GENETIC AND BIOTYPIC DIVERSITY OF GREENBUG *SCHIZAPHIS GRAMINUM* (RONDANI) POPULATIONS ON NON-CULTIVATED HOSTS

By

JAMES A ANSTEAD

Bachelor of Science

University of East Anglia

Norwich, England

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Thesis Approved:

John D. Burd
Thesis Advisor

Kevin A. Shufran

Jack W. Dillwith

Alfred Larkin
Dean of the Graduate College

PREFACE

Chapter I of this thesis is an introduction and literature review focusing on the history and biology of *Schizaphis graminum*. It concentrates on host use and biotypes. Chapters II, III and IV are formal manuscripts of the research I conducted during my M.S. program and are written in compliance with the publication policies and guidelines for manuscript preparation with the appropriate journals.

I sincerely thank my major professor Dr. John Burd for all his advice and assistance throughout my project. I would also like to thank my committee members Drs. Kevin Shufran, Kris Giles and Jack Dillwith for their advice. In particular I would like to thank Dr. Kevin Shufran for his laboratory training and his help in preparing this manuscript.

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CHAPTER I

Introduction and literature review

Introduction

Schizaphis graminum, the greenbug is an important pest of wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*) and a number of other graminaceous crops. On crop hosts it reproduces parthenogenically leading to large populations that cause considerable economic loss. Damage is typically through loss of chlorophyll leading to chlorosis, necrosis and eventually plant death (Webster and Phillips 1912). Host plant resistance has been used to attempt to control greenbug damage, however, its effectiveness has been compromised by virulent biotypes. With the exception of biotype D, which was characterized on the basis of insecticide resistance (Teetes *et al.* 1975), biotypes A-K have been characterized by their ability to differentially damage certain resistant sources (Porter *et al.* 1997). Each biotype is defined by its "virulence profile", i.e. it shows a unique pattern of virulence or avirulence against a set of resistant plant entries. Virulence against susceptible varieties results in characteristic chlorosis and necrosis, which leads to plant death (Wadley 1931). The widespread assumption that these biotypes evolved because of selection pressure from resistant field crops has recently come under scrutiny (Porter *et al.* 1997). Porter *et al.* (1997) found no correspondence between the introduction of wheat cultivars and the emergence of biotypes virulent to them and only a weak correspondence between the release of resistant sorghum cultivars and the characterization of new biotypes resistant to them. They instead proposed that the greenbug species may be a complex of host-adapted races that evolved on non-cultivated hosts.

In contrast to almost all studies of the greenbug to date, which have focused exclusively on populations on crop hosts, this study concentrated on non-cultivated hosts. The aim of this study was to document the phenotypic and genotypic diversity of greenbugs found on non-cultivated grass hosts, to look for evidence linking phenotype, genotype and host, and to show which non-cultivated grasses were hosts for greenbugs. Phenotypic variation was assessed by determining the biotypic status of greenbugs, i.e. how an array of plant differentials responded to feeding. Genotypic diversity was measured by comparing sequence divergence of mitochondrial DNA.

My hypothesis was that the greenbug populations utilizing wheat and sorghum are only a subset of the total diversity found on other hosts. I measured the diversity found on non-cultivated hosts and compared this with the diversity that had been found on crop hosts previously. I also examined which non-cultivated hosts greenbugs use. I measured the amount of genetic diversity within single biotypes, which past studies had either ignored or only superficially addressed.

The results of this thesis are important in a number of ways. If greenbug diversity is high on grass hosts, plant breeders may have to consider using grass-collected greenbugs when screening for resistance. The biotypic composition on grasses may indicate the host range of each biotype and whether or not they overlap. If new biotypes are found on grasses, then the use of the biotype characterization system used in greenbugs may need revision. If these new biotypes are not found on crop plants, should they be given a new

alphabetic designation as a biotype, or should they be identified by some other designation (host race for example)? The relationship between host, genotype and phenotype was explored, in particular to look for evidence of host races. Information about genetic variance within each biotype will also be useful in determining whether biotypes are reproductively isolated and whether biotypic designations have any evolutionary or taxonomic status in this system.

Study sites with a high diversity of grasses were chosen to maximize the potential genetic diversity of greenbugs present. Grass-host diversity was measured by constructing an α-diversity curve for each site (Southwood 1978). Phenotypic diversity of the greenbug was measured by comparing the virulence of greenbug clones on different sources of resistance in wheat, barley, rye and sorghum. Genotypic diversity was measured by comparing sequence data from the mitochondrial DNA cytochrome oxidase subunit I (COI) gene. This sequence data was used to construct a phylogenetic tree of biotypes and field populations, as well as to estimate the degree of genetic similarity among them.

Objectives

I. Determine the biotypic composition of greenbugs on non-cultivated grass hosts.
II. Determine the genetic diversity of greenbugs on non-cultivated grass hosts.
III. Determine the fecundity and reproductive investment of a single aphid clone on a number of hosts.

Methods

1. Three study areas were selected and a species curve of graminaceous greenbug hosts was generated.
2. Live greenbugs were collected from non-cultivated grasses and nearby crops at these sites, and from other sites on a contingency basis. The latter was necessary because greenbug populations at selected sites may suffer local extinction, a common event for aphids. Clonal laboratory colonies of these collections were established to provide material for experimentation.
3. The biotype of each clone was determined by a characteristic response on standard plant differentials. On wheat, barley and rye the plant entries were simply scored alive or dead when the susceptible control died. On sorghum differentials, a damage score relative to the control was given. If more than 60% of the plant was chlorotic or dead it was considered susceptible. If under 30% was damaged it was considered resistant. Intermediate scores were re-tested.
4. A virulence profile for each greenbug clone was then generated. The diversity of virulence profiles (i.e. biotype) found was determined.
5. The genetic diversity of populations was determined by sequencing the mtDNA COI gene in each clone. After extracting the total DNA from each greenbug clone, the COI gene was PCR amplified, extracted, and direct sequenced. Sequence divergence was then compared and contrasted between the clones collected from different grass species, and from the standard laboratory biotypes (Shufran *et al.* 2000). This data was then used

to generate a phylogeny as well as to estimate genetic distances among clones and biotypes and within biotypes. To determine if any specific biotype and host associations exist, host information was overlaid upon the generated phylogeny.

6. The fecundity and reproductive investment of a single greenbug isolate was tested to see if it had a specific host association. Seven-day fecundity and ovariole number were measured on four different hosts.

Literature Review

The earliest record of the occurrence of the greenbug, *Schizaphis graminum* (Rondani), concern an outbreak in Parma Italy in 1847 (Rondani 1847). In 1852, Rondani recorded another outbreak and noted that greenbugs were found only on graminaceous hosts.

Greenbugs were first detected in the US in Culpepper, Virginia in 1882 (Webster 1909). The greenbug's geographical range in North America was initially recorded as ranging from the Mexican border across the whole Western coast into Canada and across the whole of the continental USA excluding the NE (Webster and Phillips 1912). However, since then it has also been reported from Pennsylvania, New Jersey, New York, Connecticut (Leonard 1968), Wisconsin (Orlob and Medler 1961), New Brunswick (Orlob 1961) and Manitoba (Robinson and Hsu 1963).

In the US, the aphid reproduces by apomictic parthenogenesis in areas where the daylength does not decrease early enough for the induction of the sexual cycle (Wadley 1931). North of the 35th parallel, sexual reproduction is

induced during autumn. Decreased daylength is the primary trigger, but low temperatures can further promote the production of oviparae (Puterka and Slosser 1986). The greenbugs in these areas diapause and overwinter as an egg, primarily on grasses in the genus *Poa* (Dixon and Kundu 1994). In the spring, a fundatrix emerges from the egg. She is always wingless (Wadley 1931) and must therefore establish a new colony on the grass on which she emerged. Alates able to infest new hosts were not found in Indiana until May (Webster and Phillips 1912) or in Minnesota until June (Wadley 1931). Eggs may be laid and hatched on wheat, however, they do not normally contribute to fall infestation in the next year because the wheat is harvested before the first alates appear (Wadley 1931). In areas with a favorable microclimate and in areas South of the 35th parallel greenbugs overwinter as viviparous females, on both cultivated and non-cultivated hosts (Wadley 1931).

In economic terms, greenbugs have been a serious pest of grain crops in North America, particularly wheat since the 1880's and of sorghum since 1968 (Harvey and Hackerott 1969). Various control tactics have been used. Organophosphorous chemicals were successfully used to control greenbug populations from 1948-75 (Teetes *et al.* 1975). However, in 1975 greenbug populations resistant to disulfoton caused a failure of chemical control (Teetes *et al.* 1975). Since that time greenbugs have exhibited resistance to a number of chemicals including oxydemeton-methyl, dimenthoate, parathion and chlorpyrifos-methyl (Peters *et al.* 1975; Archer and Bynum 1978; Sloderbeck *et al.* 1991). A combination of resistance to chemical control and the low value of

crops make chemical control economically unfeasible in many cases, therefore interest has focused on the deployment of greenbug resistant varieties.

Dahms (1948) first conducted tolerance screening of cereal crop lines. The first source of greenbug resistance identified was "Dickinson selection 28A", selected from durum wheat (*T. turgidum* var. durum) (Dahms *et al.* 1954). DS 28A was resistant to aphids collected from field populations (Porter *et al.* 1997), however it was soon found to be damaged by a greenbug population found in the greenhouse (Wood 1961). This virulent population was designated biotype B, therefore the avirulent field population at the time was, *a priori*, biotype A. It has been postulated that biotype A is no longer recoverable from the field and is presumed extinct (Porter *et al.* 1997), however, since it was only defined in terms of not being biotype B, this conclusion may be unfounded. Biotype C was described in 1969 when greenbugs first caused extensive damage to sorghum (Harvey and Hackerott 1969). Biotype D was characterized on the basis of its resistance to organophosphate pesticides (Teetes *et al.* 1975). Biotype E was described in the late 1970's. It damaged the biotype C-resistant wheat Amigo and several resistant sorghum sources (Starks and Burton 1977, Porter *et al.* 1982). In 1986 an isolate collected from *Poa compressa* was found that differed from biotype E in its ability to damage a variety of this grass called "Reubens". This isolate was designated biotype F (Kindler and Spoomer 1986). Biotypes G and H were described by Puterka *et al.* (1988). Biotype G was unusual in that it was virulent to the known resistant sources in wheat but not to "Wintermalt" barley, a variety susceptible to all other biotypes at the time. Biotype H was

unique in its virulence to "Post" barley and its avirulence to all sorghums. In 1991 greenbugs able to overcome biotype E resistant sorghum was found and described as Biotype I (Harvey *et al.* 1991). Beregovoy and Peters (1994) described biotype J, it is avirulent to all plant differentials. The most recent biotype was described in 1997, when greenbugs virulent to I resistant sorghum were characterized as biotype K (Harvey *et al.* 1997). For a summary of test-plant differentials and feeding reactions, see Table 1.1 (p.29).

The term biotype has been defined in a number of different ways. Eastop (1973) suggested biotype referred to a subpopulation recognized by biological function rather than by morphological characters. Similarly, Saxena and Barrion (1989) defined biotype as an infraspecific category of insect populations with similar genetic composition for a biological attribute. Diehl and Bush (1984) recommended the division of the term biotype into five categories depending on the mechanisms underlying biotype differentiation; "a) nongenetic polyphenisms, b) polymorphic or polygenic variation within populations, c) geographic races, d) host races and e) species". In the case of the greenbug, virulence (see definition, p. 2) on certain hosts is the determining factor. Under the system of classification of Diehl and Bush (1984), greenbug biotypes could be described as a case of polymorphic variation within populations, host race, geographic races or possibly even species. Currently the taxonomic and evolutionary status of greenbug biotypes has not been determined.

The taxonomic and evolutionary status of greenbug biotypes has been the subject of some controversy. Have biotypes recently evolved after selection by

the introduction of resistant wheat and sorghum, or are they pre-adapted opportunists, taking advantage of a new host when it is introduced? Porter *et al.* (1997) addressed this question and found no correspondence between the development of resistant wheat cultivars and the emergence of biotypes virulent to them. Interestingly, in all cases resistant wheat cultivars could not have caused the appearance of new biotypes because the cultivars were never present in the field when a biotype virulent to them was discovered. For instance, biotype B was found to be virulent to DS 28A wheat, but this cultivar was never used commercially (Porter *et al.* 1997). They did find some correspondence between the release of resistant sorghum cultivars and the characterization of new biotypes resistant to them, but were unable to confirm a direct cause and effect relationship.

The hypothesis that greenbug biotypes are pre-adapted populations taking advantage of new hosts would fit theories of plant defense in agronomic crops. Rosenthal and Dirzo (1997) showed that in maize and some of its relatives, the perennial relative had best insect defense, followed in descending order by the wild annual, a land race, and a modern high yielding variety. They attributed this effect to the re-partitioning of resources from plant defense to increased yield. It seems possible that as wheat and sorghum are bred for increased yield, resources for aphid defense are re-partitioned to yield increase, making the crop more susceptible to attack. Sympatric races of pea aphid (*Acryrthosiphon pisum*) have been characterized by their ability to utilize different host species (Via 1999). They are believed to be reproductively isolated as a result of localized

host adaptation. Habitat choice lead to assortive mating, providing a barrier to gene flow and subsequent selection lead to the formation of two host races. There was, however little genetic diversity found among these biotypes using RFLP markers (Birkle and Douglas 1999) or mitochondrial DNA sequences (Boulding 1998). This has lead some to reject the idea of host races in the pea aphid (Boulding 1998) and others to conclude the separation into races is very recent (Birkle and Douglas 1999).

