al.*, 2014).

The object of the present study was to assess virulence to *H13* in the field by collecting Hf males on sticky traps, isolating their DNA, and using molecular markers for *vH13* to score virulence based on the known size variant alleles for avirulence and virulence in the southeastern US.

5.3 Materials and Methods

Insect collection

Hf pheromone lures preloaded into polyethylene dispensers were obtained from Pheronet (Alnarp, Sweden) and kept at -20˚C until used. Lures were attached to sticky inserts that were then loaded into a Delta trap (Trécé Inc.). Traps placed more than 30cm above the canopy will not collect Hf as the insect does not fly at such height; therefore, each trap was suspended from a wooden stake so that it was within or just above the wheat canopy (Anderson *et al.*, 2009). Both sticky inserts and lures were replaced after 7 days in the field.

Multiple location sites were selected across the southeastern United States. In Alabama, collections were made in Colbert, Hale, and Marengo counties. Collections were made in Florence and Lee counties of South Carolina. In North Carolina, collections were made in Alamance, Onslow, Tyrell, and Union counties. Sticky inserts were removed from the traps, covered with wax paper, and stored in a plastic freezer bag at -20˚C. Flies were removed from the sticky inserts in a toluene bath with gentle shaking for 10 minutes. Hf were morphologically identified with a light microscope and stored in individual tubes for later analysis.

Molecular Analyses

DNA was isolated from individual flies using the DNeasy 96 Blood and Tissue kit (Qiagen, Valencia, CA) in order to have stable DNA that would allow samples to be archived. Samples were eluted with the provided buffer in a 100µl volume. PCR was performed in a DNA Engine Dyad PTC-220 thermocycler (BioRad, Hercules, CA). Two

separate PCR reactions were performed per sample: *vH13*F/*vH13*R and *vH13*F/*vH13*5kbR (Table 5.1). PCR was performed using 5µl of DNA template; 5X GoTaq Flexi Green Buffer, 50mM $MgCl_2$, 2.5U GoTaq Flexi (Promega, Madison, WI); 10 mM PCR nucleotide mix (Promega); and 10µM of both forward and reverse primers (Eurofins MWG Operon, Huntsville, AL) in a 25µl reaction volume. The amplification cycle was as follows: 94°C/1min; 35 cycles of 94°C/30s, 55°C/30s, 72°C/30s; 72°C/10min. PCR products were visualized on 2% agarose gels. Alleles were scored with the aid of VisionWorksLS visualization and analysis software (UVP, Upland, CA). Alleles and their sizes are described as follows: *Avr1* with 3 IR = 411bp, *Avr2* with 2 IR = 383bp, and *Avr3* with 1 IR= 329bp, *vir1* with the 461bp insert = 872bp, *vir2* with the 256bp insert = 663bp, and *vir3* with the 5kb insert = 551bp.

5.4 Results and Discussion

The Hessian fly, like other cecidomyiids, has a complicated system of chromosomal inheritance. Females are diploid for the sex chromosomes (X1X2/X1X2) whereas males are hemizygous and inherit the maternal copy of the sex chromosomes (X1X2/OO) (Harris *et al.*, 2015). As *vH13* is located on the short arm of X2, male Hf will produce a single amplicon during PCR (Rider *et al.*, 2002; Lobo et al., 2006). This makes pheromone traps ideal for sampling new locations and improves upon previous collection methods that require large volumes of infested straw to be shipped to test the reaction of adults to an array of *H* genes in flat tests (Cambron *et al.*, 2010).

Alleles of *vH13* were scored for size after PCR band separation on 2% agarose gels (Table 5.2). A total of 1,383 Hf males were surveyed from 9 counties across three

states. Of the sampled Hf, 90% were found to contain one of the three avirulence alleles while 10% contained a virulent allele. The most frequent avirulence allele detected was *Avr2* (45%) while *Avr1* and *Avr3* were found at 28% each. The most common virulence allele (*vir3*) contained the 5kb insertion into exon 1 (88%). *Vir1* (1%) and *vir2* (10%) were rare.

Alternative control practices including seed treatments, foliar insecticides, and planting cover crops that are not alternative hosts of Hf are extensively used in North Carolina (Reisig *et al.*, 2013). However, recent years have seen a large increase in yield loss due to Hf forcing more growers to utilize varieties that contain one or more resistance genes. Samples from four counties identified 105 avirulent and 4 virulent Hf. *Avr2* was the most frequently occurring allele; all virulent individuals contained *vir2*. According to the most recent survey of field collections, *H13* is a viable form of resistance (Cambron *et al*., 2010). Virulence was detected in two counties, Cleveland in the west and Robeson to the south, but the frequency was at such a low level that *H13* would still be efficacious in the field. Our results support this analysis as Onslow and Tyrell counties are adjacent to a known 100% resistant region while Union County is equidistant from the two counties where virulent Hf were identified.

Pockets of virulent Hf were discovered in South Carolina, and *H13* is no longer a viable source of resistance in Orangeburg and Sumter counties (Cambron *et al.*, 2010). These locations are adjacent to the pheromone trapping sites in Lee and Florence. By using the PCR assay, we were able to detect virulent individuals in low levels at both sites. Florence, a previously 100% resistant county, indicates that virulence to *H13* is slowing spreading throughout the central region of South Carolina.

Alabama commonly deploys cultivars that contain multiple *H* genes as successive generations of Hf occur in the warmer, wetter climate. The previous survey of the state indicated that *H13* would still be effective north of the coastal plains (32˚N latitude) (Cambron *et al.*, 2010). The PCR testing of virulence to *H13* supported this assessment. Colbert County, near the northern border with Tennessee, had 78 avirulent and 5 virulent Hf. In Hale and Marengo counties, 88% of Hf males were avirulent. This is congruent with the greenhouse testing of Hf from these counties that reported 80% resistance conferred by *H13* at this location (Cambron *et al.*, 2010). All three avirulence alleles were identified with *Avr2* the most common (42%). Likewise, all three virulent alleles were identified; however, *vir3* was overwhelmingly frequent in the samples.

The use of pheromone traps to collect Hf males and the identification of a reliable marker for *vH13* has allowed the creation of a novel assay to survey Hf virulence in the field. We show here that the results of a simple PCR analysis are in agreement with the most recent survey conducted in the green house from field samples of live insects. As additional avirulence genes in Hf are cloned and characterized, primers for each Hf specific gene can be created to conduct similar PCR assays to assess virulence to the associated *H* gene in wheat. This will reduce the resources and Hf expertise required to conduct field surveys. Utilizing an inexpensive and fast DNA extraction protocol, virulence assessments on hundreds of samples from multiple locations can be accomplished in a fraction of the time with only basic molecular biology techniques and equipment (Chen *et al.*, 2014). This novel assay will allow growers up-to-date analyses of virulence in local populations reducing the guesswork of picking a wheat cultivar with the proper combination of Hf resistance.

