APD-PCR to detect genetic polymorphisms among geographically-dispersed populations of Cephus cinctus

by Kuifu Lou

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Crop and Soil Science
Montana State University

Abstract:
Wheat stem sawfly, Cephus cinctm Norton, is the most destructive chronic insect pest of wheat in the northern Great Plains. However, little is known about the extent and distribution of genetic variability in the species. Over the past 20 years in Montana, C. cinctus has changed from an insect pest exclusive to spring wheat to a pest which causes major economic, damage in both spring and winter wheat. Preliminary examination suggests that phenology and maturity of wheat cultivars grown in Montana has not changed enough over this period to account for the difference in insect virulence. Knowledge of the genetic variability within endemic populations of C. cinctus is important for developing management and resistance-breeding strategies. Our objective in this study was to assay the genomic variability within and among geographically-dispersed collections of C. cinctus from the northern U.S. Great Plains using RAPD-PCR markers. Overwintering sawfly larvae were collected from wheat stubble at eight sites in Montana, six sites in North Dakota, and one site in Wyoming. DNA was extracted and evaluated from individual larvae from each collection site. Sixty-two random decamer primers were screened and 20 of them consistently produced well-amplified and reproducible polymorphic bands. The size of amplified DNA fragments produced by these primers ranged from 200-1900 bp, with individual primers generating from two to nine bands. Genetic distances among 186 individuals based on 60 RAPD loci were calculated using similarity index, 1-M (where M is the fraction of matches). Each sawfly individual was a unique RAPD multiband phenotype. Based on UPGMA cluster analysis all Montana sawflies clustered separately from all North Dakota and Wyoming sawflies. Principal coordinate analysis based on the band frequency within each population showed a similar result. Analysis of molecular variance partitioned the RAPD variation into the among- and within-population components. The within-population component accounted for 71.6% of the variation and was significantly different from zero at the 1% probability level. The among-population and among-region components accounted for 6.3% and 22.1% of the total variation, respectively. Subset analyses of MT and ND populations showed that there were significant differences among populations in MT but not in ND. Pairwise tests for the homogeneity of the RAPD variance between populations. suggested significant divergences among 81 of the 105 (77%) population pairs including all but three of the MT . pairwise comparisons. A dendrogram based on the Euclidean distance among populations showed that all the Montana populations were grouped together, with all North Dakota populations in another group. The high degree of structuring in Montana populations suggests the high degree of reproductive isolation among geographically-separated populations is contributing to development of geographic and/or host races due to adaptation to local environment conditions and/or host differences.

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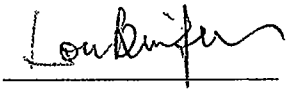
Signature 

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ABSTRACT

Wheat stem sawfly, *Cephus cinctus* Norton, is the most destructive chronic insect pest of wheat in the northern Great Plains. However, little is known about the extent and distribution of genetic variability in the species. Over the past 20 years in Montana, *C. cinctus* has changed from an insect pest exclusive to spring wheat to a pest which causes major economic damage in both spring and winter wheat. Preliminary examination suggests that phenology and maturity of wheat cultivars grown in Montana has not changed enough over this period to account for the difference in insect virulence. Knowledge of the genetic variability within endemic populations of *C. cinctus* is important for developing management and resistance-breeding strategies. Our objective in this study was to assay the genomic variability within and among geographically-dispersed collections of *C. cinctus* from the northern U.S. Great Plains using RAPD-PCR markers. Overwintering sawfly larvae were collected from wheat stubble at eight sites in Montana, six sites in North Dakota, and one site in Wyoming. DNA was extracted and evaluated from individual larvae from each collection site. Sixty-two random decamer primers were screened and 20 of them consistently produced well-amplified and reproducible polymorphic bands. The size of amplified DNA fragments produced by these primers ranged from 200-1900 bp, with individual primers generating from two to nine bands. Genetic distances among 186 individuals based on 60 RAPD loci were calculated using similarity index, 1-M (where M is the fraction of matches). Each sawfly individual was a unique RAPD multiband phenotype. Based on UPGMA cluster analysis all Montana sawflies clustered separately from all North Dakota and Wyoming sawflies. Principal coordinate analysis based on the band frequency within each population showed a similar result. Analysis of molecular variance partitioned the RAPD variation into the among- and within-population components. The within-population component accounted for 71.6% of the variation and was significantly different from zero at the 1% probability level. The among-population and among-region components accounted for 6.3% and 22.1% of the total variation, respectively. Subset analyses of MT and ND populations showed that there were significant differences among populations in MT but not in ND. Pairwise tests for the homogeneity of the RAPD variance between populations suggested significant divergences among 81 of the 105 (77%) population pairs including all but three of the MT pairwise comparisons. A dendrogram based on the Euclidean distance among populations showed that all the Montana populations were grouped together, with all North Dakota populations in another group. The high degree of structuring in Montana populations suggests the high degree of reproductive isolation among geographically-separated populations is contributing to development of geographic and/or host races due to adaptation to local environment conditions and/or host differences.

I. INTRODUCTION

Average annual wheat (*Triticum aestivum* L.) production in Montana exceeded 128 million bushels harvested from 4.8 million acres in the 1981 to 1990 cropping years (Sands and Lund, 1991). Wheat stem sawfly (Hymenoptera: Cephidae: *Cephus cinctus* Norton) has remained a chronic and important pest of wheat in Montana, North Dakota, and southern portions of Canada since the 1930s (Weiss and Morrill, 1992). Farmers in six of Montana's major wheat-producing counties (Cascade, Chouteau, Hill, Liberty, Teton, Yellowstone) estimated they lost over $13 million in 1995 because of the infestation of *C. cinctus*. That loss is projected at over $30 statewide following the 1996 harvest (Peck, 1996). Control of *C. cinctus* through development of cultivars with host plant resistance has major implications on the profitability and sustainability of wheat production in the northern great plains of North America. Currently the damage in the spring wheat is partially controlled by solid-stem varieties, but resistant winter wheat cultivars have only recently been deployed (Bruckner et al., 1997; Carlson et al., 1997).