Eisenbach and Mittler (1987b) believed crossing experiments they performed showed biotype E virulence to biotype C resistant sorghum was inherited in an extra-nuclear manner. This was subsequently refuted by Puterka and Peters (1989) who studied the inheritance of virulence to resistance in wheat, in biotypes C, E and F. The data for virulence to *Gb2* and *Gb3* resistance genes gave the best fit with a duplicate gene-modifier gene inheritance model, where virulence is recessive. Puterka and Peters (1995) also studied the inheritance of virulence to resistant in sorghum, in biotypes C, E and F. Virulence to F-resistant "Piper" (a variety of *Sorghum sudanense*) was controlled by a duplicate dominant gene-modifier gene, and was dominantly inherited. Virulence to C and E resistant "Pioneer 8493" (resistance from PI 264453), was also controlled by a duplicate dominant gene-modifier gene, but was recessive. Resistance to biotype C in " Pioneer 8515" (resistance from SA 7536-1) was recessive and simply inherited. Greenbug virulence and sorghum resistance interactions were found to fit a gene-for-gene model, however wheat resistance did not. These two studies showed that genetic recombination during meiosis in sexual reproduction

could produce biotypic diversity. In fact, crossing experiments produced new laboratory biotypes with different virulence profiles (Puterka and Peters 1995).

The greenbug is without doubt a highly polyphagous species. Webster and Philips (1912) recorded its presence on 60 host species. Hunter (1909) and Moore (1914) added a few new hosts to the list. Patch (1938) published the next attempt at an exhaustive list of grass hosts, 62 species were included but there was no information on survivorship. Dahms *et al.* (1954) published a list of 21 species on which greenbugs increased in a greenhouse. Interestingly, the two different populations of greenbug (one from Stillwater, OK and one from Manhattan, KS) he used showed different host association, with the Stillwater greenbugs unable to live on *Echinochloa crusgalli* but able to live on *Elymus canadensis,* and the Manhattan greenbugs able to live on *E. crusgalli* but unable to live on *E. canadensis.* The "Canada Wild Rye isolate" recently characterized in Oklahoma (Burd, unpublished data) was able to reproduce on Virginia wild rye (*Elymus virginicus*) but unable to reproduce on western wheatgrass (*Agropyron smithii*) (Anstead *et al.* 2000). These findings suggest individual greenbug clones have a more limited host range than the species as a whole.

The most recent publication confirms this large host range; i.e. 70 confirmed host plants in 29 genera (Michels 1986). It should be noted that greenbug densities are considerably lower on wild grass hosts (less than 5 individuals per leaf) than on wheat, sorghum, barley or oats, where infestations can run into 1000's of individuals (Wadley 1931). Grass hosts are considered important oversummering hosts for the greenbug. There is a period during the summer

when wheat is not available to the greenbug. During this time period greenbugs may be found on grasses (Daniels 1961). Greenbugs were found oversummering on 23 grass species in the Texas panhandle from 1953-59 (Daniels 1960). *Agropyron smithii* was considered the most important host. The greenbug extended its host range to sorghum in the late 1970's, however oversummering on sorghum does not seem to occur. Harvey *et al.* (1982) report two periods of infestation in sorghum, an initial seedling infestation that dies out by mid June and a later population that appears in late July and early August. Grasses are also important during the sexual cycle North of the 35th parallel. The fundatrix emerges onto non-cultivated grasses in the spring (Wadley 1931). This occurs primarily on grasses in the genus *Poa* (Dixon and Kundu 1994). The fundatrix is apterous, so both she and her offspring (before maturity) must be able to feed successfully on host they emerge on. Non-cultivated hosts are very important in this context, because any eggs laid on wheat do not contribute to the next years populations as the wheat is generally harvested before any alates are produced (Wadley 1931). For instance, in Indiana a fundatrix hatched on March 28th, but her oldest progeny was not fully developed until May 9th (Webster and Phillips 1912). In areas with a favorable microclimate and in areas South of the 35th parallel greenbugs overwinter as asexual females, on both cultivated and non-cultivated hosts (Wadley 1931).

There are numerous publications that describe the presence, absence and densities of greenbug biotypes geographically. In 1984, biotypes C and E were present in Kansas, Nebraska, Oklahoma and Texas, with biotype C

predominating (Kindler *et al.* 1984). This was also true in Arkansas (Dumas and Mueller 1986). The most recent survey found biotypes B, C, E, F, G and I present on field crops in Oklahoma between 1991 and 1996, with biotype E predominating and I second most common (Peters *et al.* 1997). In a similar survey of a wider geographical area (but much smaller sample size) Kansas, Nebraska, Texas, Colorado and Oklahoma were found to have populations mostly consisting of Biotype E with some areas in each state containing biotype I (Peters *et al.* 1997). This difference in occurrence and density of greenbug biotypes on crop plants may be a reflection of the competitiveness of these biotypes on these hosts. Although well characterized on crops, there is little information concerning which biotypes occur on wild grass hosts as these were seldom, if ever, surveyed.

There is a substantial body of work dealing with variation between greenbug biotypes. Many researches have demonstrated differences in life history parameters and other characters of greenbug biotypes. Early work showed that biotype C had better survival and fecundity at temperature extremes than biotypes A and B (Wood and Starks 1972, Starks *et al.* 1973). Some interesting work was carried out looking at mate preference (Eisenbach and Mittler 1987). Biotype E males, when given a choice, strongly preferred biotype E females and biotype C males preferred biotype C females (although not as strongly). Mate choice may be a factor in reproductively isolating these biotypes which are often found together. Other researchers focused on the ability of greenbug biotypes to produce sexuals. Under a 11 hour photoperiod, biotypes C, E and I readily

produced large numbers of sexuals, G and F produced lower numbers of sexuals, and biotypes B, H and J produced no sexuals (Puterka and Peters 1990, Ullah and Peters 1996). Differences in feeding behavior have been noticed. Montllor *et al.* (1983) found biotype E aphids initiated phloem feeding more quickly than biotype C on biotype C resistant (E susceptible) sorghum and also had a higher reproductive rate. Hays *et al.* (1999) showed that on resistant barley, biotype E greenbugs probed more and spent much less time feeding than Biotype H greenbugs. Very little work has focused on variation within biotypes, but Michels *et al.* (1987) showed differences in progeny production and developmental periods between biotype E greenbugs collected from corn and wheat. McCauley *et al.* (1990) showed similar results, i.e. corn-reared aphids were more fecund than sorghum raised aphids when fed on either host.

Recently more direct methods have been used to assess variation between greenbug clones. Morphometric studies showed that biotypes B, C and E could be differentiated (Inayatullah *et al.* 1987b and Fargo *et al.* 1986). These studies also indicated biotype B was more divergent than E or C. They were able to separate all three biotypes based on four morphs (males, apterous viviparous, apterous oviparous and alate/viviparous females) (Inayatullah *et al* 1987a). Alate biotype C greenbugs generally had more antennal sensoria than biotypes B and E (Inayatullah *et al* 1991).

Isozyme analysis failed to provide any polymorphism between biotypes (Beregovoy and Starks 1986). Therefore, most work has focused on genotypic diversity in both mitochondrial and genomic DNA. Powers *et al.* (1989) studied

mitochondrial DNA restriction patterns and related these to divergence among biotypes B, C, E and F. Their results showed a divergence level indicating 300,000-600,000 years had passed since biotypes B and C shared a common mitochondrial DNA ancestor. This suggested that it was unlikely these biotypes evolved as a response to selection pressure from modern crop cultivars, since 300,000 years is well before the beginnings of human agriculture. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to detect DNA polymorphisms in four aphid species including greenbugs (Black *et al.* 1992). Differences were detectable between greenbug biotypes, with the exception of biotypes C and E, which were indistinguishable.

A number of studies have used the length of the intergenic spacer (IGS) region of the rRNA cistron as a molecular fingerprinting probe to study diversity within and between greenbug populations. It has been used to study clonal diversity, spatially and temporally on sorghum and wheat (Shufran *et al.* 1991). They found that most of the variation in a greenbug population within a single field could be found from sampling a single leaf. In a similar study using the IGS as a marker 93.1% of the clonal diversity was found to be present in a single field of overwintering greenbugs (Shufran and Wilde 1994). Black (1993) found differences in the IGS subrepeat structure, which were variable enough to separate biotypes. He also found some variation within biotype E, but concluded that each biotype tested (B, C and E) probably evolved from a single population or maternal lineage. Shufran *et al.* (1992), however, showed biotype E populations in the field were comprised of many clonal lineages.

Shufran *et al.* (1997) studied the inheritance of IGS variants during sexual reproduction and noted rearrangements and the generation of new size variants. They concluded that recombination during meiosis in the sexual reproductive cycle generates and maintains genetic variability in greenbugs.

Mitochondrial DNA has been used to examine the sequence divergence between greenbug biotypes and infer phylogenetic relationships (Shufran *et al.* 2000). All presently known biotypes were included (B, C, E, F, G, H, I, J and K) along with a possible example of biotype A (New York isolate), an unique isolate collected from Canada Wild Rye (Burd, unpublished data) and an isolate from Europe. All biotypes and isolates grouped into 3 clades, with the exception of biotype H and the outgroup *S. rotundiventris* which fell outside the *S. graminum* group. The three clades had significant sequence divergence between them. This divergence supported the conclusions of Powers *et al.* (1989) that diversity in the greenbug arose before the advent of modern agriculture. The greenbug biotypes most commonly found on crops (C, E, I and K) were the most homogeneous group and were found in clade 1. Clade 2 contained biotypes F, G and the New York isolate. Both biotype F, and the New York isolate may represent the population Wood (1961) referred to as biotype A (Kindler and Spomer 1986, Shufran *et al.* 2000). Biotype G was collected from wheat, but is seldom found on crops (Bush *et al* 1987, Ullah 1993). Clade 3 contained biotype B, the Canada Wild Rye isolate and the European isolate. These results indicated that the ability to successfully utilize crop hosts is only present in part of the greenbug species. However a shortcoming of this and other studies was

that no measure of diversity within biotypes was made, as only a single laboratory clone of each was included.

In summary, it appears that some greenbug biotypes may be distinct populations that can be separated morphologically biologically and genetically. However, little work has been produced dealing with intra-biotypic variation. It is therefore still not clear how these biotypes evolved and whether there are barriers to gene flow between them.

Review of Research methodology

Mitochondrial DNA has been very useful in the study of inter and intra-specific variation in insects. In studies of 13 and 17 year cicadas (*Magicicada septendecim* and *M. tredecim*) two distinct mitochondrial haplotypes were found in each year class which differed by 2.5% (Martin and Simon 1990, Simon *et al.* 1993). In the honeybee (*Apis mellifera*), PCR was used to amplify mtDNA regions which were subsequently sequenced (Garnery *et al.* 1992). Nineteen haplotypes were found which revealed three clades. These clades corresponded with three geographical populations (African, North Mediterranean and South Mediterranean). Mitochondrial DNA has also been used with some success to study aphid populations. Barrette *et al.* (1994) carried out a restriction digest analysis of the mitochondria of *Acryrthosiphon pisum*. They only found two variable sites, but showed all four haplotypes. Martinez *et al.* (1990 and 1992) digested *Rhopalosiphum padi* mtDNA with 20 restriction endonucleases but found little restriction site polymorphism. Simon *et al.* (1998) amplified a 2.2 kb

portion of three mitochondrial genes. Using restriction endonucleases they found low variation but it was sufficient to divide *R. padi* into three haplotypes.

Mitochondrial DNA has been successfully utilized to solve phylogenetic questions. Moran *et al.* (1999) used sequences from three mitochondrial regions and one nuclear gene to examine the phylogenetics and evolution of *Uroleucon* spp. They concluded that the most plausible view of the radiation of *Uroleucon* spp. was that acquisition of new hosts occasionally lead to sympatric speciation. Clements *et al.* (2000) used sequences from the cytochrome oxidase subunit II (COII) gene and elongation factor-1 alpha (EF-1α) to examine variation within the *Myzus persicae* complex. The low variation within this complex led them to conclude that *M. nicotianae* and *M. persicae* were synonymous.

The advantage of using a mitochondrial gene sequence is that many of the processes governing its evolution and inheritance are well understood. It is maternally inherited and is not recombinant which makes the construction of phylogenetic trees easier. Mitochondrial DNA also evolves approximately 10 times faster than genomic DNA (Miyata *et al* 1982) making it particularly useful for separating closely related individuals or clones. It should be noted, however, that using a mitochondrial gene sequence does not guarantee the correct phylogeny will be generated. Cases of shared ancestral polymorphisms and multiple substitutions at a single nucleotide site can occur (Simon *et al* 1994).

The cytochrome oxidase I (COI) gene codes for subunit I of the cytochrome oxidase enzyme, that transfers electrons to oxygen, the final electron acceptor in some electron transport chains. It is the most conserved region in the

mitochondrial genome (Simon *et al.* 1994). The slow rate of evolution of this gene may make it less suitable for intra-specific studies, however, there is a lower chance of an incorrect phylogeny being caused by multiple substitutions at a single site. One potential drawback with the COI gene is the possibility that some sequences may be transposed out of the mitochondria DNA into nuclear genomic DNA. This has been recorded in *Sitobion* aphids (Sunnucks and Hales 1996). Patterns of evolution are different in mtDNA and nuclear genes and this could lead to the generation of an incorrect phylogeny. However, given the high comparative copy number of mitochondrial versus genomic DNA I consider this unlikely. The COI and its relative the COII gene have already been used in a number of recent phylogenetic studies. They have been used estimate the phylogeny of hepialid moths of the genus *Wiseana* in New Zealand (Brown *et al.* 1999a), and to aid identification of these species as larvae (Brown *et al.* 1999b). Also, they have been used to study sympatric speciation in the bee genus *Lasioglossum* (Danforth 1999) and to determine the phylogeny and sequence of invasion of islands by tenebrionid beetles of the genus *Hegeter* (Juan *et al.* 1996).

In aphids, COI gene sequences were used to reject the hypothesis that there were host races in the pea aphid (*Acyrthosiphon pisum*) (Boulding 1998). For an alternative view see Via (1999). Moran *et al.* (1999) used COI sequences (and other markers) to determine the phylogeny of aphids in the *Uroleucon* genus. A combination of RAPD-PCR and COII sequences were used by Sunnucks *et al.* (1997a) to separate the aphid *Therioaphis trifolii* into host-restricted biotypes.

Sunnucks *et al.* (1997b) used COI sequences in combination with a microsatellite marker to divide *Sitobion avenae* into three distinct genotypes, which had little gene flow between them. Host specialization was found, with one of the lineages found only on wheat, one found only on *Dactylis glomerata,* and the other found on both. The aim of my study is to use the COI gene in combination with non-cultivated host data and the phylogenetic relationships already shown between the known greenbug biotypes (Shufran *et al.* 2000) to determine whether there are any differences in host specialization and infer the evolutionary status of greenbug biotypes.