5.5 Acknowledgements

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5.7 Tables

Table 5.1. Primer sequences used for amplification of the *vH13* gene are listed.

Name	Primer Sequence (5'-3')
vH13 F	GGT TGC TTT TAT GGT TTT GG
vH13 R	CTT CTC CTT CTT GGC TGT C
vH13 5kb R	TTG AAT GTG CCG CGA GAG C

Table 5.2. Frequency of *vH13* alleles in males collected from pheromone traps. Percentages for each allele within the population are given in parenthesis.

Location	**vH13 Allele Numbers (%)**						
	Avr1	Avr2	Avr3	vir1	vir2	vir3	Total
Colbert County, AL	52 (28.7)	50 (27.6)	72 (39.8)	1 (0.55)	1 (0.55)	5 (2.8)	181
Hale County, AL	97 (20.6)	213 (45.2)	107 (22.7)	0	0	54 (11.5)	471
Marengo County, AL	133 (34)	134 (34)	81 (21)	0	0	44 (11)	392
Alamance County, NC	13 (17.8)	37 (50.7)	21 (28.8)	0	2 (2.7)	0	73
Onslow County, NC	4 (18.2)	16 (72.8)	1 (4.5)	0	1 (4.5)	0	22
Tyrell County, NC	1 (8.8)	3 (35.3)	13 (52.9)	0	0	0	17
Union County, NC	3 (8.8)	12 (35.3)	18 (53.0)	0	1 (2.9)	0	34
Florence County, SC	7 (22.6)	15 (48.4)	4 (12.9)	0	0	5 (16.1)	27
Lee County, SC	37 (22.8)	76 (46.9)	30 (18.5)	0	9 (5.6)	10 (6.2)	162
Total	340 (25.1)	541 (40.2)	343 (25.1)	1 (.1)	14 (1.0)	113 (8.5)	1379

APPENDIX

APPENDIX

Supplemental Tables and Figures for Chapter 3

Table A3.1. Microarray data expressed in log_2 fold change in comparison to Biotype GP for family 1. Adjusted *p* values are listed. Expressed sequence tag (EST).

EST	Accession Number	Field Collection	Log_2 Fold Change	Adjusted *p* Value
MDEST720	EV466597	Israel	0.113381642	0.704470809
		Alabama	0.124862028	0.407835402
		Georgia	-0.183565168	0.364861185
		Texas	-0.165085316	0.407963829
		Colorado	-0.030790174	0.993074635
		Kansas	-0.146666158	0.738514327
MDEST700	EV466472	Israel	0.077733468	0.658054201
		Alabama	0.022047536	0.805244614
		Georgia	0.042207697	0.731511199
		Texas	-0.017914909	0.912146533
		Colorado	0.060313076	0.993074635
		Kansas	-0.032557243	0.999944651
MDEST754	EV466582	Israel	0.045086281	0.830895436
		Alabama	0.042094482	0.709689373
		Georgia	0.127541027	0.400166087
		Texas	-0.032889369	0.872039964
		Colorado	-0.143739513	0.746285688
		Kansas	-0.318618783	0.040176125
G10E4	JZ482473	Israel	0.114898676	0.809958521
		Alabama	0.519374983	0.049643835
		Georgia	-0.270565139	0.423765403
		Texas	-0.273433023	0.409319641
		Colorado	-0.097874799	0.993074635
		Kansas	-0.060642384	0.999944651
SSGP-1A1	ACZ26299	Israel	0.010905207	0.969274453
		Alabama	0.239742788	0.045601823

		Georgia	0.034014271	0.851368086
		Texas	-0.007454685	0.984760893
		Colorado	0.068453357	0.993074635
		Kansas	0.018733883	0.999944651
S6E5	JZ482474	Israel	0.188346758	0.332991327
		Alabama	0.235048904	0.076683319
		Georgia	0.108338632	0.547617588
		Texas	-0.005496108	0.984760893
		Colorado	0.053061555	0.993074635
		Kansas	-0.027841183	0.999944651
G22D5	JZ482475	Israel	0.104547549	0.704470809
		Alabama	0.439864584	0.013200428
		Georgia	0.104675261	0.586337644
		Texas	-0.016450901	0.967650973
		Colorado	0.042252872	0.993074635
		Kansas	0.020608474	0.999944651
G16H10	JZ482476	Israel	0.145323735	0.705282956
		Alabama	0.368236989	0.073174334
		Georgia	0.128920951	0.636345869
		Texas	-0.027433187	0.959181449
		Colorado	0.009372873	0.993074635
		Kansas	-0.014024273	0.999944651
SSGP-1B1	ACZ26297	Israel	0.173053756	0.146866547
		Alabama	0.197837558	0.034878039
		Georgia	0.339959999	0.006661992
		Texas	0.254625775	0.056697794
		Colorado	0.207980939	0.160170752
		Kansas	0.041827542	0.999944651
S17A12	JZ482477	Israel	0.506935659	0.016579292
		Alabama	0.146980046	0.370665968
		Georgia	0.126627289	0.569658715
		Texas	0.045654005	0.872039964
		Colorado	0.018890744	0.993074635
		Kansas	0.103610976	0.919637715
S12G7	JZ482478	Israel	0.210091224	0.455995427
		Alabama	0.523521737	0.014482079
		Georgia	0.264458162	0.241234555