One of the ways that insects cope with their environment is through the formation of biotypes, an important evolutionary process involving ecological divergence of populations in local areas. Plant resistance may be overcome by development of biotypes resulting from inherent genetic variability within the insect population and selection pressure from plant populations or an environment which forces insect populations to

evolve for survival (Smith, 1989). Populations within insect species that have the ability to damage plant genotypes normally resistant to that insect are considered biotypes (Puterka and Peters, 1990). This kind of biotypic variation has direct implications on plant breeding strategy for development of host plant resistance.

C. cinctus is considered a pest native to the North American plains (Mills, 1944), but it has recently been reported that it was introduced from former USSR (Ivie, 1997). As wheat acreage increased in the plains region the sawfly became firmly established as a pest of spring wheat, and later in the 1980's as a pest of winter wheat. Because *C. cinctus* produces both by arrhenotoky and thelytoky, is sexually dimorphic, and has high reproductive potential (Holmes, 1982), it may develop biotypes which overcome host plant resistance. Stem solidness, the genetic resistance mechanism of wheat to *Cephus*, is allopatric in nature, and because it evolved in the absence of the insect, likely is a more durable resistance than monogenic, wheat antibiosis genes conditioning resistance to Hessian fly, *Mayetiola destructor* (Say) and greenbug, *Schizaphis graminum* (Rondani) (Smith, 1989). Biotypic diversity has been reported for greenbug (Michels, 1986), Hessian fly (Patterson et al., 1992), and Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Puterka et al., 1992). Potential for biotypes development has obvious implications for sawfly management, biological control and development of resistant varieties, as the failure to recognize distinct populations can have costly and frustrating consequences (Bush and Hoy, 1983; Gonzales et al., 1979; Rosen, 1978).

Modern reduced-tillage management practices which minimize stubble disturbance and reduce soil erosion have enhanced sawfly overwintering survival and may have contributed to higher population densities. Historically, economically important

infestations and crop losses to *C. cinctus* were confined to spring wheat; however since the mid-1980's winter wheat has also been heavily damaged in Montana (Morrill et al., 1992a, 1992b). Infestation rate in "resistant" winter wheat genotypes ranged from 16 to 47% in 1991 (Morrill et al., 1992a). Evidence for diversity in *C. cinctus* is reported for parthenogenic reproductive behavior (Farstad, 1938) and virulence among two Canadian sawfly populations (Holmes et al., 1957). Biotypes may be distinguished by virulence, morphological differences, color, insecticidal susceptibility, behavior, host preference (Smith, 1989), isozyme analysis (Abid et al., 1989), and RAPD analysis (Black et al., 1992).

Our limited knowledge of *C. cinctus* hinders our ability to differentiate biotypes in the North American plains. Because of their small size and morphological attributes which can be easily changed by environment, they can not be easily differentiated from one another based on morphological criteria. Within the native habitat, the extent of genetic variation between geographical populations depends on several factors, including gene flow between populations and time since separation (Templeton, 1990). Genetic differences within introduced population can be the results of genetic variation in the founder populations, the number of founding events and selection pressure (Baker and Stebbins, 1965).

With the advent of molecular techniques, DNA-based approaches have been suggested for examination of genetic diversity. Elucidation of genetic variation in geographical populations can be an important aspect of pest studies (Kambhampati et al., 1990; Roehrdanz and Johnson, 1988), providing insight into the geographical origin of colonized populations (Kambhampati et al., 1991). The lack of informative primer sets

designed from known sequence data provided the justification for the use of RAPD-PCR (amplification of random DNA sequences using the polymerase chain reaction) (Williams et al., 1990) as an alternative to RFLP's. RAPD-PCR has recently been successfully used to identify genetic variation, examine phylogenetic relationships, and differentiate species in mosquitos (*Aedes* spp.) (Ballinger-Crabtree et al., 1992; Kambhampati et al., 1992), greenbug (*Schizaphis graminum*) (Rondani), Russian wheat aphid, other aphid species (*Acyrthosiphon pisum* and *Uroleucon ambrosiae*) (Black et al., 1992; Puterka et al., 1993), and parasitic wasps, *Anaphes* spp. and *Trichogramma* spp. (Landry et al., 1993). In these studies, the number of RAPD primers necessary to provide adequate polymorphic bands for differentiation ranged from two (Kambhampati et al., 1992) to thirteen (Landry et al., 1993).

The objectives of this study are:

1. to test whether RAPD-PCR could provide a way to differentiate genetic variation among different *C. cinctus*.

2. to provide basic information regarding the genomic polymorphism among and within geographically dispersed populations of *C. cinctus*.

II. LITERATURE REVIEW

History of *C. cinctus*

C. cinctus is reportedly native to North America (Ainslie, 1920; Callenbach and Hansmeier, 1945; Criddle, 1922; Farstad, 1940; Mills, 1944). The insect was first reported mining grass stems near Alameda, California in 1890 (Ainslie, 1920). In 1895, adults were found feeding in the Canadian Northwest Territories (Ainslie, 1929). In 1906, larvae were found feeding in wheat near Kulm, North Dakota and in various grasses, chiefly *Agropyron* species, in Wyoming (Ainslie, 1920 and 1929). Infested wheat stems were identified in 1910, in northeastern Montana near Bainville (Montana Agriculture Experiment Station, 1946). In 1908, larvae were found in grasses in Oregon, and from 1911 to 1915 the species was found in native grasses of Utah and other states (Ainslie, 1929). An alternative hypothesis suggests *C. cinctus* was introduced from the former USSR and dispersed through expansion of the U.S. rail system in the late 19th century (Ivie, 1997). *C. pygmaeus* and *Trachelus* spp., are distributed throughout wheat and barley producing regions of North Africa and West Asia (Miller et al., 1993).

Understanding the development of the pest might help us to understand the possible mechanisms of its speciation. *C. cinctus* became a major economic pest of wheat in the northern Great Plains in the 1930s (Wallace and McNeal, 1966) as wheat acreage increased. Development of large scale farming resulted in movement of the insect from

prairie grasses to wheat (Davis, 1955; Munro, 1945). During the late 30s and early 40s, millions of bushels of wheat per year were lost to this insect.