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Table 1.1: Plant response to greenbug feeding.

	Plant Differential											
	Wheat							Rye		Barley		
Biotype	Custer	DS 28A	Amigo	CI 17882	CI 17959	Largo	GRS1201	Elbon Rye	Insave Rye	Wintermalt	Post 90	PI 426756
A	S	R										
B	S	S	R	S	S	S	R	S	R	S	R	R
C	S	S	R	R	R	R	R	S	R	S	R	R
E	S	S	S	R	R	R	R	S	R	S	R	R
F	S	R	S	S	S	S	S	S	S	S	R	R
G	S	S	S	S	S	S	R	S	R	R	R	R
H	S	S	S	S	R	R	S	S	S	S	S	S
I	S	S	S	R	R	R	R	S	R	S	R	R
J	R	R	R	R	R	R	R	R	R	R	R	R
K	S	S	S	R	R	R	R	S	R	S	R	R
CWR	S	S	R	S	S	S	S	S	R	S	R	R

(S = susceptible plant response, R = Resistant plant response)

CWR = Canada wildrye isolate.

(Woods 1961, Porter *et al.* 1982, Kindler and Spoomer 1986, Puterka *et al.*1988, Harvey *et al.* 1991, Beregovoy and Peters 1994, Harvey *et al.* 1997).

CHAPTER II

Biotypic diversity of *Schizaphis graminum* (Homoptera: Aphididae) populations on non-cultivated grass hosts.

J. A. Anstead[1], J. D. Burd[2] and K. A. Shufran,[2]

[1]Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, USA. [2]USDA-ARS, Plant Science and Water Conservation Research Laboratory, Stillwater, OK, USA.

ABSTRACT The greenbug (*Schizaphis graminum* (Rondani)) has a large host range comprised primarily of non-cultivated grasses. Despite this, most studies and biotype surveys have focused on greenbug populations on crop hosts. Consequently the role of non-cultivated grass hosts in maintaining biotypes is unclear. Greenbugs were collected from non-cultivated hosts in Oklahoma, Kansas and Colorado, and their biotype and host association were determined. Biotype I was the predominant biotype and was present on all host species that greenbugs were found on. Biotypes E and K were also found on a number of grass hosts. However, almost all biotype G isolates collected were from *Agropyron smithii* Rydberg and *A. intermedium* Beauvois. A new biotype, virulent to "Largo", but otherwise having the same virulence profile as biotype I was found on *A. smithii* in Hays, KS. The Hays KS site had the lowest number of grass species, but the greatest frequency of greenbugs and the highest biotypic diversity. There was no apparent link between grass host diversity and biotypic diversity, indicating that greenbug biotypes have limited host ranges.

KEY WORDS Greenbug, *Schizaphis graminum*, biotype, host association

THE GREENBUG (*Schizaphis graminum* (Rondani)) is an important pest of wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* (L.) Moench), barley (*Hordeum vulgare* L.) and a number of other graminaceous crops. It reproduces primarily by parthenogenesis leading to large populations, causing considerable economic loss. Damage is caused by both loss of photosynthate and a virulence reaction typified as loss of chlorophyll leading to chlorosis, necrosis and eventually death (Webster and Phillips 1912). Host plant resistance has been used to reduce greenbug damage, however its effectiveness has been compromised by the occurrence of virulent biotypes (for a review, see Porter et al. 1997). Greenbug biotypes are characterized by their ability to damage certain resistance sources and have been given the letter designations A through K [Except D whose designation was based on insecticide resistance (Teetes et al.1975)].

The distribution and abundance of greenbug biotypes has been well documented on crops. In 1984, biotypes C and E were present in Kansas, Nebraska, Oklahoma and Texas, with biotype C predominating (Kindler et al. 1984). This was also true in Arkansas (Dumas and Mueller 1986). In Oklahoma a survey in 1991 found biotypes B, C, E, F and G present. Biotype E predominated, and all other biotypes were present at frequencies less than 5% (Ullah 1993). Biotypes B, C, E, F, G and I were present on field crops in Oklahoma between 1991 and 1996, with biotype E predominating followed closely by I (Peters et al. 1997). Kansas, Nebraska, Texas and Colorado were also found to have populations mostly consisting of biotype E, with some areas

in each state containing biotype I (Peters et al. 1997). While these surveys represented extensive characterization of the biotype composition on crops, the biotypic diversity on non-cultivated hosts was not considered.

Cultivated crop species represent a very small proportion of available greenbug hosts. The host range of the greenbug includes 70 host species in 29 genera, most of which are non-cultivated species (Michels 1986). Moreover, various populations show differential host associations. Dahms et al. (1954) showed that greenbugs collected from Stillwater, OK were unable to live on *Echinochloa crusgalli* (L.) but were able to live on *Elymus canadensis* L., while greenbugs from Manhattan, KS were able to live on *Ec. crusgalli* but unable to live on *El. canadensis*. Similarly, the "Canada wildrye isolate" collected from *El. canadensis* (J. D. Burd unpublished data) was able to reproduce on *El. canadensis, El. virginicus* L. and Otis barley, but was unable to reproduce on *Agropyron smithii* (Anstead et al. 2000c).

Non-cultivated hosts are an important component of greenbug population dynamics. Grass hosts are considered especially important for greenbug oversummering. There is a period during the summer when wheat is not available to the greenbug and sorghum is an unsuitable host (Daniels 1961). During this time, greenbugs must survive on non-cultivated grasses. Greenbugs were found oversummering on 23 grass species in the Texas panhandle from 1953-59 and *Agropyron smithii* was considered its most important summer host (Daniels 1960). Although the greenbug can successfully exploit sorghum complete oversummering on sorghum may not always occur. Harvey et al.

(1982) reported two periods of infestation in sorghum, an initial seedling infestation that dies out by mid June and a later population that appears in late July and early August.

Wadley (1931) explained the importance of grasses during the sexual cycle North of the 35th parallel. Oviparae is induced in the autumn as daylength decreases. Eggs are laid primarily on grasses, as wheat has not emerged. In this region greenbugs oviposit primarily on *Poa spp.* (Dixon and Kundu 1994). The fundatrix emerges onto non-cultivated grasses in the spring and must establish a colony. Alates are produced that disperse to wheat where new colonies are established. However when the wheat is harvested only greenbugs on grasses remain to restart the cycle.

Besides acting as temporal and spatial reservoirs, non-cultivated grasses may also serve as reservoirs of biotypic and genetic diversity. Shufran et al. (1991) found a high degree of clonal diversity on wheat and sorghum and suggested that this diversity was maintained on non-cultivated grasses. Porter et al. (1997) found no correspondence between the introduction of wheat cultivars and the emergence of virulent biotypes and only weak correspondence between the release of resistant sorghum cultivars and the characterization of new virulent biotypes. Moreover, they proposed that the greenbug species may be a complex of host-adapted races that evolved on non-cultivated hosts. Evidence for this was reported by Kindler and Hays (1999) who found that biotype F had significantly higher fecundity on Canada bluegrass, *Poa compressa* L. than on several other cool-season grass hosts. They concluded that the development of

biotype F was driven by its association with this non-cultivated grass host. Shufran et al. (2000) showed that based on mtDNA sequences, the greenbug species could be divided into 3 clades. The isolates collected regularly from crop hosts were confined to one clade and the other clades contained isolates rarely collected on crop hosts or collected from non-cultivated hosts (Shufran et al. 2000). The authors postulated that these clades may have diverged as host-adapted races on non-cultivated grasses. Anstead *et al.* (2000b) showed even stronger relationships between the divergence of these clades and host use, and supported the conclusion that the greenbug species is comprised of host-adapted races.

The objectives of this study were to assess biotypic diversity on non-cultivated grasses and to examine biotype-host associations.

Materials and Methods

Sampling. Aphid collections were made from three sites; Hays, KS (N38°50'32", W99°18'09"), Redrock, OK (N36°26'34", W97°06'84") and Marshall, OK (N36°06'53", W97°36'08"). The site at Hays was in a riparian area, bordering Big Creek. The site sloped about 10 feet down across its width and was partially shaded. It had been grazed previously. The wheat field was bordered by a considerable area with volunteer wheat as well as an area that was tilled. The area in Redrock was in a low lying area adjacent to a stock pond, with little shade and it too had been grazed. It bordered a wheat field on one side and a railway right-of-way on the other. The site at Marshall was not grazed but had

been mown regularly but infrequently prior to the experiment. It bordered county road NS303 and a wheat field. There was a transition from a dry area next to the road, to a wetter, lower area beside the field fenceline. This site was considerably drier than the other sites. The areas were chosen for their diversity of non-cultivated grasses and because each site bordered a cultivated wheat field. Each study area was 40m by 10m.

To estimate species richness an α-diversity curve of graminaceous hosts for each area was created in June 1999. The fenceline between the non-cultivated and cultivated areas served as a transect. At 1 meter intervals, a transect was laid at a 90° angle from the fenceline to the perpendicular border of the site (10 meters). Each grass species touching the line was identified and recorded. The cumulative number of grass species found was plotted against the cumulative distance examined. When this curve reached a plateau, this represented the α-diversity in the plot (Southwood 1978). All grasses in the plot were included. If a grass could not be identified in its vegetative state, it was either marked and identified at a later growth stage or a small amount was removed to the laboratory for identification. Grasses were identified using Hitchcock and Chase (1971). Cool season annuals were included by identifying them from the previous seasons culms and inflorescences, and were included in the curve. During subsequent sampling all species identified were found again and only a single example of an additional grass was found. A single *Aegilops cylindrica* Host plant was found in the Hays Kansas study site and is included in Table 2.1.

Aphids were sampled between June 1999 and February 2000 at approximately one month intervals. Sampling was carried out randomly by casting a 0.25 m^2 quadrant into the plot. Twenty culms of each grass species in the quadrant were examined manually for aphids. On each sampling date twenty quadrants were examined. At Hays, the neighboring field contained considerable volunteer wheat and presence/absence of greenbugs was recorded separately (Table2.2). Greenbugs were also collected from a number of other sites in Oklahoma, Kansas and Colorado as opportunity arose (Table 2.4). Individual greenbugs were collected alive and kept separately.

In the laboratory, single greenbugs were used to start clonal colonies. Each colony was maintained on "Otis" barley grown in a 3.65 cm by 21 cm cone-tainer™ (Cone-tainer Company, Canby, Oregon, USA) and covered with a cylindrical plastic cage. The cages are manufactured from plastic tubing (3.5 cm diameter), with several cloth covered ventilation holes in the sides. The major cause of greenbug mortality after colonies were established was fungal disease. To avoid this, barley plants were grown in fritted clay (near-sterile media) and were watered and fertilized from the base of the cone-tainer. This was achieved by placing the Cone-tainers in a rack and placing this in a tray of water containing Peters Professional™ 20-20-20 fertilizer at one teaspoon per gallon. After the aphids and cage were placed on the barley, water was withheld to reduce humidity in the cage and help prevent fungal disease. The plants were then kept in a growth chamber at 17±3°C with a photoperiod of 14:10 (L:D)

hours. Because greenbugs are virulent to "Otis barley", they required new barley plants every two to three weeks.

Biotyping; determination of biotypes A, B, C, F, G, H and J. The biotype of each clone was determined using the plant differentials from Anstead (2000). Large numbers of aphids were produced clonally for each test. The aphids were allowed to reproduce freely on seedling barley in 4 rectangular pots (15cm by 8cm). This produced approximately 40 heavily infested barley plants, which provided between 400-800 aphids for each test. For each test, 3 sets of 3 plants for each differential were randomized in a 30 cm by 30 cm flat of polystyrene wells containing fritted clay. Planting dates were staggered between the differentials to ensure all entries emerged at the same time. "Wintermalt", "Post 90", "Insave" and "GRS1201" were planted first. 24 h later, "DS28A", "Amigo", CI17959 and CI17882 were planted, and 24 h later "Largo", PI264453 and "Custer" were planted. The plants were infested at the two-leaf stage by placing infested barley leaves across the test plants to ensure efficient transfer. After 3-4 d, the barley leaves were removed. The tests were maintained under artificial light (incandescent and fluorescent) with a photoperiod of 14:10 (L:D) h, at 20 ± 5°C. After the susceptible control, "Wintermalt" barley, was killed (usually about 7 d), the test was terminated and the remaining plants rated for damage. All plants were scored as alive or dead. If more than 75% of a particular test plant was dead, then it was rated susceptible. If none were dead, it was resistant. If any entry didn't meet these criteria it was re-tested. This new protocol is a simplification of the standard 1-9 (1=no damage, 9=plant death) damage rating

used for greenbug resistance screening (for standard protocol see Puterka et al. 1988). Initially the traditional rating system was used, however a rating of 9 (dead) was obtained for all susceptible entries and therefore the protocol was simplified to save time.

Biotyping; determination of biotypes E, I and K. Testing with sorghum was necessary to separate biotypes E, I and K, however all isolates were tested to allow us to detect differences in sorghum virulence for the other "non-sorghum" biotypes (B, C, F, G, H, J). For each test, 3 replicates of 3 seedlings were grown in fritted clay, in Cone-tainers™ covered with cages. When the sorghum seedlings reached the 2-leaf stage they were infested with equal numbers of aphids (between 15 and 20 in each test). The aphids were placed directly onto the plant with a paintbrush to reduce transfer mortality.

Three sorghum entries were used to ascertain biotypic composition; "Shallu" susceptible to biotypes E, I and K; PI 264453 resistant to biotype E, but susceptible to biotypes I and K; and PI 550610 resistant to E and I, but susceptible to K. Therefore if there was no damage the greenbug was biotype C, if it damaged only "Shallu" it was biotype E. If it damaged Shallu and PI 550610 it was biotype I and if it damaged all three entries it was biotype K (Harvey et al. 1997). After 14 d the sorghum plants were visually rated for damage. If more than 60% of the plant was chlorotic or dead it was considered susceptible. This would correspond to a score of $\geq$7 on the traditional 1-9 scale of damage (1=0-10%, 9=81-100%) (Harvey et al. 1991 and 1997). If under 30% was damaged

(score of ≤4 on the old scale) it was considered resistant. Intermediate scores were re-tested.