		Texas	0.042395909	0.899974741
		Colorado	0.027265769	0.993074635
		Kansas	-0.074606983	0.999944651
G21E12	JZ482479	Israel	0.273443896	0.184440827
		Alabama	0.430852182	0.015507444
		Georgia	0.328853295	0.082350331
		Texas	0.230054338	0.232674966
		Colorado	0.113981061	0.993074635
		Kansas	-0.065080971	0.999944651
G8E12	JZ482480	Israel	0.526295831	0.336617888
		Alabama	0.748946178	0.051928977
		Georgia	0.388069192	0.432388003
		Texas	0.114704514	0.860561105
		Colorado	0.045100851	0.993074635
		Kansas	-0.172846132	0.999944651
G21G4	JZ482481	Israel	0.707387921	0.257637423
		Alabama	0.257693218	0.537248804
		Georgia	0.395393348	0.490544962
		Texas	0.212576094	0.754060085
		Colorado	-0.068777824	0.993074635
		Kansas	-0.562234806	0.536209618
S12A11	JZ482482	Israel	0.300984349	0.590975698
		Alabama	1.116377423	0.005149744
		Georgia	0.461807421	0.241234555
		Texas	0.158982178	0.735607373
		Colorado	0.302569438	0.846274702
		Kansas	0.136941757	0.999944651
MDEST934	EV466384	Israel	0.517299583	0.302501382
		Alabama	0.842516856	0.026138518
		Georgia	0.399576819	0.383018396
		Texas	0.076470024	0.904686748
		Colorado	0.362974778	0.809180569
		Kansas	0.231514344	0.919637715
SSGP-1A2	ACZ26298	Israel	0.204135009	0.509674328
		Alabama	0.752455084	0.002591331
		Georgia	0.483161314	0.043437515
		Texas	0.288879103	0.231729984

		Colorado	0.442939067	0.160170752
		Kansas	0.353457617	0.174629103
SSGP-1C1	ACZ26296	Israel	0.699769292	0.116737277
		Alabama	0.711314299	0.042841024
		Georgia	0.723359372	0.089510446
		Texas	0.328726904	0.451130148
		Colorado	0.248969548	0.993074635
		Kansas	-0.042940291	0.999944651
G8F2	JZ482483	Israel	0.036373589	0.973104677
		Alabama	1.328591004	0.012754677
		Georgia	0.147813982	0.827113237
		Texas	-0.020539627	0.984760893
		Colorado	0.198159697	0.993074635
		Kansas	-0.024036948	0.999944651
G22E11	JZ482484	Israel	-0.738807442	0.014642269
		Alabama	0.331274368	0.156483036
		Georgia	0.447794894	0.141309028
		Texas	-0.101575931	0.794063097
		Colorado	-0.088834365	0.993074635
		Kansas	-0.155999414	0.919637715
SSGP-1D1	ACZ26295	Israel	0.748085489	0.291175132
		Alabama	0.680863942	0.156154509
		Georgia	0.949718235	0.127759927
		Texas	1.162062793	0.077038297
		Colorado	0.200606181	0.993074635
		Kansas	-0.315945839	0.919637715
SSGP-1C2	ACZ26293	Israel	0.101908143	0.753082716
		Alabama	-0.347357058	0.051928977
		Georgia	-0.571835906	0.017155361
		Texas	-0.431144531	0.068529154
		Colorado	-0.246629024	0.653210469
		Kansas	0.144030072	0.804651923
G8A9	JZ482485	Israel	0.258002968	0.536280703
		Alabama	-0.848884506	0.007229346
		Georgia	-0.783185432	0.019307881
		Texas	-0.387791132	0.227952805
		Colorado	-0.495491584	0.304505463

		Kansas	-0.196790804	0.819944582
MDEST868	EV466449	Israel	-0.196821642	0.345875694
		Alabama	-0.516053625	0.005149744
		Georgia	-0.759884306	0.000310509
		Texas	-0.304063538	0.105537791
		Colorado	-0.298836092	0.277045775
		Kansas	-0.444097431	0.025957879
MDEST866	EV466451	Israel	-0.115581253	0.601555286
		Alabama	-0.239162127	0.051928977
		Georgia	-0.465317144	0.006200999
		Texas	-0.131430407	0.398968711
		Colorado	-0.053806372	0.993074635
		Kansas	-0.242863063	0.152780493
MDEST954	EV466364	Israel	0.503532615	0.704470809
		Alabama	-0.148882865	0.824490039
		Georgia	-0.509438032	0.574823931
		Texas	-0.127083422	0.918794644
		Colorado	-0.810594054	0.789757887
		Kansas	-1.397145952	0.148528819
SSGP-1E1	ACZ26294	Israel	0.263557495	0.634488529
		Alabama	0.037639633	0.894837259
		Georgia	-0.830862215	0.031270228
		Texas	0.347109277	0.367963639
		Colorado	0.383869956	0.714791235
		Kansas	0.884200956	0.027415456
MDEST798	EV466519	Israel	0.382834123	0.679597922
		Alabama	-1.120323992	0.027835518
		Georgia	-2.117407727	0.001450557
		Texas	-0.224268014	0.759704215
		Colorado	0.147308884	0.993074635
		Kansas	0.721278658	0.347249099

Table A3.2. Microarray data expressed in log_2 fold change in comparison to Biotype GP for family 2. Adjusted *p* values are listed. Expressed sequence tag (EST).

EST	Accession Number	Field Collection	Log_2 Fold Change	Adjusted *p* Value
MDEST884	EV466433	Israel	-0.539370446	0.720983043
		Alabama	-2.164726031	0.020637307
		Georgia	-1.392972956	0.178135609
		Texas	-0.683886315	0.537627146
		Colorado	-0.369708207	0.993074635
		Kansas	-0.062105313	0.999944651
MDEST776	EV466541	Israel	-0.828657848	0.629923621
		Alabama	-2.762054267	0.013287252
		Georgia	-1.475441094	0.203194614
		Texas	-0.895639707	0.449321232
		Colorado	-0.823875813	0.895616214
		Kansas	-0.335793449	0.999944651
MDEST823	EV466494	Israel	-0.718010639	0.522934536
		Alabama	-1.471584782	0.037823867
		Georgia	-1.342215725	0.117392633
		Texas	-0.969836604	0.257325827
		Colorado	-0.836405938	0.746285688
		Kansas	-0.314011367	0.999944651
MDEST1040	EV466278	Israel	0.167698844	0.905257728
		Alabama	-1.568223667	0.060997258
		Georgia	-2.259842351	0.036543499
		Texas	-1.412888727	0.183896584
		Colorado	-0.205678378	0.993074635
		Kansas	0.771290221	0.750908045
W2E12	JZ482487	Israel	-1.560602325	0.455995425
		Alabama	-2.366892203	0.072474453
		Georgia	-3.561105761	0.036543499
		Texas	-0.780813338	0.689635199
		Colorado	-0.446964338	0.993074635