A number of factors are responsible for the *C. cinctus* population reaching economic proportions but three are of major importance; deployment of stem rust-resistant varieties, surface tillage, and strip cropping (McGinnis, 1950). Prior to development of rust-resistant varieties sawfly populations were reduced to low levels following each rust epidemic. When resistant wheat varieties replaced the susceptible ones the rust epidemics ceased. Thus the sawfly population escaped the devasting effects of these epidemics and continued to thrive.

To reduce losses from wind erosion new cultural methods were developed. While these practices provided control for wind erosion they afforded little control over the sawfly. The mouldboard plow had effectively controlled the sawfly population through deep burial of the stubs. With the introduction of surface tillage implements, this control was lost and the sawfly situation became more critical. As a further measure in water conservation and control of soil erosion, summer fallow and strip farming was introduced, which helped maintain undisturbed sawfly overwintering sites. This change immediately increased the losses caused by the *C. cinctus* (Holmes, 1982). It was thought that only the margins of wheat fields suffer serious loss. With large blocks being replaced by narrow strips, the losses increased in the same ratio as did the field margins; where very narrow strips were used, infestations upward of 90% occurred.

More recently, reduced tillage and chemical fallow conservation practices have enhanced sawfly overwinter survival. *C. cinctus* populations can be reduced by tillage systems. Reduced tillage and no-till management systems may increase *C. cinctus* survival

rate (Farstad and Jacobson, 1945; Weiss et al., 1987). Sawfly larvae overwinter in wheat stubble, therefore tillage practices which push infested stubble to the soil surface increase sawfly larval mortality by reducing overwinter survival (Holmes, 1982; Holmes and Farstad, 1956; Weiss et al., 1987).

As the severity of infestations increased, adequate control measures were sought. Early cultural controls methods proved only partially satisfactory (Criddle, 1911; Weiss and Morrill, 1992). Although nine parasitoid species have been found in the northern Great Plains (Holmes et al., 1963) and several exotic species were released (Luginbill and McNeal, 1954a, 1954b), biological control efforts have been unsuccessful (Weiss and Morrill, 1992). Accordingly, in 1932, a co-operative project between the Cereal Division at the Dominion Experimental Station, Swift Current, Saskatchewan, and the Dominion Entomological Laboratory, Lethbridge, Alberta, was initiated to study sawfly resistance in wheat and to develop resistant varieties (McGinnis, 1950).

Solid-stem varieties of *Triticum vulgare* and *T. durum* showed marked resistance to sawfly attack (Kemp, 1934). Resistance to *C. cinctus* in wheat is positively correlated with stem solidness (Farstad, 1940; Kemp, 1934; Luginbill and Knipling, 1969; Platt and Farstad, 1946; Platt et al., 1948). The resistance results from the hindrance of egg development, first-instar larval development, or older larval development (Roberts, 1954). Population levels of *C. cinctus* are greatly reduced by the use of solid-stem spring wheat cultivars (Holmes and Peterson, 1957). Consequently a breeding program was initiated to combine stem solidness with high quality, thus producing a suitable resistant variety. Hybrid lines were selected and tested for resistance to *C. cinctus* attack. Replicated uniform nurseries were established at various points in the sawfly-infested regions of the

Canadian prairies. The index of resistance was based on two factors, extent of cutting and percentage emergence. 'Rescue' was the first solid-stem spring wheat variety with resistance to *C. cinctus*. It was reported to have yield potential almost equal to hollow-stem susceptible cultivars at the time of release (Montana Agricultural Experiment Station, 1946). Although partially controlled by the development of solid-stemmed spring wheat cultivars, *C. cinctus* has remained a persistent and important pest of wheat in Montana, North Dakota, and southern portions of Alberta and Saskatchewan (Weiss and Morrill, 1992). During 1989, extensive damage was observed in winter wheat in central Montana, for which losses were estimated at 80% (Morrill et al., 1992a and 1992b; Morrill and Kushnak, 1996). At present, the *C. cinctus* is an economically significant pest of cereal crops in the northern Great Plains, especially in northern and eastern Montana, western North Dakota and areas of Saskatchewan, Manitoba and Alberta, Canada. Cereal grains currently affected include bread and durum wheat, although barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.) are sometimes infested to some degree.

Biology of *C. cinctus*

Wheat stem sawfly is an insect with complete metamorphosis: adult-egg-larva-pupa-adult (Morrill, 1995). Its life cycle is closely synchronized with the physiological development of its host plants. The sawfly spends most of its life within the stem of the host plant, effectively avoiding attempts at biological or chemical control. *C. cinctus* has been collected from areas of every state west of the Mississippi in the northern and central plains of North America where the annual precipitation is 250-500 mm, as well as from

Manitoba, Saskatchewan, Alberta, and British Columbia (Davis 1953).

Adults emerge from infested stubble in early summer and can survive for about one week (Morrill, 1995). Wasp emergence coincides with stem elongation of wild grasses. The date of emergence depends on temperature, soil type, and the depth that the infested wheat stubble is buried (Luginbill and McNeal, 1955). *C. cinctus* is a relatively weak flier. Although females have been observed to disperse at least 2.2 km, the female will usually deposit eggs in stems near the emergence site (Ainslie, 1920; Criddle, 1911; Holmes, 1975). Adults fly on warm calm days, but flights may cease during cloudy weather. Wasps cling to the plants during windy conditions (Butcher, 1946; Seamans, 1945). Farstad and Platt (1946) noted that wheat is preferred over other small grains. Holmes and Peterson (1960) demonstrated that females prefer to oviposit in the elongating (uppermost) internode, and spring wheat is preferred to winter wheat. Females usually deposit a single egg in each stem and then fly to another stem (Ainslie, 1920), though other females also may deposit an egg in the same stem (Weiss et al., 1987). Ainslie (1920) and Mills (1944) found that adult females may lay 30 to 50 eggs. The number of eggs deposited in the host stems may be affected by longevity of the female, host availability, and size of the female (Wall, 1952). If more than one egg hatches in the same stem, a struggle occurs among the larvae until only one survives (Davis, 1955; Munro, 1945; Seamans et al., 1938).