Results

The grasses present in each study site were identified to species. An α-diversity curve was plotted for each area (Figure 2.1). The curves were slightly distorted by the presence of bunch grasses i.e. those with a cespitose growth habit such as *Panicum virgatum L.*, *Chloris virgata* Swartz or *Elymus virginicus*, which were not randomly distributed within the plot. There were 11 grass species present at Marshall, 10 at Redrock and 9 at Hays (Table 2.1). *Cynodon dactylon* (L.) was the only species present at all three sites.

The collection data is summarized in Table 2.2. Because Hays was the only site where greenbugs were collected regularly, more detailed information about host use was tabulated (Table 2.3). Greenbugs were present in Hays throughout the study period, initially on non-cultivated grasses and later on volunteer and cultivated wheat. At the other sites, greenbugs were only found once on non-cultivated hosts, but had become established on fall-planted wheat by November. At Hays greenbugs were found on *Agropyron smithii*, *Bromus tectorum* L. and *Setaria viridis* (L.) They were not found on *Elymus virginicus*, *Sporobolus sp.*, *Cynodon dactylon* or *Poa pratensis*, which were abundant (Table 2.3). Host plants and biotypes of greenbugs collected from random locations in Oklahoma and Colorado are listed in Table 2.4. *Sorghum halpense* was the most common host on which greenbugs were found in Oklahoma

whereas *A. intermedium* was the only grass host that they were collected from in Colorado.

Table 2.5 summarizes all the collection data. Overall biotype I was the predominant biotype. It was present on every host and accounted for 63% of the greenbugs collected, followed by biotypes E (17%), G (13%) and K (7%). It should be noted that all biotype G isolates showed a biotype I virulence profile on sorghum. Therefore screening for biotypes using sorghum only, would have lead to the misclassification of these isolates and an overestimation of the frequency of biotype I.

A new biotype was also found, i.e. it killed a set of plant differentials which had not previously been described. It was collected from *Agropyron smithii* in Hays, Kansas, in June 1999. It showed the same virulence profile as biotype I except it was virulent against "Largo" wheat.

Other aphid species were also collected from non-cultivated hosts during the course of this study. This data is presented in the appendix.

Discussion

On non-cultivated grasses, greenbug densities were low and very patchy, both temporally and spatially. The differences in host species richness at each site did not appear to influence greenbug abundance. At Redrock and Marshall, even when several suitable greenbug hosts were present, greenbugs were not found (Table 2.2). Greenbugs were collected once from *Sorghum halpense,* but were not found again. There are a number of possible reasons local extinctions

like this occur in aphids, such as host quality, natural enemies and abiotic factors such as temperature (Hales et al. 1997). Interestingly, even when greenbugs were present in the wheat field, these grasses were not colonized. This may suggest that they couldn't colonize these grasses because they were not adapted to these particular hosts. Phylogenetic studies based on mtDNA sequences indicate that greenbugs utilizing crop hosts are a very homogenous group that appear divergent from those greenbugs that occur primarily on non-cultivated hosts (Anstead et al. 2000a). Consequently, the results from the present study indicating that the greenbugs on wheat at the Marshall and Redrock study sites could not use the non-cultivated species present in the study suggest that they originated from outside the local area and lack the genetic diversity to utilize the grasses present.

At the Hays study site, greenbugs over-summered on non-cultivated grasses. This shows the grasses have a direct role in the re-colonization of wheat in the autumn. Interestingly at Hays greenbugs were present in cultivated wheat 2 months before the wheat in Marshall and Redrock, even though planting dates were much earlier at these locations. This earlier infestation was a direct result of local movement from oversummering grasses by local greenbug populations. versus later more regional greenbug movement in Redrock and Marshall. At Hays, greenbugs were found on only 3 of the 9 grasses sampled (Table 2.2), even though all 9 of them have previously been listed as suitable greenbug hosts (Michels 1986). Again, this suggests that the local population was only adapted to a subset of the grasses present. This is in agreement with earlier

work (Dahms et al. 1954, Kindler and Hays 1999, Anstead et al. 2000b), that showed individual aphid clones differed in their ability to survive on different grass species. Host specialization has also been found in other aphid species. Work by De Barro et al. (1995) on *Sitobion avenae* F. showed particular clones preferred to colonize, and performed better on wheat than other grasses. In another study *S. avenae* lineages separated by a single-locus microsattelite and a mitochondrial marker, were found to exhibit host specialization (Sunnucks et al. 1997b). The same authors found three host-adapted races within *Therioaphis trifolii* (Monell) (Hemiptera: Aphididae) (Sunnucks et al. 1997a).

Biotype I was present on all hosts (Table 2.5). There appears to be no specialization by host for this biotype. This conclusion is supported by Anstead et al. (2000a), where three clades within the greenbug species were identified according to a mitochondrial gene tree. They suggested that these clades may represent host-adapted races. Biotype I was present in all three clades and therefore would be expected to have a large host range.

The presence of biotype G at such high densities was unexpected. In previous surveys it accounted for a maximum of 2-3% of greenbugs collected from crop hosts (Bush et al., 1987; Ullah, 1993). In this survey it made up 13% of the isolates collected and was found in Kansas. It had previously only been found in Texas and Oklahoma. Furthermore, biotype G was only found on *Agropyron* spp. It is possible biotype G is best adapted to non-cultivated grass hosts. However, further collections and host range studies would be needed to confirm this.

Genetic recombination during meiosis in the sexual reproduction cycle can lead to the formation of new biotypes (Puterka and Peters 1990). Therefore the discovery of a "new biotype" was not totally unexpected. Hays is North of the 35th parallel, where sexual reproduction is known to occur (Wadley, 1931) and therefore greater biotypic diversity would be predicted. The "new biotype" showed the same virulence profile as biotype I except it was virulent to "Largo". Biotype I greenbugs were present at this site, as were biotype G aphids which are virulent to "Largo". Based on mtDNA sequences this isolate was found to be more closely related to other biotype I isolates than biotype G isolates (Anstead et al. 2000a). This provides a good example of how virulence genes present in population on non-cultivated hosts may move into crop adapted races, thereby exposing crops to new biotypes.

In summary, biotype G was the only biotype collected that showed a specific host specialization and was limited to *Agropyron* spp. Biotypes E, I and K were found on multiple hosts. Biotype I was found on all hosts which harbored greenbug. Biotypes E, I and K did not appear to show any host specialization for the grasses that were sampled. The presence of an isolate with a new virulence profile, showed that biotypic diversity can be maintained on non-cultivated grasses and these grasses may serve as reservoirs for biotypic diversity. Moreover, non-cultivated grass hosts have the potential to serve as temporal bridges between the growing seasons of crops.

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Table 2.1. Non-cultivated grasses present at 3 study sites surveyed for greenbugs.

Site	Scientific name	Common name
Hays, KS	*Aegilops cylindrica* *	jointed goatgrass
	*Agropyron Smithii**	Western wheatgrass
	Bouteloua curtipendula (Michaux)	side-oats gramma [C2]
	*Bromus tectorum**	downy brome [C2]
	Cynodon dactylon *	Bermuda grass [C3]
	Elymus virginicus *	Virginia wildrye
	Poa pratensis *	Kentucky bluegrass
	Setaria viridis *	Green foxtail [C2]
	*Sporobolus sp.**	dropseed
Redrock, OK	*Bromus catharticus* Vahl*	rescue grass [C2]
	Bromus tectorum *	downy brome [C2]
	Cynodon dactylon *	Bermuda grass [C3]
	Elymus canadensis *	Canada wildrye [C2]
	Eragrostis sp. *	lovegrass [C2]
	Hordeum pusillum Nuttal*	little barley
	Panicum virgatum *	switchgrass
	Phalaris canariensis L.*	canary grass
	Sorghum halpense *	Johnsongrass
	Tridens flavus (L.)	purpletop
Marshall, OK	*Aristida oligantha* Michaux	annual 3-awn
	Bothriochloa saccarhoides (Rydberg)*	silver bluestem
	Bouteloua curtipendula	side-oats gramma [C2]
	Bromus catharticus *	rescue grass [C2]
	*Chloris virgata**	feather fingergrass
	Cynodon dactylon *	Bermuda grass [C3]
	*Echinochloa crusgalli**	prairie cupgrass
	Elymus canadensis *	Canada wildrye [C2]
	Eragrostis spectabilis (Pursch)	purple lovegrass [C2]
	Scheddonardus paniculatis (Nuttal)	Tumblegrass
	*Setaria viridis**	green foxtail [C2]
	Sorghum halpense *	Johnsongrass [C2]

*Recorded greenbug host (Michels 1986)
[C2] Common to two sites
[C3] Common to three sites

Table 2.2. Summary of collection data for 3 study sites 1999-2000

Site	Month	Host species	Number of colonies found	GB present on crop	GB present on volunteer
Hays, KS	May	*Bromus tectorum.*	3	N	N
		Agropyron Smithii	2		
	June	*Agropyron Smithii*	7	N	N
		Bromus tectorum	9		
	July		0	N	N
	August	*Setaria*	1	N	Y
	September		0	Y	Y
	October		0	Y	Y
	November		0	Y	Y
	December	*Bromus tectorum*	1	Y	Y
	March		0	Y	Y
Redrock, OK	May		0	N	
	June		0	N	
	July	*Sorghum halpense*	1	N	
	August		0	N	
	September		0	N	
	October		0	N	
	November		0	N	
	December		0	Y	
	January		0	Y	
Marshall, OK	May	*Sorghum halpense*	3	N	
	June		0	N	
	July		0	N	
	August		0	N	
	September		0	N	
	October		0	N	
	November		0	N	
	December		0	Y	
	January		0	Y	

Table 2.3. Grasses examined and greenbug densities, Hays KS.

Host	No. of culms examined	Number of culms with aphids.	Biotypes present
Agropyron smithii	977	7	G, I, K & New Biotype
Bromus tectorum	410	10	G & I
Elymus virginicus	218	0	
Sporobolus sp.	106	0	
Setaria viridis	104	1	Unknown
Cynodon dactylon	74	0	
Poa pratense	32	0	
Bouteloua curtipendula	2	0	
Aegilops cylindrica	1	0	

*Data for May collections not included in this table. The single greenbug from *S. viridis* died before its biotype could be established. For complete collection data see Appendix.

Table 2.4. Other collections made on an ad-hoc basis.

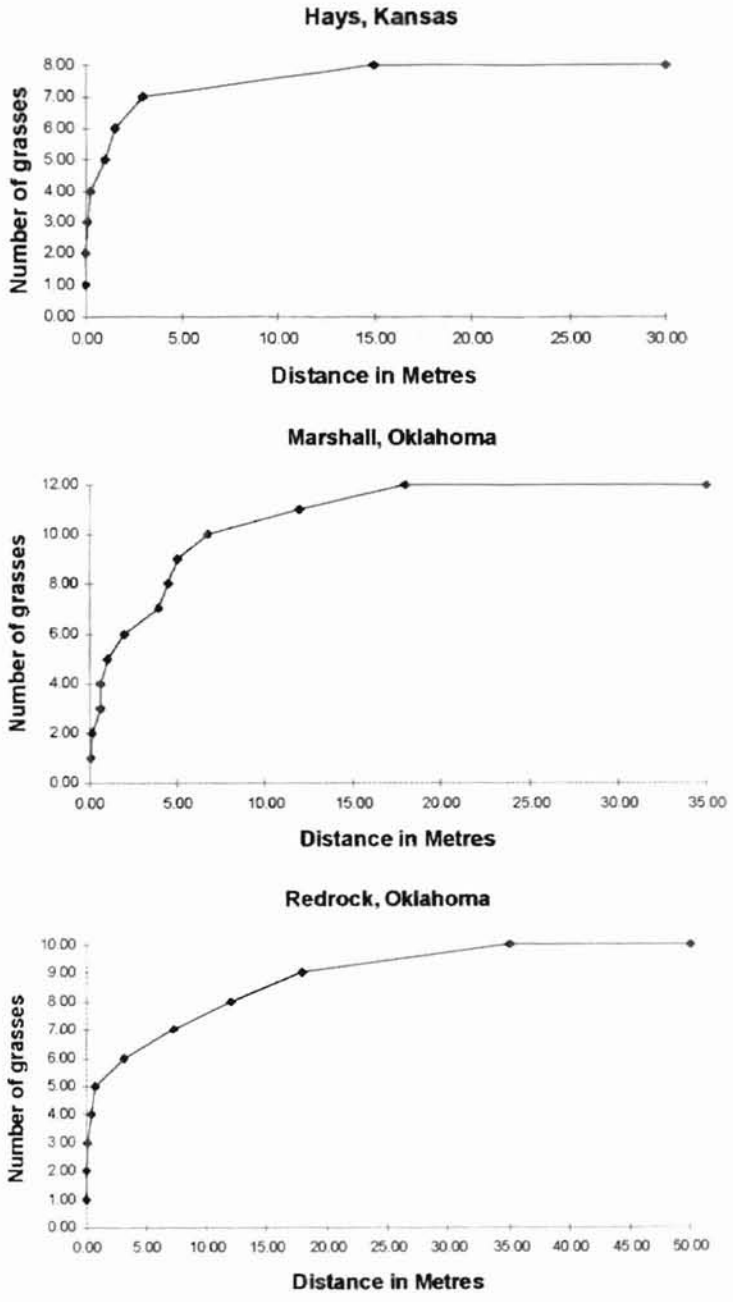
State	Host	No. of colonies found	Biotypes present
OK	*Sorghum halpense*	15	E & I
OK	*Sorghum bicolor*	8	E & I
OK	*Echinochloa crusgalli*	4	I
CO	*Agropyron intermedium*	3	G & I
OK	*Chloris verticulata* Nuttal	1	Unknown
CO	*Chloris sp.*	1	Unknown

The greenbugs collected from *Chloris spp.* died before their biotypes could be determined. Full collection data is shown in the appendix.