		Kansas	0.857722629	0.919637715
G15F11	JZ482488	Israel	-0.839577269	0.713735317
		Alabama	-2.851534138	0.028352823
		Georgia	-2.460148549	0.106678859
		Texas	-1.330501566	0.393570614
		Colorado	-0.489800064	0.993074635
		Kansas	-0.032687662	0.999944651
MDEST689	EV466628	Israel	0.358062704	0.830895436
		Alabama	-2.606239269	0.015338583
		Georgia	-5.383489305	7.16913E-05
		Texas	-0.260119533	0.872039964
		Colorado	0.327699189	0.993074635
		Kansas	0.734141467	0.804651923
MDEST982	EV466336	Israel	-2.958281413	0.061703253
		Alabama	-3.375665449	0.017743786
		Georgia	-3.644384987	0.024560445
		Texas	-3.760037106	0.044530695
		Colorado	-2.764449526	0.184391032
		Kansas	-0.557000262	0.999944651
G21G10	JZ482489	Israel	-0.041262856	0.976475672
		Alabama	-0.987858411	0.096177346
		Georgia	-0.457942709	0.566345091
		Texas	0.359830441	0.688688903
		Colorado	0.981468854	0.513303427
		Kansas	0.799825901	0.516496686
MDEST789	EV466528	Israel	1.462647746	0.010728413
		Alabama	-0.267677929	0.537248804
		Georgia	-0.050016236	0.942520605
		Texas	0.543929649	0.352512393
		Colorado	1.216869944	0.084707368
		Kansas	1.047370283	0.096166388
MDEST734	EV466583	Israel	-0.315008912	0.748283163
		Alabama	-1.214657569	0.031759572
		Georgia	-0.904504812	0.178118839
		Texas	-0.253727353	0.759704215
		Colorado	0.159662301	0.993074635
		Kansas	0.100690019	0.999944651

G14E6	JZ482490	Israel	-0.316780456	0.739319654
		Alabama	-0.938637586	0.064705277
		Georgia	-0.630794809	0.340011605
		Texas	-0.203412014	0.801353606
		Colorado	0.053035196	0.993074635
		Kansas	0.005229072	0.999944651
G12A2	JZ482491	Israel	-0.389905159	0.704470809
		Alabama	-0.926569569	0.082386507
		Georgia	-0.538570729	0.445902343
		Texas	-0.265955292	0.745598489
		Colorado	-0.099711196	0.993074635
		Kansas	0.378036134	0.880437122
L7D5	JZ482492	Israel	-0.426608955	0.809958521
		Alabama	-1.833044979	0.054068197
		Georgia	-0.532575579	0.674128477
		Texas	-0.555516459	0.689635199
		Colorado	0.052065782	0.993074635
		Kansas	0.055497373	0.999944651
G17A11	JZ482493	Israel	-0.180102918	0.897851969
		Alabama	-1.610412013	0.046152507
		Georgia	-0.944235825	0.352308138
		Texas	-0.357042939	0.770945926
		Colorado	0.184787367	0.993074635
		Kansas	0.113854961	0.999944651
MDEST849	EV466468	Israel	-0.792771028	0.108664631
		Alabama	-1.287629037	0.006550405
		Georgia	-0.421703442	0.384993964
		Texas	-0.161028807	0.787606036
		Colorado	-0.142301902	0.993074635
		Kansas	-0.311285242	0.804479112
MDEST790	EV466527	Israel	-0.864529171	0.087498681
		Alabama	-1.231535249	0.01036237
		Georgia	-0.499003333	0.316992533
		Texas	-0.152881028	0.801353606
		Colorado	-0.140397715	0.993074635
		Kansas	-0.298177136	0.826461329
MDEST761	EV466556	Israel	-1.087796675	0.174313098

		Alabama	-1.129047809	0.056601901
		Georgia	-1.026740601	0.165049527
		Texas	-0.437274395	0.595854848
		Colorado	-0.348524734	0.993074635
		Kansas	-0.216989417	0.999944651
L7F4	JZ482494	Israel	-0.192176276	0.473428446
		Alabama	-0.505338953	0.013287252
		Georgia	-0.157790929	0.473176898
		Texas	-0.178925272	0.403594002
		Colorado	-0.209481007	0.746285688
		Kansas	-0.330795328	0.157474042
MDEST588	EV466728	Israel	-0.354383754	0.232376907
		Alabama	-0.534420288	0.021536043
		Georgia	-0.087163295	0.766201064
		Texas	-0.082224757	0.802944479
		Colorado	-0.133855697	0.993074635
		Kansas	-0.130062635	0.919637715
G3C11	JZ482495	Israel	0.062950143	0.899327283
		Alabama	-0.372417891	0.189227381
		Georgia	-0.261583122	0.491616187
		Texas	0.008213721	0.984760893
		Colorado	0.163041396	0.993074635
		Kansas	-0.354574322	0.567224308
G12G12	JZ482496	Israel	0.095650408	0.900718291
		Alabama	-1.082475202	0.027448882
		Georgia	-0.370552192	0.547488776
		Texas	0.018576882	0.984760893
		Colorado	0.030817168	0.993074635
		Kansas	-0.587677393	0.535994683
MDEST933	EV466385	Israel	0.229922963	0.726068064
		Alabama	0.810961234	0.031759572
		Georgia	0.703619431	0.117899191
		Texas	0.217840941	0.688214003
		Colorado	0.139156539	0.993074635
		Kansas	0.066429027	0.999944651
MDEST772	EV466545	Israel	0.411400209	0.262137567
		Alabama	0.645313659	0.023522963

		Georgia	0.691374632	0.038017454
		Texas	0.989132408	0.008933928
		Colorado	0.747350082	0.054915123
		Kansas	-0.217478612	0.804479112
S14F7	JZ482497	Israel	0.585403703	0.302501382
		Alabama	1.226021775	0.012754677
		Georgia	1.254911445	0.018782609
		Texas	0.728279308	0.153110617
		Colorado	0.566183675	0.637667476
		Kansas	0.499300947	0.544061062
L7E9	JZ482498	Israel	-0.586623157	0.704470809
		Alabama	1.623034727	0.050789861
		Georgia	0.837078231	0.431927545
		Texas	0.912531799	0.388367874
		Colorado	0.050099965	0.993074635
		Kansas	-0.482026708	0.937378509
MDEST910	EV466407	Israel	-0.927368217	0.519241361
		Alabama	1.362598884	0.110449694
		Georgia	1.358627617	0.212421315
		Texas	1.124370752	0.315706616
		Colorado	-0.071284496	0.993074635
		Kansas	-0.654558754	0.841301422
S3E10	JZ482499	Israel	4.294479383	0.014642269
		Alabama	3.626898077	0.021081909
		Georgia	5.424690906	0.005814065
		Texas	2.467253751	0.164727924
		Colorado	0.761856259	0.993074635
		Kansas	0.115681795	0.999944651
S20B4	JZ482500	Israel	4.812618901	0.007578226
		Alabama	3.764416323	0.020637307
		Georgia	5.957124208	0.003088202
		Texas	2.485906705	0.175134024
		Colorado	0.865846488	0.993074635
		Kansas	0.014474771	0.999944651
S8D5	JZ482501	Israel	4.615139294	0.018426697
		Alabama	4.392467892	0.016741918
		Georgia	5.625659497	0.009226963