Eggs hatch in about seven days (Ainslie, 1920) and larvae feed on parenchyma and vascular tissue within the stem (Holmes, 1954). There are four to five instars depending on the host (Farstad, 1940). As the crop ripens, the larva moves downward. Early researchers hypothesized that larval movement down the stem was a response to decreasing stem moisture (Davis, 1955); however, Holmes (1975) suggested that the light

penetrating the stem walls of maturing host plants may initiate the downward movement. Upon completion of larval development, the larva will girdle the inside of a stem with a V-shaped notch, the height above the soil surface depends on soil and stem moisture (Holmes, 1975). Cutting begins when the stems contain approximately 50% moisture (Holmes, 1975) and the kernel is 40 to 50% moisture (Holmes and Peterson, 1965). Immediately below the notch, the larva plugs the stem with frass. The stem usually breaks at the notch, forming a "stub" that serves as an overwintering chamber. The stub is hollow and allows the larva to overwinter below the soil surface, thus protecting it against the severe winter climate. Here it forms a hybernaculum (looks like cocoon) in which it overwinters and pupates the next spring. The following summer, the adult chews an emergence exit through the plug and flies to nearby fields (Davis, 1955; Seamans et al., 1938).

Analysis of the damage caused by *C. cinctus* larvae shows that the insect causes economic losses in two ways. Physiological damage occurs as the *C. cinctus* larva tunnel through the stem destroying the vascular bundles, and thereby reducing the flow of water and nutrients to the developing kernels. This results in fewer kernels, lower test weights, and lower protein content in the harvested grain (Holmes, 1977; Weiss et al., 1987). Physical damage occurs when the sawfly larva notches the stem, causing the weakened stem to lodge and making it difficult or impossible to harvest.

Reproductive Mechanisms of *C. cinctus*

Like most other species of Hymenoptera, the reproductive mechanism in *C.*

cinctus is still not well understood (Crozier, 1977). The basic reproductive mode is arrhenotoky. Based on 20 years of extensive surveys throughout sawfly-infested areas of Alberta and Saskatchewan, Mackay (1955) reported that the population of *C. cinctus* is largely bisexual. The cytological study showed that the *C. cinctus* follows the basic chromosome pattern in Hymenoptera. In oogenesis of females exhibiting facultative parthenogenesis, pairing and chiasma formation take place in the usual manner. Females have two of each morphologically distinguishable chromosome of a haploid set and are therefore diploid. Males are haploids, stabilized by adaptive modifications of male meiosis, that is, absence of synapsis and suppression of the first division. This reduction of the meiotic process to a single equational division ensures the production of normal haploid spermatozoa.

Thelyotoky, i.e. diploid parthenogenesis, was also reported by Farstad (1938) in an exceptional, localized population. For eight years no males were found among large numbers of adult sawflies that emerged in the laboratory from collections made yearly in that locality; nor were any male sawflies found during the flight periods of the adult in the field. In this thelyotokous population, unfertilized eggs developed into diploid females, the diploid chromosome number was presumably regained during the maturation of the egg, as has been shown in other thelyotoky forms (Sanderson, 1933; Smith, 1941). Unfortunately none of the spanandric (rarely-occurring) males were examined cytologically but the possibility of occurrence of diploid males is considered to be slight It is possible that thelyotoky in *C. cinctus* arose by mutation (Smith, 1938) from an ancestral bisexual race that was indigenous in the native grasses.

However, more different mechanisms have been observed. According to limited

data obtained from the laboratory, Peterson (cited by Mackay, 1955) found that males usually arise from unfertilized eggs but that, occasionally, in the bisexual population females also arise from eggs that are not fertilized. Both male and female offspring are obtained from eggs laid by mated females; the males develop from unfertilized eggs by facultative parthenogenesis.

As in some other known insects, *C. cinctus* may adopt different reproductive mechanisms in different environments. Farstad (1938) pointed out, according to different reports, that it's quite possible that *Cephus* is arrhenotokously parthenogenetic in Europe while the species in Canada may actually be thelyotokously parthenogenetic, which is the case of *Diprion polytomum* Htg. (Smith, 1938).

Inheritance of Stem Solidness of Sawfly Resistant Wheat

A relatively effective way to control *C. cinctus* has been development of solid stem wheat varieties. Stem solidness is caused by the development of pith (undifferentiated parenchymous cells) inside the stem. Although it has never been demonstrated that pith is the only factor causing a variety to be resistant, many observations show that when a variety is less solid it is also less resistant (Wallace and McNeal, 1966). Stem solidness is a highly heritable character. Lebsock and Koch (1968) reported that heritability of stem solidness ranged from 60 to 95%.

Biffen (1905), in one of the first reports on the inheritance of stem solidness, studied crosses of 'Rivet' (*Triticum aestivum*), which is solid in the top internode, with hollow-stemmed cultivars of *T. aestivum*. He reported a ratio of three hollow segregates

to one solid in the F2 generation and concluded that hollow stem was dominant. He also suggested that stem solidness is not morphologically a simple character. Engledow and Hutchinson (1925) studied crosses of 'Rivet' with 'Chinese Spring' and concluded stem solidness was dominant and controlled by one gene. Yamashita (cited by Platt et al., 1941) used an extensive series of crosses involving several species of *Triticum* to study the genetics of the solid stem character. He indicated the presence of several genes that varied in number and effect with each species. Putnam (1942) studied the inheritance of stem solidness in tetraploid wheats. He split the stems length-wise and recorded them as solid, intermediate, or hollow. His results in crosses of *T. durum* and *T. turgidum* varieties to 'Golden Ball' (solid-stemmed wheat) indicated that the inheritance of stem solidness was controlled by one partially dominant factor. McNeal (1956) studied inheritance of stem solidness in a cross of 'Rescue' by 'Thatcher'. He found that Thatcher and Rescue were differentiated by one major gene and several modifying genes for stem solidness. The major gene was found to have an effect equal to two and one-half times that of all minor modifying genes.