Table 2.5. Summary of total collections and biotype

Grass	No. colonies	No. biotyped	Biotypes
Agropyron smithii	7	4	I, G, K ,New
Agropyron intermedium	3	2	I, G
Bromus tectorum	10	3	I, G
Chloris verticulata	4	3	I, E
Echinochloa crusgalli	1	1	I
Setaria viridis	1	1	I
Sorghum halpense	18	7	I, E
Volunteer sorghum	5	3	I
Volunteer wheat	20	10	E, I, K

Figure 2.1. α-diversity of grasses at three study sites



Molecular Ecology

CHAPTER III

MtDNA sequence divergence among *Schizaphis graminum* (Homoptera: Aphididae) isolates from non cultivated hosts: further evidence for host-adapted races

J. A. Anstead[1], J. D. Burd[2] and K. A. Shufran[2]

[1]Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, USA. [2]USDA-ARS, Plant Science and Water Conservation Research Laboratory, Stillwater, OK, USA.

Abstract

A 1043 base pair region of the mitochondrial cytochrome oxidase subunit I (COI) gene of the greenbug *Schizaphis graminum* was sequenced for 27 field collected isolates from non-cultivated and cultivated hosts, an isolate from Syria, and an isolate collected from South Carolina. *S. rotundiventris* (Signoret) was used as an outgroup. Maximum likelihood, maximum parsimony and neighbor-joining phylogenies were estimated for these isolates. All tests produced trees with identical topologies. The presence of 3 clades within the greenbug species was confirmed. The isolates showed no segregation according to biotype. Clade 1 contained all the isolates collected from cultivated hosts and five of the six collected from *Sorghum halpense* L., and was the most homogenous clade. Four of the five isolates collected from *Agropyron* spp. were found in Clade 2. Clade 3 contained isolates from a number of different hosts, but none from cultivated hosts. Greenbugs using crop plants are one relatively homogenous race of the greenbug. There are 3 distinct clades each adapted to a different set of non-cultivated hosts. These clades probably evolved as host-adapted races on non-cultivated and wild grasses prior to human agriculture.

Keywords: *Schizaphis graminum*, cytochrome oxidase I gene, biotype, molecular phylogeny

Introduction

The greenbug (*Schizaphis graminum* (Rondani)) is an important pest of wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* Moench), barley (*Hordeum vulgare* L.) and a number of other graminaceous crops. It reproduces parthenogenically leading to large populations that cause considerable economic loss. Host plant resistance has been used to control greenbug damage, however its effectiveness has been compromised by the occurrence of virulent biotypes (for a full review, see Porter *et al.* 1997). While the distribution of greenbug biotypes is known to some extent (Peters *et al.* 1997; Dumas & Mueller 1986), their origin and evolutionary status remain opaque (Porter *et al.* 1997).

Recent phylogenetic analysis of greenbug biotypes based on sequence data from the mtDNA cytochrome oxidase subunit 1 (COI) gene, revealed 3 clades within *S. graminum* (Shufran *et al.* 2000). Each clade had a significant amount of divergence between them. Distances, (in % sequence divergence) between clades ranged from 4% to 6.8%. One clade contained the "agricultural biotypes", C, E, I, K and J. A second clade contained biotypes F, G and the "New York" isolate. The third clade contained biotype B, an isolate collected from Canada wildrye and an isolate collected from wheat in Europe. Biotype H fell outside the rest of these clades alongside the outgroup *Schizaphis rotundiventris*. These results are in agreement with the findings of Powers *et al.* (1989) who suggested that biotypes were probably host races, which diverged prior to the beginnings of human agriculture. However, both studies only examined a single individual of

each biotype and therefore did not consider intra-biotypic diversity, which has been documented by Shufran *et al.* (1992). They found that biotype E populations in the field were comprised of multiple clonal lineages.

Greenbugs utilize a large number of non-cultivated hosts, including 70 species in 29 genera (Michels 1986). However, most genetic studies have concentrated on greenbug populations collected from cultivated hosts or laboratory clones. Recently it has been shown that individual greenbug clones and localized greenbug populations cannot use the full range of documented greenbug hosts, suggesting the presence of host-species races (Anstead *et al.* 2000c; Dahms *et al.* 1954). Grass hosts serve as oversummering bridges for the greenbug during the period when wheat is not available and sorghum is unsuitable. During this time period, greenbugs are restricted to grasses (Daniels 1961). Greenbugs were found oversummering on 23 grass species in the Texas panhandle from 1953-59 (Daniels 1960). *Agropyron smithii* Rydberg was considered the most important host. Grasses are also important during the sexual cycle, which occurs primarily North of the 35th parallel. Eggs are oviposited on non-cultivated grasses in the autumn and in the spring the fundatrix emerges onto non-cultivated grasses, which are primarily in the *Poa* genus (Wadley 1931).

The objective of this study was to assess the genetic diversity of greenbugs on non-cultivated hosts. DNA sequences of the COI gene in the mtDNA were used to estimate the degree of relatedness 25 clones collected from a variety of hosts. These results were compared with those of laboratory isolates reported in

a previous study (Shufran *et al.* 2000). In addition, greenbug isolates from South Carolina and Syria were included. A phylogeny was constructed using these sequences, which allowed the inference of evolutionary relationships. The relationships between phylogeny, biotype and host plant were examined. We also estimated variation within some biotypes.

Materials and Methods

Insect Collection

Aphids collected from a number of sites in Oklahoma, Kansas and Colorado were used for this study (Anstead *et al.* 2000a) After biotype determination (Anstead *et al.* 2000a), a sample was frozen and stored at -80°C for genetic analysis. An isolate from South Carolina (collected from wheat, Dec. 1995 and obtained from S.Gray, USDA-ARS, Ithaca, NY) was also included. It showed susceptible reactions on "Custer", CI17882, "Largo", "GRS1201" and "Wintermalt" and resistant reactions on "Amigo" and "Post 90", however the reaction on "DS28A" was inconclusive (Stewart M. Gray, personal communication). Based on these host differences, it is a unique biotype when compared to the published biotypes B, C, E, F, G, H, I, J and K (Anstead 2000, Table 1.1). The Syrian isolate was collected on 6th March 2000, from wheat in Tel Hayda, about 30 KM South of Aleppo by Mustapha El Bouhssini, (ICARDA, P.O. box 5466, Aleppo Syria), and was preserved in 95% ethanol, therefore no biotype information exists.

DNA Extraction PCR Amplification and Sequencing

DNA was extracted from 1 to 3 individuals of each clone using the procedure of Black *et al.* (1992). Amplification was carried out according to the procedure of Shufran *et al.* (2000), except 1.5 U *Taq* DNA polymerase (Gibco-BRL) was used in each reaction. Amplification of the correct PCR product was confirmed by electrophorising 10μl of the reaction mixture in a 1.2% agarose gel and staining with ethidium bromide. Once the appropriate band was detected, contaminants such as primer-dimers and amplification primers were removed from the remaining 40μl of the reaction using the Wizard PCR Prep Kit (Promega, Madison WI). The product was sequenced using the amplification primers used in the PCR and a set of internal primers (4861 and 4862, Shufran *et al.* 2000). The PCR products were direct sequenced by the Recombinant DNA/Protein Resource Facility (Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK) using a Perkin-Elmer (Applied Biosystems) model 373 XL DNA Sequencing System incorporating an ABI-373 Automated DNA Sequencer.

Sequence analysis

Sequences were aligned using the MAP program (Huang 1994). This was a multiple sequence alignment with a mismatch score of –15, a gap open penalty of 30, and a gap extension penalty of 3. Alignments were first carried out on each isolate separately to give a complete sequence for each isolate. Isolates were then aligned and manually corrected for discrepancies and gaps.

The 1043 bases that had sequence data for both strands were used in the phylogenetic reconstruction. This is less than the 1200 bases used by Shufran *et al.* (2000) (Genbank AF220511-AF220523), but produced the same phylogenetic trees as with the longer sequence. *Schizaphis rotundiventris* (Shufran *et al.* 2000) was used as the outgroup for all tests. The analysis were carried out using the MEGA statistical package (Kumar *et al.* 1993) and PAUP version 4.0b2 (written by David Swafford). We used the same statistical analyses as Shufran *et al.* (2000). Distances were estimated by the method of Tamura and Nei (1993) with a gamma correction factor of α=0.3 (estimated by maximum likelihood procedure in PAUP), because there were unequal rates in the number and types of transitions and transversions. Distances were also estimated using the 2-parameter method (Kimura 1980). A dendrogram produced with neighbor-joining (NJ) analysis (Saitou & Nei 1987) was based on the above Tamura and Nei (1993) distances with 1000 bootstrap replications. Maximum parsimony (MP) analysis was performed by bootstrapping method (1000 replications) with heuristic search, using a 95% majority rule consensus. A maximum likelihood

(ML) dendrogram was produced according to the method of Hasegawa *et al.* (1985).

All sequences were submitted to Genbank and have the consecutive accession numbers AF285893-AF285916. PAUP command lines are shown in the appendix.

Results

Among the *S. graminum* biotypes and isolates there were 128 variable sites. Eighty five were 3rd codon substitutions (66%). There were 92 silent substitutions and 36 replacement substitutions. *S. rotundiventris* differed from *S. graminum* by 8.8% to 10.01% (K-2 parameter distance) or by 12.7-15.9% (Tamura-Nei gamma distance, α=0.3). Biotype H differed from the rest of *S. graminum* by 5.1% - 6.5% (K-2 parameter distance) or by 6.5% - 8.5% (Tamura - Nei gamma distance, α=0.3). The maximum divergence within the species (excluding biotype H) was 5.1% (K-2 parameter distance) or 6.26% (Tamura - Nei gamma distance, α=0.3). Divergence within biotypes was also estimated (Table 3.2). Maximum parsimony, maximum likelihood and neighbor joining analysis produced the same three clades as reported by Shufran *et al.* (2000) (Figure 3.1). The topologies produced by all three methods were identical; therefore only the maximum likelihood results are shown (Figures 3.1 and 3.2). There was more diversity between the 3 clades than within them (see Table 3.1).

The phylogeny generated according to biotype is shown in Figure 3.1, and includes the biotypes and isolates used by Shufran *et al* (2000). Biotype I was

present in all clades. Biotypes G and K were present in clades 1 and 2. The two new examples of biotype E collected were in the same clade as the previous E. The "new biotype" from *Agropyron smithii* (Anstead *et al.* 2000b) was in Clade 1.

Figure 3.2. shows the same phylogeny but according to host. Clade 1 contained all the isolates collected from wheat and sorghum. It also contained four of the five isolates collected from *Sorghum halpense,* two isolates from *Agropyron* spp. and one from *Bromus tectorum* L. Clade 2 contained four of the six isolates collected from *Agropyron spp.*, and one from *Bromus tectorum.* Clade 3 contained three isolates from three separate grass species (*Sorghum halpense*, *Chloris verticulata* Nuttal and *Echinochloa crusgalli* L.

Discussion

Our results confirm those of Shufran *et al.* (2000), i.e. there are 3 clades (COI haplotypes) within the greenbug species. The addition of 22 taxonomic units further confirmed there was greater diversity between clades than within them. Distances estimates were similar to those of Shufran *et al.* (2000), for instance biotype H was 5.1% - 6.5% (K-2 parameter distance) divergent from the rest of the greenbugs in this study compared with 5.06% - 6.17% reported by Shufran *et al.* (2000). In both cases biotype H was grouped outside the rest of the isolates and therefore may represent a separate *Schizaphis* species. However other events, such as a mitochondrial colonization that affected only this biotype, or even just this clone, could account for this divergence. These mitochondrial sweeps have been used to explain unusual divergence in mitochondrial

sequences in *Anopheles dirus* (Walton *et al.* 2000) and have been linked with the spread of symbionts such as *Wolbachia* (Shoemaket *et al.* 1999). The divergence of biotype H could have been caused by such a sweep because biotype H appears to be anholocyclic. If a new mitochondria with a slight advantage colonized a biotype H clone it would be restricted to its progeny.

The "South Carolina" isolate grouped with the agricultural isolates, as might be expected, as it was collected from wheat. The "new biotype" from *A. smithii* also grouped in this clade. Divergence within the species (excluding biotype H) was over 5%. This is much greater than the 0.4% found in a study using the same gene, that rejected the hypothesis that there were host races in the pea aphid (*Acyrthosiphon pisum*) (Boulding 1998). The highly divergent nature of these clades indicates that they haven't shared a common mitochondrial ancestor for a long time. Molecular clocks for other mitochondrial genes in arthropods give a substitution rate of approximately 2% per million years (Brower 1994, Juan *et al.* 1996). Using this rate, the distances between clades (2-4%) indicate they haven't shared a common mitochondrial ancestor for between 1 and 2 million years. This clock may not be completely accurate in this case, but even if it was ten-fold inaccurate, these clades would have last shared a common ancestor long before the beginnings of wheat cultivation in the fertile crescent over 10,000 years ago. Because mitochondrial DNA is inherited in a strictly maternal lineage, this result could have been caused in one of two ways, geographic isolation at the time of divergence or sympatric isolation on separate hosts.

There is strong evidence that these clades may have diverged as a result of sympatric isolation. Clade 1 contains all the agricultural biotypes and all the field-collected isolates from wheat and sorghum. It contained the Syrian and South Carolina isolates (both collected from wheat). It also contained 5 out of the six isolates collected from *Sorghum halpense,* which is a close relative of sorghum. Clade 1 was the most homogeneous despite containing the most isolates. There was only 1.1% sequence divergence among the 21 isolates in this clade. Clade 1 contained a group of greenbugs able to exploit crop hosts that may also use close relatives such as *Sorghum halpense.*

Four of the five isolates added to Clade 2 were collected from two closely related *Agropyron* spp. This suggests Clade 2 may be divergent as a result of host specialization. Clade 3 contained single isolates collected from *Sorghum halpense*, *Chloris verticulata* and *Echinochloa crusgalli.* Further collections would be needed to see if isolates from these hosts partitioned into this clade.