		Texas	3.189372137	0.110130943
		Colorado	0.993630095	0.993074635
		Kansas	0.100881222	0.999944651
S18E7	JZ482502	Israel	2.736770708	0.234683433
		Alabama	3.707338609	0.031759572
		Georgia	4.674392739	0.028398352
		Texas	3.073121713	0.144517393
		Colorado	0.637172871	0.993074635
		Kansas	-0.156075729	0.999944651
S12G8	JZ482503	Israel	2.378306423	0.098626815
		Alabama	2.330064138	0.040320642
		Georgia	3.893259954	0.009556944
		Texas	1.110232265	0.432188359
		Colorado	0.044173039	0.993074635
		Kansas	0.071707087	0.999944651

Table A3.3. Microarray data expressed in log_2 fold change in comparison to Biotype GP for family 4. Adjusted *p* values are listed. Expressed sequence tag (EST).

EST	Accession Number	Field Collection	Log_2 Fold Change	Adjusted *p* value
MDEST729	EV466588	Israel	0.326173792	0.089614013
		Alabama	0.241554197	0.094751942
		Georgia	0.455017263	0.020056854
		Texas	0.054338834	0.819681876
		Colorado	0.104260595	0.993074635
		Kansas	0.061675304	0.999944651
G20G4	JZ482504	Israel	0.354300293	0.023329478
		Alabama	0.176351243	0.147752852
		Georgia	0.377465513	0.022533919
		Texas	0.332665747	0.059466914
		Colorado	0.177521995	0.642143206
		Kansas	0.101702325	0.804651923
S6B12	JZ482505	Israel	0.267678306	0.128902447
		Alabama	0.379822483	0.015507444
		Georgia	0.203286502	0.225768653
		Texas	0.365330526	0.057940003
		Colorado	0.307985348	0.160170752
		Kansas	0.223551427	0.251412247
L3A9	JZ482506	Israel	0.642942007	0.001826927
		Alabama	0.207947076	0.194219963
		Georgia	0.347360437	0.094308689
		Texas	0.480978011	0.055725163
		Colorado	0.116901067	0.993074635
		Kansas	-0.112680642	0.904653152
S4B3	JZ482507	Israel	0.233967202	0.743135497
		Alabama	0.449657084	0.226426496
		Georgia	0.303020613	0.553104232
		Texas	0.437791973	0.380005028
		Colorado	-0.169442663	0.993074635

		Kansas	-0.725367093	0.172617333
S16F7	JZ482508	Israel	0.072264128	0.824406935
		Alabama	0.341751049	0.051808219
		Georgia	0.410617204	0.060704706
		Texas	0.453571627	0.059466913
		Colorado	0.048157145	0.993074635
		Kansas	-0.258991582	0.363576836
L7C7	JZ482509	Israel	-0.329664404	0.014642269
		Alabama	-0.004664789	0.964195229
		Georgia	0.069318549	0.624561258
		Texas	-0.141059781	0.310243009
		Colorado	-0.048129582	0.993074635
		Kansas	-0.096210776	0.765222320
MDEST817	EV466500	Israel	-0.309475207	0.028606413
		Alabama	-0.076787128	0.481008771
		Georgia	-0.006210489	0.966748524
		Texas	-0.085317029	0.591915171
		Colorado	-0.127456972	0.790979835
		Kansas	-0.193863967	0.246924389
G8G4	JZ482510	Israel	-0.358842772	0.190559985
		Alabama	0.205640853	0.286783546
		Georgia	-0.111780929	0.674128477
		Texas	0.076898424	0.809935074
		Colorado	-0.112921919	0.993074635
		Kansas	-0.372378018	0.180726031
S18E2	JZ482511	Israel	0.125316698	0.704470809
		Alabama	0.257619591	0.120511563
		Georgia	0.218045198	0.322242082
		Texas	-0.085850601	0.737708407
		Colorado	-0.170038864	0.842546369
		Kansas	-0.172512085	0.678602891
S10D6	JZ482512	Israel	0.120289769	0.775924979
		Alabama	0.291363747	0.193155827
		Georgia	0.159507179	0.604559271
		Texas	-0.125728696	0.717089181
		Colorado	-0.217189437	0.872247928
		Kansas	-0.251655781	0.647037735

S6B3	JZ482513	Israel	0.079630628	0.830895436
		Alabama	0.356553082	0.073866693
		Georgia	0.388024019	0.122936487
		Texas	-0.000591227	0.998143729
		Colorado	-0.228655986	0.790979834
		Kansas	-0.38935622	0.158583672
LG1E5	GR557757	Israel	-0.260604714	0.332991327
		Alabama	0.134608765	0.451476138
		Georgia	0.559810364	0.022533919
		Texas	0.136583334	0.602457617
		Colorado	0.286355391	0.573123166
		Kansas	-0.025736094	0.999944651
S3B8	JZ482514	Israel	-0.309051715	0.117650438
		Alabama	0.178688774	0.220678247
		Georgia	0.362603741	0.059288434
		Texas	0.147605488	0.446655768
		Colorado	0.053938192	0.993074635
		Kansas	-0.443967163	0.027440918
G16C11	JZ482515	Israel	-0.557049332	0.020966459
		Alabama	0.088248327	0.632425779
		Georgia	0.285273863	0.243601897
		Texas	0.078635993	0.801353606
		Colorado	0.028243113	0.993074635
		Kansas	-0.527782676	0.040176125
S3H1	JZ482516	Israel	-0.254587498	0.713735317
		Alabama	0.282046814	0.425117724
		Georgia	0.427449899	0.373906099
		Texas	0.125708202	0.840600811
		Colorado	-0.164037868	0.993074635
		Kansas	-0.784568033	0.127419471
L6G11	JZ482517	Israel	-0.522557308	0.067847681
		Alabama	0.189548394	0.378084257
		Georgia	0.348337829	0.212421315
		Texas	-0.459864305	0.109409849
		Colorado	-0.772243018	0.014349935
		Kansas	-1.011487064	0.000907156
MDEST1000	EV466318	Israel	0.299031618	0.439069832