Holmes (1984) reported that the Portuguese spring wheat 'S-615' is a parent of all currently grown solid-stemmed bread wheats. Platt et al. (1941), in a study of inheritance of solid stem, crossed hollow stem varieties 'Renown' and Thatcher with solid-stemmed selections of S-615-9 and S-633-3. They reported that three genes were involved in the expression of solidness and that the solid condition resulted when all three genes were recessive. The authors suggested that the genes act cumulatively, and that four or more dominant genes would produce phenotypically hollow plants. McNeal et al. (1957) examined F_2 plants from crosses between Rescue and four solid-stemmed wheat

introductions from Portugal. They concluded that each of the Portuguese wheats possessed the same major gene, or genes, for stem solidness that occur in Rescue. However three of the Portuguese wheats differed slightly from Rescue. This was ascribed to the action of minor genes affecting stem solidness. McKenzie's findings (1965) agreed with the study by McNeal (1956) in Rescue x Thatcher material concerning the presence of a single major gene. McKenzie (1965) studied inheritance of stem solidness in two hollow-stemmed (Red Bobs and Redan) by two solid-stemmed ('C.T.715' and S-615) spring wheats and hypothesized that the varieties in each cross differed by four genes for stem solidness. One major gene was indicated in both crosses and the other three genes within each cross were similar in their influence on solidness.

Larson (1959) found in monosomic lines of S-615 that top and bottom internode solidness was controlled by genes at different loci. After examining aneuploids of Rescue it was found that chromosome 3D had genes on the long arm for a solid top internode and genes for solid lower internode on the short arm (Larson and MacDonald, 1962). Larson (1959) also found that chromosome 3D of Rescue had a gene, or genes, inhibiting the production of pith, especially in the top internode, but in S-615, a parent of Rescue, the 3D chromosome promoted pith production in the top internode. Larson (1959) also showed that an aneuploid of Rescue has fewer chromosomes influencing solid stem than has its solid-stemmed parent, S-615.

Formation of Insect Biotypes

Speciation is the consequence of selection, isolation, drift, and time and is one of

the ways living organisms adapt to the diversity of environments available to them (White, 1978). Biotypes are different insect groups which can be most commonly distinguished by survival and development on a particular host or by host preference for feeding, oviposition, or both. Other insect biotypes differ in diurnal or seasonal activity patterns, size, shape, color, insecticide resistance, migration and dispersal tendencies, pheromone differences, or disease vector capacities (Eastop, 1973; Russell, 1978).

Theoretically, three main sets of variables are involved in speciation (White, 1978). First, there are the underlying genetic mechanisms, which may consist solely of allelic changes at individual gene loci or may include one or more chromosomal rearrangements. Second, genetic isolating mechanisms can play a primary role in initiating speciation. Finally, there is a geographic component that may range from complete geographic isolation of the diverging populations (strict allopatry) (White, 1978) to no isolation (complete sympatry) (Mayr, 1947).

The basis of isolation may involve genetically based differences in host preference (when mating occurs on the host) or many other factors such as allochronic barriers that arise as a direct result of phenological differences among hosts (Bush, 1969; Bush and Diehl, 1982; Huettel and Bush, 1972). Nongenetic differences such as induced host preference or seasonal mating times may also contribute to reproductive isolation, although, by themselves, are unlikely to bring about substantial isolation. Because there is at least a potential for occasional gene flow between races, selection must be responsible for the development and maintenance of host race differences in ecology and behavior (Bush and Diehl, 1982). Many divergent selective forces may simultaneously act on each host race. These include temporal differences in host availability, chemical

differences among hosts that affect survival ability, as well as host-associated variation in rates of parasitism, predation, competition, disease, and interactions with microorganisms (Diehl and Bush, 1984).

The frequency and suitability of host plant species encountered by insect herbivores can vary in space and time because of heterogeneity in the environment, disturbance, colonization and intra or interspecific interactions (Singer, 1986; Thompson, 1985). Such variability could favor change in host-selection behavior, and result in genetically-based divergence in diet among populations of plant-feeding insects (Courtney, 1982; Jaenike and Holt, 1991; Ward, 1987). The potential of a population to undergo selective change in host-selection behavior is determined by four factors: (i) the amount of phenotypic variation in host-selection behavior, (ii) the genetic basis of such variation, (iii) the relationship between host-selection behavior and fitness (Jaenike, 1990; Singer et al. 1989); and (iv) migration that must be low enough to allow genetic divergence of the norm of reaction for host response (Bossart and Scriber, 1995; Futuyma and Peterson, 1985). Low insect mobility combined with host plant isolation could foster genetic differentiation of populations by reducing gene flow (Alstad and Corbin, 1990).

After a pest-resistant variety has been grown for some time resistance may appear to break down. In fact this is due to the development of a strain of the pest (biotype) which is able to overcome the plant's resistant properties rather than to any change in the plant itself (Diehl and Bush, 1984). The best understood case of genetic polymorphism in biotypes concerns the interactions between virulence genes in the Hessian fly, and resistance genes in wheat. Hybridization of Hessian fly biotypes has been claimed to indicate evidence of a "gene-for-gene" interaction between the fly and its wheat host

(Gallun, 1978; Hatchett and Gallun, 1970) similar to the genetic interactions that have been extensively studied in plant-parasitic fungi and other disease organisms (Day, 1981). Because wheat is a hexaploid, the chance of resistance genes occurring at different (nonallelic) loci may be enhanced, since the plant begins with a minimum of at least three duplicated genes after hybridization. Also different wheat plants may adopt different resistant mechanisms to insects, the survival group of insects on different resistant wheats might form different biotypes (Diehl and Bush, 1984).

From its historical perspective, *C. cinctus* has extended it adaptation from the native grasses to spring wheat, from spring wheat to winter wheat, and partly to resistant solid-stem varieties. During this adaptation period it is quite possible that *C. cinctus* adapted to new habitats through gradual development of new biotypes. Parthenogenetic reproduction is known to occur in *C. cinctus* (Farstad, 1938). Bisexual reproduction is also recognized (Mackay, 1955). These two conditions undoubtedly contribute to the final sex ratio of a given population. Furthermore, *C. cinctus* is obliged to complete its development within a single stem in a single season, having no opportunity to move from an unfavorable environment. Consequently it is expected that the host plant may strongly influence the developing pest. The sex ratio of sawfly is likely to be affected by different host plant varieties in different regions, and also by different climates (McGinnis, 1950). The sawfly has the ability to utilize a large number of grass species in addition to cereals, increasing the likelihood of establishment in new habitats. Furthermore, both arrhenotokous and the thelyotokous reproduction is know in sawfly populations which would enables rapid establishment of existing *C. cinctus* genotypes. Sexual reproduction also produces new genetic recombinants that may be better adapted to survive in new

habitats (Blackman, 1985). Sexual reproduction is a factor in host race or biotype development in aphids (Briggs, 1965; Puterka and Peters, 1989, 1990; Puterka and Burton, 1991) and which has been documented in *D. noxia* (Puterka et al., 1992).