There appears to be exchange of genetic material between clades conditioning for virulence to crops. Biotype I is present in all 3 clades and biotypes G and K are present in two clades. There is no segregation into clades according to biotype. This suggests that the present system of assigning biotypes is not an indication of evolutionary origin, i.e. biotype has no taxonomic or evolutionary status. This was confirmed by estimates of divergence within biotypes. Biotypes I, G and K had divergences within them higher than within the three clades. Of the biotypes for which we had multiple isolates, only E had divergence lower than that of the clades. This was however based on only three

isolates. The addition of sequences from further collections would probably add to this divergence. Most previous authors have treated biotypes as discrete populations, even when variation was found in a biotype. Black (1993) found variation in the IGS subrepeat structure within biotype E but concluded that each biotype tested (B, C and E) probably evolved from a single population or maternal lineage. Unfortunately, there still exists an assumption that virulence to resistant crop species must mean something in evolutionary terms; e.g. "the development of biotype F is driven by native grasses" (Kindler & Hays 1999). Where as biotypes may be associated with particular hosts, it is not accurate to say their formation is driven by them. Our results show that whilst virulence to crops is important to researchers, farmers, and breeders, biotypes are not discrete populations that evolutionary forces can act on.

This study showed there was greater diversity amongst greenbugs isolates collected from non-cultivated grass hosts than among those collected from cultivated hosts. Non-cultivated hosts are therefore reservoirs for both genetic and biotypic diversity. The presence of the same biotypes in each clade indicates there is exchange of virulence genes between greenbug populations. This means that virulence genes present in any of these populations could easily be transferred into the greenbug race commonly found on crops. This further suggests resistance screening against greenbugs from non-cultivated hosts would be invaluable.

This study provides further evidence that *S. graminum* evolved three host-adapted races on wild grass hosts, as first suggested by Porter *et al.* (1997) and

by Shufran *et al.* (2000). These races use different sets of hosts but should not be considered separate species, as there appears to be gene flow between them.

Further work is needed in this system. This study used only a single marker. Genomic markers will be required to confirm the relationships elucidated by mtDNA studies. This combined with a comprehensive phylo-geographical survey of greenbugs on non-cultivated hosts in North America would allow the confirmation of these results.

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This study is part of James Anstead's MS thesis research on biotypic and genetic diversity of *Schizaphis graminum* on non-cultivated hosts. This work was carried out under the guidance of Dr John Burd whose research covers the IPM of cereal aphids, and is part of a larger project addressing the genetic variation and evolution of aphid biotypes.

Table 3.1. Sequence analysis of a 1.043 kb portion of the COI gene of *S. graminum*, maximum distances within clades and distance between clades (% sequence divegence)

	Within clades			Between clades		
	1	2	3	1 &2	2 & 3	1 & 3
Kimura 2- parameter distance	1.07	1.2	1.16	2.05	3.77	3.66
Tamura-Nei gamma distances (α=0.3)	1.11	1.27	1.24	2.21	4.47	4.28

Table 3.2. Sequence analysis of a 1.043 kb portion of the COI gene of *S. graminum*, maximum divergence within biotypes (% sequence divergence)

Biotype	No. examined (n)	Kimura 2- parameter distance	Tamura-Nei gamma distances (α=0.3)
E	3	0.87	0.91
G	4	3.05	3.52
I	15	4.89	5.87
K	3	2.15	2.34

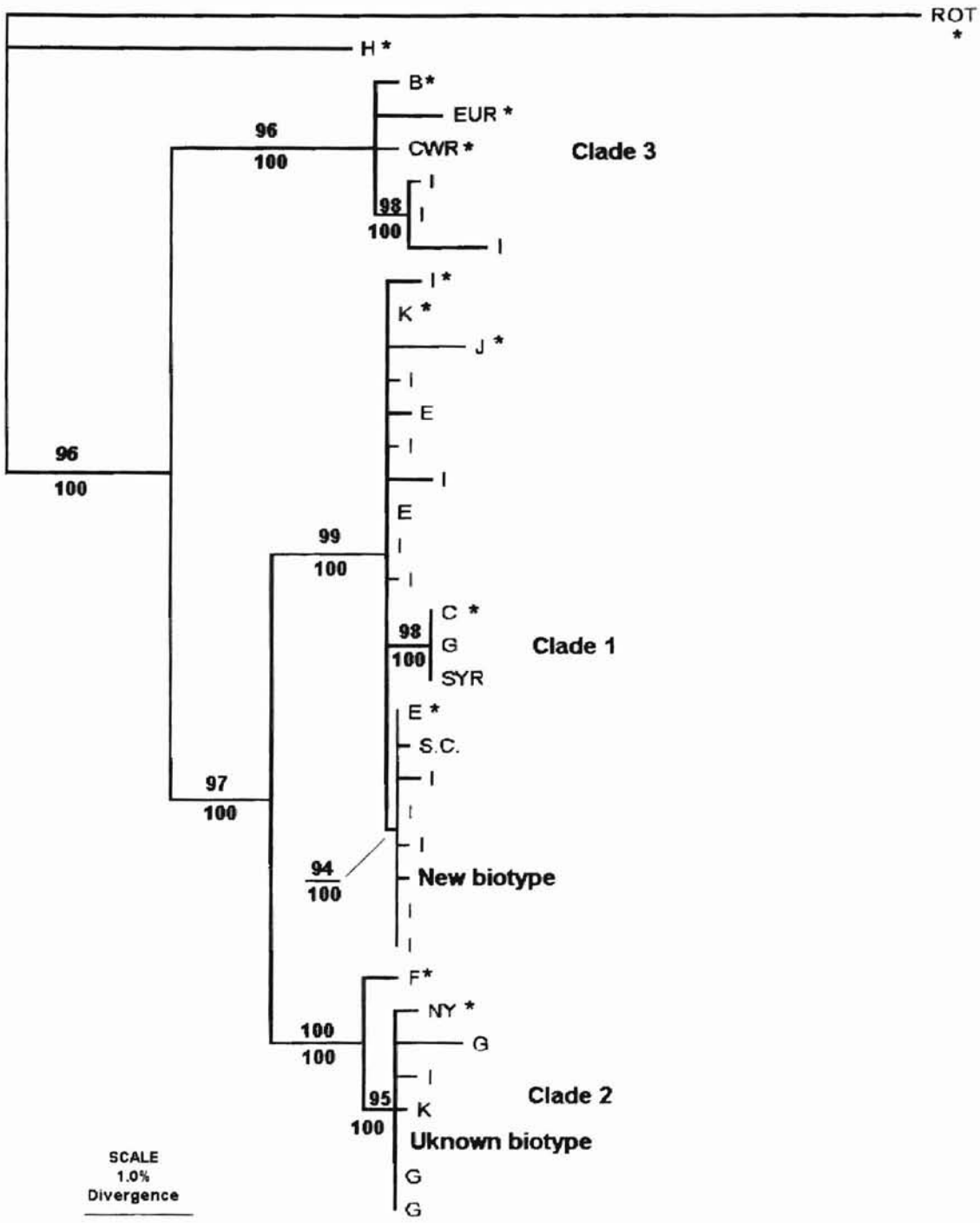

Figure 3.1: Maximum likelihood tree of greenbug isolates by biotype (*sequences from Shufran *et al.* 2000), produced from nucleotide sequences from a 1043 kb portion of the COI gene. For both distance/neighbour-joining and maximum parsimony analysis, 1000 bootstrap replications were performed. The percentage of replications supporting each branch are shown. The top value represents neighbour-joining, while the bottom number represents maximum parsimony.

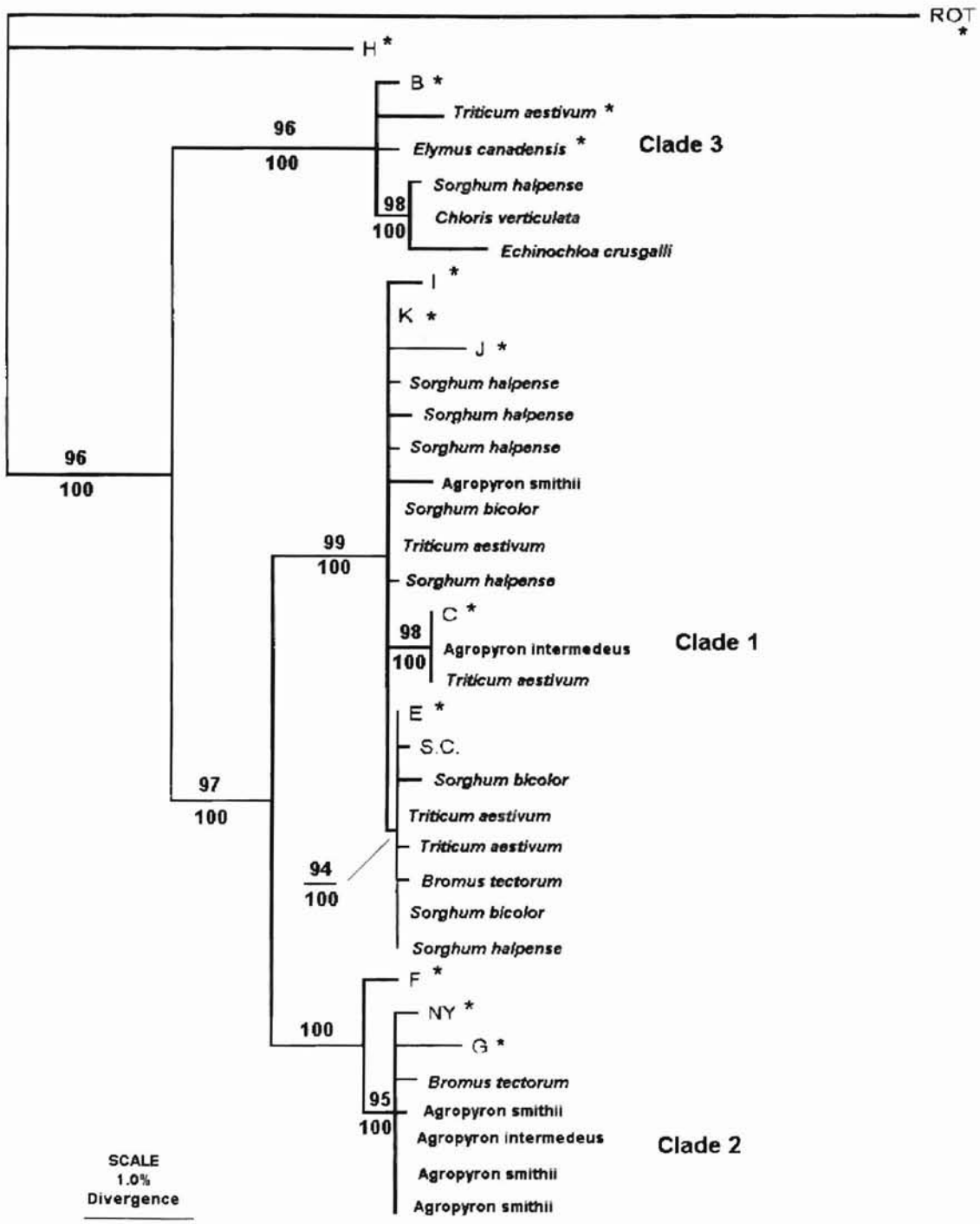

Figure 3.2: Maximum likelihood tree of greenbug isolates by host, (*sequences from Shufran *et al.* 2000), produced from nucleotide sequences from a 1043 kb portion of the COI gene. For both distance/neighbour-joining and maximum parsimony analysis, 1000 bootstrap replications were performed. The percentage of replications supporting each branch are shown. The top value represents neighbour-joining, while the bottom number represents maximum parsimony.

CHAPTER IV

REPRODUCTIVE CAPABILITIES OF A UNIQUE *SCHIZAPHIS GRAMINUM* [1] ISOLATE COLLECTED FROM *ELYMUS CANADENSIS L.* (CANADA WILDRYE)

J. A. Anstead[1], B. Bextine[1], J. D. Burd[2] and K. A. Shufran,[2]

[1]Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, USA. [2]USDA-ARS, Plant Science and Water Conservation Laboratory, Stillwater, OK, USA.

[1] HOMOPTERA: APHIDIDAE

ABSTRACT

The fecundity of a unique *Schizaphis graminum* (Rondani) isolate collected from *Elymus canadensis* (L.)(Canada wildrye) was tested. Its biotypic status is unique because it is virulent to the wheat variety "GRS 1201", but avirulent to Amigo. Seven-day fecundity and ovariole numbers were measured on three different hosts and a susceptible control ("Otis" barley). Fecundity was significantly higher on *E. canadensis*, *E. virginicus*, and Otis barley than on *Agropyron smithii*. Ovariole numbers were also significantly lower on *Agropyron smithii* than the other entries. The differences in the fecundity of the Canada wildrye isolate on these hosts supports the theory that this isolate has a different host range than previously reported populations.

INTRODUCTION

Greenbugs (*Schizaphis graminum*) were first detected in the US in Culpepper, Virginia in 1882 (Webster 1909). Greenbugs have been an economic pest of grain crops in North America, particularly wheat, since the 1880's and of sorghum since 1968 (Harvey and Hackerott 1969). Crop varieties resistant to greenbug damage have been deployed in an attempt to reduce greenbug damage. This strategy has been undermined by the appearance of greenbugs able to overcome this resistance. The first source of resistance was a selection from Durum wheat called "Dickinson selection 28A" (DS 28A) (Dahms *et al.* 1954). This was resistant to aphids in the field at that time (Dahms *et al.* 1954). However it was soon found to be damaged by a strain of greenbug found in the greenhouse (Wood 1961). This strain was designated biotype B, (the field population at the time was therefore "a priori" biotype A). Subsequently a system by which greenbug biotypes are characterized by their ability to damage certain resistant sources has been developed and biotypes A-K have been described (except D whose designation was based on insecticide resistance (Teetes *et al.* 1975)).

A unique isolate was recently collected from *Elymus canadensis* (Canada wildrye) from Payne county, Oklahoma in 1997 (J. D. Burd unpublished data). It is unique in being virulent to the wheat variety "GRS 1201", but avirulent to "Amigo". In all other virulence tests it is equivalent to biotype B (Anstead *et al.* 2000a). It has not yet been assigned an alphabetical biotype designation and

will be referred to as the Canada wildrye isolate in this paper. Following its collection it was maintained for 6 months on Canada wildrye without causing any of the virulence reactions produced on susceptible crop hosts (J. D. Burd unpublished data). It has never been recorded on any other host.