		Alabama	0.558330824	0.032326692
		Georgia	0.707296698	0.028398352
		Texas	-0.319896314	0.315706616
		Colorado	-0.571902248	0.160170752
		Kansas	-0.612487014	0.069123062
S8A3	JZ482518	Israel	1.486031054	0.000452935
		Alabama	0.634000592	0.055320462
		Georgia	2.513540395	4.04E-06
		Texas	0.943275829	0.055764583
		Colorado	0.210749525	0.993074635
		Kansas	-0.773248978	0.087733601
G28D4	JZ482519	Israel	0.716751676	0.067048544
		Alabama	0.106326844	0.717142871
		Georgia	2.027590472	2.29E-05
		Texas	0.471085772	0.228033434
		Colorado	-0.120648724	0.993074635
		Kansas	-0.401439987	0.516496686
MDEST773	EV466544	Israel	1.454072923	1.93E-05
		Alabama	1.831334094	4.36E-06
		Georgia	0.538506986	0.082350331
		Texas	2.078672192	4.97E-07
		Colorado	1.842023516	3.96E-06
		Kansas	1.837984623	2.05E-06
MDEST744	EV466573	Israel	-0.349781276	0.116737277
		Alabama	-0.482226209	0.015507444
		Georgia	-0.251245112	0.241234555
		Texas	-0.362681921	0.101628075
		Colorado	-0.225698957	0.678977911
		Kansas	-0.774136461	0.000907156
G8A3	JZ482520	Israel	-0.295878216	0.257637423
		Alabama	-0.496270875	0.019736909
		Georgia	-0.486043447	0.040424096
		Texas	-0.440930198	0.073813697
		Colorado	-0.264904926	0.637667476
		Kansas	-0.657187227	0.007519409
MDEST922	EV467317	Israel	-0.489958495	0.036586092
		Alabama	-0.622910098	0.008512212

		Georgia	-0.445662768	0.060704706
		Texas	-0.505560978	0.059466913
		Colorado	-0.294833137	0.552522817
		Kansas	-0.997915872	0.000185029
MDEST795	EV466522	Israel	-0.432654667	0.146866547
		Alabama	-0.552294628	0.023566866
		Georgia	-0.321126068	0.262003118
		Texas	-0.439362587	0.124652216
		Colorado	-0.130940119	0.993074635
		Kansas	-0.375845228	0.251412247
L4H12	JZ482521	Israel	-1.031303523	1.25E-07
		Alabama	-0.407981382	0.005149744
		Georgia	-0.417343881	0.008369144
		Texas	-0.636291694	0.000190814
		Colorado	-0.124015576	0.790979831
		Kansas	-0.338761757	0.027415454
MDEST838	EV466479	Israel	-0.850736592	0.062887459
		Alabama	-0.939499033	0.020637307
		Georgia	-0.638954659	0.152167434
		Texas	-0.662933592	0.145054301
		Colorado	-0.278388829	0.959059078
		Kansas	-0.327436495	0.750908045
S19C4	JZ482522	Israel	-0.205319551	0.854394202
		Alabama	-1.108765545	0.059501515
		Georgia	-0.900531962	0.225768653
		Texas	-0.585181628	0.441616807
		Colorado	-0.127995436	0.993074635
		Kansas	0.182924381	0.999944651
MDEST960	EV466358	Israel	-0.405153233	0.597629701
		Alabama	-1.113318894	0.020637307
		Georgia	-0.424196356	0.441858983
		Texas	-0.670605735	0.219466495
		Colorado	-0.295126731	0.993074635
		Kansas	0.214566857	0.999944651
MDEST842	EV466475	Israel	-0.400704294	0.704470809
		Alabama	-0.956650651	0.076683319
		Georgia	-0.602470809	0.398190747

		Texas	-1.166368377	0.101628075
		Colorado	-0.325299929	0.993074635
		Kansas	0.204229002	0.999944651
Lg3C11	GR305978	Israel	-0.433356497	0.473428446
		Alabama	-1.355866892	0.005149744
		Georgia	-1.068219398	0.029956896
		Texas	-0.579106952	0.232674966
		Colorado	-0.343144844	0.895616245
		Kansas	-0.170354971	0.999944651
MDEST1207	EV466111	Israel	-0.006188252	0.988694316
		Alabama	-0.214646087	0.410653181
		Georgia	-1.296787022	0.000748673
		Texas	-0.548750583	0.110130943
		Colorado	-0.104689749	0.993074635
		Kansas	0.131625937	0.999944651
S15G10	JZ482524	Israel	-0.363189474	0.601555286
		Alabama	-0.046675404	0.896554608
		Georgia	-0.791685368	0.097024523
		Texas	-0.776009843	0.110130943
		Colorado	-0.635301924	0.480189166
		Kansas	0.300774583	0.819944582
L1C12	JZ482525	Israel	-0.077335836	0.911434576
		Alabama	-0.987776482	0.029283271
		Georgia	-1.450593696	0.011676509
		Texas	-0.905975666	0.101628075
		Colorado	-0.377085827	0.901510803
		Kansas	0.450796893	0.647566789
G9B3	JZ482526	Israel	-0.124056978	0.897851969
		Alabama	-1.135888685	0.033024889
		Georgia	-1.125670307	0.076089575
		Texas	-0.797254683	0.223549314
		Colorado	-0.284011499	0.993074635
		Kansas	0.421562967	0.804479112
S19E7	JZ482527	Israel	0.275401364	0.769455659
		Alabama	-1.162793512	0.031759572
		Georgia	-0.893708518	0.164561583
		Texas	-0.981169422	0.133522841

		Colorado	-0.551729372	0.790979834
		Kansas	0.660500042	0.535994157
S16C4	JZ482528	Israel	-0.237542037	0.782442499
		Alabama	-1.437519759	0.013521393
		Georgia	-1.734392761	0.009226963
		Texas	-1.167733493	0.070942924
		Colorado	-0.573659055	0.774939847
		Kansas	0.367592556	0.839726474
MDEST16	EV467299	Israel	-2.247583134	0.109568994
		Alabama	-2.736836666	0.021536043
		Georgia	-2.791181851	0.040424096
		Texas	-1.553398563	0.253241628
		Colorado	-0.429015338	0.993074635
		Kansas	0.226422966	0.999944651
MDEST747	EV466570	Israel	-0.287153977	0.881519011
		Alabama	-2.724266649	0.017191357
		Georgia	-2.955720298	0.022533919
		Texas	-1.393828877	0.257325827
		Colorado	-0.118984453	0.993074635
		Kansas	0.733232293	0.841301422

Table A3.4. Microarray data expressed in log_2 fold change in comparison to Biotype GP for family 11. Adjusted *p* values are listed. Expressed sequence tag (EST).