Although we are not sure whether or how the process of speciation has occurred in *C. cinctus*, possible biotypes of *C. cinctus* have been reported. Evidence for biotypic diversity in *C. cinctus* is reported for parthenogenic reproductive behavior (Farstad, 1938), virulence among two Canadian sawfly populations (Holmes et al., 1957), and emergence date (Morrill and Kushnak, 1996). Sawflies from Lethbridge and Regina differed in their abilities to infest and cut Rescue and other varieties of spring wheat. The two groups of sawflies differed significantly in the percentages of infested stems cut in the durum variety Golden Ball. The higher percentage of cut stems of Golden Ball by the Lethbridge sawflies apparently resulted from genetic differences between the sawflies from the two locations (Holmes et al., 1957).

RAPD-PCR Applications in Insect Genetic Studies

Until recently most of the progress in genetic systems has relied on a phenotypic assay of genotype. Because the efficiency of a selection scheme or genetic analysis based on phenotype is a function of the heritability of the trait, factors like the environment, multigenic and quantitative inheritance, or partial and complete dominance often confound the expression of a genetic trait. Many of the complications of a phenotype-based assay can be mitigated through direct identification of genotypes with a DNA-based diagnostic assay. For this reason, DNA-based genetic markers are being integrated into several insect

system studies and are expected to play an important role in the future of insect resistance breeding (Williams et al., 1992).

The utility of DNA-based diagnostic markers is determined to a large extent by the technology that is used to reveal DNA-based polymorphisms. Prior to the development of RAPD (Random Amplified Polymorphic DNA), the DNA markers most commonly used were RFLP (restriction fragment length polymorphisms) (Paterson et al., 1991). Anonymous low copy number genomic clones are frequently used to visualize polymorphisms. Detection of RFLPs by Southern blot hybridizations is laborious and incompatible with the high throughout required for many applications. Other polymorphism assays that are based on the polymerase chain reaction (PCR), require target DNA sequence information for the design of amplification primers. The time and cost of obtaining this sequence information is prohibitive for many large scale genetic mapping applications (Innis et al., 1990; Krawetz, 1989).

During 1990, a new genetic assay was developed independently by two different laboratories (Welsh and McClellan, 1990; Williams et al., 1990). This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence, and the polymorphisms function as genetic markers, which can be applied to study insect genetics (Williams et al., 1990).

When a single primer is mixed with genomic DNA and thermostable polymerase, and subjected to temperature cycling under conditions resembling those of the PCR, a DNA amplification product is generated for each genomic region that happens to be flanked by a pair of 10-base priming sites in the appropriate orientation, which are within 5,000 base pairs of each other. Amplification products are analysed by electrophoresis

(Operon Tech., 1994). RAPD is carried out in a series of cycles, each of which begins with a denaturation step to render the target nucleic acid single-stranded. This is followed by an annealing step during which the primers anneal to their complementary sequences so that their 3' hydroxyl ends face the target. Finally each primer is extended through the target region by the action of DNA polymerase. These three-step cycles are repeated over and over until a sufficient amount of product is produced. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocyclic amplification. The presence of each amplification product identifies complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer at each end of the amplified product (Arnheim and Erlich, 1992). Genomic DNA from two different individuals often produce different amplification fragment patterns. A particular DNA fragment which is generated for one individual but not for another represents a DNA polymorphism and can be used as a genetic marker. These markers are inherited in a Mendelian fashion (Williams et al., 1990). On average, each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphism between individuals. For example, the frequency of finding RAPD polymorphisms has been shown to be 0.3 per primer in *Arabidopsis thaliana*, 0.5 per primer in soybean (*Gliricidia*), 1 per primer in corn (*Zea Mays*), and 2.5 per primer in *Neurospora crassa* (Waugh and Powell, 1992). The advantage is that only one primer is needed and no prior information of the genomic DNA is required. The protocol is also relatively quick and easy to perform and uses fluorescence in lieu of radioactivity. Because

the RAPD technique is an amplification-based assay, only nanogram quantities of DNA are required, and automation is feasible (Waugh and Powell, 1992).

RAPD is quite complex even though there are a limited number of reagents used. In addition to a genomic DNA sample usually containing less than 1 amol of specific target sequence, the 25-100 microliter volume includes 20 nmol of each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 10 to 100 picomol of primer, appropriate salts and buffers, and DNA Taq polymerase. Amplification of random genomic sequences in a reproducible way is only possible with rigorously optimized reaction conditions, including temperature control and timing, and concentrations of template DNA, primer DNA, Taq DNA polymerase, and nucleotides (Arnheim and Erlich, 1992; Devos and Gale, 1992; Operon Tech., 1994; Williams et al., 1990).

The development of RAPD markers provided a powerful tool for the investigation of genetic variation in insects. The RAPD procedure works with anonymous genomic markers, requires only small amounts of DNA, and is simpler, less costly, and less labour intensive than other DNA marker methodologies. The marker itself is phenotypically neutral, independent of the allelic and nonallelic interactions, and also independent of environmental effects. RAPD markers were first used to create DNA fingerprints for the study of individual identity of the bacterial species and strains (Welsh and McClelland, 1990). Since then, a lot of research has been carried out using RAPD markers to create DNA fingerprints for the studies of individual identification and taxonomic relationships (Goodwin, 1991; He et al., 1992; Hedrick, 1992; Joshi and Nguyen, 1993; Waugh and Powell, 1992). RAPDs are widely used as genetic markers in insect population genetics.