In a study of DNA sequence divergence in the greenbug, the Canada wildrye isolate was found to reside in a clade along with three other greenbugs collected from non-cultivated grasses (Anstead *et al.* 2000b). If this clade represents a host-adapted race or group of races, the Canada wildrye isolate should exhibit a greater fitness on specific non-cultivated hosts. We tested this hypothesis by measuring its fecundity on four different hosts; *Elymus canadensis, Elymus virginicus, Agropyron smithii* and "Otis" variety barley. These hosts were chosen as to include the host the Canada Wildrye isolate was collected from, another host-species in the same genus, an unrelated non-crop greenbug host (Daniels 1961) and a susceptible crop host (Otis).

MATERIALS AND METHODS

Greenbugs were maintained on "Otis" barley grown in 3.65 cm by 21 cm Cone-tainers™ (Cone-tainer company, Canby, Oregon, USA) covered with ventilated 3.5 cm diameter cylindrical plastic cages (Anstead *et al.* 2000a). Barley plants were grown in fritted clay in the greenhouse. They were watered and fertilized (Peters Professional™ 20-20-20) from the base of the cone-tainer to reduce disease. After the aphids and cage were placed on the barley watering

ceased. This reduced humidity in the cage and helped prevent fungal disease. The plants were then kept in a growth chamber at 20 ± 5°C with a photoperiod of 14:10 (L:D) hours. Because greenbugs are virulent to Otis barley, they required new barley plants every 10-20 days. *Elymus virginicus* and *E. canadensis* seeds were collected from wild populations in Stillwater, OK. *A. smithii* seeds were field collected from Hays, KS. Colonies of the Canada wildrye isolate greenbugs were started on each of the plant entries. Four plants of each entry were grown in six-inch pots covered with a cage. These plants provided host-acclimatized greenbugs for experimentation and were infested with greenbugs two weeks before the start of the experiments. Mortality on *A. smithii* was high and this colony had to be restarted several times. Fecundity was measured in two ways. Firstly, we measured seven-day fecundity which has been found to be correlated to total fecundity (Shufran *et al.* 1992) and secondly we counted ovariole number. Ovariole number has been found to be influenced by host quality (Walters *et al.* 1988).

Seven-day fecundity. For each of the four treatments a single plant was grown in fritted clay in a four-inch pot. This was repeated five times. Greenbugs were individually placed on a blade of grass at the four-leaf stage. The insect and grass blade were secured in a plastic cage. These cages were constructed by taking a 5cm diameter petri dish and cutting holes in each side so that a grass blade could be laid across it. This caused no damage to the leaf blade. A section of the petri dish was cut out and replaced with screening to allow ventilation. Two rubber bands were used to seal the cage.

One randomly selected greenbug nymph was placed on a single grass blade in each cage, one cage per plant. Greenbugs were monitored daily. At the appearance of the first offspring, the count was begun. All offspring were removed daily, minimizing a greenbug induced change in host quality and ensuring no other greenbugs reached maturity and reproduced. After seven-days the count was terminated.

Ovariole production. Approximately 10 acclimatized greenbugs were caged on four plants at the four-leaf stage, grown in fritted clay in six-inch diameter pots in a no-choice test. There were 3 pots for each plant entry. After several generations of reproduction (in this case 10 days), 30 randomly chosen adult apterous greenbugs were removed from each grass host. Each greenbug was dissected in insect saline according to the protocol of Walters and Dixon (1983) and its ovarioles were counted.

Statistics. For each experiment, Statistical Analysis Software (SAS Institute 1988) was used to analyze the data. An analysis of variance was conducted using ProcMix, ($P<0.05$). This includes a Least Squares Means (LSMeans) test to determine which treatments were significantly different from each other ($P<0.05$).

RESULTS

Seven-Day fecundity. Seven-day fecundity was highest on *E. canadensis*, with an average of 26 nymphs (Figure 4.1). This was not significantly different from the mean number of nymphs produced on *E. virginicus* (P=0.07). The fecundity of this isolate on *E. virginicus* was not significantly different from that on "Otis" barley (P=0.48). However seven-day fecundity was significantly higher on *E. canadensis* than on "Otis" barley (P<0.0001) (Figure 1). Greenbug fecundity on *A. smithii* was zero, the five greenbugs placed on it were unable to reproduce.

Ovariole production. Mean ovariole numbers were highest in greenbugs raised on "Otis" barley, with an average of 5.1 ovarioles (Figure 2). Mean ovariole numbers were 3.9, 2.9, and 0.8 respectively on *E. canadensis, E. virginicus* and *A. smithii.* All mean ovariole numbers were significantly different from each other (P<0.02). Most of the greenbugs on *A. smithii* failed to reproduce, Some of the greenbugs collected from *A. smithii* did not appear to have any ovarioles, indicating they are unable to complete development on this host.

DISCUSSION

Greenbugs raised on *Elymus canadensis* were well adapted to their host, as evidenced by high seven-day fecundity and ovariole numbers. Based on seven-day fecundity, *E. canadensis* was the most suitable host. Ovariole numbers also showed that *E. canadensis* was the most suitable host apart from the susceptible control. These results support the hypothesis that the Canada wildrye collected from *E. canadensis* was adapted to that host.

Elymus virginicus is closely related to *E. canadensis* and will readily hybridize with it (Hitchcock and Chase 1971). We would, therefore, expect greenbugs collected from *E. canadensis* to exhibit similar biological responses and have a relatively high fecundity on *E. virginicus.* This was shown to be correct. Seven-day fecundity was not significantly different from that on *E. canadensis.* Ovariole numbers were significantly lower on *E. virginicus* than *E. canadensis*, but were significantly higher than in the greenbugs raised on *A. smithii.* These results show *E. virginicus* is a less suitable host than *E. canadensis* for the Canada wildrye isolate.

Agropyron smithii has been considered to be the most important greenbug oversummering host in Texas and Oklahoma (Daniels 1960 and 1961). However, it was a completely unsuitable host for this isolate. Seven-day fecundity was zero and ovariole numbers were significantly lower than for all other hosts, having a mean of less than one per greenbug. Aphids are known to

produce fewer ovarioles under various environmental stresses, including stress imposed by being on a poor quality host (Walters *et al.* 1988).

These experiments clearly show that the Canada wildrye isolate has a more limited host range than the greenbug species as a whole. Similar conclusions were reached by Dahms *et al.* (1954) for a different isolate. They showed that greenbugs, (of unknown biotype) collected from Stillwater, OK were unable to live on *Echinochloa crusgalli* (L.) Beauv. but were able to live on *Elymus canadensis,* whereas greenbugs from Manhattan, KS were able to live on *Ec. crusgalli* but unable to live on *El. canadensis*.

In addition greenbugs collected from *A. smithii* included in a mitochondrial sequence study (Anstead *et al.* 2000b) were found to be highly divergent from the Canada wildrye isolate.

The differences in the fecundity of the Canada wildrye isolate on these hosts supports the theory that greenbug isolates have limited host ranges and that the species may be a complex of host-adapted races.

ACKNOWLEDGEMENT

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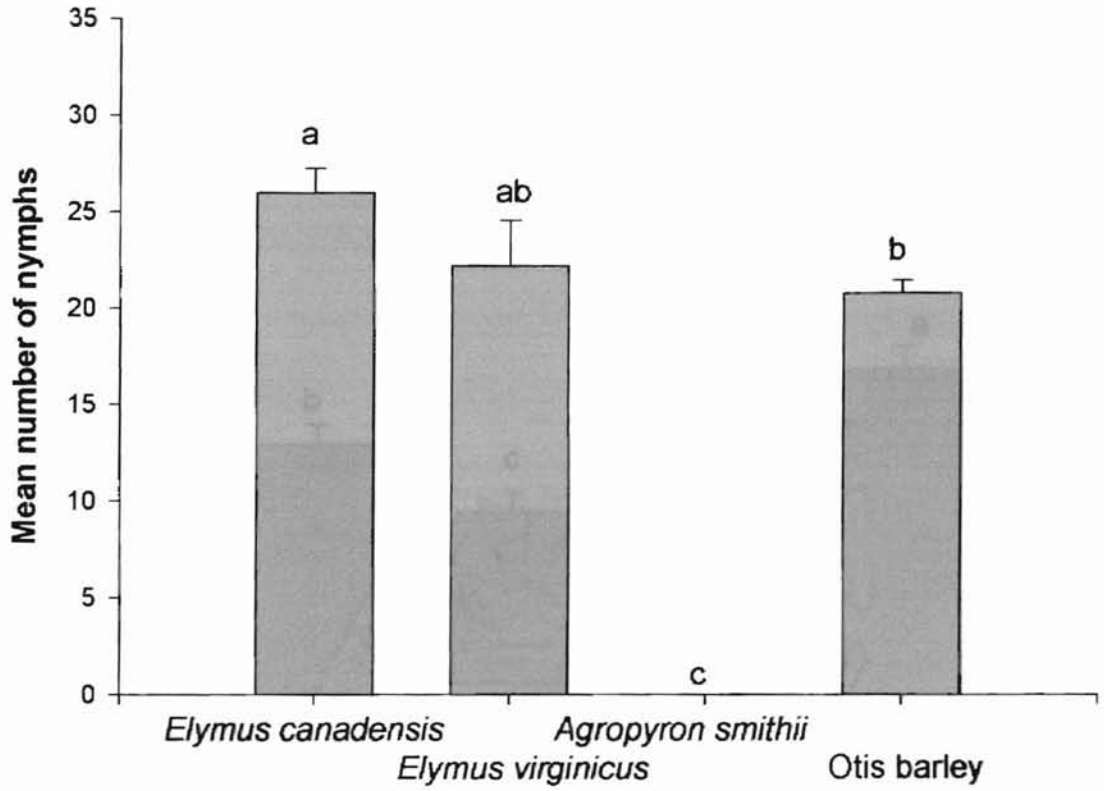

Figure 4.1: Mean seven-day fecundity of "Canada wildrye isolate" greenbugs on four different grass hosts. Values with the same letter are not significantly different ($p>0.05$) according to the LSMeans test (SAS institute 1985).

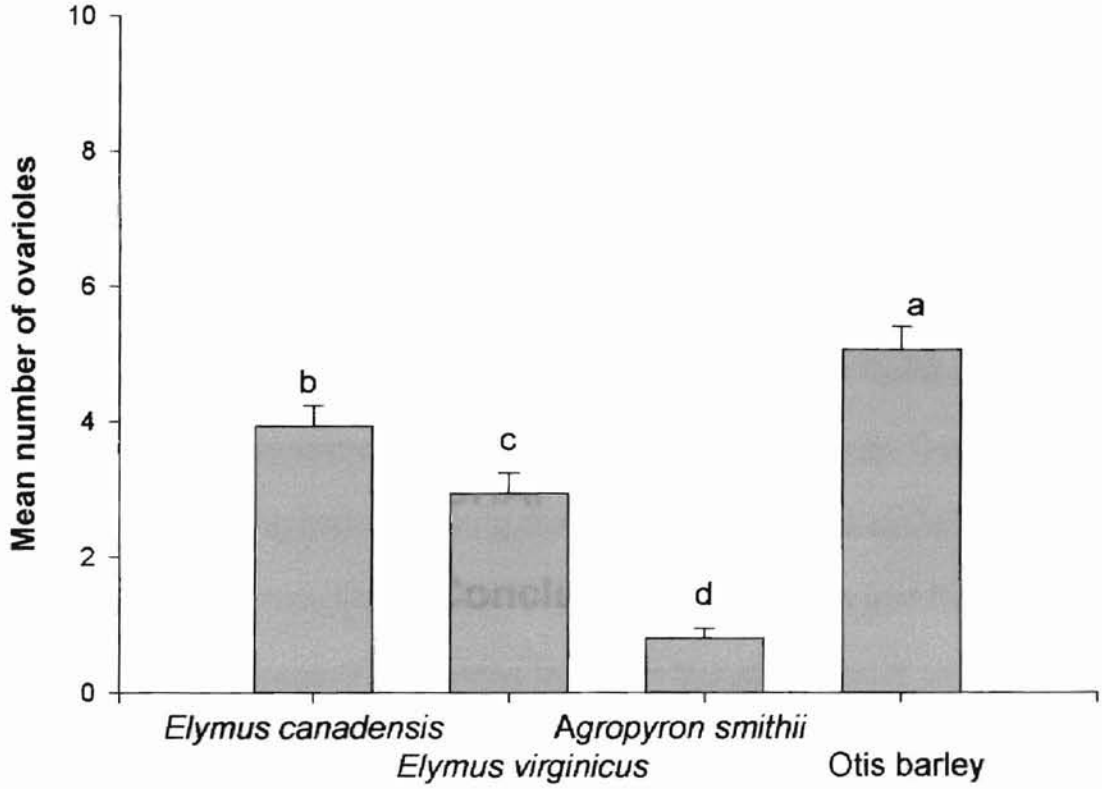

Figure 4.2: Mean ovariole numbers for "Canada wildrye isolate" greenbugs on four different hosts. Values with the same letter are not significantly different ($p>0.05$) according to the LSMeans test (SAS institute 1985).

CHAPTER V

Conclusions

These studies answered a number of important questions about the relationships between *Schizaphis graminum* and its non-cultivated hosts. I found significant biotypic diversity on non-cultivated grasses. Of the 10 greenbug biotypes (A-K) only C, E, I and K are regularly found on crops (Peters *et al.* 1997). On non-cultivated hosts I found biotypes E, I, K, G and a "new biotype". Biotype G was found at higher than previously recorded densities, 13% compared to the 2-3% found on crop hosts (Bush *et al.* 1987, Ullah 1993). The "new biotype", an isolate with a unique virulence profile was found on *A. smithii*. Non-cultivated grasses provide a reservoir of biotypic diversity. Genetic recombination during meiosis during the sexual reproductive cycle has been shown to generate "new" biotypes in the laboratory (Puterka and Peters 1990). This is likely the source of the unique isolate in this study and is probably the mechanism that maintains biotypic diversity, generating new biotypes that are virulent to resistant crop varieties and may be virulent to them.