EST	Accession Number	Field Collection	Log_2 Fold Change	Adjusted *p* Value
SSGP11C2	AY828563	Israel	0.741484661	9.77E-06
		Alabama	0.607544651	0.824490039
		Georgia	0.560839539	0.674128477
		Texas	0.307977366	0.688688903
		Colorado	0.381123751	0.993074635
		Kansas	0.228847607	0.597258574
MDEST1025	EV466293	Israel	0.785904356	0.302501381
		Alabama	0.396444322	0.439402032
		Georgia	0.429006621	0.547617588
		Texas	0.084605068	0.940980477
		Colorado	0.237829021	0.993074635
		Kansas	0.47506787	0.765222325
SSGP11C1	AY828563	Israel	0.319793191	0.000873273
		Alabama	0.211247182	0.000140052
		Georgia	0.172950997	4.04E-06
		Texas	0.175970821	0.070942924
		Colorado	0.416042074	0.993074635
		Kansas	0.444192339	0.999944651
MDEST685	EV466632	Israel	1.227504498	0.302501384
		Alabama	0.042517634	0.307817809
		Georgia	-0.111158045	0.547617588
		Texas	0.118463963	0.547222133
		Colorado	0.139080735	0.323432281
		Kansas	0.234566853	0.137105501
MDEST1048	EV466270	Israel	-1.337873533	0.340982451
		Alabama	-1.743631184	0.244163716
		Georgia	-2.406430858	0.426562643
		Texas	-0.764068986	0.696237562
		Colorado	0.009765357	0.993074635
		Kansas	-0.000425817	0.999944651

Table A3.5. Primers for quantitative real-time PCR (qRT-PCR) including the melting temperature (Tm). Forward primers are labeled F, and reverse primers are labeled R.

Name	Primer Sequence (5'-3')	Tm (°C)
18S_F	ATCTATGGGTGGTGGTGCAT	60.4
18S_R	CCAGACAAATCACTCCACGA	60.4
MDEST700_F	CGCCAACAGCCCAATCA	59.6
MDEST700_R	CCAATCTAGCATGGAAAGATCGT	61.0
G8F2_F	AATGCGGGAGATGCTAATGG	60.4
G8F2_R	TTTTGCGGCTGTCGGTTT	57.6
MDEST798_F	GGCAGGAAAACCAACAAAACC	60.6
MDEST798_R	TTTGGTGGCCTTTTCCATGT	58.4
MDEST689_F	ACATTCATTGCTACGCCAAAGA	58.9
MDEST689_R	CCAATGCGGTTGAAGGTTCT	60.4
L7D5_F	CGGACTCACTGAACGGATAAACC	64.6
L7D5_R	CGATGTCCTCATCCACGACTCT	64.5
S20B4_F	TTTGCCCACCAGCCATGA	59.9
S20B4_R	TGGATTTTCGACGACGTTCCT	60.6
MDEST747_F	TGGACAAAATAGTATGCAGAAACGA	59.7
MDEST747_R	AAGGCGGCATAACTGCTTTTAA	58.9
S8A3_F	GGCTGCAAGTTTCGCTGAAG	62.4
S8A3_R	ATCTGATACCGCACGCCTTT	60.4
MDEST817_F	TGCTTCATCGTCAACCTCATGAT	61.0
MDEST817_R	ACCGAAGATACCAAAAAAAAATCGA	58.0
G7E6_F	TGGCAGTGATAGCTGTAGCATCA	62.8
G7E6_R	TGGGTCGGTTTCTAGCTTCTCA	62.7
L7A12_F	AAATCGACCCAGCAGGAGATG	62.6
L7A12_R	CTTGAAGCCCGAGACTGGAAA	62.6
11C1_F	GAAAGAAACGACCCAGCAGAA	62.7
11C1_R	CTTGAAGCCCGAGACTGGAAA	62.6

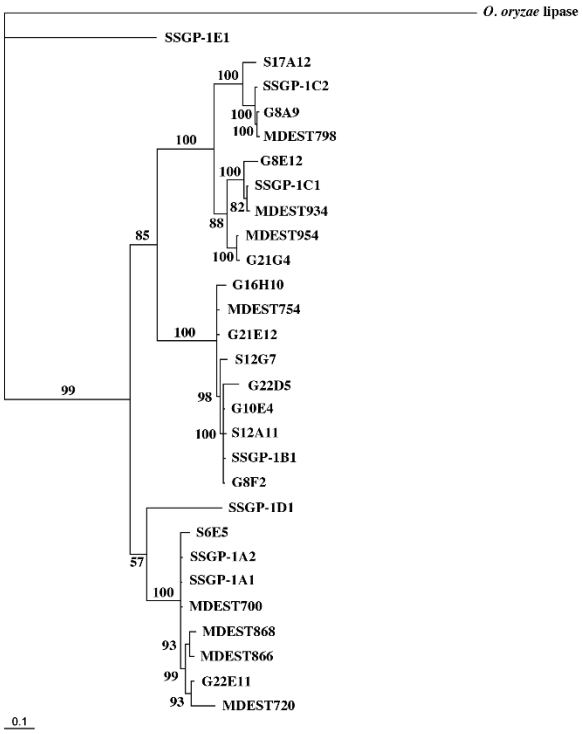

Figure A3.0.1. Bayesian phylogenetic tree of secreted salivary gland protein (SSGP) transcripts in Family 1. The phylogenetic reconstruction is rooted using the secreted salivary lipase-like gene from the Asian rice gall midge, *Orseolia oryzae* (Wood-Mason) as an outgroup, posterior probability values are located at the nodes, and clades are indicated by Roman Numerals. SSGPs in this family separated into two clades. However, there is no correlation between transcript abundance and phylogeny.