Several groups have reported on the utility of RAPD markers as a source of

phylogenetic information. Since Chapco et al. (1992) carried out a feasibility study of the use of RAPD in the natural population genetics and systematics of grasshoppers (Acrididae: Melanoplinae and Acrididae: Oedipodinae), RAPD-PCR has recently been successfully used to identify genetic variation, examine phylogenetic relationships, and differentiate species in mosquitos (Ballinger-Crabtree et al., 1992; Kambhampati et al., 1992), greenbug, other aphid (Black et al., 1992; Puterka et al., 1993), and parasitic wasps (Landry et al., 1993). RAPD-PCR has also been used to differentiate strains of the Indianmeal moth (Lepidoptera: Pyralidae) (Dowdy and McGawghey, 1996), and identify biotypes of Russian wheat aphid (Black et al., 1992). In these studies, the number of RAPD primers necessary to provide adequate polymorphic bands for differentiation ranged from two (Kambhampati et al., 1992) to thirteen (Landry et al., 1993). RAPD markers have also been used effectively to assess the amount of genetic diversity in germplasm collections. Dawson et al. (1995) reported that by using RAPD assay, a direct relationship between the variation and the geographical distance between subpopulations had been revealed by cluster analysis. Williams et al. (1994) successfully determined the geographical origin of the insect pest, *Listronotus bonariensis* (Kuschel), by RAPD analysis.

Statistics Analysis of RAPD Data

Population genetics studies require analysis of multiple genetic markers in many individuals. Once the informative primers have been identified and variation has been estimated by sampling individuals in different geographic locations, this information must

be analysed properly. One of the easiest and most popular methods used is cluster analysis using RAPDPLOT (Black, 1995). There are three basic steps involved in cluster analysis. The first involves comparing all pairs of individuals in a study. A measure of distance is calculated for each pairwise comparison. If there are n individuals in a study, then there are n(n - 1)/2 distance measures. Second, all distance scores are placed into a matrix. Third, the distance matrix is collapsed using one of several algorithms to produce a dendrogram. There are two most commonly used ways to generate the distance matrix. The first matrix contains distance measures derived from the Nei and Li (1985) similarity index:

$$S = 2N_{AB}/(N_A + N_B)$$

where N_{AB} is the number of fragments that individuals A and B share in common, N_A is the number of fragments in individual A and N_B is the number of fragments in individual B. The distance between A and B is simply 1 - S. This is the measure that is widely used when comparing restriction maps and VNTR (variable numbers of tandem repeats) patterns among individuals. A second distance measure is based on the shared presence or absence of a fragment (Apostol et al., 1993). The shared absence of a fragment actually provides more information regarding genetic similarity between individuals (both homozygote recessives) than does the shared presence of that fragment (heterozygote or homozygote dominant). As a second distance, RAPDPLOT estimates the fraction of matches (M) using the formula:

$$M = N_{AB}/N_T$$

where N_{AB} is the total number of matches in individuals A and B (i.e., both fragments absent or present) and N_T is the number of loci scored in the overall study. Unlike the

similarity index, the denominator for M is fixed. An M value of 1 indicates that two individuals have identical fragment patterns; a value of 0 indicates that two individuals had completely different patterns. As with VNTR markers, RAPD fragments that comigrate are assumed to arise from identical alleles. However in using M, it was also assumed that the absence of a fragment in two individuals arose from the identical ancestral mutation (i.e., recessive alleles are identical in state). This may not be true because there are potentially many point mutations at the primer sites that could interrupt annealing. The assumption that recessive alleles are identical in state is valid among full siblings but may overestimate relatedness among nonsiblings. The assumption is completely invalid above the species level. For these reasons, it was recommended that only Nei and Li's similarity index be used for molecular taxonomy (Black, 1995). However, in most applications, the dendrograms produced by the two measures are quite similar.

The second popular statistical methods for RAPD data analysis is multivariate analysis because RAPD bands at multiple loci are compared among individuals. RAPD requires a special form of multivariate analysis because they are scored as a "1" or a "0" and not as continuous variables that follow a normal distribution. Ballinger-Crabtree et al. (1992) described the use of nearest neighbor discriminant analysis in examination of RAPD variation among individual *A. aegypti* belonging to two subspecies. This method does not assume that each variable follows a normal distribution. Euclidean or Mahalanobis distances were calculated among all pairs of individuals. Individuals are placed into groups with identical or similar individuals. These are an individual's "nearest neighbors". Each time a new individual is evaluated, it is placed in the cluster that contains its nearest neighbor. An individual that is equidistant from individuals in different clusters

is placed into an "other" category. The technique was found to accurately place individuals into the correct subspecies cluster most of the time. However, the output of nearest neighbor discriminant analysis is a misclassification table that was not as simple to interpret as the dendrograms examined in the same study. Furthermore, classification of individuals that fell within the "other" cluster was difficult.

The most recently developed RAPD analysis method was introduced from human genetics. As in other genetic study areas, the application of molecular markers in human genetics is far ahead of similar applications in insect and plant genetics. Prior to the development of PCR, population geneticists relied almost exclusively on biochemical polymorphisms as genetic markers, in which little or no allozyme variability had been detected in many insect taxa (Black, 1995). However, the knowledge of human population genetic diversity has improved considerably since 1980s, with the application of molecular techniques to population genetic studies (Excoffier et al., 1992). Larger numbers of haplotypic markers defined within each sample have greatly improved quantitative resolution. Because no precise analytic model for the full population of molecular differences among a set of interconnected haplotypes was known, different studies had tried to translate information on DNA haplotype into estimates of the magnitude of intraspecific subdivision in different ways. Most methods involved nonlinear transformation of the original data set into estimates of genetic diversity. Several assumptions on the underlying evolution of the molecule were required. But they were neither always met nor generally verifiable. There was an obvious need for a more general methodology that did not depend so critically on the specific assumptions. So Excoffier et al. (1992) designed an alternative methodology that made use of the available molecular

information gathered in population surveys, while remaining flexible enough to accommodate different types of assumptions about the evolution of the genetic system In 1993, Huff et al. first successfully applied the AMOVA (analysis of molecular variance) to the analysis of genetic variation in plants by treating a RAPD profile as a haplotype. Since then, AMOVA has been extensively used to classify genetic variation (Nesbitt et al., 1995; Vicario et al., 1995; Yeh et al., 1995).

AMOVA builds upon classical analysis of variance to compute molecular variance components at different hierarchical levels. It uses the fact that a sum of squared deviation between individual observations and their mean is equal to a double sum of squared differences (distances) between pairs of observations.