This study supports the conclusions of Porter *et al.* 1997 that biotype formation was not driven by the development of resistant crop varieties. Biotypes, as defined in the greenbug do not appear to have any evolutionary and taxonomic status. Rather this study confirms the presence of three genetically distinct mtDNA clades (Shufran *et al.* 2000). The distances between clades indicate a substantial time since they had shared a common mitochondrial ancestor. At 2% divergence per million years (Brower, 1994, Juan *et al.* 1996) this would be between 1 and 2 million years. This is long before the advent of agriculture, so 20th century crop hosts could not have driven the

divergence of these populations. The populations in these clades differ in the grass hosts they use. Clade 1 contained greenbugs found predominantly on crop plants and *Sorghum halpense*. Clade 2 populations appeared to show some specialization for *Agropyron* spp. Clade 3 contained greenbugs from a number of non-cultivated grasses, but none from crop hosts. These clades are likely to represent host-adapted races. However, to elucidate the relationship between clade and host thoroughly would require a much larger survey, both in terms of numbers of host species examined and geographical area.

Given the limitations of mtDNA markers, confirmation of these relationships would also require the use of a genomic marker. There is a possibility that these phylogenetic patterns could have arisen from historical geographic isolation and not from sympatric isolation. Use of a genomic marker would solve this issue.

In summary, the greenbug species is composed of 3 host-adapted races that probably evolved sympatrically on non-cultivated grass hosts. One of these races is able to easily exploit agricultural cropping systems. The other races are mostly limited to non-cultivated hosts, but provide a genetic reservoir able to produce biotypes that may overcome crop resistance.

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Appendix A: Field collection data

Hays, KS

Day/month/year	1/6/1999		8/7/1999		11/8/1999	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Agropyron Smithii	200	7	180	0	175	0
Bromus tectorum	175	9	0	0	0	0
Poa pratense	0	0	0	0	20	0
Elymus virginicus	70	0	88	0	0	0
Cynodon dactylon	0	0	0	0	20	0
Sporobolus sp.	0	0	60	0	46	0
Bouteloua curtipendula	0	0	2	0	0	0
Aegilops cylindrica	3	0	0	0	0	0
Setaria viridis	0	0	18	0	40	1

Day/month/year	10/9/1999		6/10/1999		13/11/1999	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Agropyron Smithii	115	0	120	0	28	0
Bromus tectorum	17	0	60	0	57	0
Poa pratense	0	0	12	0	0	0
Elymus virginicus	0	0	0	0	60	0
Cynodon dactylon	0	0	4	0	0	0
Sporobolus sp.	0	0	0	0	0	0
Bouteloua curtipendula	0	0	0	0	0	0
Aegilops cylindrica	0	0	0	0	0	0
Setaria viridis	34	0	3	0	2	0

Day/month/year	2/12/1999		6/3/2000	
Grass	No. culms	No. GB	No. culms	No. GB
Agropyron Smithii	73	0	86	0
Bromus tectorum	45	1	56	0
Poa pratense	0	0	0	0
Elymus virginicus	0	0	0	0
Cynodon dactylon	15	0	35	0
Sporobolus sp.	0	0	0	0
Bouteloua curtipendula	0	0	0	0
Aegilops cylindrica	0	0	0	0
Setaria viridis	6	0	1	0

Redrock, OK

Day/month/year	15/6/99		21/7/99		24/8/99	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Bromus catharticus	15	0	0	0	0	0
Bromus tectorum	34	0	0	0	0	0
Cynodon dactylon	20	0	40	0	25	0
Elymus canadensis	15	0	14	0	0	0
Hordeum pusillum	3	0	0	0	0	0
Phalaris canariensis	0	0	0	0	0	0
Sorghum halepense	5	0	22	0	6	1
Eragrostis sp.	20	0	30	0	23	0
Panicum virgatum	30	0	40	0	35	0
Tridens flavus	33	0	80	0	56	0

Day/month/year	9/9/99		25/9/99		16/10/99	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Bromus catharticus	0	0	3	0	12	0
Bromus tectorum	0	0	0	0	12	0
Cynodon dactylon	13	0	32	0	24	0
Elymus canadensis	0	0	0	0	0	0
Hordeum pusillum	0	0	0	0	0	0
Phalaris canariensis	0	0	0	0	0	0
Sorghum halepense	5	0	13	0	0	0
Eragrostis sp.	12	0	0	0	0	0
Panicum virgatum	35	0	24	0	23	0
Tridens flavus	43	0	20	0	10	0

Day/month/year	25/11/99		18/1/00		23/2/00	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Bromus catharticus	23	0	11	0	16	0
Bromus tectorum	24	0	27	0	34	0
Cynodon dactylon	27	0	0	0	45	0
Elymus canadensis	0	0	0	0	0	0
Hordeum pusillum	0	0	0	0	0	0
Phalaris canariensis	0	0	0	0	0	0
Sorghum halepense	0	0	0	0	0	0
Eragrostis sp.	0	0	0	0	2	0
Panicum virgatum	14	0	6	0	12	0
Tridens flavus	7	0	3	0	3	0

Marshall, OK

Day/month/year	13/7/99		3/8/99		1/9/99	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Aristida oligantha	0	0	0	0	0	0
Bouteloua curtipendula	0	0	0	0	0	0
Bromus catharticus	0	0	0	0	3	0
Chloris virgata	4	0	0	0	7	0
Cynodon dactylon	5	0	4	0	6	0
Echinochloa crusgalli	10	0	2	0	3	0
Elymus crusgalli	4	0	0	0	4	0
Eragrostis spectabilis	37	0	45	0	54	0
Scheddonardus paniculatis	3	0	0	0	0	0
Sorghum halepense	8	3	0	0	3	0
Setaria viridis	37	0	23	0	21	0
Bothriochloa saccharoides	34	0	15	0	12	0

Day/month/year	25/9/99		26/10/99		24/11/99	
Grass	No. culms	No. GB	No. culms	No. GB	No. culms	No. GB
Aristida oligantha	0	0	0	0	0	0
Bouteloua curtipendula	0	0	0	0	0	0
Bromus catharticus	0	0	0	0	0	0
Chloris virgata	3	0	0	0	0	0
Cynodon dactylon	12	0	8	0	18	0
Echinochloa crusgalli	0	0	0	0	0	0
Elymus crusgalli	4	0	5	0	0	0
Eragrostis spectabilis	32	0	17	0	23	0
Scheddonardus paniculatis	0	0	0	0	0	0
Sorghum halepense	2	0	0	0	0	0
Setaria viridis	17	0	12	0	14	0
Bothriochloa saccharoides	13	0	23	0	34	0

Day/month/year	18/I/00		22/II/00	
Grass	No. culms	No. GB's	No. culms	No. GB's
Aristida oligantha	0	0	0	0
Bouteloua curtipendula	0	0	0	0
Bromus catharticus	2	0	17	0
Chloris virgata	1	0	8	0
Cynodon dactylon	4	0	23	0
Echinochloa crusgalli	0	0	0	0
Elymus crusgalli	2	0	0	0
Eragrostis spectabilis	33	0	25	0
Scheddonardus paniculatis	0	0	1	0
Sorghum halepense	0	0	0	0
Setaria viridis	20	0	24	0
Bothriochloa saccharoides	552	0	17	0

Appendix B: Collection details for isolates biotyped

Isolate	Date Collected	State	Closest town	Host	Biotype	Genbank Accession no.
1-00-11	13/I/00	KS	Hays	*Triticum aestivum*	E	
8-99-14*	10/VIII/99	CO	Mead	*Agropyron intermedium*	?	AF285900
8-99-15	8/X/99	CO	Mead	*Agropyron intermedium*	I	
8-99-18*	8/X/99	CO	Prospect valley	*Agropyron intermedium*	G	AF285915
8-99-30*	10/VIII/99	KS	Winona	*Triticum aestivum*	I	AF285909
6-99-11*	1/VI/99	KS	Hays	*Agropyron smithii*	I	AF285901
9-99-12*	10/IX/99	KS	Hays	*Triticum aestivum*	I	AF285906
11-99-2*	13/XI/99	KS	Hays	*Triticum aestivum*	I	AF285910
6-99-4*	1/VI/99	KS	Hays	*Bromus tectorum*	G	AF285911
6-99-5*	1/VI/99	KS	Hays	*Bromus tectorum*	I	AF285893
6-99-7*	1/VI/99	KS	Hays	*Agropyron smithii*	K	AF285905
6-99-16*	1/VI/99	KS	Hays	*Agropyron smithii*	G	AF285903
6-99-18*	1/VI/99	KS	Hays	*Agropyron smithii*	G	AF285904
10-99-3	6/X/99	KS	Hays	*Triticum aestivum*	E	
11-99-1	13/XI/99	KS	Hays	*Triticum aestivum*	K	
8-98-2*	4/VIII/98	KS	S. Haven	*Sorghum bicolor*	I	AF285908
7-98-01	7/VII/98	NE	Ceresco	*Sorghum bicolor*	I	
7-98-27*	31/VII/98	OK	Balko	*Sorghum halpense*	I	AF285896
8-98-10*	7/VIII/98	OK	Guymon	*Chloris verticulata*	I	
8-98-9*	6/VIII/98	OK	Guymon	*Echinochloa crusgalli*	I	AF285899
8-98-6	6/VIII/98	OK	Guymon	*Chloris verticullata*	I	
8-98-10	7/VIII/98	OK	Guymon	*Chloris verticullata*	E	AF285898
7-98-23	30/VII/98	OK	Guymon	*Chloris verticullata*	I	
6-99-20*	1/VI/99	OK	Marshall	*Sorghum halpense*	I	AF285894
6-99-22*	13/VII/99	OK	Marshall	*Sorghum halpense*	E	AF285895
8-98-13	18/VIII/98	OK	Morrison	*Sorghum halpense*	E	
7-99-1*	21/VII/99	OK	Redrock	*Sorghum halpense*	I	AF285897
7-99-3*	1/VII/99	OK	Sumner	*Sorghum bicolor*	E	AF285902
7-99-7*	21/VII/99	OK	Sumner	*Sorghum halpense*	I	AF285912
7-99-8*	21/VII/99	OK	Sumner	*Sorghum bicolor*	I	AF285913
7-99-10*	21/VII/99	OK	Sumner	*Sorghum halpense*	I	AF285914

*sequenced and used in subsequent phylogenetic analysis.

Appendix C: Other aphids collected

Host	Aphid	No. of colonies
Sorghum halpense	*Rhopalosiphum maidis*	8
	Macrosiphum euphorbiae	4
Bromus tectorum	*Rhopalosiphum padi*	3
Elymus virginicus	*Sitobion avenae*	3
Eragrostis cilianensis	*Macrosiphum euphorbiae*	2
	Sipha flava	1
Agropyron smithii	*Diuraphis noxia*	1
	Diuraphis tritici	1
	Rhopalosiphum padi	1
Sporobolus cryptandrus	*Rhopalosiphum maidis*	1
	Macrosiphum euphorbiae	1
Echinochloa crusgalli	*Rhopalosiphum maidis*	1
	Macrosiphum euphorbiae	1
Setaria sp.	*Rhopalosiphum maidis*	1
Chloris sp.	*Rhopalosiphum maidis*	1
Chloris virgata	*Sipha flava*	1
Agropyron desertorum	*Sipha elegans*	1

Appendix D: PAUP commands

Maximum Likelihood

```
#nexus
[Subset data]

BEGIN DATA;
   DIMENSIONS NTAX=37   NCHAR= 1041;
     FORMAT
       INTERLEAVE
          MISSING=?  GAP=-  MATCHCHAR=.
              DATATYPE=DNA
;
MATRIX
             [ Data    ]
;
ENDBLOCK;

begin paup;
outgroup 1;
log file=gbml.out;
set criterion=likelihood increase=auto;
hsearch;
likelihoods 1/ rates=gamma shape=estimate;
savetrees file=GBml.tre brlens;
endblock;
```

Neighbour joining

```
#nexus
[Subset data]

BEGIN DATA;
   DIMENSIONS NTAX=37   NCHAR= 1041;
     FORMAT
       INTERLEAVE
          MISSING=?  GAP=-  MATCHCHAR=.
              DATATYPE=DNA
;
MATRIX
             [ data    ]

;
ENDBLOCK;

begin paup;
 outgroup 1;
 log file=gbd.out;
 set criterion=distance;
 dset di=tamnei rates=gamma shape=0.300097;
 nj;
bootstrap method=nj nreps=1000;
 contree;
describe;
endblock;
```

Maximum parsimony

```
#nexus
[Subset data]

BEGIN DATA;
   DIMENSIONS NTAX=37   NCHAR= 1041;
     FORMAT
       INTERLEAVE
          MISSING=?  GAP=-  MATCHCHAR=.
              DATATYPE=DNA
;
MATRIX
            [  data  ]
;
ENDBLOCK;

begin paup;
 outgroup 1;
 log file=gbp.out;
 set criterion=parsimony;
 hsearch swap=tbr;
 lik /k;
describe;
bootstrap method=heuristic nreps=1000;
endblock;
```

VITA 2

James A Anstead

Candidate for the Degree of

Master of Science

Thesis: GENETIC AND BIOTYPIC DIVERSITY OF GREENBUG *SCHIZAPHIS GRAMINUM* (RONDANI) POPULATIONS ON NON-CULTIVATED HOSTS

Major Field: Entomology

Biographical:

Education: Graduated from Sawston Village college, Cambridge, England in 1990; received three A-levels in Biology, Chemistry and Physics from Long Road Sixth Form College, Cambridge, England in 1992; received an upper second class Bachelor of Science degree in Ecology from The University Of East Anglia, Norwich England in 1996. Completed the requirements for the Master of Science degree in Entomology from Oklahoma State University, Stillwater, Oklahoma in July 2000.

Experience: Research Assistant, Dept. of Entomology and Plant Pathology, Oklahoma State University, June 1998 to present; Laboratory Assistant, AgrEvo UK Ltd., October 1996 to May 1998.

Professional memberships and awards: Entomological Society of America 1998 to present. 2nd prize for invited papers, International Plant Resistance to Insects meeting, 2000. 2nd prize for student papers, Southwest Branch of the Entomological Society of America, 1999.