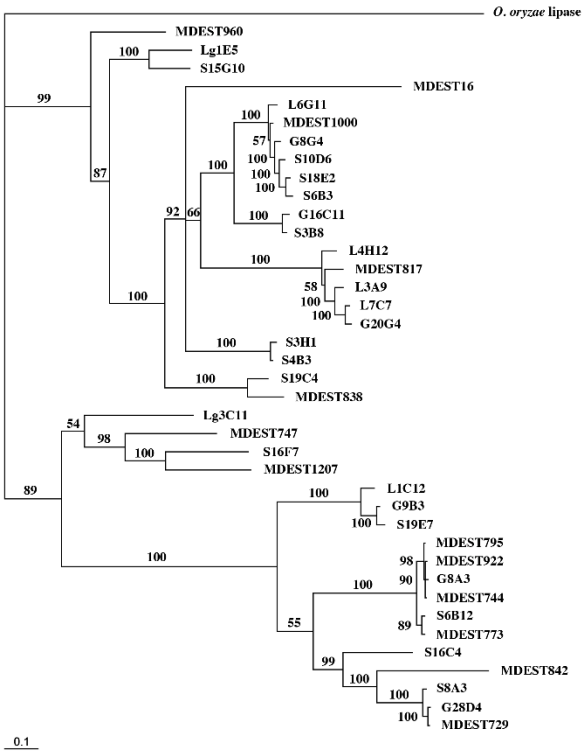

Figure A3.0.2. Bayesian phylogenetic tree of secreted salivary gland protein (SSGP) transcripts in Family 4. The phylogenetic reconstruction is rooted as in Fig. S1; posterior probability values and clades are indicated. Significant variability in transcript abundance is dispersed throughout the tree, and no pattern between transcript abundance and phylogeny is shown.

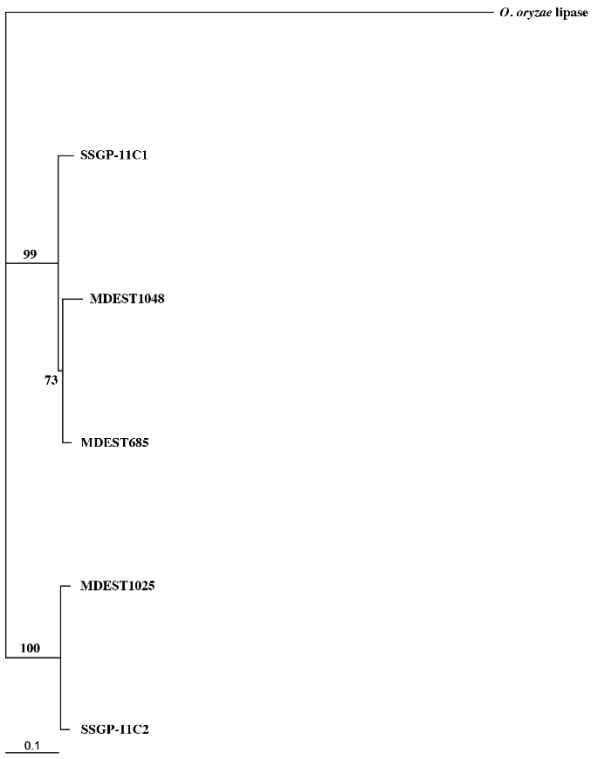

Figure A3.3. Bayesian phylogenetic tree of secreted salivary gland protein (SSGP) transcripts in Family 11. The phylogenetic reconstruction is rooted as in Fig. S1; posterior probability values and clades are indicated. No correlation of transcript abundance variation and phylogeny could be seen within this small family of SSGPs.

VITA

VITA

Alisha Janelle Johnson

Education

Ph.D. in Entomology, Department of Entomology, Purdue University, West Lafayette, Indiana. Major Field: Molecular biology. Research interests: plant-insect interactions, population structure. Dissertation: *Characterization of Hessian fly from Israel*. Major Professor: Dr. Richard H. Shukle. May, 2015.

Master of Science, Department of Entomology, Purdue University, West Lafayette, Indiana. Major Field: Molecular biology. Research interests: population genetics of an invasive pest. Thesis: *A first assessment of the phylogenetics of Hessian fly using mitochondrial and nuclear markers*. Major Professor: Dr. Richard H. Shukle. May, 2006.

Bachelor of Arts, Biology, DePauw University, Greencastle, Indiana. May, 2001.
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Publications

Johnson, A.J., R.H. Shukle, M.-S. Chen, S. Srivasta, s. Subrmanyam, B.J. Schemerhorn, P.G. Weintraub, H.E.M. Abdel Moniem, K.L. Flanders, G.D. Buntin, and C.E. Williams. 2015. Differential expression of candidate salivary effector proteins in field collections of Hessian fly, *Mayetiola destructor*. *Insect Molecular Biology* 24(2): 191-202. doi: 10.1111/imb.12148.

Zhao, C., L.N. Escalante, …, A.J. Johnson, …, J.J. Stuart, and S.Richards. 2015. A massive expansion of effector genes underlies gall-formation in the wheat pest *Mayetiola destructor*. *Current Biology* 25: 613-620.

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Johnson, A.J. P.K. Morton, B.J. Schemerhorn, and R.H. Shukle. 2011. Use of a nuclear marker to assess population structure in Hessian fly (*Mayetiola destructor*). *Annals of the Entomological Society of America* 104: 666-674.

Shukle, R.H., M. Yoshiyama, P.K. Morton, A.J. Johnson, and B.J. Schemerhorn. 2008. Tissue and developmental expression of a gene from Hessian fly encoding an ABC-active-transport protein: Implications for Malpighian tubule function during interactions with wheat. *Journal of Insect Physiology* 54(1): 146-154.

Johnson, A.J., B.J. Schemerhorn, and R.H. Shukle. 2004. A first assessment of mitochondrial DNA variation and geographic distribution of haplotypes in the Hessian fly (Diptera: Cecidomyiidae). *Annals of the Entomological Society of America* 97(5): 940-948.

Professional Experience

2001- Present
Laboratory Technician GS8-6
USDA- ARS, Crop Production and Pest Control Unit, West Lafayette, Indiana
Duties: conduct research, participate in the writing of manuscripts for peer reviewed journals, rear Hessian fly on wheat plants, supervise undergraduate student research, maintain laboratory supplies and equipment including placing orders and scheduling repairs, and oversee safety, health, and environmental compliance to Purdue and USDA-ARS policies.

July, 2001-November, 2001
Laboratory Technician
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Professional Activities

June, 2014 Web APOLLO manual annotation workshop, Dr. Monica Munoz-Torres (Berkley Bioinformatics Open-Source Projects), University of Illinois Champaign

June, 2011 Poster: Translational insect genomics: pheromone traps and molecular markers genotype Hessian fly resistance gene *H13* in wheat. Plant Genomics Series.

March, 2011 Poster: Genotyping virulence to *H13* wheat in field collections of Hessian fly from the southeastern United States. Entomological Society of America, North Central Branch Meeting.

March, 2009 Poster: Analyzing the diversity of secreted salivary gland transcripts in populations from Israel and the United States. Entomological Society of America, North Central Branch.

Collaborators and Affiliations

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Other Activities

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Corporation Relations Advisor
New Member Educator Advisor