The central idea of AMOVA is to convert the inter-individual distance matrix into an equivalent analysis of variance. The inter-individual distance matrix can be defined in the following two ways. The first was a slightly modified distance metric from Nei and Li (1985)

$$D=(\delta_{xy}^2)=100[1-\frac{2n_{xy}}{n_x+n_y}]$$

where n_x and n_y are the numbers of markers observed in individuals x and y, respectively, and $2n_{xy}$ is the number of markers shared by two individuals; multiplication by 100 merely puts the number on the same scale as the second measure. The second measure was the Euclidean metric of Excoffier et al. (1992), defined here (in analogous terms) as:

$$E=(\xi^2_{xy})=n[1-\frac{2n_{xy}}{2n}]$$

where n is the total number of polymorphic sites. This latter measure amounts to a tally of band differences between individuals. A classic variance-components extraction yields the variance components of interest (Excoffier et al., 1992). Significance level for variance component estimates were computed by non-parametric permutational procedures. Both the non-Euclidean D and Euclidean E distance matrices were subjected to analysis. Software to conduct AMOVA has been developed and refined for use on PC's (Excoffier et al., 1992).

III. MATERIALS AND METHODS

C. cinctus Collection Sites

Overwintering *C. cinctus* larvae were randomly collected from wheat stubble in the field at 15 sites in Montana, North Dakota, and Wyoming in 1993 and 1994 (Table 1). Figure 1 shows the relative geographic distribution of these collection sites in Montana. *C. cinctus* larvae were stored at 4°C until DNA extraction. Additionally, one caged family (four individuals) which originated from the MT-B collection site was reared in the greenhouse. Sex of *C. cinctus* was discriminated according to pupal morphology in 1993 collections but not in subsequent collections in 1994. Adult wasps of sawflies (taxomy undetermined) collected in Syria (kindly provided by Dr. M. Ivie) were used for comparative purposes.

C. cinctus Genomic DNA Preparation

Genomic DNA was extracted using a modification of a previously reported method (Ballinger-Crabtree et al., 1992). Individual sawflies (larva, pupa, or adult) were ground to powder in liquid nitrogen with pestle grinders, then resuspended in 300 μl of lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% sodium dodecyl sulfate, 0.15 mM spermine, 0.5 mM spermidine) and 5 μl of a 20 mg/ml solution of proteinase K. Suspensions

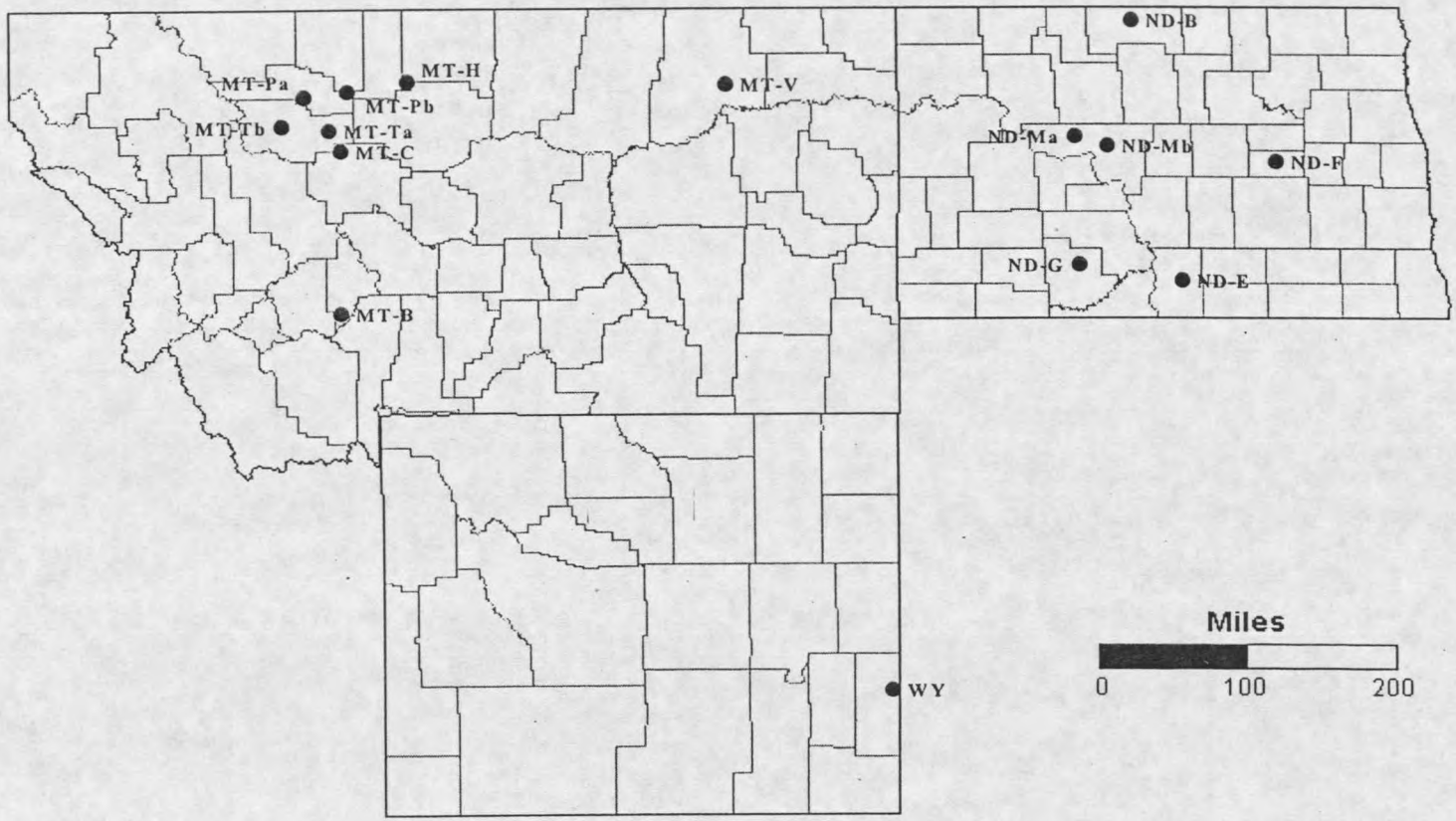

Figure 1. Collection sites of *C. cinctus* larvae sampled for RAPD-PCR studies in Montana, North Dakota and Wyoming in 1993 and 1994.